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Microbiological approaches for the implementation of algae in food: exploitation and safety evaluation

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Gentlemen, it is the microbes who will have the last word.

Louis Pasteur

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Abstract

In order to guarantee food security for the future decades, food scientists are focusing their studies on the search for novel food sources with a low impact on the environment and that at the same time could represent a valuable source of nutrients. In these respects, seaweeds and microalgae have the right characteristics. Algae are a heterogeneous group of organisms characterized by the ability to perform photosynthesis. Algae are commonly divided into macro and microalgae. Macroalgae are eukaryotic pluricellular organisms while microalgae are unicellular prokaryotic or eukaryotic organisms. Given their composition, algae are an appropriate food source for consumers because they present low calories content and a high presence of nutrients such as essential amino acids, proteins, dietary fibers, vitamins, minerals and bioactive compounds. Their abundance in bioactive compounds confers to algae a great potential for food, nutraceutical and pharma industries. Several bioactivities have been accredited to algae: antimicrobial, antioxidant, antidiabetic, anti-inflammatory and immunomodulation action, and the use as functional ingredients of these organisms for the development of therapeutic agents is widely studied. For those reasons, greater employment of microalgae and seaweeds in food production could be a possible solution to guarantee food security for the next decades. Considering this premise and the great potential of this group of organisms, the aim of this thesis is to study three different aspects connected to the use of algae in food formulations. In particular, was studied the use of food-grade seaweed and microalgae extracts as natural preservatives in foods assessed through in vitro and in situ tests. An overview of food safety aspects connected to the use of algae in food was reported. Interactions of microalgae and lactic acid bacteria were evaluated in order to produce novel food productions or food supplements.

Introduction and aim of the thesis

Algae and Novel foods

According to the data reported by FAO and by many studies (Grafton et al., 2015; McKenzie & Williams, 2015), in 2050 the world population will reach 9.7 billion people. In order to guarantee food security for everyone, food production must be doubled. Though, increasing food accessibility and food production is not enough. Is necessary to produce food in ways that enable continuous provisions, by not degrading our ability to produce food in the next years and do not compromise significant ecosystems (Grafton et al., 2015). An irresponsible food production, and industrialization, could damage irreversibly the planet in terms of loss of water supplies, climate change and loss in terms of animal and plant species. In this regard, even if known and used for centuries, interest in algae (seaweeds and microalgae) as a new food source has increased in recent decades. Unlike plants and other food products, seaweeds do not require the use of fresh water and can grow in the seas thus occupy three quarters of the earth's surface. Also microalgae are a more sustainable production if compared to traditional crops: they ensure valuable proteins source without occupying cultivable land.

Though, food production is changing, not just because of the need for more sustainable productions, but also because of new food choices.

Rising cultural diversity, globalization and the search for sustainable and healthy foods are the main reason for these changes and constant research for new food sources.

For that reason, many “novel foods” are approved lately by EFSA.

Many algae and its derivatives are defined as "novel food". These were governed by EU food legislation with Regulation (EC) 258/97, applied to all those food products and compounds for which there is no demonstrable "significant" consumption until May 15, 1997 within the European Union. This Regulation introduced the concept of "novel food" but was later replaced by the new Regulation (EU) 2015/2283, effective from 1 January 2018,

which makes simpler the terms for introducing new foods on the EU while guaranteeing food safety for consumers. The purpose of the current regulation is to ensure the effective functioning of the internal market while ensuring a high level of protection of human health and consumer interests. The term novel food covers food obtained from new sources, new ingredients used in food formulations as well as not traditionally used technologies for food production.

Although, many species of seaweeds and microalgae are already sold and produced in Europe without the "Novel food" wording because significantly consumed before 1997.

Algae are increasingly being marketed as "bio-functional or bio-nutraceutical" foods; these terms do not have legal status in many countries but describe foods that contain bioactive or phytochemical compounds that can bring health benefits beyond the role of basic nutrition (Hafting et al., 2012). Because of their composition, algae are a food source appropriate for every kind of consumer because of their low calories content and the high presence of nutrients such as essential amino acids, proteins, dietary fibers, vitamins, minerals and bioactive compounds. Their richness in bioactive compounds confers them a great potential for the food and pharma industries. Indeed, these organisms are used as food supplements, nutraceuticals, functional food ingredients and food hydrocolloids. Moreover, studies about the exploitation of algae are growing fast, not only in food and in pharma industries, but also in the energetic sector. Algae are the main population in aquatic environments, from billions of years their photosynthetic ability to absorb CO_2 and produce O_2 allow the survival on earth exerting the most important oxygenating action.

Several activities have been credited to these organisms: antimicrobial, antioxidant, antidiabetic, anti-inflammatory and immunomodulation action and the use as functional ingredients of these classes of organisms for the development of therapeutic agents is studied. For these reasons, implementing the use of algae in food production could be the first step to guarantee food security for the next decades.

Seaweeds

Because of the abundance along the coasts of Asia, seaweeds are part of traditional diets and are widely consumed by the eastern population. Numerous algal species are used as food in Asian countries, and China and Indonesia are by far the countries that most contribute to global production (Buschmann et al., 2017). What distinguishes seaweeds from plants is the lack of differentiated organs, the absence of vascularization useful for the transport of nutrients and the lack of specialized tissues, therefore they do not have the characteristic distinction in stem, leaves and roots. Their multicellular structure is defined as thallus (Thallophyte) and can be unicellular or multicellular, with microscopic or macroscopic structures, the dimensions, therefore, vary from a few centimeters to several meters. They are photoautotrophic organisms, very heterogeneous and ubiquitous: in fact, they reside in different habitats such as seas, rivers and lakes.

Because of the need to survive several stresses, they have a strong and flexible structure composed of carbohydrates, making up 76% of the dry weight (Holdt & Kraan, 2011).

One interesting nutritional property of algae is the content of polyphenols, which constitute the largest group of secondary metabolites; they include a diversified number of chemical compounds, consisting of a hydroxyl group bonded to an aromatic hydrocarbon group (Cox et al., 2010). Polyphenols' structures can vary from more simple molecules, such as phenolic acids, to bigger and more complex such as phlorotannins.

The potential of seaweeds as food also lies in their protein content. Seaweeds are generally composed of 10 to 47% of dry weight of proteins, and it depends on the growing season and on species (Pangestuti & Kim, 2015). Algal bioactive proteins and peptides have been shown to have antihypertensive, anticoagulant and antioxidant activity (Harnedy & FitzGerald, 2011). An important role is played by lectin and phycobiliprotein. The lipid content in algae is lower compared to other nutrients; such organisms can contain up to 1-5% by dry weight

of total lipids (Miyashita et al., 2013). However, it is a powerful functional lipid source due to the high value of omega-3, such as eicosapentaenoic acid and stearidonic acid, and omega-6 (Holdt & Kraan, 2011).

Given the heterogeneous characteristics and the number of algae, their classification is constantly being revised. The most common classification is based on the type of pigments contained in the photosynthetic system and, depending on these, there are three important divisions: red algae (*Rhodophyta*), green algae (*Chlorophyta*) and brown algae (*Phaeophyta*) (Ferrara, 2020).

Red algae are characterized by a typical red-violet color of the thallus, due to the presence of three main pigments: two phycobiliproteins like phycoerythrins (red pigment) and phycocyanins (blue pigment) that, associated to chlorophyll, generate the typical red/purple color. Studies have shown that the nutrient content of this kind of seaweed can be exploited in human health applications, with purported benefits such as antiviral, anti-inflammatory and anticoagulant properties (Holdt & Kraan, 2011).

Red algae are characterized by a variable composition in amino acids and proteins such as phycobiliproteins, high presence of sulfated polysaccharides such as carrageenans, agarans and galactans, minerals, polyphenols and lipids (Cian et al., 2015; Ferrara, 2020). The most important role that red seaweed play in food industries is for the extraction of polysaccharides such as Agarose and Carrageenan that are used as stabilizing and gelling agents, in particular in yogurts and jams, and in many other food productions.

Red seaweeds present a protein concentration that varies from 10 to almost 50% and are the macroalgae with the highest amount of proteins. These proteins have proved a series of important bioactivities such as antioxidant, anti anemic and antidiabetic (Admassu et al., 2018).

Chlorophyta, known as green algae, is a heterogeneous group that includes both multicellular and single-celled organisms. The green color that characterizes them is due to the presence of chlorophylls mixed with xanthophylls. These seaweeds are consumed as food because of

their very varied and valuable composition. The presence of fatty acids (Omega 3); Vitamin B and C, carotenoids and minerals make them a valuable food supplement. The most consumed species is *Ulva lactuca,* a bright green seaweed that populates the Mediterranean Sea and cold-temperate waters. Green algae are also constituted by ulvans, a sulfated polysaccharide that has exerted many bioactivities (Jaulneau et al., 2010).

The *Phaeophyta* (brown algae) are marine algae that present a great diversity in shapes and sizes of the thallus. The brown color is due to the presence of a high variety and quantity of xanthophylls (fucoxanthin) and other pigments (phlorotannins). Fucoxanthin is the most copious of all carotenoids that compose *Phaeophyta* and plays a vital role in the protection and in photosynthetic functions (Peng et al., 2011). The presence of xanthophylls allows the organisms to perform photosynthesis at high depths in the oceans. They also contain chlorophyll a and c and β-carotene. The presence of sulfated polysaccharides like fucoidan is also really important. Fucoidan consists of sulfated L-fucose and small portions of glucose, galactose, xylose, mannose and uronic acids. It has a complex chemical nature and shows different structures depending on the type of alga from which it is isolated. It is a fundamental part of the algal cell wall. Various biological properties have been accredited to fucoidans including anti-inflammatory, antiproliferative, antiviral and anticoagulant (Elizondo-Gonzalez et al., 2012; Li et al., 2008).

Microalgae

Microalgae are single-celled organisms that live in all the aquatic environments such as, oceans and seas, rivers and ponds, hypersaline lakes and even in extreme ecosystems like deserts and arctic (Rathinam et al., 2014). These microorganisms can be subdivided into eukaryotic and prokaryotic algae, but the common characteristic is the ability to perform photosynthesis and convert light energy and carbon dioxide (CO_2) into biomass and O_2. Because of a simple cellular structure, easy access to water sources, CO_2 and other nutrients, their photosynthetic ability is much more efficient in converting solar energy into biomass than land-based plants. Differently from the traditional feedstock, microalgae have higher productivity in terms of biomass. For those reasons and their valuable composition, they are considered one of the most promising sources of food for the future. Furthermore, microalgae do not need to be grown on arable land, can be grown on seawater or on residual nutrients.

But what makes microalgae one of the most interesting sources as a food of the future is their composition in nutrients. Overall, protein is the most important organic component. Numerous eukaryotic microalgae and cyanobacteria can synthesize high protein content, (*C. vulgaris* 51–58%, and *A. platensis* 60–65%). What makes the protein composition even more interesting, is the ability, for some microalgae like *A. platensis,* to synthesize essential amino acids (Ghaeni et al., 2014). The second most abundant components of microalgae are carbohydrates. In particular, polysaccharides are fundamental to store energy mainly as cellulose, starch and glycogen. Many microalgae species are also able to produce exopolysaccharides that confer to cells a mucilaginous structure making them more resistant to stress. Interest in microalgae carbohydrates is growing due to many related bioactivities and to industrial and biotechnological applications. Another important component of microalgae cells are lipids. The lipid content of microalgae is variable and it

can reach 40% in some species. Generally, algal oils are composed of polyunsaturated fatty acids that can be commercialized for nutraceutical and pharmaceutical applications such as omega-3 (ω-3) α-linolenic (ALA, C18: 3), eicosapentaenoic (EPA, C20: 5) and docosahexaenoic (DHA, C22: 6) acids and omega-6 (ω-6) including linoleic (LA, C18: 2), ɣ-linolenic (GLA, C18: 3) and arachidonic (ARA, C20: 4) acids (Koller et al., 2014). The ability of certain microalgae to produce lipids has brought scientists to study these organisms not only as a source of food and feed. Many studies focus on the possibility to use microalgal oils to substitute fossil oils. Other fundamental components of microalgae are pigments. Three major groups of algal pigments, responsible for their color, are found in microalgae and are essential for light-harvesting, protection of cells against overexposure to light, CO_2 fixation. The three classes of pigments are chlorophylls, which provide a green coloration, carotenoids (in particular, carotenes and xanthophylls are responsible for orange and yellow pigmentation respectively), and phycobilins (phycocyanin and phycoerythrin) (Koller et al., 2014). Chlorophylls are green pigments essential for photosynthesis and play an important role in food production as a food colorant (E140). Carotenoids are light-harvesting pigments that support chlorophylls to absorb light. They play another essential action as antioxidants able to deactivate reactive oxygen species (ROS) formed by overexposure to too many solar radiations (Matos, 2017). The presence of carotenoids in microalgae has gained the attention of the food and pharma industries. The most important carotenoids in microalgae, from an industrial point of view, are β carotene and astaxanthin. These two pigments are extracted from two green microalgae, *Dunaliella salina* and *Hematococcus pluvialis.* These compounds are used as antioxidants in food and food supplements, as feed for aquaculture to confer to fishes a more pleasant coloration, and in pharmacological products because of bioactivities. Phycobilins (phycocyanin and phycoerythrin) are bilins responsible for capture light in many microalgal phyla. These compounds are useful in many biotechnological and food applications. At the moment, phycobiliproteins are the compounds resultant from microalgae with the most important market values (Koller et al., 2014). Phycocyanins are

used as a blue natural colorant in the food industry, as a constituent of functional foods, and also as an antioxidant in cosmetic products (Ariede et al., 2017).

Some species of microalgae, mostly Dynoflagellates and cyanobacteria, can produce algal toxins. These potent toxins can be found both in seawater and in freshwater contaminating drinking water or fish and molluscs. Because of their stability to high temperatures, these toxins are still present after cooking. Algal toxins are generally present consequently to algal blooms in oceans and seas all over the world and can lead to harming of humans, through food or directly in contact, fish die-offs and close of fisheries. Algal toxins can cause many poisoning syndromes like: diarrhoeic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), amnesic shellfish poisoning (ASP), ciguatera fish poisoning (CFP), and paralytic shellfish poisoning (PSP). Because of climate change, algal blooms is expected to increase the frequency in coastal areas. This growing phenomenon will have negative impacts on food security, human health but also on tourism and the local economy (Gobler, 2020).

Microalgae are for sure one of the best resources for future food production and biotechnology, but because of climate change, they will become also a possible threat to human health. For that reason, is important to study algae in the food system not only as a resource but also as a possible threat for consumers.

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References

Admassu, H., Gasmalla, M. Abdalbasit. A., Yang, R., & Zhao, W. (2018). Bioactive Peptides Derived from Seaweed Protein and Their Health Benefits: Antihypertensive, Antioxidant, and Antidiabetic Properties. *Journal of Food Science*, *83*(1), 6–16. https://doi.org/10.1111/1750-3841.14011

Ariede, M. B., Candido, T. M., Jacome, A. L. M., Velasco, M. V. R., de Carvalho, J. C. M., & Baby, A. R. (2017). Cosmetic attributes of algae - A review. *Algal Research*, *25*, 483–487. https://doi.org/10.1016/j.algal.2017.05.019

Buschmann, A. H., Camus, C., Infante, J., Neori, A., Israel, Á., Hernández-González, M. C., Pereda, S. V., Gomez-Pinchetti, J. L., Golberg, A., Tadmor-Shalev, N., & Critchley, A. T. (2017). Seaweed production: overview of the global state of exploitation, farming and emerging research activity. *European Journal of Phycology*, *52*(4), 391–406. https://doi.org/10.1080/09670262.2017.1365175

Cian, R., Drago, S., de Medina, F., & Martínez-Augustin, O. (2015). Proteins and Carbohydrates from Red Seaweeds: Evidence for Beneficial Effects on Gut Function and Microbiota. *Marine Drugs*, *13*(8), 5358–5383. https://doi.org/10.3390/md13085358

Cox, S., Abu-Ghannam, N., & Gupta, S. (2010). An Assessment of the Antioxidant and Antimicrobial Activity of Six Species of Edible Irish Seaweeds. *International Food Research Journal*, *17*, 205–220. https://doi.org/10.21427/d7hc92

Elizondo-Gonzalez, R., Cruz-Suarez, L. E., Ricque-Marie, D., Mendoza-Gamboa, E., Rodriguez-Padilla, C., & Trejo-Avila, L. M. (2012). In vitro characterization of the antiviral activity of fucoidan from *Cladosiphon okamuranus* against Newcastle Disease Virus. *Virology Journal*, *9*(1), 307. https://doi.org/10.1186/1743-422X-9-307

Ferrara, L. (2020). Seaweeds: A Food for Our Future. *Journal of Food Chemistry & Nanotechnology*, *6*(2). https://doi.org/10.17756/jfcn.2020-084

Ghaeni, M., Roomiani, L., & Moradi, Y. (2014). Evaluation of Carotenoids and Chlorophyll as Natural Resources for Food in *Spirulina* Microalgae. *Applied Food Biotechnology*, *2*(1), 39–44. https://doi.org/DOI: 10.22037/afb.v2i1.7210

Gobler, C. J. (2020). Climate Change and Harmful Algal Blooms: Insights and perspective. *Harmful Algae*, *91*, 101731. https://doi.org/10.1016/j.hal.2019.101731

Grafton, R. Q., Daugbjerg, C., & Qureshi, M. E. (2015). Towards food security by 2050. *Food Security*, *7*(2), 179–183. https://doi.org/10.1007/s12571-015-0445-x

Hafting, J. T., Critchley, A. T., Cornish, M. L., Hubley, S. A., & Archibald, A. F. (2012). On-land cultivation of functional seaweed products for human usage. *Journal of Applied Phycology*, *24*(3), 385–392. https://doi.org/10.1007/s10811-011-9720-1

Harnedy, P. A., & FitzGerald, R. J. (2011). Bioactive proteins, peptides, and amino acids from macroalgae. *Journal of Phycology*, *47*(2), 218–232. https://doi.org/10.1111/j.1529-8817.2011.00969.x

Holdt, S. L., & Kraan, S. (2011). Bioactive compounds in seaweed: functional food applications and legislation. *Journal of Applied Phycology*, *23*(3), 543–597. https://doi.org/10.1007/s10811-010-9632-5

Jaulneau, V., Lafitte, C., Jacquet, C., Fournier, S., Salamagne, S., Briand, X., Esquerré-Tugayé, M.-T., & Dumas, B. (2010). Ulvan, a Sulfated Polysaccharide from Green Algae, Activates Plant Immunity through the Jasmonic Acid Signaling Pathway. *Journal of Biomedicine and Biotechnology*, *2010*, 1–11. https://doi.org/10.1155/2010/525291

Koller, M., Muhr, A., & Braunegg, G. (2014). Microalgae as versatile cellular factories for valued products. *Algal Research*, *6*, 52–63. https://doi.org/10.1016/j.algal.2014.09.002

Li, B., Lu, F., Wei, X., & Zhao, R. (2008). Fucoidan: Structure and Bioactivity. *Molecules*, *13*(8), 1671–1695. https://doi.org/10.3390/molecules13081671

Matos, Â. P. (2017). The Impact of Microalgae in Food Science and Technology. *Journal of the American Oil Chemists' Society*, *94*(11), 1333–1350. https://doi.org/10.1007/s11746-017-3050-7

McKenzie, F. C., & Williams, J. (2015). Sustainable food production: constraints, challenges and choices by 2050. *Food Security*, *7*(2), 221–233. https://doi.org/10.1007/s12571-015-0441-1

Miyashita, K., Mikami, N., & Hosokawa, M. (2013). Chemical and nutritional characteristics of brown seaweed lipids: A review. *Journal of Functional Foods*, *5*(4), 1507–1517. https://doi.org/10.1016/j.jff.2013.09.019

Pangestuti, R., & Kim, S.-K. (2015). Seaweed proteins, peptides, and amino acids. In *Seaweed Sustainability* (pp. 125–140). Elsevier. https://doi.org/10.1016/B978-0-12-418697-2.00006-4

Peng, J., Yuan, J.-P., Wu, C.-F., & Wang, J.-H. (2011). Fucoxanthin, a Marine Carotenoid Present in Brown Seaweeds and Diatoms: Metabolism and Bioactivities Relevant to Human Health. *Marine Drugs*, *9*(10), 1806–1828. https://doi.org/10.3390/md9101806

Rathinam, R., Hemaiswarya, S., Venkatesan, G., & Carvalho, I. S. (2014). Biomass from Microalgae: An Overview. *Oceanography: Open Access*, *02*(01). https://doi.org/10.4172/2332-2632.1000118

Aim of the thesis

This Ph.D. project was aimed to exploit the impact of algae in food application. The study covered various aspects of the use of algae in food ranging from safety aspects to new application perspectives for product improvements.

- **Chapter 1- Antimicrobial activity of algae and food applications-** aims to assess the antimicrobial activity of food-grade extracts obtained from seaweeds and microalgae. A novel approach based on impedometric technique and fluorescence microscopy was applied to study the minimal inhibitory concentration and the minimal bactericidal concentration of algae extracts towards pathogenic and spoilage microorganisms. It was also evaluated how fermentation and high pressure can influence the antimicrobial activity of a seaweed extract.

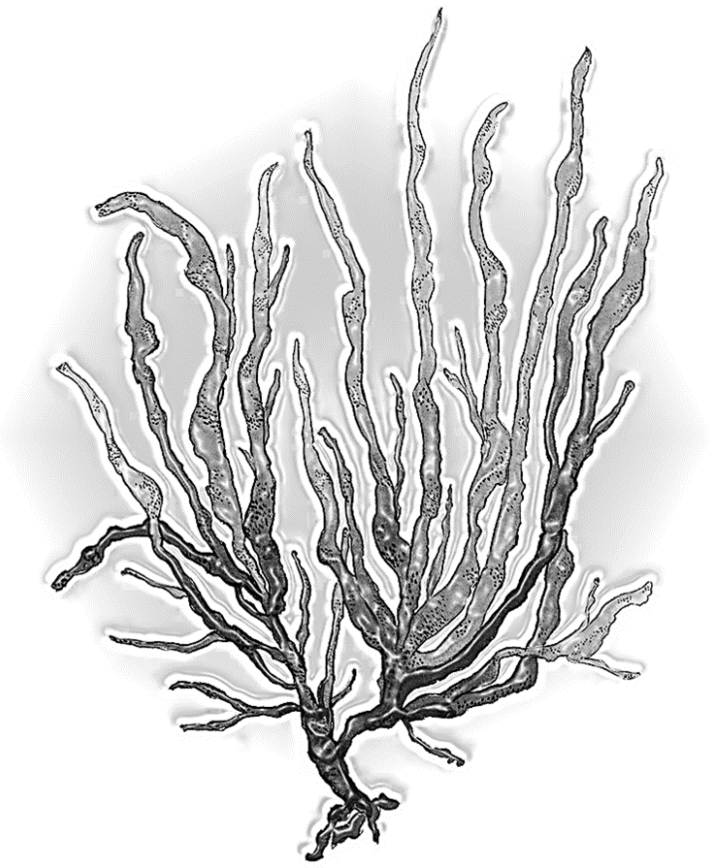
- In **Chapter 2 - Algae and food safety-** are addressed food safety aspects related to algae. In particular, it is evaluated if algae-based products, already present in the market as Ready to Eat foods, may arise microbiological food safety issues for consumers. Moreover, the interaction among lactic acid bacteria with two marine toxins, an algal and a bacterial toxin as common seafood contaminants, was investigated with the aim to open up perspectives for a bioremediation process.

- **Chapter 3- Microalgae and lactic acid bacteria: interactions in formulations-** aims to study the interaction of lactic acid bacteria and microalgae in formulations. The stimulatory activity of *A. platensis* on lactic acid bacteria used as starter for fermented foods production was assessed. It was also investigated the production of a fermented food supplement based on *A. platensis* focusing on changes in the volatile profiles of the products due to fermentation. Finally, with the aim to evaluate if lactic

acid fermentation is a good tool to enhance the bioactivity of algae, it is presented a study in which was investigated the lipid reduction activity of fermented microalgae extracts through the Nile red zebrafish fat metabolism assay.

Chapter 1:

Antimicrobial activity of algae and food applications



Edible seaweeds and spirulina extracts for food application: *in vitro* and *in situ* evaluation of antimicrobial activity towards foodborne pathogenic bacteria

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Abstract

Research is every day more focused on studying and selecting food preservatives of natural origin. In this scenario, algae are an excellent source of bioactive compounds, among which antimicrobials, whose presence is really variable depending on the algal species and environmental conditions. The aim of the present study was to obtain, by a food-grade approach, antimicrobial extracts from 5 species already approved as foods and to test their efficacy *in vitro* (agar well diffusion assay) and *in situ* (microbial challenge test) towards foodborne pathogenic bacteria. Moreover, the total phenolic compounds of the extracts were determined in order to evaluate possible correlations with the antimicrobial activity. Strains belonging to *Salmonella* spp., *Listeria monocytogenes, Escherichia coli, Staphylococcus aureus,* and *Bacillus cereus* were considered. Overall, the extracts showed good antimicrobial activity *in vitro* towards all the tested microorganisms, especially *L. monocytogenes* (15 mm of inhibition diameter). The effect of inhibition was monitored during 24, 48 and 120 hours showing a good persistence in time. *Arthrospira platensis* exerted the highest efficacy, further revealed towards *L. monocytogenes* on salmon tartare as bacteriostatic using 0.45% of the extract and bactericidal using 0.90% of the extract. The presence of phenolic compounds could be related to the antimicrobial activity but was not revealed as the main component of this activity. The extract with the highest phenolic content (18.79 ± 1.90 mg GAE/g) was obtained from *Himanthalia elongata*. Anyway, the efficacy confirmed also in a food matrix might open perspectives for their applications as food preservatives.

Introduction

Seaweeds and microalgae are becoming progressively popular as food in western countries, and some species in particular are recognized as a valuable source of healthy compounds (Gupta & Abu-Ghannam, 2011; Holdt & Kraan, 2011; Samarakoon & Jeon, 2012). Algae are indeed a good supply of bioactive and functional constituents for diet, such as essential amino acids, polyunsaturated fatty acids, fibers, vitamins, phytosterols and minerals (Niccolai et al., 2019). Twenty million tons of seaweeds are worldwide harvested every year and half of the production is destined for human consumption in form of dried products (Buschmann et al., 2017). Also *Arthrospira platensis*, an edible cyanobacterium commercially known as spirulina, is commonly employed as food supplement because of its nutritional value (Andrade, 2018; Batista et al., 2017). Many bioactivities have been attributed to algae including antioxidant, antidiabetic, anti-inflammatory and immunomodulation actions. Lately, many studies involving the implementation of algae in food formulations to obtain or enhance functional and nutritional properties of food have been presented (Camacho et al., 2019; Torres et al., 2017; Zouari et al., 2011).

Antimicrobial activity, among the other properties, has been proved for some algae (Alghazeer et al., 2013; Hongayo et al., 2012; Pérez et al., 2016; Saleh & Al-Mariri, 2017; Zbakh et al., 2012) even if at a variable extent according to several factors such as: i. species, *Phaeophyceae*, for instance, are described as the most effective against foodborne pathogenic bacteria; ii. chemical composition, which varies according to collecting area and season; iii. type of solvent used in the extraction process, indeed ethanolic and methanolic extracts are very effective against Gram-negative and Gram-positive bacteria; iv. the concentration of the extract (Pina-Pérez et al., 2017). Some algal species and bioactive molecules extracted from them fall into the category of "novel food" introduced by Regulation (EC) 258/97. This applies to all food products and substances that weren't significantly consumed until May 15, 1997 within the European Union. This Regulation was subsequently replaced by the new Regulation (EU) 2015/2283, which simplifies the conditions for introducing new foods on

the EU market maintaining a high level of food safety. (Zarbà et al., 2020) The algal species used in this work are already approved as food by EFSA, making easier the use of their extract in food formulations.

The proliferation of foodborne bacterial pathogens in foods endanger consumers' safety since these microorganisms are responsible for infections or intoxications. Preservatives are commonly used in food products to inhibit microbial growth but consumers' concern due to their possible harmful effects on health has generated an increasing demand for foods free from synthetic additives (Souza et al., 2006). Therefore, the role of algal extracts as antimicrobial agents against foodborne pathogens might guarantee food safety by combining the requests of consumers for products with a "clean labels" status.

In this context the aim of the present study was to: i) obtain antimicrobial extracts from algae approved as food, such as *Himanthalia elongata, Laminaria spp., Palmaria palmata* and *Undaria pinnatifida*, using a food-grade extraction, and test their efficacy *in vitro* towards the main foodborne pathogens (*Salmonella* spp., *L. monocytogenes*, *E. coli*, *S. aureus*, *and B. cereus*), by agar well diffusion assay; ii) evaluate *in situ*, on salmon tartare as food model, the efficacy of the most performing extract by microbiological challenge test; iii) evaluate the role of total phenolic compounds of the extracts on the antimicrobial activity.

Materials and Methods

Algae samples

Four species of seaweeds (*H. elongata, Laminaria* spp., *P. palmata* and *U. pinnatifida*) and a cyanobacterium (*A. platensis)* were overall considered. Dried samples were purchased on the Italian market considering two different producers for each species, in order to evaluate variability within the same. The 10 samples were grinded with Oster 890-48H mixer (Recampro, Spain) and maintained at room temperature in darkness until use.

Extraction process

With the purpose to extract compounds with potential antimicrobial activity such as small peptides, polyphenols and organic acids, an extraction process was carried as described by Martelli et al (2020)(Martelli, Favari, et al., 2020). In particular, 10 g of grinded sample were extracted with 100 mL of ethanol/water (70:30 v/v) acidified with 1% formic acid (CH_2O_2). A double extraction was performed, alternating twice a shaking cycle in an HS 501 digital shaker (IKA) (Staufen- Germany) at 200 strikes/minute to a sonication one in Ultrasonic Cleaner sonicator (VWR, United States), each lasting 15 minutes. The sample was then centrifuged (Eppendorf 5800 Centrifuge, Model 5810R, Hamburg, Germany) at 12857xg for 10 minutes at 10 °C. The solution was filtered on a paper filter to recover the solid part so as to proceed to the second extraction. The two extracts obtained were combined and dried under vacuum on a rotary evaporator Strike 300 (Steroglass, Italy, PG) at 4xg with a bath temperature of 40 °C. The dried extracts obtained were dissolved in sterile bi-distilled water to obtain 250 mg/ml solutions. For each species, two extracts were obtained (I and II). All the extractions were carried out in duplicate in order to test the reliability of the extraction method.

Foodborne pathogenic strains

The antimicrobial activity of the extracts was tested towards 14 strains belonging to the main foodborne pathogenic bacteria: *Salmonella spp*. (*S. enterica* ATCC 14028; *S. enterica* serotype *Rissen* and *Salmonella spp.* suini), *Listeria monocytogenes* (LM30; LMG 21264 and LMG 13305), *Escherichia coli* (DSM 9025; DSM 10973 and POM 1048), *Staphylococcus aureus* (NCTC 9393; ATCC 6538 and ATCC 19095) and *Bacillus cereus* (31 and 33). These strains belong to the collection of the Department of Food and Drug (University of Parma, Italy) and to international collections: National Collection of Type Cultures (NCTC), Belgian Coordinated Collection of Microorganisms (BCCM), American Type Culture Collection (ATCC), and Deustsche Sammlung von Mikroorganismen (DSM). Bacteria were kept at -80 °C in Tryptone Soya Broth (TSB) (Oxoid, Basingstoke, UK) supplemented with 12.5% glycerol (v/v). Before use, they were revitalized twice by inoculum (3% v/v) in TSB (Oxoid) supplemented with 0.6% of yeast extract and then incubated for 16 hours at 37 °C in aerobic conditions.

Evaluation of the in vitro antimicrobial activity

An agar well diffusion assay was carried out in order to evaluate the antimicrobial activity of the extracts (Martelli, et al., 2020 a; Ricci et al., 2019). The pathogenic bacteria were diluted to a concentration of 8 Log CFU/mL and seeded on Tryptone Soya Agar (TSA) (Oxoid) by using sterile swabs. Then, wells having a diameter of 7 mm were created by means of sterile tips in the agar and filled with 30 μL of each extract. The antimicrobial activity was evaluated by measuring the diameter of the inhibition zone (mm) after 24, 48 and 120 hours of incubation at 37 °C in aerobic conditions. Water was used as negative control. Average values ± standard deviations were reported and the analyses were performed in triplicate.

Microbiological Challenge Test

Specific studies are recommended to gather experimental data in case the behavior of a microorganism in a product is not well known (Codex Alimentarius., 2002). For this purpose, microbiological challenge test (MCT) allows to evaluate if an artificially inoculated organism can survive or develop in a specific product, determining the eventual reaching of unacceptable levels (Notermans & in't Veld, 1994). In the case of *L. monocytogenes,* the refrigerated storage cannot be considered a sufficient parameter to inhibit its growth (Chan & Wiedmann, 2008) and MCT becomes an important tool to monitor its behavior in ready-to-eat (RTE) foods and to evaluate the efficacy process parameters or ingredients used as preservatives. In the present work, the extract that has shown the highest *in vitro* efficacy was selected to test the antimicrobial activity *in situ*. The test was conducted as described by Bernini et al. (2013) with some modifications (Bernini et al., 2013). A MCT was designed in a salmon tartare, chosen as a food model, considering two different extract concentrations, 0.45 and 0.9% (v/w), corresponding to 4.5 mg/g and 9.0 mg/g respectively in the product. Negative samples, consisting of salmon tartare without any addition of the extract, were also considered. The antimicrobial activity was tested towards a mixture of the three *L. monocytogenes* strains used for the *in vitro* test (LM30, LMG 21264 and LMG 13305) and revitalized as previously described (2.3). The salmon tartare, added or not with the extract, was then contaminated in order to obtain a final concentration of 4 Log CFU/g of *L. monocytogenes* in the product. Tartare was then mixed to distribute evenly the contamination, portioned and stored at refrigeration temperatures for four days to simulate domestic conservation. *L. monocytogenes* was monitored just after inoculum (T0), after 24h (T1), 48h (T2), 72h (T3) and 96h (T4) of shelf life by plate count on Agar Listeria acc. to Ottaviani & Agosti (ALOA) (Biolife, Milan, Italy) after incubation at 37°C for 24 hours. Analysis were performed in triplicate and average values ± standard deviations were reported.

Chemicals

Ethanol, methanol and formic acid used for extraction procedures were obtained from Sigma-Aldrich (St. Louis, MO, USA), while bi-distilled water was in house produced by a Millipore Alpha Q purification system (Waters, Billerica, MA, USA). Sodium carbonate and gallic acid utilized for the determination of phenol content were purchased from Sigma-Aldrich (St. Louis, MO, USA), while Folin-Ciocalteu's phenol reagent solution was obtained from VWR (Milano, Italy).

Total phenolic amount determination

The determination of the total phenolic content was performed following the protocols reported by Cox et al. (2010) with some modifications. In particular, 0.10 g of seaweed extract were weighed, added with 10 mL of a methanol/bi-distilled water solution (70/30, v/v) and extracted at room temperature by constant shaking on a shaker HS 501 digital (IKA, Staufen, Germany) at 200 strokes/minute for 30 minutes. Then, the solutions were centrifuged at 5000 rpm for 10 minutes at room temperature using a Centrifugette 4206 centrifuge (Alc International, Pévy, France). 200 μL of sample extracted were then added with 1 mL of Folin-Ciocalteu's phenol reagent solution, previously diluted in bi-distilled water (1/10, v/v), and 2 mL of aqueous sodium carbonate (20 %, w/v), and incubated in the dark for 30 minutes. After this step, the absorbance of the solution was measured at 760 nm by a JASCO V-530 spectrophotometer (Easton, MD, USA). Blank was prepared and analyzed following the same procedure. The total phenolic amount was calculated as mg gallic acid equivalent per g (mg GAE/g) from a calibration curve obtained by measuring the absorbance at 760 nm of 5 gallic acid solutions at different concentrations: 0.01, 0.025, 0.05, 0.075 and 0.1 mg/g. All the extractions and analyses were repeated twice, while for each extract and standard solution the absorbance measurement was performed in triplicate.

Statistical analysis

The statistical analysis was carried out by testing a univariate multifactorial ANOVA using the GLM software SAS University edition. With the significance test (P value <0.05) a first analysis was carried out in which the inhibition halo was placed as a dependent variable, while extract, contact time and algae species corresponded to the independent variables. Subsequently, a post-hoc TEST (Tukey) was carried out. The antimicrobial activity of the different extracts obtained from the same algae species (I and II) was compared and evaluated against each pathogenic strain at different contact times. Statistical elaborations of the results obtained from total phenolic content determination were performed with IBM SPSS Statistics 23. In particular, two tailed T-test for independent samples was applied to determine a significant difference ($p<0.05$) between extracts I and II corresponding to different samples of the same algae species. Conversely, to define analogies and/or differences among samples belonging to different species, one-way ANOVA analysis was applied using Tukey test and the results were considered different for values of $p < 0.05$.

Results

In vitro antimicrobial activity

The *in vitro* antimicrobial activity of the extracts was evaluated by agar well diffusion assay as described by Martelli et al. (2020) (Martelli, Favari, et al., 2020) i. This test is based on the measurement of the inhibition zone on a layer of bacterial cells, after the spreading in the culture medium of the extracts to be tested. Fourteen foodborne pathogenic strains belonging to *Salmonella* spp., *L. monocytogenes, E. coli, S. aureus,* and *B. cereus* were considered. The extracts activities were determined by evaluating the dimension of the inhibition zone (mm in diameter) around the well after 24, 48, and 120 h of incubation at 37°C. In Figure 1 the heatmap representing in color scale the inhibition effect towards each foodborne pathogen along time is reported.

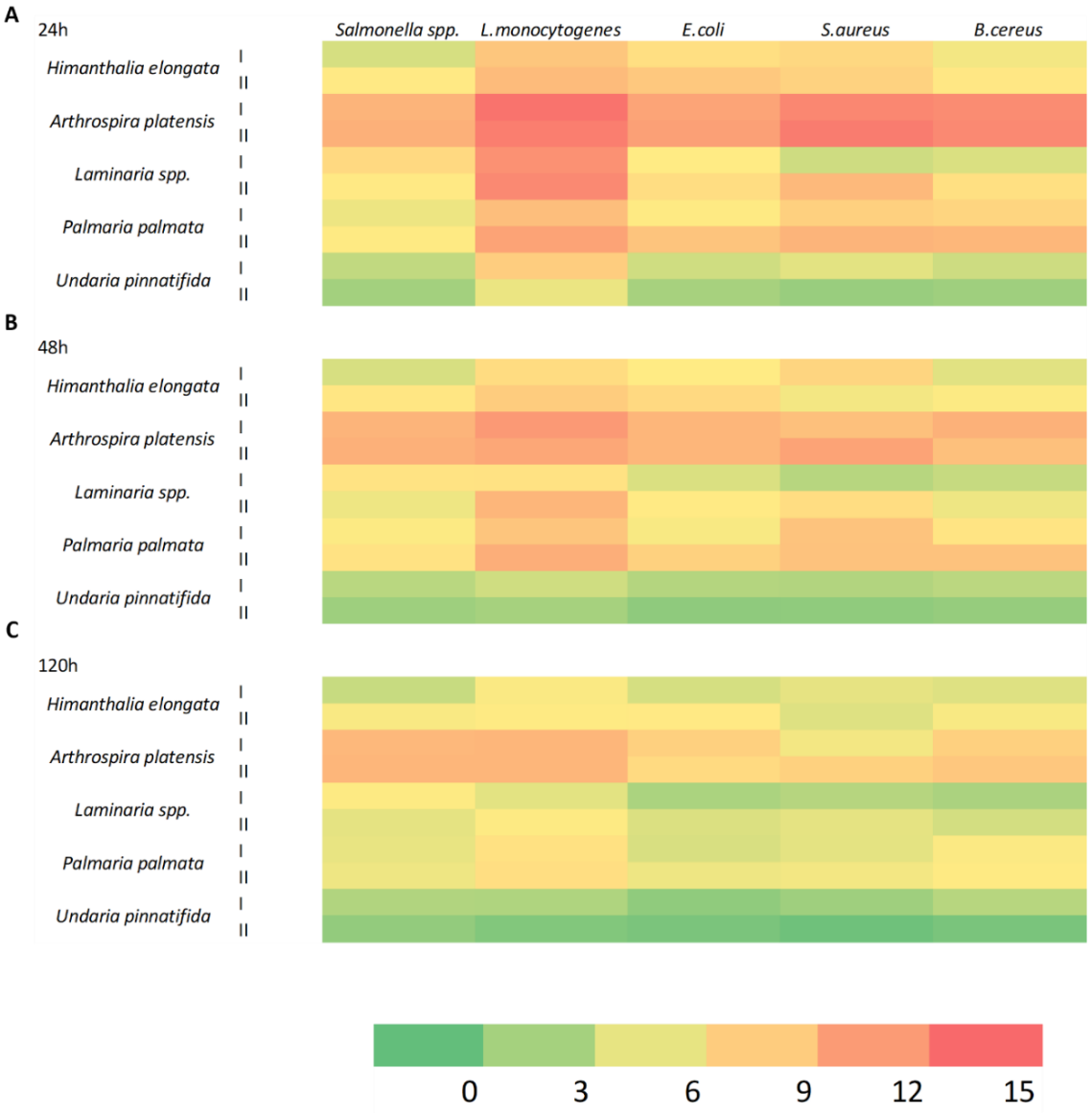

Figure 1. Antimicrobial activity of algae extracts towards *Salmonella* spp., *L. monocytogenes*, *E. coli*, *S. aureus* and *B. cereus* represented by heatmap. A scale ranging from a minimum of 0 mm (green) to a maximum of 15 mm (red) was used to represent the size of inhibition diameter calculated, as average values of triplicates, after 24 h (**A**), 48 h (**B**), and 120 h (**C**) of incubation at 37 °C. I and II represent two different extracts referred to the same algae species.

Overall, extracts obtained from *A. platensis* resulted as the most effective. By comparing the antimicrobial activity after 24 and 48 hours (Figure 1A and 1B) it was observed an overall loss of effectiveness during incubation time of almost all the extracts. After 120 hours (Figure 1C) some of them, in particular those deriving from *U. pinnatifida*, were not active toward the considered food pathogens anymore.

In the case of *H. elongata, Laminaria* spp., *P. palmata* and *U. pinnatifida*, according to the statistical model, the antibacterial activity varied among extracts I and II obtained by the same seaweed species as according to incubation time and to the pathogenic species (P value < 0.05). Only in the case of *A. platensis* the extract antibacterial activity didn't vary significantly (P value > 0.05).

In Figure 2 the activity of each extract against the tested foodborne pathogens is reported.

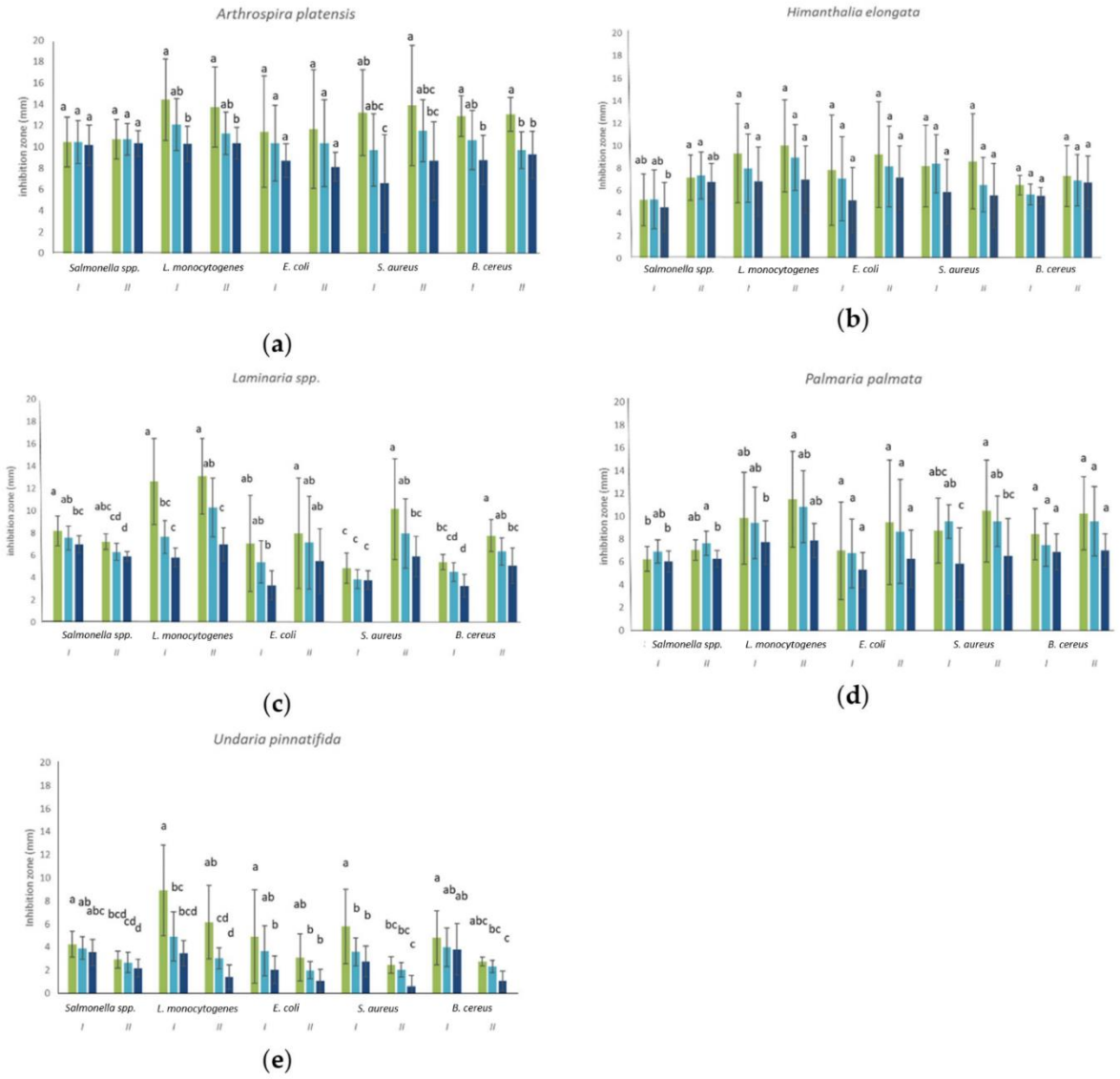

Figure 2. Antimicrobial activity of algae extracts along time: 24 h (Green line), 48 h (light blue line) and 120 h (Blue line). (**a**) *Arthrospira platensis* extracts; (**b**) *Himanthalia elongata* extracts. (**c**) *Laminaria spp.* extracts. (**d**) *Palmaria palmata* extracts. (**e**) *Undaria pinnatifida* extracts. The means with different letters are significantly different ($p < 0.05$).

The high standard deviation values underlined the heterogeneous efficacy of the extracts inside the same population. The inhibition of *Salmonella* spp. by both *A. platensis* extracts did not significantly differ, and the effect remained constant during time (Figure 2a). The same behavior was observed regarding *E. coli*. In the case of *L. monocytogenes*, both extracts led to a significant decrease in the inhibition zone along time, resulting in a loss of effectiveness ($p < 0.05$). The behavior of *S. aureus* and *B. cereus* was very similar to *L. monocytogenes* when *A. platensis* extracts were used. However, extracts I and II differently exerted inhibitory effects on *S. aureus* and, in particular, a significant difference in the first 24 hours occurred ($p < 0.05$). The inhibitory zone of *H. elongata* extracts towards *Salmonella* spp. remained constant after 24 and 48 hours of incubation, then decreased after 120 hours, highlighting a loss of effectiveness in the last three days of observation (Figure 2b). Besides, a significant difference between the activity of extracts was highlighted ($p < 0.05$). The antibacterial activity of *H. elongata* extracts against all the other food pathogens (*L. monocytogenes, E. coli, S. aureus and B. cereus*) didn't show any significant differences either as a function of incubation time or according to the origin of the sample. The antimicrobial activity of *Laminaria* spp. extracts was particularly high towards *L. monocytogenes*, but it decreased significantly over time ($p < 0.05$) (Figure 2c). When referred to *Salmonella spp*. and *B. cereus* it could be noticed also a significant difference between the effectiveness of the two extracts ($p < 0.05$). The inhibition of *L. monocytogenes* was different considering extracts I and II at 48 hours. In Figure 2c it can also be observed that the inhibition of *E. coli* remained constant in extract I for the first 48 hours, and then decreased. The two extracts proved a significantly different activity towards *S. aureus* ($p < 0.05$). The inhibition of *Salmonella* spp. due to *P. palmata* extracts I and II was constant in the first 48 hours (Figure 2d). Different was the *L. monocytogenes* case: the inhibitory zone remained constant in the first 48 hours for extract I and then decreased, while in the presence of extract II after a halo first degrowth at 24 hours, it remained stable. The inhibition of *P. palmata* extracts against *E. coli* and *B. cereus* was constant along time with any significant difference.

Extract I behaved towards *S. aureus* in a similar way to *Salmonella*. *U. pinnatifida* extracts proved the lower antimicrobial activity compared to all the tested extracts (Figure 2e).

Antimicrobial activity in situ

In order to evaluate the antimicrobial effect of an algal extract *in situ*, a contamination simulation with a mixture of *L. monocytogenes* strains was performed on salmon tartare chosen as a food model. For this purpose, *A. platensis* extract II, which showed the highest antimicrobial activity *in vitro*, was selected as a useful ingredient for the improvement of microbiological food safety. Two concentrations of extract were considered in the formulation: 0.45 and 0.9% (v/w).

In the control sample (C), without extract addition, *L. monocytogenes*, artificially inoculated in order to reach an initial contamination of 4 Log cfu/g in the product, remained at the inoculum level during the first 24 hours and then rapidly grew by two logarithms (Figure 3). So a completely different behavior of the pathogen in the presence of 0.45% and 0.9% (v/w) of the extract was evident (Figure 3). Indeed, in these samples the contamination was maintained at the inoculum level during 4 days of storage, proving a bacteriostatic activity of the extract.

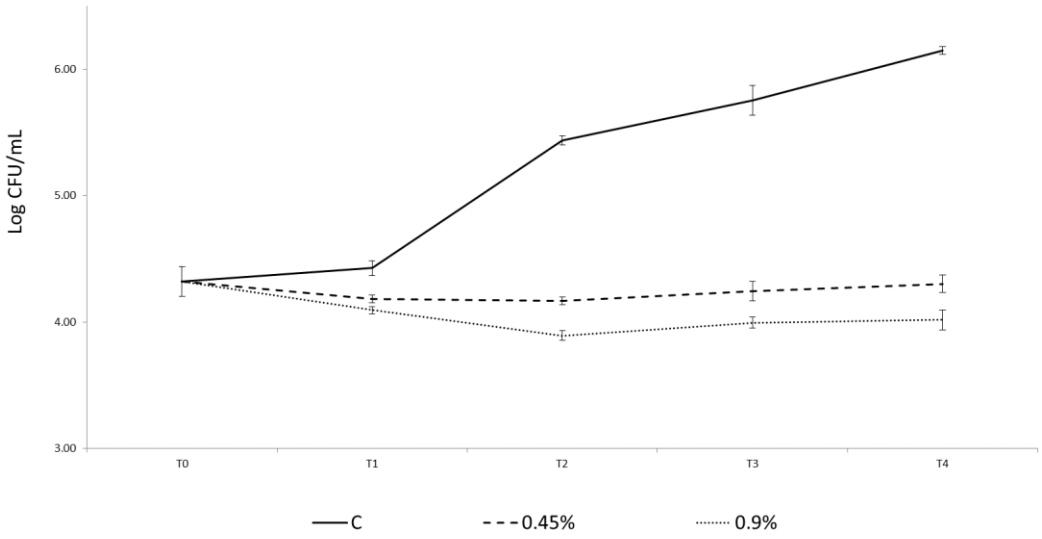

Figure 3. Behavior of *Listeria monocytogenes* during a 4 days' shelf life at refrigeration temperatures in salmon tartare without extract addition (C) (—), with 0.45% (v/w) (---) and 0.9% (v/w) (·····) of *A. platensis* extract.

Phenolic content

The amount of total phenolic compounds of the different algae extracts was reported in Table 1. Among the samples tested, *U. pinnatifida* presented the lowest phenolic content, while the highest was found in *H. elongata* extracts. Regarding this latter, a significant higher phenolic content was found in extract II respect to all the other considered samples (18.79 ± 1.90 mg GAE/g). Extracts obtained from the red seaweed specie *P. palmata* and the cyanobacterium *A. platensis* showed comparable phenolic content, and resulted the second group in terms of phenol abundance, with values of 2.45 ± 0.06 – 4.56 ± 0.41 mg GAE/g and 3.18 ± 0.48 – 3.40 ± 0.42 mg GAE/g respectively. Significant lower quantities of phenols were found in *Laminaria* spp. and in *U. pinnatifida* extracts, both pertaining to the group of brown algae. In particular, *U. pinnatifida* contained 10 times less phenolic compounds in respect to all the other seaweed species considered, with values of 0.15 ± 0.01 mg GAE/g (Table 1).

Table 1. Total phenolic content of seaweed extracts expressed as mg GAE/g. Stars indicate a significant difference ($p<0.05$) between samples pertaining to the same specie (T-test), while letters refer to differences and/or analogies among different species (one way Anova).

Algae species	TPC (mg GAE/g)
Himanthalia elongata I	$1.52^{d} \pm 0.38$
Himanthalia elongata II	$18.79^{a} \pm 1.90$*
Arthrospira platensis I	$3.18^{bc} \pm 0.48$
Arthrospira platensis II	$3.40^{bc} \pm 0.42$
Laminaria spp. I	$1.15^{d} \pm 0.25$
Laminaria spp. II	$1.55^{d} \pm 0.92$
Palmaria palmata I	$4.56^{b} \pm 0.41$*
Palmaria palmata II	$2.45^{c} \pm 0.06$
Undaria pinnatifida I	$0.16^{e} \pm 0.01$
Undaria pinnatifida II	$0.14^{e} \pm 0.01$

Discussion

Thanks to the good nutritional value and high bioactive compound content, the use of seaweeds in food and feed formulation is everyday growing (Ak et al., 2016; Swinscoe et al., 2020). Seaweeds and derived compounds have proved a series of bioactivities that could significantly enhance the use of these organisms in food formulation in order to guarantee a healthy diet for consumers (Peñalver et al., 2020). In particular, antioxidant, anti-inflammatory, anti-cancer, and anti-diabetic properties are attributed to compounds extracted from seaweeds and microalgae. Favoring the requests of corporations and consumers for clean label products, researchers are every day more interested in the compounds extracted from plants and, among these, to new antimicrobial agents (Ríos & Recio, 2005). In this context, currently, seaweeds and algae may represent a promising resource, even if many of the extracts obtained since now are derived by species not recognized as edible. So, the possibility to obtain novel antimicrobial compounds from seaweeds and microalgae already approved by EFSA as food enhance their applications in food formulations. Furthermore, these extracts could not only work as antimicrobial compounds but also as a source of flavors. Different works have focused on the *in vitro* antimicrobial activity of several algae extracts against *Salmonella* spp. (Cox et al., 2014; Elshouny et al., 2017; Moubayed et al., 2017; Navarro et al., 2017; Rao et al., 2010; Sivakumar & Santhanam, 2011; Vinay Kumar, 2011), *L. monocytogenes* (Cox et al., 2010; Kim et al., 2018; Martelli, Favari, et al., 2020; Nshimiyumukiza, Ossiniel et al., 2015), *E. coli* (Maadane et al., 2017; Mendiola et al., 2007; Radhika et al., 2014; Shaieb et al., 2014), *S. aureus* (El Shafay et al., 2016; Kadam et al., 2015; S. Santoyo et al., 2009; Susana Santoyo et al., 2006) and *B. cereus* (Velichkova et al., 2018), demonstrating different effectiveness depending on the algae species as on the extraction process. Others reported antimicrobial activity referred to the algae species tested in the present study, even if a different extraction process was followed. The extraction protocol used in this study proved a good antimicrobial activity, contrary to other studies stating that the use of a methanolic solution or dichloromethane are better to extract antimicrobial compounds from seaweeds

(Bansemir et al., 2006). Moreover, at the best of our knowledge, the efficacy of the extracts *in situ* by microbiological challenge test has not yet been tested. Numerous macro and microalgae have been discussed for their content in bioactive compounds (Pina-Pérez et al., 2017; Sivakumar & Santhanam, 2011) among which polysaccharides, proteins and amino acids, polyunsaturated fatty acids (PUFAs) and antioxidants (polyphenols, carotenoids and flavonoids) mainly demonstrated antimicrobial activity (Al-Saif et al., 2014; Sanmukh, 2014; Stabili et al., 2014). This last group of secondary metabolites has been associated to a broad spectrum of *in vitro* antibacterial activity, specifically against *Bacillus* spp. and *S. aureus*. Phlorotannins are the most important phenolic compounds characteristic of brown seaweeds. Other phenolic compounds, such as bromophenols, flavonoids, phenolic acids, anthraquinones, coumarins, rutin, quercetin, and kaempferol, present in different algae species, also demonstrated antimicrobial potential (Al-Saif et al., 2014; Pina-Pérez et al., 2017; Rodríguez-Bernaldo de Quirós et al., 2010). The antimicrobial activity of algae against both Gram-positive and Gram-negative bacteria was also traced back to fatty acids (Vello et al., 2014) that represent a defense against bacteria, viruses and protozoans (Leflaive & Ten-Hage, 2009).

Each type of algae, because of various classes and quantity of compounds, has a different inhibitory activity on diverse classes of bacteria (Mishra, 2018). In this study, all the algae species considered (*A. platensis, H. elongata, Laminaria* spp., *P. palmata* and *U. pinnatifida*) have generally shown a good antimicrobial activity (Figure 2). Anyway, differences in the efficacy of the extracts against Gram-positive and Gram-negative bacteria have arisen. The cyanobacterium extracts proved the highest antimicrobial activity against Gram-positive bacteria (*L. monocytogenes, S. aureus* and *B.cereus*) as already demonstrated (El-Sheekh et al., 2014), but an interesting effect was also observed on Gram-negative bacteria. *H. elongata* extracts were highly effective against the Gram-positive bacteria *L. monocytogenes* but strong differences between Gram-positive and Gram-negative weren't noticed. Similarly, another *Phaeopyceae, U. pinnatifida*, proved the highest efficacy against *L. monocytogenes. Laminaria* spp extracts mainly inhibited two Gram-positive bacteria, *L. monocytogenes and S. aureus.* A higher antimicrobial activity on Gram-positive respect to Gram-negative ones was also observed on

the *Rhodophyta P. palmata* extracts. These results confirm what is stated by other studies, in which the highest antimicrobial activity of seaweed extracts is found against Gram-positive bacteria (El Shafay et al., 2016; Stirk et al., 2007). *A. platensis* is studied as a valuable source of antimicrobial, antiviral and antioxidant compounds but its activity is really variable and dependent on the extractive solvent (LewisOscar et al., 2017; Mala et al., 2009; Rania & Hala, 2008). This cyanobacterium, commercially known as Spirulina, is used in food formulations or as a food supplement (Batista et al., 2017; Martelli, Alinovi, et al., 2020) as in healthy products (Golmakani et al., 2019; Majdoub et al., 2009; Mohammadi-Gouraji et al., 2019; Yamaguchi et al., 2019), because of its composition rich in proteins and small peptides. The antimicrobial potential of small peptides is well known and the activity of several algae is related to their high protein content. Also the great antimicrobial activity of *A. platensis* extracts I and II could definitively be linked to the large amount of proteins and small peptides of this cyanobacterium (Figure 2a). The application of an *A. platensis* extract as a preservative, as far as the authors are aware, was never tested *in situ* and could find applications for instance in seafood products due to its sea-like smell (Figure 3). In particular, these products are frequently subjected to *L. monocytogenes* contaminations, because of its psychrotrophic behavior. Just in the first 6 months of 2020, RASFF (The Rapid Alert System for Food and Feed) has issued 7 warnings regarding *L. monocytogenes* contaminating salmon based products (RASFF 2020.0525, 2020.0683, 2020.0876, 2020.1049, 2020.1631, 2020.2276 and 2020.2409). The behavior and the growth ability of this microorganism in salmon based food is widely studied (Beaufort et al., 2007). Salmon is generally preserved under pressure packaging or CO_2 atmosphere packaging, but the use of natural origin preservatives is a well-considered possibility (Rørvik, 2000). Recently, the efficacy of essential oils (EOs) obtained by plants and fruits has been evaluated with interesting results in terms of MIC (Minimal Inhibitory Concentration) and MBC (Minimal Bactericidal Concentration) referred to *L. monocytogenes* strains isolated from salmon (Pedrós-Garrido et al., 2020). Though, from a sensory point of view, the use of EOs wasn't very compatible with salmon as they masked any fish or seafood odor and flavor. For that reason, the use of an alternative with a sea-like flavor could be really applicable apart from being effective. Indeed, an *A. platensis* extract

obtained by using the extraction protocol of the present study was tested against *Listeria innocua* and a MIC of 0.20% and a MBC of 0.30% was found, proving a really good efficacy (Bancalari et al., 2020). *A. platensis* extracts have already been tested against several food pathogenic bacteria. Tough, those obtained in this work proved a higher antimicrobial activity against *B. cereus* if compared to other work (Shaieb et al., 2014). In fact, both ethanolic and aqueous extract didn't exert any inhibition on the pathogenic bacteria.

Phaeophyceae is described in the literature as the class of seaweeds with the most relevant antimicrobial activity. Promising groups of polysaccharides, such as Fucoidans and other sulfated polysaccharides extracted from *Phaeophyta*, have demonstrated an antimicrobial potential. In particular, sulfated polysaccharides and, among them, alginates, fucoidans and laminarian, inhibited the growth of *E. coli* and *S. aureus* (de Jesus Raposo et al., 2015). Fucoidans have proven not only antimicrobial activity against food pathogenic bacteria but also antiviral, antioxidant, immunomodulatory, anticoagulant, antithrombotic, antitumor and anti-inflammatory activities (Li et al., 2008; Marudhupandi & Kumar, 2013). In this study three species belonging to *Phaeophyceae* have been tested. The antimicrobial activity of *H. elongata* extract has been previously studied with similar results (Martelli, Favari, et al., 2020; Plaza et al., 2010; Rajauria et al., 2013) while the extracts obtained in the present work have been more effective against *L. monocytogenes* and *Salmonella* spp. than those obtained by Rajauaria and colleagues (Rajauria et al., 2013) (Figure 2b). In fact, the extract produced an inhibition halo of 9.95 mm, while the extract obtained in the present work led to a halo of more than 10 mm. Also the genus *Laminaria*, characterized by *Phaeophyceae* of large dimensions, proved a good antimicrobial potential towards *L. monocytogenes, S. aureus* and *Salmonella ebony* (Cox et al., 2010; Kadam et al., 2015), even if a different extraction protocol was used. In fact, the methanolic *Laminaria* spp. and *H. elongata* extracts obtained by Cox and colleagues (2010) (Cox et al., 2010), have proved a very valuable antimicrobial activity against the food pathogenic bacteria tested. These algal extracts were able to inhibit the growth of *L. monocytogenes* of 100%. Indeed, the food-grade extraction method applied in the present work was never tested on this species and led to interesting results (Figure 2c). Otherwise, *U. pinnatifida* didn't reveal a satisfactory antimicrobial activity and this could be due to the

extraction process (Figure 2e). Its antimicrobial activity has been connected to the essential oils content (Patra et al., 2017) but in this case a polar solvent, water and ethanol, was in fact applied.

