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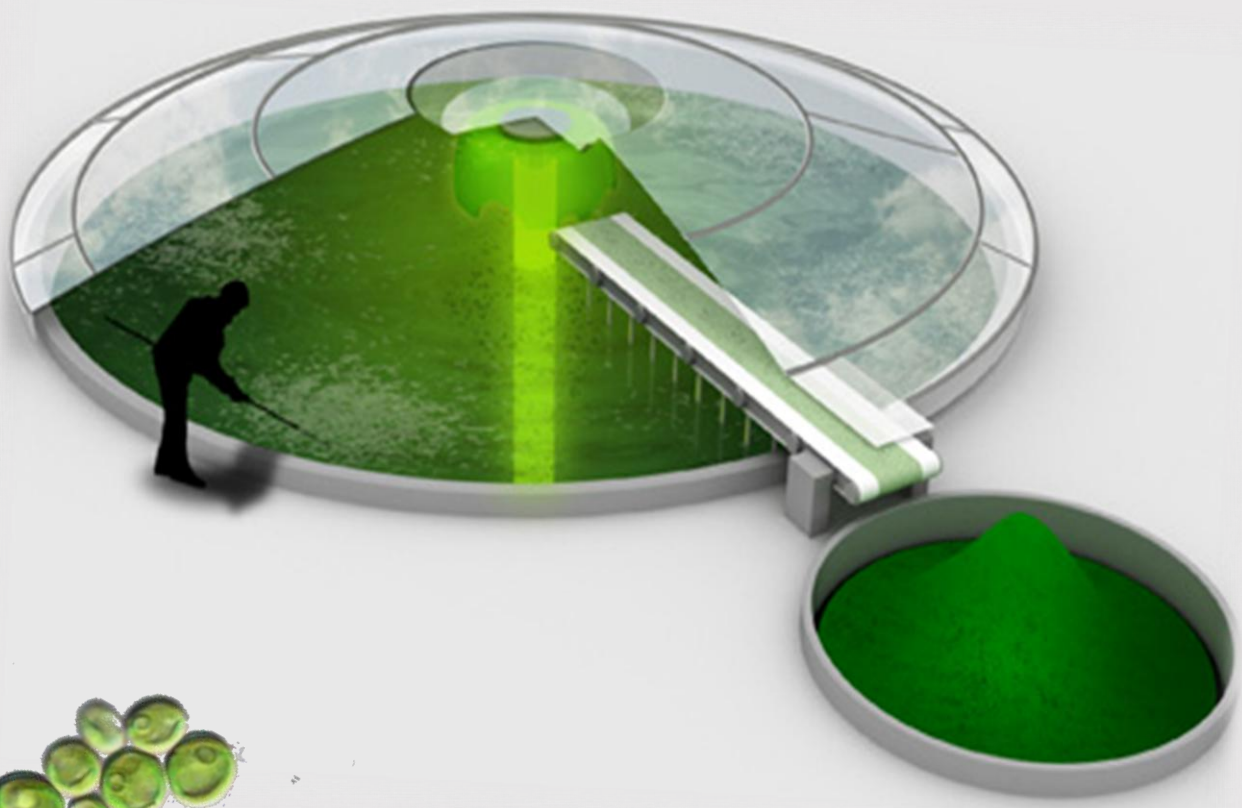
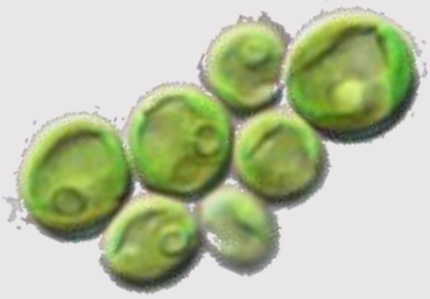


Microalgae as source of innovative foods and nutraceuticals



ALBERTO NICCOLAI

UNIVERSITÀ
DEGLI STUDI
FIRENZE

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Scienze Agrarie e Ambientali

CICLO XXIX

COORDINATORE Prof. Simone Orlandini

Microalgae as source of innovative foods and nutraceuticals

Settore Scientifico Disciplinare AGR/16

Dottorando

Dott. Alberto Niccolai

Tutore

Prof. Mario R. Tredici

Co-tutore

Dott. Liliana Rodolfi

Coordinatore

Prof. Simone Orlandini

Anni 2014 / 2016

Dichiarazione

Con la presente affermo che questa tesi è frutto del mio lavoro, svolto nell'ambito di un gruppo di ricerca e quindi con la collaborazione di altri colleghi e sotto la guida del tutore e del co-tutore, e che, per quanto io ne sia a conoscenza, non contiene materiale precedentemente pubblicato o scritto da un'altra persona né materiale che è stato utilizzato per l'ottenimento di qualunque altro titolo o diploma dell'Università o altro istituto di apprendimento, a eccezione del caso in cui ciò venga riconosciuto nel testo.

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Alberto Niccolai, 29/11/2016

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Alberto Niccolai, 29/11/2016

Parole chiave: Microalghe, cianobatteri, cibo, nutraceutici

Riassunto

Obiettivo: Le microalghe (compresi i cianobatteri) rivestono un grande potenziale come fonte di composti naturali, da utilizzare come ingredienti funzionali in preparazioni alimentari. Fino ad oggi, diverse specie di microalghe e cianobatteri sono stati identificati, ma solo pochi di loro sono stati approvati e applicati nella produzione alimentare. Per aumentare il numero di ceppi idonei allo sfruttamento nell'industria alimentare, si rende necessaria un'approfondita indagine sulla loro sicurezza e sulla loro qualità nutrizionale.
L'obiettivo generale del progetto di tesi è stato quello di valutare l'idoneità di alcune biomasse microalgali e cianobatteriche che potrebbero essere proposte per la produzione di alimenti innovativi e nutraceutici. Per perseguire questo obiettivo, numerose biomasse di microalghe e di cianobatteri sono state prodotte e testate per valutarne: i) composizione biochimica, ii) tossicità *in vitro*, iii) digeribilità *in vitro*, iv) sicurezza e potenziali effetti salutistici *in vivo*, v) effetti sulle qualità organolettiche e sulle potenzialità antiossidanti dopo addizione in alimenti convenzionali, vi) idoneità alla fermentazione.
Metodi e Risultati: Il **Capitolo 3** riporta i test utilizzati per indagare la possibile tossicità *in vitro* di ceppi selezionati di microalghe e cianobatteri. Gli estratti metanolici e acquosi delle biomasse microalgali e cianobatteriche sono stati testati su fibroblasti dermici di origine umana e su *Artemia salina*. Sebbene in alcuni casi i due modelli abbiano fornito risultati contrastanti, questo lavoro ha confermato la validità dei pre-screenings di tossicità. Questi modelli *in vitro*, specialmente nel caso dell'estratto metanolico dopo 48 ore di incubazione, sono stati in grado di rilevare la potenziale tossicità di substrati microalgali e potrebbero servire come linee guida per prove *in vivo* su mammiferi, necessarie per richiedere l'applicazione per *novel food* nell'Unione Europea.
L'analisi della composizione biochimica e la valutazione della digeribilità sono analisi fondamentali per fornire informazioni sulla qualità e sulla biodisponibilità degli elementi nutritivi nelle biomasse algali. Nel **Capitolo 4**, vengono studiate la composizione biochimica e la digeribilità *in vitro* di alcune biomasse di microalghe e cianobatteri. La composizione biochimica equilibrata e la buona digeribilità, in particolare per i cianobatteri, evidenziano il potenziale di questi microrganismi come alimenti funzionali. Per migliorare la digeribilità

in vitro, è stato anche valutato un pretrattamento di sonicazione su due biomasse liofilizzate appartenenti al phylum delle Chlorophyta. I dati ottenuti in questo studio confermano l'effetto positivo della sonicazione sulla dispersione cellulare e quindi sull'efficienza dell'azione enzimatica durante il processo di digestione.
I risultati ottenuti dai modelli *in vitro* sono preliminari e devono essere confermati da studi *in vivo* sui mammiferi effettuati sull'intera biomassa algale.
I **Capitoli 5** e **6** si focalizzano sulla valutazione della sicurezza *in vivo* su ratti alimentati con biomasse di *Arthrospira platensis* F&M-C256 e *Tisochrysis lutea* F&M-M36. Le diete integrate (al 20%) con le due biomasse sono state ben tollerate. Le osservazioni cliniche e dei pesi corporei non hanno evidenziato effetti negativi e non sono stati osservati aumenti della pressione sanguigna o danni ossidativi renali. Il colesterolo LDL è risultato invariato, ed è stato osservato un aumento significativo del colesterolo HDL in entrambi i gruppi alimentati con *A. platensis* F&M-C256 *e T. lutea* F&M-M36. Queste due biomasse sarebbero probabilmente sicure anche a più alti dosaggi e potrebbero rappresentare una fonte promettente di alimenti funzionali per la prevenzione di diverse malattie.
Nel **Capitolo 7** al fine di esplorare la potenziale applicazione di microalghe e cianobatteri come ingredienti alimentari, sono stati analizzati biscotti contenenti *A. platensis* F&M-C256, *Chlorella vulgaris* Allma, *Tetraselmis suecica* F&M-M33 e *Phaeodactylum tricornutum* F&M-M40 in termini di parametri fisico-chimici, bioattività e digeribilità *in vitro*. Le proprietà fisiche dei biscotti sono state valutate per 8 settimane e non state trovate differenze significative in termini di stabilità nella struttura. Inoltre, non sono state trovate differenze significative nella digeribilità *in vitro* tra i biscotti a base di microalghe ed il controllo. I biscotti a base di microalghe hanno invece presentato maggior potere antiossidante e contenuto fenolico totale rispetto al controllo.
Nel **Capitolo 8**, al fine di migliorare l'accettabilità delle microalghe da parte del consumatore in termini di qualità organolettica e digeribilità, e per valutare la potenziale applicazione per la produzione di alimenti funzionali, le biomasse di *A. platensis* F&M-C256 e *T. suecica* F&M-M33 sono state fermentate con *Lactobacillus plantarum*. Oltre a migliorare la digeribilità, la fermentazione acido-lattica ha incrementato anche l'attività antiossidante. Questo studio ha evidenziato il potenziale di specie microalgali per la produzione di alimenti funzionali e probiotici.

Nel **Capitolo 9**, sono riportate le discussioni generali, dove i risultati dei diversi capitoli vengono discussi approfonditamente rispetto ai dati di letteratura.

Conclusioni: I risultati dell'analisi della composizione biochimica e dei test *in vitro* ed *in vivo* dimostrano il potenziale delle microalghe e dei cianobatteri come candidati per la produzione di alimenti funzionali e nutraceutici. Inoltre, le biomasse di *A. platensis* F&M-C256, *C. vulgaris* Allma, *T. suecica* F&M-M33 e *P. tricornutum* F&M-M46 si sono dimostrate idonee per lo sviluppo di prodotti funzionali a base di microalghe (ad esempio biscotti). Il processo di fermentazione ha poi migliorato la qualità delle biomasse di *A. platensis* F&M-C256 e *T. suecica* F&M-M33 in termini di qualità organolettiche e digeribilità.

Keywords: Microalgae, cyanobacteria, food, nutraceuticals

Summary

Aim: Microalgae (including cyanobacteria) have great potential as a source of natural compounds, to be used as functional ingredients in food preparations. Up to date, several species of microalgae and cyanobacteria were classified but only few of them are approved and applied in food production. To increase the number of suitable strains for the food industry, a deep investigation about their safety and quality is fundamental.

The general aim of the thesis project was to evaluate the suitability of some microalgal and cyanobacterial biomasses that might be proposed for the production of innovative foods and nutraceuticals. To pursue this aim, i) chemical composition, ii) *in vitro* toxicity, iii) *in vitro* digestibility, iv) *in vivo* safety evaluation and potential health effects, v) the effects on organoleptic and antioxidant quality after the inclusion of microalgal and cyanobacterial biomass in conventional foods, and vi) suitability to fermentation were investigated.

Methods and Results: Chapter 3 reports the tests which investigated the possible *in vitro* toxicity of selected microalgal and cyanobacterial strains. Methanolic and aqueous extracts of the microalgal biomasses were tested on human dermal fibroblasts and on *Artemia salina* models. Although in some cases the two models provided contrasting results, this work confirmed their validity for preliminary screening of toxicity. These *in vitro* models, especially in the case of methanolic extract after 48 hours of incubation, were able to detect the potential toxicity of microalgal substrates and may well serve as guidelines for *in vivo* tests on mammals, which are necessary to apply for novel food in the European Union.

The biochemical composition and the evaluation of digestibility are fundamental analyses to provide information about the quality and bioavailability of nutritional components of microalgal biomasses. In **Chapter 4**, the biochemical composition and the *in vitro* digestibility of some selected microalgal and cyanobacterial biomasses are reported. A balanced biochemical composition and a good digestibility, in particular for cyanobacteria, indicated these microorganisms as promising functional foods. To enhance the *in vitro* digestibility, a sonication pre-treatment on two lyophilised biomasses belonging to Chlorophyta phylum was also evaluated. The data obtained in this study

confirm the positive effect of sonication on cellular dispersion and consequently on the efficiency of the enzymes action during the digestion process.

Results obtained from *in vitro* models are preliminary and must be confirmed by *in vivo* studies on mammals performed on the whole algal biomass. **Chapters 5** and **6** focused on a safety evaluation of one month feeding of *Arthrospira platensis* F&M-C256 and *Tisochrysis lutea* F&M-M36 in rats. The microalgae supplemented diets (20%) were well-tolerated. Clinical observations and body weights did not reveal negative effects and no increase in blood pressure or renal oxidative damage were observed. LDL cholesterol levels were unchanged, but a significant increase in HDL was found in the *A. platensis* F&M-C256 and *T. lutea* F&M-M36 fed groups. Microalgae as *A. platensis* F&M-C256 and *T. lutea* F&M-M36 are probably safe even at higher dosage and they may represent a promising source of functional foods for the prevention of several diseases.

To explore the potential food application of microalgae and cyanobacteria, *A. platensis* F&M-C256, *Chlorella vulgaris* Allma, *Tetraselmis suecica* F&M-M33 and *Phaeodactylum tricornutum* F&M-M40-based cookies were analysed in terms of physicochemical parameters, bioactivity and *in vitro* digestibility (**Chapter 7**). The cookies physical properties were evaluated along 8 weeks and no significant differences were found in terms of texture stability. No significant difference in *in vitro* digestibility between microalgae cookies and the control was found. On the other hand, microalgae cookies presented significantly higher *in vitro* antioxidant capacity and total phenolic content than the control.

In **Chapter 8**, in order to improve the acceptability of microalgae by the consumer in terms of organoleptic quality and digestibility, and to evaluate the potential application for the production of functional foods, *Lactobacillus plantarum* was used to ferment *A. platensis* F&M-C256 and *T. suecica* F&M-M33 biomasses. In addition to enhancing digestibility, lactic acid fermentation also increased antioxidant activity. This study highlighted the potential of microalgae species for the production of economically valuable functional food and probiotics.

Chapter 9 reports the general discussion in which the findings of the different chapters are discussed together with a deepened comparison with literature results.

Conclusions: The results of the analysis of biochemical composition and *in vitro* and *in vivo* tests demonstrate the potential of microalgae and cyanobacteria as candidates for the production of functional foods and nutraceuticals.

A. platensis F&M-C256, *C. vulgaris* Allma, *T. suecica* F&M-M33 and *P. tricornutum* F&M-M46 have been also found suitable for the development of functional microalgae-based products (e.g. cookies). Fermentation process also enhanced the quality of *A. platensis* F&M-C256 and *T. suecica* F&M-M33 biomasses in terms of organoleptic quality and digestibility.

Scientific production

Papers related to the thesis

Niccolai A., Bigagli E., Biondi N., Rodolfi L., Cinci L., Luceri C., Tredici M. R. (2016) In vitro toxicity of microalgal and cyanobacterial strains of interest as food source. *Journal of Applied Phycology*, 1-11.

Niccolai A., Shannon E., Abu-Ghannam N., Biondi N., Rodolfi L., Tredici M. R. *Lactobacillus plantarum* fermentation of microalgal and cyanobacterial biomasses as a potential application for the production of functional foods. *In preparation.*

Niccolai A., Chini Zittelli G., Biondi N., Rodolfi L., Tredici M. R. Biochemical composition and *in vitro* digestibility of microalgal and cyanobacterial strains of interest as food source. *In preparation.*

Bigagli E., Cinci L., **Niccolai A.**, Tredici M. R., Biondi N., Rodolfi L., Lodovici M., Mori G., Luceri C. Safety evaluation and effects on lipid metabolism of a diet rich in *Arthrospira platensis* F&M-C256 biomass, in rats. *Submitted.*

Batista A.P., **Niccolai A.**, Fradinho P., Fragoso S., Bursic I., Rodolfi L., Biondi N., Tredici M.R., Sousa I., Raymundo A. Microalgae cookies – Sensory, physical and chemical properties, antioxidant activity and *in vitro* digestibility. *In preparation.*

Bigagli E., Cinci L., **Niccolai A.**, Tredici M. R., Biondi N., Rodolfi L., Lodovici M., Mori G., Luceri C. Safety evaluation and hypopotriglyceridemic activity of a diet rich in the marine microalga *Tisochrysis lutea* (T-ISO) F&M-M36: a sub-acute study in rats. *In preparation.*

Presentations at Congresses

Chini Zittelli G., Tredici M. R., Tibaldi E., Poli B. M., Rodolfi L., Biondi N., **Niccolai A.** (2014) Production and use of microalgae biomass for aquaculture feeds. Conference: Algae Europe 2014. 1st EABA and EC Contractors' Conference and the 8th International Algae Congress, at Florence (Italy). *Oral presentation.*

Niccolai A., Biondi N., Bigagli E., Luceri C., Cinci L., Rodolfi L., Tredici M. R. (2015) In vitro safety assessment of microalgal and cyanobacterial strains of interest as food ingredients. Conference: Algae Europe 2015. 2nd EABA and EC Contractors' Conference and the 9th International Algae Congress, at Lisbon (Portugal). *Poster.*

Niccolai A., Shannon E., Abu-Ghannam N., Biondi N., Rodolfi L., Tredici M. R. (2016) *Lactobacillus plantarum* fermentation of microalgal and cyanobacterial biomasses as a potential application for the production of functional foods. Conference: Algae Europe 2016. 3nd EABA and EC Contractors' Conference and the 10th International Algae Congress, at Madrid (Spain). *Poster*.

Bigagli E., Cinci L., **Niccolai A.**, Tredici M. R., Biondi N., Rodolfi L., Lodovici M., Mori G., Luceri C. (2016) Safety evaluation and hypolipidemic effects of one month feeding of *Arthrospira platensis* F&M-C256 in rats. Conference: Algae Europe 2016. 3nd EABA and EC Contractors' Conference and the 10th International Algae Congress, at Madrid (Spain). *Poster*.

Batista A. P., **Niccolai A.**, Fradinho P., Fragoso S., Sousa I., Raymundo A., Rodolfi L., Biondi N., Tredici M. R. (2016) Microalgae Biscuits – physicochemical, bioactive and *in-vitro* digestibility aspects. Conference: Algae Europe 2016. 3nd EABA and EC Contractors' Conference and the 10th International Algae Congress, at Madrid (Spain). *Oral presentation.*

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A. Niccolai

Microalgae as source of innovative foods and nutraceuticals

250 pages.

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Chapter 1

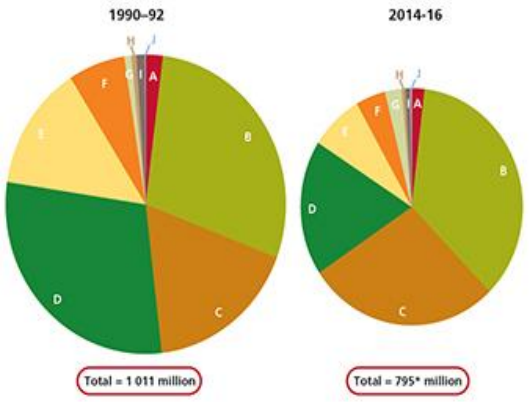
General introduction

1

1. Introduction

The pressure on natural resources is becoming increasingly unsustainable, as the food supply problem continues to grow in many parts of the world, therefore, in addition to the financial and energy crises, it is necessary to shift the focus on food security. Indeed, the global energy crisis that is increasing by almost 50 years suggests that we should consider new alternatives for solving these problems (Goswami and Kreith, 2015). Increasing population also increases the global demand for energy and, due to the exhaustion of conventional energy sources, notably petroleum, it also increases the price, thus decreasing the capacity of people to purchase primary goods (Sorrell, 2015).

To live a healthy and balanced life, people must have food of good quality and in quantities needed to meet their energy and nutrients requirements. Without adequate nutrition, adults and children will encounter difficulty in maintaining optimal physical condition, making them more susceptible to disease (Stipanuk, 2013). Nowadays, not all people have access to sufficient quantities of food (Fig. 1). About 795 million people are chronically undernourished and are not able to have enough food to meet their energy needs (Fig. 1) (FAO, 2015). Malnutrition in the form of deficiency of vitamins, minerals and essential fatty acids continues to be, on a global scale, the leading cause of serious illnesses and death of millions of people per year (McGuire, 2015).



	Number (millions)		Regional share (%)	
	1990–92	2014–16	1990–92	2014–16
A Developed regions	20	15	2.0	1.8
B Southern Asia	291	281	28.8	35.4
C Sub-Saharan Africa	176	220	17.4	27.7
D Eastern Asia	295	145	29.2	18.3
E South-Eastern Asia	138	61	13.6	7.6
F Latin America and the Caribbean	66	34	6.5	4.3
G Western Asia	8	19	0.8	2.4
H Northern Africa	6	4	0.6	0.5
I Caucasus and Central Asia	10	6	0.9	0.7
J Oceania	1	1	0.1	0.2
Total	1 011	795*	100	100

Figure 1 The changing distribution of hunger in the world: numbers and shares of undernourished people by region, 1990–92 and 2014–16
Source: FAO, 2015

It is expected that in 2050 the world population will reach 9 billion with a particularly high concentration in urban areas (Fig. 2) (Antal and Van Den Bergh, 2016), and that the demand for food and raw materials is not directly proportional to the increase of the population.

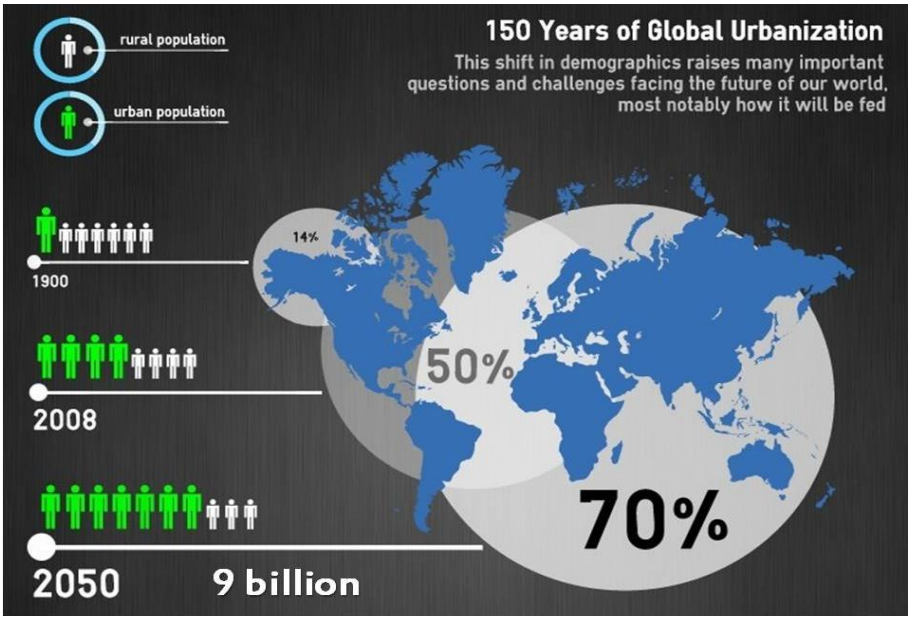


Figure 2 Word population expected in 2050
Adapted from Website Population Reference Bureau, 2013

The economic development that some emerging countries are experiencing, allowing for richest diets, especially in animal protein, leads to an increase in the demand for food. To meet future demands, the global production of food and raw materials should highly increase over the next 40 years (Antal and Van Den Bergh, 2016). Such production could be reached thanks to the intensification of agricultural programs implemented in recent years in most industrialized countries (Antal and Van Den Bergh, 2016), but we have to consider some factors that might counteract the achievement of this objective. For the first time in history, the demand for freshwater in the urban areas has exceeded that used for agriculture (Cosgrove and Loucks, 2015). It is expected that in 2050, the cities will consume about half of the available freshwater, reducing by one-third that available for agricultural production, and increasing the areas of the planet where there is not a safe source of drinking water for human consumption (Cosgrove and Loucks, 2015).

It is estimated that freshwater shortage will have a negative impact on agricultural production, calculating a loss in terms of food of around 350 million tons per year, an equal amount to the current global rice production (Cribb, 2010). The challenge for the next generation of farmers will be to double the productivity of the fields using only two-thirds of the water available today. This implies an increase of about 200% efficiency of water use (Cribb, 2010).

From all these issues then a question arises, from which the next challenge comes: "how to increase the production of biomass with a minimal increase in terrestrial area and with a limited use of water and fossil energy and at the same time reduce or avoid adverse effects on natural resources and ecosystems?". It is clear that to rely on molecular biology or the introduction of new types of fertilizers or pesticides, as was done in the second half of the last Century to face food needs from emerging countries is not enough. Rather, it is necessary to find new sources of energy, and reduce to a minimum the impact on ecosystems. Microalgae should be part of the solution towards a new economy based on new biological sources (Tredici, 2014). Indeed, microalgae cultivation has several advantages compare to traditional crops, especially in terms of sustainability: i) they do not need fertile soil and pesticides or herbicides for their cultivation, limiting the water pollution by these products; ii) many microalgae can be cultivated in saline, sea or brackish water, avoiding the competition for freshwater resources; iii) they can grow on wastewaters iv) they require CO_2 sources for their growth, thus they can absorb this gas from flue gases (Tredici, 2016; Chini Zittelli et al., 2013a,b; Rodolfi et al., 2009).

1.1 Microalgae and biotechnology: photosynthesis, taxa, and cultivation

1.1.1 Photosynthesis of microalgae

Microalgae, as well as cyanobacteria, are usually phototrophic microorganisms, which obtain the energy directly from sunlight, using inorganic elements as a source of nutrients for growth. These microorganisms are present in all aquatic ecosystems, both marine and freshwater, where are the starting point of the food chains. They can be found, also in the surface layers of many lands, organized in colonies, in filaments or solitary (Tredici, 2010).

Microalgae (including cyanobacteria) are directly responsible for about half of the photosynthetic activity on Earth (Tredici, 2010). The capacity of these organisms to grow in extreme environments, including deserts, hypersaline

lakes and glaciers is related to their particular abilities to produce reserve substances, in their high biodiversity, which makes them suitable also for extreme climates (Varshney et al., 2015). Generally, large filamentous cyanobacteria colonize first, most probably because of their possession of thick extracellular sheaths or mucus layers, which improve the water retention properties (Tomaselli and Giovannetti, 1993). Some microalgal and cyanobacterial species can also grow on organic substrates (sugars, alcohols or organic acids) in the presence or absence of light, which makes them even more adaptable to the different environmental conditions (Varshney et al., 2015).

The photoautotrophic organisms, through photosynthesis, convert inorganic compounds (H_2O, CO_2, N, and P) into organic compounds as carbohydrates (sugars), proteins and lipids (oils) by using solar energy. This process is divided into two phases: light phase, which occurs in thylakoids, and leads to the formation of ATP and $NADPH_2$ in addition to the release of O_2; and, dark phase, which takes place in the stroma, in which $NADPH_2$ and ATP are used to reduce carbon dioxide into carbohydrates in the Calvin-Benson cycle. The spectrum of visible light, between 350 (violet) and 750 (far red) nm corresponds to that of Photosynthetically Active Radiation (PAR), which is the radiation used in photosynthesis. Chloroplasts are the seat of the photosynthetic apparatus and are constituted by a series of said thylakoid vesicles and by a matrix or stroma. Prokaryotic cells, such as those of cyanobacteria, have a more simple structure. Chlorophyll *a* is present in all oxygenic photoautotrophic organisms mainly in the reaction center, while chlorophyll *b* or *c* and some carotenoids act as antenna pigments (Tredici, 2010).

1.1.2 Microalgal taxa

Microalgae commercially exploited and their products represent a very small number of species compared to the high number present in nature, from 30,000 to 1 million species according to different sources (Guiry, 2012).

The main microalgae classification system was based on morphological characteristics, type of reserve substance, nature of pigments and constituents of the cell wall (Christensen, 1964; Tomaselli, 2008). In recent years molecular analyses are providing new characters well beyond the morphology-based systematics of the past, leading to a much better understanding of what may constitute a species, as well as the phylogenetic and evolutionary relationships between species. In the future developments of proteomics and metabolomics will impact on our understanding of what constitutes a species (Borowitzka,

2016). However, thanks to several taxonomic databases, especially AlgaeBase (http://www.algaebase.org/) it is now relatively easy for a researcher to check the current as well as the past names (synonyms) of a particular species.
The main algal groups are shown below:

- Cyanobacteria: they are the unique group of prokaryotes with oxygenic photosynthesis, they have glycogen as main reserve substance, their cell wall is of Gram-negative type, they synthesize chlorophyll and phycobiliproteins and, in the case of Prochlorales, also chlorophyll *b*. They include *Microcystis*, *Arthrospira* (commonly called spirulina), *Nostoc* and nitrogen-fixing cyanobacteria, such as *Anabaena azollae* (symbiont of Azolla fern), which are a valuable source of nitrogen in the rice fields (Stanier and Bazine, 1977; Balloni et al., 1980);
- Rhodophyta (red algae): they have chlorophyll *a* and *d* and phycobiliproteins, they synthesize starch of Floridee and amorphous polysaccharides. They may contain high amounts of polyunsaturated fatty acids (as *Porphyridium*) (Dixon, 1973);
- Chlorophyta (green algae): they include a large variety of organisms with considerable morphological differences. They all have chlorophyll *a* and *b* and synthesize starch. Some species can synthesize and accumulate carotenoids outside of the chloroplast in the case of stress (Huss et al., 1999). *Chlorella*, *Dunaliella*, *Haematococcus* and *Tetraselmis* are cultivated at large-scale for aquaculture, or for the production of pigments and dietary supplements (Chini Zittelli et al., 2006);
- Dinophyta (dinoflagellates): they synthesize chlorophyll *a* and *c*, and peridinin, accumulate starch and are a potential source of polyunsaturated fatty acids (such as n-3). They can generate extended blooms and are producers of algal toxins. In addition to the environmental impact, these toxins can also create problems in aquaculture (Lee, 1999a);
- Prymnesiophyta: their cells contain chlorophyll *a* and *c* and fucoxanthin, can produce chrysolaminarin as a reserve substance and their cells can be coated with calcium carbonate flakes (coccolithophorids) (Lee, 1999b). To this group belong important microalgae commonly used in aquaculture (*Isochrysis*, *Pavlova*) (Chini Zittelli et al., 2004) and others which can produce ichthyotoxins, such *Prymnesium* (Lee, 1999b);
- Bacillariopyhta (diatoms): they are the group with the largest number of known species, they can have unicellular organization, form colonies or short chains. Their characteristic brown colour is due to the combination of

chlorophyll *a*, *c*, and fucoxanthin. Their principal stock product is chrysolaminarin, although lipids can be present in considerable amounts. Diatoms are divided into pennate, with bilateral symmetry, and centric, with radial symmetry. They live in aquatic environments and in the surface layers of soil and play an important ecological role being responsible for 40% of all primary production in the oceans (Falkowski and La Roche, 1991). Commercial uses of the diatoms are mainly related to aquaculture (*Phaeodactylum*), and to the significant presence of polyunsaturated fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Lee, 1999c);

- Eustigmatophyta: This class includes single-celled organisms that live in marine waters and on the surface of many soils. They contain only chlorophyll *a* (Lee, 1999d). Most of the Eustigmatophyceae are used in aquaculture (*Nannochloropsis*) and for the extraction of polyunsaturated fatty acids (EPA) (Chini Zittelli et al., 2003).

1.1.3 Cultivation of microalgae

Photobioreactors are reactors in which phototrophic microorganisms can grow or where they can perform photobiological reactions. The bioreactors can be open, if the surface of the culture is in direct contact with the atmosphere or closed, if the surface of the culture is not in direct contact with the atmosphere (Tredici, 2004).

In this work, we tested strains/products belonging to species already approved as food in the EU (*C. vulgaris*, *A. flos-aquae* from Klamath Lake blooms, *A. platensis*), and species not approved at present (*C. sorokiniana* and *Tetraselmis suecica*), but belonging to genera of approved species. Other microalgae were also tested: *Nostoc sphaeroides*, an edible cyanobacterium consumed in China and other Asian countries (Han et al. 2013), *Tisochrysis lutea*, *Nannochloropsis oceanica*, and *Phaeodactylum tricornutum*, widely used (as well as *Tetraselmis* spp.) in aquaculture (Tredici et al. 2009; Muller-Feuga 2013), and *Porphyridium purpureum*, rich in EPA, but to date used only in cosmetics (Arad and Levy-Ontman 2010; Rajasulochana and Preethy 2015). The algae produced in this thesis were cultivated in closed photobioreactors (Tredici et al. 2011; Chini Zittelli et al. 2013a,b) in semi-batch mode. Closed photobioreactors can be defined as culture systems where most of the light has to across the reactor walls before arriving at the cells. In this way, it decrease the possibility of contamination by other organisms, it can ensure greater control of the culture

and it can optimize the production process. To obtain high performance it is necessary to optimize the constructive criteria such as the Is/V (Illuminated surface/Volume) ratio, which determines the amount of light that enters within the system per unit of volume (Tredici, 2004). These photobioreactors can produce biomass with natural and artificial light, making possible the production of biomass throughout the year, and at all latitudes even if they are characterized by a fairly high cost. Thanks to the production of a high-quality biomass, such as *Nannochloropsis* (Chini Zittelli et al., 2003; Rodolfi et al., 2003; Freire et al., 2016) *Tetraselmis* (Moheimani, 2013) and *Isochrysis* (Thu et al., 2015) they are successfully used in aquaculture. Photobioreactors can be classified according to their design in: (i) flat or tubular; ii) horizontal, inclined, vertical or spiral; iii) manifold or serpentine; iv) hybrid; v) floating; vi) biofilm reactors.

The reactors used for the cultivation of strains in the present thesis were of the flat panel type. The photobioreactor used is denominated "Green Wall Panel" (GWP®), characterized by a good scalability at the industrial level and by a low cost of realization and management. The first Green Wall Panel type (GWP®-I, Fig. 3 left, Tredici and Rodolfi, 2004) was followed by the second model, the GWP®-II (Fig. 3 center) (Tredici et al., 2011), characterized by a greater simplicity of construction and easiness of operation and by the third model the GWP®-III (Tredici, 2016) (Fig. 3 right).

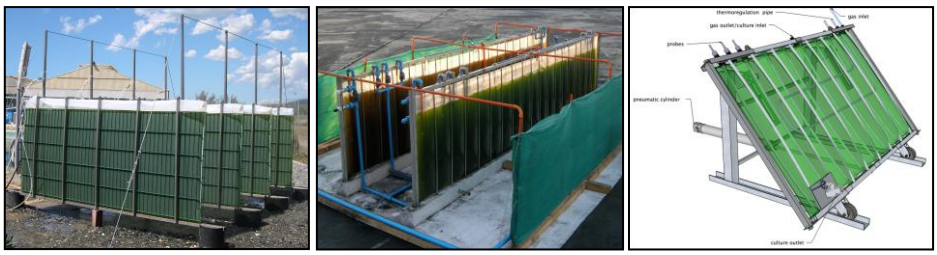


Figure 3 Green Wall Panel (GWP®) photobioreactors: GWP®-I (left), GWP®-II (center), GWP®-III (right)

Source: Website Fotosintetica & Microbiologica S.r.l., 2016

1.2 Microalgae as new foods and nutraceuticals

1.2.1 Definition of "food" and "nutraceutical"

FAO defines "food" any substance consumed to provide nutritional support for the body (http://www.fao.org/home/en/).

The term "nutraceutical" is derived from "nutrition" and "pharmaceutical". Therefore, nutraceuticals may be defined as "natural bioactive, chemical compounds that have health promoting, disease preventing or medicinal properties" (Sawalha, 2014). Nutraceuticals products may include isolated nutrients, dietary supplements, nutritious biomass, herbal products, genetically modified food, fortified foods with essential vitamins, minerals, amino acids, antioxidants, essential fatty acids and high value-added bioactive molecules. A nutraceutical product is expected to possess specific health benefits, due to the presence of either antioxidant compounds, signalling-pathway modulators or other bioactive molecules (Saha et al., 2015).

The term "dietary supplements" are defined as any product that can be taken by mouth that contains an ingredient intended to supplement a diet. Dietary ingredients in these products may include vitamins, minerals, herbs, amino acids, and substances such as enzymes, organ tissues, and metabolites (Website Frost and Sullivan, 2011). "Functional foods and beverages" are similar in appearance to, or may be a conventional food and beverages that are consumed as part of the usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions. These products help to prevent a disease or compromised health condition and/or to improve physical or mental performance (Website Frost and Sullivan, 2011). Moreover, terms such as "dietary supplements", "food supplements", "foods for special dietary use", "foods for specific health use" and "health foods" differentially classify nutraceuticals in the United States, the European Union, India, Japan and China, respectively (Table 1) (Website Frost & Sullivan, 2011).

Table 1 Varying definitions and regulations for nutraceuticals around the world

Country	Given label	Definition	Regulating body
United States of America	Dietary supplements	Any of the following which have a beneficial nutritional effects - Vitamins - Botanicals and their extracts - Aminoacids - Concentrates and extracts	Food and Drug Authority (Website FDA,
European Union	Food supplements	- Concentrated sources of nutrients - Other substances with a beneficial nutritional effect	Europe and Food Standard Safety Authority (Website EFSA, 2016)
India	Food for special dietary use	- Plants/botanicals: whole or extracts - Minerals, vitamins, aminoacids, enzymes - Substances of animal origin	Food Safety and Standards Act (Website FSSAI, 2015)
Japan	Foods for specific health use	Any food that can provide and/or supplement any of the following - Nutrition - Sensory satisfaction - Beneficial physiological effect	Japan Health and Nutrition Food Association (Website MHLWJ, 2016)
China	Health foods	Any food that: - Should be suitable for specific group(s) of people - Should also be able to adjust body functions and - Should not aim at curing diseases	State Food and Drug Administration (Website CFDA, 2016)

Adapted from Website Frost & Sullivan, 2011

1.2.2 Microalgae as a source of food

Microalgae are an enormous biological resource in terms of biodiversity, representing one of the most promising microbial groups for new products and applications (Pulz and Gross, 2004). They can be used to enhance the nutritional value of food and animal feed, due to their well-balanced chemical composition. Moreover, they are cultivated as a source of highly valuable molecules such as polyunsaturated fatty acids, pigments, antioxidants, pharmaceuticals and other

biologically active compounds. The application of microalgal biomass and/or metabolites is an interesting and innovative approach for the development of healthier food products (Gouveia et al., 2008b).

The use of micro- and macroalgae as food is known since centuries (Powell et al., 1962; Mabeau and Fleurence, 1993) and even today they are used in a variety of commercial products with many more in development (Griffiths et al., 2016).

Macroalgae, including *Porphyra*, *Enteromorpha intestinalis*, *Laminaria japonica*, *Undaria pinnatifida* and *Monostroma nitidum*, are common components in the diets of many people, especially from the Atlantic Europe and Asia (Becker, 2004). In Scandinavia and Scotland, *Rhodymenia palmata* is frequently collected and used for food purposes, while in India is prepared a soup made by *Gracilaria* (Krauss, 1962).

Various indigenous people use cyanobacteria such as *Arthrospira* and *Nostoc* grown in nature, to integrate poor diets poor in proteins, vitamins and minerals. For example the Kanembou in Chad collect spirulina in natural lakes, where special conditions of pH and salinity create a monoalgal bloom (Abdulqader et al., 2000). In Myanmar, the natural blooms of *Arthrospira* occur in small volcanic basins in some periods of the year, are collected, subjected to simple processing and consumed as food (Potts, 2000). The Klamath Lake in Oregon (USA), is known for the exploitation of seasonal blooms of *Aphanizomenon flos-aquae*. From this cyanobacterium is obtained a dietary supplement consumed in the United States and since 15 years it is also known in Europe (Hu, 2004). In Asia *Nostoc* (a filamentous cyanobacterium) is collected from arid environments to be used as a ritual food (Potts, 2000).

The microalgae cultivation at large-scale started in around 1940 thanks to new knowledge about biotechnology and the demand for microalgae from aquaculture world (Vonshak, 1996). Starting from 1950, microalgae have been investigated for the production of protein-rich biomass for supplying the overpopulated areas of the planet (Spoehr, 1951). Only with the first experiments aimed to evaluate the acceptability and the digestibility of the algae there were the first concerns about their use as the unique source of food. Powell et al. (1962) at the conclusion of their study carried out on men that had been fed with large amounts of *Chlorella* and *Scenedesmus*, verified that high quantities of microalgae in diets (> 100 g day^{-1}) can give digestive and nutrient absorption problems. Therefore, microalgae for human consumption should be considered more as a food supplement and not as a food to be consumed as the

only portion of a diet. Gross et al. (1978) noted a considerable increase in weight in infants fed with small doses of *Scenedesmus* sp. (10 g day^{-1}), integrated to the normal food ration, attributing this increase in weight to the higher protein intake than children fed without microalgae.
Numerous combinations of microalgae or mixtures with other health foods can be found in the market in the form of tablets, powders, capsules and pastilles as nutritional supplements (Gouveia et al., 2008b) (Fig. 4).

Figure 4 Tablets, powders, capsules, and pastilles from microalgae
Adapted from Webiste e-sportshop.cz; Website Ankit Pulps & Boards Pvt. Ltd.; Website Spirulina Powder; Website GKwell

They can also be incorporated into food products (e.g. pastas, biscuits, bread, snack foods, candies, yoghurts, soft drinks), providing health-promoting effects

that are associated with microalgal biomass, probably related to a general immune-modulating effect (Gouveia et al., 2008b) (Fig. 5).

Figure 5 Products with different microalgal biomasses: biscuits (a), pasta (b), bread (c)
Adapted from Gouveia et al., 2008b; Fradique et al., 2010; Webiste Culinary Pen; Website AlgiTect; Webiste Gourmetmom

1.2.3 Microalgae as a source of nutraceuticals

The potential of microalgae to offer a competitive commercial alternative source of nutraceuticals is a reality (Table 2).

Table 2 Nutraceutical products, divided into 11 categories with typical commercial products outlined

Nutraceutical category	Example
Amino acid and (poly)-peptide	Arginine, bioactive peptides, digestive enzymes, glutamate, immunoglobulins, isoleucine, lactoferrin, leucine, lysine, valine
Fibres	β-glucan, digestion resistant starch and maltodextrin, gum, insoluble fibre, inulin, pectin, polydextrose
Mineral	Calcium, iron, magnesium, potassium, selenium, sodium, zinc
Phytochemical	Isoflavone, isothiocyanate, lignan, phytosterol, polyphenol, tocotrienol
Pigment and carotenoid	Astaxanthin, β-carotene, lutein, lycopene, zeaxanthin
Polyol	Isomalt, lactitol, maltitol, xylitol
Polyunsaturated fatty acid	Arachidonic acid, conjugated linoleic acid, docosahexaenoic acid, eicosapentaenoic acid, γ-linoleic acid
Prebiotic	Fructooligosaccharides, glucooligosaccharides, inulin, polydextrose, soya bean oligosaccharides, xylooligosaccharides
Probiotic	Living organisms and/or bacterial cultures
Vitamin	A, B1, B2, B6, B12, Biotin, C, D, E, Folic acid, K, niacin, pantothenic acid
No set category	Chondroitin, coenzyme Q10, glucosamin, inositol, lipoic acid

Source: Saha et al., 2015

Microalgae are used for different purposes in the field of human nutrition, the largest of which, in quantitative terms is probably the use as protein supplements (from *Arthrospira*, *Aphanizomenon*, *Chlorella*) and carotenoids (β-carotene from *Dunaliella* and astaxanthin from *Haematococcus*) (Becker, 2004; Koller et al., 2014).

Cyanobacteria contain β-carotene (80% of total carotenoids) as the major carotenoid, followed by zeaxanthin, while the richest commercial source of β-carotene is the green microalga *Dunaliella salina* (Sajilata et al., 2008; Gateau

et al., 2016). *Haematococcus pluvialis*, a green microalga, is the richest known natural source of carotenoid astaxanthin, which has antioxidant activities much higher than β-carotene and vitamin E (Kang et al., 2007). From a commercial point of view, microalgal carotenoids have long been used as natural colouring materials. Aquatic feed containing β-carotene is known to enhance the flesh colour of salmon. Fowl feed enriched in β-carotene is also used to enhance the colour and appeal of egg yolks and, overall, enhance the nutritional and health benefit of the egg. β-carotene incorporated into feed has also been reported to contribute to the improved health and fertility of cattle (Thajuddin & Subramanian, 2005). Carotenoids have also been reported to be physiologically important to a variety of metabolic functions in humans. One molecule of β-carotene can be enzymatically converted into two molecules of vitamin A within the body. This leads to all the health benefits of vitamin A being acquired, such as improved eyesight, enhanced immune response, and the protection against certain cancers through the vitamin's ability to scavenge damaging free radicals (Paiva & Russell, 1999; Bowen et al., 2015).

The lipid content of microalgae is variable among the species and in the same species according to the method of cultivation, and in special cases may also reach 60% of the dry weight of the cell (Rodolfi et al., 2009). The microalgal fatty acids are typically composed of carbon chains from C12 to C20 (saturated or with varying degrees of unsaturation) (Becker, 2004). The fatty acids that have more influence on the quality of microalgal biomass are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). It is recommended a daily assumption of EPA and DHA of approximately 100-150 mg day^{-1} for children of 2-4 years and 200-250 mg day^{-1} for children of 6-10 years. For pregnant and lactating women the daily assumption should be up to 0.3 g day^{-1} (FAO, 2010). Studies have determined that DHA is one of the major fatty acids of the brain and retina. DHA has been referred to as an IQ-associated fatty acid, due to its involvement with brain development and functioning throughout life, especially in relation to visual and cognitive development (Auestad et al., 2003). Studies have also shown that EPA and DHA assist in the prevention of coronary heart disease, stroke, hypertension, dementia, Alzheimer's and depression (Kris-Etherton et al., 2002; Ruxton et al., 2005; Swanson et al., 2012; Das, 2008; Ryckebosh et al., 2014). Recognized microalgal sources of essential fatty acids are: the cyanobacterium *Arthrospira platensis* for γ-linolenic acid (GLA); the red microalga *Porphyridium* sp. for arachidonic acid (ARA); the dinoflagellate microalga *Crypthecodinium cohnii* and the heterotrophic microalga

Schizochytrium sp. (Spolaore et al., 2006), and the microalga *Isochrysis galbana* for DHA (Alonso et al., 1992; Molino Grima et al., 1992); the green microalga *Nannochloropsis* spp. and the diatoms *Phaeodactylum tricornutum* and *Nitzschia* sp. for EPA (Spolaore et al., 2006). GLA is one of the essential ω-6 fatty acids that help to lower low-density lipoprotein in hypocholesteromic patients. It relieves from symptoms of pre-menstrual syndrome and helps in treating atopic eczema. It has pro-inflammatory and anti-inflammatory properties (Gang-Guk et al., 2008). ARA is a biogenetic precursor of prostaglandin and leucotrienes, which play major roles in circulatory and central nervous systems. It is important for visual sharpness and valuable ingredient in baby food formulations (Pratoomyot et al., 2005).

The high protein content (even >70%) of many microalgal biomasses is one of the main features that leads to consider these microorganisms as a promising unconventional source of proteins (Lupatini et al., 2016). Many algal biomasses have a high protein quality, especially in relation to the composition and digestibility of amino acids (Becker, 2004). Some microalgal species have a higher content of sulfur amino acids (methionine and cysteine), essential amino acids that must be integrated with the diet, compared to conventional vegetable protein sources (Giordano and Prioretti, 2016). Over five decades ago, *Chlorella* and *Arthrospria* were recommended for human and animal dietary consumption (Saha et al., 2015). Microalgal proteins favourably compare to conventional vegetable proteins in terms of their nutritional attributes and digestibility. In 1973, the WHO/FAO recommended microalgae consumption based on the balance of their essential amino acids (Becker, 2007). Ten amino acids: including arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine are considered as essential for human and animal nutrition (Saha et al., 2015). Interestingly, all these ten essential amino acids can be sourced from microalgae protein derived from *Chlorella vulgaris*, *Dunaliella bardawil*, *Scenedesmus obliquus*, *Arthrospira maxima* and *Arthrospira platensis*. Of commercial interest is the fact that the market for algal protein has started to develop over the last five years, as scientific studies reporting on the nutritional, functional and nutraceutical properties of algal proteins have increased (Saha et al., 2015).

Microalgae contain an array of vitamins including A, B1, B2, B3 (Niacin), B6, B12, C, E, folic acid and pantothenic acid, which have been reported to be present at high levels compared with most routine foods (Becker, 2004). The water soluble B-complex vitamins have wide-ranging functions that ultimately

facilitate the processes involved in yielding energy from carbohydrates. B-complex vitamins are essential for healthy skin, hair, eyes, and the proper functioning of the liver and nervous systems. *Arthrospira platensis, Chlorella pyrenoidoisa* and *Scenedesmus quadricauda* are among the richest sources of A, E, B1, Vit B2, B3, B6, folic acid, B12, C and pantothenic acid vitamins (Becker, 2004). A diet or supplement containing biomass from a combination of carefully selected microalgal species could provide vitamins of high bio-availability (Saha et al., 2015).

Microalgae compounds are also recognised as hypotensive, immunomodulatory and antiproliferative (Saha et al., 2015). Hypertension is a controllable risk factor associated with cardiovascular disease, and the renin-angiotensin-aldosterone system is a target for blood pressure control (FitzGerald et al., 2004). Cleavage of angiotensinogen by renin produces angiotensin I, which is subsequently hydrolyzed by angiotensin-I-converting enzyme (ACE) to angiotensin II (a potent vasoconstrictor). Research has demonstrated that the ACE enzyme is subject to inhibition by peptides possessing the appropriate amino acid sequence. Several peptides from *Chlorella vulgaris* were reported to possess angiotensin I-converting enzyme (ACE) inhibitory activities (Suetsuna and Chen, 2001). Also, peptides from *Arthrospira platensis* were reported to show ACE-I inhibitory activities (Suetsuna and Chen, 2001). In some instances, ACE-I inhibitory compounds may also act as immunomodulators, as the inhibition of ACE results in the formation of bradykinin, which mediates the acute inflammatory process (FitzGerald et al., 2004). Chicken and mice fed *Arthrospira* biomass demonstrated increased phagocytic activity and increased natural killer cell-mediated antitumor activities (Singh et al., 2011). *In vitro* studies with human blood cells incubated with *Arthrospira* extracts showed elevated interferon (13.6-fold) and interleukin (IL)-1β and IL-4 (three-fold) levels (Singh et al., 2011). Other studies with cyanobacterial extracts containing microcystin demonstrated enhanced murine immunosuppressive activities and cytokine modulation *in vivo* (Singh et al., 2011). A murine *in vivo* study demonstrated that extracellular sulphated polysaccharides from *Porphyridium cruentum* showed both immune enhancing activity and the inhibition of S180 tumour cell proliferation (Sun et al., 2012). The peptide VECYGPNRPQF, derived from protein hydrolysates of *Chlorella vulgaris*, was also found to possess anti-proliferative activities (Sheih et al., 2010). A recent review (Wu et al., 2016) investigated immunomodulatory, and anti-inflammatory activities of *Arthrospira* in both animals and humans, along with the underlying

mechanisms. *Arthrospira* activates cellular antioxidant enzymes, inhibits lipid peroxidation and DNA damage, scavenges free radicals, and increases the activity of superoxide dismutase and catalase. Clinical trials show that *Arthrospira* prevents skeletal muscle damage under conditions of exercise-induced oxidative stress and can stimulate the production of antibodies and up- or downregulate the expression of cytokine-encoding genes to induce immunomodulatory and anti-inflammatory responses. The molecular mechanism(s) by which *Arthrospira* induces these activities is unclear, but phycocyanin and β-carotene are important molecules. Moreover, Spirulina effectively regulates the ERK1/2, JNK, p38, and IκB pathways (Wu et al., 2016).

Plant-based functional foods, containing sterols and fibres, are widely recommended as an alternative non-pharmacological approach to lower plasma cholesterol levels. A study with hypercholesterolemic patients showed a reduction in cholesterol and improved lipid profiles following a *Arthrospira* feeding trial (Ramamoorthy and Premakumari, 1996). A cholesterol-lowering activity was also reported in hamsters following a feeding trial with *Chlorella pyrenoidosa* biomass powder (Cherng and Shih, 2005). It was proposed that water-soluble fibres, vegetable protein, phospholipids, Vitamin C, Vitamin E and β-carotene were the possible active ingredients involved. Of note is that the ratio of arginine to lysine for *Chlorella* protein is deemed better than proteins from soya bean. Plant proteins with low lysine contents have been reported as advantageous towards lowering serum cholesterol levels (Cherng and Shih, 2005). A hypothesis was also proposed from a separate study that the Advanced Glycation End product-inhibitory property of *Chlorella* prevents atherosclerosis *in vivo* (Yamagishi et al., 2005).

