UNIVERSITÀ
degli STUDI
di CATANIA

Department of Biomedical and Biotechnological Sciences

Ph.D. in Biotechnology

Curriculum in Agri-Food Sciences

XXXVI Cycle

PARIDE SALVATORE OCCHIPINTI

Microbial Resource for Waste Water Treatment in Agriculture and Foods

Ph.D. Thesis

Tutor: *Prof.ssa Cinzia Caggia*

Co-*Tutor:* *Prof.ssa Cinzia Lucia Randazzo*

Coordinator: *Prof. Vito De Pinto*

ACADEMIC YEARS 2020/2023

"*È sapiente solo chi sa di non sapere, non chi s'illude di sapere e ignora così perfino la sua stessa ignoranza*."
Socrate

This PhD project was financed by a grant from the University of Catania and was carried out at the Department of Agriculture, Food and Environment.
Parts of the Ph.D. course was hosted by the company Algreen B.V., at laboratories of Wageningen University & Research (The Netherlands), and by Prof. Antonino Pollio at Algae Biology Laboratory, Department of Biology of the Federico II University of Naples.
PhD project benefited from the funds of European projects: Water4Agrifood and Samothrace.

ABSTRACT

In the latest decades the demand for freshwater is drastically increased due to several reasons, and it is expected to grow due to the growing population, which it is expected to rise by 20–30% by 2050. Hence, massive water bodies as well as land exploiting must be revised to avoid desertification phenomena. The total water consumption is mainly due to agriculture practices (for almost 50%), to industry and to municipalities. In this context, the technologies for wastewaters treatment represent an important strategy to obtain reclaimed water (RW) which could play a key role in the agriculture practices or in food production. The biotechnologies can provide an eco-sustainable solution to obtain larger amount of RW. Bacteria, yeast, mould and microalgae are able to exploit waste, as agri-food by-products, urban as well as industrial wastewaters obtaining RW and simultaneously high-value compounds. In particular, microalgae have a great potential, being able to exploit non-arable land, non-potable water, capturing CO_2 and solar energy useful to combat the green-house gas and global warming problems and thanks their high content of bioactive molecules, microalgae are seen as a potential renewable source and as an effective biological system for treating several varieties of wastewaters.

The present PhD thesis was aimed to explore the application of biotechnologies, as phycoremediation and/or constructed wetland (CV), or industrial technologies, as tangential membrane filtration system, as strategy for RW. Through the research activities reported in the present thesis, the effectiveness of the application of useful microorganisms was evaluated in three types of wastewaters of particular interest, especially in the Mediterranean regions.

The **first chapter** includes two papers concerning one of the most important by product of Mediterranean agri-food industry, the olive mill waste water (OMWW). In the first paper, the OMWW were treated to obtain a new functional beverage with a health–promoting effect, through filtration and/or microfiltration and fermentation, using strains belonging to *Lactiplantibacillus plantarum*, *Candida boidinii* and *Wickerhamomyces anomalus*. In the second paper, the recovery of phenol from OMWW through a tangential membrane filtration system was obtained and the concentrates, characterized by antioxidant activity and antimicrobial effects, were added into a commercial blood orange juice as fortifying. Moreover, the permeates of the filtration system were assess as irrigation water.

In the **second chapter**, urban waste water from a Sicilian farm was treated with a CV system and used to irrigate crops (lettuce and tomatoes) and the effect of such a treatment alone, combined with UV and with different drip lines was evaluated in removing microbial load in RW. Moreover, microbiological traits of irrigated crop and soil samples were detected by culturable and unculturable approaches.

The **third chapter** reports a microalgae literature review to highlight the biotechnological applications of microalgae pointing out on traits of strengths, as well as weakness, of each single application field, as food, feed, nutraceutical, cosmetic, biofuel industry and wastewater treatment. Also the production system was

deeply studied.

The **fourth chapter** reports two cases study upon microalgae-based wastewater treatment for irrigation purposes. In the first case study the treatment was based on an autochthonous Microalgal Pool (MP) and compared to *Chlorella vulgaris* and *Scenedesmus quadricauda* for *Escherichia coli* removal efficacy. In the second case study the wastewater treatment was performed by an autochthonous microalga species, *Klebsormidium* sp. K39, and its effect compared to those of *C. vulgaris* and *S. quadricauda* concerning BOD, COD, nitrogen, phosphorous and *E. coli* removal efficacy.

Lastly, the **fifth chapter** is focused on the poly-extremophile microalga species, *Galdieria sulphuraria*, in order to assess its ability to grow on different buttermilk-based media for biomass producing and for natural pigment accumulation.

KEYWORDS

- **Biotechnology**
- **Water management**
- **Microalgae**
- **Crop irrigation**
- **Wastewater**
- **Reclaimed water**

CHAPTER 1. OLIVE MILL WASTEWATER AS A RESOURCES FOR FOOD INDUSTRIES

Olive mill wastewater fermented with microbial pools as a new potential functional beverage.

Paola Foti[1], **Paride S. Occhipinti[1]**, Nunziatina Russo[1,2] Antonio Scilimati[3], Morena Miciaccia[3]Maria Grazia Perrone[3], Cinzia Caggia[1,2,4]*, Cinzia L. Randazzo[1,2,4], Flora V. Romeo[5]

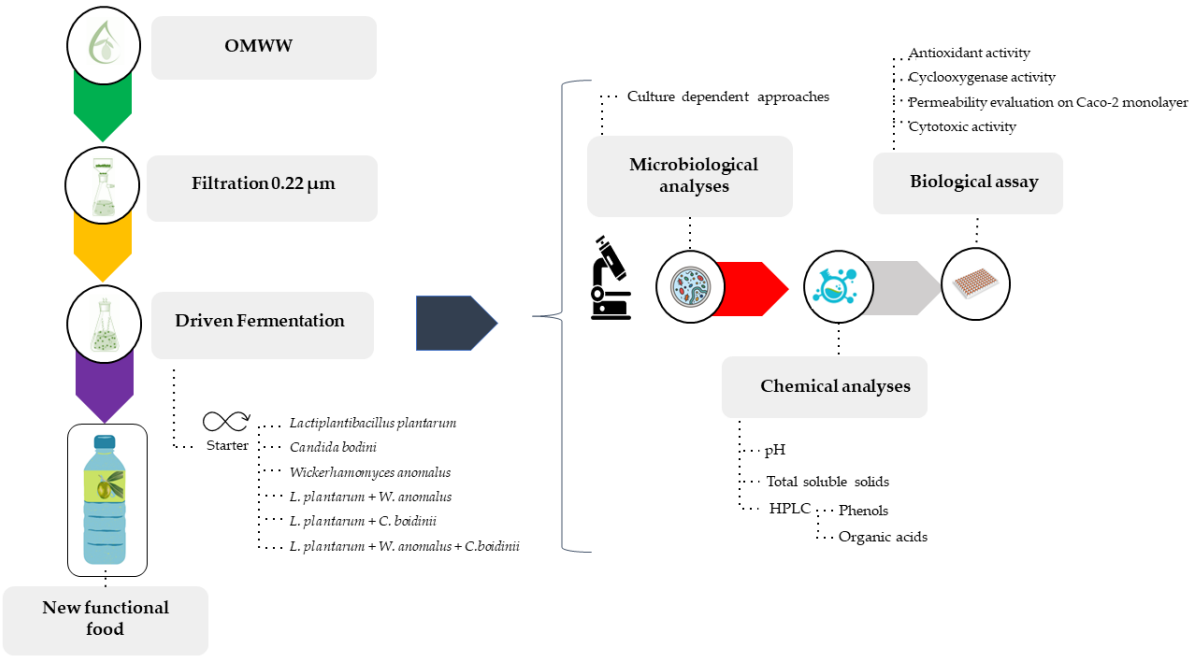
[1] Dipartimento di Agricoltura, Alimentazione e Ambiente - Di3A, Università degli Studidi Catania, Catania (Italy)

[2] ProBioEtna srl, Spin off of University of Catania, via S. Sofia 100, 95123 Catania (Italy)[3] Department of Pharmacy-Pharmaceutical Sciences, University of Bari, Via E. Orabona4, 70125 Bari (Italy)

[4] CERNUT (Interdepartmental Research Centre in Nutraceuticals and Health Products), University of Catania, Via le A. Doria 6, 95125 Catania (Italy)

[5] Centro di ricerca olivicoltura, frutticoltura e agrumicoltura - CREA, Acireale (Italy)

This chapter has been published in Molecules-MPDI, 2023, 28, 646



Abstract

Olive mill wastewater (OMWW) represents a by–product but also a source of biologically active compounds, and their recycling is a relevant strategy to recover income and to reduce environmental impact. The objective of the present study was to obtain a new functional beverage with a health–promoting effect starting from OMWW. Fresh OMWW were pre–treated through filtration and/or microfiltration and subjected to fermentation using strains belonging to *Lactiplantibacillus plantarum*, *Candida boidinii* and *Wickerhamomyces anomalus*. During fermentation, phenolic content and hydroxytyrosol were monitored. Moreover, the biological assay of microfiltered fermented OMWW was detected versus tumor cell lines and as anti-inflammatory activity. The results showed that in microfiltered OMWW, fermentation was successfully conducted, with the lowest pH values reached after 21 days. In addition, in all fermented samples, an increase in phenol and organic acid contents was detected. Particularly, in samples fermented with *L. plantarum* and *C. boidinii* in single and combined cultures, the concentration of hydroxytyrosol reached values of 925.6, 902.5 and 903.5 mg/L, respectively. Moreover, biological assays highlighted that fermentation determines an increase in the antioxidant and anti–inflammatory activity of OMWW. Lastly, an increment in the active permeability on Caco-2 cell line was also revealed. In conclusion, results of the present study confirmed that the process applied here represents an effective strategy to achieve a new functional beverage.

Introduction

Olive oil by-products, while representing a management problem for olive oil companies, may actually represent a source of high value–added compounds that can be used in pharmaceuticals, food, feed and cosmetics for their health properties [1–3]. In fact, olive mill wastewater (OMWW) is a resource rich in phenols including hydroxytyrosol (HT) and tyrosol (TYR), characterized by high antioxidant, anti–inflammatory, antimicrobial and anticarcinogenic activities [4]. The scientific community has proposed several strategies for the valorization of this by–product including solvent extraction techniques, selective resins, membrane filtration or enzymatic applications [5]. These techniques allow the extraction and/or concentration of bioactive compounds in order to increase the nutraceutical component and produce new products or functional ingredients, thus, responding to the demand of consumers who are now aware of the beneficial role that these natural products play in human and animal diets. In the food industry, OMWW have been proposed as an added functional ingredient in meat, dairy, fish, bakery products and juices [6–8]. As a matter of fact, the addition of such phenolic components in food matrices has been shown not only to fulfil a technological function (i.e., to extend the shelf life) but also to improve the health and safety properties of the food. Although the interest of the scientific community in the use of microorganisms in the bioprocessing of agro-industrial waste has grown in recent years [9], only a few microbial applications have been proposed for the valorization of this matrix. Authors have reported that the use of live microorganisms increases the content and bioavailability of the phenolic compounds, and especially of HT and TYR [10,11]. In addition, the driven microbial fermentation provides several advantages by preserving and improving food safety and shelf life due to the formation of organic acids, such as lactic, acetic, formic, propionic acids, etc. [12]. The diversities of acids are dynamic among different alcoholic beverages and fermented food, as are the synergistic effects of abiotic and biotic factors [13]. Functional microorganisms, such as lactic acid bacteria (LAB) and yeasts, are responsible for the metabolism of organic acids. Therefore, the use of selected microorganisms, especially yeasts and LAB isolated from spontaneous similar fermented matrices such as table olives, could represent a low–cost strategy to stabilize and improve the nutraceutical and sensory traits of OMWW. In detail, *Lactiplantibacillus plantarum* strains from fermented olives have been largely associated with the metabolism of phenolic compounds as they can produce degradation enzymes, such as β-glucosidase, esterase, tannase, decarboxylase [14]. Moreover, some of them have been proposed due to their potential probiotic activity [15]. With regard to yeasts, several species show β-glucosidasic, lipasic and esterasic activity and have been used for their ability to improve sensorial profile through production of esters from fatty acids and free fatty acids. Among yeasts, *Candida boidinii* and *Wickerhamomyces anomalus* are the most commonly used as starters [16]. In addition, yeasts isolated from oil matrices, especially strains of *W. anomalus*, have demonstrated several probiotic characteristics, among which the most known is the in vitro cholesterol removal capacity [17]. Today, the functional beverage sector is steadily increasing worldwide thanks to its high nutritional value and the possibility to add flavors. Furthermore, nutraceutical

beverages with added probiotics and prebiotics are of considerable interest to the consumer [18], as this matrix was shown to inhibit proliferation and induce apoptosis in several tumor cells, prevent DNA damage and exert anti–inflammatory activity [19]. The aim of this study was to set up a process to obtain a new functional beverage with a health–promoting effect starting from OMWW. For this purpose, OMWW were pre–treated through filtration and microfiltration and then subjected to fermentation with selected microbial pools, isolated from spontaneously fermented table olives. During fermentation, the biotechnological aptitude of the different strain combinations, their effect on the fermentation parameters, the increase of the phenolic content, especially as HT increase, were evaluated. Furthermore, a biological characterization to evaluate the safety profile and the antioxidant activity was performed on treated OMWW samples. Finally, the ability to cross Caco-2 cell monolayers, as a model of gastrointestinal tract absorption, was performed.

Results

Chemico-physical characterization of sample of different trials

The OMWW belonging to Trial I were monitored at different times (0, 8, and 30 days), through the detection of pH, total soluble solids (TSS), total phenol content and single phenols by HPLC (Table S1). Regarding pH, any significant difference was observed at the beginning of fermentation, and the lowest pH value (4.45) was reached at T8 in sample inoculated with *C. boidinii* in single culture. The TTS at the beginning of fermentation showed values between 7.08 and 8.32, reaching values between 5.60 and 6.34 at T30. During the fermentation, the total phenol content showed, to some extent, a constant trend, reaching the highest concentration at T30 in samples fermented with *W. anomalus* in single culture, with a value of 3241.9 mg/L. The results obtained by HPLC confirmed this increase, as samples treated with *W. anomalus* showed the highest concentration of HT, equal to 2630.4 mg/L. Regarding TYR, an increase during fermentation was observed, reaching, after 30 days, values between 508.6 and 679.4 mg/L in all treated samples. The chemical analyses performed on Trial I were repeated on Trial II (Table S1). The pH decreased during fermentation, showed the lowest values at T8. In detail, all inoculated samples showed a lower pH than the control sample. In particular, the lowest value was found in the samples with *L. plantarum* and *W. anomalus* in single culture, but also in the combination *L. plantarum* and *C. boidinii* and, finally, with the mix of the three strains with values ranging from 3.97 to 3.99. With regard to TSS, the greatest decrease occurred with the combination of *L. plantarum* in association with *W. anomalus*, going from a value of 7.84 at T0 down to 5.50 at T8. At T30, almost all samples maintained the value showed at T8 of fermentation. In addition, total phenols at T8 and T30, in all inoculated samples, showed a higher content over time compared with the control sample. In detail, the samples with significantly higher phenolic content were the three–strain association (3379.5 mg/L) and *W. anomalus* in single combination (3261.9 mg/L) at T8, while at T30 was the sample inoculated with *L. plantarum* with a value of 3577.6 mg/L. The results obtained by HPLC showed a decrease at T8 of HT in

all samples except in the samples with *L. plantarum* where there was an increase of 115 mg/L of HT, and the sample inoculated with *L. plantarum* with *W. anomalus* which showed an increase equal to 262 mg/L of HT. All samples inoculated up to T30 had higher HT content. Opposingly, the TYR decreased during fermentation from an average range of values from 319.7 mg/L to 136 mg/L. In Trial III, microfiltration resulted in a clear and sterile matrix. Before starting the final fermentation, a preliminary test was carried out in a reduced volume (100 mL) to ascertain if any difference could be revealed between trials with the addition of glucose, peptone and yeast extract (added at the same concentrations) and the trials without any additions. The results showed the same pH values and cell density during fermentation. Moreover, the addition of these compounds made the OMWW turbid (data not shown). For these reasons, to improve the acceptability of the product to consumers, the thesis without additions was chosen for the final test. During fermentation, pH, TSS and total phenol content were monitored (Table 1). Regarding pH, no significant difference was found at the beginning of fermentation. The pH at T0 was in a range of 5.12 and 5.19. Fermentation stopped at T21 for all samples examined. The end of fermentation was revealed by the stabilization of the pH value that was evaluated every three days of fermentation (data not shown). In particular, the samples inoculated with *W. anomalus* in single culture and in association with *L. plantarum* reached a pH value of 4.54 and 4.49 at T21, respectively. In addition, although slower than the previous theses, the theses containing *L. plantarum* and *C. boidinii* in single culture also reached at T21 a pH of 4.65 and 4.60, respectively. Total soluble solids showed no significant difference at any of the fermentation times. Initial values ranged from 8.30 to 10.85 °Brix, while values between 5.32 and 8.17 °Brix were reached at the end of fermentation. The sample used as a control during fermentation maintained its pH and TSS values. Regarding the content of total phenols, the highest values at the beginning of fermentation were found in the sample containing the *L. plantarum* and *W. anomalus* combination, a value that decreased during the fermentation process. In contrast, the sample with the three–strains combination showed an increase in total phenol content up to T14 with a value of 4015 mg/L, and then decreased at T21 reaching a value of 1543 mg/L.

Table 1. Chemical parameters detected in samples of trial III.

Sample	Time	pH	TSS (°Brix)	Total phenol (mg/L)
Control		5.18 ± 0.01	8.30 ± 0.77	3627.4 ± 0.54^{c}
L. plantarum		5.16 ± 0.01	10.60 ± 0.78	3711.2 ± 4.89^{b}
C. boidinii		5.18 ± 0.01	10.28 ± 0.70	3539.8 ± 0.54^{d}
W. anomalus		5.12 ± 0.08	9.76 ± 1.53	3172.9 ± 1.63^{f}
L.p+ W.a		5.13 ± 0.06	8.56 ± 0.80	4135.0 ± 4.89^{a}
L.p+C.b		5.19 ± 0.02	10.04 ± 1.13	3474.7 ± 1.09^{e}
L.p+W.a+C.b		5.18 ± 0.01	8.88 ± 1.39	2967.1 ± 2.18^{g}
		n.s	n.s	**
Control		5.17 ± 0.01^{a}	8.30 ± 0.78	1985.8 ± 3.26^{g}
L. plantarum		5.04 ± 0.03^{b}	10.30 ± 0.98	3032.5 ± 2.18^{b}
C. boidinii		4.97 ± 0. 01^{bcd}	8.88 ± 2.18	3020.6 ± 0.54^{c}
W. anomalus		4.87 ± 0.02^{e}	9.14 ± 1.77	2395.3 ± 1.63^{f}
L.p+ W.a		4.88 ± 0.04^{de}	7.52 ± 1.17	2897.2 ± 0.01^{e}
L.p+C.b		5.00 ± 0.01^{bc}	9.50 ± 0.32	2991.0 ± 5.44^{d}
L.p+W.a+C.b		4.94 ± 0.01^{cde}	8.24 ± 1.29	3268.2 ± 1.63^{a}
		**	n.s	**
Control	14	5.18 ± 0.02^{a}	8.30 ± 0.78	1809.5 ± 0.54^{f}
L. plantarum	14	4.68 ± 0.01^{cd}	9.90 ± 0.99	3282.9 ± 0.54^{c}
C. boidinii	14	4.77 ± 0.01^{b}	7.99 ± 2.82	2443.8 ± 1.63^{d}
W. anomalus	14	4.67 ± 0.04^{cd}	8.28 ± 1.44	3539.7 ± 0.54^{b}
L.p+ W.a	14	4.62 ± 0.09^{d}	6.64 ± 1.52	2199.2 ± 3.81^{e}
L.p+C.b	14	4.89 ± 0.01^{b}	9.03 ± 1.12	3545.1 ± 53.84^{b}
L.p+W.a+C.b	14	4.82 ± 0.02^{bc}	7.62 ± 1.36	4015.0 ± 2.72^{a}
		**	n.s	**
Control	21	5.19 ± 0.01^{a}	8.20 ± 0.61	1009.4 ± 0.54^{f}
L. plantarum	21	4.65 ± 0.03^{c}	8.15 ± 1.20	3392.1 ± 0.54^{a}
C. boidinii	21	4.60 ± 0.01^{d}	5.60 ± 0.57	3005.2 ± 0.54^{b}
W. anomalus	21	4.54 ± 0.04^{e}	6.36 ± 0.37	2394.2 ± 1.09^{c}
L.p+W.a	21	4.49 ± 0.01^{de}	5.32 ± 0.04	1914.6 ± 1.63^{d}
L.p+C.b	21	4.84 ±0.02^{b}	8.00 ± 1.41	3403.2 ± 10.88^{a}
L.p+W.a+C.b	21	4.82 ± 0.01^{b}	5.99 ± 0.01	1543.6 ± 1.09^{e}

Data are expressed as mean ± standard deviations. Mean values with different letters within the same column at the same time interval are statistically different. N.s. not significant; **Significance at P< 0.01.

Microbiological analyses

Results on microbiological analyses (Table S2) are referred at the same sampling times reported for chemical analyses. Overall, for samples of Trial I, high microbial densities were detected for aerobic mesophilic bacteria, enterobacteria, and yeast in all sampling times. Regarding LAB, an increase of 1 Log unit at T8 was detected, and the values were quite constant until T30, with some exceptions. In detail, at the beginning of fermentation, the sample treated with *L. plantarum* showed a significantly higher cell density, with a value of 5.85 log CFU/mL, whilst at T30 the highest LAB densities were detected in samples treated with the combination of the three strains, namely of *L. plantarum* and *C. boidinii, W. anomalus* in samples inoculated with *W. anomalus* in single culture, and in samples treated with *L. plantarum* and *C. boidinii* in mixed cultures. Yeasts and molds also showed a similar trend in all samples. In fact, cell density increased at T8 of fermentation and then decreased at T30, when an average value of 6.45 Log CFU/mL were detected. Aerobic mesophilic bacteria counts showed only a slight variation during fermentation, reaching a final mean value of 6.25 Log CFU/mL, whereas Enterobacteriaceae and Staphylococci showed a significant decrease during fermentation. At the beginning of fermentation, the latest microbial groups showed an initial average value of 6.63 and 3.05 Log CFU/mL, respectively. These values decreased significantly during fermentation in the inoculated samples, reaching values under the detection limit. In Trial II, the LAB and yeast counts increased during fermentation (Table S2). In detail, the LAB mean value starting from 4.27 Log CFU/mL reached, after 30 days, a mean value of 7.34 Log CFU/mL, whilst in samples inoculated with W. anomalus it reached the lowest cell density. A similar trend was observed for yeasts that at the 30th day exhibited a mean cell density of 9 Log CFU/mL in the sample inoculated with *L. plantarum* and *C. boidinii* in mixed culture. Aerobic mesophilic bacteria were found at high density, until the end of fermentation when a final average value of 7.52 Log CFU/mL was counted. Different trends were observed for Enterobacteriaceae and Staphylococci, for which after a slight increase a significant decrease was detected after 30 days in all samples. Regarding Trial III, before starting fermentation, the microfiltered OMWWs were subjected to microbiological analyses to confirm the achieved sterility. The following microbial groups were searched: LAB, yeasts, Staphylococci, total mesophilic aerobic bacteria, Enterobacteriaceae and *Clostridium perfringens*. All used media and conditions are reported in Section 4. Once the OMWWs were analyzed, the selected strains were inoculated at a cell density of 108 and 107 CFU/mL for L. plantarum and yeasts, respectively. As shown in Figure 1, a different growth pattern between the two yeasts and the LAB strains was observed during fermentation. In fact, while in all inoculated samples LAB showed an initial decrease, during the first 14 days they increased until the 21st day; the yeasts increased their cell density during the first 18 days, when they reached values between 7.03 and 7.78 Log CFU/mL**.**

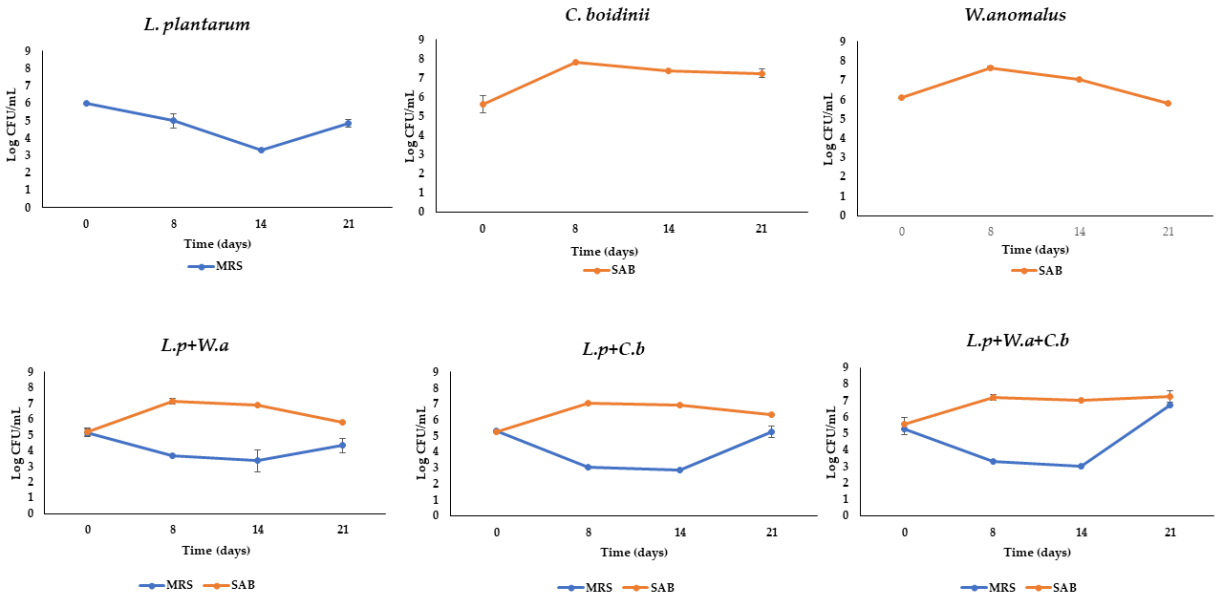


Figure 1. Microbial counts detected in MRS and SAB during fermentation in microfiltered OMWW differently inoculated. Data are expressed as means of Log CFU/mL ± standard deviation.

Phenol and organic acid detection

Regarding phenolic content, HT and TYR were the main detected compounds, found at high concentration by HPLC during fermentation (Figure 2). As for HT, at the beginning of fermentation a concentration between 341.7 and 469.1 mg/L was found. At the end of fermentation, an exponential increase of HT in all inoculated samples was observed. Particularly in the samples treated with *L. plantarum* and *C. boidinii* in single and in combined cultures, the HT concentration was found as 925.6, 902.5 and 903.5 mg/L, respectively. A slowly increase in concentration of TYR was observed during fermentation, reaching values between 315.6 and 544.7 mg/L in all inoculated samples. In contrast, the control samples showed a significant decrease in HT along fermentation, reaching values of 170.6 mg/L and a slight increase in TYR, reaching final value of 303.7 mg/L**.**

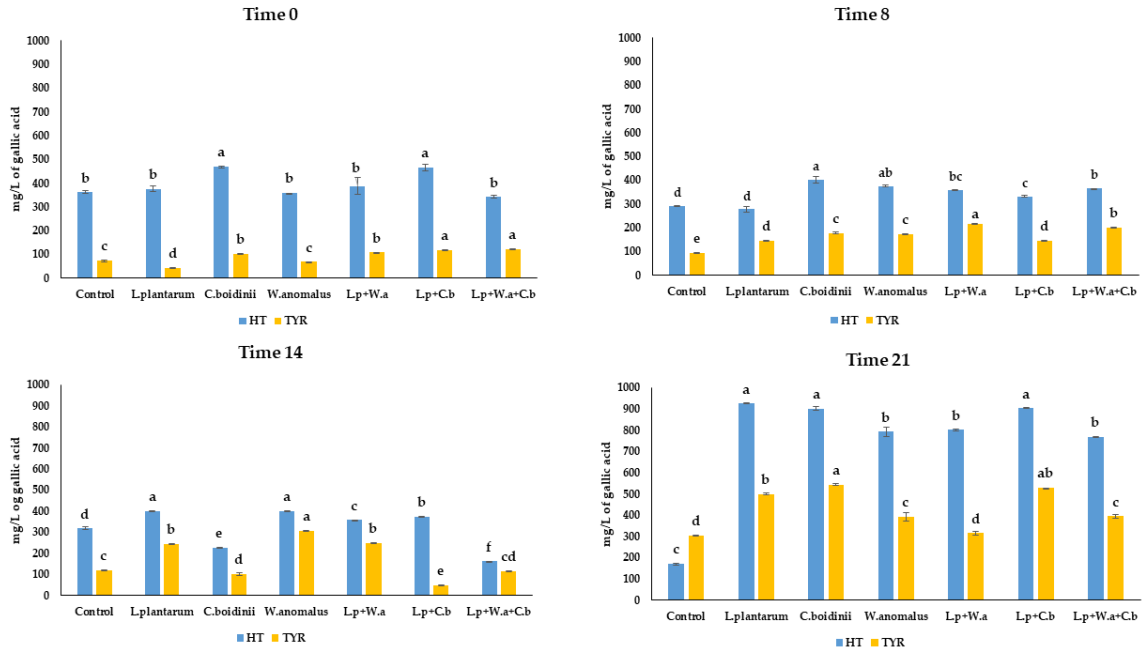


Figure 2. Concentration of hydroxytyrosol (HT) and tyrosol (TYR) during fermentation in microfiltered OMWW differently inoculated. Different letters indicate statistical differences within the columns for the same compound (significance at P≤0.01).

In addition, organic acids were evaluated at the end of fermentation in all samples. The control sample, at the beginning of fermentation, was used as an initial control (Table 2). The control, analyzed at both the beginning and at the end of fermentation, showed a constant value of acids except for butyric, for which a concentration of 566.4 mg/L was detected only at T21. For all samples inoculated with the different microbial combinations, on the other hand, an acid increase during fermentation was observed, except for isobutyric acid that decreased in sample inoculated with *W. anomalus* and in all the inoculated combinations. In detail, the sample inoculated with *L. plantarum* showed the highest increase for all the detected acids.

Table 4. Organic acids (mg/L) detected by HPLC

Sample	Time days	Citric acid	Lactic acid	Acetic acid	Propionic acid	Isobutyric acid	Butyric acid
Control	0	4172.9 ± 96.54	1606.6 ± 99.00	416.8 ± 97.31	3865.9 ± 268.47	3136.9 ± 188.31	0.00 ± 0.00
Control	21	4529.3 ± 100.00^{de}	1219.2 ± 18.03^{f}	326.3 ± 78.89^{e}	3743.1 ± 34.21^{g}	1654.9 ± 15.21^{d}	566.4 ±48.79^{d}
L. plantarum	21	7033.4 ± 15.76^{a}	4512.6 ± 18.07^{a}	7212.8 ± 82.59^{a}	9802.4 ± 12.82^{a}	3235.3 ± 5.51^{a}	4666.4 ± 103.03^{a}
C. boidinii	21	6624.4 ± 87.69^{b}	4123.3 ± 20.03^{b}	4568.4 ± 58.78^{c}	9153.8 ± 19.41^{b}	3202.7 ± 27.72^{a}	4393.3 ± 44.23^{a}
W. anomalus	21	5214.4 ± 121.00^{c}	3774.5 ± 99.00^{c}	6214.7 ± 168.83^{b}	8219.2 ± 41.95^{c}	2072.0 ± 77.52^{b}	4239.4 ± 176.96^{a}
L.p+ W.a	21	4126.9 ± 106.79^{f}	2846.0 ± 35.53^{e}	6188.4 ± 85.52^{b}	6831.2 ± 10.08^{f}	1626.7 ± 1.41^{e}	3682.8 ± 26.00^{b}
L.p+C.b	21	4744.1 ± 16.31^{d}	3167.5 ± 33.49^{d}	4366.7 ± 132.82^{c}	7913.0 ± 24.25^{d}	2096.8 ± 16.35^{b}	2995.9 ± 54.51^{c}
L.p+W.a+C.b	21	4381.7 ± 20.88^{ef}	3075.3 ± 31.95^{d}	3334.0 ± 8.10^{d}	7342.0 ± 116.15^{e}	1810.2 ± 5.28^{c}	3550.1 ± 25.58^{b}

Data are expressed as mg/L of means ± standard deviations. Different letters indicate statistical differences within the same column (Significance at p≤0.01).

Biological assay

Cell culture and transepithelial transport of samples through Caco-2 cell monolayers

The intestinal permeability values, estimated with the Caco-2 cell experimental model, correlate well with human in vivo absorption data for many drugs and chemicals. Caco-2 cells are a human colon epithelial cancer cell line that, when cultured as a monolayer, differentiate to form tight junctions between cells to serve as a model of paracellular movement of compounds across the monolayer. The monolayer represents the human intestinal epithelial cell barrier and by this assay, the measured endpoint is intestinal permeability (expressed as apparent permeability—Papp value) (Table 3).

Table 3. Apparent permeability of different sample of OMWW.

Samples	Concentration of HT (mg/L)	Papp BA (nm/s) Passive transport	Papp AB (nm/s) Active transport	BA/AB	λ (nm)	ε
Control	1.70	2581	457	4.22	275	0.80
L.planturum	9.50	4015	1014	3.95	285	0.20
C. boidinii	9.00	2540	575	4.41	275	0.09
W. anomalus	7.90	1958	367	5.34	285	0.22
L.p + W.a	8.00	1905	335	5.67	284	0.19
L.p + C.b	9.00	2912	1125	2.58	275	0.19
L.p + W.a + C.b	7.70	2587	522	4.95	283	0.20

All samples were tested at a dilution of 1:100. In the table BA indicates basolateral to apical transport; AB indicates apical to basolateral transport; BA/AB values are from Papp AP−BL/Papp BL−AP.

The flux from the apical part of the monolayer to the basolateral side (BA) is referred to the passive transport, while the measurement of active transport is obtained by measuring the reversed flow (AB), since Caco-2 cells express efflux pumps in the apical side. The smaller the BA/AB ratio value, the greater the contribution of the active transport to the membrane crossing. In all tested samples, the contribution of active transport to the membrane crossing was always lower than that due to passive diffusion, as demonstrated by high values of Papp AB. This occurs mainly for samples inoculated with *L. plantarum* and *C. boidinii*, which showed the highest value (as 1125 nm/s). This value could be related to a synergic effect between the LAB and *the C. boidinii* strains, that also in single cultures showed Papp values of 1014 and 575 nm/s, respectively. This result is confirmed by the lowest BA/AB value (2.58), detected in samples fermented with *L. plantarum* and *C. boidinii*. OMWW samples and HT pure (used as a control) at the opportune dilution (1:25) have been evaluated on different cell lines, normal (HepG2) and tumoral (Caco-2), in order to evaluate their toxicological profile [20]. Since no cytotoxic effect was detected (data not shown), they resulted to be safe at a dilution of 1:25, while with higher concentrations (as such and 1:10) a cytotoxic effect was registered. These results are in agreement with data reported by Di Mauro et al. [21], confirming that the use of higher concentrations (as such and 1:10) induced a reduction in cell viability in a dose–dependent manner, while lower concentrations did not affect cell viability.

Activity on COX-1 and COX-2

COX, also called Prostaglandin H synthase (PGHS), is a key enzyme in the inflammatory cascade. It catalyzes the conversion of arachidonic acid (AA) in prostanoids, bioactive lipids mediating numerous physiological and pathological processes in the body. Prostanoids include thromboxane A2 (TXA2), prostaglandins (PGD2, PGE2, PGF2α) and prostacyclin (PGI2). Two COX isoforms are known, COX-1 and COX-2, encoded by different genes. The two isoforms show 60% homology in their amino acid sequence. COX-1 is the isoform constitutively expressed in most tissues and responsible for maintaining normal physiological functions such as gastric protection, modulation of platelet function, and renal homeostasis. COX-2, differently from COX-1, is the inducible isoform upon pro–inflammatory stimuli. The possibility of finding anti–inflammatory properties in nutraceutical compounds would make the products under study extremely interesting, thus, the OMWW fermented sample inhibition of ovine COX-1 (oCOX-1) and human COX-2 (hCOX-2) enzyme activity was investigated and HT was used as positive control. Pure HT showed, at a concentration of 40 mg/L, inhibition activity on oCOX-1 and hCOX- 2 with a percentage of 6.41 and 26.11, respectively (Figure S1). The control OMWW sample did not show any anti–inflammatory activity, while low anti–inflammatory activity was found for the different OMWW samples. In detail, samples fermented with *L. plantarum, C. boidinii* and *W. anomalus* in single culture showed a moderate inhibitory activity towards both isoforms (Table S3). In particular, the sample inoculated with L. plantarum in single culture showed a selective inhibition of oCOX-1, whereas samples treated with *C. boidinii* showed an inhibition towards both oCOX-1 and hCOX-2 with the percentage of inhibition reaching 15.96% and 12.95%, respectively. In addition, the sample inoculated with a combo of *L. plantarum* and *C. boidinii* preserves a selective inhibition towards oCOX-1, with an inhibition of 8.20%. It could be hypothesized that *C. boidinii* produces some metabolites with a greater affinity and selectivity towards oCOX-1 isoform.

Antioxidant Activity

Diabetes, cardiovascular diseases, arthritis and joint diseases, allergies and chronic obstructive pulmonary diseases are classified, according to the World Health Organization, as specific inflammation–mediated chronic diseases. The processes underlying these diseases are many, but oxidative stress is undoubtedly involved in their pathogenesis and in the development and establishment of a sustained inflammatory state. All selected samples were evaluated for their antioxidant activity by measuring their reactivity with 1,1-diphenyl-2-picrylidrazyl (DPPH), a purple–colored stable radical that strongly absorbs at $\lambda = 517$ nm, in order to determine their efficacy as scavengers of stable free radicals. Testing was carried out to compare the effect of fermented OMWW samples with the known antioxidant activity of HT (Figure 3). The data showed that the OMWW control exhibited lower antioxidant capacity, at all dilutions tested, compared with both that exerted by HT and fermented samples. In particular, at the lower tested volume (12.5 μL), the best antioxidant activity was obtained in the sample inoculated with *C. boidinii*, reaching a % RSA value higher than pure

HT. The same behavior was observed for samples inoculated with L. plantarum and W. anomalus, in single culture.

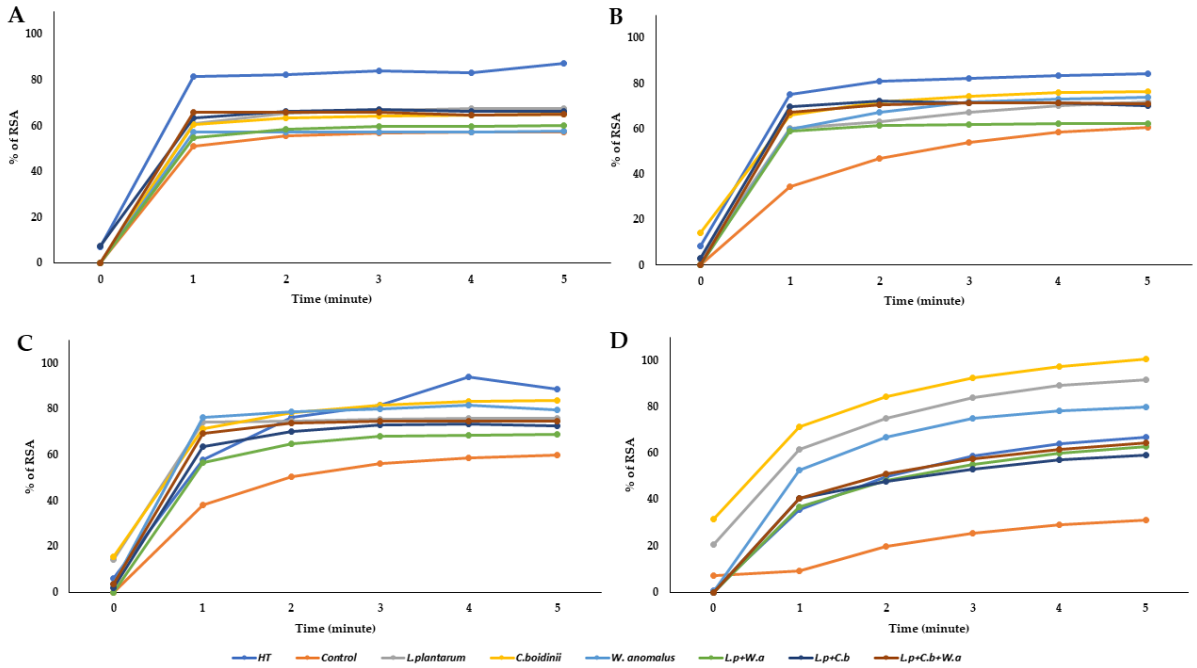


Figure 4. Evaluation of antioxidant action expressed as % RSA. Each graph corresponds to a volume (µL) used for each sample: A) 50 µL of samples; B) 37.50 µL of samples; C) 25 µL of samples; D) 12.5 µL of sample.

Discussion

Fermentation is widely considered a low–cost strategy to recovery and valorize agro-industrial by–products [22]. In this study, in order to obtain a suitable matrix to be fermented with selected microbial pools, different Trials were set up. For this purpose, fresh OMWWs were collected at two successive seasonal years. Samples obtained from Trial I, untreated fresh OMWW, appeared very turbid and rich in unwanted solids at both the beginning and end of fermentation. Therefore, in Trial II, the OMWWs were subjected to on farm filtration using carton filters with different porosity. To date, such a technique is used to remove unwanted solid components from the matrix, maintaining the nutritional compounds as phenolic fraction (Figure S2) [23]. Results of Trial II showed that although the OMWWs (filtered through cardboard filters) visually appeared as clear from a physical point of view, at both the beginning and end of fermentation, they were not microbiologically suitable, in relation to the high total aerobic mesophilic bacteria densities. According to the European Regulation (EC) No. 1441/2007, the absence of pathogens, such as *Salmonella* spp. and *L. monocytogenes*, is considered an essential criterion for the microbiological safety of vegetable products, while no mandatory microbiological criterion is fixed for total aerobic mesophilic bacterial count. However, some guidelines include *Escherichia coli* and total aerobic mesophilic count as quality parameters, fixing the following thresholds (as CFU/g): *E. coli* < 10 for satisfactory; between 10 and $\leq 10^2$ for acceptable; and $>10^2$ as not acceptable [24]. The same authors, for total aerobic mesophilic count, proposed the following thresholds: $\leq 10^4$ for satisfactory, between 10^4 and lower or the same of 10^6 for acceptable, and $>10^6$ not acceptable, respectively [24]. Therefore, OMWW obtained through the last cardboard filter, with a porosity

between 0.20 and 0.40 μm, were afterwards subjected to microfiltration (0.22 μm) in the laboratory. This procedure resulted in a microbiologically sterile, clear matrix mainly composed of phenols (Figure S3). To date, the microfiltration technique is successfully applied in food industries, such as the dairy industry, as it induces an improvement in the microbial quality of the final product [25]. In the present study, the application of such a strategy allowed the evaluation of the biotechnological aptitude of the strains, used as single or mixed cultures, and enabled an understanding of how they interact with the matrix. The results showed that the use of microbial starters drove fermentation by lowering the pH to values as low as 4.49 and inducing an increase in the phenolic compounds. In detail, the combinations of *L. plantarum* and *C. boidinii*, both in single and in mixed cultures, resulted, at the end of fermentation, in the highest HT content, with values of 925.6, 902.5 and 903.5 mg/L, respectively. No oleuropein was detected at any sampling time as found by other authors [5,26]. Although *L. plantarum* is mainly known for its β-glucosidase activity or its probiotic potential [27], in all the tests carried out, there was a slight decrease of LAB count in sample with *L. plantarum* that showed an increase only after t14 of fermentation (Figure 1). This suggests that these strains are able to utilize certain metabolic pathways to survive in difficult matrices, which is why there is an increased activity in the last sampling time. An interesting study that may explain the adaptation of *L. plantarum* is proposed by Reveròn et al. [28], who propose a study of transcriptomics and the mechanism of action of *L. plantarum* in response to treatment with pure HT. *C. boidinii* strain used as a potentially resistant strain to several hurdles present in the matrix. Recently, De Melo Pereira et al. [29] reported that the genus *Candida* is commonly found in many fermented foods and beverages obtained by the main types of fermentation (alkaline, alcoholic, acetic, lactic, and mixed processes). In addition to its ubiquitous trait, the *Candida* genus also possesses a complex metabolic mechanism that allows it to survive, compete, and sometimes dominate fermentation processes [30]. Furthermore, it is known that a selected culture, besides the ability to control the fermentation process, should show the ability to survive in the fermentation environment and to exert acidifying activity through the production of organic acids. In the present study, results highlighted that *L. plantarum* inoculated samples exhibited the highest values of all detected acids. In a functional beverage, organic acids can play an additional role in protecting phenols, such as HT and TYR, from oxidation. In addition, different studies revealed that a lactic acid concentration of 0.5% (v/v) produced by LAB prevents pathogens' growth, such as *Salmonella* species, *Escherichia coli*, and *Listeria monocytogenes* [31,32]. This result confirmed results previously reported, namely, that the fermentation driven by LAB leads to the production of mono–, di–, and tri– carboxylic acids, i.e., acetic, lactic, and propionic acids as intermediaries of biosynthetic metabolic pathways and amino acid metabolism. In detail, Okoye et al. [33] demonstrated through genome study that LAB contain unique and shared secondary metabolite biosynthetic gene clusters with bio preservative potential and a transcription factor, namely CRP (cyclic AMP receptor protein) endowed with novel binding sites involved in organic acid metabolism. Zooming in on biological activity, results obtained from tested microfiltered fermented OMWW and from pure HT, when tested at a 1:25 dilution, were found to be safe on chosen cell lines. In the present study, the

choice of cell lines was based on taking into consideration that the HepG2 is one of the most reliable experimental models for prediction human liver toxicity. Indeed, the liver is responsible for most of the orally administered xenobiotic metabolism, for its anatomical proximity to the gastrointestinal tract and for its histological structure [34], whereas the Caco-2 cell line has been chosen as the most suitable in vitro model to rapidly assess the intestinal permeability and for xenobiotic transport studies [35,36]. Caco-2 cells exhibit a well–differentiated brush border on the apical surface and tight junctions, and express typical small–intestinal microvillus hydrolases and nutrient transporters. The crossing of biological membranes must be taken into account because it correlates with the ability of a pharmacologically active compound to reach the target site where performing the biological function. The intestinal transport of polyphenols seems to be strongly influenced by several factors such as food matrix, biotransformation and conjugation that occur during absorption [37,38]. Many studies have focused on the uptake of individual phenols, such as HT and TYR, which have shown good absorption across the cell membrane, while the uptake of a phytocomplex and how its different composition may affect the transport mechanism has been less explored [39]. In a recent study, Bartolomei et al. [40], demonstrated that a phenolic pool, extracted from extra virgin olive oil (EVOO), induced a protective effect against H2O2 induced oxidative stress on Caco-2 and HepG2 cell lines. This observation demonstrated a selective transepithelial transport of certain oleuropein derivatives by Caco-2 cells, confirming that the phytocomplex could be transported with different mechanisms than those involved for single phenolic compounds, separately tested. According to results previously reported both phytocomplex composition and used starter cultures can significantly influence cell membrane crossing. In the present study, microbial cultures differently modulated the response of anti–inflammatory and antioxidant activity. It has been widely reported that phenols contained in EVOO reduce the reactive oxygen species (ROS) and malondialdehyde production, the nitric oxide release and the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) [41]. Results obtained in the present study confirmed that OMWW samples affected the inhibitory activity towards COX-1 and COX-2, by a modulation of COX-2, according to previous in vitro reports on human monocytes [42]. The same authors demonstrated that HT attenuated ROS–mediated COX-2 transcription induced by bacterial lipopolysaccharide (LPS). COX catalyzes the first step in the biosynthesis of prostaglandins (PG), prostacyclin and thromboxane starting from free arachidonic acid (AA) [43]. Among prostaglandins, PGE2 is involved in inflammation, angiogenesis and in promoting the growth of several solid tumors, such as breast, ovarian, head and neck cancer, renal cell carcinoma and hematological cancers [44–46]. COX-1 and COX-2 are of great interest because they are targets of non–steroidal anti–inflammatory drugs (NSAIDs), which, when binding to the active site of COX, prevent the AA from reaching the catalytic pocket and, thus, the biosynthesis of prostaglandins. COX inhibition is therefore important in reducing the inflammatory response, tumorigenesis and cancer progression. Many of the recognized anti–cancer properties of HT are related to other activities, such as ability to modulate the antioxidant system and ROS scavenge [47,48]. Ramirez–Tortosa et al. [49] demonstrated that a supplementation with HT (15 mg/day) is effective into downregulate several

transcriptional factors, as described for other antioxidant agents, able to induce, at plasma level, a decrease of metalloproteinase in women with breast cancer. HT, as reported by the European Food Safety Authority but in general the phytocomplex present both in olive oil and in by–products, has a beneficial effect on human health. The interaction between phenols and microorganisms used as starters plays a key role in understanding the mechanism of action and how they can modulate the anti–inflammatory and antioxidant response in the development of degenerative diseases [50].