P. palmata is a red seaweed characteristic of the Atlantic Ocean and it also demonstrated good antimicrobial activity against *L. monocytogenes*, *Salmonella ebony, Enterococcus faecalis* and *Pseudomonas aeruginosa* (Cox et al., 2010). No information about the antimicrobial effect of *P. palmata* extracts on *E. coli, B. cereus* or *S. aureus* are reported in the literature (Figure 2d). It can be supposed that the extraction procedure applied in this study led to obtain extracts rich in polyphenolic compounds. The tested algae and the extraction process can also indicate that the antimicrobial activity could be due to small peptides, polysaccharides and phenolic compounds that act synergistically. Indeed, phenolic content appears not to be the main component of the activity we observed, though some relations could be noticed. *H. elongata* extract II, having the highest phenolic content (18.79 ± 1.90 mg GAE/g), had a significantly higher antimicrobial activity ($p < 0.05$) if compared to *H. elongata* extract I (1.52 ± 0.38 mg GAE/g) towards *Salmonella* spp., presenting a difference in inhibition halo higher than 2 mm (Figure 2b). *A. platensis* extracts didn't prove a significant difference ($p > 0.05$) on the antimicrobial activity (Figure 2a) and neither in the phenolic content (Table 1). Regarding *Laminaria* extracts the differences on the antimicrobial activity can't be related to the phenolic content that did not differ (Figure 2c). Otherwise, the different effects of *P. palmata's* extracts towards *L. monocytogenes, Salmonella spp.* and *S. aureus* ($p < 0.05$) (Figure 2d) might be linked to their phenolic content which significantly differs ($p < 0.05$) (4.56 ± 0.41 and 2.45 ± 0.06 mg GAE/g respectively for extract I and II) (Table 1). Finally, the almost absence of phenolic compounds in *U. pinnatifida* extracts corresponded to low antimicrobial activity of these samples (Figure 2e). The protocol used to obtain the extracts doesn't involve the use of high temperature, for that reason, the phenolic content of the matrix shouldn't be affected during the extraction process (Dordevic et al., 2020).

The results obtained showed that the phenolic content of seaweed seems to be related to the species, as also reported in previous studies. Cox and colleagues (2010) analyzed the phenolic content in seaweed samples belonging to different species, obtaining values ranging from

about 37 mg GAE/g for *L. digitata* to 151 mg GAE/g for *H. elongata*. In that case, the authors speculated that total phenolic content could vary among brown, red or green seaweeds. Brown seaweeds have been described as rich in polyphenols (Chandini et al., 2008) and *H. elongata* was found as the species with the highest total phenolic amount (Rodríguez-Bernaldo de Quirós et al., 2010), as reported also in the present study. In addition to that, it has to be taken into account that the concentration of phenols may present a wide range of variability in this kind of matrices. It was indeed demonstrated that the total phenolic amount was strongly influenced by several factors as seasonality, temperature, light intensity, and water salinity (Marinho et al., 2019). This may explain why *H. elongata* extracts I and II showed a significant different phenolic content (Table 1). Even if to a less extent, a similar trend was observed in the case of the red seaweed *P. palmata*: extract I presented a significant higher phenolic content (4.56 ± 0.41 mg GAE/g) compared to extract II in which this concentration resulted about the half (2.45 ± 0.06 mg GAE/g) ($p < 0.05$). Taking onto account that samples were provided by two different suppliers, it can be supposed that considered seaweed pertaining to *H. elongata* and *P. palmata* have been subjected to diverse stabilization treatments before commercialization or have been collected from different area and/or in different seasons. Concerning *A. platensis*, *Laminaria* spp. and *U. pinnatifida* no differences were found between total phenolic amount of the two extract inside the specie, but the content resulted significantly different among the species. In particular, phenolic content of *A. platensis* extracts of 3.29 ± 0.45 mg GAE/g was comparable with data recently obtained by da Silva and colleagues (2017) (da Silva et al., 2017) who reported a total amount of 3.32 ± 0.08 mg GAE/g in samples of *A. platensis* extracted with high pressure/temperature extraction (HPTE) method. Total phenolic content of *Laminaria* spp. calculated in the present work (I:1.15 ± 0.25 and II:1.55 ± 0.92 mg GAE/g) (Table 1) resulted in line with data reported by Rodríguez-Bernaldo de Quirós et al. (2010), that showed an amount of 1.3 ± 0.6 – 1.4 ± 1.0 expressed as mg Phloroglucinol/g. Conversely, the same authors reported a value of 6.0 ± 2.2 mg Phloroglucinol/g for *U. pinnatifida*, while lower amounts were found in this study for the same variety (0.15 ± 0.01 mg GAE/g).

Conclusions

In order to ensure microbiological food safety and improve quality according to consumer preferences for "clean labels" products, food corporations are looking for new compounds to be used as an alternative solution to traditional preservatives. This study highlighted that: (i) extracts obtained from different species of edible algae exerted antimicrobial activity towards the main foodborne pathogens such as *Salmonella spp., L. monocytogenes, E. coli, S. aureus,* and *B. cereus*; (ii) *A. platensis* extract showed an interesting antibacterial activity *in situ* towards *L. monocytogenes*; (iii) a great variability in the phenolic content of the tested seaweed was noticed, and this content was not always related to the antimicrobial activity observed, emphasizing the possibility of a synergic effect involving other compounds. In this context, a deep investigation of extract composition in order to evaluate the effect of compounds responsible for the antimicrobial activity will surely be of great interest. Overall, good effectiveness of algae extracts was confirmed even if results suggested that antibacterial activity can be influenced by various factors: it varies according to algal species, producers, contact time and foodborne pathogens. In particular, the growth inhibitory efficacy toward *L. monocytogenes* of *A. platensis* extract, highlighted by the microbiological challenge test, could open up perspectives of applications for food preservation. Indeed, antimicrobial extract might work as a food preservative and antioxidant not only in fish products but also in other products that need to guarantee safety criteria and to preserve the freshness by slowing down microbial growth.

References

Ak, B., Avşaroğlu, E., Işık, O., Özyurt, G., Kafkas, E., & Uslu, L. (2016). Nutritional and Physicochemical Characteristics of Bread Enriched with Microalgae *Spirulina platensis*. *Int. Journal of Engineering Research and Application*, *6*(12), 9.

Alghazeer, R., Whida, F., Abduelrhman, E., Gammoudi, F., & Azwai, S. (2013). Screening of antibacterial activity in marine green, red and brown macroalgae from the western coast of Libya. *Natural Science*, *5*(1), 7–14. https://doi.org/10.4236/ns.2013.51002

Al-Saif, S. S. A., Abdel-Raouf, N., El-Wazanani, H. A., & Aref, I. A. (2014). Antibacterial substances from marine algae isolated from Jeddah coast of Red sea, Saudi Arabia. *Saudi Journal of Biological Sciences*, *21*(1), 57–64. https://doi.org/10.1016/j.sjbs.2013.06.001

Andrade, L. M. (2018). *Chlorella* and *Spirulina* Microalgae as Sources of Functional Foods, Nutraceuticals, and Food Supplements; an Overview. *MOJ Food Processing & Technology*, *6*(1), 45–58. https://doi.org/10.15406/mojfpt.2018.06.00144

Bancalari, E., Martelli, F., Bernini, V., Neviani, E., & Gatti, M. (2020). Bacteriostatic or bactericidal? Impedometric measurements to test the antimicrobial activity of *Arthrospira platensis* extract. *Food Control*, 107380. https://doi.org/10.1016/j.foodcont.2020.107380

Bansemir, A., Blume, M., Schröder, S., & Lindequist, U. (2006). Screening of cultivated seaweeds for antibacterial activity against fish pathogenic bacteria. *Aquaculture*, *252*(1), 79–84. https://doi.org/10.1016/j.aquaculture.2005.11.051

Batista, A. P., Niccolai, A., Fradinho, P., Fragoso, S., Bursic, I., Rodolfi, L., Biondi, N., Tredici, M. R., Sousa, I., & Raymundo, A. (2017). Microalgae biomass as an alternative ingredient in cookies: Sensory, physical and chemical properties, antioxidant activity and in vitro digestibility. *Algal Research*, *26*, 161–171. https://doi.org/10.1016/j.algal.2017.07.017

Beaufort, A., Rudelle, S., Gnanou-Besse, N., Toquin, M. T., Kerouanton, A., Bergis, H., Salvat, G., & Cornu, M. (2007). Prevalence and growth of *Listeria monocytogenes* in naturally

contaminated cold-smoked salmon. *Letters in Applied Microbiology*, *44*(4), 406–411. https://doi.org/10.1111/j.1472-765X.2006.02096.x

Bernini, V., Bottari, B., Dalzini, E., Sgarbi, E., Lazzi, C., Neviani, E., & Gatti, M. (2013). The presence, genetic diversity and behaviour of *Listeria monocytogenes* in blue-veined cheese rinds during the shelf life. *Food Control*, *34*(2), 323–330. https://doi.org/10.1016/j.foodcont.2013.04.015

Buschmann, A. H., Camus, C., Infante, J., Neori, A., Israel, Á., Hernández-González, M. C., Pereda, S. V., Gomez-Pinchetti, J. L., Golberg, A., Tadmor-Shalev, N., & Critchley, A. T. (2017). Seaweed production: Overview of the global state of exploitation, farming and emerging research activity. *European Journal of Phycology*, *52*(4), 391–406. https://doi.org/10.1080/09670262.2017.1365175

Camacho, F., Macedo, A., & Malcata, F. (2019). Potential Industrial Applications and Commercialization of Microalgae in the Functional Food and Feed Industries: A Short Review. *Marine Drugs*, *17*(6), 312. https://doi.org/10.3390/md17060312

Chan, Y. C., & Wiedmann, M. (2008). Physiology and Genetics of *Listeria Monocytogenes* Survival and Growth at Cold Temperatures. *Critical Reviews in Food Science and Nutrition*, *49*(3), 237–253. https://doi.org/10.1080/10408390701856272

Chandini, S. K., Ganesan, P., & Bhaskar, N. (2008). In vitro antioxidant activities of three selected brown seaweeds of India. *Food Chemistry*, *107*(2), 707–713. https://doi.org/10.1016/j.foodchem.2007.08.081

Codex Alimentarius. (2002). Report of the thirty-fourth session of the Codex Committe on food hygiene (ALINORM 03/13). *Report of the Thirty-Fourth Session of the Codex Committe on Food Hygiene (ALINORM 03/13).*

Cox, S., Abu-Ghannam, N., & Gupta, S. (2010). An Assessment of the Antioxidant and Antimicrobial Activity of Six Species of Edible Irish Seaweeds. *International Food Research Journal*, *17*, 205–220. https://doi.org/10.21427/d7hc92

Cox, S., Hamilton Turley, G., Rajauria, G., Abu-Ghannam, N., & Jaiswal, A. K. (2014). Antioxidant potential and antimicrobial efficacy of seaweed (*Himanthalia elongata*) extract in model food systems. *Journal of Applied Phycology*, *26*(4), 1823–1831. https://doi.org/10.1007/s10811-013-0215-0

Da Silva, M. F., Casazza, A. A., Ferrari, P. F., Aliakbarian, B., Converti, A., Bezerra, R. P., Porto, A. L. F., & Perego, P. (2017). Recovery of phenolic compounds of food concern from *Arthrospira platensis* by green extraction techniques. *Algal Research*, *25*, 391–401. https://doi.org/10.1016/j.algal.2017.05.027

de Jesus Raposo, M., de Morais, A., & de Morais, R. (2015). Marine Polysaccharides from Algae with Potential Biomedical Applications. *Marine Drugs*, *13*(5), 2967–3028. https://doi.org/10.3390/md13052967

Dordevic, D., Kushkevych, I., Jancikova, S., Zeljkovic, S. C., Zdarsky, M., & Hodulova, L. (2020). Modeling the effect of heat treatment on fatty acid composition in home-made olive oil preparations. *Open Life Sciences*, *15*(1), 606–618. https://doi.org/10.1515/biol-2020-0064

El Shafay, S. M., Ali, S. S., & El-Sheekh, M. M. (2016). Antimicrobial activity of some seaweeds species from Red sea, against multidrug resistant bacteria. *The Egyptian Journal of Aquatic Research*, *42*(1), 65–74. https://doi.org/10.1016/j.ejar.2015.11.006

El-Sheekh, M. M., Daboor, S. M., Swelim, M. A., & Mohamed, S. (2014). Production and characterization of antimicrobial active substance from *Spirulina platensis*. *Iranian Journal of Microbiology, 6*(2), 8.

Elshouny, W. A. E.-F., El-Sheekh, M. M., Sabae, S. Z., Khalil, M. A., & Badr, H. M. (2017). Antimicrobial activity of *Spirulina platensis* against aquatic bacterial isolates. *Journal of Microbiology, Biotechnology and Food Sciences*, *6*(5), 1203–1208. https://doi.org/10.15414/jmbfs.2017.6.5.1203-1208

Golmakani, M.-T., Soleimanian-Zad, S., Alavi, N., Nazari, E., & Eskandari, M. H. (2019). Effect of Spirulina (*Arthrospira platensis*) powder on probiotic bacteriologically acidified feta-

type cheese. *Journal of Applied Phycology*, *31*(2), 1085–1094. https://doi.org/10.1007/s10811-018-1611-2

Gupta, S., & Abu-Ghannam, N. (2011). Recent developments in the application of seaweeds or seaweed extracts as a means for enhancing the safety and quality attributes of foods. *Innovative Food Science & Emerging Technologies*, *12*(4), 600–609. https://doi.org/10.1016/j.ifset.2011.07.004

Holdt, S. L., & Kraan, S. (2011). Bioactive compounds in seaweed: Functional food applications and legislation. *Journal of Applied Phycology*, *23*(3), 543–597. https://doi.org/10.1007/s10811-010-9632-5

Hongayo, M. C., Larino, R. C., & Malingin, D. L. (2012). Antibacterial and Antioxidant Effects of Brown Alga *Padina australis* Hauck Crude Extract. *IAMURE International Journal of Science and Clinical Laboratory*, *2*(1), 13. https://doi.org/10.7718/iamure.ijscl.v2i1.388

Kadam, S., O'Donnell, C., Rai, D., Hossain, M., Burgess, C., Walsh, D., & Tiwari, B. (2015). Laminarin from Irish Brown Seaweeds *Ascophyllum nodosum* and *Laminaria hyperborea*: Ultrasound Assisted Extraction, Characterization and Bioactivity. *Marine Drugs*, *13*(7), 4270–4280. https://doi.org/10.3390/md13074270

Kim, H.-J., Dasagrandhi, C., Kim, S.-H., Kim, B.-G., Eom, S.-H., & Kim, Y.-M. (2018). In Vitro Antibacterial Activity of Phlorotannins from Edible Brown Algae, *Eisenia bicyclis* Against Streptomycin-Resistant *Listeria monocytogenes*. *Indian Journal of Microbiology*, *58*(1), 105–108. https://doi.org/10.1007/s12088-017-0693-x

Leflaive, J., & Ten-Hage, L. (2009). Chemical interactions in diatoms: Role of polyunsaturated aldehydes and precursors. *New Phytologist*, *184*(4), 794–805. https://doi.org/10.1111/j.1469-8137.2009.03033.x

LewisOscar, F., Nithya, C., Bakkiyaraj, D., Arunkumar, M., Alharbi, N. S., & Thajuddin, N. (2017). Biofilm Inhibitory Effect of *Spirulina platensis* Extracts on Bacteria of Clinical

Significance. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *87*(2), 537–544. https://doi.org/10.1007/s40011-015-0623-9

Li, B., Lu, F., Wei, X., & Zhao, R. (2008). Fucoidan: Structure and Bioactivity. *Molecules*, *13*(8), 1671–1695. https://doi.org/10.3390/molecules13081671

Maadane, A., Merghoub, N., El Mernissi, N., Ainane, T., & Amzazi, S. (2017). Antimicrobial activity of marine microalgae isolated from moroccan coastlines. *Journal of Microbiology, Biotechnology and Food Sciences*, *6*(6), 1257–1260. https://doi.org/10.15414/jmbfs.2017.6.6.1257-1260

Majdoub, H., Mansour, M. B., Chaubet, F., Roudesli, M. S., & Maaroufi, R. M. (2009). Anticoagulant activity of a sulfated polysaccharide from the green alga *Arthrospira platensis*. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *1790*(10), 1377–1381. https://doi.org/10.1016/j.bbagen.2009.07.013

Mala, R., Sarojini, M., Saravanababu, S., & Umadevi, G. (2009). Screening for antimicrobial activity of crude extracts of *Spirulina platensis*. *Journal of Cell and Tissue Research*, *9*(3), 1951–1955.

Marinho, G. S., Sørensen, A.-D. M., Safafar, H., Pedersen, A. H., & Holdt, S. L. (2019). Antioxidant content and activity of the seaweed *Saccharina latissima*: A seasonal perspective. *Journal of Applied Phycology*, *31*(2), 1343–1354. https://doi.org/10.1007/s10811-018-1650-8

Martelli, F., Alinovi, M., Bernini, V., Gatti, M., & Bancalari, E. (2020). *Arthrospira platensis* as Natural Fermentation Booster for Milk and Soy Fermented Beverages. *Foods*, *9*(350), 15. https://doi.org/doi:10.3390/foods9030350

Martelli, F., Favari, C., Mena, P., Guazzetti, S., Ricci, A., Rio, D. D., Lazzi, C., Neviani, E., & Bernini, V. (2020). Antimicrobial and Fermentation Potential of *Himanthalia elongata* in Food Applications. *Microorganisms*, *8*(2), 15. https://doi.org/doi:10.3390/microorganisms8020248

Marudhupandi, T., & Kumar, T. T. A. (2013). Antibacterial effect of fucoidan from *Sargassum wightii* against the chosen human bacterial pathogens. *International Current Pharmaceutical Journal*, *2*(10), 156–158. https://doi.org/10.3329/icpj.v2i10.16408

Mendiola, J. A., Jaime, L., Santoyo, S., Reglero, G., Cifuentes, A., Ibañez, E., & Señoráns, F. J. (2007). Screening of functional compounds in supercritical fluid extracts from *Spirulina platensis*. *Food Chemistry*, *102*(4), 1357–1367. https://doi.org/10.1016/j.foodchem.2006.06.068

Mishra, A. K. (2018). *Sargassum*, *Gracilaria* and *Ulva* Exhibit Positive Antimicrobial Activity against Human Pathogens. *OALib*, *5*(e4258), 1–12. https://doi.org/10.4236/oalib.1104258

Mohammadi-Gouraji, E., Soleimanian-Zad, S., & Ghiaci, M. (2019). Phycocyanin-enriched yogurt and its antibacterial and physicochemical properties during 21 days of storage. *LWT*, *102*, 230–236. https://doi.org/10.1016/j.lwt.2018.09.057

Moubayed, N. M. S., Al Houri, H. J., Al Khulaifi, M. M., & Al Farraj, D. A. (2017). Antimicrobial, antioxidant properties and chemical composition of seaweeds collected from Saudi Arabia (Red Sea and Arabian Gulf). *Saudi Journal of Biological Sciences*, *24*(1), 162–169. https://doi.org/10.1016/j.sjbs.2016.05.018

Navarro, F., Forján, E., Vázquez, M., Toimil, A., Montero, Z., Ruiz-Domínguez, M. del C., Garbayo, I., Castaño, M. Á., Vílchez, C., & Vega, J. M. (2017). Antimicrobial activity of the acidophilic eukaryotic microalga *Coccomyxa onubensis*: Antimicrobial activity of *Coccomyxa*. *Phycological Research*, *65*(1), 38–43. https://doi.org/10.1111/pre.12158

Niccolai, A., Shannon, E., Abu-Ghannam, N., Biondi, N., Rodolfi, L., & Tredici, M. R. (2019). Lactic acid fermentation of *Arthrospira platensis* (spirulina) biomass for probiotic-based products. *Journal of Applied Phycology*, *31*(2), 1077–1083. https://doi.org/10.1007/s10811-018-1602-3

Notermans, S., & in't Veld, P. (1994). Microbiological challenge testing for ensuring safety of food products. *International Journal of Food Microbiology*, *24*(1–2), 33–39. https://doi.org/10.1016/0168-1605(94)90104-X

Nshimiyumukiza, Ossiniel, Kang, Shin-Kook, Kim, Hyo-Jung, Lee, Eun-Hye, Han, Hae-Na, Kim, Yunhye, Kim, Deok-Hoon, Kim, Ji-Hoon, Eom, Sung-Hwan, & Kim, Young-Mog. (2015). Synergistic Antibacterial Activity of *Ecklonia cava* (*Phaeophyceae*: *Laminariales*) against *Listeria monocytogenes* (*Bacillales*: *Listeriaceae*). *Fisheries and Aquatic Sciences*, *18*(1), 1–6. https://doi.org/10.5657/FAS.2015.0001

Patra, J. K., Lee, S.-W., Park, J. G., & Baek, K.-H. (2017). Antioxidant and Antibacterial Properties of Essential Oil Extracted from an Edible Seaweed *Undaria Pinnatifida*: Antioxidant Potential of Essential Oil from *Undaria Pinnatifida*. *Journal of Food Biochemistry*, *41*(e12278), 10. https://doi.org/10.1111/jfbc.12278

Pedrós-Garrido, S., Clemente, I., Calanche, J. B., Condón-Abanto, S., Beltrán, J. A., Lyng, J. G., Brunton, N., Bolton, D., & Whyte, P. (2020). Antimicrobial activity of natural compounds against *Listeria* spp. And their effects on sensory attributes in salmon (S*almo salar*) and cod (Gadus morhua). *Food Control*, *107*, 106768. https://doi.org/10.1016/j.foodcont.2019.106768

Peñalver, R., Lorenzo, J. M., Ros, G., Amarowicz, R., Pateiro, M., & Nieto, G. (2020). Seaweeds as a Functional Ingredient for a Healthy Diet. *Marine Drugs*, *18*(6), 301. https://doi.org/10.3390/md18060301

Pérez, M., Falqué, E., & Domínguez, H. (2016). Antimicrobial Action of Compounds from Marine Seaweed. *Marine Drugs*, *14*(3), 52. https://doi.org/10.3390/md14030052

Pina-Pérez, M. C., Rivas, A., Martínez, A., & Rodrigo, D. (2017). Antimicrobial potential of macro and microalgae against pathogenic and spoilage microorganisms in food. *Food Chemistry*, *235*, 34–44. https://doi.org/10.1016/j.foodchem.2017.05.033

Plaza, M., Santoyo, S., Jaime, L., García-Blairsy Reina, G., Herrero, M., Señoráns, F. J., & Ibáñez, E. (2010). Screening for bioactive compounds from algae. *Journal of Pharmaceutical and Biomedical Analysis*, *51*(2), 450–455. https://doi.org/10.1016/j.jpba.2009.03.016

Radhika, D., Veerabahu, C., Priya, R., & Mohaideen, A. (2014). A comparative study of biopotential of crude and fractionated extracts of some sea weeds from tuticorin coast. *International Journal of Phytopharmacology*, *5*(1), 27–30.

Rajauria, G., Jaiswal, A. K., Abu-Gannam, N., & Gupta, S. (2013). Antimicrobial, antioxidant and free radical-scavenging capacity of brown seaweed *Himanthalia elongata* from western coast of Ireland: Antimicrobial and antioxidant properties of irish seaweed. *Journal of Food Biochemistry*, *37*(3), 322–335. https://doi.org/10.1111/j.1745-4514.2012.00663.x

Rania, A., & Hala, T. (2008). Antibacterial and Antifungal Activity of Cyanobacteria and Green Microalgae. Evaluation of Medium Components by Plackett-Burman Design for Antimicrobial Activity of S*pirulina platensis*. *Global Journal of Biotechnology & Biochemistry*, *3*(1), 22–31.

Rao, A. R., Reddy, A. H., & Aradhya, S. M. (2010). Antibacterial properties of *Spirulina platensis, Haematococcus pluvialis, Botryococcus braunii* micro algal extracts. *Current Trends in Biotechnology and Pharmacy*, *4*(3), 809–819.

Ricci, A., Bernini, V., Maoloni, A., Cirlini, M., Galaverna, G., Neviani, E., & Lazzi, C. (2019). Vegetable By-Product Lacto-Fermentation as a New Source of Antimicrobial Compounds. *Microorganisms*, *7*(607), 11. https://doi.org/10.3390/microorganisms7120607

Ríos, J. L., & Recio, M. C. (2005). Medicinal plants and antimicrobial activity. *Journal of Ethnopharmacology*, *100*(1–2), 80–84. https://doi.org/10.1016/j.jep.2005.04.025

Rodríguez-Bernaldo de Quirós, A., Frecha-Ferreiro, S., Vidal-Pérez, A. M., & López-Hernández, J. (2010). Antioxidant compounds in edible brown seaweeds. *European Food Research and Technology*, *231*(3), 495–498. https://doi.org/10.1007/s00217-010-1295-6

Rørvik, L. (2000). *Listeria monocytogenes* in the smoked salmon industry. *International Journal of Food Microbiology*, *62*(3), 183–190. https://doi.org/10.1016/S0168-1605(00)00334-2

Saleh, B., & Al-Mariri, A. (2017). Antimicrobial Activity of the Marine Algal Extracts against Selected Pathogens. *J. Agr. Sci. Tech.*, *19*, 1067–1077.

Samarakoon, K., & Jeon, Y.-J. (2012). Bio-functionalities of proteins derived from marine algae—A review. *Food Research International*, *48*(2), 948–960. https://doi.org/10.1016/j.foodres.2012.03.013

Sanmukh, S. (2014). Bioactive Compounds Derived from Microalgae Showing Antimicrobial Activities. *Journal of Aquaculture Research & Development*, *03*(03). https://doi.org/10.4172/2155-9546.1000224

Santoyo, S., Rodríguez-Meizoso, I., Cifuentes, A., Jaime, L., García-Blairsy Reina, G., Señorans, F. J., & Ibáñez, E. (2009). Green processes based on the extraction with pressurized fluids to obtain potent antimicrobials from *Haematococcus pluvialis* microalgae. *LWT - Food Science and Technology*, *42*(7), 1213–1218. https://doi.org/10.1016/j.lwt.2009.01.012

Santoyo, Susana, Herrero, M., Señorans, F. J., Cifuentes, A., Ibáñez, E., & Jaime, L. (2006). Functional characterization of pressurized liquid extracts of S*pirulina platensis*. *European Food Research and Technology*, *224*(1), 75–81. https://doi.org/10.1007/s00217-006-0291-3

Shaieb, F. A., Issa, A. A., & Meragaa, A. (2014). Antimicrobial activity of crude extracts of cyanobacteria N*ostoc commun*e and *Spirulina platensis*. *Archives of Biomedical Sciences*, *2*(2), 34–41.

Sivakumar, J., & Santhanam, P. (2011). Antipathogenic activity of *spirulina* powder. *Recent Research in Science and Technology*, *3*(4), 158–161.

Souza, E. L. de, Stamford, T. L. M., & Lima, E. de O. (2006). Sensitivity of spoiling and pathogen food-related bacteria to *Origanum vulgare L. (Lamiaceae)* essential oil. *Brazilian Journal of Microbiology*, *37*(4), 527–532. https://doi.org/10.1590/S1517-83822006000400023

Stabili, L., Acquaviva, M. I., Biandolino, F., Cavallo, R. A., De Pascali, S. A., Fanizzi, F. P., Narracci, M., Cecere, E., & Petrocelli, A. (2014). Biotechnological potential of the seaweed *Cladophora rupestris (Chlorophyta, Cladophorales*) lipidic extract. *New Biotechnology*, *31*(5), 436–444. https://doi.org/10.1016/j.nbt.2014.05.002

Stirk, W. A., Reinecke, D. L., & van Staden, J. (2007). Seasonal variation in antifungal, antibacterial and acetylcholinesterase activity in seven South African seaweeds. *J Appl Phycol*, 6.

Swinscoe, I., Oliver, D. M., Ørnsrud, R., & Quilliam, R. S. (2020). The microbial safety of seaweed as a feed component for black soldier fly (*Hermetia illucens*) larvae. *Food Microbiology*, *91*, 103535. https://doi.org/10.1016/j.fm.2020.103535

Torres, M. D., Arufe, S., Chenlo, F., & Moreira, R. (2017). Coeliacs cannot live by gluten-free bread alone—Every once in awhile they need antioxidants. *International Journal of Food Science & Technology*, *52*(1), 81–90. https://doi.org/10.1111/ijfs.13287

Velichkova, K., Sirakov, I., Rusenova, N., Beev, G., Denev, S., Valcheva, N., & Dinev, T. (2018). In vitro antimicrobial activity on l*emna minuta, chlorella vulgaris* and *spirulina* sp. extracts. *Fresenius Environmental Bulletin*, *27*(8), 5736–5741.

Vello, V., Phang, S.-M., Chu, W.-L., Abdul Majid, N., Lim, P.-E., & Loh, S.-K. (2014). Lipid productivity and fatty acid composition-guided selection of *Chlorella* strains isolated from Malaysia for biodiesel production. *Journal of Applied Phycology*, *26*(3), 1399–1413. https://doi.org/10.1007/s10811-013-0160-y

Vinay Kumar. (2011). Antibacterial activity of crude extracts of *Spirulina platensis* and its structural elucidation of bioactive compound. *Journal of Medicinal Plants Research*, *5*(32), 7043–7048. https://doi.org/10.5897/JMPR11.1175

Yamaguchi, S. K. F., Moreira, J. B., Costa, J. A. V., de Souza, C. K., Bertoli, S. L., & Carvalho, L. F. de. (2019). Evaluation of Adding S*pirulina* to Freeze-Dried Yogurts Before Fermentation and After Freeze-Drying. *Industrial Biotechnology*, *15*(2), 89–94. https://doi.org/10.1089/ind.2018.0030

Zarbà, C., Chinnici, G., & D'Amico, M. (2020). Novel Food: The Impact of Innovation on the Paths of the Traditional Food Chain. *Sustainability*, *12*(2), 555. https://doi.org/10.3390/su12020555

Zbakh, H., Chiheb, H., Bouziane, H., Sánchez, V. M., & Riadi, H. (2012). Antibacterial activity of benthic marine algae extracts from the Mediterranean coast of Morocco. *Journal of Microbiology, Biotechnology and Food Sciences*, *2*(1), 219–228.

Zouari, N., Abid, M., Fakhfakh, N., Ayadi, M. A., Zorgui, L., Ayadi, M., & Attia, H. (2011). Blue-green algae (*Arthrospira platensis*) as an ingredient in pasta: Free radical scavenging activity, sensory and cooking characteristics evaluation. *International Journal of Food Sciences and Nutrition*, *62*(8), 811–813. https://doi.org/10.3109/09637486.2011.582461

Bacteriostatic or bactericidal? Impedometric measurements to test the antimicrobial activity of *Arthrospira platensis* extract

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Abstract

In recent years, increasing consumer's demand for cleaner labels, functional, safe and high-quality products has led to searching for new antimicrobial agents of natural origin that can improve quality and safety by reducing the impact on the product composition. In this scenario, the use of algae extracts in food formulations as antimicrobial agents is taking more and more interest. In particular, the antimicrobial potential of *Arthrospira platensis* has already been tested in vitro, using the agar well diffusion method or the broth dilution method, which can provide a quantitative estimation of minimum inhibitory concentration (MIC). Even if several methods are already in use to test the MIC and minimum bactericidal concentration (MBC) of antimicrobials, the aim of the present research was to propose impedance measurement as a valid method for the in vitro evaluation of MIC and MBC of a natural antimicrobial extract of *A. platensis*. To this purpose, six different concentrations of *A. platensis* extract (0.1%, 0.15%, 0.2%, 0.25%, 0.3% and 0.5% v/v) were tested on *Pseudomonas fluorescens* and *Serratia liquefaciens*, two species commonly involved in food spoilage, and *Listeria innocua*, as representative of the human foodborne pathogenic species *Listeria monocytogenes*. The results obtained confirmed the in vitro antimicrobial potential of *A. platensis* extract, but also highlighted how MIC and MBC could be different depending on both the concentration of antimicrobial and the tested strain. Furthermore, the proposed method allowed the identification of MIC and MBC values in a new way never used before for this purpose.

Introduction

Preservation techniques such as heat treatment, salting, acidification and drying are commonly used to prevent the growth of spoilage and pathogenic bacteria in foods (Lucera, Costa, Conte, & Del Nobile, 2012). Those methods certainly lead to a modification of the natural composition of products, often affecting food quality standards such as nutritional, sensory, odor, color and texture (Lucera et al., 2012; Pisoschi et al., 2018). For these reasons, in a hurdle technology approach, antimicrobial compounds have been extensively used by industries to ensure food safety and protect products from spoilage along with preparation, storage and distribution. However, the addition of compounds of synthetic origin in food formulations fights with the increasing consumer's demand for even more clean labels, functional, and high-quality products. In recent years the interest in searching for new antimicrobial agents of natural origin that can improve quality and safety with reducing the impact on the product is growing (Ricci et al., 2019). Furthermore, natural preservatives could also constitute a valid alternative to address the problem of the growing microbial resistance to antibiotics (Lucera et al., 2012; Pisoschi et al., 2018). To date, several natural antimicrobials exerting different roles are in use by food industries, and they are commonly grouped by the diverse origin: plants, animals, microbes and fungi (Pisoschi et al., 2018). Among the ingredients with high technological potential, that can be used as natural preservatives, seaweeds and microalgae are gaining a lot of attention (Martelli, et al 2020 b; Pina-Pérez, Rivas, Martínez, & Rodrigo, 2017). Among them, the use of *Arthrospira platensis* in food formulation is taking more and more interest because of consumer's request (Barkallah et al., 2017; Batista et al., 2017; Beheshtipour, Mortazavian, Haratian, & Darani, 2012; De Caire & Parada, 2000; Golmakani, Soleimanian-Zad, Alavi, Nazari, & Eskandari, 2019; Martelli et al., 2020b; Varga & Molnár-Ásványi, 2012; Zouari et al., 2011; Ásványi-Molnár, Sipos-Kozma, Tóth, Ásványi, & Varga, 2009). *A. platensis*, commercially known as Spirulina, is a fresh-water cyanobacterium that has attracted a lot of attention due to its growing employment as human foodstuff and for its potential functional properties. This

cyanobacterium is one of the most important among microalgae showing antimicrobial activity towards many bacterial pathogens and fungi. It represents a novel source for antimicrobials because of its high level of amino acids and small peptides, phycocyanobilin, polyphenols, carotenoids, chlorophyll and other compounds that have proven an in vitro antimicrobial activity (Amaro, Guedes, & Malcata, 2011; Elshouny, El-Sheekh, Sabae, Khalil, & Badr, 2017; Kumar, SaranTirumalai, Singh, Bhatnagar, & Shrivastava, 2013; Mala, Sarojini, Saravanababu, & Umadevi, 2009). Until now, the antimicrobial potential of algae in general and *A. platensis* in particular has already been tested in vitro, using the agar well diffusion method (Cakmak, Kaya, & Asan-Ozusaglam, 2014; Manivannan, Karthikai devi, Anantharaman, & Balasubramanian, 2011; Martelli et al., 2020a) or the broth dilution method (Gupta, Rajauria, & Abu-Ghannam, 2010) that can provide a quantitative estimation of minimum inhibitory concentration (MIC). In general, the methods for in vitro evaluation of antimicrobial activity have been widely reviewed by Balouiri, Sadiki, and Ibnsouda (2016) who listed and discussed in detail all the advantages and limitations of the methods actually in-use. Nowadays, the disk-diffusion and broth or agar dilution methods are the most used, for ease of application, cost-effectiveness and immediacy of results interpretation. On the other hand, they are time-consuming and they could be subjected to manual undertaking and risk of errors by the users (Balouiri et al., 2016). The absorbance measurement of cell cultures is commonly associated with such mentioned methods, however, it presents some limitation, such as the need for a calibration step, in order to correlate the results with viable counts, or the absence of cell physiological state consideration (Chorianopoulos et al., 2006). Other techniques such as time-kill test or flow cytometry and bioluminescent methods are also used but, associated with the high performances, they have the disadvantages of needing specific equipment and user's training (Balouiri et al., 2016). In addition, not all these methods enable the evaluation of MIC and minimum bactericidal concentration (MBC) with the same and unique approach. Considering all these facts, the aim of the present research was to propose impedance measurement as a valid method for the in vitro evaluation of MIC and MBC of an *A. platensis*

antimicrobial extract. This method, initially used as growth index of lactic acid bacteria in milk (LAB) (Lanzanova, Mucchetti, & Neviani, 1993) was recently reassessed by Bancalari, Bernini, Bottari, Neviani, and Gatti (2016) for the evaluation of the in vitro growth kinetics of LAB. Conversely, in the present research, it was used to test the effect of six different concentrations of *A. platensis* extract (0.1%, 0.15%, 0.2%, 0.25%, 0.3%, 0.5% v/v) on *Pseudomonas fluorescens* and *Serratia liquefaciens*, two species commonly involved in food spoilage, and on *Listeria innocua* as representative of the human foodborne pathogen species *Listeria monocytogenes*.

Material and methods

Arthrospira platensis extraction process

An extraction process from dried *A. platensis*, kindly provided by S.a.Ba.r. (Novellara, RE, Italy), was carried out as described by Martelli et al., (2020a). Briefly, 100 mL of ethanol/water (70:30 v/v) acidified with 1% formic acid (CH_2O_2) solvent was used for extraction of 10 g of *A. platensis*. A double extraction was carried out, alternating twice a shaking cycle to a sonication one in Ultrasonic Cleaner sonicator (VWR, United States). The sample was then centrifuged (Eppendorf 5800 Centrifuge, Model 5810R, Hamburg, Germany) at 12,857×g for 10 min at 10 °C. The solution was filtered with filter paper to recover the solid part so as to proceed to a second extraction. The two extracts obtained were combined and concentrated until fully dried with a rotary evaporator Strike 300 (Steroglass, Italy) at 4×g at a bath temperature of 40 °C. The concentrated extract was then suspended in sterile water to recover the soluble part and then stored at −80 °C until use for antimicrobial activity test. The obtained extract had a concentration of 235 mg/mL (23.5% v/v). The final pH of the obtained extract was 4.0.

Bacterial strains and culture conditions

P. fluorescens 5026, *S. liquefaciens* 5006, and *L. innocua* Lin6 strains were used for the experiments. The strains, belonging to the Food Microbiology unit collection of the Food and Drug Department (University of Parma), have been previously isolated from different food matrixes and identified by 16S rRNA sequencing. The strains, maintained as frozen stock cultures in Tryptic Soy Broth (TSB) (Oxoid, Ltd., Basingstoke, United Kingdom) broth containing 20% (v/v) glycerol at −80 °C, were recovered in TSB by two overnight sub-culturing (2% v/v) at 37 °C for *L. innocua* and 30 °C for *P. fluorescens* and *S. liquefaciens*.

Experimental design and growth conditions

The activity of *A. platensis* extract on the tested strains was evaluated following the experimental design reported in Figure 1.

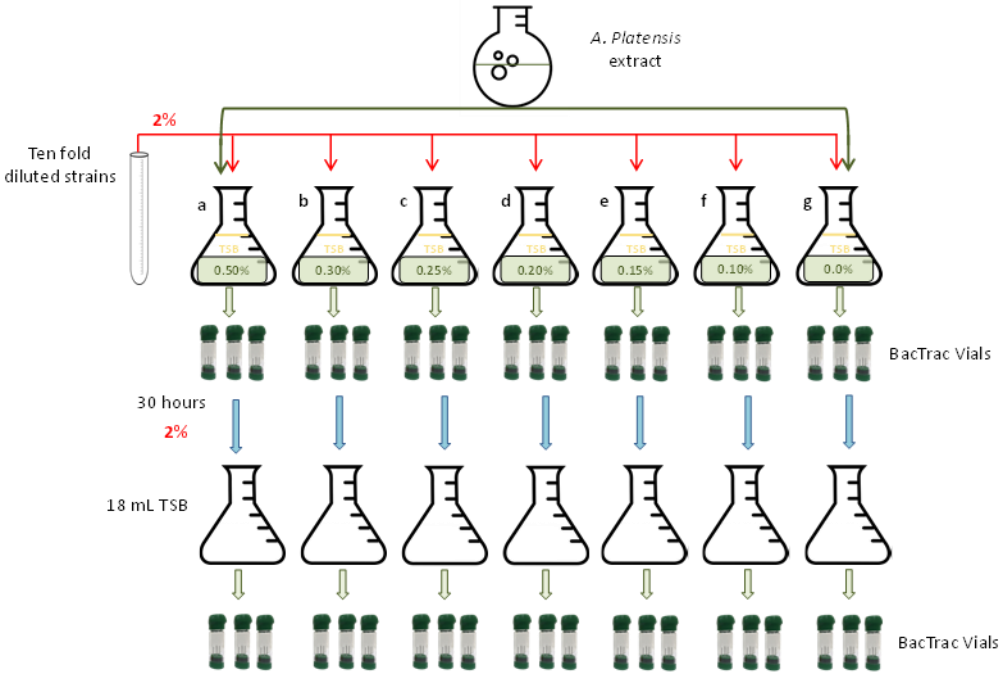

Figure 1. Schematic representation of the experimental design.

TSB (Oxoid) was reconstituted to 30 g/L (w/v) and sterilized at 121 °C for 20 min. *A. platensis* extract was then added to reach a final concentration (v/v) in the medium of: a) 0.50%; b) 0.30%; c) 0.25%; d) 0.20%; e) 0.15%; f) 0.10%; g) 0% (negative control sample without any addition of extract) (Fig. 1). The last sub-culturing step of each bacterial culture was ten-fold diluted in Ringer solution (Oxoid) and inoculated (2% v/v) in 18 mL of the growth media supplemented, respectively and separately, with all the different concentrations of *A. platensis* extract (Fig. 1). A 6 mL aliquot of each inoculated medium was then transferred into three sterilized BacTrac 4300® vials (SY-LAB, Neupurkersdorf, Austria), and incubated at the optimal growth temperature of each strain (37 °C for *L. innocua* and 30 °C for *P. fluorescens* and *S. liquefaciens*). After 30 h of incubation, the content of the three vials for each strain was aseptically mixed and used to inoculate the fresh TSB medium in the flask. All the analysis were carried out in triplicate and monitored for 30 h by measuring the impedometric signal every 10 min.

Impedometric measurement

Impedance measurements were performed by means of BacTrac 4300® Microbiological Analyzer system (SY-LAB, Neupurkersdorf, Austria). requiring the use of dedicated glass measuring cells (vials) with 4 electrodes. The specific impedance E% value was measured and recorded every 10 min for 30 h (Bancalari et al., 2016, 2019). Each experiment was replicated twice and each analytical variable was measured in triplicate. The results of the impedometric measurements were analyzed as previously reported by Bancalari et al. (2016) and the Lag and yEnd values, together with the observation of impedometric curves, were considered to evaluate the bacteriostatic or bactericidal activity of the antimicrobial extract on the tested strains, defining MIC and MBC.

Evaluation of MIC and MBC

The MIC value was defined as the lowest concentration able to inhibit the growth of the tested strains. In our case, no growth means that no Lag values were recorded in 30 h. The MBC was determined by sub-culturing the cells exposed to different concentrations of the antimicrobial extract used to evaluate MIC, in fresh TSB medium and defined when no Lag values were recorded in 30 h (Fig. 1).

Culture-independent viable counts

Fluorescence microscopy count was obtained by using the LIVE/ DEAD® Baclight ™ Bacterial Viability kit (Molecular Probes, Oregon, USA) and Nikon Eclipse 80i epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a C-SHG1 100 W mercury lamp. Nikon filter set B2A FITC was used for Lbh1 FITC labelled probe (excitation wavelength, 450–490 nm; emission wavelength, 500–520 nm). Nikon filter set G-2E⁄C was used for St4 Cy3-labelled probe (excitation wavelength, 540⁄25 nm; emission wavelength, 605⁄55 nm). Pictures of each field were taken and then superimposed through the Nis

Elements software (ver. 2.10 Nikon). (Bottari, Santarelli, Neviani, & Gatti, 2010; Gatti, Bernini, Lazzi, & Neviani, 2006; Santarelli, Bottari, Lazzi, Neviani, & Gatti, 2013). The analysis was performed on the sub-cultured cells after 30 h of incubation. One mL of ten-fold diluted sub-cultures was stained with LIVE/DEAD® and after 15 min filtered onto black polycarbonate filters (0.2 μm pore size) (Millipore Corp., Billerica, MA, USA). Then it was visualized by epifluorescence microscope (Nikon 80i, Tokyo, Japan) and cells were counted as previously described (Bottari et al., 2010; Gatti et al., 2006; Santarelli et al., 2013). A minimum of five separate counts were performed for each sample. Results were reported as average values ± standard deviation of total, viable and non-viable cells referred to one ml.

Results and discussion

Evaluation of A. platensis extract MIC by impedometric measurement

The impedometric analysis was performed by means of BacTrac 4300® that enables the detection of bacteria activity in real-time via the decrease of the impedance in an alternating current (AC) field. In fact, during duplication, bacteria viable cells break down sugars present in the medium into smaller molecules that make the medium more conductive, decreasing the overall resistance and total impedance. This variation is due only to the presence and duplication of bacteria, and thus it is used as a measure of their metabolism (Bancalari et al., 2016). The instrument is able to register, during the incubation time, two specific impedance values for every single measurement: i) the conventional conductance value (M-value) that corresponds to the overall medium impedance, and ii) the capacitance value (E-value) which is the measure of electrochemical double layer impedance in the vicinity of the electrodes. Both these values, simultaneously recorded every 10 min, are shown as relative changes compared to a starting value and expressed as M% and E%. Furthermore, they are also visualized in realtime in a capacitance or conductance curve (Bancalari et al., 2019). As the value of the double-layer capacitance is more sensitive to any slight alteration in the nearby of the electrodes, it was more suitable for the measurement in TSB, and therefore E-value was chosen for this investigation (SY-LAB microbiology). Differently from the method already proposed and available in the literature (Chorianopoulos et al., 2006; Puttaswamy, 2013), at the end of the analysis all recorded capacitance data (E%) were used in two different ways: i) were fitted by the Gompertz equation, following the method previously reported by Bancalari et al. (2016) to obtain the kinetic parameter Lag and yEnd; ii) were used to build a graphical representation of the original capacitance curve (Fig. 2).

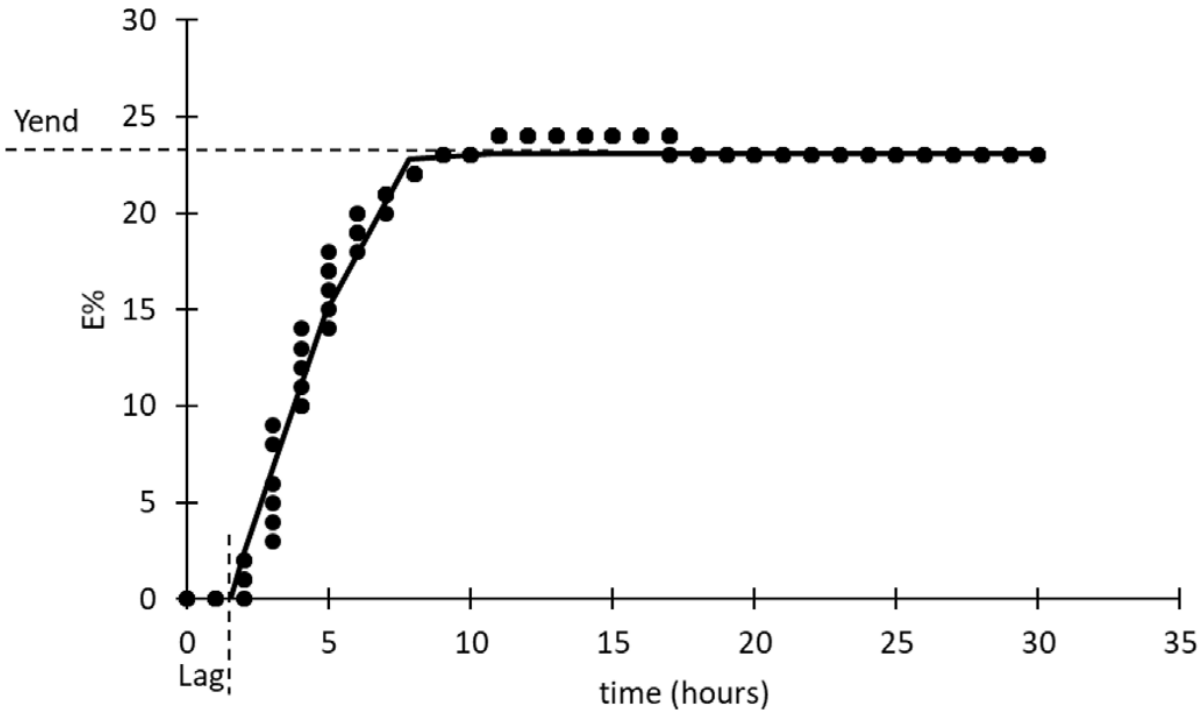

Figure 2. Example of capacitance curves fitted to the Modified Gompertz equation (Gibson et al., 1988) using DMfit version 2.1 Excel add-in (http://www.combase.cc/index.php/en/tools). Black circles symbols are the y values that DMfit uses to represent the E% data recorded by the BacTrac4300 each 10 minutes for 30 hours of incubation. Solid line is the fitted curve described by Modified Gompertz equation. The two parameters used in this experiments can be calculated by the ComBase too: i) lag time (Lag), and iii) maximum value of E% (Yend) (Bancalari et al., 2019).

Lag value is an adjustment period measured in hours, and the greater the value, the bigger the time that the strains need to adapt before growing. yEnd is the highest variation of impedance recorded and it has been interpreted as the maximum acidifying capacity of the LAB strains (Fig. 2) (Bancalari et al., 2016). In this case, for the bacterial species considered in this study, it could be interpreted as metabolic capacity (Sauer, Herskovits, & O'Riordan, 2019; Silby, Winstanley, Godfrey, Levy, & Jackson, 2011; Yang & Bashir, 2008). The calculated Lag and yEnd values, allowed to estimate and display the bacteriostatic activity, quantified as the Minimum Inhibitory Concentration (MIC) and bactericidal activity, as Minimum Bactericidal Concentration (MBC), of *A. platensis* extract on the tested strains. At first, MIC was evaluated by inoculating the strains in the medium in presence of different

concentrations of *A. platensis* extract and without any addition as a negative control (0%) as schematized in Figure 1. By comparing the Lag values of the strains grown with different percentages of *A. platensis* extract to those obtained in the negative control, we were able to evaluate the presence or not of an inhibitory effect on strains growth. In particular, if the Lag values, in presence of the extract, were equal to the negative control ones, no effect was detected. On the other hand, if the Lag values increased along with the concentration of A. platensis extract used, an effect on the bacterial growth took place. When detected, Lag values were expressed in hours, while, when no growth was detected, "nd" was reported, which means that no variation of capacitance (E%) values were recorded in 30 h (Table 1).

Table 1. Lag and yEnd parameters of the three considered strains evaluated in TSB in presence of different *A. platensis* extract concentration for the determination of Minimum inhibitory concentration (MIC).

***A. platensis* extract in the medium**	***L. innocua Lin6***		***P. fluorescens 5026***		***S. liquefaciens 5006***	
	Lag	Yend	Lag	Yend	Lag	Yend
0%	<1±0.01	50±0.12	<1±0.01	25±0.088	<1±0.01	40±0.05
0.10%	<1±0.01	44.04±0.09	<1±0.01	16±0.09	<1±0.01	25±0.07
0.15%	1.21±0.05	34.01±0.18	27±0.08	3±0.1	4.3±0.05	14±0.10
0.20%	nd	nd	nd	nd	19±0.12	18±0.09
0.25%	nd	nd	nd	nd	nd	nd
0.30%	nd	nd	nd	nd	nd	nd
0.50%	nd	nd	nd	nd	nd	nd

Lag: is reported in hours; nd: not detected within 30 hours.

Values are the mean ± SD of, at least two separate experiments in which each variable was measured in triplicate

In the case of *L. innocua*, the Lag values were<1 h, both for the control and 0.10% of *A. platensis* extract, so in this case, no effect on the growth was observed (Table 1). On the other

hand, by increasing the concentration of antimicrobial extract, an initial, even if small, effect was detected when 0.15% was used. This concentration prolonged the Lag time until 1.21 h, but at the same time also affected the metabolic capacity of the strain, by lowering the yEnd value (Table 1). This means that the cells were in greatest difficulty as compared to the control, first of all at the beginning of their growth phase (> Lag), but also they were not able anymore to reach their maximum metabolic capacity. This effect is clearly visible also from the conductance curves (Fig. 3A1). Moving forward to the highest concentration of *A. platensis* extract, no growth was detected anymore, thus MIC value was established at 0.20% (Table 1). The same effect observed for *L. innocua* at 0.10% of extract in the medium was found also for *P. fluorescens* and *S. liquefaciens*, even if these last showed a lower Yend value underling a lower metabolic capacity in TSB respect to *L. innocua* (Fig. 3B1 and 3C1, Table 1).

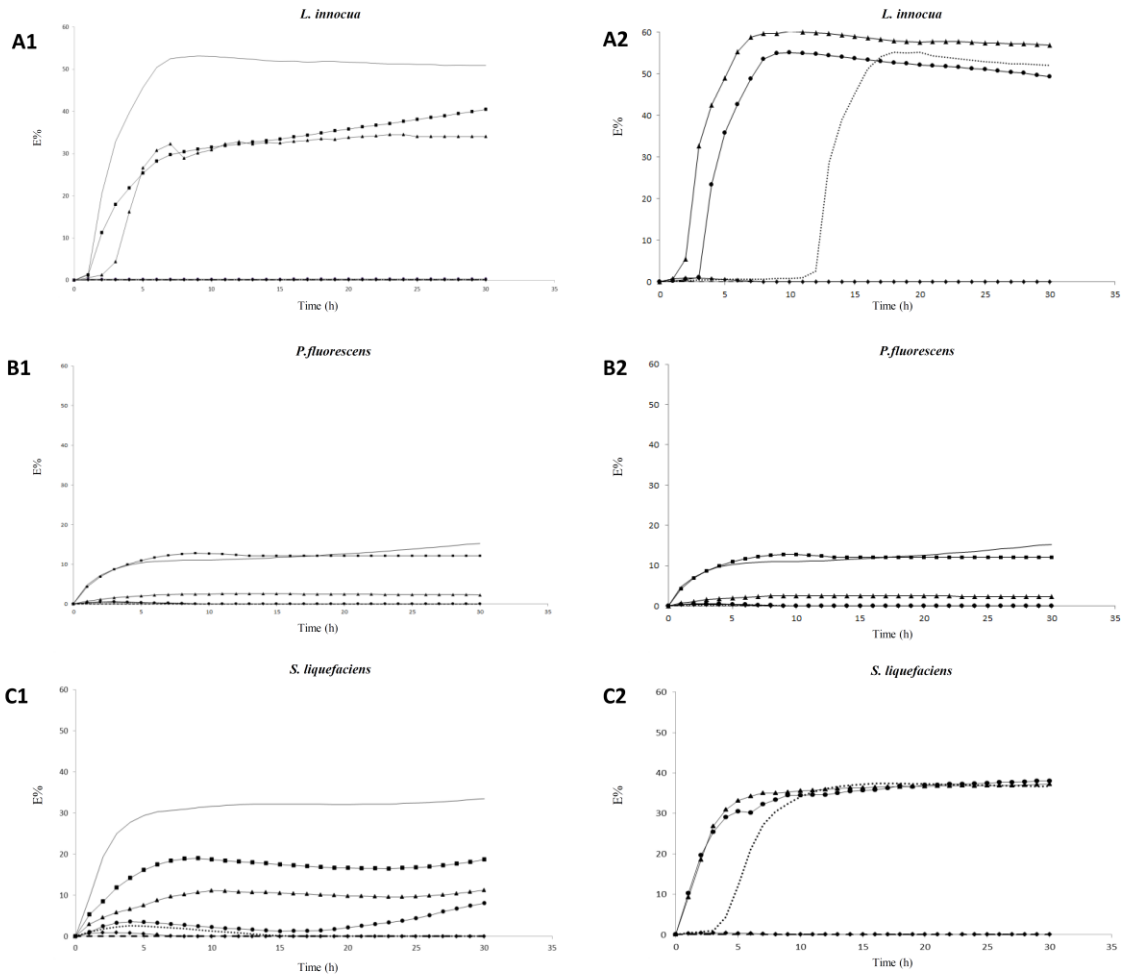

Figure 3. Capacitance curve of the first inoculum (A1,B1,C1) and the sub-culturing (A2,B2,C2) of the three strains tested in presence different antimicrobial concentrations

Antimicrobial concentrations used for each analysis:

(—) 0%, (■) 0.1%, (▲) 0.15, (●)0.2%, (···) 0.25%, (◆) 0.3%, (– –) 0.5%

Moreover, *P. fluorescens* growth was more affected by the addition of 0.15% of extract compared to *L. innocua*, as it showed a higher increase of the Lag phase, which reached 27 h, with a yEnd values of 3. This result means that a little amount of cells was able to grow, in fact they took a lot of time to leave the Lag phase. Consequently, they were not able to impact on the overall capacitance of the media and, therefore, also a lower value of yEnd

was recorded (Table 1 and Figure 3B1) (D'Incecco, Bancalari, Gatti, Ranghetti, & Pellegrino, 2020). However, as for *L. innocua*, MIC value of *P. fluorescens* was 0.20% (Table 1). *S. liquefaciens*, showed a highest resistance to *A. platensis* extract, as compared to the other strains its Lag phase was 19 h at 0.20%, and it still maintained a good metabolic capacity, with a yEnd value of 18 (Table 1). MIC values for *S. liquefaciens* was 0.25% (Table 1). This difference among species was already observed by Whiting and Buchanan (2007) who pointed out that the growth range and/or inactivation characteristics of bacteria may vary significantly between species and sometimes also within the same species. This underlines the importance of using a fast, easy and reproducible method to test as many variables as possible to understand the behavior of the strains, ensuring the safety of food processes.

Evaluation of A. platensis extract MBC by impedometric measurement

To determine the MBC of the tested strains, the same method as for MIC was used. In addition, to better underline the bactericidal effect, the trend of capacitance curves obtained by the sub-cultured strains were also compared. After 30 h of incubation, the three vials of each strain tested for MIC were mixed and sub-cultured in a fresh TSB medium and incubated at the same condition in BacTrac 4300® (Fig. 3A2, 3B2, 3C2). This procedure to sub-culturing the strains into a fresh medium without the antimicrobial addition is commonly used to determine whether the cells are still alive or not, thus establishing if the effect on cells is bacteriostatic or bactericidal (Balouiri et al., 2016). As the 0.10% of A. platensis extract did not affect the growth of any of the bacteria tested (Table 1), it was not considered for the sub-culturing. By determining the Lag and yEnd values of the sub-cultured strains, it was possible to observed a high variability within the tested species (Table 2).

Table 2. Lag and yEnd parameters of the sub-cultures of the three considered strains arising from different *A. platensis* extract concentrations for the determination of Minimum bactericidal concentration (MBC).

Antimicrobial concentration of the sub-culture	***L. innocua Lin6***		***P. fluorescens 5026***		***S. liquefaciens 5006***	
	Lag	Yend	Lag	Yend	Lag	Yend
0.15%	1.02±0.05	60±0.1	7.28±0.7	14±0.05	<1±0.01	37±0.8
0.20%	3.1±0.08	51±0.08	14±0.11	11.59±0.12	<1±0.03	38±0.11
0.25%	10.5±0.16	50.4±0.18	27±0.17	10.32±0.15	2.18±0.19	36.5±0.2
0.30%	nd	nd	nd	nd	nd	nd
0.50%	nd	nd	nd	nd	nd	nd

Lag: is reported in hours; nd: not detected after 30 hours.

Values are the mean ±SD of, at least two experiments in duplicate.

In particular, *L. innocua* and *P. fluoresces*, having both MIC values of 0.20%, when sub-cultured in TSB were able to grow even with 0.25% of *A. platensis* extract. This means that some alive cells were still present at the concentration of extract defined as MIC and gave rise to a higher MBC and corresponding to 0.30% of extract. This MBC value was stated also for *S. liquefaciens* even if it had higher MIC values compared to the other two strains (Table 2). Going deeper in the results, we can observe that *L. innocua*, even if showed a longer Lag phase when a growing amount of *A. platensis* extract was used, still maintained a very high metabolic capacity as revealed from conductance curve observation (Figure 3A2) and quantified from Yend value (Table 2). It is interesting to note that the activity of subcultures is at least equal, or higher than that of the control (Table 1). This means that, even if the number of alive cells decreased (> Lag), their metabolic activity, after 30 h of contact with the *A. platensis* extract, has not been compromised or reduced at all. *S. liquefaciens* showed the lowest Lag values and constant yEnd (Table 2, Figure 3C2). This could mean that a greater number of alive cells were present when sub-culturing. In fact, as we can observe in Figure 3, the curves obtained using the first three concentrations (Figure 3C2) were

comparable to those obtained for the control (Fig. 3C1). Although MBC referred to *P. fluorescens* is 0.30%, as for the other two strains, observing the Lag and Yend values (Table 2) and the curves (Fig. 3B2), the contact with *A. platensis* extract for 30 h, affected *Pseudomonas* cells on a higher extent compared to the other strains considered. The in vitro antimicrobial effect of *A. platensis* extract has been already investigated on different spoilage and pathogenic bacteria (Kumar et al., 2013) and its activity was mainly attributed to the presence of lipids, tocopherols, C-phycocyanin and extracellular polysaccharides. Considering that the above mentioned extraction method is comparable with our method, we can attribute to the same compounds the antimicrobial effect observed in the present study. In any case, further studies need to be carried out to confirm this hypothesis. More in detail, Sarada, Sreenath Kumar, and Rengasamy (2011) reported that up to 40% of A. platensis total protein is represented by the protein-pigment complexes as C-phycocyanin (C-PC) that was demonstrated to be able to control the growth of some Gram-negative bacteria. Despite few informations are present in literature about the specific action of all tocopherols, lipids and extracellular polysaccharides in *A. platensis* extract, their antimicrobial effect has already been proved for other natural antimicrobials (Mariod, Matthäus, Idris, & Abdelwahab, 2010; Ulusoy, Boşgelmez-Tınaz, & Seçilmiş-Canbay, 2009; Yue, Shang, Zhang, & Zhang, 2017).

Culture-independent viable counts

The fluorescence microscopy count was used to estimate the cell number of the sub-cultured strains, to have a deeper view of the effect of *A. platensis* extract and to confirm the result obtained with the impedometric method. After 30 h of incubation of sub-cultured strains into the fresh TSB, the total (red + green), viable (green) and dead (red) cells were counted (Bottari et al., 2010; Fakruddin, Mannan, & Andrews, 2013). In Table 3 the results of the cell counts are reported for each subcultured strain with 0.10%, 0.20% and 0.30% of *A. platensis* extract.