The extraction of multiple high value-added biomolecules from microalgal biomass may promote a more sustainable, cost-effective process, leading to the better utilization of microalgae (Draaisma et al., 2013; Website Frost and Sullivan, 2011).

1.2.4 Microalgae authorised as food and producing companies

Arthrospira platensis, *Chlorella luteoviridis*, *Chlorella pyrenoidosa*, *Chlorella vulgaris* and *Aphanizomenon flos-aquae* from Klamath Lake (Oregon, USA) were used prior to May 1997 in Europe and thus are authorized as food in Europe (Website European Union, Novel Food catalogue). *Odontella aurita* was authorized successively (European Union, 2005) and, in 2014, also the

chlorophyta *Tetraselmis chuii* was approved as food, following the application by the Spanish company Fitoplancton Marino S.L. (AESAN, 2013; AECOSAN, 2014). Astaxanthin from *Haematococcus pluvialis* was recently authorized (EFSA 2014). To be used as new food, with the exception of the microalgae already cited, microalgae and cyanobacteria have to follow the novel food regulation (European Union, 2015). In the United States, *C. protothecoides*, *A. platensis*, *Dunaliella bardawil*, and astaxanthin from *Haematococcus* are included in the GRAS list (FDA, GRAS Notices). In Australia, all *Chlorella* (including *C. sorokiniana*) and *Arthrospira* species and derivatives for which a novel food application was submitted have been so far considered as traditional food, whereas *A. flos-aquae* was considered as novel food and safety assessment was required due to the potential presence of cyanobacterial toxins such as microcystins and nodularin (FSANZ, 2016; Niccolai et al., 2016).
In the field of human nutrition microalgae are marketed in different forms, principally tablets, capsules and liquids (Griffiths et al., 2016). They can also be incorporated into pastas, beverages, snacks, and candies (Gouveia et al., 2008a). Thanks to their different chemical properties, they can act as a nutritional supplement or represent a source of natural food colorants (de Jesus Raposo et al., 2013). The microalgae commercial production is dominated by four strains: *Arthrospira*, *Chlorella*, *Dunaliella salina* and *Aphanizomenon flos-aquae* (Table 3).

Table 3 Present state of microalgae production

Alga	Annual production (t/year)	Producer country	Applications and products
Arthrospira (spirulina)	5,000	China, India, USA, Myanmar, Japan	Human and animal nutrition , cosmetics (pycobiliproteins, powders, extracts, tablets, beverages, chips, pasta, liquid extracts)
Chlorella sp.	2,000	Taiwan, Germany, Japan	Human nutrition, aquaculture, cosmetics (tablets, powders, nectar, noodles)
Dunaliella salina	1,200	Australia, Israel, USA, China	Human nutrition, cosmetics (β-carotene, powders)
Aphanizomenon flos-aquae	500	USA	Human nutritions (capsules, crystals, powders)
Haematococcus pluvialis	300	USA, India, Israel	Aquaculture, astaxanthin
Crypthecodinium cohnii	240	USA	DHA oil
Shyzochytrium sp.	10	USA	DHA oil

Adapted from Spolaore et al., 2006; Gouveia et al., 2008b; and Norsker et al., 2011

A significant amount of *Arthrospira* is produced in China and India. The world's largest producer of algal powder (200 t/year) is Hainan Simai Enterprising Ltd. located in the Hainan province of China (Priyadarshani and Rath, 2012). The largest plant in the world is owned by Earthrise Farms (located at Calipatria, CA, USA) and covers an area of 440,000 m^2 (Sastre, 2013). Earthrise Farms produces *Arthrospira*-based tablets and powder that are distributed in more than 20 countries around the world (Earthrise LLC, http://www.earthrise.com/). Moreover, other smaller companies sell a large variety of nutraceuticals products made from *Arthrospira*. The Myanmar Spirulina Factory (Yangon, Myanmar) sells tablets, chips, pasta and liquid extract, and Cyanotech Corp. (a plant in Kona, Hawaii, USA) produces products ranging from pure powder to packaged bottles under the name Spirulina pacifica (Cyanotech Corp., http://www.cyanotech.com/). In Brazil, the Olson Microalgas Macronutrição company (Camaquã, Rio Grande do Sul) produces Spirulina sp. LEB 18 capsules for sale as a dietary supplement (Olson Ltda, http://www.olson.com.br/). In 2005, the production value (in terms of annual turnover) of *Arthrospira* was equal to 40 million US $ (Table 4). *Chlorella* is produced by more than 70 companies. Taiwan Chlorella Manufacturing and Co. (Taipei, Taiwan) is the largest producer of dried biomass (400 t) (Taiwan Chlorella Manufacturing & Co., LTD, http://www.taiwanchlorella.com/). A significant production is also achieved by Algomed in Klötze, Germany (130 – 150 t dry biomass per year) with tubular photobioreactors (Roquette Klötze GmbH & Co. KG, http://www.algomed.de/). In 2006, the production value (in terms of annual turnover) of *Chlorella* was equal to 38 million US $ (Table 4). *D. salina* is exploited for its β-carotene content that can reach 12% of dry weight (Del Campo et al., 2007). For human consumption, Cognis Nutrition and Health (Cognis Nutrition and Health Ltd, http://www.cognis.com/), the world's largest producer of this strain, offers *Dunaliella* powder as ingredient in dietary supplements and functional foods. In 2013, the market value of β-carotene from *D. salina* was 270 million US $ (Rodríguez, 2014). In 1998, *A. flos-aquae* from Klamath Lake, mainly commercialized in forms of capsules, crystals or powders for human consumption was estimated to have a market value equal to 100 million US $ (Charmicael et al., 2000).

In 2004, the production value (in term of annual turnover) of ω-3 fatty acids was estimated to be 300 million US $, followed by astaxanthin, mainly extracted from *H. pluvialis* (10 million US $ estimated in 2004). Alternative sources of β-carotene and phycobiliprotein (including phycocyanin) assumed

another importance value in microalgae-based products market (285 US $ estimated in 2012 and 50 million US $ estimated in 2004, respectively) (Vigani et al., 2015, Table 4).

Table 4 Market figures of microalgae-based products.

Microalgae-based product	Number of producers	Production value (annual turnover, mio US $)	Production value of alternative sources (annual turnover, mio US $)
Whole dryed microalgae:			
Arthrospira	15	40 (2005)	no alternative
Chlorella	70	38 (2006)	no alternative
High-value molecules:			
Astaxanthin	8	10 (2004)	200 (2004)
Phycobiliprotein (incl. phycocyanin)	2	na	50 (2004)
ω-3 fatty acids	4	300 (2004)	14390 (2009)
β-carotene	10	na	285 (2012)

na: not available, mio: millions
Note: In brackets the year for which the estimation was done
Source: Enzing et al., 2014

1.3 Overview of international food regulatory procedures

To provide a general overview of major international approaches to the regulation of novel foods/ingredients and nutritive substances, the regulatory frameworks in the European Union (EU), the United States of America (USA), Canada, Australia and New Zealand are described.

1.3.1 European Union

The legislation for the authorisation and use of novel foods and novel ingredients in the EU consists of two regulations:

- Regulation (EC) No 258/97 of the European Parliament and of the Council concerning novel foods and novel food ingredients (European Union, 1997);
- Commission Regulation (EC) No 1852/2001 laying down detailed rules for making certain information available to the public and for the

protection of information submitted pursuant to European Parliament and Council Regulation (EC) No 258/97 (European Union, 2001).

The Regulation (EC) No 258/97 was adopted in 1997 and applies to the placing on the market within the Community of foods and/or food ingredients which have not yet been used for human consumption to a significant degree within the Community. This Regulation includes foods and food ingredients:

- with a new or intentionally modified primary molecular structure;
- that consist of or are isolated from microorganisms, such as fungi or algae;
- that consist of or are isolated from plants and food ingredients isolated from animals;
- whose nutritional value, metabolism or level of undesirable substances has been significantly changed by the production process.

The Regulation was not applicable to food additives, flavourings or extraction solvents. Food additives are substances added intentionally to foodstuffs to perform certain technological functions, for example to colour, to sweeten or to help preserve foods. In the European Union all food additives are identified by an E number. The most common additives to appear on food labels are antioxidants (to prevent deterioration caused by oxidation), colours, emulsifiers, stabilisers, gelling agents and thickeners, preservatives and sweeteners (Website EFSA, Food Additives).

The general criteria applied to novel foods and ingredients are that they must not:

- mislead the consumer;
- present a danger for the consumer;
- differ from foods or food ingredients which they are intended to replace to such an extent that their normal consumption would be nutritionally disadvantageous for the consumer.

In Fig. 6 the premarket authorisation process applied to novel foods and novel food ingredients is illustrated.

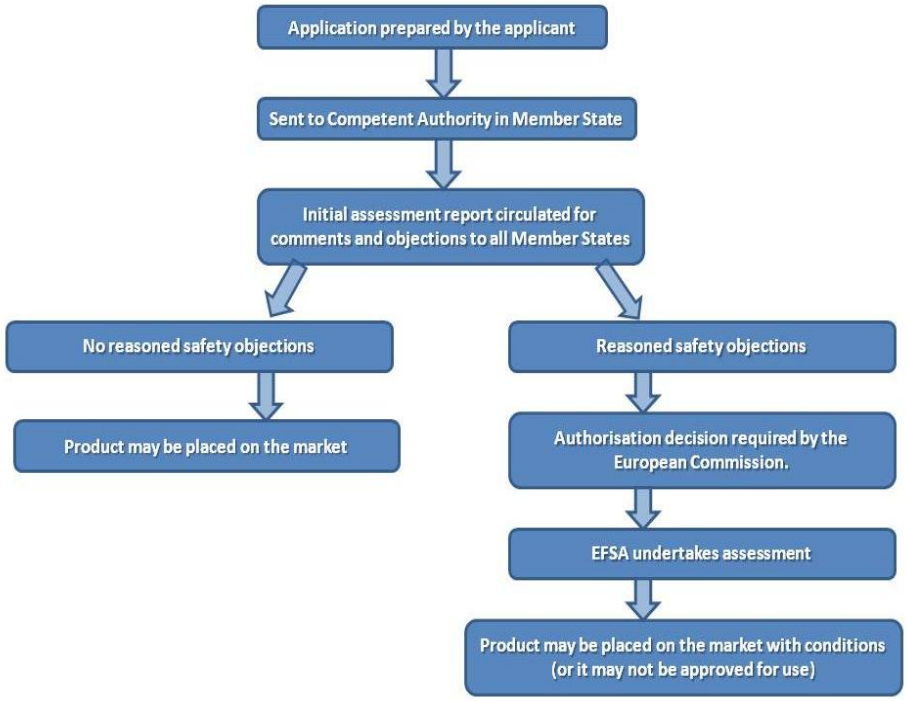


Figure 6 Premarket authorisation process applied to novel foods and novel food ingredients in the EU

The approval to market of the novel food or novel food ingredient is granted to the applicant (i.e. individual authorisation). However, another applicant may notify the European Commission the placing on the market of a food that is substantially equivalent to the authorised food; this notification must be substantiated by scientific evidence.

In December 2013, the European Commission adopted a proposal for a Regulation of the European Parliament and of the Council on novel foods (Website European Union, Novel Foods). In November 2015, the Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amended Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealed Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001 (OJ L 327, 11.12.2015, pp. 1-22). The Regulation aims to ensure food safety and protection of public health, and to secure the functioning of the internal market for food, while supporting innovation for the food sector (European Union, 2015).

The same general criteria currently applied to novel foods and ingredients are applicable to the replacement regulation: such foods and ingredients should be safe; their use should not mislead the consumer; and where a novel food is intended to replace another food, it should not differ from that food in a way that would be nutritionally less advantageous for the consumer.

The Regulation provides a simplified procedure for the pre-market approval of novel foods and novel food ingredients. Identified key changes are:

- centralised EU-level procedure that separates risk assessment and risk management; all applications are submitted to the European Commission directly, rather than through individual EU member states as per the current arrangements;
- the definitions (e.g. of novel food) are clarified and updated, although the 1997 cut-off date will remain;
- individual authorisations are replaced with generic authorisations, removing the current procedure based on substantial equivalence;
- the applicant can be a Consortium;
- a simplified process for 'traditional foods from third countries' that is more proportionate to risk. In this case, if a history of safe food use in a third country for at least 25 years can be demonstrated by an applicant, and if member states or EFSA do not present reasoned safety objections, the food may be added to the EU list of novel foods (and can be sold on the EU market). If reasoned safety objections are presented, EFSA will conduct an assessment and the standard novel food authorisation procedure will be followed (with shorter deadlines).

A cut-off date is an objective parameter that can provide a clear beginning point in determining whether a food or substance is subject to particular legislative requirements. However, not all foods are likely to require regulatory pre-market assessment and a cut-off date requires supporting criteria to determine which new foods should be subject to additional regulation. The EU definition of novel food does provide some additional clarification. The Regulation of novel foods in the EU introduces a simplified process for "traditional foods from third countries", whereby establishing a history of safe use of a food may be sufficient to establish the safety of consumption by the food in the general population (FSANZ, Supporting document 4).

1.3.2 United States of America

In the USA, the term "food additive means any substance the intended use of which results…, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of… food" (Federal Food, Drug, And Cosmetic Act, Section 201). Food additives (that do not include substances generally recognized as safe (GRAS), substances used prior to January 1958, and those included in the exemptions of the Federal Food, Drug, and Cosmetic Act), require pre-market approval aimed at demonstrating safety under the intended conditions of use. The Food and Drug Administration (FDA) issues regulations, based on data submitted through petitions or obtained by its own initiative, specifying the conditions under which an additive has been demonstrated to be safe and, therefore, may be lawfully used (FDA 2014) (Niccolai et al., 2016).

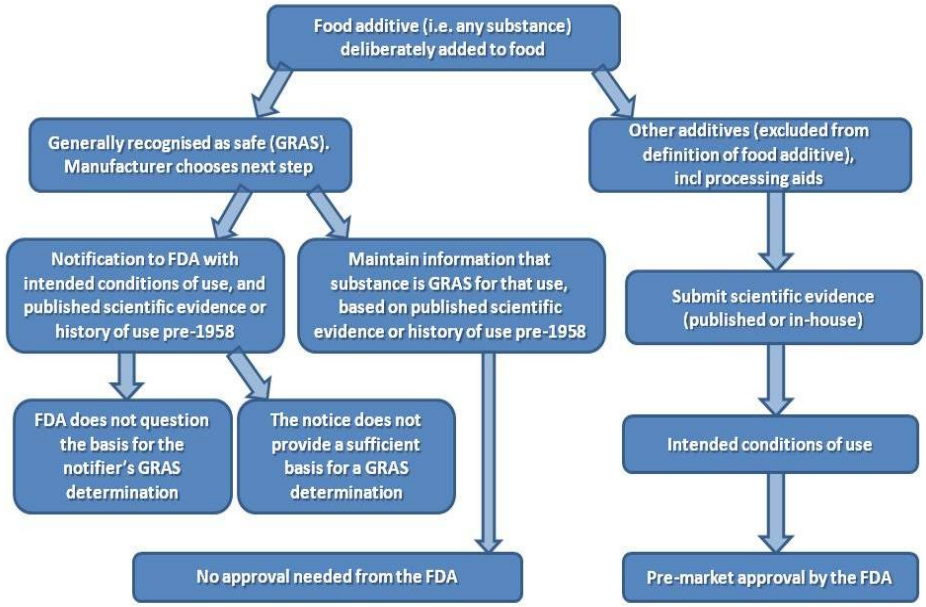


Figure 7 Premarket approval process applied to food additive in the USA

Food businesses can commission a panel of experts to assess the GRAS status of a substance. A food business is not obliged to notify the FDA of its intention to market a GRAS substance; however they can do so if they wish. Upon receipt of a GRAS notification, the USFDA will provide an indication of whether the basis of the GRAS determination appears reasonable or not. While not a

decision, or full assessment, by the USFDA, this response provides an indication of the quality of the GRAS assessment undertaken by the food business. These USFDA responses are publicly accessible and a list of GRAS notifications is maintained on the USFDA website.

Some published reviews of the GRAS system, noting that industry does not have to notify the USFDA of a GRAS determination, have expressed concern about the lack of transparency associated with these assessments of safety that result in an industry GRAS determination without the oversight of the USFDA.

Although a food additive may have GRAS status, the USFDA may at any time prohibit its use or conduct further studies to determine its safety if new evidence suggests that a product already in use may be unsafe or if consumption levels have changed (FSANZ, Supporting document 4).

1.3.3 Canada

In Canada, any foods sold must meet the requirements of the Food and Drugs Act (Website Canada's Food and Drug Act) and the Food and Drug Regulations (Website Canada's Food and Drug Regulations).

Part B, Division 28 of the Food and Drug Regulations regulates novel foods. The regulations define "novel food" as:

- a substance, including a microorganism, that does not have a history of safe use as a food;
- a food that has been manufactured, prepared, preserved or packaged by a process that has not been previously applied to that food and causes the food to undergo a major change;
- a food that is derived from a plant, animal or microorganism that has been genetically modified such that: the plant, animal or microorganism exhibits characteristics that were not previously observed in that plant, animal or microorganism; the plant, animal or microorganism no longer exhibits characteristics that were previously observed in that plant, animal or microorganism; one or more characteristics of the plant, animal or microorganism no longer fall within the anticipated range for that plant, animal or microorganism.

Therefore, the Canadian "novel food" requirements apply to both novel and nutritive substances as regulated in the Food Standards Code. The approval process applied to novel food in the Canada is illustrated in Fig. 8.

Health Canada assesses the safety of all genetically-modified and other novel foods proposed for sale in Canada. Companies are required to submit detailed

scientific data for review and approval by Health Canada, before such foods can be sold. The process (B28.002) requires the manufacturer to notify the Director in writing of their intention to sell or advertise for sale the novel food; and receive a written notice from the Director.

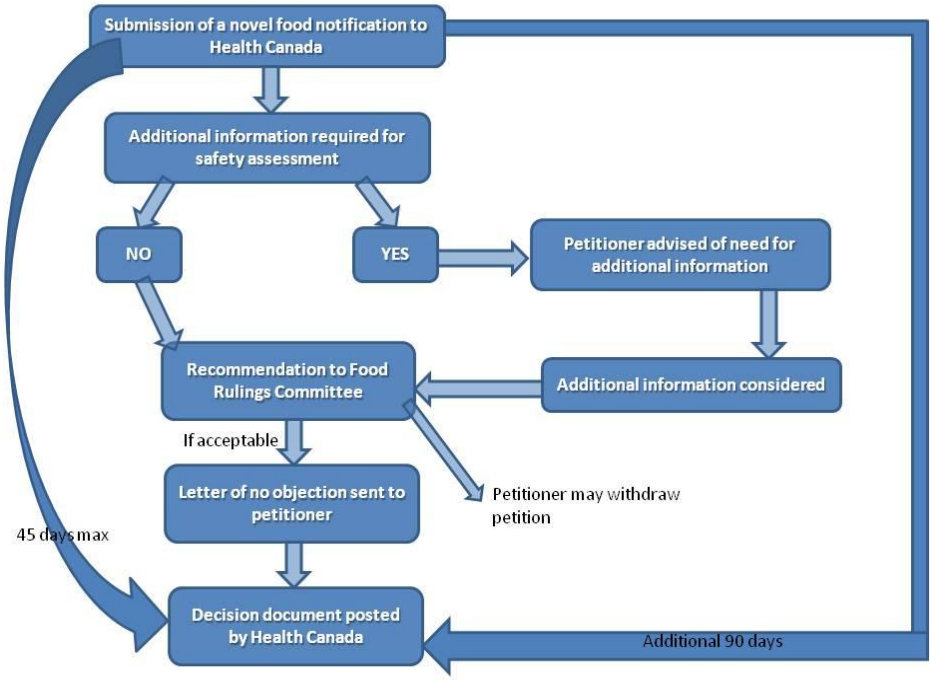


Figure 8 Premarket approval process applied to novel food in Canada

The Canadian novel food regulation shares some commonalities with the Code's novel food standard (novel foods are prohibited and defined) and with the US GRAS notification system (notification to Health Canada and letter of no objection to petitioner (if acceptable).

Health Canada has developed "Guidelines for the Safety Assessment of Novel Foods" (Website Safety Assessment of Novel Foods) which includes consideration of a history of safe use of a food in another country as part of the evidence to support the safety of a novel food. The guidelines set out the type of information that would be needed to support a claim that a product has a history of safe use. The Health Canada guidelines have been noted as an example of the type of information that industry would need to hold to demonstrate a history of safe use (FSANZ, Supporting document 4).

1.3.4 Australia and New Zealand

The Food Standards Australia New Zealand (FSANZ) considers as novel food all non-traditional food or its derivatives and requires safety assessment (Commonwealth of Australia Gazette, 2007). The regulatory reforms present in Australia and New Zealand introduced to ensure the safety of novel foods have lead to the development and implementation of three standards addressing each of the innovations summarised in Table 5.

Table 5 Standars for novel foods

	Novel foods/food ingredients*
Purpose	Ensure that novel foods undergo a risk based safety assessment prior to introduction to the food supply
Foods captured	Non-traditional foods which there is insufficient knowledge in the broad community to enable safe use
Key requirements	Sale prohibited unless explicitly permitted Subject to pre-market and approval process Special conditions may apply (e.g. labelling, restrictions on sale)

* Standard 1.5.1 novel foods, Australia New Zealand Food Standards Code. Source: adapted from Healy et al., 2003

The authorisation process applied to novel foods in Australia New Zealand is illustrated in Fig. 9. A key requirement of the authorisation process has been a pre-market safety assessment and approval process to assess the potential health impacts of the novel foods and food produced using novel technologies in both the short and long term. It is also recognised that the general community may wish to exercise a choice as to whether or not to accept the use of novel technologies and the food produced using them.

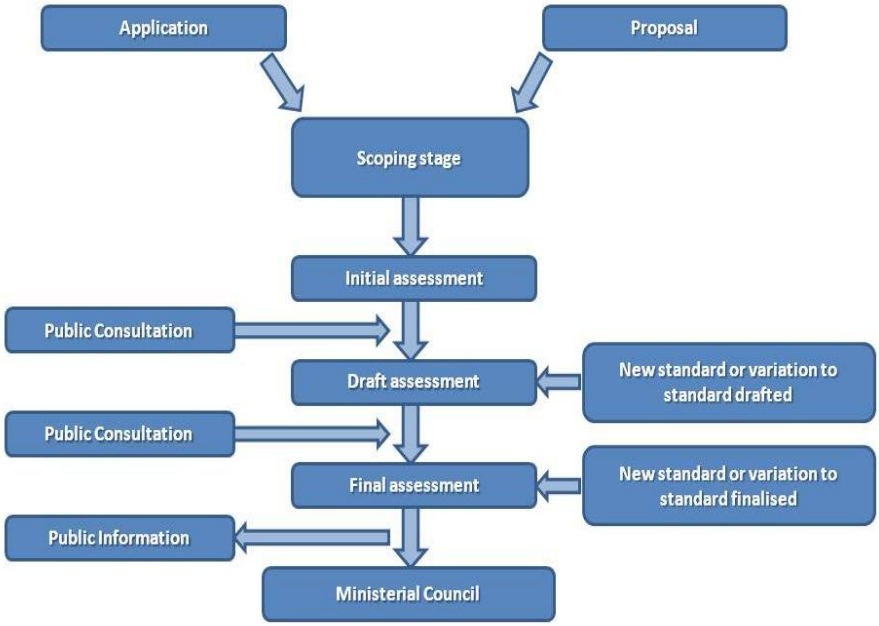


Figure 9 Authorisation process applied to novel foods in Australia and New Zealand

Currently, FSANZ, with the Proposal P1024 (Website FSANZ, Proposal P1024), is reviewing the requirements for novel food applications (Website Standards 1.5.1). The Proposal seeks to improve the regulation of nutritive substances and novel foods to ensure appropriate pre-market safety assessment of these foods before they are sold in Australia and New Zealand.

In conclusion, despite microalgae and cyanobacteria are considered a potential source of functional foods and nutraceuticals thanks to their valuable and balanced biochemical composition, the use of these microorganisms as food ingredient is still poorly developed in Europe due to high cost, low demand and strict Novel Foods legislation.
The enormous biodiversity of microalgae in nature and in culture collections still needs accurate evaluation in terms of quality and safety before exploitation. An accurate selection of the best microalgae and cyanobacteria is the starting point for the future development of microalgae-based products.

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Chapter 2

Aim of the thesis and structure of the work

2

2. Aim of the thesis and structure of the work

2.1 Aim of the thesis

The general aim of the thesis project was to evaluate the suitability of some microalgal and cyanobacterial biomasses for the production of innovative foods and nutraceuticals. To evaluate their potential, selected microalgae and cyanobacteria were analysed in terms of biochemical composition, *in vitro* toxicity and *in vitro* digestibility. To enhance the *in vitro* digestibility, a sonication pre-treatment was also investigated. In order to evaluate the *in vivo* safety and the potential health effects of two promising strains (*Arthrospira platensis* F&M-C256 and *Tisochrysis lutea* F&M-M36), a sub-chronic *in vivo* study with rats was carried out. The two organisms were chosen for their balanced biochemical composition and content of particular pigments (phycocyanin and fucoxanthin), essential aminoacids, vitamins, and minerals.
To explore the potential application of microalgae and cyanobacteria in baked foods, *A. platensis* F&M-C256, *Chlorella vulgaris* Allma, *Tetraselmis suecica* F&M-M33 and *Phaeodactylum tricornutum* F&M-M40-based biscuits were prepared and analysed in terms of sensory and physical properties, bioactivity and *in vitro* digestibility.
In addition, to improve the acceptability of microalgae by the consumer in terms of organoleptic quality and digestibility, *A. platensis* F&M-C256 and *Tetraselmis suecica* F&M-M33 were fermented. The study focused on these two microalgae because *A. platensis* and *Tetraselmis chuii* are already approved by EU as food, and, therefore, the results obtained would possibly have an immediate applicability.

2.2 Structure of the work

Chapter 1, the introductive chapter, describes microalgae photosynthesis and cultivation, main microalgal taxa, and the possible applications of microalgae, especially in the food and nutraceutical fields. The food regulatory procedures in the European Union, United States of America, Canada, Australia and New Zealand are also reported. **Chapter 2** reports the aim of the thesis and the structure of the work.
To use microalgal biomass for food purposes, there is a need to determine certain characteristics. Of major importance: the presence of toxic substances and digestibility. **Chapter 3** reports the results of the *in vitro* toxicity of

microalgal and cyanobacterial strains selected as a possible new food source. In addition, when the potential application of new matrices for the production of functional foods is being investigated, the study of the nutritive function and the evaluation of digestibility are fundamental. To this purpose, in **Chapter 4** the biochemical composition and the *in vitro* digestibility of some selected strains are evaluated.

Results obtained from *in vitro* models are preliminary and must be confirmed by *in vivo* studies on mammals with the use of the whole algal biomass. These pre-screenings (Chapter 3 and 4) allow reducing the number of candidate strains to be selected for further *in vivo* studies on mammals that are necessary, in most cases, to fulfill the mandatory requirement of sub-chronic toxicity data for novel food applications in the European Union. To this purpose, in **Chapters 5** and **6** the potential toxic and health effects of *Arthrospira platensis* F&M-C256 and *Tisochrysis lutea* F&M-M36 in rats is reported.

After the food safety assessment and the nutritional evaluations, in **Chapter 7** is explored the potential application of microalgae as functional ingredients within conventional foods, e.g. cookies. Microalgae-based cookies are analysed for their sensory and physical properties, bioactivity and *in vitro* digestibility.

In **Chapter 8** the use of *Lactobacillus plantarum* to ferment *Arthrospira platensis* F&M-C256 and *Tetraselmis suecica* F&M-M33 biomasses, is reported. The study aimed at improving the acceptability of microalgae-based products in terms of organoleptic quality and digestibility, and to evaluate the potential application for the production of functional foods.

In **Chapter 9** a general discussion is reported, which analyses the prospects of microalgae as a possible source of innovative foods and nutraceuticals in the light of the results of the research.

Chapter 3

In vitro toxicity of microalgal and cyanobacterial strains of interest as food source

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Abstract

The general objective of the present work was to evaluate the toxicity of 11 microalgal strains and one natural bloom, selected as potential food or food ingredients due to their nutritional quality, in two models, human dermal fibroblasts and *Artemia salina*. Methanolic and aqueous extracts of the biomasses were tested on *A. salina* for 24 and 48 hours at concentrations up to 12.5 g L^{-1} of extracted biomass. Only aqueous extracts were tested on fibroblasts for 24 hours.

Chlorella vulgaris Roquette, *C. vulgaris* Allma, *Tetraselmis suecica* F&M-M33 and *Porphyridium purpureum* F&M-M46 showed no toxicity towards *A. salina* and fibroblasts. Only Klamath powder was toxic to both models with all types of extracts. *Tisochrysis lutea* (T-ISO) F&M-M36, *C. sorokiniana* F&M-M49 grown in BG11 and *C. sorokinina* IAM C-212 showed toxicity, even if to different extents, to fibroblasts, and only with the methanolic extract to *A. salina*. The remaining strains showed no toxicity towards *A. salina*, but were toxic to fibroblasts: *A. platensis* M2 and *Nannochloropsis oceanica* F&M-M24 exhibited low toxicity, *Nostoc sphaeroides* F&M-C117 medium toxicity and *Phaeodactylum tricornutum* F&M-M40 high toxicity. Although in some cases the two models provided contrasting results, this work confirms their validity for preliminary screening of toxicity. The models are able to indicate organisms and substrates of potential toxicity and may well serve as guidelines for *in vivo* tests on mammals, which are necessary to apply for novel food in the EU.

Keywords: Brine shrimp, bioassay, Fibroblasts, Toxicity, Microalgae, Food

1. Introduction

Microalgae (including cyanobacteria) are oxygenic photosynthetic microorganisms that have long been recognized as a potential source of food. The high potential of microalgae as food is due to their balanced biochemical composition and high nutritional value (Tredici et al. 2009). They can have a high protein content with a balanced amino acid composition (Becker 2007), a good content of vitamins, minerals, short polyunsaturated fatty acids, including linoleic and linolenic acids and long-chain polyunsaturated fatty acids such as docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids (Bishop and Zubeck 2012; Batista et al. 2013). Some strains are also good sources of carotenoids

like lutein, astaxanthin and β-carotene (Del Campo et al. 2007). *Chlorella*, *Arthrospira*, *Dunaliella*, and *Haematococcus* are microalgae largely employed for human consumption (Tredici et al. 2009; Ibañez and Cifuentes 2013).

In the EU, novel foods and food ingredients are defined as those that "have not been used to a significant degree for human consumption within the Union before 15th May 1997" (European Union 1997). With the Regulation (EC) No 258/97 the European Union, through the European Food Safety Authority (EFSA), guarantees that novel foods and food ingredients are subject to a single safety assessment through a unified procedure in order to protect public health. Foods and food ingredients to be authorized must not present a danger for the consumer, mislead the consumer and differ from foods that they are intended to replace to such an extent that their normal consumption would be nutritionally disadvantageous for the consumer (European Union 1997). In the US, if a substance intended for food use is not generally recognized as safe (GRAS) and is not included in the exemptions, it is considered a food additive, i.e. a substance "becoming a component of food or otherwise affecting the characteristics of food" (US Congress 2016). Food additives require premarket approval aimed at demonstrating safety under the intended conditions of use. The Food and Drug Administration (FDA) issues regulations, based on data submitted through petitions or obtained by its own initiative, specifying the conditions under which an additive has been demonstrated to be safe and, therefore, may be lawfully used (FDA 2014). The GRAS standard requires the industry to provide scientific evidence that the substance is not harmful under its intended conditions of use and once certified exempts the substance from pre-market approval (FDA 2014). The Food Standards Australia New Zealand (FSANZ) considers as novel food all non-traditional food or its derivatives and requires safety assessment (Commonwealth of Australia Gazette, 2007). Currently, FSANZ is reviewing the requirements for novel food applications (Standards 1.5.1). Algae not listed in the Standards need approval as novel food and thus safety assessment (FSANZ, Proposal P1024).

The microalgae used prior to May 1997 in Europe and thus authorized as food in the EU are: *Aphanizomenon flos-aquae* from Klamath Lake, *Arthrospira platensis*, *Chlorella luteoviridis*, *Chlorella pyrenoidosa* and *Chlorella vulgaris* (European Union, Novel Food catalogue). The diatom *Odontella aurita* was authorized successively (European Union 2005) and, in 2014, also *Tetraselmis chuii* was approved as food, following the application by the Spanish company Fitoplancton Marino S.L. (AESAN 2013; AECOSAN 2014). Astaxanthin from

Haematococcus pluvialis was also recently authorized (EFSA 2014). To be used as food, with the exception of the species mentioned above, microalgae have to follow the novel food regulation (European Union 2015). In the US, *C. protothecoides*, *A. platensis*, *D. bardawil*, and astaxanthin from *Haematococcus* are included in the GRAS list (FDA, GRAS Notices). In Australia, all *Chlorella* (including *C. sorokiniana*) and *Arthrospira* species and derivatives for which a novel food application was submitted have been so far considered as traditional food, whereas *A. flos-aquae* was considered as novel food and safety assessment was required due to the potential presence of cyanobacterial toxins such as microcystins and nodularin (FSANZ 2016). These approved microalgae (and microalgal products) represent a very small number of species compared to the high number present in nature, from 30,000 to 1 million according to different sources (Guiry 2012).

In this work, we tested three strains/products belonging to species already approved as food in the EU (*C. vulgaris*, *A. flos-aquae* from Klamath Lake blooms, *A. platensis*), and two species not approved at present (*C. sorokiniana* and *T. suecica*), but belonging to genera of approved species. Five other microalgae were also tested: *Nostoc sphaeroides*, an edible cyanobacterium consumed in China and other Asian countries (Han et al. 2013), *Tisochrysis lutea*, *Nannochloropsis oceanica* and *Phaeodactylum tricornutum*, widely used (as well as *Tetraselmis* spp.) in aquaculture (Tredici et al. 2009; Muller-Feuga 2013) and *Porphyridium purpureum*, rich in EPA, but to date used only in cosmetics (Arad and Levy-Ontman 2010; Rajasulochana and Preethy 2015). These strains as well as the natural bloom were chosen for their high potential as food sources thanks to a high protein content (*A. platensis* M2, Klamath powder, *N. sphaeroides* F&M-C117, *T. suecica* F&M-M33, *C. sorokiniana* F&M-M49 and IAM C-212, *C. vulgaris* Allma and Roquette) or for their ability to produce high amounts of polyunsaturated fatty acids (*N. oceanica* F&M-M24, *P. tricornutum* F&M-M40, *P. purpureum* F&M-M46, *T. lutea* F&M-M36), besides having interesting amounts of protein.

The first concern when proposing microalgae as food or food ingredients is to establish their lack of toxicity towards the consumer. In this regard, several species of different algal groups have been reported to produce neurotoxins, hepatotoxins, diarrheic toxins, dermotoxins, etc., which are usually active at very low doses (Landsberg 2002). Besides biotoxins, microalgae and cyanobacteria are able to produce other secondary metabolites (cytotoxins) that may have noxious effects on human health (Carmichael 1992; Tredici et al.

2009). Finally, it is to consider that also molecules that do not enter in the category of *toxins* (Carmichael 1992), can have toxic effects if provided at excessively high dosages. This concept provides the rational behind legislation, which requires that the toxicity data provided be related to the dose of the biomass/extract/component that is intended for use.

Toxicity tests for novel food applications require time-consuming and expensive procedures. Moreover, the EU strongly encourages the "3Rs" principle (replacement, reduction and refinement) for sparing animals used for scientific purposes (European Union 2010). *In vitro* tests and bioassays on invertebrates have the advantage of being less expensive and shorter in duration, offering a simplified model for a preliminary screening to be used as a guideline for further *in vivo* studies. To determine microalgal toxicity (intended in all the meanings reported above), model organisms such as crustaceans (*Artemia* or *Daphnia*) are often used (Vezie et al. 1996; Guilhermino et al. 2000; Biondi et al. 2004). In this study, we adopted the brine shrimp (*Artemia salina*) assay, an easy and low-cost toxicity test (Solis et al. 1993; Piccardi et al. 2000). It is to note that, despite it targets a whole organism, the brine shrimp assay is considered by several authors as an *in vitro* test (Sleet and Brendel 1985; Lagarto Parra et al. 2001; Carballo et al. 2002). Besides invertebrates, toxicity is often evaluated on mammalian cells, mainly cancer cell lines (Jaki et al. 1999; Carballo et al. 2002; Hisem et al. 2011). Some authors report a good correlation between the toxic activity measured with brine shrimps and that observed with tumor cell lines (Anderson et al. 1991). Here, we report the toxicity of 13 microalgal extracts on *A. salina* and on human fibroblasts in order to evaluate the potential health risks of these microbial strains if used as food or food ingredients.

2. Materials and methods

2.1 Experimental plan

Microalgal biomasses were extracted in methanol and/or water and tested against normal human dermal fibroblasts (NHDF) and the nauplii of the crustacean *Artemia salina* to evaluate their potential toxicity. The experimental design is reported in Table 1.

Table 1. Plan of the experiments

	Extracts		Effect of salt
	MeOH	**H_2O**	
Fibroblasts (NHDF)		✓	✓
Artemia	✓	✓	

2.2 Microalgal strains and biomass production

The algae tested are listed in Table 2. A positive reference extract was prepared from *Nostoc* sp. M1, known from previous experiments carried out in our laboratory to be strongly cytotoxic. A negative reference extract was also prepared using organic lettuce (*Lactuca sativa* var. *capitata*).

Table 2. Algae and algal products selected for toxicity screening

Strain	Type of culture medium	Biomass obtention
CYANOBACTERIA		
Arthrospira platensis M2	alkaline	in-house cultivation
Klamath powder	fresh	commercial product (Erbologica SAS)
Nostoc sphaeroides F&M-C117	fresh	in-house cultivation
Nostoc sp. M1	fresh	in-house cultivation
CHLOROPHYTES		
Chlorella sorokiniana F&M-M49	fresh/marine	in-house cultivation
Chlorella sorokiniana IAM C-212	fresh	in-house cultivation
Chlorella vulgaris Roquette	fresh	commercial product (Roquette Frerès)
Chlorella vulgaris Allma	fresh	commercial product (Allma Microalgae)
Tetraselmis suecica F&M-M33	marine	in-house cultivation
RHODOPHYTES		
Porphyridium purpureum F&M-M46	marine	in-house cultivation
DIATOMS		
Phaeodactylum tricornutum F&M-M40	marine	in-house cultivation
HAPTOPHYTES		
Tisochrysis lutea (T-ISO) F&M-M36	marine	in-house cultivation

EUSTIGMATOPHYTES		
Nannochloropsis oceanica F&M-M24	marine	in-house cultivation

Most of the tested microalgae were produced at the facilities of Fotosintetica & Microbiologica S.r.l. or of the Institute of Ecosystem Study of the CNR, both in Sesto Fiorentino, Florence. The algae were produced in GWP®-II photobioreactors (Tredici et al. 2011; Zittelli et al. 2013) in semi-batch mode and the biomasses were harvested by centrifugation, frozen, lyophilized and powdered. The powdered biomasses were stored at -20°C until use. Only *A. plantesis* M2 biomass was washed with physiological saline solution to remove extracellular sodium bicarbonate and carbonate before freezing. All the freshwater strains were cultivated in BG11 (Rippka et al 1979) and all the marine strains in F (Guillard and Ryther 1962) medium, while *A. platensis* was cultivated in Zarrouk medium (Zarrouk 1966). *C. sorokiniana* F&M-M49 was cultivated in both BG11 and F medium (Guccione et al. 2014). The F medium-grown biomass (unwashed) was used to test the effect on fibroblasts of sea salt residues (see below). *T. suecica* F&M-M33 was grown in the standard F medium as well as in the same medium deprived of nitrogen to induce carbohydrate accumulation. *C. vulgaris* Allma (Allma Microalgae, Lisbon, Portugal), *C. vulgaris* Roquette (Roquette Frères, Lestrem, France) and Klamath powder (Erbologica SAS, Serina, Bergamo, Italy) are commercial products. Klamath powder is obtained from a natural bloom mainly composed of *A. flos-aquae*, harvested from Upper Klamath Lake (Oregon, USA).

2.3 In vitro *cytotoxicity on human fibroblasts*

2.3.1 Preparation of microalgal extracts

Lyophilized microalgae were suspended (25 g L^{-1}) in Dulbecco's Modified Eagle Medium (DMEM) without phenol red and sonicated for two cycles of 3 minutes in ice, followed by a 3 minutes resting interval by using an ultrasonic homogeniser (Sonopuls HD 2070, Bandelin Electronic, Berlin, Germany), set at 100% power. Microalgal suspensions were then centrifuged for 15 minutes at 15,000 x g at 4 °C, filtered through a 0.2 µm filter and stored at -20 °C.

2.3.2 Cell cultures

Human dermal fibroblasts (N°CC-2509), kindly provided by Prof. Lisa Giovannelli (NEUROFARBA, University of Florence), were maintained in DMEM without phenol red, supplemented with 10% fetal bovine serum (FBS), glutamine (2 mM), penicillin (100 IU mL^{-1}) and streptomycin (100 µg mL^{-1}) in a humidified incubator at 37 °C under a 5% CO_2 atmosphere.

2.3.3 Determination of cytotoxic activity of microalgal aqueous extracts on human fibroblasts

NHDF cells of passages 12-14 were seeded in 96-well plates (5×10^3 per well), kept for 24 hours and then treated with microalgal extracts for 24 hours. The concentrations tested were 12.5, 5.0, 2.5 and 0.5 g of extracted biomass per liter for 24 hours. Viability analysis was performed using Cell Titer 96™ Aqueous One solution cell proliferation assay kit (Promega, Madison, WI, USA). At the end of the incubation period, the treated cells were exposed to Aqueous One solution (Giovannelli et al. 2014) and incubated for 2 hours at 37 °C. The product of the reaction was measured at 490 nm using a spectrophotometer (Multilabel Counter 1240 Victor 3, Perkin Elmer, Waltham, MA, USA).

2.4 Effect of salt interference

To evaluate the interference of marine salts on fibroblast viability, artificial seawater (Tropic Marine, Adriatic Sea Equipment & Co, Rimini, Italy) at a salinity of 60 g L^{-1} was used to prepare culture media for NHDF at different salinities, from 30 to 0.6 g L^{-1}. The experiment was set up and performed as reported above for the algal extracts. To further verify the effect of the salts that may remain in the algal biomass after centrifugation, *C. sorokiniana* F&M-M49 grown in freshwater and in seawater was extracted and tested as previously described.
To quantify the salt content of the biomasses, ashes were determined by incineration in a muffle furnace at 450 °C.

2.5 Determination of cytotoxic activity of microalgal extracts on A. salina

2.5.1 Extraction of biomasses

Biomass extraction for *A. salina* tests was performed following two different protocols. One set of experiments was performed on methanolic extracts.

For each algal strain, as well as for the negative reference lettuce, an aliquot of 0.5 g of dry biomass was extracted in 60 mL of methanol overnight at room temperature. The solvent was then separated from biomass by filtration on paper. The biomass residue was extracted again with 30 mL of methanol at 37 °C for 4 hours, then the exhausted biomass was removed by filtration on paper. The two extracts (60+30 mL) were mixed and then evaporated under vacuum. The dry residues were suspended in 2.5 mL of methanol to obtain a final concentration of the extracts of 200 g L^{-1} of extracted biomass. Glassware and plastic-ware were kept in 0.27 M NaOH in 9:1 ethanol: water for 24 hours before usage, to prevent cross-contamination (Golakoti et al. 1995), and then washed with deionized water.
Another set of experiments on *A. salina* was performed on aqueous extracts of microalgae and lettuce. Aliquots of the dry biomasses (0.2 g) were dispersed in 1.5 mL of sterile water and sonicated for 3 minutes, and then the probe was washed with 0.3 mL of sterile water. The sonicated material was frozen at -20°C. As soon as the material was taken out of the freezer, 0.2 mL of an antibiotic solution containing 3,200 µg mL^{-1} of streptomycin sulphate (chemist's preparation) and 6,000 µg mL^{-1} of ampicillin (Amplital, Pfizer) were added to each sonicated biomass to prevent bacterial growth. After thawing, each sonicated biomass was homogenized and an aliquot was transferred into a sterile 1.5 mL test tube and centrifuged for 15 min at 18,000 x g at 4 °C. The supernatant was finally transferred to a new sterile test tube. The final extract concentration was of 100 g L^{-1} of extracted biomass. For this type of extraction only disposable material was used.

2.5.2 Lethality tests on A. salina

Cysts of *A. salina* (Premium, SHG, Ovada, Alessandria, Italy) were transferred for hatching in a glass bottle containing sterile artificial seawater (Tropic Marine) at 30 g L^{-1} salinity. The inoculated seawater was bubbled with air sterilized through 0.2 µm porosity filters. The incubation was carried out under constant illumination of 80 µmol photons m^{-2} s^{-1} at 25-27 °C. Once the cysts hatched, the nauplii were used to set up the experiments.
The experiments were performed in 96-well microtiter plates on a final test volume of 100 µL. For methanolic extracts, the concentrations tested varied from 12.5 to 0.1 g L^{-1} of extracted biomass. An aliquot (12.5 µL) of the extract was transferred into the first well and the solvent let to evaporate. After evaporation, 20 µL of pure DMSO:water 1:10 were added to prepare the final

extract, that was then diluted in the other wells 1:2 in 10 µL of DMSO:water 1:10 till the last concentration to be tested. Controls were prepared with 10 µL of DMSO:water 1:10. Each extract concentration and the control were tested in six replicates. Reference extracts were set-up with biomasses expected to produce a positive (*Nostoc* sp. M1) and a negative (lettuce) response in the test.

For aqueous extracts, the concentrations tested were from 12.5 to 0.1 g L^{-1} of extracted biomass. An aliquot (25 µL) of the extract was transferred into the first well of the microtiter plate and diluted 1:2 in the other wells in 50 µL of artificial seawater (30 g L^{-1} salinity) containing antibiotics. The final concentration of antibiotics in each well was 160 µg mL^{-1} of streptomycin and 300 µg mL^{-1} of ampicillin. Control was prepared with sterile artificial seawater (30 g L^{-1} salinity) containing antibiotics at the same concentrations as in the test wells. Each extract concentration and the control were tested in three replicates. Reference extracts were set-up with *Nostoc* sp. M1 and lettuce that were treated in the same way as the test extracts.

After the test and reference extract dilutions and the control were prepared in the microtiter plates, 90 µL of seawater containing 5 nauplii were added to each well to reach the final test volume of 100 µL. The plates were then incubated at room temperature and the number of dead nauplii was counted under a direct light microscope (Nikon Eclipse E200, Nikon, Tokyo, Japan) at 40 magnifications after 24 and 48 hours of incubation.

Nauplii were considered as dead when no movement was observed in the reasonable time of the observation (Carballo et al. 2002, Lopes et al. 2011). Mortality was calculated according to Abbott (Abbot 1987):

$$M\ (\%\ vs\ control)\ =\ \frac{(L_C - L_T)}{L_T} \times 100$$

where M is mortality; L_C, living nauplii in the control after 24 or 48 hours; L_T, living nauplii in the test or reference extract after 24 or 48 hours.

2.6 Calculation of IC_{50} values

For all microalgal extracts tested against NHDF and *A. salina* dose-response curves were obtained. The concentration (expressed as grams of extracted biomass per liter) which inhibits 50% of the population (IC_{50}) was calculated by a linear regression model using log transformed data when necessary. When the experimental data did not include the 50% inhibition, IC_{50} values were

estimated, still through linear regression. For some extracts (indicated as "not determinable") it was not possible to calculate an IC_{50} value because no toxicity was detected at the concentrations tested. IC_{50} values higher than 15 g L^{-1} were not indicated in extenso, as this amount was considered the threshold of non-toxicity.

The extracts were divided into four toxicity categories according to their IC_{50} value: high toxicity ($IC_{50} \leq 5$ g L^{-1}); medium toxicity ($5 < IC_{50} \leq 10$ g L^{-1}); low toxicity ($10 < IC_{50} \leq 15$ g L^{-1}) and no toxicity ($IC_{50} > 15$ g L^{-1}). The latter category included also those extracts for which an IC_{50} value was not available.

3. Results

Extracts from 13 test biomasses were evaluated as well as those from two reference materials (*Nostoc* M1 and lettuce). Among the test biomasses, only for *P. purpureum* F&M-M46 calculation of the IC_{50} was not possible due to lack of toxicity in the tested range (Table 3).

Table 3. IC_{50} values calculated or estimated for microalgal aqueous or methanolic extracts (expressed as g of extracted biomass per liter) on human fibroblasts (NHDF) and *A. salina* after 24 and 48 hours of exposure; data are reported as mean ± SD

Strain	IC_{50} value (g L^{-1})				
	Fibroblasts	*Artemia*			
	H_2O extract	H_2O extract		MeOH extract	
	24 h	24 h	48 h	24 h	48 h
Nostoc sp. M1 (positive reference)	9.3 ± 2.9	nc	7.2 ± 0.2	8.2 ± 2.3	0.4 ± 0.1
Lettuce (negative reference)	nc	>15*	>15*	nc	nc
A. platensis M2	11.5 ± 5.7	>15*	>15*	nc	nc
Klamath powder	8.8 ± 0.4	>15*	4.7 ± 1.9	1.7 ± 0.0	1.6 ± 0.1
N. sphaeroides F&M-C117	7.8 ± 1.3	>15*	>15*	nc	nc
C. sorokiniana F&M-M49 BG11	12.6 ± 4.0*	nc	>15*	13.6 ± 2.9*	13.4 ± 2.3*
C. sorokiniana F&M-M49 F	2.7 ± 1.0	-	-	-	-
C. sorokiniana IAM C-212	3.7 ± 0.09	nc	nc	6.0 ± 0.6	5.6 ± 0.6
C. vulgaris Roquette	>15*	nc	>15*	nc	nc
C. vulgaris Allma	nc	>15*	>15*	nc	nc
T. suecica F&M-M33	>15*	nc	>15*	>15*	>15*
P. purpureum F&M-M46	nc	nc	nc	nc	nc
P. tricornutum F&M-M40	2.3 ± 0.4	nc	>15*	>15*	>15*
T. lutea F&M-M36	6.0 ± 2.2	>15*	> 15*	6.0 ± 0.5	5.6 ± 0.9
N. oceanica F&M-M24	11.2 ± 2.1	nc	nc	nc	nc

* estimated value; nc, not calculable due to absence of toxicity in the tested range; -, not tested

For *C. vulgaris* Roquette, *C. vulgaris* Allma and *T. suecica*, as well as for the negative reference lettuce, it was possible, for at least one of the extracts, to estimate the IC_{50}, which value, however, was always higher than 15 g L^{-1} (Table 3). These three biomasses as well as that of *P. purpureum* were considered as non toxic (Fig. 1).

The remaining nine test biomasses and the positive reference *Nostoc* M1 showed IC_{50} below 15 g L^{-1} (hence toxicity) for at least one extract and one model (NHDF or *A. salina*). Only Klamath powder showed an IC_{50} below 15 g L^{-1} for all the extracts with both models. The IC_{50} value of the water extract of Klamath powder against NHDF was in the range that we classified as medium toxicity (Table 3, Fig. 1), whereas both water and methanolic extracts showed IC_{50} against *A. salina* in the range that we considered of high toxicity (Table 3, Fig. 1). It is to note that the toxicity of the Klamath powder aqueous extract against *A. salina* was much higher after 48 hours of exposure, while the methanolic extract acted almost at its full potency already after 24 hours. A similar behavior was observed for the positive reference *Nostoc* M1 (Table 3, Fig. 1). The main difference was that the methanolic extract of *Nostoc* M1 behaved like the aqueous one in terms of toxicity increase with exposure time. In fact, after 24 h the IC_{50} value of the methanolic extract was 8.2 g L^{-1} and decreased to 0.4 g L^{-1} after 48 h; the aqueous extract was not toxic after 24 hours and showed an IC_{50} of 7.2 g L-1 after 48 hours (Table 3).

For *C. sorokiniana* F&M-M49 grown in BG11, *C. sorokiniana* IAM C-212 and *T. lutea* F&M-M36 an IC_{50} below 15 g L^{-1} could be calculated for both models, although in the case of *A. salina* only for the methanolic extracts. IC_{50} values for *T. lutea* F&M-M36 extracts were in the range considered of medium toxicity (Table 3, Fig.1), as well as the methanolic extract from *C. sorokiniana* IAM-C212, which aqueous extract showed instead an IC_{50} in the range of high toxicity against NHDF (Table 3, Fig. 1). IC_{50} values were in the range of low toxicity for both extracts from *C. sorokiniana* F&M-M49 grown in BG11 (Table 3, Fig. 1).

The remaining five biomasses were active only against NHDF. *A. platensis* M2 and *N. oceanica* F&M-M24 extracts showed an IC_{50} higher than 11 g L^{-1} and laid in the low toxicity range; the IC_{50} of *N. sphaeroides* F&M-C117 was in the range of medium toxicity, whereas extracts from *C. sorokiniana* F&M-M49 grown in F medium and *P. tricornutum* F&M-M40 showed an IC_{50} lower than 3 g L^{-1} and were considered of high toxicity (Fig. 1, Table 3).

To assess the contribution of artificial seawater salt to the cytotoxic effects observed, also considering the results obtained with *C. sorokiniana* F&M-M49 grown in BG11 and F medium, we tested a range of salt concentrations (from 30 to 0.6 g L^{-1}) on NHDF. All the hypertonic concentrations (30, 15, 12 g L^{-1}) strongly reduced cell survival as expected, 6 g L^{-1} caused a modest reduction, whereas 3 and 0.6 g L^{-1} did not show any cytotoxicity (data not shown). An IC_{50}

of 11.2 ± 0.6 g L^{-1} was calculated. We can exclude toxicity of artificial seawater salt in our trials since the maximum salt concentration in microalgal extracts was estimated to be <3 g L^{-1}.