Materials and Methods

OMWW sampling

The OMWW samples used in the present study were obtained by a three–phase olive oil extraction system at the Consoli oil company (Adrano, Italy) and collected during a two–year period. In detail, for Trial I OMWW was collected in the 2019–2020 season and for Trials II and III OMWW samples were collected in the 2020–2021 season. All the Trials are described in Figure 3. For Trial I the fresh produced OMWW was immediately stored at −20 °C at the Di3 A, University of Catania.

For Trial II, the OMWWs were stored at room temperature in the company facilities, until further treatments. To obtain a clear matrix, OMWW samples were subjected to filtration using Oenopad® XF1, XF7 and XFSS filters (OENO S.R.L., Erbusco, Italy) suitable for food matrices and consisting of cellulose, diatomaceous earth and perlite. Different fractions were obtained: the as is sample (prefiltered or PF sample); and the three fractions (F1, F2, F3) obtained by sequential filtration with filters at different porosity, as: the "XF1" filter (8.0–20 µm) to eliminate solid particulates; the "XF7" filter (2.0–4.0 µm) for clarifying step; the "XFSS" filter (0.20–0.40 µm) for final sterilization. All fractions were collected and stored at −20 ◦C.

In addition, the Trial III was obtained from the F3 sample, in turn obtained from Trial II,
by microfiltration using the Sartoclear Dynamics® kit (Sartorius, Varedo, Italy), connected to a vacuum pump. The latest process allows both the clarification/filtration and cold sterilization in a single step, as the used bottle presented a 0.22-µm polyethersulfone (PES) filter membrane. After processing samples needed for subsequent tests were frozen at −20 °C.

Set-up of fermentation process

In order to set up the fermentation process some components, such as yeast extract at 1% (w/v), peptone and glucose at 2% (w/v), were added into the fresh OMWW samples and to the F3 samples right before fermentation. All components were purchased from Liofilchem (Roseto degli Abruzzi, Italy). The fermentation process was started through the inoculum of microbial pools, consisting of yeast and lactic acid bacteria strains, belonging to the microbial culture collection of the Department of Agricultural, Food and Environmental Sciences (Di3 A) and to ProBioEtna srl, Spin off of University of Catania. In details, the

Candida boidinii F3 30.1, *Wickerhamomyces anomalus* F5 60.5 and *Lactiplantibacillus plantarum* F 3.5 (DSM 34190) strains were used. All the strains were previously isolated from naturally fermented table olives [51]. One hundred microliters of each yeast inoculum and *L. plantarum* were spotted in Yeast Peptone Dextrose broth (YPD, Sigma-Aldrich, Milano, Italy) and de Man, Rogosa, and Sharpe broth (MRS, Oxoid, UK) and allowed to incubate overnight at a selective temperature of 25 °C and 32 °C, respectively. Then, the strains were inoculated at 0.5%, which corresponded to an initial cell density of 107 CFU/mL for yeasts and 108 CFU/mL for *L. plantarum* (Figure 4). Seven experimental samples were set up for each Trial: the un–inoculated samples (controls); three single culture inoculated samples; two samples inoculated with each yeast strain in mixed culture with the *L. plantarum* strain; one three–strain mixed culture sample. All tests were conducted in triplicate in an OMWW total volume of 400 mL. For Trial I and II, the fermentation process was monitored at regular intervals: at T0 (after about 7 h of microbial inoculation); T8 (after 8 days of fermentation); T30 (after 30 days of fermentation). For Trial III, fermentation parameters were monitored at T0, T8, T14 (after 14 days of fermentation) and T21 (end of fermentation). All fermentations were carried out at room temperature (20 ± 4 °C).

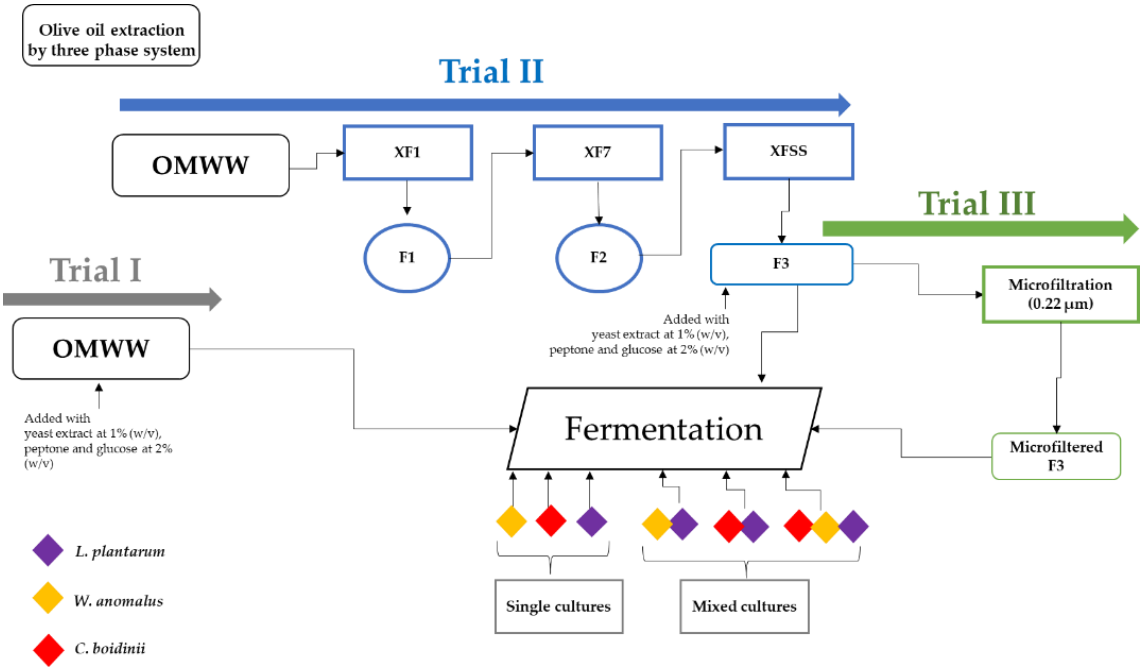


Figure 4. Fermentation process and OMWW obtained trials

Chemical analysis

The pH, the TSS and the total phenol content were monitored for all samples during fermentation. The pH was measured with a Mettler DL25 pH meter (Mettler–Toledo International Inc., Columbus, OH, USA) and the total soluble solids (TSS), expressed as °Brix, were measured using a refractometer (Atago, RX-5000, Milano, Italy). In addition, the total phenolic content was determined according to the Folin–Ciocalteu's colorimetric method (FC). The tested samples were mixed with 5 mL of commercial FC reagent (Labochimica, Campodarsego, Italy) diluted with water (1:10 v/v) and added with 4 mL of a 7.5% sodium

carbonate solution. Subsequently, samples were left in the dark at room temperature. After 2 h, the absorbance was measured spectrophotometrically at 765 nm (Cary 100 Scan UV-Visible, Agilent, CA, USA). The total phenolic content was expressed as mg gallic acid equivalent (GAE)/L of sample).

Microbiological analyses

Samples of Trial I, II and III were serially diluted and poured into agar plates containing specific media and incubated under specific conditions: de Man, Rogosa, and Sharpe Agar (MRSA, Oxoid, Milano, Italy) for lactic acid bacteria counts, incubated at 32 °C for 48 h under anaerobic conditions; Plate Count Agar (PCA, Oxoid, Milano, Italy) for mesophilic aerobic bacteria counts, incubated at 25 °C for 48 h; Violet Red Bile Glucose Agar (VRBGA, Liofilchem, Roseto degli Abruzzi, Italy IT) for the determination of Enterobacteriaceae, incubated aerobically at 30–35 °C for 18–24 h; Sabouraud Dextrose Agar (SAB, Bio-Rad, Hercules, CA, USA) for yeast counts, incubated at 25 °C for 48 h. Mannitol Salt Agar (MSA, Oxoid) for staphylococci counts, incubated at 32 ◦C for 72 h. At the end of fermentation, the presence/absence of *Clostridium perfringens* was also determined in Sulphite-Polymyxin-Sulphadiazine Agar (SPS, Oxoid, UK), incubated at 35–37 °C for 18–48 h, under anaerobic conditions. Moreover, for starter cultures monitoring, samples of Trial III were subjected to additional counting, in MRS agar and in SAB agar media, for *L. plantarum* and yeast determination, respectively. All microbiological analyses were performed in triplicate and the results were expressed as Log CFU/mL.

HPLC analysis

Detection of phenols

The HPLC analyses of fermented OMWW samples were performed by directly injecting the filtered samples (0.45 μm PTFE filters, Merck, Darmstadt, Germany) into the HPLC chromatographic system, i.e., Waters Alliance 2695 HPLC liquid chromatography equipped with a Waters 996 photodiode array (PDA) detector set at 280 nm and managed through the Waters Empower software (Waters Corporation, Milford, MA, USA). The column used was a Luna C18 (250 mm × 4.6 mm i.d., 5 m, 100 Å; Phenomenex, Torrance, CA, USA) maintained in an oven at 40 °C. A flow rate of 1 mL/min was used. Chromatographic separation was performed according to Romeo et al. [11]. The internal standard (I.S.), 50 mM pure gallic acid (Fluka, Buchs Switzerland), was used to quantify the phenolic compounds. The identification of phenolic compounds was obtained by comparing the peak retention time with those of pure standards of tyrosol (TYR), oleuropein (OLE), hydroxytyrosol (HT) chlorogenic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, verbascoside, luteolin-7-o-glucoside, o-coumaric acid, rutin, oleuropein, apigenin-7-o-glucoside, luteolin-4-glucoside, quercetin, luteolin, apigenin (Extrasynthese, Genay, France). All analyses were performed in triplicate for each sample.

Detection of organic acids

The determination of organic acids was carried out at the end of fermentation in trial III. Each sample was filtered through a 0.45 μm PTFE syringe filter (Merck, Germany) before being injected into HPLC (HPLC instruments were described in the previous section) with a DAD detector set at 210 nm (and with spectrum acquisition from 200 to 400 nm). Isocratic elution with 5 mM sulfur acid was performed on a Rezex ROA Organic Acid H+ column (Phenomenex, CA, USA). The run time was set to 50 minutes at 0.6 mL/min. For calibration, pure standards of lactic, citric, acetic, propionic, isobutyric and butyric acids (all purchased from Sigma-Aldrich, Italy) were injected at different concentrations. All analyses were performed in triplicate for each sample.

Biological assays

Cell culture and cytotoxic activity

Caco-2 cells were grown in Dulbecco's Modified Eagle Medium high glucose (DMEM high glucose, Euroclone S.p.A., Pero, Italy) supplemented with 10% Fetal Bovine Serum (FBS, Euroclone S.p.A., Pero, Italy), 2 mM glutamine (Euroclone S.p.A., Pero, Italy), 100 U/mL of penicillin and 0.1 mg/mL of streptomycin (Euroclone). Caco-2 cells were kindly supplied from Dr. Aldo Cavallini and Dr. Caterina Messa from the Laboratory of Biochemistry National Institute for Digestive Diseases. "S. de Bellis", Bari (Italy). Human hepatocellular liver carcinoma (HepG2) cell line was purchased from American Type Culture Collection (ATCC). HepG2 cells were cultured in Eagle's Minimum Essential Medium (MEM, Euroclone), supplemented with 10% FBS, 2 mM glutamine (Euroclone), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Euroclone S), 1% Non–Essential Amino Acids (NEAA, Euroclone). Cultured cells were maintained at 37 ◦C in atmosphere containing 95% of air and 5% of CO_2. Cells were sub-cultivated every 48 h by trypsine–EDTA solution. Determination of cell growth was performed using the 3-(4.5-dimethylthiazol-2-yl)-2.5- diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, Milan, Italy), 10.000 cells/well were seeded into 96-well plates at a volume of 100 μL. After 24 h, 100 μL of microfiltered fermented OMWW samples were added at the appropriate dilution: as such, 1:10, 1:25, 1:50 and 1:100 in triplicate. After 72 h incubation time with extracts, the plates containing the cells were incubated with MTT for 3–4 h at 37 ◦C and 5% of CO_2. At the end of incubation time, MTT was aspirated, and the formazan crystals were solubilized by using 100 μL of dimethyl sulfoxide/ethanol (1:1) (Sigma–Aldrich). The absorbance values at $\lambda = 570$ nm were determined on the Victor Microplate Reader (PerkinElmer, Roma, Italy). Pure HT (Phytolab, Vastenbergsgreuth, Germany) was used as a positive control.

Transport Caco-2 Monolayer

Caco-2 cells were seeded onto a Millicell-96 assay system (Millipore, Burlington, MA, USA) in which a cell monolayer was set in between a filter cell and a receiver plate at a density of 20.000 cells/well. The culture medium was replaced every 48 h and the cells were kept for 21 days in culture. The trans epithelial electrical resistance (TEER) of the monolayers was measured daily before and after the experiment by using an epithelial voltohmmeter (Millicell–ERS). Generally, TEER values greater than 1000 Ω for a 21-day culture are considered optimal. After 21 days of Caco-2 cell growth, the medium was removed from the filter wells and the receiver plate, and they were filled with fresh Hank's balanced salt solution (HBSS) buffer (Invitrogen, Waltham, MA, USA). This procedure was repeated twice, and the plates were incubated at 37 °C for 30 min. After the incubation time, the HBSS buffer was removed and OMWW samples (dilution 1:100) were added to the filter well whereas fresh HBSS was added to the receiver plate. The plates were incubated at 37 °C for 120 min. Afterward, samples were removed from the apical (filter well) and basolateral (receiver plate) side of the monolayer to measure the permeability. The apparent permeability (Papp) referred to HT in units of nm/second was calculated using the following Equation (1):

$$P_{app} = \left(\frac{V_A}{Area \times time}\right) \times \left(\frac{[sample]acceptor}{[sample]initials}\right)$$

VA = the volume (in mL) in the acceptor well; Area = the surface area of the membrane (0.11 cm^2 of the well); time = the total transport time in seconds (7200 s); [sample]acceptor = the concentration of the sample measured by U.V. spectroscopy; [sample]initial = the initial sample concentration (1 × 10−4 M) in the apical or basolateral wells.

Cyclooxygenase Activity Inhibition

Preliminarily, the fermented OMWW samples obtained from Trial III were evaluated for their ability to inhibit ovineCOX-1 or humanCOX-2 enzymes, measuring the extent (%) of enzyme activity inhibition at 50 μM, at dilution 1:25. The inhibition of the enzyme was evaluated by using a colorimetric COX inhibitor screening assay kit (Catalog No. 7601050, Cayman Chemicals, Ann Arbor, MI, USA) following the manufacturer's instructions. COX is a bifunctional enzyme exhibiting both cyclooxygenase and peroxidase activities. The cyclooxygenase component catalyzes the conversion of arachidonic acid into the hydroperoxide PGG2 and then peroxidase component catalyzes PGG2 reduction into the corresponding alcohol PGH2, the precursor of PGs, thromboxane, and prostacyclin. The COX inhibitor screening assay colorimetrically measures the peroxidase activity of the cyclooxygenases monitoring the appearance of oxidized N,N,N0,N0 -tetramethyl-p-phenylenediamine (TMPD) at λ = 590 nm on the Victor Microplate Reader (PerkinElmer, Italy). Stock solutions of tested samples were dissolved in deionized distillated water.

Antioxidant activity

The radical scavenging activity was determined as percentage of RSA (radical scav- enging activity), according to Palmeri et al., 2022. The values were expressed by using the following equation (2):

$$RSA\ \% = \left(\frac{\text{Blank Absorbance} - \text{Sample Absorbance}}{\text{Blank Absorbance}}\right) \times 100$$

Different dilutions of samples were added to the mixture of methanolic solution and 2,2-diphenyl-1-picrylhydrazyl radical 10−4 M. The DPPH absorbance values were evaluated at λ = 517 nm by monitoring the kinetics for 5 min with spectrophotometer (Shimadzu UV-1800, Denmark). Pure HT (Phytolab, Germany) was used as a positive control.

Statistical analysis

Statistical analysis of the obtained results was performed by means of one-way analysis of variance (ANOVA) and Tukey's HSD post hoc test for separation of means at a significance level of $P \leq 0.05$. For data processing, SPSS software (version 21.0, IBM Statistics, NY, USA) was used for data processing.

Conclusions

The microfiltration process resulted in a suitable strategy to obtain a OMWW matrix able to be fermented. The use of selected microbial pools in single and co–cultures showed an increase in HT and TYR contents at the end of fermentation, compared with the control sample. Biological analyses showed that fermentation increases the antioxidant and inflammatory activity of OMWW that resulted to be safe in HepG2 and Caco-2 cell lines. In detail, the phenolic pattern associated to starter microorganisms exhibited an increase of active permeability on Caco-2 monolayer, and a moderate inhibition towards oCOX-1 and hCOX-2 was observed. The results confirm that fermented OMWW can be proposed as a new beverage and/or functional ingredient that could include the addition of compounds as flavorings and probiotic microorganisms. Despite the interesting results obtained at lab scale, perspective studies should aim to replay the process at the industrial scale to standardize phenol concentration at each obtained new formulation.

Supplementary material

Table S1. Chemical parameters detected in samples of Trial I and Trial II.

Sample	Time (days)	pH	TSS (°Brix)	Total phenol (mg/L)	HT (mg/L)	TYR (mg/L)
Trial I						
Control	0	5.05 ± 0.01	7.77 ±0.02^{b}	2449.8 ± 0.25^{d}	410.1 ± 4.43^{a}	76.4 ± 0.46^{a}
L. plantarum	0	5.02 ± 0.01	7.72 ±0.01^{b}	2596.0 ± 1.42^{b}	285.4 ± 42.61^{bc}	68.2 ± 0.82^{bc}
C. boidinii	0	5.01 ± 0.01	7.73 ±0.08^{b}	2515.9 ± 0.15^{c}	415.2 ± 14.38^{a}	54.7 ± 1.49^{d}
W. anomalus	0	5.01 ± 0.01	8.21 ±0.02^{a}	2638.4 ± 0.59^{b}	373.9 ± 14.19^{a}	52.5 ± 3.28^{d}
L.p+ W.a	0	5.02 ± 0.02	7.08 ±0.01^{c}	2459.4 ± 0.79^{c}	345.2 ± 14.90^{ab}	61.5 ± 0.52^{c}
L.p+C.b	0	5.03 ± 0.01	8.32 ± 0.08^{a}	2654.7 ± 1.80^{b}	105.2 ± 2.66^{d}	54.3 ± 2.29^{d}
L.p+W.a+C.b	0	5.10 ± 0.14	8.19 ± 0.11^{a}	2744.9 ± 0.02^{a}	229.5 ± 6.62^{c}	74.6 ± 1.01^{ab}
		n.s	**	**	**	**
Control	8	4.85 ± 0.01^{a}	6.27 ± 0.08^{a}	2037.5 ± 2.08^{d}	252.5 ± 1.05^{b}	89.8 ± 6.42
L. plantarum	8	4.76 ± 0.03^{ab}	5.65 ± 0.06^{b}	1584.7 ± 0.41^{e}	174.6 ± 2.06^{d}	88.8 ± 3.14
C. boidinii	8	4.45 ± 0.07^{c}	5.79 ± 0.02^{b}	2893.4 ± 7.62^{a}	305.2 ± 14.17^{a}	98.2 ± 7.38
W. anomalus	8	4.82 ± 0.03^{a}	5.73 ± 0.09^{b}	2336.3 ± 0.41^{b}	224.4 ± 0.93^{c}	113.1 ± 16.44
L.p+ W.a	8	4.69 ± 0.01^{b}	5.19 ±0.01^{c}	2363.6 ± 3.39^{b}	267.9 ± 2.20^{b}	112.7 ± 4.63
L.p+C.b	8	4.76 ± 0.01^{ab}	6.19 ±0.05^{a}	2047.2 ± 6.69^{d}	266.8 ± 3.65^{b}	103.2 ± 1.76
L.p+W.a+C.b	8	4.64 ± 0.01^{b}	6.16 ± 0.08^{a}	2191.8 ± 2.49^{c}	300.0 ± 3.08^{a}	108.9 ± 2.12
		**	**	***	*	n.s
Control	30	4. 99 ± 0.02^{b}	6.31 ± 0.14^{a}	2642.5 ± 1.30^{b}	1283.6 ± 23.21^{c}	439.8 ± 67.33^{b}
L. plantarum	30	5.51 ± 0.14^{a}	5.99 ± 0.01^{ab}	2485.1 ± 12.90^{c}	1516.6 ± 153.76^{bc}	511.5 ± 40.51^{ab}
C. boidinii	30	4.81 ± 0.01^{bc}	5.60 ± 0.28^{b}	3135.5 ± 5.23^{ab}	2190.2 ± 155.64^{ab}	679.4 ± 9.71^{a}
W. anomalus	30	4.59 ± 0.01^{cd}	5.95 ± 0.08^{ab}	3241.9 ± 0.13^{a}	2630.4 ± 44.05^{a}	537.8 ± 9.71^{ab}
L.p+ W.a	30	4.55 ± 0.07^{d}	6.07 ± 0.09^{ab}	2555.9 ± 4.30^{b}	1622.1 ± 80.47^{bc}	529.0 ± 12.03^{ab}
L.p+C.b	30	4.71 ± 0.01^{cd}	6.05 ± 0.07^{ab}	2335.6 ± 5.34^{d}	1560.0 ± 80.26^{bc}	508.6 ± 5.84^{ab}
L.p+W.a+C.b	30	4.63 ± 0.05c^{d}	6.34 ± 0.01^{a}	3129.7 ± 4.63^{ab}	1543.3 ± 244.36^{bc}	539.4 ± 3.24^{ab}
		**	*	**	*	*
Trial II						
Control	0	5.36 ± 0.02^{ab}	7.88 ± 0.16^{bc}	3773.7 ± 7.26^{a}	727.6 ± 39.38^{bc}	382.5 ± 3.54^{b}
L. plantarum	0	5.30 ± 0.14^{ab}	7.67 ± 0.06^{c}	3655.2 ± 0.55^{b}	479.3 ± 31.42^{d}	262.0 ± 7.25^{cd}
C. boidinii	0	5.30 ± 0.01^{ab}	8.05 ± 0.07^{b}	3271.4 ± 14.62^{c}	616.6 ± 60.40^{c}	245.4 ± 12.34^{d}
W. anomalus	0	5.47 ± 0.03^{a}	8.01 ± 0.01^{b}	3778.9 ± 12.59^{a}	402.8 ± 0.08^{d}	528.4 ± 0.39^{a}
L.p+ W.a	0	5.20 ± 0.01^{b}	7.84 ± 0.08^{bc}	3653.1 ± 5.48^{b}	802.3 ± 12.26^{ab}	332.8 ± 47.82^{bc}
L.p+C.b	0	5.37 ± 0.01^{ab}	8.56 ± 0.01^{a}	2957.8 ± 10.03^{e}	874.3 ± 3.34^{a}	245.1 ± 10.16^{d}
L.p+W.a+C.b	0	5.32 ± 0.02^{ab}	8.06 ± 0.08^{b}	3077.2 ± 1.13^{d}	765.9 ± 33.78^{ab}	299.8 ± 5.06^{cd}
		*	**	**	**	**
Control	8	4.35 ± 0.07^{a}	6.47 ± 0.04^{a}	2992.6 ± 1.85^{d}	526.2 ± 4.64^{bcd}	166.2 ± 3.79^{c}
L. plantarum	8	3.97 ± 0.01^{c}	5.64 ± 0.06^{e}	3005.1 ± 7.29^{c}	594.7 ± 0.49^{bc}	211.2 ± 22.84^{b}
C. boidinii	8	4.05 ± 0.01^{c}	6.05 ± 0.07^{cd}	3201.8 ± 1.12^{d}	306.1 ± 0.16^{d}	120.0 ± 0.05^{d}
W. anomalus	8	3.97 ± 0.01^{c}	5.89 ± 0.01^{d}	3261.9 ± 4.15^{b}	393.2 ± 5.78^{cd}	121.7 ± 1.11^{d}
L.p+ W.a	8	4.18 ± 0.01^{b}	5.50 ± 0.01^{e}	3200.7 ± 3.78^{d}	1064.9 ± 15.22^{a}	280.8 ± 4.01^{a}
L.p+C.b	8	3.98 ± 0.01^{c}	6.16 ± 0.08^{bc}	3024.1 ± 28.40^{c}	314.2 ±146.37^{d}	171.8 ± 4.39^{c}
L.p+W.a+C.b	8	3.99 ± 0.01^{c}	6.36 ± 0.05^{ab}	3379.5 ± 26.15^{a}	666.9 ± 12.83^{b}	142.8 ± 3.70^{cd}
		**	**	**	**	**
Control	30	6.29 ± 0.01^{a}	6.50 ± 0.01^{a}	2796.7 ± 1.85^{e}	330.7 ± 2.17^{d}	129.5 ± 0.34^{b}
L. plantarum	30	4.15 ± 0.07^{e}	5.64 ± 0.02^{c}	3577.6 ± 12.40^{a}	840.3 ± 6.68^{b}	126.0 ± 1.38^{b}
C. boidinii	30	5.57 ± 0.01^{b}	5.84 ± 0.06^{bc}	3267.2 ± 2.28^{c}	979.2 ± 8.26^{b}	87.0 ± 0.27^{cd}
W. anomalus	30	5.64 ± 0.06^{b}	5.94 ± 0.08^{b}	3160.4 ± 41.87^{d}	596.3 ± 89.00^{c}	141.4 ± 1.63^{b}
L.p+ W.a	30	5.04 ± 0.06^{c}	5.68 ± 0.11^{c}	3364.1 ± 25.20^{bc}	1235.6 ± 38.93^{a}	306.2 ± 12.98^{a}
L.p+C.b	30	4.54 ± 0.06^{d}	6.05 ± 0.07^{b}	3426.8 ± 16.75^{b}	810.7 ± 87.83^{b}	67.5 ± 0.85^{d}
L.p+W.a+C.b	30	5.59 ± 0.01^{b}	6.36 ± 0.01^{a}	3348.4 ± 39.05^{bc}	827.2 ± 12.99^{b}	100.3 ± 0.11^{c}
		**	**	**	**	**

Data are expressed as mean ± standard deviations. Mean values with different letters within the same column at the same time interval are statistically different. n.s. not significant; *Significance at $P < 0.05$; **Significance at $P < 0.01$.

Table S2. Main microbial groups counted in Trial I and Trial II samples during fermentation

Sample	Time (days)	LAB	Yeasts	Aerobic mesophilic bacteria	Enterobacteriaceae	Staphylococci
Trial I						
Control	0	5.23 ± 0.01^{d}	6.50 ± 0.04^{b}	6.29 ± 0.16^{d}	6.90 ± 0.01^{a}	1.06 ± 0.03^{e}
L. plantarum	0	5.85 ± 0.02^{a}	5.69 ± 0.01^{e}	7.36 ± 0.03^{a}	6.94 ± 0.01^{a}	1.06± 0.03^{e}
C. boidinii	0	5.31 ± 0.01^{c}	5.49 ± 0.01^{f}	5.96 ± 0.01^{e}	6.79 ± 0.01^{b}	3.98 ± 0.03^{b}
W. anomalus	0	4.78 ± 0.04^{f}	5.48 ± 0.01^{f}	7.06 ± 0.08^{b}	6.57 ± 0.03^{c}	2.63 ± 0.21^{d}
L.p+W.a	0	5.48 ± 0.02^{b}	6.19 ± 0.02^{d}	6.85 ± 0.01^{bc}	6.33 ± 0.01^{d}	4.79 ± 0.01^{a}
L.p+C.b	0	5.30 ± 0.04^{c}	6.96 ± 0.01^{a}	6.72 ± 0.03^{c}	6.36 ± 0.02^{d}	3.30 ± 0.01*c*
L.p+W.a+C.b	0	5.15 ± 0.12^{e}	6.28 ± 0.03^{c}	7.50 ± 0.04^{a}	6.57 ± 0.01^{c}	4.59± 0.16^{a}
		**	**	**	**	**
Control	8	6.74 ± 0.03^{a}	8.15 ± 0.21^{a}	8.15 ± 0.21^{a}	5.00± 0.01^{bc}	1.63± 0.40
L. plantarum	8	6.60 ± 0.01^{c}	6.88 ± 0.04^{b}	6.83 ± 0.^{01b}	4.61± 0.01^{c}	3.30 ± 0.43
C. boidinii	8	6.59 ± 0.01^{c}	7.06 ± 0.05^{b}	6.43 ± 0.07^{b}	0.00± 0.01^{d}	4.29± 0.01
W. anomalus	8	6.37 ± 0.02^{e}	6.85 ± 0.01^{b}	6.77 ± 0.01^{c}	6.02± 0.03^{ab}	3.00 ± 0.41
L.p+W.a	8	6.74 ± 0.05^{a}	7.28 ± 0.16^{b}	5.08 ± 0.01^{d}	4.85± 0.01^{c}	3.88 ± 0.03
L.p+C.b	8	6.62 ± 0.08^{b}	7.28 ± 0.31^{b}	6.57 ± 0.01^{bc}	5.34± 0.06^{abc}	4.37 ± 0.27
L.p+W.a+C.b	8	6.48 ± 0.01^{d}	6.98 ± 0.01^{b}	5.35 ± 0.01^{d}	6.17± 0.74*a*	4.12± 0.07
		**	*	**	**	n.s
Control	30	6.43± 0.01^{a}	6.56 ± 0.17^{b}	6.07 ± 0.01^{c}	2.23 ± 0.34^{a}	3.05 ± 0.06^{a}
L. plantarum	30	5.32± 0.05^{bc}	6.35 ± 0.15b^{c}	7.00 ± 0.01^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
C. boidinii	30	4.78 ± 0.06^{c}	6.04 ± 0.15b^{c}	5.72 ± 0.03^{d}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
W. anomalus	30	6.89 ± 0.09^{a}	6.33 ± 0.01b^{c}	6.23 ± 0.02^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
L.p+W.a	30	6.05 ±0.02^{ab}	5.77 ± 0.10^{d}	6.05 ± 0.08^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
L.p+C.b	30	6.44 ± 0.04^{a}	6.41 ± 0.08^{bc}	6.07 ± 0.10^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
L.p+W.a+C.b	30	7.03 ± 0.01^{a}	7.71 ± 0.01^{a}	6.64 ± 0.01^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
		*	**	**	**	**
Trial II						
Control	0	1.30 ± 0.01^{e}	2.48 ± 0.01^{c}	5.38 ± 0.55^{bc}	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}
L. plantarum	0	5.80 ± 0.17^{a}	5.41 ± 0.12^{ab}	4.15± 0.21^{e}	2.14 ± 0.09^{ab}	1.06 ± 0.03^{e}
C. boidinii	0	5.75 ± 0.03^{a}	5.51 ± 0.77^{ab}	4.23 ± 0.34^{de}	2.88 ± 0.02 ^{a}	3.97 ± 0.03^{b}
W. anomalus	0	2.00 ± 0.01^{d}	4.82 ± 0.01^{ab}	5.26 ± 0.38^{cde}	1.09 ± 0.12c	2.65 ± 0.21^{d}
L.p+ W.a	0	4.77 ± 0.01^{c}	4.64 ± 0.29^{ab}	5.65 ± 0.11^{ab}	2.77 ± 0.49 ^{a}	4.79 ± 0.01^{a}
L.p.+C.b	0	5.00 ± 0.00^{bc}	4.20 ± 0.04^{c}	7.62 ± 0.04^{a}	1.60 ± 0.09^{bc}	3.30 ± 0.01^{c}
L.p+W.a+C.b	0	5.31 ± 0.20^{b}	5.58 ± 0.19^{a}	6.71 ± 0.02^{ab}	1.02 ± 0.03^{c}	4.84 ± 0.28^{a}
		*	**	**	**	**
Control	8	5.43 ± 0.45^{b}	5.92 ± 0.04^{b}	7.38 ± 0.55^{a}	5.26 ± 0.58	4.60 ± 0.16^{b}
L. plantarum	8	7.91 ± 0.01^{a}	7.84 ± 0.09^{a}	5.82 ± 0.18^{b}	4.17 ± 0.21	0.00 ± 0.00^{d}
C. boidinii	8	7.50 ± 0.04 ^{a}	7.61 ± 0.01^{a}	7.36 ± 0.51^{a}	4.20 ± 0.24	3.00 ± 0.00^{c}
W. anomalus	8	7.91 ± 0.01 ^{a}	7.50 ± 0.65^{a}	7.84 ± 0.09^{a}	4.39 ± 0.43	3.39 ± 0.43^{c}
L.p+W.a	8	7.58 ± 0.17 ^{a}	7.47 ± 0.49^{a}	7.49 ± 0.02^{a}	4.38 ± 0.41	4.38 ± 0.41^{b}
L.p.+C.b	8	7.62 ± 0.01 ^{a}	7.00 ± 0.21^{ab}	7.00 ± 0.01^{a}	4.60 ± 0.01	3.90 ± 0.03^{bc}
L.p+W.a+C.b	8	7.61 ± 0.02 ^{a}	7.85 ± 0.19^{a}	5.30 ± 0.01^{b}	4.04 ± 0.06	5.72 ± 0.03^{a}
		**	*	**	n.s	**
Control	30	7.77 ± 0.01 ^{a}	7.84 ± 0.01^{d}	7.75 ± 0.01^{b}	0.00 ± 0.00	0.00 ± 0.00
L. plantarum	30	7.36 ± 0.08 ^{a}	7.29 ± 0.02^{f}	7.30 ± 0.01^{c}	0.00 ± 0.00	0.00 ± 0.00
C. boidinii	30	7.72 ± 0.03 ^{a}	8.00 ± 0.01^{c}	7.31 ± 0.01^{c}	0.00 ± 0.00	0.00 ± 0.00
W. anomalus	30	5.00 ±0.01^{b}	6.00 ± 0.01^{g}	7.86 ± 0.03^{a}	0.00 ± 0.00	0.00 ± 0.00
L.p+W.a	30	8.44 ± 0.66 ^{a}	8.77 ± 0.02^{b}	7.30 ± 0.01^{c}	0.00 ± 0.00	0.00 ± 0.00
L.p+C.b	30	7.80 ± 0.04 ^{a}	9.00 ± 0.01^{a}	7.86 ± 0.01 ^{a}	0.00 ± 0.00	0.00 ± 0.00
L.p+W.a+C.b	30	7.30 ± 0.01 ^{a}	7.43 ± 0.03^{e}	7.31 ± 0.03^{c}	0.00 ± 0.00	0.00 ± 0.00
		**	**	**	n.s	n.s

Data are expressed as Log CFU/mL mean ± standard deviations. Mean values with different letters within the same column at the same time interval are statistically different. n.s. not significant; *Significance at $P < 0.05$; **Significance at $P < 0.01$.

Table S3. Evaluation of tested samples inhibition (as %) on COXs enzymes.

Samples	Concentration of HT (mg/L)	Inhibition *o*COX-1 (%)	Inhibition *h*COX-2 (%)
Control	6.80	0.00	0.00
L.planturum	37.02	5 .09	0.00
C. boidinii	36.09	15.96	12.95
W. anomalus	31.72	1.32	3.27
L.p + W.a	32.06	0.19	0.86
L.p + C.b	36.13	8.20	0.00
L.p + W.a + C.b	30.71	0.00	5.78

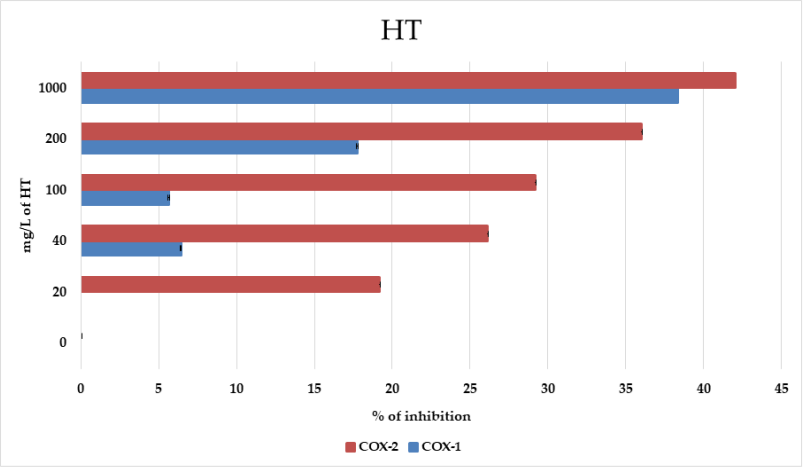


Figure S1. Evaluation of inhibition (as %) of different HT concentrations on *o*COX-1 and *h*COX-2.

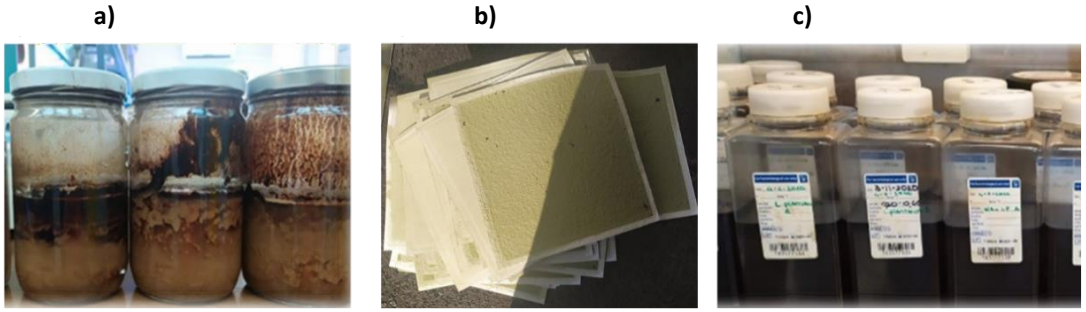


Figure S2 a) Trial I at the end of fermentation; b) Cartons filters after the spinning processand samples from trial II; c) Sample of Trial II after the fermentation process

Figure S3. OMWW samples microfiltered at 0.22 µm.

Acknowledgments: This study was conducted within a Ph.D. research program in Biotecnologie (XXXV

cycle) by Paola Foti who received a grant ‘Dottorato innovativo con caratterizzazione industriale, PON RI 2014-2020’, titled ‘Olive oil by-products as a new functional food and source of nutritional food ingredients’ from the Department of Agriculture, Food and Environment (Scientific Tutors: C.C. Cinzia Caggia; Flora V. Romeo and Cinzia L. Randazzo). Authors thank the Azienda Olearia Consoli Pasquale & F.lli s.n.c (Adrano, CT), partner of the doctoral programme, to kindly supply the POC. Author thanks Dr. Aldo Cavallini and Dr. Caterina Messa, from the Laboratory of Biochemistry National Institute for Digestive Diseases, “S. de Bellis”, Bari (Italy), to kindly supply the Caco-2 cells.

References

1. Gullon, P.; Gullon, B.; Astray, G.; Carpena, M.; Fraga-Corral, M.; Prieto, M.A.; Simal-Gandara, J. Valorization of by-products from olive oil industry and added-value applications for innovative functional foods. Food Res. Int. 2020, 137, 109683.

2. Flamminii, F.; Di Mattia, C.D.; Difonzo, G.; Neri, L.; Faieta, M.; Caponio, F.; Pittia, P. From by-product to food ingredient: Evaluation of compositional and technological properties of olive-leaf phenolic extracts. J. Sci. Food Agric. 2019, 99, 6620–6627.

3. Lama-Muñoz, A.; del Mar Contreras, M.; Espínola, F.; Moya, M.; Romero, I.; Castro, E. Extraction of oleuropein and luteolin-7-O-glucoside from olive leaves: Optimization of technique and operating conditions. Food Chem. 2019, 293, 161–168.

4. Robles-Almazan, M.; Pulido-Moran, M.; Moreno-Fernandez, J.; Ramirez-Tortosa, C.; Rodriguez-Garcia, C.; Quiles, J.L.; Ramirez Tortosa, M. Hydroxytyrosol: Bioavailability, toxicity, and clinical applications. Food Res. Int. 2018, 105, 654–667.

5. Dermeche, S.; Nadour, M.; Larroche, C.; Moulti-Mati, F.; Michaud, P. Olive mill wastes: Biochemical characterizations and valorization strategies. Process. Biochem. 2013, 48, 1532–1552.

6. Servili, M.; Esposto, S.; Sordini, B.; Veneziani, G.; Urbani, S. L’acqua di Vegetazione dei Frantoi Oleari: Una Risorsa da Valorizzare; Accademia dei Georgofili: Florence, Italy, 2021; pp. 317–337.

7. Foti, P.; Occhipinti, P.S.; Romeo, F.V.; Timpanaro, N.; Musumeci, T.; Randazzo, C.L.; Caggia, C. Phenols recovered from olive mill wastewater as natural booster to fortify blood orange juice. Food Chem. 2022, 393, 133428.

8. Cedola, A.; Cardinali, A.; D’Antuono, I.; Conte, A.; Del Nobile, M.A. Cereal foods fortified with by-products from the olive oil industry. Food Biosci. 2019, 33, 100490.

9. Hadj Saadoun, J.; Bertani, G.; Levante, A.; Vezzosi, F.; Ricci, A.; Bernini, V.; Lazzi, C. Fermentation of Agri-Food Waste: A Promising Route for the Production of Aroma Compounds. Foods 2021, 10, 707.

10. Aponte, M.; Ungaro, F.; d’Angelo, I.; De Caro, C.; Russo, R.; Blaiotta, G.; Dal Piaz, F.; Calignano, A.; Miro, A. Improving in vivo conversion of oleuropein into hydroxytyrosol by oral granules containing probiotic Lactobacillus plantarum 299v and an Olea europaea standardized extract. Int. J. Pharmaceut. 2018, 543, 73–82.

11. Romeo, F.V.; Granuzzo, G.; Foti, P.; Ballistreri, G.; Caggia, C.; Rapisarda, P. Microbial application to improve olive mill wastewater phenolic extracts. Molecules 2021, 26, 1944.

12. Ruiz Rodríguez, L.G.; Zamora Gasga, V.M.; Pescuma, M.; Van Nieuwenhove, C.; Mozzi, F.; Sánchez Burgos, J.A. Fruits and fruit by-products as sources of bioactive compounds. Benefits and trends of lactic acid fermentation in the development of novel fruit-based functional beverages. Food Res. Int. 2021, 140, 109854.

13. Miao, Z.; Hao, H.; Yan, R.; Wang, X.; Wang, B.; Sun, J.; Li, Z.; Zhang, Y.; Sun, B. Individualization of Chinese alcoholic beverages: Feasibility towards a regulation of organic acids. LWT 2022, 172, 114168.

14. Starzy ´nska-Janiszewska, A.; Fernández-Fernández, C.; Martín-García, B.; Verardo, V.; Gómez-Caravaca, A.M. Solid state fermentation of olive leaves as a promising technology to obtain hydroxytyrosol and elenolic acid derivatives enriched extracts. Antioxidants 2022, 11, 1693.

15. Benítez-Cabello, A.; Calero-Delgado, B.; Rodríguez-Gómez, F.; Garrido-Fernández, A.; Jiménez-Díaz, R.; Arroyo-López, F.N. Biodiversity and multifunctional features of lactic acid bacteria isolated from table olive biofilms. Front. Microbiol. 2019, 10, 836.