Table 3. Results of microscopy fluorescence count. For each strains the viable, not-viable and total count are reported as Log cell / mL. The inhibition effect of the extract was reported as %.

		0%	10%	20%	30%	%inhibition* 10%	%inhibition* 20%	%inhibition* 30%
L.innocua Lin6	Viable	6.94	6.73	5.92	2.47	38.43%	90.48%	99.99%
	Non-viable	4.44	4.4	4.9	5.47			
	Total	6.94	6.73	5.96	5.96			
P.fluorescens 5026	Viable	6.97	5.91	5.28	3.03	91.17%	97.93%	99.99%
	Non-viable	4.76	5.36	5.45	6.3			
	Total	6.97	6.02	5.67	5.79			
S.liquefaciens 5006	Viable	6.74	6.34	5.61	3.14	60.46%	92.71%	99.98%
	Non-viable	4.86	5.06	5.53	5.79			
	Total	6.75	6.36	5.87	5.87			

*The % of inhibition was calculated on viable cell count as: [(Log CFU/mL of control- Log CFU/mL of the antimicrobial)/ (Log CFU/mL of control)]*100 (K.H. Cho et al., 2005)

The antimicrobial effect was variable within the species considered, but evident on both live and dead cells (Table 3). In fact, the number of live cells decreased along with the increasing concentration of extract, and, on the other hand, dead cells increased with the increasing amount of antimicrobial in the medium. In particular, regarding *L. innocua*, the number of live cells fell from 6.94 Log CFU/ml of the control to 2.47 Log CFU/ml of the sub-cultured cells that were grown with the 0.30% of *A. platensis* extract. This lead to a 99.99% of inhibition corresponding to 4 decimal reductions, allowing to confirm that 0.30% was the MBC for *L. innocua* (Table 3). The same results were obtained for *P. fluorescens* who showed a good reduction of viable cells, from 6.97 Log CFU/ml to 3.03 Log CFU/ ml, with 99.99% of inhibition in the presence of 0.30% of *A. platensis* extract, confirming this value as the MBC for *P. fluorescens* (Table 3). Slight differences were observed for *S. liquefaciens*, who showed

the highest viable cell count of 3.14 Log CFU/ml with 0.30% of *A. platensis* extract, highlighting a lower antimicrobial inhibition of 99.98%, but reasonably acceptable the MBC as 0.30% (Table 3). It was therefore interesting to note that also the lowest concentrations of *A. platensis* extract showed different effects depending on the strains. In fact, the % of inhibition calculated for *L. innocua* in presence of 0.10% of the extract was the lowest if compared to the other two strains. This was in agreement with the impedometric results that showed how the sub-cultured cells from 0.10% of *A. platensis* extract had the best metabolic capacity, resulting less affected by the antimicrobial. Conversely, the sub-cultured cells of *P. fluorescens* and *S. liquefaciens* were more affected by the extract, showing an inhibition of 91.17% and 60.46% respectively (Table 3). To give a better view of the effect of *A. platensis* extract on cell viability, the fluorescence microscopy images are reported (Figure 4). For all the strains, the cells in contact with a higher amount of *A. platensis* extract, despite their sub-culturing in a fresh medium, showed a high percentage of mortality (Figure 4D). This further confirm the 0.3% of *A. platensis* extract as the MBC for all the tested strains. Conversely, the lowest concentration of *A. platensis* extract used (Figure 4B) seems to not affect cells viability, confirming the data obtained from the impedometric analysis.

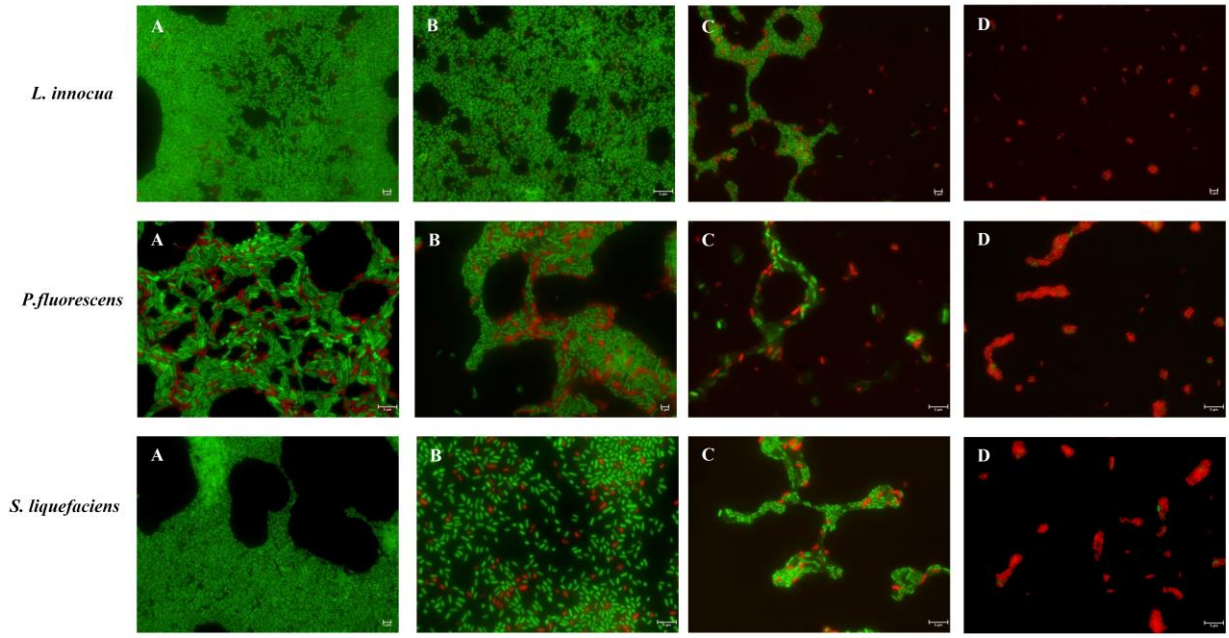

Figure 4. Microscopy fluorescence images of the sub-cultured cells in presence of the diverse antimicrobial concentrations, reported as: A) control 0%, B) 0.10%, C) 0.20% and D) 0.3%.

Conclusions

The results obtained from this research have a double impact: firstly, we confirmed the in vitro antimicrobial activity of *A. platensis* against two strains representative of food spoilage species, *P. fuorescens* and *S.* liquefaciens, and a *L. innocua* strain representative of the pathogenic species *L. monocytogenes*. In fact, even if we did not analyze the composition of the *A. platensis* extract, we can hypothesize that its activity could be related to the presence of small peptides, lipids, tocopherols, phycocianin and extracellular polysaccharides. MIC and MBC values could differ depending on the concentration of antimicrobial and the strain tested. Indeed, while MIC values varied for the three species considered, 0.20% for *L. innocua* and *P. fluorescens* and 0.25% for *Serratia*, MBC was 0.30% for all the species. Secondly, with the proposed impedometric method we were able to assess MIC and MBC values in a new way never used before for this purpose. In fact, by evaluating the obtained parameters, Lag time (hours) and the metabolic capacity (yEnd), we were able to observe how every single strain differently replies to the variation of antimicrobial concentration. Considering all that, the applications of this approach could be very interesting and impactful because by measuring the changing of capacitance values, even any small variation caused by microorganism metabolism are detected, making it a promising method. Furthermore, it would represent a good strategy to test other antimicrobial compounds than *A. platensis* as it allows the analysis of many variables or many samples at the same time and in addition, it gives a fast, objective, reliable and easy-to-interpret result as compared to some of the conventional methods.

References

Amaro, H. M., Guedes, A. C., & Malcata, F. X. (2011). Antimicrobial activities of microalgae: An invited review. *Science against Microbial Pathogens: Communicating Current Research and Technological Advances*, 1272–1280.

Ásványi-Molnár, N., Sipos-Kozma, Z., Tóth, Á., Ásványi, B., & Varga, L. (2009). Development of functional dairy food enriched in spirulina (*Arthrospira platensis*). *Tejgazdaság*, 69, 15–22.

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, 6, 71–79. https://doi.org/10.1016/j.jpha.2015.11.005.

Bancalari, E., Bernini, V., Bottari, B., Neviani, E., & Gatti, M. (2016). Application of impedance microbiology for evaluating potential acidifying performances of starter lactic acid bacteria to employ in milk transformation. *Frontiers in Microbiology*, 7, 11. https://doi.org/10.3389/fmicb.2016.01628.

Bancalari, E., D'Incecco, P., Savo Sardaro, M. L., Neviani, E., Pellegrino, L., & Gatti, M. (2019). Impedance microbiology to speed up the screening of lactic acid bacteria exopolysaccharide production. *International Journal of Food Microbiology*, 306, 10. https://doi.org/10.1016/j.ijfoodmicro.2019.108268.

Barkallah, M., Dammak, M., Louati, I., Hentati, F., Hadrich, B., Mechichi, T., et al. (2017). Effect of *Spirulina platensis* fortification on physicochemical, textural, antioxidant and sensory properties of yogurt during fermentation and storage. *Lebensmittel- Wissenschaft und -Technologie*, 84, 323–330. https://doi.org/10.1016/j.lwt.2017.05.071.

Batista, A. P., Niccolai, A., Fradinho, P., Fragoso, S., Bursic, I., Rodolfi, L., et al. (2017). Microalgae biomass as an alternative ingredient in cookies: Sensory, physical and chemical properties, antioxidant activity and in vitro digestibility. *Algal Research*, 26, 161–171. https://doi.org/10.1016/j.algal.2017.07.017.

Beheshtipour, H., Mortazavian, A. M., Haratian, P., & Darani, K. K. (2012). Effects of *Chlorella vulgaris* and *Arthrospira platensis* addition on viability of probiotic bacteria in yogurt and its biochemical properties. *European Food Research and Technology*, 235, 719–728. https://doi.org/10.1007/s00217-012-1798-4.

Bottari, B., Santarelli, M., Neviani, E., & Gatti, M. (2010). Natural whey starter for parmigiana reggiano: Culture-independent approach. *Journal of Applied Microbiology*, 108, 1676–1684. https://doi.org/10.1111/j.1365-2672.2009.04564.x.

Cakmak, Y. S., Kaya, M., & Asan-Ozusaglam, M. (2014). Biochemical composition and bioactivity screening of various extracts from *Dunaliella salina*, a green microalga. *EXCLI Journal*, 13, 679–690.

Chorianopoulos, N. G., Lambert, R. J. W., Skandamis, P. N., Evergetis, E. T., Haroutounian, S. A., & Nychas, G.-J. E. (2006). A newly developed assay to study the minimum inhibitory concentration of *Satureja spinosa* essential oil. *Journal of Applied Microbiology*, 100, 778–786. https://doi.org/10.1111/j.1365-2672.2006.02827.x.

De Caire, G. Z., & Parada, J. L. (2000). Effect of *Spirulina platensis* biomass on the growth of lactic acid bacteria in milk. *World Journal of Microbiology and Biotechnology*, 16, 563–565.

D'Incecco, P., Bancalari, E., Gatti, M., Ranghetti, A., & Pellegrino, L. (2020). Low-temperature centrifugation of milk for manufacture of raw milk cheeses: Impact on milk debacterization and cheese yield. *Lebensmittel-Wissenschaft und -Technologie*, 118, 108789. https://doi.org/10.1016/j.lwt.2019.108789.

Elshouny, W. A. E.-F., El-Sheekh, M. M., Sabae, S. Z., Khalil, M. A., & Badr, H. M. (2017). Antimicrobial activity of *Spirulina platensis* against aquatic bacterial isolates. *Journal of Microbiology, Biotechnology and Food Sciences*, 6, 1203–1208. https://doi.org/10.15414/jmbfs.2017.6.5.1203-1208.

Fakruddin, M., Mannan, K. S. B., & Andrews, S. (2013). Viable but nonculturable bacteria: Food safety and public health perspective. *ISRN Microbiology*, 2013, 1–6. https://doi.org/10.1155/2013/703813.

Gatti, M., Bernini, V., Lazzi, C., & Neviani, E. (2006). Fluorescence microscopy for studying the viability of micro-organisms in natural whey starters. *Letters in Applied Microbiology*, 42, 338–343. https://doi.org/10.1111/j.1472-765X.2006.01859.x.

Gibson, A. M., Bratchell, N., & Roberts, T. A. (1988). Predicting microbial growth: Growth responses of *salmonellae* in a laboratory medium as affected by pH, sodium chloride and storage temperature. *International Journal of Food Microbiology*, 6, 155–178. https://doi.org/10.1016/0168-1605(88)90051-7.

Golmakani, M.-T., Soleimanian-Zad, S., Alavi, N., Nazari, E., & Eskandari, M. H. (2019). Effect of Spirulina (*Arthrospira platensis*) powder on probiotic bacteriologically acidified feta-type cheese. *Journal of Applied Phycology*, 31, 1085–1094. https://doi.org/10.1007/s10811-018-1611-2.

Gupta, S., Rajauria, G., & Abu-Ghannam, N. (2010). Study of the microbial diversity and antimicrobial properties of Irish edible brown seaweeds. *International Journal of Food Science and Technology*, 45, 482–489. https://doi.org/10.1111/j.1365-2621.2009.02149.x.

Kumar, V., SaranTirumalai, P., Singh, A., Bhatnagar, A. K., & Shrivastava, J. N. (2013). Natural compounds from algae and *Spirulina platensis* & its antimicrobial activity. *Indo Global Journal of Pharmaceutical Sciences*, 3, 212–223.

Lanzanova, M., Mucchetti, G., & Neviani, E. (1993). Analysis of conductance changes as a growth index of lactic acid bacteria in milk. *Journal of Dairy Science*, 76(1), 20.

Lucera, A., Costa, C., Conte, A., & Del Nobile, M. A. (2012). Food applications of natural antimicrobial compounds. *Frontiers in Microbiology*, 3, 1–13. https://doi.org/10.3389/fmicb.2012.00287.

Mala, R., Sarojini, M., Saravanababu, S., & Umadevi, G. (2009). Screening for antimicrobial activity of crude extracts of *Spirulina platensis*. *Journal of Cell and Tissue Research*, 9(3), 1951–1955.

Manivannan, K., Karthikai devi, G., Anantharaman, P., & Balasubramanian, T. (2011). Antimicrobial potential of selected brown seaweeds from Vedalai coastal waters. Gulf of Mannar. *Asian Pacific Journal of Tropical Biomedicine*, 1, 114–120. https://doi.org/10.1016/S2221-1691(11)60007-5.

Mariod, A. A., Matthäus, B., Idris, Y. M. A., & Abdelwahab, S. I. (2010). Fatty acids, tocopherols, phenolics and the antimicrobial effect of *sclerocarya birrea* kernels with different harvesting dates. *Journal of the American Oil Chemists' Society*, 87, 377–384. https://doi.org/10.1007/s11746-009-1510-4.

Martelli, F., Alinovi, M., Bernini, V., Gatti, M., & Bancalari, E. (2020). B. *Arthrospira platensis* as natural fermentation booster for milk and Soy fermented beverages. *Foods*, 9, 15. https://doi.org/10.3390/foods9030350.

Martelli, F., Favari, C., Mena, P., Guazzetti, S., Ricci, A., Rio, D. D., Lazzi, C., Neviani, E., & Bernini, V. (2020). Antimicrobial and Fermentation Potential of *Himanthalia elongata* in Food Applications. *Microorganisms*, *8*(2), 15. https://doi.org/doi:10.3390/microorganisms8020248.

Pina-Pérez, M. C., Rivas, A., Martínez, A., & Rodrigo, D. (2017). Antimicrobial potential of macro and microalgae against pathogenic and spoilage microorganisms in food. *Food Chemistry*, 235, 34–44. https://doi.org/10.1016/j.foodchem.2017.05.033.

Pisoschi, A. M., Pop, A., Georgescu, C., Turcuş, V., Olah, N. K., & Mathe, E. (2018). An overview of natural antimicrobials role in food. *European Journal of Medicinal Chemistry*, 143, 922–935. https://doi.org/10.1016/j.ejmech.2017.11.095.

Puttaswamy, S. (2013). Novel electrical method for the rapid determination of minimum inhibitory concentration (MIC) and assay of bactericidal/bacteriostatic activity. *Journal of Biosensors & Bioelectronics*, 4, 6. https://doi.org/10.4172/2155-6210.S2- 003.

Ricci, A., Bernini, V., Maoloni, A., Cirlini, M., Galaverna, G., Neviani, E., et al. (2019). Vegetable by-product lacto-fermentation as a new source of antimicrobial compounds. *Microorganisms*, 7, 11. https://doi.org/10.3390/microorganisms7120607.

Santarelli, M., Bottari, B., Lazzi, C., Neviani, E., & Gatti, M. (2013). Survey on the community and dynamics of lactic acid bacteria in Grana Padano cheese. *Systematic & Applied Microbiology*, 36, 593–600. https://doi.org/10.1016/j.syapm.2013.04.007.

Sarada, D. V. L., Sreenath Kumar, C., & Rengasamy, R. (2011). Purified C-phycocyanin from *Spirulina platensis* (nordstedt) geitler: A novel and potent agent against drug resistant bacteria. *World Journal of Microbiology and Biotechnology*, 27, 779–783. https://doi.org/10.1007/s11274-010-0516-2.

Sauer, J. D., Herskovits, A. A., & O'Riordan, M. X. D. (2019). Metabolism of the grampositive bacterial pathogen *Listeria monocytogenes*. *Microbiology Spectrum*, 7(4), https://doi.org/10.1128/microbiolspec.GPP3-0066-2019 Jul.

Silby, M. W., Winstanley, C., Godfrey, S. A. C., Levy, S. B., & Jackson, R. W. (2011). *Pseudomonas* genomes: Diverse and adaptable. *FEMS Microbiology Reviews*, 35, 652–680. https://doi.org/10.1111/j.1574-6976.2011.00269.x.

SY-LAB. https://microbiology.sylab.com/products/p/show/Product/product/bactrac-4300.html Last access on 9th April,2020.

Ulusoy, S., Boşgelmez-Tınaz, G., & Seçilmiş-Canbay, H. (2009). Tocopherol, carotene, phenolic contents and antibacterial properties of rose essential oil, hydrosol and absolute. *Current Microbiology*, 59, 554–558. https://doi.org/10.1007/s00284-009-9475-y.

Varga, L., & Molnár-Ásványi, N. (2012). Manufacturing technology for a *Spirulina*-enriched mesophilic fermented milk. *International Scientific Conference on Sustainable Development & Ecological Footprint*, 1–6.

Whiting, R. C., & Buchanan, R. L. (2007). Progress in microbiological modeling and risk assessment. In M. P. Doyle, & L. R. Beuchat (Vol. Eds.), *Food microbiology: Fundamentals and frontiers*: Vol. 3, (pp. 953–969).

Yang, L., & Bashir, R. (2008). Electrical/electrochemical impedance for rapid detection of foodborne pathogenic bacteria. *Biotechnology Advances*, 26, 135–150. https://doi.org/10.1016/j.biotechadv.2007.10.003.

Yue, X.-F., Shang, X., Zhang, Z.-J., & Zhang, Y.-N. (2017). Phytochemical composition and antibacterial activity of the essential oils from different parts of sea buckthorn (*Hippophae rhamnoides L.). Journal of Food and Drug Analysis*, 25, 327–332. https://doi.org/10.1016/j.jfda.2016.10.010.

Zouari, N., Abid, M., Fakhfakh, N., Ayadi, M. A., Zorgui, L., Ayadi, M., et al. (2011). Bluegreen algae (*Arthrospira platensis*) as an ingredient in pasta: Free radical scavenging activity, sensory and cooking characteristics evaluation. *International Journal of Food Sciences & Nutrition*, 62, 811–813. https://doi.org/10.3109/09637486.2011.582461.

Antimicrobial and fermentation potential of *Himanthalia elongata* for food applications

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Abstract

Himanthalia elongata is a brown oceanic seaweed rich in bioactive compounds. It could play an important role in food production because of its antimicrobial and antioxidant properties. Three strains belonging to *Lactobacillus casei* group (*Lactobacillus casei, Lactobacillus paracasei* and *Lactobacillus rhamnosus*) and a *Bacillus subtilis* strain were used for solid state fermentation of commercial se**a**weeds and bacterial growth was monitored by plate counts method. A High-Pressure Processing (HP) was also employed (6,000 bar, 5 minutes, 5°C) before extraction. The antimicrobial activity of the extracts was tested towards the main food pathogenic bacteria (*Salmonella* spp., *Listeria moncytogenes, Escherichia coli, Staphylococcus aureus* and *Bacillus cereus*), the phenolic content was estimated using the Folin-Ciocalteau method while targeted UHPLC-MS^2 methods were used to unravel the profile in phlorotannins. *H. elongata* allowed the growth of *L. casei* group strains and *B. subtilis*, showing the fermentability of this substrate. A significant antimicrobial activity towards *L. monocytogenes* was observed in the extracts obtained from unfermented samples, but both fermentation and HPP did not enhance the natural antimicrobial activity of this seaweed species. The content in phenolic compounds decreased because of the fermentation process and the amount of phenolics in both unfermented and fermented *H. elongata* extracts being very low. Despite phlorotannins have been related to the natural antimicrobial activity of this brown seaweed, these results did not support this association. Conclusion: Even if fermentation and HPP have not proved to be effective tools to enhance useful compounds from *H. elongata*, the seaweed showed to be a suitable substrate for *L. casei* group strains as well as for *B. subtilis* growth and its extract highlighted antimicrobial activity towards foodborne pathogens.

Introduction

Over the centuries, various preservation techniques have been developed to increase food safety and avoid spoilage during storage and distribution. In recent years, consumers, concerned about the potential side effects of synthetic antimicrobials, are requesting food that contains preservatives of natural origin (Tajkarimi et al., 2010).

This consumer choice has raised the interest in compounds extracted from natural sources, which are increasingly investigated for their antimicrobial activity. Among them, algae represent an interesting raw material, as they are rich in bioactive compounds, and macroalgae have already proved a broad range of applications as antibacterial (Alghazeer et al., 2013; Hongayo et al., 2012; Saleh & Al-Mariri, 2017; Zbakh et al., 2012). Different studies attributed the antimicrobial and antioxidant capacity of seaweed extracts to the presence of bioactive compounds such as phlorotannins, flavonoids, steroids and sulfated polysaccharides (Chandini et al., 2008; Heo et al., 2005; Holdt & Kraan, 2011; Watson & Cruz-Rivera, 2003). These secondary metabolites allow the synthesizing organism a good defense against pathogens, as well as survival in stressful conditions. Moreover, many of these compounds have been proven able to inhibit bacterial growth (Hintz et al., 2015). Some plants peptides might also exert antimicrobial activity, and could be divided in two groups: (i) endogenous peptides, which are already present in the organism, and (ii) peptides generated by enzymatic hydrolysis and/or fermentation (Chai et al., 2019). Moreover, antimicrobial compounds like organic acids can be produced through fermentation (Schnürer & Magnusson, 2005).

Seaweed fermentations are poorly reported in the literature, even if lactic acid bacteria (LAB) and yeasts have been employed, after agar-agar extraction, in red seaweed waste fermentation in order to produce fertilizers (Ennouali et al., 2006). Fermentation of *Undaria pinnatifida* (brown seaweed) has been reported as an alternative feed for aquaculture (Uchida & Murata, 2002), whereas, in the framework of human consumption, a fermented beverage from *Gracilaria fisheri* has been developed (Prachyakij et al., 2008).

Fermentation of *Himanthalia elongata*, a brown seaweed belonging to the order of *Fucales* growing spontaneously on the coast of the Atlantic Ocean, was attempted without success, as neither heat-processed nor raw seaweed was able to support the growth of *Lactobacillus plantarum* (Gupta et al., 2011). However, its antimicrobial activity toward *Escherichia coli* and *Staphylococcus aureus* was demonstrated (Plaza et al., 2010). Eom and colleagues attributed the strong antimicrobial activity of marine brown algae to phlorotannins (Eom et al., 2012), a subclass of phenolic compounds originated from the polymerization of phloroglucinol (1,3,5-trihydroxy benzene), or to sulfated polysaccharides, such as fucoidans. High concentrations of fucose and sulfate, as well as their particular arrangement in brown algal fibers, are probably responsible for the resistance to bacterial fermentation (Michell et al., 1996).

High pressure improved extraction of active compounds by disrupting tissues, cell walls, membranes and organelles, consequently increasing the mass contact of the solvents with the samples. This strategy could represent an opportunity to obtain higher extraction yields with no deleterious effects on the activity and structure of potentially bioactive components (Cox et al., 2014; Jun, 2011, 2013). Despite the evidence on the antimicrobial activity of algae and the role of fermentation in the production of bioactive compounds, the attempt to screen different bacterial species for fermentation and to produce antimicrobial compounds from fermented seaweed has never been tested. For this reason, the aim of the present study was to evaluate i) the capacity of *L. casei* group bacteria and *B. subtilis* to ferment *H. elongate*, ii) the antimicrobial activity of *H. elongata* extract toward foodborne pathogenic bacteria and ii) the effect of biological (fermentation) and technological (HPP) processes on the antimicrobial activity and phenolic composition of *H. elongata*.

Materials and Methods

Sample preparation

H. elongata samples were purchased as dried from ALGAMAR (Pontevedra, Spain) (*HE* I) and Nuova Terra (Prato, Italy) (*HE* II) in order to evaluate variability within the species. They were grinded with Oster 890-48H mixer (Recampro, Spain) and maintained at room temperature in darkness until use.

Standards and reagents

All chemicals and solvents, purchased from Sigma–Aldrich Co. (St. Louis, MO, USA), were of analytical grade. Ultrapure water from MilliQ system (Millipore, Bedford, MA, USA) was used throughout the experiment.

Bacterial strains used for fermentation

Four bacterial strains isolated from food matrices and belonging to different species were used to ferment *HE* I: *Lactobacillus casei* (2240) and *Lactobacillus rhamnosus* (1473) isolated from Parmigiano Reggiano cheese, *Lactobacillus paracasei* (4186) isolated from Pecorino Toscano cheese and *Bacillus subtilis* (5002) isolated from rice. The strains, belonging to the collection of the Department of Food and Drug of the University of Parma, were maintained at -80 °C in de Man, Rogosa and Sharpe (MRS) (Oxoid, Basingstoke, UK) (for LAB) and in Nutrient Broth (Oxoid) (NB) (for *B. subtilis*), added with 12.5% glycerol (v/v).

Set up of fermentation conditions and monitoring

Before fermentation, the frozen cultures were revitalized twice in MRS broth (Oxoid) (inoculum of 3% v/v) incubated overnight at 37 °C under suspended conditions for LAB and in NB incubated overnight at 30°C under shaking for *B. subtilis*. Afterward, LAB and *B. subtilis* were inoculated (3% v/v) in MRS and NB and incubated at the specific temperatures of the species, for 16 h, in order to obtain a concentration of 9 Log cfu/mL. The grown cell cultures were collected by centrifugation (12857xg for 10 minutes at 4 °C), washed twice in

Ringer solution (Oxoid, Milan, Italy) and resuspended in sterile bidistilled water. *HE* I was rehydrated with 75% of water and then inoculated individually with each bacterial suspension to obtain a final concentration of 7 Log cfu/mL. The microbial concentration was evaluated just after the inoculum (T0), after 24 h (T1) and 72 h (T2) of fermentation at the optimal temperature for each strain. Ten fold serial dilutions in Ringer (Oxoid) were plated on MRS agar or Nutrient Agar (NA) for LAB and *B. subtilis*, respectively, and then incubated for 72 h in aerobic conditions at the optimal temperature for each strain. Fermentations were carried out in duplicate and for each sampling time analyses were performed in duplicate. Average values ± standard deviations were reported. After the process, fermented seaweeds were lyophilized.

High-pressure processing (HPP)

High-pressure treatments were performed using the equipment of HPP Italia (Traversetolo, Parma, Italy) on *HE* I. Seaweeds were first rehydrated for 30 minutes with 75% of water at room temperature, put under vacuum in bags and then placed into containers in the hyperbaric chamber that was then filled with cold water for the treatment. The process was carried out at 6,000 bars, which was for 5 minutes at 5 °C. After the treatment, the product was lyophilized. Samples were treated and analyzed in duplicate. Average values ± standard deviations were reported.

Extraction process

In order to extract molecules with potential antimicrobial activity such as polyphenols, small peptides and acids, an extraction process from *HE* I (unfermented, fermented and HPP treated) and *HE* II (unfermented) was carried out. In particular, 100 mL of ethanol/water (70:30 v/v) acidified with 1% formic acid (CH_2O_2) were added to 10 g of lyophilized sample. A double extraction was carried out, alternating twice a shaking cycle to a sonication one in an ultrasonic bath, each lasting 15 minutes. An HS 501 digital shaker (IKA) (Staufen- Germany) was used for the shaking cycle (200 rpm), while the sonication was carried out by means of the Ultrasonic Cleaner sonicator (VWR, United States). The

sample was then centrifuged (Eppendorf 5800 Centrifuge, Model 5810R, Hamburg, Germany) at 12857xg for 10 minutes at 10 °C. The solution was filtered with filter paper to recover the solid part so as to proceed to the second extraction. The two extracts obtained were combined and concentrated by the rotary evaporator Strike 300 (Steroglass, Italy, PG) at 4xg with a bath temperature of 40 °C until fully dried. The concentrated extract was then suspended using sterile water to recover the soluble part and then stored at -80 °C until use. With the aim to test the stability at high temperature, the *HE* I extract underwent a treatment at 121° for 15 minutes by autoclave.

Pathogenic strains

The antimicrobial activity of the extracts obtained from unfermented, fermented and HPP treated *HE* I and unfermented *HE* II was tested towards 14 pathogenic strains belonging to: *Salmonella* spp. (S1: *S. enterica* ATCC 14028; S2: *S. enterica* serotype *Rissen* and S3: *Salmonella* spp. suini), *Listeria monocytogenes* (L1: LM30; L2: LMG 21264 and L3: LMG 13305), *Escherichia coli* (E1: DSM 9025; E2: DSM 10973 and E3: POM 1048), *Staphylococcus aureus* (A1: NCTC 9393; A2: ATCC 6538 and A3: ATCC 19095) and *Bacillus cereus* (C1: 31 and C2: 33). These strains are part of the collection of the Department of Food and Drug (University of Parma, Italy) and of international collections: National Collection of Type Cultures (NCTC), Belgian co-ordinated collection of microorganisms (LMG), American Type Culture Collection (ATCC), Deustsche Sammlung von Mikroorganismen (DSM). They were stored at -80 °C in Tryptic Soy Broth (TSB) (Oxoid) supplemented with 12.5% glycerol (v/v). Before use, bacteria were revitalized twice by inoculum (3% v/v) in TSB added with 0.6% yeast extract followed by incubation for 16 hours at 37 °C in aerobic conditions.

Evaluation of antimicrobial activity in vitro

The evaluation of the antimicrobial activity of the extracts was carried out according to the agar well diffusion assay (Kim et al., 2013) with little modifications. The pathogenic strains were diluted to a concentration of 8 Log cfu/mL and seeded on Tryptone Soy Agar (TSA) (Oxoid) by means of sterile swabs. Then, using sterile tips, wells with a diameter of 7 mm were created in the agar and filled with 30 μL of each extract. Plates were incubated at 37 °C in aerobic conditions and the antimicrobial activity was evaluated by measuring the total inhibition zone (mm) observable after 24, 48 and 120 hours of incubation. Analyses were performed in triplicate and average values ± standard deviations were reported. Water was used as a negative control.

Total phenolic content

The total phenolic content (TPC) of the extracts obtained from unfermented, fermented and HPP treated *HE* I and unfermented *HE* II was determined using the Folin-Ciocalteau method as outlined by Medina-Remón and colleagues (Medina-Remón et al., 2009) with slight modifications (Mena et al., 2013). Briefly, 15 μL of diluted sample were mixed with 170 μL of double-distilled water in a 96-well microplates (Sarstedt AG & Co., Nümbrecht, Germany), then added with 12 μL of Folin–Ciocalteau's reagent and 30 μL of sodium carbonate (200 g/L). The mixtures were kept at room temperature in darkness for 1 h. After the reaction period, 73 μL of double-distilled water were added and absorbance was recorded at 765 nm in a Sunrise™ microplate reader (Tecan, Grödig, Austria). Samples quantification was performed using gallic acid as standard and results were expressed as mg of gallic acid equivalents (GAE) per gram of dry weight (DW). Analyses were performed in triplicate and average values ± standard deviations were reported.

UHPLC-ESI-MS^2 analysis

The extracts obtained from unfermented, fermented and HPP treated *HE* I and unfermented *HE* II were analyzed by ultra-high-performance liquid chromatography (UHPLC) coupled with mass spectrometry (MS) to investigate the presence of phlorotannins. In order to be analyzed, extracts were centrifuged at 15294xg for 10 min, diluted in 0.1% formic acid in water (1:2, v/v), vortexed, centrifuged once again at 10625xg for 10 min at 4 °C, and finally filtered (0.45 μm nylon filter). An Accela UHPLC 1250 apparatus equipped with a linear ion trap MS (LIT-MS) (LTQ XL, Thermo Fisher Scientific Inc., San José, CA, USA) was used. The separation of the compounds was carried out by means of an Acquity UPLC HSS T3 column (100 x 2.1 mm, 1.8 μm particle size, Waters, Milford, MA, USA). For UHPLC, the mobile phase was pumped at a flow rate of 0.3 mL/min, consisting of a mixture of acidified acetonitrile 0.1% formic acid (solvent A) and 0.1% formic acid in water (solvent B). Following 1.50 min of 1% solvent A in B, the proportion of A was linearly increased to 38.3% over 7.10 min, reaching 90% solvent A at 10 min, followed by 3 min at 90% solvent A and then 4 min at the initial condition to re-equilibrate the column. The injection volume was 5 μL and the column was thermostated at 40 °C. The MS worked in negative ionization mode with a capillary temperature set at 275 °C and the source at 250 °C. The sheath gas (N_2) flow was 40 units, while the auxiliary gas (N_2) flow was 10 units. The source voltage was 4 kV, while the capillary voltage and the tube lens voltage were –50 V and –142.75 V, respectively. Targeted MS^2 analyses were carried out to identify phlorotannins, by fragmentation of specific molecular ions (*m*/*z*). Identification was performed by comparison with spectral data reported in the literature.

Statistical analysis

The analyses were conducted separately for *Salmonella* spp, *E. coli*, *S. aureus*, *L. monocytogenes*, *B. cereus* and for each of them two different analyses were performed: one for the extract obtained by non-fermented seaweeds (time of fermentation=0) seaweeds and one for the extracts obtained after fermentation. For both, the effect of different parameters on the inhibition zone was studied by means of linear mixed-effects models (LMM). The radius of inhibition zone was measured at 24, 48 and 120 hours after inoculum. For the statistical analysis of the non-fermented extracts [i) *HE* I *and HE* II, ii) *HE* I HPP treated, iii) *HE* I sterilized], the effect of the incubation time, the effect on the pathogenic bacterial strains as well as the interaction between time of incubation and the kind of extract were considered as "fixed effects", while the effects of the plate and the one of the section nested within the plate were considered as random effects. For the analysis of the extracts obtained after fermentation, the time of fermentation (24 and 72 hours), the effect of the different strains used for fermentation (*B. subtilis* took as reference), the effect on the pathogenic strains, the effect of incubation time (24, 48 and 120 hours) and the interaction between time of incubation and type of extracts were considered as fixed effects. The plate, the section nested within the plate and also the batch of fermentation were considered as a possible source of non-independency. All the analyses were conducted with R (*R: The R Project for Statistical Computing*, n.d.), the LMM model analyses were conducted using the "lme4" package (Bates et al., 2015). For analyses related to the extracts phenolic content, the SPSS statistical package (version 25, SPSS, Inc., Chicago, IL, U.S.A.) was used. One-way analysis of variance (ANOVA) with Duncan post hoc tests as well as t-tests were carried out.

Results and discussion

Himanthalia elongata fermentation

All strains showed a different, but overall good growth capacity on *H. elongata* (Figure 1). Contrary to Gupta et al. (Gupta et al., 2011), who observed that neither raw nor thermally processed *H. elongata* were able to support the growth of *L. plantarum*, in the present work the strains used for fermentation (*L. casei* 2240, *L. paracasei* 4186, *L. rhamnosus* 1473, *B. subtilis* 5002) have demonstrated the capacity to grow in this matrix, opening new perspectives for novel fermented foods based on algae. To the best of our knowledge, the species tested in the present study were never employed for *H. elongata* fermentation. However, *Weissella* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Streptococcus* spp., *L. rhamnosus* were employed for the fermentation of different algal species showing good growth ability (Gupta et al., 2012; Lee et al., 2015; Uchida & Miyoshi, 2013).

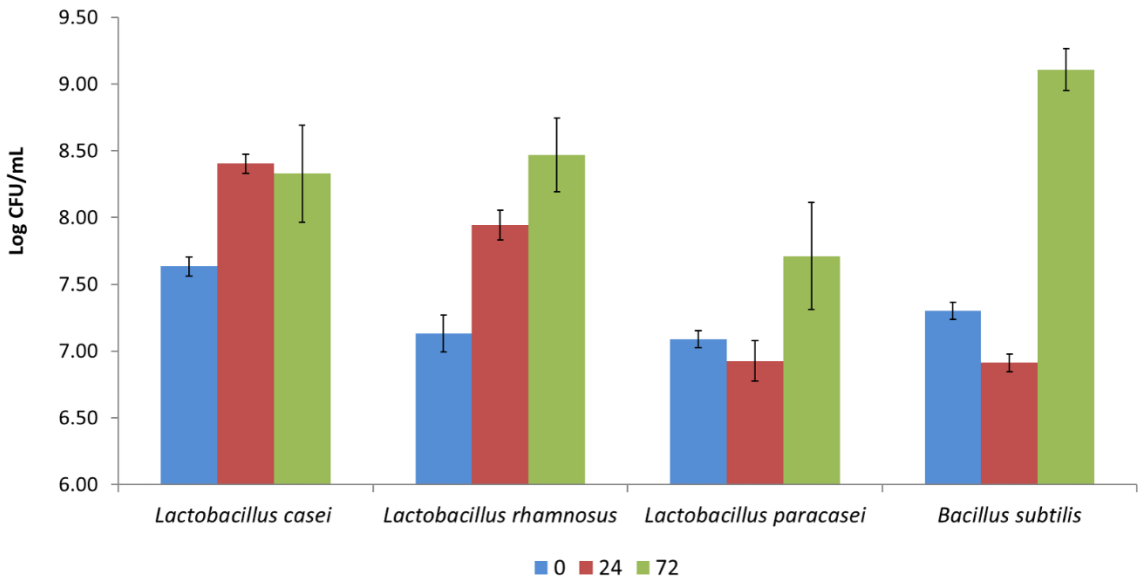

Figure 1. *L. casei* group and *B. subtilis* strains growth ability (Log cfu/mL) in *Himanthalia elongata* after 24 and 72 hours of fermentation at optimal temperatures.

Antimicrobial activity toward foodborne pathogens

Currently, the antimicrobial potential of seaweed toward the main foodborne pathogens is one of the most stimulating fields of research in the area of "marine vegetables". It has been demonstrated that brown algae extracts are the most effective against foodborne pathogens (Pina-Pérez et al., 2017). Several seaweed species have been studied for their rich content in bioactive compounds and their antimicrobial activity has been well known for many years (Sridhar & Vidyavathi, 1991). Each class of seaweed (*Phaeophyceae, Rhodophyta* and *Chlorophyta*), because of the different composition, shows a different degree of antimicrobial activity and different target microorganisms (Mishra, 2018). *Ecklonia cava,* a brown seaweed, was tested toward *L. monocytogenes* and a good antimicrobial activity was found (Nshimiyumukiza, Ossiniel et al., 2015). Several *Sargassum* species were found to be strong antimicrobial agents mainly against Gram-positive bacteria but also towards *Salmonella* spp. (Mishra, 2018). Seaweeds offer opportunities for obtaining new types of bioactive compounds that could be used by the food industry as preservatives; though, the mechanism of inhibition of seaweeds extracts are not always clear. The antimicrobial activity of *H. elongata* compounds has been previously studied (Cox et al., 2014; Plaza et al., 2010; Rajauria et al., 2013), but the effect of fermentation and of HPP on these algae has never been considered. In the present work, the inhibitory activity of algae extracts against the main food pathogenic bacteria was evaluated. As a first observation, the extracts obtained from non-fermented seaweeds differently inhibited target pathogens (Figure 2).

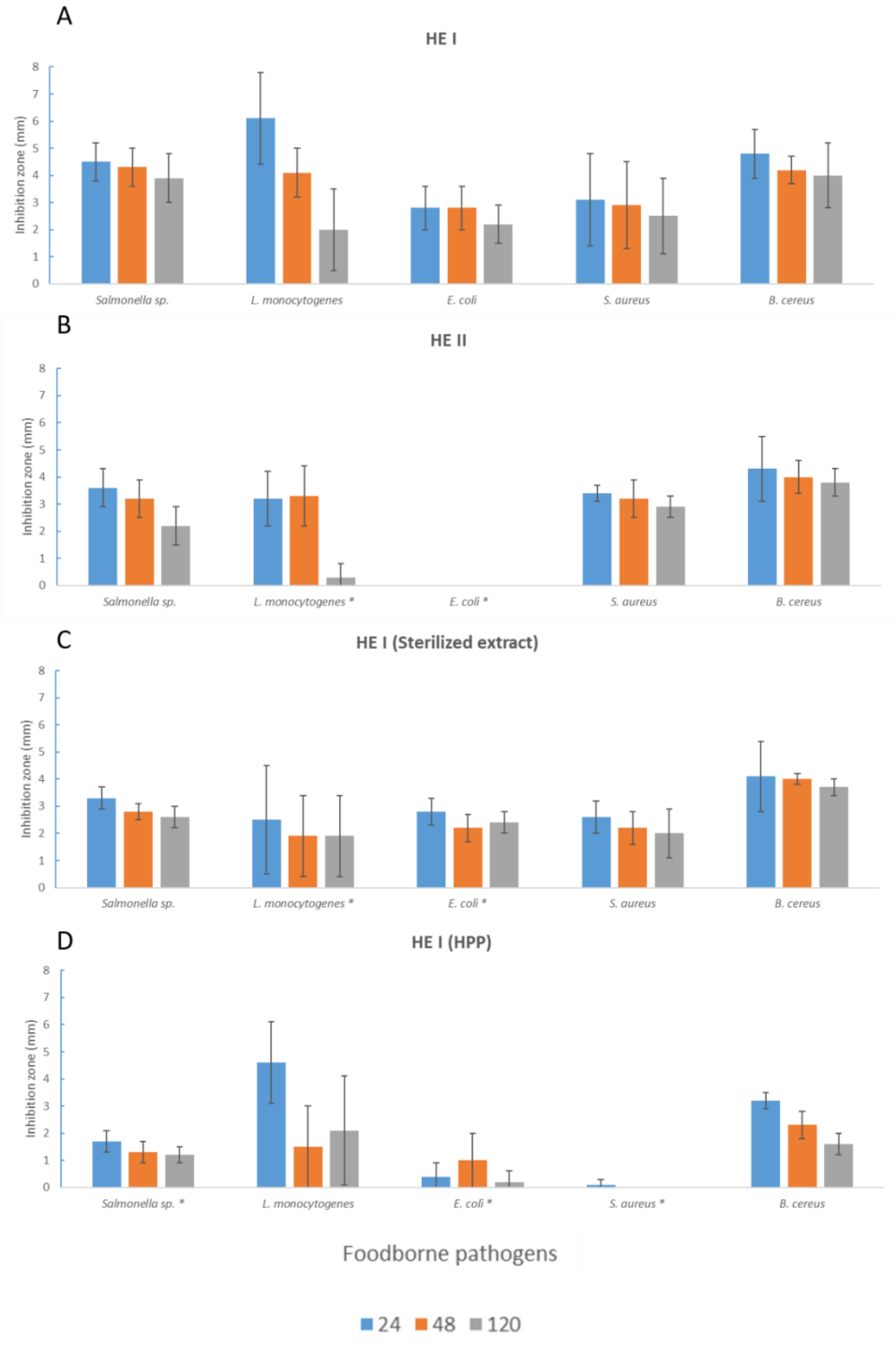

Figure 2. Inhibition radius (mm) of extracts obtained from unfermented seaweed towards foodborne pathogens. Measurements were taken after 24, 48 and 120 hours. Values ± standard deviations are reported. With * expresses the significance ($p < 0.05$) between the antimicrobial activity of *HE* I and of the other extracts (*HE* II, *HE* I (Sterilized Extract) and *HE* I (HPP)) for each time point (24, 48, and 120 h).

HE I and *HE* II were provided from different suppliers in order to evaluate a possible variability within the same species. Indeed, the two extracts showed a significantly different behavior against *L. monocytogenes* ($p<0.05$ and *E. coli* ($p<0.001$). However, most of the pathogenic species tested were affected by both the extracts. Antimicrobial activity towards different pathogens could be related to the wide number of compounds observed in seaweeds (polysaccharides, polyunsaturated fatty acids, phlorotannins, other phenolic compounds, carotenoids, etc.) (Pina-Pérez et al., 2017). Nevertheless, many natural factors, such as the environmental condition (light, temperature and salinity), the life stage (as the age of seaweed), the geographical location and seasonality of growth and harvesting can influence it (Hollants et al., 2013; Pérez et al., 2016). Since HE I explained better antimicrobial performances compared to HE II, it was subjected to different treatments such as heat temperature, fermentation and HPP, in order to check their effects on antimicrobial activity. *HE* I extract showed a good heat resistance, as the thermal treatment induced a significant reduction in antimicrobial activity only towards *L. monocytogenes* ($p<0.05$). The resistance of the extract to high temperature could be useful for applications in thermally processed products, to prevent the occurrence of post-processing contamination. In order to study the possibility to enhance seaweed's antimicrobial activity, a high-pressure treatment, considered a promising strategy for extraction of bioactive ingredients from plant material (Jun, 2013), was performed. However, the antimicrobial activity didn't increase but, on the contrary, the efficacy against *Salmonella* spp. ($p<0.001$), *E. coli* ($p<0.001$ and *S. aureus* ($p<0.05$) was reduced after HPP treatment. All the extracts by non fermented samples, regardless of the seaweed and the treatment, lost the inhibitory activity for all the tested microorganisms after 120 h of incubation at the optimum growth temperature, and this was already significant after 48 h for *L. monocytogenes* ($p=0.001$). *HE* I was also tested after *L. casei, L. rhamnosus L. paracasei* fermentation to evaluate the contribution of microorganisms and their metabolic activity (Figure 3 and 4).

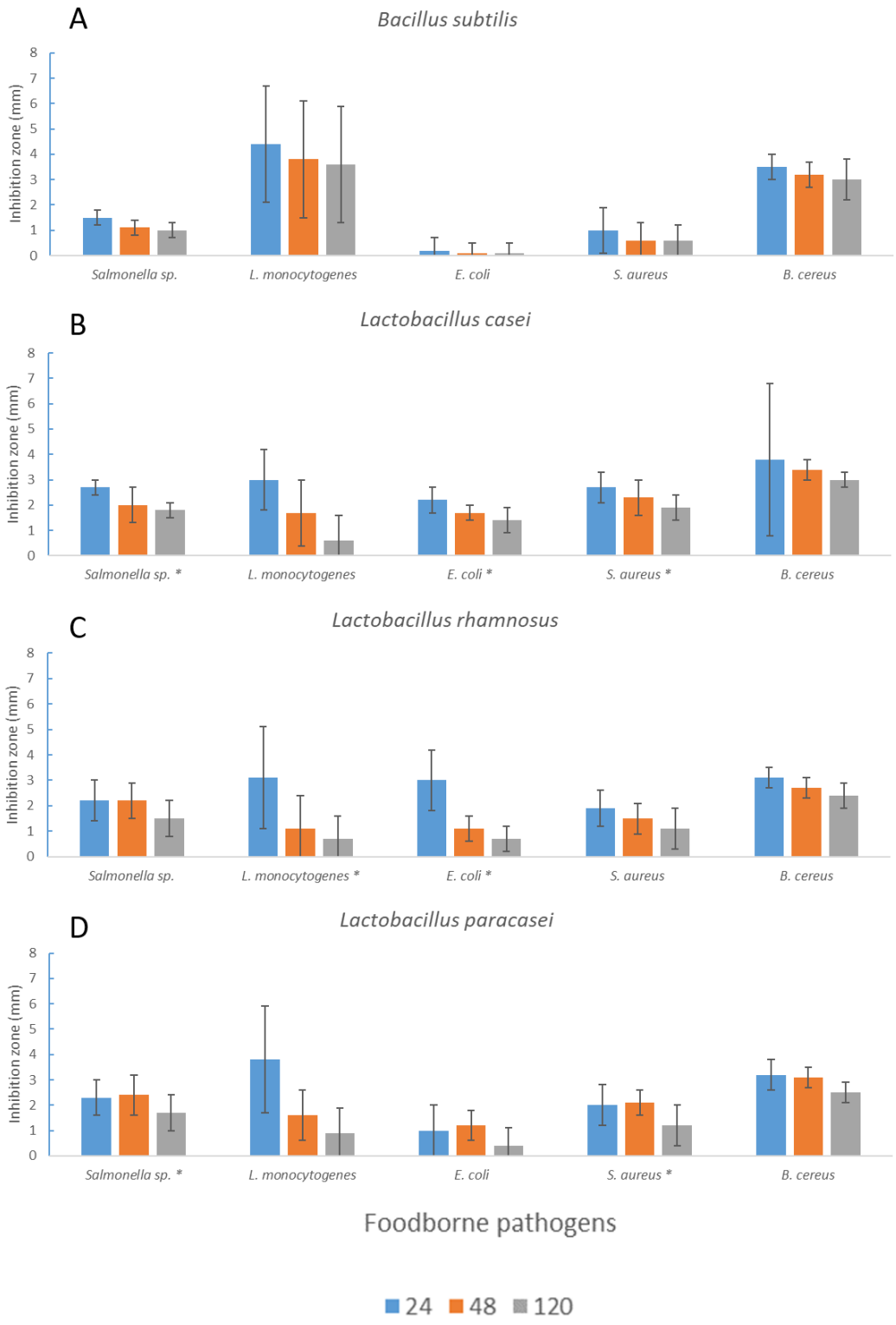

Figure 3. Inhibition radius (mm) of fermented *HE* I extracts after 24 hours fermentation. Measurements were taken after 24, 48 and 120 hours. Average values ± standard deviations are reported. With * is expressed the significance ($p < 0.05$) between *H. elongata* fermented with *B. subtilis* and the same seaweed fermented with *L. casei* group (*L. casei, L. rhamnosus* and *L. paracasei*) strains.

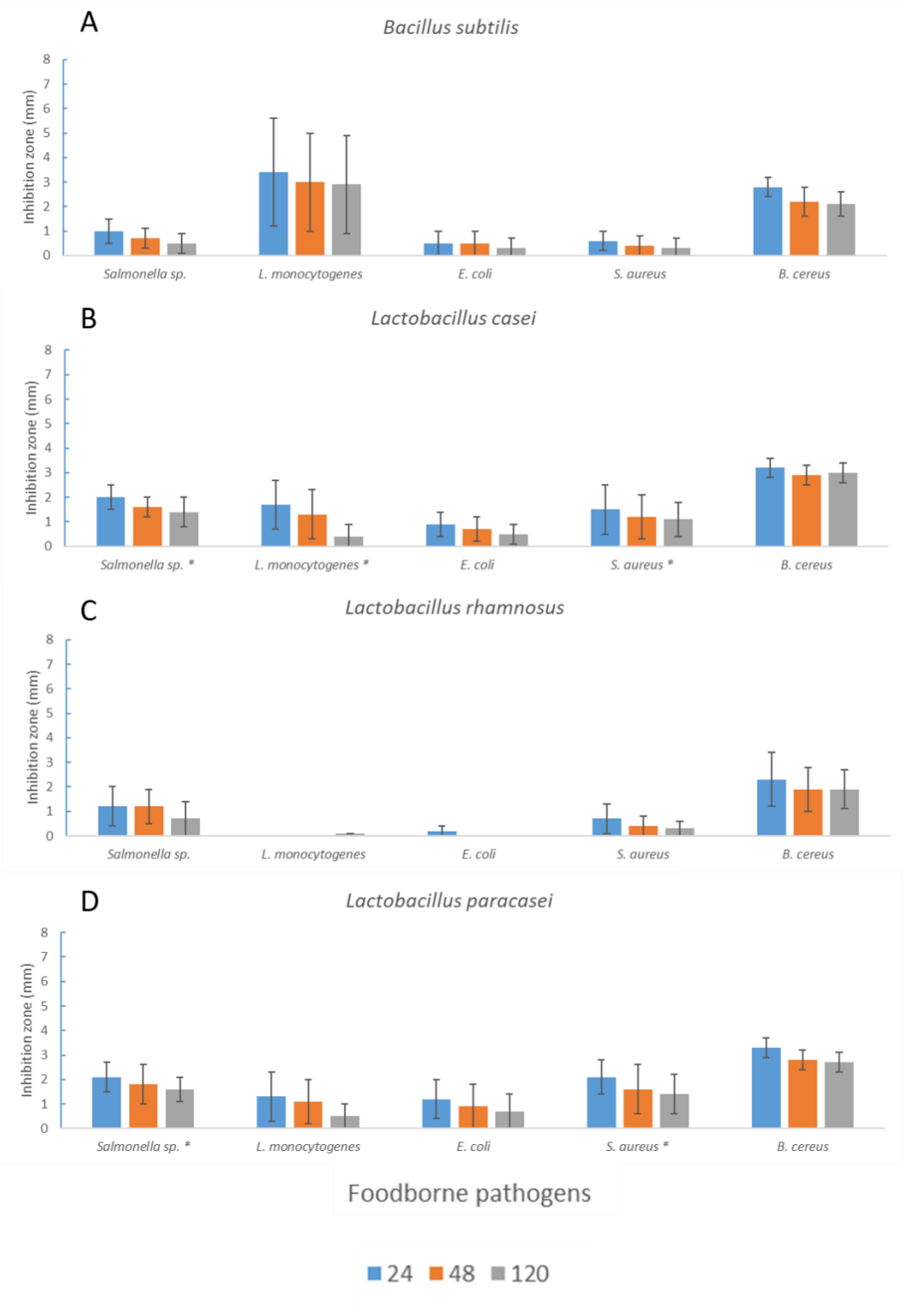

Figure 4. Inhibition radius (mm) of *HE* I extracts after 72 hours fermentation. Measurements were taken after 24, 48 and 120 hours. Average values ± standard deviations are reported. With * is expressed the significance ($p < 0.05$) between *H. elongata* fermented with *B. subtilis* and the same seaweed fermented with *L. casei* group (*L. casei, L. rhamnosus* and *L. paracasei*) strains.

Extracts after LAB fermentation gave rise to a stronger antimicrobial activity compared with those obtained after fermentation with *B. subtilis* (with an exception for *L. monocytogenes)*. The extracts obtained after *L. casei* and *L. paracasei* fermentation showed significantly higher activity towards *Salmonella* spp. and *S. aureus*, whereas in the case of *L. rhamnosus,* a significantly lower antimicrobial activity on *L. monocytogenes* ($p<0.05$) but a significantly higher one towards *E. coli* ($p<0.05$) could be observed. Overall, extracts obtained after seaweed fermentation significantly lost their activity towards all the tested pathogenic bacteria (except for *E. coli*) over time (Figure 3 and 4). The comparison between the antimicrobial activity (considered as an average value) of extracts derived from fermentation or not is reported in Figure 5. Fermentation did not enhance the natural antimicrobial activity of this brown seaweed. Recent papers regarding plant fermentation have shown a significant enhancement of antimicrobial activity towards pathogens (Chai et al., 2019; Kantachote et al., 2008; Moayedi et al., 2016), but this was not confirmed in the case of *H. elongata* in the present work. Possibly, some bacterial catabolic activity broke down the compounds originally exerting the antimicrobial activity in the raw extracts, resulting in a lower efficacy.

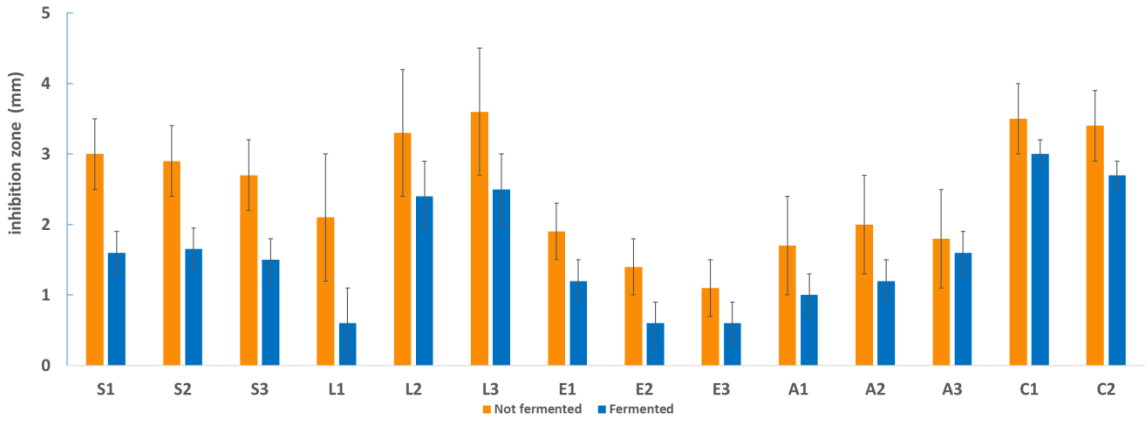

Figure 5. Antimicrobial activity of extract from not fermented and fermented samples (considered as average values of inhibition radius measurements) towards each strain of foodborne pathogenic bacteria, *Salmonella* spp. (S1: *S. enterica* ATCC 14028; S2: *S. enterica* serotype *Rissen* and S3: *Salmonella* spp. suini), *Listeria monocytogenes* (L1: LM30; L2: LMG 21264 and L3: LMG 13305), *Escherichia coli* (E1: DSM 9025; E2: DSM 10973 and E3: POM 1048), *Staphylococcus aureus* (A1: NCTC 9393; A2: ATCC 6538 and A3: ATCC 19095) and *Bacillus cereus* (C1: 31 and C2: 33).

Total phenolic content

TPCs were similar for *HE* I and *HE* II extracts obtained from unfermented samples, with values of 2.94 ± 0.28 mg GAE/g DW and 3.22 ± 0.21 mg GAE/g DW, respectively. Results from the Folin-Ciocalteau assay showed that TPCs were significantly higher for extracts deriving from non-fermented samples compared to fermented ones (Table 6). Quantitatively, fermentation caused a 10-fold decrease of TPC values, while the time of fermentation (24 and 72 hours) did not affect TPCs. After 24 hours of fermentation, *L. casei* led to a smaller reduction in the TPC compared to the other microorganisms, while *L. casei* and *L. paracasei* were responsible for a greater TPC reduction after 72 hours of fermentation. Regarding HPP, the extract deriving from HPP-treated seaweed had a total content in phenolic compounds slightly lower (2.72 ± 0.03 mg GAE/g DW) than the corresponding untreated extract (2.94 ± 0.28 mg GAE/g DW), but the difference was not statistically significant. This might indicate that HPP does not influence the seaweed extract TPC. In general, it should be noted that the amount in TPC of all these seaweed extracts was quite low, in line with the literature (Cox et al., 2014; Marinho et al., 2019; Rodríguez-Bernaldo de Quirós et al., 2010). High variability in the TPC of seaweeds and, in particular, of *H. elongata* had been previously reported, due to multiple factors like collection season, geographical region, etc. (Holdt & Kraan, 2011; Marinho et al., 2019).

Table 1. Total phenolic content of *H. elongata* extracts obtained after fermentation. Values are presented as mean ± SD (n=3). TPC is expressed as mg gallic acid equivalents/g dry weight. Means within each row with different letters (a–b) differ significantly ($p < 0.05$), while means within column 24 h and 72 h with different letters (A-B) differ significantly ($p < 0.05$)

		***HE* I**	***24h***	***72h***
TPC (mg GAE/g DW)	***Lactobacillus casei***	2.94 ± 0.28^{a}	0.27 ± 0.01$^{b, A}$	0.21 ± 0.02$^{b, B}$
	Lactobacillus paracasei	2.94 ± 0.28^{a}	0.20 ± 0.00$^{b, B}$	0.20 ± 0.02$^{b, B}$
	Lactobacillus rhamnosus	2.94 ± 0.28^{a}	0.22 ± 0.01$^{b, B}$	0.32 ± 0.08$^{b, A}$
	Bacillus subtilis	2.94 ± 0.28^{a}	0.20 ± 0.02$^{b, B}$	0.36 ± 0.00$^{b, A}$

Phlorotannin identification by UHPLC-MS² analysis

The UHPLC-MS² targeted analyses used allowed the tentative identification of a total of 20 phlorotannins (Table 7). The compounds were tentatively identified based on the interpretation of their mass spectral behavior obtained from MS² experiments and by comparison with literature (Hermund et al., 2018; Lopes et al., 2018; Vissers et al., 2017). The identified phlorotannins exhibited distinct molecular weights (370–870 Da) and degree of polymerization (3–7 phloroglucinol units). Interestingly, all the compounds were identified in *HE* II (Table 7), while none was found in *HE* I. This could be due to many factors occurring during seaweed cultivation and growth, but quite possibly to a lesser exposure of *HE* I to sources of stress, such as microbial infections or UV-radiation (Arnold & Targett, 2003; Lopes et al., 2018). Quantification was not carried out due to the lack of proper commercially available standards for this particular group of hydrolyzable tannins.

Table 2. Mass spectral characteristic of tentatively identified phlorotannins in *Himantalia elongata* (*HE*II)

Compound	RT (min)	$[M–H]^-$ (*m*/*z*)	MS^2 ion fragments (*m*/*z*)	Ref
Trimer	4.56	369	351	a
Trimer	5.16	369	279, 351, 325, 307	a
Trimer	5.34	369	351, 295, 325, 307	a
Trimer	5.65	369	351, 279, 325, 307	a
Trimer	7.00	369	238	a
Trimer	7.14	369	238	a
Trimer	1.50	373	355, 207, 329, 165	b
Trimer (Phlorethol)	5.39	373	231, 355	c
Trimer (Fucophlorethol)	5.75	373	233, 247, 229, 355, 125	c
Tetramer	5.22	497	479, 407, 371	
Tetramer	5.40	497	479, 353, 371, 335	b
Tetramer	5.85	497	235	a
Tetramer	6.36	497	355, 371, 479	a
Tetramer	6.69	497	373, 371, 233, 353, 238, 479	b
Pentamer	5.95	621	603, 339, 337	a, b
Pentamer	6.22	621	603, 339, 357, 337, 229	a, b
Pentamer (Fucol)	7.16	621	495, 371, 497, 477, 229, 603	c
Pentamer (Fuhalol)	8.06	651	509, 465, 413, 607, 339, 582	c
Hexamer	6.51	745	727, 601	a
Heptamer	7.12	869	851, 842, 833	b, c

RT: retention time, $[M–H]^-$: molecular ion

Fragment ions are listed in order of relative abundances

[a] Tentatively identified based on the mass spectral data reported by (Lopes et al., 2018)

[b] Tentatively identified based on the mass spectral data reported by (Hermund et al., 2018)

[c] Tentatively identified based on the mass spectral data reported by (Vissers et al., 2017) extract.