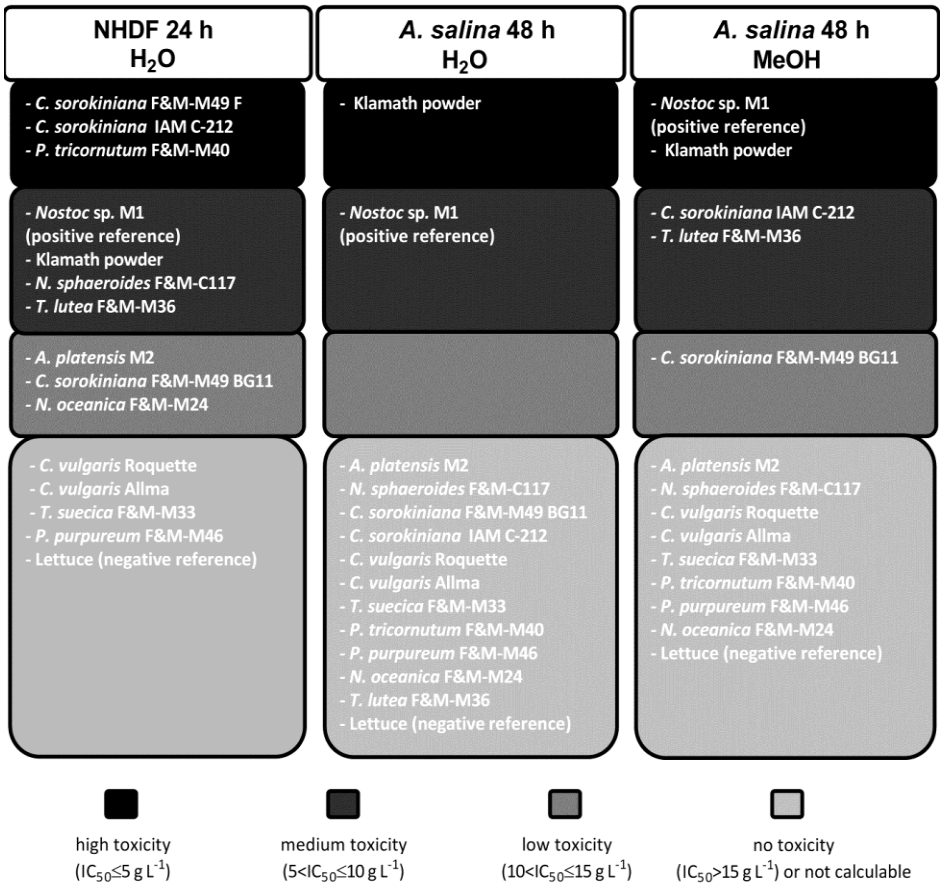

Fig. 1. Grouping of microalgal extracts according to their toxicity level, based on IC_{50} values. Aqueous extracts on NHDF after 24 hours, and aqueous and methanolic extracts on *A. salina* after 48 hours are reported. The toxicity levels are displayed in decreasing order (from darker to lighter color)

4. Discussion

Although the number of microalgal species worldwide is vast, only few have been isolated and studied with regard to biochemistry and physiology, and even less are exploited for commercial applications (Tredici et al. 2009). Less than a dozen microalgal species are presently accepted in the EU as food or food

ingredients (European Union 1997) and a similar situation is recorded also in the US and Australia. To increase this number it is necessary to go through the novel food approval regulations that require the assessment of the safety of the candidate food ingredient (European Union 2015; FDA 2014; FSANZ Proposal P1024). Starting from this perspective, this research aimed to evaluate, as preliminary step, the toxicity of microalgal biomasses of interest as food sources due to their nutritional qualities, using *A. salina* and human dermal fibroblasts as models.

As far as toxicity on cells is concerned, most of the literature deals with tests against cancer cell lines (Jaki et al. 1999; Carballo et al. 2002; Bechelli et al. 2011; Hisem et al. 2011) and only few studies, to our knowledge, focus on the cytotoxic effects of microalgae on non-cancerous cells (Prestegard et al. 2009; Lopes et al. 2011; Goh et al. 2014). *In vitro* cytotoxicity tests are performed to determine the intrinsic ability of a compound to cause cell death as a consequence of damage to the basic cellular functions. In this way, a screening for cytotoxicity using normal cells might be regarded as a general bioassay (Ekwall et al. 1990). Moreover, the use of a normal human cell lines, that present a non-aberrant phenotype, is an added value with respect to the use of cancerous cells that more typically show chromosome and phenotype aberrations (Jallepalli and Lengauer 2001; Fackental and Godley 2008). It is worth noting that there is little literature on toxicity of eukaryotic microalgae on cell lines and *Artemia*, while a wider number of papers deal with cyanobacteria.

Six of the tested algal biomasses (*T. lutea* F&M-M36, Klamath powder, *N. sphaeroides* F&M-C117, *C. sorokiniana* F&M-M49 grown in F medium, *C. sorokiniana* IAM C-212 and *P. tricornutum* F&M-M40) showed a similar or even higher cytotoxicity on fibroblasts compared to the positive reference *Nostoc* M1, while only Klamath powder showed a toxicity similar to *Nostoc* M1behavior on *A. salina*. The toxic effects on fibroblasts of *Nostoc* M1, a cyanobacterium containing cryptophycin, a powerful antimitotic agent (Panda et al. 1997), were lower than expected from our previous data on antifungal activity of this strain (unpublished) and on cytotoxicity of other cryptophycin-containing *Nostoc* strains (Piccardi et al. 2001). On *A. salina*, the toxic action of the aqueous extract was observed only after 48 hours of exposure, whereas with the methanolic extract the effect was more marked and present already after 24 hours. This behavior, in both models, can be explained by due to the low solubility of cryptophycin (a cyclic depsipeptide) in water, and to the low division rate of fibroblasts compared to tumor cell lines and of nauplii cells that

might slows the action of the antimitotic venom. The IC_{50} value shown by *Nostoc* M1 on human fibroblasts in this study (9.3 g L^{-1}) is much higher than that reported by Hrouzek et al. (2016) for the most cytotoxic *Nostoc* strain against murine fibroblasts (2.8 g L^{-1}).

Nostoc is one of the cyanobacterial genera with the highest frequency of cytotoxic metabolites. Among about 80 *Nostoc* strains tested, cytotoxicity frequency to different target cells was found to vary for the same extracts from 26 to 76% (Hrouzek et al. 2016). Biondi et al. (2008) found 5 out of 6 Antarctic *Nostoc* isolates to be cytotoxic to HeLa cells. Cytotoxicity against murine tumour cell lines was found in 33% (Hrouzek et al. 2011), 60% (Hrouzek et al. 2016) and 65% (Hisem et al. 2011) of the *Nostoc* strains tested. On *A. salina*, Piccardi et al. (2000) found toxicity in 24% of 50 *Nostoc* extracts, mainly aqueous. The same frequency of toxicity was found by Hisem at al. (2011) in 29 methanolic extracts. It is worth noting that the genus *Nostoc* includes, besides highly toxic strains, edible species (as *N. sphaeroides* and *N. flagelliforme*) and that, in this study, the edible *N. sphaeroides* F&M-C117 showed medium toxicity to fibroblasts and no toxicity to *A. salina*. A similar toxicity pattern was shown by the aqueous extract from *P. tricornutum* F&M-M40. Toxicity of aqueous extracts from this algal species is reported by Prestegard et al. (2009) on rat hepatocytes (>30% death at 4 g L^{-1} of extracted biomass after one hour of exposure) and IPC leukemia cells (>30% death at 1.5 g L^{-1} after 18 hours). No literature data are available on toxicity of this alga to *A. salina*.

Among the algae approved as food in the EU, the two commercial *C. vulgaris* strains were found not toxic (neither to fibroblasts nor to *A. salina*). *A. platensis* (the so-called spirulina), that on the basis of its longtime history (centuries) of human consumption is considered a safe food, in our work showed a low cytotoxic effect on fibroblasts and no toxicity to *A. salina*. Bechelli et al. (2011) found toxicity at 2 g L^{-1} of extracted biomass with ethanolic extracts of *A. platensis* on leukemia cells and no toxicity to normal bone marrow cells. One of the microalgae approved as food in the EU is *A. flos-aquae* from Klamath Lake (European Union, Novel Food catalogue). In our study medium toxicity to fibroblasts was observed with the aqueous extract from the biomass harvested from the natural bloom dominated by this alga in Klamath Lake, whereas high toxicity to *A. salina* was found in both aqueous and methanolic extracts. Our results confirm the findings from Bechelli et al. (2011) who observed a high cytotoxicity of the ethanolic extract of this bloom against both leukemia and normal bone marrow cells at concentrations lower than those found toxic in our

work. It is necessary to point out that, although some strains of *Aphanizomenon* are known to produce saxitoxin and neosaxitoxin (Ferreira et al. 2001; Ballot et al. 2010a, b), as well as mueggelone, responsible for neurotoxic and ichthyotoxic activity (Papendorf et al. 1997), so far *Aphanizomenon flos-aquae* from Klamath Lake has never been found to produce these toxins (Carmichael 1992; Heussner et al. 2012). Up to now this cyanobacterium from Upper Klamath Lake (Oregon, US) has not been stably isolated, thus the only way to test its biomass is to harvest blooms from this lake. The toxicity of the bloom observed in our work could be due to: i) compounds produced by *A. flos-aquae*; ii) compounds produced by other microalgae present in the population, as for example *Microcystis* spp., often occurring in these blooms (Gilroy et al. 2000), although the microcystins would have affected *A. salina* (Vezie et al. 1996) but not fibroblasts (Matsushima et al. 1990); iii) compounds from biogenic contaminants (derived from plants, animals, and other microorganisms) or non-biogenic sources (Chamorro-Cevallos et al. 2007).

Among the other algae tested, only in few cases it is possible to compare our data with literature. We found toxicity of *N. oceanica* F&M-M24 aqueous extracts to fibroblasts at the highest concentration (low toxicity). Goh et al. (2014) found no cytotoxicity of *N. oculata* extracts to mouse fibroblasts. In our study, *P. purpureum* F&M-M46 did not exert toxic effects at the concentrations tested. Gardeva et al. (2012) found that the polysaccharide from *P. purpureum* reduced the viability of MCF7, HeLa and Graffi tumour cells. The methanolic extract of *T. suecica* F&M-M33 showed no toxicity to *A. salina* (our work), while the methanolic extracts of *T. suecica* CCAP 66/22D caused a 100% mortality on *Artemia* nauplii (Lincoln et al. 1996).

The different toxicity patterns often observed in methanolic and aqueous extracts may derive from different molecules being extracted by the different solvents (Piccardi et al. 2000), or from the same component that is extracted by the solvents with a different efficiency (see the case of *Nostoc* sp. M1).

In general, our results show that the sensitivity of fibroblasts towards microalgal aqueous extracts is higher compared to that of *A. salina* and only the extract from Klamath powder is toxic in both models. With the aqueous extracts a higher toxicity on *A. salina* was observed after 48 hours of exposure, thus further confirming the lower sensitivity of this model compared to fibroblasts, this is not necessarily a drawback. In accordance with our study, Hisem et al. (2011) report a lower sensitivity of *A. salina* compared to the cell model. The results obtained in our work with aqueous extracts on fibroblasts are more

coherent with those obtained with methanolic extracts on *A. salina*, although the fibroblasts higher sensitivity allowed to detect a larger number of potentially toxic biomasses.

Tests on cell lines appear more adequate when the objective is to determine cytotoxic activity, as also stated by Hisem et al. (2011). These authors attribute the higher sensitivity of cells with respect to *A. salina* to the fact that cyanobacterial metabolites generally affect basal metabolic pathways present in the eukaryotic cell rather than systems in complex multi-cellular organisms. However, fibroblasts appear too sensitive for use in general toxicity screenings, while *A. salina* lethality test, in particular using a universal solvent (e.g. methanol) and an exposure time of at least 48 hours, appears far more reliable. Considering its easiness of realization and low cost, our work confirms the validity of this test in pre-screenings.

5. Conclusions

This work presents an *in vitro* analysis of the toxicity of 11 microalgal strains and one natural bloom of potential interest for food applications. Taken together, these data indicate that extracts from microalgae have a different pattern of inhibition (and thus toxicity) on *A. salina* and fibroblasts. Although these two models are useful and widely employed tools to screen the potential toxic effect, the attainment of results not fully in accordance, probably due to the different sensitivity and complexity of the models, should be taken into serious consideration. Clearly, results obtained from *in vitro* models or invertebrate bioassays are preliminary and must be confirmed by *in vivo* studies on mammals performed on the whole algal biomass and not only on extracts. These pre-screenings allow reducing the number of candidate strains to be selected for further *in vivo* studies on mammals that are necessary, in most cases, to fulfill the mandatory requirement of subchronic toxicity data for novel food applications, at least in the EU. In conclusion, from this study the two commercial *C. vulgaris* strains, *T. suecica* F&M-M33 and *P. purpureum* F&M-M46 emerged as non-toxic and other two strains, *A. platensis* M2 and *N. oceanica* F&M-M24, as substantially non-toxic. *T. lutea* F&M-M36, the two *C. sorokiniana* strains, *N. sphaeroides* F&M-C117 and *P. tricornutum* F&M-M40 showed some toxicity and need further investigation. Finally, Klamath powder was found to be the most toxic among the biomasses tested, inhibiting both models.

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Conflict of interest

The authors declare that they have no competing interests.

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Chapter 4

Biochemical composition and *in vitro* digestibility of microalgal and cyanobacterial strains of interest as food source

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A. Niccolai, G. Chini Zittelli, N. Biondi, L. Rodolfi, L., M. R. Tredici
Biochemical composition and *in vitro* digestibility of microalgal and cyanobacterial strains of interest as food source

The work reported in this chapter was carried out at CNR-ISE, Sesto Fiorentino under the guidance of Dr. Graziella Chini Zittelli and of my supervisors, Prof. Mario R. Tredici and Dr. Liliana Rodolfi, and in collaboration with Dr. Natascia Biondi

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Abstract

Microalgae are considered a fundamental source for the development of new food products and can be used to enhance the nutritional value of conventional foods, due to their valuable and balanced biochemical composition.

The aim of this study is to investigate the biochemical composition and the *in vitro* digestibility of several microalgal and cyanobacterial strains of interest as food source.

Arthrospira platensis F&M-C256, Klamath powder, and *Nostoc sphaeroides* F&M-C117 presented high protein (51-69%) and low lipid content (6-15%). Marine species contain high concentrations of PUFAs-ω3, mainly C20:5 ω3 (EPA) and C22:6 ω3 (DHA) along with substantial amounts of C16:1, C18:1 ω9 among MUFAs and C16:0 among SFA, whereas the freshwater algae contained high concentrations of the PUFA C18:3 ω3 (ALA) and an even higher amount of the SFA C16:0. The nutritional quality of lipid profile evaluated by different indexes indicated the high potential of these microalgae for human health.

Cyanobacteria, showed the highest digestibility in terms of dry matter (DMD), organic matter (OMD), carbohydrate (CD) and crude protein (CPD). While, the green algae *Chlorella sorokiniana* F&M-M49 and *Tetraselmis suecica* F&M-M33 and the marine *Phaeodactylum tricornutum* F&M-M40 and *Porphyridium purpureum* F&M-M46 showed lower digestibility values, mainly because of their robust cell wall or for the presence of exopolysaccharides that could have limited the action of digestive enzymes.

Fiber can also entrap proteins in the cellular matrix, rendering them less bioavailable to enzymatic hydrolysis. No significant direct correlation between CPD and dietary fiber was found, and microalgae rich in fiber always reported lower DMD, OMD, and CD values.

C. sorokiniana F&M-M49 and *C. vulgaris* Allma were pre-treated by sonication before pepsin incubation and the DMD obtained was compared to that of the untreated samples. The data obtained in this study confirm the positive effect of the sonication pretreatment and of the continuous stirring on cell dispersibility but without improving the digestibility of these *Chlorella* strains.

Keywords: Microalgae, Biochemical composition, Digestibility, Food

1. Introduction

Microalgae (including cyanobacteria) as food source and food supplements are known for centuries and they are produced in Asian countries, USA, and Australia from several decades (Lee, 1997; Singh and Gu, 2010). Microalgae are mainly applied as food supplements, in the cosmetics industry, or as animal feed (Dufossé et al., 2005; Huntley et al., 2015; Singh and Gu, 2010). In Western countries the use as food or food ingredients is still rather limited compared to Asian countries.

Arthrospira, *Chlorella*, *Dunaliella*, *Aphanizomenon flos-aquae* from Klamath Lake and *Haematococcus pluvialis* are microalgae largely employed for human consumption due to their balanced biochemical composition and high nutritional value (Tredici et al., 2009). Indeed, microalgae can contain high amount of proteins, vitamins, minerals (Becker, 2007; Liu et al., 2013) carotenoids (Del Campo et al., 2000; Wu et al., 2009), and long-chain polyunsaturated fatty acids (Becker, 2004).

The microalgae business sector is currently very dynamic with several companies starting every year. In 2015, more than 100 companies with different sizes mainly producing *Arthrospira* (the so called spirulina) have been spread through all Europe, mostly in France. The products from microalgae have currently only three possible forms: paste (for aquaculture), dried powder (for food and feed), and extracts (for nutraceuticals) (Verdelho Vieira, 2015).

In the EU, novel foods and food ingredients are defined as those that "have not been used to a significant degree for human consumption within the Union before 15th May 1997" (EU, 1997). With the Regulation (EC) No 258/97, the European Union, through the European Food Safety Authority (EFSA), guarantees that novel foods and food ingredients are subject to a single safety assessment through a unified procedure in order to protect public health (Niccolai et al., 2016). According to this Regulation, the European Union include microalgae in the novel food category. *A. flos-aquae* from Klamath Lake, *A. platensis*, *C. luteoviridis*, *C. pyrenoidosa* and *C. vulgaris* are microalgae already authorized as food in the EU because used prior to May 1997 (European Union, Novel Food catalogue). *Odontella aurita*, *Tetraselmis chuii* and *H. pluvialis* (for astaxanthin production) were also successively approved as food or food ingredient (EU, 2005; AECOSAN, 2014; AESAN, 2013; EFSA, 2014).

In this work, strains belonging to species already approved as food (*A. platensis*, *C. vulgaris* and *A. flos-aquae* from Klamath Lake) and species not approved but belonging to the same genus already authorized (*C. sorokiniana* and *T. suecica*), species used in aquaculture as *Isochrysis*, *Nannochloropsis* and *Phaeodactylum* (Atalah et al., 2007; Priyadarshani, 2012) or in cosmetic field as *Porphyridium* (Marcati et al., 2014) were tested.

Although the number of microalgal species worldwide is vast, only few have been isolated and studied with regard to biochemistry and physiology, and even less are exploited for commercial applications (Tredici et al., 2009). In this regard, knowledge of the chemical composition is mandatory as a first step in a screening methodology, since it will help to target valuable compounds (in particular antioxidants, pigments, and essential fatty acids), in the studied microalga (Batista et al., 2013). Environmental factors and growth conditions, such as temperature, illumination, CO_2 supply, salinity, pH-value, mineral content, population density, and physiological status, can modify chemical composition (Tredici et al., 2009). Therefore, the growing conditions could be optimized to maximize the production for the biomolecules of interest (Batista et al., 2013). In this regard, the selection of microalgae species with balanced nutritional profiles is fundamental for successful novel foods development. A detailed physicochemical characterization of the microalgae is an essential stage that will allow determining which algae are best suited for different applications and purposes (Batista et al., 2013).

When it wants to evaluate a possible application as food or food ingredients of new sources and predicting their nutritional quality, information on the digestibility of the various nutrients is of utmost important (Boisen and Eggum, 1991). *In vitro* digestion models are widely used to study the structural changes, digestibility and release of food components under simulated gastrointestinal conditions (Hur et al., 2011; Minekus at al., 2014). Mainly plants, meats, fish, dairy, and emulsion-based foods were studied for their digestibility and the most frequently biological molecules used in the digestion models were digestive enzymes (pancreatin, pepsin, trypsin, chymotrypsin, peptidase, α-amylase and lipase), especially from porcine, rabbit or human origin (Minekus et al., 2014), bile salts, and mucin (Hur et al., 2011). It is worth nothing that differences in pH, mineral type, ionic strength and digestion time, which alter enzyme activity, may also considerably alter the results obtained between different studies (Minekus et al., 2014). In seaweed especially, phenolic molecules and polysaccharides are compounds that can limit the digestibility (Fleurence,

1999). As stated by Fleurence (1999), the algal polysaccharides behave like soluble or insoluble fibers. Studies performed on brown algae show the strong inhibitory action of soluble fibers on *in vitro* pepsin activity and their negative effects on protein digestibility (Horie et al., 1995). According by Becker (2004), microalgae with higher fiber content are scarcely suitable for human nutrition.

It is necessary to point out what is intended to dietary fiber. Most of the authors agree with Trowell et al. (1976) that defined dietary fiber as "the sum of lignin and others polysaccharides that are not digested by endogenous secretions of the digestive tract of man". Dhingra et al. (2012) showed the classification of dietary fiber components based on water solubility/fermentability. Cellulose, hemicellulose and lignin are water insoluble and less fermented fibers, on the contrary pectin, gums and mucilages are water soluble and well fermented fibers (Dhingra et al., 2012).

In humans, a high content of fiber in cereals, fruits and vegetable have a positive effect on health, even if they could decrease the digestibility of the ingested food containing fibers. The recommended dietary fiber intakes for children and adults are 14 g/1000 kcal (Anderson et al., 2009). Principally, the positive effect of fiber consumption is related to: i) increase the volume of fecal bulk and decrease the time of intestinal transit, cholesterol and glycaemic levels; ii) trap substances that can be dangerous for the human organism (mutagenic and carcinogenic agents); iii) stimulate the proliferation of the intestinal flora (Beecher, 1999).

In our study, Boisen and & Fernández (1997) method was used to investigate the *in vitro* digestibility of microalgal and cyanobacterial strains. Recently, many authors used Boisen & Fernández method (1997) to study the *in vitro* digestibility of food, feed, or of organic matrices (Giuberti et al., 2013, Huang et al., 2014, Malumba et al., 2015; Serena and Bach Knudsen, 2007).

The main problem to deal with, when microalgae are being used as food, is the robust cell wall which restricts the access of the digestible enzymes to the cell components (Janczyk et al., 2005). With the exception of the cyanobacteria *Arthrospira* sp., *A. flos-aquae* and *Nostoc* sp., most of the other microalgae of commercial importance (mainly Chlorophyceae, especially *Chlorella* genus) have rigid indigestible cell walls, which, in many cases, makes necessary to break the cell wall (Doucha and Lívanský, 2008). Many authors described the sonication treatment as efficient method to destroy microalgal cells (McMillan et al., 2013; Safi et al., 2014; Wang et al., 2014). According to Janczyk et al. (2005) and in order to facilitate the enzymes access to the algal cell

components, a sonication processes on two *Chlorella* species was applied in this study.

The aim of this work was to evaluate the biochemical composition and the *in vitro* digestibility of 10 microalgal strains and one natural bloom. A possible increase *in vitro* digestibility using a sonication treatment was also investigated with two *Chlorella* strains.

2. Materials and methods

2.1 Microalgal strains and biomass production

The investigated algae are listed in Table 1.

Table 1. Algae tested for biochemical characterization and *in vitro* digestibility

Strain	Sample abbreviation	Type of culture medium	Biomass obtention
Arthrospira platensis F&M-C256	Ap	alkaline	in-house cultivation
Klamath powder	K	fresh	commercial product (Erbologica S.A.S.)
Nostoc sphaeroides F&M-C117	Ns	fresh	in-house cultivation
Chlorella sorokiniana F&M-M49	CsM49	fresh	in-house cultivation
Chlorella sorokiniana IAM C-212	CsIAM	fresh	in-house cultivation
Chlorella vulgaris Allma	CvA	fresh	commercial product (Allma Microalgae)
Tetraselmis suecica F&M-M33 (starved)	TsS	marine	in-house cultivation
Tetraselmis suecica F&M-M33 (nutrient replete medium)	TsNR	marine	in-house cultivation
Porphyridium purpureum F&M-M46	Pp	marine	in-house cultivation
Phaeodactylum tricornutum F&M-M40	Pt	marine	in-house cultivation
Tisochrysis lutea (T-ISO) F&M-M36	Tiso	marine	in-house cultivation
Nannochloropsis oceanica F&M-M24	No	marine	in-house cultivation

Most of the biomasses tested in this study were produced at the facilities of Fotosintetica & Microbiologica S.r.l. or of the Institute of Ecosystem Study of the CNR, both located in Sesto Fiorentino, Florence (Italy). The algae were cultivated in GWP®-II photobioreactors (Tredici et al., 2011) in semi-batch mode, then the biomasses were harvested by centrifugation, frozen, lyophilized and powdered. The powdered biomasses were stored at -20°C until analysis. Only *A. plantesis* F&M-C256 biomass was washed with physiological solution during harvesting to remove bicarbonate excess. All the freshwater strains were cultivated in BG11 (Rippka et al., 1979) and all the marine strains in artificial seawater enriched with F medium nutrients (Guillard & Ryther, 1962). *A. platensis* F&M-C256 was cultivated in Zarrouk medium (Zarrouk, 1966). *T. suecica* F&M-M33 was also grown in standard F medium deprived of nitrogen source to induce carbohydrate accumulation. *C. vulgaris* Allma (Allma Microalgae, Portugal) and Klamath (Erbologica S.A.S.) powders were commercial products. In particular, Klamath powder is a natural bloom, mainly containing *A. flos-aquae*, harvested from Upper Klamath Lake (Oregon, USA). Twelve microalgal biomasses (see Table 1) were analyzed for their *in vitro* digestibility using an enzymatic method. Two of the tested biomasses (*C. sorokiniana* F&M-M49 and *C. vulgaris* Allma) were also used to study the effect of a sonication pre-treatment on *in vitro* digestibility.

2.2 Biochemical composition

All strains were analyzed for protein, carbohydrate, lipid, dietary fiber, ash and moisture. Elemental analysis was performed on dry biomass using a CHNSO Analyzer (Flash EA, 1112 Series, Thermo Electron Corporation, Massachusetts, USA) (Gnaiger and Bitterlich, 1984).
Total protein content was estimated as N x 6.25, where N is the nitrogen content determined through the elemental analysis. Carbohydrate was determined following Dubois et al. (1951) and lipid following Marsh & Weinstein (1966). Dietary fiber was determined by AOAC Method 985.29 (AOAC Official Method 985.29). Moisture, ashes and fatty acids was analyzed following ISTISAN protocols (ISTISAN Report 1996/34, method B, page 7; ISTISAN Report 1996/34, pages 77-78; ISTISAN Report 1996/34, page 47, respectively). The extracts for the total phenolic content determination were prepared according to the procedure used by Hajimahmoodi et al. (2010). The assay was carried out according to Rajauria et al., 2013, using the Folin Ciocalteu assay.

All the analyses were performed in triplicate. Data are expressed as mean ± standard deviation.

2.2.1 Lipid nutritional quality indexes

The nutritional quality of the lipid fraction was estimated by three different indexes that were calculated based on the concentration of saturated fatty acids (SFA, lauric C12:0, myristic C14:0, palmitic C16:0, and stearic C18:0), monounsaturated fatty acids (MUFA, oleic C18:1 ω9), and polyunsaturated fatty acids (PUFA, linoleic C18:2 ω6, linolenic C18:3 ω3, arachidonic C20:4 ω6 and eicosapentaenoic C20:5 ω3):

(1) Atherogenicity index (AI) = [(C12:0 + (4 × C14:0) + C16:0)]/(ΣMUFA + Σω6 + Σω3) (Ulbricht and Southgate, 1991);

(2) Thrombogenicity index (TI) = (C14:0 + C16:0 + C18:0)/[(0.5 × ΣMUFA) + (0.5 × Σω6) + (3 × Σω3) + (Σω3/Σω6)] (Santos-Silva et al., 2002);

(3) Fatty acids hypocholesterolemic/hypercholesterolemic ratios (H/H) = (C18:1ω9 + C18:2ω6 + C20:4ω6 + C 18:3ω3 + C20:5ω3)/(C14:0 + C16:0) (Santos-Silva et al., 2002).

2.3 In vitro *digestibility*

The evaluation of *in vitro* digestibility was performed according to Boisen & Fernández (1997), modified to adapt it to our necessities. This *in vitro* analysis reproduces the enzymatic attack occurring in the proximal tract of the digestive system (gastric and pancreatic juice). Biomasses of 10 microalgal strains and one natural bloom were analyzed (Table 1). Casein (Acros Organics, Geel, Belgium) was used as reference material for protein with 100% digestibility (Fleurence, 2004). The analysis was performed in triplicate. Data are expressed as mean ± standard deviation.

Step 1

Samples of 1 g of finely ground material (particle size ≤1 mm) were weighed and transferred in 250 mL conical flasks. To each flask, 25 mL of phosphate buffer (0.1 M, pH 6.0) were added and mixed, then 10 mL 0.2 M HCl were added and pH was adjusted to a value of 2.0 by addition of 1 M HCl or 1 M NaOH. Then 3 ml of a freshly prepared pepsin water solution containing 30 mg

of porcine pepsin with an activity of 0.8 FIP-U/mg (Applichem, Darmstdat, Germany) were added. A blank was also prepared with the same reactive but without algal biomass. The flasks were closed with a rubber stopper and placed on multipoint stirrers (IKA® KS 260 basic, Königswinter, Germany) at 150 rpm in a thermostatically-controlled heating chamber (ProCLIMATIC, Imola, Italy) at 39 °C. The samples were stirred for six hours.

Step 2

After six hours at 39 °C, 10 mL of phosphate buffer (0.2 M, pH 6.8) and 5 mL of a 0.6 M NaOH solution were added to the samples and to the blank. The pH was adjusted to a value of 6.8 by addition of 1 M HCl or 1 M NaOH. Then 10 mL of a freshly prepared pancreatin 50%-ethanol solution containing 500 mg of porcine pancreatin with an activity of 42362 FIP-U/g (Applichem, Darmstdat, Germany) were carefully mixed with the sample or blank. After closing with a rubber stopper, the flasks were placed again on the multipoint stirrers at 150 rpm and further incubated at 39 °C for 18 hours. The undigested residues were then collected by centrifugation (J2-21M/E centrifuge, Beckman, Minnesota, USA) at 8000 rpm for 30 minutes. Then, the supernatant was filtered on 47 mm glass-fiber membranes with nominal porosity of 1.2 µm (FILTER-LAB, Barcelona, Spain), to determine any residual biomass and/or undissolved reagents re-suspended from the pellet or not sedimented. The undigested material (sedimented pellet) was washed with deionised water to remove any salts of the buffer, and then centrifuged with the same parameters mentioned above. The supernatant was removed after the second centrifugation (filtered, dried and weighed to be added to the weight of the undigested material), then the pellet and the filters were dried at 80 °C for six hours and then at 45 °C until constant weight. The blank was treated following the same steps mentioned above.

Calculation

The percentage of *in vitro* dry matter (DM), organic matter (OM), crude protein (CP) or carbohydrate (C) digestibility, was calculated according to Boisen & Fernández (1997):

$$D_{DM/OM/CP/C}\ (\%) = \frac{SW_{DM/OM/CP/C} - [(UW_{DM/OM/CP/C} + RP) - B]}{SW_{DM/OM/CP/C}} \text{ x } 100$$

where:

$D_{DM/OM/CP/C}$ is the *in vitro* digestibility of dry matter or organic matter or crude protein or carbohydrate, expressed as percentage

$SW_{DM/OM/CP/C}$ is the weight of the starting material (dry matter or organic matter or crude protein or carbohydrate) expressed in grams

$UW_{DM/OM/CP/C}$ is the weight of the undigested material (dry matter or organic matter or crude protein or carbohydrate) or the amount of protein or carbohydrate expressed in grams

RP is the residual particulate, any residual biomass and/or undissolved reagents re-suspended from the pellet or not sedimented expressed in grams

B is the blank (all reagents without biomass) expressed in grams as dry weight, organic matter or crude protein or carbohydrate content

The weight of the starting material of dry matter and organic matter were corrected for the moisture values.

2.3.1 Sonication procedure to improve digestibility

A sonication pre-treatment was tested with *C. sorokiniana* F&M-M49 and *C. vulgaris* Allma biomasses. We decided to test two strains of the *Chlorella* genus, one with a thick cell wall (*C. sorokiniana* F&M-M49) and one with a thin cell wall (*C. vulgaris* Allma) to evaluate a different effect on *in vitro* digestibility.
One gram of sample prepared as described above, before pepsin addition was sonicated with an ultrasonic homogenizer (Microson™ XL2000, Misonix Inc., Farmingdale, New York, USA) set at a frequency of 20 kHz and a power of 130 W for 30 minutes, maintaining the temperature below 30 °C by immerging the sample flask in an ice bath. After sonication, pepsin was added and the rest of the analysis performed as already described. Micrographs before and after sonication were taken at 400 magnifications by an optical microscope (Zeiss West, Oberkochen, Germany) equipped with a digital camera and software (Infinity Lite and Infinity Capture, Lumenera, Ottawa, Canada), to verify the effect of the pre-treatment on cell aggregation and cell disruption.

2.4 Statistical and data analysis

The results are expressed as mean values ± standard deviation. The differences of the *in vitro* dry matter digestibility among the two strains sonicated and not sonicated were analyzed with one-way ANOVA and Tukey's multiple comparison test with a level of significance of $P<0.05$. For statistical correlation analysis, Pearson's correlation coefficient was employed and a significant correlation at the 0.05 level was identified. GraphPad Prism 6.01 was used for these aims.

3. Results and discussion

3.1 Biochemical composition

Table 2 shows the biochemical composition of 10 microalgae strains and one natural bloom. Proteins are considered fundamental macro-element of microalgal biomass to evaluate these microorganisms as a suitable food source. The estimations of crude protein in microalgae include other nitrogenous constituents like nucleic acids, glucosamides, amines, and cell wall materials (Becker, 2004). Five strains (*A. platensis* F&M-C256, Klamath powder, *N. sphaeroides* F&M-C117, *C. sorokiniana* F&M-M49, *C. vulgaris* Allma) showed protein values between 51 and 69%, with *A. platensis* F&M-C256 and Klamath powder (the two cyanobacteria) showing the highest values (68.9 and 62.4%, respectively). *A. platensis* F&M-C256 protein content was comparable to the value found by Coca et al. (2015) and Shimamatsu (2009) (70%) but higher than reported for this alga by other authors (Batista et al., 2013; Kent et al., 2015; Ogbonda et al., 2007) (40–50%). The protein content of Klamath powder was exactly comparable than the one found by Becker (2007) for *A. flos-aquae* (62%); while, the protein content found for *N. sphaeroides* F&M-C117 was lower than that reported as maximum protein content by Lv et al. (2014) for *N. flagelliforme* (58%). *C. sorokiniana* F&M-M49 and *C. vulgaris* Allma showed protein values rather comparable than that showed by Tibbets et al. (2015) for *C. vulgaris* (50%) and by Guccione et al. (2014) for *C. sorokiniana* (45%). The marine species and one freshwater strain (*T. suecica* F&M-M33 grown in nutrient replete medium, *P. purpureum* F&M-M46, *P. tricornutum* F&M-M40, *T. lutea* F&M-M36, *N. oceanica* F&M-M24 and *C. sorokiniana* IAM C-212) exhibited a content of protein ranged from 34 to 43%. *N. oceanica* F&M-M24 showed higher protein content compared to the values

found by Kent et al. (2015) for *Nannochloropsis* sp. and by Tibbets et al. (2015) for *N. granulata* (around 30%). As expected, the lowest value of protein (18%) was observed in starved *T. suecica* F&M-M33. It is interesting to note as most of the freshwater and cyanobacteria species reported higher values of protein (up to 69% in *A. platensis* F&M-C256). The protein content of almost every microalga compares favorably with that of the reference indicated by FAO/WHO and other food proteins (wheat, chickpea, milk powder) (FAO/WHO, 1991).

The carbohydrate and total dietary fiber (TDF) contents in the microalgal biomasses were found to be very diverse. In most of the microalgae studied (*A. platensis* F&M-C256, Klamath powder, *N. sphaeroides* F&M-C117, *C. sorokiniana* F&M-M49, *C. sorokiniana* IAM C-212, *P. purpureum* F&M-M46, *P. tricornutum* F&M-M40, *T. suecica* F&M-M33 grown in nutrient replete medium, and *N. oceanica* F&M-M24) total carbohydrate varied between 10 and 19%, which was within a 10–20% range found in literature (Batista et al., 2013; Feller et al., 2015; Guccione et al., 2014; Kent et al., 2015; Pugh et al., 2001). The carbohydrate content found in our study for *N. sphaeroides* F&M-C117 showed a lower value (15%) compared to Becker (2004) and Tekenaka et al. (1998) for *N. flagelliforme* (>20%). *C. vulgaris* Allma, *T. suecica* F&M-M33 grown in nutrient replete medium, and *T. lutea* F&M-M36 exhibited the lowest content of carbohydrate (from 6 to 10%), which were lower values compared to the range found in literature (Abiusi et al., 2014; Batista et al., 2013; Feller et al., 2015) (13-22%). It is worth nothing that starved *T. suecica* F&M-M33 showed the highest value for carbohydrate together with the lowest value for protein.

Intakes of dietary fiber equal to 14 g/1000 kcal can stimulate the proliferation of intestinal flora and can decrease cholesterol and glycaemic levels (Beecher, 1999). Only Klamath powder seems to contain negligible quantities of TDF. The Company Earth's essential Elements (E3Live™) also found for the commercial frozen *A. flos-aquae* a low amount of TDF (1.5%). *C. sorokiniana* IAM C-212, *A. platensis* F&M-C256, and *C. vulgaris* Allma were found to contain the lowest amounts of TDF (5.2, 6.2 and 7.1%, respectively), which were values usually reported in literature for these strains (Cheong et al., 2010; Belay, 2007; Gutiérrez-Salmeán et al., 2015; Matos et al., 2016). The other two freshwater strains (*N. sphaeroides* F&M-C117 and *C. sorokiniana* F&M-M49) and all the marine species showed higher TDF values between 10-18% (Table 2). Most of these strains exhibited values of TDF comparable to the contents

found by Matos et al. (2016), while the contents of TDF indicated by the Company Algaen Corporation (AlgaeBerry™) for *Nostoc* sp., by Fitoplancton Marino S.L. for *T. chuii* (AESAN, 2013) and by Natrah et al. (2007) for *I. galbana* were much lower (≤ 5%).

It is interesting to note that the carbohydrate content was not correlated with the TDF (R^2 = 0.002; P >0.05). In particular, among the species grown under nutrient sufficient conditions, the marine *P. purpureum* F&M-M46 showed high total carbohydrate content (17%) and high TDF content (15.4%) which could be associated with its content in sulfated exopolysaccharides (Rebolloso Fuentes et al., 2000). In contrast, Klamath powder exhibited an almost nil TDF content at the front of a carbohydrate level of 19%. In the case of *T. suecica*, a significant increase in carbohydrate and a 30% reduction of TDF were observed under nutrient deficiency. Under stress conditions, this microalga typically produces large amounts of carbohydrate as energy and carbon reserves (Bondioli et al., 2012).

As far as the lipid content of biomass is concerned, Klamath powder showed the lowest lipid content (6%) compared to the other microalgae, which was similar to the value of lipid found by Kushak et al. (2000) and Becker (2007) for *A. flos-aquae* (5% and 3%, respectively). Four microalgal biomass (*A. platensis* F&M-C256, *C. vulgaris* Allma, *P. purpureum* F&M-M46 and *P. tricornutum* F&M-M40) exhibited a lipid content in range of 11-21%, which was comparable to the range of values found in literature (Feller et al., 2015; Tibbets et al., 2015) (14-19%). Hori et al. (1990) reported a much lower lipid content (1%) for *N. commune* compared to *N. sphaeroides* F&M-C117 (15%). Six strains (*C. sorokiniana* F&M-M49, *C. sorokiniana* IAM C-212, starved *T. suecica* F&M-M33, *T. suecica* F&M-M33 grown in nutrient replete medium, *T. lutea* F&M-M36, and *N. oceanica* F&M-M24) exhibited values of lipid from 23 to 29%, which were rather comparable to the range of lipid found by other authors (Abiusi et al., 2014; Batista et al., 2013; Guccione et al., 2014; Tibbets et al., 2015) (22-24%) (Table 2).

Ash contents were generally low (4-9.4%) in freshwater species, on the contrary, marine strains exhibited significantly higher values up to 22% for *P. purpureum* F&M-M46 (Table 2). This information refers to high inorganic compounds presented in marine microalgae species, which is associated with the high salinity presented in the culture medium (Feller et al., 2015).

Table 2. Biochemical composition of 10 microalgal strains and one natural bloom. Data are expressed as % of dry weight including humidity. Mean values ± SD (n=3) are reported except for dietary fiber

S	CP (%)	CHT (%)	L (%)	TDF (%)	A (%)	M (%)
Ap	68.9 ± 1.0^{a}	12.8 ± 0.21^{f}	10.7 ± 0.56^{g}	6.2	6.1 ± 0.10^{f}	7.9 ± 0.20^{c}
K	62.4 ± 5.19^{a}	18.8 ± 0.15^{b}	6.1 ± 0.84^{h}	0.1	6.2 ± 0.32^{f}	6.8 ± 0.24^{d}
Ns	50.8 ± 1.45^{c}	14.5 ± 0.53^{e}	15.1 ± 1.19^{e}	12.1	4.0 ± 0.25^{g}	7.8 ± 0.28^{c}
CsM49	51.3 ± 0.48^{c}	15.5 ± 0.08^{d}	22.7 ± 2.00^{d,e}	11.2	5.4 ± 0.11^{f}	8.5 ± 0.24^{b}
CsIAM	39.9 ± 0.94^{e,f}	10.7 ± 0.90^{g,h}	27.9 ± 1.30^{a}	5.2	9.4 ± 0.37^{e}	7.5 ± 0.30^{b,c}
CvA	56.8 ± 2.70^{b}	5.9 ± 0.25^{i}	16.9 ± 2.83^{d}	7.1	9.3 ± 1.47^{e}	4.9 ± 0.17^{f}
TsS	18.3 ± 0.10^{h}	36.8 ± 1.46^{a}	22.4 ± 1.15^{b}	12.6	14.8 ± 0.47^{b}	6.1 ± 0.26^{e}
TsNR	40.2 ± 0.51^{d,e,f}	10.2 ± 0.20^{h}	28.5 ± 1.16^{a}	18.1	15.7 ± 0.20^{b}	7.2 ± 0.14^{c,d}
Pp	34.2 ± 0.10^{g}	17.0 ± 1.72^{c}	13.1 ± 1.12^{f}	15.4	22.0 ± 0.88^{a}	10.0 ± 0.39^{a}
Pt	38.8 ± 0.11^{f}	11.0 ± 0.70^{g}	20.5 ± 0.54^{c}	14.7	14.8 ± 0.12^{b}	8.0 ± 0.23^{c}
Tiso	42.9 ± 0.42^{d,e}	8.6 ± 0.89^{h}	27.9 ± 3.25^{a}	11.9	11.5 ± 0.27^{d}	6.3 ± 0.26^{e}
No	43.1 ± 0.10^{d}	14.3 ± 0.19^{e}	28.2 ± 2.04^{a}	9.5	12.9 ± 0.84^{c}	7.2 ± 0.21^{c,d}

Different letters in the same row correspond to significant differences (P<0.05). S Strain; CP Crude protein; CHT Total carbohydrate; L Lipid; TDF Total dietary fiber; A Ash; M Moisture; Ap *A. platensis* F&M-C256; K Klamath powder; Ns *N. sphaeroides* F&M-C117; CsM49 *C. sorokiniana* F&M-M49; CsIAM *C. sorokiniana* IAM C-212; CvA *C. vulgaris* Allma; TsS *T. suecica* F&M-M33 (starved); TsNR *T. suecica* F&M-M33 (nutrient replete medium); Pp *P. purpureum* F&M-M46; Pt *P. tricornutum* F&M-M40; Tiso *T. lutea* F&M-M36; No *N. oceanica* F&M-M24

Table 3 shows microalgae fatty acid content and profile. The proportion of total saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA)-ω3 and -ω6 fatty acids were also reported.

Palmitic acid (C16:0) was the most abundant SFA (1–6.7%) followed by myristic acid (C14:0, 0.02–3.1%), lauric acid (C12:0, 0.1-1.6%) and stearic acid (C18:0, 0.04–0.6%) (Table 3). The sum of all identified SFAs ranged from 18 to

47% of the total fatty acid content, which was in agreement with the values reported in literature (Abiusi et al., 2014; Feller et al., 2015; Matos et al., 2016). It has been found that *T. lutea* F&M-M36 contains 47% SFA, mainly comprised of C14:0, followed by *A. platensis* F&M-C256 with 45% SFA, predominantly C16:0; *C. vulgaris* Allma and *P. purpureum* F&M-M46 have intermediate SFA content (31 and 30%, respectively), while *C. sorokiniana* IAM C-212 and starved *T. suecica* F&M-M33 have the lowest SFA content (18%).

As found by Matos et al. (2016), the highest monounsaturated fatty acids (MUFA) content was found in *N. oceanica* F&M-M24, representing 41% of total fatty acid content, followed by *P. tricornutum* F&M-M40 (30%) and *C. sorokiniana* F&M-M49 (29%). Whereas, *A. platensis* F&M-C256, *C. sorokiniana* IAM C-212, and *P. purpureum* F&M-M46 contained very low concentrations of MUFA (4-10%). The main MUFA detected in all species were palmitoleic acid (C16:1) and oleic acid (C18:1 ω9), with a high variation in quantity among the different species. C16:1 was the main MUFA found in the most marine species studied, ranging from 0.8 to 5.8%, except for the two *T. suecica* F&M-M33 and for *P. purpureum* F&M-M46 that had a very low content (from 0.04 to 0.2%) (Table 3). These results were comparable with those obtained by Feller et al. (2015) and Matos et al. (2016), who demonstrated that *P. cruentum* contains low levels of palmitoleic acid and higher levels of arachidonic acid (ARA, C20:4 ω6). The Expert Consultation of FAO stated that replacing SFA (C12:0-C16:0) as well as carbohydrates with MUFA reduces LDL and increases HDL cholesterol concentrations (FAO/WHO, 2008). Our data suggested that the marine species *P. tricornutum* F&M-M40 and *C. sorokiniana* F&M-M49 had optimal compositions due to the low SFA content (~27% of total fatty acids) compared to the other microalgae, a low carbohydrate content (11 and 16%, respectively), and a high MUFA content (30%).

Among PUFAs composition, α-linolenic (ALA, C18:3 ω3) and eicosapentaenoic (EPA, C20:5 ω3) acids were the predominant PUFAs- ω3, while γ-linolenic (GLA C18:3 ω6), arachidonic (ARA, C20:4 ω6) and linoleic (LA C18:2 ω6) acids were the prevalent PUFAs-ω6. *C. sorokiniana* IAM C-212, *P. purpureum* F&M-M46 and starved *T. suecica* F&M-M33 contained high PUFAs (73, 68, and 67% of total fatty acids). High proportions of PUFAs-ω3 were observed in *N. sphaeroides* F&M-C117 (49%), *C. sorokiniana* IAM C-212 (39%), starved *T. suecica* F&M-M33 (38%), *P. purpureum* F&M-M46 (37%) and *P. tricornutum* F&M-M40 (36%). While high concentrations of

PUFAs-ω6 were found in *A. platensis* F&M-C256 (47%), *C. sorokiniana* IAM C-212 (34%) and *P. purpureum* F&M-M46 (30%) of the total fatty acids.
These PUFAs were rather comparable with the values found in the literature (Abiusi et al., 2014; Batista et al., 2013; Feller et al., 2015; Gugger et al., 2002). Klamath powder and *A. platensis* F&M-C256 contained very low concentrations of PUFA (2 and 3%, respectively) and *A. platensis* F&M-C256 had the least favourable ω3/ω6 ratio (0.004) among all microalgae (Table 3). This is generally typical for freshwater microalgae/cyanobacteria and makes them poor sources of nutritionally-essential long-chain PUFA, arachidonic acid (ARA, C20:4 ω6), eicosapentaenoic acid (EPA, C20:5 ω3) and docosahexaenoic acid (DHA, C22:6 ω3) (Matos et al., 2016). A well-balanced ratio between the ω3/ω6 PUFA is advantageous for human health and 1:4 is considered the recommended ratio to prevent cardiovascular diseases (Simopoulos, 2012). Except for *A. platensis* F&M-C256 and *T. suecica* F&M-M33 grown in nutrient replete medium, all other microalgae showed ω3/ω6 ratio of ≥1.0 (Table 3). Indeed, in cyanobacteria (e.g., *A. platensis*) the unsaturated double bonds are preferentially in the ω6 position while in Chlorophyceae they are mainly in the ω3 position (Batista et al., 2013; Matos et al., 2016).
As found by Matos et al. (2016), *A. platensis* F&M-C256 was rich in γ-linolenic acid (GLA, C18:3 ω6) (1.7%). GLA has several beneficial health effects and GLA oils are mainly used for their antiinflammatory effects (Van Hoorn et al., 2008). Clinical studies have shown that GLA regulates transepidermal water loss (TEWL), whereby, it prevents dryness and helps heal eczema and xeroderma. GLA is also shown to stimulate apoptosis of cancer cells without affecting healthy cells and can prevent weight regain after weight loss (Van Hoorn et al., 2008).
Studies have shown that EPA and DHA, found in fish, crustaceans, and algae, are important for proper fetal development, including neuronal, retinal, and immune function. EPA and DHA may affect many aspects of cardiovascular function including inflammation, peripheral artery disease, major coronary events, and anticoagulation. Moreover, they have been linked to promising results in prevention, weight management, and cognitive function in those with very mild Alzheimer's disease (Bucy et al., 2012; Swanson et al., 2012). Considering the health benefits related to the consumption of EPA and DHA, *N. ocenica* F&M-M24 and *P. tricornutum* F&M-M40 are interesting for nutrition applications because they were able to synthesize high amounts of EPA (3.2 and

4%, respectively). In addition, *T. lutea* can synthesize DHA, and thus these microalgae could be also used to enrich foods with this ω3 fatty acid.

As shown in Table 3, *N. sphaeroides* F&M-C117, *C. sorokiniana* F&M-M49 and *C. vulgaris* Allma have showed high α-linolenic acid (ALA, C18:3 ω3) compared to the other microalgae that contributed to an increased ω3/ω6 ratio up to 1.9. Since ALA plays an important role reducing the level of platelets stickiness due to the alteration of the aggregating potential, the importance of this fatty acid to human-cell functioning is evident. Indeed, the assumption of ALA can decrease the probability of thrombotic occlusion and reduce the atherosclerotic process (Connor, 1999).

Table 3. Fatty acids content and profile (% of dry weight) of the different microalgae

	Cyanobacteria		**Chlorophyta**						**Rhodophyta**	**Bacillariophyta**	**Haptophyta**	**Eustigmatophyta**
	Ap	K	Ns	CsM49	CsIAM	CvA	TsS	TsNR	Pp	Pt	Tiso	No
C12:0	-	-	0.20	1.55	-	0.55	-	0.59	0.14	0.51	1.21	0.35
C14:0	-	0.25	0.04	0.05	-	0.06	-	0.03	0.02	0.57	3.09	0.93
C16:0	2.56	1.24	3.02	1.74	1.26	1.99	1.33	0.95	1.26	1.27	1.62	6.73
C18:0	0.12	-	0.15	0.11	-	0.26	-	0.60	0.04	0.05	0.14	0.35
Other SFA	0.02	-	0.13	0.05	-	0.14	-	0.73	0.04	0.60	1.04	0.24
Σ SFA	2.70	1.49	3.50	3.50	1.26	3.00	1.33	2.90	1.50	3.00	7.10	8.60
C16:1	0.29	-	1.23	0.15	0.17	0.44	0.04	0.04	0.16	2.42	0.79	5.82
C18:1 ω9	0.18	-	0.48	2.52	0.52	1.81	1.95	1.37	0.02	0.90	2.91	3.06
Other MUFA	0.03	-	0.004	1.43	-	0.05	-	0.49	0.02	0.08	0.10	0.04
Σ MUFA	0.50	-	1.75	3.90	0.69	2.30	1.37	1.90	0.20	3.40	3.80	8.92
C16:3 ω3	-	0.23	-	-	0.87	-	-	-	-	-	-	-
C16:4 ω3	-	-	-	-	-	-	1.02	-	-	-	-	-
C18:3 ω3 (ALA)	0.01	1.51	2.14	3.44	1.93	2.51	1.76	1.56	-	0.07	1.60	0.02
C20:5 ω3 (EPA)	-	-	-	-	-	-	-	0.39	1.87	3.97	-	3.15
C22:6 ω3 (DHA)	-	-	-	-	-	-	-	-	-	-	1.01	-
Other PUFA ω3	-	-	-	0.07	-	-	-	-	-	-	-	-
ΣPUFA ω3	0.01	1.74	2.14	3.51	2.80	2.51	2.78	1.95	1.87	4.04	2.61	3.17
C16:2 ω6	-	-	-	-	0.56	-	-	-	-	-	-	-
C18:2 ω6	1.15	0.33	1.08	2.38	1.85	1.85	1.19	1.88	0.35	0.53	1.13	0.28
C18:3 ω6 (GLA)	1.66	-	0.03	0.04	-	0.01	-	0.03	0.01	0.08	0.04	0.07
C20:2 ω6	-	-	-	0.04	-	-	-	-	0.03	0.23	-	0.04
C20:4 ω6 (ARA)	-	-	0.03	-	-	-	-	0.11	1.06	0.07	-	0.48
Other PUFA ω6	0.01	-	-	-	-	-	-	-	0.07	-	-	0.09
ΣPUFA ω6	2.82	0.33	1.14	2.46	2.41	1.86	1.19	2.09	1.52	0.91	1.17	0.96

ΣPUFA ω3 + ω6	2.83	2.07	3.28	5.97	5.21	4.37	4.87	3.97	3.39	4.95	3.78	4.13
ω3/ω6	0.004	5.27	1.88	1.43	1.16	1.35	1.33	0.93	1.23	4.44	2.23	3.30
TFA	6.0	3.56	8.5	13.4	7.16	9.7	7.29	8.5	5.0	11.3	15.1	21.7

- traces or 0 value; Ap *A. platensis* F&M-C256; K Klamath powder; Ns *N. sphaeroides* F&M-C117; CsM49 *C.sorokiniana* F&M-M49; CsIAM *C. sorokiniana* IAM C-212; CvA *C. vulgaris* Allma; TsS *T. suecica* F&M-M33 (starved); TsNR *T. suecica* F&M-M33 (nutrient replete medium); Pp *P. purpureum* F&M-M46; Pt *P. tricornutum* F&M-M40; Tiso *T. lutea* F&M-M36; No *N. oceanica* F&M-M24

The nutritional quality of lipid profiles observed in the algae species was evaluated by different indexes as shown in Table 4. Foods with polyunsaturated to saturated fatty acid (P/S) ratio below 0.45 are considered by the FAO/WHO to be undesirable in the human diet (FAO/WHO, 2008), due to their potential to induce increases in blood cholesterol. The P/S ratio of beef is typically about 0.1, and this ratio decreases with an increase of meat fatness (Scollan et al., 2006). The P/S ratio in the microalgae studied in this work varied from 0.48 (*N. oceanica* F&M-M24) to 4.13 (*C. sorokiniana* IAM C-212), with presumable positive health effects in humans.