16. Lanza, B.; Di Marco, S.; Bacceli, M.; Di Serio, M.G.; Di Loreto, G.; Cellini, M.; Simone, N. *Lactiplantibacillus plantarum* used as single, multiple, and mixed starter combined with Candida boidinii for table olive fermentations: Chemical, textural, and sensorial characterization of final products. Fermentation 2021, 7, 239.

17. Zullo, B.A.; Ciafardini, G. Evaluation of physiological properties of yeast strains isolated from olive oil and their in vitro probiotic trait. Food Microbiol. 2019, 78, 179–187.

18. Nazhand, A.; Durazzo, A.; Lucarini, M.; Guerra, F.; Souto, S.B.; Souto, E.B.; Santini, A.I. Nutraceuticals and functional beverages: Focus on Prebiotics and Probiotics active beverages. In Future Foods; Academic Press: Cambridge, MA, USA, 2022; pp. 251–258.

19. Parra-Perez, A.M.; Pérez-Jiménez, A.; Gris-Cárdenas, I.; Bonel-Pérez, G.C.; Carrasco-Díaz, L.M.; Mokhtari, K.; García-Salguero, L.; Lupiáñez, J.A.; Rufino-Palomares, E.E. Involvement of the PI3K/AKT intracellular signaling pathway in the anticancer activity of hydroxytyrosol, a polyphenol from Olea europaea, in hematological cells and implication of HSP60 levels in its anti-inflammatory activity. Int. J. Mol. Sci. 2022, 23, 7053.

20. Perrone, M.G.; Vitale, P.; Ferorelli, S.; Boccarelli, A.; Coluccia, M.; Pannunzio, A.; Campanella, F.; Di Mauro, G.; Bonaccorso, C.; Fortuna, C.G.; et al. Effect of mofezolac-galactose distance in conjugates targeting cyclooxygenase (COX)-1 and CNS GLUT-1 carrier. Eur. J. Med. Chem. 2017, 141, 404–416.

21. Di Mauro, M.D.; Fava, G.; Spampinato, M.; Aleo, D.; Melilli, B.; Saita, M.G.; Centonze, G.; Maggiore, R.; D'Antona, N. Polyphenolic fraction from olive mill wastewater: Scale-up and in vitro studies for ophthalmic nutraceutical applications. Antioxidants 2019, 8, 462.

22. Ricci, A.; Bernini, V.; Maoloni, A.; Cirlini, M.; Galaverna, G.; Neviani, E.; Lazzi, C. Vegetable by-product lacto-fermentation as a new source of antimicrobial compounds. Microorganisms 2019, 7, 607.

23. Alfano, A.; Corsuto, L.; Finamore, R.; Savarese, M.; Ferrara, F.; Falco, S.; Santabarbara, G.; De Rosa, M.; Schiraldi, C. Valorization of olive mill wastewater by membrane processes to recover natural antioxidant compounds for cosmeceutical and nutraceutical applications or functional foods. Antioxidants 2018, 7, 72.

24. Arienzo, A.; Murgia, L.; Fraudentali, I.; Gallo, V.; Angelini, R.; Antonini, G. Microbiological quality of ready-to-eat leafy green salads during shelf-life and home-refrigeration. Foods 2020, 9, 1421.

25. France, T.C.; Kelly, A.L.; Crowley, S.V.; O'Mahony, J.A. Cold microfiltration as an enabler of sustainable dairy protein ingredient innovation. Foods 2021, 10, 2091.

26. Allouche, N.; Fki, I.; Sayadi, S. Toward a high yield recovery of antioxidants and purified hydroxytyrosol from olive mill wastewaters. J. Agric. Food Chem. 2004, 52, 267–273.

27. Vaccalluzzo, A.; Pino, A.; De Angelis, M.; Bautista-Gallego, J.; Romeo, F.V.; Foti, P.; Caggia, C.; Randazzo, C.L. Effects of different stress parameters on growth and on oleuropein-degrading abilities of *Lactiplantibacillus plantarum* strains selected as tailored starter cultures for naturally table olives. Microorganisms 2020, 8, 1607.

28. Reverón, I.; Plaza-Vinuesa, L.; Santamaría, L.; Oliveros, J.C.; de Las Rivas, B.; Muñoz, R.; López de Felipe, F. Transcriptomic evidence of molecular mechanisms underlying the response of *Lactobacillus plantarum* WCFS1 to hydroxytyrosol. Antioxidants 2020, 9, 442.

29. De Melo Pereira, G.V.; Maske, B.L.; de Carvalho Neto, D.P.; Karp, S.G.; De Dea Lindner, J.; Martin, J.G.P.; de Oliveira Hosken, B.; Soccol, C.R. What is Candida doing in my food? A review and safety alert on its use as starter cultures in fermented foods. Microorganisms 2022, 10, 1855.

30. Palareti, G.; Legnani, C.; Cosmi, B.; Antonucci, E.; Erba, N.; Poli, D.; Testa, S.; Tosetto, A. Comparison between different D-dime cutoff values to assess the individual risk of recurrent venous thromboembolism: Analysis of results obtained in the DULCIS study. Int. J. Lab. Hematol. 2016, 38, 42–49.

31. Wang, C.; Chang, T.; Yang, H.; Cui, M. Antibacterial mechanism of lactic acid on physiological and morphological properties of Salmonella Enteritidis*, Escherichia coli* and *Listeria monocytogenes*. Food Control. 2015, 47, 231–236.

32. Bangar, S.P.; Suri, S.; Trif, M.; Ozogul, F. Organic acids production from lactic acid bacteria: A preservation approach. Food Biosci. 2022, 46, 101615.

33. Okoye, C.O.; Dong, K.; Wang, Y.; Gao, L.; Li, X.; Wu, Y.; Jiang, J. Comparative genomics reveals the organic acid biosynthesis metabolic pathways among five lactic acid bacterial species isolated from fermented vegetables. New Biotechnol. 2022, 70, 73–83.

34. Ramirez, T.; Strigun, A.; Verlohner, A.; Huener, H.A.; Peter, E.; Herold, M.; Bordag, N.; Mellert, W.; Walk, T.; Spitzer, M.; et al. Prediction of liver toxicity and mode of action using metabolomics in vitro in HepG2 cells. Arch. Toxicol. 2018, 92, 893–906.

35. Colabufo, N.A.; Contino, M.; Cantore, M.; Capparelli, E.; Grazia, M.; Cassano, G.; Gasparre, G.; Leopoldo, M.; Berardi, F.; Perrone, R. Naphthalenyl derivatives for hitting P-gp/MRP1/BCRP transporters. Bioorg. Med. Chem. 2013, 21, 1324–1332.

36. Riganti, C.; Contino, M.; Guglielmo, S.; Perrone, M.G.; Salaroglio, I.C.; Milosevic, V.; Giampietro, R.; Leonetti, F.; Rolando, B.; Lazzarato, L.; et al. Design, biological evaluation, and molecular modeling of tetrahydroisoquinoline derivatives: Discovery of a potent P-glycoprotein ligand overcoming multidrug resistance in cancer stem cells. J. Med. Chem. 2019, 62, 974–986.

37. D'Archivio, M.; Filesi, C.; Varì, R.; Scazzocchio, B.; Masella, R. Bioavailability of the polyphenols: Status and controversies. Int. J. Mol. Sci. 2010, 11, 1321–1342.

38. Soler, A.; Romero, M.P.; Macia, A.; Saha, S.; Furniss, C.S.M.; Kroon, P.A.; Motilva, M.J. Digestion stability and evaluation of the metabolism and transport of olive oil phenols in the human small-intestinal epithelial Caco 2/TC7 cell line. Food Chem. 2010, 119, 703–714.

39. Serreli, G.; Deiana, M. Extra virgin olive oil polyphenols: Modulation of cellular pathways related to oxidant species and inflammation in aging. Cells 2020, 9, 478.

40. Bartolomei, M.; Bollati, C.; Bellumori, M.; Cecchi, L.; Cruz-Chamorro, I.; Santos-Sánchez, G.; Ranaldi, G.; Ferruzza, S.; Sambuy, Y.; Arnoldi, A.; et al. Extra virgin olive oil phenolic extract on human hepatic HepG2 and intestinal Caco-2 Cells: Assessment of the antioxidant activity and intestinal trans-epithelial transport. Antioxidants 2021, 10, 118.

41. Abdallah, M.; Marzocco, S.; Adesso, S.; Zarrouk, M.; Guerfel, M. Olive oil polyphenols extracts inhibit inflammatory markers in J774A.1 murine macrophages and scavenge free radicals. Acta Histochem. 2018, 120, 1–10.

42. Scoditti, E.; Nestola, A.; Massaro, M.; Calabriso, N.; Storelli, C.; De Caterina, R.; Carluccio, M.A. Hydroxytyrosol suppresses MMP-9 and COX-2 activity and expression in activated human monocytes via PKCα and PKCβ1 inhibition. Atherosclerosis 2014, 232, 17–24.

43. Perrone, M.G.; Scilimati, A.; Simone, L.; Vitale, P. Selective COX-1 inhibition: A therapeutic target to be reconsidered. Curr. Med. Chem. 2010, 17, 3769–3805.

44. Calvello, R.; Lofrumento, D.D.; Perrone, M.G.; Cianciulli, A.; Salvatore, R.; Vitale, P.; De Nuccio, F.; Giannotti, L.; Nicolardi, G.; Panaro, M.A.; et al. Highly selective cyclooxygenase-1 inhibitors P6 and mofezolac counteract inflammatory state both in vitro and in vivo models of neuroinflammation. Front Neurol. 2017, 9, 251.

45. Pati, M.L.; Vitale, P.; Ferorelli, S.; Iaselli, M.; Miciaccia, M.; Boccarelli, A.; Di Mauro, G.D.; Fortuna, C.G.; Souza Domingos, T.F.; Rodrigues Pereira da Silva, L.C.; et al. Translational impact of novel widely

pharmacological characterized mofezolac-derived COX-1 inhibitors combined with bortezomib on human multiple myeloma cell lines viability. Eur. J. Med. Chem. 2019, 164, 59–76.

46. Perrone, M.G.; Vitale, P.; Miciaccia, M.; Ferorelli, S.; Centonze, A.; Solidoro, R.; Munzone, C.; Bonaccorso, C.; Fortuna, C.G.; Kleinmanns, K.; et al. Fluorochrome selection for imaging intraoperative ovarian cancer probes. Pharmaceuticals 2022, 15, 668.

47. Rosignoli, P.; Fuccelli, R.; Sepporta, M.V.; Fabiani, R. In vitro chemo-preventive activities of hydroxytyrosol: The main phenolic compound present in extra-virgin olive oil. Food Funct. 2016, 7, 301–307.

48. El-Azem, N.; Pulido-Moran, M.; Ramirez-Tortosa, C.L.; Quiles, J.L.; Cara, F.E.; Sanchez-Rovira, P.; Granados-Principal, S.; Ramirez-Tortosa, M.C. Modulation by hydroxytyrosol of oxidative stress and antitumor activities of paclitaxel in breast cancer. Eur. J. Nutr. 2019, 58, 1203–1211.

49. Ramirez-Tortosa, C.; Sanchez, A.; Perez-Ramirez, C.; Quiles, J.L.; Robles-Almazan, M.; Pulido-Moran, M.; Sanchez-Rovira, P.; Ramirez-Tortosa, M. Hydroxytyrosol supplementation modifies plasma levels of tissue inhibitor of metallopeptidase 1 in women with breast cancer. Antioxidants 2019, 8, 393.

50. Sanlier, N.; Gokcen, B.B.; Sezgin, A.C. Health benefits of fermented foods. Crit. Rev. Food Sci. Nutr. 2019, 59, 506–527.

51. Pino, A.; Vaccalluzzo, A.; Solieri, L.; Romeo, F.V.; Todaro, A.; Caggia, C.; Arroyo-López, F.N.; Bautista-Gallego, J.; Randazzo, C.L. Effect of sequential inoculum of beta-glucosidase positive and probiotic strains on brine fermentation to obtain low salt Sicilian table olives. Front. Microbiol. 2019, 10, 174.

52. Palmeri, R.; Siracusa, L.; Carrubba, M.; Parafati, L.; Proetto, I.; Pesce, F.; Fallico, B. Olive leaves, a promising byproduct of olive oil industry: Assessment of metabolic profiles and antioxidant capacity as a function of cultivar and seasonal change. Agronomy 2022, 12, 2007.

Phenols recovered from olive mill wastewater as natural booster to fortify blood orange juice

Paola Foti[1,2], **Paride S. Occhipinti**[1], Flora V. Romeo[2,] *, Nicolina Timpanaro[2], Teresa Musumeci[4,5], Cinzia L. Randazzo[1,3,5], Cinzia Caggia[1,3,5]

[1]Department of Agriculture, Food and Environment (Di3A), University of Catania, Via Santa Sofia 100, 95123, Catania, Italy.

[2]Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA), Centro di Ricerca Olivicoltura, Frutticoltura e Agrumicoltura, Corso Savoia 190, 95024 Acireale, Italy.

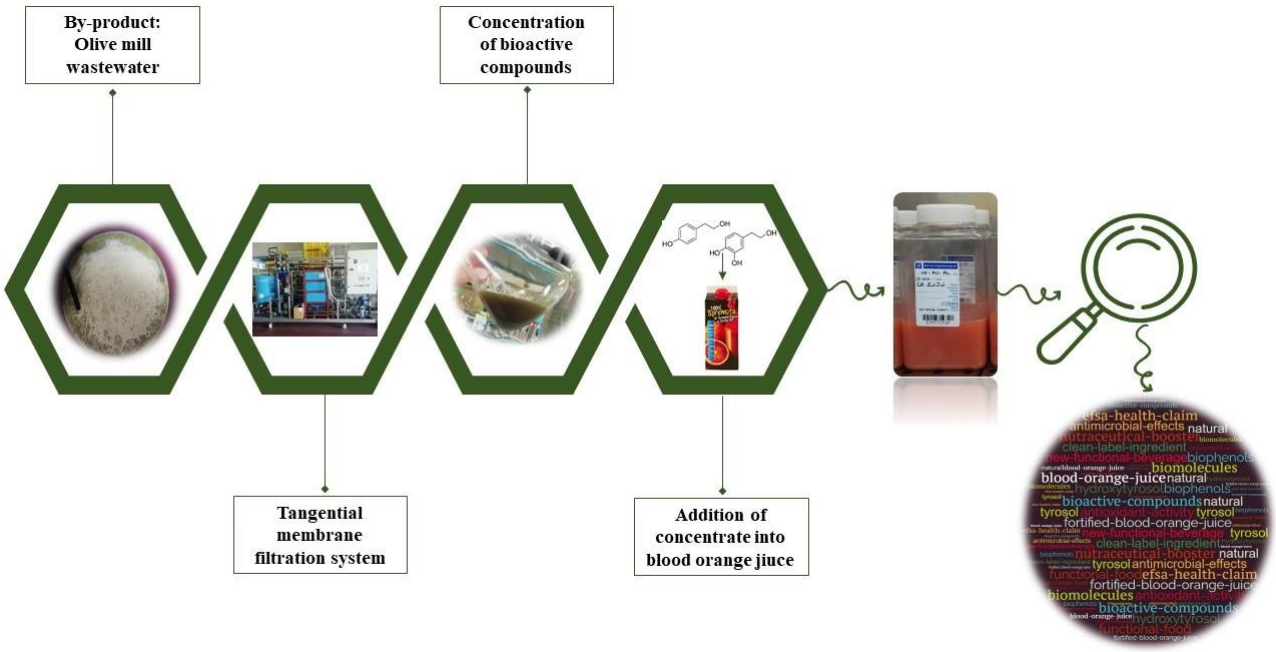
[3]ProBioEtna srl, Spin-off of University of Catania, Via S. Sofia, 100, 95123 Catania, Italy[4]Department of Drug Sciences and Health, University of Catania, Via Santa Sofia, 64, 6,95125, Catania, Italy.

[5]CERNUT, Interdepartmental Research Centre in Nutraceuteuticals and Health Products. University of Catania, viale A. Doria 6, 95125 Catania, Italy.

This chapter has been published in Food Chemistry-ELSEVIER, *2022*, 393, 133428.

Abstract

In the present study, a tangential membrane filtration system was applied to recover phenols from olive mill wastewater. The obtained concentrates were characterised for physico-chemical traits, antioxidant activity and antimicrobial effects. Results indicated that the highest concentration of hydroxytyrosol (7203.7 mg/L) was detected in the concentrate obtained by reverse osmosis, which also showed the highest antioxidant and antimicrobial activity. Moreover, the same concentrate was added, at different ratio, up to 4:250 v/v, into a commercial blood orange juice. The fortified juice with the addition of the concentrate, up to 2:250 v/v ratio, did not show off-flavour and off-odour comparedto the control. Furthermore, after 60 days of refrigerated storage, the fortified juice exhibited a hydroxytyrosol content still complying with the daily intake recommended byEFSA health claim. The obtained results can be industrially useful in producing orange juice added with a natural antioxidant concentrate as a 'clean label' ingredient.



Introduction

In the last years, consumers have becoming more and more aware about the ingredients in food and started actively scrutinizing the product labels. At the same time, increasing attention has been paid to the valorisation of agro-industrial waste and to the utilization of by-product, promoting their reuse to develop new functional food. In particular, vegetable by-products are considered valuable sources for the formulation of new natural food additives. Their exploitation represents a low-cost and environmentally friendly strategy that can provide alternatives to synthetic chemical compounds in food industries (Faustino et al., 2019). Olive oil production represents the main agro-industrial activity in Mediterranean countries, and it is associated with the generation of a large amount of both liquid and solid by-products (Berbel et al., 2018). The olive mill wastewater (OMW), a liquid waste mainly obtained by the 3-phase extraction system, still represents a relevant management problem, above all for small olive oil companies but, at the same time, a high added value resource, being rich in bioactive compounds, such as hydroxytyrosol, tyrosol, oleuropein, flavonoids and others (Romeo et al., 2021). The strong antioxidant activity of these compounds turns olive oil by-products into an inexpensive source of natural antioxidants with recognised healthy effects. Hydroxytyrosol has been proven to show anti- inflammatory and antimicrobial activities, to play a role in preventing and combating cardiovascular diseases and metabolic syndromes, with neuroprotective, anticancer and chemomodulatory effects (Robles-Almazan et al., 2018). The European Food Safety Authority (EFSA), indeed, confirmed the health claim related to olive polyphenols at dose of 5 mg of hydroxytyrosol or its derivatives, corresponding to a daily consumption of 20 g of extra virgin olive oil. Furthermore, a recent study highlighted that the addition of olive by-products to foods exhibited an effect in extending the shelf life and in inhibiting the growth of pathogens (Di Nunzio et al., 2020). The recovery of phenols from OMW can be performed through conventional techniques, such as filtration trough membranes, solvent extractions and through emerging non- thermal technologies that reduce sensory alteration and nutritional deprecation of final product (Galanakis et al., 2018a; Barba et al., 2015). Among them, the membrane extraction technique is one of the most evaluable methods mainly at industrial scale (Caporaso et al., 2019). Furthermore, the membrane filtration technique is characterized by a low energy consumption, good operating conditions and high efficiency in component separation. This technology, based on the capacity of substances to cross the polymeric or inorganic semipermeable membrane at different rates, allows a cost-effective purification of phenolic pool present in OMWs, thanks to the low operating temperature (Cassano et al., 2013). The filtration technique involves microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) processes (Mallamaci et al., 2021). Furthermore, the fractions obtained from OMW can be added into food formulations as concentrated stabilised extracts and, in some cases, microencapsulated

(Foti et al., 2021a). Therefore, this treatment makes of a by-product an alternative resource of biologically active phenols that can be used to fortify foods and/or beverages (Caporaso et al., 2019; Galanakis et al., 2017). Orange juice is a natural functional beverage thanks to the high content of vitamin C and flavonoids, the most abundant phenolic compounds present in Citrus fruits (Ballistreri et al., 2019). Red orange fruits represent the most important citrus product of Southern Italy. From these fruits, the obtained blood orange juice is characterized by high anthocyanin levels. Thanks to its acidity, orange juice is a suitable matrix to deliver nutraceutical molecules or probiotics and represents a promising candidate for the formulation of new functional beverages (Bonaccorso et al., 2021). The aim of this study was to select the best concentrate, among the OMW fractions, obtained through ultrafiltration and reverse osmosis at industrial level, for fortification of blood orange juice. For this purpose, physico-chemical, microbiological, antioxidant and antimicrobial properties of different concentrates were evaluated. In addition, the most promising concentrate was added to a commercial blood orange juice, at different amounts, and its effect on physico-chemical, chemical, microbiological and sensory traits was evaluated up to 60 days of refrigerated conditions.

Materials and methods

Olive mill wastewater sampling

The OMW was kindly supplied by olive oil company “Azienda Olearia Consoli Pasquale & F.lli s.n.c” (Adrano, Sicily). The OMW samples, acidified with 0.6 % of food grade hydrochloric acid, were treated at farm level using the 'Permeaprocess' plant (Permeare s.r.l., Italy). The system consists of a tangential filtration based on selective membranes suitable for purification, fractionation, and concentration of compounds. This physical method allows the elimination of water at room temperature by means of a semi-permeable membrane, capable of overcoming osmotic pressure. This technique separates the water contained in the samples, concentrating all the components present, including phenols and organic acids. Three concentrates were obtained: the ultrafiltration concentrate (C1 sample), the first osmosis concentrate (C2 sample) and the second osmosis concentrate (C3 sample). Moreover, as showed in Figure 1, the P1 sample was obtained from the C1, the P2 from the C2 and the P3 from the C3. All obtained fractions were stored at + 4 °C before analyses.

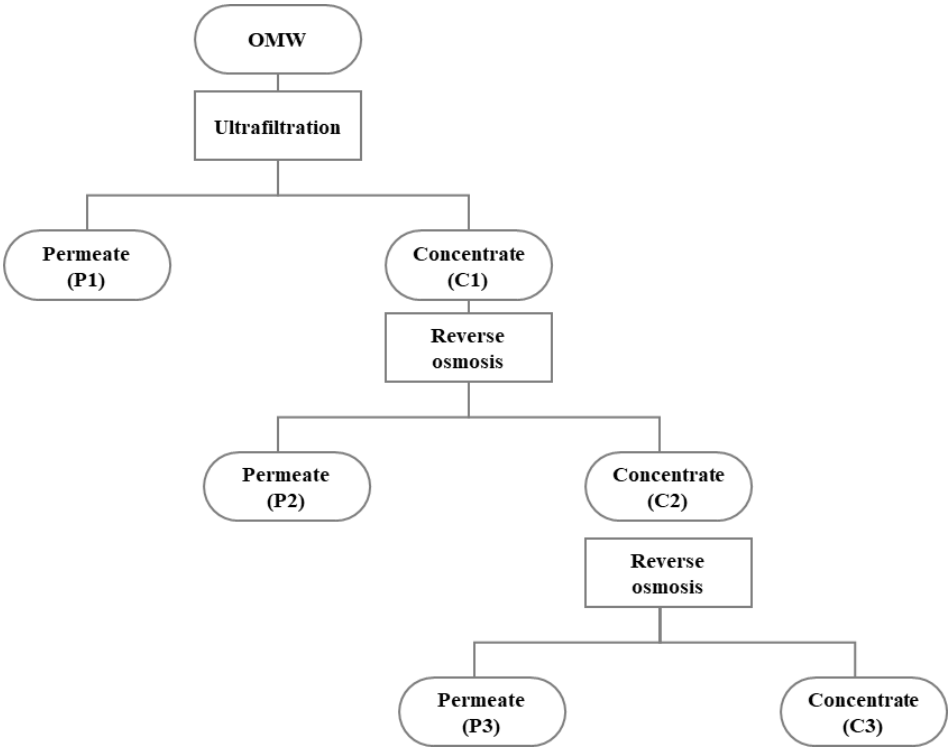


Fig. 1. Flowsheet of OMW filtration process.

Chemical analyses of OMW and obtained fractions

The pH of OMW and obtained fraction samples was measured using a Mettler DL25 pH meter (Mettler-Toledo International Inc., Columbus, OH, USA). In addition, total soluble solid (TSS) value was determined using a refractometer (Atago, RX-5000) and expressed as °Brix. The total phenolic content was detected according to the Folin-Ciocalteu's (FC) colorimetric method. Samples were mixed with 5 mL of FC commercial reagent (Labochimica, Italy) diluted with water 1:10 v/v, added of 4 mL of a 7.5% sodium carbonate solution and left at room temperature away from light. The absorbance of samples was spectrophotometrically measured at 765 nm (Cary 100 Scan UV-Visibile, Agilent, CA, USA). The total phenolic content was expressed as mg of gallic acid equivalents (GAE)/L of sample.

HPLC analyses

Phenol detection

The HPLC analysis of OMW, concentrates, and the P1 permeate was performed by directly injecting the filtered samples (0.45 □m PTFE filters, Merck, Germany) into the chromatographic HPLC system. The system consisted of a liquid chromatography Waters Alliance 2695 HPLC equipped with a Waters 996 photodiode array detector (PDA) set at 280 nm and with Waters Empower software (Waters Corporation, MA, USA). The column was a Luna C18 (250 mm X 4.6 mm i.d., 5 m, 100 Å; Phenomenex, Torrence, CA, USA) maintained in an oven at 40°C. A flow of 1 mL/min was used. The chromatographic separation was performed according to Romeo et al. (2021). The internal standard (I.S.), a 50 mM pure gallic acid (Fluka, Switzerland), was used to quantify the phenolic compounds. The identification of phenolic compounds was obtained by comparing retention time with pure tyrosol (TYR), oleuropein (OLE) and hydroxytyrosol (HT) (Extrasynthese, Genay, France). All the analyses were carried out in triplicate for each sample.

Organic acid detection

For the determination of organic acids, samples were diluted with ultrapure water, at different ratios: the OMW, the C1 and the P1 samples at 1:1 v/v; the C2 and the C3 samples at 1:10 v/v; while the P2 and the P3 samples were used as they were. Each sample was then filtered, through a 0.45 μm PTFE syringe filter (Merck, Germany), before being injected into HPLC (the HPLC instruments were described in the previous section) with a DAD detector set at 210 nm (and with spectrum acquisition from 200 to 400 nm). Isocratic elution with 5 mM sulphuric acid was performed on a Rezex ROA Organic Acid H+ column (Phenomenex, Torrence, CA, USA). The run time was set at 50 minutes at 0.6 mL/min. For calibration, pure standards of lactic, citric, acetic, propionic, isobutyric and butyric acids (all purchased from Sigma-Aldrich, Italy) were injected at different concentrations. All the analyses were carried out in triplicate for each sample.

Microbiological analyses

Microbiological analyses of OMW and concentrate samples

The concentrates were serially diluted and poured into agar plates contained specific media and incubated at specific conditions: de Man, Rogosa, and Sharpe Agar (MRSA, Oxoid, UK) for Lactobacilli count, incubated at 32 °C for 48 h under anaerobic conditions; Plate Count Agar (PCA, Oxoid, UK) for aerobic mesophilic bacteria count, incubated at 25 °C for 48 h; Sulphite-Polymyxin-Sulphadiazine Agar (SPS, Oxoid, UK) for the detection of *Clostridium perfringens*, incubated at 35–37 °C for 18–48 h under anaerobic conditions; Sabouraud Dextrose Agar (SAB, Bio-Rad, CA) for yeasts counting, incubated at 25 °C for 48 h. Microbiological analyses were performed in triplicate and results expressed as Log CFU/mL ± standard deviation (SD).

Microbiological analyses of permeate samples

The two permeate samples, P2 and P3, were microbiologically analysed following the membrane filtration method (Standard Methods for the Examination of Water and Wastewater: APHA, 2012). In detail, for the detection and counting of *Escherichia coli*, 100 mL of sample were filtered on membrane filters (0.45 µm pores, Cellulose, Merck, Germany) and poured in RAPID' E. coli 2 Agar plates (Bio-Rad, Italy), incubated at 37°C for 24 h. For detection of *Clostridium* spores, 1 mL of sample was poured into sterile 50 mL tubes, added with 24 mL of sterile distilled water and tubes heated at 75 ± 5 °C for 10 minutes. Then, 25 mL of liquid Sulphite Polymyxin Sulphadiazine (SPS) at 45°C, at double (2X) concentration, were added and tubes incubated at 37 °C for 24 h. For detection of sulphite-reducing bacteria, 1 mL of sample was poured, by inclusion, on SPS plates and plates anaerobically incubated at 37 °C for 24 h. The counting of somatic coliphages was carried out following the "ISO 10705-2:2000(E) - Water quality- Detection and enumeration of bacteriophages - Part 2: Enumeration of somatic coliphages" protocol. The detection of intestinal nematodes (helminth eggs) was carried out following the "Official method suppl. ord. g.u. n. 87" of 13 April 2000, which foresees a sedimentation phase and a series of centrifugations followed by flotation and observation under microscope. The detection and enumeration of *Legionella* spp. was carried out following the ISO 11731:2017 Water quality - Enumeration of *Legionella* procedure.

Antioxidant activity of OMW and fractions

Different dilutions of samples were added to the mixture of methanolic solution and 2,2- Diphenyl-1-picrylhydrazyl radical 10-4 M (DPPH, Merck, Germany). The absorbance was evaluated at 517 nm and the results expressed as a percentage decrease, compared to the control. Antioxidant activity was expressed with respect to sample volume and the concentration at which 50% radical scavenging occurred (IC50). Stronger radical quenching results at a lower IC50 value. Inhibition percentage for each sample was calculated as follows:

$$\%\ inhibition = \frac{A_0 - A_x}{A_0}\ 100$$

where A0 is the absorbance of a DPPH blank and Ax is the sample absorbance.

Antimicrobial activity of OMW and obtained fractions

The inhibitory activity of OMW and obtained fractions (C1, P1, C2 and C3) was tested, according to Foti et al. (2021b), against pathogenic strains*: Listeria monocytogenes* ATCC 19114, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 25213, *Pseudomonas aeuroginosa* ATCC 9027, *Salmonella typhimurium* ATCC 14028, *Bacillus subtilis* ATCC 19659, *Clostridium sporogenes* ATCC 11437, and *Enterococcus faecalis* ATCC 29212 (American Type Culture Collection). In addition, the same fractions were tested on probiotic strains: *Lacticaseibacillus rhamnosus* CRL1505, *Lacticaseibacillus paracasei* 101/37, *Bifidobacterium animalis* subsp. lactis BLC1 (purchased from Sacco S.r.l., Italy) and *Propionibacterium freudenreichii* DSM 4902 (Leibniz-Institute DSMZ, German collection). The test was performed on: Potato Dextrose Agar (PDA, Likson, Italy) for *Candida albicans*; MRSA, for Lactobacilli; Muller Hinton Agar Base (MHA, Liofichem, Italy) for other bacteria. For the probiotic strains, each individual culture was standardised using Mc Farland 0.5 solution, which corresponded to a cell density of approximately 1.5x108 CFU/mL, while the standardised pathogenic strains were diluted to a cell density of 1x106 CFU/mL. In each plate, containing the selective medium, 1 mL of cell suspension was spatulated, allowed to dry, and then sterile cellulose discs (Ø 6 mm) imbibed with each tested sample, at different dilution rates, were placed. The samples were tested as they were and at different dilution ratio (from 1:2 to 1:16). Distilled water was used as negative control. Plates were incubated at specific temperatures for 48 h and results expressed as diameter of the inhibition halo (mm).

Blood orange juice fortification

In the present study, a 100% blood orange juice, without any addition of sugar or preservatives and with an estimated shelf life of 60-65 days, was kindly provided by the Oranfrizer Company (located in Scordia, Sicily). The concentrate C2, filtered at 0.22 μm (PTFE filters, Merck, Germany), was added to the blood orange juice at different ratio [1:250 v/v (FBOJ1); 2:250 v/v (FBOJ2); 3:250 v/v (FBOJ3); 4:250 v/v (FBOJ4)] and the obtained fortified blood orange juice (FBOJ) samples were stored at +4 °C for 60 days. The FBOJ samples were analysed at different times (0, 15 and 60 days) to evaluate chemical, microbiological, and sensory traits compared to the commercial juice as control. All analyses were carried out in triplicate.

Microbiological analysis of FBOJ

The FBOJ samples, obtained by addition of the C2 concentrate, were microbiologically analysed at 0, 15 and 60 days of storage on the following media: MRS, for the determination of Lactobacilli; PCA, for mesophilic aerobic bacteria count; SAB, for yeasts and moulds. The culture conditions were the same as described in 2.4.1 section. Microbiological analyses were performed in triplicate and results were expressed as Log CFU/mL ± SD.

Total phenolic content and HPLC assay of FBOJ

The FBOJ samples obtained by addition of the C2 concentrate, were tested for total phenolic content as described in 2.2 section and for the quali-quantitative determination of single phenol, by direct injection into HPLC, as described in 2.3.1 section.

Colorimetric assay of FBOJ

The colour of the FBOJ samples, obtained by adding at different ratio the C2 concentrate, was determined at different storage times, as previously reported by Timpanaro et al. (2021). The coordinates L* (brightness), a* (green-red component), b* (blue-yellow component), were determined as the average of two transmittance measurements using a spectrophotometer CM-5 (Minolta, Milan, Italy). The parameters L*, a*, b* were determined using the illuminant D65, according to the CIELAB scale.

Sensory analysis of FBOJ

The standard ISO 13299:2016 provides guidelines for developing a sensory profile, which can be obtained for any products that can be evaluated by sight, smell, taste, tact, or hearing. The profile method was performed by a trained panel (EN ISO 8586:2014), and ten panellists (4 males and 6 females, aged between 28 and 45 years) were selected among the staff of CREA - Research Centre for Olive, Fruit and Citrus Crops, located in Acireale, Italy. During the training period, the judges selected the attributes to describe the colour (light orange to deep red), odour of orange, off-odour, acidity, sweetness, flavour, off- flavour, and bitterness using orange fresh juice as control. Judges evaluated the intensity of each attribute by assigning a score between 1 (absence of the sensation) and 9 (extremely intense) on a numerical unipolar scale (ISO 4121:2003). Sensory analyses were performed at the same day of C2 addition. All sensory tests were performed at the sensory analysis laboratory devised in accordance with UNI EN ISO 8589: 2014.

Statistical analyses

All analyses were performed in triplicate. SPSS software (version 21.0, IBM Statistics, Armonk, NY, USA) was used for data processing. Statistical analysis of the results was performed using one-way analysis of variance (ANOVA), and Tukey's HSD post hoc test for means separation at a significance level of $P \leq 0.05$.

Results

Physico-chemical traits of OMW and obtained fractions

The OMW and the obtained fractions were analysed for pH, TSS and total phenolic content. The pH ranged from 3.41 to 3.96. The TSS and total phenols values increased proceeding from ultrafiltration to reverse osmosis, reaching values of 15.17 °Brix and 8523.23 mg/L in the C3 sample (Table 1).
The P2 and P3 permeates showed the lowest values of both TSS (0.19 and 0.36 °Brix, respectively) and total phenol contents (19.42 mg/L and 55.05 mg/L, respectively).

Table 1.Physico-chemical traits of OMW and obtained fractions.

Samples	pH	TSS (°Brix)	Total phenols (mg/L)
OMW	3.92±0.07[a]	5.40±0.02[d]	2983.39±0.31[d]
C1	3.91±0.08[a]	6.29±0.07[c]	3244.11±0.21[c]
P1	3.94±0.06[a]	5.05±0.07[d]	2888.02±0.02[e]
C2	3.96±0.05[a]	10.35±0.24[b]	6207.41±0.12[b]
C3	3.90±0.14[a]	15.17±0.04[a]	8523.23±0.03[a]
P2	3.45±0.01[b]	0.19±0.04[e]	19.42±0.01[g]
P3	3.41±0.01[b]	0.36±0.28[e]	55.05±0.01[f]
	**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. **Significance at $P \leq 0.01$.

Phenols, antioxidant activity and organic acid detection

Zooming on phenolic compounds, except for other phenols present in traces, HT and TYR were the only phenols detected by HPLC analysis, reaching the highest concentration in the C2 and C3 samples, with 7203.7 and 6936.2 mg/L (HT) and 1046.6 mg/L and 1613.9 (TYR), respectively (Table 2). It is interesting to point out that the C3 sample, despite the highest total phenolic content, showed a slight lower content of HT.

Table 2 Phenols and antioxidant activity detected in OMW and in the obtained fractions.

Sample	HT (mg/L)	TYR (mg/L)	IC_{50}
OMW	3321.07±61.73[c]	508.02±20.40[c]	87.67± 0.17[a]
C1	3415.15±65.94[c]	494.37±0.14[c]	84.00±0.10[ab]
P1	3327.68±42.58[c]	499.11±4.07[c]	80.18±0.18[b]
C2	7203.67±54.85[a]	1046.62±2.50[b]	41.17±0.02[d]
C3	6936.27±43.82[b]	1613.97±6.87[a]	50.95±0.16[c]
	**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. **Significance at $P \leq 0.01$.

Results on antioxidant activity, evaluated by the DPPH method, showed that the proton removal activity of fractions was positively related to the concentration of free phenolic compounds. Lower IC50 values are related to a stronger radical quenching activity. As expected, the lowest IC50 values were detected for the C2 (41.17 IC50) and the C3 samples (50.95 IC50), as reported in Table 2. These results confirmed that the C2 concentrate sample, with the highest HT content, exhibited the highest antioxidant activity. Looking at organic acids, for lactic, acetic and propionic acids a general increase in their concentrations during the filtration process was observed (Table 3), whereas isobutyric acid was detected only in the OMW and C1.

Table 3. Detected organic acids.

Sample	Lactic acid (mg/L)	Acetic acid (mg/L)	Propionic acid (mg/L)	Isobutyric acid (mg/L)
OMW	3583.7±135.80^{c}	6680.9±94.59^{c}	0.00±0.00^{c}	13187.3±507.60^{a}
C1	3554.3±58.78^{c}	6540.6±40.10^{c}	0.00±0.00^{c}	12621.7±374.88^{b}
P1	3733.7±10.35^{c}	6714.7±33.29^{c}	0.00±0.00^{c}	0.00±0.00^{c}
C2	7953.7±7.93^{b}	12137.2±7.38^{b}	2984.4±89.77^{b}	0.00±0.00^{c}
C3	11860.8±107.20^{a}	17612.0±343.96^{a}	5393.9±500.56^{a}	0.00±0.00^{c}
P2	98.6±4.43^{d}	1177.0±135.83^{e}	0.00±0.00^{c}	0.00±0.00^{c}
P3	150.8±4.53^{d}	2460.4±49.56^{d}	0.00±0.00^{c}	0.00±0.00^{c}
	**	**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. **Significance at $P \leq 0.01$.

The highest concentration of lactic acid was detected in the C3 and C2 samples, at 11860.8 and 7953.7 mg/L, respectively, while in the other samples the mean concentration value was 3600 mg/L (in OMW, C1 and P1 samples) and 99 and 151 mg/L in P2 and P3, respectively. The C3 and C2 samples showed the highest concentrations of acetic acid, reaching values of 17612.0 and 12137.2 mg/L, respectively, and were the only samples in which propionic acid was found (5393.9 and 2984.4 mg/L, respectively). Citric and butyric acids were never detected in any samples.

Microbiological analyses of OMW and obtained fractions

Overall, lactobacilli and *Clostridium perfringens* were not detected in OMW, C1, P1, C2 and C3 samples, whereas a mesophilic aerobic bacteria count, ranging between 4.00 and 4.35 Log CFU/mL, was detected in all samples. Furthermore, yeasts and moulds were not found in the P1 and in the C2 samples, whereas a mean value of 3.83 Log CFU/mL was counted in the other samples (Table S1, supplementary material).Regarding the P2 and P3 permeates, *Escherichia coli* (in 100 mL), *Clostridium* spores, sulphite-reducing bacteria, somatic coliphages, intestinal nematodes (helminth eggs) and *Legionella* spp. were not detected and the results were found to comply with the limits imposed by Regulation (EU) 2020/741 on minimum requirements for water reuse (EU, 2020).

Antimicrobial activity

The antimicrobial activity of the OMW and the obtained fractions on pathogenic and probiotic strains was assessed by evaluation of inhibition zones. Overall, all the tested samples did not show any effect on probiotic

tested strains, namely as *L. rhamnosus* CRL1505, *L. paracasei* 101/37, *Bif. animalis* subsp. lactis BLC1 or *P. freudenreichii* DSM 4902. Among the tested samples, the C2 and the C3 concentrates showed inhibitory activity against *E. coli* and *P. aeuruginosa*, with an inhibition zone of 12 and 14 mm, respectively (Table S2, supplementary material). Both the C2 and the C3 concentrates exhibited a dose-dependent antimicrobial activity against pathogens (Table S2). No inhibitory activity was observed against the other tested pathogens (*L. monocytogenes*, *C. albicans*, *St. aureus* or *Salmonella thyphimurium*).

Microbiological, chemical and sensory traits of FBOJ

The C2 concentrate, the richest fraction in the bioactive compound HY, was included in blood orange juice to fortify the nutraceutical component of the product. The C2 concentrate was added to 250 mL commercial orange juice, at a ratio of 1:250 v/v (FBOJ1), 2:250v/v (FBOJ2), 3:250 v/v (FBOJ3), 4:250 v/v (FBOJ4), and the obtained FBOJ analysed at 0, 15 and 60 days of storage, at refrigerated conditions. Regarding microbiological results, in all samples (both FBOJ and controls) the searched microbial groups (as lactobacilli, mesophilic aerobic bacteria and yeasts and moulds) were not detected at any sampling times, except in controls and FBJO1 which at 60 days of storage showed yeasts and moulds densities of 3.3 and 3.0 Log CFU/mL, respectively (data not shown). Looking at physico-chemical results, no significant differences in pH and TSS values was found between control and FBOJ samples (Table 4), at any sampling points. Regarding the total phenolic content, data showed that increasing the volume of the C2 addition, a higher phenol content was found in the fortified juices.

Table 4 Chemical parameters of FBOJ samples fortified with different additions of C2 concentrate.

Samples	Time (days)	pH	TSS (°Brix)	Total phenols (mg/L)	HT (mg/L)	TYR (mg/L)
Commercial juice	0	3.38 ± 0.01	11.60 ± 0.08	3142.2 ± 0.54^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}
FBOJ1	0	3.37 ± 0.01	11.55 ± 0.01	3553.2 ± 2.66^{d}	26.92 ± 0.67^{d}	43.21 ± 4.14^{b}
FBOJ2	0	3.38 ± 0.05	11.67 ± 0.01	3643.6 ± 0.54^{c}	67.43 ± 3.77^{c}	52.65 ± 3.81^{ab}
FBOJ3	0	3.39 ± 0.05	11.65 ± 0.01	3715.2 ± 1.63^{b}	82.23 ± 0.75^{b}	58.08 ± 1.62^{a}
FBOJ4	0	3.39 ± 0.00	11.56 ± 0.01	3893.2 ± 1.09^{a}	100.87 ± 1.42^{a}	56.50 ± 0.86^{b}
		n.s.	n.s.	**	**	**
Commercial juice	15	3.3 ± 0.01^{d}	11.36 ± 0.01^{c}	2900.0 ± 0.54^{e}	0.00 ± 0.00^{e}	0.00±0.00^{b}
FBOJ1	15	3.34 ± 0.01^{c}	11.75 ± 0.01^{a}	3147.9 ± 1.63^{d}	24.28 ± 0.65^{d}	60.20 ± 0.22^{a}
FBOJ2	15	3.35 ± 0.01^{bc}	11.66 ± 0.01^{b}	3174.1 ± 3.26^{c}	48.22 ± 0.38^{c}	58.89 ± 0.97^{a}
FBOJ3	15	3.38 ± 0.01^{a}	11.65 ± 0.00^{b}	3396.8 ± 0.55^{b}	81.01 ± 0.59^{b}	57.75 ± 2.44^{a}
FBOJ4	15	3.36 ± 0.01^{b}	11.67 ± 0.02^{b}	3410.6 ± 0.54^{a}	105.52 ± 1.87^{a}	59.49 ± 0.30^{a}
		**	**	**	**	**
Commercial juice	60	3.31 ± 0.00^{c}	11.70 ± 0.01^{a}	2545.0 ± 0.01^{d}	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}
FBOJ1	60	3.32 ± 0.00^{bc}	11.56 ± 0.06^{ab}	2623.1 ± 0.54^{d}	21.67 ± 0.05^{d}	50.11 ± 0.16^{ab}
FBOJ2	60	3.36 ± 0.02^{a}	11.66 ± 0.04^{a}	2851.1 ± 1.09^{c}	46.29 ± 0.56^{c}	53.60 ± 0.85^{a}
FBOJ3	60	3.35 ± 0.00^{ab}	11.29 ±0.13b	2894.6 ± 0.56^{b}	75.35 ± 2.27^{b}	52.24 ± 0.20^{ab}
FBOJ4	60	3.35 ± 0.01^{abc}	11.26 ± 0.06^{b}	3100.0 ± 1.10^{a}	94.58 ± 2.91^{a}	49.20 ± 2.05^{b}
		*	**	**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column at the same time interval are statistically different. N.s. not significant; *Significance at $p \leq 0.05$; **Significance at $p \leq 0.01$.

In FBOJ4 sample, where the ratio C2/juice was 4:250 v/v, at the same day of fortification (T0), the total phenolic content was 750 mg/L higher than that detected in the control juice. Furthermore, it is interesting to underline that after 60 days of storage, the FBOJ4 sample showed almost the same total phenolic content (3100 mg/L) detected in the control juice at initial time (Table 4). Zooming at content of bioactive molecules, monitored at different sampling times, the FBOJ samples showed a proportional increase in HT and TYR. It is interesting to highlight that the FBOJ1 sample, obtained by adding the lowest volume of C2 extract (1 mL of C2 to 250 mL of juice), exhibited an initial concentration of HT and TYR of 26.92 and 43.21 mg/L, and a concentration of 21.67 and 60.10 mg/L of HT and TYR, respectively, after 60 days of storage. This data allows us to state that the FBOJ1 provides, up to the end of estimated shelf life, the recommended health beneficial intake of HT, as recognised by European Foods Safety Authority (EFSA). Regarding the colour coordinates, the brightness (L*) showed an increasing trend during the shelf life in all tested sample (Table S3 supplementary material). In addition, a significant decrease in the red coordinate (a*), right after 15 days of refrigerated storage, was observed, while the yellow coordinate (b*) remains unchanged over time, reaching the highest values in FBOJ3 and FBOJ4 samples (Table S3, supplementary material). Looking at the sensory parameters, overall, only flavour, off-flavour, bitterness and off- odour showed significant differences (Table 5). The flavour reached the highest score in FBOJ1 sample, but the statistical differences

among samples were not related to the concentrate addition. Flavour descriptor decreased in FBOJ3 and FBOJ4 samples, while the perceived bitterness was higher in FBOJ4 sample, compared to other samples. Off- flavour and off-odour descriptors statistically increased in FBOJ3 and FBOJ4 samples (Figure S4, supplementary material).