Conclusions

Seaweeds are an underestimated and suitable source of food and food ingredients. Their richness in bioactive compounds makes them a possible source of food preservatives and other useful molecules. The results highlighted that *H. elongata* extracts were more efficient against *L. monocytogenes* and *B. cereus*. The extracts obtained from *H. elongata* showed antimicrobial activity against various food pathogenic bacteria. A great difference was observed between *HE* I and *HE* II: despite being the same species a great variability, probably due to environmental factors or to treatments suffered before commercialization, has emerged. The tested treatments (sterilization, HPP and fermentation) negatively affected the antimicrobial activity of *H. elongata*. In conclusion: i) *H. elongata* demonstrated to be a suitable substrate for *L. casei* group bacteria and *B. subtilis* growth, ii) its extract highlighted antimicrobial activity towards foodborne pathogens, iii) fermentation was not an appropriate technology to obtain innovative antimicrobial compounds from *H. elongata*, iv) HPP, often used as a tool to improve extraction of bioactive compounds from plant matrixes, didn't enhanced the natural antimicrobial activity and the phenolic content of this seaweed, v) the content in phenolic compounds decreased as a consequence of the fermentation process. Further studies are required to better understand what may be the compounds behind the antimicrobial activity of *H. elongata* extracts.

References

Alghazeer, R., Whida, F., Abduelrhman, E., Gammoudi, F., & Azwai, S. (2013). Screening of antibacterial activity in marine green, red and brown macroalgae from the western coast of Libya. *Natural Science*, 5(1), 7–14. https://doi.org/10.4236/ns.2013.51002

Arnold, T. M., & Targett, N. M. (2003). To grow and defend: lack of tradeoffs for brown algal phlorotannins. *Oikos*, 100(2), 406–408. https://doi.org/10.1034/j.1600-0706.2003.11680.x

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, 67(1), 48. https://doi.org/10.18637/jss.v067.i01

Chai, T.-T., Tan, Y.-N., Ee, K.-Y., Xiao, J., & Wong, F.-C. (2019). Seeds, fermented foods, and agricultural by-products as sources of plant-derived antibacterial peptides. *Critical Reviews in Food Science and Nutrition*, 59(1), 162–177. https://doi.org/10.1080/10408398.2018.1561418

Chandini, S. K., Ganesan, P., & Bhaskar, N. (2008). In vitro antioxidant activities of three selected brown seaweeds of India. *Food Chemistry*, 107(2), 707–713. https://doi.org/10.1016/j.foodchem.2007.08.081

Cox, S., Hamilton Turley, G., Rajauria, G., Abu-Ghannam, N., & Jaiswal, A. K. (2014). Antioxidant potential and antimicrobial efficacy of seaweed (*Himanthalia elongata*) extract in model food systems. *Journal of Applied Phycology*, 26(4), 1823–1831. https://doi.org/10.1007/s10811-013-0215-0

Ennouali, M., Ouhssine, M., Ouhssine, K., & Elyachioui, M. (2006). Biotransformation of algal waste by biological fermentation. *African Journal of Biotechnology*, 5(13), 1233–1237.

Eom, S.-H., Kim, Y.-M., & Kim, S.-K. (2012). Antimicrobial effect of phlorotannins from marine brown algae. *Food and Chemical Toxicology*, 50(9), 3251–3255. https://doi.org/10.1016/j.fct.2012.06.028

Gupta, S., Abu-Ghannam, N., & Rajauria, G. (2012). Effect of heating and probiotic fermentation on the phytochemical content and antioxidant potential of edible Irish brown seaweeds. *Botanica Marina*, 55(5), 527–537. https://doi.org/10.1515/bot-2011-0052

Gupta, S., Abu-Ghannam, N., & Scannell, A. G. M. (2011). Growth and kinetics of *Lactobacillus plantarum* in the fermentation of edible Irish brown seaweeds. *Food and Bioproducts Processing*, 89(4), 346–355. https://doi.org/10.1016/j.fbp.2010.10.001

Heo, S., Park, E., Lee, K., & Jeon, Y. (2005). Antioxidant activities of enzymatic extracts from brown seaweeds. *Bioresource Technology*, 96(14), 1613–1623. https://doi.org/10.1016/j.biortech.2004.07.013

Hermund, D. B., Plaza, M., Turner, C., Jónsdóttir, R., Kristinsson, H. G., Jacobsen, C., & Nielsen, K. F. (2018). Structure dependent antioxidant capacity of phlorotannins from Icelandic *Fucus vesiculosus* by UHPLC-DAD-ECD-QTOFMS. *Food Chemistry*, 240, 904–909. https://doi.org/10.1016/j.foodchem.2017.08.032

Hintz, T., Matthews, K. K., & Di, R. (2015). The Use of Plant Antimicrobial Compounds for Food Preservation. *BioMed Research International*, 2015, 1–12. https://doi.org/10.1155/2015/246264

Holdt, S. L., & Kraan, S. (2011). Bioactive compounds in seaweed: functional food applications and legislation. *Journal of Applied Phycology*, 23(3), 543–597. https://doi.org/10.1007/s10811-010-9632-5

Hollants, J., Leliaert, F., De Clerck, O., & Willems, A. (2013). What we can learn from sushi: a review on seaweed-bacterial associations. *FEMS Microbiology Ecology*, 83(1), 1–16. https://doi.org/10.1111/j.1574-6941.2012.01446.x

Hongayo, M. C., Larino, R. C., & Malingin, D. L. (2012). Antibacterial and Antioxidant Effects of Brown Alga *Padina australis* Hauck Crude Extract. *IAMURE International Journal of Science and Clinical Laboratory*, 2(1), 13. https://doi.org/10.7718/iamure.ijscl.v2i1.388

Jun, X. (2011). Micromechanism of ultrahigh pressure extraction of active ingredients from green tea leaves. *Food Control*, 22, 1473–1476.

Jun, X. (2013). High-Pressure Processing as Emergent Technology for the Extraction of Bioactive Ingredients From Plant Materials. *Critical Reviews in Food Science and Nutrition*, 53(8), 837–852. https://doi.org/10.1080/10408398.2011.561380

Kantachote, D., Charernjiratrakul, W., & Umsakul, K. (2008). Antibacterial Activities of Fermented Plant Beverages Collected in Southern Thailand. Antibacterial Activities of Fermented Plant Beverages Collected in Southern Thailand, *Journal of Biological Sciences*, 8(8), 1280–1288. https://doi.org/10.3923/jbs.2008.1280.1288

Kim, S.-J., Cho, A. R., & Han, J. (2013). Antioxidant and antimicrobial activities of leafy green vegetable extracts and their applications to meat product preservation. *Food Control*, 29(1), 112–120. https://doi.org/10.1016/j.foodcont.2012.05.060

Lee, S.-J., Lee, D.-G., Park, S.-H., Kim, M., Kong, C.-S., Kim, Y.-Y., & Lee, S.-H. (2015). Comparison of biological activities in *Sargassum siliquanstrum* fermented by isolated lactic acid bacteria. *Biotechnology and Bioprocess Engineering*, 20(2), 341–348. https://doi.org/10.1007/s12257-015-0112-2

Lopes, G., Barbosa, M., Vallejo, F., Gil-Izquierdo, Á., Andrade, P. B., Valentão, P., Pereira, D. M., & Ferreres, F. (2018). Profiling phlorotannins from *Fucus* spp. of the Northern Portuguese coastline: Chemical approach by HPLC-DAD-ESI/MS and UPLC-ESI-QTOF/MS. *Algal Research*, 29, 113–120. https://doi.org/10.1016/j.algal.2017.11.025

Marinho, G. S., Sørensen, A.-D. M., Safafar, H., Pedersen, A. H., & Holdt, S. L. (2019). Antioxidant content and activity of the seaweed *Saccharina latissima*: a seasonal perspective. *Journal of Applied Phycology*, 31(2), 1343–1354. https://doi.org/10.1007/s10811-018-1650-8

Medina-Remón, A., Barrionuevo-González, A., Zamora-Ros, R., Andres-Lacueva, C., Estruch, R., Martínez-González, M.-Á., Diez-Espino, J., & Lamuela-Raventos, R. M. (2009). Rapid Folin–Ciocalteu method using microtiter 96-well plate cartridges for solid phase extraction to assess urinary total phenolic compounds, as a biomarker of total polyphenols intake. *Analytica Chimica Acta*, 634(1), 54–60. https://doi.org/10.1016/j.aca.2008.12.012

Mena, P., Martí, N., Saura, D., Valero, M., & García-Viguera, C. (2013). Combinatory Effect of Thermal Treatment and Blending on the Quality of Pomegranate Juices. *Food and Bioprocess Technology*, 6(11), 3186–3199. https://doi.org/10.1007/s11947-012-0961-z

Michell, C., Lahaye, M., Bonnet, C., Mabeau1, S., & Barry, J.-L. (1996). In vitro fermentation by human faecal bacteria of total and purified dietary fibres from brown seaweeds. *British Journal of Nutrition*, 75(2), 263–280. https://doi.org/10.1017/BJN19960129

Mishra, A. K. (2018). *Sargassum, Gracilaria* and *Ulva* Exhibit Positive Antimicrobial Activity against Human Pathogens. *OALib*, 5(e4258), 1–12. https://doi.org/10.4236/oalib.1104258

Moayedi, A., Hashemi, M., & Safari, M. (2016). Valorization of tomato waste proteins through production of antioxidant and antibacterial hydrolysates by proteolytic *Bacillus subtilis*: optimization of fermentation conditions. *Journal of Food Science and Technology*, 53(1), 391–400. https://doi.org/10.1007/s13197-015-1965-2

Nshimiyumukiza, Ossiniel, Kang, Shin-Kook, Kim, Hyo-Jung, Lee, Eun-Hye, Han, Hae-Na, Kim, Yunhye, Kim, Deok-Hoon, Kim, Ji-Hoon, Eom, Sung-Hwan, & Kim, Young-Mog. (2015). Synergistic Antibacterial Activity of *Ecklonia cava (Phaeophyceae: Laminariales)* against *Listeria monocytogenes (Bacillales: Listeriaceae)*. *Fisheries and Aquatic Sciences*, 18(1), 1–6. https://doi.org/10.5657/FAS.2015.0001

Pérez, M., Falqué, E., & Domínguez, H. (2016). Antimicrobial Action of Compounds from Marine Seaweed. *Marine Drugs*, 14(3), 52. https://doi.org/10.3390/md14030052

Pina-Pérez, M. C., Rivas, A., Martínez, A., & Rodrigo, D. (2017). Antimicrobial potential of macro and microalgae against pathogenic and spoilage microorganisms in food. *Food Chemistry*, 235, 34–44. https://doi.org/10.1016/j.foodchem.2017.05.033

Plaza, M., Santoyo, S., Jaime, L., García-Blairsy Reina, G., Herrero, M., Señoráns, F. J., & Ibáñez, E. (2010). Screening for bioactive compounds from algae. *Journal of Pharmaceutical and Biomedical Analysis*, 51(2), 450–455. https://doi.org/10.1016/j.jpba.2009.03.016

Prachyakij, P., Charernjiratrakul, W., & Kantachote, D. (2008). Improvement in the quality of a fermented seaweed beverage using an antiyeast starter of *Lactobacillus plantarum* DW3 and partial sterilization. *World Journal of Microbiology and Biotechnology*, 24(9), 1713–1720. https://doi.org/10.1007/s11274-008-9662-1

R: The R Project for Statistical Computing. (n.d.). Retrieved October 16, 2019, from https://www.r-project.org/

Rajauria, G., Jaiswal, A. K., Abu-Gannam, N., & Gupta, S. (2013). Antimicrobial, antioxidant and free radical-scavenging capacity of brown seaweed *Himanthalia elongata* from western coast of Ireland: antimicrobial and antioxidant properties of irish seaweed. Journal of Food Biochemistry, 37(3), 322–335. https://doi.org/10.1111/j.1745-4514.2012.00663.x

Rodríguez-Bernaldo de Quirós, A., Frecha-Ferreiro, S., Vidal-Pérez, A. M., & López-Hernández, J. (2010). Antioxidant compounds in edible brown seaweeds. *European Food Research and Technology*, 231(3), 495–498. https://doi.org/10.1007/s00217-010-1295-6

Saleh, B., & Al-Mariri, A. (2017). Antimicrobial Activity of the Marine Algal Extracts against Selected Pathogens. *J. Agr. Sci. Tech*., 19, 1067–1077.

Schnürer, J., & Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science & Technology*, 16(1–3), 70–78. https://doi.org/10.1016/j.tifs.2004.02.014

Sridhar, K. R., & Vidyavathi, N. (1991). Antimicrobial Activity of Seaweeds. *Acta Hydrochimica et Hydrobiologica*, 19(5), 455–496. https://doi.org/10.1002/aheh.19910190502

Tajkarimi, M. M., Ibrahim, S. A., & Cliver, D. O. (2010). Antimicrobial herb and spice compounds in food. *Food Control*, 21(9), 1199–1218. https://doi.org/10.1016/j.foodcont.2010.02.003

Uchida, M., & Miyoshi, T. (2013). Algal Fermentation—The Seed for a New Fermentation Industry of Foods and Related Products. *JARQ*, 47(1), 53–63.

Uchida, M., & Murata, M. (2002). Fermentative preparation of single cell detritus from seaweed, *Undaria pinnatifida,* suitable as a replacement hatchery diet for unicellular algae. *Aquaculture*, 207, 345–357. https://doi.org/10.1016/S0044-8486(01)00792-X

Vissers, A. M., Caligiani, A., Sforza, S., Vincken, J.-P., & Gruppen, H. (2017). Phlorotannin Composition of *Laminaria digitata*. *Phytochemical Analysis*, 28(6), 487–495. https://doi.org/10.1002/pca.2697

Watson, S. B., & Cruz-Rivera, E. (2003). Algal chemical ecology: an introduction to the special issue. *Phycologia*, 42(4), 319–323. https://doi.org/10.2216/i0031-8884-42-4-319.1

Zbakh, H., Chiheb, H., Bouziane, H., Sánchez, V. M., & Riadi, H. (2012). Antibacterial activity of benthic marine algae extracts from the Mediterranean coast of Morocco. *Journal of Microbiology, Biotechnology and Food Sciences*, 2(1), 219–228.

Chapter 2:

Algae and food safety

Ready to eat algae can represent a food safety issue? A focus on microbial contamination and foodborne pathogens behavior by microbiological challenge test

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Abstract

Consumption of seaweeds is becoming progressively popular in western countries where seaweeds are often categorized as a superfood. Algae can be marketed fresh, but they are usually sold dehydrated to ensure longer shelf life. The growing trend of consumption, often as ready to eat, open up possible risks for public health because of food pathogenic bacteria that can contaminate the raw material during harvesting or manipulation. In this study, fourteen ready to eat foods based on dehydrated algae, representative of the most consumed species, were considered. Plate counts were carried out to investigate the microbial content of food products and *Bacillus cereus* strains were isolated and identified by 16s rRNA gene sequencing. The microbiological quality was heterogeneous among the samples and, in particular, the presence of marine bacteria, *B. cereus* and coliforms were detected. To contribute to related risk assessment, the ability of *B. cereus* strains to proliferate into seaweed-based food was evaluated, to our knowledge for the first time, by microbiological challenge test on two ready to eat foods based on *Undaria pinnatifida* and *Palmaria palmata*. Despite this study demonstrated the inability of *B. cereus* strains to grow in seaweed-based food, their presence in dehydrated foodstuffs cannot rule out the replication after rehydration before consumption, making it necessary to shed light on the possible risks for consumers.

Introduction

Algae are an important component of the daily diet in some countries such as China, Korea, Japan, Indonesia and the Philippines with traditional consumption as dried or fresh form. Even though seaweeds are considered a superfood and consumers' interest is growing (Palmieri & Forleo, 2020), their use is not yet usual in western countries, with some exceptions along the Atlantic coast such as Ireland, France, Maine and Nova Scotia (Birch et al., 2019; Chapman et al., 2015; Fleurence et al., 2012; Holdt & Kraan, 2011). Thanks to their composition, algae are considered a good source of functional ingredients and bioactive compounds like phytosterols, polyunsaturated fatty acids, fibers, vitamins, minerals and amino acids. More than twenty million tons of seaweeds are globally harvested each year. The main producers are China and Indonesia, and half of the production is used for human consumption as dried products (Buschmann et al., 2017). Seaweeds can be marketed fresh but, usually, they are air-dried at temperatures ranging from 50 to 60 °C (Gupta & Abu-Ghannam, 2011; López-Pérez et al., 2017), then packed and consumed after hydration and cooking or as ready to eat food without any further heat treatment or processing (Moore et al., 2002). The growing tendency in consumption of dried algae also in Western countries, often as ready to eat food or as ingredients in complex formulations, opens up new scenarios concerning public health because of food pathogenic bacteria that can contaminate the raw material during harvesting and following manipulation. Indeed, many contaminants may occur along the process and clear relationships between macroalgae and bacteria that colonize their surface have been demonstrated: seaweeds provide nutrients while the bacterial community promotes algal growth and protects the host against pathogens (Hollants et al., 2013). The drying process exhibits a selective effect on seaweed-associated bacteria consortium by reducing water activity, losing moisture, and thus promoting the survival of spore-forming bacteria (del Olmo et al., 2017). The Bacillales order is one of the most recognized on these products and in particular *Bacillus cereus* has been isolated in dehydrated samples (del Olmo et al., 2017; Hollants et al., 2013). When stress conditions are

overcome, spore-forming bacteria can return to vegetative form, colonizing, and then developing in hydrated seaweeds in domestic conditions (Schoeni & Lee Wong, 2005).

To shed light on the possible biological hazards associated with novel foods, the main objective of the present study was to: I) investigate the microbial populations present in commercial ready to eat dehydrated algae, focusing on pathogenic bacteria; II) evaluate *B. cereus* behavior in commercial *Undaria pinnatifida* and *Palmaria palmata* ready to eat food during refrigerated storage by microbiological challenge tests.

Materials and Methods

Algae samples

Food samples were purchased on the Italian market, considering different brands and different producers. Only ready to eat (RTE) products were chosen and analyzed. A total of fourteen dehydrated samples, representing seven species of algae were selected. Ready to eat food containing *Undaria pinnatifida, Palmaria palmata, Arthrospira platensis, Porphyra* spp., *Laminaria* spp., *Ulva* spp. *and Hizikia fusiformis* were purchased considering two different suppliers (I and II).

Microbial plate counts

Dehydrated samples were homogenized (Seward Stomacher, 400 circulator, UK) with sterile Ringer solution (Oxoid, Basingstoke, UK), tenfold diluted, and plated in duplicate on different culture media. Total mesophilic bacteria were determined on Plate Count Agar (PCA) (Oxoid, Bagsintoke, UK) incubated at 30° C for 48 h. Marine bacteria were determined on Marine broth 2216 (BD Difco, Heidelberg, Germany) added with bacteriological agar (Oxoid, Basingstoke, UK) (1,5 % w/v) and incubated at 30° C for 72 h. Total coliforms and *Escherichia coli* were quantified on Chromocult Coliform Agar (Merck, Darmstadt, Germany) after incubation at 37° C for 24 h. Moulds and yeasts were enumerated on Yeast Extract-Dextrose-Chloramphenicol (YEDC, Remel, San Diego, California, Usa) incubated at 30° C for 72 hours. ALOA (Agar Listeria acc. to Ottaviani & Agosti, Biolife, Milan, Italy) incubated at 37° C for 24 hours was used for the enumeration of *Listeria* spp. and *Listeria monocytogenes*. For the research of aerobic spore-forming bacteria, the homogenized samples were at first treated at 80° C for 10 minutes and, after dilution, plated on Nutrient Agar (VWR, Radnor, Pennsylvania, Usa) and on Brilliance Bacillus cereus (Oxoid, Basingstoke, UK) for the selection of *B. cereus*. Plates were incubated under aerobic conditions. Microbial counts were performed in duplicate. Average values ± standard deviations were reported as Log cfu/g.

Isolation and identification of B. cereus strains

Five colonies grown on Brilliance Bacillus cereus agar were isolated through four purification steps on the same media. The purified strains were examined under optical microscope Olympus BX51 (Olympus, Waltham, United States) to evaluate morphology, and then stored at – 80° C in Tryptic Soy Broth (TSB) (Oxoid, Basingstoke, UK) plus 20% glycerol (v/v) until use.

Before genotypic identification, strains were grown twice at 37 °C for 18 h in TSB (Oxoid) in aerobic conditions. The DNA was extracted and purified by using DNeasy and Blood Tissue Kit (Qiagen, Germany) and checked on agarose gel with TAE 1X running buffer (1% w/v). Then, the 16S rRNA gene was amplified by PCR (Martelli, Bancalari, et al., 2020). The amplification products were sequenced (Macrogen Europe Inc.) and sequence comparisons were performed against the NCBI database using BLASTN (Basic Local Alignment Tool, BLAST, p://www. ncbi.nlm.nih.gov/blast.cgi).

Microbiological Challenge Test

Two ready to eat foods based on *U. pinnatifida* and *P. palmata* were selected for two experimental trials designed to evaluate the behavior of *B. cereus* artificially contaminating the products. Samples were rehydrated with 75% of sterile water just before contamination that was performed by using three *B. cereus* strains isolated and identified in the previous step. Each frozen stock culture was transferred twice (2% inoculum v/v) into TSB (Oxoid) and incubated at 37°C for 18 h in aerobic conditions. The grown cell cultures were collected by centrifugation (12,857× g for 10 min at 4 °C), washed twice in Ringer solution (Oxoid), and suspended in equal volumes in sterile bidistilled water used for seaweed rehydration to obtain contamination ranging between 2 and 3 Log cfu/g. Rehydrated but not contaminated seaweeds were considered as control samples. Aliquots of rehydrated seaweeds, both contaminated and not, were prepared and stored at refrigeration temperatures for four days to simulate domestic conservation. *B. cereus* was monitored by plate count on Brilliance Bacillus cereus (Oxoid) just after inoculum (T0) and after 24h (T1), 48h (T2), 72h (T3) and 96h

(T4) of storage. Analysis were performed in triplicate for each sampling time. Data were reported as average values of Log cfu/g ± standard deviations.

Statistical analysis

Two tailed t-test for independent samples was applied to determine a significant difference ($p<0.05$) between sample I and II of the same algal species.

Results and Discussion

Microbial contamination of algae

The consumption of algae, also as RTE products, is growing (Martelli, Alinovi, et al., 2020) and the evaluation of their microbiota with particular focus on the presence and behaviour of food pathogens is of fundamental importance to contribute to safety assessment. In the present work, commercial RTE samples based on algae were analyzed by plate counts to investigate their microbial content. The results are reported in Figure 1.

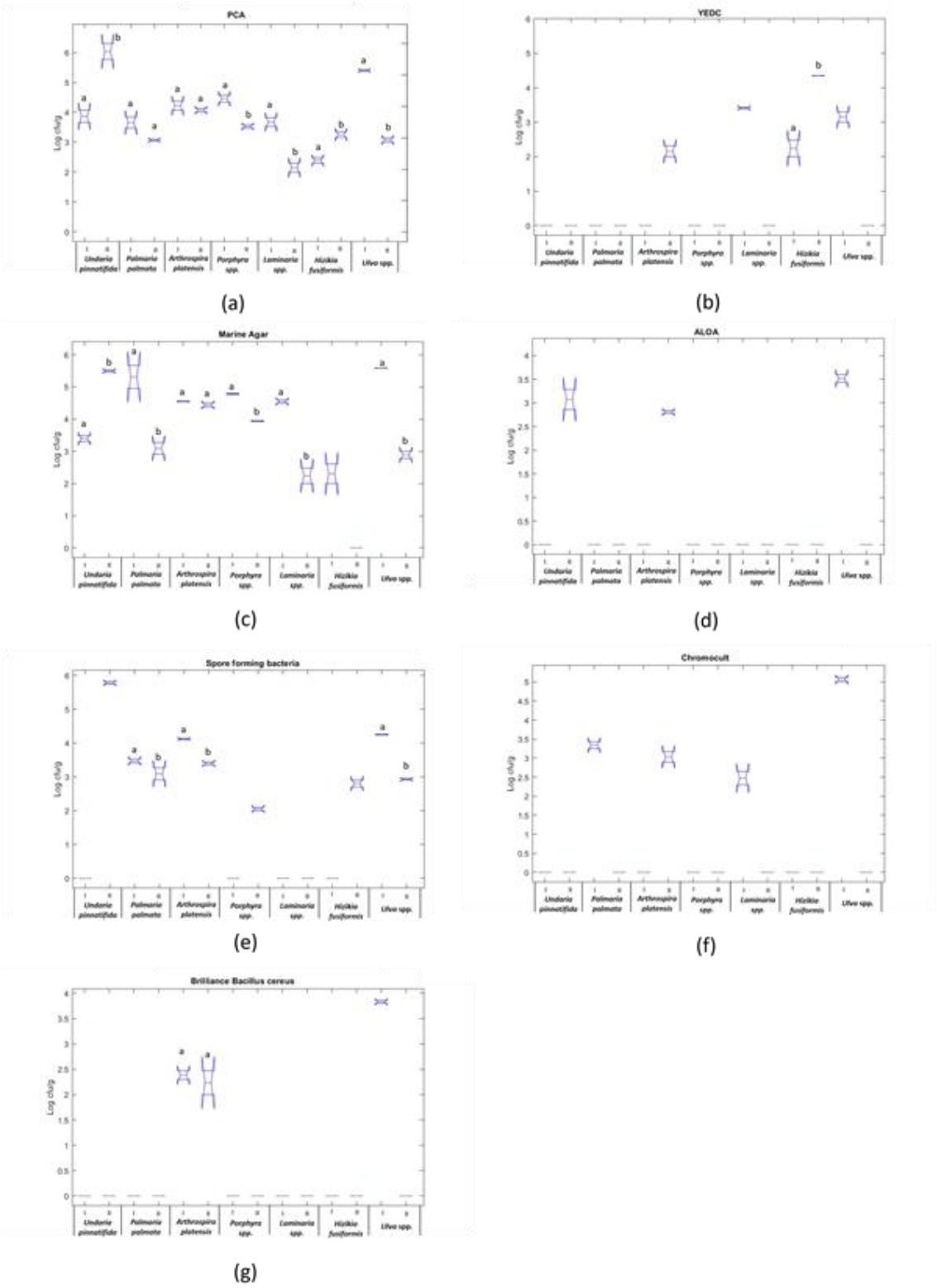

Figure 1. Microbial plate counts of dehydrated ready to eat algae-based products determined on different culture media (a. PCA; b. YEDC; c. Marine agar; d. ALOA; e. Nutrient Agar; f. Chromocult; g. Brilliance Bacillus cereus). Values are reported as Log cfu/g ± standard deviation. Significant differences between the count of the two replicates ($p<0.05$) are expressed with letters (a,b).

Products based on *U. pinnatifida* highlighted a highly heterogeneous microbial load, confirming previous data (del Olmo et al., 2017). In *U. pinnatifida* I, a low number of microorganisms, never exceeding 4 Log cfu/g, was detected, differently from sample II characterized by a significantly higher charge of microorganisms (6.03 ± 0.37 Log cfu/g). In the same sample, colonies were counted on ALOA, but they were not attributable to *L. monocytogenes* species. Also 5.78 ± 0.05 Log cfu/g referred to spore-forming bacteria were found in the sample. Significant differences between samples I and II were also found considering Marine Agar ($p< 0.05$) plate counts. Quantification of microorganisms in *P. palmata* didn't show significant differences among samples: only marine bacteria were 2 Log cfu/g higher in *Palmaria palmata* I than in *Palmaria palmata* II ($p<0.05$). Two products based on *A. platensis* were also analyzed. Commercially known as Spirulina, *A. platensis* is a cyanobacterium widely consumed for its valid composition in proteins, vitamins, essentials amino acids, polyunsaturated fatty acids, minerals, polyphenols, carotenoids and chlorophyll (Niccolai et al., 2019). Both samples presented a similar microbial load in PCA and in Marine Agar. *A. platensis* I had a significantly higher number of spore-forming bacteria while *A. platensis* II presented 2.15 ± 0.21 Log cfu/g in YEDC, 3.03 ± 0.19 Log cfu/g in Chromocult and 2.80 ± 0.04 Log cfu/g in ALOA (not attributable to *L. monocytogenes*) that were not detected in sample I. Raceway ponds are the most common cultivation method of these kinds of microalgae, but it can be problematic because bacterial strains, mainly including *Bacillus* spp., *Alteromonas* spp., *Flavobacterium* spp. and *Pseudomonas* spp. are reported as contaminants during cultivation and could be maintained in the dried biomass (Wang et al., 2013). *B. cereus* was indeed found in both samples at levels ranging between 1.61 Log cfu/g and 2.67 Log cfu/g (Figure 1g). *Porphyra* spp., commonly known as Nori, is the most known and consumed seaweed. In both samples, only marine bacteria and total mesophilic bacteria were detected, while coliforms, *Listeria* spp. or spore-forming bacteria were below the detection limit (1 Log cfu/g) (Figure 1). In the sample I total mesophilic bacteria and marine bacteria were higher than 4 Log cfu/g, while lower numbers ($p<0.05$) were detected in sample II.

Laminaria spp. showed significant differences between the two samples considered. As previously reported for *Porphyra* spp., total mesophilic bacteria and marine bacteria were significantly higher in sample I respect to sample II ($p<0.05$). Moreover, in the first one 2.47 ± 0.24 Log cfu/g in chromocult and 3.41 ± 0.04 Log cfu/g in YEDC were also detected. *H. fusiformis* samples had significant differences in PCA and YEDC ($p<0.05$) plate counts. *Ulva* spp. (I) showed a higher microbial load than what was observed in other studies referred to *Ulva lactuca* (del Olmo et al., 2017). In particular, a high number of total coliforms and spore-forming bacteria were detected (Figure 1e and 1f), probably reflecting fecal contamination that occurred during manipulation at harvesting or conveyed by cultivation water. Colonies were able to grow on ALOA, but none of them was attributable to *L. monocytogenes*. In the same sample, *B. cereus* was detected at a concentration of about 4 Log cfu/g (Figure 1g) though, a significantly lower charge was found in *Ulva* spp. (II). That values agree with previous reports on *Ulva lactuca* sample (del Olmo et al., 2017).

In general, the highest amount of bacteria was detected both on PCA, as representative of the total aerobic mesophilic bacteria, and on Marine agar. In Marine agar, the presence of halophilic microorganisms, characteristic of the marine habitat, was detected in almost all samples with variable concentrations ranging from 2 to 5 Log cfu/g. The bacteria able to grow on this media are lower than what was found in another study (del Olmo et al., 2017). The main reason could be the different collecting area and season. Overall, the presence of yeasts and moulds was variable as also found by Del Olmo and colleagues (*4*) (Figure 1b).

Characteristic *Listeria* spp. colonies, even if not belonging to *L. monocytogenes* specie, were revealed on ALOA in 5 samples. Total coliforms were detected at the maximum concentration of 5 Log cfu/g in *Ulva* spp. I and this amount is higher than what is found in the literature (*4*). This data could reflect a low quality of cultivation water or a contamination during the dehydration or further processing steps. The presence of spore-forming microorganisms was relevant in most of the samples, reaching more than 4 Log cfu/g in some of them (Figure 1e). Only in 3 samples, blue-green colonies were found on Brillance Bacillus cereus Agar. Overall microbial contamination in commercial products was not generally high but the presence of sporogenic pathogens like *B. cereus* and coliforms may open risk scenarios for consumers.

B. cereus identification

Five *B. cereus* strains were isolated from two different species of algae: *A. platensis* (I and II) (concentration of about 2.5 Log cfu/g) and from *Ulva* spp. (I) with a concentration of 3.83 ± 0.04 Log cfu/g. *B. cereus* is generally recognized as ubiquitous in nature and particularly in a marine environment (Liu et al., 2017), soil, dust, and plants (Schoeni & Lee Wong, 2005). During the last decades, an increasing number of psychrotolerant *B. cereus* strains have been described. This species is characterized by strains ables to grow below 7 °C and produce endospores (Stenfors & Granum, 2001). Spores can be passively diffused and contaminate also foods. Norwegian surface waters have been studied for the presence of *B. cereus* spores and cytotoxic strains have been isolated from several rivers (Ostensvik et al., 2004). This suggests the possibility that water supply may be a source for *B. cereus* entering the food processing chain.

The presence of *B. cereus* in the analyzed samples arises food safety issues and implies possible risks for human health related to RTE algae consumption. Moreover, considering that seaweeds and cyanobacteria may represent worldwide a future source of nutrients, it can be referred to a global concern. So, microbiological control of collection waters or during cultivation steps, such as cleaning and drying of algae according to good hygienic practices, could surely reduce the presence of environmental contaminants among which undesirable microorganisms. Microbiota of wild seaweeds is deeply being investigated from an ecological point of view (Hollants et al., 2013) but determination and quantification of food-related bacteria contaminating dehydrated algae have not been yet sufficiently detailed. This raises the need for specific investigations concerning safety issues about dried seaweeds and cyanobacteria consumption. Indeed, many products containing raw algae are also commercialized as RTE foods, which makes it even more important to evaluate the presence and the behavior of pathogenic bacteria on this type of foods that are attracting more and more consumers.

The behavior of B. cereus in seaweed-based products during refrigerated storage

Algae sold in the dehydrated form are microbiologically stable because of the low water content but, although the growth of pathogens is greatly discouraged, it cannot be excluded after rehydration and during food storage, also considering their use as ingredients in many recipes. Moreover, some *B. cereus* strains have demonstrated to be able to grow at low temperatures and it is of fundamental importance to study their behavior during shelf life at refrigerated conditions (Ivy et al., 2012).

Taking into account that *B. cereus* strains were isolated from the commercial food products analyzed, the growth ability of this pathogen was challenged, for the first time to our knowledge, on RTE seaweed salads. In particular, two seaweed products based on *U. pinnatifida* and *P. palmata* were considered in a scenario of rehydration and storage for domestic consumption.

In figure 2 is illustrated the behavior of a mixture of three strains of *B. cereus* during storage at refrigeration temperature. The bacterial charge was evaluated just after artificial contamination of the product (T0) and daily for 4 days of shelf life (T1, T2, T3, and T4).

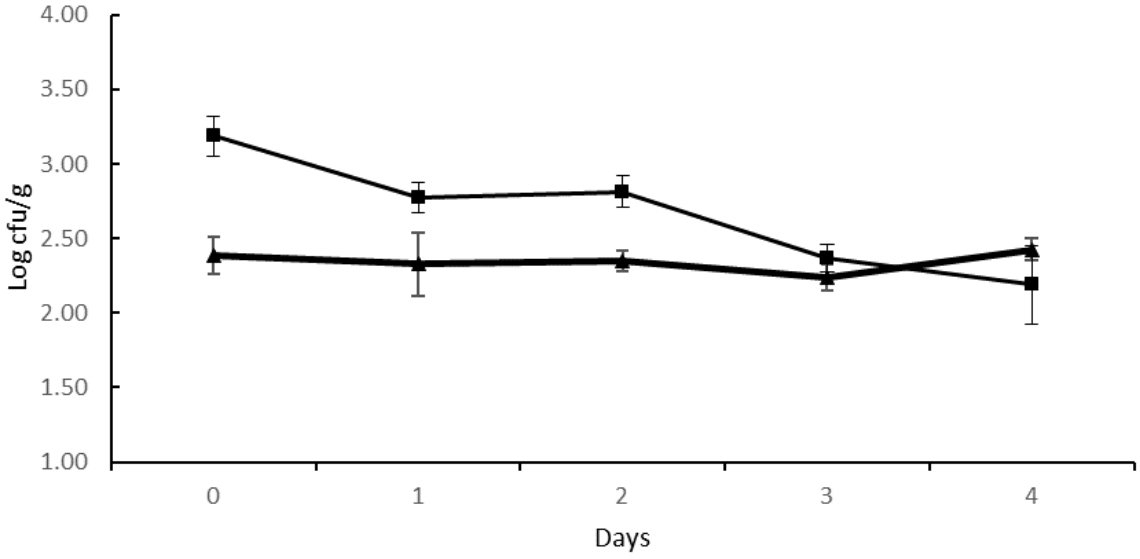

Figure 2. The behavior of *B. cereus* in seaweeds food products based on *U. pinnatifida* (■) and *P. palmata* (▲) evaluated by Microbiological challenge test during a 4 days shelf life at refrigeration conditions.

The reduction of the microbial load of *B. cereus*, artificially contaminating samples with 3.18 ± 0.14 Log cfu/g, was evident in *U. pinnatifida*. After a first decrease during the first 24 hours of shelf life, the number of cells remained stable up to 48 h. Then *B. cereus* still decreased up to reach a concentration of 2.19 ± 0.27 Log cfu/g at the end of the storage, with an overall reduction of 1 Log cfu/g compared to the initial artificial contamination. *B. cereus* did not find favorable conditions for growth, maybe due to the compositional characteristics of *U. pinnatifida* (Patra et al., 2017). In *P. palmata* it was observed a quite different behavior. Indeed, the initial artificial contamination with 2.39 ± 0.12 Log cfu/g of *B. cereus* did not decrease but remained stable along the shelf life considered.

Anyway, even if *U. pinnatifida* had a bactericidal effect, also *P. palmata* didn't allow the growth of *B. cereus* during a four days' shelf-life at refrigerated conditions demonstrating that, even considering *B. cereus* strains adapted to this niche, the growth of this pathogen was discouraged in the condition here tested.

The probable reason is the natural antimicrobial activity of seaweeds, traceable to the composition of the organism itself (Martelli, Favari, et al., 2020), but also to the epiphytic bacteria that populate the surface of seaweeds (Horta et al., 2014). From this study transpires that microbiological hazards cannot be excluded, and for that reason, it is necessary to deepen these aspects to contribute to a more complete assessment of the risk associated with different algae consumption habits.

References

Birch, D., Skallerud, K., & Paul, N. A. (2019). Who are the future seaweed consumers in a Western society? Insights from Australia. *British Food Journal*, *121*(2), 603–615. https://doi.org/10.1108/BFJ-03-2018-0189

Buschmann, A. H., Camus, C., Infante, J., Neori, A., Israel, Á., Hernández-González, M. C., Pereda, S. V., Gomez-Pinchetti, J. L., Golberg, A., Tadmor-Shalev, N., & Critchley, A. T. (2017). Seaweed production: Overview of the global state of exploitation, farming and emerging research activity. *European Journal of Phycology*, *52*(4), 391–406. https://doi.org/10.1080/09670262.2017.1365175

Chapman, A. S., Stévant, P., & Larssen, W. E. (2015). Food or fad? Challenges and opportunities for including seaweeds in a Nordic diet. *Botanica Marina*, *58*(6), 423–433. https://doi.org/10.1515/bot-2015-0044

del Olmo, A., Picon, A., & Nuñez, M. (2017). The microbiota of eight species of dehydrated edible seaweeds from North West Spain. *Food Microbiology*, *70*, 224–231. https://doi.org/10.1016/j.fm.2017.10.009

Fleurence, J., Morançais, M., Dumay, J., Decottignies, P., Turpin, V., Munier, M., Garcia-Bueno, N., & Jaouen, P. (2012). What are the prospects for using seaweed in human nutrition and for marine animals raised through aquaculture? *Trends in Food Science & Technology*, *27*(1), 57–61. https://doi.org/10.1016/j.tifs.2012.03.004

Gupta, S., & Abu-Ghannam, N. (2011). Recent developments in the application of seaweeds or seaweed extracts as a means for enhancing the safety and quality attributes of foods. *Innovative Food Science & Emerging Technologies*, *12*(4), 600–609. https://doi.org/10.1016/j.ifset.2011.07.004

Holdt, S. L., & Kraan, S. (2011). Bioactive compounds in seaweed: Functional food applications and legislation. *Journal of Applied Phycology*, *23*(3), 543–597. https://doi.org/10.1007/s10811-010-9632-5

Hollants, J., Leliaert, F., De Clerck, O., & Willems, A. (2013). What we can learn from sushi: A review on seaweed-bacterial associations. *FEMS Microbiology Ecology*, *83*(1), 1–16. https://doi.org/10.1111/j.1574-6941.2012.01446.x

Horta, A., Pinteus, S., Alves, C., Fino, N., Silva, J., Fernandez, S., Rodrigues, A., & Pedrosa, R. (2014). Antioxidant and Antimicrobial Potential of the *Bifurcaria bifurcata* Epiphytic Bacteria. *Marine Drugs*, *12*(3), 1676–1689. https://doi.org/10.3390/md12031676

Ivy, R. A., Ranieri, M. L., Martin, N. H., den Bakker, H. C., Xavier, B. M., Wiedmann, M., & Boor, K. J. (2012). Identification and Characterization of Psychrotolerant Sporeformers Associated with Fluid Milk Production and Processing. *Applied and Environmental Microbiology*, *78*(6), 1853–1864. https://doi.org/10.1128/AEM.06536-11

Liu, Y., Lai, Q., Du, J., & Shao, Z. (2017). Genetic diversity and population structure of the *Bacillus cereus* group bacteria from diverse marine environments. *Scientific Reports*, *7*(689), 11. https://doi.org/10.1038/s41598-017-00817-1

López-Pérez, O., Picon, A., & Nuñez, M. (2017). Volatile compounds and odour characteristics of seven species of dehydrated edible seaweeds. *Food Research International*, *99*, 1002–1010. https://doi.org/10.1016/j.foodres.2016.12.013

Martelli, F., Alinovi, M., Bernini, V., Gatti, M., & Bancalari, E. (2020). *Arthrospira platensis* as Natural Fermentation Booster for Milk and Soy Fermented Beverages. *Foods*, *9*(350), 15. https://doi.org/doi:10.3390/foods9030350

Martelli, F., Bancalari, E., Neviani, E., & Bottari, B. (2020). Novel insights on pink discoloration in cheese: The case of Pecorino Toscano. *International Dairy Journal*, *111*, 104829. https://doi.org/10.1016/j.idairyj.2020.104829

Martelli, F., Favari, C., Mena, P., Guazzetti, S., Ricci, A., Rio, D. D., Lazzi, C., Neviani, E., & Bernini, V. (2020). Antimicrobial and Fermentation Potential of *Himanthalia elongata* in Food Applications. *Microorganisms*, *8*(2), 15. https://doi.org/doi:10.3390/microorganisms8020248

Moore, J. E., Xu, J., & Millar, B. C. (2002). Diversity of the microflora of edible macroalga (*Palmaria palmata*). *Food Microbiology*, *19*(2–3), 249–257. https://doi.org/10.1006/fmic.2001.0467

Niccolai, A., Shannon, E., Abu-Ghannam, N., Biondi, N., Rodolfi, L., & Tredici, M. R. (2019). Lactic acid fermentation of *Arthrospira platensis* (spirulina) biomass for probiotic-based products. *Journal of Applied Phycology*, *31*(2), 1077–1083. https://doi.org/10.1007/s10811-018-1602-3

Ostensvik, O., From, C., Heidenreich, B., O'Sullivan, K., & Granum, P. E. (2004). Cytotoxic *Bacillus* spp. Belonging to the *B. cereus* and *B. subtilis* groups in Norwegian surface waters. *Journal of Applied Microbiology*, *96*(5), 987–993. https://doi.org/10.1111/j.1365-2672.2004.02226.x

Palmieri, N., & Forleo, M. B. (2020). The potential of edible seaweed within the western diet. A segmentation of Italian consumers. *International Journal of Gastronomy and Food Science*, *20*, 1–9. https://doi.org/10.1016/j.ijgfs.2020.100202

Patra, J. K., Lee, S.-W., Park, J. G., & Baek, K.-H. (2017). Antioxidant and Antibacterial Properties of Essential Oil Extracted from an Edible Seaweed *Undaria Pinnatifida*: Antioxidant Potential of Essential Oil from *Undaria Pinnatifida*. *Journal of Food Biochemistry*, *41*(e12278), 10. https://doi.org/10.1111/jfbc.12278

Schoeni, J. L., & Lee Wong, A. C. (2005). *Bacillus cereus* Food Poisoning and Its Toxins. *Journal of Food Protection*, *68*(3), 636–648. https://doi.org/10.4315/0362-028X-68.3.636

Stenfors, L. P., & Granum, P. E. (2001). Psychrotolerant species from the *Bacillus cereus* group are not necessarily *Bacillus weihenstephanensis*. *FEMS Microbiology Letters*, *197*(2), 223–228. https://doi.org/10.1111/j.1574-6968.2001.tb10607.x

Wang, H., Zhang, W., Chen, L., Wang, J., & Liu, T. (2013). The contamination and control of biological pollutants in mass cultivation of microalgae. *Bioresource Technology*, *128*, 745–750. https://doi.org/10.1016/j.biortech.2012.10.158

Mitigation of marine toxins by bacterial fermentation: the case of okadaic acid and tetrodotoxin

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Toxins

Abstract

Harmful algal bloom and marine toxins are actual and overgrowing problems. Okadaic acid (OA), produced by several species of dinoflagellates, is the main toxin responsible for diarrheic shellfish poisoning in humans. Tetrodotoxin (TTX) is a tremendously strong neurotoxin, and is considered an emergent toxin in Europe because, over recent years, it became more recurrent in temperate waters. Given their high toxicity, strategies to obtain a mitigation of these toxins are every day more fundamental, in the optic of preserve consumer health, and, on the other hand, in giving supports to shellfisheries in selling their products. In this context, the application of microbial metabolism to biodegrade toxins and "undesired" substances, may represent a promising strategy in terms of toxin reduction. In the present study, the ability of lactic acid bacteria (LAB) and *Paracoccus* spp. to reduce OA and TTX *in vitro* was evaluated using HPLC/MS-MS technique. Different mechanisms of interaction were observed. Some species of LAB were able to reduce significantly the amount of toxin while in viable state (*Lacticaseibacillus rhamnosus* 1473 = 30 % OA reduction), but also a binding effect of the toxins was observed. Contrarily, *Paracoccus* spp. was able to reduce significantly the amount of toxin while in a cell-free extract form, in particular *Paracoccus aerius 5038* could reduce the presence of TTX of about 24%. As a general remark, this study provided a sound line of evidence for the possible future use either of specific bacteria or selected bacterial components to reduce the level of harmful marine toxins in aquaculture and food production. Interesting future studies could also be conducted directly in bivalves or in food matrix to check whether the use of bacteria described in this work could mitigate the level of toxins in real world-like condition.

Introduction

Harmful algal blooms (HAB) and phycotoxins represent a current safety issue strictly connected to global climate change (Gobler, 2020). HAB phenomena, consisting in the overgrowth of marine phytoplankton, are caused by about 300 recognized microalgae species of which more than 100 are producers of toxins responsible for intoxication or even death in animals and humans (Visciano et al., 2016). Marine toxins can be classified on the basis of their poisoning symptoms: diarrheic shellfish poisoning (DSP), paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP), and ciguatera fish poisoning (CFP). Besides, HAB and marine toxins are also an economic problem for shellfisheries causing an impact on profitability due to site closures and the related consequences (Theodorou et al., 2020).

Among marine toxins, tetrodotoxin (TTX) is a tremendously strong neurotoxin (it blocks voltage-gated sodium (Na+) channels in nerve and muscle tissues) named after the *Tetraodontidae* pufferfish family from which it was first isolated. This compound is considered an emergent toxin in Europe because, over recent years, it became more recurrent in temperate waters (Silva et al., 2015). From a food safety viewpoint, this toxin has been responsible for many fatalities over the years due to the consumption of marine foods (Lago et al., 2015). TTX can also be found in different taxa not closely related, such as marine invertebrates but also terrestrial and marine vertebrates (Silva et al., 2019). The widespread presence in many organisms is due to its exogenous origin; it is indeed produced by several bacterial strains belonging to *Bacillus, Shewanella, Roseobacter, Alteromonas, Aeromonas, Nocardiopsis, Vibrio*, and *Pseudomonas* (EFSA , 2017). TTX has been associated to *Prorocentrum* blooms, proving a high risk for shellfisheries and consequently for consumers (Magarlamov et al., 2017). Some species of this Dinoflagellata phylum are well known to be phycotoxins producers.

Okadaic acid (OA) is the main toxin responsible for DSP in humans, causing diarrhea, nausea and vomiting, but it is not considered lethal (Fu et al., 2019). It has moreover been

shown to be neurotoxic, hepatotoxic and cytotoxic (Kamat et al., 2013). OA is produced by several species of *Dinophysis*, *Phalacroma* and *Prorocentrum* (Arapov, 2013). The high persistence of these phytoplankton populations in many geographic areas highlights their importance from an economic point of view given that their presence above the legal limit results in a large number of harvesting closures (Blanco et al., 2019).

Given their high toxicity, mitigation strategies to both ensure consumer health and support shellfisheries are of increasing interest. In this context, the application of microbial metabolism to biodegrade toxins and "undesired" substances, called bioremediation, may represent a promising strategy in terms of toxin reduction. Indeed different studies involve the use of lactic acid bacteria (LAB) as a tool to biodegrade or remove toxins (Dalié et al., 2010; El-Nezami et al., 1998; Fuchs et al., 2008; Halttunen et al., 2007; Ogawa et al., 2001; Zhao et al., 2015). In particular, this approach was widely studied to reduce mycotoxins amount in food and feed (Dalié et al., 2010; Fuchs et al., 2008; Perczak et al., 2018; Shetty & Jespersen, 2006), but limited are the researches aimed to investigate the mitigation of marine toxins and cyanotoxins (Meriluoto et al., 2005; Ramani et al., 2012).

Given the high toxicity of marine toxins, their increasing spread leading to a relevant occurrence in fish-based foods, and the promising results in terms of reduction of some toxic compounds obtained by LAB, this research aimed to investigate the effect of interaction between different bacteria and two marine toxins, in order to evaluate a possible reduction. In particular, different LAB and marine isolated bacteria strains were considered and the effect of viable, not viable and lysed cells was tested *in vitro* on okadaic acid and tetrodotoxin.

Results

Microbial growth

In order to evaluate the effect of bacterial growth and activity on the level of OA and TTX, bacterial counts of the metabolically active bacteria were determined. In supplementary table 1 are represented the growth of lactic acid bacteria without toxins. The tested strains showed different attitudes of growth. In table 1 is reported the behavior of bacteria just after inoculum (t0) and after 48 hours (tF) of incubation in the presence of OA (100 μg/L) and TTX (50 μg/L).

Table 1. Microbial counts before and after contact between Okadaic acid (100 μg/L) and Tetrodotoxin (50 μg/L) and microorganisms. Stars highlight statistical differences within the strains. * = $p<0.05$. The values are reported as mean ± SD.

	Okadaic acid (100 μg/L)			**Tetrodotoxin (50μg/L)**		
Strain	**t0 Log CFU /ml**	**tf Log CFU/ml**	**Δ Log CFU/ml**	**t0 Log CFU /ml**	**tf Log CFU/ml**	**Δ Log CFU/ml**
L. casei 2240	8.78 ± 0.16	8.88 ± 0.14	0.10	8.78 ± 0.16	8.83 ± 0.09	0.05
L. rhamnosus 2411	9.58 ± 0.10	8.98 ± 0.10	-0.60	9.58 ± 0.10	8.86 ± 0.06	-0.72
L. fermentum 11441	9.22 ± 0.04	8.43 ± 0.05	-0.79*	9.22 ± 0.04	7.98 ± 0.06	-1.24*
L. brevis 6906	9.12 ± 0.12	8.42 ± 0.01	-0.70*	9.12 ± 0.12	8.97 ± 0.01	-0.15
L. rhamnosus 1473	8.12 ± 0.01	9.16 ±0.02	1.04*	8.12 ± 0.01	9.05 ± 0.01	0.93*
L. paracasei 4186	7.79 ± 0.16	7.49 ± 0.58	-0.30	7.79 ± 0.16	7.43 ± 0.12	-0.36
P. marcusii 5034	8.46 ± 0.01	8.64 ± 0.04	0.18	8.46 ± 0.01	8.85 ± 0.04	0.39*
P. aerius 5038	8.32 ± 0.04	8.76 ± 0.01	0.44*	8.32 ± 0.04	8.65 ± 0.02	0.33*
P. aestuarii 5039	7.53 ± 0.13	8.49 ± 0.02	0.96*	7.53 ± 0.13	8.39 ± 0.04	0.86*

Regarding OA samples, *Lacticaseibacillus casei* 2240 and *Lacticaseibacillus paracasei* 4186 remained at the inoculum level during the 48 hours' incubation. Differently, *Lacticaseibacillus rhamnosus* 2411, *Limosilactobacillus fermentum* LMG 11441 and

Levilactobacillus brevis LMG 6906 decreased during incubation, probably entering in death phase. Contrarily, *Lacticaseibacillus rhamnosus* 1473 increased by more than 1 Log CFU/mL respect to the inoculum level ($p< 0.05$), proving that the bacteria stayed in exponential phase during the incubation. All the *Paracoccus* spp. strains incubated together with OA presented a very similar behavior to each other. In fact, they stayed in an exponential phase keeping growing during the 48 hours' incubation. This was more evident for *Paracoccus aerius* 5038 and *Paracoccus aestuarii* 5039 that statistically increased their concentrations (Table 1). In table 1 is also shown the behavior of the tested strains during 48 hours of incubation with 50 μg/L of TTX, which showed results similar to those observed for OA. Only *L. rhamnosus* 1473 and *Paracoccus* spp. strains increased their concentration respect to the inoculum level ($p<0.05$) while *L. casei 2240* concentration didn't change and the other LAB strains entered a death phase. In supplementary table 2 are compared the growth of lactic acid bacteria after 48 hours (tF) with and without the toxins (OA and TTX). Significant differences were found in the growth of LMG 6906 and 1473 in presence of OA and in the growth of LMG 11441 and 4186 in presence of TTX.

Bacteria and toxins interaction

In order to evaluate the effect of interaction between marine toxin and lactic acid or marine bacteria, all the samples were analyzed and the toxin content was calculated. In negative control samples, the two toxins resulted absent and no interferences were evidenced. To highlight a reduction of toxin amount due to bacteria activity, analyte concentration calculated in the samples were compared with those found in positive controls. The results were expressed as percentages. Changes of OA concentrations in MRS broth after 48 hours' incubation in samples inoculated with LAB strains, as viable or lysed cells are shown in Table 2.

Table 2. Results of interaction between toxins and lactic acid bacteria and respective distribution of the toxins. Data are expressed as percentages, considering as 100% the control samples. Stars underline statistical differences ($p<0.05$) with the controls. (n.d. = not detectable).

		MRS		PBS		
	ID	**Pellet**	**Supernatant**	**Pellet**	**Supernatant**	**Cell-free extract**
Okadaic acid (100 μg/L)	2240	9.88 ± 0.36	90.12 ± 1.20	5.63 ± 0.39	94.37 ± 0.44	100
	2411	11.10 ± 0.30	88.90 ± 0.20	5.36 ± 0.12	94.64 ± 0.19	100
	LMG 11441	12.72 ± 0.32	87.28 ± 0.29	5.55 ± 0.19	94.45 ± 0.25	100
	LMG 6906	11.93 ± 0.81	88.07 ± 0.10	5.44 ± 0.09	94.56 ± 0.58	100
	1473	n.d	68.65 ± 2.00*	8.08 ± 8.08	85.11 ± 3.09	100
	4186	n.d	88.90 ± 11.10	n.d	90.60 ± 3.00	100
Tetrodotoxin (50 μg/L)	2240	1.41 ± 1.41	98.59 ± 1.41	9.75 ± 0.58	81.75 ± 4.94	100
	2411	1.90 ± 1.90	81.10 ± 2.83*	n.d	100	92.50 ± 2.50
	LMG 11441	n.d	100	n.d	94.00 ± 1.41*	100
	LMG 6906	n.d	100	5.80 ± 5.80	94.20 ± 5.80	96.50 ± 3.50
	1473	14.67 ± 0.12	85.33 ± 1.95	7.36 ± 7.36	73.60 ± 1.48*	100
	4186	n.d	82.50 ± 1.00*	7.13 ± 7.13	92.87 ± 7.13	87.00 ± 2.00*

Not all interactions led to a modification of OA concentration. In particular, cell free extracts of lactic acid bacteria did not exert any effect on the initial toxin content. Rather, a statistically significant reduction of toxin amount was proved by metabolically active *L. rhamnosus* 1473 cells (MRS). In fact, the percentage of toxin after incubation was 68.65 ± 2.00% referred to the control (100%), showing a reduction of about 30% of the initial content. In MRS, LAB strains seemed to have a slight binding activity toward OA, in particular regarding 4 out of 6 strains (2240, 2411, LMG 11441 and LMG 6906) 10% of the toxins were found in the pellet. The same effect was observed in PBS samples even if to a lesser extent. In fact, about 5% of OA was found in the pellet (Table 2). Regarding TTX, heterogeneous interactions with the different LAB strains were also noted (Table 2). A reduction of 20% in the amount of TTX in MRS when *Lacticaseibacillus rhamnosus* 2411 and *Lacticaseibacillus paracasei* 4186 were inoculated was observed ($p< 0.05$). A binding interaction resulted only

in sample inoculated with *L. rhamnosus* 1473, in which about 15% of TTX was found in the pelleted cells. The capability to reduce the TTX level was also assessed in metabolically inactive bacteria. To do so, growth media were substituted with PBS buffer to reduce any metabolic activity of bacteria. In this condition, *Lacticaseibacillus casei* 2240, *Limosilactobacillus fermentum*1141 and *Lacticaseibacillus rhamnosus* 1473 were able to reduce TTX concentration in the samples: the greatest effect was exerted by *L. rhamnosus* 1473 with a 20% TTX reduction. Interaction between cells and TTX was observed in PBS regarding 4 out of 6 strains (Table 2). The 9.75 ± 0.58% of the toxin was detected in *L. casei* 2240 pellet. Differently from what was observed in OA samples, Cell-free extracts of lactic acid bacteria, and in particular *L. rhamnosus* 2411, *L. brevis* 6906 and *L. paracasei* 4186 strains, showed an interaction with TTX. Cell-free extracts of *L. paracasei* 4186 could reduce the presence of TTX by more than 10% ($p < 0.05$). Differently from what was observed for LAB species, bacteria isolated from marine environment showed completely different behavior. In Table 3 the percentages of OA found after the incubation with metabolically active (Marine broth), not metabolically active (PBS) and cell-free extracts of *Paracoccus* spp. strains are reported.

Table 3. Results of interaction between toxins and *Paracoccus* sp. and respective distribution of the toxins. Data are expressed as percentages, considering as 100% the control samples. Stars underline statistical differences ($p<0.05$) with the controls. (n.d. = not detectable)

		Marine broth		PBS		
	ID	**Pellet**	**Supernatant**	**Pellet**	**Supernatant**	**Cell-free extract**
Okadaic acid (100 μg/L)	5034	n.d	92.70 ± 7.30	n.d	100	87.15 ± 11.85
	5038	n.d	100	n.d	100	83.05 ± 9.25
	5039	n.d	97.00 ± 2.00	n.d	100	78.20 ± 3.39*
Tetrodotoxin (50 μg/L)	5034	n.d	100	n.d	100	81.50 ± 3.54*
	5038	n.d	100	n.d	100	76.00 ± 1.41*
	5039	n.d	100	n.d	100	83.50 ± 10.61

Differently from Lactic acid bacteria behavior, only *Paracoccus* spp. strains lysed cells were able to interact with the toxin. The most important reduction of OA amount was obtained with 5039 strain, in which cell-free lysed extract the 78.20 ± 3.39% of the toxin was collected ($p<0.05$). The same strain proved a slight interaction while metabolically active (97.00 ± 2.00%). Also, *Paracoccus marcusii* 5034 and *Paracoccus* aerius 5038 lysed cells showed an interaction with OA: 87.15 ± 11.85 % and 83.05 ± 9.25 % of the toxin was respectively detected in the free cell extract. Contrary to what happened with LAB strains, no interaction between pelleted cells and OA was observed. Regarding TTX the effect of *Paracoccus* spp. with TTX was similar to what was observed with OA (Table 3). In fact, only lysed cells could act on the toxin and reduce the amount of TTX detected. In particular, *P. aerius 5038* free cell extract could reduce the toxin of about 24%, similarly to *P. marcusii* 5034 lysed cells that led to a TTX decrease of about 20% As already noted with OA, no interaction between pelleted cells and TTX was detected.

Discussion

The contamination of seafood with marine toxins poses concerns for human health and represents a severe threat for aquaculture (Brown et al., 2019). Climate change and eutrophication are growing phenomena with a great impact on the prevalence and distribution of marine toxins also in biological niches that were considered not suitable for toxins accumulation in the past. As an example, TTX has been recently found contaminating bivalve molluscs like mussels and Pacific oysters harvested in Europe (Turner et al., 2015), indicating the capability to migrate in a broad range of animal species – including some intended for wide human consumption. This changing scenario is undergoing rapid changes and, exposes consumers of seafood to serious health threats. Therefore, in order to guarantee consumers' safety, is necessary to study a possible solution to mitigate the presence of these compounds either at a pre-harvest stage or along the food production chain. This study focused on biological strategies for the mitigation of toxins potentially occurring in different food products such as shellfish and fish-based food. For this purpose, the effect of interactions between bacteria (LAB and *Paracoccus* spp. strains isolated from the marine environment) and toxins (OA and TTX) was investigated by considering both metabolically active or not active cells and cell-free extracts (lysed cells). The bacterial species used in the present study have not been tested previously for their ability to remove marine toxins such as OA and TTX.

Different effects have been observed due to the interaction of the two toxins tested and the bacterial strains considered both as metabolically active, non-active and lysed cells. The potential ability of these bacteria may be the capacity to help to reduce the presence of these toxins by metabolically transforming them or with a binding mechanism as observed for other toxins (Fuchs et al., 2008; Lemes et al., 2008). In this study, different efficacy and type of interaction in the removal or binding of OA acid by LAB were noted. In literature, the interaction between LAB and other microbial toxins such as mycotoxins is well documented (Dalié et al., 2010; Fuchs et al., 2008; Perczak et al., 2018). Researches about this topic have

led to the conclusion that binding rather than metabolism was the mechanism by which mycotoxins were removed (El-Nezami et al., 2002). It was suggested that toxins, like aflatoxin B1, could bound to the bacteria by weak noncovalent interactions, such as associating with hydrophobic pockets on the bacterial surface (Haskard et al., 2001). In this study, a very similar behavior could be supposed for OA that was found associated with bacterial pellet. In both the tested media (MRS and PBS), a binding effect by LAB was noticed tough to a little extent (5-10%). The binding activity of LAB toward OA is not yet demonstrated but it can be supposed that interaction between bacterial cell wall and toxin occurred. Differently to what observed towards mycotoxins and microcystin, the ability of *L. rhamnosus* 1473, in a metabolically active state to remove OA from the media was observed (Meriluoto et al., 2005). This strain duplicated during the 48 hours of incubations, increasing of 1.04 ± 0.02 Log CFU/ml and probably for that reason it was able to metabolize the toxin causing a reduction of the initial concentration. Only using this strain indeed a transformation of the toxin was feasible; in the samples involving this strain a decrease of toxin initial amount was noticed in MRS supernatant ($p< 0.05$) compartments but, at the same time, the presence of OA was not evidenced in the pelleted cells.