The ratio hypocholesterolemic/hypercholesterolemic fatty acids (H/H) is an index based on current knowledge of the effects of individual fatty acids on cholesterol metabolism (Santos-Silva et al., 2002; Simat et al., 2015). Nutritionally, higher H/H values are considered more beneficial for human health because is related to a high PUFA content (Matos et al., 2016). The highest H/H values were found in *N. oceanica* F&M-M24, Klamath powder and in *T. lutea* F&M-M36 species (0.83, 1.08 and 2, respectively). These results are in agreement with H/H values for marine fish such as sardine and mackerel (2.46) reported by Fernandes et al. (2014). The fatty acids from microalgae, that are highly polyunsaturated, could have beneficial effects on cholesterol.

Two other indexes (atherogenicity index, AI and thrombogenicity index, TI) are used to evaluate the potential for stimulating platelet aggregation according to Turan et al. (2007). Lower AI and TI values indicate a greater potential to protect against coronary artery disease. In our study, AI values ranged from 0.10 (*P. tricornutum* F&M-M40) to 0.45 (*N. oceanica* F&M-M24); while *A. platensis* F&M-C256 showed the highest AI (1.58) (Table 4). Simat et al. (2015) reported AI values of 0.59–0.92 for the omnivorous fish bogue (*Boops boops*). The lowest TI value was observed in the marine *N. oceanica* F&M-M24 (0.91), which is higher compared to the sardine TI value (0.20) reported by Fernandes et al (2014).

Table 4. Nutritional quality indexes of the lipid fraction of 10 microalgae strains and one natural bloom

Microalga	P/S	H/H	AI	TI
A. platensis F&M-C256	1.04	0.77	1.58	0.52
Klamath powder	1.39	1.08	0.14	1.23
N. sphaeroides F&M-C117	0.94	0.64	0.33	1.22
C.sorokiniana F&M-M49	1.71	0.35	0.13	4.66
C. sorokiniana IAM C-212	4.13	0.21	0.11	3.41
C. vulgaris Allma	1.46	0.42	0.21	3.01
T. suecica F&M-M33 (S*)	3.66	0.25	0.12	3.68
T. suecica F&M-M33 (NR*)	1.37	0.28	0.18	5.42
P. purpureum F&M-M46	2.26	0.41	0.17	2.58
P. tricornutum F&M-M40	1.65	0.49	0.10	3.01
T. lutea F&M-M36	0.53	2.00	0.39	1.20
N. oceanica F&M-M24	0.48	0.83	0.45	0.91

* starved; ** grown in nutrient replete medium; P/S polyunsaturated/saturated; H/H Σ hypercholesterolemic/Σ hypocholesterolemic; AI atherogenicity index; TI thrombogenicity index

Fig. 1 shows the total phenolic content (TPC) of the examined microalgae. Phenolic compounds, including simple phenols, flavonoids, phenylproponoids, tannins, lignins, phenolic acids, and their derivatives, synthesized as secondary metabolites are considered the major contributors to antioxidant capacity (Abd El-Baky et al., 2009). Cyanobacteria exhibited higher TPC values (from 13.1 to 18.9 mg GAE g^{-1}) compared to the other microalgae. In particular, *A. platensis* F&M-C256 showed the highest TPC (19 mg GAE g^{-1}). TPC reported in the literature for *Arthrospira* varied from 2.4 to 49.8 mg GAE g^{-1} depending on culture medium (Abd El-Baky et al., 2009), extraction methods (Chaiklahan et al., 2013) and culture conditions (Kepekçi and Saygideger, 2012). *P. purpureum* F&M-M46 and *N. oceanica* F&M-M24 also showed high TPC (around 12 mg GAE g^{-1}). These two microalgae exhibited higher TPC compared to the values found by Goiris et al. (2012) for *P. cruentum* and *N. oculata* ethanol/water extract (1.2 and 2 mg GAE g^{-1}, respectively). *P. tricornutum* F&M-M40, *T. lutea* F&M-M36 and all Chlorophyta strains exhibited TPC from 3.6 mg GAE g^{-1} until 9.2 mg GAE g^{-1}. The range of TPC found in our study were higher compared to the range of values found by Goiris et al. (2012) (1.7-3.8 mg GAE g^{-1}), except for *C. sorokiniana* (not analysed by Goiris et al., 2012). Peng et al.

(2009) found for *C. sorokiniana* ethanol extract a TPC of 26.7 mg GAE g^{-1} higher compared to that obtained in our study with *C. sorokiniana* F&M-M49 and *C. sorokiniana* IAM C-212 (3.6 and 4.3 mg GAE g^{-1}, respectively).

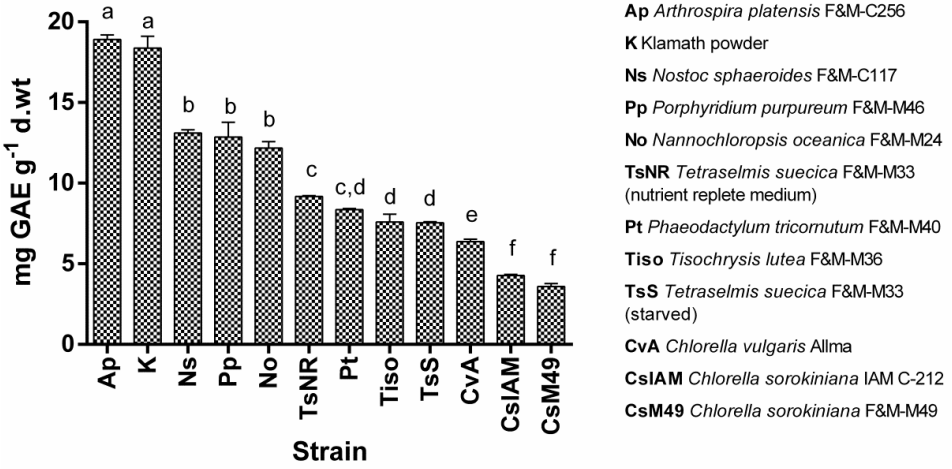

Fig. 1. Total phenolic content (expressed as mg GAE g^{-1}) in decreasing order of 10 microalgal strains and one natural bloom. The analysis was performed in triplicates. Data are reported as mean ± SD. Different letters between columns correspond to significant differences (P<0.05)

3.2 In vitro *digestibility*

Until now, few microalgal species are presently accepted in the EU as food or food ingredients (EU, 1997). To extend this number is necessary to follow the novel food approval regulation that requires specific data to approve a new food (EU, 2015).

The evaluation of *in vitro* digestibility is a fundamental analysis to provide information about the nutritional bioavailability of microalgal biomasses. As far as digestibility of microalgae is concerned, most of the literature deals with tests for macroalgae (Fleurence, 1999; Paiva et al., 2014; Tibbets et al., 2016) and only few studies, to our knowledge, focus on the digestibility of microalgae (Mišurcová et al., 2010; Machů et al., 2014; Tibbets et al., 2012). Moreover, it is difficult to compare the results of digestibility from various studies in the literature due to different algal samples, in term of strains and type of matrix (e.g. dried, frozen) and different methods of digestibility determination (Mišurcová, 2011).

The *in vitro* digestibility of 10 microalgal biomass and one natural bloom was determined by an enzymatic method using pepsin and pancreatin. Dry matter (DMD), organic matter (OMD), carbohydrate (CD) and crude protein (CPD) digestibilities were reported in Fig. 2.

None of the strains exhibited digestibility values lower than 45%. Overall, the trends of DMD, OMD and CD are rather superimposable. On the contrary, CPD digestibility follows a completely different trend (Fig. 2). In particular, as far as DMD is concerned, values of digestibility between 47 and 68% were found in nine microalgal biomasses (*P. purpureum* F&M-M46, *P. tricornutum* F&M-M40, *T. suecica* F&M-M33 grown in nutrient replete medium, starved *T. suecica* F&M-M33, *N. oceanica* F&M-M24, *C. sorokiniana* F&M-M49, *T. lutea* F&M-M36, *N. sphaeroides* F&M-C117, Klamath powder) with *P. purpureum* F&M-M46 as the least digestible (47%). The differences in DMD values between the microalgal and cyanobaterial samples could be related to the different structure of their cell walls (Mišurcová, 2011). Diatoms have cell walls made of opaline silica and green algal cell walls are typically composed of cellulose, hemicellulose, pectic compounds, and glycoproteins (Andersen, 2013). Corteggiani Carpinelli et al. (2014) found *N. gadiatana* has set of genes for the synthesis and incorporation in the cell wall of cellulose and sulfated fucans and it is able to store carbon in polymers of β-1,3- and β-1,6-linked glucose called chrysolaminarin. These compounds contribute to maintaining the rigidity of the cell wall limiting the digestive action of enzymes such as pepsin and pancreatin (Becker, 2007).

Besides cell walls components, polysaccharides can form stable complexes with proteins that become inaccessible for proteolytic enzymes during the digestibility process (Mišurcová, 2011). *Porphyridium*, the least digestible microalga, typically contain many polysaccharides (De Jesus Raposo et al., 2013).

C. vulgaris Allma, *C. sorokiniana* IAM C-212, and *A. platensis* F&M-C256 showed the highest DMD values (from 70 to 78%) (Fig. 2). The highest digestibility of *A. platensis* F&M-C256 (78%) is related to its cell wall structure, typical of Gram-negative microorganisms. The low concentration (<1%) of β-1,2-glucan (first layer), a polysaccharide with low digestibility, the small thickness (12 nm) of the first layer, and the proteic and lipopolysaccharidic nature of the second layer resulted in a higher digestibility of this alga (Tomaselli, 1997). The easy breakage of its cell walls allowed a higher accessibility to its intracellular content by enzymes, and for this reason,

chemical or physical processing steps are not required in order to make its biomass more digestible (Balloni et al., 1980; Tomaselli, 1997). Devi et al. (1981) and Mišurcová et al. (2010) found an even higher DMD values for *A. platensis* (84% and 94%, respectively) compared to our study. The DMD showed by *C. sorokiniana* IAM C-212 was comparable to the value obtained by Mišurcová et al. (2010) for *C. pyrenoidosa* (75%). To our knowledge, no literature is available concerning DMD of the other investigated microalgae.

CD followed the same trend of DMD and also in this case *A. platensis* F&M-C256 was the most digestible strain (79%) and *P. purpureum* F&M-M46 the least digestible microalga (about 51%). Also the behavior of OMD was very similar to that of DMD, with the difference that seven strains (*P. purpureum* F&M-M46, *P. tricornutum* F&M-M40, *T. suecica* F&M-M33 grown in nutrient replete medium, starved *T. suecica* F&M-M33, *N. oceanica* F&M-M24, *C. sorokiniana* F&M-M49, *T. lutea* F&M-M36) showed values between 51 and 65% and four strains (*N. sphaeroides* F&M-C117, *C. vulgaris* Allma, *C. sorokiniana* IAM C-212, *A. platensis* F&M-C256) plus Klamath powder exhibited OMD from 71 to 86% (Fig. 2).

Concerning CPD, *N. oceanica* F&M-M24 and *C. sorokiniana* F&M-M49 exhibited the least values (50 and 55%), while most of the strains (*T. suecica* F&M-M33 grown in nutrient replete medium, *T. lutea* F&M-M36, *C. sorokiniana* IAM C-212, starved *T. suecica* F&M-M33, *P. tricornutum* F&M-M40, *P. purpureum* F&M-M46), including Klamath powder showed values from 62 to 70%. *C. vulgaris* Allma, *A. platensis* F&M-C256, and *N. sphaeroides* F&M-C117 were the most digestible strains (76, 81, 82%, respectively), which compares favourably with the true protein digestibility values found for beans, oats, and wheat (FAO/WHO, 2007). Many authors reported very diverse CPD values for different species of *Chlorella* (from 44 to 97%, Janczyk et al., 2005; Morris et al., 2008; Mišurcova et al., 2010), *Arthrospira* (from 70 to 84%, Devi et al., 1981; Becker, 2004), and *Nostoc* (45–50%, Hori et al. 1990). Tibbets et al. (2012) reported higher CPD for *P. tricornutum*, *N. granulata*, *T. chuii* and *P. aerugineum* (83-97%) compared to the values found in our study for these genera. This large variability may be related to species differences but more likely due to differences in methods used such as differences in enzyme mixtures, sample processing, assay conditions, and the extent of algal cell wall disruption (Mišurcova, 2011).

Despite no significant linear correlation ($R^2 = 0.10$; $P > 0.05$) between CPD and dietary fiber was found, reduced CPD of algae can also be attributed to

relatively high levels of fiber that can entrap proteins in the cellular matrix, rendering them less bioavailable to enzymatic hydrolysis (Marrion et al. 2005). A significant negative correlation (R^2 = 0.63; P<0.05) between DMD, OMD, and CD and the fiber content was found. Microalgae rich in fiber always reported lower DMD, OMD, and CD. When the low digestibility was not attributable to the high fiber contents (as for e.g. the case of *C. sorokiniana* F&M-M49), probably the nature of the cell wall caused the limited digestibility (Table 2, Fig. 2).

Besides their antioxidant potential TPC was also important because of their potential negative effects on protein quality. Plant-based phenolic compounds have the ability to precipitate dietary protein, causing reduced protein digestibility and amino acid availability (Julkunen-Tiitto, 1985). The mechanism by which these compounds may reduce protein digestibility is through hydrogen-binding and/or oxidation of proteins and amino acids rendering them unavailable to proteolytic enzyme hydrolysis and subsequent intestinal absorption (Tibbets et al., 2015). For the majority of microalgae studied here, CPD was >60% and TPC was generally low (<19 mg GAE g^{-1}; Fig. 1); however, no significant correlation between TPC and CPD was found (R^2 = 0.16, P>0.05). As such, it seems unlikely that TPC in microalgal biomass will contribute to any significant detrimental effects on protein bioavailability.

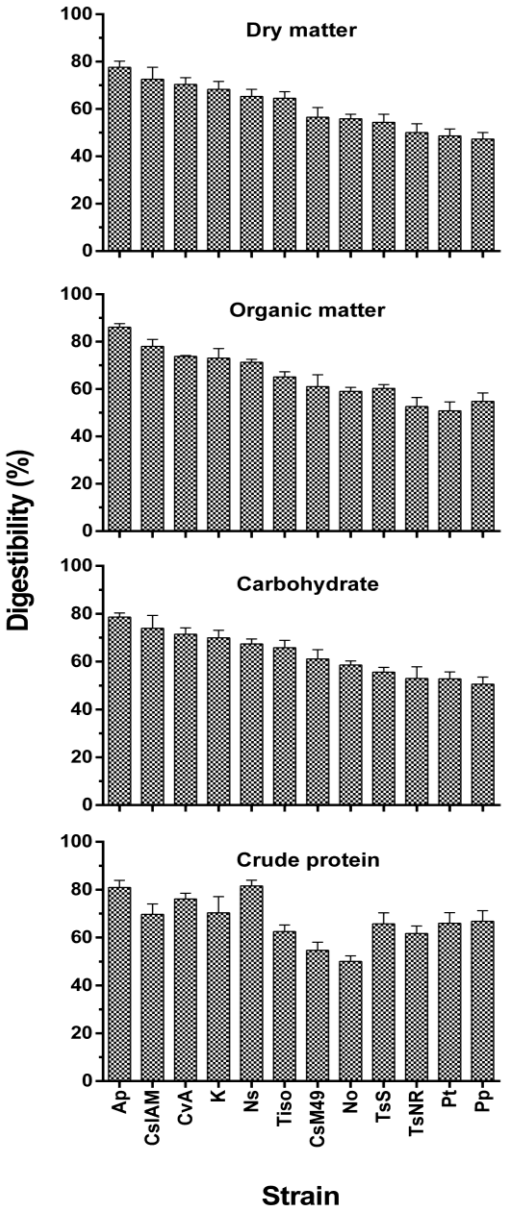

Fig. 2. Dry matter (in order of decreasing digestibility), organic matter, carbohydrate and crude protein digestibility (expressed as %) of 10 microalgal strains and one natural bloom. The analyses were performed in triplicate. Data are reported as mean value ± SD. Ap *A. platensis* F&M-C256; K Klamath powder; Ns *N. sphaeroides* F&M-C117; CsM49 *C.sorokiniana* F&M-M49; CsIAM *C. sorokiniana* IAM C-212; CvA *C. vulgaris* Allma; TsS *T. suecica* F&M-M33 (starved); TsNR *T. suecica* F&M-M33 (nutrient replete medium); Pp *P. purpureum* F&M-M46; Pt *P. tricornutum* F&M-M40; Tiso *T. lutea* F&M-M36; No *N. oceanica* F&M-M24

3.2.1 Sonication effect on in vitro *digestibility of two* Chlorella *strains*

The robust microalgae cell wall, which restricts the access of the gut enzymes to the cell components, is one of the main reason which makes microalgae low digestible (Janczyk et al., 2005). Microalgae of commercial importance (mainly Chlorophyceae) have rigid indigestible cell walls and many authors described the sonication treatment as efficient method to open microalgal cell (McMillan et al., 2013; Safi et al., 2014; Wang et al., 2014).

The dry biomass of two *Chlorella* strains, *C. sorokiniana* F&M-M49 and *C. vulgaris* Allma, were pre-treated by sonication before pepsin incubation and the DMD obtained was compared to that of the untreated samples. Untreated *C. sorokiniana* F&M-M49 and *C. vulgaris* Allma showed DMD values of 55 and 70%, respectively, significantly different among them ($P<0.05$). A significant difference ($P<0.05$) also emerged when digestibility of the two sonicated strains was compared. Although the pre-treatment led to an increase of dry matter digestibility for both *C. sorokiniana* F&M-M49 and *C. vulgaris* Allma (Table 5), these values were not significantly different ($P>0.05$) compared to those of the untreated samples.

Table 5. *In vitro* DMD of two *Chlorella* strains with and without sonication pre-treatment. Analyses were performed in triplicate and data are reported as mean value ± SD

Microalga	**Dry matter digestibility (%)**
C. sorokiniana F&M-M49	54.7 ± 4.02^{a}
C. sorokiniana F&M-M49 sonicated	59.7 ± 2.84^{a}
C. vulgaris Allma	69.5 ± 3.02^{b}
C. vulgaris Allma sonicated	73.1 ± 2.11^{b}

Different superscript letters represent significant differences ($P<0.05$) between treatments

Microscope observations performed on suspensions of *Chlorella* biomass before and after sonication are showed in Figs 3 and 4. Sonication dissolved entirely the aggregates in *C. sorokiniana* F&M-M49 (Fig. 3b) and significantly reduced their dimension in *C. vulgaris* Allma (Fig. 4b). Few cells were completely disintegrated after sonication in both microalgae, although a cell weakening due to sonication, cannot be ruled out. From the microscopic observations, it is possible to underline that the sonication treatment dispersed the cells but did not

improve significantly the digestibility, on the other hand also the digestion process without sonication treatment allowed complete cells dispersion (Fig. 3c) or reduced aggregate dimensions (Fig. 4c) thanks to the adoption of a mixing regimen. To our knowledge, no literature is available concerning sonication methods for increasing the DMD of microalgae.

The data obtained in this study confirm the positive effect of the sonication pretreatment and of the continuous stirring on cell dispersibility without a significant improvement in digestibility of these *Chlorella* strains.

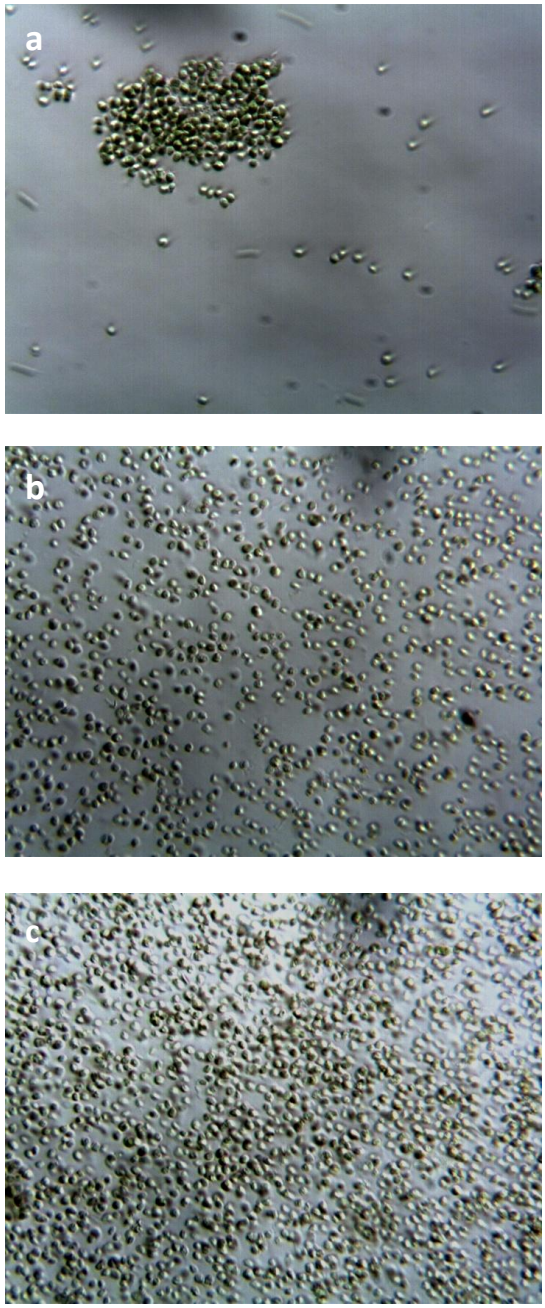

Fig. 3. Microscopic aspect of *C. sorokiniana* F&M-M49 suspensions before sonication (a), after sonication without digestion (b) and after digestion without sonication treatment (c). Magnification 400x. Photomicrograph by the authors

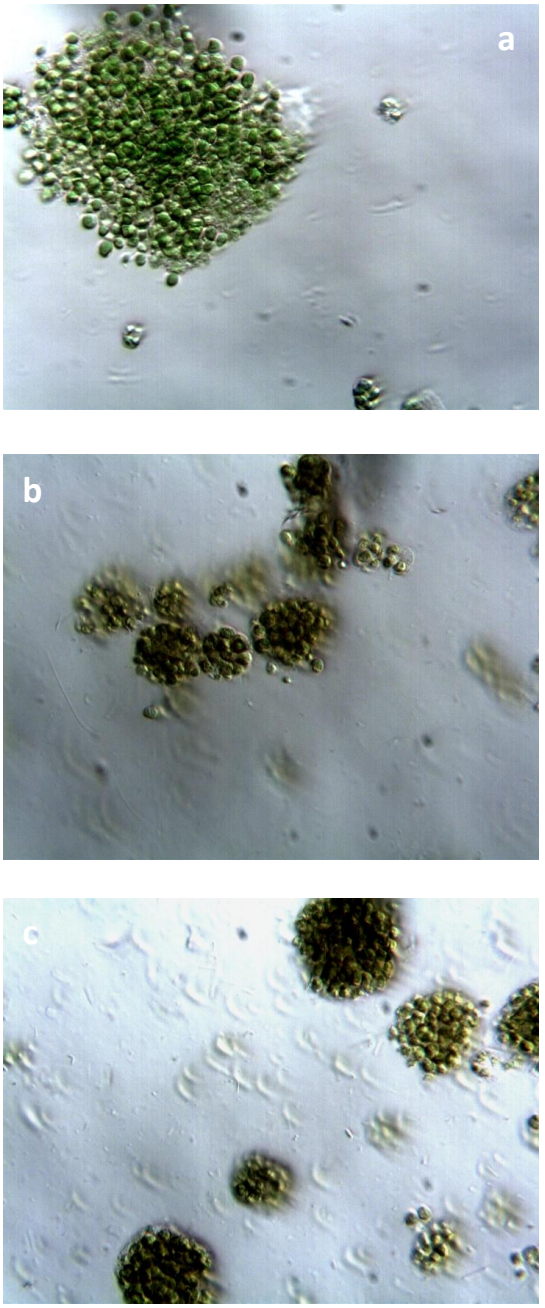

Fig. 4. Microscopic aspect of *C. vulgaris* Allma suspensions before sonication (a), after sonication without digestion (b) and after digestion without sonication treatment (c). Magnification 400x. Photomicrograph by the authors

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Conflict of interest

The authors declare that they have no competing interests.

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Chapter 5

Safety evaluation and effects on lipid metabolism of a diet rich in *Arthrospira platensis* F&M-C256 biomass, in rats.

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Safety evaluation and effects on lipid metabolism of a diet rich in *Arthrospira platensis* F&M-C256 biomass, in rats.

The work reported in this chapter was carried out at NEUROFARBA Dpt., University of Florence under the guidance of my supervisors, Prof. Mario R. Tredici and Dr. Liliana Rodolfi, and in collaboration with Dr. Natascia Biondi, Dr. Elisabetta Bigagli, Dr. Lorenzo Cinci, Prof. Maura Lodovici, Master student Giulia Mori, and Dr. Cristina Luceri

5

Abstract

Arthrospira platensis (*A. platensis*) is a nutritious food source with several promising health promoting activities. We investigated the dietary safety of *A. platensis* F&M-C256 in rats, explored its potential health promoting effects and the underlying mechanisms.

Male Sprague-Dawley rats were fed AIN-76 diet (control) or the same diet containing 20% *A. platensis* F&M-C256 for 1 month. *A. platensis* F&M-C256 supplemented diet was well-tolerated: no behavioral abnormalities were observed, and food consumption, clinical observations and body weights were not affected (except for liver weight). The high salt content of the algal biomass resulted in an increased water consumption and urine excretion, but not in raised Na^+ plasma levels. No histopathological alterations of the kidney, increased blood pressure or renal oxidative damage were observed. The digestibility of *A. platensis* F&M-C256 diet was slightly lower compared to controls and this was accompanied by an increased feces production and fecal water content. *A. platensis* F&M-C256 DNA content (about 4%) did not increase urinary uric acid excretion. No changes in organs weights or histopathology were observed. Clinical biochemistry parameters did not indicate any renal or hepatic impairment. Total cholesterol and LDL were unchanged, but a significant increase in HDL was found in the *A. platensis* F&M-C256 fed group. The reduction of liver weight was associated to significantly decreased plasma triglycerides, increased excretion of fecal lipids and to the induction of PPAR-α expression in the liver.

A. platensis F&M-C256 is likely to be safe even at high dosages and may represent a promising source of functional foods for the prevention of dyslipidemias.

Keywords: Microalgae; Food; Safety; Arthrospira platensis F&M-C256; Hypolipidemic effects; Dyslipidemias

1. Introduction

Arthrospira platensis, formerly known as *Spirulina platensis*, is a planktonic cyanobacterium that thrives in tropical and subtropical water bodies characterized by high levels of carbonate and bicarbonate and high pH (up to 11) in Africa, Asia, Central and South America (Tomaselli, 1997). *A. platensis* is cultivated worldwide and is the microalga with the largest annual biomass

production, in excess of 5000 t (Borowitzka, 2013). Taking advantage of the selective (high alkalinity) culture medium, most of the *Arthrospira* biomass is produced in open systems, mainly raceway ponds (Belay, 2008; Milledge, 2011), while cultivation in photobioreactors, being more expensive, is mainly limited to research (Tredici & Chini Zittelli, 1997). The largest production facilities are located in China, United States, and India, although diffuse small scale production facilities are common in many countries, including France.

In terms of nutrition, *A. platensis* is a source of macro and micronutrients including proteins, iron, γ-linolenic acid, vitamins, minerals, sulfated polysaccharides and phycocyanin and a number of unexplored bioactive compounds (Kulshreshtha et al., 2008). Protein content and aminoacidic composition are comparable to those of soybean, thus *A. platensis* represents an alternative source of high-quality protein that could meet the needs of malnourished people (Becker, 2007). Currently, *A. platensis* is mainly used as dietary supplement in the health food market, in the form of powder, capsules or tablets. *A. platensis* is also increasingly used as a food ingredient, incorporated into pasta, biscuits, bread, candies, yogurt and soft drinks (Christaki et al. 2011; Pulz and Gross 2004).

Although the safety of *Arthrospira* for human consumption is supported by its long history of use as food (Abdulqader et al., 2000; Belay, 2008) also recognized in the food regulations of most countries (e.g. Europe, Australia, US) (Borowitzka, 2013; Niccolai et al., 2016), further risk assessment should be conducted. Several toxicity studies have been performed both in *in vitro* and *in vivo* models. Although *in vitro* models are faster, easier and less expensive they can only be adopted in pre-screenings, as they are unable to provide information on the interactions in complex organisms, thus studies in *in vivo* models are still necessary (Niccolai et al., 2016). Few *in vivo* toxicological studies are available in the literature. Salazar et al. (1998) in a sub-chronic toxicity study found no adverse effects in mice fed *Arthrospira maxima* up to 30% in the diet. Among the few reports on *A. platensis*, Hutadilok-Towatana et al. (2008) tested the acute and sub-chronic effects of dietary supplementation in mice and rats, respectively, observing no adverse effects.

In addition to its nutritional value, *Arthrospira* shows several activities of pharmacological interest such as antioxidant (Khan et al., 2005), anti-inflammatory (Mao et al., 2000; Ali et al., 2015). Hypolipidemic effects have been demonstrated for *A. maxima* and *Arthrospira* spp. in various animal models (Torres-Durán et al., 1999; Blé-Castillo et al., 2002; Moura et al., 2011;

Muga et al., 2014). Hypolipidemic activity of *A. platensis* have been also demonstrated in mice and in rats (Kato et al., 1984; Iwata et al., 1990) and in rabbits (Colla et al., 2008; Kim et al., 2010). In all these studies, the hypolipidemic effects were investigated in chemically or dietary-induced experimental models of hyperlipidemias. To date, the knowledge of *A. platensis* effects in a balanced, normo-fat diet is limited. The aim of this study was therefore to investigate the dietary safety of 20% *Arthrospira platensis* F&M-C256 in a 4-week feeding trial in rats, verify its effects on lipid metabolism and explore the underlying mechanisms.

2. Materials and methods

2.1 Biomass production, preparation and gross composition

Arthrospira plantesis F&M-C256 (*A. platensis* F&M-C256) from the F&M Culture Collection of Microalgae and Cyanobacteria was cultivated in GWP® photobioreactors (Tredici et al., 2004) in semi-batch mode. The biomass was harvested by centrifugation, washed with NaCl solution, frozen, lyophilized and powdered. The powdered biomass was stored at -20°C until use. Elemental analysis was performed on dry biomass using a CHNSO Analyzer (Flash EA, 1112 Series, Thermo Electron Corporation, Massachusetts, USA) (Gnaiger and Bitterlich, 1984). Total protein content was estimated as N x 6.25, where N is the nitrogen content determined through the elemental analysis. Carbohydrate was determined following Dubois et al. (1956) and lipid following Marsh & Weinstein (1966). Humidity was analyzed following ISTISAN protocols (ISTISAN Report 1996/34, Method B, Page 7). Fiber was determined according to AOAC (AOAC Official method 985.29). Protein, carbohydrate, lipids and fiber content were 59%, 16%, 7% and 12.4%, respectively.

2.2 Diet preparation

Dietary components (Piccioni, Gessate, Milan, Italy) were based on the American Institute of Nutrition (AIN)-76 diet containing 5% fat (corn oil). The microalga-supplemented diet was modified to compensate for proteins, lipids, carbohydrate and fibers deriving from *A. platensis* F&M-C256 biomass (**Table 1**).

Table 1. Composition of the experimental diets

g/100 g of diet (d.w.)	AIN-76 (control diet)	*A. platensis* F&M-C256 supplemented AIN-76
Dry frozen biomass	-	**20**
Corn oil	5	**3.6**
Sucrose	50	**50**
Starch	15	**11.8**
Casein	20	**8.2**
Cellulose	5	**2.5**
Mineral Mix AIN 76	3.5	**3.5**
Vitamin Mix AIN 76	1	**1**
Coline	0.2	**0.2**
DL Methionine	0.3	**0.3**

2.3 Animals and treatment design

All procedures were carried out in agreement with the European Union Regulations on the Care and Use of Laboratory Animals (OJ of ECL 358/1, 12/18/1986), according to Italian regulations on the protection of animals used for experimental and other scientific purposes (DM 116/1992), after approval from the Italian Ministry for Scientific Research. After 1 week of acclimatization in plastic cages, male Sprague-Dowley rats, aged 6-8 weeks, were divided in two experimental groups. Rats were fed AIN-76 (control diet) or *A. platensis* F&M-C256 supplemented AIN-76 *ad libitum* for 1 month (N = 8/diet group). The microalga-supplemented diet contained 20% of lyophilized microalgal biomass. Water was available *ad libitum*, the lighting regime was a standard 12 h light and 12 h dark, temperature was maintained constant at 21±2°C. At the end of the study, rats were euthanized by inhalation of CO_2. Following the sacrifice, selected organs and tissues (liver, kidney, colon, caecum, bladder, stomach, heart) were weighed and stored for macroscopic and histopathology examination, for the determination of oxidative stress parameters and gene expression. Blood was collected for clinical analyses (lipid profile, kidney and liver function).

2.4 Clinical observations and body weights

All animals were observed daily for morbidity and mortality. Detailed physical examinations were conducted on all animals weekly, beginning one week prior to the beginning of the experiments and one week prior to necropsy. Individual

animal body weights were recorded weekly starting from the first day of the experiment. Mean body weights and mean body weight changes were calculated for the corresponding intervals. Final body weights were recorded prior to sacrifice.

2.5 Feed consumption and digestibility of the diets

During the third week of treatment, the animals were placed in metabolic cages for one day in order to collect 24-hour urine and feces, to assess the palatability of the diet by measuring food daily consumption, to measure water daily consumption and the diet digestibility. Samples of feed and fecal samples were collected, weighed and oven-dried at 55°C until constant weight to determine humidity. The coefficient of digestibility was calculated as follows: Digestibility % = [feed dry weight – 24-hours feces dry weight /feed dry weight] x 100.

2.6 Clinical pathology

Blood samples were collected from all animals prior to scheduled necropsy. Plasma and urinary chemical parameters were performed at the General Laboratory of the Azienda Ospedaliero Univesitaria di Careggi, Florence, Italy, and included: urea, creatinine, alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol, LDL, HDL, uric acid, and sodium.

2.7 Macroscopic and microscopic examinations

A complete necropsy was conducted on all rats. The necropsy included an examination of the external surface, of all orifices, and of the cranial, thoracic, abdominal and pelvic cavities, including viscera. Selected tissues and organs were collected and placed in 10% neutral-buffered formalin. At necropsy, the following organs of all animals were weighed: brain, heart, kidneys, liver, bladder, caecum, colon, spleen.

2.8 Histological analysis

For histological evaluations tissue samples of heart, kidneys, liver, urinary bladder and brain were collected and fixed in 4% phosphate buffered formalin for 12 hours. Samples were dehydrated in ethanol and embedded in paraffin. 5-µm thick sections were hematoxilin-eosin stained and observed for

morphological analysis. Regarding heart, the thickness of the ventricular wall was measured. In detail, five microscopical fields per animal were registered by a digitizing camera applied to a light microscope with a 20x objective, each field corresponding to a test area of 141,100 μm^2. On the digitized images measurements of ventricular wall thickness were carried out using ImageJ 1.33 image analysis software (http://rsb.info.nih.gov/ij) by two independent observers in blind fashion. 5-µm thick sections of frozen liver and feces were obtained and stained with Sudan Black (Sigma Aldrich, Milan Italy) in order to evaluate the presence of neutral triglycerides and lipids.

2.9 Blood Pressure Measurements

Systolic, diastolic blood pressure, mean arterial pressure (MAP) and hearth frequency were monitored in conscious rats by noninvasive computerized tail-cuff method (Visitech BP-2000 Series II Blood Pressure Analysis System, Virginia, USA) at week 3 of treatment after two days of training.

2.10 Fecal water content

Fresh fecal samples were harvested when the rats were killed and frozen at -20°C until analyses. To determine the degree of diarrhea, fecal samples were dried in a dehumidified oven at 50°C until constant weight and the water content was expressed as a percentage of the fresh fecal weight (Castagnini et al., 2009).

2.11 Tibio-tarsal joint edema assessment

The thickness of the tibio-tarsal joint of rats fed *A. platensis* F&M-C256 supplemented AIN-76 diet was measured by using a micrometer (screwgauge) and compared to the joint thickness of the control group.

2.12 RT-PCR

RNA was extracted from tissue homogenates by using a commercially available kit following manufactures' instructions (Macherey-Nagel, Bethlehem, USA). For first-strand cDNA synthesis, 1 µg of total RNA from each sample was reverse-transcribed by using the RevertAid RT Kit (Thermo Scientific, Waltham, MA, USA). Primers were designed on the basis of the rat GenBank sequences (**Table 2**). For each target gene, the relative amount of mRNA in the

samples was calculated as the ratio of each gene to b-actin mRNA (Luceri et al., 2002).

Table 2. Primer sequences

Gene	Primer forward	Primer reverse	Base pair
b-actin	ACCACAGCTGAGAGGGAAATC	AGAGGTCTTTACGGATGTCAACG	281
OGG1	CCTGGCTGGTCCAGAAGTAG	TTTCCCAGTTCTTTGTTGGC	345
APEX	GCTCAGAGAACAAACTCCCG	TTGTTTCCTTTGGGGTTACG	385
UNG	TCCGGACCCCGACTCCTGGC	GCGGGGGTGGAACTGGCCTC	419
SOD2	TTAACGCGCAGATCATGCAGC	TGGCCTTATGATGACAGTGAC	807
PPAR-a	GTCCTCTGGTTGTCCCCTTG	CCTCTCCGAGGGACTGAGAA	389
HMGCR	CAGCTGTACCATGCCGTCTA	AAAGAGCCAGAAACCAAGCA	433

2.13 Microalgal DNA content

DNA was extracted from 50 mg of microalgal biomass by sonication in 500 µl Lysis buffer (TrisHCl 0.1M; EDTA 5.10-2M ; SDS 0.2%; NaCl 0.2M). Then, 100 µl of Proteinase K (10 mg/ml, Qiagen, Hilden, Germany) were added to 100 µl of lysate and the samples were incubated overnight at 56°C and gently shacked at 300 rpm. At the end of incubation, 20 µl of sodium acetate (3M pH 5.2 Sigma Aldrich, Milan Italy) and 500 µl of absolute ethanol were added and incubated overnight at -80°C. The samples were centrifuged at 14,000 rpm for 30 min. The supernatant was discharged and the pellet was washed twice (300 µl of 70% ethanol) and then centrifuged as described above. Finally, the ethanol was removed and the pellet was re-suspended in 20 µl of H_2O.

2.14 Fecal lipid content

Dried fecal samples were re-suspended in 500 µl of normal saline. Then 500 µl of chloroform-methanol (2:1, v/v) were added in order to extract the lipids. The suspension was then centrifuged at 1,000 g and the solvent was aspirated, and evaporated. The residue was weighed and fecal lipids were expressed as a percentage of fecal dry weight (http://www.bio-protocol.org/e1375).

2.15 Carbonyl residues (CO) and ferric reducing ability of plasma (FRAP)

CO levels in the liver and kidneys were determined by the method of Correa-Salde and Albesa (2009). The FRAP values were measured according to the method used by Benzie and Strain (1996) as described by Lodovici et al. (2015).

2.16 Statistical analysis

Data were analyzed using statistical methods and values were presented as mean with the standard error (SE) and the number of animals (N) used to calculate the mean. Body weight gain, necropsy body weight, clinical chemistry and gene expression were analyzed using un-pair t-test with Mann Whitney correction.

3. Results

3.1 Survival, clinical observations, body weights

All animals survived until the time of sacrifice. At necropsy, in the bladder of 1 out of the eight rats fed *A. platensis* F&M-C256 diet an amorphous formation was found. Excretion of dark-red feces was not observed throughout the feeding period and there were no other treatment-related clinical manifestations. The growth curves of the animals during the study were consistent with historical background data (**Figure 1**, panel A). Compared to the control group, no treatment-related statistically significant effects of the diet rich in microalgal biomass were noted on body weight or weight gain (**Figure 1**, panel A and B, 51.0 ± 6.6 g in control group vs 65.5 ± 8.4 g in *A. platensis* F&M-C256 group).

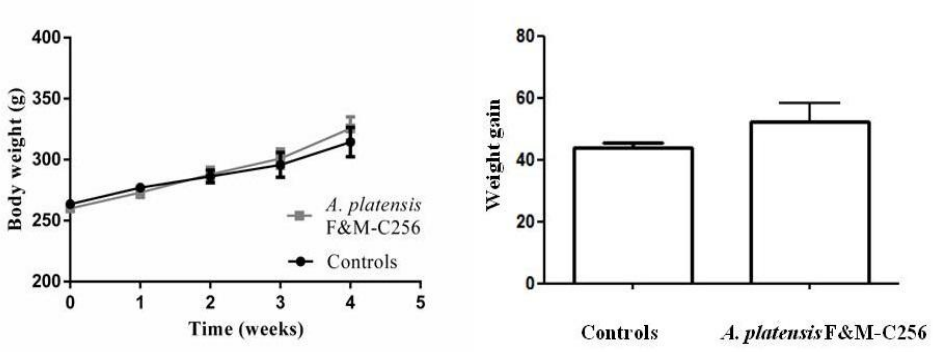

Figure 1. Effect of feeding rats with a 20% *A. platensis* F&M-C256 diet on body weight and weight gain

3.2 Food and water consumption, digestibility of the diet and feces production

The overall feed consumption of animals receiving the *A. platensis* F&M-C256 diet was similar to that of the controls. The palatability of the diets was good since animals completely consumed the food administered daily. The digestibility of the *A. platensis* F&M-C256 diet was quite lower (-0.9%) compared to the control diet. In rats fed the *A. platensis* F&M-C256 rich diet, a tendency (+ 68%) to an increased production of feces was observed. Besides, the fecal water content increased compared to controls (60% vs 50%). It is worth nothing that the treatment with *A. platensis* F&M-C256 was also associated to a significant increase in water consumption compared to control rats (**Table 3**) and this effect was accompanied by an augmented urine excretion.

Table 3. Effects of *A. platensis* F&M-C256 rich diet on water daily consumption, feces production, fecal water content and urine production

	Water daily consumption (ml)	**Urine daily production (ml)**	**Feces daily production (g)**	**Water fecal content (%)**
Control diet (n=4)	21.53 ± 1.68	7.00 ± 1.29	1.85 ± 0.38	50.42
***A. platensis* F&M-C256 diet (n=8)**	27.18 ± 1.24*	12.63 ± 0.99**	2.71 ± 0.38	60.32

*p<0.05; ** p<0.01

3.3 Organ weight

No significant changes in brain, heart, spleen, caecum, kidney and bladder weight to body weight was observed. A significant reduction of liver weight was observed in *A. platensis* F&M-C256 fed rats when compared to controls ($p<0.01$) (**Table 4**).

Table 4. Effects of *A. platensis* F&M-C256 rich diet on group mean organ weight adjusted to necropsy body weight in rats

	brain weight/ body weight (g)	heart weight/ body weight (g)	spleen weight/ body weight (g)	liver weight/ body weight (g)	caecum weight/ body weight (g)	kidney weight/ body weight (g)	bladder weight/ body weight (g)
Control diet (n=4)	0.005	0.004	0.002	0.047	0.009	0.007	0.000
***A. platensis* F&M-C256 diet (n=8)**	0.0052	0.0036	0.002	0.033**	0.0090	0.0067	0.0005

*$p<0.05$

3.4 Effects of A. platensis F&M-C256 *on clinical chemistry parameters*

Serum creatinine values, urea, aspartate transaminase (GOT), alkaline phosphatase and alanine aminotransferase (ALT) did not indicate any renal or hepatic impairment. Compared to control group, total cholesterol concentration was unchanged in the *A. platensis* F&M-C256 fed group, but a significant increase in HDL was found. Triglycerides significantly diminished in *A. platensis* F&M-C256 fed rats (from 187 to 52.5 mg/dL) (**Table 5**). The urinary uric acid content was similar in the two groups (10.8 ± 1.3 mg/dL in the control group vs 11.9 ± 2.9 mg/dL in the *A. platensis* F&M-C256 group). No differences in sodium excretion were observed (74 ± 10.7 mEq/L vs 82.3 ± 5 mEq/L in controls and *A. platensis* F&M-C256 fed rats respectively).

Table 5. Effect of *A. platensis* F&M-C256 on clinical chemistry parameters

	Urea (g/L)	**Creatinin (mg/dL)**	**AST U/L**	**ALT U/L**	**Alkaline phosphatase (U/L)**	**Triglycerides (mg/dL)**
Control diet (n=4)	0.42±0.04	0.34±0.06	290.5±176.5	75.0±16	155.0±28.0	187±62
***A. platensis* F&M-C256 diet (n=8)**	0.27±0.02*	0.35±0.01	147.3±14.0	48.8±2.3*	147.0±16.4	52.5±10.9*

*$p<0.05$

Table 5. Continued

	Total cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)
Control diet (n=4)	73.0±4.0	55.0±5.0	5.0±0.01
***A. platensis* F&M-C256 diet (n=8)**	76.2±2.2	72.7±2.4*	5.0±0.01

*p<0.05

3.5 Blood pressure

Systolic and diastolic blood pressure as well as heart rate and MAP were not significantly affected by *A. platensis* F&M-C256 rich diet (**Table 6**).

Table 6. Blood pressure parameters

	Systolic (mm Hg)	Diastolic (mm Hg)	Heart rate (beats/min)	MAP (mm Hg)
Control diet (n=4)	142.9 ± 5.3	81.1 ± 7	386.2 ± 32.3	100.8 ± 6.6
***A. platensis* F&M-C256 diet (n=8)**	135.8 ± 14.5	79.7 ± 6.7	387.5 ± 28.2	99.2 ± 9.7

MAP= mean arterial pressure

3.6 Tibio-tarsal joint edema assessment

No significant difference was found in the mean tibio-tarsal joint thickness between groups, data not shown.

3.7 Histological analysis

Histological analyses conducted on sagittal sections of whole brain did not show any sign of morphological damage in brain or in cerebellar areas. The liver showed a normal morphological structure. In hepatocytes no clear cyto-pathological signs such as steatosis, hemosiderine accumulation or biliary stasis were present. In the bladder of 1 out of the eight rats fed *A. platensis* F&M-C256 diet areas with crystalloid appearance included within amorphous areas were observed. In one kidney of the same animal, a eosinophilic amorphous content was found in the lumen of proximal and distal convoluted tubules (**Figure 2**). The kidneys of the remaining animals did not show any morphological damage.

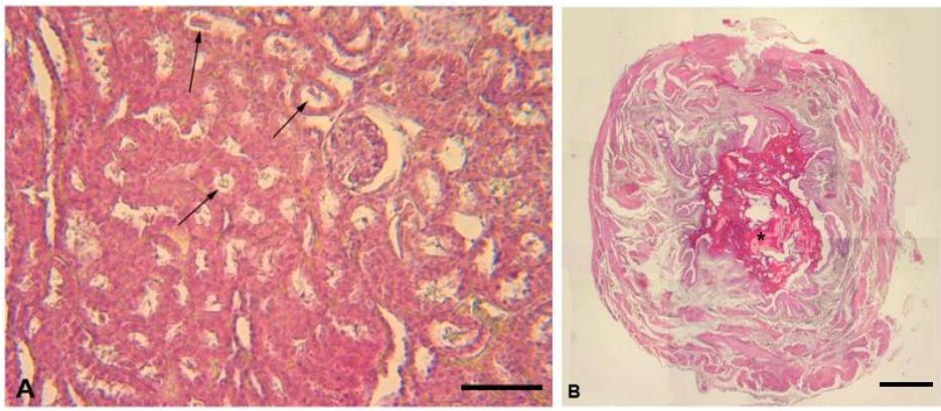

Figure 2. Representative images of amorphous areas in kidney and urinary bladder of one rat fed *A. platensis* F&M-C256. Panel A: digitalized image of kidney with amorphous content in proximal and distal convolute tubules (black arrows). Panel B: digital reconstruction of whole urinary bladder. Amorphous eosinophilic mass with some crystalloid areas was evident in the lumen (black asterisk). Scale bar: 100 μm

3.8 Oxidative stress and antioxidant status in the liver and kidney

FRAP and carbonyl residues assays were performed in order to evaluate the antioxidant status and protein oxidative damage in the liver and kidney. No significant differences were found (data not shown).

3.9 Expression of oxidative stress-related genes in the kidney

To investigate oxidative stress related parameters in the kidney, the expression of the DNA repair enzymes UNG, OGG1, APEX and that of the antioxidant enzyme SOD2, was investigated; the expression of OGG1 was slightly but significantly reduced in *A. platensis* F&M-C256 fed rats compared to controls. No significant treatment related difference in the expression of the other enzymes was observed (**Table 7**).

Table 7. Expression of UNG, OGG1, APEX and SOD2 in the kidney of *A. platensis* F&M-C256 fed rats

	UNG	OGG1	APEX	SOD2
Control diet (n=4)	0.48 ± 0.12	0.52 ± 0.04	0.91 ±0.17	0.94 ± 0.13
***A. platensis* F&M-C256 diet (n=8)**	0.53 ± 0.05	0.38 ± 0.04*	0.71 ± 0.08	0.81± 0.12

*p<0.05. The relative amount of mRNA in the samples was calculated as the ratio of each gene to the housekeeping gene, β-actin.

3.10 Expression of lipid metabolism-related genes in the liver

To explore the possible molecular basis of the observed effects on lipid metabolism, we analyzed the expression of PPAR-α and HMGCR genes in the liver. We did not find any differences in HMGCR expression between the two groups (0.63 ± 0.04 in the controls vs 0.61 ± 0.02 in *A. platensis* F&M-C256 fed rats), but we found a significant over expression of PPAR-α in the liver of rats fed *A. platensis* F&M-C256 diet (**Figure 3**).

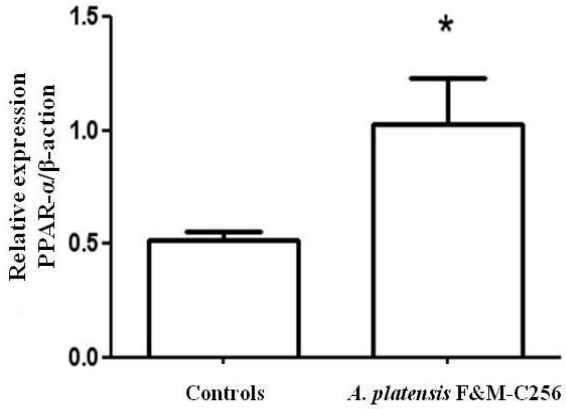

Figure 3. Expression of PPAR-α in the liver of *A. platensis* F&M-C256 fed rats and in rats fed standard diet (Controls).*= p<0.05

3.11 Fecal lipids excretion

Compared to the control group, rats fed *A. platensis* F&M-C256 diet showed an increased (+56%) fecal lipid excretion (**Table 8**).

Table 8. Percentage of lipids in stool samples of *A. platensis* F&M-C256 fed rats and in rats fed control diet

	Fecal lipid excretion (%)
Control diet (n=4)	13.7 ± 2.5
***A. platensis* F&M-C256 diet (n=8)**	21.4 ± 2.7*

*$p<0.05$

3.12 Microalgal DNA content

DNA content in *A. platensis* biomass was 4% of dry weight.