Table 5 Sensory traits of FBOJ samples fortified with different additions of C2 concentrate.

	Commercial juice	**FBOJ1**	**FBOJ2**	**FBOJ3**	**FBOJ4**	
Colour	5.62 ± 0.44	5.65 ± 0.44	5.75 ± 0.38	5.68 ± 0.37	5.75 ± 0.38	n.s.
Odour of orange	5.62 ± 0.44	5.42 ± 0.57	5.18 ± 0.59	5.00 ± 0.46	5.06 ± 0.68	n.s.
Acidity	5.00 ± 0.27	5.14 ± 0.20	5.12 ± 0.23	5.06 ± 0.32	5.31 ± 0.37	n.s.
Sweetness	4.50 ± 0.38	4.40 ± 0.25	4.69 ± 0.53	4.19 ± 0.26	4.31 ± 0.37	n.s.
Flavour	5.69 ± 0.37^{ab}	5.85 ± 0.62^{a}	5.50 ± 0.38^{ab}	5.12 ± 0.64^{b}	5.25 ± 0.38^{ab}	*
Off-flavour	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	2.62 ± 0.52^{a}	2.69 ± 0.46^{a}	**
Bitterness	1.31 ± 0.26^{b}	1.37 ± 0.23^{ab}	1.38 ± 0.23^{ab}	1.44 ± 0.18^{ab}	1.69 ± 0.26^{a}	*
Off-odour	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	1.81 ± 0.59^{a}	2.06 ± 0.42^{a}	**

Data are expressed as means ± SD. Mean values with different letters within the same row are statisticallydifferent: ** significance at $p \leq 0.01$; * significance at $p \leq 0.05$; n.s., not significant.

Discussion

Polyphenols from olive fruit, olive mill wastewater or olive oil, *Olea europaea* L. extract and leaf, standardised for their content of HT, possess the health claim approved by EFSA under Article 13 (Health Claims Regulation 1924/2006), in relation to the protection of blood lipids from oxidative damage, which is known to adversely affect cardiovascular health (EFSA, 2011; EC, 2012). Furthermore, in a recent study, the safety and the effects of HT purified (99.5%) from OMW were assessed by administering HT at a daily dose of 45 mg for 8 weeks to volunteers with mild hyperlipidaemia (Lopez-Huertas & Fonolla, 2017). In particular, the authors demonstrated that the administration of HT did not affect markers of cardiovascular disease, blood lipids, inflammatory status, liver or kidney function and that electrolyte balance with vitamin C increased two fold at 4 and 8 weeks, compared to baseline levels (Lopez-Huertas & Fonolla, 2017). In the present study, the tangential membrane filtration technique produced fractions differently concentrated in bioactive compounds. The concentrate C2 showed the highest concentration of HT, known for its antioxidant activity and for playing a role as an intracellular and extracellular scavenger of reactive oxygen species (ROS) (Robles-Almanaz et., 2018). Indeed, in the present study, the higher concentration of HT was positively related to a greater antioxidant activity. Several studies have reported that the addition of OMW phenolic fraction induces a fortification of the nutraceutical component and increases the shelf life of foods (Mikdame et al., 2020; Servili et al., 2011). As a matter of facts, phenolic compounds show wide antimicrobial activity,

such as antibacterial, antiviral and antifungal effects (Marković et al., 2019). Although HT has been reported to in vitro inhibit the growth of several pathogens, included *L. monocytogenes*, *St. aureus*, *Salmonella enterica*, *Yersinia* or beneficial microorganisms, as *L. acidophilus* and *Bifidobacterium bifidum* (Marković et al., 2019), in the present study no inhibitory activity was observed against the probiotic tested strains or against *L. monocytogenes*, *St. aureus*, and *Salmonella* spp. In the present study, the C2 and C3 concentrates showed inhibitory activity against *P. aeuruginosa* and *E. coli*, otherwise Medina et al. (2016) reported that a MIC value of 400 μg/mL of HT was able to affect the growth of *E. coli*, while MIC values higher than 1000 μg/mL were required to affect the growth of *P. aeuruginosa*. Among the tested samples, only the C2 showed antimicrobial activity against *B. subtilis*, Cl. sporogenes and *E. faecalis*. For *B. subtilis*, the results agreed with those reported by Tafesh et al. (2011) and by Galanakis et al. (2018b) who showed the antimicrobial effect of OMW phenolic extracts, in combination with other antioxidants, against *B. subtilis*, *E. coli*, and *P. aeruginosa*. In addition, the two concentrates C2 and C3 did not exhibit any antagonist effect against *C. albicans*. This finding could be due to the hydrophilic nature of OMW concentrates, being the more lipophilic constituents partitioned into the olive oil during processing. Diallinas and co-workers (2018) reported that a lower hydrophilic/lipophilic balance could increase the cellular uptake enhancing the antioxidant or antimicrobial activities. However, the exact mechanism by which HT exerts its antimicrobial activity remains not completely understood (Wei et al., 2018) and Reverón and co-workers (2020) suggested an involvement of ROS overproduction as a mechanism of antimicrobial activity. The widest antimicrobial activity of the C2 concentrate could be related to the higher antioxidant activity even although the complex chemical composition of concentrates includes wide range of phytochemicals with synergistic effects. Nevertheless, it is relevant to underline that the controversial results reported in literature could be due to the lack of a standard method or evaluation criteria for screening antimicrobial activity in plant extracts (Nostro et al., 2000). Differences in antimicrobial assay, growth media, bacterial strains, inoculum size and cell density of the target microorganism make comparisons of antimicrobial data of plant extracts from different sources very difficult. The addition of the C2 concentrate in blood orange juice has been here proposed to obtain a functional beverage with a high content of both flavonoids and HT. Indeed, orange juice is a natural source of vitamin C, and a 200 mL dose provides up to 80% of recommended daily intake (Klimczak et al., 2007). Zooming on the effect of the C2 addition on chemical composition of juice, the results here reported confirmed that the nutraceutical value of the juice was increased and the HT was still revealed up to 60 days of storage at refrigerated conditions. Furthermore, no microbiological differences were observed in samples at any C2 additions, although the yeast and mould growth were detected after 60 days in control and in fortified juice with the lowest addition of C2 (FBOJ1 sample). The addition of any compound to food could have a detrimental effect on sensory and technological properties (Marinelli et al., 2015). The colour of

orange juice influences consumers' choice, above all for blood orange juices that are rich in anthocyanins, responsible of the dark red colour. In this study, the colour of FBOJ was monitored at different times, revealing significant statistical differences on L*, a* and b* parameters. Sensory analyses were carried out at the same day of fortification in order to assess the threshold of perception of the C2 addition to the juice. This parameter is an important preliminary step to investigate the proper concentration of a fortifying agent in designing a new functional food or beverage. Moreover, results of present study confirmed that membrane filtration techniques produce permeate fractions (the P2 and P3) suitable for irrigation, being compliant with limits imposed by Regulation (EU) 2020/741 on minimum requirements for water reuse (EU, 2020) and with the legal limits for releasing into the aquatic system (Cassano et al., 2013; Paraskeva et al., 2007; Russo 2007).

Conclusions

Many consumers currently require supplement with vitamins, minerals and other nutrients, and as never before research-based evidence are required to correctly inform consumer, mainly on natural compounds. At the same time, an increasing interest has been posed on the ingredients used in food products, with a major challenge for 'clean label' ingredient. The present study confirmed that the tangential membrane filtration, an eco-friendly technique, represents a suitable valorisation strategy of OMW. This extraction technology on an industrial scale can effectively represent an income-generating solution for the olive oil industry by creating a collaboration with local food and beverage companies for the formulation of new products with high nutraceutical value. In order to overcome the seasonality of olive oil by-products, a crucial point could be the creation of a continuous production, realized from different industrial by-products, to obtain a supply cycle of phenolic concentrates for food industry. The concentrates rich in HT and TYR, obtained by reverse osmosis, exhibited antimicrobial and antioxidant activity, whereas the last two permeates, thanks to the low chemical load and for complying with the limits fixed by Regulation (EU) 2020/741 on minimum requirements for water reuse (EU, 2020), are suitable for the context of a circular economy. The addition of these concentrates in orange juice formulation implies an increase of phenolic content and provides the suitable amount of molecules with healthy effect on consumer. In detail, the FBOJ samples obtained by adding 2 mL of concentrate into 250 mL of juice showed a higher nutraceutical content without any sensory change. The OMW phenol concentrate and blood orange juice combined in a new functional beverage highlight the beneficial effect of the Mediterranean diet.

Supplementary material

Table S1 Microbiological traits of OMW and obtained fractions.

Sample	Mesophilic aerobic bacteria	Yeasts and moulds
OMW	4.35±0.06	3.93±0.03[a]
C1	4.34±0.05	4.34±0.05[a]
P1	4.00±0.00	<1[c]
C2	4.04±0.05	<1[c]
C3	4.15±0.21	3.24±0.33[b]
	n.s.	**

Data are expressed as Log_{10} CFU/mL ± SD; n.s. not significant; **Significance at $P \leq 0.01$.

Table S2 Antimicrobial activity of C2 and C3 against pathogens.

Target strains	C2			C3		
	raw	1:2	1:4	raw	1:2	1:4
						-
Escherichia coli ATCC 25922	12	7	-	10	8	-
Pseudomonas aeruginosa ATCC 9027	14	8	-	14	8	-
Bacillus subtilis ATCC 19659	12	8	-	-	-	-
Clostridium sporogenes ATCC 11437	12	9	-	-	-	-
Enterococcus faecalis ATCC 29212	8	-	-	-	-	-

Data are expressed as halo diameter of inhibition (Ø mm).

Table S3 Colour parameters of FBOJ samples fortified with different additions of C2 concentrate.

Samples	Time (days)	L*(D65)	a*(D65)	b*(D65)
Commercial juice	T0	48.78 ± 0.12^{b}	42.31 ± 0.13^{a}	52.15 ± 0.21^{c}
FBOJ1	T0	52.45 ± 0.07^{a}	41.48 ± 0.28^{b}	49.89 ± 0.03^{d}
FBOJ2	T0	48.61 ± 0.08^{b}	40.73 ± 0.08^{cd}	55.72 ± 0.08^{a}
FBOJ3	T0	47.59 ± 0.28^{c}	40.51 ± 0.01^{d}	53.57 ± 0.04^{b}
FBOJ4	T0	52.31 ± 0.14^{a}	41.21 ± 0.16^{bc}	51.83 ± 0.21^{c}
		**	**	**
Commercial juice	T15	53.25 ± 0.07^{b}	38.96 ± 0.03^{a}	48.07 ± 0.04^{c}
FBOJ1	T15	57.45 ± 0.63^{a}	37.97 ± 0.02^{b}	45.58 ± 0.13^{d}
FBOJ2	T15	51.20 ± 0.14^{c}	37.20 ± 0.20^{c}	50.71 ± 0.11^{b}
FBOJ3	T15	51.79 ± 0.15^{c}	36.49 ± 0.01^{d}	52.81 ± 0.12^{a}
FBOJ4	T15	51.60 ± 0.28^{c}	35.68 ± 0.03^{e}	52.50 ± 0.14^{a}
		**	**	**
Commercial juice	T60	63.49 ± 0.26^{e}	16.58 ± 0.14^{a}	51.04 ± 0.08^{b}
FBOJ1	T60	70.78 ± 0.31^{a}	14.74 ±0.21^{b}	47.93 ± 0.09^{c}
FBOJ2	T60	69.49 ± 0.26^{b}	14.48 ±0.12^{b}	48.09 ± 0.01^{c}
FBOJ3	T60	68.46 ± 0.13^{c}	14.77 ± 0.14^{b}	51.48 ± 0.06^{a}
FBOJ4	T60	66.85 ± 0.06^{d}	14.94 ±0.06^{b}	51.55 ± 0.02^{a}
		**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column arestatistically different. **Significance at $P \leq 0.01$.

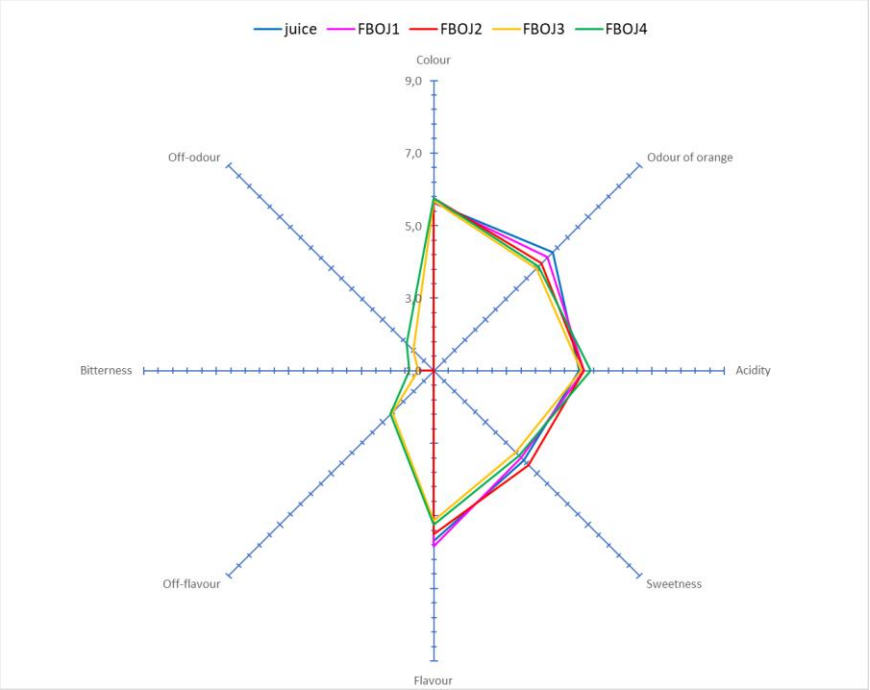


Figure S4 Spider plot of the FBOJ samples fortified with different additions of C2 concentrate.

Acknowledgments: This study was conducted within a Ph.D. research program in Biotecnologie (XXXV cycle) by Paola Foti who received a grant 'Dottorato innovativo con caratterizzazione industriale, PON RI 2014-2020', titled 'Olive oil by-products as a new functional food and source of nutritional food ingredients' from the Department of Agriculture, Food and Environment (Scientific Tutors: C.C. Cinzia Caggia; Flora V. Romeo and Cinzia L. Randazzo). We thank the Azienda Olearia Consoli Pasquale & F.lli s.n.c (Adrano, CT), partner of the doctoral programme, to kindly supply the OMW samples and the filtration system equipment and the Oranfrizer Company (Scordia, Sicily) for kindly supplying the blood orange juice samples.

References

Ballistreri, G., Fabroni, S., Romeo, F. V., Timpanaro, N., Amenta, M. & Rapisarda P. (2019). Anthocyaninsand other polyphenols in citrus genus: biosynthesis, chemical profile, and biological activity. In R. R. Watson (Ed.), *Polyphenols in plants*. *Isolation, purification and extract preparation* (pp.191–216). 2nd edition. Academic Press (Elsevier).

Barba, F. J., Galanakis, C. M., Esteve, M. J., Frigola, A., & Vorobiev, E. (2015). Potential use of pulsed electric technologies and ultrasounds to improve the recovery of high-added value compounds from blackberries. *Journal of Food Engineering*, *167*, 38-44.

Berbel, J., & Posadillo, A. (2018). Review and analysis of alternatives for the valorisation of agro-industrialolive oil by-products. *Sustainability*, *10*(1), Article 237.

Bonaccorso, A., Russo, N., Romeo, A., Carbone, C., Grimaudo, M. A., Alvarez-Lorenzo, C., Randazzo, C.L., Musmeci, T., & Caggia, C. (2021). Coating *Lacticaseibacillus rhamnosus* GG in alginate systems:An emerging strategy towards improved viability in orange juice. *AAPS Pharmaceutical Science and Technology*, *22*(3), 1-14.

Caporaso, N., Formisano, D., & Genovese, A. (2018). Use of phenolic compounds from olive mill wastewater as valuable ingredients for functional foods. *Critical reviews in food science and nutrition*,*58*(16), 2829-2841.

Cassano, A., Conidi, C., Giorno, L., & Drioli, E. (2013). Fractionation of olive mill wastewaters by membrane separation techniques. *Journal of Hazardous Materials*, *248*, 185-193.

Di Nunzio, M., Picone, G., Pasini, F., Chiarello, E., Caboni, M. F., Capozzi, F., Gianotti, A., & Bordoni,

A. (2020). Olive oil by-product as functional ingredient in bakery products. Influence of processing and evaluation of biological effects. *Food Research International*, *131*, Article 108940.

Diallinas, G., Rafailidou, N., Kalpaktsi, I., Komianou, A. C., Tsouvali, V., Zantza, I., Mikros, E., Skaltsounis, A. L., & Kostakis, I. K. (2018). Hydroxytyrosol (HT) analogs act as potent antifungals bydirect disruption of the fungal cell membrane. *Frontiers in Microbiology*, *9*, Article 2624.

EFSA Journal 2011;9(4):2033. Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance of normal blood HDL-cholesterol concentrations (ID 1639), maintenance of normal blood pressure (ID 3781), "anti-inflammatory properties" (ID 1882), "contributes to the upperrespiratory tract health" (ID 3468), "can help to maintain a normal function of gastrointestinal tract" (3779), and "contributes to body defences against external agents" (ID 3467) pursuant to Article 13(1)of Regulation (EC) No 1924/20061.

European Community. Council Regulation No. 432/2012 of 16 May 2012 establishing a list of permitted health claims made on foods, other than those referring to the reduction of disease risk and to children'sdevelopment and health.

Off. J. Eur. Communities 2012, L 136, 1−40.EU Regulation 2020/741 of theEuropean Parliament and of the Council of 25 May 2020 on minimum requirements for water reuse, related to the prescription on the quality of water refined for irrigation purposes in agriculture. OfficialJournal of the European Union, L 177/32.

Faustino, M., Veiga, M., Sousa, P., Costa, E. M., Silva, S., & Pintado, M. (2019). Agro-food byproducts asa new source of natural food additives. *Molecules*, *24*(6), Article 1056.

Foti, P., Romeo, F. V., Russo, N., Pino, A., Vaccalluzzo, A., Caggia, C., & Randazzo, C. L. (2021a). Olivemill wastewater as renewable raw materials to generate high added-value ingredients for agro-food industries. *Applied Sciences*, *11*(16), Article 7511.

Foti, P., Ballistreri, G., Timpanaro, N., Rapisarda, P., & Romeo, F. V. (2021b). Prebiotic effects of citrus pectic oligosaccharides. *Natural Product Research*, 1-4.

Galanakis, C. M. (2018a). Phenols recovered from olive mill wastewater as additives in meat products. *Trends in Food Science & Technology*, *79*, 98-105.

Galanakis, C. M., Tsatalas, P., Charalambous, Z., & Galanakis, I. M. (2018b). Control of microbial growthin bakery products fortified with polyphenols recovered from olive mill wastewater. *Environmental Technology & Innovation*, *10*, 1-15.

Galanakis, C. M., Yücetepe, A., Kasapoğlu, K. N., & Özçelik, B. (2017). High-value compounds from oliveoil processing waste. In Smain Chemat (Ed.), *Edible Oils, Extraction, Processing, and Applications* (pp. 179-204). CRC Press.

Klimczak, I., Małecka, M., Szlachta, M., & Gliszczyńska-Świgło, A. (2007). Effect of storage on the content of polyphenols, vitamin C and the antioxidant activity of orange juices. *Journal of food composition and analysis, 20*(3-4), 313-322.

International Organization for Standardization (2000). Water Quality - Detection and Enumeration of Bacteriophages - Part 2: Enumeration of Somatic Coliphages ISO 10705-2:2000.

International Organization for Standardization (2017). Water Quality – Enumeretion of *Legionella* ISO 11731:2017.ISO 4121 (2003). Sensory Analysis - Guidelines for the use of Quantitative Response Scales.

Lopez-Huertas, E. & Fonolla, J. (2017). Hydroxytyrosol supplementation increases vitamin C levels in vivo. A human volunteer trial. *Redox Biology, 11,* 384–389.

Mallamaci, R., Budriesi, R., Clodoveo, M. L., Biotti, G., Micucci, M., Ragusa, A., & Franchini, C. (2021).Olive tree in circular economy as a source of secondary metabolites active for human and animal healthbeyond oxidative stress and inflammation. *Molecules*, *26*(4), Article 1072.

Marković A. K., Torić J., Barbarić M., & Brala C. J. (2019). Hydroxytyrosol, tyrosol and derivatives and their potential effects on human health. *Molecules*, *24*(10), Article 2001.

Marinelli, V., Padalino, L., Nardiello, D., Del Nobile, M. A., & Conte, A. (2015). New approach to enrichpasta with polyphenols from grape marc. *Journal of Chemistry*, 1, 1-8.

Medina-Martínez, M. S., Truchado, P., Castro-Ibáñez, I., & Allende, A. (2016). Antimicrobial activity of hydroxytyrosol: A current controversy. *Bioscience, Biotechnology, and Biochemistry*, *80*, 801-810.

Mikdame, H., Kharmach, E., Mtarfi, N. E., Alaoui, K., Ben Abbou, M., Rokni, Y., ... & Rais, Z. (2020). By-products of olive oil in the service of the deficiency of food antioxidants: The case of butter. *Journal of Food Quality*, *2020*, Article 6382942.

Nostro, A., Germano, M.P., D'angelo, V., Marino, A., & Cannatelli, M.A. (2000). Extraction methods andbioautography for evaluation of medicinal plant antimicrobial activity. *Letters in AppliedMicrobiology, 30*, 379–384.

Paraskeva, C. A., Papadakis, V. G., Tsarouchi, E., Kanellopoulou, D. G., & Koutsoukos, P. G. (2007). Membrane processing for olive mill wastewater fractionation. *Desalination*, *213*(1-3), 218-229.

Reverón, I., Plaza-Vinuesa, L., Santamaría, L., Oliveros, J. C., de Las Rivas, B., Muñoz, R., & López de Felipe, F. (2020). Transcriptomic evidence of molecular mechanisms underlying the response of *Lactobacillus plantarum* WCFS1 to hydroxytyrosol. *Antioxidants, 9*(5), Article 442.

Robles-Almazan, M., Pulido-Moran, M., Moreno-Fernandez, J., Ramirez-Tortosa, C., Rodriguez-Garcia, C., Quiles, J. L., & Ramirez-Tortosa, M. (2018). Hydroxytyrosol: Bioavailability, toxicity, and clinical applications. *Food Research International*, *105*, 654-667.

Romeo, F. V., Granuzzo, G., Foti, P., Ballistreri, G., Caggia, C., & Rapisarda, P. (2021). Microbial application to improve olive mill wastewater phenolic extracts. *Molecules*, *26*(7), Article 1944.

Russo, C. (2007). A new membrane process for the selective fractionation and total recovery of polyphenols, water and organic substances from vegetation waters (VW). *Journal of Membrane Science*, *288*(1-2), 239-246.

Servili, M., Rizzello, C. G., Taticchi, A., Esposto, S., Urbani, S., Mazzacane, F., Di Maio, I., Sevaggini, R.,Gobbetti, M., & Di Cagno, R. (2011). Functional milk beverage fortified with phenolic compounds extracted from olive vegetation water and fermented with functional lactic acid bacteria. *InternationalJournal of Food Microbiology, 147*(1), 45-52.

Tafesh, A., Najami, N., Jadoun, J., Halahlih, F., Riepl, H., & Azaizeh, H. (2011). Synergistic antibacterialeffects of polyphenolic compounds from olive mill wastewater. *Evidence-Based Complementary and Alternative Medicine*, *2011*, Article 431021.

Timpanaro, N., Strano, M. C., Allegra, M., Foti, P., Granuzzo, G., Carboni, C., & Romeo, F. V. (2021). Assessing the effect of ozonated water on microbial load and quality of Nocellara Etnea table olives. *Ozone: Science & Engineering*, 1-8.

UNI EN ISO 13299, 2016. Sensory Analysis – Methodology – General Guidance for Establishing a Sensory Profile.

UNI EN ISO 8586, 2014. Sensory Analysis - General Guidelines for the Selection, Training and Monitoringof Selected Assessors and Expert Sensory Assessors.

UNI EN ISO 8589, 2014. Sensory Analysis - General Guidance for the Design of Test Rooms.

Weia, J., Wangd, S., Peib, D., Qud, L., Lia, Y., Chena, J., Dib, D., & Gao, K. (2018). Antibacterial Activity of Hydroxytyrosol Acetate from Olive Leaves (Olea Europaea L.). *Natural Product Research, 32*(16),1967–1970.

CHAPTER 2. RECLAIMED WATER FOR CROP IRRIGATION

Treated urban wastewater for lettuce (Lactuca sativa var. Canasta) and tomato (Lycopersicon esculentum, var. Rio) crop irrigation.

Paride Salvatore Occhipinti[1], Nunziatina Russo[1], Alessandra Pino[1], Cinzia L. Randazzo[1,2], Cinzia Caggia[1,2]*

[1]Department of Agriculture, Food and Environment, University of Catania, Via S. Sofia, 100, 95123 Italy;
[2]CRIAB: Centro di Ricerca Interdipartimentale per l'implementazione dei processi di monitoraggio fisico, chimico e biologico nei sistemi di biorisanamento e di acquacoltura, Department of Medical sciences, Surgicals and Advanced Technologies, Hygiene and Public Health "G.F. Ingrassia" - University of Catania 95123 Catania, Italy

*Correspondence: ccaggia@unict.it

This study is still under experimentation. Thus, the reported results and observations are preliminary data which need to be confirmed by further tests and trials.

Abstract

The use of alternative water sources to overcome the scarcity of water demand for agriculture can play a key role to alleviate the pressure on freshwater sources. Despite reclaimed wastewater for irrigation scopes can be an interesting strategy, the potential issues related to environment and the public health, due to the transmission of pathogenic microorganisms must be considered. The present study was aimed at evaluate the impact of irrigation with RW, obtained by different approaches, on microbial composition in vegetables crops. Tomato and lettuce were irrigated with phyto-remediated, phyto-remediated and UV treated wastewater and conventional water. The evaluation in microbial shifts was evaluated through denaturing gradient gel electrophoresis (DGGE). Results showed no significant differences in terms of microbiological traits compared to crops irrigated with conventional water. DGGE analyses showed that in lettuce and tomato irrigated with RW no shift in composition of microbial community was observed. Despite the removal efficacy of each system was considerable, *Escherichia coli, Clostridium* spores and sulphobacteria sporigenes were detected in RW whereas no pathogenic indicators were detected in crop and soil samples. Moreover, in soil samples differently irrigated no significant difference in microbiological traits was observed, showing that irrigation with RW distributed by drip line is suitable for vegetable crops, complying the UE Regulation in terms of absence of pathogenic indicators in vegetable samples.

Introduction

The need for water resources is a consequence of population growth, economic development, climate change and pollution (FAO, 2016). Globally, 70% of fresh water resources are used for irrigation (Hong et al., 2013) and it has been estimated that in some regions of the world, water use has grown much faster than the human population. In this context the reuse of treated wastewater represents an extremely valid strategy (WHO, 2006) able to converte wastewater from waste to a valuable resource. Agricultural irrigation with treated wastewater is already taking place in numerous European and non-European countries (EPA, 2012). However, despite the aforementioned benefits, wastewater reuse still poses risks to both human health and the environment. The most important effects concern the soil microbial community and, as a consequence, structure and osmotic potential of soil (Wong et al., 2014). Reclaimed water (RW) represents a strategy extremely useful. However, based on the quality of the treated effluent, the use of RW can cause risks for plants, soils and for humans. The use of treated wastewater for irrigation may cause disturbances within these communities and impacts soil fertility and productivity. These microbiological alterations in the soil involve a variety of complex variables such as climate, soil, and wastewater characteristics (Lopes et al., 2015).

Microorganisms in soil respond to wastewater irrigation in several ways, including an increase in microbial activities and biomass (Adrover et al., 2012; Becerra-Castro et al., 2015). The quality of the wastewater in terms of nutrients and organic matter may have stimulated different metabolic pathways or microbial activities within the soil (Becerra-Castro et al., 2015). Soil characteristics and processes may influence the extent to which treated wastewater can influence microbial activities. The majority of the processes occurring in soils (80–90%) are facilitated by microbes and hence an increase could lead to an improvement in soil fertility (Hidri et al., 2010). Untreated wastewater contains different kinds of pathogens such as *Salmonella* spp., *Escherichia coli* (*E. coli*), intestinal nematodes and *Legionella* spp. The World Health Organisation and European Union guidelines fixed safety criteria for irrigation purposes, for which RW must comply with standard criteria as specific physico-chemical and microbiological parameters (Ofori et al., 2021). Indeed, regulations and guidelines are enacted by countries, regions and city authorities in the reuse of treated wastewater for irrigation. Examples of such regulations and guidelines are Water Recycling Criteria (California-USA), United States (US) Environmental Protection Agency Guidelines for Water Reuse, World Health Organization Guidelines for Safe Use of Wastewater, Excreta and Greywater, and the European Union (EU) Regulation No. 2020/741. Nevertheless, observations by Gatta et al. (2016), reported *E. coli* threshold far above the limit required by Italian law, as well as in an experimental trial in Lebanon, the irrigation water in most cases had higher fecal coliforms than the World Health Organization proposed limit of 1000 CFU/ 100 mL (Mcheik et al., 2017). In all these studies and many other cases, pathogenic contamination of plant's edible parts was not observed. This was because there was no direct contact between the plants (edible part) and the irrigation water. Indeed, as reported by Cirelli et al. (2012) and Melloul et al. (2001) for fruit trees or vegetables grown on vines and not in direct contact with irrigation water, transmission risks may be lower than for vegetables growing in direct contact with soil and irrigated wastewater. In a study by Nogueira et. al., 2013, where they analysed a lettuce crop irrigated with potable water and treated wastewater, they reported that several crops can be irrigated by wastewater treated with the appropriate care, as the presence of different species could be found. In a study by Christou et al. (2014), in a tomato crop irrigated by a tertiary treated effluent, the results showed no evidence of microbiological contamination of edible parts Although, the entire population of microorganisms, “microbiota” as epiphytes, on the surface, or as endophytes, within tissue, is an important component can be influenced by soil because the plants concurrently absorb both nutritive substances and microorganisms and what else is present in the soil (Hidri et al., 2010). In this context, irrigation waters play a key role in microbiota composition, because they are carriers of microorganisms both pathogenic and not. Wastewater, hence, can affect the composition of the microbiota, both directly, carrying extraneous microorganisms, and indirectly, by promoting bacteria growth through

improving the carbon content in the soil (Hidri et al., 2010). In the crops, it has been seen that the microbial diversity of fruits decreases with increasing distance from the soil; therefore, the greater the distance between soil and fruit, the lower the microbial diversity found (Becerra-Castro et al., 2015). In addition, no less relevant are the indirect effects produced by wastewater. Some of the bacteria in water can be transmitted to the fruit and thus to consumers, mainly through raw vegetables. Opportunistic human pathogens described as endophytic bacteria include members of the genus *Salmonella* and the Enterobacteriaceae family (Markova et al., 2005; Opelt et al., 2007; Rosenblueth and Martínez-Romero, 2006; Wang et al., 2006). The aim of the study is to assess the microbiological quality of tomato and lettuce irrigated with different treated wastewater and conventional water. Contextually, also shifts in terms of tomato and lettuce microbiota will be evaluated through the technique of denaturing gradient gel electrophoresis (DGGE).

Results

Microbiological analyses of water samples

Results of microbiological analyses of irrigation water and untreated wastewater samples are reported in Table 1. The *E. coli* load, which was of 7.29 ± 0.15 Log cells $100mL^{-1}$ in the Imhof tank, was reduced through the phyto-remediation of about 5.5 Log cells $100mL^{-1}$, and further with phyto-remediation coupled to UV lamp treatment till showing a *E. coli* removal of about 6.7 Log cells $100mL^{-1}$. *Clostridium* spores load as well as showed a reduction of about 1.2 Log spore mL-1 with phyto-remediation treatment, whereas was not detected in water samples Phyto-remediated and UV treated. Regarding the Sulphobacteria sporigenes load, showed a reduction of about 3.7 Log CFU mL^{-1} with phyto-remediation treatment, and of about 4.8 7 Log CFU mL^{-1} with phyto-remediation coupled to UV treatment. Overall, *E. coli, Clostridium* spores and sulphobacteria sporigenes were not detected in control water samples. Anyway, presence of *Legionella* spp. was not observed in any water samples.

Table 1. Microbiological indicators detected in water samples taken at inlet (Imhof tank), at outlet point of phyto-remediation system (phyto-remediated water), at outlet point of UV treatment (phyto-remediated water + UV) and from freshwater sources of the agritourist structure.

		Imhof tank wastewater	Phyto-remediated water	Phyto-remediated water + UV	Conventional water
E. coli	Log cells 100 mL^{-1}	7.29 ± 0.15	1.75 ± 0.25	0.5 ± 0.5	ND*
Clostridium spores	Log spore mL^{-1}	2.23 ± 0.11	0.95 ± 0.05	ND	ND*
Sulphobacteria sporigenes	Log CFU mL^{-1}	4.89 ± 0.55	1.14 ± 0.15	0.9 ± 0.10	ND*

*: not detected

Microbiological analyses of lettuce

Results of microbiological analyses of lettuce are reported in Table 2. Overall *E. coli* and *Salmonella* spp. were never detected in lettuce samples. The Enterobacteriaceae count showed the highest value in the samples irrigated with phyto-remediated wastewater thought drip line yellow (4.17 ± 0.39 UFC g^{-1}), even though it was not significantly different than values registered both in samples irrigated with phyto-remediated and UV treated wastewater and in control samples. The same observations were highlighted in total coliform, yeast and mould, and total aerobic mesophilic load, for which values of 5.15 ± 0.64, 4.85 ± 0.76 and 4.98 ± 0.40 UFC g^{-1} were detected for samples irrigated with phyto-remediated wastewater, respectively. The same trend was observed for enterococci, for which the highest value was detected in samples irrigated with phyto-remediated wastewater thought yellow dripline (4.95 ± 0.18 UFC g^{-1}), with values significantly higher than those detected in control samples with yellow dripline (3.99 ± 0.79 UFC g^{-1}) and in samples irrigated with phyto-remediated and UV treated wastewater – yellow dirpline (3.67 ± 0.47 UFC g^{-1}). Anyway, no significant differences were shown using different driplines for the same water sample.

Table 2. Microbiological analyses of lettuce samples irrigated with phyto-remediated water (Phyto), phyto-remediated and UV treated water (Phyto + UV) and conventional water (Control) through yellow and blue driplines (treated with bacteriostatic substances 1 and 2, respectively) and green dripline (not treated).

Samples		Enterobacteriaceae	Aerobic Mesophilic Bacteria	Total Coliform	Enterococci	Yeast and Mould
Phyto	Green	4.16 ± 0.35^{a}	4.71 ± 0.21^{a}	4.77 ± 0.30^{a}	4.10 ± 0.01^{ab}	4.69 ± 0.27^{a}
	Yellow	4.17 ± 0.39^{a}	4.98 ± 0.40^{a}	4.37 ± 0.62^{a}	4.95 ± 0.18^{a}	4.47 ± 0.71^{a}
	Blue	4.05 ± 0.41^{a}	4.76 ± 0.38^{a}	5.15 ± 0.64^{a}	3.76 ± 0.11^{abc}	4.85 ± 0.76^{a}
Phyto+ UV	Green	3.56 ± 0.54^{a}	4.01 ± 0.54^{a}	3.37 ± 0.64^{a}	3.49 ± 0.63^{abc}	3.65 ± 0.35^{a}
	Yellow	3.51 ± 0.31^{a}	3.99 ± 0.72^{a}	3.11 ± 1.55^{a}	3.67 ± 0.47^{bc}	3.99 ± 1.54^{a}
	Blue	3.83 ± 0.21^{a}	4.33 ± 0.61^{a}	4.39 ± 0.37^{a}	2.84 ± 0.63^{c}	4.31 ± 0.76^{a}
Control	Green	4.09 ± 0.89^{a}	4.69 ± 0.31^{a}	4.59 ± 0.63^{a}	3.47 ± 0.41^{bc}	4.06 ± 1.22^{a}
	Yellow	3.90 ± 0.20^{a}	4.91 ± 0.30^{a}	4.77 ± 0.60^{a}	3.99 ± 0.79^{bc}	4.80 ± 0.50^{a}
	Blue	3.97 ± 0.82^{a}	4.76 ± 0.13^{a}	4.60 ± 0.40^{a}	4.04 ± 0.66^{abc}	4.48 ± 0.32^{a}

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. Significance at $p \leq 0.03$.

Microbiological analyses of tomato

Results of microbiological analyses of tomato are reported in Table 3. Overall, *E. coli, Salmonella* spp., total coliform and enterococci were never detected in tomato samples. The Enterobacteriaceae count showed the highest value in control samples thought drip line green (1.29 ± 0.17 UFC g^{-1}), even though it is not significantly different than values registered in samples irrigated with phyto-remediated and with and without UV treatment. Also the yeast and mould count showed the highest value in control samples, but thought drip line yellow, however not significantly different than values registered in samples irrigated with phyto-remediated and with and without UV treatment. Instead, in aerobic mesophilic bacteria count was observed the highest value in samples irrigated with phyto-remediated water, in particular thought drip line blue (1.56 ± 0.38 UFC g^{-1}), although, they are not significantly different than values registered in control samples and in samples irrigated with phyto-remediated and UV treated water.

Table 3. Microbiological analyses of tomato samples irrigated with phyto-remediated water (Phyto), phyto-remediated and UV treated water (Phyto + UV) and conventional water (Control) through yellow and blue driplines (treated with bacteriostatic substances 1 and 2, respectively) and green dripline (not treated).

Samples		Enterobacteriaceae	Aerobic Mesophilic Bacteria	Yeast and Mould
Phyto	Green	1.08 ± 0.16^{a}	1.32 ± 0.14^{a}	1.49 ± 0.37^{a}
	Yellow	1.07 ± 0.20^{a}	1.38 ± 0.10^{a}	1.17 ± 0.11^{a}
	Blue	1.10 ± 0.17^{a}	1.56 ± 0.38^{a}	1.25 ± 0.16^{a}
Phyto+ UV	Green	1.01 ± 0.14^{a}	1.01 ± 0.24^{a}	1.25 ± 0.15^{a}
	Yellow	1.22 ± 0.11^{a}	1.02 ± 0.52^{a}	1.29 ± 0.24^{a}
	Blue	1.35 ± 0.20^{a}	1.11 ± 0.61^{a}	1.31 ± 0.16^{a}
Control	Green	1.29 ± 0.17^{a}	1.29 ± 0.31^{a}	1.06 ± 0.52^{a}
	Yellow	1.11 ± 0.34^{a}	1.31 ± 0.30^{a}	1.80 ± 0.50^{a}
	Blue	1.01 ± 0.14^{a}	1.16 ± 0.13^{a}	1.70 ± 0.52^{a}

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. Significance at $p \leq 0.03$.

Microbiological analyses of soil samples

Results of microbiological analyses of soil samples are reported in Figure 1. Overall, results showed that *E. coli* and *Salmonella* sp. were never detected. Moreover, any significant difference among cell densities of Enterobacteriaceae, aerobic mesophilic bacteria, total coliform, enterococci and yeast and mould was revealed among samples differently treated in trems of irrigation water.

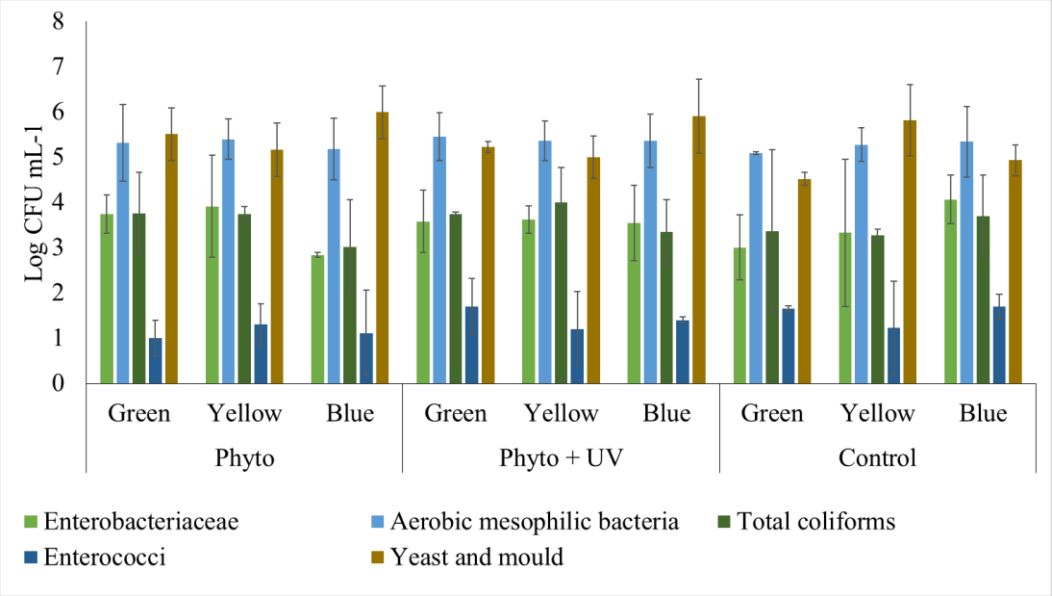


Figure 1. Microbiological results of soil samples irrigated with phyto-remediated water (Phyto), phyto-remediated and UV treated water (Phyto + UV) and conventional water (Control) through yellow and blue driplines (treated with bacteriostatic substances 1 and 2, respectively) and green dripline (not treated).
Data are expressed as means ± SD. Mean values with different letters are statistically different. Values of same microbiological indicator followed by different lowercase letters are significantly different ($p \leq 0.03$).

PCR-DGGE results

In order to highlight any shifts in the microbial community (both eukariotic and procariotic), samples of lettuce and tomato irrigated through drip line containing 2 different bacteriostatic compounds (yellow and blue) with RW water, phytoremediated and UV treated RW were subjected to PCR-DGGE analysis and the obtained profiles compared. The prokariotic and eukariotic profiles of lettuce and tomato samples are reported in Figure 2. In details, in both 16S and 18S DGGE gels, reported in panel A and B, comparing each other the band obtained in the single line (Line 1, 2, 3, 4, 5, 6) is possible to hihgligh how the microbial community relative to lettuce samples differently irrigated, did not show anys shifhts. Focusing on tomato DGGE analyses, prokariotic and eukariotic profiles of tomato samples (Panel C and D respectively), reported a number of bands lower compared to the profiles found in lettuce DGGE analyses. Contextually, as already observed in lettuce DGGE

analysis, comparing each profile obtained from tomato samples differently irrigated, no significant shifts were found.

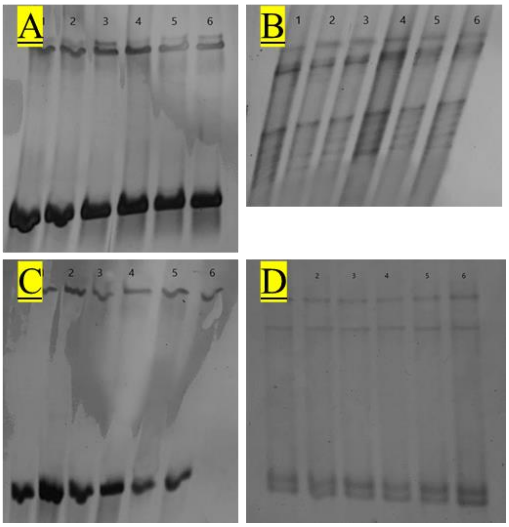


Figure 2. Denaturing gradient gel electrophoresis (DGGE) patterns of lettuce samples 16S rRNA gene fragments amplified using 16S primers set [(F-968-GC, R-1401] (Panel A). DGGE patterns of lettuce samples 18S rRNA gene fragments amplified using 18S primers set [NL1GC, LS2] (Panel B). DGGE patterns of tomato samples 16S rRNA gene fragments amplified using 16S primers set [F-968-GC, R-1401] (Panel C). DGGE patterns of tomato samples 18S rRNA gene fragments amplified using 18S primers set [NL1GC, LS2]. Line 1: lettuce sample irrigated with conventional water through dripline treated with bacteriostatic substance 1 (dripline yellow); Line 2: lattuce sample irrigated with conventional water through dripline treated with bacteriostatic substance 2 (blue dripline); Line 3: lettuce sample irrigated with water from the phytodepuration system with dripline treated with bacteriostatic substance 1 (dripline yellow); Line 4: lettuce sample irrigated with water from the phytodepuration system through drip line treated with bacteriostatic substance 2 (blue drip line); Line 5: lettuce sample irrigated with water from the phytodepuration system coupled to UV lamp through drip line treated with bacteriostatic substance 1 (drip line yellow); Line 6: lettuce sample irrigated with water from the phytodepuration system coupled to UV lamp through drip line treated with bacteriostatic substance 2 (drip line green).