A different attitude of LAB against TTX was observed. The reduction of TTX through a spontaneous fermentation of pufferfish has been previously tested (Anraku et al., 2013). The study proved that during a few years' fermentation periods under salt, the amount of TTX contained in pufferfishes ovaries decreased to 1/50–1/90 times respect to the fresh ovary. The reduction of TTX by LAB has also been noticed. In that case, exopolysaccharides were involved in the reduction of toxins amount (Tu et al., 2018). The reduction of this toxin has never been related to lactic acid bacteria metabolism. In the present study, a TTX reduction in MRS was observed for the first time. *L. rhamnosus 2411* and *L. paracasei 4186* proved an interaction with the toxin showing a reduction in its amount. This phenomenon is probably related to a metabolization of the toxin that has been demonstrated by marine bacteria concerning Paralytic shellfish toxins (Donovan et al., 2009).

The results of this study evidently show the strain differences in the removal of TTX. The most efficient reduction was obtained by 1473 (in MRS) and 2240 (in PBS) strains, while no

removal by binding was achieved by *Paracoccus* spp. strains. This fact suggests that the bacterial ability to remove toxins is dependent on the bacterial cell wall structure. As already portrayed by other studies, the ability of bacteria to bind toxins and other compounds, could be strictly connected to the conformation of the bacterial cell walls and consequently to gram-positive or gram-negative bacteria (El-Nezami et al., 2002). Confirming this statement, also in this study no bonding effect was observed due to a Gram-negative genus (*Paracoccus)* differently to gram-positive bacteria (LAB). A variable binding phenomenon was observed in PBS and in MRS, that reflect a metabolically non-active and active cells state respectively. The three *Paracoccus* spp. strains considered portrayed a similar behavior towards the two tested toxins. In fact, all these species did not demonstrate a capture activity or a metabolization activity on both marine toxins. A toxin reduction activity was recorded only when bacteria had undergone a lysing treatment. This attitude means a reductive effect on these toxins closely related to enzymes and endocellular materials. In the last decades, several *Paracoccus* spp. strains have been reported to degrade and metabolize many kinds of toxic aromatic compounds (Bai et al., 2008). This genus is widely studied in the field of bioremediation, in particular for the ability of some strains to degrade insecticide residuals (pyridine, fipronil, carbofuran and Chlorpyrifos) (Bai et al., 2008; Kumar et al., 2012; Li et al., 2011; Peng et al., 2008), pyrene (Yang et al., 2013), and other compounds (Nisha et al., 2015, 2016; Siddavattam et al., 2011; Xu et al., 2008; Zhang et al., 2011). What emerged in this study may thus open up to further researches on the application of lyses *Paracoccus* cells as a possible tool to biodegrade undesired compounds in general, and not only marine toxins. Several studies show effective interactions between LAB and mycotoxins. For instance, *L. casei* group bacteria were very effective in removing aflatoxin B1, with more than 80% of the toxin trapped (Haskard et al., 2001). Otherwise, as presented in this study, the ability of LAB to "trap" marine toxins were lower. This behavior can be linked to the different toxins structures but also to a substantial environmental difference. LAB and moulds producing mycotoxins are microorganisms typical of the same environment and this can be the reason why the bonding interactions is more efficient.

Conclusions

This study aimed to shed light on the possibility to use LAB and marine isolated bacteria to reduce or bond two marine toxins. At the best of our knowledge, it is the first time that interaction between LAB or other bacteria and OA is studied. Even if slight, some interesting interactions were noticed and it is clear that LAB and *Paracoccus* spp. had a different effect on toxins. Gram-positive bacteria (LAB) are able to metabolize or bond on the cell walls the tested toxins, while Gram-negative bacteria could only reduce the amount of toxin after a lysis treatment of bacterial cells. Further researches need to be conducted in order to deeply investigate the role of bacterial metabolism, intracellular enzymes or cell wall involved in this mechanism. To this purpose contact time and bacterial concentration may be interesting variables to be considered. As a general remark, this study provided a sound line of evidence for the possible future use either of specific bacteria or selected bacterial components to reduce the level of harmful marine toxins in aquaculture and food production. Interesting future studies could also be conducted directly in bivalves or in food matrix to check whether the use of bacteria described in this work could mitigate the level of toxins in real world-like conditions.

Materials and Methods

Microbial strains

Six lactic acid bacteria strains, belonging to different species, and three wild *Paracoccus* strains were used (Table 4).

Table 4. Bacterial strains used in the experiments

ID	Species	Temperature	Source	Isolation matrix
2240	*Lacticaseibacillus casei*	37°C	UNIPR	Parmigiano Reggiano cheese
2411	*Lacticaseibacillus rhamnosus*	37°C	UNIPR	Parmigiano Reggiano cheese
LMG 11441	*Limosilactobacillus fermentum*	37°C	BCCM	Not available
LMG 6906	*Levilactobacillus brevis*	30°C	BCCM	Human feces
1473	*Lacticaseibacillus rhamnosus*	37°C	UNIPR	Parmigiano Reggiano cheese
4186	*Lacticaseibacillus paracasei*	37°C	UNIPR	Pecorino Toscano cheese
5034	*Paracoccus marcusii*	30°C	UNIPR	Seaweeds
5038	*Paracoccus aerius*	30°C	UNIPR	Seaweeds
5039	*Paracoccus aestuarii*	30°C	UNIPR	Seaweeds

[1] UNIPR, University of Parma; BCCM, BCCM/LMG Collection Laboratorium voor Microbiologie, Universiteit Gent.

Limosilactobacillus fermentum LMG 11441 and *Levilactobacillus brevis* LMG 6906 were purchased from Belgian Coordinated Collection of Microorganisms (BCCM) (Universiteit Gent, Gent, Belgium). The remaining strains belong to the collection of the Department of Food and Drug (University of Parma, Italy) where they have been previously isolated from different food matrixes and identified by 16S rRNA sequencing. LAB strains were maintained as frozen stock cultures in MRS broth (Oxoid, Ltd., Basingstoke, United Kingdom) containing 20% (v/v) glycerol at -80 °C. *Paracoccus* strains were kept as frozen stock cultures in Marine broth 2216 (BD Difco, Heidelberg, Germany) containing 20% (v/v) glycerol at -80 °C. The strains were revitalized in MRS broth (LAB) and in Marine broth 2216 (*Paracoccus* spp.) by three overnight sub-culturing (2% v/v) at their optimum temperature (Table 4).

Chemicals

Analytical standards of OA and TTX were obtained from Cayman Chemicals (Michigan, USA). For each toxin, stock solutions at the concentration of 100 mg/L were prepared in appropriate solvents and stored at -80°C until use. HPLC grade acetonitrile and formic acid used for sample extractions and dilutions, as for UHPLC analyses, were supplied from Sigma Aldrich (Milano, Italy), while bi-distilled water was in house produced utilizing an Alpha-Q system (Millipore, Marlborough, MA, USA). Phosphate Buffer Solution (PBS), was laboratory-made (1.6 g of NaCl, 0.24 g of Na_2HPO_4, 0.04 g of KH_2PO_4 and 0.04 g of KCl and 160 mL of bi-distilled water, pH 7 with HCl 1N) then sterilized in autoclave at 121°C for 20 minutes.

Experimental design

The experiments were conducted adapting the protocol reported by Zaho et al. (2005) for mycotoxins. Two working solutions were previously prepared for OA and TTX by drying an opportune aliquot of each toxin stock solution (10 μL) under a gentle nitrogen flow and dissolving the residue in 1 mL of sterilized PBS solution, obtaining a final concentration of 1000 μg/L. One mL of each revitalized bacterial solution was centrifuged at 12,857* g for 10 min at 4°C. The supernatant was then discarded and the bacterial cells were suspended in 1 mL of sterile PBS. This cells washing step was carried out twice, to guarantee the removal of residual culture medium. Bacterial cells were then suspended with 900 μL of MRS broth (LAB) or Marine broth 2216 (*Paracoccus*) or PBS and 100 μL of OA solution or (TTX solution were added, obtaining a final toxin concentration of 100 μg/L and 50 μg/L for OA and TTX respectively. The samples were homogenized in a vortex and incubated at the specific growth temperature for each strain (Table 1) for 48 hours. After incubation samples were centrifuged for 10 minutes at 4 °C at 12,857* g. Supernatant and pellet were separately extracted with 0.5 mL of acetonitrile. For this purpose, samples were stirred on a vortex for 2 min to ensure optimum homogenization of the extracting solution with the sample. A final centrifuge at (12,857* g for 10 min at 4°C) was carried out in order to separate supernatant which was transferred in HPLC vials.

To obtain cell free extracts, 1 mL of revitalized bacterial solution was centrifuged at 12,857* g for 10 min at 4°C, then the supernatant was discarded and the bacterial pellet was suspended in 1 mL of PBS. In order to guarantee the removal of the media present in the revitalization process, a total of two cells washings steps were applied. After these passages, 1 mL of each bacterial cells culture suspended in PBS, was transferred in BeadBug™ prefilled tubes, 2.0 mL capacity with 0.1 mm Silica glass beads (Sigma- Aldrich, USA), and treated with Mini-Beadbeater (Biospec products, US/ Canada) alternating 2 minutes of mixing and 1 minute in ice, 3 times. After the last treatment, the supernatant with cell-free extracts was used for the experiment. To confirm the absence of alive bacterial cells, counts on MRS (LAB strains) and in Marine broth (*Paracoccus* strains) were performed. As positive controls, OA or TTX were added to not inoculated MRS, Marine broth 2216 or PBS maintaining the same proportions considered for the inoculated samples, while negative controls were constituted of toxins free media/PBS and bacteria cultures or cell-free extracts. All the experiments were replicated twice and then analyzed.

Reported data are expressed as percentages obtained by comparing the concentration of toxins calculated in samples, based on calibration curves, with that present in the positive control samples, taken as 100%.

The experimental design is schematized in Figure 1.

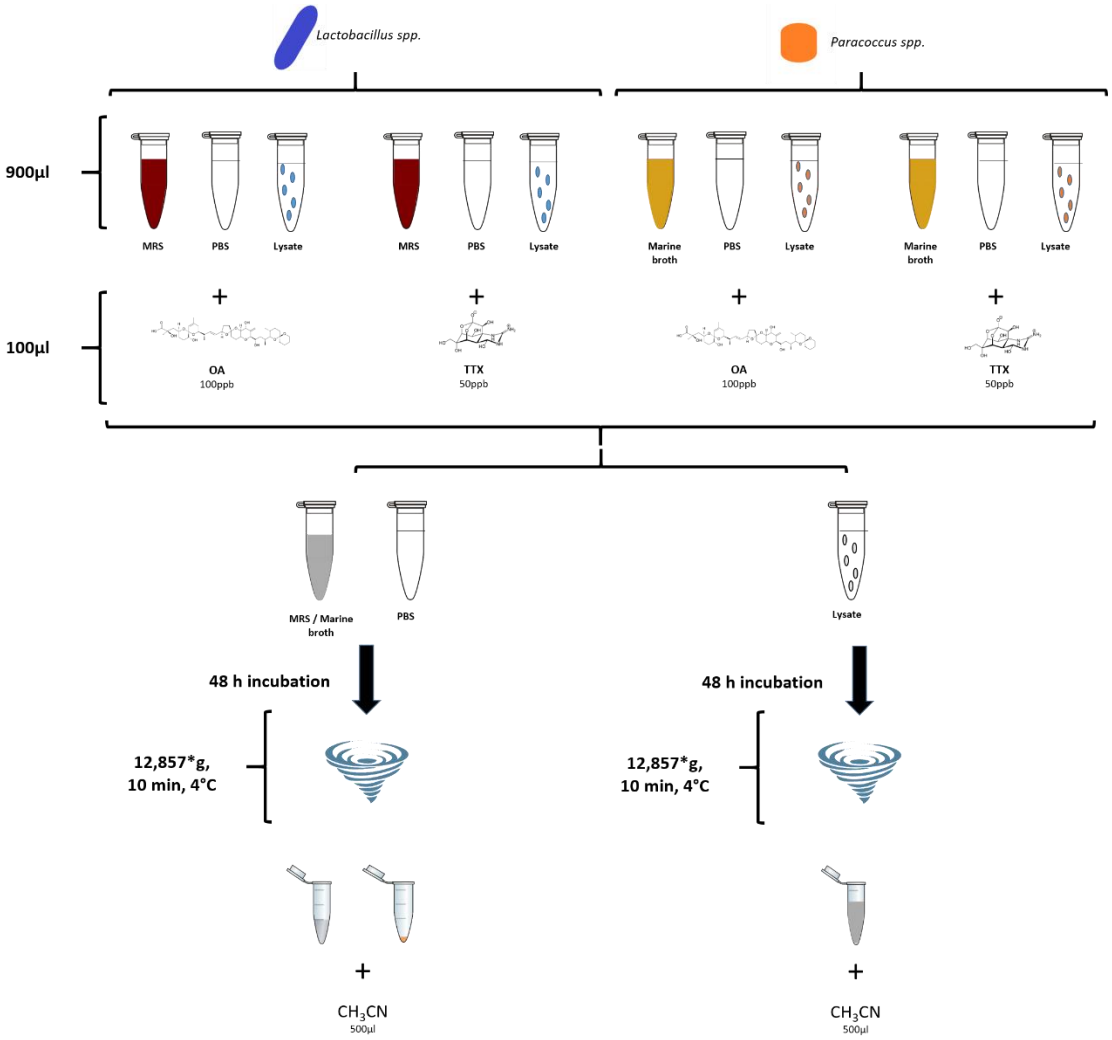

Figure 1. Experimental design

Microbial counts

Samples were incubated for 48 h at the optimal temperature for each strain, then 100 µl of each suspension containing bacteria and toxins and controls, were serial diluted and plated in order to evaluate bacterial concentration. Lactic acid bacteria and *Paracoccus* spp. were counted on MRS agar and Marine agar respectively, at the beginning (t0) and after 48 hours (tF) of incubation at their optimum temperature.

UHPLC-MS/MS analysis and quantification of toxins

All the UHPLC-MS/MS analyses were conducted on a UHPLC Dionex Ultimate 3000 separation module coupled with a triple quadrupole mass spectrometer (TSQ Vantage; Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with an electrospray source (ESI). Since the two analytes, OA and TTX. considered in this study presented different Physico-chemical characteristics, in terms of polarity and ionization potential, two methods of analysis were applied, as follows.

For the separation of OA a RP-C18 EVO Kinetex column (Phenomenex, Torrance, CA, USA) (2.10×100 mm, particle size 2.6 μm) was used. Water (eluent A) and aqueous acetonitrile (95%) (eluent B), both acidified with 0.1% formic acid, were utilized as eluents applying a gradient elution: initial conditions were set at 2% of B, that was increased to 100% in 10 minutes. The column was flashed for 2 min, then initial conditions were restored. The total run time resulted of 23 min. The column oven was maintained at 40 °C, 5 μL of samples extract were injected; the flow rate was maintained at 0.350 mL/min. For the detection, the ESI source was operated in negative ionization mode; spray voltage was set at 3000 V, the capillary temperature at 270 °C, vaporizer temperature at 200 °C, sheath gas flow at 50 units and the auxiliary gas flow at 5 units. Detection was performed in SRM mode, monitoring the following transitions were considered: m/z 803.0 → 562.9 (CE = 45 eV) and m/z 803.0 → 255.1 (CE = 49 eV).

The separation of TTX was conducted using a XBridge BEH Amide column (Waters Co., Milford, MA, USA) (2.10×100 mm, particle size 2.5 μm). Water (eluent A) and aqueous acetonitrile (95%) (eluent B), both acidified with 0.1% formic acid, were utilized as eluents applying a gradient elution: initial conditions were set at 95% of B, that was decreased to 20% in 10 minutes. The column was flashed for 2 min and in 1 min the initial conditions were restored. The total run time resulted of 23 min. The column oven was maintained at 40 °C, 5 μL of samples extract were injected; the flow rate was maintained at 0.350 mL/min. The detection of TTX was performed using the ESI source in positive ionization mode with the spray voltage set at 3500 V, the capillary temperature at 270 °C, vaporizer temperature at 200 °C, sheath gas flow at 50 units and the auxiliary gas flow at 5 units. Also in this case,

SRM detection mode was chosen, monitoring the following transitions: m/z 319.9 → 301.9 (CE = 24 eV) and m/z 319.9 → 161.9 (CE = 38 eV).

Relative concentrations of OA and TTX were calculated on the basis of two different calibration curves; in particular acetonitrile standard solutions at the concentration of 50, 75, 100, 150 and 200 μg/L were prepared for OA, while 20, 30, 50, 75 and 100 μg/L were the amounts considered for TTX. Good linearity ($R^2 > 0.99$) for both calibration ranges was obtained.

Statistical analysis

Data were statistically elaborated using SPSS Statistics 23.0 software (SPSS Inc., Chicago, IL). In particular, an independent-samples *t*-test was applied to compare microbial counts before and after the addition of toxins, as data obtained for the positive control samples with those obtained for incubated samples with a statistical difference when $p < 0.05$. Other independent-samples *t*-tests were applied to confront bacterial growth in presence of toxins (OA and TTX) with bacterial growth without toxins ($p<0.05$).

References

Anraku, K., Nonaka, K., Yamaga, T., Yamamoto, T., Shin, M.-C., Wakita, M., Hamamoto, A., & Akaike, N. (2013). Removal of Toxin (Tetrodotoxin) from Puffer Ovary by Traditional Fermentation. *Toxins*, *5*(1), 193–202. https://doi.org/10.3390/toxins5010193

Arapov, J. (2013). A review of shellfish phycotoxin profile and toxic phytoplankton species along Croatian coast of the Adriatic Sea. *Acta Adriatica*, *54*(2), 283–298.

Bai, Y., Sun, Q., Zhao, C., Wen, D., & Tang, X. (2008). Microbial degradation and metabolic pathway of pyridine by a *Paracoccus* sp. Strain BW001. *Biodegradation*, *19*(6), 915–926. https://doi.org/10.1007/s10532-008-9193-3

Blanco, Arévalo, Correa, & Moroño. (2019). Lipophilic Toxins in Galicia (NW Spain) between 2014 and 2017: Incidence on the Main Molluscan Species and Analysis of the Monitoring Efficiency. *Toxins*, *11*(10), 612. https://doi.org/10.3390/toxins11100612

Brown, A. R., Lilley, M., Shutler, J., Lowe, C., Artioli, Y., Torres, R., Berdalet, E., & Tyler, C. R. (2019). Assessing risks and mitigating impacts of harmful algal blooms on mariculture and marine fisheries. *Reviews in Aquaculture*, raq.12403. https://doi.org/10.1111/raq.12403

Dalié, D. K. D., Deschamps, A. M., & Richard-Forget, F. (2010). Lactic acid bacteria – Potential for control of mould growth and mycotoxins: A review. *Food Control*, *21*(4), 370–380. https://doi.org/10.1016/j.foodcont.2009.07.011

Donovan, C. J., Garduno, R. A., Kalmokoff, M., Ku, J. C., Quilliam, M. A., & Gill, T. A. (2009). *Pseudoalteromonas* Bacteria Are Capable of Degrading Paralytic Shellfish Toxins. *Applied and Environmental Microbiology*, *75*(21), 6919–6923. https://doi.org/10.1128/AEM.01384-09

EFSA Panel on Contaminants in the Food Chain (CONTAM). (2017). Risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods. *EFSA Journal*, 65. https://doi.org/doi: 10.2903/j.efsa.2017.4752

El-Nezami, H., Kankaanpaa, P., Salminen, S., & Ahokas, J. (1998). Ability of Dairy Strains of Lactic Acid Bacteria to Bind a Common Food Carcinogen, A¯atoxin B. *Food and Chemical Toxicology*, *36*, 321–326.

El-Nezami, H., Polychronaki, N., Salminen, S., & Mykkanen, H. (2002). Binding Rather Than Metabolism May Explain the Interaction of Two Food-Grade *Lactobacillus* Strains with Zearalenone and Its Derivative -Zearalenol. *Applied and Environmental Microbiology*, *68*(7), 3545–3549. https://doi.org/10.1128/AEM.68.7.3545-3549.2002

Fu, L., Zhao, X., Ji, L., & Xu, J. (2019). Okadaic acid (OA): Toxicity, detection and detoxification. *Toxicon*, *160*, 1–7. https://doi.org/10.1016/j.toxicon.2018.12.007

Fuchs, S., Sontag, G., Stidl, R., Ehrlich, V., Kundi, M., & Knasmüller, S. (2008). Detoxification of patulin and ochratoxin A, two abundant mycotoxins, by lactic acid bacteria. *Food and Chemical Toxicology*, *46*(4), 1398–1407. https://doi.org/10.1016/j.fct.2007.10.008

Gobler, C. J. (2020). Climate Change and Harmful Algal Blooms: Insights and perspective. *Harmful Algae*, *91*, 101731. https://doi.org/10.1016/j.hal.2019.101731

Halttunen, T., Collado, M. C., El-Nezami, H., Meriluoto, J., & Salminen, S. (2007). Combining strains of lactic acid bacteria may reduce their toxin and heavy metal removal efficiency from aqueous solution: Toxin removal with LAB combination. *Letters in Applied Microbiology*, *46*(2), 160–165. https://doi.org/10.1111/j.1472-765X.2007.02276.x

Haskard, C. A., El-Nezami, H. S., Kankaanpaa, P. E., Salminen, S., & Ahokas, J. T. (2001). Surface Binding of Aflatoxin B1 by Lactic Acid Bacteria. *Applied and Environmental Microbiology*, *67*(7), 3086–3091. https://doi.org/10.1128/AEM.67.7.3086-3091.2001

Kamat, P. K., Rai, S., & Nath, C. (2013). Okadaic acid induced neurotoxicity: An emerging tool to study Alzheimer's disease pathology. *NeuroToxicology*, *37*, 163–172. https://doi.org/10.1016/j.neuro.2013.05.002

Kumar, R., Singh, B., & Gupta, V. K. (2012). Biodegradation of Fipronil by *Paracoccus* sp. In Different Types of Soil. *Bulletin of Environmental Contamination and Toxicology*, *88*(5), 781–787. https://doi.org/10.1007/s00128-012-0578-y

Lago, J., Rodríguez, L., Blanco, L., Vieites, J., & Cabado, A. (2015). Tetrodotoxin, an Extremely Potent Marine Neurotoxin: Distribution, Toxicity, Origin and Therapeutical Uses. *Marine Drugs*, *13*(10), 6384–6406. https://doi.org/10.3390/md13106384

Lemes, G. A. F., Kersanach, R., da S. Pinto, L., Dellagostin, O. A., Yunes, J. S., & Matthiensen, A. (2008). Biodegradation of microcystins by aquatic *Burkholderia* sp. From a South Brazilian coastal lagoon. *Ecotoxicology and Environmental Safety*, *69*(3), 358–365. https://doi.org/10.1016/j.ecoenv.2007.03.013

Li, K., Wang, S., Shi, Y., Qu, J., Zhai, Y., Xu, L., Xu, Y., Song, J., Liu, L., Rahman, M. A., & Yan, Y. (2011). Genome Sequence of *Paracoccus* sp. Strain TRP, a Chlorpyrifos Biodegrader. *Journal of Bacteriology*, *193*(7), 1786–1787. https://doi.org/10.1128/JB.00014-11

Magarlamov, T., Melnikova, D., & Chernyshev, A. (2017). Tetrodotoxin-Producing Bacteria: Detection, Distribution and Migration of the Toxin in Aquatic Systems. *Toxins*, *9*(5), 166. https://doi.org/10.3390/toxins9050166

Meriluoto, J., Gueimonde, M., Haskard, C. A., Spoof, L., Sjövall, O., & Salminen, S. (2005). Removal of the cyanobacterial toxin microcystin-LR by human probiotics. *Toxicon*, *46*(1), 111–114. https://doi.org/10.1016/j.toxicon.2005.03.013

Nisha, K. N., Devi, V., Varalakshmi, P., & Ashokkumar, B. (2015). Biodegradation and utilization of dimethylformamide by biofilm forming *Paracoccus* sp. Strains MKU1 and MKU2. *Bioresource Technology*, *188*, 9–13. https://doi.org/10.1016/j.biortech.2015.02.042

Nisha, K. N., Sridhar, J., Varalakshmi, P., & Ashokkumar, B. (2016). Draft Genome Sequence of Paracoccus sp. MKU1, a New Bacterial Strain Isolated from an Industrial Effluent with Potential for Bioremediation. *Journal of Genomics*, *4*, 13–15. https://doi.org/10.7150/jgen.14847

Ogawa, M., Shimizu, K., Nomoto, K., Tanaka, R., Hamabata, T., Yamasaki, S., Takeda, T., & Takeda, Y. (2001). Inhibition of in vitro growth of Shiga toxin-producing *Escherichia coli* O157:H7 by probiotic *Lactobacillus* strains due to production of lactic acid. *International Journal of Food Microbiology*, *68*(1–2), 135–140. https://doi.org/10.1016/S0168-1605(01)00465-2

Peng, X., Zhang, J. S., Li, Y. Y., Li, W., Xu, G. M., & Yan, Y. C. (2008). Biodegradation of insecticide carbofuran by *Paracoccus* sp. YM3. *Journal of Environmental Science and Health, Part B*, *43*(7), 588–594. https://doi.org/10.1080/03601230802234492

Perczak, A., Goliński, P., Bryła, M., & Waśkiewicz, A. (2018). The efficiency of lactic acid bacteria against pathogenic fungi and mycotoxins. *Archives of Industrial Hygiene and Toxicology*, *69*(1), 32–45. https://doi.org/10.2478/aiht-2018-69-3051

Ramani, A., Rein, K., Shetty, K. G., & Jayachandran, K. (2012). Microbial degradation of microcystin in Florida's freshwaters. *Biodegradation*, *23*(1), 35–45. https://doi.org/10.1007/s10532-011-9484-y

Shetty, P. H., & Jespersen, L. (2006). *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents. *Trends in Food Science & Technology*, *17*(2), 48–55. https://doi.org/10.1016/j.tifs.2005.10.004

Siddavattam, D., Karegoudar, T. B., Mudde, S. K., Kumar, N., Baddam, R., Avasthi, T. S., & Ahmed, N. (2011). Genome of a Novel Isolate of *Paracoccus denitrificans* Capable of Degrading N,N-Dimethylformamide. *Journal of Bacteriology*, *193*(19), 5598–5599. https://doi.org/10.1128/JB.05667-11

Silva, M., Pratheepa, V., Botana, L., & Vasconcelos, V. (2015). Emergent Toxins in North Atlantic Temperate Waters: A Challenge for Monitoring Programs and Legislation. *Toxins*, *7*(3), 859–885. https://doi.org/10.3390/toxins7030859

Silva, M., Rodríguez, I., Barreiro, A., Kaufmann, M., Neto, A. I., Hassouani, M., Sabour, B., Alfonso, A., Botana, L. M., & Vasconcelos, V. (2019). Tetrodotoxins Occurrence in Non-

Traditional Vectors of the North Atlantic Waters (Portuguese Maritime Territory, and Morocco Coast). *Toxins*, *11*(6), 306. https://doi.org/10.3390/toxins11060306

Theodorou, J. A., Moutopoulos, D. K., & Tzovenis, I. (2020). Semi-quantitative risk assessment of Mediterranean mussel *(Mytilus galloprovincialis L.*) harvesting bans due to harmful algal bloom (HAB) incidents in Greece. *Aquaculture Economics & Management*, 1–21. https://doi.org/10.1080/13657305.2019.1708994

Tu, N., Dat Van, N., Canh Le van, & Doan, H. V. (2018). Detection of the Potential Inactivation of Tetrodotoxin by Lactic Acid Bacterial Exopolysaccharide. *Toxins*, *10*(7), 288. https://doi.org/10.3390/toxins10070288

Turner, A., Powell, A., Schofield, A., Lees, D., & Baker-Austin, C. (2015). Detection of the pufferfish toxin tetrodotoxin in European bivalves, England, 2013 to 2014. *Eurosurveillance*, *20*(2), 21009. https://doi.org/10.2807/1560-7917.ES2015.20.2.21009

Visciano, P., Schirone, M., Berti, M., Milandri, A., Tofalo, R., & Suzzi, G. (2016). Marine Biotoxins: Occurrence, Toxicity, Regulatory Limits and Reference Methods. *Frontiers in Microbiology*, *7*, 1051. https://doi.org/10.3389/fmicb.2016.01051

Xu, G., Zheng, W., Li, Y., Wang, S., Zhang, J., & Yan, Y. (2008). Biodegradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol by a newly isolated *Paracoccus* sp. Strain TRP. *International Biodeterioration & Biodegradation*, *62*(1), 51–56. https://doi.org/10.1016/j.ibiod.2007.12.001

Yang, X., Ye, J., Lyu, L., Wu, Q., & Zhang, R. (2013). Anaerobic Biodegradation of Pyrene by *Paracoccus denitrificans* Under Various Nitrate/Nitrite-Reducing Conditions. *Water, Air, & Soil Pollution*, *224*(5), 1578. https://doi.org/10.1007/s11270-013-1578-1

Zhang, J., Zheng, J.-W., Liang, B., Wang, C.-H., Cai, S., Ni, Y.-Y., He, J., & Li, S.-P. (2011). Biodegradation of Chloroacetamide Herbicides by *Paracoccus* sp. FLY-8 in Vitro. *Journal of Agricultural and Food Chemistry*, *59*(9), 4614–4621. https://doi.org/10.1021/jf104695g

Zhao, L., Jin, H., Lan, J., Zhang, R., Ren, H., Zhang, X., & Yu, G. (2015). Detoxification of zearalenone by three strains of *Lactobacillus plantarum* from fermented food in vitro. *Food Control*, *54*, 158–164. https://doi.org/10.1016/j.foodcont.2015.02.003

Supplementary Materials

Supplementary Table 1. Microbial counts of bacteria without the presence of toxins. Stars highlight statistical differences within the strains. * = p<0.05. The values are reported as mean ± SD.

Strain	t0 Log CFU /ml	tf Log CFU/ml	Δ Log CFU/ml
L. casei 2240	8.78 ± 0.16	9.05 ± 0.07	0.27
L. rhamnosus 2411	9.58 ± 0.10	8.69 ± 0.12	-0.89
L. fermentum 11441	9.22 ± 0.04	8.58 ± 0.01*	-0.63
L. brevis 6906	9.12 ± 0.12	8.94 ± 0.13	-0.18
L. rhamnosus 1473	8.12 ± 0.01	8.99 ± 0.05*	0.86
L. paracasei 4186	7.79 ± 0.16	8.17 ± 0.08	0.39
P. marcusii 5034	8.46 ± 0.01	8.72 ± 0.17	0.26
P. aerius 5038	8.32 ±0.04	8.73 ± 0.05	0.42
P. aestuarii 5039	7.53 ± 0.13	8.63 ± 0.09	1.11

Supplementary Table 2. Comparison between microbial growths of bacteria without (Controls) and with toxins (OA and TTX) after 48 hours. Stars highlight statistical differences within the strains. * = p<0.05. The values are reported as mean ± SD.

	Controls	Okadaic acid (100 μg/L)	Tetrodotoxin (50μg/L)
Strain	tf Log CFU/ml	tf Log CFU/ml	tf Log CFU/ml
L. casei 2240	9.05 ± 0.07	8.88 ± 0.14	8.83 ± 0.09
L. rhamnosus 2411	8.69 ± 0.12	8.98 ± 0.10	8.86 ± 0.06
L. fermentum 11441	8.58 ± 0.01	8.43 ± 0.05	7.98 ± 0.06*
L. brevis 6906	8.94 ± 0.13	8.42 ± 0.01*	8.97 ± 0.01
L. rhamnosus 1473	8.99 ± 0.05	9.16 ± 0.02*	9.05 ± 0.01
L. paracasei 4186	8.17 ± 0.08	7.49 ± 0.58	7.43 ± 0.12*
P. marcusii 5034	8.72 ± 0.17	8.64 ± 0.04	8.85 ± 0.04
P. aerius 5038	8.73 ± 0.05	8.76 ± 0.01	8.65 ± 0.02
P. aestuarii 5039	8.63 ± 0.09	8.49 ± 0.02	8.39 ± 0.04

Chapter 3:

Microalgae and lactic acid bacteria: interactions in formulations

Arthrospira platensis as natural fermentation booster for milk and soy fermented beverages

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Abstract

Arthrospira platensis, commercially known as Spirulina, is a fresh-water cyanobacterium that is gaining even more attention in the last years due to its high biological and nutritional value. For this reason, it has been employed in several food applications, to obtain or enhance functional and technological properties of cheese, yogurt, bread, cookies or pasta. The aim of this work was to evaluate the potential boosting effect of two different concentrations (0.25% and 0.50%) of *A. platensis* on the fermentation capability of several starter LAB strains, 1 probiotic and 4 commercial mix culture. These strains were used to ferment three different substrates and their fermentation behaviors were evaluated by impedance analyses together with rheological and color measurements. In tryptic soy broth (TSB), the *A. platensis* boosting effect was significantly higher if compared to yeast extract for all the starter LAB strains except for *Lb. casei* who was equally stimulated. Different results were found when the same LAB strains were cultivated in SSM. The most evident boosting effect of *A. platensis* was found for *S. thermophilus* and *Lb. casei.* LAB growth was promoted by *A. platensis*, confirming that it could be a useful tool in the production of novel functional fermented dairy foods. The potential boosting effect was evaluated on four commercial mix cultures used to produce milk and soy fermented beverages. It was demonstrated that the booster effect took place, but it was variable and dependent not only on the mix culture used, but also on the substrate and *A. platensis* concentration. Also, rheological and color modifications were found to be dependent on these factors.

Introduction

A. platensis is a fresh-water cyanobacterium that has several biological activities and a great nutritional value because it contains high levels of proteins (more than 60% on dry basis), essentials amino acids, vitamins, polyunsaturated fatty acids, minerals, polyphenols, carotenoids and chlorophyll (Ghaeni et al., 2014). Due to the presence of the pigment C-phycocyanin, the color of *A. platensis* is green-blue (Park et al., 2018). This microalga, commercially known as Spirulina, has different industrial applications: e.g. as a food supplement (Andrade, 2018; Batista et al., 2017), in feed (Holman & Malau-Aduli, 2013), in cosmetics (Ariede et al., 2017) and in health products (Majdoub et al., 2009). In several studies *A. platensis* has been employed in food formulations in order to obtain or enhance the functional and technological properties of cheese (Golmakani et al., 2019), yogurt (Mohammadi-Gouraji et al., 2019; Yamaguchi et al., 2019), soft drink beverages (Camacho et al., 2019), bread, cookies and pasta (Ak et al., 2016; Massoud et al., 2017; Zouari et al., 2011). Moreover, the growing demand for fermented, functional or vegan products opens new frontiers for its use as a natural ingredient and/or as a natural growth stimulator of starter bacteria in fermented food production. In fact, the increasing consumers' demand for functional and natural food has led to the continuous research of new natural ingredients to be used in food formulations (Bhowmik et al., 2009). Some studies reported that *A. platensis* stimulated lactic acid bacteria (LAB) growth in vitro condition (Bhowmik et al., 2009; Parada, 1998; Plaza et al., 2009) moreover, in milk products such as yogurt and ayran, Spirulina contributed to the preservation of LAB viability during storage (Barkallah et al., 2017; Çelekli et al., 2019; Guldas & Irkin, 2010).

This aspect could be very important especially for probiotic cultures that, after ingestion, are believed to play a significant role in the intestinal tract against pathogenic microorganisms (Gardiner et al., 2002). To perform this activity, a sufficient number of viable microorganisms must be present throughout the shelf life of the product (Parada,

1998). Moreover, as it is well known that LAB strains have a high nutritional requirement and that their fermentation time could be influenced by numerous factors, (especially in fermented milk-related products) (Gueimonde et al., 2004; Korbekandi et al., 2009; Mortazavian et al., 2007; Shafiee et al., 2010; Shah, 2000), in some cases energy boost is required in order to promote growth, fermentation capability and viability of the strains (Varga et al., 2002). As it has been already observed, *A. platensis* biomass could increase the rate and the survival of several strains in fermented dairy products (Vijay Pratap Singh, 2014). In this context, *A. platensis*, could play a multiple role in fermented foods: as a functional ingredient but also as a booster to enhance the technological performances of starter LAB. The impacts of *A. platensis* on the viability s of some LAB strains in fermented milk products have been already investigated by some authors (Barkallah et al., 2017; Hannane Beheshtipour et al., 2012), who used *A. platensis* at the end of fermentation, without warring about the problems related to the microbial contaminations brought by *A. platensis.* In fact, the addition after fermentation, as in the case of yogurt at refrigeration temperatures slow down or prevent microbial cell duplication during storage. On the other hand, as our purpose was to investigate the potentiality of *A. platensis* as a fermentation booster, it was therefore necessary to add it at the beginning of fermentation. In this case, to avoid any growth of contaminant microorganisms during fermentation that can compromise the success of fermentation and the safety of the final product, a sterilization step of *A. platensis* was necessary. Considering all these reasons, the aim of the present research was to i) evaluate the potentiality of sterilized *A. platensis* as natural growth booster for acidifying starter LAB strains, a probiotic strain and also commercially used acidifying starter cultures, by an innovative approach based on impedance analysis (Bancalari et al., 2016) ii) study the impact of *A. platensis* addition on the overall color and rheological characteristics of the fermented milk and fermented soy-based beverage.

Materials and Methods

Strains and commercial acidifying starter cultures

Seven wild LAB strains, belonging to different species commonly used as acidifying starter for milk fermentation, the probiotic strain *Lactobacillus rhamnosus* GG and 4 commercial acidifying mix cultures (Mix I, II, III, and IV) were used. The acidifying LAB strains, belonging to the collection of the Laboratory of Food Microbiology of the Department of Food and Drug (University of Parma), have been previously isolated from different food matrixes and identified by 16S rRNA sequencing (Tab. 1), while the commercial mix cultures were provided by Sacco Srl (Cadorago, CO, Italy). The acidifying LAB strains and *Lactobacillus rhamnosus* GG, maintained as frozen stock cultures in MRS (Oxoid, Ltd., Basingstoke, United Kingdom) (*Lactobacillus*), or M17 (Oxoid, Ltd., Basingstoke, United Kingdom) (*Lactococcus* and *Streptococcus*) broth containing 20% (v/v) glycerol at −80°C, were recovered in Tryptic Soy Broth (TSB) (Oxoid, Ltd., Basingstoke, United Kingdom) by two overnight sub-culturing (2% v/v) at 42°C for *Lactobacillus* and *Streptococcus*, and 30°C for *Lactococcus* (Table 1). Mix I, II, III and IV were maintained in lyophilized form at 4°C until the use.

Table 1. Lactic acid bacteria strains (LAB) and commercial mix cultures used in this study: *UNIPR: University of Parma; ATCC: American Type Culture Collection*

Species	Strain	Abbreviation	Source	Incubation temperature
Lactobacillus delbrueckii subsp. bulgaricus	2214	*Lbd. bulgaricus*	UNIPR	42°C
Lactobacillus casei	4339	*Lb. casei*	UNIPR	37°C
Lactococcus lactis subsp. cremoris	1978	*Lcl. cremoris*	UNIPR	30°C
Lactococcus lactis subsp. lactis	2269	*Lcl. lactis*	UNIPR	30°C
Leuconostoc	4456	*Leuconostoc sp.*	UNIPR	30°C
Weissella	4458	*Weissella minor*	UNIPR	30°C
Streptococcus thermophilus	518	*St. thermophilus*	UNIPR	42°C
Lactobacillus rhamnosus GG	GG	*Lb. rhamnosus*	ATCC 53103	37°C
Streptococcus thermophilus multistrains	I	*MixI*	Sacco Srl, Cadorago, Italy	37°C
Streptococcus thermophilus and Lactobacillus delbrueckii ssp. bulgaricus	II	*MixII*	Sacco Srl, Cadorago, Italy	37°C
Streptococcus thermophilus and Lactobacillus delbrueckii ssp. bulgaricus	III	*MixIII*	Sacco Srl, Cadorago, Italy	37°C
Streptococcus thermophilus, Lactobacillus delbrueckii spp. lactis and Lactobacillus helveticus.	IV	*MixIV*	Sacco Srl, Cadorago, Italy	37°C

Experimental design

The potential boosting effect of *A. platensis* on all the chosen strains and the commercial mix acidifying starter cultures was evaluated following the experimental design reported in Figure 1. Three different growth substrates were considered: Tryptic Soy Broth (TSB) (Oxoid, Ltd., Basingstoke, United Kingdom), reconstituted skim milk powder (SSM) (Oxoid), and a commercial soy-based beverage (SBB). TSB, reconstituted to 30 g/L (w/v), was sterilized at 121°C for 20 min. SSM reconstituted to 10% (w/v), was sterilized at 100°C for 10 min. SBB, composed of water, hulled soya beans (8%), sugar, calcium carbonate, acidity regulator (Potassium phosphates), flavoring, salt, stabilizer (Gellan gum), vitamins (B2, B12, D2), iodine (Potassium Iodide), was aseptically opened and immediately used. The dehydrated organic *A. platensis*, kindly provided by S.A.Ba.R (Novellara, RE, Italy), was rehydrated in water (5% w/v) and then sterilized at 121°C for 20 min. Sterilized *A. platensis* (SP) (5%) was added at 0.25% and 0.50% (w/v) to each substrate (Figure 1). Yeast extract (Oxoid) (YE) a common LAB growth stimulant, was evaluated as a positive control (C+). It was rehydrated in water (5% w/v), sterilized at 121°C for 20 minutes and added at 0.25% and 0.50% (w/v) to each substrate (Figure 1).

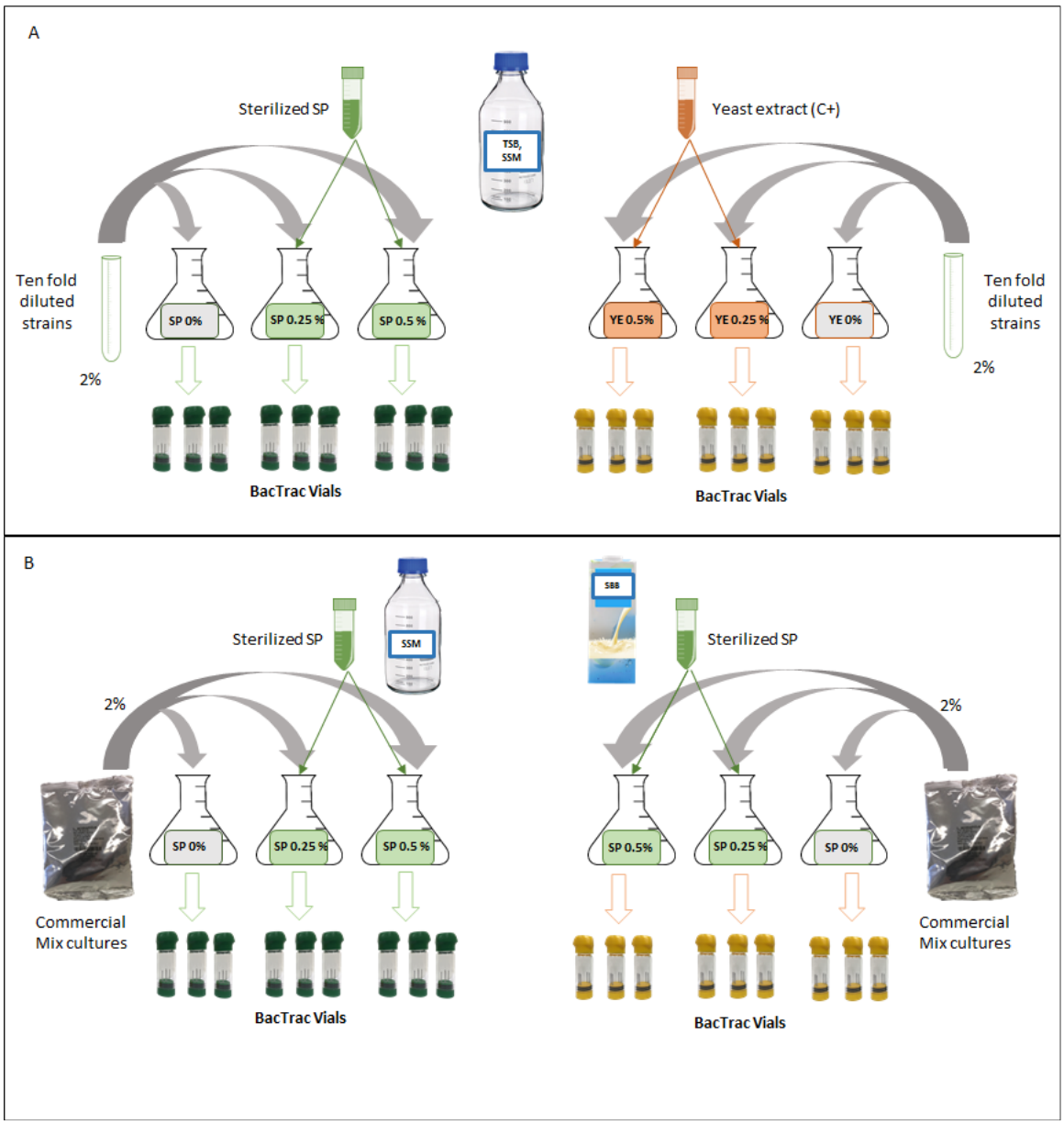

Figure 1. Schematic representation of the experimental design of this study.

Set up of fermentation conditions of LAB strains

The acidifying and growth performances of LAB strains and *Lactobacillus rhamnosus* GG (Table 1) were evaluated in TSB and SSM. For TSB, the last sub-culturing step of each bacterial culture was tenfold diluted in Ringer solution (Oxoid) and inoculated (2% v/v) in 18mL of the growth media supplemented, respectively and separately, with 0.25% and 0.50% of SP or YE (Figure 1). A negative control, without any addition of YE and SP, was

also considered. For the evaluation of acidifying and growth performances in SSM, other 24 h sub-culturing (2 % v/v) of each strain in SSM were performed before use. The inoculated media were transferred into sterilized BacTrac 4300® vials (SY-LAB, Neupurkersdorf, Austria), 6 mL in each vial, and incubated at the optimal growth temperature of each strain (Table 1). All the fermentations were carried out in triplicate and monitored for 48 h by measuring the impedometric signal every 10 min, by BacTrac 4300® Microbiological Analyzer (SY-LAB, Neupurkersdorf, Austria).

Commercial mix culture fermentation

The four commercial mix bacterial cultures (Mix I, II, III and IV) (Table 1) were directly inoculated in SSM and in SBB according to the manufacturer's instructions. As described above, the inoculated SSM and SBB were transferred into sterilized BacTrac 4300®, 6 mL in triplicate, and incubated at 37°C. All the fermentations were monitored for 48 hours as previously described.

Impedance Measurements

Impedance measurements were performed by mean of BacTrac 4300® Microbiological Analyzer system requiring the use of dedicated glass measuring cells (vials) with 4 electrodes. The system is based on the impedance splitting method and is able to register two specific impedance values for every single measurement: i) the conventional conductance value (M) that corresponds to the media impedance, and ii) capacitance value (E) which is the electrochemical double layer of the electrodes-electrolyte impedance (Bancalari et al., 2019). Both these values, simultaneously recorded by the instrument, are shown as relative changes compared to a starting value and expressed as M% and E% (Bancalari et al., 2016, 2019). The continuous plotting of the changes in E and M-values are visualized as E% and M% over time resulting in capacitance or conductance curves. Once

aseptically filled with the samples, the sterilized vials were located in the appropriate position inside the BacTrac 4300® incubators at the optimal growth temperature of each strain (Table 1). The two specific impedance values M% and E%, were measured and recorded every 10 min for 48 h (Bancalari et al., 2016, 2019). The results of the impedometric measurements were analyzed as previously reported by Bancalari (Bancalari et al., 2016) and the Lag value was considered in this study as an indicator of the potential booster effect given by SP during fermentation.

pH measurement

pH was measured after 24 h of fermentation by means of pH meter (Beckman Instrument mod Φ350, Furlenton, CA, USA) and a glass electrode (Hamilton, Bonaduz, Switzerland).

Rheological characterization of fermented SSM and SBB

The flow behavior of SSM and SBB was evaluated, before and after fermentation, by means of an MCR 102 rheometer (Anton Paar, Graz, Austria) equipped with a 50 mm cone-plate geometry (1° angle). An aliquot of sample was placed on the lower plate and the upper cone of the rheometer was lowered to a fixed height of 104 μm. Before analyses, samples were subjected to a pre shear to diminish structural differences among samples caused by different treatments (200 s^{-1} for 60 s). Flow behavior was tested by applying a linear ramp of shear rates from 10 to 200s^{-1}. The temperature was set at 4°C, and it was controlled by means of a Peltier device. The flow behavior of the samples was described by the Power law equation of the Ostwald de Waele model, as follows:

$$\tau=k(\gamma)^n$$

Where τ represents the shear stress (Pa), γ is the shear rate (s^{-1}), and k and n are a consistency factor (Pa s^n) and the flow behavior index, respectively. Analyses were performed in triplicate before fermentation (t0) and after the fermentation process (t24).

Color measurements of fermented SSM and SBB

Color measurements were performed, before and after fermentation of SSM and SBB inoculated with four commercial mix starters, using a CR-2600d spectrophotometer (Minolta Co., Osaka, Japan) equipped with a standard illuminant D65. The instrument was calibrated prior to each analysis using a white color tile standard. An aliquot of sample (10 mL) was transferred in a 55-mm petri dish, and colorimetric measurements were performed by placing the spectrophotometer lens in direct contact with the bottom of the petri dish. The lightness of color (L* that ranges between 100 of white to 0 of black), redness (a*, that ranges between +120 of red to -120 of green), yellowness (b*, that ranges between +120 of yellow to -120 of blue) were measured in SCI mode by considering the CIE L*a*b* color space. Analyses were performed in quintuplicate in random points of the bottom surface of the petri dish. According to rheological characterization, the color was measured before fermentation (t0) and after the fermentation process (t24).

Statistical analysis

To investigate the effect of concentrations (0, 0.25, 0.50%) of *A. platensis* and the effect of substrate (SSM and SBB), a two-way ANOVA model was performed using PRC GLM of SAS (SAS Inst. Inc., NC, USA). In the case of pH, color coordinates and rheological parameters, the fermentation time was considered for the statistical analysis (0h, corresponding to the inoculated and non-fermented sample; 24h, corresponding to the fermented sample) and in this case, a three-way ANOVA model was considered.

Results and discussion

Measurement of the boosting effect of sterilized A. platensis on LAB strains by impedance analysis

In this study, the potential boosting effect of *A. platensis* (SP) was evaluated by mean of impedometric method, by comparing the behavior of the acidifying starter LAB strains and *Lactobacillus rhamnosus* GG in presence or in absence of different concentration of SP in TSB and SSM. Furthermore, the potential effect of SP has been compared to that of a common LAB growth stimulant (YE) as a positive control. As the capacitance (E%) is a more sensible measurement, it was used for impedance measure in TSB, on the other hand, in SSM the data were recorded as M% (Bancalari et al., 2019). Capacitance or conductance data were analyzed by using an excel add-in and the Gompertz equation, following the method previously reported by Bancalari (Bancalari et al., 2016). This allowed to obtain the kinetic parameter Lag that was used in this study to describe the potential boosting effect of *A. platensis*. Lag is measured in hours, and the greater the Lag value, the bigger the time that the strains need to adapt to the growth conditions. To better highlight this effect, the Δlag was calculated as difference between the Lag mean value of the negative control (C-) and the Lag mean value measured with SP and YE addition for each strain. The bigger the value, the higher the stimulation effect of SP and/or YE on bacterial growth (Figure 2).

The high variability of Δlag values observed for the analyzed strains means that they were differently stimulated depending on the SP and YE concentration and on the medium used (Figure 2). In figure 2A, the results of the analysis in TSB are reported. It was possible to observe that both the stimulators (SP and YE) had a different and variable boosting effect depending on the species. The most relevant boosting effect (bigger Δlag value) was found for *St. thermophilus* who was the fastest when SP was added to TSB at both concentrations (Figure 2A). Conversely, the effect of YE was significantly lower ($P<0.001$) and negligible. The YE boosting effect was significantly lower ($p <0.001$) if compared to SP also for the other starter LAB strains except for *Lb. casei* who was equally stimulated by SP and YE (Figure 2A). These results are in agreement with a previous study (Parada, 1998) where a

stimulation of starter LAB growth promoted by *A. platensis* in synthetic media was observed. For *Lb. rhamnosus* GG the stimulator effect was dependent on the SP concentration used; the greater effect was found at 0.50% rather than 0.25% (Figure 2A). These results are in accordance with Beheshtipour and colleagues (H. Beheshtipour et al., 2013), who observed that *A. platensis* had a stimulatory effect on *Lb. rhamnosus* GG, by acting as a prebiotic factor, enhancing the growth of such microorganisms and also promoting acid production during fermentation (Parada, 1998). Different results were found when the same LAB strains were cultivated in SSM. Out of all strains tested, *Weissella* and *Leuconostoc* were not able to grow (Figure 2B). The reason of their incapability to metabolize lactose could be found in the fact that they were isolated from sourdough, where lactose is absent (Fusco et al., 2015; Kandler, 1983) (Figure 2B).

The most evident boosting effect of SP in SSM was found for *S. thermophilus* and *Lb. casei* (Figure 2B). While *S. thermophilus* was equally stimulated by YE and SP, independently by the concentration, *Lb. casei* was more stimulated by YE. Interestingly, *Lb. casei* 4339 (Table 1) that has been proposed as a secondary culture because of its longer Lag phase (Bancalari et al., 2016), in this case, with the addition of SP, has grown as fast as *St. thermophilus.* On the other hand, *Ld. bulgaricus* was equally boosted by both YE or SP (Figure 2B). Probiotic *Lb. rhamnosus* GG strain was equally stimulated by YE (both concentration) and the lowest concentration of SP (Figure 2B). The probiotic microorganisms are often added to yogurt or yogurt-like products where sometimes are in a lower number than the minimum required for a probiotic product, because they grow slowly in milk and often show loss of viability during storage (Zhao et al., 2006). To overcome this problem and enhance the growth and survival of these bacteria, some stimulators such as microalgae have already been proposed (Ásványi-Molnár et al., 2009; Gyenis et al., 2005; Varga et al., 2002; Zhao et al., 2006). Our results are generally in agreement with some researchers (de Caire & Parada, 2000; Varga et al., 1999) who observed that LAB growth was promoted by SP, confirming that it could be a useful tool in the production of novel functional fermented dairy foods (Varga et al., 1999). Anyway, despite some papers have already investigated the ability of *A. platensis* as a

growth stimulator, the approach proposed in the present study was completely different. Indeed, the impedometric method enabled the quantification (in minutes) of the time saved by using *A. platensis* as a growth booster for both starter strains and mix cultures, those representing a novelty of great technological interests. On the other hand, our research differs from the others available in the literature, because *A. platensis* has been sterilized before use. This was done because the powder has its own microbial contamination (Wang et al., 2013) and its addition at the beginning of a fermentation process could lead to the germination of spores or duplication of food borne pathogenic bacteria during fermentation or storage. To avoid this problem and to ensure safety of the fermented foods, it was decided to sterilize SP.

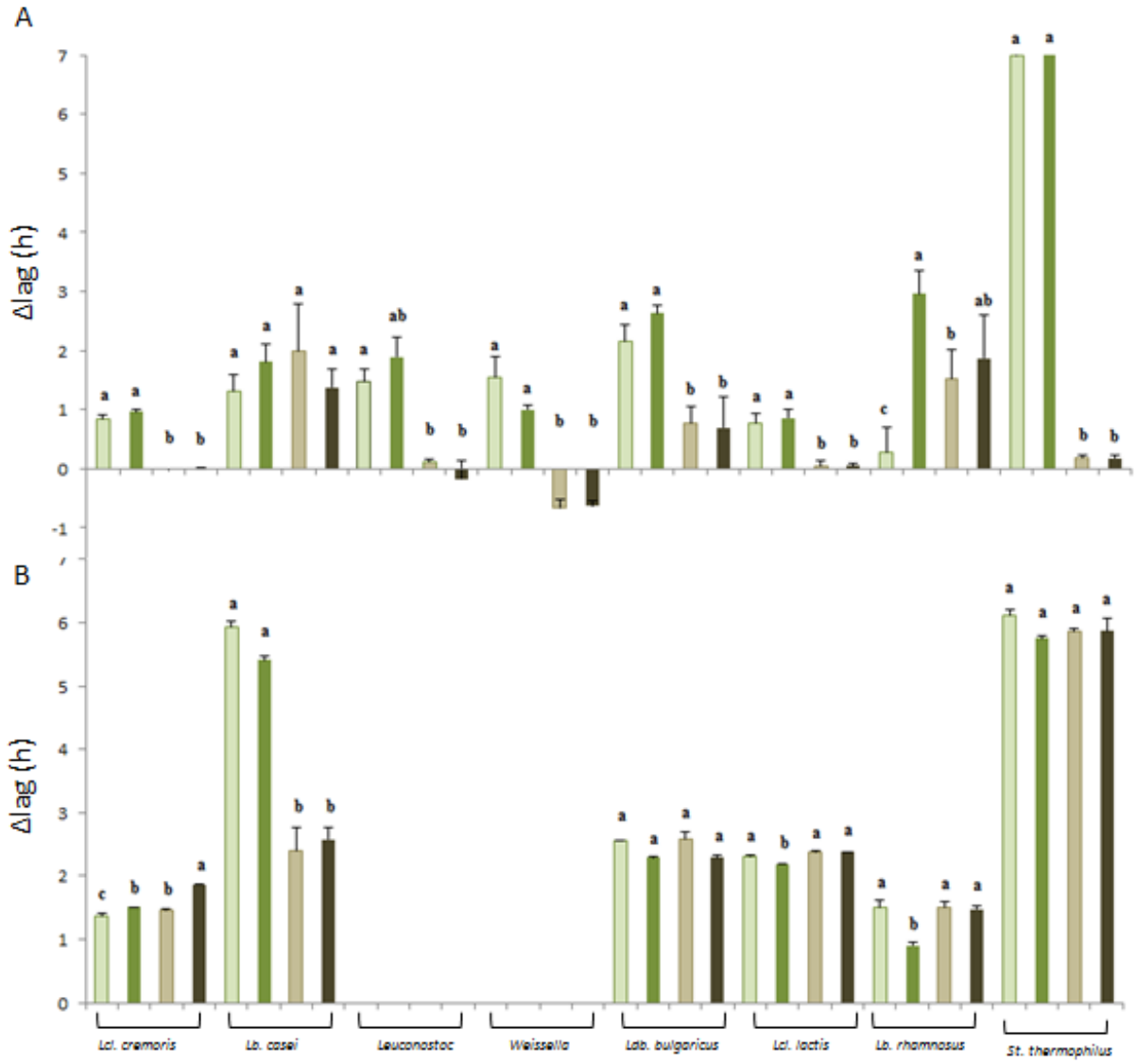

Figure 2. Boosting effect of *A. platensis* showed as mean Δlag values in Tryptic Soy Broth (TSB) (A) and in reconstituted skim milk powder (SSM) (B). Light green bars represent 0.25% of SP and dark green 0.5%; light and dark brown bars represent respectively 0.25% and 0.5% of YE. [a-b] Different lowercase letters by column within the same mix culture indicate the presence of significant differences according to ANOVA ($P<0.001$).

Impedance analysis of commercial mix cultures

The potential boosting effect was evaluated on four commercial mix cultures used to produce fermented SSM and SBB. As far as the authors are aware, no literature data are available about the addition of SP to boost microorganisms in the production of fermented soy-based beverages. For this reason, our result can be useful, even considering the growing interest in the vegetal substitutes of fermented milk (Cao et al., 2019). In table 2 the Lag values are reported as the mean of 3 replicates and the boosting effect is also reported as the difference between the Lag values obtained by fermentation using SP and the control (0% SP). This parameter was expressed in minutes and, if positive, a boosting effect took place. On the other hand, a negative value indicates a growth slowdown (Table 2). To further investigate the potential effect of SP on the fermentation process, also the pH of fermented beverages was measured at the end of each fermentation (Table 2). For the mix culture I (Mix I) no significant differences ($P>0.05$) in the parameter Lag were found when the mix was used to ferment SSM rather SBB without SP addition, which means that the lyophilized bacterial cells need at least the same time to duplicate and start to grow, despite the different environment (Table 2). Moreover, with the addition of both concentrations of SP, no significant differences were found in SSM. Conversely, the addition of increasing concentration of SP in SBB prolonged the Lag time (growth slowdown) of approximately 35 minutes with 0.25% SP and 46 minutes with 0.50% SP (Table 2). pH measured for the SSM fermented with mix I was significantly ($P<0.001$) lower when 0.25% of SP was used. This means that probably SP, despite not being able to boost the culture at the beginning of growth, still enhances the fermentation capability of strains (lower pH) belonging to *S. thermophilus* species (Table 2). Interestingly, a slight improvement of fermentation capability of Mix I (lower pH) was also observed when SP was added to SBB at both concentrations and to SSM with 0.50% of SP added ($P<0.001$). These results are in agreement with previously reported by Molnar and colleagues (2005) (Molnár et al., 2005) who observed that the addition of *A. platensis* caused a reduction of pH in yogurt samples, probably due to its biomass stimulatory effect on the *Ld. bulgaricus* growth. Comparing the Lag values of

the controls of Mix II in SSM and SBB, the parameter Lag was found to be significantly higher ($P<0.001$) in SBB rather than in SSM; thus in this case, the growth of bacterial cells was slowed down, probably due to a difficult adaptation to the environment (Table 2). Furthermore, significant differences in Lag values ($P<0.001$) due to SP concentrations were found when the Mix II was inoculated in SSM. In fact, a boosting effect was observed especially when 0.25% of SP was used. Even if SP addition seems to not really influence the fermentation capability of Mix II, a small improvement (24 min) was observed in SSM especially when 0.25% of SP was used. Moreover, a significantly lower pH value was found when 0.50% of SP was added. These results are in agreement with Varga and colleagues (Varga et al., 1999) who observed that pH values in milk containing *A. platensis* and inoculated with the mixed culture of *S. thermophilus* and *Ld. bulgaricus* decreased to an higher extent compared to control samples.