4. Discussion

In the last years, there has been a growing interest in functional foods because of the beneficial health effects that they can promote and their potential to prevent chronic diseases such as diabetes, obesity, cardiovascular diseases and metabolic syndrome. Microalgae are an almost unexplored natural source of bioactive ingredients of high nutritional and nutraceutical value. The first concern when proposing microalgae as food or food ingredients for human consumption is to establish their lack of toxicity towards the consumer. In this regard, several species of different algal groups have been reported to produce neurotoxins, hepatotoxins, diarrheic toxins, dermotoxins, which are usually active at very low doses (Landsberg, 2002). Besides toxins, microalgae are able to produce other secondary metabolites that may have noxious effects on human health (Tredici et al., 2009). We assessed the tolerability of a diet containing a relatively high amount of *Arthrospira platensis* F&M-C256 biomass in rats. Overall our results indicate that a diet supplemented with 20% algal biomass was well tolerated during the 30 day feeding period and no treatment-related morbidity or mortality was detected. Some previous studies on the safety of microalgae tested from 1% to 20% biomass in the diet (Stewart et al., 2008). The high amount of biomass in our experimental diet (20%) was chosen to evaluate the potential of microalgae as foods or food components for human use. Data reported so far indicate a general safety profile of *A. platensis* in animals at dosages up to 120 mg/kg. The novelty of this study is in the fact that the sub-chronic toxicity of *A. platensis* F&M-C256 was investigated at doses compatible with dietary supplementation and did not take into account the historical use of this microalga as food whose dosage might vary from 10-40 g

per person a day (as Dih´e biscuits in Africa) (Ciferri, 1983; Marles et al., 2011).

The NOAELs (No Observed Adverse Effect Level) appear to be over 10 g/kg BW/d for *A. platensis* and 30% in the diet for *Arthrospira* sp. (corresponding to 30 g/kg BW/d) (Chamorro et al. 1988). Human studies also showed that *A. platensis* at a dose of 1.7 g/kg BW did not result in any adverse effects in children (Simpore et al., 2005). Yamani et al. (2009) indicated that an intake of about 10 g of spirulina per day for 6 months, did not induce adverse effects in HIV patients. However, some adverse effects were reported as reviewed in Marles et al. (2011).

The nucleic acid content is an important concern since the biochemical degradation of leads to uric acid, which can produce kidney stones and induce gout attacks in the long term. *Arthrospira* content of nucleic acids is about 4-6% of its dry weight (Gutiérrez-Salmeán et al., 2015). The World Health Organization recommends that the daily total nucleic acid consumption in humans should not exceed 4 g, which represents the nucleic acid content of about 80 g of dry *Arthrospira* biomass. Feeding a diet containing 20% of *A. platensis* F&M-C256 in rats corresponds to feeding 3 g of biomass a day; given that the DNA content of *A. platensis* F&M-C256 is about 4% we can estimate that the total content of nucleic acids (DNA+RNA) is around 10%, which translates into 0.3 g of nucleic acid per day. It is therefore reasonable to consider that the nucleic acid content of *A. platensis* F&M-C256 does not pose health problems, even at high dosages. The amorphous neo-formation found in the bladder of 1 out of 8 *A. platensis* F&M-C256 fed rats, suggestive of an uric acid crystal and possibly related to the DNA content of microalgae was therefore interpreted as a finding of sporadic occurrence since no other morphological alterations at bladder or kidney level were found. Feeding the diet containing *A. platensis* F&M-C256 did not affect food consumption, clinical observations and body weights indicating that the overall health status of the animals was not affected. To our knowledge, no detailed data have been reported on *in vivo* digestibility of a diet containing *A. platensis* F&M-C256. We observed that the diet containing spirulina was slightly less digestible than controls and that the fecal water content was higher suggesting that *A. platensis* F&M-C256 might have a probiotic effect. This is supported by previous findings (Parada et al., 1998).

Since *A. platensis* F&M-C256 has a quite high Na^+ content (555 mg/100 g), long-term consumption of this alga especially in a large amount would

probably lead to hypernatremia and its related disorders. In our sub-acute toxicity study, we could not detect any increases in Na^+ plasma levels. This indicates no accumulation of Na^+ in the circulation due to continuous intake of *A. platensis* even at high dosage. Moreover, no microscopic histopathological alterations of the kidney, neither blood pressure or oxidative stress related damage in the kidney were observed. Of course, we cannot exclude that the high Na^+ content could represent a potential harm of *A. platensis* F&M-C256 over long term consumption. At organ level, no macroscopic and/or histopathological observations suggestive of adverse effects due to the treatment were noted, including any signs of steatosis or hepatocellular damage. Similarly, we did not observe any change of biological significance in organ weights with the exception of the liver. In our experiment, the reduction of liver weight was associated to a significant reduction in the levels of triglycerides and an increased excretion of fecal lipids: 126 mg in rats fed control diet and 342 mg in rats fed *A. platensis* F&M-C256.

Data from preclinical studies with various animal models, demonstrated the hypolipidemic activity of *Arthrospira*. A high fat diet supplemented with *Arthrospira* resulted in a significant reduction in total serum cholesterol, LDL, VLDL cholesterol and increased HDL in mice and in rats (Kato et al., 1984; Iwata et al., 1990) in hamsters (Riss et al., 2007) and rabbits (Colla et al., 2008). The ingredients responsible for this hypolipidaemic activity remain to be identified, but C-phycocyanin has been suggested as the active component (Nagaoka et al., 2005). All these studies were performed in experimentally-induced hypercolesterolemia or by using pro-atherogenic, high risk diets. Our results demonstrate that spirulina is able to reduce risk factors for cardiovascular diseases even in a well balanced dietary regimen. This result is of particular interest because it suggests that the dietary use of this microalga might be useful in the prevention of dyslipidemias other than in the cure of such disorder.

The lipid-lowering effects of *Arthrospira* have been described in type 2 diabetes (Lee et al., 2008), nephrotic syndrome (Samuels et al., 2002), and in hypercholesterolemic patients (Park et al., 2008). Recently, *Arthrospira* supplementation at a dose of 1 g per day for 12 weeks, resulted in a significant reduction of triglycerides concentration in dyslipidaemic patients (Mazokopakis et al., 2014). A recent meta-analysis revealed a significant effect of supplementation with spirulina in reducing plasma concentrations of total cholesterol, LDL-C and triglycerides, and increasing those of HDL-C (Serban et al., 2016).

Our and previous results suggest that *A. platensis* influence lipid metabolism in the liver but to date, the underlying mechanisms for the hypolipidemic effect, are not known. Some experimental studies have attributed the beneficial effects of spirulina to its antioxidant capacity (Kim et al., 2010). However in our experimental settings, total antioxidant status in the plasma or liver oxidative damage were unchanged. We therefore analyzed the hepatic expression of PPAR-α gene encoding for proteins that control lipoprotein metabolism. Treatment with fibrates, a widely used class of lipid-modifying agents, results in a substantial decrease in plasma triglycerides and is usually associated with an increase in HDL cholesterol concentrations through the activation of the peroxisome proliferator-activated receptors (PPAR-α). Very interestingly, our results demonstrate for the first time that feeding a diet containing *A. platensis* F&M-C256 for one month induces the expression of PPAR-α in the liver, thus providing a mechanistic explanation of the observed hypolipidemic effect.

5. Conclusions

Feeding rats a diet containing 20% of *A. platensis* F&M-C256, which results in daily dose of 12 g/kg, is without significant adverse effects. The extrapolated ADI (Acceptable Daily Intake) for humans results to be 120 mg/Kg which is in line of any anticipated human consumption (8.4 g/d) suggesting that the use of *A. platensis* F&M-C256 as food or food component for humans, is likely to be safe even at high dosage. Our observations further support previous findings indicating that consumption of *A. platensis* F&M-C256 is without significant adverse effects and that this microalga exerts beneficial effects on risk factors for cardiovascular diseases. Besides, *A. platensis* F&M-C256 might represent an emerging and promising source of functional foods or nutraceuticals for the prevention of dyslipidemias.

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Conflict of interest

The authors declare that they have no competing interests.

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Chapter 6

Safety evaluation and hypotriglyceridemic activity of a diet rich in the marine microalga *Tisochrysis lutea* (T-ISO) F&M-M36: a sub-acute study in rats

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Safety evaluation and hypotriglyceridemic activity of a diet rich in the marine microalga *Tisochrysis lutea* (T-ISO) F&M-M36: a sub-acute study in rats

The work reported in this chapter was carried out at NEUROFARBA Dpt., University of Florence under the guidance of my supervisors, Prof. Mario R. Tredici and Dr. Liliana Rodolfi, and in collaboration with Dr. Natascia Biondi, Dr. Elisabetta Bigagli, Dr. Lorenzo Cinci, and Dr. Cristina Luceri

Abstract

Tisochrysis lutea is a marine microalga, mainly used in aquaculture, which contains biologically active compounds with potential applications in functional foods and nutraceuticals. We investigated the dietary safety of 1 month feeding *Tisochrysis lutea* F&M-M36 in rats, explored its potential heath promoting effects and the underlying mechanisms.

Male Sprague-Dawley rats were fed AIN-76 diet (control) or the same diet containing 20% *T. lutea* F&M-M36 for 1 month. *T. lutea* F&M-M36 supplemented diet was well-tolerated since food consumption, clinical observations and body weights were not affected. The high salt content of this microalga (6.1%) resulted in an increased water consumption and in a significative raised urinary Na^+. No significant changes in brain, spleen, caecum, kidney, bladder weight to body weight, increase blood pressure or renal oxidative damage were observed. While, a significant increase in heart and liver weight was observed in *T. lutea* F&M-M36 treated group. The digestibility of *T. lutea* F&M-M36 diet was slightly less compared to controls (-4%) and this was accompanied by an increased feces production and fecal water content. *T. lutea* F&M-M36 DNA content was 4% and caused slightly urinary uric acid excretion. Clinical biochemistry parameters did not indicate any renal or hepatic impairment, but a significant increase in total cholesterol and HDL was found in *T. lutea* F&M-M36 group. An increase expression of HMGCR in the liver of the *T. lutea* F&M-M36 group compared to control diet fed rats was observed.

T. lutea F&M-M36 could represent a promising source of functional foods and nutraceuticals.

Keywords: Safety; Microalgae; Food; Tisochrysis lutea F&M-M36

1. Introduction

Microalgae are considered one of the most promising sources for new functional foods or nutraceuticals thanks to their balanced biochemical composition, high nutritional value and their capacity to produce several bioactive compounds (Bishop and Zubeck, 2012). Depending on the species, microalgae contain vitamins, essential elements, essential amino acids and polyunsaturated fatty acids (PUFAs) (Batista et al., 2013). Alternative sources of PUFAs are clearly desirable, and some microalgae which synthesise these

fatty acids are particularly attractive, especially because these ingredients are naturally encapsulated, retarding oxidation processes (Borowitzka, 2013).
Notably, microalgae also contain phycobiliproteins and a wide variety of carotenoids such as fucoxanthin known for its ability to reduce the risk of atherosclerosis, diabetes, cancer and metabolic syndrome (Chacon-Lee and Gonzalez-Marino, 2010) and astaxanthin, an antioxidant, anti-inflammatory and insulin sensitizing molecule (Yuan et al., 2011). Furthermore, the yield, quality and diversification of phytochemicals may be easily maintained or changed by controlling their culture media and growth conditions, which may be improved to ameliorate the concentrations of specific compounds (de Jesus Raposo and de Morais, 2015). Although microalgae are rich sources of these functional compounds, more research is needed before their ingredients can be used in foods (Niccolai et al., 2016). The marine microalga *Isochrysis galbana* is a rich source of PUFAs, mainly eicosapentaenoic acid (EPA; 20:5ω3) (Christaki et al., 2011; Custódio et al., 2014) and of fucoxanthin (Kim et al., 2012), a promising ingredient in the food and feed industries (Batista et al., 2013; Custódio et al., 2014; Thu et al., 2015). The possibility to use *I. galbana* microalga biomass as a new functional ingredient in biscuits and pasta has been evaluated by Gouveia et al. (2008) and Fradique et al. (2013), with good results in terms of color, texture and fatty acid profile (Gouveia et al., 2008; Fradique et al., 2013). Herrero et al. (1993) performed a one month study to assess the nutritional properties of 12% protein *I. galbana* in rats and found out that microalga rich diet reduced triglycerides levels, a significantly reduced weight increase and increased water consumption compared to control diet. Orally administered *I. galbana* (50 mg/day) for 2 months also promoted body weight loss in control rats and maintained weight in diabetic rats (Nuño et al., 2013). These findings suggest that *I. galbana* might represent a new source of healthy food for human consumption. In this regard, no comprehensive study on the dietary safety of *Tisochrysis lutea* has been conducted so far. We, therefore, performed a sub-acute toxicity study in rats fed 20% *T. lutea* F&M-M36 in the diet.

2. Materials and methods

2.1 Biomass production, preparation, and gross composition

Tisochrysis lutea (T-ISO) F&M-M36 wad provided from Archimede Ricerche S.r.l. in Camporosso, Imola (Italy). The microalga was cultivated in GWP® photobioreactors (Tredici and Rodolfi, 2004) in semi-batch mode, then the

biomasses were harvested by centrifugation, frozen, lyophilized and powdered. The powdered biomasses were stored at -20°C until analysis.

Elemental analysis was performed on dry biomass using a CHNSO Analyzer (Flash EA, 1112 Series, Thermo Electron Corporation, Massachusetts, USA) (Gnaiger and Bitterlich, 1984). Total protein content was estimated as N x 6.25, where N is the nitrogen content determined through the elemental analysis. Carbohydrate was determined following Dubois et al. (1956) and lipid following Marsh & Weinstein (1966). Humidity was analyzed following ISTISAN protocols (ISTISAN Report 1996/34, Method B, Page 7). Fiber was determined according to AOAC (AOAC Official method 985.29). Protein, carbohydrate, lipids and fiber content were 42%, 4%, 15%, and 18%, respectively. Fatty acid composition was evaluated by Laboratori CLODIA (Verona, Italy). Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), ω-3, ω-6, and ω-9 were 7.1%, 3.8%, 3.8%, 2.6%, 2.7%, and 2.9% of dry weight, respectively.

2.2 Diet preparation, animals and treatment design

Dietary components (Piccioni, Gessate, Milan, Italy) were based on the American Institute of Nutrition (AIN)-76 diet containing 5% fat (corn oil). The microalga-supplemented diet was modified to compensate for proteins, lipids, carbohydrate and fibers deriving from *Tisochrysis lutea* F&M-M36 biomass (**Table 1**).

Table 1. Composition of the experimental diets

g/100 g of diet (d.w.)	AIN-76 (control diet)	*Tisochrysis lutea* F&M-M36 supplemented AIN-76
Dry frozen biomass	-	**20**
Corn oil	5	**1.7**
Sucrose	50	**50**
Starch	15	**13.3**
Casein	20	**11.7**
Cellulose	5	**1.4**
Mineral Mix AIN 76	3.5	**3.5**
Vitamin Mix AIN 76	1	**1**
Coline	0.2	**0.2**
DL Methionine	0.3	**0.3**

All procedures were carried out in agreement with the European Union Regulations on the Care and Use of Laboratory Animals (OJ of ECL 358/1, 12/18/1986), according to Italian regulations on the protection of animals used for experimental and other scientific purposes (DM 116/1992), after approval from the Italian Ministry for Scientific Research.
We used 6- to 8-week male Sprague-Dawley rats. The animals were housed in plastic cages with wire tops and maintained at a temperature of 22°C, with a 12:12-h light-dark cycle, according to the European Union Regulations on the Care and Use of Laboratory Animals [European Community (1986) European community regulations on the care and use of laboratory animals (Law 86/609/EC)]. After their arrival from the supplier, animals were quarantined for a few days, during which they were fed a standard lab chow. Rats were then randomly allocated to two experimental groups: Rats fed AIN-76 diet (controls) or a microalgae supplemented AIN-76 *ad libitum* for 1 month (N = 8).
At the end of the study, rats were euthanized by inhalation of CO_2. Following the sacrifice, selected organs and tissues (liver, kidney, colon, caecum, bladder, stomach, heart) were weighed and stored for macroscopic and histopathology examination, for the determination of oxidative stress parameters and gene expression. Blood was collected for clinical biochemistry (lipid profile, kidney and liver function).

2.3 Clinical observations and body weight

All animals were observed daily for morbidity and mortality. Detailed physical examinations were conducted on all animals weekly, beginning one week prior to the beginning of the experiments and one week prior to necropsy. Individual animal body weights were recorded weekly starting from the first day of the experiment. Mean body weights and mean body weight changes were calculated for the corresponding intervals. Final body weights were recorded prior to sacrifice.

2.4 Feed consumption and digestibility of the diet

During the third week of treatment, the animals were placed in metabolic cages for one day in order to collect 24-hour urine and feces, to assess the palatability of the diet by measuring food daily consumption, to measure water daily consumption and the diet digestibility. Samples of feed and fecal samples were collected, weighed and oven-dried at 55°C until constant weight to determine

humidity. The coefficient of digestibility was calculated as follows: Digestibility % = [feed dry weight – 24-hours feces dry weight /feed dry weight] x 100.

2.5 Clinical pathology

Blood samples were collected from all animals prior to scheduled necropsy. Plasma chemistry parameters were performed at the General Laboratory of the Azienda Ospedaliero Univesitaria di Careggi, Florence, Italy and included: urea, creatinine, alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol, LDL, and HDL.

2.6 Macroscopic and microscopic examinations

A complete necropsy was conducted on all rats. The necropsies included an examination of the external surface, all orifices, and the cranial, thoracic, abdominal and pelvic cavities, including viscera. Selected tissues and organs were collected and placed in 10% neutral-buffered formalin. At necropsy, the following organs were weighed from all animals: brain, heart, kidneys, liver, bladder, caecum, colon, spleen.

2.7 Histological analysis

For histological evaluations tissue samples (heart, kidney, liver, urinary bladder, and brain) were collected and fixed in phosphate buffered 4% formalin for 12 hours. Samples were dehydrated in ethanol and embedded in paraffin. 5 µm tick sections were hematoxilin-eosin stained and observed for morphological analysis. Regarding heart, the thickness of ventricular wall was measured. In detail, five microscopical fields per animal were registered by a digitizing camera applied to a light microscope with a 20x objective, each field corresponding to a test area of 141,100 μm^2. On digitized images measurements of ventricular wall thickness were carried out using ImageJ 1.33 image analysis software (http://rsb.info.nih.gov/ij) by two independent observers in blind fashion. 5 µm tick sections of frozen liver and feces were obtained and stained with Sudan Black (Sigma Aldrich, Milan, Italy) in order to evaluate the presence of neutral triglycerides and lipids.

2.8 Blood Pressure Measurements

Systolic, diastolic blood pressure, mean arterial pressure (MAP) and frequency were monitored in conscious rats by noninvasive computerized tail-cuff method (Visitech BP-2000 Series II Blood Pressure Analysis System) at week 3 of treatment after two days of training.

2.9 Fecal water content

Fresh fecal samples were harvested when the rats were killed and frozen at -20°C until analyses. To determine the degree of diarrhea, fecal samples were dried in a dehumidified oven at 50°C until stable in weight and the water content was expressed as a percentage of the fresh fecal weight (Castagnini et al., 2009).

2.10 Tibio-tarsal joint edema assessment

The thickness of the tibio-tarsal joint of rats fed *T. lutea* F&M-M36 supplemented AIN-76 diet was measured by using a micrometer (screwguage) and compared to the joint thickness of control group.

2.11 RT-PCR

RNA was extracted from tissue homogenates by using a commercially available kit following manufactures' instructions (Macherey-Nagel, Bethlehem, USA). For first-strand cDNA synthesis, 1 µg of total RNA from each sample was reverse-transcribed by using the RevertAid RT Kit (Thermo Scientific, Waltham, MA USA). Primers were designed on the basis of the rat GenBank sequences (**Table 2**). For each target gene, the relative amount of mRNA in the samples was calculated as the ratio of each gene to b-actin mRNA (Luceri et al., 2002).

Table 2. Primer sequences

Gene	Primer forward	Primer reverse	Base pair
b-actin	ACCACAGCTGAGAGGGAAATC	AGAGGTCTTTACGGATGTCAACG	281
OGG1	CCTGGCTGGTCCAGAAGTAG	TTTCCCAGTTCTTTGTTGGC	345
APEX	GCTCAGAGAACAAACTCCCG	TTGTTTCCTTTGGGGTTACG	385

UNG	TCCGGACCCCGACTCCTGGC	GCGGGGGTGGAACTGGCCTC	419
SOD2	TTAACGCGCAGATCATGCAGC	TGGCCTTATGATGACAGTGAC	807
PPAR-a	GTCCTCTGGTTGTCCCCTTG	CCTCTCCGAGGGACTGAGAA	389
HMGCR	CAGCTGTACCATGCCGTCTA	AAAGAGCCAGAAACCAAGCA	433

2.12 Microalgal DNA content

DNA was extracted from 50 mg of microalgal biomass sonicated in 500 µl Lysis buffer (TrisHCl 0.1M; EDTA 5.10-2M ; SDS 0.2%; NaCl 0.2M). Then, 100 µl of Proteinase K (10mg/ml Qiagen,) were added to 100 µl of lysate and the samples were incubated overnight at 56°C and gently shacked at 300 rpm. At the end of incubation, 20 µl of sodium acetate (3M pH 5.2, Sigma Aldrich, Milan, Italy) and 500 µl of absolute ethanol were added and incubated overnight at -80°C. The samples were centrifuged at 14,000 rpm for 30 min. The supernatant was discharged and the pellet was washed twice (300 µl of 70% ethanol) and then centrifuged as described above. Finally, the ethanol was removed and the pellet was re-suspended in 20 µl of H2O.

2.13 Fecal lipid content

Dried fecal samples were re-suspended in 500 µl of normal saline. Then 500 µl of chloroform-methanol (2:1, v/v) were added in order to extract the lipids. The suspension was then centrifuged at 1,000 g and the solvent was aspirated, and evaporated. The residue was weighed and fecal lipids were expressed as a percentage of fecal dry weight (http://www.bio-protocol.org/e1375).

2.14 Carbonyl residues (CO) and ferric reducing ability of plasma (FRAP)

CO levels in the liver and kidneys were determined by the method of Correa-Salde and Albesa (2009). The FRAP values were measured according to the method used by Benzie and Strain (1996) as described by Lodovici et al. (2015).

2.15 Statistical analysis

Data were analyzed using statistical methods and values were presented as mean with the standard error (SE) and the number of animals (N) used to calculate the mean. Body weight gain, necropsy body weight, clinical chemistry

and gene expression were analyzed using un-pair t-test with Mann Whitney correction.

3. Results

3.1 Survival, clinical observations, body weights

All animals survived at the time of sacrifice. At necroscopy, the abdominal fat, but not the brain from rats fed *T. lutea* F&M-M36 were colored in orange. In the bladder from 1 out of the eight rats fed *T. lutea* F&M-M36 an amorphous formation was found. Excretion of dark-red feces was not observed throughout the feeding period and there were no other treatment-related clinical observations. The growth curves of the animals fed *T. lutea* F&M-M36 were similar to those of the controls (**Figure 1**, panel A). However, when we compared the body weight gain of rats fed *T. lutea* F&M-M36 to that in the control group, we observed a tendency to a lower growth (p=0.06) (**Figure 1**, panel B).

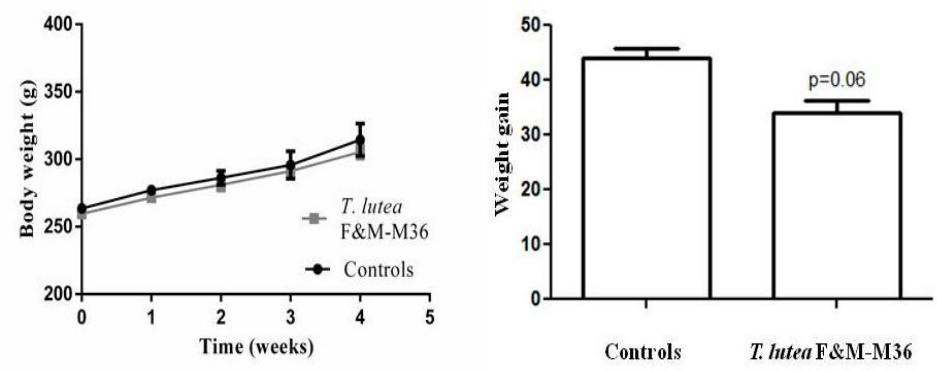

Figure 1. Effect of feeding rats with 20% *T. lutea* F&M-M36 biomass on body weight (panel A) and weight gain (panel B)

3.2 Food and water consumption, digestibility of the diet and feces production

The overall feed consumption of animals receiving *T. lutea* F&M-M36 biomass was similar to that of the controls and animals completely ended the diet administered daily indicating a good palatability of the microalga supplemented diet. The digestibility of the diet was reduced in *T. lutea* F&M-M36 (-4%) fed rats compared to control diet. In rats fed *T. lutea* F&M-M36 rich diet, the

amount of feces produced daily was significantly ($p<0.05$) augmented compared to control rats as well as the percentage of fecal water content. *T. lutea* F&M-M36 supplemented diet led to a doubled water daily consumption and triplicated urine daily production (**Table 3**).

Table 3. Effects of *T. lutea* F&M-M36 on water daily consumption, feces production fecal water content and urine production

	Water daily consumption (ml)	**Urine daily production (ml)**	**Feces daily production (g)**	**Water fecal content (%)**
Control diet (n=4)	21.53 ± 1.68	7.00 ± 1.29	1.85 ± 0.38	50.42
***T. lutea* F&M-M36 diet (n=8)**	42.01 ± 1.90**	21.56 ± 1.13**	3.80 ± 0.36*	64.51*

*p<0.05; ** p<0.01

3.3 Organ weight

No significant changes in brain, spleen, caecum, kidney and bladder weight to body weight was observed. A significant increase in heart weight was observed in *T. lutea* F&M-M36 treated group when compared to control diet ($p<0.05$). A significant reduction of liver weight was also observed in *T. lutea* F&M-M36 fed rats when compared to control diet ($p<0.05$) (**Table 4**).

Table 4. Effects of *T. lutea* F&M-M36 on group mean organ weights adjusted to necropsy body weight in rats

	brain weight/ body weight (g)	heart weight/ body weight (g)	spleen weight/ body weight (g)	liver weight/ body weight (g)	caecum weight/ body weight (g)	kidney weight/ body weight (g)	bladder weight/ body weight (g)
Control diet (n=4)	0.005	0.004	0.002	0.047	0.009	0.007	0.000
***T. lutea* F&M-M36 diet (n=8)**	0.006	0.005*	0.002	0.037*	0.011	0.008	0.001

*p<0.05

3.4 Effects of T. lutea *F&M-M36 on clinical chemistry parameters*

Serum creatinine values, urea, aspartate transaminase (GOT), alkaline phosphatase and alanine aminotransferase (ALT) did not indicate any renal or

hepatic impairment. Compared to control group, total cholesterol concentration was significantly higher in animals fed *T. lutea* F&M-M36 but this effect was mainly ascribed to the augmented HDL (+212%). Triglycerides were also significantly diminished in *T. lutea* F&M-M36 fed rats (-73%) ($p<0.05$) (**Table 5**). The urinary content of uric acid was slightly reduced in *T. lutea* F&M-M36 compared to controls (10.8 ± 1.3 mg/dL in the control group vs 7.1 ± 0.48 mg/dL in *T. lutea* F&M-M36). A significant increase in urinary Na^+ was found (74 ± 10.7 mEq/L vs 266.3 ± 19.07 mEq/L in controls and *T. lutea* F&M-M36 rats respectively).

Table 5. Effect of *T. lutea* F&M-M36 on clinical chemistry parameters

	Urea (g/L)	**Creatinin (mg/dL)**	**AST U/L**	**ALT U/L**	**Alkaline phosphatase (U/L)**	**Triglycerides (mg/dL)**
Control diet (n=4)	0.42±0.04	0.34±0.06	290.5±176.5	75.0±16	155.0±28.0	187±62
***T. lutea* F&M-M36 diet (n=8)**	0.33±0.02	0.34±0.01	180.0±67.5	59.2±4.5	173.7±12.9	52.3±5.2*

Table 5. Continued

	Total cholesterol (mg/dL)	**HDL (mg/dL)**	**LDL (mg/dL)**
Control diet (n=4)	73.0±4.0	55.0±5.0	5.0±0.01
***T. lutea* F&M-M36 diet (n=8)**	131.5±6.2**	117.9±5.8**	7.0±1.29

*p<0.05; ** p<0.01

3.5 Blood pressure

Systolic and diastolic blood pressure, as well as pulse and MAP, were not significantly affected by microalgae rich diets. However, a tendency toward a reduction of these parameters were observed in the group fed *T. lutea* F&M-M36 (**Table 6**).

Table 6. Blood pressure parameters

	Systolic	**Diastolic**	**Pulse**	**Mean arterial pressure**
Control diet (n=4)	142.9 ± 5.3	81.1 ± 7	386.2 ± 32.3	100.8 ± 6.6
***T. lutea* F&M-M36 diet (n=8)**	130.2 ± 7.6	66.8 ± 14.4	351.3 ± 27.2	88 ± 12.7

3.6 Tibio-tarsal joint edema assessment

No significant difference was found in the mean tibio-tarsal joint thickness between groups, data not shown.

3.7 Histological analysis

Histological analysis conducted on sagittal sections of whole brain did not highlight any sign of morphological damage nor in brain nor in cerebellar areas. The liver in all experimental groups showed a normal morphological structure. In hepatocyte no clear cytopathological signs such as steatosis, hemosiderine accumulation or biliary stasis were present. In the urinary bladder of 1 out of the eight rats fed *T. lutea* F&M-M36 an eosinophilic formation was found. This mass occupying the entire lumen of urinary bladder was characterized by the presence of areas with crystalloid appearance inserted in amorphous areas. In the kidney of the same animals, an eosinophilic amorphous content was found in the lumen of proximal and distal convoluted tubule (**Figure 2**). The kidneys of remaining animals did not show any morphological damage. The Sudan Black staining has not revealed the presence of neutral triglycerides and lipids in liver and feces in any of the experimental groups (data not shown).

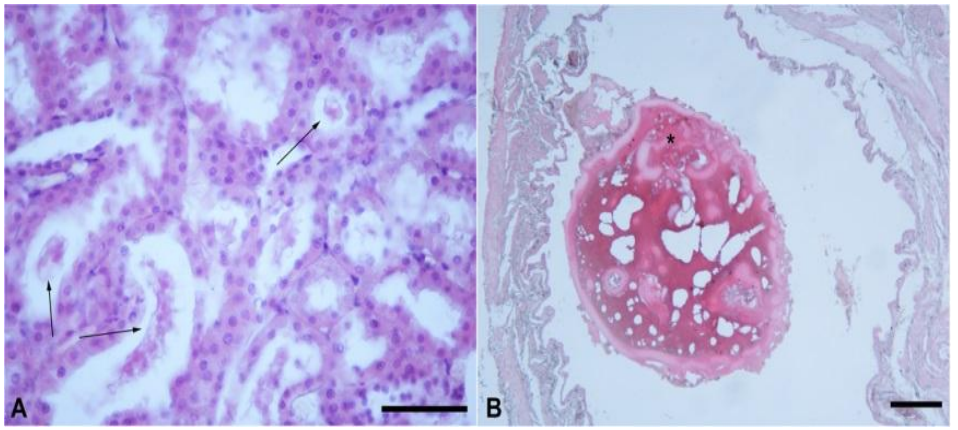

Figure 2. Representative images of amorphous substance in kidney and urinary bladder. Panel A (x10 objective): digitalized image of kidney with amorphous content in proximal and distal convolute tubules (black arrows). Panel B (x4 objective): lumen of urinary bladder. Amorphous eosinophilic mass with some crystalloid areas was evident in the lumen (black asterisk). Scale bar: 100 µm

3.8 Kidney oxidative stress and antioxidant status

To investigate oxidative stress related parameters in the kidney, the expression of the DNA repair enzymes UNG, OGG1, APEX was performed as an indirect method to measure DNA damage at kidney level. None of these parameters differed significantly comparing rats fed *T. lutea* F&M-M36 to controls. Antioxidant status was evaluated by measuring FRAP and SOD2 expression and no significant treatment related difference was observed. Similarly, carbonyl residues as a measure of protein oxidative damage, was not affected (**Table 7**).

Table 7. Expression of UNG, OGG1, APEX and SOD2 in the kidney of *T. lutea* F&M-M36 fed rats

	UNG	**OGG1**	**APEX**	**SOD2**
Control diet (n=4)	0.48 ± 0.12	0.52 ± 0.04	0.91 ±0.17	0.94 ± 0.13
***T. lutea* F&M-M36 diet (n=8)**	0.40 ± 0.05	0.41 ± 0.05	0.93 ± 0.11	0.69 ± 0.07

3.9 Expression of lipid metabolism-related genes in the liver

To explore the possible molecular basis of the effects on lipid metabolism, we analyzed the expression of PPAR-α, PPAR-γ and HMGCR genes in the liver. We observed an increase expression of HMGCR in the liver of the *T. lutea* F&M-M36 group ($p<0.05$) compared to control diet fed rats. The increased expression of PPAR-γ in *T. lutea* F&M-M36 group did not reach the statistical significance.

3.10 Fecal lipids excretion

Compared to control diet, rats fed *T. lutea* F&M-M36 showed an increased (+ 75%) fecal lipid excretion (**Table 8**).

Table 8. Percentage of lipids in stool samples of *T. lutea* F&M-M36 fed rats and in rats fed control diet

	Fecal lipids excretion (%)
Control diet (n=4)	13.7 ± 2.5
***T. lutea* F&M-M36 diet (n=8)**	24.7 ± 3.5*

*$p<0.05$

3.11 Microalgal DNA content

DNA content in *T. lutea* F&M-M36 biomass was 4% of dry weight biomass.

4. Discussion

Microalgae are aquatic, photosynthetic microorganisms with many advantages over other crops since they can be grown in hostile climatic conditions such as desert and coastal areas or in highly controlled close-culture systems (Mata et al., 2010). *Chlorella*, *Arthrospira*, *Dunaliella*, and *Haematococcus* are microalgae largely employed for human consumption due to their balanced biochemical composition and their high nutritional value (Ibañez and Cifuentes, 2013). The marine microalga *Isochrysis* is mainly used in aquaculture (Patil et al., 2007). *Isochrysis* contains biologically active compounds with potential applications in functional foods and nutraceuticals (Buono et al., 2014; Custódio et al., 2014; Luo et al., 2015). In particular, the peculiar content in eicosapentaenoic acid (EPA; 20:5ω3) (Custodio et al., 2014) and fucoxanthin (Kim et al., 2012; Crupi et al., 2013) allows considering this microalga an interesting ingredient for the development of healthy food products. Environmental conditions, such as light intensity and nitrogen availability, are able to affect the cellular chemical composition of *I. galbana*, mainly lipid content and fatty acid composition (Sukenik and Wahnon, 1991).

Overall 20% *T. lutea* F&M-M36 in the diet was well-tolerated during the 30 days feeding period and no treatment-related mortality was detected. This coincides with the reported absence of adverse effects in albino and male Sprague–Dawley rats administered *I. galbana* (Herrero et al., 1993; Nuño et al., 2013, respectively). Food consumption, clinical observations and body weights indicate that the overall health status was not affected. However, it is to note that rats fed *T. lutea* F&M-M36 showed a tendency to a reduction in body weight gain compared to controls. These observations are limited to the 30 days feeding but we cannot exclude significant effects in longer feeding periods. As stated by Zeng et al. (2004), *I. galbana* polymers are shaped by different monomers, the beta-glucosidic bonds of which may allow them to act as dietary fiber in the gastrointestinal tract. Dietary fiber affects satiety, energy, and body composition, reducing appetite and energy intake, consequently leading to weight reduction (Nuño et al., 2013). Moreover, Maeda et al. (2005) indicate that fucoxanthin can promote fat burning within fat cells, increasing the

expression of thermogenin. The increased expression of thermogenin due to the consumption of a fucoxanthin rich diet in rats is activated from fatty acids in adipocytes of brown tissue (Maeda et al., 2005). In a study of Abidov et al. (2010), fucoxanthin also increased the resting energy expenditure (REE) in obese non-diabetic women.

Despite the general health status of rats was not affected, *T. lutea* F&M-M36 high salt content is a safety concern. Due to the relevant issue of salt content, the study specifically focused on the hydro saline balance, blood pressure and renal damage. The hydro saline imbalance was supported by the increased water consumption accompanied by urinary levels increase. Blood pressure was not affected over one month feeding, but it is not possible to exclude long term effects. Notoriously, data from the literature report an increased risk of cardiovascular events in high salt diets (Graudal et al., 2014). In the *T. lutea* F&M-M36 group, an increase in hearth weight was noted. This increase appears to be para-physiological since it lacked correlating to both macroscopic and microscopic changes at hearth or kidney level or to blood pressure measurement. Clinical biochemistry parameters and histopathology also did not show any impairment at kidney levels. The amorphous neo-formation found in the bladder of both microalgae fed rats was interpreted as a uric acid crystal of incidental/sporadic occurrence and possibly related to the DNA content of microalgae (Jin et al., 2012).

Salinity is, therefore, a big safety issue which must be solved by using strategies to reduce its content, e.g. washing the biomass after the harvest (Harun et al., 2010), or by using lower doses of this microalga.

By virtue of the claimed health promoting activities of fucoxanthin, it was also explored kidney oxidative stress and antioxidant status but none expression of the tested repair enzymes (UNG, OGG1, APEX), FRAP, and SOD2 differed significantly comparing rats fed *T. lutea* F&M-M36 to controls.

The orange coloration of the abdominal fat in *T. lutea* F&M-M36 fed rats was interpreted as the result of the accumulation of a lipophilic pigment. Similar findings were reported by Stewart et al. (2008) and by Takahashi et al. (2005) in rats fed an astaxanthin rich diet.

The reduction of liver weight observed was not related to signs of steatosis or other morphological signs of hepatocellular damage. Interestingly, the reduction of liver weight, was associated with a significant reduction in the levels of triglycerides groups. Moreover, fecal neutral sterol content was increased suggesting that the reduced levels of triglycerides might be due to increased

excretion of lipids. This effect was further explored by analyzing the expression of genes implicated in the control of lipid metabolism such as PPAR-α, PPAR-γ, and HMGCR in the liver. In our experimental condition, *T. lutea* F&M-M36 group displayed significantly augmented total cholesterol levels and an increased expression of hepatic HMGCR. However, the raise in total cholesterol observed *T. lutea* F&M-M36 group was mainly due to a significant increase in good cholesterol HDL. In our study, *T. lutea* F&M-M36 contains a high amount of polyunsaturated fatty acids (PUFAs) (around 4%). PUFAs are able to reduce cholesterol and triglycerides levels and their daily consumption has been associated with the prevention of cardiovascular diseases, atherosclerosis, metabolic syndrome and diabetes (Endo and Arita, 2016).

5. Conclusions

Feeding a diet containing 20% of *T. lutea* F&M-M36 results in daily dose of 12 g/kg is without significant adverse effects. Our observations further support the previous findings indicating the dietary *T. lutea* F&M-M36 is able to exert beneficial effects on risk factors for cardiovascular diseases. *T. lutea* F&M-M36 might thus represents an emerging and promising source of functional foods or nutraceuticals.

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Conflict of interest

The authors declare that they have no competing interests.

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Chapter 7

Microalgae cookies – Physical and chemical properties, antioxidant activity and *in vitro* digestibility

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A. P. Batista, A. Niccolai, P. Fradinho, S. Fragoso, I. Bursic, L. Rodolfi, N. Biondi, M. R. Tredici, I. Sousa, A. Raymundo
Microalgae cookies – Physical and chemical properties, antioxidant activity and *in vitro* digestibility

This chapter is carried under the guidance of my supervisors, Prof. Mario R. Tredici and Dr. Liliana Rodolfi, and in collaboration with Dr. Natascia Biondi, and, at LEAF, Universidade de Lisboa with Dr. Ana Paula Batista, PhD student Patrícia Fradinho, Master student Solange Fragoso, Master student Ivana Bursic, Prof. Isabel Sousa, and Prof. Anabela Raymundo

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Abstract

Microalgae use as food ingredients is still poorly developed in Europe due to high cost, low demand, and strict Novel Foods legislation. The aim of this work was to evaluate microalgae (*Arthrospira platensis* F&M-C256, *Chlorella vulgaris* Allma, *Tetraselmis suecica* F&M-M33 and *Phaeodactylum tricornutum* F&M-M40) as a mean to enhance functional properties of cookies. Two biomass levels were tested and compared to control: 2% (w/w) typically used in algal-based products and a significantly higher content, 6% (w/w), to provide algal-bioactives in a higher amount. The cookies physical properties were evaluated during eight weeks after production and no significant differences were found in terms of colour and texture stability. Cookies prepared with *A. platensis* and *C. vulgaris* presented significantly higher protein content. *A. platensis* also provided a significant structuring effect, in terms of cookies texture. All microalgae-based cookies showed significantly higher total phenolic content (TPC) compared to the control. *P. tricornutum* cookies exhibited the highest TPC. Microalgal cookies presented significantly higher *in vitro* antioxidant capacity (AC) compared to the control. No significant difference in *in vitro* digestibility (IVD) between microalgal cookies and the control was found. In general, increasing microalgae content from 2% to 6% resulted in a significant increase in the cookies TPC and AC, while digestibility was slightly lower.
Keywords: Microalgae; Novel foods; Digestibility; Antioxidants; Phenolics

1. Introduction

Microalgae are an innovative and promising food ingredient, rich in nutrients such as high value proteins, long-chain polyunsaturated fatty acids, carotenoids, vitamins, minerals, and phenolics as well as other bioactive molecules (Plaza et al., 2009). In the last years, increasing attention has been drawn to this alternative ingredient by the food industry, with algae identified in the top food trend list for 2016 by the Global Food Forum (2016). Different companies are investing in this sector, such as Terravia (ex-Solazyme, USA), currently producing and commercializing algal food ingredients such as protein isolates and culinary oils (Terravia, 2016). Another example is Dulcesol Group, leader in baked products and pastries sector in Spain, which has also invested in a

microalgae production unit for developing a healthy baked product line (Dulcesol, 2016). Moreover, many other smaller companies are starting to pay attention to this area as consumers are increasingly aware of the benefits of alga inclusion in a healthy diet (Vigani et al., 2015). Microalgae have been also considered to have a great potential as a sustainable feedstock for food commodities (Draisma et al., 2013). However, the use of microalgae as a food source is still poorly developed in Europe, which has been mainly attributed to technical difficulties related to their cultivation, low demand in European countries compared to Asian markets and to strict European legislation regarding Novel Foods (Vigani et al., 2015).

In the last years some papers have been published on the development and characterization of food products integrated with microalgal biomass (Gouveia et al., 2007, 2008; Batista et al., 2012; Fradique et al., 2013; Singh et al., 2015), commonly with contents below 3% (w/w). Also, many papers deal with the bioactive properties of microalgal biomass and/or extracts (e.g. Plaza et al., 2009; Liu et al., 2011; Custodio et al., 2014), but very few deal with the bioactive compounds of microalgae foods and their resistance to different processing steps (e.g. Fradique et al., 2013). There is a lack of knowledge on how the food processing conditions influence digestibility, bioavailability and bioactive properties of the microalgal functional ingredients in different food matrixes.

Cookies are considered a convenient nutrient dense snack food, widely consumed by European citizens from all age groups. The aim of this work was to study microalgae as a mean to enhance functional properties of this baked food matrix, especially at higher biomass incorporation levels. It was intended to use significantly higher concentrations than the ones found in commercial products (typically below 3% w/w), in order to provide higher level of bioactive compounds, whilst not compromising digestibility. Four microalgal strains were tested: *Arthrospira platensis* F&M-C256, *Chlorella vulgaris* Allma, *Tetraselmis suecica* F&M-M33, and *Phaeodactylum tricornutum* F&M-M40.

The Novel Foods Regulation (EC) nº 258/97, recently reviewed by Regulation (EU) nº 2283/2015, determines that any food that has not been used to a significant degree for human consumption within the European Union before 15th May 1997 must be submitted to a safety assessment, in order to protect public health, through a unified procedure led by the European Food Safety Authority (EFSA).

A. platensis (commonly known as spirulina), consumed by human populations since ancient times (Abdulqader et al., 2000), and *C. vulgaris*, *C. luteoviridis* and *C. pyrenoidosa* have been consumed in Europe for several decades and are thus authorized as food in the European Union. *A. platensis* has been widely consumed as nutritional supplement due to its associated health benefits, such as high protein (up to 60%), vitamin B12, γ-linolenic acid (GLA) and phycocyanin content (Hongsthong & Bunnag, 2009). *Chlorella* is also rich in protein, as well as pigments and glucans which can act as immunostimulant (Reyes-Suárez et al., 2008; Tabarsa et al., 2015). In the present work a commercial *C. vulgaris* biomass (Allma, Lisbon, Portugal) was used.

Tetraselmis chuii has recently been authorized for commercialization as novel food ingredient through an application by the company Fitoplancton Marino S.L. (Cadiz, Spain) (AESAN 2013; AECOSAN 2014). In the present study, another species belonging to the same genus, *T. suecica*, was used. This marine chlorophyte is characterized by high content of polyunsaturated fatty acids and α-tocopherol (Pérez-Lopéz et al., 2014).

P. tricornutum is a marine diatom which has not yet been submitted to novel food application. Nevertheless, it was included in the present study considering its high content in eicosapentaenoic acid (EPA 20:5 ω3) as well as in fucoxanthin, a carotenoid associated with antioxidant, anti-diabetes and anti-obesity effects (Gilbert-López et al., 2016; Mikami & Hosokawa, 2013).

Moreover, previous *in vitro* toxicity tests by Niccolai et al. (2016) showed no adverse effects of methanolic and aqueous extracts of these biomasses on *Artemia salina*.

2. Materials and methods

2.1 Microalgal strains and biomass production

Arthrospira platensis F&M-C256 and *Tetraselmis suecica* F&M-M33 were provided by Archimede Ricerche S.r.l., located in Camporosso, Imperia (Italy) and *Phaeodactylum tricornutum* F&M-M40 was produced at the facility of Fotosintetica & Microbiologica S.r.l., located in Sesto Fiorentino, Florence (Italy). The algae were cultivated in GWP®-I (Chini Zittelli et al., 2013) or GWP®-II photobioreactors (Tredici et al., 2015; Tredici et al., 2016) in semi-batch mode, then the biomasses were harvested by centrifugation, frozen, lyophilized and powdered. The powdered biomasses were stored at -20 °C until analysis. *A. platensis* F&M-C256 biomass was washed with tap water to remove

excess bicarbonate before being frozen. *Chlorella vulgaris* Allma was purchase from Allma. The two marine strains (*Tetraselmis suecica* F&M-M33 and *Phaeodactylum tricornutum* F&M-M40) were cultivated in F medium (Guillard and Ryther, 1962), while *A. platensis* F&M-C256 was cultivated in Zarrouk medium (Zarrouk, 1966).
The biochemical composition of the four microalgal biomasses is presented in Table 1.

Table 1. Biochemical composition of the four microalgal biomasses used in the experiments (% dry weight). Results are expressed as average ± standard deviation.

	Protein (%)	**Carbohydrate (%)**	**Lipid (%)**	**Ash (%)**
Arthrospira platensis F&M-C256	68.9 ± 1.0	12.8 ± 0.2	10.7 ± 0. 6	6.1 ± 0.1
Chlorella vulgaris Allma	56.8 ± 2.7	5.9 ± 0.3	16.9 ± 2.8	9.3 ± 1.5
Tetraselmis suecica F&M-M33	40.2 ± 0.5	10.2 ± 0.2	28.5 ± 1.2	15.7 ± 0.2
Phaeodactylum tricornutum F&M-M40	38.8 ± 0.1	11.0 ± 0.7	19.3 ± 1.7	14.8 ± 0.1

2.2 Cookies preparation

Cookies were prepared according to a previously optimized formulation (Gouveia et al., 2007, 2008), using wheat flour, sugar, baking powder, margarine, and microalgae biomass, as indicated in Table 2. All percentages are presented in w/w. The ingredients were mixed in a food processor (Bimby, Vorwerk, Germany), kneading 15 seconds at speed 4. The cookies were then moulded into 48 mm diameter and 4 mm height circles and baked at 120 ºC for 40 min. After cooling, sample cookies were stored in hermetic containers, protected from light. Some of the cookies were immediately crushed to powder (using an electric mill) and used for chemical composition, antioxidant capacity and *in vitro* digestibility analyses.

Table 2. Cookies formulations (%).

Ingredients	F1 (control) g/100g	F2 g/100g	F3 g/100g
Wheat flour	49	47	43
Sugar	20	20	20
Margarine	20	20	20
Water	10	10	10
Baking powder	1	1	1
Microalga	0	2	6

F1 control cookies formulation; F2 2% cookies formulation; F3 6% cookies formulation

2.3 Cookies analyses

2.3.1 Colour analysis

The colour of sample cookies was measured instrumentally using a Minolta CR-400 (Japan) colorimeter with standard illuminant D65 and a visual angle of 2°. The results were expressed in terms of L∗, lightness (from 0 to 100%); a∗, redness to greenness (60 to −60 positive to negative values, respectively); b∗, yellowness to blueness (60 to −60 positive to negative values, respectively), according to the CIELab system. Hue angle, h^{o}_{ab}, was also calculated, as defined by: $h^{o}_{ab}=\arctan(b^{*}/a^{*})$. The total colour difference between sample cookies along storage time (up to eight weeks), as well as between raw and cooked samples, was determined according to: $\Delta E^{*}=[(\Delta L^{*})^{2}+(\Delta a^{*})^{2}+(\Delta b^{*})^{2}]^{1/2}$. The measurements were conducted under the same light conditions, using a white standard (L*=94.61, a*=-0.53, b*=3.62), under artificial fluorescent light at room temperature, replicated 10 times for each sample.

2.3.2 Texture analysis

The cookie texture was measured using a texturometer TA.XTplus (Stable MicroSystems, UK) in penetration mode with a cylindrical aluminium probe of 2 mm diameter plunged 3 mm at 1 mm s^{-1}. The resistance to penetration, or hardness, was measured by the total area below the force vs. time curve,

corresponding to the penetration work (N·s). Measurements were repeated 10 times for each sample, along time (up to eight weeks).

2.3.3 Chemical composition

All chemical composition analyses were repeated, at least in triplicate, according to the methods described below.

2.3.3.1 Moisture content

Cookie moisture content was determined gravimetrically using an automatic moisture analyser PMB 202 (aeADAM) at 130 °C, until constant weight.

2.3.3.2 Total Ash

Total ash content was determined gravimetrically by incineration at 550 ºC in a muffle furnace.

2.3.3.3 Crude Protein

Crude protein was determined by the Kjeldhal method according to the AOAC 950.36 official method for baked products. The determined total nitrogen content was multiplied by a conversion factor of 5.7 to obtain the cookie crude protein content.

2.3.3.4 Crude fat content

The cookie crude fat content was determined according to the procedure used for cereals and derived products in the Portuguese standard method NP4168 (1991). This procedure is based on the hydrolysis of the bonds between lipids, proteins, and carbohydrates by using hydrochloric acid, ethanol and formic acid, followed by filtration and extraction with *n*-hexane in a Soxhlet extractor for 6 h. The crude fat residue was determined gravimetrically, after solvent evaporation in a rotary evaporator and oven drying.

2.3.3.5 Fatty acid profile

The cookies fatty acid profile was determined by GC/MS. The lyophilized biomasses were extracted and methylated according to the studies of Pal et al. (2011) and of Recht et al. (2012). Samples of lyophilized biomass (10-20 mg) were weighed in a test tube with screw cap, with 350 µL of a 1 mg mL^{-1}

solution of internal standard (fatty acid C19:0), previously evaporated. Then, 2.5 mL of a 2% (v/v) solution of H_2SO_4 into MeOH were added and heating to 85 °C for 1.5 hours, if necessary applying a little mixing. Thereafter, the reaction was turned off by adding 1 mL of MilliQ water. Then, 1 mL of hexane was added, and the solution was vortexed and centrifuged for 5 min. After centrifugation, the supernatant (hexane) was collected and transferred to another tube and this step was repeated 3-4 times. Then the hexane extract was evaporated under a stream of N_2. Finally, the dry residues were recovered in 1 mL of hexane and injected into the GC/MS.

GC/MS was composed by an HP5890 gas chromatograph coupled to an HP5970 mass selective detector (Hewlett Packard, USA) operating in EI mode, 70 eV. The gas chromatograph was equipped with a Zebron ZB-WAXplus capillary column (Phenomenex, Bologna, Italy), 60 m x 0.25 mm, 0.25 µm d.f. The injector temperature was maintained at 255 °C and injections were made in split mode (split ratio 1:20, injection volume 1 µL). The oven temperature program started at 70 °C and raised to 150 °C at 16 °C min^{-1}, then to 210 °C at 5 °C min^{-1}, then to 250 °C at 1.5 °C min^{-1} and hold for 5 min. The carrier gas was helium at 165 kPa head pressure. Mass spectrometer operated in scan mode in positive ion, in 41–650 m/z range (1.33 scan s^{-1}). Fatty acids were identified by comparing retention times with those of authentic standards (37 Component FAME Mix, Supelco, Italy) and the experimental EI mass spectra with those of the NIST mass spectral database, version 05. Fatty acids were quantified using the methyl ester of C19:0 fatty acid, not present in the samples, as the internal standard, making a single point calibration curve for less abundant fatty acid, corrected for the fatty acid amount in the mix, or a six points one for the more abundant.

2.3.3.6 Phycocyanin content

Phycocyanin content was determined in *A. platensis* cookie and dough samples, according to the method developed by Boussiba & Richmond (1979) modified by Reis et al. (1998). This method is based on the extraction of these water soluble pigments with phosphate buffer at pH 7, 0.1M at low temperatures and spectrophotometrically quantification at 620 nm (*C*-Phycocyanin) and 650 nm (*C*-allophycocyanin).