Discussion

The use of treated waste water as irrigation water can play a role in overcoming the scarcity of water. However, as reported by several authors (Armon et al., 1994; Howard and Hutcheson, 2003; Ibenyassine et al., 2006; Tyler and Triplett, 2008) a positive correlation between plant contamination and wastewater irrigation has been described, suggesting that wastewater can be an important source of bacteria that can colonise plants. Hence, due to transmissible pathogens, including several genera, as *Salmonella* and genera belonging to Enterobacteriaceae (Markova et al., 2005; Opelt et al., 2007; Rosenblueth and Martínez-Romero, 2006), the risk for human health cannot be ignored. In this context, to protect public health, regulations and guidelines are enacted by countries, regions and city authorities in reuse of treated wastewater for irrigation. In particular, UE regulation mandates all forms of treated wastewater or reclaimed water use for irrigation to be disinfected prior application to crops (European Comission, 2020). Great emphasis is placed on the compliance of microbiological

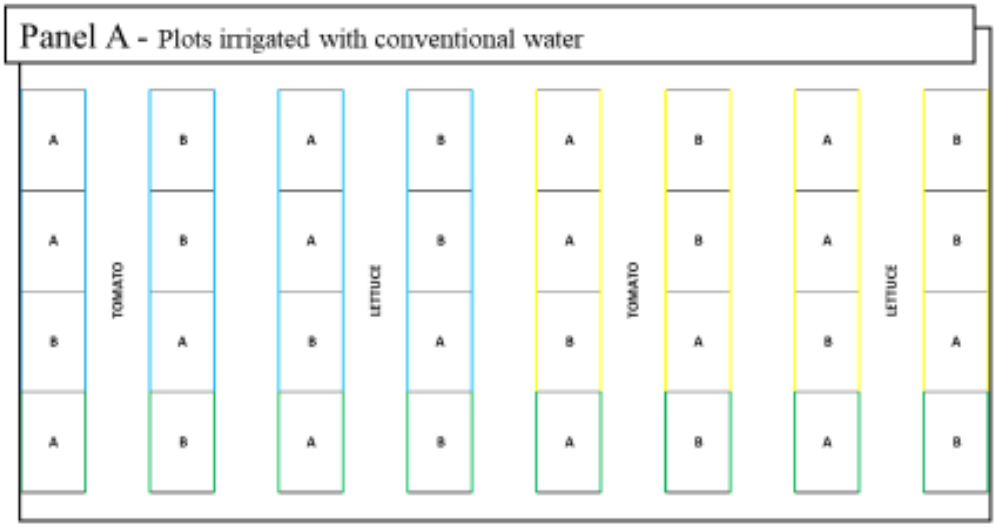
parameters, recommending farmers to adopt barriers that prevent direct contact of irrigation water with food. Sprinkler and furrow irrigation using treated wastewater during fruiting or before the harvesting should be discouraged, since the possibility of water having contact with fruits or edible parts is high, preferring localised drip irrigation, in order to avoid any contact between water and vegetable surface. In light of this, the microbiological analyses on vegetable crops did not show any pathogenic contamination, both in lettuce samples, with their edible part in contact with soil and water, and tomato, with the edible part not in contact with soil. Moreover, also the larger microbial community did not show any significant shifts, both in terms of composition (as reported by the DGGE analyses), and in terms of distribution, where the microbiological analyses did report any significant difference on microbial densities among vegetables irrigated with different treated wastewaters. Indeed, water is not in contact with the edible part of the vegetable because the irrigation is localized, with reduced flow and distributed through drip line at soil level. Soil is described as a matrix that hosts a large variety of cells harboring a great variety of cell types, including intact, viable, non-viable and partially or fully disintegrated (i.e., autolyzed) cells which contribute to its physico-chemical and microbiological characteristics. In the present study microbiological analyses of soil samples showed that the irrigation with RW did not induced pathogenic contamination neither an increase of microbial detected load. In this case, the buffering effect of the soil, which is described as resilient to disturbing effect as pathogenic or external microorganisms' contamination. A similar observation was made by Gatta et al. (2016) and Mcheik et al., (2017), where despite the irrigation water used by them in most cases had higher fecal coliforms, pathogenic contamination of plant's edible parts was not observed. This was because there was no direct contact between the plants (edible part) and the irrigation water.

Material and methods

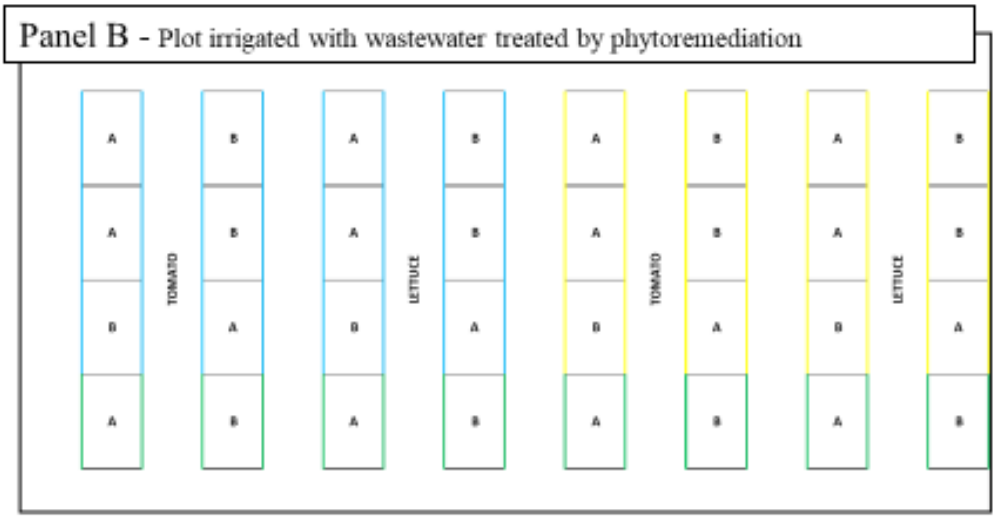
Experimental design

The lettuce (*Lactuca sativa* var. Canasta) and tomato (*Lycopersicon esculentum*, var. Rio) were produced at the experimental field located at the 'Valle dei Margi' farm in Grammichele (Italy), and irrigated with conventional water, wastewater from the farm facilities treated by phytoremediation and with wastewater treated by phytoremediation and UV. At the same time, two different treatments with bacteriostatic substances (confidential information from Irritec spa) applied to drip lines used for crop watering were evaluated. The experimental field was plotted into 9 different representative plots (Figure 3 - Panel A, B and C): plot 1- Irrigated with conventional water (Control) with dripline treated with bacteriostatic substance 1 (dripline yellow); plot 2- Irrigated with conventional water (Control) with dripline treated with bacteriostatic substance 2 (blue dripline); plot 3- Irrigated with

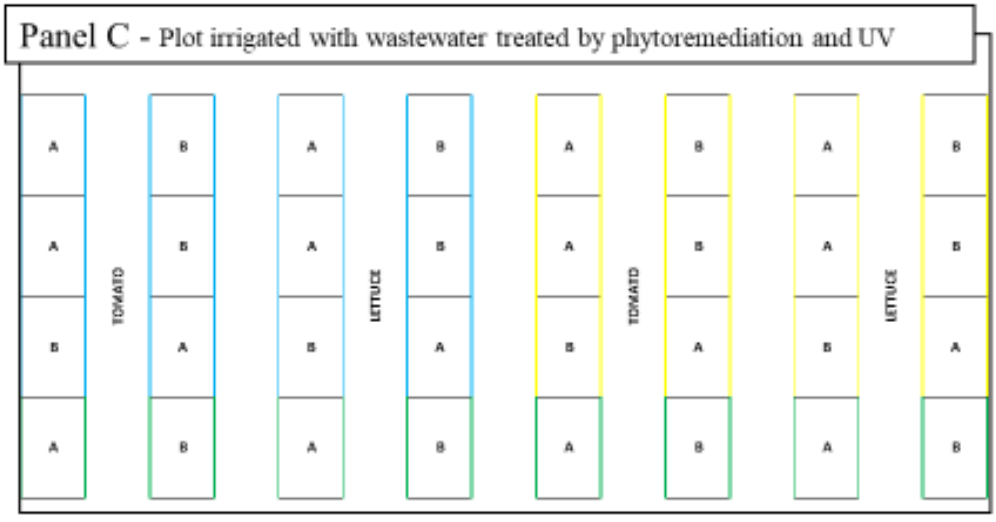
conventional water (Control) with dripline not treated with bacteriostatic substance 2 (dripline green); plot 4 - Irrigated with water from the phytodepuration system with dripline treated with bacteriostatic substance 1 (dripline yellow); plot 5- Irrigated with water from the phytodepuration system with drip line treated with bacteriostatic substance 2 (blue drip line); plot 6- Irrigated with water from the phytodepuration system with dripline not treated with bacteriostatic substance 1 (dripline green); plot 7- Irrigated with water from the phytodepuration system treated with UV, with drip line treated with bacteriostatic substance 1 (drip line yellow); plot 8- Irrigated with water from the UV-treated phytodepuration system, with drip line treated with bacteriostatic substance 2 (blue drip line); plot 9- Irrigated with water from the UV-treated phytodepuration system, with drip line treated with bacteriostatic substance 2 (drip line green)



Legenda. Yellow lines: plot 1; Blue lines: plot 2; Green lines: plot 3. The capital letters A and B represent two replica.



Legenda. Yellow lines: plot 4; Blue lines: plot 5; Green lines: plot 6. The capital letters A and B represent two replica.



Legenda. Yellow lines: plot 7; Blue lines: plot 8; Green lines: plot 9. The capital letters A and B represent two replica.

Figure 3. Graphical representation of experimental fields for lettuce and tomato production.

Water samples

Water samples were analysed to validate the bacterial removal performance (as logarithmic unit reduction) of phytoremediation system and for the classification of treated water quality, according to EU Regulation 2020/741, which establishes minimum requirements for water reuse. Microbiological analyses were performed on water samples collected from: 1- Imhoff tank outlet; 2- Horizontal sub-surface flow outlet; 3- Surface flow outlet; 4- UV-lamp system outlet and one sample for each plot as described above, using the membrane filtration method (Standard Methods for the Examination of Water and Wastewater: APHA, 2017). In detail, for the detection and count of *E. coli*, 100 mL of water were filtered through membrane filters (0.45 µm pores) and placed in Bio-Rad's RAPID's *E. coli* ™2 Agar plates, incubated at 37 °C for 24h. For *Clostridium* spore detection 1 mL of sample was placed in sterile 50 mL tubes, 24 mL of sterile distilled water were added and the tubes soaked at 75 ± 5 °C for 10 minutes. Then 25 mL of liquid Sulphite Polymyxin Sulphadiazine (SPS) (45 °C), prepared at double (2X) concentration, were added and the tubes incubated at 37 °C for 24h. The detection and enumeration of *Legionella* spp. was conducted following the procedure ISO 11731:2017 Water quality - Enumeration of *Legionella*.

Vegetables and soil samples

Two samples of lettuce, tomato and relative soil samples were collected from each plot and transferred, under refrigerated conditions, to Agri-Food Microbiology Laboratory at Di3A. Microbiological analyses were immediately performed. In detail, for each sample, 25 g were appropriately cut and diluted in 225 mL of sterile saline (0.9 % NaCl) solution in placed in a stomacher for 6 min. Microbiological determinations were performed using the streaking technique; in detail, 100 µL of sample suspension were plated on the following culture media and under the following incubation conditions: Kanamycin Aesculin Azide (KAA) Agar at 37°C for 48 h for enterococcal counts; Sabouraud Dextrose Agar (SDA) at 25°C for 48 h for yeast and mould counts; Bio-Rad's RAPID' *E. coli* ™2 Agar at 37°C for 24 h for total coliform and *E. coli* counts; Plate Count Agar (PCA) at 37°C for 48 h for determination of the total aerobic mesophilic load; Violet Red Bile Glucose Agar (VRBGA) at 37°C for 48 h for enumeration of Enterobacteriaceae. The detection of *Salmonella* was conducted following the protocol ISO6579-1-2017.

Total DNA extraction

The lettuce, the tomato and the soil samples (50 g) were propriately cut, diluted in 100 mL of sterile saline (0.9 % NaCl) and placed in a stomacher for 6 min. The solution was then placed into sterile 50

mL tubes, which were then centrifuged 10 min at 4700 rpm under refrigerated conditions (4 °C). The collected pellet was resuspended in sterile saline (0.9 % NaCl), and the solution was transferred into sterile 2 mL tubes, which were then centrifuged for 10 min at 14,000 rpm under refrigerated conditions (4 °C). The pellet obtained was then subjected to total DNA extraction following the CTAB method (Doyle and Doyle, 1990). For each treated sample, in sterile 1.5 mL tubes, 30 mg of pellets were added to 700 µL of CTAB buffer [20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 2% CTAB] and placed for 30 minutes at 65°C and then for 5 minutes at 20°C. 300 µL of phenol-chloroform-isoamyl (24:25:1) was added and vortex mixed. After centrifugation for 20 min at 6800 rpm, the supernatant was recovered, approximately 400 µL, and transferred to a new 1.5 mL tube, adding 400 µL of isopropanol, and then centrifuged for 5 minutes at 10,000 rpm. Discarding the supernatant and resuspending the pellet in 300 µL 70% ethanol, it was centrifuged for 5 minutes at 10,000 rpm. The obtained pellet was freed from the supernatant and left to dry, then resuspended in 40 µL of ultrapure water. DNA extracts were analysed by electrophoresis in 1.5% (wt/vol) agarose gels with TAE 1X buffer (40 mMTriseHCl pH 7.4, 20 mM sodium acetate, 1.0 mM Na2-EDTA), running at 100 V for 15 min, and stored at -20 °C until they were used.

PCR-denaturing gradient gel electrophoresis (DGGE) analysis

PCR was performed in a final volume of 50 µL, containing 0.2 mM of each primer for yeasts (NL1GC, LS2; El Sheikha et al., 2009) and for bacteria (F-968-GC, R-1401; Nubel, U. et al., 1996) using kit DreamTaq DNA Polymerase 2X buffer, dATP, dCTP, dGTP and dTTP, 0.4 mM each, and 4 mM MgCl2, 2 µL extracted DNA (≈30 ng) and Water nuclease-free up to 50 µL. The amplification was carried out as follows: an initial denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 60 s, 52 °C for 2 min, 72 °C for 2 min and a final extension at 72 °C for 7 min for yeasts; an initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 60 s, 63 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 5 min for bacteria. Amplification products were analyzed by electrophoresis in 1.5% (wt/vol) agarose gels with TAE 1X buffer (40 mMTriseHCl pH 7.4, 20 mM sodium acetate, 1.0 mM Na2-EDTA), running at 100 V for 45 min, and stored at -20 °C until they were used. The PCR products were analyzed by DGGE by using a Bio-Rad Dcode universal mutation detection system (Bio-Rad Laboratories, USA), using the procedure first described by El Sheikha (2010) for yeast and Muyzer (1993) for bacteria. Thirty microliter of PCR amplicons were loaded into 8% (w/v) polyacrylamide gels (acrylamide/N,N0-methylene bisacrylamide, 37.5/1, Promega) in 1x TAE buffer (40mMTriseHCl pH 7.4, 20mMsodium acetate, 1.0mM Na2-EDTA). Yeast electrophoresis experiments were performed at 60 °C using a denaturing gradient ranging from 30-60% (100% corresponded to 7 M urea and 40% [v/v] formamide). The gels were electrophoresed at 200 V for 10 min and then at 80 V for 12 h. Bacteria electrophoresis experiments were performed at 60 °C using a

denaturing gradient ranging from 30-60%, (100% corresponded to 7 M urea and 40% [v/v] formamide). The gels were electrophoresed at 200 V for 10 min and then at 85 V for 16 h.

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's HSD post hoc test for means separation were performed using the STATISTICA ETL software (version 10, StatSoft. inc., Tulsa, OK, USA). The significance level was set at $p \leq 0.03$.

Conclusion

The lettuce and tomato crops irrigated with RW treated by phyto-remediation system or phyto-remediation coupled with UV treatment showed not significant differences in terms of microbiological traits compared to crops irrigated with conventional water. Contextually, the genomic analyses of lettuce and tomato microbiota, revealed as the irrigation water did not affect the composition of microbial community. Despite the removal efficacy of both system was considerable, the water samples reported the presence of the pathogenic indicators, as *E. coli, Clostridium* spores and sulphobacteria sporigenes. Although no detection of pathogenic indicators was registered in microbiological analyses both of the crops and of the soil. Moreover, as already observed in vegetable microbiological analyses, neither the soil samples differently irrigated did not highlight any significant differences in microbiological quality. Results of present study confirmed that RW can be proposed for irrigation distributed by drip line are suitable for vegetable crops, complying, at the same time, the UE regulation due to the absence of pathogenic indicator in any vegetable samples.

References

Adrover, M., Farrús, E., Moyà, G., & Vadell, J. (2012). Chemical properties and biological activity in soils of Mallorca following twenty years of treated wastewater irrigation. Journal of environmental management, 95, S188-S192.

APHA/AWWA/WEF (2017) Standard Methods for the Examination of Water and Wastewater (23rd edition), American Public Health Association, American Water Works Association, Water Environment Federation, Denver.

Armon, R., Dosoretz, C. G., Azov, Y., & Shelef, G. (1994). Residual contamination of crops irrigated with effluent of different qualities: a field study. Water Science and Technology, 30(9), 239.

Becerra-Castro, C., Lopes, A. R., Vaz-Moreira, I., Silva, E. F., Manaia, C. M., & Nunes, O. C. (2015). Wastewater reuse in irrigation: A microbiological perspective on implications in soil fertility and human and environmental health. *Environment international*, *75*, 117-135.

Christou, A., Maratheftis, G., Eliadou, E., Michael, C., Hapeshi, E., & Fatta-Kassinos, D. (2014). Impact assessment of the reuse of two discrete treated wastewaters for the irrigation of tomato crop on the

soil geochemical properties, fruit safety and crop productivity. Agriculture, ecosystems & environment, 192, 105-114.

Cirelli, G. L., Consoli, S., Licciardello, F., Aiello, R., Giuffrida, F., & Leonardi, C. (2012). Treated municipal wastewater reuse in vegetable production. Agricultural Water Management, 104, 163-170.

Doyle, J.J., Doyle, J.L. (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

El Sheikha, A. F. (2010). Determination of geographical origin of Shea tree and Physalis fruits by using the genetic fingerprints of the microbial community by PCR/DGGE. Analysis of biological properties of some fruit extracts. Analysis of biological properties of some fruit extracts.

El Sheikha, A. F., Condur, A., Métayer, I., Le Nguyen, D. D., Loiseau, G., & Montet, D. (2009). Determination of fruit origin by using 26S rDNA fingerprinting of yeast communities by PCR–DGGE: preliminary application to Physalis fruits from Egypt. Yeast, 26(10), 567-573.

FAO (Food and Agriculture Organization of the United Nations), 2015. Aquastat Information System on Water and Agriculture. FAO, Rome, Italy. http://www.fao.org/WAICENT/FAOINFO/AGRICULT/AGL/aglw/aquastat/main/index.stm

Gatta, G., Libutti, A., Beneduce, L., Gagliardi, A., Disciglio, G., Lonigro, A., & Tarantino, E. (2016). Reuse of treated municipal wastewater for globe artichoke irrigation: Assessment of effects on morpho-quantitative parameters and microbial safety of yield. Scientia horticulturae, 213, 55-65.

Hidri, Y., Bouziri, L., Maron, P. A., Anane, M., Jedidi, N., Hassan, A., & Ranjard, L. (2010). Soil DNA evidence for altered microbial diversity after long-term application of municipal wastewater. *Agronomy for sustainable development*, *30*(2), 423-431.

Howard, M. B., & Hutcheson, S. W. (2003). Growth dynamics of Salmonella enterica strains on alfalfa sprouts and in waste seed irrigation water. Applied and environmental microbiology, 69(1), 548-553.

Ibenyassine, K., AitMhand, R., Karamoko, Y., Cohen, N., & Ennaji, M. M. (2006). Use of repetitive DNA sequences to determine the persistence of enteropathogenic Escherichia coli in vegetables and in soil grown in fields treated with contaminated irrigation water. Letters in Applied Microbiology, 43(5), 528-533.

International Organization for Standardization. (2017). ISO 11731: 2017 Water Quality—Enumeration of Legionella.

International Standard Organization. (2017). ISO 6579-1: 2017: Microbiology of the food chain–Horizontal method for the detection, enumeration and serotyping of Salmonella–Part 1: Detection of Salmonella spp.

Lopes, A. R., Becerra-Castro, C., Vaz-Moreira, I., Silva, M. E. F., Nunes, O. C., & Manaia, C. M. (2015). Irrigation with treated wastewater: Potential impacts on microbial function and diversity in agricultural soils. *Wastewater Reuse and Current Challenges*, 105-128

Markova, Y. A., Romanenko, A. S., & Dukhanina, A. V. (2005). Isolation of bacteria of the family Enterobacteriaceae from plant tissues. Microbiology, 74(5), 575-578.

Mcheik, M., Toufaily, J., Haj Hassan, B., Hamieh, T., Abi Saab, M. T., Rouphael, Y., ... & Al Hadidi, L. (2017). Reuse of treated municipal wastewater in irrigation: a case study from Lebanon and Jordan. Water and Environment Journal, 31(4), 552-558.

Melloul, A. A., Hassani, L., & Rafouk, L. (2001). *Salmonella* contamination of vegetables irrigated with untreated wastewater. World Journal of Microbiology and Biotechnology, 17(2), 207-209.

Muyzer, G., De Waal, E. C., & Uitterlinden, A. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and environmental microbiology, 59(3), 695-700.

Nogueira, S. F., Pereira, B. F. F., Gomes, T. M., De Paula, A. M., Dos Santos, J. A., & Montes, C. R. (2013). Treated sewage effluent: Agronomical and economical aspects on bermudagrass production. Agricultural Water Management, 116, 151-159

Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R. I., ... & Backhaus, H. (1996). Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. Journal of Bacteriology, 178(19), 5636-5643.

Ofori, S., Puškáčová, A., Růžičková, I., & Wanner, J. (2021). Treated wastewater reuse for irrigation: Pros and cons. Science of The Total Environment, *760*, 144026.

Opelt, K., Berg, C., & Berg, G. (2007). The bryophyte genus Sphagnum is a reservoir for powerful and extraordinary antagonists and potentially facultative human pathogens. FEMS Microbiology Ecology, 61(1), 38-53.

Regulation (EU), 2020. 741 of the european parliament and of the council of 25 May 2020 on minimum requirements for water reuse. Official Journal of the European Union. L 177/32, 5.6.2020.

Rosenblueth, M., & Martínez-Romero, E. (2006). Bacterial endophytes and their interactions with hosts. Molecular Plant-Microbe Interactions, 19(8), 827-837.

Sun, Y., Chen, Z., Wu, G., Wu, Q., Zhang, F., Niu, Z., Hu, H.-Y. 2016. Characteristics of water quality of municipal wastewater treatment plants in China: implications for resources utilization and management. Journal of Cleaner Production, 131, 1-9

Tyler, H. L., & Triplett, E. W. (2008). Plants as a habitat for beneficial and/or human pathogenic bacteria. Annu. Rev. Phytopathol., 46, 53-73.

Wang, E. T., Tan, Z. Y., Guo, X. W., Rodríguez-Duran, R., Boll, G., & Martínez-Romero, E. (2006). Diverse endophytic bacteria isolated from a leguminous tree Conzattia multiflora grown in Mexico. Archives of Microbiology, 186(4), 251-259.

Wang FH, Qiao M, Lv ZE, Guo GX, Jia Y, Su YH, et al., (2014). Impact of reclaimed water irrigation on antibiotic resistance in public parks, Beijing, China. Environ. Pollut. 184, 247–253.

CHAPTER 3. MICROALGAE LITTERATURE REVIEW

Current challenges of microalgae applications: exploiting the potential of non-conventional microalgae species.

Paride Salvatore Occhipinti[1], Nunziatina Russo[1,2], Paola Foti[1], Irene Maria Zingale[1], Alessandra Pino[1,2], Flora Valeria Romeo[4], Cinzia L. Randazzo[1,2,3] Cinzia Caggia[1,2,3]*

[1]Department of Agriculture, Food and Environment, University of Catania, Via S. Sofia, 100, 95123 Italy;

[2]ProBioEtna srl, Spin off University of Catania, via S. Sofia 100, 95123 Catania, Italy

[3]CERNUT, Interdepartmental Research Centre in Nutraceuticals and Health Products. University of Catania, viale A. Doria 6, 95125 Catania, Italy

[4]Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA), Centro di Ricerca Olivicoltura, Frutticoltura e Agrumicoltura, Corso Savoia 190, 95024 Acireale, Italy

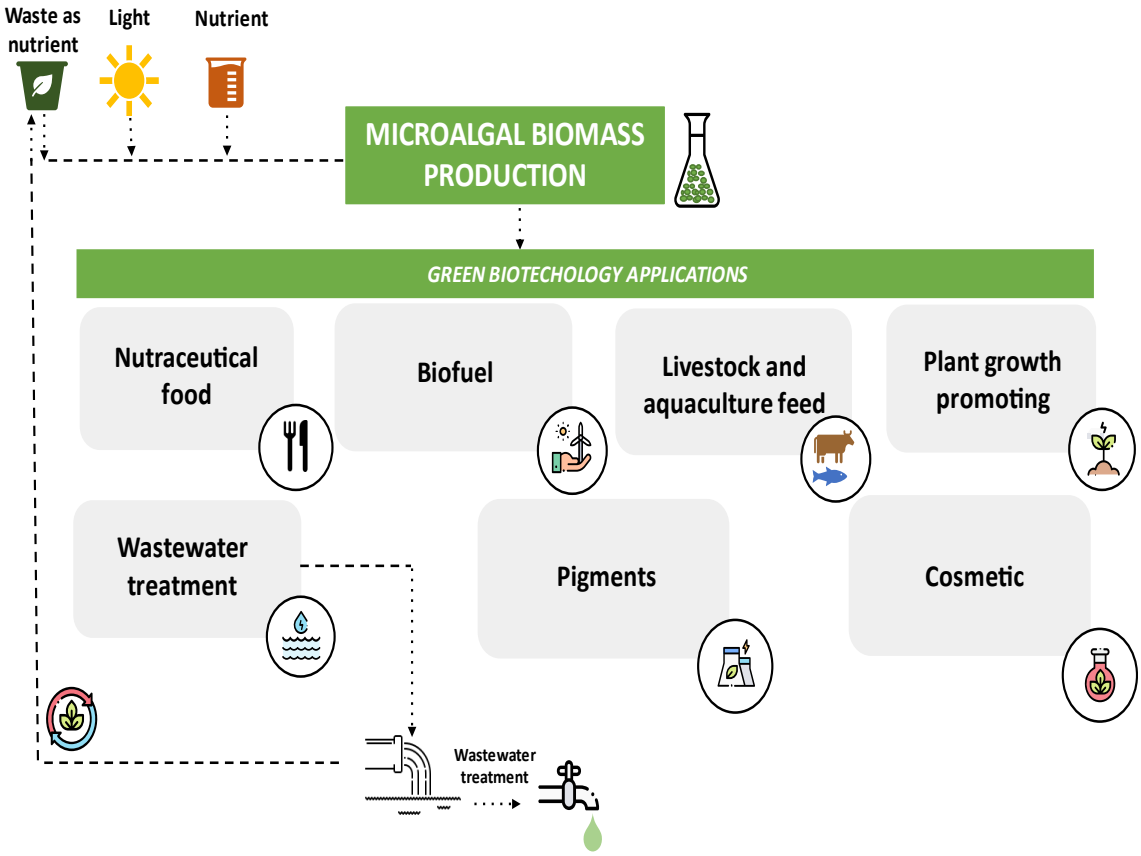
*Correspondence: ccaggia@unict.it

This chapter is has been accepted for publication to *Journal of the Science of Food and Agriculture*. (ID number JSFA-23-1749.R2)

Abstract:

The intensified attention to health, the growing of elderly population, the changing lifestyles, and the medical discoveries have increased demand for natural and nutrient-rich foods, shaping the popularity of microalgae products. Microalgae thanks to their metabolic versatility represent a promising solution for a "green" economy, exploiting non-arable land, non-potable water, capturing CO_2 and solar energy. The interest in microalgae is justified by their high content of bioactive molecules, such as amino acids, peptides, proteins, carbohydrates, polysaccharides, polyunsaturated fatty acids (as ω-3 fatty acids), pigments (as β-carotene, astaxanthin, fucoxanthin, phycocyanin, zeaxanthin and lutein), or mineral elements. Such molecules are of interest for human and animal nutrition, cosmetic and biofuel production, for which microalgae are potential renewable sources. Microalgae, also, represent effective biological systems for treating a variety of wastewaters and can be used as a CO_2 mitigation approach, helping to combat the green-house gas and global warming emergencies. Recently a growing interest is focusing on extremophilic microalgae species, which are easier to cultivate axenically and represent good candidate for the open pond cultivation. In some cases, the cultivation and/or harvesting systems are still immature, but novel techniques appear as promising solutions to overcome such barriers. This review provides an overview on the actual microalgae cultivation systems and the current state of their biotechnological applications to obtain high value compounds or ingredients. Moreover, potential and future research opportunities for environment, human and animal benefits are pointed out.

Keywords: microalgae; natural compounds; high valuable compounds; nutraceuticals.



1. Introduction

The term "algae" does not refer to a specific taxonomic group but it is commonly used to indicate micro and macroscopic photosynthetic organisms, including three wide groupings: macroalgae, microalgae and cyanobacteria. [1] Actually, the term "microalgae" lacks a clear taxonomic value, it refers to unicellular, colonial or filamentous organisms, prokaryotic or eukaryotic, which are estimated to be between 200.000 and several millions of species. [2] Microalgae are considered the most primitive and dominant photosynthetic organisms in the Earth's surface. It has been estimated that they occupy the Earth's surface from more than three billion years, contributing to create the current terrestrial atmospheric composition and being responsible for fixing 40% of CO_2. [3,4] Prokariotic microalgae include cyanobacteria, traditionally known as blue-green algae (divisions Cyanophyta and Prochlorophyta), which are gram-negative bacteria; whereas eukaryotic microalgae, for which the systemic classification is essentially based on their pigment composition, include Chlorophyceae (green algae), Phaeophyceae (brown algae), Pyrrophyceae (dinoflagellates), Chrysophyceae (golden brown algae), Bacillariophyceae (diatoms), Rhodophyceae (red algae), Euglenophyta, Cryptophyta, Haptophyta, Dinophyta and Xantophyceae. [5] Microalgae synthetize a broad range of molecules with different structures and functional roles, a large amount of proteins are produced both for biological and structural functions, whereas secondary metabolites are accumulated to interact with external environmental conditions. Moreover, microalgae structural (polar) and

reserve (neutral) lipids have a diversified composition of fatty acids, often related to the ecological niches, including polyunsaturated fatty acids ω-3 and ω-6. [6] Carbohydrates, obtained through photosynthesis, are a wide category encompassing sugars (monosaccharides) and their polymers (di-, oligo-, and polysaccharides) and the most abundant are glucose, rhamnose, xylose, and mannose. [7] Regarding pigments, they comply with the light capture ability in the first phases of photosynthesis and chlorophylls (five types: a, b, c, d and e), carotenoids (carotenes and xanthophylls) and phycobilins are the three main classes produced by microalgae. [8] A key relevant aspect of microalgae is their metabolic versatility. They can grow heterotrophically, autotrophically or photoheterotrophically, namely mixotrophically [9], and this makes microalgae as interesting solutions for treatment of wastewater coming from several productive sectors. Contextually, agricultural facilities and agro-industries encounter serious problems due to the co-products and by-products generated during their production processes. The recovery of such products to obtain microalgae biomass would mean to exploit agricultural by-products as growth substrate, being, in many cases quite similar to media for microbial growth. For instance, the vinasse from ethanol distillation (from beet and cane molasses fermentation) is a brown liquid containing mostly organic matter and a high amount of inorganic salts. [10] Indeed, several studies have been performed on microalgal cultivation systems or on treatment of industrial and domestic wastewaters, [11] whereby they are widely used for secondary or tertiary treatments. Nevertheless, the contemporary presence of bacteria, fungi and other microorganisms, considered as contaminants, could result adverse for microalgal performance affecting productivity, or in the worst-case scenario, causing culture crash, as documented in a 10-years comprehensive study in Singapore. [12] An interesting strategy is based on using extremophilic microalgae. The extreme pH, temperature or salinity conditions limit the growth of competitors and predators, as bacteria, improving the efficacy of microalgae-based treatment. [13] The aim of this review is to explore the use of un-conventional microalgae species and their cultivation systems pointing out the current state of their applications, with a view on potential and future research opportunities.

2. Microalgal biomass production

Since 1953, when "Algae Culture, from Laboratory to Pilot Plant", edited by J.S. Burlew, [14,15] was published "where were Brought almost all of the work done including the first larger scale outdoor trials made to date in the USA, Germany, Japan and Israel", many designs have been developed. They can be classified essentially into two categories: traditional open systems and enclosed photobioreactors (PBRs), and their main traits are listed in Table 1. Recently, new designed multi-technology (hybrid) systems have been introduced, sharing the common feature to utilize suspended cultures in an aquatic environment. [11, 16] The most typical open system, extensively used since the

1950s for algae cultivation, is the raceway pond. The algal culture is constantly mixed and circulated around the raceway track, in either concrete or compacted earth, by paddlewheels, where the flow is driven around bends by baffles placed in the flow channel. During daylight, in front of the paddlewheel, where the flow begins, the culture is continuously fed, while, broth is harvested behind the paddlewheel, at the end of the circulation loop. [17] The pond is usually about 0.3 m deep to provide a sufficient sunlight for photosynthesis. Currently, the most commercial scale algae cultivation systems are open ponds, being relatively inexpensive to build and easy to scale up. Nevertheless, numerous limitations, such as: temperature fluctuations between day and night and among seasons as well as geographic location, evaporative water loss, low CO_2 and high O_2 concentrations, large optically dark zones or contaminations with unwanted algae or microorganisms, make the open system much less efficient than photobioreactors (PBR). [18] Nowadays, PBRs are successfully used for producing large quantities of microalgal biomass from single-species culture, thanks to the possibility of maintaining optimal parameters, avoiding contamination, by continuously adjusting light intensity, CO_2 and O_2 concentrations, pH and temperature values, etc. There are many available configurations for PBRs systems: such as typical closed reactors, that include flat plate reactors, tubular PBRs, and bag systems. [19] Tubular PBRs are the most commonly used at industrial scale. A tubular PBR consists of an array of straight transparent tubes, generally 0.1 m or less in diameter, usually in plastic or glass, adapt to capture sunlight. Microalgal culture is kept constantly circulating from a reservoir to the solar collector and back to the reservoir, by highly turbulent flow produced using either a mechanical pump or a gentler airlift pump. Despite biomass yield obtained in PBRs is generally 30 times higher than that obtained in raceways, [17] the cultivations, at commercial scale production, require several crucial considerations, such as: design, cost, risk of contamination and cleaning. An alternative strategy consisting of a hybrid system is obtained by coupling open and closed systems in a two-stage cultivation system. The first stage uses closed PBRs to culture the inoculum for the second stage where algae are cultivated in open pond. In this way, microalgae are cultivated in optimal conditions for cell growth before being transferred into a growth-limited environment, as open pond which, for instance, can enhance lipid production. [19, 20] However, large-scale applications have been limited by the cost of the first stage. [11] Bilad and co-workers used both a closed PBR and a hybrid system, and the membrane photobioreactor (MPBR) for microalgal cultivation. [21] The MPBR consists of an additional filtration tank where a membrane provides the retention of microalgal cells, preventing the wash out and increasing biomass concentration, while the medium passes as permeate. This system achieved 9 times higher biomass productivity with a 77% smaller footprint than PBR. [21] Overall, microalgae cultivations for commercial biofuel production are usually performed in raceway ponds, whereas PBR designs are suitable for productions of high value products. [22]

Table 1. Microalgae cultivation: open vs closed systems

Parameters	Open systems	Enclosed photobioreactors (PBRs)	References
Biomass production (kg m^{-3})	0.14 (raceway pond)	4 (tubular photobioreactor)	[17]
Operation costs	Low	High	[18]
Light utilization efficiency	Poor	Highly efficient	[23]
Process control	Difficult	Accurate	[18]
Species control	Difficult	Possible	[24]
Contamination risk	High	Low	[24]
Value of produced biomass	Low	High	[22]

2.1 Cultivation strategy

Depending on the selected species and objectives to be achieved a proper cultivation system is required. Despite the disadvantage of CO_2 requiring, O_2 accumulation, light-growth limitation, [23,25] the most common strategy for microalgae cultivation is the photoautotrophic way. Alternatively, heterotrophic cultures are commonly performed in conventional fermenters (stirred tank fermenter) where the O_2 requiring is obtained by an intensive aeration. [26] Nevertheless, heterotrophic growth has been observed exclusively within few microalgal species, and its specific growth rate is still much less efficient than that obtained by *Candida utilis*, which although presents about the same size, shows a maximum specific growth rate (0.19 per h) about 2–5 times faster than *Chlorella*, which is mainly heterotrophically produced using glucose or acetic acid. [26,27] Moreover, darkness can lead to reduced pigmentation, limiting the potential of heterotrophic cultivation for phytochemicals large-scale production. In myxotrophy, the simultaneous presence of two energy sources (light and reduced organic carbon) can significantly increase biomass productivity because of both heterotrophic and autotrophic metabolisms operate concurrently within a single microalgal monoculture, overcoming both autotrophic and heterotrophic limitations. [28] Nevertheless, mixotrophic cultivation cannot be adapted in open cultivation systems because the presence of organic carbon improves bacterial contaminant growth, holding closed system the only practical possibility. Recently, Abiusi and co-workers have designed an oxygen balanced mixotrophic process that does not require any gas exchange. [29,30] *Chlorella sorokiniana*, cultivated in enclosed PBR, as both autotrophic and heterotrophic cultures, was supplied with an optimal rate of acetic acid, showing a doubled biomass production, as the sum of the two metabolisms. Extreme growth conditions aid in preventing contamination and predation of microalgae, therefore facilitating their outdoor cultivation. During the last two decades, particular attentions have been paid toward acidophilic and acid tolerant microalgae and their biotechnological application, for example for production of pigments, as phycocyanin, [31] and most of the researches on acidophilic microalgae has been focused on *Galdieria* genus.

Interesting results were reached using *Galdieria sulphuraria* in mixotrophic cultivation, where at pH 1.7 the biomass productivity was 1.8 times higher than in autotrophic culture, and the culture remained axenic for the whole experiment. [31]

2.2 Microalgal harvesting

Microalgae are grown in large water volumes and for the harvesting process a concentration step, a process with intensive energy demand, is required. Therefore, the selection of harvesting technologies depends on microalgae specie and on the economically and energetically suitable process. [32] The choice for microalgal harvesting has to take into account the cell structure, the growth rate and the lipid content. Several techniques could be adopted for harvesting and thickening: screening, coagulation–flocculation and sedimentation, flotation, centrifugation, magnetic separation, electrophoresis and dewatering and drying. [33] The screening consists of introducing microalgal biomass onto a screen of given aperture size. The efficiency of the screening operation depends on the size of screen hole and algal particle dimensions. Microstrainers and vibrating screens are commonly employed as screening devices. [34] The harvesting through coagulation and flocculation is based on negative surface charge of microalgal cells, density near to the growth medium, in dispersed state, results in a stable system with a slow natural sedimentation. [33] The coagulation-flocculation of microalgal cells, useful at large scale with a wide range of microalgal species, can be induced by using chemicals, namely flocculants, causing aggregation of microalgal cells to form larger clumps, which are easier filtered and/or settle. [35] In the flotation process, air or gas bubbles are used to move to the top of growth medium the suspended matter that were then collected by skimming process. [36] The filtration culture suspension is based on forcing to flow across filter medium using driving force derived from gravity, vacuum, pressure, or magnets. [37] The harvesting through centrifugation is generally characterized by high separation efficiency, >90% at 13000 g, as reported by Heasman and co-workers. [38] However, Dassey and Theegala demonstrated that high biomass separation efficiency could be sacrificed when large volume of culture is processed, resulting in a lower energy intake. [39] Indeed, the large energy consumption, the long treatment time make the process very expensive, mainly for large-scale applications. [40] Due to the negative charge on microalgal cell surface, harvesting based on electrical approaches, as electrophoresis, electroflocculation, or electroflotation can be adoptable strategies. Exposing the medium to an electric field by metallic electrodes energized with a DC voltage, microalgal cells can thicken close to electrode (electrophoresis), to the bottom (electroflocculation) or to medium surface (electroflotation). [32] Alternatively, the use of natural coagulant in microalgae harvesting are studied and proven to exceed the alum. [41] Biopolymers derived from plant wastes and fruit pieces, as nirmali, moringa and surjana seed, maize seed, Cactaceae, etc.

had showed significant coagulant capacities, and recently the moringa native to Sudan, has received the greatest level of attention. [41,42] Proteoglycan coagulant, produced by *Bacillus mojavensis* strain 32A has showed an interesting flocculating activity, as 96% at pH 10. [43] Lastly, chitosan-based compounds, derived from marine crustaceans, are also potentially eco-friendly coagulants and flocculants in harvesting process. Generally, the mechanism involved in the harvesting process of chitosan is based on bridging and chitosan is commonly used in laboratory for harvesting, for example, *Chlorella* sp. from cultivation medium. [44]

3 Biotechnological applications

Microalgae are fast-growing organisms able to survive in several environmental conditions. The biomass production is faster and higher than that of high plants, with a less seasonal variation, producing abundant raw materials characterized by easy biomolecule extraction processes. As well as for the biofuel production, for which microalgae are a potential renewable source, different commercial applications are possible, such as: wastewater treatment and CO_2 capturing, human nutrition, feed for animal and aquatic life, active ingredients for cosmetic industry, high-value compounds, pigments like astaxanthin, β-carotene, and phycobiliproteins, stable isotope production, biofertilizer, or pharmaceutical means, as antimicrobial, antiviral, antibacterial and anticancer drugs. In Table 2 an overview on microalgae biotechnological applications is shown.

3.1 Microalgae for production of nutraceutical compounds

Microalgae, according to the belonging species, are source of several biological molecules, such as proteins, polyunsaturated fatty acids, peptides, minerals and pigments with high nutraceutical value. [1] Since the early 1950s, microalgae have been explored as an alternative protein source to face global food demand, and their large-scale production has been successfully established since the 1980s, in several countries. [45] As already established by the World Health Organization (WHO) and Food and Agriculture Organizations (FAO) of the United Nations, microalgae are eligible as substitutes of animal proteins. [46] The Cyanobacterium *Arthrospira platensis* presents a calcium content higher than 180% than milk, proteins higher than 670% than tofu, β-carotene higher than 3100% than carrot, and iron higher than 5100% than spinach, reasons why microalgae has been promoted as "superfood" by WHO. [47] Both *A. platensis* and *Arthrospira. maxima* are the species most commonly and intensively investigated. They are rich in polyunsaturated fatty acids, such as γ-linolenic acid (18:3 ω-6), arachidonic acid (ARA, 20: ω-6) eicosapentaenoic acid (EPA, 20:5 ω-3), and docosahexaenoic acid (DHA, 22:6 ω-3). [48] Many microalgae species are reported to be producer of edible oil. *Isochrysis*

galbana, Nannochloropsis sp., *Tetraselmis* sp. and *Phaeodactylum tricornutum* are EPA-producers, while *Porphyridium cruentum* is an arachidonic acid (AA) producer. [49,50] Furthermore, as largely reported by Spolaore and co-workers, [51] for the high content of B vitamins and phycobiliproteins these species are considered as healthy promoters with antioxidant, cholesterol-lowering and other beneficial effects. Moreover, *Arthrospira* has gained significant popularity in the health and food industry, as primary food source in Asian countries, mainly in China, Korea, and Japan, while in other parts of the world it has been used as nutrition supplement. The green algae *Chlorella vulgaris* is the second most relevant species for human nutrition, to be rich in proteins (48% of dry weight) and phosphorous (1,761.5 mg/100 g of dry weight biomass). Furthermore, its nutraceutical benefits are related to β-1,3-glucan, macromolecules with immunostimulant effects. [52] *Dunaliella salina*, containing carotenoids (9-cis-β-carotene) known to prevent intracellular oxidative damage, has been consumed as dietary supplements for human health in form of pills, capsules, and fortified nutritional mixtures, or as a natural food and beverages. [53] Other species have been investigated for their nutraceutical value, as the halophilic *Picochlorum* sp. for its folate content, that appeared higher than that detected in *Chlorella.* [54] The biomass of *Picochlorum* sp. showed a total folate content of 6,470 μg/100 g, which is currently the highest total folate content detected in algae, reported as 1,700 and 2,600 μg/100 g. [55] Similar results were reported by Fujii and co-workers that, in microalgae collected from Japanese ponds, found total folate contents in the range between 1,500 and 3,600 μg/100 g in dry biomass. [56] Within the halophilic *Dunaliella tertiolecta* species, known for production of carotenoid from natural seawater, the strain *D. tertiolecta* mp3 was found able to accumulate zeaxanthin, under different growth conditions. [57]

Furthermore, a recent study, carried out by Fields and co-workers, revealed that consumption of *Chlamydomonas reinhardtii* mitigated weight loss in a murine model of acute colitis and positively affected gastrointestinal health in humans. [58]

3.2 Microalgae for production of active compounds for cosmetic applications

If macroalgae are already widely exploited in cosmetic industry, microalgae are still less used. Among the different pharmacological activities, some microalgae compounds could be applied in cosmetics. [59,60] Pigments, such as β-carotene, astaxanthin, lutein, have been largely described as natural ingredients to be incorporated into moisturizing, antiaging, photoprotection, and skin lightening products. [61,62] β-carotene is known for its provitamin A activity and it is largely used in antiaging products. In particular, the halotolerant *D. salina* species is described as main producer of β-carotene, up to 10% of its dry weight. [61] Astaxanthin is also applied in antiaging products because of its remarkable antioxidant properties, which are much greater than that detected for tocopherol. [63]

H. pluvialis is the richest source of natural astaxanthin (it can accumulate more than 3 g of astaxanthin by kg of dry biomass) and nowadays it is produced at industrial scale. [61] Lutein has been used for skin combating sunburn, reducing wrinkles, and for other cosmetic benefits. Within the product-type segment, the lutein segment accounts for the largest value, sharing around the 19%. Furthermore, lutein produced by *Scenedesmus* sp. has been successfully used to slow eye macular degeneration. The specific composition and quantity of lipids are species-dependent and the most common oleaginous microalgae are *Chlorella* sp., *Nannochloropsis* sp., *Scenedesmus* sp., and *Dunaliella* sp. [64] A fundamental aspect in pigment production is downstream processing, in particular their extraction from microalgal cells. The high consumption of toxic solvents can be a burden to the environment and longer processing time result on lower profit. [65] The downstream processing technique, proposed by Chia et al., [66] to extract C-phycocyanin from *Arthrospira* spp., based on a sonication treatment coupled to a liquid biphasic system (to purify the phycobiliproteins), was described as able to achieve a purification fold of 6.17 and a recovery yield of 94.89%. At the same time, for extraction of astaxanthin from *H. pluvialis*, an alternative solution, to overcome the criticisms explained above, has been proposed by Khoo et al. [67] In particular, the use of CO_2-based alkyl carbamate ionic liquids has been proposed and results stated that DIMCARB (dimethylammonium dimethylcarbamat) gave the highest yield of astaxanthin (27.99 ± 1.01 mg/g of astaxanthin) under the optimized extraction conditions, namely 100% (w/w) of DIMCARB, 75 min of incubation, at 45 °C.