When Mix III was used to ferment SSM and SBB, no significant differences ($P>0.05$) in Lag values were observed. A significant ($P<0.001$) reduction of Lag was found only when SP was added to SSM in a concentration of 0.25%. In this case, the boosting effect was approximately 14 min. On the other hand, a negative effect of both concentrations of SP on Mix III in SBB was observed, with a growth slowdown of about 78 and 101 min respectively for the lowest and highest concentrations of SP (Table 2). Mix III seems to be suitable for fermentation of soy-based beverage without the use of SP, that can be however useful to improve the fermentation time in SSM. For the fourth mix considered (Mix IV), Lag values of the controls (0% SP) in SSM and SBB were not found to be statistically different ($P>0.05$). SP addition has led to a decrease of Lag value in SBB that was significant ($P<0.001$) for both concentrations. A boosting effect of 27 min was calculated in SBB when 0.25% SP was used. Therefore, mix IV seems to be a suitable starter for the fermentation of soy-based beverages. Conversely, in SSM a slowdown of 63 and 76 min was observed when 0.25% and 0.50% were respectively added (Table 2); in this case, the slowdown was also probably associated to lower growth and acidifying ability, as it is possible to observe from the higher pH values, if compared to the control, reported in table 2. To sum up, a diverse and variable trend was

observed when the four mix cultures were used to ferment SSM and SBB. The differences depend on the mix culture, on the substrate and on the diverse concentration of SP added, highlighting that each mix culture could have a specific application depending on the intended use. In particular, the impedometric method used to detect Lab growth allowed, firstly, to evaluate in which fermented beverage the diverse mix cultures grew faster and, secondly, to verify if the addition of SP had a boosting effect. In particular, this method allowed to observe that the mix with the best fermentation performances in SSM were Mix I and Mix III. On the other hand, Mix IV seems to be the best choice to ferment soy-based beverages when SP was added.

Table 2. Boosting effect of the three concentrations of *A. platensis* on mix culture

Mix cultures	SP %	Lag±SD (h)	Booster effect (min)	pH±SD after 24h
I in SSM	0	1.74^{b}±0.02	-	4.2^{c}±0.0
	0.25	1.68^{b}±0.03	3.67	3.9^{e}±0.0
	0.50	1.73^{b}±0.02	0.62	4.1^{d}±0.0
I in SBB	0	1.59^{b}±0.04	-	4.5^{a}±0.0
	0.25	2.17^{a}±0.24	-35.10	4.3^{b}±0.0
	0.50	2.36^{a}±0.08	-46.29	4.3^{b}±0.0
II in SSM	0	2.86^{b} ±0.01	-	4.3^{a}±0.0
	0.25	2.46^{d}±0.04	23.73	4.3^{a}±0.0
	0.50	2.59^{c}±0.06	16.25	4.1^{b}±0.0
II in SBB	0	5.47^{a}±0.01	-	3.8^{d}±0.0
	0.25	5.46^{a}±0.02	0.85	4.0^{c}±0.0
	0.50	5.37^{a}±0.05	6.32	3.8^{d}±0.0
III in SSM	0	1.96^{c}±0.16	-	4.5^{a}±0.0
	0.25	1.72^{d}±0.02	14.15	4.3^{b}±0.0
	0.50	1.98^{c}±0.06	-1.28	4.3^{b}±0.0
III in SBB	0	1.97^{c}±0.09	-	4.0^{d}±0.0
	0.25	3.65^{a}±0.08	-101.31	4.2^{c}±0.0
	0.50	3.27^{b}±0.09	-78.37	4.2^{c}±0.0
IV in SSM	0	1.80^{c}±0.04	-	4.0^{a}±0.0
	0.25	2.85^{b}±0.03	-63.02	4.0^{a}±0.0
	0.50	3.07^{a}±0.03	-76.55	4.0^{a}±0.0
IV in SBB	0	1.83^{c}±0.02	-	3.6^{b}±0.0
	0.25	1.38^{d}±0.04	27.06	3.6^{b}±0.0
	0.50	1.45^{d}±0.07	22.97	3.6^{b}±0.0

$^{a\text{-}d}$ Different lowercase letters by column within the same mix culture indicate the presence of significant differences.

Lag values are reported as mean of 3 replicates for each Mix cultures and concentration (0%, 0.25%, 0.50%). The boosting effect (min) is also reported for each mix culture as the difference between the Lag value obtained from the fermentations using SP and the control (0% SP). The pH value is reported as a mean value of three replicates.

Rheological properties of fermented samples

Change in rheological properties of fermented beverages can be due to different chemical and physical modifications such as pH and proteins' conformational changes and exopolysaccharides production (Li et al., 2014; Penna et al., 2006). The aim of our evaluation was to assess if the addition of *A. platensis* could indirectly improve the viscosity of fermented beverages by possibly stimulating the production of thickening molecules or the acidifying capacity of LAB cultures. The flow behavior of SSM and SBB added with the two concentrations of SP, was evaluated, before (t0) and after fermentation (t24) (Table 3). Power law derived rheological parameters k and n are reported in Table 3. Considering k, all the main effects, corresponding to the evaluated growth media, SP concentrations and fermentation times, were significant ($P<0.05$). Also, n showed significant main effects ($P<0.05$) in almost all the cases with the exception of SP concentration in the case of mix culture I and II. In particular, the effect of the type of beverage before fermentation on rheological properties was mainly related to the presence of thickening agents in the SBB formulation (gellan gum), that generated a more viscous liquid than SSM ($k = 0.033 \pm 0.008$ and 0.009 ± 0.004 Pa s^n for SBB and SSM before fermentation, respectively), also characterized by a more pseudoplastic behavior ($n = 0.71 \pm 0.04$ and 0.81 ± 0.08 for SBB and SSM before fermentation, respectively). As expected, the fermentation process significantly improved the viscoelastic properties of all samples ($P<0.05$). Moreover, the change in rheological properties promoted by the fermentation was significantly influenced ($P<0.05$) by the concentration of SP and by the mix culture used.

Consistency index k decreased or did not change for all mix cultures in SSM and SBB, with the addition of 0.25% and 0.50% SP; the decrease of k can be related to a decrease of viscosity

of the treatments compared to the control samples. Accordingly, flow behavior index n increased or did not change, and this can be related to a decrease of pseudoplastic behavior of the treatments in comparison with the control samples. The only exception was represented by mix III at a SP concentration of 0.25%, which showed a significant increase of viscosity and pseudoplastic behavior (an increase of k index and decrease of n index). Accordingly, in the case of SBB, mix III showed a strong increase of k index and a decrease of n index at both 0.25% and 0.50% SP. Moreover, while in the case of mix I, II and IV the enhancement of viscosity in the samples was not observed, in the case of mix III low concentrations of SP (0.25%) possibly showed the potential ability to improve and stimulate the production of thickening molecules (e.g. EPS) both in SSM and in SBB.

Table 3. Results of colorimetric coordinates L*, a*, b* and power-law, rheological parameters n and k derived from equation 1, of each mix culture inoculated in skimmed milk (SSM) and soymilk (SBB).

			L*		a*		b*		k (Pa s^n)		n	
mix culture	medium	SP (%)	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24
I	SSM	0	83.72aB ± 0.11	87.73aA ± 0.07	-4.07dB ± <0.01	-2.24fA ± 0.04	1.25dB ± 0.04	4.23eA ± 0.17	0.008bB ± 0.002	4.732aA ± 0.039	0.839abA ± 0.038	0.150dB ± 0.005
		0.25	65.17dB ± 1.13	74.41cA ± 0.04	2.83aA ± 0.75	1.64cB ± 0.09	21.08aA ± 0.09	17.10cB ± 0.15	0.010bB ± 0.006	2.562bcA ± 0.221	0.839abA ± 0.120	0.262abB ± 0.015
		0.50	61.48fB ± 0.18	68.47eA ± 0.05	2.69aA ± 0.09	2.68aA ± 0.02	19.70bA ± 0.30	19.81aA ± 0.14	0.008bB ± 0.001	1.777cA ± 0.110	0.862aA ± 0.033	0.270aB ± 0.014
	SBB	0	81.45bB ± 0.01	84.47bA ± 0.02	-2.73cB ± <0.01	-2.12eA ± 0.03	15.23cA ± <0.01	14.43dB ± 0.07	0.034aB ± <0.001	4.513aA ± 0.034	0.705cA ± 0.003	0.182cdB ± 0.021
		0.25	70.16cB ± 0.06	73.66dA ± 0.09	0.86bB ± 0.03	1.29dA ± 0.01	18.11bB ± 0.06	18.77bA ± 0.10	0.033aB ± 0.001	4.321aA ± 0.579	0.722bcA ± 0.004	0.194cdB ± 0.033
		0.50	63.91eB ± 0.17	68.46eA ± 0.04	2.21aA ± 0.13	2.05bA ± 0.02	19.21bA ± 0.24	19.67aA ± 0.03	0.039aB ± 0.008	3.092bA ± 0.716	0.704cA ± 0.022	0.22bcB ± 0.012
II	SSM	0	83.23aB ± 0.07	85.18aA ± 0.08	-4.01dB ± <0.01	-2.23eA ± 0.08	0.84eB ± <0.01	2.87fA ± 0.13	0.007bB ± 0.002	3.618aA ± 0.629	0.873aA ± 0.027	0.207bB ± 0.062
		0.25	67.29dB ± 0.44	74.06dA ± 0.03	2.23bA ± 0.32	1.39cA ± 0.01	17.78cA ± 1.08	16.79dA ± 0.03	0.016bB ± 0.007	1.667bA ± 0.462	0.763abA ± 0.092	0.31aB ± 0.018
		0.50	60.65fB ± 0.26	66.76fA ± 0.43	3.72aA ± 0.16	2.82aB ± 0.07	21.51aA ± 0.55	20.42aA ± 0.30	0.009bB ± 0.005	1.062bA ± 0.033	0.847aA ± 0.070	0.362aB ± 0.001
	SBB	0	79.30bB ± 0.01	84.55bA ± 0.06	-3.64dB ± 0.01	-2.17eA ± 0.03	11.70dB ± 0.03	14.75eA ± 0.11	0.030aB ± 0.004	3.100aA ± 0.855	0.717bA ± 0.016	0.198bB ± 0.049
		0.25	70.76cB ± 0.04	74.78cA ± 0.08	0.90cB ± 0.06	1.14dA ± 0.02	18.01bcB ± <0.01	18.25cA ± 0.02	0.033aB ± 0.001	3.333aA ± 0.380	0.720bA ± 0.008	0.158bB ± 0.034
		0.50	64.83eB ± 0.28	69.36eA ± 0.19	2.32bA ± 0.21	1.97bA ± 0.04	19.19bA ± 0.42	19.73bA ± 0.21	0.038aB ± 0.001	3.529aA ± 0.084	0.712bA ± 0.008	0.124bB ± <0.001
III	SSM	0	83.56aB ± <0.01	85.61aA ± 0.21	-3.83eA ± 0.01	-3.83eA ± <0.01	1.02eB ± 0.04	1.78dA ± 0.02	0.009bB ± 0.001	1.325dA ± 0.225	0.757aA ± 0.008	0.520aB ± 0.033
		0.25	69.73cB ± 0.32	71.92cA ± 0.01	1.85cA ± 0.05	1.53cA ± 0.23	17.82cA ± 0.05	17.78bA ± 0.66	0.011bB ± 0.005	2.684cA ± 0.288	0.687aA ± 0.030	0.366cB ± 0.001
		0.50	61.27fB ± 0.71	64.48eA ± 0.04	2.77bA ± 0.04	3.30aA ± 0.44	19.93aA ± <0.01	19.69aA ± 0.48	0.008bB ± 0.002	1.529dA ± 0.247	0.691aA ± 0.029	0.439bB ± <0.001
	SBB	0	81.99bB ± 0.04	82.78bA ± 0.02	-3.07dB ± 0.01	-2.66dA ± 0.01	13.30dB ± 0.08	14.54cA ± 0.12	0.026aB ± 0.010	2.786cA ± 0.049	0.735aA ± 0.147	0.306dB ± <0.001
		0.25	67.39dB ± 0.05	69.52dA ± 0.01	2.84bA ± 0.02	1.28cB ± 0.02	19.74aA ± 0.06	16.76bB ± 0.36	0.035aB ± 0.005	7.419aA ± 1.963	0.696aA ± 0.030	0.132eB ± 0.018
		0.50	61.95eB ± <0.01	63.39fA ± 0.07	3.32aA ± 0.04	2.45bB ± 0.01	19.31bA ± 0.23	17.74bB ± 0.02	0.025aB ± 0.005	5.038bA ± 0.293	0.744aA ± 0.035	0.277dB ± 0.011
IV	SSM	0	81.59aB ± 0.01	84.65aA ± 0.02	-3.48fB ± 0.02	-2.20dA ± 0.01	-1.15fB ± 0.03	2.75eA ± 0.01	0.012dB ± 0.004	2.455bcA ± 0.27	0.781bA ± 0.049	0.294abB ± 0.016
		0.25	67.56cB ± 0.04	71.48dA ± 0.03	0.75dB ± 0.01	1.88bA ± 0.04	15.05dB ± <0.01	17.81cA ± 0.01	0.008dB ± 0.001	1.532cA ± 0.101	0.869aA ± 0.039	0.311aB ± 0.004
		0.50	60.17eB ± 0.04	64.75fA ± 0.17	1.80bB ± 0.02	2.97aA ± 0.04	16.92cB ± 0.11	20.88aA ± 0.08	0.007dB ± <0.001	1.294cA ± 0.110	0.894aA ± 0.017	0.317aB ± 0.008
	SBB	0	80.32bB ± 0.08	84.07bA ± 0.02	-3.41eB ± <0.01	-2.36eA ± 0.04	13.09eB ± 0.10	14.03dA ± 0.09	0.025aB ± 0<0.001	4.625aA ± 1.223	0.746bcA ± 0.001	0.150dB ± 0.021
		0.25	67.44cB ± 0.10	72.02cA ± 0.06	0.80cB ± 0.02	1.12cA ± 0.05	17.93bA ± 0.13	17.90cA ± <0.01	0.035bB ± <0.001	3.223bA ± 0.156	0.702cA ± 0.014	0.220cB ± 0.039
		0.50	61.97dB ± 0.06	66.29eA ± 0.05	1.93aA ± 0.03	1.81bB ± 0.01	18.70aB ± 0.02	19.18bA ± 0.05	0.044cB ± 0.007	2.63bcA ± 0.461	0.677cA ± 0.030	0.250bcB ± 0.013

Observations were made before (t0) and after 24 hours (t24) the beginning of the fermentation.

[a-f] Different lowercase letters indicate differences in samples fermented by a mix culture (I, II, III, IV), having different growth media (SSM, SBB) and percentages of added SP% (0, 0.25% 0.50%) and compared at the same time (t0 or t24).

[A-B] Different uppercase letters indicate differences in the same sample (row) compared before (t0) and after (t24) fermentation.

Color characteristics of fermented milks

Despite the original color of *A. platensis* is green-blue (Park et al., 2018), the thermal treatment leads to a degradation of pigments changing the color change to a dark-green or brownish color (Antelo et al., 2008). Therefore, the impact of the thermally treated *A. platensis* on the overall color characteristics of the fermented SSM and SBB has been evaluated. The results of colorimetric analyses are reported in Table 3. As it is possible to observe from the reported L*, a*, b* values, the addition of SP had a drastic impact over colorimetric characteristics ($P<0.001$, in all mix cultures). The color of SSM and SBB changed from a whitish color of the controls (higher L* value) to a more yellowish and reddish color (higher b* and a* values) as shown in Figure 3, with the addition of 0.25% and 0.50% SP, as in the case of mix culture I. Comparing the initial color of the two substrates, SBB was characterized by different L*, a*, b* values ($P<0.001$) in all the mix cultures (data not shown) and, in particular, by a more yellowish color than SSM (Figure 3). The fermentation significantly changed the color characteristics of the samples (Table 3), and the modification was also noticeable by direct observation (Figure 3). In general, the fermentation caused an increase of L* values, if compared with the non-fermented sample ($P<0.05$ in all the comparisons), and this result was in accordance with Rankin & Brewer (1998) (Rankin & Brewer, 1998). In particular, it was possible to notice that the increase of lightness was more marked in most of the cases for samples added with 0.25% and 0.50% SP (on average, +4.1 ± 1.6%, +4.0 ± 2.1%, +2.3 ± 1.6% for sample with 0.50%, 0.25% and 0% of SP, respectively). This can be due to the breakdown of pigment molecules such as carotenoids, xanthophylls, phycobiliproteins and chlorophylls (Danesi & Rangel-Yagui, 2002), as a consequence of reduced stability at low pH and microbial growth (Chaiklahan et al., 2012; de Marco Castro et al., 2019) or to the production of molecules that can modify the colorimetric characteristics of fermented milk (Rankin & Brewer, 1998). Considering a* and b* values, the change of both parameters did not show a consistent trend concerning fermentation for none of the considered factors. The differences among samples were possibly conditioned by the different responses of the four commercial mix cultures in relation to growth substrates characteristics and the concentrations of SP added. For example, Mix III in SBB showed a

significant and evident decrease of both a* and b* values when 0.25% and 0.50% of SP were used. Conversely, when Mix III was used to ferment SSM, no significant changes between fermented and non-fermented samples were observed with the only exception of b* value in 0% SP sample (Table 3). This different behavior could be related to the different growing capacity and metabolic activities of LAB strains in different media fortified with SP.

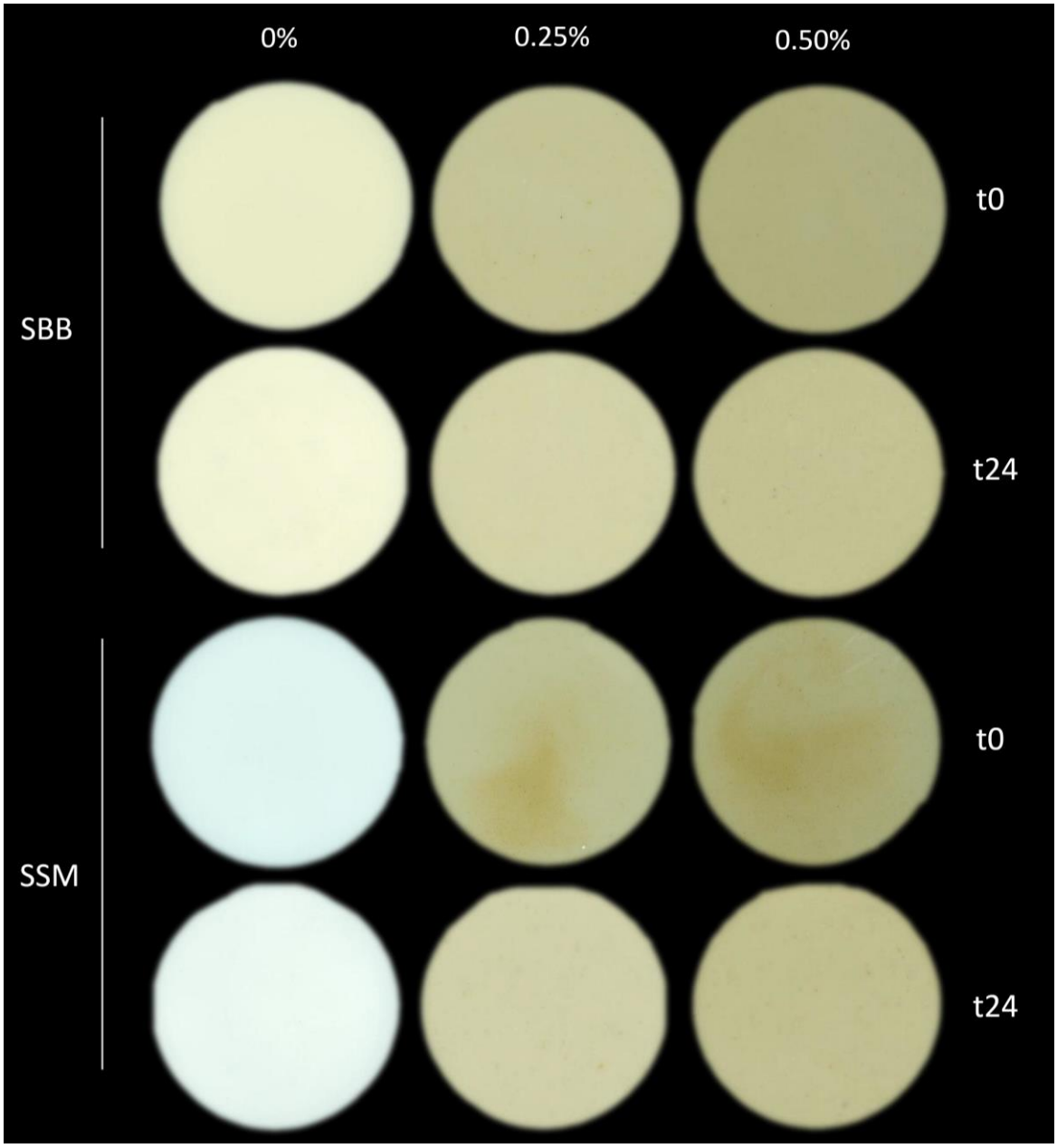

Figure 3. A representative picture of skimmed milk (SSM) and soy-based beverage (SBB) beverages inoculated with commercial mix culture I, before (t0) and after 24 hours (t24) of fermentation. Samples were fermented with the addition of 0, 0.25, and 0.5% (v/v) lysate of *Arthrospira platensis*.

Conclusions

The results obtained in this research allow us to conclude that *Spirulina*, besides being a well-known source of beneficial compounds that can be converted into sustainable functional compounds, can also be added as a natural ingredient to produce fermented milk and soy beverages with the aim to boost the fermentation performances of LAB and or improve their viability in the final product. Considering that this type of use requires its sterilization, the results obtained in our study take on great technological relevance also because of the innovative impedometric method used. It allowed to specifically estimate the impact of the addition of different concentrations of sterilized SP on the fermentation's behaviors of diverse acidifying LAB, probiotic strain and commercially used mix cultures. To a different extent, the strains and commercial mix starter cultures were stimulated by the addition of SP. The encountered variability in the boosting effect of *A. platensis* was dependent on the mix culture, the substrate used, and on the diverse concentration added. This variability was also reflected on the rheological and color characteristics of fermented milk, whose change was dependent on the different growth abilities and microbial specificity of LAB cultures highlighting that each of them could have specific employment. Regarding the concentration of SP used, the lowest one (0.25%) showed the best boosting effect on the strains and mix culture. On the other hand, the highest concentration used (0.5%) showed an inconstant effect on LAB, displaying in some cases a stimulatory effect, or no effect or even a slowdown of LAB growth.

Knowing this variability, the SP effect should be studied depending on the strain, culture and their application, to find the right balance between the technological advantage in terms of decreasing the production times and the functional effect of spirulina addition.

References

Ak, B., Avşaroğlu, E., Işık, O., Özyurt, G., Kafkas, E., & Uslu, L. (2016). Nutritional and Physicochemical Characteristics of Bread Enriched with Microalgae *Spirulina platensis*. *Int. Journal of Engineering Research and Application*, *6*(12), 9.

Andrade, L. M. (2018). Chlorella and Spirulina Microalgae as Sources of Functional Foods, Nutraceuticals, and Food Supplements; an Overview. *MOJ Food Processing & Technology*, *6*(1), 45–58. https://doi.org/10.15406/mojfpt.2018.06.00144

Antelo, F. S., Costa, J. A. V., & Kalil, S. J. (2008). Thermal degradation kinetics of the phycocyanin from *Spirulina platensis*. *Biochemical Engineering Journal*, *41*(1), 43–47. https://doi.org/10.1016/j.bej.2008.03.012

Ariede, M. B., Candido, T. M., Jacome, A. L. M., Velasco, M. V. R., de Carvalho, J. C. M., & Baby, A. R. (2017). Cosmetic attributes of algae - A review. *Algal Research*, *25*, 483–487. https://doi.org/10.1016/j.algal.2017.05.019

Ásványi-Molnár, N., Sipos-Kozma, Z., Tóth, Á., Ásványi, B., & Varga, L. (2009). Development of functional dairy food enriched in spirulina (*Arthrospira platensis). Tejgazdaság*, *69*(2), 15–22. CABDirect.

Bancalari, E., Bernini, V., Bottari, B., Neviani, E., & Gatti, M. (2016). Application of Impedance Microbiology for Evaluating Potential Acidifying Performances of Starter Lactic Acid Bacteria to Employ in Milk Transformation. *Frontiers in Microbiology*, *7*(1628), 11. https://doi.org/10.3389/fmicb.2016.01628

Bancalari, E., D'Incecco, P., Savo Sardaro, M. L., Neviani, E., Pellegrino, L., & Gatti, M. (2019). Impedance microbiology to speed up the screening of lactic acid bacteria exopolysaccharide production. *International Journal of Food Microbiology*, *306*(108268), 10. https://doi.org/10.1016/j.ijfoodmicro.2019.108268

Barkallah, M., Dammak, M., Louati, I., Hentati, F., Hadrich, B., Mechichi, T., Ayadi, M. A., Fendri, I., Attia, H., & Abdelkafi, S. (2017). Effect of *Spirulina platensis* fortification on

physicochemical, textural, antioxidant and sensory properties of yogurt during fermentation and storage. *LWT*, *84*, 323–330. https://doi.org/10.1016/j.lwt.2017.05.071

Batista, A. P., Niccolai, A., Fradinho, P., Fragoso, S., Bursic, I., Rodolfi, L., Biondi, N., Tredici, M. R., Sousa, I., & Raymundo, A. (2017). Microalgae biomass as an alternative ingredient in cookies: Sensory, physical and chemical properties, antioxidant activity and in vitro digestibility. *Algal Research*, *26*, 161–171. https://doi.org/10.1016/j.algal.2017.07.017

Beheshtipour, H., Mortazavian, A. M., Mohammadi, R., Sohrabvandi, S., & Khosravi-Darani, K. (2013). Supplementation of *Spirulina platensis* and *Chlorella vulgaris* Algae into Probiotic Fermented Milks: Algae addition into probiotic fermented milk. *Comprehensive Reviews in Food Science and Food Safety*, *12*(2), 144–154. https://doi.org/10.1111/1541-4337.12004

Beheshtipour, Hannane, Mortazavian, A. M., Haratian, P., & Darani, K. K. (2012). Effects of *Chlorella vulgaris* and *Arthrospira platensis* addition on viability of probiotic bacteria in yogurt and its biochemical properties. *European Food Research and Technology*, *235*(4), 719–728. https://doi.org/10.1007/s00217-012-1798-4

Bhowmik, D., Dubey, J., & Mehra, S. (2009). Probiotic Efficiency of *Spirulina platensis* - Stimulating Growth of Lactic Acid Bacteria. *World Journal of Dairy & Food Sciences*, *4*(2), 160–164.

Camacho, F., Macedo, A., & Malcata, F. (2019). Potential Industrial Applications and Commercialization of Microalgae in the Functional Food and Feed Industries: A Short Review. *Marine Drugs*, *17*(312), 32. https://doi.org/10.3390/md17060312

Cao, Z.-H., Green-Johnson, J. M., Buckley, N. D., & Lin, Q.-Y. (2019). Bioactivity of soy-based fermented foods: A review. *Biotechnology Advances*, *37*(1), 223–238. https://doi.org/10.1016/j.biotechadv.2018.12.001

Çelekli, A., Alslibi, Z. A., & Bozkurt, H. üseyin. (2019). Influence of incorporated *Spirulina platensis* on the growth of microflora and physicochemical properties of ayran as a functional food. *Algal Research*, *44*, 101710. https://doi.org/10.1016/j.algal.2019.101710

Chaiklahan, R., Chirasuwan, N., & Bunnag, B. (2012). Stability of phycocyanin extracted from *Spirulina* sp.: Influence of temperature, pH and preservatives. *Process Biochemistry*, *47*(4), 659–664. https://doi.org/10.1016/j.procbio.2012.01.010

Danesi, E. D. G., & Rangel-Yagui, C. de O. (2002). An investigation of e ect of replacing nitrate by urea in the growth and production of chlorophyll by *Spirulina platensis*. *Biomass and Bioenergy*, *23*, 261–269.

de Caire, G. Z., & Parada, J. L. (2000). Effect of *Spirulina platensis* biomass on the growth of lactic acid bacteria in milk. *World Journal of Microbiology & Biotechnology*, *16*, 563–565.

de Marco Castro, E., Shannon, E., & Abu-Ghannam, N. (2019). Effect of Fermentation on Enhancing the Nutraceutical Properties of *Arthrospira platensis* (Spirulina). *Fermentation*, *5*(1), 28. https://doi.org/10.3390/fermentation5010028

Fusco, V., Quero, G. M., Cho, G.-S., Kabisch, J., Meske, D., Neve, H., Bockelmann, W., & Franz, C. M. A. P. (2015). The genus *Weissella*: taxonomy, ecology and biotechnological potential. *Frontiers in Microbiology*, *6*(155), 22. https://doi.org/10.3389/fmicb.2015.00155

Gardiner, G. E., Bouchier, P., O'Sullivan, E., Kelly, J., Kevin Collins, J., Fitzgerald, G., Paul Ross, R., & Stanton, C. (2002). A spray-dried culture for probiotic Cheddar cheese manufacture. *International Dairy Journal*, *12*(9), 749–756. https://doi.org/10.1016/S0958-6946(02)00072-9

Ghaeni, M., Roomiani, L., & Moradi, Y. (2014). Evaluation of Carotenoids and Chlorophyll as Natural Resources for Food in Spirulina Microalgae. *Applied Food Biotechnology*, *2*(1), 39–44. https://doi.org/DOI: 10.22037/afb.v2i1.7210

Golmakani, M.-T., Soleimanian-Zad, S., Alavi, N., Nazari, E., & Eskandari, M. H. (2019). Effect of Spirulina (*Arthrospira platensis*) powder on probiotic bacteriologically acidified feta-

type cheese. *Journal of Applied Phycology*, *31*(2), 1085–1094. https://doi.org/10.1007/s10811-018-1611-2

Gueimonde, M., Delgado, S., Mayo, B., Ruas-Madiedo, P., Margolles, A., & de los Reyes-Gavilán, C. G. (2004). Viability and diversity of probiotic *Lactobacillus* and *Bifidobacterium* populations included in commercial fermented milks. *Food Research International*, *37*(9), 839–850. https://doi.org/10.1016/j.foodres.2004.04.006

Guldas, M., & Irkin, R. (2010). Influence of *Spirulina platensis* powder on the microflora of yoghurt and acidophilus milk. *Mljekarstvo*, *60*(4), 237–243.

Gyenis, B., Szigeti, J., Molnár, N., & Varga, L. (2005). Use of dried microalgal biomasses to stimulate acid production and growth of *Lactobacillus plantarum* and *Enterococcus faecium* in milk. *Acta Agraria Kaposváriensis*, *9*(2), 53–59.

Holman, B. W. B., & Malau-Aduli, A. E. O. (2013). *Spirulina* as a livestock supplement and animal feed: *Spirulina* supplementation in livestock. *Journal of Animal Physiology and Animal Nutrition*, *97*(4), 615–623. https://doi.org/10.1111/j.1439-0396.2012.01328.x

Kandler, O. (1983). Carbohydrate metabolism in lactic acid bacteria. *An Tonie van Leeuwenhoek*, *49*, 209–224.

Korbekandi, H., Jahadi, M., Maracy, M., Abedi, D., & Jalali, M. (2009). Production and evaluation of a probiotic yogurt using *Lactobacillus casei* ssp. *casei*. *International Journal of Dairy Technology*, *62*(1), 75–79. https://doi.org/10.1111/j.1471-0307.2008.00436.x

Li, C., Li, W., Chen, X., Feng, M., Rui, X., Jiang, M., & Dong, M. (2014). Microbiological, physicochemical and rheological properties of fermented soymilk produced with exopolysaccharide (EPS) producing lactic acid bacteria strains. *LWT - Food Science and Technology*, *57*(2), 477–485. https://doi.org/10.1016/j.lwt.2014.02.025

Majdoub, H., Mansour, M. B., Chaubet, F., Roudesli, M. S., & Maaroufi, R. M. (2009). Anticoagulant activity of a sulfated polysaccharide from the green alga *Arthrospira platensis*.

Biochimica et Biophysica Acta (BBA) - General Subjects, *1790*(10), 1377–1381. https://doi.org/10.1016/j.bbagen.2009.07.013

Massoud, R., Khosravi-Darani, K., Nakhsaz, F., & Varga, L. (2017). Evaluation of physicochemical, microbiological and sensory properties of croissants fortified with *Arthrospira platensis (Spirulina*). *Czech Journal of Food Sciences*, *34*(4), 350–355. https://doi.org/10.17221/289/2015-CJFS

Mohammadi-Gouraji, E., Soleimanian-Zad, S., & Ghiaci, M. (2019). Phycocyanin-enriched yogurt and its antibacterial and physicochemical properties during 21 days of storage. *LWT*, *102*, 230–236. https://doi.org/10.1016/j.lwt.2018.09.057

Molnár, N., Gyenis, B., & Varga, L. (2005). Influence of a powdered *Spirulina platensis* biomass on acid production of lactococci in milk. *Milchwissenschaft*, *60*(4), 380–382.

Mortazavian, A. M., Ehsani, M. R., Mousavi, S. M., Rezaei, K., Sohrabvandi, S., & Reinheimer, J. A. (2007). Effect of refrigerated storage temperature on the viability of probiotic micro-organisms in yogurt. *International Journal of Dairy Technology*, *60*(2), 123–127. https://doi.org/10.1111/j.1471-0307.2007.00306.x

Parada, J. (1998). Lactic acid bacteria growth promoters from *Spirulina platensis*. *International Journal of Food Microbiology*, *45*(3), 225–228. https://doi.org/10.1016/S0168-1605(98)00151-2

Park, J. H., Lee, S. I., & Kim, I. H. (2018). Effect of dietary S*pirulina (Arthrospira*) *platensis* on the growth performance, antioxidant enzyme activity, nutrient digestibility, cecal microflora, excreta noxious gas emission, and breast meat quality of broiler chickens. *Poultry Science*, *97*(7), 2451–2459. https://doi.org/10.3382/ps/pey093

Penna, A. L. B., Gurram, S., & Barbosa-Cánovas, G. V. (2006). Effect of high hydrostatic pressure processing on rheological and textural properties of probiotic low-fat yogurt fermented by different starter cultures. *Journal of Food Process Engineering*, *29*(5), 447–461. https://doi.org/10.1111/j.1745-4530.2006.00076.x

Plaza, M., Herrero, M., Cifuentes, A., & Ibáñez, E. (2009). Innovative Natural Functional Ingredients from Microalgae. *Journal of Agricultural and Food Chemistry*, *57*(16), 7159–7170. https://doi.org/10.1021/jf901070g

Rankin, S. A., & Brewer, J. L. (1998). Color of Nonfat Fluid Milk as Affected by Fermentation. *Journal of Food Science*, *63*(1), 178–180. https://doi.org/10.1111/j.1365-2621.1998.tb15704.x

Shafiee, G., Mortazavian, A. M., Mohammadifar, M. A., Koushki, M. R., Mohammadi, A., & Mohammadi, R. (2010). Combined effects of dry matter content, incubation temperature and final pH of fermentation on biochemical and microbiological characteristics of probiotic fermented milk. *Afr. J. Microbiol. Res.*, *4*(12), 1265–1274.

Shah, N. P. (2000). Effects of milk-derived bioactives: an overview. *British Journal of Nutrition*, *84*(S1), 3–10. https://doi.org/10.1017/S000711450000218X

Varga, L., Szigeti, J., Kovács, R., Földes, T., & Buti, S. (2002). Influence of a S*pirulina platensis* Biomass on the Microflora of Fermented ABT Milks During Storage (R1). *Journal of Dairy Science*, *85*(5), 1031–1038. https://doi.org/10.3168/jds.S0022-0302(02)74163-5

Varga, L., Szigeti, J., & Ordog, V. (1999). Effect of a *Spirulina platensis* biomass enriched with trace elements on combinations of starter culture strains employed in the dairy industry. *Milchwissenschaft*, *54*(5), 247–248.

Vijay Pratap Singh, P. M. (2014). *Spirulina* and its Nutritional Importance: A Possible Approach for Development of Functional Food. *Biochemistry & Pharmacology: Open Access*, *03*(06). https://doi.org/10.4172/2167-0501.1000e171

Wang, H., Zhang, W., Chen, L., Wang, J., & Liu, T. (2013). The contamination and control of biological pollutants in mass cultivation of microalgae. *Bioresource Technology*, *128*, 745–750. https://doi.org/10.1016/j.biortech.2012.10.158

Yamaguchi, S. K. F., Moreira, J. B., Costa, J. A. V., de Souza, C. K., Bertoli, S. L., & Carvalho, L. F. de. (2019). Evaluation of Adding *Spirulina* to Freeze-Dried Yogurts Before Fermentation

and After Freeze-Drying. *Industrial Biotechnology*, *15*(2), 89–94. https://doi.org/10.1089/ind.2018.0030

Zhao, Q.-Z., Wang, J.-S., Zhao, M.-M., Jiang, Y.-M., & Chun, C. (2006). Effect of Casein Hydrolysates on Yogurt Fermentation and Texture Properties during Storage. *Food Technol. Biotechnol*, *44*(3), 429–434.

Zouari, N., Abid, M., Fakhfakh, N., Ayadi, M. A., Zorgui, L., Ayadi, M., & Attia, H. (2011). Blue-green algae (*Arthrospira platensis*) as an ingredient in pasta: free radical scavenging activity, sensory and cooking characteristics evaluation. *International Journal of Food Sciences and Nutrition*, *62*(8), 811–813. https://doi.org/10.3109/09637486.2011.582461

Solid state fermentation of *Arthrosphira platensis* to implement new food products: evaluation of stabilization treatments and bacterial growth on the volatile fraction

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Abstract

Arthrospira platensis is a cyanobacterium widely used in food formulation and mainly consumed as a food supplement because of its high amount of proteins, vitamins and minerals. Different probiotic food supplements are present in the market and a lactic acid fermented food product like dried spirulina, could be useful not only to introduce lactic acid bacteria (LAB) with beneficial effects to consumer's diet, but also to improve or change the aromatic profile of the substrate. Therefore, the aim of this study was the evaluation of lactic acid fermentation of *A. platensis* biomass focusing on the consequent changes in the aromatic profile. For this purpose, two different stabilization treatments (UV light treatment and sterilization) were applied prior to fermentation with two LAB strains, *Lacticaseibacillus casei* 2240 and *Lacticaseibacillus rhamnosus* GG. The biomass proved to be a suitable matrix for solid state fermentation showing a LAB growth of more than 2 Log CFU/g in 48h. The fermentation process was also useful for off flavors reduction. In particular, the fermentation process significantly influenced the concentration of those compounds responsible for aldehydic/ ethereal, buttery/waxy (acetoin and diacetyl), alkane and fermented aromatic notes (isoamyl alcohol). The heat treatment of the matrix, in addition to guarantee safety for consumers, led to an improved aroma after fermentation. In conclusion, according to the heat treatment applied, a fermented spirulina powder with different aromatic profile was obtained. Fermentation with lactic acid bacteria can be an interesting tool to obtain cyanobacterial biomass with more pleasant sensory properties for potential use in food formulation.

Introduction

Arthrospira platensis, commercially known as Spirulina, is a cyanobacterium commonly consumed as a food supplement because of its high nutritional value (Andrade et al., 2018; Batista et al., 2017). It is characterized by a high percentage of proteins (60%), conferring all the essential aminoacids, followed in abundance by carbohydrates and polyunsaturated fatty acids (ω-3 and ω-6). Moreover, discrete quantities and varieties of minerals, vitamins and pigments (C- phycocyanin, chlorophyll and carotenoids) (Niccolai et al., 2019) are present. To date, microalgae and spirulina, defined as the Novel food of the future (Zarbà et al., 2020), are used for various purposes in nutraceutical, cosmetic (Ariede et al., 2017), feed (Holman & Malau-Aduli, 2013) and pharma (Hamed et al., 2015) sectors and they continue to increasingly attract the interest of consumers and companies because of several bioactivities that this cyanobacterium is proving in many studies (Bancalari et al., 2020; Martelli, Cirlini, et al., 2020). Applications aimed to confer macro and micronutrients, and improve colour are increasing: and many food products, among which bread, cookies and pasta (Ak et al., 2016; Massoud et al., 2017; Zouari et al., 2011), cheeses (Golmakani et al., 2019), yogurt (Mohammadi-Gouraji et al., 2019; Yamaguchi et al., 2019) and beverages (Camacho et al., 2019), supplemented with *A. platensis* have been developed. To date most of the algal biomass produced is consumed in the form of powder or tablets as proteic and micronutrients rich supplements. Positive results have also been obtained using this cyanobacterium as a prebiotic for lactic acid bacteria (LAB). Indeed it was able not only to preserve LAB viability in food matrixes (Barkallah et al., 2017; Guldas & Irkin, 2010) but also to stimulate their growth in broth(Bhowmik et al., 2009; Martelli, Alinovi, et al., 2020; Parada, 1998). The effect of *A. platensis* biomass on LAB could be exploited especially for the production of probiotic food supplements or ingredients. Fortification of foods with probiotic strains shows beneficial effects such as anti-inflammatory, antioxidant, immunomodulatory, protection against colitis and damage to epithelial cells (Bron et al., 2017). Moreover, probiotics, once ingested, are believed to play an important role in the

intestinal tract against foodborne pathogens (Gardiner et al., 2002) and can reduce symptoms due to antibiotic therapies, to relieve food allergies and reduce atypical dermatitis (Wong et al., 2013). A lactic acid fermentation process applied to spirulina dried powder could be useful not only to supply consumers of LAB with beneficial effects to consumer diet, but also to improve or change the aromatic profile as seen in the case of vegetable matrices (Ricci, Cirlini, Maoloni, et al., 2019). Indeed, a biological process to reduce unpleasant smells may represent an important tool for applications of algal biomass in complex foods avoiding off flavors. During fermentation, the metabolic activity of LAB leads not only to rapid acidification of the substrate and fast consumption of easily fermentable sugars, with a competitive advantage in the use of LAB in nutrient-rich environments, but also to the production of volatile compounds belonging to different chemical classes such as: alcohols, aldehydes, ketones, acids, esters and sulfur compounds. These compounds mainly derive from the catabolism of citrate and from the degradation of proteins and lipids. The formation of aromatic compounds is a complex process in which precursors are initially generated and subsequently converted into aromatic compounds (Smid & Kleerebezem, 2014).

On the other hand, complex issues arise in the production of spirulina-based products, that can be considered safe for consumer health. Because of cultivation conditions and manipulation during downstream processing, *A. platensis* can be contaminated by alterative and pathogenic bacteria (Wang et al., 2013) that may duplicate during fermentation compromising quality and safety.

The aromatic fraction of *A. platensis* is characterized by several volatiles present in different concentrations and with different odor thresholds, formed in the matrix during growth and maturation(Aguero et al., 2003) . The most abundant volatiles identified as aromatic components of *A. platensis* are hydrocarbons, especially heptadecane, followed by furanic compounds, pyrazines, sulfur compounds, aldehydes, ketones and alcohols (Aguero et al., 2003; Bao et al., 2018; Cuellar-Bermúdez et al., 2017). All these substances, naturally present in the matrix and ascribed to amino acids and fatty acids microorganism metabolism, contribute to the typical fishy odor that characterizes algae and algae derived products

(Fink, 2007; Högnadóttir, 2000) . This unpleasant flavor could be reduced by applying a fermentation step on the product, as described in recent works in which yeasts and bacteria were used to ferment different algae matrices (Bao et al., 2018; Seo et al., 2012).

On the basis of these considerations, the present study was aimed to evaluate solid state fermentation of *A. platensis* biomass considering two LAB species, *Lacticaseibacillus rhamnosus* and *Lacticaseibacillus casei*, belonging to *Lacticaseibacillus casei* group, for the production of a lactic acid fermented food product. To reach this goal, to stabilize biomass, two different treatments based on UV light irradiation and thermal sterilization were applied prior to fermentation. The effects on LAB growth were evaluated and the aromatic fraction of *A. platensis* was characterized in order to assess improvements in the volatile profile given by LAB fermentation.

Materials and Methods

Arthrospira platensis stabilization treatments

The dehydrated *A. platensis*, kindly provided by Bertolini Farm (Fidenza, PR, Italy), was used as a substrate for fermentation. It is a commercial product cultivated in raceway pond and marketed as "Organic Spirulina" according to EU Organic Aquaculture Regulation (EC No. 834/07). In order to reduce the microbial contamination present on the biomass, two different treatments were applied separately. The first consisted in UV light irradiation for 15 minutes to reduce the microbial total charge applied under a Faster BH-EN 2004 Class II Microbiological Safety Cabinet (S/N 1113) (Richmond scientific, Great Britain) with a lamp emitting light at an intensity of 253.7 nm, in the spectrum of UV-C (UV), while the second was based on a thermal treatment of 121°C for 20 minutes applied in autoclave (3870MLV, Tuttnauer, NY, US) to sterilize the algal biomass (ST). The time for the sterilization cycle was of approximately 50 minutes. To reach the temperature of 121 °C, 15 minutes were necessary, 20 minutes to perform the sterilization process and further 15 minutes to cool the autoclave after treatment. To evaluate the efficacy of the stabilization treatments, microbial plate counts were performed just after treatments and after 48 hours of incubation at 37 ° C in order to determine the residual microbial load. To this purpose, samples were ten fold serially diluted in Ringer solution (Oxoid, Basingstoke, USA) and plated on Plate Count Agar (PCA) (Oxoid, Basingstoke, USA) incubated at 37 °C for 48h. Analyses were performed in duplicate and average values ± standard deviations were reported as Log CFU/g.

Lactic acid bacteria strains

Two LAB strains were used to ferment *A. platensis* biomass: *Lacticaseibacillus casei* 2240, isolated from Parmigiano Reggiano cheese, belonging to the collection of the Department of Food and Drug (University of Parma), and *Lacticaseibacillus rhamnosus* GG, a probiotic

commercial strain. They were maintained at -80 °C in de Man, Rogosa and Sharpe (MRS) cultivation medium (Oxoid, Basingstoke, UK), added with 12.5% glycerol (v/v) before use.

Arthrospira platensis biomass fermentation

LAB strains were revitalized twice in MRS broth (Oxoid) (inoculation of 3% v/v) incubated for 16 h at 37 °C under aerobic conditions. They were then inoculated in fresh MRS broth (3% v/v) and incubated for 15 h at 37 °C, to obtain a bacterial concentration of 9 Log CFU /ml. After centrifugation (Eppendorf centrifuge 5810 R, Eppendorf, Hamburg, Germany) (12,857x g for 10 minutes at 4 °C), cells were collected, washed twice in Ringer solution (Oxoid, Milan, Italy) and suspended in sterile bidistilled water.

A. platensis biomass was rehydrated with 70% w/w of sterile water and then inoculated individually with each bacterial suspension in order to obtain an estimated LAB concentration of 7 Log CFU/ ml in each sample. LAB concentration was evaluated just after the inoculation (T0), and after 24 hours (T1) and 48 hours of fermentation (T2). Serial dilutions of the samples in Ringer (Oxoid) were plated on MRS agar (Oxoid) and incubated for 48 h at 37 °C in aerobic conditions. pH of all the samples, before and after the fermentation step, was also measured (Mettler Toledo, Greifensee, Switzerland). Fermentations were carried out in duplicate and for each sampling time analyses were performed in duplicate. Colonies were counted manually and to calculate the CFU/ g concentration the following equation was applied:

$$\frac{\Sigma c}{(1 * na + 0.1 * nb + 0.01 * nc)d}$$

Where ∑C is the summation of all the counted colonies, *na* is the number of plates of the first countable serial dilution, *nb* is the number of plates of the second countable serial dilution, *nc* is the number of plates of the third countable serial dilution and *d* is the serial dilution factor of the first countable serial dilution.

Average values ± standard deviation were reported as Log CFU/g. Treated but not inoculated samples were also incubated at 37°C and analyzed at the same sampling times as control samples. The 48 h fermented and stabilized but not fermented samples were then lyophilized by a Freeze dryer Lio-5P (5Pascal, Milano, Italy) for 48h and then LAB concentration was determined by plate counting on MRS.

HS-SPME/GC-MS analysis

The characterization of the volatile fraction was conducted on all the fermented and stabilized but not fermented *A. platensis* samples. To this purpose, the protocol reported by Ricci et al. 2019 (Ricci, Cirlini, Maoloni, et al., 2019) was applied with some modifications. Briefly, 2 g of biomass and 5 μL of an aqueous Toluene standard solution (100 μg/mL in 10 mL) were used for the analyses. The headspace of the samples was extracted by a Divinylbenzene–Carboxen–Polydimethylsiloxane (DVB/Carboxen/PDMS) SPME fiber (Supelco, Bellefonte, PA, USA) for 30 min at 40 °C, after an equilibration time of 15 min at the same temperature. The separation of the volatile compounds was achieved using a SUPELCOWAX 10 capillary column (Supelco, Bellefonte, PA, USA; 30 m ×0.25 mm× 0.25 μm) placed on a Thermo Scientific Trace 1300 gas chromatograph coupled with a Thermo Scientific ISQ single quadrupole mass spectrometer equipped with an electronic impact (EI) source. All the parameters applied for the analyte separation and detection as injector, transfer line and column compartment temperatures, injection mode and gas carrier flow were the same reported by Ricci et al. 2019 (Ricci, Cirlini, Maoloni, et al., 2019). The detected volatile compounds were then identified on the basis of their Linear Retention Indexes, calculated using as reference a C8–C20 alkane solution analysed under the same chromatographic conditions applied for sample analysis, as by the comparison of the registered mass spectra with those reported in the instrument libraries (NIST 14). The semi-quantification of all the identified volatiles was achieved on the basis of the use of a reference compound (Toluene). Data were reported as μg/g of wet weight.

Statistical analysis

In order to determine the actual growth of tested LAB strains in *A. platensis* samples, results obtained from microbial counts were statistically treated applying one-way ANOVA test, comparing different growing times and lyophilized samples.

One-way ANOVA was carried out also considering data obtained from semi-quantification of all the detected volatiles to underline analogies and differences among the considered samples, in terms of production/diminution and/or release of volatiles. Moreover, two-way ANOVA was applied in order to evaluate the influence of two different factors, fermentation and stabilization treatment, on the volatile profile. All the analyses were performed using IBM SPSS Statistics 23.0 software (SPSS Inc., Chicago, IL) applying Tukey's test as post hoc test ($p \leq 0.05$).

Results and discussion

Arthrospira platensis fermentation

One of the aims of the study was the evaluation of solid state fermentation of *A. platensis* with *L. casei* group bacteria that could lead not only to the implementation of new lactic acid fermented food products but also to modifications of algae biomass aromatic fraction. Fermenting a matrix having a high bacterial charge could negatively affect the finished product considering that also spoilage and/or pathogenic bacteria may grow during the process. In this specific case, the biomass utilized for the experiment was cultivated in a raceway pond. This type of cultivation is associated with undesired microflora, like *Bacillus spp.*, *Alteromonas spp.*, *Flavobacterium spp.* and *Pseudomonas spp.* (Wang et al., 2013), and previous studies have reported a variable concentration of bacteria, ranging from 2 to 7 Log CFU/g, in this type of matrix (Niccolai et al., 2019). For that reason, prior to fermentation, carried for 48 hours in sterilized glass cans, samples were subjected to a UV radiation treatment, that doesn't seem to affect the *A. platensis* composition (Pala & Toklucu, 2011), or a sterilization treatment in an autoclave in order to reduce the presence of microbiological contaminants. The UV treatment was ineffective for the reduction of microbial contamination that was maintained at the same level of untreated samples (5.10 ± 0.2 Log CFU/g). To expect UV disinfection rates on food surfaces is necessary to consider interactions between microorganisms and surface materials, trying to avoid shielding effects from incident UV and predict the dependency on the surface structure or topography (Koutchma, 2008). For that reason, UV treatment did not reduce the initial microbial contamination because of an uneven distribution of rays in the dehydrated samples. Furthermore, the presence of a high amount of pigments, phenolic compounds and other components with a protective effect against UV rays, may contribute to microbial survival (de Jesus Raposo et al., 2016). After the UV treatment, a fermentation process of 48 hours was conducted considering two *Lacticaseibacillus casei* group bacteria. *L. casei* 2240 and *L. rhamnosus* GG concentration was determined just after the inoculation, and the growth was

determined after 24 and 48 hours of incubation at 37°C. The endogenous contamination didn't affect the counts given a much higher LAB inoculation of 7 Log CFU/g.

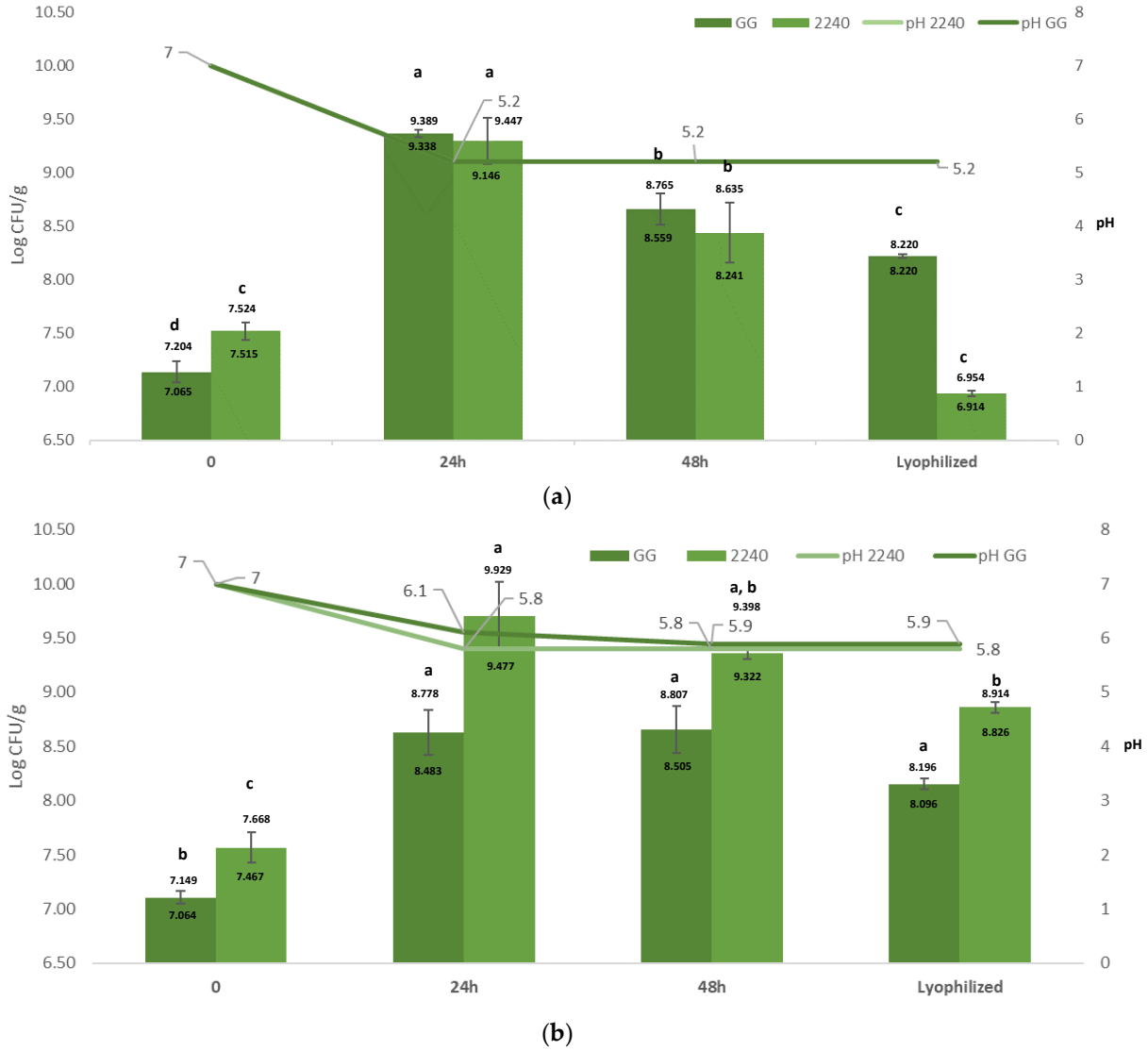


Figure 1. LAB growth (Log CFU/g) and pH values in *A. platensis* biomass after 24 and 48 h of fermentation at 37°C for *L. rhamnosus* GG (GG) and *L. casei* 2240 (2240) bacteria: a) UV treated samples. b) heat treated samples. Data are represented as average ± SD (bars), 2 replicates for each sample were measured. Letters indicate significant differences ($p<0.05$).

As shown in Figure 1A, the two strains showed a similar attitude to grow in the UV treated matrix. After 24 hours of fermentation, both species showed good replication capacity reaching a concentration higher than 9 Log CFU/g. In particular, *L. rhamnosus*

GG increased by 2.23 Log CFU/g (from 7.13 ± 0.09 Log CFU/g to 9.36 ± 0.03 Log CFU/g) ($p<0.05$), and to an higher extent than *L. casei* 2240 with a growth of 1.78 Log CFU/g (from 7.52 ± 0.06 Log CFU/g to 9.30 ± 0.21 Log CFU/g). After 48 hours of fermentation, a decrease in the concentration of both strains was noticed. *L. casei* 2240 decreased of 0.86 Log CFU/g (from 9.30 ± 0.21 Log CFU/g to 8.44 ± 0.28 Log CFU/g) while *L. rhamnosus* GG of 0.70 Log CFU/g (from 9.36 ± 0.03 Log CFU/g to 8.66 ± 0.14 Log CFU/g) (Figure 1A) ($p<0.05$). However, the values remained higher than the inoculation. *L. casei* 2240 strain was already used to ferment other matrixes, like *Himanthalia elongata*, but growth ability on this brown seaweed was lower than what observed in this study (Martelli, Favari, et al., 2020). The best capacity of this strain to grow on *A. platensis* can be linked to the high amount of proteins and small peptides (Andrade et al., 2018). *L. rhamnosus* GG is a probiotic commonly supplemented to fermented milk (Jia et al., 2016) but applications to other matrix have also been evaluated. For instance, a sausage supplemented with *L. rhamnosus* GG has been produced (Rubio et al., 2013) though this strain grew to a lesser extent than in *A. platensis*. During fermentation, the initial pH of the hydrated *A. platensis* biomass decreased from 7.0 ± 0.1 to 5.2 ± 0.1 in the first 24 hours for both samples and remained stable until the end of the fermentation process. This decrease in pH during fermentation reflects LAB strains growth with consequent production of organic acids, mainly lactic acid. Considering the possibility to use lactic acid fermented *A. platensis* in a food supplement formulation or as an ingredient, the survival of LAB was evaluated after a lyophilization process. *L. rhamnosus* GG was reduced at 8.22 ± 0.02 Log CFU/g ($p<0.05$), and *L. casei* 2240 was reduced by more than 1 Log CFU/g (from 8.44 ± 0.28 Log CFU/g to 6.9 ± 0.03 Log CFU/g) ($p<0.05$) (Figure 1A). The second fermentation process was carried out on *A. platensis* samples treated at 121°C for 20 minutes in order to eliminate the presence of any microbial contamination. The absence of residual contamination after treatment was confirmed by plate count below the detection limit (1 Log CFU/g). The sterilization treatment involves the use of a particular apparatus, the autoclave, and high energy to reach the temperatures and pressures required by the process. This treatment is therefore longer and more

expensive than that based on UV rays, but the results show that it is certainly more effective in reducing the microbial concentration of the product. Following the heat treatment, the characteristic green-blue color of *A. platensis* changed to a dark green, brownish color. This may be due to the degradation of pigments such as carotenoids, xanthophylls, phycocyanins and chlorophyll caused by the high temperatures reached (Antelo et al., 2008; Dutta et al., 2006; Weemaes et al., 1999). The growth trend of LAB strains is presented in figure 1B. In particular, after 24 hours *L. casei* 2240 increased by 2.13 Log CFU/g (from 7.57 ± 0.142 Log CFU/g to 9.70 ± 0.320 Log CFU/g) ($p<0.05$) and *L. rhamnosus* GG by 1.5 Log (from 7.10 ± 0.06 Log CFU/g to 8.63 ± 0.21 Log CFU/g) ($p<0.05$) and then remained constant (with no significant differences) up to 48h. The ability of these *Lacticaseibacillus* species to grow on different matrixes has been challenged over the years, with different results (Pereira et al., 2011; Ricci, Cirlini, Maoloni, et al., 2019; Rubio et al., 2013; Wu et al., 2010). In particular *L. casei* and *L. rhamnosus* strains grown on sterilized vegetables by-products increased more than what was observed on *A. platensis* (Ricci, Bernini, et al., 2019). However, the ability of this cyanobacterium to boost the growth of LAB is known in literature (Martelli, Alinovi, et al., 2020) and because of its richness in small peptides and proteins, *A. platensis* can be considered a good matrix to allow the growth of LAB species like *L. casei* and *L. rhamnosus*. After fermentation of the sterilized biomass, a lower acidification rate was observed compared to the UV treated fermented samples. The pH value of the sterilized fermented biomasses after 24 hours was 5.8 ± 0.1 and 6.1± 0.1 respectively for *L. casei* 2240 and *L. rhamnosus* GG and maintained stable also at 48 hours of fermentation (Figure 1B). Analogously to the UV treated samples, a lyophilization process was applied to the sterilized products after fermentation. It was observed a decrease in the microbial concentration of 0.5 Log CFU/g for both strains; in particular, *L. rhamnosus* GG was reduced to 8.15 ± 0.05 Log CFU/g and *L. casei* 2240 to 8.86 ± 0.05 Log CFU/g ($p<0.05$) (Figure 1B). *L. rhamnosus* GG better survived the lyophilization process compared to *L. casei* 2240, whose viability decrease of more than 1 Log CFU/g. The survivability of freeze-dried strains is of particular importance for the production of foods containing live cells and for this

reason many studies focus on enhancing the viability of LAB after this process (Giulio et al., 2005; Reddy et al., 2009). Better survivability of *L. casei* 2240 to lyophilization in sterilized biomass was noticed compared to UV treated samples, on the basis of statistical model applied (Figure 1). Several authors (Beheshtipour et al., 2012; Guldas & Irkin, 2010; Varga et al., 2002) tested the addition of *A. platensis* in dairy products such as yogurt, cheese and fermented milk with positive results, which included an increase in the number of LAB and an improvement in the nutritional quality of the fermented product during storage. The fermentation process leads to increased production of phenolic compounds and phycocyanobilins and consequently increased radical scavenger properties of the cyanobacterium (de Marco Castro et al., 2019). Lactic acid fermentation is an appropriate technology to enhance the functional properties of spirulina and also to integrate consumers' diet with beneficial bacteria, giving further advantages to the final product. However, to meet the definition of probiotic products, microorganisms must be viable for the entire shelf life of the product and in such quantities as to multiply and integrate the intestinal flora. The activity of *A. platensis* to enhance LAB vitality, such as *L. casei, Streptococcus thermophilus, Lacticaseibacillus acidophilus*, and *Bifidobacteria* has been documented (Bhowmik et al., 2009; Martelli, Alinovi, et al., 2020; Niccolai et al., 2019; Parada, 1998). There is no consensus regarding the minimum quantity of probiotic microorganisms to be ingested to guarantee their functionality in the human intestine. Usually to observe a positive effect on health, from 6 to 7 Log CFU/g of live probiotic microorganisms, able to colonize the intestine, should be consumed daily (Dave & Shah, 1997). All the fermented biomasses obtained in this study presented a sufficient amount of bacteria, allowing the production of fermented foods with a high functional value. A picture of the fermented biomasses produced after lyophilisation, is presented in figure 2.