2.3.3.7 Total phenolic content

The extracts were prepared according to the procedure used by Hajimahmoodi et al. (2010). Samples (0.1 g) were mixed with 2 mL deionised water solution (80 °C) and the microalgae cell walls disrupted using an ultrasonic homogenizer (Microson^{TM} XL2000, Misonix Inc., Farmingdale, New York, USA) set at a frequency of 20 kHz and a power of 130 W for 30 min at room temperature. Samples were then centrifuged (5,000×g, 10 min, at 25 °C) and the supernatants recovered. The extraction was repeated three times and the supernatants combined and filtered on 47 mm membranes with a nominal porosity of 1.2 μm (FILTER-LAB, Barcelona, Spain). All extracts were stored at -20 °C.
The total phenolic content assay was carried out according to Rajauria et al., 2013, using the Folin Ciocalteu assay. To each sample (100 μL of the extract), 2% sodium carbonate (2 mL; Sigma-Aldrich, Italy) was added. After 2 min, 50% Folin Ciocalteu reagent (100 μL; Sigma-Aldrich, Italy) was added. The reaction mixture was incubated in darkness at 25 °C for 30 min. The absorbance of each sample was measured at 720 nm using a UV-Vis spectrophotometer reader (Cary® 50 UV-Vis spectrophotometer, VARIAN Inc., California, USA). Results were expressed in gallic acid equivalents (mg GAE/g) of dry microalgal biomass and cookies, through a calibration curve with gallic acid (0 to 500 μg mL^{-1}, Sigma, Italy).

2.3.4 Antioxidant capacity

The antioxidant capacity of the cookies and microalgae samples was assessed by direct quencher procedure, as optimized by Serpen et al. (2008, 2012) for cereal products. The quantification method was FRAP (Ferric Reducing Antioxidant Power), characterized by the reduction of Fe^{3+} to Fe^{2+} depending on the available reducing species, followed by the alteration of colour from yellow to blue measured spectrophotometrically (Benzie & Strain, 1996). Thirty (30) mg of powdered samples were weighed into a centrifuge tube (15 mL capacity). The reaction was started by adding 3.3 mL of FRAP working solution, composed of sodium acetate buffer 0.3M (pH3.6) : TPTZ 10 mM (in HCl 40 mM) : $FeCl_3$ 20 mM in a proportion of 10:1:1 (v/v/v) . The tube was shaken rigorously in a vortex and incubated at 37 ºC for 10 min in the dark. Centrifugation was performed at 4,000×g for 5 min and the supernatant filtered by 0.45 μm syringe filter membranes. Absorbance values were measured at 595 nm and were subtracted by blank assay values (A_0 – blank without sample with

reagents; Ae – blank with sample without reagents). Standard calibration curves were constructed by plotting colour formation at 595 nm against different concentrations of Trolox standard solutions that were submitted to the same FRAP protocol. The antioxidant capacity of the samples was expressed in terms of mmol of Trolox Equivalent Antioxidant Capacity (TEAC) per kilogram of sample.

2.3.5 In vitro digestibility

The cookies *in vitro* digestibility was assessed by the Boisen & Fernández (1997) method. The *in vitro* analysis reproduces the chemical-enzymatic catalysis that occurs in the proximal tract of the mammalian digestive system.
Biomass and cookies were powdered before analysis. Samples of 1 g of finely ground material (particle size ≤ 1 mm) were weighed and transferred in 250 mL conical flasks. To each flask, phosphate buffer (25 mL, 0.1 M, pH 6.0) was added and mixed, followed by HCl (10 mL, 0.2 M). The pH was adjusted to 2.0 by the addition of HCl (1 M) or NaOH (1 M). A freshly prepared pepsin water solution (3 mL; Applichem, Darmstadt, Germany) containing 30 mg of porcine pepsin with an activity of 0.8 FIP-U/mg was added. The flasks were closed with a rubber stopper and placed on multipoint stirrers at 150 rpm, for 6 h, in a thermostatically-controlled heating chamber (ProCLIMATIC, Imola, Italy) at 39 °C. Following incubation, phosphate buffer (10 mL, 0.2 M, pH 6.8) and NaOH solution (5 mL, 0.6 M) were added to each sample. The pH was adjusted to 6.8 by addition of HCl (1 M) or NaOH (1 M). A freshly prepared pancreatin ethanol:water solution (10 mL, 50:50 v/v) containing 500 mg of porcine pancreatin with an activity of 42362 FIP-U/g (Applichem, Darmstdat, Germany) was added to each sample. After closing with a rubber stopper, the flasks were incubated again at 39 °C, 150 rpm, for 18 h. A reagent blank without biomass was also prepared.
The undigested residues were collected by centrifugation (ROTANTA 460 R centrifuge, Hettich Zentrifugen, Tuttlingen, Germany) at 18,000×g for 30 min. The undigested material (sedimentation pellet) was washed with deionised water until the pellet was completely covered by water to remove any salts from the buffer and then centrifuged with the same parameters reported above.
The supernatant was removed after the second centrifugation, and filtered on 47 mm glass-fiber membranes with a pore diameter of 1.2 μm (FILTER-LAB, Barcelona, Spain). The pellet and filter papers were dried at 80 °C for 6 h, and then at 45 °C until a constant mass was reached. To account for any residual

biomass or undissolved reagents not sedimented during centrifugation, the mass of the dried residue on each filter paper was added to the mass of the undigested material (pellet). The reagent blank was processed in the same manner. The percent *in vitro* digestibility of dry matter was calculated according to Boisen and Fernández (1997):

$$D\ (\%) = \frac{SW - [(UW + RP) - B]}{SW} \text{ x } 100$$

where:

D is the *in vitro* digestibility of dry matter expressed as percentage

SW is the mass of the starting material expressed in grams

UW is the mass of the undigested material expressed in grams

RP is the residual particulate, any residual biomass and/or undissolved reagents re-suspended from the pellet or not sedimented expressed in grams

B is the blank (all reagents without biomass) expressed in grams

2.4 Statistical analysis

Statistical analysis of the experimental data was performed using STATISTICA from StatSoft (version 8.0), through variance analysis (one way ANOVA), by the Scheffé test – Post Hoc Comparison at a significance level of 95% ($p<0.05$). All results are presented as average ± standard deviation.

3. Results and discussion

The cookies with microalgal biomass incorporation presented visual attractive and unusual appearances (Fig. 1). Innovative green tonalities varied, depending on the microalga used, from a blueish-green (*A. platensis*) to a brownish-green (*P. tricornutum*). The microalgae cookies presented an average diameter of 47.1 mm and an average thickness of 7.5 mm while the control cookies were slightly thicker (8.3 mm on average).

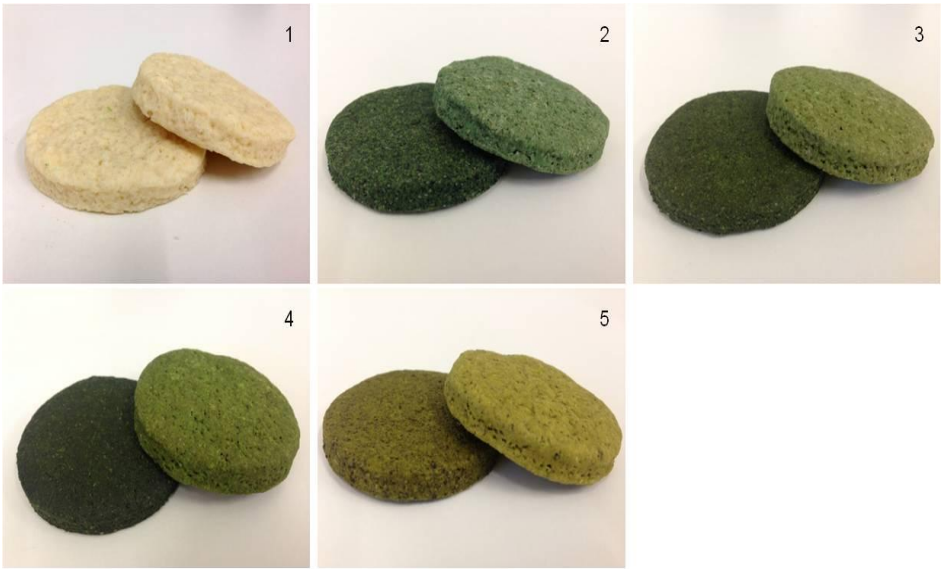

Fig. 1 Control cookie (1) and cookies with 2% (w/w) and 6% (w/w) microalgal biomass (2 - *A. platensis*, 3 – *C. vulgaris*, 4 – *T. suecica*, 5 – *P. tricornutum*)

3.1 Colour stability

The results obtained for the cookie colour parameters L*, a*, b* and h^{o} are presented in Fig. 2. Regarding the luminosity parameter L*, a reduction in luminosity with increasing alga concentration can be observed.

An increase in microalgal concentration has also led to lower values of the chromatic parameters a* and b* (in modulus), while the hue tonality remains constant (100^{o}-120^{o} between yellow and green).

These results may seem unexpected, considering that in Fig. 1, the cookies with 6% alga seem to have more intense green colours. Apparently, the darkening,

associated with tonality maintenance of hue tonality "mask" the colour intensity reduction. In previous studies, a similar effect was found for *C. vulgaris* (Gouveia et al., 2007) and *Isochrysis galbana* (Gouveia et al., 2008) cookies, where a reduction in a* and b* parameters upon increasing microalgal biomass concentration from 0.5% to 3.0% (w/w) was observed. This effect may be related to a higher pigment degradation along baking process or with a colour saturation effect.

Cookies with 2% *C. vulgaris* and *T. suecica* presented the highest a* values (in modulus) and intermediate b* values (22.8-25.3) (Fig. 2), which is in agreement with the high chlorophyll content that characterizes chlorophyte algae (Plaza et al., 2009). *A. platensis* cookies presented tonalities similar to the chlorophyte cookies, although with less intensity (lower a* and b* values), reflecting the lower chlorophyll and carotenoid content generally present in this alga (Batista et al., 2013). On the other hand, *P. tricornutum* cookies presented low a* values (in modulus) and the highest b* values, resulting in a hue angle tonality of 100º, closer to yellow (90º) than to green (180º). These results should be related to the presence of fucoxanthin, a carotenoid usually present in high concentrations in this marine diatom (Gilbert-López et al., 2016).

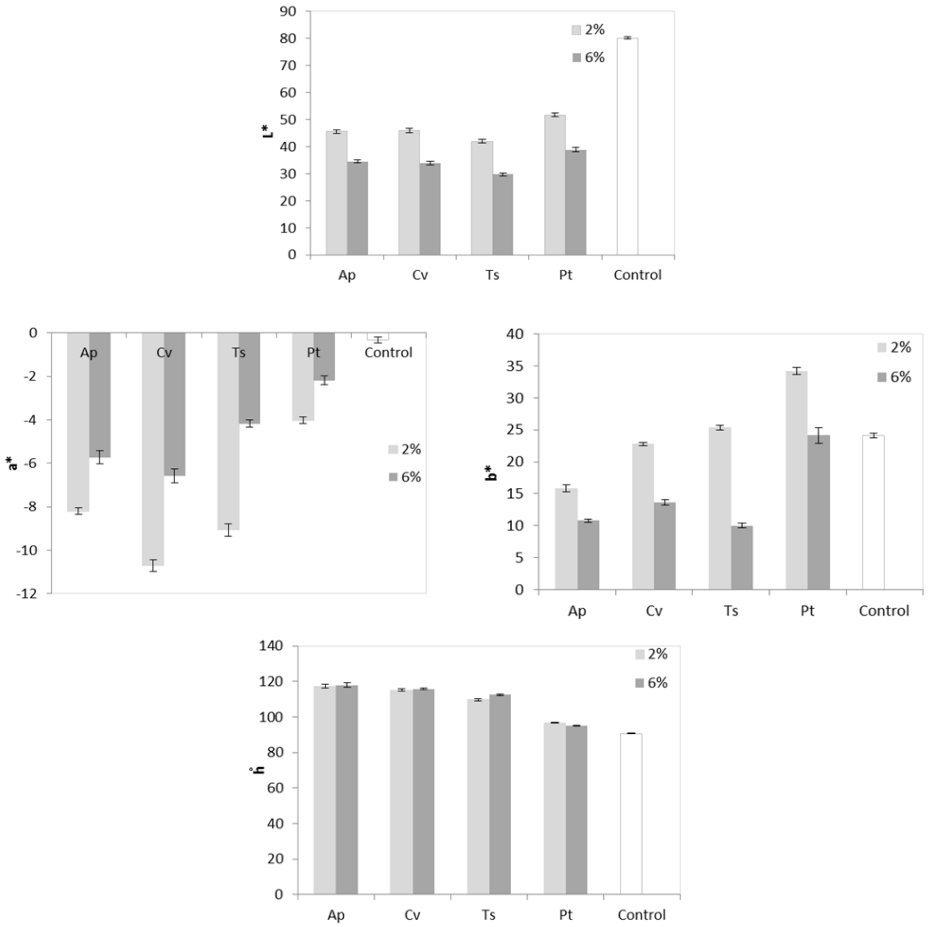

Fig. 2 Colour parameters, L*, a*, b* and hº of cookies with 2% and 6% (w/w) microalgal biomass incorporation, in week 0 (Ap – *A. platensis*, Cv – *C. vulgaris*, Ts – *T. suecica*, Pt – *P. tricornutum*).

Table 3 presents the total colour differences (ΔE*) between baked and raw (dough) sample cookie. Microalgal cookies show significantly higher colour differences upon baking than the control cookie (ΔE*=7.6), with the highest values for *P. tricornutum* cookie samples (ΔE*=19-24). These differences result mainly from a general increase in luminosity (probably associated to water evaporation) and an accentuated hue angle tonality decrease in the case of *P. tricornutum* (results not shown) which should be related to pigment loss upon baking.

The colour stability along conservation time can also be observed in Table 3 through the calculation of total colour difference of each sample along time in

relation to week 0. In all cases ΔE* is lower than 5 (except for *P. tricornutum* 6% in week 8: 5.42) which means that the cookie colour differences are not detected by normal human vision (Castellar et al., 2006). Therefore, it can be concluded that the developed cookies present stable colorations along eight weeks storage.

Table 3. Total colour variation (ΔE*) between cooked and raw cookie samples and colour stability along conservation time (ΔE* in relation to week 0).

Total colour difference (ΔE*)		Raw vs. Cooked	Week 1 vs. Week 0	Week 2 vs. Week 0	Week 3 vs. Week 0	Week 4 vs. Week 0	Week 8 vs. Week 0
Control		7.63	0.84	0.86	1.23	1.55	1.89
A. platensis	**2%**	16.01	0.60	0.66	1.16	1.63	1.86
	6%	15.58	0.73	0.89	0.94	0.94	0.77
C. vulgaris	**2%**	11.22	0.70	1.17	0.96	0.74	1.12
	6%	12.58	0.75	1.26	1.11	1.32	3.13
T. suecica	**2%**	15.93	1.02	1.73	2.43	2.49	2.78
	6%	10.85	1.83	2.12	2.40	3.80	4.69
P. tricornutum	**2%**	18.97	1.50	2.03	2.48	2.37	4.19
	6%	23.63	1.31	2.57	2.37	3.35	5.42

3.2 Texture stability

The cookies texture was evaluated by penetration tests (as described in section 2.3.2), and the resulting hardness, expressed by resistance to penetration work (total area below force vs. time curve), was calculated from the texturograms and presented in Fig. 3.

No significant differences ($p>0.05$) were found between the cookies with 2% alga when compared to the control (and between different algae), which means that adding 2% biomass does not promote cookie structural changes that can alter the resistance to probe penetration. Increasing microalgae concentration from 2% to 6% causes significant ($p<0.05$) hardness increase, from 24-29 N·s (2% cookies) to 37-38 N·s for 6% *C. vulgaris* and *T. suecica* cookies, to 50 N·s for *P. tricornutum* and 63 N·s for *A. platensis* cookies.

These results can be related to previous water and oil absorption tests carried out on the same microalgae (Fragoso, 2016), in which significantly higher ($p<0.05$) absorption values (4.4-5.2 g_{H20}/g_{alga} and 0.8-1.2 g_{oil}/g_{alga}) were attained in relation to wheat flour (2.1 g_{H20}/g_{flour} and 0.7 g_{oil}/g_{flour}). The highest values were attained for *A. platensis*, followed by *P. tricornutum*, *C. vulgaris* and *T. suecica*, which can be directly related to the different nature of these algae cell walls (peptidoglycan, silica and cellulose/hemicellulose, respectively). It is assumed that, when microalgae are added to the cookie dough, they absorb more water and oil/fat, reinforcing the cookie internal structure. These data suggest that it would be possible to increase the water content and reduce the flour content, resulting cookies with the same texture properties than the control cookie.

These results are also in agreement with previous studies where it was observed a linear increase in cookies hardness with *C. vulgaris* (Gouveia et al., 2007) and *I. galbana* (Gouveia et al., 2008) at concentrations of 0.5% and 3.0%. Singh et al. (2015) also observed that the level of *A. platensis*, from 1.6 to 8.4%, had positive effect on the hardness of sorghum flour biscuits. The same *"texturing"* or *"structuring"* effect of microalgae has been described also in other type of food products, such as fresh pastas with *A. maxima* and *C. vulgaris* (Fradique et al., 2010).

The evolution of cookies hardness along time can also be observed in Fig. 3. The control and 2% alga cookies do not present significant ($p>0.05$) changes in hardness along eight weeks, except for 2% *A. platensis* cookie which increased significantly ($p<0.05$). For the 6% cookies, in general, the texture results are more variable along time, with significant differences ($p<0.05$) observable in most cases, without a clear variation tendency.

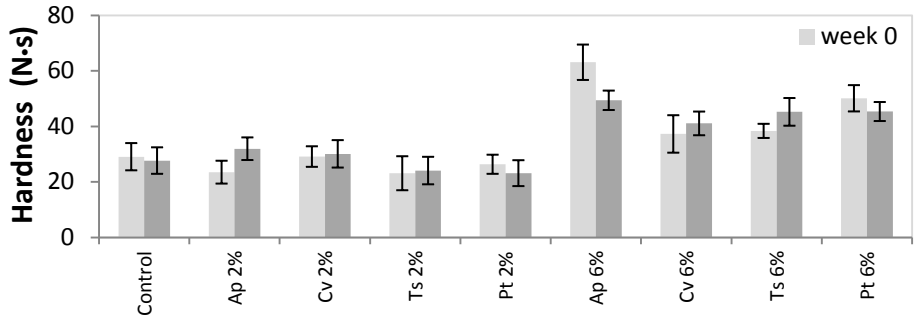

Fig. 3 Texture, expressed by penetration work (hardness, area N·s), of cookies with 2% and 6% (w/w) microalgal biomass incorporation, along time (Ap – *A. platensis*, Cv – *C. vulgaris*, Ts – *T. suecica*, Pt – *P. tricornutum*).

3.3 Chemical composition

Table 4 presents the chemical composition of the cookies prepared with microalgal biomass incorporation. All cookies presented moisture values ranging from 3.2 to 5.0%, which is typical for this type of dried foods.

Table 4. Biochemical composition (g/100g) of cookies with 2% and 6% (w/w) microalgal biomass incorporation. Results are expressed as average ± standard deviation. Different letters in the same column correspond to significant diferences ($p<0.05$).

		Moisture (g/100g)	Total Ash (g/100g)	Crude Fat (g/100g)	Crude Protein (g/100g)	Carbohydrates* (g/100g)	Energy Value (Kcal/100g)
Control		3.8 ± 0.2 ^{ab}	2.7 ± 0.2 ^{a}	16.1 ± 0.1 ^{a}	4.9 ± 0.5 ^{a}	69.4	448
A. platensis	**2%**	3.8 ± 0.1 ^{ab}	2.6 ± 0.4 ^{a}	16.1 ± 0.5 ^{a}	6.1 ± 0.2 ^{abc}	64.7	441
	6%	5.0 ± 0.2 ^{d}	2.3 ± 0.1 ^{a}	16.1 ± 0.1 ^{a}	7.8 ± 0.3 ^{de}	60.2	434
C. vulgaris	**2%**	3.2 ± 0.1 ^{a}	2.3 ± 0.1 ^{a}	16.3 ± 0.2 ^{a}	5.9 ± 0.5 ^{abc}	66.1	447
	6%	4.8 ± 0.3 ^{cd}	2.6 ± 0.1 ^{a}	16.9 ± 0.4 ^{a}	8.0 ± 0.6 ^{e}	59.5	439
T. suecica	**2%**	3.4 ± 0.2 ^{ab}	2.4 ± 0.2 ^{a}	16.1 ± 0.1 ^{a}	5.2 ± 0.1 ^{a}	66.9	445
	6%	3.3 ± 0.1 ^{a}	3.2 ± 0.1 ^{a}	16.3 ± 0.4 ^{a}	6.9 ± 0.4 ^{cd}	63.4	442
P. tricornutum	**2%**	3.9 ± 0.1 ^{ab}	2.3 ± 0.2 ^{a}	16.1 ± 0.1 ^{a}	5.1 ± 0.2 ^{ab}	65.2	441
	6%	4.3 ± 0.2 ^{bc}	3.0 ± 0.1 ^{a}	16.2 ± 0.1 ^{a}	6.6 ± 0.4 ^{bc}	62.5	437

* Carbohydrates were calculated by difference

No significant changes were observed on the cookies mineral content upon microalgal addition (2.3-3.2%) neither on the crude fat content (16.1-16.9%). Regarding the fatty acid profile no major differences were found between the control and microalgae cookies, with predominance of palmitic acid (16:0), followed by oleic acid (C18:1) and linoleic acid (C18:2) (Table 5).

Table 5. Main fatty acids present in the cookies enriched with different levels of microalgae (% dry weight). SFA – Saturated Fatty Acids; MUFA – Monounsaturated Fatty Acids; DUFA – Diunsaturated Fatty Acids; PUFA – Polyunsaturated Fatty Acids; TFA – Total Fatty Acids

	A. platensis		***C. vulgaris***		***T. suecica***		***P. tricornutum***		**Control**
	2%	**6%**	**2%**	**6%**	**2%**	**6%**	**2%**	**6%**	
C12:0	0.19	0.25	0.28	0.32	0.20	0.30	0.17	0.22	0.26
C14:0	0.16	0.19	0.20	0.20	0.16	0.20	0.17	0.28	0.20
C16:0	5.10	5.25	5.44	5.32	4.92	5.10	5.28	5.14	5.25
C18:0	0.87	0.83	0.90	0.85	0.85	0.79	0.88	0.78	0.82
C18:1	5.44	5.44	5.77	5.72	5.40	5.55	5.62	5.55	5.62
C18:2	4.36	3.70	3.90	4.05	4.27	3.79	4.56	3.84	3.76
C18:3 ω3	-	0.28	0.22	0.36	0.18	0.32	-	0.25	0.19
∑ SFA	6.32	6.52	6.82	6.69	6.13	6.39	6.50	6.42	6.53
∑ MUFA	5.44	5.44	5.77	5.72	5.40	5.55	5.62	5.55	5.62
∑ DUFA	4.36	3.70	3.90	4.05	4.27	3.79	4.56	3.84	3.76
∑ PUFA	-	0.28	0.22	0.36	0.18	0.32	-	0.25	0.19
TFA	16.12	15.94	16.71	16.82	15.98	16.05	16.68	16.06	16.10

Despite the fact that the biomass of the marine microalgae *T. suecica* and *P. tricornutum* contain long chain fatty acids (≥C20), especially 4% EPA (C20:5ω3) in *P. tricornutum* (Table 6), there are no traces of these fatty acids in the cookies. This could be due to thermal degradation upon baking at 120 ºC for 40 min.

Table 6. Main fatty acids present in the microalgal biomass (% dry weight). SFA – Saturated Fatty Acids; MUFA – Monounsaturated Fatty Acids; DUFA – Diunsaturated Fatty Acids; PUFA – Polyunsaturated Fatty Acids; TFA – Total Fatty Acids.

	A. platensis	***C. vulgaris***	***T. suecica***	***P. tricornutum***
C12:0	-	0.55	0.59	0.51
C14:0	-	0.06	0.03	0.57
C14:1	0.01	0.02	-	-
C16:0	2.56	1.99	0.95	1.27
C16:1	0.29	0.44	0.04	2.42
C18:0	0.12	0.26	0.60	0.05
C18:1	0.18	1.81	1.37	0.90
C18:2	1.15	1.85	2.56	0.53
C18:3 ω3	0.01	2.51	1.53	0.07
C18:3 ω6	1.66	0.01	0.03	0.08
C 20:0	0.02	0.03	-	0.01
C 20:1	-	-	0.06	-
C 20:2	-	-	-	0.23
C20:3 ω6	0.01	-	-	-
C 20:4	-	-	0.11	0.07
C20:5	-	-	0.39	3.97
C 24:1	-	-	-	0.05
∑ SFA	2.70	2.89	2.17	2.41
∑ MUFA	0.48	2.27	1.47	3.32
∑ DUFA	1.15	1.85	2.56	0.76
∑ PUFA	1.68	2.52	2.06	4.19
TFA	6.01	9.53	8.26	10.68

The main chemical composition changes arising from microalgae incorporation in cookies are related to protein content (Table 4). The protein content of microalgae cookies was always higher than the control cookie (4.9%). 2% algal cookies ranged from 5.1 to 6.1% protein while 6% cookies ranged from 6.6 to 8.0% protein. The highest values were attained for *A. platensis* and *C. vulgaris* cookies. It can be concluded that the inclusion of microalgae in cookies, particularly *A. platensis* and *C. vulgaris*, can be regarded as an effective nutritional fortification in terms of protein.

In the case of *A. platensis*, it should also be noted the presence of phycocyanin, a blue pigment with associated nutraceutical properties (Fernández-Rojas et al., 2014). Even after thermal treatment, the cookies presented 172 mg/kg and 363 mg/kg phycocyanin for 2% and 6% incorporation level, respectively (in microalgal biomass: 8% w/w). In previous studies (Batista et al., 2006),

phycocyanin has been used as colouring and functional ingredient in oil-in-water food emulsions, proving also to be a powerful structuring agent.

Fig. 4 presents the phenolic content of microalgae cookies and microalgae biomass. The addition of microalgae results in an effective supplementation of phenolic compounds, which are practically absent in the control cookie. *A. platensis* 6% cookie presented the highest phenolic content (0.90 mg GAE/g), which is in agreement with this algae composition (19 mg GAE/g), followed by *P. tricornutum* 6% cookie (0.62 mg GAE/g). Both *A. platensis* and *P. tricornutum* 2% cookies still showed much higher phenolic content than the chlorophyte algae at the highest concentration (*C. vulgaris* and *T. suecica*). Other authors have highlighted the potential of phenolic compounds production from *A. platensis* (Kepekçi & Saygideger, 2012) and *P. tricornutum* (Santana-Casiano et al., 2014), as well as their correlation with the antioxidant activity of these algae extracts.

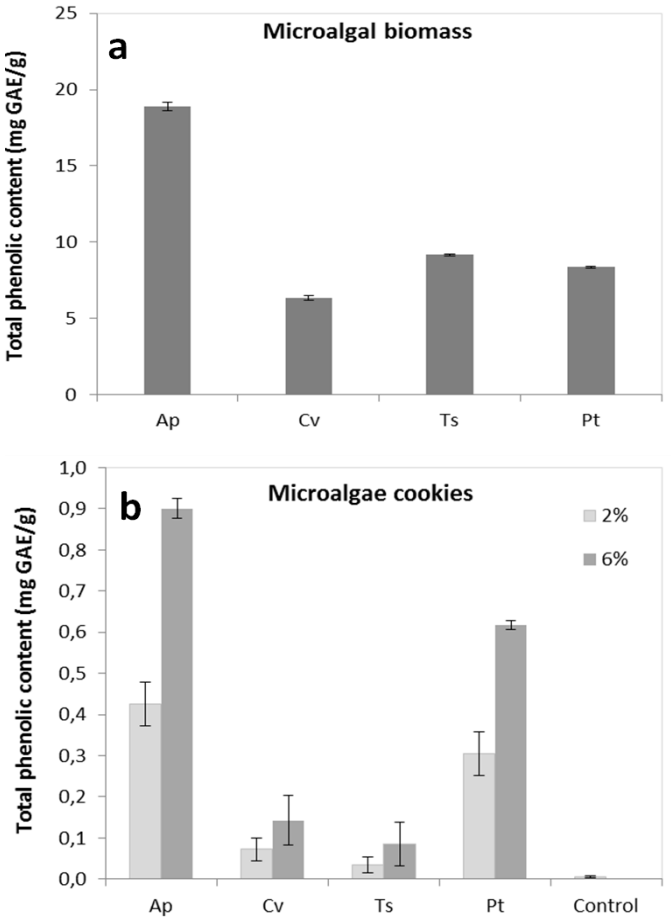

Fig. 4 Total phenolic content (expressed as gallic acid equivalents mg g^{-1} dry weight) of four microalgal strains (a) and in cookies enriched with different levels of microalgae (b) (Ap – *A. platensis*, Cv – *C. vulgaris*, Ts – *T. suecica*, Pt – *P. tricornutum*).

3.4 Antioxidant capacity

The antioxidant capacity of microalgae-enriched cookies was tested through the FRAP method. From Fig. 5 it is evident that adding microalgae increases antioxidant capacity of the cookies.

P. tricornutum microalgal biomass presented the highest ($p<0.05$) antioxidant capacity (248 mmol TEAC/kg), followed by *C. vulgaris* (193 mmol TEAC/kg) and by *A. platensis* and *T. suecica* (about 160 mmol TEAC/kg). The antioxidant capacity of the microalgae cookies is in agreement with the microalgae biomass

results, although no significant differences were found for cookies prepared with different algae, at the same concentration. In the case of *P. tricornutum*, this could be due to antioxidants loss upon baking, namely fucoxanthin pigment. In fact, from the colour difference results (Table 3) it was already noticed colour loss on this sample upon cooking.

For all the microalgae studied, a significant ($p<0.05$) increase in antioxidant capacity was observed when increasing biomass concentration from 2% to 6%. Overall, cookies with 2% alga showed values around 7.0 and 9.5 mmol TEAC/kg while 6% cookies showed values around 11.8 to 15.4 mmol TEAC/kg.

Some other authors have studied the antioxidant capacity of *A. platensis* enriched cookies. El Baky et al. (2015) also observed increasing antioxidant activity for biscuits containing 0.3 to 0.9% *A. platensis* biomass. Singh et al. (2015) presented a linear response surface methodology (RSM) model with linear positive correlation between *A. platensis* concentration in sorghum flour biscuits (1.6 to 8.4%), and antioxidant activity (DPPH and ABTS inhibition).

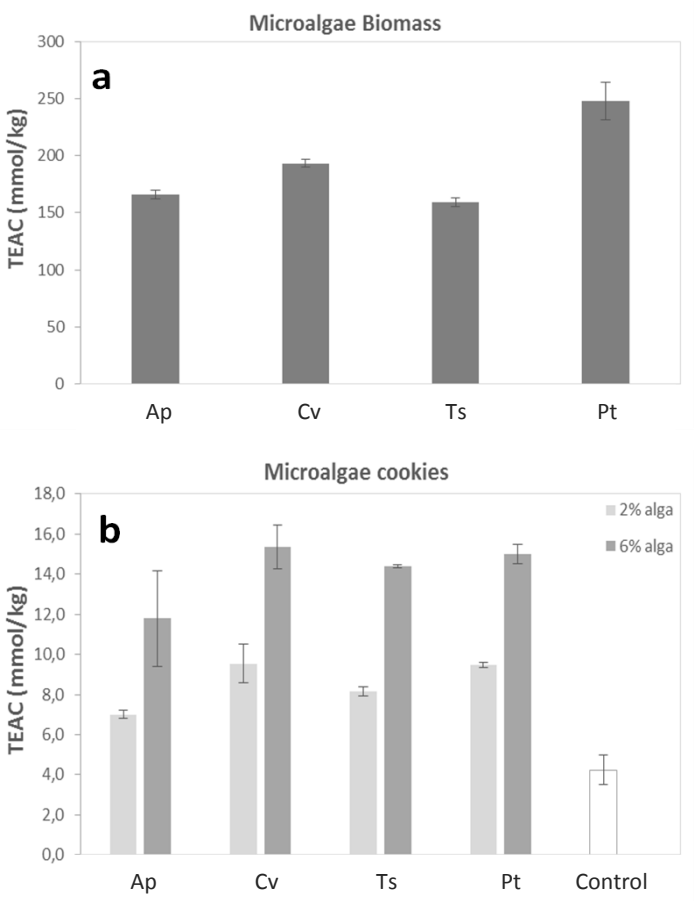

Fig. 5 Antioxidant capacity (expressed as mmol of Trolox Equivalent Antioxidant Capacity, TEAC, per kg) of four microalgal strains (a) and in cookies enriched with different levels of microalgae (b) (Ap – *A. platensis*, Cv – *C. vulgaris*, Ts – *T. suecica*, Pt – *P. tricornutum*).

3.5 In vitro digestibility

As far as digestibility of microalgae is concerned, most of the literature deals with tests for macroalgae (Fleurence, 1999; Paiva et al., 2014; Tibbets et al., 2016) and only few studies focus on the digestibility of microalgae (Mišurcová et al., 2010; Machů et al., 2014; Tibbets et al., 2012). To our knowledge, no literature is available concerning *in vitro* digestibility of microalgae-based cookies.

The *in vitro* digestibility (IVD) results are presented in Fig. 6. *T. suecica* and *P. tricornutum* microalgae biomass presented the lowest IVD (around 50%). The differences in IVD values between the different microalgae could be related to the different structure of microalgae cell walls (Mišurcová, 2011). As expected, cookies added with 6% Chlorophyceae biomass exhibited the lowest digestibility (87%), although no significant difference in IVD between microalgae cookies and the control (IVD 95%) were found.

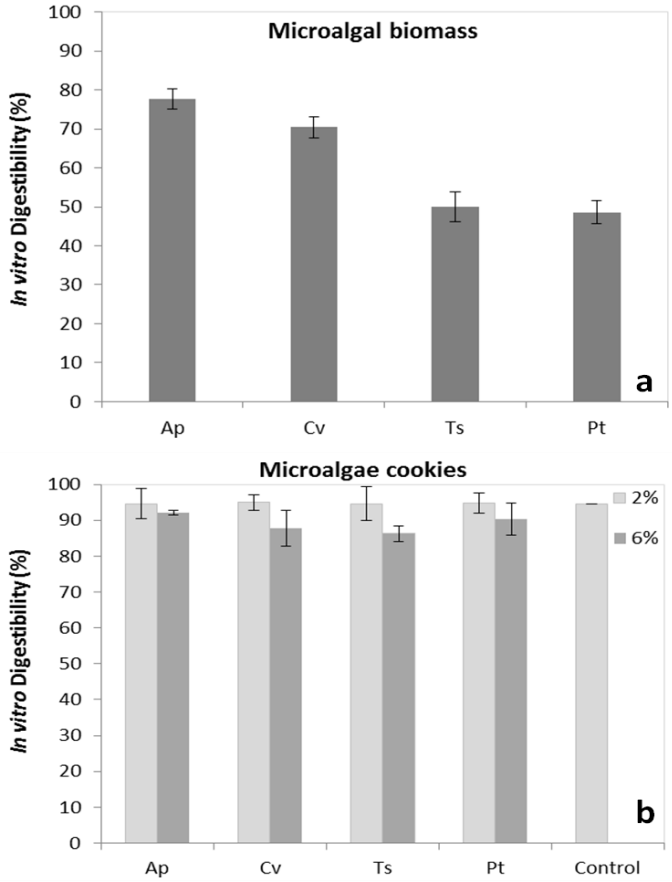

Fig. 6 *In vitro* digestibility (%) of four microalgal strains (a) and in cookies enriched with different levels of microalgae (b) (Ap – *A. platensis*, Cv – *C. vulgaris*, Ts – *T. suecica*, Pt – *P. tricornutum*).

4. Conclusions

The addition of microalgal biomass as natural ingredient resulted in cookies with an attractive and innovative appearance. Innovative and stable green tonalities varied, depending on the microalga used, from a blueish-green (*A. platensis*) to a brownish-green (*P. tricornutum*). *A. platensis* provided a significant structuring effect, in terms of cookies texture. In general, increasing microalgae content from 2% to 6% resulted in a significant increase in the cookies TPC (on average +115%) and AC (on average +66%), while digestibility was slightly lower (-5.9% on average).

From this study, there are prospects for considering microalgae-based cookies functional nutrient foods, which could be widely consumed on a daily basis by European citizens from all age groups.

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Conflict of interest

The authors declare that they have no competing interests.

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Chapter 8

Lactobacillus plantarum fermentation of microalgal and cyanobacterial biomass as a potential application for the production of functional foods

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Lactobacillus plantarum fermentation of microalgal and cyanobacterial biomasses as a potential application for the production of functional foods

The work reported in this chapter was carried out at Dublin Institute of Technology, Dublin under the guidance of Prof. Nissreen Abu-Ghannam and of my supervisors, Prof. Mario R. Tredici and Dr. Liliana Rodolfi, and in collaboration with Dr. Natascia Biondi and PhD student Emer Shannon

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Abstract

The first objective of this study was to evaluate the cyanobacterium *Arthrospira platensis* F&M-C256 and the green alga *Tetraselmis suecica* F&M-M33 (lyophilised and frozen biomasses) as substrates for lactic acid fermentation using *Lactobacillus plantarum* ATCC 8014. Within 48 h of the fermentation process, 10 and 9 log CFU mL^{-1} concentration for the lyophilised and frozen substrates, respectively, were reached. Microalgal biomass appears as a suitable substrate for *L. plantarum* growth in both forms. Considering that most of the photosynthetic microorganisms of commercial importance (mainly Chlorophyceae, such as *T. suecica*) have rigid cell walls that reduce their digestibility, the second objective was to investigate if fermentation was able to enhance *in vitro* digestibility of the two examined microalgae. Fermentation increased digestibility of both microalgae, but only in the frozen form (+10.5% for *A. platensis* and +11.1% for *T. suecica*). Lactic acid fermentation increased the antioxidant activity and the total phenolic content in the broths with *A. platensis* biomass (both frozen and lyophilised). Even if the characteristics of both lyophilised and frozen biomasses improved after fermentation, it is worth noting that the freezing process is significantly more economically viable than lyophilisation on an industrial scale. This study highlighted the potential of *A. platensis* and *T. suecica* biomasses for the production of economically valuable functional food products.
Keywords: Fermentation; Digestibility; Microalgae; Arthrospira; Tetraselmis, Food

1. Introduction

A number of technologies are employed to improve the organoleptic properties, nutritional profile, and shelf life of raw vegetable products while maintaining safe microbial levels (Jaiswal and Abu-Ghannam, 2013). Fermentation by lactic acid-producing bacteria is one such technology commonly used. Lactic acid fermented products from milk are well known, but a demand for dairy-free alternatives has arisen. This is driven by increasing intolerance to lactose, and concerns regarding the saturated fat content of dairy products and their effect on LDL cholesterol (Luckow and Delahunty, 2004). Plants, mainly vegetables, and macroalgae, therefore, represent a viable alternative for the production of fermented foods due to their availability and high nutritional value (Gupta et al.,

2011). Microalgae (including cyanobacteria) could represent interesting substrates for the production of new fermented foods. Carbohydrates in microalgae are allocated in different compartments of the cells mainly as reserve polymers (in the cytoplasm in cyanobacteria and in the plastids in microalgae), and structural components of the cell wall (Domozych et al., 2007; Xia et al., 2001), providing an excellent substrate for fermentation (Ho et al., 2012). Cyanobacteria synthesize glycogen as their primary energy reserve, and peptidoglycan as the major cell wall component (Tomaselli, 1997), while in green microalgae starch is the storage carbohydrate (Rismani-Yazdi et al., 2011) and cellulose the major component of the cell wall (Domozych et al., 2007). Microalgal polysaccharides have the potential to be effectively fermented (Gupta et al., 2011), thus possibly leading to the development of fermented functional foods. Reports of functional foods produced from fermented microalgae are limited in comparison to those relating to terrestrial plants (Gupta et al., 2011). These few studies focus particularly on the cyanobacterium *Arthrospira platensis* (commonly called spirulina) (Bhowmik et al., 2009).

Microalgal matrices can be provided for the fermentation process in different forms: dry (e.g. sun-dried, spray-dried, lyophilised) or frozen. The form in which microalgal matrix is presented could affect the fermentation process. From several decades, freezing and lyophilisation are two among the best known conservation processes for microalgae. The lyophilisation process ensures the best product preservation although it is economically disadvantageous and time-consuming compared to the freezing process (Ratti, 2001). Lyophilisation may negatively affect the antioxidant potential of organic matrices (Michalczyk et al., 2009). On the other hand, the freezing process followed by the thawing phase can damage cells, thus probably improving digestibility.

Besides the presence of fermentable carbohydrates, a balanced biochemical composition along with the high nutritional value, makes microalgae new functional food candidates (Tredici et al., 2009). Microalgae can contain high levels of proteins (50–70% dry mass), vitamins, and minerals (Becker, 2007). The nutritive value of microalgal proteins is of particular interest, due to the presence of all the essential amino acids such as leucine, isoleucine, and valine (Becker, 2007). Microalgae may also contain high levels of carotenoids (Del Campo et al., 2000), short-chain (Chacón-Lee et al., 2010) and long-chain polyunsaturated fatty acids (Becker, 2013). The cyanobacterium *A. platensis* is one of the microorganisms already approved under Regulation (EC) No 258/97

of the European Union concerning novel foods and novel food ingredients, due to its consumption prior to May 1997 (European Union, 1997). The green microalga *T. suecica*, not being consumed prior to May 1997, requires approval as novel food before commercialization. However, another species within this genus, *T. chuii*, has been approved (AECOSAN 2014). Thus, *Tetraselmis* and *Arthrospira* represent two of the most promising microalgal genera for the development of functional foods (Niccolai et al., 2016).

When the potential application of new matrices for the production of functional foods is studied, *in vitro* evaluation of digestibility is a fundamental analysis to provide information on their nutritional bioavailability (Boisen and Eggum, 1991). Microalgae can have high digestibility, as in the case of *Arthrospira* that being a cyanobacterium present a cell wall of easy breakage (Becker, 2007). In other cases, mainly green algae, such as *Tetraselmis*, the digestibility is generally low due to the robust cell wall which restricts the access of the gut enzymes to the cell components (Janczyk et al., 2005). In this case, an increase of enzyme accessibility to the cell content is necessary to improve digestibility (Doucha and Lìvansky, 2008) and this could be achieved through lactic acid fermentation (Harun et al., 2010). Fermentation process could also favour the release of a higher amount of intracellular proteins most likely as a result of disruption of the cell walls, dissociation of the nutrient-matrix complexes, or transformation into more active molecular structures (Parada and Aguilera, 2007). In accordance to Windey et al. (2012), the process can also break the amino acid chains increasing protein bioavailability.

Microalgae have potent antioxidant activity, due to the presence of compounds such as polyphenols and pigments (Tredici et al., 2009; Liu et al., 2011). This property is highly valuable for the production of functional foods. The radical scavenging abilities of *Arthrospira* and *Tetraselmis* have been evaluated in recent years (Liu et al., 2011; Ulloa et al., 2012). Liu et al. (2011) found that DPPH radical scavenging ability and total phenolic content of *Arthrospira* increased significantly after fermentation with lactic acid bacteria. This was attributed to the lysis of the cell wall during fermentation, which released unidentified phenolics which increase antioxidant capacities (Liu et al., 2011). Findings such as these show the potential of fermentation to improve the bioactivity of microalgae-based foods.

This study was designed to investigate one cyanobacterium (*A. platensis* F&M-C256) and one Chlorophyta (*T. suecica* F&M-M33) as substrates for *Lactobacillus plantarum* fermentation and the fermented products were

evaluated in terms of *in vitro* digestibility, antioxidant capacity, and total phenolic content. These parameters are important attributes for the development of functional food products for which microalgae could be considered as interesting candidates.

2. Materials and methods

2.1 Microalgal material

Biomasses of the green microalga *Tetraselmis suecica* F&M-M33 and of the cyanobacterium *Arthrospira platensis* F&M-C256 were provided by Archimede Ricerche S.r.l., Camporosso, Imperia, Italy. Both strains were cultivated in GWP®-I photobioreactors (Tredici et al., 2004) in semi-batch mode. *A. platensis* was cultivated in Zarrouk medium (Zarrouk, 1966), harvested by filtration while the biomass was washed with saline solution to remove excess bicarbonate before being frozen. *T. suecica* was cultivated in F medium (Guillard and Ryther, 1962), harvested by centrifugation, and the algae paste obtained frozen. Aliquots of each biomass were lyophilised and powdered. The frozen and powdered biomasses were stored at -20 °C until use. In the literature, *A. platensis* is often incorrectly reported with the name *Spirulina platensis*. To avoid confusion with the true *Spirulina* genus, we have always adopted the correct denomination *A. platensis*.

2.2 Bacterial inoculum preparation

Lactobacillus plantarum ATCC 8014 was purchased from Cruinn Diagnostics Ltd, Dublin, Ireland. The culture was maintained at -80 °C (Thermo Scientific Revco Elite PLUS Freezer ULT2586-10-A, Massachusetts, U.S.A.) in 20% glycerol stocks.

For the preparation of the inoculum, de Man, Rogosa and Sharpe (MRS) broth (Scharlau Chemie, Spain) was autoclaved (15 min, 121 °C) (Tomy SS-325, Tomy Seiko Co. Ltd, Japan). 25 mL of the cooled, sterile broth were inoculated with 1 mL of thawed *L. plantarum* stock culture and incubated at 37 °C for 24 h (Gallenkamp, Weiss Technik, U.K.). This inoculum was then serially diluted one hundred times to obtain a working culture containing 6-7 log CFU mL^{-1} as determined by plate counts.

2.3 Fermentation

In order to determine the applicability of *A. platensis* and *T. suecica* as growth substrate for *L. plantarum,* lyophilised and frozen biomasses of each species were tested according to a modified method by Gupta et al. (2011). 5 g of each frozen or lyophilised *T. suecica* and *A. platensis* biomasses were transferred in a 100 mL Erlenmeyer flask and 48 mL deionised water was added. Under sterile conditions, the water and biomass broths were inoculated with 2 mL of *L. plantarum* culture. The flasks were incubated at 37 °C and 100 rpm in an orbital shaker (Innova 42, Mason Technology, Ireland). Samples were taken at 0, 24, 48, and 72 h for microbiological analysis. According to Gupta et al. (2011), a total of 72 h of fermentation was considered a sufficient time to observe the responses of the investigated fermentation parameters.

The pH of fermented samples was measured (Orion^{TM} 2 Star^{TM} pH meter Benchtop, Singapore) at 0 and 72 h.

To evaluate the post-fermented *in vitro* digestibility, antioxidant capacity and total phenolic content of each sample, aliquots (15 mL) were taken after 72 h of incubation and stored at 4 °C (Lec AC150, 119 L, U.K.) until analysis.

2.4 Analytical methods

2.4.1 Microbiological analysis

Viable cell counts in the microalgal broth (log CFU mL^{-1}) were performed by the standard plate method with MRS agar (Scharlau Chemie, Spain). Fermentation broth was serially diluted (1:10) in Maximal Recovery Diluent (Sigma–Aldrich, Germany) before plating . The plates were incubated at 37 °C for 36-48 h for cell enumeration. Viable cell counts were carried out at the start and after 24, 48, and 72 h of incubation.

2.4.2 HPLC analysis of lactic acid

Samples were collected from the fermented broths at the start and after 24, 48, and 72 h, diluted 1:10 with water and centrifuged at 16,211 x g for 15 min at 4 °C (ROTANTA 460 R centrifuge, Hettich Zentrifugen, Germany). Cell-free supernatant was used to quantify the lactic acid content. Analyses of lactic acid content were carried out with a high performance liquid chromatography (HPLC) system (Waters Alliance, e2695 Separation module, Massachusetts, U.S.A.) equipped with an auto sampler and dual pump controller. The detection

system consisted of a Waters-2996 UV detector (210 nm) and a differential refractometer (Waters-410) connected in series. The column (Rezex ROA-Organic acid 5 H+ (8%) 350.0 mm x 7.8 mm; Phenomenex, U.K.) was maintained at a temperature of 65 °C and preceded by a guard column (50.0 mm 7.8 mm; Phenomenex, U.K.). An isocratic programme was used with a flow rate of 0.9 mL min^{-1} with a mobile phase of ammonium phosphate monobasic (0.05 M) ($NH_4H_2PO_4$) (Sigma–Aldrich, Germany) adjusted to pH 2.4 with concentrated phosphoric acid (H_3PO_4) (Sigma–Aldrich, Germany). An injection volume of 20 µL was used. Each sample was injected twice. Samples and mobile phases were pre-filtered using a 0.22 µm Millipore filter (Millipore, Massachusetts, USA). Data acquisition and integration were performed using Empower™ 4.0 software. Sodium lactate standards (Sigma-Aldrich, Germany) were used to identify and quantify lactic acid in the samples.

2.4.3 Total carbohydrates

Samples were collected from the fermented broths and centrifuged as per Section 2.4.2. Total carbohydrates of the solid and liquid phases were determined in the pellet and supernatant fractions, respectively, of the centrifuged broth. Total carbohydrates were estimated by the phenolsulphuric acid method (Dubois et al., 1956) and compared to a glucose (Sigma–Aldrich, Germany) standard curve.

2.4.4 In vitro *digestibility*

The *in vitro* digestibility was performed on the unfermented and fermented biomass after 72 h of incubation. An adapted method developed by Boisen and Fernández (1997) was used to evaluate *in vitro* digestibility. The *in vitro* analysis reproduces the chemical-enzymatic catalysis (gastric and pancreatic juice) that occurs in the proximal tract of the monogastric digestive system.
After 72 h of fermentation, the collected samples were lyophilised for 24 h and carefully powdered before analysis. Samples of 1 g of finely ground material (particle size ≤1 mm) were weighed and transferred in 250 mL conical flasks. To each flask, phosphate buffer (25 mL, 0.1 M, pH 6.0) was added and mixed, followed by HCl (10 mL, 0.2 M). The pH was adjusted to 2.0 by the addition of HCl (1 M) or NaOH (1 M). A freshly prepared pepsin water solution (3 mL; Applichem, Germany) containing 30 mg of porcine pepsin with an activity of 0.8 FIP-U/mg was added. The flasks were closed with a rubber stopper and

placed on multipoint stirrers at 150 rpm for 6 h in a thermostatically-controlled heating chamber (Innova® 42 Incubator shaker, Mason Technology, Ireland) at 39 °C. Following incubation, phosphate buffer (10 mL, 0.2 M, pH 6.8) and NaOH solution (5 mL, 0.6 M) were added to each sample. The pH was adjusted to 6.8 by addition of HCl (1 M) or NaOH (1 M). A freshly prepared pancreatin ethanol : water solution (10 mL, 50:50 v/v) containing 500 mg of porcine pancreatin with an activity of 42362 FIP-U/g (Applichem, Germany) was added to each sample. After closing with a rubber stopper, the flasks were again incubated for 18 h at 39 °C, 4 x g. A reagent blank without biomass was also prepared.

The undigested residues were collected by centrifugation (ROTANTA 460 R centrifuge, Hettich Zentrifugen, Germany) at 10,375 x g for 30 minutes. The undigested material (sedimentation pellet) was washed with deionised water to remove any salts from the buffer, and then centrifuged as per the parameters above.

The supernatant was removed after the second centrifugation, and filtered on 47 mm glass-fiber membranes with a pore diameter of 1.2 µm (FILTER-LAB, Spain). The pellet and filter papers were dried at 80 °C for 6 h, and then at 45 °C until a constant mass was reached. To account for any residual biomass or undissolved reagent not sedimented during centrifugation, the mass of the dried residue on each filter paper was added to the mass of the undigested material (pellet). The reagent blank was processed in the same manner. The percentage *in vitro* digestibility of dry matter was calculated according to Boisen and Fernández (1997):

$$D\ (\%)\ = \frac{SW - [(UW\ +\ RP)\ -\ B]}{SW}\ \text{x}\ 100$$

where:

D is the *in vitro* digestibility of dry matter expressed as percentage

SW is the mass of the starting material expressed in grams

UW is the mass of the undigested material expressed in grams

RP is the residual particulate, any residual biomass and/or undissolved reagent re-suspended from the pellet or not sedimented expressed in grams

B is the blank (all reagents without biomass) expressed in grams

2.4.5 DPPH assay

In order to evaluate enhancements in radical scavenging abilities due to a release of antioxidant components after fermentation for both frozen and lyophilised *A. platensis* and *T. suecica*, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was carried out according to Rajauria et al. (2013).
Briefly, the assay was performed in a 96-well (non-sterile) round-bottom microtiter plate (Greiner Bio-One International GmbH, Germany) with 1:1 ratio of 100 µL of DPPH radical solution (165 µM, in methanol, Aldrich, Ireland) and 100 µL of sample (equal to a final extract concentration of 0.2 mg mL^{-1} methanol:water 1:5). The reaction mixtures were incubated in darkness at 30 °C for 30 min. The absorbances were measured at 517 nm using a UV-Vis spectrophotometric plate reader (BioTek PowerWave with Gen5 Data Analysis software, Vermont, U.S.A.). The following equation was used to calculate the DPPH radical scavenging capacity:

$$\text{Scavenging capacity (\%)} = \left[1 - \left(\frac{A_{\text{sample}} - A_{\text{sample blank}}}{A_{\text{control}}}\right)\right]$$

where $A_{control}$ is the absorbance of the control (DPPH solution without sample), A_{sample} is the absorbance of the test sample (DPPH solution plus test sample) and $A_{sample\ blank}$ is the absorbance of the sample only (sample without DPPH addition).