3.3 Microalgae for production of food ingredients

Microalgae have a great potential to be used as ingredients in innovative and sustainable food products, improving protein content, valuable nutrients, such as phenolic compounds, vitamins and minerals, or as pigments in food dyes, such as astaxanthin (red), lutein (yellow), chlorophyll (green), or phycocyanin (bright blue). *C. vulgaris* has been used as food coloring or antioxidant agent, while *I. galbana* as a ω-3 PUFAs provider. [68] As reported by Hossain et al., [69] when 15% (w/w) of astaxanthin from *H. pluvialis* was added to cookies, a significant reduction in glucose releasing, in *in vitro* digestion, and an increase in total phenolic content and antioxidant capacity was observed. Furthermore, when *I. galbana* and *Diacronema vlkianum* biomass were added to pasta a significant increase of polyunsaturated fatty acids, in particular EPA and DHA, both in raw and cooked pastas were detected. [70] In 2016, a study explored the effect of adding 10% of *A. platensis* biomass to bread and an increase, from 7.40% to 11.63%, in protein and mineral contents, especially calcium, magnesium, and iron was noted. [71] In 2019, supplementation of 2, 6, and 10% of *Arthrospira* biomass in crostini, a bakery product, was evaluated and results showed higher protein and antioxidant content

along with the increase of microalgal addition. [72] Nevertheless, digestibility of microalgal biomass is still a crucial aspect because the robust cell walls, composed of peptidoglycan or cellulose, or the high amount of nucleic acid or neurotoxins that could represent a risk for human health.[73-75] To overcome this aspects, numerous pre-treatments are available, such as bead milling, high pressure homogenization, heat treatment and many others, however further studies are required to find higher efficient and cost-effective technologies to increase digestibility without hindering quality of the high value compounds. [76] Furthermore, consumer and government acceptance play a key role in developing and marketing food products containing microalgae. Moreover, application of microalgae in functional foods is still restricted since limited data are available about allergenic compounds or effect on human health. [77] Contextually, due to the considerable amount of microalgae biomass added to explicate their benefits, often adverse effects on food taste and texture occurred. [78] Different microalgal properties affect their potential use as food ingredients, such as gelation, emulsification, and miscibility. [79,80] Incorporation of *Chlorella* in processed cheese led to increase hardness and reduced meltability, [81] while addition of *Arthrospira* into pasta enhanced the firmness and the cohesiveness, without affecting cooking properties. [82] Nevertheless, sensorial data revealed that consumers are generally positive about both green color and marine taste. Moreover, it is interesting to highlight that the addition of *A. platensis* and *C. vulgaris* into probiotic fermented milks resulted in an enhancing of probiotic viability. [83] Currently, many of these products are present on the global market. New and unconventional food, such as vegetable creams (with higher protein content), are becoming very popular thanks to the increased demand for healthy products, and some new recipes meet the criteria to be labelled as "high-protein content" following the current EU legislation. [84] Unfortunately, commercial companies do not clarify the used microalgae species and, in most cases, the label only describes the microalgae genus.

3.3.1 Safety concerns and legislation

Like other microorganisms, including yeast and bacteria, some species of microalgae are safe for human consumption and have obtained the GRAS (Generally Recognized as Safe) status from the US FDA. In such a case the purification costs are significantly reduced and potentially expanded the applications as valuable food and/or feed ingredients. The few microalgae that have obtained the GRAS status are: *A. platensis, C. reinhardtii, Auxenochlorella protothecoides, C. vulgaris, D. bardawil*, and *Euglena gracilis*. In European Union (EU), the European Food Safety Authority (EFSA), following the "precautionary principle" approach, stated that foods that have been consumed within the EU before May of 1997 are deemed safe to be consumed, whilst any other food have to be labelled as "novel food" and must undergo a safety assessment by the EFSA, before being marketed.

[85] In EU the approved species are only *A. platensis, C. pyrenoidesa*, and *C. vulgaris.* [85] Nevertheless, among the thousands existing microalgae species, around 200 showed concerns about safety traits and about 100 species have been proven to produce toxins. [86,87] A comprehensive data on toxic species can be found at www.marinespecies.org, an updated list of microalgae species producing or suspected to produce toxins or toxic effects (IOC Harmful Algal Bloom Programme and the World Register of Marine Species). In the last years, important advances have been achieved towards the development of more specific, sensitive, and rapid methodologies that allow the identification of different microalgae species and toxins. [88] In addition, legislations and regulatory aspects on the commercialization of carotenoids from microalgae biomass are recently described for food and cosmetic products in USA, Japan, China, and Europe. [89] Thus, microalgal-derived astaxanthin, β-carotene, and chlorophyll are regulated and approved by the FDA, based on their non-toxic and non-carcinogenic properties. [90] Moreover, astaxanthin from *H. pluvialis* has been approved as a color additive in Europe, USA, and in Japan. In details, the EFSA Panel on Nutrition, Novel Foods, and Food Allergens concluded that an intake of 8 mg of astaxanthin through food supplements is safe for adults [85] and the FDA approved it for direct human consumption. [91]

3.4 Aquaculture and livestock feed

Aquaculture is an important sector as terrestrial agriculture that provides food for the human population. Fishmeal is usually supplied as feed in fish farming and it is produced from small fishes or fish waste which are cooked, pressed, dried and ground to form a solid. [92] An environmentally and economically sustainable alternative for replacing fishmeal can be the microalgae-based feed, which showed significant results in production of zooplankton, mollusks, crustaceans, shrimp and fish farming, [93] providing a high value nutrition, improving the color of aquatic organisms and disease resistance. [94] However only some microalgal species are proven species to be used as feed in aquaculture, in particular microalgae belonging to the genera *Isochrysis, Pavlova, Nannochloropsis, Arthrospira, Chlorella, Scenedesmus, Dunaliella, Haematococcus,* and *Schizochytrium*. [52,95] For instance, the carotenoids, as astaxanthin from *H. pluvialis*, or β-carotene from *D. salina*, phycocyanin from *Arthrospira*, are used as source of natural pigments for culturing salmonid fish, prawns, and ornamental fish. [96] Besides aquafeed, livestock feed for pets, horses, broilers and ruminant animals are other potential microalgal applications. At low percentages, microalgal biomass, has been recommended to be included in feed for animals such as pigs, cows, sheep, chicken and other domestic animals, in order to improve their immune systems, lipid metabolism and gut functions. [97] However, ruminants are the most suitable animals to feed algae because they are even able to digest unprocessed microalgal biomass. [97] However, the technology to produce microalgae is still immature

and the main drawbacks and challenges are the high production costs, [96] the low digestibility of cell walls, for some microalgae, and the presence of contaminants. [98]

3.5 Wastewater treatment

Wastewater treatment by means of microalgal systems is a technology that has been applied for longer than 60 years. Microalgae-based wastewater treatment has been recently intensively studied, with a focus on the production of algal biomass and the associated removal of inorganic nutrients from a wide variety of wastewaters [99]. The pioneering studies of Oswald, in California, set the fundamentals of wastewater treatment in the so-called "high-rate algal ponds" (HRAPs), originally focused on removing organic matter and nutrients [100; 101]. Nowadays, the biotransformation of pollutants from wastewater like xenobiotics, nutrients and CO_2 from polluted air by macro- or microalgae is known as phyco-remediation. The main aim of the phyco-remediation is to depurate wastewater for irrigation or other aims and concurrently, exploiting wastewater as growing medium based on the high concentration of N and P [102]. Also the organic-rich anaerobic digestate which is difficult to be purified by conventional techniques is appropriate to be used as a low-cost nutrient source for the economic viability and sustainability of microalgae production [103]. *C. vulgaris* has been extensively exploited for biomass production from food waste, sludge extracts, corn steep liquor, cheese whey, vinasse, tofu wastewater, and industrial dairy effluents [104; 105; 106]. Recently the ability of *A. platensis* to accumulate lipids during mixotrophic growth on dairy wastewater has been evaluated [107], obtaining about 5 g L^{-1} of total biomass concentration with about 30% of lipid content. *A. platensis* has also been applied to treat piggery wastewater, confectionary effluent, composite media made of mineral medium, beet vinasse, and distillery wastewater [106]. Typically, treatment of municipal and agricultural wastewaters by microalgae is performed in outdoor systems, without any adjustment of temperatures and pH. However, the wastewater parameters can be widely different, from highly acidic pH values and temperatures (spanning from 10 to 40 °C), high organic loads (>100 g L^{-1}) and a high load of contaminant population. An interesting strategy to successfully treat a wide type of wastewater can be the extremophile microalgae. *Galdieria sulphuraria*, known as *Cyanidium caldarium*, has been exploited for its interesting extremophilic growth properties, being able to grow both in neutral and highly acidic conditions, down to pH value of 1.8 [108], acidifying the medium by an active proton efflux, reducing the costs of pH control and, in turns, the risk of contamination [109]. Moreover, the versatile metabolism, able to grow auto-, hetero- and mixotrophically, exploiting more the 50 different sugars and alcohols, makes *G. sulphuraria* a promising candidate for treating wastewater [110; 111]. As reported in a recent study [112], *G. sulphuraria* showed to grow heterotrophically and mixotrophically on cultivation medium containing

a byproduct of the dairy industry (buttermilk) as carbon source. A further promising acidophilic microalgal species is *Chlamydomonas acidophila*, isolated from acidic river in a mining area, at pH values ranging from 1.7 to 3.1 [113]. It has been shown that *C. acidophila* can grow mixotrophically without CO_2 addition by using different carbon sources, such as glucose, glycerol or starch, at acidic conditions (pH 2.5) removing NH_4 [113; 114]. *Chlorella sorokiniana*, a well-studied thermophilic green microalga, has revealed high photoautotrophic growth rates up to 43 °C [115]. Despite microalgal feedstock for biofuel use is considered an ideal scenario many disadvantages must be solved, as for example the expensive harvesting process required in a HRAP, for microalgal biomass, avoid that the biomass settles to the bottom of the ponds, where it decomposes, releasing methane and degrading water quality [116].

3.6 Bio-fertilizers and bio-stimulants for promoting plant growth

Microalgae can be utilized for sustainable agriculture by partial substituting chemical fertilizers. Recently detailed insights on algal biochar as a potential fertilizer for sustainable agriculture have been produced. [117] The mechanism responsible for biofertilization is still unclear, biomass is provided to soil, but it should be available for plants and their roots. The most accredited theory, explained by Perin and Morosinotto, [118] is that microalgal biomass could be degraded by soil microbiota present in rhizosphere, thus releasing nutrients over a prolonged period. Alternatively, symbiotic interactions could be established, as in cyanobacterium nitrogen fixators case, where bioavailable forms of nitrogen are released in return for carbonic compounds from plants. Anyway, after providing biomass in soil, the nutrient status, water retention capacity, pH and electrical conductivity result improved. [1] Among the most reported responses, an increased content in proteins, carbohydrates and photosynthetic pigments has been registered in plants treated with microalgal extract, in particular from *S. quadricauda.* [119-121] However, the microalgae biomechanisms in the plant physiology and the different effects for each bioactive compound remain still unclear, reasons why the effect of the microalgal biomass is considered as a complex of reactions. [122,123] In addition, biochemical composition of microalgal cells, rich in micro and macro-nutrients, makes their biomass a promise source for biofertilizer. Unfortunately, only few microalgal genera are industrially exploited as microalgae-based commercial products, [124,125] confirming how young is the sector, and of less is known on microalgal species, considering they are several million.

3.7 Biofuel production

Many efforts have been done to find biofuel production technologies, but both the 1st and the 2nd generation has been discovered as not suitable for environmental incompatibility for requiring more arable agricultural lands and modern innovations, respectively. [126] For this reason, the attention is now shifting to the 3rd generation technology which uses defined species of microalgae as feedstocks, thanks to the high oil content and biodiesel yield, the low land area needed and absence of lignin absence, that is recalcitrant and needs several pre-treatment processes for carbohydrates realizing. [127,128] Many microalgae species can supply several different types of renewable biofuels such as biomethane, produced by anaerobic digestion of algal biomass; bioethanol, produced via fermentation and anaerobic digestion of the remaining algal biomass. [129] Microalgal species widely investigated belong to the green algae genera *Chlorella, Scenedesmus, Dunaliella, Porphyridium* and *Euglena*, that show particular attitudes for bioethanol production, thanks to their high polysaccharide content. [127] *Microcystis aeruginosa*, a freshwater blue green alga (cyanobacterium), and *Scenedesmus obliquus* (green alga), with lipids content as 28% and 40%, respectively, [129] were considered the most promising specie for biodiesel production. To produce biodiesel, as reported by Leong et al., [130] it seems very promising the use of microalgal-bacterial consortium. Also biohydrogen, currently produced by techniques, such as steam reforming or electrolysis, not entirely free from the involvement of fossil fuels, [131] can be produced by cultivation of Cyanobacteria and green algae, trough directly and indirectly pathways, as deeply explained by Show et al. [132] Unlike other well-established biofuels, as biodiesel and bioethanol, biohydrogen from microalgae is still at its preliminary stage of development. Criticisms in microalgal biohydrogen centered on its practicality and sustainability. [132] There are still various difficulties in biofuel production from microalgal biomass feedstock. Current data indicate that the cost of biofuel production from microalgal biomass is still higher than that of different other sources, and actually, it is not yet proven to be an economically sustainable source of biofuel. [126] A great challenge is to reduce the harvest cost, which is estimated as 40% of the whole production cost. Anyway, designing efficient and innovative oil extracting methods could improve the biodiesel yield from microalgal cells. Nguyen et al. showed the highest fatty acid ester yield (96.0 % w/w) under wet microalgae conditions with 650 mol/mol and 10,000 mol/mol of H_2SO_4 and methanol concentrations. [133] Furthermore, employing transgenic strains with producing high-value products and using residual byproducts to boost production economics, microalgal production can be scaled-up to an industrial level. [126]

4. Conclusion and future perspectives

According to the FAO report on "The future of Food and Agriculture: trends and challenges", [134] about one third of global food produced is still lost or wasted along the food chain, from production to consumption highlighting an inefficiency of current food systems. At the same time, increasing healthcare spending, growing geriatric population, food innovations, changing lifestyles, and medical discoveries have benefited demand for superfoods. Furthermore, the increasing awareness regarding superfoods as natural, nutrient-rich agro-foods containing vitamins and minerals are some of the key aspects shaping the popularity of microalgae products in the world. In this scenario, microalgae represent a promising candidate for both food/feed and energy production as well as for valorization of by-products aimed to create a virtuous recycling system, in accordance with the United Nation 2030 Agenda goals. [135] As Pikaar et al. [136] theorized in a model simulation, microbial sources of food and feed hold great promise for achieving a future food production system that is both more sustainable and resilient. In particular, it would be feasible to replace 10–19% of conventional crop-based protein feed with microbial biomass by 2050, with significant reductions in global cropland area, nitrogen leakage and agricultural emission. Despite several species are already commercially used, they are still not produced in high-enough quantities or in a cost-effective manner, required for fuels and feeds. Nowadays, total soy oil and meal production, estimated to be around 200 million t/year, with a current price below 0.5 €/kg, is far away from the global microalgae production, which they amount about 25,000 t/year with a market price of 20–50 €/kg. Although, it has been estimated that, if production reach 10,000 t of biomass per year, the cost price will fall below 5 €/kg, and further industrialization could reduce it below 1 €/kg. [97,137,138] Hence, an optimizing firstly on the manufacturing and on commercialization of microalgae products is required. In this context, several strategies can be adopted to overcome these limits: open pond cultivation systems based on poly-extremophile microalgae can be a strategy to cut down production costs; innovative and natural methods to harvest, extract and process microalgae represent opportunities to develop the most promising sectors such as food, energy and cosmetic productions. Finally, microalgae can be a great opportunity to develop new production systems to complement or improve traditional agriculture in order to satisfy the world's food and feed demand.

Table 2. A comprehensive overview on microalgae biotechnological applications

Application	Main genera or species	Valuable compounds	Weakness	Strengths	Reference
Food and nutraceutical	*Arthrospira spp.; Chlorella spp; Scendesmus spp.; Dunaliella spp.; Hematococcus spp.*	Proteins; β-carotene; iron; acid γ-linolenic (ω-3 fatty acid), B vitamins	Adverse effects on food taste and texture High amount of nucleic acid if used directly as food	High nutritional value Several sale formats	[73; 139]
Animal feeding in aquaculture	*Chlorella vulgaris; Isochrysis galbana; Phaeodactylum tricornutum; Chaetoceros* spp; *Nannochloropsis oceanica; Skeletonema spp; Haematococcus pluviali.*	Proteins; β-carotene; lutein; ω-3 fatty acid	High production cost Low digestibility of cell walls for some microalgae	Better growth rate and health of fishes High protein feed for animals	[5; 140]
Wastewater treatment	*Chlorella sp.; Anabaena sp.; Arthrospira sp.; Botryococcus sp.; Chlamydomonas sp.; Dunaliella sp.; Haematococcus sp.; Isochrysis sp.; Nannochloropsis sp.; Neochloris sp.; Microcystic sp.; Oscillatoria sp.; Phormidium sp.; Scenedesmus sp.; Synechococcus sp.*	Fresh biomass; treated wastewater	Expensive harvesting method Low quality biomass due to contaminant	Cheap nutrient sources Two products obtained: Treated wastewater and microalgae biomass	[141; 16]
Plant growth promoting	*Scendesmus quadricauda*; *Arthrospira plantensis*; *Chlorella spp.*	Phytohormones; amino acids; vitamins; polysaccharides; carbohydrate; polyamine; polyphenols	Unclear biomechanisms of microalgae in plant physiology Few microalgal genus exploited	Improved plant growth Bio-fertilizers	[188]
Biofuels	*Chlorella spp; Scendesmus spp.; Dunaliella spp.; Porphyridium spp.; Euglena; Microcystis*	Polysaccharide; lipids	Several pre-treatment processes needed Industrial production scale not yet economically convenient	Clean energy Highest socio-economic significance	[127; 129]
Biorefenery and cosmetic	*Dunaliella salina; Haematococcus pluvialis; Spirulina spp; Porphydrium spp.; Nannochloropsis spp.*	β-carotene; astaxanthin; fucoxanthin; phycobiliproteins; zeaxanthin; lutein.	Low production efficiency Highly specialized refinement processes	High-value products, potential microalgal production, scaled-up to industrial level	[57 ;142; 143]

Author Contributions: Conceptualization, C.C. and C.L.R.; methodology, C.C.; software, A.P.; validation, F.V.R., C.L.R.; investigation, P.S.O. and I.M.Z.; resources, F.V.R.; data curation, A.P..; writing—original draft preparation, P.S.O.; writing—review and editing, C.C.; visualization, N.R.; supervision, C.C. and C.L.R.; project administration, C.C.; funding acquisition, C.L.R. All authors have read and agreed to the published version of the manuscript.

Funding: This study has been partially supported by PON "RICERCA E INNOVAZIONE" 2014–2020, Azione II—Obiettivo Specifico 1b—Progetto "Miglioramento delle produzioni agroalimentari mediterranee in condizioni di carenza di risorse idriche—WATER4AGRIFOOD and partially funded by European Union (NextGeneration EU), through the MUR-PNRR project Sustainable management of natural resources in agriculture: SAMOTHRACE (ECS00000022).

Acknowledgments: This study was conducted within a Ph.D. research program in Biotecnologie (XXXVI cycle) by Paride Salvatore Occhipinti who received a grant from the University of Catania, Department of Agriculture, Food and Environment (Scientific Tutors: Cinzia Caggia and Cinzia L. Randazzo).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results".

References

1. Rizwan, M., Mujtaba, G., Memon, S. A., Lee, K., & Rashid, N. (2018). Exploring the potential of microalgae for new biotechnology applications and beyond: a review. Renewable and Sustainable Energy Reviews, 92, 394-404.
2. Norton, T. A., Melkonian, M., & Andersen, R. A. (1996). Algal biodiversity. Phycologia 35, 308–326.
3. Williams, R. (2013). Microscopic algae produce half the oxygen we breathe. ABC News. October.
4. Ansari, F. A., Gupta, S.K., & Bux, F. (2019) Microalgae: a biorefinary approach to the treatment of aquaculture wastewater. In Application of Microalgae in Wastewater Treatment. Gupta, S.K., & Bux, F. (Ed) Volume 2: Biorefinery Approaches of Wastewater Treatment. Springer, Cham, 69–83

5. Hemaiswarya, S., Raja, R., Ravi Kumar, R., & Carvalho, I. S. (2013). Microalgae taxonomy and breeding. In Biofuel Crops: Production, Physiology and Genetics. Singh B.P. (Ed), CAB International, 44-53.

6. Khozin-Goldberg, I. (2016). Lipid metabolism in microalgae. In The Physiology of Microalgae. Borowitzka, M. A., Beardall, J., & Raven, J. A. (Ed) Springer Nature Switzerland, 413-484.

7. Markou, G., Angelidaki, I., & Georgakakis, D. (2012). Microalgal carbohydrates: an overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels. Applied Microbiology and Biotechnology, 96, 631-645.

8. Begum, H., Yusoff, F. M., Banerjee, S., Khatoon, H., & Shariff, M. (2016). Availability and utilization of pigments from microalgae. Critical Reviews in Food Science and Nutrition, 56, 2209-2222.

9. Oesterhelt, C., Schnarrenberger, C., & Gross, W. (1999). Characterization of a sugar/polyol uptake system in the red alga Galdieria sulphuraria. European Journal of Phycology, 34, 271-277.

10 Gonzalez-Benito, G., Barrocal, V., Bolado, S., Coca, M., & Garcia-Cubero, M. T. (2009). Valorisation of by-products from food industry, for the production of single cell protein (SCP) using microalgae. New Biotechnology, (25), S262

11 Cai, T., Park, S.Y., Li, Y., 2013. Nutrient recovery from wastewater streams by microalgae: status and prospects. Renewable and Sustainable Energy Reviews, 19, 360–369.

12 Taiganides, E. P. (1992). Pig waste management and recycling: the Singapore experience. IDRC, Ottawa, ON, CA.

13. Abiusi, F., Trompetter, E., Pollio, A., et al (2022) Acid tolerant and acidophilic microalgae: An underexplored world of biotechnological opportunities. Frontiers in Microbiology, 13, 2022.820907.

14. Burlew, J.S. (Ed.) (1953a) Algae culture: from laboratory to pilot plant. Carnegie Institution of Washington, Washington, DC, 1–357

15. Burlew, J.S. (1953b) Current status of large-scale culture of algae. In Algal Culture: from Laboratory to Pilot Plant. Burlew, J.S. (Ed) Carnegie Institution, Washington, DC, 3–23

16. Li, K., Liu, Q., Fang, F., Luo, R., Lu, Q., Zhou, W., ... & Ruan, R. (2019). Microalgae-based wastewater treatment for nutrients recovery: A review. Bioresource Technology, 291, 121934.

17. Chisti, Y. (2007) Biodiesel from microalgae. Biotechnol Advances, 25, 294–306.

18. Zuccaro, G., Yousuf, A., Pollio, A., & Steyer, J. P. (2020). Microalgae cultivation systems. Microalgae cultivation for biofuels production, 11-29.

19. Su, C. H., Chien, L. J., Gomes, J., Lin, Y. S., Yu, Y. K., Liou, J. S., & Syu, R. J. (2011). Factors affecting lipid accumulation by Nannochloropsis oculata in a two-stage cultivation process. Journal of Applied Phycology, 23, 903-908.

20. Narala, R. R., Garg, S., Sharma, K. K., Thomas-Hall, S. R., Deme, M., Li, Y., & Schenk, P. M. (2016). Comparison of microalgae cultivation in photobioreactor, open raceway pond, and a two-stage hybrid system. Frontiers in Energy Research, 4, 29.

21. Bilad, M. R., Discart, V., Vandamme, D., Foubert, I., Muylaert, K., & Vankelecom, I. F. (2014). Coupled cultivation and pre-harvesting of microalgae in a membrane photobioreactor (MPBR). Bioresource Technology, 155, 410-417.

22. Suparmaniam, U., Lam, M. K., Uemura, Y., Lim, J. W., Lee, K. T., & Shuit, S. H. (2019). Insights into the microalgae cultivation technology and harvesting process for biofuel production: A review. Renewable and Sustainable Energy Reviews, 115, 109361.

23. Tredici, M. R. (2010). Photobiology of microalgae mass cultures: understanding the tools for the next green revolution. Biofuels, 1, 143-162.

24. Mata, T. M., Martins, A. A., & Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: a review. Renewable and sustainable energy reviews, 14(1), 217-232.

25. Langley, N. M., Harrison, S. T. L., & Van Hille, R. P. (2012). A critical evaluation of CO2 supplementation to algal systems by direct injection. Biochemical Engineering Journal, 68, 70-75.

26. Lee, Y. K. (2001). Microalgal mass culture systems and methods: their limitation and potential. Journal of Applied Phycology, 13, 307-315.

27. Iwamoto, H. (2004). Industrial production of microalgal cell-mass and secondary products - Major industrial species: *Chlorella*. In Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Amos R. (Ed) Blackwell Publishing Ltd, 255–263.

28. Wang, J., Yang, H., & Wang, F. (2014). Mixotrophic cultivation of microalgae for biodiesel production: status and prospects. Applied Biochemistry and Biotechnology, 172, 3307-3329.

29. Abiusi, F., Wijffels, R. H., & Janssen, M. (2020a). Oxygen Balanced Mixotrophy under Day–Night Cycles. ACS Sustainable Chemistry & Engineering, 8, 11682-11691.

30. Abiusi, F., Wijffels, R. H., & Janssen, M. (2020b). Doubling of microalgae productivity by oxygen balanced mixotrophy. ACS Sustainable Chemistry & Engineering, 8, 6065-6074.

31. Abiusi, F., Fernández, P. M., Canziani, S., Janssen, M., Wijffels, R. H., & Barbosa, M. (2022). Mixotrophic cultivation of *Galdieria sulphuraria* for C-phycocyanin and protein production. Algal Research, 61, 102603.

32. Chen, C.Y., Yeh, K.L., Aisyah, R., Lee, D.J., Chang, J.S., 2011. Cultivation photobioreactor design and harvesting of microalgae for biodiesel production: a critical review. Bioresour. Technol. 102 (1), 71-81

33. Gutiérrez, R., Passos, F., Ferrer, I., Uggetti, E., & García, J. (2015). Harvesting microalgae from wastewater treatment systems with natural flocculants: effect on biomass settling and biogas production. Algal research, 9, 204-211.

34. Show, K. Y., Yan, Y. G., & Lee, D. J. (2019). Algal biomass harvesting and drying. In Biofuels from Algae (pp. 135-166). Elsevier.

35. Grima, E. M., Belarbi, E. H., Fernández, F. A., Medina, A. R., & Chisti, Y. (2003). Recovery of microalgal biomass and metabolites: process options and economics. Biotechnology advances, 20(7-8), 491-515.

36. Singh, A., Nigam, P. S., & Murphy, J. D. (2011). Mechanism and challenges in commercialisation of algal biofuels. Bioresource technology, 102(1), 26-34.

37. Milledge, J. J., & Heaven, S. (2013). A review of the harvesting of micro-algae for biofuel production. Reviews in Envi-ronmental Science and Bio/Technology, 12(2), 165-178.

38. Heasman, M., Diemar, J., O'connor, W., Sushames, T., & Foulkes, L. (2000). Development of extended shelf-life microalgae concentrate diets harvested by centrifugation for bivalve molluscs–a summary. Aquaculture Research, 31(8-9), 637-659

39. Dassey, A. J., & Theegala, C. S. (2013). Harvesting economics and strategies using centrifugation for cost effective separation of microalgae cells for biodiesel applications. Bioresource technology, 128, 241-245.

40. Mata, T. M., Martins, A. A., & Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: a review. Renewable and sustainable energy reviews, 14(1), 217-232.

41. Ang, T. H., Kiatkittipong, K., Kiatkittipong, W., Chua, S. C., Lim, J. W., Show, P. L., ... & Ho, Y. C. (2020). Insight on extraction and characterisation of biopolymers as the green coagulants for microalgae harvesting. Water, 12(5), 1388.

42. Yin, C. Y. (2010). Emerging usage of plant-based coagulants for water and wastewater treatment. Process Biochemistry, 45(9), 1437-1444.

43. Ben Rebah, F., Mnif, W., & M. Siddeeg, S. (2018). Microbial flocculants as an alternative to synthetic polymers for wastewater treatment: a review. Symmetry, 10(11), 556.

44. Ummalyma, S. B., Mathew, A. K., Pandey, A., & Sukumaran, R. K. (2016). Harvesting of microalgal biomass: Efficient method for flocculation through pH modulation. Bioresource technology, 213, 216-221.

45. Vigani, M., Parisi, C., Rodríguez-Cerezo, E., Barbosa, M. J., Sijtsma, L., Ploeg, M., & Enzing, C. (2015). Food and feed products from micro-algae: Market opportunities and challenges for the EU. Trends in Food Science & Technology, 42, 81-92

46. Food, J., Organisation, A., & World Health Organisation Ad Hoc Expert Committee. (1973). Energy and protein requirements. In FAO Nutrition Meetings Report Series (No. 52).

47. Khan, Z., Bhadouria, P., & Bisen, P. S. (2005). Nutritional and therapeutic potential of *Spirulina*. Current Pharmaceutical Biotechnology, 6, 373-379.

48. Becker, E. W. (2013). Microalgae for human and animal nutrition. Handbook of microalgal culture: applied phycology and biotechnology, 461-503.

49. Matos, Â. P. (2017). The impact of microalgae in food science and technology. Journal of the American Oil Chemists' Society, 94, 1333-1350.

50. Paterson, S., Gómez-Cortés, P., de la Fuente, M. A., & Hernández-Ledesma, B. (2023). Bioactivity and digestibility of microalgae *Tetraselmis* sp. and *Nannochloropsis* sp. as basis of their potential as novel functional foods. Nutrients, 15(2), 477.

51. Spolaore, P., Joannis-Cassan, C., Duran, E., & Isambert, A. (2006). Commercial applications of microalgae. Journal of Bioscience and Bioengineering, 101, 87-96.

52. Yaakob, Z., Ali, E., Zainal, A., Mohamad, M., & Takriff, M. S. (2014). An overview: biomolecules from microalgae for animal feed and aquaculture. Journal of Biological Research-Thessaloniki, 21, 1-10.

53. Junior, W. G. M., Gorgich, M., Corrêa, P. S., Martins, A. A., Mata, T. M., & Caetano, N. S. (2020). Microalgae for biotechnological applications: Cultivation, harvesting and biomass processing. Aquaculture, 528, 735562.

54. Woortman, D. V., Fuchs, T., Striegel, L., Fuchs, M., Weber, N., Brück, T. B., & Rychlik, M. (2020). Microalgae a superior source of folates: quantification of folates in halophile microalgae by stable isotope dilution assay. Frontiers in Bioengineering and Biotechnology, 7, 481.

55. Brown, M. R., Mular, M., Miller, I., Farmer, C., & Trenerry, C. (1999). The vitamin content of microalgae used in aquaculture. Journal of Applied Phycology, 11, 247-255.

56. Fujii, K., Nakashima, H., & Hashidzume, Y. (2010). Isolation of folate-producing microalgae, from oligotrophic ponds in Yamaguchi, Japan. Journal of Applied Microbiology, 108, 1421-1429.

57. Kim, M., Ahn, J., Jeon, H., & Jin, E. (2017). Development of a *Dunaliella tertiolecta* strain with increased zeaxanthin content using random mutagenesis. Marine Drugs, 15, 189.

58. Fields, F. J., Lejzerowicz, F., Schroeder, D., Ngoi, S. M., Tran, M., McDonald, D., ... & Mayfield, S. (2020). Effects of the microalgae *Chlamydomonas* on gastrointestinal health. Journal of Functional Foods, 65, 103738.

59. Mourelle, M. L., Gómez, C. P., & Legido, J. L. (2017). The potential use of marine microalgae and cyanobacteria in cosmetics and thalassotherapy. Cosmetics, 4, 46.

60. Couteau, C., & Coiffard, L. (2018). Microalgal application in cosmetics. In Microalgae in Health and Disease Prevention. Levine I. A. & Fleurence J. (Ed). Academic Press, 317-323.

61. Coiffard, L., Couteau, C., 2012. Quoi de neuf dans le domaine de l'anti-âge. Medical staff dermatologie, 83, 3–7.

62. Baby, A. R., & Morocho-Jácome, A. L. (2021). Dermocosmetic applications of microalgal pigments. In Advances in Applied Microbiology (Vol. 117, pp. 63-93). Academic Press.

63. Terao, J., 1989. Antioxidant activity of beta-carotene-related-carotenoids in solution. Lipids 24, 659–661.

64. Ma, R., Wang, B., Chua, E. T., Zhao, X., Lu, K., Ho, S. H., ... & Chen, J. (2020). Comprehensive utilization of marine microalgae for enhanced co-production of multiple compounds. Marine Drugs, 18, 467.

65. Vernès, L., Granvillain, P., Chemat, F., & Vian, M. (2015). Phycocyanin from *Arthrospira platensis*. Production, extraction and analysis. Current Biotechnology, 4(4), 481-491.

66. Chia, S. R., Chew, K. W., Show, P. L., Xia, A., Ho, S. H., & Lim, J. W. (2019). *Spirulina platensis* based biorefinery for the production of value-added products for food and pharmaceutical applications. Bioresource technology, 289, 121727.

67. Khoo, K. S., Ooi, C. W., Chew, K. W., Foo, S. C., Lim, J. W., Tao, Y., ... & Show, P. L. (2021). Permeabilization of *Haematococcus pluvialis* and solid-liquid extraction of astaxanthin by CO2-based alkyl carbamate ionic liquids. Chemical Engineering Journal, 411, 128510.

68. Gouveia, L., Coutinho, C., Mendonça, E., Batista, A. P., Sousa, I., Bandarra, N. M., & Raymundo, A. (2008). Functional biscuits with PUFA-ω3 from *Isochrysis galbana*. Journal of the Science of Food and Agriculture, 88, 891-896.

69. Hossain, A. M., Brennan, M. A., Mason, S. L., Guo, X., Zeng, X. A., & Brennan, C. S. (2017). The effect of astaxanthin-rich microalgae "*Haematococcus pluvialis*" and wholemeal flours incorporation in improving the physical and functional properties of cookies. Foods, 6, 57.

70. Fradique, M., Batista, A. P., Nunes, M. C., Gouveia, L., Bandarra, N. M., & Raymundo, A. (2013). *Isochrysis galbana* and *Diacronema vlkianum* biomass incorporation in pasta products as PUFA's source. LWT-Food Science and Technology, 50(1), 312-319.

71. Ak, B., Avsaroglu, E., Isik, O., Özyurt, G., Kafkas, E., & Etyemez, M. (2016). Nutritional and physicochemical characteristics of bread enriched with microalgae *Spirulina platensis*. Int. J. Eng. Res. Appl, 6(9).

72. Niccolai, A., Zittelli, G. C., Rodolfi, L., Biondi, N., & Tredici, M. R. (2019). Microalgae of interest as food source: Biochemical composition and digestibility. Algal Research, 42, 101617.

73. Gantar, M., & Svirčev, Z. (2008). Microalgae and cyanobacteria: food for thought 1. Journal of Phycology, 44, 260-268.

74. Shimizu, Y. (2003). Microalgal metabolites. Current Opinion in Microbiology, 6, 236-243.

75. Gan, Q., Jiang, J., Han, X., Wang, S., & Lu, Y. (2018). Engineering the chloroplast genome of oleaginous marine microalga *Nannochloropsis oceanica*. Frontiers in plant science, 9, 439.

76. Van De Walle, S., Broucke, K., Baune, M. C., Terjung, N., Van Royen, G., & Boukid, F. (2023). Microalgae protein digestibility: How to crack open the black box?. Critical Reviews in Food Science and Nutrition, 1-23.

77. Ampofo, J., & Abbey, L. (2022). Microalgae: Bioactive composition, health benefits, safety and prospects as potential high-value ingredients for the functional food industry. Foods, 11(12), 1744.

78. Hosoglu, M. I. (2018). Aroma characterization of five microalgae species using solid-phase microextraction and gas chromatography–mass spectrometry/olfactometry. Food Chemistry, 240, 1210-1218.

79. Caporgno, M. P., & Mathys, A. (2018). Trends in microalgae incorporation into innovative food products with potential health benefits. Frontiers in Nutrition, 5, 58.

80. Fradique, M., Batista, A. P., Nunes, M. C., Gouveia, L., Bandarra, N. M., & Raymundo, A. (2010). Incorporation of *Chlorella vulgaris* and *Spirulina maxima* biomass in pasta products. Part 1: Preparation and evaluation. Journal of the Science of Food and Agriculture, 90, 1656-1664.

81. Jeon, J. K. (2006). Effect of *Chlorella* addition on the quality of processed cheese. Journal of the Korean Society of Food Science and Nutrition, 35, 373-377.

82. De Marco, E. R., Steffolani, M. E., Martínez, C. S., & León, A. E. (2014). Effects of *Spirulina* biomass on the technological and nutritional quality of bread wheat pasta. LWT-Food Science and Technology, 58, 102-108.

83. 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. Comprehensive Reviews in Food Science and Food Safety, 12, 144-154.

84. Boukid, F., Comaposada, J., Ribas-Agustí, A., & Castellari, M. (2021). Development of high-protein vegetable creams by using single-cell ingredients from some microalgae species. Foods, 10, 2550

85. Molino, A., Mehariya, S., Di Sanzo, G., Larocca, V., Martino, M., Leone, G. P., ... & Musmarra, D. (2020). Recent developments in supercritical fluid extraction of bioactive compounds from microalgae: Role of key parameters, technological achievements and challenges. Journal of CO2 Utilization, 36, 196-209.

86. Hallegraeff, G. M. (2014). Harmful algae and their toxins: progress, paradoxes and paradigm shifts. In Rossini G.P. (Ed) Toxins and Biologically Active Compounds from Microalgae, 2, CRC Press.

87. Caruana, A. M., & Amzil, Z. (2018). Microalgae and toxins. In Microalgae in Health and Disease Prevention, Levine I. A. & Fleurence J. (Ed). Academic Press, pp. 263-305.

88. Penna, A., Galluzzi, L., & Rossini, G. P. (2014). Detection and identification of toxic microalgae by the use of innovative molecular methods. Toxins and biologically active compounds from microalgae, 1, 51-96.89.

89 Novoveská, L., Ross, M. E., Stanley, M. S., Pradelles, R., Wasiolek, V., & Sassi, J. F. (2019). Microalgal carotenoids: A review of production, current markets, regulations, and future direction. Marine drugs, 17(11), 640.

90. Morocho-Jácome, A.L., Ruscinc, N., Martinez, R.M. et al. (2020). (Bio)Technological aspects of microalgae pigments for cosmetics. Applied Microbiology and Biotechnology, 104, 9513–9522.

91. Davinelli, S., Nielsen, M. E., & Scapagnini, G. (2018) Astaxanthin in skin health, repair, and disease: a comprehensive review. Nutrients 10, 522.

92. Miles, R., & Chapman, F. (2015). The Benefits of Fish Meal in Aquaculture Diets. Retrieved March 18, 2019, from http://edis.ifas.ufl.edu/fa122.

93. Dineshbabu, G., Goswami, G., Kumar, R., Sinha, A., & Das, D. (2019). Microalgae–nutritious, sustainable aqua-and animal feed source. Journal of Functional Foods, 62, 103545.

94. Batista, A. P., Gouveia, L., Bandarra, N. M., Franco, J. M., & Raymundo, A. (2013). Comparison of microalgal biomass profiles as novel functional ingredient for food products. Algal Research, 2(2), 164–173. https://doi.org/10.1016/J.ALGAL.2013.01.004.

95. Madeira, M. S., Cardoso, C., Lopes, P. A., Coelho, D., Afonso, C., Bandarra, N. M., & Prates, J. A. M. (2017). Microalgae as feed ingredients for livestock production and meat quality: A review. Livestock Science, 205(June), 111–121. https://doi.org/10. 1016/j.livsci.2017.09.020.

96. Shah, M. R., Lutzu, G. A., Alam, A., Sarker, P., Chowdhury, K., Parsaeimehr, A., ... & Daroch, M. (2018). Microalgae in aquafeeds for a sustainable aquaculture industry. Journal of Applied Phycology, 30, 197-213.

97. Fernández, F. G. A., Reis, A., Wijffels, R. H., Barbosa, M., Verdelho, V., & Llamas, B. (2021). The role of microalgae in the bioeconomy. New Biotechnology, 61, 99-107.

98. Al-Batshan, H. A., Al-Mufarrej, S. I., Al-Homaidan, A. A., & Qureshi, M. A. (2001). Enhancement of chicken macrophage phagocytic function and nitrite production by dietary *Spirulina platensis*. Immunopharmacology and Immunotoxicology, 23, 281-289.

99. Aravantinou, A. F., Theodorakopoulos, M. A., & Manariotis, I. D. (2013). Selection of microalgae for wastewater treatment and potential lipids production. Bioresource Technology, 147, 130-134

100. Oswald, W.J. (1962). The coming industry of controlled photosynthesis. American Journal of Public Health 52, 235–242.

101. Oswald, W.J. (1973). Productivity of algae in sewage disposal. Solar Energy 15, 107–117.

102. La Bella, E., Occhipinti, P. S., Puglisi, I., Fragalà, F., Saccone, R., Russo, N., ... & Baglieri, A. (2023). Comparative Phycoremediation Performance of Three Microalgae Species in Two Different Magnitude of Pollutants in Wastewater from Farmhouse. Sustainability, 15(15), 11644.

103. Chong, C. C., Cheng, Y. W., Ishak, S., Lam, M. K., Lim, J. W., Tan, I. S., ... & Lee, K. T. (2022). Anaerobic digestate as a low-cost nutrient source for sustainable microalgae cultivation: A way forward through waste valorization approach. Science of The Total Environment, 803, 150070.

104. Kong, W., Kong, J., Ma, J., Lyu, H., Feng, S., Wang, Z., ... & Shen, B. (2021). *Chlorella vulgaris* cultivation in simulated wastewater for the biomass production, nutrients removal and CO2 fixation simultaneously. Journal of Environmental Management, 284, 112070.

105. Guo J, Qi M, Chen H, Zhou C, Ruan R, Yan X, Cheng P. (2022) Macroalgae-derived multifunctional bioactive substances: The potential applications for food and pharmaceuticals. Foods, 31,11, 3455.

106. Wollmann, F., Dietze, S., Ackermann, J. U., Bley, T., Walther, T., Steingroewer, J., & Krujatz, F. (2019). Microalgae wastewater treatment: Biological and technological approaches. Engineering in Life Sciences, 19, 860-871.

107. Hena, S., Znad, H., Heong, K. T., & Judd, S. (2018). Dairy farm wastewater treatment and lipid accumulation by *Arthrospira platensis*. Water Research, 128, 267-277

108. Merola, A., Castaldo, R., Luca, P. D., Gambardella, R., Musacchio, A., & Taddei, R. (1981). Revision of *Cyanidium caldarium*. Three species of acidophilic algae. Plant Biosystem, 115(4-5), 189-195.

109. Delanka-Pedige, H. M. K., Munasinghe-Arachchige, S. P., Cornelius, J., Henkanatte-Gedera, S. M. et al., (2019). Pathogen reduction in an algal-based wastewater treatment system employing. *Galdieria sulphuraria*. Algal Research, 39, 101423.

110. Gross, W., & Schnarrenberger, C. (1995). Heterotrophic growth of two strains of the acid-thermophilic red alga *Galdieria sulphuraria*. Plant and Cell Physiology, 36, 633-638.

111. Sloth, J. K., Jensen, H. C., Pleissner, D., & Eriksen, N. T. (2017). Growth and phycocyanin synthesis in the heterotrophic microalga *Galdieria sulphuraria* on substrates made of food waste from restaurants and bakeries. Bioresource Technology, 238, 296-305.

112. Occhipinti, P. S., Del Signore, F., Canziani, S., Caggia, C., Mezzanotte, V., & Ferrer-Ledo, N. (2023). Mixotrophic and heterotrophic growth of *Galdieria sulphuraria* using buttermilk as a carbon source. Journal of Applied Phycology, 1-13.

113. Cuaresma, M., Casal, C., Forján, E., & Vílchez, C. (2011). Productivity and selective accumulation of carotenoids of the novel extremophile microalga *Chlamydomonas acidophila* grown with different carbon sources in batch systems. Journal of Industrial Microbiology and Biotechnology, 38, 167-177.

114. Escudero, A., Blanco, F., Lacalle, A., & Pinto, M. (2014). Ammonium removal from anaerobically treated effluent by *Chlamydomonas acidophila*. Bioresource Technology, 153, 62-68.

115. Varshney, P., Beardall, J., Bhattacharya, S., & Wangikar, P. P. (2018). Isolation and biochemical characterisation of two thermophilic green algal species-*Asterarcys quadricellulare* and *Chlorella sorokiniana*, which are tolerant to high levels of carbon dioxide and nitric oxide. Algal Research, 30, 28-37.

116. Chaiprasert, P. (2011). Biogas production from agricultural wastes in Thailand. Journal of Sustainable Energy and Environment. Spec. Issue, 63-65.

117. Mona, S., Malyan, S. K., Saini, N., Deepak, B., Pugazhendhi, A., & Kumar, S. S. (2021). Towards sustainable agriculture with carbon sequestration, and greenhouse gas mitigation using algal biochar. Chemosphere, 275, 129856.

118. Perin, G., & Morosinotto, T. (2019). Potential of microalgae biomass for the sustainable production of bio-commodities. In Progress in Botany. Vol. 81 Springer, Cham, pp. 243-276.

119. Santini, G., Biondi, N., Rodolfi, L., & Tredici, M. R. (2021). Plant biostimulants from cyanobacteria: An emerging strategy to improve yields and sustainability in agriculture. Plants, 10, 643.