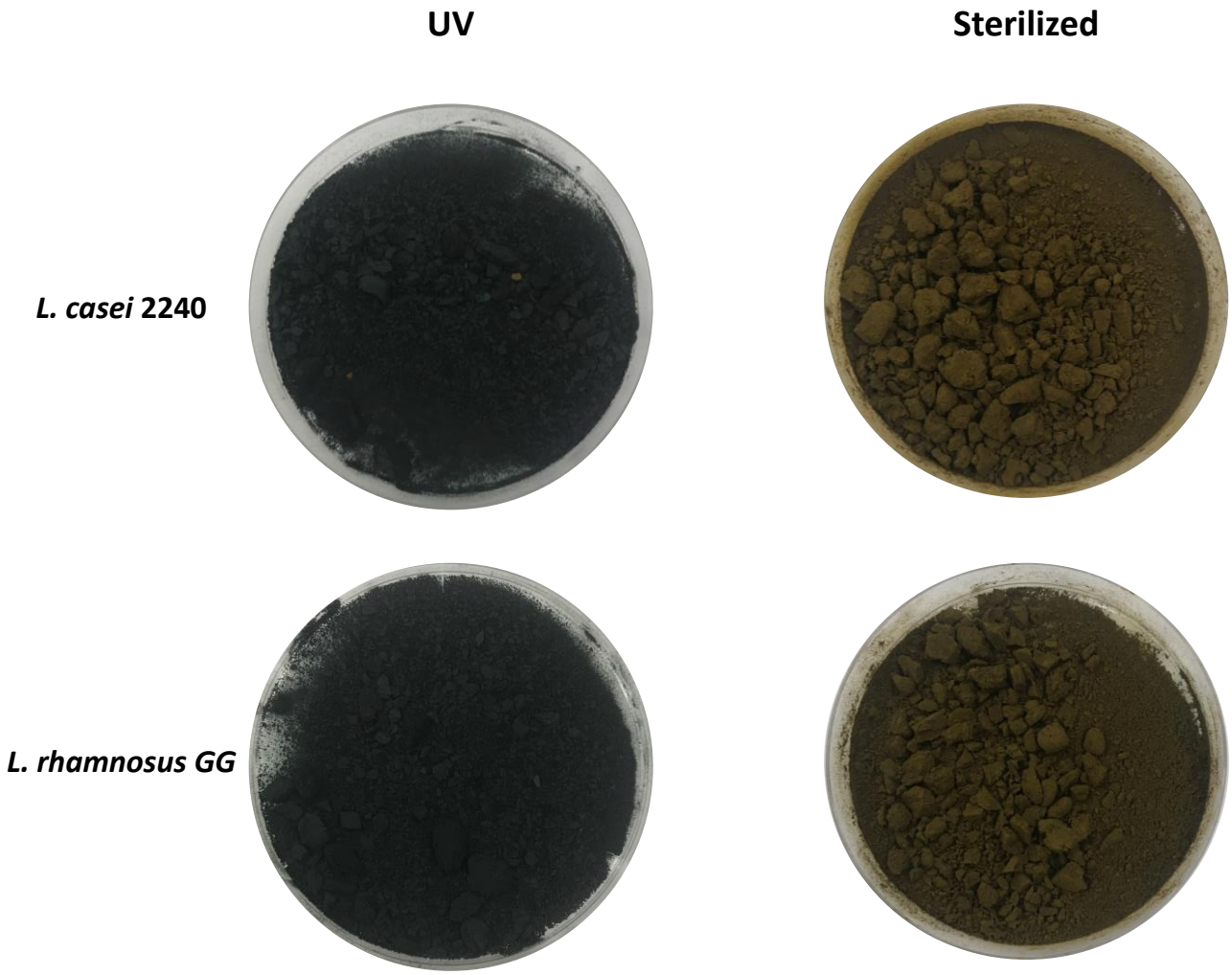


Figure 2. Representative picture of fermented and lyophilized Arthrospira platensis biomasses, subjected to UV and sterilization treatments.

Volatile profile characterization of A. platensis and changes in volatile components after fermentation

Recently, several studies have been conducted on the characterization of the aromatic fraction of *A. platensis*, and in particular, researches aimed to studying how to reduce the characteristic fishy odor of this product in order to use it as additive or ingredient in food (Bao et al., 2018; Cuellar-Bermúdez et al., 2017; Seo et al., 2012). The reduction of unpleasant aromatic notes can be achieved by solvent extraction (Cuellar-Bermúdez et al., 2017), as by fermentation using fungi or bacteria (Bao et al., 2018; Seo et al., 2012). In the present study, lactic acid bacteria fermentation was applied to stabilized *A. platensis* materials and changes in the volatile fraction were determined.

A total of 61 different volatile compounds were identified in the volatile fraction of treated but not fermented and fermented *A. platensis* samples (Table 1). In particular, 7 aldehydes, 9 ketones, 4 esters, 9 terpenes/norisoprenoids, 7 alcohols, 4 furans, 11 hydrocarbons, 7 pyrazines and 3 sulfur compounds were detected. These results are consistent with data reported by Bao et al. (2018), who detected the same classes of volatile compounds in spirulina samples fermented with different strains of *L. plantarum*, *L. acidophilus and Bacillus subtilis* (Bao et al., 2018).

Table 1. Volatile compounds found on *Arthrospira platensis* treated but not fermented and fermented samples. For each volatile compound aromatic note, calculated and tabulated linear retention indices (LRIs), references ad effect given by treatments, stabilization and fermentation (statistical difference = positive = p; no statistical difference = negative = n; not determinable = nd), are reported.

Chemical class, Compound name	Odor type	LRI	Reference LRI	Identification method	Reference	Effect of stabilization	Effect of fermentation
Aldehydes							
Isobutyraldehyde	aldehydic	805	814	MS + LRI	(Bianchi et al., 2007)	n	n
2-Methylbutanal	chocolate	904	903	MS + LRI	(sherena.johnson@nist.gov, 2012)	nd	nd
Isovaleraldehyde	aldehydic	907	888	MS + LRI	(Ricci et al., 2018)	p	p
Hexanal	green	1075	1086	MS + LRI	(M. Cirlini et al., 2012)	n	n
Methional	vegetable	1452	1468	MS + LRI	(Goodner, 2008)	p	p
Benzaldehyde	fruity	1523	1537	MS + LRI	(M. Cirlini et al., 2012)	n	n
2,5-Dimethyl benzaldehyde		1733	1705	MS + LRI	(sherena.johnson@nist.gov, 2012)	p	p
Ketones							
Acetone	solvent	810	901	MS + LRI	(Bianchi et al., 2007)	p	p
2-Butanone	ethereal	894	901	MS + LRI	(Bianchi et al., 2007)	p	p
diacetyl	buttery	971	973	MS + LRI	(Ricci et al., 2018)	n	p
6-Methyl-2-heptanone	camphoreous	1229	1236	MS + LRI	(Tanaka et al., 2003)	p	p
3-Octanone	herbal	1245	1261	MS + LRI	(Mena et al., 2016)	n	p
2-Octanone	earthy	1277	1287	MS + LRI	(sherena.johnson@nist.gov, 2012)	p	n
Acetoin	buttery	1282	1300	MS + LRI	(Ricci et al., 2018)	n	p
2,2,6-Trimethylcyclohexanone	thujonic	1306	1308	MS + LRI	(Yamamoto et al., 2014)	p	n
Sulcatone	citrus	1329	1335	MS + LRI	(Ricci, Cirlini, Guido, et al., 2019)	p	n
Esters							
Ethyl acetate	ethereal	872	869	MS + LRI	(Dall'Asta et al., 2011)	n	p
Ethyl caprylate	waxy	1430	1438	MS + LRI	(Dall'Asta et al., 2011)	nd	nd
Ethyl decanoate	waxy	1628	1645	MS + LRI	(Dall'Asta et al., 2011)	p	n
Phenethyl acetate	floral	1804	1803	MS + LRI	(Ong & Acree, 1999)	nd	nd
Terpenes, norisoprenoids and similar							
p-Xylene		1133	1149	MS + LRI	(Yanagimoto et al., 2004)	p	n
Myrcene	spicy	1155	1143	MS + LRI	(Ricci et al., 2018)	n	n
α-Cyclocitral	citrus	1427	1420	MS + LRI	(sherena.johnson@nist.gov, 2012)	p	p
β-Cyclocitral	tropical	1609	1612	MS + LRI	(Yamamoto et al., 2014)	p	n
Safranal	herbal	1635	1637	MS + LRI	(Yamamoto et al., 2014)	p	p
α-Ionene	fruity	1675		MS		n	n
α-Ionone	floral	1841	1848	MS + LRI	(Babushok et al., 2011)	p	p
β-Ionone	floral	1918	1935	MS + LRI	(Babushok et al., 2011)	n	n
β-Ionone-5,6-epoxide	fruity	1950	1989	MS + LRI	(sherena.johnson@nist.gov, 2012)	p	n
Alcohols							
Ethanol	alcoholic	923	903	MS + LRI	(Ricci et al., 2018)	p	p

Isobutyl alcohol	ethereal	1080	1100	MS + LRI	(Ricci, Cirlini, Guido, et al., 2019)	n	n
Isoamyl alcohol	fermented	1195	1210	MS + LRI	(Ricci, Cirlini, Guido, et al., 2019)	p	p
1-Pentanol	fermented	1239	1260	MS + LRI	(M. Cirlini et al., 2012)	p	n
1-Hexanol	herbal	1341	1357	MS + LRI	(Dall'Asta et al., 2011)	p	n
1-Octen-3-ol	earthy	1437	1455	MS + LRI	(M. Cirlini et al., 2012)	p	n
Benzyl alcohol	floral	1882	1896	MS + LRI	(Dall'Asta et al., 2011)	n	n
Furans							
2-Methylfuran	chocolate	853	876	MS + LRI	(Bianchi et al., 2007)	p	p
3-Methylfuran		881	877	MS + LRI	(sherena.johnson@nist.gov, 2012)	n	n
2-Butylfuran	spicy	1123	1140	MS + LRI	(Brunton et al., 2002)	p	p
2-Pentylfuran	fruity	1220	1232	MS + LRI	(Babushok et al., 2011)	p	n
Hydrocarbons							
1,2,4,4-Tetramethylcyclopentene		920		MS		n	n
2,2,4,6,6-Pentamethylheptane		944		MS		n	n
Ethyl benzene		1119	1127	MS + LRI	(Martina Cirlini et al., 2016)	n	n
Tridecane	alkane	1300	1300	MS + LRI	(Goodner, 2008)	p	p
Tetradecane	waxy/alkane	1396	1400	MS + LRI	(Goodner, 2008)	p	p
2,6,10-Trimethyltridecane		1434	1442	MS + LRI	(sherena.johnson@nist.gov, 2012)	n	n
Pentadecane	waxy	1492	1500	MS + LRI	(Goodner, 2008)	p	p
Hexadecane	alkane	1590	1600	MS + LRI	(Goodner, 2008)	p	p
N-acetyl-4(H)-Pyridine		1644		MS		p	p
Heptadecane	alkane	1687	1700	MS + LRI	(Goodner, 2008)	p	p
6,9-Heptadecadiene		1743				p	p
Pyrazines							
2-Methylpyrazine	nutty	1267	1267	MS + LRI	(Bianchi et al., 2007)	n	n
2,5-Dimethylpyrazine	chocolate	1318	1321	MS + LRI	(Bianchi et al., 2007)	p	n
2-Methyl-5-ethylpyrazine	coffee	1368	1406	MS + LRI	(Bianchi et al., 2007)	n	n
2-Ethyl-6-methylpyrazine	potato	1383	1402	MS + LRI	(Bianchi et al., 2007)	p	p
Trimethyl pyrazine	nutty	1398	1401	MS + LRI	(Bianchi et al., 2007)	p	p
2,3-Dimethyl-5-ethylpyrazine	burnt	1452	1460	MS + LRI	(sherena.johnson@nist.gov, 2012)	p	n
Tetramethyl pyrazine	nutty	1468	1474	MS + LRI	(Bianchi et al., 2007)	p	n
Sulfur compounds							
Dimethyl disulfide	sulfurous	1063	1073	MS + LRI	(Ricci et al., 2018)	p	n
2-Ethyl-4-methylthiazole	nutty	1336	1322	MS + LRI	(Bianchi et al., 2007)	n	n
Dimethyl trisulfide	alliaceous	1369	1375	MS + LRI	(Ricci et al., 2018)	n	n

The class that quantitatively mainly represents the aromatic fraction of *A. platensis*, both before and after the fermentation process, is that of hydrocarbons (Supplementary table S1), as demonstrated also in previous studies(Bao et al., 2018). The concentration of these compounds resulted indeed higher in respect to the amount of all the other components. Hydrocarbons release was significantly different comparing fermentations ($p= 0.007$) and opposing technological treatments ($p<0.001$).

Statistical differences were indeed observed in hydrocarbon quantity between UV and heat treated samples ($p<0.001$), as among samples subjected to the same process, between fermented and not fermented ones ($p=0.007$), but no interaction between factors (type of treatment and fermentation) was noticed (Figure 3, Supplementary table S1).

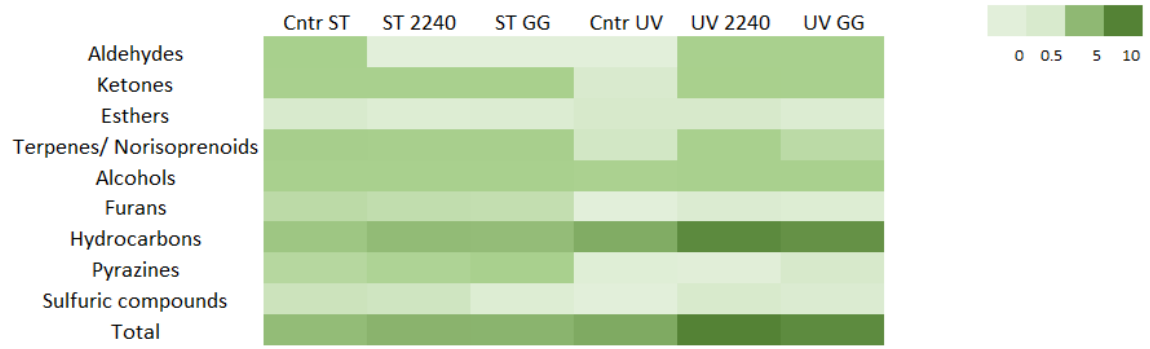


Figure 3. Heat map performed on volatile chemical classes concentration detected in sterilized (ST) or UV treated (UV) *A. platensis* biomasses fermented with *L. casei* 2240 (2240), *L. rhamnosus* GG (GG) and treated but unfermented biomasses (Cntr). A scale ranging from a maximum of 10 μg/g (Dark green) and a minimum of 0 μg/g (light green) was used.

Pentadecane, hexadecane and heptadecane are hydrocarbons derived from the decarboxylation of palmitic and stearic acids, respectively (Cuellar-Bermúdez et al., 2017; Schneider et al., 1970), and they resulted the most representative among all the hydrocarbons identified in this work. In particular, heptadecane was the volatile found in the highest amount in all the considered samples as observed in a recent study focused on the fermentation of spirulina using *Lacticaseibacillus plantarum* and *Bacillus subtilis* (Bao et al., 2018). The presence of this compound could contribute to the off-flavor of algae, associated with crude fish notes, even if it presents a high odor threshold (Cuellar-Bermúdez et al., 2017). Heptadecane resulted significantly lower in the sterilized samples compared to the UV-treated ones, even if the fermentation process seemed to increase its concentration in both cases; indeed both considered factors, stabilization treatment ($p<0.001$) and fermentation ($p=0.006$), resulted to influence its release/formation. In other studies considering *L. plantarum* and *B. subtilis,* the fermentation brought to a reduction by 27% of the relative content of heptadecane, but no information about the actual quantity found in samples was reported (Bao et al., 2018). The LAB species considered in the present work, *L. casei* and *L. rhamnosus*, have given significantly different results in the production of

heptadecane in the differently treated substrates, indeed linkable to LAB fermentation (Stoyanova et al., 2012). It can be speculated that the production and/or release of this compound may depend on the LAB specie applied. The same phenomenon can be observed also for pentadecane. A higher amount was detected in UV treated samples if compared to sterilized ones. It can be noticed that the quantity of hydrocarbon grew consequently to the fermentation when both the strains were used (Supplementary table S1). The significant difference observed in the fermented biomasses (i.e. heptadecane: UV 2240= 7.65 ± 1.95 μg/g; ST 2240= 2.53 ± 0.66 μg/g) has to be ascribed to the different stabilization process applied: the higher concentration of these volatiles in the UV-treated biomass could be ascribed to the metabolism of the endogenous microflora typical of the spirulina biomass. Since the UV treatment, differently from heat treatment, did not completely eliminated contaminating microflora, this behavior can be ascribed to the residual microflora deriving from cultivation in open ponds.

Terpenes, the second most important class in terms of concentration, were positively influenced by sterilization treatment, and an interaction between the two factors was observed ($p=0.013$). Terpenes and norisoprenoids are fundamental compounds in food aroma. β-cyclocitral and β-ionone, with pleasant fruity and floral notes, are two volatiles typical of cyanobacteria. The formation of these compounds is due to the oxidation of carotenoids following carotene oxygenases. Norcarotenoids are an important group of compounds formed by several species of cyanobacteria, generated by enzymatic degradation of carotenes and carotenoids. For example, the oxygenase reaction of carotenoids was first described in Microcystis. β- cyclocitral is formed by the cleavage reaction of β –carotene catalysed by enzyme (Yamamoto et al., 2014). β-ionone and norcarotenoids of the β-ionone-type are an important group of compounds that were found in axenic cyanobacterial cultures and a monoxenic culture of *Phormidium* sp. (Höckelmann & Jüttner, 2005). Significant differences in the presence of β- cyclocitral, the compound that main characterized the class of terpenes and norisoprenoids, were found because of the biomass stabilization treatment ($p<0.001$), while no significant differences were found in β-ionone production.

Regarding aldehydes and ketones production and/or release, both fermentations and treatments have produced significant differences in samples ($p < 0.02$); in addition, a strong interaction was noticed between the two factors for these two volatile classes. Ketone concentration resulted mainly affected by fermentation, even if differences were noted also among the two stabilization treatments ($p=0.012$) (Figure 3, supplementary table S1). In particular for aldehydes, a reduction after the fermentation of the sterilized biomass using both the tested strains was noticed. Conversely, by fermentation of the UV treated biomass, the amount of aldehydes increased. This behavior could be due to the presence of the epiphytic bacteria typical of the spirulina biomass. Considering this data, it can be supposed that fermentative LAB can reduce the amount of these compounds, but the typical microflora of *A. platensis* can produce aldehydes conferring to the fermented biomass a different aroma profile. Hexanal was found in several microalgae (Achyuthan et al., 2017; do Nascimento et al., 2020; Isleten Hosoglu, 2018; Van Durme et al., 2013). Fermentation of sterilized biomass by the both strains lead to a significant diminution of hexanal content ($p<0.01$), while only *L. rhamnosus* GG strain fermentation seemed to produce this compound in UV-treated samples. Since the presence of C6 aldehydes may be associated with fish odor (Fink, 2007), that can be considered an undesired aromatic notes especially in certain food preparations such as dairy products, fermentation could bring to a valuable improvement of the aromatic profile of the sample. Methional was found in both the UV treated fermented samples suggesting, as already emerged by other studies (Amàrita et al., 2001; Amárita et al., 2002), that this aldehyde is produced by LAB. This compound was also previously isolated from *Rhodomonas*, another microalgae (Van Durme et al., 2013). Both fermentation and treatments applied prior to it produced significant differences in methional production and also the combination of both factors ($p<0.001$). Benzaldehyde is a typical aroma formed in several species of microalgae by enzymatic and chemical degradation of phenylalanine (generating benzaldehyde) (Van Durme et al., 2013). This compound is often associated with pleasant notes of almond. It was found in the sterilized *A. platensis* biomass and it was produced by the fermentation process applied to the UV-stabilized samples, even without differences among the two strains used. A similar trend was noticed for isovaleraldehyde

(Supplementary table S1), an aldehyde produced by LAB because of the metabolization of the amino acid leucine (Helinck et al., 2004).

Regarding ketones, a growing amount of these compounds was noticed following the fermentation of both the tested biomasses. In general, the number of ketones in sterilized biomass was higher than in the UV treated biomass, proving the role of thermal treatment in the formation of these compounds (Supplementary table S1). Diacetyl and acetoin are two typical aromatic compounds produced during fermentation with the characteristic buttery aroma (de Melo Pereira et al., 2020). Fermentation produced significant differences in the presence of these molecules. In particular, the fermentation with *L. casei* 2240 has produced a higher amount of diacetyl compared to *L. rhamnosus* GG ($p< 0.05$), both in UV and sterilized materials. Production of these ketones derives by metabolization of pyruvate and citrate and is dependent on the strains used for fermentation (El-Gendy et al., 1983). Sulcatone is a citrus-like aroma. This compound was found after sterilization of the biomass with a significant difference if compared to UV treated samples, and its concentration was maintained after the fermentation step with both the strains. Interestingly, the presence of this aroma was never linked to *A. platensis* biomass or microalgae in general.

Fermentation was the only factor affecting alcohols and esters total content. Alcohols were found in all the analyzed samples. The most representative was 1-octen-3-ol, with the typical mushroom aroma, already isolated from microalgae aromatic fraction (Isleten Hosoglu, 2018). The statistical model underlined that this compound was significantly greater after sterilization if compared to UV treated but not fermented samples (0.13 ± 0.03 μg/g and 0.04 ± 0.00 μg/g respectively), but fermentation did not affect its amount. Significant differences were found for ethanol. A significantly higher amount of ethanol was found in the UV treated biomasses compared to the heat-treated samples ($p=0.001$). The higher amount of this alcohol in the samples could be linked to the sterilization treatment that has affected the composition of the starting biomass, therefore the presence of precursors for the production of alcohols, or to the presence of epiphytic bacteria and yeasts that survived the UV treatment. Significant differences were also found in the production of ethanol during fermentation ($p=0.03$). Both *L. casei* and *L. rhamnosus* are heterofermentative species and

have produced slight ethanol amounts in the biomass during fermentation. Isoamyl alcohol, a volatile compound typical of beverages and fermented foods, is formed from leucine during fermentation (Isleten Hosoglu, 2018). This alcohol was found in sterilized samples consequently to lactic acid fermentation. Though isoamyl alcohol has also been reported in phototrophic cultures of *Chlorella vulgaris* (Van Durme et al., 2013) but in this study was not found in treated but unfermented *A. platensis* biomass.

The UV treatment and the sterilization process have produced significant differences in the total amount of furans, pyrazines and sulfur compounds.

Four furans were found as a component of the aromatic profile of fermented *A. platensis* biomass. 2-Pentylfuran is an important product of lipid degradation and is responsible for beany and licorice-like sensory qualities in various food products (Milovanović et al., 2015); it has been indeed associated with the typical beany, green and metallic odor of spirulina and it was detected in considerable percentages, about 10%, in fermented spirulina samples in a previous study (Bao et al., 2018). In our case, this furan was formed consequently to the sterilization of the biomass (0.10 ± 0.04 μg/g), but it decreased consequently to fermentation, while in UV treated samples it was not detected in the not fermented substrate but it increases after fermentation.

Pyrazines are molecules typical of roasted and thermally treated foods. Seven pyrazines were found in the analyzed samples and most of them were connectable to the sterilization process. For example, 2,5-dimethylpyrazine, a pyrazine with a typical chocolate aroma, was found in the sterilized biomass and not in the UV treated samples. Interestingly, some of these compounds were also produced consequently to lactic acid fermentation. Small amounts, of 2- methylpyrazine were produced by *L. rhamnosus* GG (0.02 ± 0.00 μg/g) in the sterilized biomass. It is not the first time that the production of pyrazine by *L. casei* group bacteria was underlined (Li et al., 2012). Among all the pyrazines detected in the tested samples, 2,5-dimethylpyrazine and 2 – methylpyrazine are of particular interest because these compounds have been associated with the off-flavor of *A. platensis,* (Bao et al., 2018).

A similar behavior was observed for sulfur compounds, prevalently found in the sterilized samples. Dimethyl disulfide is a sulfuric compound formed consequently to thermal

oxidation of other volatile sulfur compounds such as methanethiol (Isleten Hosoglu, 2018). The number of sulfur compounds seemed to be related especially to the stabilization treatment: the concentration of these compounds was higher in the sterilized samples in respect to UV treated ones ($p=0.019$). Generally speaking, sulfur compounds may contribute to the unpleasant odor of algae products, because of their low threshold value. Seo et al. (2017) stated that sulfides concurred for about the 26 % to the total odor profile of sea tangle extract and fungal fermentation lead to a total reduction of dimethyl disulfide after two days from the inoculation (Seo et al., 2012). In our case, the fermentation step did not reduce the amount if this compound, detected only in UV treated materials (Supplementary Table S1). Esters, with floral and waxy notes, are the category that presented the lower quantities in the analyzed samples. 4 compounds have been identified and this is in line with what has been reported in other studies (Bao et al., 2018; Seo et al., 2012). The stabilization treatment did not seem to affect their content, while fermentation leads to a decrease of the initial quantity ($p=0.021$) (Supplementary Table S1).

Sensory properties of detected volatile components

In order to evaluate the modifications in sensory properties of fermented *A. platensis* in respect to the unfermented materials, as to determine changes in aromatic notes due to the different stabilization process applied, all the volatile compounds and their relative concentrations were grouped based on the odor type that they are able to confer. Ten main different aroma attributes were identified as follows: aldehydic/ethereal, sulfurous, green/herbal, buttery/waxy, spicy, fruity, floral, nutty/roasted, alkane and fermented. On the basis of this classification, analogies and differences in odor type production among different samples were noticed applying two-way ANOVA test considering as factors fermentation and stabilization treatment (Figure 4, Supplementary table S2). Lactic acid fermentation of spirulina biomass brought to an enhancement of the aromatic profile.

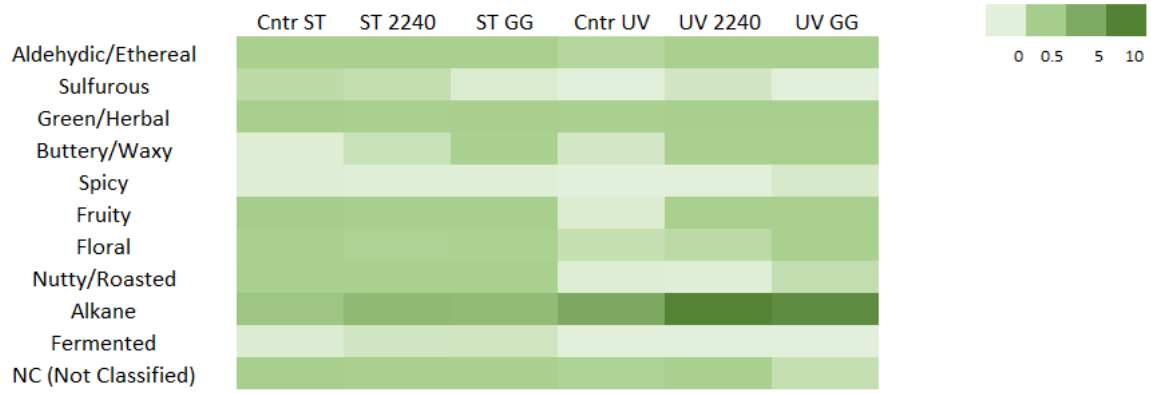


Figure 3. Heat map performed on volatile concentration, for volatile grouped on the basis of the odor type, detected in sterilized (ST) or UV treated (UV) *A. platensis* biomasses fermented with *L. casei* 2240 (2240), *L. rhamnosus* GG (GG) and treated but unfermented biomasses (Cntr). A scale ranging from a maximum of 10 μg/g (Dark green) and a minimum of 0 μg/g (light green) was used.

In particular, the fermentation process significantly influenced the concentration of those compounds responsible for aldehydic/ethereal, buttery/waxy, alkane and of course fermented aromatic notes ($p<0.01$). As expected, buttery/waxy and fermented notes increase with the fermentation process (Figure 4). These aromatic properties could be mainly associated with the presence of diacetyl, acetoin (buttery/waxy) and isoamyl alcohol (fermented). The concentration of all these volatiles showed an augment after the fermentation process, in both the stabilized materials, but while in sterilized spirulina *L. casei* 2240 produced a higher amount of diacetyl and acetoin, in UV-treated material *L. rhamnosus* GG affected the content of these compounds more prominently (Supplementary Table S1). In addition, buttery/waxy and fermented aromatic notes resulted influenced also by stabilization treatment: in particular, a statistically significant interaction was indeed observed for fermented notes for the two considered factors ($p<0.001$) (Supplementary Table S2). Also aldehydic/ethereal and alkane attributes, given mainly by the presence of aldehydes and hydrocarbons, were strongly influenced by the fermentation step, especially in UV treated samples (Figure 4). It has to be underlined that hydrocarbons, and in particular heptadecane, could be associate with the off-flavor of algae [41]. Alkane attribute concentrations were indeed influenced also by the stabilization method applied ($p<0.01$), as clearly represented in Figure 4. So, to limit the formation of these sensory attributes, sterilization has to be chosen as a stabilization treatment. On the other hand, significant differences were found in sulfurous, green/herbal, fruity, and nutty/roasted notes production ($p<0.02$), among the stabilization treatments (Supplementary Table S2). The concentrations for all these aromatic classes resulted indeed statistically higher in the sterilized samples ($p<0.02$). High temperature can cause

the formation of pyrazine and sulfur compounds, associated with nutty/roasted and sulfurous notes, as the degradation of carotenoids and fatty acids leading to an increase of hexanal, methional, 1-hexanol and 1-octen-3-ol (green/herbal and hearthy notes), as of benzaldehyde, β-cyclocitral, β-Ionone 5,6-epoxide and other compounds associated with fruity aromatic notes. In addition, a statistically significant interaction of the two factors was noted for green/herbal and fruity notes ($p<0.001$). Since fruity attributes are generally considered as pleasant aromatic properties, also in this case it is possible to speculate that sterilization may represent the better stabilization treatment because it leads to an augment of this odor class (de Melo Pereira et al., 2020; El-Gendy et al., 1983; Höckelmann & Jüttner, 2005; Yamamoto et al., 2014). In conclusion, even if sterilization may lead to some modification of the aromatic characteristic of the starting material, *A. platensis*, seemed to be the best choice to reduce the initial microbial count, naturally occurring in the tested samples. Fermentation by *L. casei* bacteria group can generate or enhance some volatile compounds responsible for pleasant aromatic notes, as fruity and creamy (buttery) notes, associated with volatiles produced by LAB metabolism.

Conclusions

In this study, *A. platensis* biomass, treated by UV or sterilization at 121 °C, was fermented by LAB (*L. casei 2240* and *L. rhamnosus GG*) in order to evaluate the fermentative and aromatic potential. The LAB growth was not affected by the treatment applied confirming *A. platensis* as a fermentable substrate that may be used for the development of new fermented foods and supplements with high functional values. Furthermore, the survivability of LAB to freeze-drying may allow the production of food products which, in addition to integrating proteins and vitamins typical of *A. platensis*, also include LAB or probiotic bacteria in the diet. Considering the importance of aroma and flavor for consumers acceptability, the main volatile compounds involved in *A. platensis* biomass fermentation were screened. An overall improvement of smell was obtained.

In particular, a greater presence of fermentation aroma was found during the fermentation of the sterilized sample highlighting that in addition to guaranteeing safety for the consumer, this process may enhance applications avoiding side effects due to off-flavor. In conclusion, according to the heat treatment applied, a fermented lyophilized spirulina powder with an interesting aromatic profile and high LAB concentration was obtained opening up perspectives for new applications in food productions.

References

Achyuthan, K., Harper, J., Manginell, R., & Moorman, M. (2017). Volatile Metabolites Emission by In Vivo Microalgae—An Overlooked Opportunity? *Metabolites*, *7*(3), 39. https://doi.org/10.3390/metabo7030039

Aguero, J., Lora, J., Estrada, K., Concepcion, F., Nunez, A., Rodriguez, A., & Pino, J. A. (2003). Volatile Components of a Commercial Sample of the Blue-Green Algae *Spirulina platensis*. *Journal of Essential Oil Research*, *15*(2), 114–117. https://doi.org/10.1080/10412905.2003.9712085

Ak, B., Avşaroğlu, E., Işık, O., Özyurt, G., Kafkas, E., & Uslu, L. (2016). Nutritional and Physicochemical Characteristics of Bread Enriched with Microalgae Spirulina platensis. *Int. Journal of Engineering Research and Application*, *6*(12), 9.

Amàrita, F., Fernàndez-Esplà, D., Requena, T., & Pelaez, C. (2001). Conversion of methionine to methional by *Lactococcus lactis*. *FEMS Microbiology Letters*, *204*(1), 189–195. https://doi.org/10.1111/j.1574-6968.2001.tb10884.x

Amárita, F., Martínez-Cuesta, C. M., Taborda, G., Soto-Yárritu, P. L., Requena, T., & Peláez, C. (2002). Formation of methional by Lactococcus lactis IFPL730 under cheese model conditions. *European Food Research and Technology*, *214*(1), 58–62. https://doi.org/10.1007/s00217-001-0420-y

Andrade, L. M., Andrade, C. J., Dias, M., Nascimento, C. A., & Mendes, M. A. (2018). Chlorella and Spirulina Microalgae as Sources of Functional Foods, Nutraceuticals, and Food Supplements; an Overview. *MOJ Food Processing & Technology*, *6*(1), 45–58. https://doi.org/10.15406/mojfpt.2018.06.00144

Antelo, F. S., Costa, J. A. V., & Kalil, S. J. (2008). Thermal degradation kinetics of the phycocyanin from Spirulina platensis. *Biochemical Engineering Journal*, *41*(1), 43–47. https://doi.org/10.1016/j.bej.2008.03.012

Ariede, M. B., Candido, T. M., Jacome, A. L. M., Velasco, M. V. R., de Carvalho, J. C. M., & Baby, A. R. (2017). Cosmetic attributes of algae—A review. *Algal Research*, *25*, 483–487. https://doi.org/10.1016/j.algal.2017.05.019

Babushok, V. I., Linstrom, P. J., & Zenkevich, I. G. (2011). Retention Indices for Frequently Reported Compounds of Plant Essential Oils. *Journal of Physical and Chemical Reference Data*, *40*(4), 043101. https://doi.org/10.1063/1.3653552

Bancalari, E., Martelli, F., Bernini, V., Neviani, E., & Gatti, M. (2020). Bacteriostatic or bactericidal? Impedometric measurements to test the antimicrobial activity of Arthrospira platensis extract. *Food Control*, 107380. https://doi.org/10.1016/j.foodcont.2020.107380

Bao, J., Zhang, X., Zheng, J.-H., Ren, D.-F., & Lu, J. (2018). Mixed fermentation of Spirulina platensis with Lactobacillus plantarum and Bacillus subtilis by random-centroid optimization. *Food Chemistry*, *264*, 64–72. https://doi.org/10.1016/j.foodchem.2018.05.027

Barkallah, M., Dammak, M., Louati, I., Hentati, F., Hadrich, B., Mechichi, T., Ayadi, M. A., Fendri, I., Attia, H., & Abdelkafi, S. (2017). Effect of Spirulina platensis fortification on physicochemical, textural, antioxidant and sensory properties of yogurt during fermentation and storage. *LWT*, *84*, 323–330. https://doi.org/10.1016/j.lwt.2017.05.071

Batista, A. P., Niccolai, A., Fradinho, P., Fragoso, S., Bursic, I., Rodolfi, L., Biondi, N., Tredici, M. R., Sousa, I., & Raymundo, A. (2017). Microalgae biomass as an alternative ingredient in cookies: Sensory, physical and chemical properties, antioxidant activity and in vitro digestibility. *Algal Research*, *26*, 161–171. https://doi.org/10.1016/j.algal.2017.07.017

Beheshtipour, H., Mortazavian, A. M., Haratian, P., & Darani, K. K. (2012). Effects of Chlorella vulgaris and Arthrospira platensis addition on viability of probiotic bacteria in yogurt and its biochemical properties. *European Food Research and Technology*, *235*(4), 719–728. https://doi.org/10.1007/s00217-012-1798-4

Bhowmik, D., Dubey, J., & Mehra, S. (2009). Probiotic Efficiency of Spirulina platensis—Stimulating Growth of Lactic Acid Bacteria. *World Journal of Dairy & Food Sciences*, *4*(2), 160–164.

Bianchi, F., Careri, M., Mangia, A., & Musci, M. (2007). Retention indices in the analysis of food aroma volatile compounds in temperature-programmed gas chromatography: Database creation and evaluation of precision and robustness. *Journal of Separation Science*, *30*(4), 563–572. https://doi.org/10.1002/jssc.200600393

Bron, P. A., Kleerebezem, M., Brummer, R.-J., Cani, P. D., Mercenier, A., MacDonald, T. T., Garcia-Ródenas, C. L., & Wells, J. M. (2017). Can probiotics modulate human disease by impacting intestinal barrier function? *British Journal of Nutrition*, *117*(1), 93–107. https://doi.org/10.1017/S0007114516004037

Brunton, N. P., Cronin, D. A., & Monahan, F. J. (2002). Volatile components associated with freshly cooked and oxidized off-flavours in turkey breast meat. *Flavour and Fragrance Journal*, *17*(5), 327–334. https://doi.org/10.1002/ffj.1087

Camacho, F., Macedo, A., & Malcata, F. (2019). Potential Industrial Applications and Commercialization of Microalgae in the Functional Food and Feed Industries: A Short Review. *Marine Drugs*, *17*(312), 32. https://doi.org/10.3390/md17060312

Cirlini, M., Dall'Asta, C., Silvanini, A., Beghè, D., Fabbri, A., Galaverna, G., & Ganino, T. (2012). Volatile fingerprinting of chestnut flours from traditional Emilia Romagna (Italy) cultivars. *Food Chemistry*, *134*(2), 662–668. https://doi.org/10.1016/j.foodchem.2012.02.151

Cirlini, Martina, Mena, P., Tassotti, M., Herrlinger, K., Nieman, K., Dall'Asta, C., & Del Rio, D. (2016). Phenolic and Volatile Composition of a Dry Spearmint (Mentha spicata L.) Extract. *Molecules*, *21*(8), 1007. https://doi.org/10.3390/molecules21081007

Cuellar-Bermúdez, S. P., Barba-Davila, B., Serna-Saldivar, S. O., Parra-Saldivar, R., Rodriguez-Rodriguez, J., Morales-Davila, S., Goiris, K., Muylaert, K., & Chuck-Hernández, C. (2017). Deodorization of Arthrospira platensis biomass for further scale-up food

applications. *Journal of the Science of Food and Agriculture*, *97*(15), 5123–5130. https://doi.org/10.1002/jsfa.8391

Dall'Asta, C., Cirlini, M., Morini, E., & Galaverna, G. (2011). Brand-dependent volatile fingerprinting of Italian wines from Valpolicella. *Journal of Chromatography A*, *1218*(42), 7557–7565. https://doi.org/10.1016/j.chroma.2011.08.042

Dave, R. I., & Shah, N. P. (1997). Viability of yoghurt and probiotic bacteria in yoghurts made from commercial starter cultures. *International Dairy Journal*, *7*(1), 31–41. https://doi.org/10.1016/S0958-6946(96)00046-5

de Jesus Raposo, M., de Morais, A., & de Morais, R. (2016). Emergent Sources of Prebiotics: Seaweeds and Microalgae. *Marine Drugs*, *14*(2), 27. https://doi.org/10.3390/md14020027

de Marco Castro, E., Shannon, E., & Abu-Ghannam, N. (2019). Effect of Fermentation on Enhancing the Nutraceutical Properties of Arthrospira platensis (Spirulina). *Fermentation*, *5*(1), 28. https://doi.org/10.3390/fermentation5010028

de Melo Pereira, G. V., da Silva Vale, A., de Carvalho Neto, D. P., Muynarsk, E. S., Soccol, V. T., & Soccol, C. R. (2020). Lactic acid bacteria: What coffee industry should know? *Current Opinion in Food Science*, *31*, 1–8. https://doi.org/10.1016/j.cofs.2019.07.004

do Nascimento, T. C., Nass, P. P., Fernandes, A. S., Vieira, K. R., Wagner, R., Jacob-Lopes, E., & Zepka, L. Q. (2020). Exploratory data of the microalgae compounds for food purposes. *Data in Brief*, *29*, 105182. https://doi.org/10.1016/j.dib.2020.105182

Dutta, D., Dutta, A., Raychaudhuri, U., & Chakraborty, R. (2006). Rheological characteristics and thermal degradation kinetics of beta-carotene in pumpkin puree. *Journal of Food Engineering*, *76*(4), 538–546. https://doi.org/10.1016/j.jfoodeng.2005.05.056

El-Gendy, S. M., Abdel-Galil, H., Shahin, Y., & Hegazi, F. Z. (1983). Acetoin and Diacetyl Production by Homo- and Heterofermentative Lactic Acid Bacteria. *Journal of Food Protection*, *46*(5), 420–425. https://doi.org/10.4315/0362-028X-46.5.420

Fink, P. (2007). Ecological functions of volatile organic compounds in aquatic systems. *Marine and Freshwater Behaviour and Physiology*, *40*(3), 155–168. https://doi.org/10.1080/10236240701602218

Gardiner, G. E., Bouchier, P., O'Sullivan, E., Kelly, J., Kevin Collins, J., Fitzgerald, G., Paul Ross, R., & Stanton, C. (2002). A spray-dried culture for probiotic Cheddar cheese manufacture. *International Dairy Journal*, *12*(9), 749–756. https://doi.org/10.1016/S0958-6946(02)00072-9

Giulio, B. D., Orlando, P., Barba, G., Coppola, R., Rosa, M. D., Sada, A., Prisco, P. P. D., & Nazzaro, F. (2005). Use of alginate and cryo-protective sugars to improve the viability of lactic acid bacteria after freezing and freeze-drying. *World Journal of Microbiology and Biotechnology*, *21*(5), 739–746. https://doi.org/10.1007/s11274-004-4735-2

Golmakani, M.-T., Soleimanian-Zad, S., Alavi, N., Nazari, E., & Eskandari, M. H. (2019). Effect of Spirulina (Arthrospira platensis) powder on probiotic bacteriologically acidified feta-type cheese. *Journal of Applied Phycology*, *31*(2), 1085–1094. https://doi.org/10.1007/s10811-018-1611-2

Goodner, K. L. (2008). Practical retention index models of OV-101, DB-1, DB-5, and DB-Wax for flavor and fragrance compounds. *LWT - Food Science and Technology*, *41*(6), 951–958. https://doi.org/10.1016/j.lwt.2007.07.007

Guldas, M., & Irkin, R. (2010). Influence of Spirulina platensis powder on the microflora of yoghurt and acidophilus milk. *Mljekarstvo*, *60*(4), 237–243.

Hamed, I., Özogul, F., Özogul, Y., & Regenstein, J. M. (2015). Marine Bioactive Compounds and Their Health Benefits: A Review: Importance of functional seafood…. *Comprehensive Reviews in Food Science and Food Safety*, *14*(4), 446–465. https://doi.org/10.1111/1541-4337.12136

Helinck, S., Le Bars, D., Moreau, D., & Yvon, M. (2004). Ability of Thermophilic Lactic Acid Bacteria To Produce Aroma Compounds from Amino Acids. *Applied and*

Environmental Microbiology, *70*(7), 3855–3861. https://doi.org/10.1128/AEM.70.7.3855-3861.2004

Höckelmann, C., & Jüttner, F. (2005). Off-flavours in water: Hydroxyketones andβ-ionone derivatives as new odour compounds of freshwater cyanobacteria. *Flavour and Fragrance Journal*, *20*(4), 387–394. https://doi.org/10.1002/ffj.1464

Högnadóttir, Á. (2000). *Flavor Perception and Volatile Compounds in Fish*. 25.

Holman, B. W. B., & Malau-Aduli, A. E. O. (2013). *Spirulina* as a livestock supplement and animal feed: *Spirulina* supplementation in livestock. *Journal of Animal Physiology and Animal Nutrition*, *97*(4), 615–623. https://doi.org/10.1111/j.1439-0396.2012.01328.x

Isleten Hosoglu, M. (2018). Aroma characterization of five microalgae species using solid-phase microextraction and gas chromatography–mass spectrometry/olfactometry. *Food Chemistry*, *240*, 1210–1218. https://doi.org/10.1016/j.foodchem.2017.08.052

Jia, R., Chen, H., Chen, H., & Ding, W. (2016). Effects of fermentation with Lactobacillus rhamnosus GG on product quality and fatty acids of goat milk yogurt. *Journal of Dairy Science*, *99*(1), 221–227. https://doi.org/10.3168/jds.2015-10114

Koutchma, T. (2008). UV Light for Processing Foods. *Ozone: Science & Engineering*, *30*(1), 93–98. https://doi.org/10.1080/01919510701816346

Li, H., Liu, L., Zhang, S., Cui, W., & Lv, J. (2012). Identification of Antifungal Compounds Produced by Lactobacillus casei AST18. *Current Microbiology*, *65*(2), 156–161. https://doi.org/10.1007/s00284-012-0135-2

Martelli, F., Alinovi, M., Bernini, V., Gatti, M., & Bancalari, E. (2020). Arthrospira platensis as Natural Fermentation Booster for Milk and Soy Fermented Beverages. *Foods*, *9*(350), 15. https://doi.org/doi:10.3390/foods9030350

Martelli, F., Cirlini, M., Lazzi, C., Neviani, E., & Bernini, V. (2020). Edible Seaweeds and Spirulina Extracts for Food Application: In Vitro and In Situ Evaluation of Antimicrobial Activity towards Foodborne Pathogenic Bacteria. *Foods*, *9*(1442), 15.

Martelli, F., Favari, C., Mena, P., Guazzetti, S., Ricci, A., Rio, D. D., Lazzi, C., Neviani, E., & Bernini, V. (2020). Antimicrobial and Fermentation Potential of Himanthalia elongata in Food Applications. *Microorganisms*, *8*(2), 15. https://doi.org/doi:10.3390/microorganisms8020248

Massoud, R., Khosravi-Darani, K., Nakhsaz, F., & Varga, L. (2017). Evaluation of physicochemical, microbiological and sensory properties of croissants fortified with Arthrospira platensis (Spirulina). *Czech Journal of Food Sciences*, *34*(4), 350–355. https://doi.org/10.17221/289/2015-CJFS

Mena, P., Cirlini, M., Tassotti, M., Herrlinger, K., Dall'Asta, C., & Del Rio, D. (2016). Phytochemical Profiling of Flavonoids, Phenolic Acids, Terpenoids, and Volatile Fraction of a Rosemary (Rosmarinus officinalis L.) Extract. *Molecules*, *21*(11), 1576. https://doi.org/10.3390/molecules21111576

Milovanović, I., Mišan, A., Simeunović, J., Kovač, D., Jambrec, D., & Mandić, A. (2015). Determination of Volatile Organic Compounds in Selected Strains of Cyanobacteria. *Journal of Chemistry*, *2015*, 1–6. https://doi.org/10.1155/2015/969542

Mohammadi-Gouraji, E., Soleimanian-Zad, S., & Ghiaci, M. (2019). Phycocyanin-enriched yogurt and its antibacterial and physicochemical properties during 21 days of storage. *LWT*, *102*, 230–236. https://doi.org/10.1016/j.lwt.2018.09.057

Niccolai, A., Shannon, E., Abu-Ghannam, N., Biondi, N., Rodolfi, L., & Tredici, M. R. (2019). Lactic acid fermentation of Arthrospira platensis (spirulina) biomass for probiotic-based products. *Journal of Applied Phycology*, *31*(2), 1077–1083. https://doi.org/10.1007/s10811-018-1602-3

Ong, P. K. C., & Acree, T. E. (1999). Similarities in the Aroma Chemistry of Gewu1 rztraminer Variety Wines and Lychee (Litchi chinesis Sonn.) Fruit. *J. Agric. Food Chem.*, *47*, 665–670.

Pala, Ç. U., & Toklucu, A. K. (2011). Effect of UV-C light on anthocyanin content and other quality parameters of pomegranate juice. *Journal of Food Composition and Analysis*, *24*(6), 790–795. https://doi.org/10.1016/j.jfca.2011.01.003

Parada, J. (1998). Lactic acid bacteria growth promoters from Spirulina platensis. *International Journal of Food Microbiology*, *45*(3), 225–228. https://doi.org/10.1016/S0168-1605(98)00151-2

Pereira, A. L. F., Maciel, T. C., & Rodrigues, S. (2011). Probiotic beverage from cashew apple juice fermented with Lactobacillus casei. *Food Research International*, *44*(5), 1276–1283. https://doi.org/10.1016/j.foodres.2010.11.035

Reddy, K. B. P. K., Awasthi, S. P., Madhu, A. N., & Prapulla, S. G. (2009). Role of Cryoprotectants on the Viability and Functional Properties of Probiotic Lactic Acid Bacteria during Freeze Drying. *Food Biotechnology*, *23*(3), 243–265. https://doi.org/10.1080/08905430903106811

Ricci, A., Bernini, V., Maoloni, A., Cirlini, M., Galaverna, G., Neviani, E., & Lazzi, C. (2019). Vegetable By-Product Lacto-Fermentation as a New Source of Antimicrobial Compounds. *Microorganisms*, *7*(607), 11. https://doi.org/10.3390/microorganisms7120607

Ricci, A., Cirlini, M., Guido, A., Liberatore, C., Ganino, T., Lazzi, C., & Chiancone, B. (2019). From Byproduct to Resource: Fermented Apple Pomace as Beer Flavoring. *Foods*, *8*(8), 309. https://doi.org/10.3390/foods8080309

Ricci, A., Cirlini, M., Levante, A., Dall'Asta, C., Galaverna, G., & Lazzi, C. (2018). Volatile profile of elderberry juice: Effect of lactic acid fermentation using L. plantarum , L. rhamnosus and L. casei strains. *Food Research International*, *105*, 412–422. https://doi.org/10.1016/j.foodres.2017.11.042

Ricci, A., Cirlini, M., Maoloni, A., Del Rio, D., Calani, L., Bernini, V., Galaverna, G., Neviani, E., & Lazzi, C. (2019). Use of Dairy and Plant-Derived Lactobacilli as Starters for Cherry Juice Fermentation. *Nutrients*, *11*(2), 213. https://doi.org/10.3390/nu11020213

Rubio, R., Aymerich, T., Bover-Cid, S., Guàrdia, M. D., Arnau, J., & Garriga, M. (2013). Probiotic strains Lactobacillus plantarum 299V and Lactobacillus rhamnosus GG as starter cultures for fermented sausages. *LWT - Food Science and Technology*, *54*(1), 51–56. https://doi.org/10.1016/j.lwt.2013.05.014

Schneider, H., Gelpi, E., Bennett, E. O., & Oró, J. (1970). Fatty acids of geochemical significance in microscopic algae. *Phytochemistry*, *9*(3), 613–617. https://doi.org/10.1016/S0031-9422(00)85701-5

Seo, Y.-S., Bae, H.-N., Eom, S.-H., Lim, K.-S., Yun, I.-H., Chung, Y.-H., Jeon, J.-M., Kim, H.-W., Lee, M.-S., Lee, Y.-B., & Kim, Y.-M. (2012). Removal of off-flavors from sea tangle (Laminaria japonica) extract by fermentation with Aspergillus oryzae. *Bioresource Technology*, *121*, 475–479. https://doi.org/10.1016/j.biortech.2012.07.007

sherena.johnson@nist.gov. (2012, December 12). *Standard Reference Data* [Text]. NIST. https://www.nist.gov/srd

Smid, E. J., & Kleerebezem, M. (2014). Production of Aroma Compounds in Lactic Fermentations. *Annual Review of Food Science and Technology*, *5*(1), 313–326. https://doi.org/10.1146/annurev-food-030713-092339

Stoyanova, L. G., Ustyugova, E. A., & Netrusov, A. I. (2012). Antibacterial metabolites of lactic acid bacteria: Their diversity and properties. *Applied Biochemistry and Microbiology*, *48*(3), 229–243. https://doi.org/10.1134/S0003683812030143

Tanaka, T., Yamaguchi, T., Katsumata, R., & Kiuchi, K. (2003). Comparison of volatile components in commercial Itohiki-Natto by solid phase microextraction and gas chromatography. *Nippon Shokuhin Kagaku Kogaku Kaishi*, *50*(6), 278–285. https://doi.org/10.3136/nskkk.50.278 .

Van Durme, J., Goiris, K., De Winne, A., De Cooman, L., & Muylaert, K. (2013). Evaluation of the Volatile Composition and Sensory Properties of Five Species of Microalgae. *Journal of Agricultural and Food Chemistry*, *61*(46), 10881–10890. https://doi.org/10.1021/jf403112k

Varga, L., Szigeti, J., Kovács, R., Földes, T., & Buti, S. (2002). Influence of a Spirulina platensis Biomass on the Microflora of Fermented ABT Milks During Storage (R1). *Journal of Dairy Science*, *85*(5), 1031–1038. https://doi.org/10.3168/jds.S0022-0302(02)74163-5

Wang, H., Zhang, W., Chen, L., Wang, J., & Liu, T. (2013). The contamination and control of biological pollutants in mass cultivation of microalgae. *Bioresource Technology*, *128*, 745–750. https://doi.org/10.1016/j.biortech.2012.10.158

Weemaes, C. A., Ooms, V., Van Loey, A. M., & Hendrickx, M. E. (1999). Kinetics of Chlorophyll Degradation and Color Loss in Heated Broccoli Juice. *Journal of Agricultural and Food Chemistry*, *47*(6), 2404–2409. https://doi.org/10.1021/jf980663o

Wong, S.-S., Quan Toh, Z., Dunne, E. M., Mulholland, E. K., Tang, M. L., Robins-Browne, R. M., Licciardi, P. V., & Satzke, C. (2013). Inhibition of Streptococcus pneumoniae adherence to human epithelial cells in vitro by the probiotic Lactobacillus rhamnosus GG. *BMC Research Notes*, *6*(1), 135. https://doi.org/10.1186/1756-0500-6-135

Wu, S.-C., Wang, F.-J., & Pan, C.-L. (2010). The comparison of antioxidative properties of seaweed oligosaccharides fermented by two lactic acid bacteria. *Journal of Marine Science and Technology*, *18*(4), 9.

Yamaguchi, S. K. F., Moreira, J. B., Costa, J. A. V., de Souza, C. K., Bertoli, S. L., & Carvalho, L. F. de. (2019). Evaluation of Adding S *pirulina* to Freeze-Dried Yogurts Before Fermentation and After Freeze-Drying. *Industrial Biotechnology*, *15*(2), 89–94. https://doi.org/10.1089/ind.2018.0030

Yamamoto, M., Baldermann, S., Yoshikawa, K., Fujita, A., Mase, N., & Watanabe, N. (2014). Determination of Volatile Compounds in Four Commercial Samples of Japanese

Green Algae Using Solid Phase Microextraction Gas Chromatography Mass Spectrometry. *The Scientific World Journal*, *2014*, 1–8. https://doi.org/10.1155/2014/289780

Yanagimoto, K., Ochi, H., Lee, K.-G., & Shibamoto, T. (2004). Antioxidative Activities of Fractions Obtained from Brewed Coffee. *Journal of Agricultural and Food Chemistry*, *52*(3), 592–596. https://doi.org/10.1021/jf030317t

Zarbà, C., Chinnici, G., & D'Amico, M. (2020). Novel Food: The Impact of Innovation on the Paths of the Traditional Food Chain. *Sustainability*, *12*(2), 555. https://doi.org/10.3390/su12020555

Zouari, N., Abid, M., Fakhfakh, N., Ayadi, M. A., Zorgui, L., Ayadi, M., & Attia, H. (2011). Blue-green algae (*Arthrospira platensis*) as an ingredient in pasta: Free radical scavenging activity, sensory and cooking characteristics evaluation. *International Journal of Food Sciences and Nutrition*, *62*(8), 811–813. https://doi.org/10.3109/09637486.2011.582461

Supplementary materials

Compounds	Content						Factors		
	Cntr ST	ST_2240	ST_GG	Cntr UV	UV_2240	UV_GG	F	S	F*S
Aldehydes									
Isobutyraldehyde	0.01086 ± 0.00077 a	n.d	n.d	n.d	0.00991 ± 0.00286 a	n.d	0.25	0.25	0.01
2-Methylbutanal	0.02264 ± 0.00332 a	n.d	n.d	n.d	n.d	n.d			
Isovaleraldehyde	0.02728 ± 0.00156 a	n.d	n.d	n.d	0.01423 ± 0.00123 a	0.01244 ± 0.00212 a	0.00	0.00	0.00
Hexanal	0.04523 ± 0.00028 a	n.d	n.d	n.d	n.d	0.05297 ± 0.00836 a	0.468	0.468	0.016
Methional	n.d	n.d	n.d	n.d	0.02289 ± 0.00137 a	0.02489 ± 0.00693 a	0.00	0.00	0.00
Benzaldehyde	0.17073 ± 0.03177 a	n.d	n.d	n.d	0.13162 ± 0.04463 a	0.10459 ± 0.03979 a	0.177	0.177	0.00
2,5-Dimethyl Benzaldehyde	0.02227 ± 0.00508 a	n.d	n.d	n.d	n.d	n.d	0.00	0.00	0.00
Ketones									
Acetone	0.01123 ± 0.00081 b	0.02461 ± 0.00831 a	0.02813 ± 0.00570 a	n.d	0.01672 ± 0.00171 a	0.02103 ± 0.00361 a	0.000	0.004	0.470
2-Butanone	0.01008 ± 0.00039 b	0.02021 ± 0.04432 a,b	0.02278 ± 0.00127a	n.d	0.02219 ± 0.00416 a	0.01859 ± 0.00415 a	0.000	0.013	0.036
Diacetyl	0.00826 ± 0.00037 c	0.04522 ± 0.00953 b	0.01092 ± 0.00049 c	0.00890 ± 0.00259 c	0.07766 ± 0.00882 a	0.03668 ± 0.01107 b	0.019	0.250	0.270
6-Methyl-2-Heptanone	0.01997 ± 0.00221 a	0.02115 ± 0.00177 a	0.01611 ± 0.00087 a	n.d	0.01649 ± 0.00236 a	0.01329 ± 0.00273 a	0.002	0.000	0.001
3-Octanone	n.d	0.00977 ± 0.00437 b	0.01493 ± 0.00125 b	0.00992 ± 0.00104 b,c	0.01973 ± 0.00201 a	0.00951 ± 0.00236 b	0.013	0.056	0.205
2-Octanone	0.01026 ± 0.00174 a	0.00875 ± 0.00058 b	0.00562 ± 0.00014 c	n.d	n.d	n.d	0.048	0.000	0.048
Acetoin	n.d	n.d	0.08577 ± 0.00331 a	n.d	0.09083 ± 0.00784 a	0.13555 ± 0.04519 a	0.006	0.053	0.053
2,2,6-Trimethylcyclohexanone	0.06032 ± 0.01269 a	0.05366 ± 0.00037 a	0.04938 ± 0.00039 a	0.00878 ± 0.00147 b,c	0.02672 ± 0.00404 b	0.01958 ± 0.00567 b	0.450	0.000	0.009
Sulcatone	0.07005 ± 0.01557 a	0.06322 ± 0.00502 a	0.05586 ± 0.00220 a	n.d	0.02003 ± 0.00166 b	0.02032 ± 0.00488 b	0.217	0.000	0.002
Esters									
Ethyl Acetate	0.01140 ± 0.00099 a	0.01519 ± 0.00094 a	0.01766 ± 0.00128 a	0.01378 ± 0.00274 a	0.02233 ± 0.00424 a	0.01843 ± 0.00328 a	0.011	0.119	0.680
Ethyl Caprylate	n.d	n.d	n.d	0.00840 ± 0.00068 a	n.d	n.d			
Ethyl Decanoate	n.d	n.d	n.d	0.01150 ± 0.00237 a	0.01017 ± 0.00214 a	n.d	0.220	0.007	0.220
Phenethyl Acetate	0.01769 ± 0.00765 a	n.d	n.d	n.d	n.d	n.d			
Terpenes/Norisoprenoids									
Para-Xylene	0.18493 ± 0.03116 a	0.13716 ± 0.04784 a	0.11965 ± 0.05426 a	n.d	0.06621 ± 0.03628 b	n.d	0.594	0.000	0.061
Myrcene	n.d	n.d	n.d	n.d	n.d	0.02083 ± 0.00970 a	0.362	0.362	0.362
α-Cyclocitral	0.03281 ± 0.00801 a	n.d	0.01957 ± 0.00194 b	n.d	n.d	n.d	0.016	0.000	0.016
β-Cyclocitral	0.12264 ± 0.03553 a	0.11031 ± 0.01463 a	0.09302 ± 0.00787 a	0.01258 ± 0.00178 b	0.03159 ± 0.00529 b	0.02949 ± 0.00622 b	0.861	0.000	0.042

Safranal	0.02946 ± 0.00566 a	0.01532 ± 0.00125 b	0.01348 ± 0.00088 b	n.d	n.d	n.d	0.000	0.000	0.000
α-Ionene	n.d	n.d	0.01743 ± 0.00140 a	n.d	n.d	n.d	0.215	0.215	0.215
α-Ionone	0.01013 ± 0.00336 ac	0.01473 ± 0.00073 a	0.01429 ± 0.00184 a	n.d	n.d	n.d	0.014	0.000	0.014
β-Ionone	0.05990 ± 0.01559 a	0.05848 ± 0.00595 a	0.06004 ± 0.00680 a	0.03442 ± 0.00188 a	0.06854 ± 0.01867 a	0.06269 ± 0.02362 a	0.114	0.304	0.101
β-Ionone 5,6-Epoxide	0.02621 ± 0.00492 a	0.02515 ± 0.00285 a	0.02666 ± 0.00081 a	n.d	n.d	n.d	0.896	0.000	0.896
Alcohols									
Ethanol	0.01358 ± 0.00236 b	0.02375 ± 0.00463 b	0.06260 ± 0.00130 a,b	0.06514 ± 0.01110 a,b	0.09513 ± 0.01047 a	0.08824 ± 0.02770 a,b	0.030	0.001	0.893
Isobutyl Alcohol	n.d	n.d	0.00951 ± 0.00174 a	n.d	n.d	n.d	0.221	0.221	0.221
Isoamyl Alcohol	n.d	0.02253 ± 0.00194 a	0.02454 ± 0.00207 a	n.d	n.d	n.d	0.000	0.000	0.000
1-Pentanol	0.01052 ± 0.00087 a	0.00907 ± 0.00012 a	0.00988 ± 0.00123 a	n.d	n.d	n.d	0.139	0.000	0.139
1-Hexanol	0.03083 ± 0.00603 b	n.d	0.05230 ± 0.00343 b	0.04333 ± 0.00407 b	0.08744 ± 0.00542 a	0.06764 ± 0.01372 a	0.233	0.021	0.126
1-Octen-3-Ol	0.12615 ± 0.03004 a	0.12107 ± 0.00564 a	0.08971 ± 0.00728 a	0.03629 ± 0.00480 b	0.06884 ± 0.00639 a	0.06283 ± 0.01333 a	0.650	0.000	0.023
Benzyl Alcohol	0.01042 ± 0.00184 a	0.01950 ± 0.00286 a	0.01971 ± 0.00396 a	0.01548 ± 0.00291 a	n.d	0.04949 ± 0.02987 a	0.523	0.722	0.998
Furans									
2-Methylfuran	0.00770 ± 0.00032 c	0.01372 ± 0.00090 b	0.02194 ± 0.00116 a	n.d	n.d	n.d	0.009	0.000	0.009
3-Methylfuran	n.d	0.01148 ± 0.00242 a	n.d	n.d	n.d	n.d	0.223	0.223	0.223
2-Butylfuran	0.00711 ± 0.00041 a	0.00599 ± 0.00102 a,b	0.00482 ± 0.00044 b	n.d	n.d	n.d	0.021	0.000	0.021
2-Pentylfuran	0.09652 ± 0.03597 a	0.06390 ± 0.02915 a	0.06414 ± 0.00848 a	n.d	0.02122 ± 0.00268 a	0.01525 ± 0.00683 a	0.455	0.000	0.020
Hydrocarbons									
1,2,4,4-Tetramethylcyclopentene	n.d	0.00929 ± 0.00062 a	n.d	n.d	n.d	n.d	0.215	0.215	0.215
2,2,4,6,6-Pentamethylheptane	0.02296 ± 0.00690 a	0.02007 ± 0.00515 a	0.01964 ± 0.00168 a	0.01729 ± 0.00582 a	0.02289 ± 0.00682 a	0.02597 ± 0.01047 a	0.620	0.893	0.222
Ethyl Benzene	n.d	n.d	n.d	n.d	0.09524 ± 0.06073 a	n.d	0.403	0.403	0.403
Tridecane	n.d	n.d	n.d	0.01100 ± 0.00145 a	n.d	n.d	0.000	0.000	0.000
Tetradecane	n.d	n.d	n.d	0.01741 ± 0.00036 b	0.04182 ± 0.00798 a	0.03469 ± 0.00746 a	0.011	0.000	0.011
2,6,10-Trimethyltridecane	n.d	n.d	n.d	n.d	n.d	0.02482 ± 0.00399 a	0.311	0.311	0.311
Pentadecane	0.15499 ± 0.07599 c	0.24355 ± 0.08369 c	0.22075 ± 0.05417 c	0.66333 ± 0.00284 b	1.49391 ± 0.27536 a	1.24060 ± 0.23169 a	0.007	0.000	0.021
Hexadecane	0.15713 ± 0.04745 b	0.22729 ± 0.05538 b	0.19335 ± 0.02903 b	0.35436 ± 0.00582 b	0.70480 ± 0.13366 a	0.60046 ± 0.10782 a	0.009	0.000	0.047
Heptadecane	1.13962 ± 0.37660 c	2.53983 ± 0.66277 c	2.43265 ± 0.33095 c	4.15299 ± 0.09135 b	7.65492 ± 1.95460 a	7.00994 ± 1.23021 a	0.006	0.000	0.190
6,9-Heptadecadiene	n.d	n.d	n.d	0.07147 ± 0.00742 a	n.d	n.d	0.000	0.000	0.000

N-Acetyl-4(H)-Pyridine	0.04755 ± 0.00854 a	0.01928 ± 0.00342 b	0.03432 ± 0.00153 a	n.d	n.d	n.d	0.011	0.000	0.011
Pyrazines									
2-Methylpyrazine	n.d	n.d	0.01654 ± 0.00083 a	n.d	n.d	n.d	0.214	0.214	0.214
2,5-Dimethylpyrazine	0.03803 ± 0.00694 a	0.03379 ± 0.00435 a	0.03696 ± 0.00272 a	n.d	n.d	n.d	0.461	0.000	0.461
2-Methyl-5-Ethylpyrazine	n.d	n.d	0.03285 ± 0.00635 a	n.d	n.d	n.d	0.221	0.221	0.221
2-Ethyl-6-Methylpyrazine	0.02741 ± 0.01126 a	0.02143 ± 0.00012 a	n.d	n.d	n.d	n.d	0.097	0.002	0.097
Trimethyl Pyrazine	0.02836 ± 0.00290 b	0.05547 ± 0.00244 a	0.05250 ± 0.00204 a	n.d	0.00541 ± 0.00085 d	0.01996 ± 0.00521 c	0.000	0.000	0.116
2,3-Dimethyl-5-Ethylpyrazine	0.01556 ± 0.00391 a	0.01944 ± 0.00326 a	0.01980 ± 0.00359 a	n.d	n.d	n.d	0.114	0.000	0.114
Tetramethyl Pyrazine	0.01873 ± 0.00355 a	0.01948 ± 0.00053 a	0.02146 ± 0.00411 a	0.00909 ± 0.00280 b	n.d	0.01411 ± 0.00340 b	0.969	0.009	0.610
Sulfur comp.									
Dimethyl Disulfide	0.05171 ± 0.02780 a	0.03838 ± 0.02634 a	0.01534 ± 0.00246 a	n.d	n.d	n.d	0.171	0.001	0.171
2-Ethyl-4-Methylthiazole	n.d	n.d	n.d	n.d	n.d	0.02147 ± 0.00262 a	0.308	0.308	0.308
Dimethyl Trisulfide	0.01310 ± 0.00482 a	0.01702 ± 0.01053 a	n.d	n.d	0.03145 ± 0.01865 a	n.d	0.575	0.765	0.315
Total aromatic compounds	3.063 ± 0.847 b	4.232 ± 0.919 b	4.176 ± 0.476 b	5.574 ± 0.048 b	11.018 ± 2.542 a	9.896 ± 1.844 a	0.008	0.001	0.670

Supplementary Table 1. Concentration (μg/g) of compounds identified in UV treated and sterilized *Arthrospira platensis* fermented with *L. casei* 2240 and *L. rhamnosus* GG and in controls (not fermented UV treated and sterilized *Arthrospira platensis*) after 48 hours. ND compounds not detected.