Results were expressed as DPPH radical-scavenging capacity (%).

2.4.6 Total phenolic content assay

The total phenolic content assay was carried out according to Rajauria et al., 2013 using the Folin Ciocalteu assay. 0.1 g of lyophilised or frozen *A. platensis* and *T. suecica* samples were dissolved in 10 mL of deionised water. To an aliquot of each sample (100 µL), 2% sodium carbonate (2 mL; Sigma-Aldrich, Ireland) was added. After 2 min, 50% Folin Ciocalteu reagent (100 µL; Sigma-Aldrich, Ireland) was added. The reaction mixture was incubated in darkness at 25 °C for 30 min. The absorbance of each sample was measured at 720 nm using a UV-Vis spectrophotometric microplate reader (BioTek PowerWave with Gen5 Data Analysis software, Vermont, U.S.A.). Results were expressed

in gallic acid equivalents (mg GAE g^{-1}) of *A. platensis* or *T. suecica* biomass, through a calibration curve of gallic acid (0 to 500 μg mL^{-1}, Sigma, Ireland).

3. Statistical analysis

All analyses were conducted in triplicate. The results were expressed as mean ± SD (standard deviation). All statistical analyses were carried out using Statgraphics Centurion XV (StatPoint Technologies Inc., Virgina, U.S.A.). Statistical differences between different fermented broths were determined using ANOVA followed by multiple range tests to determine the least significant difference (LSD). Differences were considered statistically significant when $P < 0.05$.

4. Results and discussion

4.1 Suitability of A. platensis and T. suecica for lactic acid fermentation

The growth curves of *L. plantarum* upon the utilization of *A. platensis* and *T. suecica* biomass that have been either lyophilized or frozen prior to the fermentation process are shown in Fig. 1. At the start of the process, an average bacterial concentration of 5.1 ± 0.2 log CFU mL^{-1} was found. Within 48 h of the fermentation process, a concentration of 10 log CFU mL^{-1} for the lyophilised substrates and 9 log CFU mL^{-1} for the frozen substrates was reached. Significant differences in *L. plantarum* growth ($P<0.05$) were seen for lyophilized and frozen biomasses. It is worth noting that the substrate in the frozen biomass broths was, in terms of dry material available, one-fourth (*T. suecica*) or one-fifth (*A. platensis*) of that in the lyophilised broths. With one gram of dry substrate from 11.4 to 11.6 log CFU were produced with either lyophilised or frozen biomasses; lyophilised substrates were able to produce only 1.2 (*T. suecica*) to 1.5 (*A. platensis*) times more growth than frozen biomasses. Microalgal biomass appears as a suitable substrate for *L. plantarum* growth in both forms, although frozen biomass appears more effective.

Several authors (Bergqvist et al., 2005; Gardner et al., 2001; Yoon et al., 2006) have studied the growth curves of lactic acid bacteria on conventional organic matrices, as beet, onion, carrot and cabbage. From these studies the maximum concentration reached by various species of *Lactobacillus* ranged from 9 log CFU mL^{-1} after 48 h on cabbage juice (Yoon et al., 2006) or 72 h on a vegetable

mixture (Gardner et al., 2001) to 10 log CFU mL^{-1} after 24 h on carrot juice (Bergqvist et al., 2005). However, it must be considered that in these studies *Lactobacillus* initial concentration was always higher than in our study. The results of Fig. 1 indicates that microalgae can be considered a competitive organic matrix compared to conventional fermentation substrates.

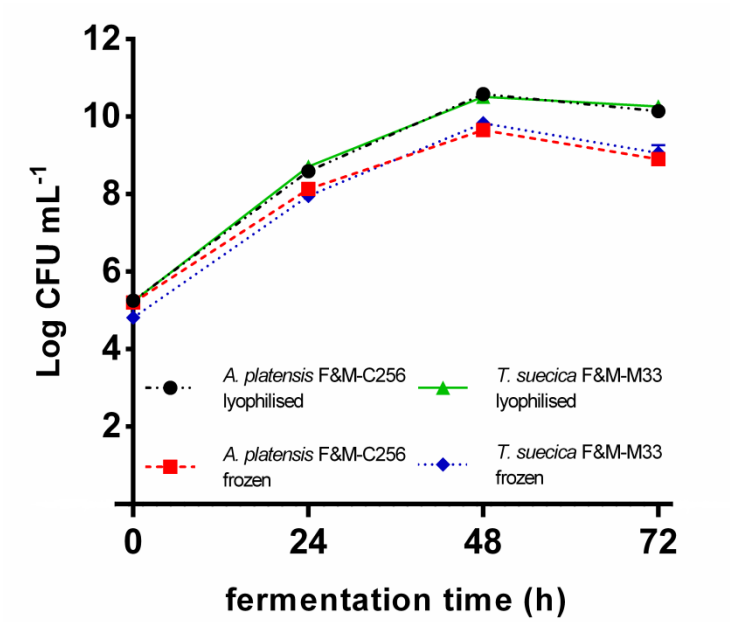

Fig. 1. Growth curve (log CFU mL^{-1}) of *L. plantarum* with lyophilised and frozen *A. platensis* and *T. suecica* biomass fermentation

The suitability of microalgae for fermentation by lactic acid bacteria has been previously evidenced (Uchida and Miyoshi, 2013). The addition of *A. platensis* (up to10 g L^{-1}) promoted the growth of different *Lactobacillus* strains up to a maximum of 7-9 log CFU mL^{-1} (according to the strain) after 10 h, starting from a concentration of about 2-3 log CFU mL^{-1} (Bhowmik et al., 2009). These values are comparable to those obtained in the present study. The positive effects of microalgae on the viability of lactic acid bacteria can be attributed to the highly nutritious substances they contain which stimulate bacterial growth and activity. These substances include exopolysaccharides, free amino acids, and essential vitamins and minerals (Parada et al., 1998).

Substrate of *Lactobacillus* growth is carbohydrate. Fig. 2 illustrates the total carbohydrate concentration of the broths prepared with *A. platensis* and *T.*

suecica biomasses before inoculation with *L. plantarum* and its partitioning between the solid and liquid fractions of the fermentation broth at the start of the process. Before inoculation, the *A. platensis* broth showed a significantly higher total carbohydrate content (P <0.05), for both lyophilised and frozen biomasses, compared to *T. suecica*. Due to the lower initial dry biomass concentration, in the broths from frozen biomasses the total carbohydrate concentration was lower than in those from lyophilised biomasses. At the start of the fermentation (inoculation with *L. plantarum*) carbohydrates partitioned between the solid (biomass) and the liquid phase, with the larger fraction remaining within the solid phase.

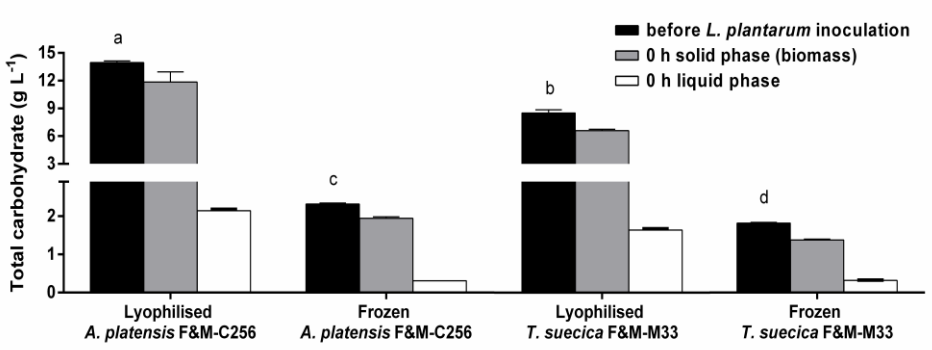

Fig. 2. Total carbohydrate concentration in the broths prepared with *A. platensis* and *T. suecica* biomasses before inoculation with *L. plantarum* and at the start of the fermentation process, when partitioning between solid phase (biomass) and liquid phase occurred. Values are expressed as mean ± SD. For the two lyophilised and the two frozen biomasses before inoculation, different letters denote a significant difference (P <0.05)

A decrease in carbohydrate concentration (60-65% for *A. platensis* and 70-75% for *T. suecica*) in the liquid phase of the broths was observed as fermentation time increased. At the same time a decrease in carbohydrate content of the solid phase was detected (about 15% in *A. plantesis* and 25% in *T. suecica*). Thus, it seems that carbohydrate in the solid phase were released in the liquid phase with increasing difficulty as the fermentation proceeded.

Lactic acid concentration in the fermentation broths during the process is reported in Fig. 3. Concentration in the two frozen biomass broths significantly increased (P <0.05) between 0 and 24 h with a production of about 0.7 g L^{-1} for

both biomasses. The maximum concentration (1.4-1.5 g L^{-1}) was reached after 48 h. In both broths with lyophilised biomasses the highest lactic acid production (about 2.0 g L^{-1} for both biomasses) occurred in the first 24 h of fermentation. Also in this case maximum concentration was reached after 48 h (3.5-3.7 g L^{-1}). The four tested broths followed overall a similar trend, however, as it emerges from Fig. 3, lyophilised biomasses were able to induce a higher lactic acid production compared to frozen biomasses. Nevertheless, when lactic acid production per unit substrate dry weight after 48 h of fermentation is considered, broths with frozen biomasses demonstrated to be the most productive, the best substrate being *A. platensis* (52.3 mg of lactic acid produced per gram of dry material, compared to 36.2 mg g^{-1} for *T. suecica*). The two lyophilized biomass broths showed lower and similar productions (about 27 mg g^{-1}). The trend observed for lactic acid concentration was similar to that of *L. plantarum* growth (Fig. 1).

A significant decrease (P <0.05) in pH to values <6 was observed in both lyophilised biomass broths (-14% and -30% for *A. platensis* and *T. suecica*, respectively), and in frozen *A. platensis* broth (-13%) during fermentation. The pH of frozen *T. suecica* broth, instead, increased until 9 at the end of the fermentation; further tests are necessary to explain this increase.

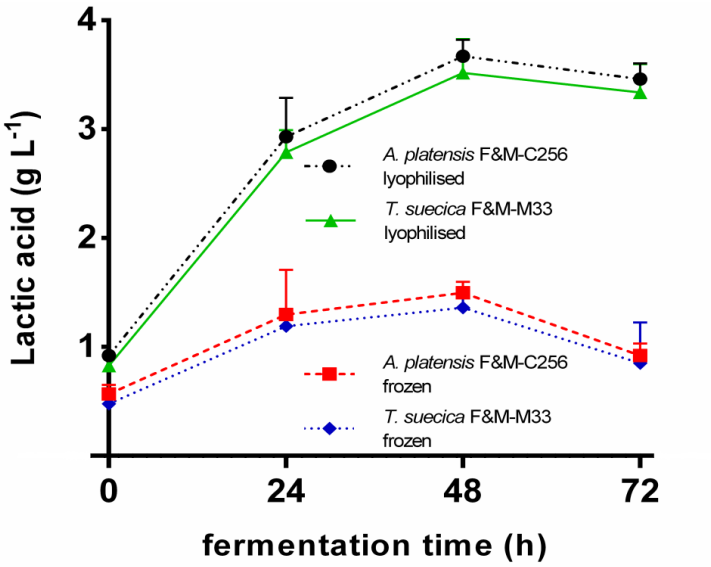

Fig. 3. Lactic acid production (g L^{-1}) in broths with lyophilised and frozen *A. platensis* and *T. suecica* biomasses at different fermentation times. Values are expressed as means ± SD

Common cereal grains have been studied for their ability to support the growth of lactic acid bacteria. For example, Tanaka et al. (2006) found that *L. delbrueckii* IFO 3202 was able to produce 28 g L^{-1} lactic acid from 100 g L^{-1} of rice bran. Although an equal concentration of substrate (100 g L^{-1} of lyophilised *Arthrospira* or *Tetraselmis*) was used in the present study, a maximum lactic acid concentration of about 3.5 g L^{-1} was obtained after 48 h of fermentation. The greater yield of lactic acid attained with rice bran may be explained by the different carbohydrate content and profile, probably more suitable to sustain *Lactobacillus* metabolism in rice bran, which contained 11.3% cellulose + hemicellulose and 46.7% starch + dextrin, whereas lyophilised *A. platensis* F&M-C256 and *T. suecica* F&M-M33 contained 13.4% and 8.2% total carbohydrates, respectively.

Also Gupta et al. (2011) for fermented seaweeds obtained lactic acid productions similar to those reached by fermenting microlagal biomasses in this work. Uchida and Miyoshi (2013) reported lactic acid production from microalgae such as *Chlorella* spp., *Tetraselmis* spp., and *Nannochloropsis* spp. in the range of 1.5 - 5.4 g L^{-1}. In addition, pre-treatment of microalgae with enzymes (as macerozyme and lactase) increased lactic acid production from 5.4 to 9.6 g L^{-1} for *Chlorella* spp., and from 1.5 to 4.3 g L^{-1} for *Tetraselmis* spp. Lactic acid production reported by these authors for *Tetraselmis* spp. is in good agreement with that obtained for lyophilised biomass in the present study, although our biomasses were not pre-treated. Degradation of the cell walls via enzymolysis could promote a greater production of lactic acid during fermentation. Moreover, the effect of this pre-treatment combined with further degradation occurring during fermentation could also increase the other investigated parameters (*in vitro* digestibility, antioxidant capacity and total phenolic content).

4.2 In vitro *digestibility*

A modified protocol based on the Boisen and Fernandez method (1997) was used to assess the *in vitro* digestibility of the unfermented and fermented (72 h) biomasses (Fig. 4). Before fermentation, lyophilised and frozen *A. platensis* biomasses had a significantly (P <0.05) greater digestibility, expressed as dry matter percentage, than *T. suecica* biomasses (+32% and +30%, respectively). The same situation (+45% and +42% for lyophilized and frozen biomass, respectively) was observed after fermentation, although with an increased difference. For frozen fermented biomasses a similar significant increase in

digestibility compared to the unfermented (P <0.05) was observed for both algae (from 77.3% to 85.4% and from 54.2% to 60.3% for *A. platensis* and *T. suecica*, respectively). Fermentation did not improve digestibility of lyophilised biomasses, as the small increases observed were not significant (P >0.05).

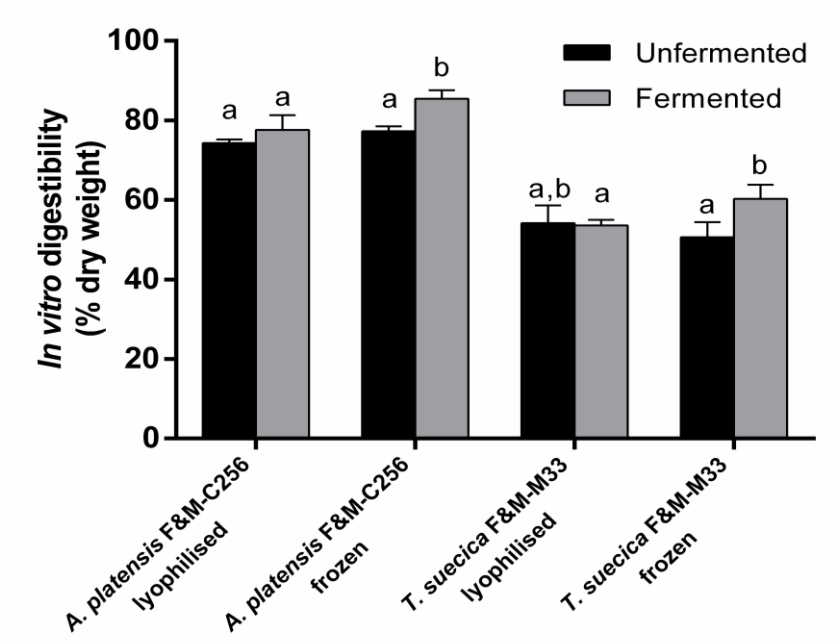

Fig. 4. *In vitro* digestibility (expressed as percent of dry matter) of lyophilised and frozen *A. platensis* and *T. suecica* biomasses before and after fermentation (72 h). Values are expressed as mean ± SD. For each strain, a different letter denotes a significant difference (P <0.05)

The cell wall (theca) of *T. suecica* is composed of polysaccharides made of acidic sugars, mainly unusual 2-keto sugars (comprising up to 65% of the theca dry matter), rather sensitive to acidic hydrolysis, and glycoproteins (Becker et al., 1994; Domozych et al., 2012). The breakage of the cell wall occurring, at last partially, during the thawing of the frozen biomasses (Mazur, 1984) resulted in an improvement in *in vitro* digestibility compared to the lyophilized biomass. *A. platensis* is a cyanobacterium, thus the cell wall structure is that of Gram negative bacteria with some modifications. The cell structure of *Arthrospira* has been well documented (Tomaselli, 1997). The low concentration (<1%) of β-1,2-glucan (first layer), a polysaccharide with low digestibility, the small

thickness (12 nm) of the first layer, and the proteic and lipopolysaccharidic nature of the second layer resulted in a higher digestibility of this alga. The easy breakage of its cell walls allowed a higher accessibility to its intracellular content by enzymes, and for this reason chemical or physical processing steps are not required in order to make its biomass more digestible (Tomaselli, 1997). Mišurcová et al. (2010) evaluated the *in vitro* digestibility of unfermented lyophilized *A. platensis* and Spirulina Pacifica® (registered mark of *A. platensis* by Cyanotech Corp, U.S.A.), finding a higher dry matter digestibility value (94.3%) both for *A. platensis* and Spirulina Pacifica® (85.6%) compared to that obtained in the present study for *A. platensis* F&M-C256 (74.3%). To our knowledge, no literature is available concerning digestibility of *T. suecica* biomass (fermented and unfermented), as well as digestibility of fermented *A. plantesis*.

With the exception of the cyanobacteria, many microalgae of commercial importance (among which several green algae, such as *T. suecica*) have rigid cell walls, thus making, in many cases, the adoption of mechanical and/or enzymatic methods to rupture cells necessary to improve digestibility. A high influence of different post-harvesting treatments on the digestibility of various algal species was demonstrated (Becker, 2013). The present study showed how the fermentation process led to a higher *in vitro* digestibility after fermentation for both frozen biomasses.

4.3 Enhanced antioxidant capacity and phenolic content post-fermentation

Arthrospira has *in vitro* and *in vivo* antioxidant, radical scavenging and anti-inflammatory activity principally due to the presence of phycocyanin. Phycocyanin contains an open chain tetrapyrrole chromophore, phycocyanobilin, which is covalently attached to the apoprotein (Bhat and Madyastha, 2001). Liu et al. (2011) hypothesized that antioxidants or phycocyanobilin released from *Arthrospira* when fermented would have better biological activity.

The comparative DPPH radical scavenging capacity of fermented and unfermented lyophilised and frozen *A. platensis* and *T. suecica* biomasses at 0.2 g L^{-1} methanol : water (1:5) extract concentration are illustrated in Fig. 5 . Lyophilised *A. platensis* exhibited 20.5% DPPH inhibition before fermentation, increased to 36.6% after fermentation (+79%). A high increase (+69%) for frozen *A. platensis* was also observed. On the contrary, *T. suecica* exhibited a

45.6% (lyophilised) and 41.3% (frozen) DPPH inhibition before fermentation, which decreased to 34.4% and 36.4%, respectively, after fermentation (Fig. 5).

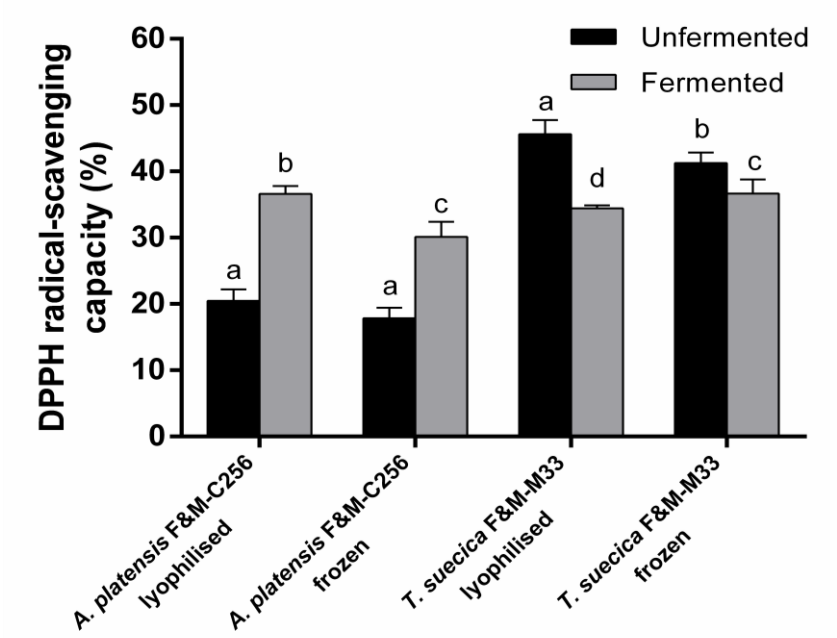

Fig. 5. DPPH inhibition (%) by lyophilised and frozen *A. platensis* and *T. suecica* biomasses before and after fermentation. Values are expressed as means ± SD. For each alga, different letters denote a significant difference (P <0.05)

Similar to the results obtained in the present study, Liu et al. (2011) reported a radical scavenging capacity of *A. platensis* biomass between 15% and 20%, although at a higher broth concentration (20 g L^{-1}). El-Baky and El-Baroty (2012) and Chu et al. (2010) found a radical scavenging ability of about 20% at concentrations from 0.005 to $2.5 \cdot 10^{-5}$ g L^{-1} for phycocyanin extracted from *Arthrospira maxima* and *A. platensis*. Correlated with the trend observed in the present study, Liu et al. (2011) found an improvement (approximately +78%) in DPPH scavenging ability after fermentation of *A. platensis* biomass.

Tetraselmis contains phenolic compounds (Goiris et al., 2012) and a high proportion of chlorophyll *a* as principal antioxidants (Pane et al., 1998). As reported by Lanfer-Marquez et al. (2005), chlorophylls are capable of inhibiting the DPPH radical. *T. suecica* also contains other antioxidant compounds, such as tocopherols (up to 125 µg g^{-1} dry weight, Chini Zittelli et al., 2006) and tocotrienols (Vismara et al., 2003; Liu et al., 2008). As a consequence, this

microalga has the potential to exert potent antioxidant effects. Some authors (Ulloa et al., 2012; Custódio et al., 2012; Jo et al. 2012) found a radical scavenging ability of 40%, 46% and 55% at higher concentrations compared to our study (30, 10 and 2 g L^{-1}, respectively) for *T. chuii* and *T. suecica*. Unexpectedly, the fermented *T. suecica* F&M-M33 biomass showed a lower percentage of inhibition compared to the unfermented sample (Fig. 5). The simultaneous actions of enzymes, weak acids, oxygen, light, and heat can lead to the formation of a large number of degradation products, including those from chlorophylls (Mangos and Berger, 1997). Moreover, tocopherols are also subject to degradation by heat and light (Allwood and Martin, 2000). Since fermentation was carried out at 37 °C for 72 h in the present study, also temperature may have contributed to degrade chlorophylls and tocopherols in *T. suecica* F&M-M33, thus decreasing its antioxidant power. To the authors' knowledge, no data has been published on DPPH inhibition of fermented *T. suecica* biomass.

The total phenolic content of lyophilised and frozen *A. platensis* and *T. suecica* biomasses during fermentation is illustrated in Fig. 6. The broths from lyophilised biomasses showed a higher phenolic content compared to those from frozen biomasses until 48 h from the start of the fermentation for both species. Unexpectedly, after 48 h the broth from frozen *A. platensis* biomass showed a higher total phenolic content compared to that from lyophilised biomass. Observing the total phenolic content in the two *T. suecica* broths, in that with lyophilised biomass a maximum of 11.9 mg GAE g^{-1} at 24 h was reached and a decrease to 5.2 mg GAE g^{-1} at 72 h was observed. A similar trend was observed in the broth with frozen *T. suecica* biomass. In the broth with lyophilised *A. platensis* biomass total phenolic content increased from 0 h (4.2 mg GAE g^{-1}) to 48 h (17.4 mg GAE g^{-1}) and then decreased at 72 h (10.0 mg GAE g^{-1}) In the broth with frozen biomass a completely different trend was observed: although the maximum value reached was slightly lower compared to that measured in the lyophilised biomass broth, the total phenolic content continued to increase from the start (1.8 mg GAE g^{-1}) to the end (11.8 mg GAE g^{-1}) of the fermentation.

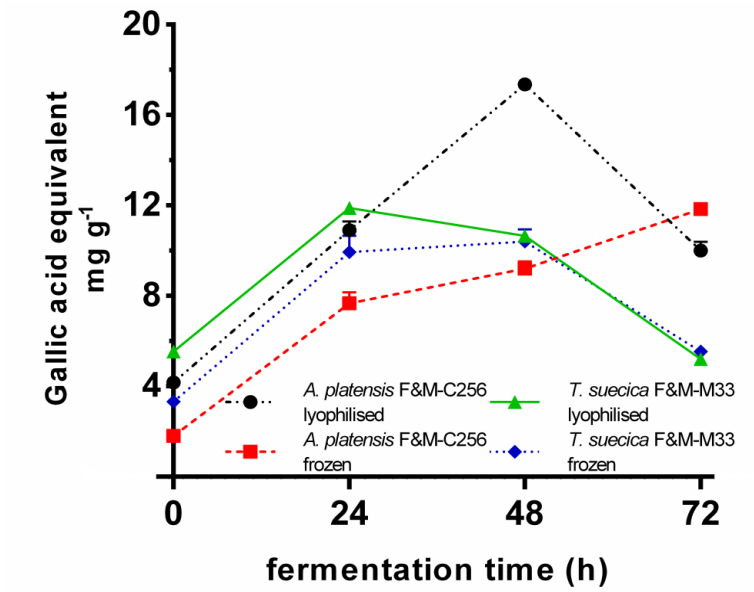

Fig. 6. Total phenolic content (expressed as gallic acid equivalents mg g^{-1} dry mass) in the broths with lyophilised and frozen *A. platensis* and *T. suecica* biomasses at different fermentation times. Values are expressed as means ± SD

Only few studies have investigated the role of phenolic compounds in microalgae (Chaiklahan et al., 2013; Hajimahmoodi et al., 2010; Li et al., 2007). Phenolic compounds, synthesized as secondary metabolites, are considered the major contributors to antioxidant capacity (Abd El-Baky et al., 2009). Total phenolic content of *Arthrospira* reported in the literature varies from 2.4 to 49.8 mg GAE g^{-1} depending on culture medium (Abd El-Baky et al., 2009), extraction methods (Chaiklahan et al., 2013) and culture conditions (Kepekçi and Saygideger, 2012). Liu et al. (2011) found that unfermented *A. platensis* powder contained 19.5 mg GAE g^{-1}, and the broth with *Arthrospira* fermented with lactic acid bacteria after 48 h incubation contained 33.6 mg GAE g^{-1}. The fermentation process could have released polyphenols such as gallic acid, or produced other metabolites (Liu et al., 2011). Since total phenols are, amongst other compounds, responsible for antioxidant activity (Abd El-Baky et al., 2009; Liu et al., 2011), the greater quantity of total phenols in fermented *A. platensis* F&M-C256 may indicate a greater antioxidant activity than unfermented biomass, which is confirmed by the DPPH data (Fig. 5).

Ahmed et al. (2014) and Custódio et al. (2014) found values for total phenolic content in *Tetraselmis* spp. aqueous extract of the hexane-extracted biomass and

in *T. suecica* ethyl acetate extract of 0.8 mg GAE g^{-1} and 1.1 mg GAE g^{-1}, respectively. Goiris et al. (2012) measured phenolic contents of 3.74 mg GAE g^{-1} and 1.71 mg GAE g^{-1} in ethanol/water extracts of *Tetraselmis* spp. and *T. suecica*, respectively. The results obtained in this work showed for T. suecica F&M-M33 aqueous extracts much higher total phenolic contents. To the authors' knowledge, no data on the total phenolic content of *T. suecica* after fermentation have been published to date.

5. Conclusions

At the end of lactic acid fermentation, cell growth of *L. plantarum* was good with both lyophilised and frozen biomasses and no differences were found between *A. platensis* and *T. suecica*, demonstrating that both lyophilised and frozen biomasses were excellent substrates. After fermentation, a significant increase in digestibility was observed for frozen but not for lyophylised biomasses. Moreover, an increase in the antioxidant activity was observed with *A. platensis*. Total phenolic content increased during fermentation, although biomass freezing had a negative impact, resulting in a lower content compared to broths from lyophilised biomasses. Frozen biomass, also considering the results per unit dry weight of substrate, appears to be a better fermentable substrate (higher lactic acid production, higher digestibility). Furthermore, it is more economically viable on an industrial scale because it obviates the need for a drying process (as lyophilisation or spray drying), that would be highly expensive in terms of energy costs, as well as time-consuming.
In conclusion, fermentation of microalgae is a promising technology and remains open to future studies. Thanks to their excellent biochemical and nutritional profile, microalgae are good candidates for the production of functional food products through fermentation.

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Conflict of interest

The authors declare that they have no competing interests.

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Chapter 9

General discussion: prospects for microalgae and cyanobacteria as source of innovative foods and nutraceuticals

9.1 General discussion

The world demand for foods is increasing and conventional agriculture shows a limited ability to face the increase in demand, especially under the changing climatic conditions and considering the increasing need of freshwater by urban areas. The problem of food scarcity and malnutrition is still unsolved today with a world population of 7 billion and expected to surpass 9 billion in 2050 (Antal and Van Den Bergh, 2016). Malnutrition occurs in people who are either undernourished or overnourished. Undernutrition is the condition that develops when the body does not get the right amount of the vitamins, minerals, and other nutrients it needs to maintain healthy tissues and organ function. While, overnutrition results from eating too much and eating too many unhealthy foods, or taking too many vitamins or other dietary replacements (Website FAO, glossary).

Alternative food sources, like microalgae, appear a possible next generation of crops for the agro-industry. Indeed: i) many microalgae show a balanced biochemical composition, with a high protein content, vitamins, minerals, carotenoids, short-chain and long-chain polyunsaturated fatty acids; ii) their cultivation can be carried out in non-arable lands using saline or sea waters, so avoiding competition with traditional crops and with humans and animals for freshwater consumption; iii) they can grow on wastewaters, reducing the water pollution; iv) they do not need of pesticides or herbicides for their cultivation limiting pollution by these products (Tredici et al., 2009; Tredici, 2015). However, only few microalgal species have been selected for large-scale food production, among these *Chlorella*, *Arthrospira* and *Dunaliella* (Norsker et al., 2011).

The general aim of the research, reported in **Chapter 2**, was to evaluate the nutritional properties of some microalgal and cyanobacterial biomasses that might be proposed for the production of innovative foods and nutraceuticals. For the use of microalgal biomass for food and nutraceutical purposes, there is a need to determine certain characteristics: i) the nutritive value of the alga; ii) the presence of toxic substances; iii) its digestibility; iv) the acceptability of the product by the consumer (Becker, 2004).

Chapter 3 reports the tests used to investigate the possible *in vitro* toxicity of selected microalgal and cyanobacterial strains. Methanolic and aqueous extracts of the microalgal biomasses were tested on human dermal fibroblasts and on *Artemia salina* models. Concerning the *in vitro* toxicity studies, most of the

literature focused on tests against cancer cell lines (Jaki et al. 1999; Carballo et al. 2002; Bechelli et al. 2011; Hisem et al. 2011) and only few studies, to our knowledge, evaluated the cytotoxic effects of microalgae on non-cancerous cells (Prestegard et al. 2009; Lopes et al. 2011; Goh et al. 2014). The use of normal human cell lines as human dermal fibroblasts, that present a non-aberrant phenotype, is an added value with respect to the use of cancerous cells that typically show chromosome and phenotype aberrations (Jallepalli and Lengauer 2001; Fackenthal and Godley 2008).

As found by Logrieco et al. (1996), that found that extracts from *Fusarium subglutinans* have a different pattern of toxicity on *A. salina* and human B lymphocytes, our data indicate that extracts from microalgae show different toxicity on *A. salina* and fibroblasts due to a different sensitivity and complexity between the two models. Moreover, the different toxicity patterns often observed in methanolic and aqueous extracts tested on *A. salina* may also derive from different molecules being extracted by the different solvents (Piccardi et al. 2000), or from the same component that is extracted by the solvents with a different efficiency (see the case of *Nostoc* sp. M1 in Chapter 3). Although in some cases the two models provided contrasting results, the work reported in Chapter 3 confirmed their validity for preliminary screening of toxicity. These *in vitro* models, especially in the case of methanolic extracts after 48 hours of incubation, are able to indicate organisms and substrates of potential toxicity and may well serve as guidelines for *in vivo* tests on mammals to be performed on the whole algal biomass, which are necessary to apply for novel food in the European Union (see Chapter 1, Subchapter 1.3.1, pp 21-24).

The biochemical composition and the evaluation of digestibility are fundamental to provide information about the quality and bioavailability of microalgae constituents. In **Chapter 4**, the biochemical composition and the *in vitro* digestibility of some selected microalgal and cyanobacterial biomasses are reported. Despite similarities in the biochemical composition, each microalga presented a typical nutrient profile allowing the selection of desired physicochemical characteristics for food applications. Knowing their characteristics is essential for the selection of the most suitable microalgae for specific food technology applications and consequently successful novel foods development (Batista et al., 2013).

Proteins are considered fundamental macroelements of microalgal and cyanobacterial biomass, which need to be characterized to evaluate these microorganisms as a new food source. The estimations of crude protein in

microalgae include other nitrogenous constituents like nucleic acids, glucosamides, amines, and cell wall materials (Becker, 2004). *A. platensis* F&M-C256, Klamath powder (a commercial product obtained from a natural bloom mainly composed of *Aphanizomenon flos-aquae*) and *C. vulgaris* Allma showed the highest values of crude protein content. It is interesting to note that, in our work, most of the marine microalgae fall in the same low range of protein content, instead, four freshwater microalgae and *A. platensis* F&M-C256, grown in alkaline media, showed higher values. The protein content of almost every microalga was always higher to the of reference indicated by FAO for other vegetable food proteins, such as lentils, faba beans, chickpeas, whole wheat, and rolled oats (25-30%) (FAO/WHO, 1991). Taking 5 g of *A. platensis* F&M-C256 or *C. vulgaris* Allma per day, the amount usually recommended by the nutrition facts for several *Arthrospira* and *Chlorella*-based products (Website Aurospirul; Website MyProtein; Website Bulk Powders; Website Health Ranger Store), a person can enrich the daily diet up to 3.5 or 2.8%, respectively of non-animal protein, with a low amount of lipids (only 0.5 and 0.8%, respectively). These numbers indicate the great potential of microalgal and cyanobacterial biomasses as new protein sources of non-animal origin. Diets high in animal protein have been shown to increase the risk of kidney problems (Reddy et al., 2002; Knight et al., 2003) osteoporosis (Abelow et al., 1992) and some types of cancer (Giovannucci et al., 1994; Willet et al., 1990) in humans. Furthermore, changes in demand for meat may increase the environmental negative impact of meat production. It has been estimated that global meat consumption may double in the period 2000 to 2050, mostly as a consequence of increasing world population, but also because of increased per capita meat consumption (FAO, 2006). In 2007, the global average meat consumption per capita per year was equal to 37.3 kg (Table 1). FAO illustrated (by forecast estimates) that emerging countries and developed countries will increase meat consumption by 1 kg per capita per year between 2016 and 2050 (Alexandratos and Bruinsma, 2012).

Table 1 World meat consumption (kg) per capita per year

Country	Meat consumption per capita per year
Africa	11.3
Emerging countries	17.1
West Asia (including the Middle East)	19.7
East and South Asia	40.8
Latin America	53.6
Developed countries	81.7
Global average	37.3

Adapted from McMichael et al., 2007

Meat production is responsible for enormous environmental impacts, mainly attributable to: i) freshwater consumption; ii) negative effects on aquatic ecosystems due to the increased discharge of phosphates and nitrates, decreased dissolved oxygen, increased temperature and turbidity that cause eutrophication events (Belsky et al., 1999); iii) greenhouse gas emissions (Gerber et al., 2013); iv) energy consumption (Canning et al., 2010).

Besides proteins, carbohydrates are fundamental macronutrients to provide the energy required by the body, for the correct functioning of the nervous system and red blood cells; and, more generally, for many of the metabolic reactions taking place inside an organism (Cummings and Englyst, 1995). Carbohydrates of microalgae can be found in the form of cellulose, starch, and other polysaccharides, as storage products or cell wall components (Becker, 2004). Carbohydrate in most of the studied microalgae were found always lower than 20%, which were comparable to the values indicated by FAO/WHO (2003) for common foods such as corn, rice and beans. Based on the effects on risk of heart disease and obesity (Tighe et al., 2010), the Institute of Medicine recommends that American and Canadian adults should take between 45–65% of dietary energy from carbohydrates (Food and Nutrition Board, 2002/2005). While, the Food and Agriculture Organization and World Health Organization jointly recommend that national dietary guidelines set a goal of 55–75% of total energy from carbohydrates, but only 10% directly from sugars (FAO/WHO, 2003).

Concerning dietary fiber, it is recommended dietary intakes of 14 g/1000 kcal (both for children and adults) in order to have a positive effect on health (Anderson et al., 2009). In particular, this intake can stimulate the proliferation of intestinal flora and decrease cholesterol and glycemic levels (Beecher, 1999).

In studies of general populations, low fiber intake is associated with increased risk of colon cancer and other malignancies (World Cancer Research, 1997), heart disease (FAO/WHO, 2003) diabetes and constipation (Anderson et al., 1995; Salmeron et al., 1997). Most of the marine algal strains showed values of total dietary fiber ≥ 13%, comparable to oat, bran, and whole wheat flour and much higher compared to fruits and vegetables (such as apricots and carrots) (Table 2).

Studies of general populations consuming diets high in fat, particularly saturated fat, have shown increased risk of cancer (World Cancer Research, 1997; Bingham et al., 2003; Cho et al., 2003) diabetes, and heart disease (FAO/WHO, 2003). In our study, only five strains (*C. sorokiniana* F&M-M49, *C. sorokiniana* IAM C-212, *Tetraselmis suecica* F&M-M33 grown in nutrient replete medium, *Tisochrysis lutea* F&M-M36 and *Nannochloropsis oceanica* F&M-M24) exhibited values of lipid from 23 to 29%. The other strains showed lipid content ≤ 22% (see Chapter 4, p. 84, Table 2), lower than common foods such as coconuts and peanuts, but higher compared to rice and vegetable such as row spinach (Table 2).

Table 2 Content of lipids and total dietary fiber in frequently consumed foods

Lipid		**Total dietary fiber**	
Food	Content (%)	Food	Content (%)
Coconut	45.6	Apricots	7.7
Spinach (raw)	0.4	Carrots (raw)	2.9
Rice	0.9	Oat bran	15.7
Roasted peanuts	49.5	Whole wheat flour	12.4
Biscuits	11.9	White bread	3.2

Adapted from Anderson and Bridges, 1988; Marlett, 1992; Rocquelin et al., 1998; Li et al., 2002

In Chapter 4, the nutritional quality of lipid profiles observed in the algae species was also evaluated by different indexes (p. 90, Table 4). Foods with polyunsaturated/saturated fatty acids ratio (P/S) below 0.45 are considered by the FAO/WHO to be undesirable in the human diet (FAO/WHO, 2008), due to their potential to induce increases in blood cholesterol. The P/S ratio of beef is typically about 0.1, and this ratio decreases with an increase of meat fatness (Scollan et al., 2006). The P/S ratio in all studied microalgae varied from 0.48 (*N. oceanica* F&M-M24) to 4.13 (*C. sorokiniana* IAM C-212), with presumable beneficial health effects. The ratio hypocholesterolemic

/hypercholesterolemic fatty acids (H/H) is an index based on current knowledge of the effects of individual fatty acids on cholesterol metabolism (Santos-Silva et al., 2002; Simat et al., 2015). Nutritionally, higher H/H values are considered more beneficial for human health because directly proportional to a high PUFA content. The result obtained for *T. lutea* F&M-M36 is quite in agreement with H/H values for marine fish such as sardine and mackerel (2.46) reported by Fernandes et al. (2014). The fatty acids from microalgae (such as *T. lutea*) that are highly polyunsaturated could have beneficial effects on cholesterol. Two other indexes are used to evaluate the potential for stimulating platelet aggregation, the atherogenicity index (AI) and thrombogenicity index (TI) (Turan et al., 2007). Lower AI and TI values indicate a greater potential to protect against coronary artery disease (Turan et al., 2007). Very interestingly, the lowest TI value was observed in the marine *N. oceanica* F&M-M24 (0.91), which is still higher compared to the value of TI found for sardine (0.20) by Fernandes et al (2014).

Fatty acids (provided either by ingestion or by triglycerides stored in fatty tissues) are distributed to cells to serve as energy for muscular contraction and general metabolism (Simopoulos, 1991). Regarding MUFA, for all Chlorophytes a general presence of oleic acid (C18:1 ω9) was found. Oleic acid (in triglyceride form) is included in the normal human diet as a part of animal fats and vegetable oils. Monounsaturated fat consumption has been associated with decreased low-density lipoprotein (LDL) cholesterol, and possibly increased high-density lipoprotein (HDL) cholesterol (Appel et al., 2005). In particular, oleic acid may be responsible for the hypotensive effects (Pitozzi et al., 2010). Concerning PUFA, *A. platensis* F&M-C256 showed a larger proportion of γ-linolenic acid (GLA C18:3 ω6, 1.7%). GLA has several beneficial health effects and GLA oils are mainly used for their antiinflammatory effects (Van Hoorn et al., 2008). GLA is also shown to stimulate apoptosis of cancer cells without affecting healthy cells and can prevent weight regain after weight loss (Van Hoorn et al., 2008). Besides γ-linolenic acid, *A. platensis* F&M-C256 was rich in linolenic acid (C18:2 ω6, 1.2%). Over the past two decades, numerous health benefits have been attributed to linoleic acid in experimental animal models including actions to reduce carcinogenesis, atherosclerosis, onset of diabetes, and body fat mass (Belury, 2002). Concerning *N. sphaeroides* F&M-C117, a higher amount in C16:0 and α-linolenic acids (ALA C18:3 ω3) than other fatty acids were found (3 and 2.1%, respectively). ALA plays an important role reducing the level of

platelets stickiness due to the alteration of the aggregating potential. This determines a decrease of the probability of thrombotic occlusion and a reduction of the atherosclerotic process (Connor, 1999). For *Porphyridium purpureum* F&M-M46, a high proportion of PUFA (3.4%), mainly eicosapentaenoic acid (EPA C20:5 ω3) and arachidonic acid (ARA C20:4 ω6) was found. ARA is the main precursor of eicosanoids, that are involved in the control and regulation of the inflammatory process (Kuehl and Egan, 1980). In our study, *N. oceanica* F&M-M24 and *Phaeodactylum tricornutum* F&M-M40 showed the highest proportion of PUFA (4 and 5%, respectively) compared to the other microalgae, mainly EPA (3 and 4%, respectively). It is worth noting that *T. lutea* F&M-M36 was the only alga which showed the presence of docosahexaenoic acid (DHA C22:6 ω3). EPA and DHA support cardiovascular health, have a beneficial effect in several forms of cancer and also in inflammatory and autoimmune disorders, and play an essential role in the brain and retina development (Bucy et al., 2012). Women during pregnancy and lactation could satisfy their recommended intake of EPA (100 mg/day, FAO, 2010), assuming around 2.5 g per day of *P. tricornutum* F&M-M40 or 3.2 g per day of *N. oceanica* F&M-M24. Microalgae (including cyanobacteria) represent one of the most attractive resources for the production of PUFAs because: i) they are primary producers in the marine food chain; ii) it is possible to change fatty acid content and profile by simply varying culture conditions; iii) PUFAs production does not suffer from the fluctuations (qualitative and quantitative) typical of that from fish oil; iv) microalgal biomass with standard content of PUFAs can be obtained in strictly controlled culture systems avoiding contamination by heavy metals; v) microalgal PUFAs are of non-animal origin (Chini Zittelli, 2013).

Thanks to the health benefits related to the fatty acids found in all investigated microalgal and cyanobacterial biomasses, these microorganisms have a great potential for the development of healthy food products and nutraceuticals.

A well-balanced ratio between the ω3/ω6 is advantageous for human health and 1:4 is considered the recommended ratio to prevent cardiovascular diseases (Simopoulos, 2002). Except for *A. platensis* F&M-C256 and *T. suecica* F&M-M33 grown in nutrient replete medium all the other microalgae studied presented a ω3/ω6 ratio higher than 1 (see Chapter 4, pp 88-89, Table 3). Indeed, in cyanobacteria the unsaturated double bounds are principally in ω6 position while, in marine species are mainly in ω3 position (Batista et al., 2013). Some foods or food ingredients of wide consumption, especially in the Western diet, contain a high amount of saturated fatty acids and high quantity of total

cholesterol (Table 3). In particular, coconut oil contains a high amount of saturated fatty acids (85%) and sunflower oil and palm oil contain a large amount of total cholesterol (up to 49%). High quantities of positive monounsaturated fatty acids for human health are identified for canola/rapeseed oil and olive oil (64 and 70%, respectively) (Website USDA, 2010). Sunflower oil and hemp oil can contain up to 75% of polyunsaturated fatty acids (FSA, 1991) (Table 3).

Table 3 Fatty acids and cholesterol composition (g/100 g) of some common dietary fats

	Saturated	**Monounsaturated**	**Polyunsaturated**	**Cholesterol**
Animal fats				
Lard	40.8	43.8	9.6	0.6
Butter	54.0	19.8	2.6	2.0
Vegetable fats				
Coconut oil	85.2	6.6	1.7	0.66
Cocoa butter	60.0	32.9	3.0	1.8
Palm oil	45.3	41.6	8.3	33.1
Soybean oil	14.5	23.2	56.5	16.3
Olive oil	14.0	69.7	11.2	5.1
Corn oil	12.7	24.7	57.8	17.2
Sunflower oil	11.9	20.2	63.0	49.0
Hemp oil	10.0	15.0	75.0	12.3
Canola/Rapeseed oil	5.3	64.3	24.8	22.2

Adapted from FSA, 1991; Website USDA, 2010

The Italian Society of human nutrition, about dietary reference intakes for fatty acids, recommended in the case of i) an adult man, 6 g per day of ω6 and 1.5 g per day of ω3 fatty acids, ii) an adult woman, 4.5 g per day of ω6 and 1 g per day of ω3 fatty acids, and iii) children (4-10 years), 4 g per day of ω6 and 1 g per day of ω3 fatty acids (Website SINU, 2012). The low quantity of saturated fatty acids and the high quantity of polyunsaturated fatty acids, and, in particular of essential fatty acids found in our work, allows considering microalgae an innovative food source useful to improve the quality of the diet of many people, especially those of Western countries.

Phenolic compounds, including simple phenols, flavonoids, tannins, lignins, phenolic acids, and their derivatives, synthesized as secondary metabolites are considered the major contributors to antioxidant capacity (Abd El-Baky et al., 2009). Cyanobacteria exhibited higher total phenolic contents (TPC) compared

to the other microalgae. In particular, *A. platensis* F&M-C256 showed the highest TPC (19 mg GAE g^{-1}) (see Chapter 4, p. 92, Fig. 1). Therefore microalgae, especially cyanobacteria, would be able not only to enrich a diet of micro and macronutrients but also in antioxidants.

In vitro digestibility test is of utmost importance to understand microalgae nutritional bioavailability. Most of the literature deals with macroalgae *in vitro* digestibility tests (Fleurence, 1999; Paiva et al., 2014; Tibbets et al., 2016) and only few studies, to our knowledge, focus on the digestibility of microalgae (Mišurcová et al., 2010; Machů et al., 2014; Tibbets et al., 2012). None of the strains exhibited digestibility values lower than 45%. The differences in dry matter digestibility values between microalgal and cyanobacterial samples could be related to the different structure of their cell walls (Mišurcová, 2011). Concerning crude protein digestibility (CPD), *C. vulgaris* Allma, *A. platensis* F&M-C256, and *N. sphaeroides* F&M-C117 were the most digestible strains (76, 81, 82%, respectively), which compares favourably with the true protein digestibility values found for beans (78%), millet (79%), wheat (77%), and rice (75%) in man (Hopkins, 1981; FAO, 1985). Many authors reported very diverse CPD values for different species of *Chlorella* (Janczyk et al., 2005; Morris et al., 2008; Mišurcova et al., 2010), *Arthrospira* (Devi et al., 1981; Becker, 2004), and *Nostoc* (Hori et al. 1990). This large variability may be related to species differences but more likely due to differences in methods used (such as different enzyme mixtures, sample processing, assay conditions, and the extent of algal cell wall disruption) (Mišurcova, 2011). Despite no significant correlation between CPD and dietary fiber was found (R^2 = 0.10), reduced CPD of algae can also be attributed to relatively high levels of fiber that can entrap proteins in the cellular matrix, rendering them less bioavailable to enzymatic hydrolysis (Marrion et al. 2005).

Some microalgae of commercial importance (mainly Chlorophyceae) have rigid indigestible cell walls and many authors consider sonication as an efficient method to open microalgal cell (McMillan et al., 2013; Safi et al., 2014; Wang et al., 2014). Although the sonication pre-treatment led to an increase of dry matter digestibility for both *C. sorokiniana* F&M-M49 and *C. vulgaris* Allma compared to those of the untreated samples (see Chapter 4, p. 97, Table 5), these values were not significantly different. The data obtained in this study confirm the positive effect of the sonication pretreatment and of the continuous stirring on cell dispersibility and consequently on the efficiency of enzyme

action, while the adoption of sonication as disruption method does not lead to a significant improvement in digestibility of these *Chlorella* strains.

Over the past 20 years, the interest in microalgae applications has grown increasingly and several authors investigated the role of microalgae in rats diet for many purposes, such as effect on serum cholesterol (Dvir et at., 2000), on oxidative stress (Carfagna et al., 2015), to prevent cardiovascular disease (Ku et al., 2013) or for other purposes, including safety assessment (Hammond et al., 2001; Cherng and Shih, 2005; Stewart et al., 2008). In **Chapters 5** and **6** a safety evaluation and the potential health effects of *A. platensis* F&M-C256 and *T. lutea* F&M-M36 in rats are described. Overall, the results shown in **Chapter 5** indicate that diets supplemented with 20% *A. platensis* F&M-C256 was well-tolerated during the 30 days feeding period and the general health status of the animal was not affected. The *A. platensis* F&M-C256 supplemented diet results in a daily dose of 12 g/kg. This amount, that mimics the potential use of microalgae as foods or food components for human consumption, showed no significant adverse effects (see Chapter 5, pp 121-122). The nucleic acid content is an important toxicological concern since the biochemical degradation of their component purines ends by producing uric acid which can produce kidney stones and gout attacks in the long term (Curhan and Taylor, 2008). The World Health Organization recommends that the daily total nucleic acid consumption should not exceed 4 g, which represent the nucleic acid content of about 80 g of dry biomass (World Health Organization, 2007). In our study, feeding a diet containing 20% of *A. platensis* F&M-C256 in rats corresponds to feeding 3 g of biomass/day. From our analysis, the DNA content of *A. platensis* F&M-C256 is 4%, then we can estimate that the total content of DNA+RNA nucleic acids is around 10%, which translates into 0.3 g of nucleic acid/day. It is, therefore, reasonable to consider that the nucleic acid content of *A. platensis* F&M-C256 does not pose health problems, even at high dosage.

In the experimental settings, treatment with fibrates, a widely used class of lipid-modifying agents, results in a substantial decrease in plasma triglycerides and is usually associated with an increase in HDL cholesterol concentrations through the activation of the peroxisome proliferator-activated receptors (PPAR-α). Very interestingly, our results demonstrate for the first time that feeding a diet containing *A. platensis* F&M-C256 for one month is able to induce the expression of PPAR-α in the liver, thus providing a mechanistic explanation of the observed hypolipidemic effect (see Chapter 5, p. 120, Table 2). As found by some authors (Singh et al., 2005; Ku et al., 2013; Vo and Kim,

2013), our results demonstrated that spirulina is able to reduce risk factors for cardiovascular diseases even in a well-balanced dietary regimen. The active ingredients responsible for this hypolipidaemic activity remain to be identified but C-phycocyanin has been suggested (Nagaoka et al., 2005). In the United States, 38% of adults (according to the National Center for Complementary and Alternative Medicine) opted for complementary and alternative medicine over conventional drugs, and herbal products are the most frequently used complementary and alternative medicine agents among patients with cardiovascular disease (Yeh et al., 2006; Nahin et al., 2007). *A. platensis* F&M-C256 might thus represent a natural functional food to be used as complementary and alternative medicine agent for the prevention of dyslipidemias.

The content in saturated fats, cholesterol, and *trans* fat of frequently consumed food in the United States is high (Table 4) and a drastic reduction of these components in a daily diet is fundamental to avoid serious illness. The American Heart Association suggests total blood cholesterol level for the population should be: < 200 mg/dL to be considered "normal blood cholesterol"; 200–239 mg/dL "borderline-high"; > 240 mg/dL "high cholesterol". Furthermore, the *trans* and saturated fats should not exceed 5 g/day and 10% of the energy of the diet, respectively (Website American Heart Association, 2009). In this respect, *A. platensis* F&M-C256 could also represent an interesting ingredient in a daily diet, not only by providing important nutrients, but also helping to reduce total cholesterol from foods high in saturated fat.