120. Puglisi, I., La Bella, E., Rovetto, E. I., Lo Piero, A. R., & Baglieri, A. (2020). Biostimulant effect and biochemical response in lettuce seedlings treated with a *Scenedesmus quadricauda* extract. Plants, 9, 123.

121. Toscano, S., Romano, D., Massa, D., Bulgari, R., Franzoni, G., & Ferrante, A. (2018) Biostimulant applications in low input horticultural cultivation systems, Italus Hortus, 25, 27-36.

122. Ronga, D., Biazzi, E., Parati, K., Carminati, D., Carminati, E., & Tava, A. (2019). Microalgal biostimulants and biofertilisers in crop productions. Agronomy, 9, 192.

123. González-Pérez, B.K., Rivas-Castillo, A.M., Valdez-Calderón, A. et al. (2022). Microalgae as biostimulants: a new approach in agriculture. World Journal of Microbiology and Biotechnology 38, 4.

124. Walker, T. L., Purton, S., Becker, D. K., & Collet, C. (2005). Microalgae as bioreactors. Plant Cell Reports, 24, 629-641.

125. Wijffels, R. H., Kruse, O., & Hellingwerf, K. J. (2013). Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae. Current Opinion in Biotechnology, 24, 405-413.

126. Chowdhury, H., & Loganathan, B. (2019). Third-generation biofuels from microalgae: a review. Current Opinion in Green and Sustainable Chemistry, 20, 39-44.

127. Amicarelli, V., Paiano, A., & Lobefaro, L. (2012). Le microalghe nel settore dei biocombustibili. Sviluppo e sostenibilità. Studi&ricerche", Università degli Studi di Bari Aldo Moro, Dipartimento di Studi Aziendali e Giusprivatistici, I Facoltà di Economia, Bari

128. Dragone, G., Fernandes, B. D., Vicente, A. A., & Teixeira, J. A. (2010). Third generation biofuels from microalgae. In Current Research Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. Mendez-Vilas A. (Ed.), Formatex, pp. 1355-1366.

129. Shuba, E. S., & Kifle, D. (2018). Microalgae to biofuels: 'Promising alternative and renewable energy, review'. Renewable and Sustainable Energy Reviews, 81, 743-755.

130. Leong, W. H., Kiatkittipong, K., Kiatkittipong, W., Cheng, Y. W., Lam, M. K., Shamsuddin, R., ... & Lim, J. W. (2020). Comparative performances of microalgal-bacterial co-cultivation to bioremediate synthetic and municipal wastewaters whilst producing biodiesel sustainably. Processes, 8(11), 1427.

131. Dawood, F., Anda, M., & Shafiullah, G. M. (2020). Hydrogen production for energy: An overview. International Journal of Hydrogen Energy, 45(7), 3847-3869.

132. Show, K. Y., Yan, Y., Zong, C., Guo, N., Chang, J. S., & Lee, D. J. (2019). State of the art and challenges of biohydrogen from microalgae. Bioresource technology, 289, 121747.

133. Nguyen, T. T., Lam, M. K., Uemura, Y., Mansor, N., Lim, J. W., Show, P. L., ... & Lim, S. (2020). High biodiesel yield from wet microalgae paste via in-situ transesterification: effect of reaction parameters towards the selectivity of fatty acid esters. Fuel, 272, 117718.

134. FAO. (2017). The Future of Food and Agriculture—Trends and Challenges; Food and Agriculture Organization of the United Nations: Rome, Italy, 2017.

135. Olabi, A. G., Shehata, N., Sayed, E. T., Rodriguez, C., Anyanwu, R. C., Russell, C., & Abdelkareem, M. A. (2023). Role of microalgae in achieving sustainable development goals and circular economy. Science of The Total Environment, 854, 158689.

136. Pikaar, I., Matassa, S., Bodirsky, B. L., Weindl, I., Humpenöder, F., Rabaey, K., ... & Popp, A. (2018). Decoupling livestock from land use through industrial feed production pathways. Environmental Science & Technology, 52, 7351-7359.

137. Ruiz J, Olivieri G, De Vree J, Bosma R, Willems P, Reith JH, et al. Towards industrial products from microalgae. Energy Environ Sci 2016;9:3036–43. https://doi.org/10.1039/c6ee01493c.

138. Tredici, M. R., Bassi, N., Prussi, M., Biondi, N., Rodolfi, L., Zittelli, G. C., & Sampietro, G. (2015). Energy balance of algal biomass production in a 1-ha "Green Wall Panel" plant: How to produce algal biomass in a closed reactor achieving a high Net Energy Ratio. Applied Energy, 154, 1103-1111.

139. Batista, A. P., Niccolai, A., Bursic, I., Sousa, I., Raymundo, A., Rodolfi, L., ... & Tredici, M. R. (2019). Microalgae as functional ingredients in savory food products: application to wheat crackers. Foods, 8, 611.

140. Becker, E. W. (2007). Microalgae as a source of protein. Biotechnology Advances, 25, 207-210.

141. Abdel-Raouf, N., Al-Homaidan, A. A., & Ibraheem, I. (2012). Microalgae and wastewater treatment. Saudi Journal of Biological Sciences, 19, 257-275.

142. Raposo, M. F. D. J., De Morais, A. M. M. B., & De Morais, R. M. S. C. (2015). Carotenoids from marine microalgae: A valuable natural source for the prevention of chronic diseases. Marine Drugs, 13, 5128-5155.

143. Sekar, S., & Chandramohan, M. (2008). Phycobiliproteins as a commodity: trends in applied research, patents and commercialization. Journal of Applied Phycology, 20, 113-136.

CHAPTER 4. MICROALGAE-BASED WASTEWATER TREATMENT

An indigenous microalgal pool from a constructed wetland as an alternative strategy for Escherichia coli removal in urban wastewater.

Paride Salvatore Occhipinti[1], Nunziatina Russo[1], Paola Foti[1], Alessandra Pino[1], Cinzia L. Randazzo[1,2], Antonino Pollio[3], Cinzia Caggia[1,2*]

[1]Department of Agriculture, Food and Environment, University of Catania, Via S. Sofia, 100, 95123 Catania, Italy;

[2] CRIAB: Centro di Ricerca Interdipartimentale per l'implementazione dei processi di monitoraggio fisico, chimico e biologico nei sistemi di biorisanamento e di acquacoltura, Department of Medical sciences, Surgicals and Advanced Technologies, Hygiene and Public Health "G.F. Ingrassia" - University of Catania 95123 Catania, Italy;

[3]Department of Biology, University of Naples Federico II, Complesso Universitario Monte Sant'Angelo, Via Cintia 4, 80126 Naples.

*Correspondence: cinzia.caggia@unict.it; Tel.: +39-3331862960

This study was submitted to *Journal of the Science of Food and Agriculture*. (ID number JSFA-23-3127.)

Abstract

An autochthonous microalgal pool (MP), isolated from a constructed wetland (CW) in Sicily, was tested for the *Escherichia coli* removal efficacy as an alternative to the secondary treatment of urban wastewater treatment. The MP removal efficacy, detected at different sampling times, was compared to those obtained using *Chlorella vulgaris* and *Scenedesmus quadricauda,* in single cultures, against intentionally added *E. coli,* at 6 or 8 Log CFU/mL, in an Imhoff tank autoclaved water (ITAW) sampled at the same CW system. Moreover, the composition and stability of the MP were detected through DGGE analysis at initial and final experimental period. Results exhibited that in ITAW samples added with *E. coli* at both densities no significant difference was detected in the removal efficacy among the tested microalgal treatments. However, in ITAW samples inoculated with *E. coli* at lower density, after 2 days, *S. quadricauda* and *C. vulgaris* induced a decrease of 2.0 unit Log of *E. coli* and the autochthonous MP of 1.8 unit Log, whereas in ITAW samples inoculated with *E. coli* at higher density, after 2 days, *S. quadricauda, C. vulgaris* and the autochthonous MP reduced the target bacteria of 2.8, 3.4 and 2.0 unit Log, respectively. Starting from the 6th day, *E. coli* was never detected in treated samples, while it reached the mean value of 7.17 Log CFU/mL in inoculated un-treated ITAW samples. Moreover, the identification of microalgal strains isolated from MP, through both DGGE analysis and sequencing, revealed the presence of *Klebsormidium* sp. K39; *C. vulgaris, Tetradesmus obliquus* and *S. quadricauda.* Although the MP composition remained quite constant, after 12 days a different distribution among the microalgal species was observed.

Keywords: Urban wastewater treatment; Microalgae; *Escherichia coli;* phycoremediation; *Klebsormidium* sp. K39.

1. Introduction

It has been estimated that a 40% of water deficit by 2030 will occur, meaning societal and economic development a formidable challenge (Sun et al., 2016). The increased need for water is a consequence of population growth, economic development, climate change, and pollution (FAO, 2012), above all in the Mediterranean region, considered a vulnerable area (Collet et al., 2015; La Jeunesse et al., 2016). Furthermore, recently Zhang et al., (2023) reported that in near-future (2021–2050) global streamflow may be lower than predicted by Earth System Models, particularly in Africa, Australia and North America, taking into account also the evapotranspiration effects. Worldwide, 70% of

freshwater resources are destined for agricultural irrigation in arid and semi-arid regions of the globe and in Southern Europe more than 50% of the total water consumption takes place in agriculture (EEA, 2009; Hong et al., 2013; Ventura et al., 2019). The International Water Management Institute (Rosegrant and Cai, 2009) estimated that by 2025, 1.8 billion people will live in countries or regions plagued by an absolute water scarcity, which means that the water availability will be lower than 100 m^3/inhabitant/year. In such a scenario, it appears urgent to rethink water resource management (Ait-Mouheb et al., 2018). Reclaimed water (RW) represents a strategy extremely useful in many countries (WHO, 2006; EPA, 2012; Ait-Mouheb et al., 2018). However, based on the quality of the treated effluent, the use of RW can cause risks for plants, soils and for humans (WHO, 2006; Ofori et al., 2021). Scientific evidences have shown that RW can contribute to the accumulation and propagation of biological (animal and human pathogens, phytopathogens), xenobiotic contaminants (drugs and metals) and antibiotic resistant genes (Luczkiewicz et al., 2010; Bouki et al., 2013; Novo et al., 2013; Rizzo et al., 2013). The most referred risk for the environment is related to the increase in organic matter and salinity which, in turn, causes alterations in the structure and function of the soil microbial community (Bell et al., 2005; Wong et al., 2008). The World Health Organisation guidelines fixed safety criteria for irrigation purposes, for which RW must comply with standard criteria as specific physico-chemical and microbiological parameters. In EU the use of RW is under the Regulation (EU) 2020/741 on minimum requirements for water reuse, which establishes a threshold of 10 CFU/100 mL (<1 Log/100 mL) of *Escherichia coli* for RW classify as class "A", useful for irrigation of food crops (Ventura et al., 2009). In Italy about 4000 ha are irrigated by RW (Ait-Mouheb et al., 2018), and in southern regions, such as Puglia and Sicily, several pilot-scale projects aimed at compensating for the lack of natural resources typical of Mediterranean areas (Lopez et al. 2006; Lonigro et al. 2015). Constructed Wetlands (CWs) are among the wastewater treatment systems more environmentally sustainable, involving the use of engineered technologies designed to utilize natural processes (US EPA, 2012). The CWs are effective in reducing Biological Oxygen Demand (BOD) and total suspended solids (TSS) and are highly recommended as a low-cost secondary treatment system, although still some limitations include: (a) limited effect on phosphorus and nitrogen removal (especially free surface type); (b) limited capacity to remove fecal coliform (Zhimiao et al., 2016) or to zero *E. coli.* Different efficacies have been reported, for instance, Green et al. (1997) reported a reduction up to 1000 colony forming units (CFU) of *E. coli*/100 mL, afterward Diaz et al. (2010) found a wide variability, between 66 and 91% of *E. coli* loads retained in the wetlands. An efficacy solution for a complete removal of fecal coliforms was tested by Russo et al. (2019a) using a UV treatment on water effluent of CW. The biotransformation of pollutants from wastewater, including xenobiotics, nutrients and CO_2 from polluted air by macroalgae is known as

phycoremediation and the microalgae-based wastewater treatment (MBWT) is one of the most promising technologies for advanced treatment. The most common microalgal species used in wastewater treatment belong to the genera *Scenedesmus, Dunaliella, Phaeodactylum, Botryococcus, Oscillatoria, Pediastrum, Nitzschia, Cosmarium, Micractinium, Chlamydomonas,* and *Actinastrum* used both as axenic culture (pure culture) or as mixed culture (Santhanam, 2009; Abinandan and Shanthakumar 2015). Among them, *Chlorella* sp. and *Scenedesmus* sp. are known to be highly resistant to different pollutants, such as polycyclic aromatic, hydrocarbons, phenolic compounds and organic solvents (Garcia et al., 2000), and naturally dominate most continuous microalgal-based treatment systems, particularly in bacterial and microalgal consortia (Muñoz et al., 2003). Furthermore, *Chlorella vulgaris,* thanks to its good acclimatization to a wide range of environmental conditions, its immobilization and biosorption capacity, with high nutrient removal rate (Moondra et al., 2020), is the most used species in bioremediation applications. The aim of the present study was to evaluate the performance of *Escherichia coli* removal efficacy of a MBWT based on an autochthonous microalgal pool (MP), obtained by a CW located in Sicily, as an alternative to the secondary treatment. In particular, the MP removal efficacy against intentionally inoculated *E. coli* was compared to both the *Chlorella vulgaris* and the *Scenedesmus quadricauda* in single cultures in an Imhoff tank autoclaved water (ITAW) sampled at the same CW system.

2. Materials and methods

2.1. Microbial strains, media and cultivation conditions

Chlorella vulgaris ACUF863, *Chlorella vulgaris ACUF110* and *Scenedesmus quadricauda* ACUF581, belonging to the Algal Collection of "Federico II" Naples University (ACUF), were cultivated photo-autotrophically in 250 mL volume sterile flask. *C. vulgaris* ACUF863 and *S. quadricauda* ACUF581 (at final cell density of 3.5 x 10^4 cells/mL) were single inoculated in 100 mL of Bold Basal Medium (BBM) broth [2.94 mM $NaNO_3$, 0.17 mM $CaCl_2{\cdot}2H_20$, 0.30 mM $MgSO_4{\cdot}7H_20$, 0.43 mM K_2HPO_4, 1.29 mM KH_2PO_4, 0.43 mM NaCl, 8.5 µM EDTA, 0.9 µM $FeSO_4$, 9 µM H_3BO_3, 1.50 µM $ZnSO_4{\cdot}7H_20$, 0.36 µM $MnCl{\cdot}4H_2O$, 0.26 µM MoO_3, 0.31 µM $CuSO_4{\cdot}5H_2O$, 0.084 µM $Co(N0_3)_2{\cdot}\ 6H_20$] (Bischoff and Bold, 1963) and incubated at 25 ± 2˚C under axenic conditions, with a photoperiod 16:8 (day:night), under LED light 25.000 lux and shaking (150 rpm). The final cell density was confirmed by using the Bürker chamber count. The microalgal pool (MP) was obtained from the free water surface (FWS) pond in a wetland plant located in an agritourist structure in Sicily, through the serial dilution method. In detail, a water sample from FWS was diluted

1:1000 in sterilized BBM medium and incubated at the conditions reported above. After 10 days, 200 µL of diluted sample were purified by streaking on BBM agar medium, supplemented with rifampicin (50 mg/L) and carbenzadin (5 µg/mL). The plates were incubated for 3 weeks at 25 ± 2˚C, with a photoperiod 16:8 (day:night) and under LED light 25.000 lux. Simultaneously, 1 mL of raw diluted (1:1000) water sample from FWS was transferred into 24-Well Flat-Bottom Plate Tissue Culture (Falcon, Becton Dickinson and Company, Franklin Lakes, NJ, USA) in order to isolate microalgae by using micropipetting technique (Andersen and Kawachi, 2005), under an inverted microscope (Fluovert, Leitz Wetzlar Germany, type 307-148.002). The microalgal isolates were purified by streaking on BBM agar medium, supplemented with rifampicin (50 mg/L) and carbenzadin (5 µg/mL) and incubated at the conditions above reported. To verify the axenicity, purified microalgal isolates and MP were streaked on BBM agar medium supplemented with glucose (18 mg/L), according to Guillard (2005), and incubated for 72 h at 37 ˚C in darkness. Finally, 10 purified microalgal isolates and purified colonies of MP were singly transferred into sterile flasks containing BBM broth and incubated at conditions reported above. To follow the microalgal dynamics of species present in the MP during the experimental trials, a visual snapshot was obtained by microscopic observations. In detail, 10 µL of undiluted water sample, before, during and after the trials were laid on a glass microscope slide and observed by an Olympus BX40 System (Olympus Italia S.r.l., Segrate Milano) at 10, 40 and 100X magnitude. The *Escherichia coli* ATCC 25922 strain was used as target microorganism in wastewater samples. The strain was revitalized in Brain Heart Infusion Broth (BHI, Scharlau Microbiology. Scharlab, Spain) and incubated overnight at 37 °C to obtain a final cell density of 9 Log CFU/mL, evaluated by serial dilutions method into Chromatic™ EC X-GLUC Agar (Liofilchem, Italy).

2.2. Wastewater sampling

Wastewater samples were obtained from the Imhoff tank of the same wetland system, located in an agrituristic structure (Sicily, Italy). Samples collected using sterile glass bottles were immediately transferred to the Laboratory of Microbiology at the Department of Agricultural, Food and Environment (University of Catania). Wastewater samples were subjected to pH, electrical conductivity (EC), total suspended solids (TSS), nitrate-nitrogen (NO^{3-} single bond N), sulphate (SO_4^{-2}), total phosphorus (TP), five-day biochemical oxygen demands (BOD_5), and salt (Na^+, K^+, and Ca^{+2}) determinations and data are reported in Table 1S (Supplementary section). Microbiological analyses were carried out following the membrane filtration method (Standard Methods for the Examination of Water and Wastewater 23th edition: APHA, 2017), and for *E. coli* detection and counting, 100 mL

of water sample were filtered on membrane filters (0.45 µm pores, Cellulose, Merck, Germany) and poured in RAPID' *E. coli* 2 Agar plates (Bio-Rad, Italy), incubated at 37 °C for 24 h.

2.3. Experimental design

Water samples, obtained from the Imhoff tank, were grossly filtered, through a 5-10 µm pore size Fisherbrand™ cellulose filter paper (Thermo Fisher Scientific. Waltham, Massachusetts, USA) and sterilized at 121 °C for 20 minutes. *C. vulgaris* ACUF863, *S. quadricauda* ACUF581, and the autochthonous MP, at final concentration of 5 Log cells/mL, were singularly inoculated into 300 mL sterile flasks containing 150 mL of the Imhoff tank autoclaved water (ITAW). In order to evaluate the removal efficacy of each microalgal culture, immediately after, *E. coli* ATCC 25922, cultured at 37 °C for 24h in Brain Heart Infusion Broth (BHI, Scharlau Microbiology, Scharlab, Spain), was inoculated in each flask at final concentrations of 6 or 8 Log CFU/mL (Figure 1). ITAW samples inoculated with single microalgal culture were used as controls whereas ITAW samples inoculated with a fresh *E. coli* ATCC 25922 culture, at final density of 6 Log CFU/mL or 8 Log CFU/mL, were used as positive controls. The flasks were kept at 25 ± 2 ºC, with a photoperiod 16:8 (day:night) under LED light 25.000 lux for 12 days. The optical density (OD) of samples, differently treated, at different sampling times, was detected using a spectrophotometer at 550 nm (Cary 100 Scan UV-Visible, Agilent, CA, USA). Each experiment was performed in triplicate. Samples were taken at 0, 2, 4, 6, 8 and 12 days after inoculum and microbiological analyses were performed, in triplicate.

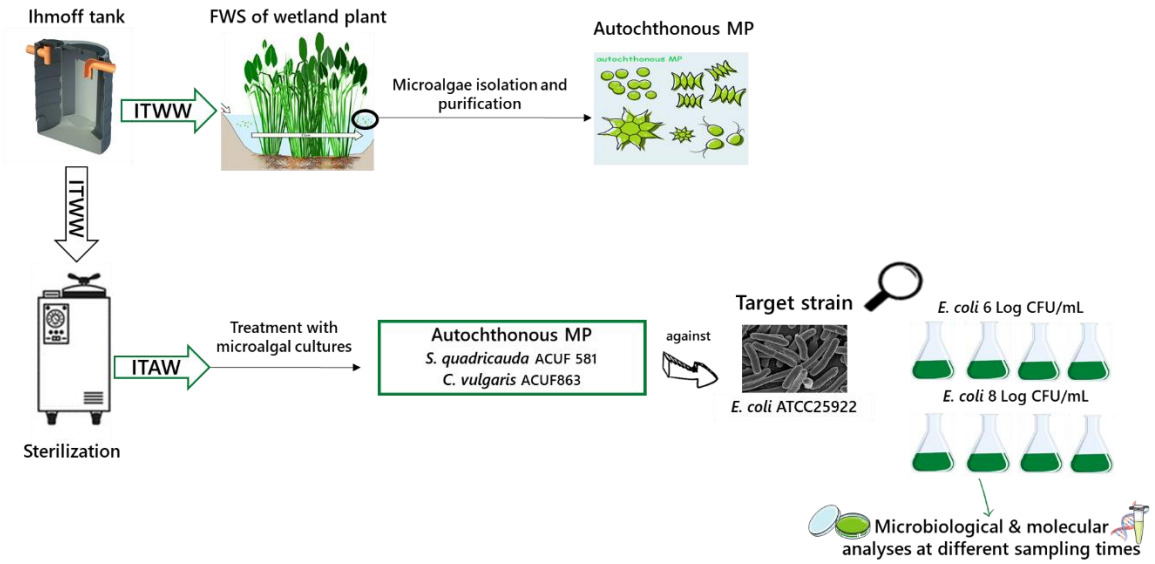


Figure 1. Experimental design (ITWW: Imhoff tank wastewater; ITAW: Imhoff tank autoclaved water; FWS: free water surface pond).

2.4. pH monitoring

ITAW samples, inoculated with the autochthonous MP, the *C. vulgaris* ACUF863, the *S. quadricauda* ACUF581, with and without the *E. coli* ATCC 25922, were taken at 0, 2, 4, 6, 8 and 12 days after inoculum, and subjected to pH determination. The pH value was detected at 25 °C using an Xs pH50 instrument. The analysis was performed in triplicate and results are reported as mean pH and standard deviation.

2.5. *E. coli* removal efficacy

In order to evaluate the bacterial removal efficacy, the ITAW samples inoculated with autochthonous MP, *C. vulgaris* ACUF863, *S. quadricauda* ACUF581 and right after with *E. coli* ATCC 25922, were taken at 0, 2, 4, 6, 8 and 12 days. The analysis was performed according to APHA guidelines (2017) and following the method reported by Milani and co-workers (2020). *E. coli* was enumerated according to the ISO 9308-1 procedures (2012), using the Chromatic^{TM} EC X-GLUC Agar media (Liofilchem, Italy) incubated at 37 ± 2 ºC for 48 h. The analysis was performed in triplicate and results expressed as mean $\log_{10}$ colony-forming units (CFU) per unit of volume and standard deviation.

2.6. Identification of strains isolated from the autochthonous MP

Based on phenotypical and microscopic traits two microalgal isolates (M1 and M2) were selected and subjected to total DNA extraction, following the CTAB method (Doyle and Doyle, 1990). DNA was amplified using the primer pairs: AV-rbcL_RH1-f (ATGTCACCACAAACAGAAACTAAAGC) and AV-rbcL_1385r (AATTCAAATTTAATTTCTTTCC), targeting the *rbcL* gene for green algae (Manhart, 1994), and primers for V0-V1_63f (CAG GCC TAA CAC ATG CAA GTC) and V6-1073r (ACGAGCTGACGACARCCATG) targeting the 16S rRNA gene for Cyanobacteria (Marchesi et al., 1998; Uroz et al., 2012). The PCR reaction was performed in a final volume of 50 µL, containing 30 ng of template DNA, 2.5 U of Taq DNA polymerase (Invitrogen, Italy), 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTPs, and 100 mM of each primer. Amplification reactions were performed using a T100 thermal cycler (BioRad, Hercules, CA, USA) as follows: 1 cycle at 94 °C for 4 min; 35 cycles at 94 °C for 60 s, 45 °C for 2 min, 65 °C for 3 min, 1 cycle at 72 °C for 5 min (for AV-rbcL_RH1-f and AV-rbcL_1385r primer pairs); 1 cycle at 95 °C for 1 min; 30 cycles at 95 °C for 60 s, 60.5 °C for 1 min, 72 °C for 1.5 min, 1 cycle at 72°C for 5 min (for V0-V1_63f (CAG GCC TAA CAC ATG CAA GTC and V6-1073r (ACGAGCTGACGACARCCATG primer pairs). Amplification products were analysed by electrophoresis in 1.0% (w/v) agarose gels in TBE 1X buffer (89 mM Tris–borate, 89 mM boric acid, 2 mM EDTA; pH 8.0), running at 100 V for 45 min, and visualized after staining with Gel Red Nucleic Acid Stain (Biotium, Inc., Fremont, CA, USA). PCR products, obtained by primer pairs targeting the *rbcL* gene, were purified using a Qiaquick PCR purification kit (Qiagen Hilden, Germany) and subjected to sequencing performed by an external service (Eurofins Genomics, Vimodrone, Italy). Taxonomic identification was assessed by sequence analysis of the *rbcL* gene using the basic local alignment search tool (BLASTn) software in the Standard databases (nucleotide collection (nr/nt)

2.7. PCR-DGGE analysis

Microalgal isolates, obtained from the autochthonous MP and from the ITAW samples inoculated with the autochthonous MP plus *E. coli* at 8 Log CFU/mL, were subjected to PCR-DGGE analysis. Total DNA was extracted following the CTAB DNA method (Doyle and Doyle, 1990). PCR products were obtained using the primer pairs Euk1A (CTGGTTGATCCTGCCAG) and Euk516r-GC (ACCAGACTTGCCCTCC-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG), amplifying a 560-bp fragment of the eukaryotic 18S (Dìez et al., 2001), and the primer pairs GC-16S353F- (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-

AGCAGTGGGGAATTTTCCGC) and CYA781RA (GACTACTGGGGT ATCTAATCCCATT), amplifying a 409-bp of the Cyanobacteria 16S ribosomal DNA (Ohkubo et al., 2006; Nubel et al., 1997). The PCR reaction was performed as previously reported (Dìez et al., 2001; Ohkubo et al., 2006). DGGE analysis of PCR amplicons was performed following the protocol described by Dìez and co-workers (2001) and by Granada-Moreno (2017), using the DCode System (Bio-Rad Laboratories, Hercules, CA). The polyacrylamide gel consisted of 8% (w/v) polyacrylamide (37.5:1 acrylamide–bisacrylamide) in 0.5x TAE buffer. Denaturing acrylamide of 100% was defined as 7 M urea and 40% [v/v] formamide. The gels were poured from the top using a gradient maker and the pump (Econopump; Bio-Rad) was set at 4.5 mL/min of speed. The gradient was set at 40-65% for the amplicons generated by the Euk1A/Euk516r-GC primes and at 30-40% for the amplicons generated by GC-16S353F/CYA781RA primers. Electrophoresis was performed for 16 h at voltage of 90-100V in a 0.5x TAE buffer at a constant temperature of 60 °C. Gels were stained with silver nitrate according to Sanguinetti et al. (1994) and Randazzo and co-workers (2015).

2.8. Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's HSD post hoc test for means separation were performed using the STATISTICA ETL software (version 10, StatSoft. inc., Tulsa, OK, USA). The significance level was set at $p \leq 0.05$.

4. Results

4.1. pH values of ITAW samples inoculated with *E. coli* ATCC 25922 at different densities and treated with different microalgal cultures

The pH values detected in ITAW samples, inoculated with *E. coli* ATCC25922 (6 Log CFU/mL) and with different microalgae (*C. vulgaris* ACUF863, *S. quadricauda* ACUF581, or autochthonous MP), after 0, 2, 4, 6, 8 and 12 days, are reported in Table 1. Overall, at each sampling time, ITAW samples inoculated with microalgae exhibited values 1.5 points higher than those observed in ITAW samples inoculated only with the *E. coli* ATCC 25922 strain. Starting from the 4th day, the ITAW sample inoculated with the autochthonous MP showed the lowest pH values, except at the 8th day when the pH was found statistically equal to those registered for ITAW sample inoculated with *S. quadricauda* ACUF581.

Table 1. pH values detected in ITAW samples inoculated with *E. coli* ATCC 25922 at 6 Log CFU/mL treated with different microalgal strains (*C. vulgaris* ACUF863*, S. quadricauda* ACUF581 and autochthonous MP) at 0, 2, 4, 6, 8, and 12 days after inoculation.

Treatment	Days					
	0	2	4	6	8	12
Control *E. coli*	7.63±0.21^{c}	7.88±0.20^{c}	8.32±0.08^{c}	8.36±0.06^{d}	8.33±0.18^{c}	8.28±0.05^{c}
S. quadricauda	8.55±0.08^{ab}	9.74±0.05^{b}	9.74±0.03^{a}	9.73±0.07^{b}	9.43±0.23^{b}	9.80±0.10^{a}
C. vulgaris	8.18±0.22^{b}	9.59±0.06^{ab}	9.82±0.07^{a}	10.14±0.10^{a}	10.10±0.6^{a}	9.82±0.06^{a}
Autochthonous MP	8.34±0.07^{a}	9.97±0.08^{a}	9.23±0.08^{b}	9.11±0.07^{c}	9.08±0.13^{b}	9.16±0.06^{b}

Data are expressed as means of three replicates ± SD. Values detected at the same time followed by different letters are significantly different ($p \leq 0.05$).

The pH values detected in ITAW samples inoculated with target bacteria at higher density, as 8 Log CFU/mL, and differently treated with the tested microalgal cultures, at 0, 2, 4, 6, 8 and 12 days after inoculum are reported in Table 2. Overall, the mean pH values of samples inoculated with microalgae were significantly higher (of about 1.5 points) than those detected in controls (samples inoculated only with *E. coli* ATCC25922), at each sampling time. Focusing on the 6th day, samples showed pH values of 9.37, 9.45, 9.10 and 8.38, respectively for *C. vulgaris* ACUF863, *S. quadricauda* ACUF581, MP and control sample (inoculated only with *E. coli*). Moreover, ITAW samples inoculated with autochthonous MP showed pH values similar to those obtained in samples inoculated with *C. vulgaris* ACUF863 (at 4, 6, 8 and 12 days after inoculum), and to samples inoculated with *S. quadricauda* ACUF58 (at 2, 6 and 8 days after inoculum).

Table 2. pH values detected in ITAW samples inoculated with *E. coli* ATCC 25922 at 8 Log CFU/mL treated with different microalgal strains (*C. vulgaris* ACUF863, *S. quadricauda* ACUF581 and autochthonous MP) at 0, 2, 4, 6, 8, and 12 days after inoculation.

Treatment	Days					
	0	2	4	6	8	12
Control *E. coli*	7.63±0.21^{c}	7.90±0.16^{c}	8.11±0.16^{d}	8.38±0.20^{c}	8.12±0.28^{c}	8.29±0.14^{c}
S. quadricauda	8.55±0.08^{ab}	9.20±0.14^{a}	9.27±0.09^{a}	9.45±0.03^{a}	9.74±0.04^{a}	9.84±0.09^{b}
C. vulgaris	8.35±0.22^{b}	8.73±0.07^{b}	9.07±0.05^{b}	9.37±0.15^{ab}	9.63±0.13^{ab}	10.06±0.12^{ab}
Autochthonous MP	8.70±0.07^{a}	9.17±0.07^{a}	8.78±0.12^{c}	9.10±0.0^{b}	9.27±0.13^{b}	10.29 ±0.13^{a}

Data are expressed as means of three replicates ± SD. Values of the same time followed by different letters are significantly different ($p \leq 0.05$).

4.2. Microalgae concentration in ITAW samples at 0, 2, 6, 8 and 12 days from inoculum

The microalgal counts, detected in ITAW samples inoculated with *C. vulgaris* ACUF863, *S. quadricauda* ACUF581 and autochthonous MP, were detected by Bürker chamber counting cell (Table 2S). Overall, no significant difference in microalgal concentration was observed between the MP and *C. vulgaris* ACUF863 inoculated samples, at any sampling time, showing a mean value of 6.00 Log cells/mL, whereas ITAW samples treated with *S. quadricauda* ACUF581 showed lower mean values (as 5.63 Log cells/mL).

4.3. Microalgal densities detected in ITAW samples inoculated with *E. coli* ATCC 25922 at different concentrations. In ITAW samples with *E. coli* ATCC 25922 (at 6 or 8 Log CFU/mL), inoculated with different microalgal cultures (*C. vulgaris* ACUF863, *S. quadricauda* ACUF581, or autochthonous MP), microalgal counts were performed, by Bürker chamber counting cell, after 0, 8 and 12 days from the microalgal inoculum (Fig. 1S). Even though high variability was detected among ITAW samples, a good growth performance was exhibited by autochthonous MP, in presence of *E. coli* ATCC 25922 at both 6 and 8 Log CFU/mL. In fact, after 8 days from inoculum, no significant difference was detected among MP, *C. vulgaris* ACUF863, and *S. quadricauda* ACUF581 counts in ITAW samples inoculated with *E. coli* at both densities. At that sampling time, it is interesting to highlight that the microalgal counts in samples inoculated with autochthonous MP reached mean values higher than 6.0 Log cells/mL, when *E. coli* ATCC 25922 was inoculated at both 6 and 8 Log CFU/mL. At the lower tested concentrations of *E. coli* ATCC25922, ITAW samples inoculated with *S. quadricauda* ACUF581 showed the lowest detected microalgal count (5.34 Log cells/mL), lower than 0.73 Log cells/mL compared to MP. Similar results were detected after 12 days, when in ITAW sample inoculated with *S. quadricauda* ACUF581, microalgal densities were found lower than those detected for MP at both *E. coli* ATCC 25922 tested densities. Overall, the microalgal densities detected in samples inoculated with MP were always statically equal to those detected in samples inoculated with *C. vulgaris* ACUF863.

4.4. Removal efficacy of *E. coli* ATCC 25922 by microalgal cultures

The cell density of *E. coli* ATCC 25922 detected in ITAW samples, un-inoculated (control) and inoculated with different microalgal cultures (*C. vulgaris* ACUF863, *S. quadricauda* ACUF581, or autochthonous MP) after 0, 2, 4, 6, 8 and 12 days from the inoculum are shown in Figure 2. Overall, a significant reduction of *E. coli* ATCC 25922 cell density was observed in all tested samples, with the exception of controls. In detail, in samples inoculated with *E. coli* ATCC 25922 at lower density

(as 6 Log CFU/mL), no significant difference was detected in the removal efficacy of the tested microalgae (Figure 2, panel A). In detail, 2 days after the microalgal inoculum, *S. quadricauda* ACUF581 and *C. vulgaris* ACUF863 induced a decrease of 2.07 unit Log of the *E. coli* ATCC 25922 viable cells, whereas autochthonous MP reduced the *E. coli* ATCC 25922 count of 1.85 unit Log. In samples of the same trial, *E. coli* ATCC 25922 viable cell was found below the detection limit starting from the 6th day. A different trend was observed in un-inoculated sample (control), where the *E. coli* density was found slightly constant till the 8th day, to reach, after 12 days, a value of 5.94 Log CFU/mL. In ITAW samples inoculated with *E. coli* ATCC25922 at higher density (as 8 Log CFU/mL), each microalgal culture performed similar removal efficacy with a significant reduction of the target bacteria (Figure 2, panel B). In detail, after 2 days from inoculum, in samples treated with *S. quadricauda* ACUF581, *C. vulgaris* ACUF863, and autochthonous MP the target bacteria was reduced by 2.8, 3.4 and 2.0 unit Log, respectively. After 6 days, *E. coli* was never detected in any microalgae treated ITAW samples, while its density was found at a mean value of 7.17 Log CFU/mL in untreated samples.

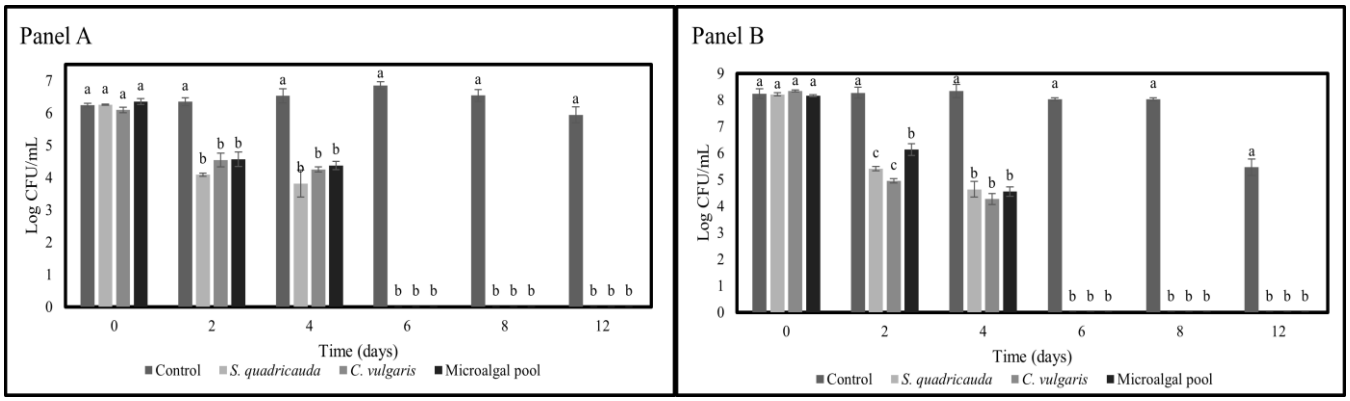


Figure 2. *E. coli* density detected (as Log CFU/mL) in ITAW samples inoculated with *E. coli* ATCC25922 at 6 Log CFU/mL (panel A) and 8 Log CFU/mL (panel B) treated with different microalgal strains (*C. vulgaris* ACUF863, *S. quadricauda* ACUF581 or autochthonous MP) at initial time and after 2, 4, 6, 8 and 12 days from inoculation. Data are expressed as means ± SD. Mean values with different letters at the same sampling time are statistically different ($p \leq 0.05$).

4.5. Sequencing data and BLAST alignment

The results of sequencing of M1 and M2 isolates were compared with the sequence databases by Basic Local Alignment Search Tool (BLAST), and the M1 strain (accession number **OQ363409**) corresponded, at 100%, to *Klebsormidium* sp. K39; whereas the M2 (accession number **OQ363408**) corresponded at 99.6% to *Tetradesmus obliquus*.

4.6. Microalgal pool community dynamic during trials

In order to highlight the dynamic of the microalgal community, the MP growth in the medium, the MP inoculated in ITAW sample added with *E. coli* at 8 Log CFU/mL and the microalgal isolates were subjected to PCR-DGGE analysis and the obtained profile compared. No amplifaction was obtained with Cyanobacteria 16S ribosomal DNA (rDNA)-specific set B primers (GC-16S353F and CYA781RA). Regarding the eukaryotic community profiles, obtained by primers 18S rRNA gene amplicons pairs Euk1A and Euk51rev, although the PCR amplification yielded a single band the DGGE analysis revealed the presence of distinct bands (Figure 3).

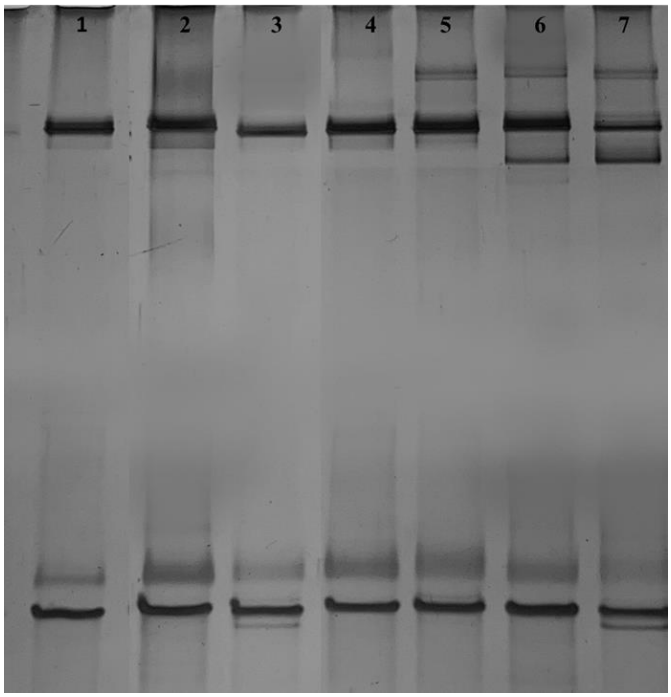


Figure 3. Denaturing gradient gel electrophoresis patterns of microalgal 18S rRNA gene fragments amplified using 18S primers set A [Euk1A and Euk516r-GC]. Line 1: M1 strain, identified as *Klebsormidium* sp. K39; Line 2: *C. vulgaris* ACUF110 strain; Line 3: M2 strain identified as *Tetradesmus obliquus*: Line 4: *C. vulgaris* ACUF863: Line 5: *S. quadricauda* ACUF581; Line 6: ITAW samples inoculated with the higher concentration of *E. coli* ATCC25922 treated with autochthonous MP after 12 days; Line 7: autochthonous MP cultured on BBM.

In details, as shown in Figure 3, the DGGE profiles confirmed the presence of species belonging to *Klebsormidium* sp. K39 and *T. obliquus* rather than *C. vulgaris* and *S. quadricauda*.

Furthermore, comparing profiles obtained by ITAW samples inoculated with the higher concentration of *E. coli* ATCC25922 treated with autochthonous MP, after 12 days (line 6, Fig. 3) with those obtained by autochthonous MP cultured on BBM (line 7, Fig. 3) it is interesting to underline the

disappearing of the lightest band corresponding to the *T. obliquus* profile (line 3, Fig. 3). These results are in accordance with microscope imagines obtained from fresh autochthonous MP, cultured BBM (panel *e*, Fig. 4), and those obtained from ITAW samples with the higher concentration of *E. coli* ATCC25922 after 12 days of treatment with the autochthonous MP (panel *f*, Fig. 4). In particular, as showed in Figure 4, when the autochthonous MP was cultured on BBM, a quite uniform distribution of each microalgal species was observed, even thought *Chlorella* sp. was found as prevalent, whilst in ITAW samples inoculated with *E. coli*, after 12 days of treatment with autochthonous MP, a different species distribution was observed, with *Klebsormidium* sp.K39 found as dominant microalga in place of *Chlorella* sp. and with reduction and disappearance of *S. quadricauda* and *T. obliquus,* respectively.

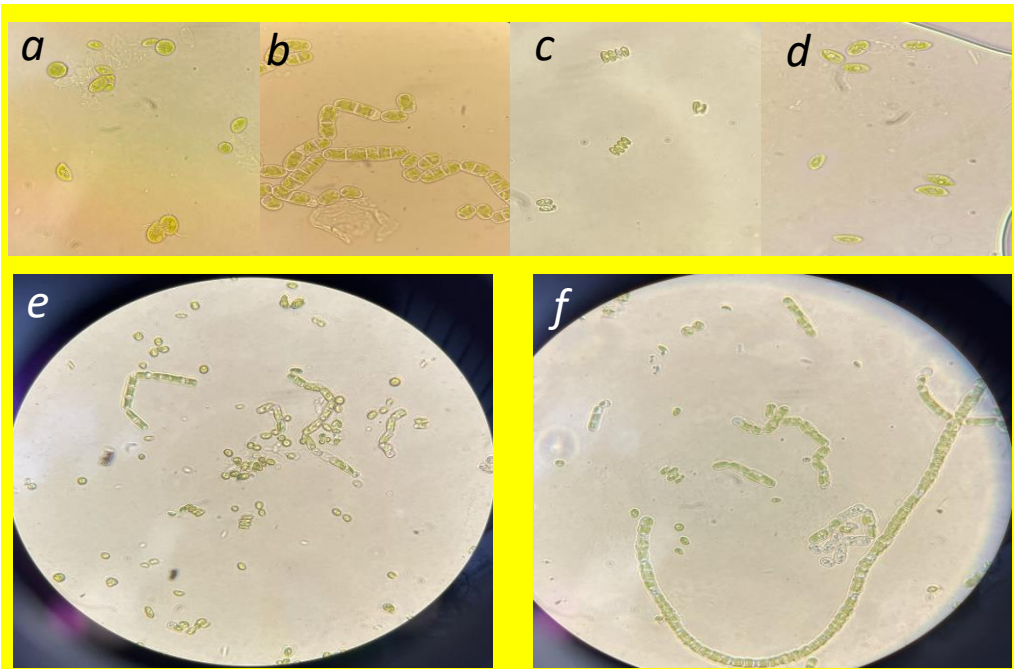


Figure 4. Microscopic images of microalgal strains and autochthonous MP used in the present study. In detail in panel *a*: particular of *C. vulgaris* at 100X magnitude; in panel *b*: particular of *Klebsormidium* sp.K39 at 100X magnitude; in panel *c*: particular of *S. quadricauda* at 100X magnitude; in panel *d*: particular of *T. obliquus* at 100X magnitude; in panel *e*: particular of autochthonous MP cultured on BBM visualizzed at 40X magnitude; in panel *f*: particulat of ITAW samples inoculated with the higher concentration of *E. coli* ATCC25922 treated with autochthonous MP after 12 days visualizzed at 40X magnitude.