	Content						Factors		
Odor type	**Cntr ST**	**ST 2240**	**ST GG**	**Cntr UV**	**UV 2240**	**UV GG**	**F**	**S**	**F*S**
Aldehydic/Ethereal	0.104 ± 0.000 a,b	0.105 ± 0.010 a,b	0.157 ± 0.004 a	0.079 ± 0.014 b	0.197 ± 0.024 a	0.172 ± 0.042 a	0.00	0.44	0.05
Sulfurous	0.065 ± 0.033 a	0.055 ± 0.037 a	0.015 ± 0.002 a	n.d	0.031 ± 0.019 a	n.d	0.65	0.02	0.16
Green/Herbal	0.302 ± 0.056 a	0.209 ± 0.011 a	0.225 ± 0.004 a	0.098 ± 0.011 b	0.226 ± 0.019 a	0.237 ± 0.046 a	0.21	0.00	0.00
Buttery/Waxy	0.008 ± 0.000 c	0.045 ± 0.010 b	0.097 ± 0.003 a	0.029 ± 0.001 b	0.179 ± 0.018 a	0.172 ± 0.056 a	0.00	0.01	0.05
Spicy	0.007 ± 0.000 a	0.006 ± 0.001 a	0.005 ± 0.000 a	n.d	n.d	0.021 ± 0.010 a	0.44	0.85	0.29
Fruity	0.519 ± 0.132 a	0.263 ± 0.052 b	0.277 ± 0.023 b	0.013 ± 0.002 c	0.204 ± 0.054 b	0.170 ± 0.053 b	0.31	0.00	0.00
Floral	0.098 ± 0.028 a	0.093 ± 0.010 a	0.094 ± 0.005 a	0.050 ± 0.005 a	0.069 ± 0.019 a	0.112 ± 0.053 a	0.37	0.21	0.26
Nutty/Roasted	0.158 ± 0.032 a	0.163 ± 0.005 a	0.202 ± 0.018 a	0.009 ± 0.003 b	0.005 ± 0.001 b	0.056 ± 0.011 b	0.18	0.00	0.93
Alkane	1.452 ± 0.500 b	3.011 ± 0.802 b	2.847 ± 0.414 b	5.199 ± 0.098 b	9.895 ± 2.368 a	8.886 ± 1.575 a	0.01	0.00	0.13
Fermented	0.011 ± 0.001 b	0.032 ± 0.002 a	0.034 ± 0.001 a	n.d	n.d	n.d	0.00	0.00	0.00
NC (not classified)	0.278 ± 0.052 a	0.197 ± 0.058 a	0.174 ± 0.003 a	0.089 ± 0.013 a	0.184 ± 0.096 a	0.051 ± 0.014 a	0.48	0.01	0.19

Supplementary Table 2. Concentration (μg/g) of odor type identified in UV treated and sterilized *Arthrospira platensis* fermented with *L. casei* 2240 and *L. rhamnosus* GG, and in controls (not fermented UV treated and sterilized *Arthrospira platensis*) after 48 hours. ND compounds not detected.

Lactic acid fermented microalgae as a new source of lipid reducing compounds: assessment through Zebrafish Nile Red Fat Metabolism Assay

Francesco Martelli, Valentina Bernini, Erasmo Neviani, Vitor Vasconcelos, Ralph Urbatzka

Abstract

Obesity is one of the most important threats to health. The use of surgery is not sufficient to contain this global issue and research is focusing their forces on the search for new bioactive compounds with a lipid reducing activity. Between the many natural resources of novel compounds, microalgae are well known for their high complexity in novel secondary metabolites that could have an activity. At the same time, lactic acid fermentation is a technique known for the high capacity to metabolize the matrix and consequently modify or produce new compounds with potential bioactivity. This work aims to study the production of fermented microalgae extracts with a lipid reducing activity and test them with Zebrafish Nile red fat metabolism assay. Three microalgal species (*Chlorella vulgaris*, *Chlorococcum* sp. and *Arthrospira platensis)* were fermented with seven strains of lactic acid bacteria belonging to 4 species (*Lactobacillus rhamnosus, Lactobacillus casei, Lactobacillus delbrueckii bulgaricus* and *Lactobacillus paracasei)*. All the selected strains were able to ferment the selected species of microalgae. The most suitable substrate for LAB growth was *Arthrospira platensis.* Thanks to fermentation, extract obtained from *Chlorella vulgaris* and *Chlorococcum* sp have shown a lipid reducing effect that was not present before the process. The most important lipid reducing effect was obtained with *Arthrospira platensis* fermented with *Lactobacillus delbrueckii bulgaricus* 1932. This work opens up to new possible sources of lipid reducing compounds obtained through lactic acid fermentation.

Introduction

According to the World Health Organization (WHO), Cardiovascular diseases are the most significant causes of death of this century. Sedimentary behavior and unhealthy eating can lead to obesity that is strictly connected to these diseases. The number of obese people is exponentially growing (Kelly et al., 2008). Obesity can be defined as a body mass index of more than 30 and is correlated with several diseases such as cancer, type 2 diabetes and cardiovascular problems (Abdelaal et al., 2017; Nguyen et al., 2008). Surgery is still the most efficient way to reduce obesity but it is an invasive technique, and a pharmacological treatment could be the answer to contain this problem (Kang & Park, 2012). To this purpose, the search for new compounds having a bioactive activity against this disease is increasing interest and microalgae may represent a possible source.

These photosynthetic organisms are rich in bioactive compounds that have proven anti-cancer, anti-inflammatory, antimicrobial and also anti-obesity activity (Bancalari et al., 2020; de Vera et al., 2018; Martelli, Cirlini, et al., 2020). Microalgae and cyanobacteria are characterized by a valid composition in amino acids that makes them an important source for food supplements (Andrade, 2018; Beheshtipour et al., 2013). Moreover, the growing consumers' interest in these organisms makes them a possible new food source for the future (Camacho et al., 2019; Draaisma et al., 2013). Because of their composition, characterized by a high percentage of amino acids, they are suitable matrixes for lactic acid fermentation. The interaction between lactic acid bacteria (LAB) and microalgae, in particular fermentation, has been studied (Bao et al., 2018; de Marco Castro et al., 2019; Martelli, Alinovi, et al., 2020; Niccolai et al., 2019).

Lactic acid fermentation is an old preservation technique that is used by humans for many centuries. Many LAB are generally recognized as safe and are studied because of their metabolism which can produce bioactive compounds such as organic acids (lactic acid), diacetyl and many other metabolites (Fernández & Zúñiga, 2006). This kind of metabolisms can influence the composition of the matrix that is fermented, by changing the bioactive

activity of the compounds (Martelli, Favari, et al., 2020). This work aimed to study the potential production of metabolites with strong bioactivities towards obesity by lactic acid fermentation of microalgae. The screening focused on anti-obesity bioactivities were tested using whole small animal models in vivo, in particular, effects on lipid homeostasis were analyzed in zebrafish (Costa et al., 2019; Noinart et al., 2017).

Materials and methods

Microalgal biomasses

Commercial algal biomasses were used for the experiments.

Dehydrated *Chlorella vulgaris* (Nutrisslim, China), dehydrated *Chlorococcum* sp. provided by Allmicroalgae (Leira, Portugal) and dehydrated organic *Arthrospira platensis*, provided by Bertolini Farm (Fidenza, PR, Italy), were used for fermentations.

Bacterial strains used for fermentation

Seven LAB strains, belonging to the collection of the Laboratory of Food Microbiology of the Department of Food and Drug (University of Parma, Parma, Italy) were used to ferment Microalgal biomass. The strains, have been previously isolated from different food matrixes, identified by 16S rRNA sequencing (Tab. 1), and maintained at -80 °C in de Man, Rogosa and Sharpe (MRS) (Oxoid, Basingstoke, UK), added with 12.5% glycerol (v/v). Lactic acid bacteria were also grown in MRS to obtain biomasses for extractions. After growth at 37°C for 48 hours, lactic acid bacteria biomasses were then centrifuged (Eppendorf centrifuge 5810 R, Eppendorf, Hamburg, Germany) (10000 rpm for 10 minutes at 4 °C), cells were collected, washed twice in sterilized water and then lyophilized by a Freeze dryer Lio-5P (5Pascal, Milano, Italy) for 48h.

Table 1. *Lactobacillus* strains used for fermentation of microalgal biomasses

ID	Species	Temperature	Isolation matrix
1473	*Lacticaseibacillus rhamnosus*	37°C	Parmigiano Reggiano
1019	*Lacticaseibacillus rhamnosus*	37°C	
2240	*Lacticaseibacillus casei*	37°C	Parmigiano Reggiano
4339	*Lacticaseibacillus casei*	37°C	Cow milk
2214	*Lb. delbrueckii bulgaricus*	37°C	Grana Padano
1932	*Lb. delbrueckii bulgaricus*	37°C	Grana Padano
4186	*Lacticaseibacillus paracasei*	37°C	Pecorino Toscano

Step up for fermentation

LAB strains were revitalized twice in MRS broth (Oxoid) (inoculum of 3% v/v) incubated for 16 h at 37 °C under aerobic conditions.

Later, each revitalized strain was inoculated in fresh MRS broth (3% v/v) and incubated for 15 h at 37°C, in order to obtain a bacterial concentration of 9 Log CFU /ml. After centrifugation (10000 rpm for 10 minutes at 4 °C), the grown cells were collected, washed twice in Ringer solution (Oxoid, Milan, Italy) and suspended in sterile bidistilled water. Then, the microalgal biomass was rehydrated with 70% of water and then inoculated individually with each bacterial suspension to obtain a final concentration of 7 Log CFU/ ml. The microbial concentration was evaluated just after the inoculum (T0), after 24 hours (T1), 48 hours (T2) and 72 hours of fermentation (T3) by plate counts

Serial dilutions in Ringer (Oxoid) of the samples were plated on MRS agar, followed by incubation for 48 h in aerobic conditions for each strain. Fermentations were carried out in duplicate and for each sampling time analyses were performed in duplicate.

After the fermentation process, the biomasses were lyophilized for 48 hours with Telstar lyoquest (Azbil Telstar, Portugal).

Average values ± standard deviations were reported.

Extraction process

Before of the extraction process, 1g of the freeze-dried sample was broken up using a porcelain mortar and pestle to enhance the extraction process. Then, the grounded biomass is transferred to a glass flat bottom round flask.

For the first extraction, 30 mL of methanol were added to the flask containing the biomass. The flask containing the biomass was then put in an ultrasonic bath FB15053 (Fisherbrand) for 5 min, taking care not to warm up above 30º C. The supernatant was decanted in a vacuum filtering system into a round bottom flask. The steps were repeated twice using 15 mL of methanol.

After the extraction process, the extract in the round bottom flask was concentrated in a rotary evaporator at 30º C until fully dried. The obtained dried extract was then suspended in 5 mL of methanol. Another filtering step was performed by using a Pasteur pipette filled with cotton (to capture any non-soluble particular matter) and the extract was transferred to a pre-weighed 20 mL vial. The solvent was then evaporated in a rotary evaporator. The vials containing dried extract were then put in a high vacuum to dry it and establish the collected extract mass. All the extracts were suspended in DMSO to obtain a concentration of 10 mg/mL.

Zebrafish Nile Red Fat Metabolism Assay

Lipid reducing activity was assessed by the zebrafish Nile red fat metabolism assay (Costa et al., 2019; Noinart et al., 2017).

Authorization by an ethics commission was not needed because any procedures were not considered as animal experimentation in line with EC Directive 86/609/EEC. Concisely, zebrafish embryos were raised from 1-day post-fertilization (DPF) in egg water (60 μg/mL marine sea salt) with 200 μM 1-phenyl-2-thiourea (PTU) to inhibit pigmentation. From 3 to 5 DPF, zebrafish larvae were exposed to extracts at a final concentration of 10 μg/mL with a daily renewal of water and fractions in a 48-well plate with a density of 6–8 larvae/well (n = 6–8). A solvent control (0.1% DMSO) and positive control (REV, resveratrol, 50 μM) were included in the assay. Neutral lipids were stained with 10 ng/mL Nile red overnight. The larvae were anesthetized with tricaine (MS-222, 0.03%) for 5 min before taking pictures on a fluorescence microscope (Olympus, BX-41, Hamburg, Germany). Fluorescence intensity was quantified in individual zebrafish larvae by ImageJ

Statistical analysis

In order to study intraspecific differences on the ability of each LAB species to ferment microalgal biomass, a paired T-test was performed ($p<0.05$). A one way ANOVA was performed on zebrafish Nile red fat metabolism assay results using Graphpad prism 8.

Results

Microalgae fermentation

Three microalgal species (*Chlorella vulgaris, Chlorococcum* sp. and *Arthrospira platensis)* were fermented with seven LAB strains belonging to 4 different species: *L. rhamnosus* (1473, 1019), *L. casei* (2240, 4339), *L. delbrueckii bulgaricus* (2214, 1932) and *L. paracasei* (4186).

The microbial growth was evaluated after 24, 48 and 72 h of incubation at 37 °C, at the optimal temperature of the tested species. Generally, all the strains showed a good growth ability in all the tested biomasses. The most suitable biomass for fermentation was *A. platensis* with an average value of 2.19 Log CFU/g reached in 72 hours' growth. The best growth performance was registered by *L. paracasei* 4186 with an increase of 2.67 Log CFU/ g (Table 1). But also *L. rhamnosus* strains were able to grow well on the biomass (2.40 ± 0.14 Log CFU/g) while *L. casei* strains showed a lower growth ability (1.80 ± 0.02 Log CFU/g). *L. delbrueckii bulgaricus* showed a good growth ability but variable between the two tested strains. In fact, *L. delbrueckii bulgaricus* 1932 (Δ= 2.65 Log CFU/g) was able to grow by more than one logarithm compared to *L. delbrueckii bulgaricus* 2214 (Δ= 1.60 Log CFU/g) (Table 1). Considering *Chlorella vulgaris*, strains 1473 and 1019, belonging to *L. rhamnosus*, showed a really inconstant ability to grow in the matrix (Δ= 2.44 and 0.67 Log CFU/g, respectively). On the other hand, *L. paracasei* 4186 highlighted the highest growth capacity (Δ= 1.99 Log CFU/ g) compared to the other strains (Table 1). Differently to what was observed during *A. platensis* fermentation, *L. belbrueckii bulgaricus* strains (2214 and 1932) showed a lower but more constant growth ability (1.71 ± 0.19 Log CFU/g).

Chlorella biomass proved to be a good substrate for LAB growth with an average value of 1.67 Log CFU/g. Fermentation of *Chlorococcum* sp. exerted the lower results with an average value of 1.36 Log CFU/g in 72h fermentation. *L. rhamnosus* strains showed the highest growth capacity (1.70 ± 0.39 Log CFU/g) while *L. delbrueckii bulgaricus* strains the lowest (0.99 ± 0.17 Log CFU/g).

Table 2. Growth ability of different lactic acid bacteria (LAB) species/strains in *Chlorella vulgaris, Chlorococcum* sp. and *Arthrospira platensis* after 72 h of fermentation at the optimal growth temperature (37°C).

	Chlorella vulgaris			*Clorococcum* sp.			*Arthrospira platensis*		
ID	T0	T3	Δ (T3-T0)	T0	T3	Δ (T3-T0)	T0	T3	Δ (T3-T0)
1473	7.50 ± 0.01	9.94 ± 0.05	**2.44**	7.23 ± 0.02	9.22 ± 0.20	**1.99**	7.10 ± 0.02	9.40 ± 0.11	**2.30**
1019	7.22 ± 0.09	7.89 ± 0.06	**0.67**	7.50 ± 0.03	8.93 ± 0.04	**1.42**	7.01 ± 0.23	9.52 ± 0.06	**2.51**
2240	7.12 ± 0.16	8.87 ± 0.04	**1.76**	7.37 ± 0.10	8.97 ± 0.03	**1.60**	7.75 ± 0.21	9.57 ± 0.06	**1.82**
4339	7.12 ± 0.12	8.56 ± 0.06	**1.44**	7.84 ± 0.05	8.92 ± 0.03	**1.08**	7.74 ± 0.06	9.53 ± 0.05	**1.79**
2214	7.33 ± 0.04	8.90 ± 0.08	**1.57**	7.75 ± 0.05	8.87 ± 0.04	**1.12**	8.07 ± 0.32	9.67 ± 0.08	**1.60**
1932	7.57 ± 0.17	9.42 ± 0.03	**1.85**	7.82 ± 0.04	8.69 ± 0.12	**0.87**	7.06 ± 0.08	9.71 ± 0.05	**2.66**
4186	7.12 ± 0.16	9.10 ± 0.14	**1.99**	7.52 ± 0.14	8.93 ± 0.05	**1.41**	7.06 ± 0.08	9.72 ± 0.05	**2.67**

Figure 1 display the growth curve representing the ability of each strain to grow in different microalgae.

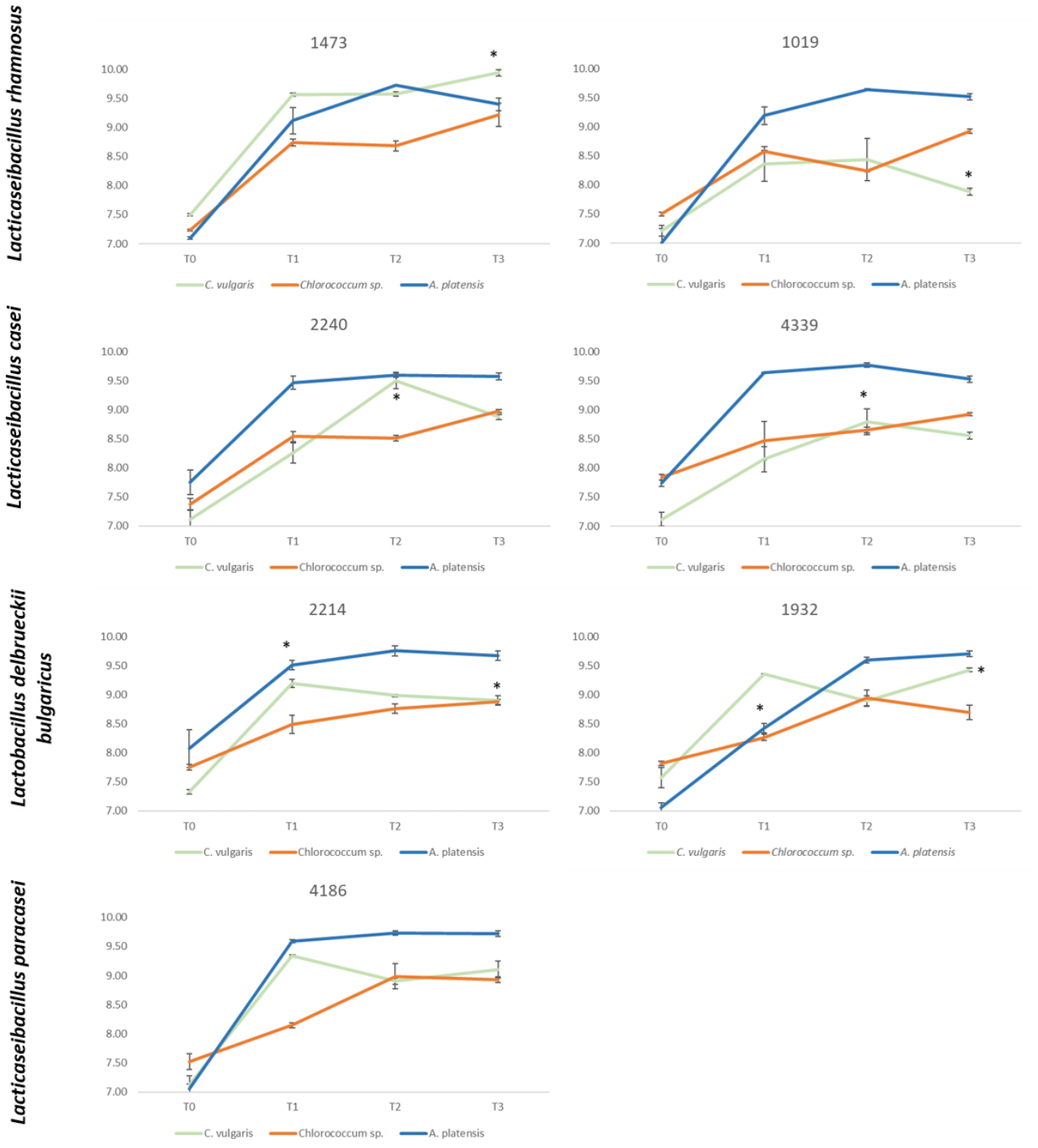


Figure 1. Growth kinetics of lactic acid bacteria strains in microalgae samples. Comparisons between the strains belonging to the same species were made and significant differences are signed with * ($p<0.05$)

Comparing the growth curves of *L. rhamnosus* on the tested microalgal biomass (Figure 1A and 1B), a different behavior on *C. vulgaris* was noticed. *L. rhamnosus* 1473, showed a higher ability to grow on the biomass. While *L. rhamnosus* 1473 keep growing until T3 (72h)

reaching a concentration of 9.94 ± 0.05 Log CFU/g, *L. rhamnosus* 1019 was able to grow until T2 (8.43 ± 0.36 Log CFU/g) and at T3 its concentration was reduced to a concentration of 7.89 ± 0.06 Log CFU/g ($p<0.05$). Regarding the behavior of the two *L. rhamnosus* strains on *Chlorococcum* sp. and on *A. platensis,* no significant differences of the tested strains during fermentation were detected. *L. casei* 2240 and 4339 presented a similar behavior on each matrix. The only significant difference was noticed at T2 during *C. vulgaris* fermentation. *L. casei* 2240 managed to grow at a concentration of 9.50 ± 0.14 Log CFU/g in 48 hours, while *L. casei* 4339 concentration was 8.79 ± 0.23 ($p< 0.05$).

Two significant differences were found studying *L. delbrueckii bulgaricus* strains behavior during algal biomass fermentation. Considering *Chlorella vulgaris,* after 3 days of fermentation, *L. delbrueckii bulgaricus* 1932 could reach a concentration of 9.42 ± 0.03 while 2214 could grow in a significantly lower amount (8.90 ± 0.08). In *A. platensis, L. delbrueckii bulgaricus* 2214 at T2 grew more than strain 1932 ($p<0.05$). Then, at the end of the fermentation time, the two strains reached an equal concentration.

Lipid reduction activity in zebrafish larvae

The fermented microalgae and the control extracts were screened for lipid reducing activity using the zebrafish Nile red fat metabolism assay. The results are shown in figure 2.

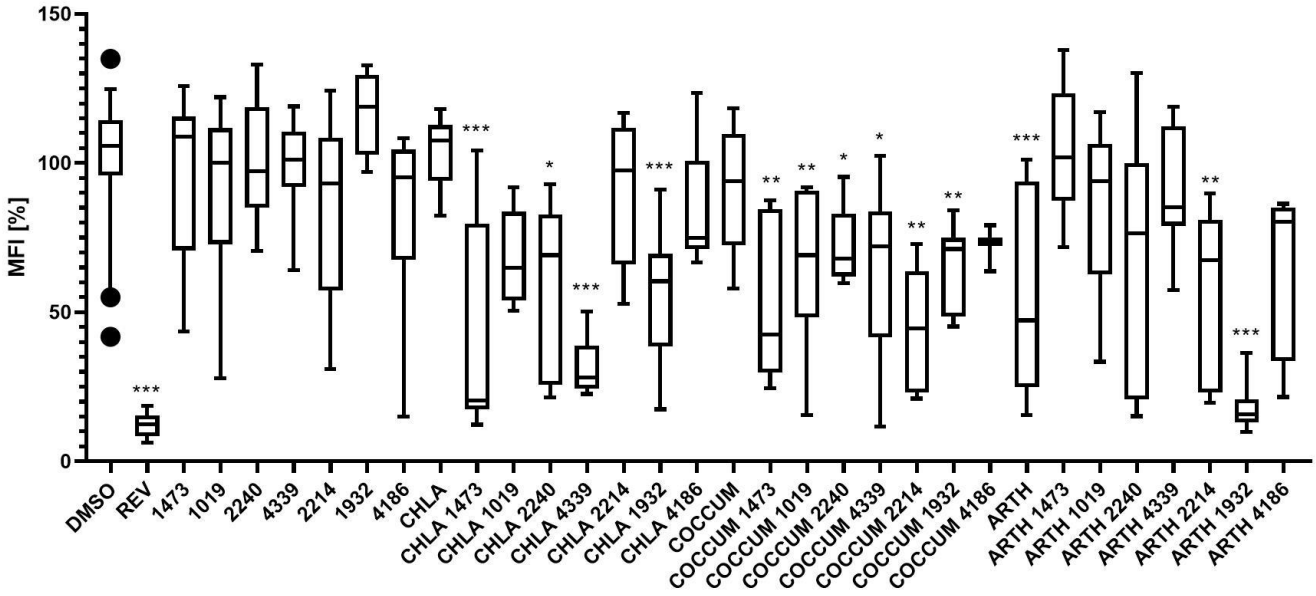


Figure 2. Data are presented as mean fluorescence intensity (MFI) relative to the solvent control (DMSO). Zebrafish at 3 days post fertilization (DPF) were exposed for 48 h to 10 μg/mL of extracts and lipids around the yolk sac and intestine were stained with Nile red.

The extracts obtained with LAB didn't produce any significant differences from the DMSO. No activity was noticed in unfermented *Chlorella vulgaris* extract (CHLA) but thanks to fermentation significant lipid reducing activities were noticed. In particular, fermentation with *Lacticaseibacillus rhamnosus* 1473 (CHLA 1473) has reduced significantly the lipid formation ($p<0.001$). The same effect was noticed for the extract obtained from biomass fermented with the two *L. casei* (CHLA 2240, $p<0.01$; CHLA 4339, $p<0.001$) and for *L. delbrueckii bulgaricus* 1932 (CHLA 1932, $p<0.001$). In particular, CHLA 4339 extract had a really strong and constant activity presenting a 32.49 ± 9.56% of MF% compared to the DMSO.

Chlorococcum sp. unfermented biomass extract didn't show any bioactivity but subsequently to fermentation significant lipid reducing activities were observed. Especially, fermentation with both *L. rhamnosus* (COCCUM 1473, $p<0.001$; COCCUM 1019, $p<0.005$), both *L. casei* (COCCUM 2240, $p<0.05$; COCCUM 4339, $p<0.05$) and both *L. delbrueckii bulgaricus* (COCCUM 2214, $p<0.001$; COCCUM 1932, $p<0.005$) have proved a lipid reduction effect in the assay. Differently to what observed in the two Eukaryotic microalgae, the cyanobacterium *A. platensis* extract has proved a significant lipid reducing effect (ARTH, $p<0.001$) and, contrary to what previously observed, all the fermentation process except for *L. delbrueckii bulgaricus* 1932 (ARTH 1932, $p<0.001$) have reduced the lipid reducing effect of *A. platensis*. In particular, the extract ARTH 1932 is the extract that proved the higher lipid reduction effect presenting a MF% of 18.14 ± 7.78%.

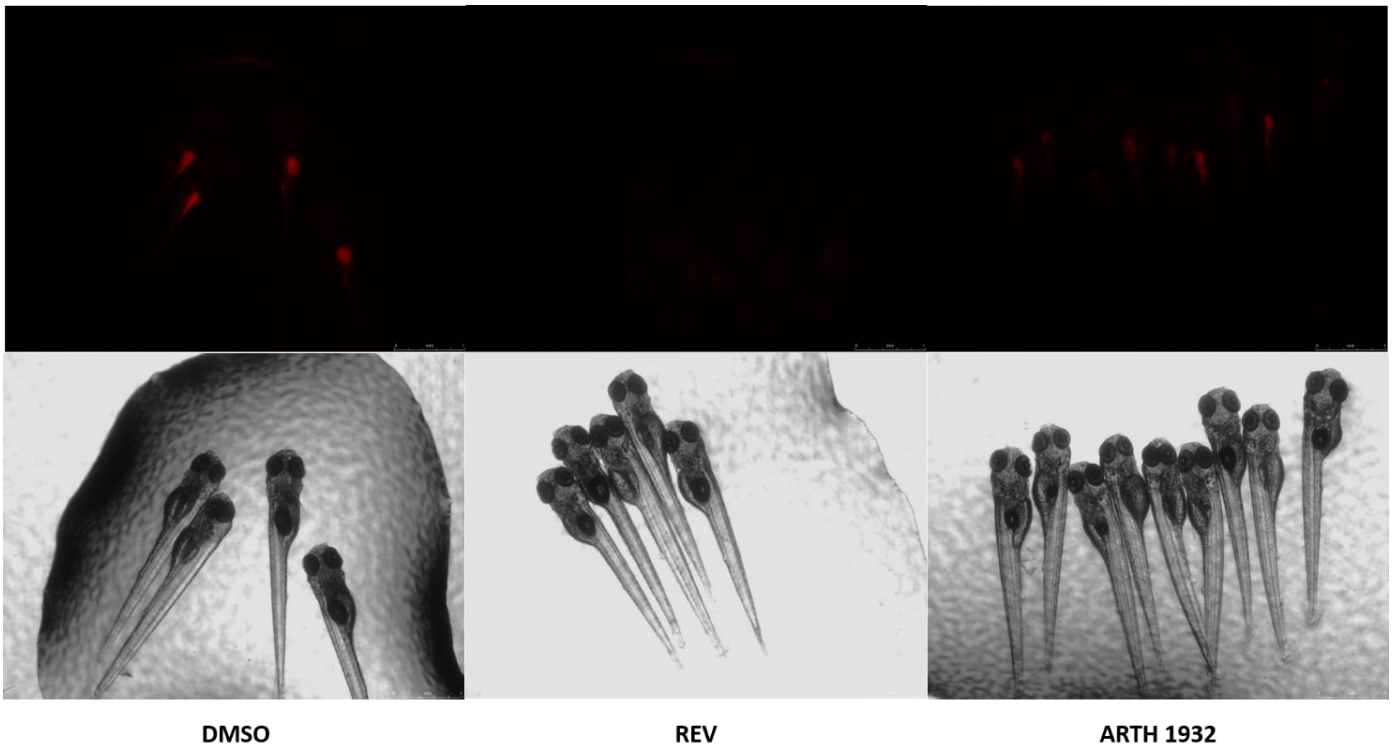


Figure 3. Representative images of zebrafish larvae (brightfield picture and red fluorescence channel). Solvent control, 0.1% dimethyl sulfoxide (DMSO); positive control, 50 μM REV and exposure to extract ARTH 1932.

Discussion

Microalgae and cyanobacteria are well known to be a source of bioactive compounds with many bioactivities (de Vera et al., 2018; Plaza et al., 2010). The use of these organisms as a food supplement and in food formulation is growing also because of their valuable composition (Andrade, 2018; Beheshtipour et al., 2013; Draaisma et al., 2013). Furthermore, the searching for new natural products that could function as anti-obesity agents had been studied in the literature, and microalgae are already considered as one precious source (Castro et al., 2016; Costa et al., 2019; Yun, 2010), however, research in this field is still underexplored and for the first time, the potential of lactic acid fermentation of microalgae as a tool to enhance the production of bioactive compounds with a lipid reducing activity is studied. Recent studies about the lipid reducing activity of fermented cauliflower have been produced with promising results (Matsuura et al., 2020). Researches in this field could bring to massive production of fermented food or food supplements that could help reducing lipid accumulation and consequently help the world population to fight obesity. For that purpose, the zebrafish Nile red fat metabolism assay as a whole small animal model in vivo for lipid reduction was used (Urbatzka et al., 2018). A total of 31 extracts were obtained from seven lactic acid bacteria biomasses, three species of microalgae and 21 fermented microalgae. Fermentation of microalgae is studied by many points of view, though solid-state fermentation of microalgae has been poorly studied and presents a lot of potentials. *Arthrospira platensis* fermentation was studied in order to try to stimulate the growth and the vitality of LAB in food products (Barkallah et al., 2017; Gyenis et al., 2005; Martelli, Alinovi, et al., 2020), to enhance the nutraceuticals properties of the algal biomass (de Marco Castro et al., 2019) or to study the production of probiotics (Niccolai et al., 2019). Also *Chlorella* sp. biomass has been studied as a fermentable matrix (Beheshtipour et al., 2013; Gyenis et al., 2005; Ścieszka & Klewicka, 2020) but fermentation of *Chlorococcum* sp. was never considered before. All the microalgae have been proved to be a very good substrate for lactic acid fermentation. Differences were found in the ability of lactic acid bacteria to

grow on the biomass. The reasons for these differences are linked to the different compositions of the microalgae selected for this study and also to the different abilities of the selected LAB strains to grow. *Arthrospira platensis* biomass proved to be the most suitable matrix for fermentation. This cyanobacterium is characterized by a high amount of proteins and small peptides that are really favorable for the growth of lactic acid bacteria (Martelli, Alinovi, et al., 2020). Also *Chlorella vulgaris* composition is a favorable substrate for the growth of lactic acid bacteria, presenting a high content of proteins and carbohydrates (Beheshtipour et al., 2013). *Chlorococcum* sp. is a *Chlorophyta* that has a valuable composition in protein, carbohydrates, lipids and bioactive compounds and is studied as a possible new source of food (Correia et al., 2020) even if is still not approved by EFSA as Novel food.

Evaluation of microalgae as a source of antiobesogenic compounds is studied with very promising results (Costa et al., 2019; Freitas et al., 2019), but for the first time is evaluated the fermentation of microalgae as a way to enhance this type of bioactivity. Fermentation is used by men for various purposes for centuries such as the extension of food's shelf-life, the increase of food safety, and the improvement of nutritional characteristics (van Boekel et al., 2010). Fermentation with *Lacticaseibacillus rhamnosus* and *Lacticaseibacillus casei* strains were able to significantly influence the lipid reducing effect of *Chlorella vulgaris* and *Chlorococcum* sp. Lactic acid fermentation is known to produce organic acids, hydrogen peroxide, CO_2, and peptides but is also well known as a way to enhance and modify the phenolic compounds present in the matrix. The two tested LAB species have presented this type of behavior during the fermentation of elderberry juice (Ricci et al., 2019). In literature, a strong lipid reducing action is attributed to phenolic compounds (Urbatzka et al., 2018). The enhancement of the activity of the *Chlorella vulgaris* and *Chlorococcum* sp. extracts after the fermentation process can be linked to a change in the phenolic composition of the microalgae. What happened to *Arthrospira platensis* extracts, in which a reduction of bioactivity was noticed after fermentation in almost all the cases except for *L. delbrueckii bulgaricus*, can be linked to metabolization of a molecule that presented a lipid reducing action. The reduction of bioactivity consequently to fermentation is not new, for instance,

after fermentation of *Himanthalia elongata,* a brown seaweed, the antimicrobial activity of extracts was reduced (Martelli, Favari, et al., 2020). The strain that has significantly influenced the lipid reducing effect after fermentation of all the three strains of microalgae is *Lactobacillus delbrueckii bulgaricus* 1932. This strain, like 2214, is a strong producer of exopolysaccharides (Bancalari et al., 2019) and, as found in studies, the lipid reducing the effect of EPS produced by LAB is well known (Zhang et al., 2016).

All the selected Lactic acid bacteria strains, even if isolated from dairy matrixes, proved a high growth ability and twelve out of twenty-one fermented extracts were able to significantly influence the lipid reducing the activity of microalgae. These results open up to new possibilities for the production of fermented extracts with interesting bioactivities.

Conclusions

Search for new compounds with a lipid reducing effect in order to reduce the obesity rate in the population is an important topic. In this study, was studied the use of fermentation to enhance or produce lipid reducing compounds. Microalgae proved to be a very good matrix to conduce a solid-state fermentation process. The fermentation process led to the production of lipid reducing effect in zebrafish Nile red fat metabolism assay. This work reveals a great potential of fermented products, in particular microalgae, to produce compounds with relevant bioactivities towards lipid formation. This work could open up to new fermented food supplements or foods that could help in reducing lipid accumulation. In future works, will be studied in deep what lactic acid fermentation has produced and the compounds responsible for bioactivity.

References

Abdelaal, M., le Roux, C. W., & Docherty, N. G. (2017). Morbidity and mortality associated with obesity. *Annals of Translational Medicine*, *5*(7), 161–161. https://doi.org/10.21037/atm.2017.03.107

Andrade, L. M. (2018). *Chlorella* and *Spirulina* Microalgae as Sources of Functional Foods, Nutraceuticals, and Food Supplements; an Overview. *MOJ Food Processing & Technology*, *6*(1), 45–58. https://doi.org/10.15406/mojfpt.2018.06.00144

Bancalari, E., D'Incecco, P., Savo Sardaro, M. L., Neviani, E., Pellegrino, L., & Gatti, M. (2019). Impedance microbiology to speed up the screening of lactic acid bacteria exopolysaccharide production. *International Journal of Food Microbiology*, *306*(108268), 10. https://doi.org/10.1016/j.ijfoodmicro.2019.108268

Bancalari, E., Martelli, F., Bernini, V., Neviani, E., & Gatti, M. (2020). Bacteriostatic or bactericidal? Impedometric measurements to test the antimicrobial activity of *Arthrospira platensis* extract. *Food Control*, 107380. https://doi.org/10.1016/j.foodcont.2020.107380

Bao, J., Zhang, X., Zheng, J.-H., Ren, D.-F., & Lu, J. (2018). Mixed fermentation of *Spirulina platensis* with *Lactobacillus plantarum* and *Bacillus subtilis* by random-centroid optimization. *Food Chemistry*, *264*, 64–72. https://doi.org/10.1016/j.foodchem.2018.05.027

Barkallah, M., Dammak, M., Louati, I., Hentati, F., Hadrich, B., Mechichi, T., Ayadi, M. A., Fendri, I., Attia, H., & Abdelkafi, S. (2017). Effect of *Spirulina platensis* fortification on physicochemical, textural, antioxidant and sensory properties of yogurt during fermentation and storage. *LWT*, *84*, 323–330. https://doi.org/10.1016/j.lwt.2017.05.071

Beheshtipour, H., Mortazavian, A. M., Mohammadi, R., Sohrabvandi, S., & Khosravi-Darani, K. (2013). Supplementation of *Spirulina platensis* and *Chlorella vulgaris* Algae into Probiotic Fermented Milks: Algae addition into probiotic fermented milk. *Comprehensive*

Reviews in Food Science and Food Safety, *12*(2), 144–154. https://doi.org/10.1111/1541-4337.12004

Camacho, F., Macedo, A., & Malcata, F. (2019). Potential Industrial Applications and Commercialization of Microalgae in the Functional Food and Feed Industries: A Short Review. *Marine Drugs*, *17*(6), 312. https://doi.org/10.3390/md17060312

Castro, M., Preto, M., Vasconcelos, V., & Urbatzka, R. (2016). Obesity: The Metabolic Disease, Advances on Drug Discovery and Natural Product Research. *Current Topics in Medicinal Chemistry*, *16*(23), 2577–2604. https://doi.org/10.2174/1568026616666160415155644

Correia, N., Pereira, H., Silva, J. T., Santos, T., Soares, M., Sousa, C. B., Schüler, L. M., Costa, M., Varela, J., Pereira, L., & Silva, J. (2020). Isolation, Identification and Biotechnological Applications of a Novel, Robust, Free-living *Chlorococcum (Oophila)* amblystomatis Strain Isolated from a Local Pond. *Applied Sciences*, *10*(9), 3040. https://doi.org/10.3390/app10093040

Costa, M., Rosa, F., Ribeiro, T., Hernandez-Bautista, R., Bonaldo, M., Gonçalves Silva, N., Eiríksson, F., Thorsteinsdóttir, M., Ussar, S., & Urbatzka, R. (2019). Identification of Cyanobacterial Strains with Potential for the Treatment of Obesity-Related Co-Morbidities by Bioactivity, Toxicity Evaluation and Metabolite Profiling. *Marine Drugs*, *17*(5), 280. https://doi.org/10.3390/md17050280

De Marco Castro, E., Shannon, E., & Abu-Ghannam, N. (2019). Effect of Fermentation on Enhancing the Nutraceutical Properties of *Arthrospira platensis (Spirulina)*. *Fermentation*, *5*(1), 28. https://doi.org/10.3390/fermentation5010028

de Vera, C., Díaz Crespín, G., Hernández Daranas, A., Montalvão Looga, S., Lillsunde, K.-E., Tammela, P., Perälä, M., Hongisto, V., Virtanen, J., Rischer, H., Muller, C., Norte, M., Fernández, J., & Souto, M. (2018). Marine Microalgae: Promising Source for New Bioactive Compounds. *Marine Drugs*, *16*(9), 317. https://doi.org/10.3390/md16090317

Draaisma, R. B., Wijffels, R. H., (Ellen) Slegers, P., Brentner, L. B., Roy, A., & Barbosa, M. J. (2013). Food commodities from microalgae. *Current Opinion in Biotechnology*, *24*(2), 169–177. https://doi.org/10.1016/j.copbio.2012.09.012

Fernández, M., & Zúñiga, M. (2006). Amino Acid Catabolic Pathways of Lactic Acid Bacteria. *Critical Reviews in Microbiology*, *32*(3), 155–183. https://doi.org/10.1080/10408410600880643

Freitas, S., Silva, N. G., Sousa, M. L., Ribeiro, T., Rosa, F., Leão, P. N., Vasconcelos, V., Reis, M. A., & Urbatzka, R. (2019). Chlorophyll Derivatives from Marine Cyanobacteria with Lipid-Reducing Activities. *Marine Drugs*, *17*(4), 229. https://doi.org/10.3390/md17040229

Gyenis, B., Szigeti, J., Molnár, N., & Varga, L. (2005). Use of dried microalgal biomasses to stimulate acid production and growth of *Lactobacillus plantarum* and *Enterococcus faecium* in milk. *Acta Agraria Kaposváriensis*, *9*(2), 53–59.

Kang, J. G., & Park, C.-Y. (2012). Anti-Obesity Drugs: A Review about Their Effects and Safety. *Diabetes & Metabolism Journal*, *36*(1), 13. https://doi.org/10.4093/dmj.2012.36.1.13

Kelly, T., Yang, W., Chen, C.-S., Reynolds, K., & He, J. (2008). Global burden of obesity in 2005 and projections to 2030. *International Journal of Obesity*, *32*(9), 1431–1437. https://doi.org/10.1038/ijo.2008.102

Martelli, F., Alinovi, M., Bernini, V., Gatti, M., & Bancalari, E. (2020). *Arthrospira platensis* as Natural Fermentation Booster for Milk and Soy Fermented Beverages. *Foods*, *9*(350), 15. https://doi.org/doi:10.3390/foods9030350

Martelli, F., Cirlini, M., Lazzi, C., Neviani, E., & Bernini, V. (2020). Edible Seaweeds and Spirulina Extracts for Food Application: In Vitro and In Situ Evaluation of Antimicrobial Activity towards Foodborne Pathogenic Bacteria. *Foods,* 9 (1442), 15.

Martelli, F., Favari, C., Mena, P., Guazzetti, S., Ricci, A., Rio, D. D., Lazzi, C., Neviani, E., & Bernini, V. (2020). Antimicrobial and Fermentation Potential of *Himanthalia elongata* in

Food Applications. *Microorganisms*, *8*(2), 15. https://doi.org/doi:10.3390/microorganisms8020248

Matsuura, N., Zang, L., Nishimura, N., & Shimada, Y. (2020). Lacto-Fermented Cauliflower Fungus (*Sparassis crispa*) Ameliorates Hepatic Steatosis by Activating Beta-Oxidation in Diet-Induced Obese Zebrafish. *Journal of Medicinal Food*, *23*(8), 803–810. https://doi.org/10.1089/jmf.2019.4571

Nguyen, N. T., Magno, C. P., Lane, K. T., Hinojosa, M. W., & Lane, J. S. (2008). Association of Hypertension, Diabetes, Dyslipidemia, and Metabolic Syndrome with Obesity: Findings from the National Health and Nutrition Examination Survey, 1999 to 2004. *Journal of the American College of Surgeons*, *207*(6), 928–934. https://doi.org/10.1016/j.jamcollsurg.2008.08.022

Niccolai, A., Shannon, E., Abu-Ghannam, N., Biondi, N., Rodolfi, L., & Tredici, M. R. (2019). Lactic acid fermentation of *Arthrospira platensis (spirulina*) biomass for probiotic-based products. *Journal of Applied Phycology*, *31*(2), 1077–1083. https://doi.org/10.1007/s10811-018-1602-3

Noinart, J., Buttachon, S., Dethoup, T., Gales, L., Pereira, J., Urbatzka, R., Freitas, S., Lee, M., Silva, A., Pinto, M., Vasconcelos, V., & Kijjoa, A. (2017). A New Ergosterol Analog, a New Bis-Anthraquinone and Anti-Obesity Activity of Anthraquinones from the Marine Sponge-Associated Fungus Talaromyces stipitatus KUFA 0207. *Marine Drugs*, *15*(5), 139. https://doi.org/10.3390/md15050139

Plaza, M., Santoyo, S., Jaime, L., García-Blairsy Reina, G., Herrero, M., Señoráns, F. J., & Ibáñez, E. (2010). Screening for bioactive compounds from algae. *Journal of Pharmaceutical and Biomedical Analysis*, *51*(2), 450–455. https://doi.org/10.1016/j.jpba.2009.03.016

Ricci, A., Cirlini, M., Calani, L., Bernini, V., Neviani, E., Del Rio, D., Galaverna, G., & Lazzi, C. (2019). In vitro metabolism of elderberry juice polyphenols by lactic acid bacteria. *Food Chemistry*, *276*, 692–699. https://doi.org/10.1016/j.foodchem.2018.10.046

Ścieszka, S., & Klewicka, E. (2020). Influence of the Microalga *Chlorella vulgaris* on the Growth and Metabolic Activity of *Lactobacillus* spp. Bacteria. *Foods*, *9*(7), 959. https://doi.org/10.3390/foods9070959

Urbatzka, R., Freitas, S., Palmeira, A., Almeida, T., Moreira, J., Azevedo, C., Afonso, C., Correia-da-Silva, M., Sousa, E., Pinto, M., & Vasconcelos, V. (2018). Lipid reducing activity and toxicity profiles of a library of polyphenol derivatives. *European Journal of Medicinal Chemistry*, *151*, 272–284. https://doi.org/10.1016/j.ejmech.2018.03.036

van Boekel, M., Fogliano, V., Pellegrini, N., Stanton, C., Scholz, G., Lalljie, S., Somoza, V., Knorr, D., Jasti, P. R., & Eisenbrand, G. (2010). A review on the beneficial aspects of food processing. *Molecular Nutrition & Food Research*, *54*(9), 1215–1247. https://doi.org/10.1002/mnfr.200900608

Yun, J. W. (2010). Possible anti-obesity therapeutics from nature – A review. *Phytochemistry*, *71*(14–15), 1625–1641. https://doi.org/10.1016/j.phytochem.2010.07.011

Zhang, Z., Zhou, Z., Li, Y., Zhou, L., Ding, Q., & Xu, L. (2016). Isolated exopolysaccharides from *Lactobacillus rhamnosus* GG alleviated adipogenesis mediated by TLR2 in mice. *Scientific Reports*, *6*(1), 36083. https://doi.org/10.1038/srep36083

Conclusions

The need for new sources of food and novel compounds is one of the most important challenges that the world will face in the next future. In this thesis were evaluated three different aspects about the use of algae in food production. Several species of seaweeds and microalgae were evaluated as a novel source of antimicrobial compounds to be used as food preservatives. The results obtained showed an antimicrobial activity both *in vitro* and *in situ*. The antimicrobial activity of the algal extracts was variable and dependent on the species and on the starting material. One of the extracts that have exerted the higher antimicrobial activity was the *Arthrospira platensis* extract. The activity of this food-grade extract was challenged in different conditions. It was challenged in an *in situ* test, through a microbiological challenge test, against the pathogenic bacteria *Listeria monocytogenes* proving a good efficiency as a natural food preservative even at low concentration. The same extract was then tested for the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) against two food alterative bacteria, *Serratia liquefaciens* and *Pseudomonas fluorescens*, and against *Listeria innocua,* as a surrogate of *Listeria monocytogenes*, through the use of impedometric technique and fluorescence microscopy. It was confirmed the efficacy of this extract at low concentrations (0.20% for MIC and 0.30% for MBC). It was also studied how fermentation and a technological treatment of the brown seaweed *Himanthalia elongata,* influenced the antimicrobial activity in order to study a possible way to enhance this action. It was possible to discover a good fermentation potential by lactic acid bacteria and by *Bacillus subtilis* of the matrix but it was also found out that both the tested treatments (Fermentation and High-pressure processing) have reduced the antimicrobial activity. Probably fermenting bacteria and high pressure have changed the composition of the matrix, reducing the efficiency of the extracts. Further tests on the composition of all the extracts obtained will be performed in order to discover what compounds have proved antimicrobial activity. This extract, as all the other seaweeds extracts obtained, could be used as food preservatives bringing new possibilities for food safety in particular to fight the antimicrobial resistance problem. For that reason, further study will be performed on the use of algae as a source of food preservatives.

In the second chapter was faced the topic of algae and food safety. The consumption of seaweeds and microalgae is exponentially growing also in Europe and for that reason is important to have a clear view of the safety level of these new food sources. Foods based on seaweeds and microalgae are generally sold as ready to eat so it is of particular importance to characterize their microbiota, in particular concerning the presence of food pathogenic bacteria. It was found out that some of the samples tested were contaminated by coliforms and by *Bacillus cereus.* Through a microbiological challenge test, it was found out that *Bacillus cereus* growth was discouraged on seaweed-based refrigerated foods. The results underlined a general safety about the consumption of seaweed-based foods, but the presence of food pathogenic bacteria can't be excluded. It was also addressed the topic of marine toxins. The aim of this work was to study, through an in vitro test, a possible interaction of bacteria (LAB and *Paracoccus sp.)* in different metabolic states with two toxins, Okadaic acid and Tetrodotoxin, to reduce their presence. It was found out that the tested bacteria behave differently but some of the bacteria tested have exerted a good reduction or bonding of the toxins. Other studies will be conducted on this topic in order to study a possible *in vivo* test and to find the reason for these interactions.

As the last topic, it was studied the interactions of lactic acid bacteria and microalgae in different formulations. It was studied how the cyanobacteria *Arthrospira platensis* could work as a booster for lactic acid bacteria for the production of fermented foods. The use of a fermentation booster can significantly reduce the time for food production and consequently enhance the quantity of food produced. The production of a fermented *Arthrospira platensis* food supplement was also evaluated. At the same time, it was studied the change of the volatile aromatic profile consequently to fermentation. Lactic acid bacteria were able to ferment *A. platensis* biomass reaching high concentrations. Consequentially to fermentation, it was found out that the aromatic profile of the biomass was more pleasant and consequently that the production of a fermented *Arthrospira platensis* food supplement was possible.

In the last work, it was studied the lipid reducing activity of fermented microalgae. The zebrafish Nile red fat metabolism assay was performed. Really promising results were discovered. It was found out that fermentation is a good tool to produce lipid reducing compounds. Other studies have to be conducted in order to find out which class of compounds has proved this activity.

To conclude, this thesis has highlighted several roles that microalgae and seaweeds could play in food industries: natural preservatives, food supplements rich in bioactive compounds and fermented food products. Were also evaluated possible safety issues connected to the presence of algae in food.

New food sources and new compounds have to be discovered in order to guarantee food security and food safety for future generations. The involvement of algae in food research is for sure one of the possible solutions to reach that aim.

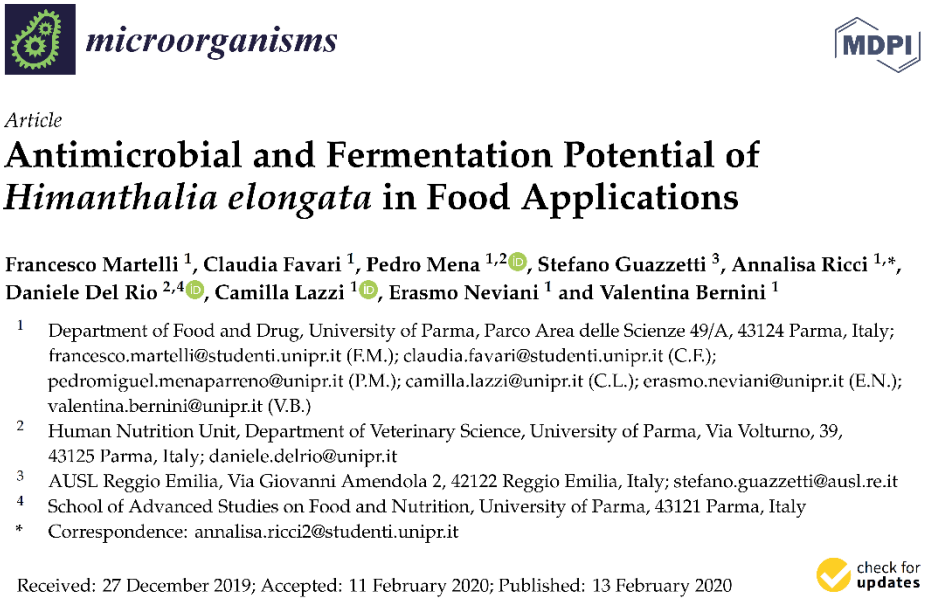
About the author

Francesco Martelli was born on May 4th, 1992 in Montecchio Emilia, Italy. He got his bachelor's degree in Food Science and Technology at the University of Parma discussing the thesis "Monitoring of human salmonellosis by pulsed-field gel electrophoresis (PFGE) from clinical cases, food and animals in Emilia Romagna region". To obtain the data he worked in Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna – Sezione diagnostica di Parma. He persecuted his study with the Master's degree program in Food Science and Technology at the University of Parma discussing the thesis "Effect of pulsed light on in vitro growth of *Listeria innocua* at different environmental conditions". The data to write the thesis were obtained in Universidad Complutense de Madrid, Departamento de Nutricion, Bromatologia y Tecnologia de los Alimentos, under the supervision of Professor Manuela Fernández Álvarez and Professor Gonzalo Doroteo García de Fernando Minguillon. After the Master's degree, he persecuted his study with the Doctoral School in Food Sciences, under the supervision of Professor Valentina Bernini and Professor Erasmo Neviani. During the Ph.D. he has worked on microbiological exploitation of seaweeds and microalgae in the food industry, carrying out also a short-term visit, of three months, at CIIMAR, Interdisciplinary Centre of Marine and Environmental Research, to deepen his knowledge on microalgae and Zebrafish Nile red fat metabolism assay, under the supervision of Professor Ralph Urbatzka and Professor Vitor Vasconcelos. The main results, achieved during the Ph.D., were reported in this thesis.

Scientific Activity

Original Papers

1) Martelli, F., Favari, C., Mena, P., Guazzetti, S., Ricci, A., Del Rio, D., Lazzi, C., Neviani, E. & Bernini, V. (2020). Antimicrobial and Fermentation Potential of Himanthalia elongata in Food Applications. *Microorganisms*, 8(2), 248. https://doi.org/10.3390/microorganisms8020248



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Antimicrobial and Fermentation Potential of *Himanthalia elongata* in Food Applications

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Arthrospira platensis as Natural Fermentation Booster for Milk and Soy Fermented Beverages

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Bacteriostatic or bactericidal? Impedometric measurements to test the antimicrobial activity of *Arthrospira platensis* extract

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4) Ricci, A., Martelli, F., Razzano, R., Cassi, D., Lazzi, C., Neviani, E., & Bernini, V. (2020). Service temperature preservation approach for food safety: Microbiological evaluation of ready meals. *Food Control*, 107297. https://doi.org/10.1016/j.foodcont.2020.107297

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Service temperature preservation approach for food safety: Microbiological evaluation of ready meals

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5) Martelli, F., Bancalari, E., Neviani, E., & Bottari, B. (2020). Novel insights on pink discoloration in cheese: The case of Pecorino Toscano. International Dairy Journal, 111, 104829. https://doi.org/10.1016/j.idairyj.2020.104829

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Short communication

Novel insights on pink discoloration in cheese: The case of Pecorino Toscano

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6) Martelli, F., Cirlini, M., Lazzi, C., Neviani, E., & Bernini, V. (2020). Edible Seaweeds and Spirulina Extracts for Food Application: In Vitro and In Situ Evaluation of Antimicrobial Activity towards Foodborne Pathogenic Bacteria. Foods, 9(10), 1442. **https://doi.org/10.3390/foods9101442**

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Edible Seaweeds and Spirulina Extracts for Food Application: In Vitro and In Situ Evaluation of Antimicrobial Activity towards Foodborne Pathogenic Bacteria

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7) Martelli, F.; Cirlini, M.; Lazzi, C.; Neviani, E.; Bernini, V. Solid-State Fermentation of *Arthrospira platensis* to Implement New Food Products: Evaluation of Stabilization Treatments and Bacterial Growth on the Volatile Fraction. Foods **2021**, 10, 67. https://doi.org/10.3390/foods10010067

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Solid-State Fermentation of *Arthrospira platensis* to Implement New Food Products: Evaluation of Stabilization Treatments and Bacterial Growth on the Volatile Fraction

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8) Ricci, A., Alinovi, M., Martelli, F., Bernini, V., Garofalo, A., Perna, G., Neviani, E., & Mucchetti, G. Heat resistance of *Listeria monocytogenes* in dairy matrices involved in Mozzarella di Bufala Campana PDO cheese. *Frontiers in Microbiology* **2020**, *11*, 3378.

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Heat Resistance of *Listeria monocytogenes* in Dairy Matrices Involved in Mozzarella di Bufala Campana PDO Cheese

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