Table 4. Lipids contents of selected food products for usual portions

Food products	Saturated (g)	Cholesterol (mg)	*Trans* (g)
Fried potatoes (100 g)	12.9	-	2.5
Steak (100 g)	13.2	92.9	0.65
Sweet biscuit (50 g)	2.1	-	1.41
Cracker (50 g)	1.8	-	2.8
Pies and cakes (60 g)	4.7	-	1.0
Whole milk (200 mL)	2.4	15.0	0.19
Hamburger (100 g)	5.9	78.5	0.67
Butter 10 g)	5.0	22.0	-
Margarine (10 g)	5.3	6.8	1.0
Cheese (50 g)	10.4	52.5	0.9
Sausage (30 g)	3.2	78.5	0.1
Total	67.0	346.2	11.2

Adapted from Pennington, 1989; Chiara and Sichieri, 2001

Besides EPA, *T. lutea* represents an interesting source of fucoxanthin, an accessory pigment present in the chloroplasts of brown algae (Kim et al., 2012). Lee et al. (2013) have shown that fucoxanthin has anti-inflammatory, antinociceptive, and anti-cancer effects. In addition, some metabolic and nutritional studies carried out on rats and mice indicate that fucoxanthin promotes fat burning within fat cells in white adipose tissue by increasing the expression of thermogenin (Maeda et al., 2005). The results reported in **Chapter 6** indicate that a diet supplemented with 20% *T. lutea* F&M-M36 was well-tolerated during the 30 days feeding period and no treatment-related mortality was detected. However, rats fed *T. lutea* F&M-M36 showed a tendency to a reduction in body weight gain compared to controls. These observations are limited to the 30 days feeding but we cannot exclude significant effects in longer feeding periods. Nuño et al. (2013) evaluated the effects of the microalgae *I. galbana* in a diabetic rat model and found that from an average initial body weight of around 220 g, rats grew up to >300 g at eight weeks with differences in body weight. In contrast, body weight remained unchanged between weeks in the untreated diabetic group and *I. galbana* diabetic group. The lower body mass of the hyperglycemic and healthy rats administered *I. galbana* compared to those that did not receive a microalgae treatment suggests the effect of its polysaccharides are shaped by different monomers acting as dietary fiber in the gastrointestinal tract (Zeng et al., 2004). Dietary fiber affects satiety, energy, and body composition, reducing appetite and energy intake up to 10%, consequently leading to weight reduction (Nuño et al., 2013). As found by Maeda et al. (2005), also fucoxanthin may have contributed to the reduction in body weight.

Given the relevant issue of *T. lutea* F&M-M36 salt content (6%), our study specifically focused on the hydro saline balance, blood pressure and renal damage. The hydro saline imbalance was supported by an increased water consumption, accompanied by increased urinary excretion. Blood pressure was not affected over one-month feeding but, as expressed above, it is not possible to exclude long term effects. Therefore, high biomass salinity is an issue which must be solved by using strategies to reduce its content (e.g. washing of the biomass after the harvest) or by using lower doses of this microalga. The amorphous neo-formation found in the bladder of both microalgae fed rats was interpreted as a uric acid crystal of sporadic occurrence and possibly related to the DNA content of microalgae.

Our observations indicate that the use of *A. platensis* F&M-C256 and *T. lutea* F&M-M36 did not have adverse effects in rats within 30 days feeding period and exerted positive health effects. Thus, the two microalgae represent promising functional foods and nutraceuticals.

Traditional foods, like biscuits, pasta, gelled desserts, mayonnaises, and breakfast cereals, largely consumed on daily basis in different European diets, can be used as vehicles of nutraceuticals (Gouveia et al., 2008). Numerous combinations of microalgae or mixtures with other health foods can be found in the market in the form of tablets, powders, capsules, pastilles and liquids, as nutritional supplements. They can also be incorporated into food products (e.g. pastas, biscuits, bread, snack foods, candies, yoghurts, and soft drinks), providing the health promoting effects that are associated with microalgal biomass, probably related to a general immune-modulating effect (Belay, 1993). *A. platensis*, *C. luteoviridis*, *C. pyrenoidosa*, and *C. vulgaris* are microalgae already accepted as food, because used prior to May 1997 in Europe (Website European Union, Novel Food catalogue). Foods supplemented with these microalgae are widely spread in the food market. Besides *Chlorella* tagliatelle and noodles, already available in retail stores, *Chlorella* powder has been studied also as an additive to wheat flower to prepare bakery products (Guoveia et al., 2008). Gouveia et al. (2008) reports that the addition of increasing amount of *C. vulgaris* dry powder to cookies brought, besides an increasing green colour, a higher firmness of the pastry. A subsequent study by Fradique et al. (2010), reports the incorporation of *C. vulgaris* and *A. maxima* biomass in pasta products. In **Chapter 7**, to explore the potential food application of microalgae as functional ingredients within conventional foods, *A. platensis* F&M-C256-, *C. vulgaris* Allma-, *T. suecica* F&M-M33-, and *P. tricornutum* F&M-M40-based cookies are analysed for their sensory and physical properties, bioactivity and *in vitro* digestibility. Two biomass levels were tested: 2% (w/w) typically used in algal-based products and a significantly higher content, 6% (w/w), to provide additional algal-bioactives. The texture profile of the biscuits was also evaluated in our work, and a significant increase of their firmness was evidenced with an increase of added microalgal biomass. These results evidence the positive effect of the alga in the biscuit structure, reinforcing the short dough system. Biscuits are considered solid emulsions of sucrose, lipids and non-gelatinized starch (Hoseney et al., 1988), being this mix of components responsible for the biscuits structure and texture. The replacement of a small amount of flour by microalgae biomass, resulted in the inclusion of a complex

biomaterial, rich in different proteins and polysaccharides. These molecules have an important role on the water absorption process, which promote the increase of biscuits firmness, resulting in more compact structures (Gouveia et al, 2008). A similar study was performed using *I. galbana* biomass (Gouveia et al., 2007). The main interest in using *I. galbana* biomass is due to its high levels of long chain ω-3 polyunsaturated fatty acids, especially EPA and DHA (Bandarra et al., 2003). As found in this study, in spite of the drastic thermal processing (high temperatures) during biscuits manufacturing, the addition of microalgal biomass leads to the presence of ω3 fatty acids (higher than control biscuits) (see Chapter 7, p. 175, Table 5). The thermal resistance of fatty acids should be due to their presence in an encapsulated form, inside the microalgal cell (Gouveia et al., 2008). These fatty acids contents indicate an important source of ω3 PUFA with a moderate biscuit consumption, as the recommendations for dietary intake in healthy adults is 500 mg/day (ISSFAL, 2004).

Very few papers (e.g. Fradique et al., 2013) deal with the bioactive compounds of microalgae foods and their resistance to different processing steps. In our study, all microalgae-based cookies showed significantly higher total phenolic content (TPC) compared to the control. *P. tricornutum* F&M-M40 cookies exhibited the highest TPC. Moreover, microalgae cookies presented significantly higher *in vitro* antioxidant capacity (AC) compared to the control (from +45% to +307%). The microalgal cookies *in vitro* digestibility were also assessed. As expected, cookies added with 6% Chlorophyceae biomass exhibited the lowest digestibility. From this study, *A. platensis* F&M-C256 *C. vulgaris* Allma, *T. suecica* F&M-M33, and *P. tricornutum* F&M-M40 cookies presented significantly higher *in vitro* antioxidant capacity and total phenolic content than the control, whilst not compromising the digestibility. Therefore, there are prospects for considering microalgae-based cookies functional nutrient foods that could be widely consumed on a daily basis worldwide from all age groups.

In recent years, consumer demand for non-dairy-based probiotics has increased (Luckow and Delahunty, 2004) due to lactose intolerance and cholesterol content associated with the consumption of fermented dairy products (Gupta et al., 2011). In this respect, several reports are available on the use of terrestrial vegetables for lactic acid fermentation such as artichokes (Ge et al., 2009), carrot (Kun et al., 2008), cabbage (Kohajdová et al., 2006), and beet root (Yoon et al., 2005). Ariahu et al. (1999) adopted natural lactic fermentation process to

enhance the organoleptic quality of one of the most frequently consumed food commodities in the world such as soybean. Beyond nutritional and sensory properties, lactic acid fermentation can also improve the shelf life of vegetables (Karovicová and Kohajdová, 2003).

During fermentation, an improvement in digestibility can be obtained due to the partial degradation of compounds such as non-starch polysaccharides, in particular cellulose, by *Lactobacillus plantarum* (Moraïs et al., 2014). *L. plantarum* is an attractive candidate for the degradation of cellulose due to its ability to metabolise the major polysaccharide constituents of cellulosic biomasses (Moraïs et al., 2014). In **Chapter 8**, in order to improve the acceptability of a microalgae-based product by consumers in terms of organoleptic quality and digestibility, *L. plantarum* was used to ferment frozen and lyophilised *A. platensis* F&M-C256 and *T. suecica* F&M-M33 biomasses. Microalgal biomass appears as a suitable substrate for *L. plantarum* in both forms, although frozen biomass appears more effective (see Chapter 8, p. 203, Fig. 1). Microalgae can be considered a suitable substrates for lactic fermentation compared to conventional foods, such as carrot, cabbage, and other vegetables (Gardner et al., 2001; Bergqvist et al., 2005; Yoon et al., 2006) (Table 5). The positive effects of microalgae on the viability of lactic acid bacteria found in our study can be attributed to the highly nutritious substances they contain which stimulate bacterial growth and activity (Parada et al., 1998).

Table 5 Comparison between the growth (log CFU mL^{-1}) of *Lactobacillus* with conventional foods and algal biomasses after 24 h of fermentation

Algae biomasses	*Lactobacillus* growth (log CFU mL^{-1})
A. platensis F&M-C256 (frozen)	8.0[1]
T. suecica F&M-M33 (frozen)	8.1[1]
Laminaria saccharina	7.7[2]
Conventional foods	***Lactobacillus* growth (log CFU mL^{-1})**
Carrot juice	9.0[3]
Cabbage juice	8.8[4]
Vegetable mixtures	8.0[5]

Sources: [1]Data by the authors; [2]Gupta et al., 2011; [3]Bergqvist et al., 2005; [4]Yoon et al., 2006; [5]Gardner et al., 2001

Despite the use of algal materials for the production of edible fermented products has been limited, a spirulina-based fermented drink is already in the market (Website Your Digestion).

For frozen fermented biomasses a similar increase in digestibility compared to the unfermented biomasses was observed for both algae; while, fermentation did not improve digestibility of lyophilised biomasses (see Chapter 8, p. 207, Fig. 4). The breakage of the cell wall occurring during thawing (Mazur, 1984) could result in an improvement of *in vitro* digestibility compared to the lyophilized biomass. *Arthrospira* has *in vitro* and *in vivo* antioxidant, radical scavenging and anti-inflammatory activity principally due to the presence of phycocyanin (El-Baky and El-Baroty, 2012). As found by Liu et al. (2011) an improvement in DPPH scavenging ability of lyophilised and frozen *A. platensis* F&M-C256 after fermentation was found. Similar to the results obtained in the present study, other authors (Liu et al., 2011; El-Baky and El-Baroty, 2012; Chu et al., 2010) reported a similar radical scavenging capacity with *A. platensis* or with phycocyanin extracted from *A. maxima* and *A. platensis*. Phenolic compounds are considered the major contributors to antioxidant capacity (Abd El-Baky et al., 2009). Similar to the trend observed in our study before and after fermentation, Liu et al. (2011) found that *A. platensis* fermented with lactic acid bacteria showed higher phenolic content compared to unfermented biomass. The fermentation process could have released polyphenols such as gallic acid, or produced other metabolites (Liu et al., 2011). Since total phenols are, amongst other compounds, responsible for antioxidant activity (Liu et al., 2011), the greater quantity of total phenols in fermented *A. platensis* F&M-C256 may indicate a greater antioxidant activity than unfermented biomass, which is confirmed by the DPPH results (see Chapter 8, pp 208-210).

Frozen biomass appears to be a better fermentable substrate (higher lactic acid production, higher digestibility). Furthermore, it is more economically viable on an industrial scale because it obviates the need for a drying process (as lyophilisation or spray drying), that would be highly expensive in terms of energy costs, as well as time-consuming.

9.2 Concluding remarks

Microalgae, as a potential source of functional foods and nutraceuticals, are a fast growing segment of the health and nutrition industry today, although well known cases of historical use of cyanobacteria as food do exist.

An accurate selection of the best microalgae and cyanobacteria is the starting point for the future development of microalgae- and cyanobacteria-based functional products. The large biodiversity of microalgae in nature and in culture collections still needs accurate evaluation and exploration of microalgae in terms of quality and safety before exploitation.

In conclusion, from the results of this research *A. platensis* F&M-C256 has proved to be one of the best strains, due to the absence of *in vitro* and *in vivo* toxicity, the balanced biochemical composition (especially the high amount of high quality proteins and the low amount of lipids), the high *in vitro* and *in vivo* digestibility and the high potential for the prevention of dyslipidemias. The high quality of *A. platensis* F&M-C256 biomass can provide valuable functional foods and nutraceuticals. Beside *A. platensis* F&M-C256, also *C. vulgaris* Allma, *T. suecica* F&M-M33 and *P. tricornutum* F&M-M46 have been found as suitable for the development of functional microalgae-based products. Lactic acid fermentation enhanced the quality of *A. platensis* F&M-C256 and *T. suecica* F&M-M33 biomasses in terms of organoleptic quality and digestibility, and we expect that also other microalgae could provide suitable fermentation substrates.

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In vitro toxicity of microalgal and cyanobacterial strains of interest as food source

Alberto Niccolai[1] · Elisabetta Bigagli[2] · Natascia Biondi[1] · Liliana Rodolfi[1] · Lorenzo Cinci[2] · Cristina Luceri[2] · Mario R. Tredici[1]

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Abstract The general objective of the present work was to evaluate the toxicity of 11 microalgal strains and one natural bloom, selected as potential food or food ingredients due to their nutritional quality, in two models, human dermal fibroblasts and *Artemia salina*. Methanolic and aqueous extracts of the biomasses were tested on *A. salina* for 24 and 48 h at concentrations up to 12.5 g L^{-1} of extracted biomass. Only aqueous extracts were tested on fibroblasts for 24 h. *Chlorella vulgaris* Roquette, *C. vulgaris* Allma, *Tetraselmis suecica* F&M-M33, and *Porphyridium purpureum* F&M-M46 showed no toxicity towards *A. salina* and fibroblasts. Only Klamath powder was toxic to both models with all types of extracts. *Tisochrysis lutea* (T-ISO) F&M-M36, *Chlorella sorokiniana* F&M-M49 grown in BG11, and *C. sorokinina* IAM C-212 showed toxicity, even if to different extents, to fibroblasts and, only with the methanolic extract, to *A. salina*. The remaining strains showed no toxicity towards *A. salina*, but were toxic to fibroblasts: *Arthrospira platensis* F&M-C256 and *Nannochloropsis oceanica* F&M-M24 exhibited low toxicity, *Nostoc sphaeroides* F&M-C117 medium toxicity and *Phaeodactylum tricornutum* F&M-M40 high toxicity. Although in some cases the two models provided contrasting results, this work confirms their validity for preliminary screening of toxicity. The models are able to indicate organisms and substrates of potential toxicity and may well serve as guidelines for in vivo tests on mammals, which are necessary to apply for novel food in the EU.

Keywords Brine shrimp bioassay · Fibroblasts · Toxicity · Microalgae · Food

✉ Natascia Biondi
natascia.biondi@unifi.it

[1] Department of Agrifood Production and Environmental Sciences (DISPAA), University of Florence, Piazzale delle Cascine 24, 50144 Florence, Italy

[2] Department of NEUROFARBA, Section of Pharmacology and Toxicology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy

Introduction

Microalgae (including cyanobacteria) are oxygenic photosynthetic microorganisms that have long been recognized as a potential source of food. The high potential of microalgae as food is due to their balanced biochemical composition and high nutritional value (Tredici et al. 2009). They can have a high protein content with a balanced amino acid composition (Becker 2007), a good content of vitamins, minerals, short-chain polyunsaturated fatty acids, including linoleic and linolenic acids, and long-chain polyunsaturated fatty acids such as docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids (Bishop and Zubeck 2012; Batista et al. 2013). Some strains are also good sources of carotenoids like lutein, astaxanthin, and β-carotene (Del Campo et al. 2007). *Chlorella*, *Arthrospira*, *Dunaliella*, and *Haematococcus* are microalgae largely employed for human consumption (Tredici et al. 2009; Ibañez and Cifuentes 2013).

In the EU, novel foods and food ingredients are defined as those that "have not been used to a significant degree for human consumption within the Union before 15th May 1997" (European Union 1997). With the Regulation (EC) No 258/97, the European Union, through the European Food Safety Authority (EFSA), guarantees that novel foods and food ingredients are subject to a single safety assessment through a unified procedure in order to protect public health. Foods and food ingredients to be authorized must not present

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a danger for the consumer, mislead the consumer, and differ from foods that they are intended to replace to such an extent that their normal consumption would be nutritionally disadvantageous for the consumer (European Union 1997). In the USA, the term "food additive means any substance the intended use of which results…, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of… food" (Federal Food, Drug, And Cosmetic Act, Section 201). Food additives (that do not include substances generally recognized as safe (GRAS), substances used prior to January 1958, and those included in the exemptions of the Federal Food, Drug, and Cosmetic Act), require pre-market approval aimed at demonstrating safety under the intended conditions of use. The Food and Drug Administration (FDA) issues regulations, based on data submitted through petitions or obtained by its own initiative, specifying the conditions under which an additive has been demonstrated to be safe and, therefore, may be lawfully used (FDA 2014). The GRAS standard requires the industry to provide scientific evidence that the substance is not harmful under its intended conditions of use and once certified exempts the substance from pre-market approval (FDA 2014). The Food Standards Australia New Zealand (FSANZ) considers as novel food all non-traditional food or its derivatives and requires safety assessment (Commonwealth of Australia Gazette 2007). Currently, FSANZ is reviewing the requirements for novel food applications (Standards 1.5.1). Algae that are not listed in the Standards need approval as novel food and thus safety assessment (FSANZ, Proposal P1024).

The microalgae used prior to May 1997 in Europe and thus authorized as food in the EU are *Aphanizomenon flos-aquae* from Klamath Lake, *Arthrospira platensis*, *Chlorella luteoviridis*, *Chlorella pyrenoidosa*, and *Chlorella vulgaris* (European Union, Novel Food catalogue). The diatom *Odontella aurita* was authorized later (European Union 2005), and, in 2014, also *Tetraselmis chuii* was approved as food, following the application by the Spanish company Fitoplancton Marino S.L. (AESAN 2013; AECOSAN 2014). Astaxanthin from *Haematococcus pluvialis* was also recently authorized (EFSA 2014). To be used as food, with the exception of the species mentioned above, microalgae have to follow the novel food regulation (European Union 2015). In the USA, *Chlorella protothecoides*, *A. platensis*, *Dunaliella bardawil*, and astaxanthin from *Haematococcus* are included in the GRAS list (FDA, GRAS Notices). In Australia, all *Chlorella* (including *Chlorella sorokiniana*) and *Arthrospira* species and derivatives for which a novel food application was submitted have been so far considered as traditional food, whereas *A. flos-aquae* was considered as novel food and safety assessment was required due to the potential presence of cyanobacterial toxins such as microcystins and nodularin (FSANZ 2016). These approved microalgae (and microalgal products) represent a very small number of species compared to the high number present in nature, from 30,000 to 1 million according to different sources (Guiry 2012).

In this work, we tested three strains/products belonging to species already approved as food in the EU (*C. vulgaris*, *A. flos-aquae* from Klamath Lake blooms, *A. platensis*), and two species not approved at present (*C. sorokiniana* and *Tetraselmis suecica*), but belonging to genera of approved species. Five other microalgae were also tested: *Nostoc sphaeroides*, an edible cyanobacterium consumed in China and other Asian countries (Han et al. 2013), *Tisochrysis lutea*, *Nannochloropsis oceanica*, and *Phaeodactylum tricornutum*, widely used (as well as *Tetraselmis* spp.) in aquaculture (Tredici et al. 2009; Muller-Feuga 2013), and *Porphyridium purpureum*, rich in EPA, but to date used only in cosmetics (Arad and Levy-Ontman 2010; Rajasulochana and Preethy 2015). These strains as well as the natural bloom were chosen for their high potential as food sources thanks to a high protein content (*A. platensis* F&M-C256, Klamath powder, *N. sphaeroides* F&M-C117, *T. suecica* F&M-M33, *C. sorokiniana* F&M-M49 and IAM C-212, *C. vulgaris* Allma and Roquette) or for their ability to produce high amounts of polyunsaturated fatty acids (*N. oceanica* F&M-M24, *P. tricornutum* F&M-M40, *P. purpureum* F&M-M46, *T. lutea* F&M-M36), besides having interesting amounts of protein.

The first concern when proposing microalgae as food or food ingredients is to establish their lack of toxicity towards the consumer. In this regard, several species of different algal groups have been reported to produce neurotoxins, hepatotoxins, diarrheic toxins, dermotoxins, etc., which are usually active at very low doses (Landsberg 2002). Besides biotoxins, microalgae and cyanobacteria are able to produce other secondary metabolites (cytotoxins) that may have noxious effects on human health (Carmichael 1992; Tredici et al. 2009). Finally, it is to consider that also molecules that do not enter in the category of *toxins* (Carmichael 1992) can have toxic effects if provided at excessively high dosages. This concept provides the rational behind legislation, which requires that the toxicity data provided be related to the dose of the biomass/extract/component that is intended for use.

Toxicity tests for novel food applications require time-consuming and expensive procedures. Moreover, the EU strongly encourages the "3Rs" (replacement, reduction, and refinement) principle for sparing animals used for scientific purposes (European Union 2010). In vitro tests and bioassays on invertebrates have the advantage of being less expensive and shorter in duration, offering a simplified model for a preliminary screening to be used as a guideline for further in vivo studies. To determine microalgal toxicity (intended in all the meanings reported above), model organisms such as crustaceans (*Artemia* or *Daphnia*) are often used (Vezie et al. 1996; Guilhermino et al. 2000; Biondi et al. 2004). In this study, we adopted the brine shrimp (*Artemia salina*) assay, an easy and

low-cost toxicity test (Solis et al. 1993; Piccardi et al. 2000). It is to note that, despite it targets a whole organism, the brine shrimp assay is considered by several authors as an in vitro test (Sleet and Brendel 1985; Lagarto Parra et al. 2001; Carballo et al. 2002). Besides invertebrates, toxicity is often evaluated on mammalian cells, mainly cancer cell lines (Jaki et al. 1999; Carballo et al. 2002; Hisem et al. 2011). Some authors report a good correlation between the toxic activity measured with brine shrimp and that observed with tumor cell lines (Anderson et al. 1991). Here, we report the toxicity of 13 microalgal extracts on *A. salina* and on human fibroblasts in order to evaluate the potential health risks of these microbial strains if used as food or food ingredients.

Materials and methods

Experimental plan

Microalgal biomasses were extracted in methanol and/or water and tested against normal human dermal fibroblasts (NHDF) and the nauplii of the crustacean *A. salina* to evaluate their potential toxicity. The experimental design is reported in Table 1.

Microalgal strains and biomass production

The algae tested are listed in Table 2. A positive reference extract was prepared from *Nostoc* sp. M1, known from previous experiments carried out in our laboratory to be strongly cytotoxic. A negative reference extract was also prepared using organic lettuce (*Lactuca sativa* var. *capitata*).

Most of the tested microalgae were produced at the facilities of Fotosintetica & Microbiologica S.r.l. or of the Institute of Ecosystem Study of the CNR, both in Sesto Fiorentino, Florence. The algae were produced in GWP®-II photobioreactors (Tredici et al. 2011; Chini Zittelli et al. 2013) in semi-batch mode, and the biomasses were harvested by centrifugation, frozen, lyophilized, and powdered. The powdered biomasses were stored at −20 °C until use. Only *A. plantesis* F&M-C256 biomass was washed with physiological saline solution to remove extracellular sodium bicarbonate and carbonate before freezing. All the freshwater strains were cultivated in BG11 (Rippka et al. 1979) and all the marine strains in F (Guillard and Ryther 1962) medium, while *A. platensis* was cultivated in Zarrouk medium (Zarrouk 1966). *C. sorokiniana* F&M-M49 was cultivated in both BG11 and F medium (Guccione et al. 2014). The F medium-grown biomass (unwashed) was used to test the effect on fibroblasts of sea salt residues (see below). *Chlorella vulgaris* Allma (Allma Microalgae, Lisbon, Portugal), *C. vulgaris* Roquette (Roquette Frères, Lestrem, France), and Klamath powder (Erbologica SAS, Serina, Bergamo, Italy) are commercial products. Klamath powder is obtained from a natural bloom mainly composed of *A. flos-aquae*, harvested from Upper Klamath Lake (OR, USA).

Table 1 Plan of the experiments

	Extracts		Effect of salt
	MeOH	H_2O	
Fibroblasts (NHDF)		✓	✓
Artemia salina	✓	✓	

In vitro cytotoxicity on human fibroblasts

Preparation of microalgal extracts Lyophilized microalgae were suspended (25 g L^{-1}) in Dulbecco's Modified Eagle Medium (DMEM) without phenol red and sonicated for 2 cycles of 3 min in ice, followed by a 3-min resting interval by using an ultrasonic homogenizer (Sonopuls HD 2070, Bandelin Electronic, Germany), set at 100 % power. Microalgal suspensions were then centrifuged for 15 min at 15,000 × *g* at 4 °C, filtered through a 0.2-μm filter and stored at −20 °C.

Cell cultures Human dermal fibroblasts (N°CC-2509), kindly provided by Prof. Lisa Giovannelli (NEUROFARBA, University of Florence), were maintained in DMEM without phenol red, supplemented with 10 % fetal bovine serum (FBS), glutamine (2 mM), penicillin (100 IU mL^{-1}), and streptomycin (100 μg mL^{-1}) in a humidified incubator at 37 °C under a 5 % CO_2 atmosphere.

Determination of cytotoxic activity of microalgal aqueous extracts on human fibroblasts NHDF cells of passages 12–14 were seeded in 96-well plates (5×10^3 per well), kept for 24 h, and then treated with microalgal extracts for 24 h. The concentrations tested were 12.5, 5.0, 2.5, and 0.5 g of extracted biomass per liter for 24 h. Viability analysis was performed using Cell Titer 96 Aqueous One solution cell proliferation assay kit (Promega, USA). At the end of the incubation period, the treated cells were exposed to Aqueous One solution (Giovannelli et al. 2014) and incubated for 2 h at 37 °C. The product of the reaction was measured at 490 nm using a spectrophotometer.

Effect of salt interference

To evaluate the interference of marine salts on fibroblast viability, artificial seawater (Tropic Marine, Adriatic Sea Equipment & Co, Rimini, Italy) at a salinity of 60 g L^{-1} was used to prepare culture media for NHDF at different salinities, from 30 to 0.6 g L^{-1}. The experiment was set up and performed as reported above for the algal extracts. To further

Table 2 Algae and algal products selected for toxicity screening

Strain	Type of culture medium	Biomass obtention
Cyanobacteria		
Arthrospira platensis F&M-C256	Alkaline	In-house cultivation
Klamath powder	Fresh	Commercial product (Erbologica SAS)
Nostoc sphaeroides F&M-C117	Fresh	In-house cultivation
Nostoc sp. M1	Fresh	In-house cultivation
Chlorophytes		
Chlorella sorokiniana F&M-M49	Fresh/marine	In-house cultivation
Chlorella sorokiniana IAM C-212	Fresh	In-house cultivation
Chlorella vulgaris Roquette	Fresh	Commercial product (Roquette Frerès)
Chlorella vulgaris Allma	Fresh	Commercial product (Allma Microalgae)
Tetraselmis suecica F&M-M33	Marine	In-house cultivation
Rhodophytes		
Porphyridium purpureum F&M-M46	Marine	In-house cultivation
Diatoms		
Phaeodactylum tricornutum F&M-M40	Marine	In-house cultivation
Haptophytes		
Tisochrysis lutea (T-ISO) F&M-M36	Marine	In-house cultivation
Eustigmatophytes		
Nannochloropsis oceanica F&M-M24	Marine	In-house cultivation

verify the effect of the salts that may remain in the algal biomass after centrifugation, *C. sorokiniana* F&M-M49 grown in freshwater and in seawater was extracted and tested as previously described.

To quantify the salt content of the biomass, ash was determined by incineration in a muffle furnace at 450 °C.

Determination of cytotoxic activity of microalgal extracts on *A. salina*

Extraction of biomasses Biomass extraction for *A. salina* tests was performed following two different protocols. One set of experiments was performed on methanolic extracts.

For each algal strain, as well as for the negative reference lettuce, an aliquot of 0.5 g of dry biomass was extracted in 60 mL of methanol overnight at room temperature. The solvent was then separated from biomass by filtration on paper. The biomass residue was extracted again with 30 mL of methanol at 37 °C for 4 h, then the exhausted biomass was removed by filtration on paper. The two extracts (60 + 30 mL) were mixed and then evaporated under vacuum. The dry residues were suspended in 2.5 mL of methanol to obtain a final concentration of the extracts of 200 g L^{-1} of extracted biomass. Glassware and plastic- ware were kept in 0.27 M NaOH in 9:1 ethanol/water for 24 h before use, to prevent cross-contamination (Golakoti et al. 1995), and then washed with deionized water.

Another set of experiments on *A. salina* was performed on aqueous extracts of microalgae and lettuce. Aliquots of the dry biomasses (0.2 g) were dispersed in 1.5 mL of sterile water and sonicated for 3 min, and then the probe was washed with 0.3 mL of sterile water. The sonicated material was frozen at −20 °C. As soon as the material was taken out of the freezer, 0.2 mL of an antibiotic solution containing 3200 μg mL^{-1} of streptomycin sulfate (chemist's preparation) and 6000 μg mL^{-1} of ampicillin (Amplital, Pfizer) were added to each sonicated biomass to prevent bacterial growth. After thawing, each sonicated biomass was homogenized, and an aliquot was transferred into a sterile 1.5 mL test tube and centrifuged for 15 min at 18,000 × *g* at 4 °C. The supernatant was finally transferred to a new sterile test tube. The final extract concentration was of 100 g L^{-1} of extracted biomass. For this type of extraction, only disposable material was used.

Lethality tests on *A. salina* Cysts of *A. salina* (Premium, SHG, Ovada, Alessandria, Italy) were transferred for hatching in a glass bottle containing sterile artificial seawater (Tropic Marine) at 30 g L^{-1} salinity. The inoculated seawater was bubbled with air sterilized through 0.2 μm porosity filters. The incubation was carried out under constant illumination of 80 μmol photons m^{-2} s^{-1} at 25–27 °C. Once the cysts hatched, the nauplii were used to set up the experiments.

The experiments were performed in 96-well microtiter plates on a final test volume of 100 μL. For methanolic extracts, the concentrations tested varied from 12.5 to 0.1 g L^{-1}

of extracted biomass. An aliquot (12.5 μL) of the extract was transferred into the first well, and the solvent let to evaporate. After evaporation, 20 μL of pure DMSO:water 1:10 was added to prepare the final extract, that was then diluted in the other wells 1:2 in 10 μL of DMSO:water 1:10 till the last concentration to be tested. Controls were prepared with 10 μL of DMSO:water 1:10. Each extract concentration and the control were tested in six replicates. Reference extracts were set up with biomasses expected to produce a positive (*Nostoc* sp. M1) and a negative (lettuce) response in the test.

For aqueous extracts, the concentrations tested were from 12.5 to 0.1 g L^{-1} of extracted biomass. An aliquot (25 μL) of the extract was transferred into the first well of the microtiter plate and diluted 1:2 in the other wells in 50 μL of artificial seawater (30 g L^{-1} salinity) containing antibiotics. The final concentration of antibiotics in each well was 160 μg mL^{-1} of streptomycin and 300 μg mL^{-1} of ampicillin. Control was prepared with sterile artificial seawater (30 g L^{-1} salinity) containing antibiotics at the same concentrations as in the test wells. Each extract concentration and the control were tested in three replicates. Reference extracts were set up with *Nostoc* sp. M1 and lettuce that were treated in the same way as the test extracts.

After the test and reference extract dilutions and the control were prepared in the microtiter plates, 90 μL of seawater containing five nauplii were added to each well to reach the final test volume of 100 μL. The plates were then incubated at room temperature, and the number of dead nauplii was counted under a direct light microscope (Nikon Eclipse E200, Nikon, Tokyo, Japan) at 40 magnifications after 24 and 48 h of incubation.

Nauplii were considered as dead when no movement was observed in the reasonable time of the observation (Carballo et al. 2002; Lopes et al. 2011). Mortality was calculated according to Abbott (1987):

$$M\,(\%\,vs\,\text{control}) = \frac{(L_C - L_T)}{L_T} \times 100$$

where M is mortality; L_C, living nauplii in the control after 24 or 48 h; L_T, living nauplii in the test or reference extract after 24 or 48 h.

Calculation of IC_{50} values

For all microalgal extracts tested against NHDF and *A. salina*, dose-response curves were obtained. The concentration (expressed as gram of extracted biomass per liter) which inhibits 50 % of the population (IC_{50}) was calculated by a linear regression model using log transformed data when necessary. When the experimental data did not include the 50 % inhibition, IC_{50} values were estimated, still through linear regression. For some extracts (indicated as "not calculable"), it was not possible to calculate an IC_{50} value because no toxicity was detected at the concentrations tested. IC_{50} values higher than 15 g L^{-1} were not indicated *in extenso*, as this amount was considered the threshold of non-toxicity.

The extracts were divided into four toxicity categories according to their IC_{50} value: high toxicity ($IC_{50} \leq 5$ g L^{-1}), medium toxicity ($5 < IC_{50} \leq 10$ g L^{-1}), low toxicity ($10 < IC_{50} \leq 15$ g L^{-1}), and no toxicity ($IC_{50} > 15$ g L^{-1}). The latter category included also those extracts for which an IC_{50} value was not available.

Results

Extracts from 13 test biomasses were evaluated as well as those from two reference materials (*Nostoc* M1 and lettuce). Among the test biomasses, only for *P. purpureum* F&M-M46 calculation of the IC_{50} was not possible due to lack of toxicity in the tested range (Table 3). For *C. vulgaris* Roquette, *C. vulgaris* Allma, and *T. suecica*, as well as for the negative reference lettuce, it was possible, for at least one of the extracts, to estimate the IC_{50}, which value, however, was always higher than 15 g L^{-1} (Table 3). These three biomasses as well as that of *P. purpureum* were considered as non-toxic (Fig. 1).

The remaining nine test biomasses and the positive reference *Nostoc* M1 showed an IC_{50} below 15 g L^{-1} (hence toxicity) for at least one extract and one model (NHDF or *A. salina*). Only Klamath powder showed an IC_{50} below 15 g L^{-1} for all the extracts with both models. The IC_{50} value of the water extract of Klamath powder against NHDF was in the range that we classified as medium toxicity (Table 3, Fig. 1), whereas both water and methanolic extracts showed an IC_{50} against *A. salina* in the range that we considered of high toxicity (Table 3, Fig. 1). It is to note that the toxicity of the Klamath powder aqueous extract against *A. salina* was much higher after 48 h of exposure, while the methanolic extract acted almost at its full potency already after 24 h. A similar behavior was observed for the positive reference *Nostoc* M1 (Table 3, Fig. 1). The main difference was that the methanolic extract of *Nostoc* M1 behaved like the aqueous one in terms of toxicity increase with exposure time. In fact, after 24 h, the IC_{50} value of the methanolic extract was 8.2 g L^{-1} and decreased to 0.4 g L^{-1} after 48 h; the aqueous extract was not toxic after 24 h and showed an IC_{50} of 7.2 g L^{-1} after 48 h (Table 3).

For *C. sorokiniana* F&M-M49 grown in BG11, *C. sorokiniana* IAM C-212, and *T. lutea* F&M-M36, an IC_{50} below 15 g L^{-1} could be calculated for both models, although in the case of *A. salina* only for the methanolic extracts. IC_{50} values for *T. lutea* F&M-M36 extracts were in the range considered of medium toxicity (Table 3, Fig. 1), as well as the methanolic extract from *C. sorokiniana* IAM-C212, which aqueous extract showed

Table 3 IC_{50} values calculated or estimated for microalgal aqueous or methanolic extracts (expressed as *g* of extracted biomass per liter) on human fibroblasts (NHDF) and *A. salina* after 24 and 48 h of exposure; data are reported as mean ± SD

Strain	IC_{50} value (g L^{-1})				
	Fibroblasts	*Artemia salina*			
	H_2O extract	H_2O extract		MeOH extract	
	24 h	24 h	48 h	24 h	48 h
Nostoc sp. M1 (positive reference)	9.3 ± 2.9	nc	7.2 ± 0.2	8.2 ± 2.3	0.4 ± 0.1
Lettuce (negative reference)	nc	>15*	>15*	nc	nc
A. platensis F&M-C256	11.5 ± 5.7	>15*	>15*	nc	nc
Klamath powder	8.8 ± 0.4	>15*	4.7 ± 1.9	1.7 ± 0.0	1.6 ± 0.1
N. sphaeroides F&M-C117	7.8 ± 1.3	>15*	>15*	nc	nc
C. sorokiniana F&M-M49 BG11	12.6 ± 4.0*	nc	>15*	13.6 ± 2.9*	13.4 ± 2.3*
C. sorokiniana F&M-M49 F	2.7 ± 1.0	–	–	–	–
C. sorokiniana IAM C-212	3.7 ± 0.09	nc	nc	6.0 ± 0.6	5.6 ± 0.6
C. vulgaris Roquette	>15*	nc	>15*	nc	nc
C. vulgaris Allma	nc	>15*	>15*	nc	nc
T. suecica F&M-M33	>15*	nc	>15*	>15*	>15*
P. purpureum F&M-M46	nc	nc	nc	nc	nc
P. tricornutum F&M-M40	2.3 ± 0.4	nc	>15*	>15*	>15*
T. lutea (T-ISO) F&M-M36	6.0 ± 2.2	>15*	>15*	6.0 ± 0.5	5.6 ± 0.9
N. oceanica F&M-M24	11.2 ± 2.1	nc	nc	nc	nc

*Estimated value

nc not calculable due to absence of toxicity in the tested range; − not tested

instead an IC_{50} in the range of high toxicity against NHDF (Table 3, Fig. 1). IC_{50} values were in the range of low toxicity for both extracts from *C. sorokiniana* F&M-M49 grown in BG11 (Table 3, Fig. 1).

The remaining five biomasses were active only against NHDF. *A. platensis* F&M-C256 and *N. oceanica* F&M-M24 extracts showed an IC_{50} higher than 11 g L^{-1} and laid in the low toxicity range; the IC_{50} of *N. sphaeroides* F&M-C117 was in the range of medium toxicity, whereas extracts from *C. sorokiniana* F&M-M49 grown in F medium and *P. tricornutum* F&M-M40 showed an IC_{50} lower than 3 g L^{-1} and were considered of high toxicity (Fig. 1, Table 3).

To assess the contribution of artificial seawater salt to the cytotoxic effects observed, also considering the results obtained with *C. sorokiniana* F&M-M49 grown in BG11 and F medium, we tested a range of salt concentrations (from 30 to 0.6 g L^{-1}) on NHDF. All the hypertonic concentrations (30, 15, 12 g L^{-1}) strongly reduced cell survival as expected, 6 g L^{-1} caused a modest reduction, whereas 3 and 0.6 g L^{-1} did not show any cytotoxicity (data not shown). An IC_{50} of 11.2 ± 0.6 g L^{-1} was calculated. We can exclude toxicity of artificial seawater salt in our trials since the maximum salt concentration in microalgal extracts was estimated to be <3 g L^{-1}.

Discussion

Although the number of microalgal species worldwide is vast, only few have been isolated and studied with regard to biochemistry and physiology, and even less are exploited for commercial applications (Tredici et al. 2009). Less than a dozen microalgal species are presently accepted in the EU as food or food ingredients (European Union 1997), and a similar situation is recorded also in the USA and Australia. To increase this number, it is necessary to go through the novel food approval regulations that require the assessment of the safety of the candidate food ingredient (European Union 2015; FDA 2014; FSANZ Proposal P1024). Starting from this perspective, this research aimed to evaluate, as preliminary step, the toxicity of microalgal biomasses of interest as food sources due to their nutritional qualities, using *A. salina* and human dermal fibroblasts as models.

As far as toxicity on cells is concerned, most of the literature deals with tests against cancer cell lines (Jaki et al. 1999; Carballo et al. 2002; Bechelli et al. 2011; Hisem et al. 2011), and only few studies, to our knowledge, focus on the cytotoxic effects of microalgae on non-cancerous cells (Prestegard et al. 2009; Lopes et al. 2011; Goh et al. 2014). In vitro cytotoxicity tests are performed to determine the intrinsic ability of a compound to cause cell death as a consequence of damage to the

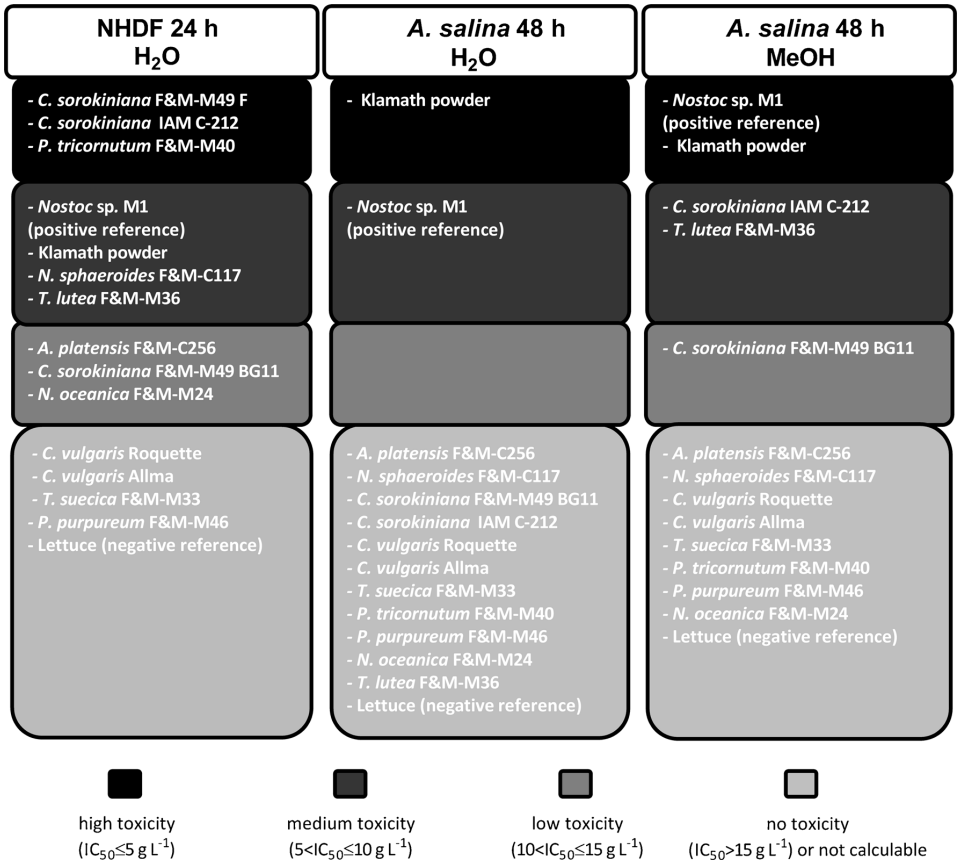

Fig. 1 Grouping of microalgal extracts according to their toxicity level, based on IC_{50} values. Aqueous extracts on NHDF after 24 h, and aqueous and methanolic extracts on *A. salina* after 48 h are reported. The toxicity levels are displayed in decreasing order (from darker to lighter color)

basic cellular functions. The use of normal human cell lines, that present a non-aberrant phenotype, is an added value with respect to the use of cancerous cells that more typically show chromosome and phenotype aberrations (Jallepalli and Lengauer 2001; Fackental and Godley 2008). It is worth noting that there is little literature on toxicity of eukaryotic microalgae on cell lines and *A. salina*, while a larger number of papers deal with cyanobacteria.

Six of the tested algal biomasses (*T. lutea* F&M-M36, Klamath powder, *N. sphaeroides* F&M-C117, *C. sorokiniana* F&M-M49 grown in F medium, *C. sorokiniana* IAM C-212, and *P. tricornutum* F&M-M40) showed a similar or even higher cytotoxicity on fibroblasts compared to the positive reference *Nostoc* M1, while only Klamath powder showed a toxicity similar to *Nostoc* M1 on *A. salina*. The toxic effects on fibroblasts of *Nostoc* M1, a cyanobacterium containing cryptophycin, a powerful antimitotic agent (Panda et al. 1997), were lower than expected from our previous data on antifungal activity of this strain (unpublished) and on cytotoxicity of other cryptophycin-containing *Nostoc* strains (Piccardi et al. 2001). On *A. salina*, the toxic action of the aqueous extract was observed only after 48 h of exposure, whereas with the methanolic extract, the effect was more marked and present already after 24 h. This behavior, in both models, can be explained by the low solubility of cryptophycin (a cyclic depsipeptide) in water, and to the low division rate of fibroblasts compared to tumor cell lines and of nauplii cells that might slow the action of the antimitotic venom. The IC_{50} value shown by *Nostoc* M1 on human fibroblasts in this study (9.3 g L^{-1}) is much higher than that reported by Hrouzek et al. (2016) for the most cytotoxic *Nostoc* strain against murine fibroblasts (2.8 g L^{-1}).

Nostoc is one of the cyanobacterial genera with the highest frequency of cytotoxic metabolites. Among 81 *Nostoc* strains tested, cytotoxicity frequency to different target cells was found to vary for the same extracts from 26 to 76 % (Hrouzek et al. 2016). Biondi et al. (2008) found five out of six Antarctic *Nostoc* isolates to be cytotoxic to HeLa cells.

Cytotoxicity against murine tumor cell lines was found in 33 (Hrouzek et al. 2011), 60 (Hrouzek et al. 2016), and 65 % (Hisem et al. 2011) of the *Nostoc* strains tested. On *A. salina*, Piccardi et al. (2000) found toxicity in 24 % of 50 *Nostoc* extracts, mainly aqueous. The same frequency of toxicity was found by Hisem et al. (2011) in 29 methanolic extracts. It is worth noting that the genus *Nostoc* includes, besides highly toxic strains, edible species (as *N. sphaeroides* and *Nostoc flagelliforme*) and that, in this study, the edible *N. sphaeroides* F&M-C117 showed medium toxicity to fibroblasts and no toxicity to *A. salina*. A similar toxicity pattern was shown by the aqueous extract from *P. tricornutum* F&M-M40. Toxicity of aqueous extracts from this algal species is reported by Prestegard et al. (2009) on rat hepatocytes (>30 % death at 4 g L^{-1} of extracted biomass after 1 h of exposure) and IPC leukemia cells (>30 % death at 1.5 g L^{-1} after 18 h). No literature data are available on toxicity of this alga to *A. salina*.

Among the algae approved as food in the EU, the two commercial *C. vulgaris* strains were found not toxic (neither to fibroblasts nor to *A. salina*). *Arthrospira platensis* (the so-called spirulina), that on the basis of its longtime history (centuries) of human consumption is considered a safe food, in our work showed a low toxic effect on fibroblasts and no toxicity to *A. salina*. Bechelli et al. (2011) found toxicity at 2 g L^{-1} of extracted biomass with ethanolic extracts of *A. platensis* on leukemia cells and no toxicity to normal bone marrow cells. One of the microalgae approved as food in the EU is *A. flos-aquae* from Klamath Lake (European Union, Novel Food catalogue). In our study, medium toxicity to fibroblasts was observed with the aqueous extract from the biomass harvested from the natural bloom dominated by this alga in Klamath Lake, whereas high toxicity to *A. salina* was found in both aqueous and methanolic extracts. Our results confirm the findings from Bechelli et al. (2011) who observed a high toxicity of the ethanolic extract of this bloom against both leukemia and normal bone marrow cells at concentrations lower than those found toxic in our work. It is necessary to point out that, although some strains of *Aphanizomenon* are known to produce saxitoxin and neosaxitoxin (Ferreira et al. 2001; Ballot et al. 2010a, b), as well as mueggelone, responsible for neurotoxic and ichthyotoxic activity (Papendorf et al. 1997), so far *A. flos-aquae* from Klamath Lake has never been found to produce these toxins (Carmichael 1992; Heussner et al. 2012). Up to now, this cyanobacterium from Upper Klamath Lake (OR, USA) has not been stably isolated; thus, the only way to test its biomass is to harvest blooms from this lake. The toxicity of the bloom observed in our work could be due to: (i) compounds produced by *A. flos-aquae*; (ii) compounds produced by other microalgae present in the population, as for example *Microcystis* spp., often occurring in these blooms (Gilroy et al. 2000), although the microcystins would have affected *A. salina* (Vezie et al. 1996) but not fibroblasts (Matsushima et al. 1990); and (iii) compounds from biogenic contaminants (derived from plants, animals, and other microorganisms) or non-biogenic sources (Chamorro-Cevallos et al. 2007).

Among the other algae tested, only in few cases it is possible to compare our data with literature. We found toxicity of *N. oceanica* F&M-M24 aqueous extracts to fibroblasts at the highest concentrations (low toxicity). Goh et al. (2014) found no cytotoxicity of *Nannochloropsis oculata* extracts to mouse fibroblasts. In our study, *P. purpureum* F&M-M46 did not exert toxic effects at the concentrations tested. Gardeva et al. (2012) found that the polysaccharide from *P. purpureum* reduced the viability of MCF7, HeLa, and Graffi tumor cells. The methanolic extract of *T. suecica* F&M-M33 showed no toxicity to *A. salina* (our work), while the methanolic extracts of *T. suecica* CCAP 66/22D caused a 100 % mortality on *Artemia* nauplii (Lincoln et al. 1996).

The different toxicity patterns often observed in methanolic and aqueous extracts may derive from different molecules being extracted by the different solvents (Piccardi et al. 2000) or from the same component that is extracted by the solvents with a different efficiency (see the case of *Nostoc* sp. M1).

In general, our results show that the sensitivity of fibroblasts towards microalgal aqueous extracts is higher compared to that of *A. salina*, and only the extract from Klamath powder is toxic with both models. With all the extracts, a higher toxicity on *A. salina* was observed after 48 h of exposure, thus further confirming the lower sensitivity of this model compared to fibroblasts. This is not necessarily a drawback. In accordance with our study, Hisem et al. (2011) report a lower sensitivity of *A. salina* compared to the cell model. The results obtained in our work with aqueous extracts on fibroblasts are more coherent with those obtained with methanolic extracts on *A. salina*, although the fibroblasts higher sensitivity allowed to detect a larger number of potentially toxic biomasses.

Tests on cell lines appear more adequate when the objective is to determine cytotoxic activity, as also stated by Hisem et al. (2011). These authors attribute the higher sensitivity of cells with respect to *A. salina* to the fact that cyanobacterial metabolites generally affect basal metabolic pathways present in the eukaryotic cell rather than systems in complex multi-cellular organisms. However, fibroblasts appear too sensitive for use in general toxicity screenings, while *A. salina* lethality test, in particular using a universal solvent (e.g., methanol) and an exposure time of at least 48 h, appears far more reliable. Considering its easiness of realization and low cost, our work confirms the validity of this test in pre-screenings.

In conclusion, this work presents an in vitro analysis of the toxicity of 11 microalgal strains and one natural bloom of potential interest for food applications. Taken together, these data indicate that extracts from microalgae have a different

pattern of inhibition (and thus toxicity) on *A. salina* and fibroblasts. Although these two models are useful and widely employed tools to screen the potential toxic effect, the attainment of results not fully in accordance, probably due to the different sensitivity and complexity of the models, should be taken into serious consideration. Clearly, results obtained from in vitro models or invertebrate bioassays are preliminary and must be confirmed by in vivo studies on mammals performed on the whole algal biomass and not only on extracts. These pre-screenings allow reducing the number of candidate strains to be selected for further in vivo studies on mammals that are necessary, in most cases, to fulfill the mandatory requirement of subchronic toxicity data for novel food applications, at least in the EU. In conclusion, from this study, the two commercial *C. vulgaris* strains, *T. suecica* F&M-M33, and *P. purpureum* F&M-M46 emerged as non-toxic and other two strains, *A. platensis* F&M-C256 and *N. oceanica* F&M-M24, as substantially non-toxic. *T. lutea* F&M-M36, the two *C. sorokiniana* strains, *N. sphaeroides* F&M-C117, and *P. tricornutum* F&M-M40 showed some toxicity and need further investigation. Finally, Klamath powder was found to be the most toxic among the biomasses tested, inhibiting both models.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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About the author

Alberto Niccolai was born in Florence, Italy on 30 October 1989. He followed his secondary education at the Agricultural Technical Institute in Pescia (Italy) where he graduated cum laude in 2008 with the thesis "Utilisation of spontaneous plant biomass or resulting from cultivation for energetic purposes in Fucecchio Marshes". He studied Agricultural Sciences and Master in Agricultural Sciences and Technologies at University of Florence (Italy), where he graduated cum laude in 2013. He specialised in Environmental Management of Rural Areas. His first thesis "Productive response and organoleptic quality of the oil as a function of fertilization, climate regime and cultivation techniques for two biological companies" and the second MSc thesis "Cultivation of *Chlorella* in a thin layer photobioreactor at high cell density" was done at the Department of Agricultural Production and Environmental Sciences (DISPAA) at Florence University. In 2013, he started his PhD research in Agricultural and Environmental Sciences at the same Department. During the international event "Feeding the Planet Energy for Life", Expo Milan 2015 he won the price for the publication of the idea "Nutra- spirulina: new functional foods and vegetable protein from spirulina culture" on a specific catalogue "TXT 14_2015, Good living in Tuscany". Within his Phd, on March 2016 he did an internship (3 months) at Dublin Institute of Technology (Ireland).
The results of his PhD research are described in this thesis.

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