5. Discussion

Conventional wastewater treatment aims to remove, mostly mechanically, suspended solids and to reduce biological oxygen demand. This biodegradation involves the breakdown of both organic and inorganic constituents (nitrogen and phosphorous compounds), which is of great importance to prevent the eutrophication of downstream waters, such as rivers and lakes. The degradation capacity of conventional technologies is limited, especially with regard to heavy metals, extremely high nutrient loads, and xenobiotics, leading to an increasing accumulation of these substances in groundwater (Wollmann et al., 2019). Because of the metabolic flexibility of microalgae, i.e. their ability to perform photoautotrophic, mixotrophic, or heterotrophic metabolism, they represent a promising biological system for treating a variety of sources of wastewater. As already reported, *C. vulgaris* has been widely exploited for biomass production from food wastes, sludge extracts, corn steep liquor, cheese whey and vinasse, textile waste effluent, tofu wastewater, and industrial dairy effluents (Kong et al., 2021; Guo et al., 2022). In this study, a wastewater treatment based on an autochthonous MP, isolated from the FWS pond of a CW plant, was compared to treatments performed by single *C. vulgaris* ACUF863 or *S. quadricauda* ACUF581 in sterilized wastewater samples taken from the same CW plant. In particular, the *E. coli* removal efficacy was tested in a 12 day-period starting from two initial bacterial densities to evaluate the effective removal effect in specific physico-chemical conditions. Results related to microalgal densities, unveil that in ITAW samples, without *E. coli*, the MP reached similar cell densities as those detected for *C. vulgaris* ACUF863, and higher than detected for *S. quadricauda* ACUF581 after 2, 6 and 12 days. These findings highlighted that the autochthonous MP is well adapted to specific local conditions and confirmed the great ability of the two species, *C. vulgaris* and *S. quadricauda,* to easily acclimatizate to environmental conditions (Moondra et al., 2020), being suited to wastewater matrix, as reported by Santhanam (2009), Abinandan and Shanthakumar (2015) and Moondra et al. (2020). Nevertheless, compared to monocultures, microalgal polycultures can resist invasive species or pathogens (Mitchell et al., 2002) and represent a more robust system (Gonçalves et al., 2017) able to adapt to environmental fluctuations. In the present study, the microalgal densities in samples inoculated with MP, at both the two concentration of *E. coli,* exhibited values quite similar to those detected in samples inoculated with *C. vulgaris* plus *E. coli* at both concentrations, while higher microalgal concentrations were found compared with detected in control samples inoculated with *S. quadricauda*, and in samples inoculated with *S. quadricauda* plus *E. coli* at both concentrations. These findings are in accordance with previous reports where the polycultures exhibited higher biomass production (Naeem et al., 1994; Cardinale et al., 2006; Cardinale, 2011). Focusing on *E. coli*

removal efficacy, in the ITAW samples inoculated with *E. coli* ATCC25922 at both densities, treated with each microalgal culture, the *E. coli* was not detected starting from the 6th day. At the same time, it is interesting to highlight that for all different treatments a negative correlation between pH values and *E. coli* densities was observed (data not shown). According to Sebastian and Nair (1984), Oswald (2003) and Muñoz and Guieysse (2006), one of main mechanism involved in *E. coli* removal is related to pH variations, mainly due to the microalgal photosynthetic activity. CO_2 uptake is involved in the pH increase up to 10–11, as already observed. In the present study, the pH values in samples treated with microalgae increased of about 2 points, compared to those detected in control samples inoculated only with *E. coli*, confirming the previous reports. Posadas et al. (2015) and Zitnik et al. (2019) reported that when the pH of medium is adjusted, in a range of 7-8, a mutualistic relationship between microalgae and *E. coli* was observed resulting in any pathogen removal effect. Overall, as extensively observed by Moawad (1968), studying the mechanisms involved in microalgae pathogenic removal, any physico-chemical parameter favourable for algal growth is mostly unfavourable for virus, amoeba, protozoa or bacterial survival. Focusing on ITAW samples inoculated with *E. coli* at lower density, all treatments exhibited a removal efficacy statistically similar. In each treatment, *E. coli* was not detected starting from the 6th day after inoculum. Similar trend was observed in ITAW samples inoculated with *E. coli* at higher density, at any sampling points, except samples treated with autochthonous MP that registered an *E. coli* density of 6.13 Log CFU/mL, 1.18 and 0.72 units higher than those detected in samples treated with *C. vulgaris* and *S. quadricauda*, respectively. These data confirmed that the removal efficacy of the autochthonous MP is comparable to the most common strains largely used in microalgae-based wastewater treatments, also in different stress conditions, as a high *E. coli* concentration, according to Colak and Kaya (1988), who reported a rate of coliform removal at 99% in high rate algal ponds (HRAPs) and in agreement with results of Abdel-Raouf et al. (2012), who reported, in the stabilization ponds, a significant removal of coliforms up to 99.6%

Focusing on PCR-DGGE analyses, results confirmed a quite stability of the autochthonous MP till the end of the experimental trial. In particular, only the 18S rDNA gene was amplified and even if for each single strain only one amplicon was obtained, the DGGE revealed multiple distinct bands, according to Lakaniemi et al. (2012a; 2012b). In details, results revealed the presence of *Klebsormidium* and *Tetradesmus* genera, the latter genus largely exploited in synthetic wastewater or municipal wastewater (Li et al., 2019). Furthermore, *Klebsormidium* sp. K39 resulted as the main microalgal species present in the autochthonous MP, according to Liu et al. (2016) who found that filamentous algae (as *Klebsormidium*) exhibits advantages in wastewater treatment over unicellular microalgae including higher resistance to predation rather than an easier harvesting. Different behaviour was observed for *T. obliquus*, that disappeared in ITAW samples inoculated with of *E. coli*

ATCC25922 and treated with autochthonous MP after 12 days. Overall, considered the efficacy of the phycoremediation, it remains still important to understand the cost and further investigations on economic and engineering nature, considering the source of income resulting from microalgal biomass (Shahid et al., 2020).

6. Conclusion

In this study a suitable solution for a wastewater treatment based on an autochthonous MP was compared to treatments based on *C. vulgaris* and *S. quadricauda*. The autochthonous MP was characterized as mainly composed by four species belonging to green algae (Chlorophyceae), namely *Klebsormidium* spp*., Chlorella* spp*., Tetradesmus* spp*., Scenedesmus* spp. and highlithed interesting *E. coli* removal efficinecy, lowering the bacterial density to values compliant with the EU regulation limits. Furthermore, the autochthonous MP showed interesting adaptation and its composing remained quite costant although a slight variation in the microalgal composition, as species ratio beteween inititial and final samplings were observed. Therefore, although the microalgal-based wastewater treament appears an evaluable alternative, further investigations are required to asses the N or P effectiveness recovery or to better explore any fluctuation within the MP and its species composition in an *in situ* trial.

CRediT authorship contribution statement

Conceptualization, C.C., A.P., and C.L.R.; Methodology, A.Pn., N.R., and P.F.; Formal analysis, P.S.O. and P.F.; investigation, P.S.O. and N.R.; resources, C.C.; Data curation, P.S.O., A.Pn. and C.C.; writing—original draft preparation, P.S.O.; writing—review and editing, C.C., N.R., and C.L.R.; visualization, P.S.O. and C.C.; supervision, C.C. and A.P.; project administration, C.C., C.L.R.; funding acquisition, C.C. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary Materials:

Table 1S. Physico-chemical and microbiological traits of Imhoff tank water samples used in the present study.

Parameters	Measure unit	Values
EC	µS/cm	3441
pH	-	6.99
TSS	mg/L	85
COD	mg/L	723
N-NH_3	mg/L	42.1
N-NO_2	mg/L	0.079
N-NO_3	mg/L	0.82
P-PO_4	mg/L	4.36
E. coli	Log CFU/100 mL	7.17
Fluorides	mg/L	0.747
Chlorides	mg/L	414.6
Sulphates	mg/L	376.5
Sodium	mg/L	762.4
Potassium	mg/L	24.8
Magnesium	mg/L	24.5
Calcium	mg/L	59.9

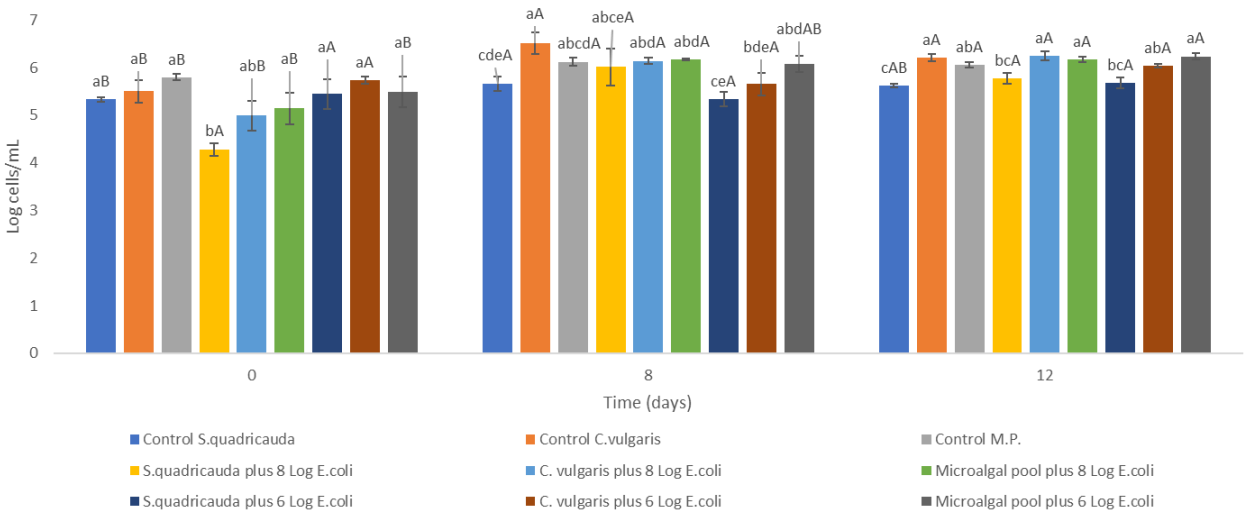


Figure 1S. Microalgal counts detected in ITAW samples inoculated with *E. coli* ATCC25922, at different cell densities, and with different microalgal cultures (*C. vulgaris* ACUF863, *S. quadricauda* ACUF581 or autochthonous MP) at initial time and after 8 and 12 days from inocula. The values are means of data and three replicates. Values of the same time followed by different lowercase letters are significantly different. Values of the same treatment followed by capital letters are significantly different ($p \leq 0.05$).

Table 2S. Growth (as Log cells/mL) of microalgae strains (*C. vulgaris* ACUF863, *S. quadricauda* ACUF581 or autochthonous MP) in ITAW samples at initial time and after 2, 6, 8 and 12 days from inocula. Data are expressed as means of three replicates ± SD. Values at the same time followed by different letters are significantly different ($p \leq 0.05$).

Treatment	0	2	6	8	12
S. quadricauda	5.33±0.05^{b}	5.44±0.03^{b}	5.80±0.06^{b}	5.66±0.15^{b}	5.62±0.04^{b}
C. vulgaris	5.50±0.23^{ab}	5.62±0.08^{a}	6.06±0.10^{a}	6.15±0.23^{a}	6.21±0.07^{a}
Autochthonous MP	5.80±0.07^{a}	5.78±0.04^{a}	6.02±0.02^{a}	6.12±0.08^{ab}	6.05±0.05^{a}

Funding: This study has been partially supported by PON "RICERCA E INNOVAZIONE" 2014–2020, Azione II—Obiettivo Specifico 1b—Progetto "Miglioramento delle produzioni agroalimentari mediterranee in condizioni di carenza di ri-sorse idriche—WATER4AGRIFOOD and partially funded by European Union (NextGeneration EU), through the MUR-PNRR project Sustainable management of natural resources in agriculture: SAMOTHRACE (ECS00000022).

Acknowledgements: This study was conducted within a Ph.D. research program in Biotechnology (XXXVI) by Paride Salvatore Occhipinti who received a grant from University of Catania at the Department of Agriculture, Food and Environment (Scientific Tutor: Cinzia Caggia and Cinzia Lucia Randazzo). The authors thank Prof. Giuseppe Luigi Cirelli for providing water samples.

Data Availability Statement: All related data and methods are presented in this paper. Additional inquiries should be addressed to the corresponding author.

References

Abdel-Raouf, N., Al-Homaidan, A. A., Ibraheem, I. (2012). Microalgae and wastewater treatment. Saudi journal of Biological Sciences, 19, 257-275. https://doi.org/10.1016/j.sjbs.2012.04.005

Abinandan, S., Shanthakumar, S. (2015). Challenges and opportunities in application of microalgae (Chlorophyta) for wastewater treatment: A review. Renewable Sustainable Energy Rev. 52: 123–132. https://doi.org/10.1016/j.rser.2015.07.086

Ait-Mouheb, N., Bahri, A., Thayer, B. B., Benyahia, B., Bourrié, G., Cherki, B., ... Harmand, J. (2018). The reuse of reclaimed water for irrigation around the Mediterranean Rim: a step towards a more virtuous cycle? Regional Environmental Change, 18, 693-705. https://doi.org/10.1007/s10113-018-1292-z

Andersen, R. A., Kawachi, M. (2005). Microalgae isolation techniques. Algal Culturing Techniques. Academic Press, 2005

APHA/AWWA/WEF (2017) Standard Methods for the Examination of Water and Wastewater (23rd edition), American Public Health Association, American Water Works Association, Water Environment Federation, Denver.

Atashgahi, S., Aydin, R., Dimitrov, M. R., Sipkema, D., Hamonts, K., Lahti, L., ... Smidt, H. (2015). Impact of a wastewater treatment plant on microbial community composition and function in a hyporheic zone of a eutrophic river. Scientific Reports, 5, 1-13. doi: 10.1038/srep17284.

Bell, T., Newman, J. A., Silverman, B. W., Turner, S. L., Lilley, A. K. (2005). The contribution of species richness and composition to bacterial services. Nature, 436(7054), 1157-1160. https://doi.org/10.1038/nature03891.

Bischoff, H.W., Bold, H.C., 1963. Phycological Studies. 4-Some Soil Algae from Enchanted Rock and Related Algal Species. Univ. Texas. N. 6318, pp. 32–36.

Bonanomi, G., De Filippis, F., Cesarano, G., La Storia, A., Ercolini, D., Scala, F. (2016). Organic farming induces changes in soil microbiota that affect agro-ecosystem functions. Soil Biology and Biochemistry, 103, 327-336. https://doi.org/10.1016/j.soilbio.2016.09.005.

Bouki, C., Venieri, D., Diamadopoulos, E. (2013). Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: a review. Ecotoxicology and environmental safety, 91, 1-9. https://doi.org/10.1016/j.ecoenv.2013.01.016.

Cardinale, B. J. (2011). Biodiversity improves water quality through niche partitioning. Nature, 472(7341), 86-89. https://doi.org/10.1038/nature09904.

Cardinale, B. J., Srivastava, D. S., Emmett Duffy, J., Wright, J. P., Downing, A. L., Sankaran, M., Jouseau, C. (2006). Effects of biodiversity on the functioning of trophic groups and ecosystems. Nature, 443(7114), 989-992. https://doi.org/10.1038/nature05202.

Colak, O., Kaya, Z. (1988). A study on the possibilities of biological wastewater treatment using algae. Doga Biyolji Serisi, 12, 18-29. https://doi.org/10.1016/j.biotechadv.2011.05.015.

Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., ... Tiedje, J. M. (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Research, 37(suppl_1), D141-D145. https://doi.org/10.1093/nar/gkn879.

Collet, L., Denis, R., Borrell Estupina, V., Dezetter, A., Servat, E. (2015). Water supply sustainability and adaptation strategies under anthropogenic and climatic changes of a meso-scale Mediterranean catchment. Science of the Total Environment, 536, 589–602. https://doi.org/10.1016/j.scitotenv.2015.07.093.

Dang, C. K., Chauvet, E., Gessner, M. O. (2005). Magnitude and variability of process rates in fungal diversity-litter decomposition relationships. Ecology Letters, 8, 1129-1137. https://doi.org/10.1111/j.1461-0248.2005.00815.x.

Díaz, F. J., O'Geen, A. T., Dahlgren, R. A. (2010). Efficacy of constructed wetlands for removal of bacterial contamination from agricultural return flows. Agricultural Water Management, 97, 1813-1821. https://doi.org/10.1016/j.agwat.2010.06.015.

Díez, B., Pedrós-Alió, C., Marsh, T. L., Massana, R. (2001). Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. Applied and Environmental Microbiology, 67, 2942-2951. https://doi.org/10.1128/AEM.67.7.2942-2951.2001.

Doyle, J.J., Doyle, J.L. (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Drury, B., Rosi-Marshall, E., Kelly, J. J. (2013). Wastewater treatment effluent reduces the abundance and diversity of benthic bacterial communities in urban and suburban rivers. Applied and Environmental Microbiology, 79, 1897-1905. https://doi.org/10.1128/AEM.03527-12.

EEA, O. (2009). Water resources across Europe—confronting water scarcity and drought (EEA Report No. 2). European Environment Agency (EEA), Office for Official Publications of the European Communities (OPOCE), Copenhagen, Denmark.

EPA (2012). Guidelines for Water Reuse. Environmental Protection Agency (EPA), Wasghinton DC (EPA/600/R-12/618).

European Commission. (2020). Regulation (EU) 2020/741 of the European Parliament and of the Council of 25 May 2020 on minimum requirements for water reuse. Official Journal of the European Union L, 177, 32-55.
FAO (2012). Coping with water scarcity. An action framework for agriculture and food security. FAO Water Reports. Food and Agriculture Organization of the United Nations (FAO), Rome. ISBN: 978-92-5-107304-9.
Federation, W. E., & Aph Association. (2005). Standard methods for the examination of water and wastewater. American Public Health Association (APHA): Washington, DC, USA, 21.
Garcia, J., Mujeriego, R., Hernandez-Marine, M. (2000). High rate algal pond operating strategies for urban wastewater nitrogen removal. Journal of Applied Phycology, 12, 331-339.
Granada-Moreno, C. I., Aburto-Medina, A., de Los Cobos Vasconcelos, D.,González-Sánchez, A. (2017). Microalgae community shifts during the biogas upgrading in an alkaline open photobioreactor. Journal of Applied Microbiology, 123, 903-915. https://doi.org/10.1111/jam.13552.
Green, M. B., Griffin, P., Seabridge, J. K., Dhobie, D. (1997). Removal of bacteria in subsurface flow wetlands. Water Science and Technology, 35(5), 109-116. https://doi.org/10.1016/S0273-1223(97)00059-0.
Guillard, R. R. (2005). Purification Methods for Microalgae. Algal Culturing Techniques, 117.
Guo, J., Qi, M., Chen, H., Zhou, C., Ruan, R., Yan, X., Cheng, P. (2022). Macroalgae-Derived Multifunctional Bioactive Substances: The Potential Applications for Food and Pharmaceuticals. Foods, 11(21), 3455. https://doi.org/10.3390/foods11213455.
Hena, S., Znad, H., Heong, K. T., Judd, S. (2018). Dairy farm wastewater treatment and lipid accumulation by Arthrospira platensis. Water Research, 128, 267–277. https://doi.org/10.1016/j.watres.2017.10.057.
Hong, P. Y., Al-Jassim, N., Ansari, M. I., Mackie, R. I. (2013). Environmental and public health implications of water reuse: antibiotics, antibiotic resistant bacteria, and antibiotic resistance genes. Antibiotics, 2(3), 367-399. https://doi.org/10.3390/antibiotics2030367.
International Organization for Standardization (ISO) (2012). Water quality—Detection and enumeration of Escherichia coli and coliform bacteria—Part 1: Membrane filtration method 9308-1:2001.
Kong, W., Kong, J., Ma, J., Lyu, H., Feng, S., Wang, Z., ... Shen, B. (2021). *Chlorella vulgaris* cultivation in simulated wastewater for the biomass production, nutrients removal and CO2 fixation simultaneously. Journal of Environmental Management, 284, 112070. https://doi.org/10.1016/j.jenvman.2021.112070.
La Jeunesse, I., Cirelli, C., Aubin, D., Larrue, C., Sellami, H., Afifi, S., ... Soddu, A. (2016). Is climate change a threat for water uses in the Mediterranean region? Results from a survey at local scale. Science of the Total Environment, 543, 981-996. https://doi.org/10.1016/j.scitotenv.2015.04.062
Lakaniemi, A. M., Intihar, V. M., Tuovinen, O. H., Puhakka, J. A. (2012a). Growth of *Chlorella vulgaris* and associated bacteria in photobioreactors. Microbial Biotechnology, 5, 69-78. https://doi.org/10.1111/j.1751-7915.2011.00298.x.
Lakaniemi, A.M., Intihar, V.M., Tuovinen, O.H., Puhakka, J.A. (2012b). Growth of *Dunaliella tertiolecta* and associated bacteria in photobioreactors, Journal of Industrial Microbiology and Biotechnology, 39, 1357–1365. https://doi.org/10.1007/s10295-012-1133-x.
Larsdotter, K. (2006). Wastewater treatment with microalgae-a literature review. Vatten, 62(1), 31.
Li, K., Liu, Q., Fang, F., Luo, R., Lu, Q., Zhou, W., ... Ruan, R. (2019). Microalgae-based wastewater treatment for nutrients recovery: A review. Bioresource Technology, 291, 121934. https://doi.org/10.1016/j.biortech.2019.121934.

Liu, J., Danneels, B., Vanormelingen, P., Vyverman, W. (2016). Nutrient removal from horticultural wastewater by benthic filamentous algae *Klebsormidium* sp., *Stigeoclonium* spp. and their communities: from laboratory flask to outdoor Algal Turf Scrubber (ATS). Water Research, 92, 61-68. https://doi.org/10.1016/j.watres.2016.01.049.

Lonigro, A., Montemurro, N., Rubino, P., Vergine, P., Pollice, A. (2015). Reuse of treated municipal wastewater for irrigation in Apulia Region: the" IN. TE. RRA" project. Environmental Engineering & Management Journal (EEMJ), 14(7).

Lopez, A., Pollice, A., Lonigro, A., Masi, S., Palese, A. M., Cirelli, G. L., ... Passino, R. (2006). Agricultural wastewater reuse in southern Italy. Desalination, 187(1-3), 323-334. https://doi.org/10.1016/j.desal.2005.04.091.

Lu, X. M., Lu, P. Z. (2014). Characterization of bacterial communities in sediments receiving various wastewater effluents with high-throughput sequencing analysis. Microbial Ecology, 67, 612-623. https://doi.org/10.1007/s00248-014-0370-0.

Łuczkiewicz, A., Jankowska, K., Fudala-Książek, S., Olańczuk-Neyman, K. (2010). Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant. Water Research, 44(17), 5089-5097. https://doi.org/10.1016/j.watres.2010.08.007.

Manaia, C. M., Vaz-Moreira, I., Nunes, O. C. (2012). Antibiotic resistance in waste water and surface water and human health implications. Emerging Organic Contaminants and Human Health, 173-212. https://doi.org/10.1007/698_2011_118.

Manhart, J. R. (1994). Phylogenetic analysis of green plant *rbcL* sequences. Molecular Phylogenetics and Evolution, 3(2), 114-127. https://doi.org/10.1006/mpev.1994.1014.

Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J., Wade, W. G. (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Applied and Environmental Microbiology, 64(2), 795-799. https://doi.org/10.1128/AEM.64.2.795-799.1998.

Marti, E., Jofre, J., Balcazar, J. L. (2013). Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a wastewater treatment plant. PloS one, 8(10), e78906. https://doi.org/10.1371/journal.pone.0078906.

Milani, M., Consoli, S., Marzo, A., Pino, A., Randazzo, C., Barbagallo, S., Cirelli, G. L. (2020). Treatment of winery wastewater with a multistage constructed wetland system for irrigation reuse. Water, 12(5), 1260. https://doi.org/10.3390/w12051260.

Mitchell, C. E., Tilman, D., Groth, J. V. (2002). Effects of grassland plant species diversity, abundance, and composition on foliar fungal disease. Ecology, 83(6), 1713-1726. https://doi.org/10.1890/0012-9658(2002)083[1713:EOGPSD]2.0.CO;2.

Moawad, S. K. (1968). Inhibition of coliform bacteria by algal population in microoxidation ponds. Environmental Health, 10(2), 106-112.

Mohsenpour, S. F., Hennige, S., Willoughby, N., Adeloye, A., Gutierrez, T. (2021). Integrating micro-algae into wastewater treatment: A review. Science of the Total Environment, 752, 142168. https://doi.org/10.1016/j.scitotenv.2020.142168.

Moondra, N., Jariwala, N. D., Christian, R. A. (2020). Sustainable treatment of domestic wastewater through microalgae. International Journal of Phytoremediation, 22(14), 1480-1486. https://doi.org/10.1080/15226514.2020.1782829.

Muñoz, R., Guieysse, B. (2006). Algal–bacterial processes for the treatment of hazardous contaminants: a review. Water Research, 40(15), 2799-2815. https://doi.org/10.1016/j.watres.2006.06.011.

Muyzer, G., De Waal, E. C., Uitterlinden, A. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology, 59(3), 695-700. https://doi.org/10.1128/aem.59.3.695-700.1993.

Naeem, S., Thompson, L. J., Lawler, S. P., Lawton, J. H., Woodfin, R. M. (1994). Declining biodiversity can alter the performance of ecosystems. Nature, 368, 734-737.

Novo, A., Andre, S., Viana, P., Nunes, O.C., Manaia, C.M. (2013). Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. Water Research 47, 1875–1887. https://doi.org/10.1016/j.watres.2013.01.010.

Nubel, U., Garcia-Pichel, F., Muyzer, G. (1997) PCR primers to amplify 16S rRNA genes from cyanobacteria. Applied and Environmental Microbiology, 63, 3327–3332. https://doi.org/10.1128/aem.63.8.3327-3332.1997.

Ofori, S., Puškáčová, A., Růžičková, I., Wanner, J. (2021). Treated wastewater reuse for irrigation: Pros and cons. Science of the Total Environment, 760, 144026. https://doi.org/10.1016/j.scitotenv.2020.144026.

Ohkubo, S., Miyashita, H., Murakami, A., Takeyama, H., Tsuchiya, T., Mimuro, M. (2006) Molecular detection of epiphytic *Acaryochloris* spp. on marine macroalgae. Applied Environmental Microbiology 72, 7912–7915 https://doi.org/10.1128/AEM.01148-06.

Oswald, W. J. (2003). My sixty years in applied algology. Journal of Applied Phycology, 15(2), 99-106.

Oswald, W.J., (1962). The coming industry of controlled photosynthesis. American Journal of Public Health 52, 235–242. http://dx.doi.org/10.2105/AJPH.52.2.235.

Oswald, W.J., (1973). Productivity of algae in sewage disposal. Sol. Energy 15, 107–117. http://dx.doi.org/10.1016/0038-092X(73)90013-3.

Posadas, E., del Mar Morales, M., Gomez, C., Acién, F. G., Muñoz, R. (2015). Influence of pH and CO_2 source on the performance of microalgae-based secondary domestic wastewater treatment in outdoors pilot raceways. Chemical Engineering Journal, 265, 239-248. https://doi.org/10.1016/j.cej.2014.12.059.

Rahube, T. O., Yost, C. K. (2010). Antibiotic resistance plasmids in wastewater treatment plants and their possible dissemination into the environment. African Journal of Biotechnology, 9(54), 9183-9190.

Randazzo, C. L., Pino, A., Ricciardi, L., Romano, C., Comito, D., Arena, E., ... Caggia, C. (2015). Probiotic supplementation in systemic nickel allergy syndrome patients: study of its effects on lactic acid bacteria population and on clinical symptoms. Journal of Applied Microbiology, 118(1), 202-211. https://doi.org/10.1111/jam.12685.

Randazzo, C. L., Torriani, S., Akkermans, A. D., de Vos, W. M., Vaughan, E. E. (2002). Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. Applied and Environmental Microbiology, 68(4), 1882-1892. https://doi.org/10.1128/AEM.68.4.1882-1892.2002.

Rani, S., Gunjyal, N., Ojha, C. S. P., Singh, R. P. (2021). Review of challenges for algae-based wastewater treatment: strain selection, wastewater characteristics, abiotic, and biotic factors. Journal of Hazardous, Toxic, and Radioactive Waste, 25(2), 03120004. https://doi.org/10.1061/(ASCE)HZ.2153-5515.0000578.

Replansky, T., Bell, G. (2009). The relationship between environmental complexity, species diversity and productivity in a natural reconstructed yeast community. Oikos, 118(2), 233-239. https://doi.org/10.1111/j.1600-0706.2008.16948.x.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., ... Fatta-Kassinos, D. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. Science of the Total Environment, 447, 345-360. https://doi.org/10.1016/j.scitotenv.2013.01.032.

Rosegrant, M. W., Cai, X. (2002). Global water demand and supply projections: part 2. Results and prospects to 2025. Water International, 27(2), 170-182. https://doi.org/10.1080/02508060208686990.

Ruas, G., Serejo, M. L., Paulo, P. L., Boncz, M. Á. (2018). Evaluation of domestic wastewater treatment using microalgal-bacterial processes: effect of CO_2 addition on pathogen removal. Journal of Applied Phycology, 30, 921-929. https://doi.org/10.1007/s10811-017-1280-6.

Russo, N., Marzo, A., Randazzo, C.L, Caggia, C., Toscano, A., Cirelli, G.L. (2019a). Constructed wetlands combined with disinfection systems for removal of urban wastewater contaminants. Science of the Total Environment. 656: 558-566. https://doi.org/10.1016/j.scitotenv.2018.11.417.

Russo, N., Pino, A., Toscano, A., Cirelli, G. L., Caggia, C., Arioli, S., Randazzo, C. L. (2019b). Occurrence, diversity, and persistence of antibiotic resistant enterococci in full-scale constructed wetlands treating urban wastewater in Sicily. Bioresource Technology, 274, 468-478. https://doi.org/10.1016/j.biortech.2018.12.017.

Sanguinetti, C. J., Simpson, A. J. (1994). Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques, 17(5), 914-921.

Santhanam, N. (2009). Oilgae guide to algae-based wastewater treatment. Tamilnadu: Home of Algal Energy.

Sebastian, S., Nair, K.V.K. (1984). Total removal of coliforms and *E. coli* from domestic sewage by high-rate pond mass culture of *Scenedesmus obliquus*. Environmental Pollution Series A, Ecological and Biological, 34(3), 197-206.

Shahid, A., Malik, S., Zhu, H., Xu, J., Nawaz, M. Z., Nawaz, S., ... Mehmood, M. A. (2020). Cultivating microalgae in wastewater for biomass production, pollutant removal, and atmospheric carbon mitigation; a review. Science of the Total Environment, 704, 135303. https://doi.org/10.1016/j.scitotenv.2019.135303.

Sun, Y., Chen, Z., Wu, G., Wu, Q., Zhang, F., Niu, Z., Hu, H. Y. (2016). Characteristics of water quality of municipal wastewater treatment plants in China: implications for resources utilization and management. Journal of Cleaner Production, 131, 1-9. https://doi.org/10.1016/j.jclepro.2016.05.068.

Uroz, S., Oger, P., Morin, E., Frey-Klett, P. (2012). Distinct ectomycorrhizospheres share similar bacterial communities as revealed by pyrosequencing-based analysis of 16S rRNA genes. Applied and Environmental Microbiology, 78(8), 3020-3024. https://doi.org/10.1128/AEM.06742-11.

US EPA. (2012). Guidelines for Water Reuse. AR-1530 EPA/600/R-12/618.

Vaz-Moreira, I., Nunes, O. C., Manaia, C. M. (2014). Bacterial diversity and antibiotic resistance in water habitats: searching the links with the human microbiome. FEMS Microbiology Reviews, 38(4), 761-778. https://doi.org/10.1111/1574-6976.12062.

Ventura, D., Consoli, S., Barbagallo, S., Marzo, A., Vanella, D., Licciardello, F., Cirelli, G. L. (2019). How to overcome barriers for wastewater agricultural reuse in Sicily (Italy)?. Water, 11(2), 335. https://doi.org/10.3390/w11020335.

WHO, A. (2006). Compendium of standards for wastewater reuse in the Eastern Mediterranean Region. Regional Office for the Eastern Mediterranean, Cairo, Egypt.

Wohl, D. L., Arora, S., Gladstone, J. R. (2004). Functional redundancy supports biodiversity and ecosystem function in a closed and constant environment. Ecology, 85(6), 1534-1540. https://doi.org/10.1890/03-3050.

Wollmann, F., Dietze, S., Ackermann, J. U., Bley, T., Walther, T., Steingroewer, J., Krujatz, F. (2019). Microalgae wastewater treatment: Biological and technological approaches. Engineering in Life Sciences, 19(12), 860-871. https://doi.org/10.1002/elsc.201900071.

Wong, V. N., Dalal, R. C., Greene, R. S. (2008). Salinity and sodicity effects on respiration and microbial biomass of soil. Biology and Fertility of Soils, 44, 943-953. https://doi.org/10.1007/s00374-008-0279-1.

World Health Organization. (2006). WHO guidelines for the safe use of wasterwater excreta and greywater (Vol. 1). World Health Organization.

Zhai, J., Li, X., Li, W., Rahaman, M. H., Zhao, Y., Wei, B., Wei, H. (2017). Optimization of biomass production and nutrients removal by *Spirulina platensis* from municipal wastewater. Ecological Engineering, 108, 83-92. https://doi.org/10.1016/j.ecoleng.2017.07.023.

Zhang, Y., Zheng, H., Zhang, X., Leung, L. R., Liu, C., Zheng, C., ... Blöschl, G. (2023). Future global streamflow declines are probably more severe than previously estimated. Nature Water, 1-11. https://doi.org/10.1038/s44221-023-00030-7.

Zhimiao, Z., Xinshan, S., Yuhui, W., Daoyuan, W., Suyan, W., Yuana, H., et al. (2016) Effects of algal ponds on vertical flow constructed wetlands underdifferent sewage application techniques. Ecological Engineering, 93, 120–128 https://doi.org/10.1016/j.ecoleng.2016.05.033.

Žitnik, M., Šunta, U., Torkar, K. G., Klemenčič, A. K., Atanasova, N., Bulc, T. G. (2019). The study of interactions and removal efficiency of Escherichia coli in raw blackwater treated by microalgae Chlorella vulgaris. Journal of Cleaner Production, 238, 117865. https://doi.org/10.1016/j.jclepro.2019.117865.

Zoetendal, E. G., Akkermans, A. D., De Vos, W. M. (1998). Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Applied and Environmental Microbiology, 64(10), 3854-3859. https://doi.org/10.1128/AEM.64.10.3854-3859.1998.

Comparative Phycoremediation Performance of Three Microalgae Species in Two Different Magnitude of Pollutants in Wastewater from Farmhouse

Paride Salvatore Occhipinti [1,†], Emanuele La Bella [1,†], Ivana Puglisi [1,*], Ferdinando Fragalà [1], Rossella Saccone [1], Nunziatina Russo [1], Cinzia Lucia Randazzo [1,2], Cinzia Caggia [1,2] and Andrea Baglieri [1]

[1]Department of Agriculture, Food and Environment, University of Catania, 95123 Catania, Italy;

[2]CRIAB: Centro di Ricerca Interdipartimentale per L'implementazione dei Processi di monitoraggio Fisico, Chimico e Biologico nei Sistemi di Biorisanamento e di Acquacoltura, Department of Medical Sciences, Surgicals and Advanced Technologies, Hygiene and Public Health "G.F. Ingrassia", University of Catania, 95123 Catania, Italy

* Correspondence: ipuglisi@unict.it

† These authors contributed equally to this work.

This article has been published *Sustainability* – MDPI. 2023, 15(15), 11644; https://doi.org/10.3390/su151511644

Abstract: The cultivation of microalgae using urban wastewater as a nutrient substrate represents a promising bio-refinery concept that can serve multiple purposes; indeed, it allows for the generation of biomass, which can be used for various applications while meanwhile removing nutrients from wastewater. In this study, the potential of urban wastewater collected at two different time periods in a farmhouse as a nutrient substrate for microalgal growth was assessed. Wastewater samples were treated on a laboratory scale, inoculating reactors with two common species, *Chlorella vulgaris* (CV) and *Scenedesmus quadricauda* (SQ), and with an autochthonous strain of *Klebsormidium* sp. K39 (Kleb), directly isolated from effluents of the same system. The main aim of the study was to compare the microalgae's performances in terms of wastewater re-mediation and biomass productivity. In the first case study, which involved an effluent with a lower pollutant level, microalgal cultivation showed removal efficiencies in the range of 57–63% for total nitrogen, 65–92% for total phosphorous, 94–95% for COD, and 100% for *E. coli*. In the second case study, involving an effluent with a higher pollutant level, the remediation performances of the three microalgae strains ranged from 93 to 96% for total nitrogen, from 62 to 74% for total phosphorous, from 96 to 97% for COD, and 100% for *E. coli*. At the end of the experimental trials, treated waters showed values of pollutants suitable for irrigation use, in accordance with environmental and national legislation, which established specific thresholds for irrigation purposes.

Keywords: municipal effluent; *Chlorella vulgaris*; *Scenedesmus quadricauda*; *Klebsormidium* sp. K39; decontamination; irrigation use

1. Introduction

In the last few years, the rapidly expanding population, coupled with global climate changes, has represented a considerable pressure on Earth's resources. Indeed, climate change negatively impacts agricultural productivity and affects the water cycle, leading to altered precipitation patterns and increasing water scarcity in some regions, as well as the increase in population putting a strain on freshwater resources [1]. A further important issue is related to the release of municipal wastewaters and, in turn, the environmental challenges they pose to receiving water bodies [2,3]. The high concentration of pollutants, such as excess nitrogen and phosphorus, may cause an important alteration in the health of the water system [4,5]. Furthermore, conventional treatment methods, such as activated sludge systems or chemical coagulation, are still very expensive and often unable to completely eliminate microcompounds or inorganic nutrients [6,7]. The use of reclaimed water (RW), a suitable strategy in agriculture for irrigation purposes, may represent a risk for plants, soils, and humans [8,9] for the accumulation and propagation of biological (animal and human pathogens, phytopathogens), xenobiotic contaminants (drugs and metals), and antibiotic-resistant genes [10–13]. The World Health Organisation guidelines established safety criteria for irrigation purposes, for which RW must comply with standard criteria. In the EU, the use of RW is under Regulation (EU) 2020/741 on minimum requirements for water reuse, which establishes a threshold of 10 CFU 100 mL^{-1} (<1 Log 100 mL^{-1}) of *Escherichia coli* for RW classifying as class "A", useful for irrigation of food crops [14]. In this context, the exploitation of microalgae is emerging as an interesting alternative green source with a low carbon dioxide (CO_2) footprint [15,16]. Microalgae are also attracting the interest of worldwide researchers, mainly due to their multipurpose applications as raw materials for the development of new agricultural products [7,17,18]. Moreover, microalgae are taken into account as important sustainable sources of valuable chemicals, pharmaceuticals, and other products [19–21]. The microalgae-based wastewater treatment process is a sustainable, eco-friendly process with no secondary pollution [22], able to recover wastewater from various organic and inorganic contaminants, ranging from aromatic hydrocarbons, food residues, solvents, plasticisers, antioxidants, washing and cleaning-related compounds, to high nutrient loads such as nitrogen and phosphorous [23]. Furthermore, previous studies have shown that microalgae-based wastewater treatment has a rate of coliform removal of up to 99% [24,25]. Microalgae may be adapted to a wide range of types of wastewater, providing a tertiary biotreatment coupled with the production of valuable biomass, a potential feedstock for the development of added-value products for the agricultural sector [25]. Among microalgae species suitable for wastewater treatment, the genera *Chlorella* and *Scenedesmus* are the most largely used [26]. However, a limitation in applying such a

strategy is related to the difficulties of maintaining monoalgal cultures with constant biomass composition [27]. The remediation abilities of these two genera are largely reported [7,28]. For instance, Wang et al. [29] demonstrated that *Chlorella* sp., employed for urban wastewater treatment, was able to remove high contents of nitrogen, ranging from 62.5 to 82.4%; phosphorus, from 83.2 to 90.6%; and heavy metals. In the same way, Wong et al. [30] investigated the lipid production and nutrient removal capabilities of *S. quadricauda* using different types of wastewater from a sewage treatment plant. The results showed interesting performances for both evaluated properties, indicating that the microalga is a viable candidate for wastewater treatment and lipid production. It is relevant to point out that the major pollutants in urban wastewater are nutrients and heavy metals; therefore, a relevant trait for the selection of microalgae strains to be used for this purpose is to detect these abilities [31]. Moreover, microalgae cultivation can provide an opportunity to produce valuable biomass, which can be utilized to obtain bioproducts for multipurpose applications. It is worth noting that research in this field is ongoing, and further studies are needed to optimize the processes, explore different microalgae species, and assess the scalability and economic feasibility of using microalgae for wastewater treatment and resource recovery. To achieve a 'win-win' solution by linking wastewater remediation and microalgae biomass accumulation, different types of wastewater could be used as a culture medium for the cultivation of different microalgae species. Based on the above perspectives, this study is aimed at evaluating the phycoremediation performance and biomass accumulation of an indigenous strain of filamentous microalga, previously identified as *Klebsormidium* sp. K39, in urban wastewater treatment, compared to *Chlorella vulgaris* (CV) and *S. quadricauda* (SQ). These performances were evaluated for two different magnitudes of pollutants in wastewater from a farmhouse.

2. Materials and Methods

2.1. Raw Wastewaters

Wastewater samples were collected from a constructed wetland active on a farm holiday in Sicily (Italy) in two different periods, as the different host affluence levels (due to the COVID emergency) caused significant differences in their composition. The collected raw wastewaters were preliminary analysed (see detailed methods below in Section 2.2) and used as growth substrates for microalgae. In Figure 1, a scheme of the phytodepuration system acting in the farm holiday is reported. The wastewater samples used for the experimental trials were collected directly from the Imhoff tank.

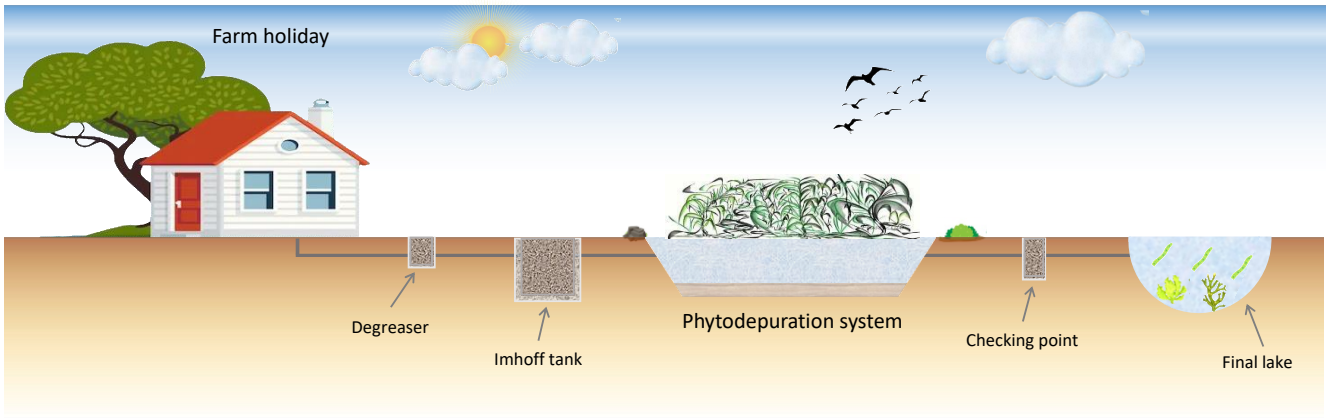


Figure 1. Phytodepuration system scheme at the farmhouse.

The characteristics of the raw wastewaters used in this study are reported in Table 1 (analyses are described in Section 2.2).

Table 1. Composition of raw wastewaters: Total Kjeldahl Nitrogen (TKN), Total Phosphorous (TP), Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD_5), and *Escherichia coli*.

	Wastewater 1 (MW 1)	**Wastewater 2 (MW 2)**
pH	7.24	7.25
EC (mS cm^{-1})	3.95	5.35
TN (mg L^{-1})	10	50.7
TP (mg L^{-1})	3.2	10.67
COD (mg L^{-1})	550	753
Zn (mg L^{-1})	nd *	nd
Cu (mg L^{-1})	nd	nd
Cd (mg L^{-1})	nd	nd
Pb (mg L^{-1})	nd	nd
Ni (mg L^{-1})	nd	nd
Hg (mg L^{-1})	nd	nd
E. coli (log CFU 100 mL^{-1})	235	440

* nd: not detected.

2.2. Chemical Analyses and Nutrient Removal Rate Determination

The wastewater samples were first centrifuged at 4000× *g* for 5 min, and the supernatants were collected [32]. Measurements of EC and pH values were performed using an XS Cond 7 and an XS pH 80+ DHS, respectively. In order to evaluate the preliminary composition of wastewaters and the nutrient removal ability of microalgae, chemical characterization by monitoring several parameters, including TKN, TP, heavy metals, COD, and BOD_5, was performed following the standard methods recommended by the American Public Health Association [33]. TKN was performed by the Kjeldahl method in 50 mL of sample. In a test tube, 2 catalyst tablets were added, each containing 3.5 g of K_2SO_4 and 3.5 mg of Se, and 10 mL of concentrated sulphuric acid. The tubes were placed in the digestor and treated for 60 min at 200 °C and 120 min at 370 °C. After digestion, samples were treated with an acid solution and boiled in concentrated sulfuric acid. The samples were then distilled according to a pre-defined method of the instrument (Method n° 26, VELP UDK 130 A). The distillation of the samples was performed by adding an excess of 35% NaOH to the acid digestion mixture to convert NH_4^+ to NH_3, followed by boiling and condensation of the ammonia (NH_3) gas in a receiving solution (4% H_3BO_3). Finally, to quantify the amount of ammonia in the receiving solution, the water samples were titrated. For the titration, to each sample were added 10 drops of Tashiro's indicator (0.75 g L^{-1} methyl red sodium salt + 0.375 g L^{-1} methylene blue in ethanol 50% (*v/v*), denatured) and 0.2 N HCl until the endpoint of the titration. Analysis to determine TP contents was based on the persulfate oxidation under acidic conditions of the samples [33], converting the various forms of phosphate and phosphorus to the orthophosphate form. The phosphorus contents were determined by putting 50 mL of sample, or a diluted amount of 50 mL, into an Erlenmeyer flask, adding 1 drop of phenolphthalein indicator, and 5 M sulphuric acid or 2 M sodium hydroxide until the samples developed a red colour. The next steps were the addition of 1 mL of 10 M sulphuric acid and 0.4 g of potassium persulphate, followed by the transfer of the samples into an incubator at 95–100 °C for 2 h. After cooling, the samples were added to 1 drop of phenolphthalein and neutralized to a faint pink colour with 2 M sodium hydroxide, made up to 100 mL with distilled water. Then, at each sample, 10 mL of a mixed reagent was added, composed of 100 mL of 30 g L^{-1} ammonium molybdate solution, 250 mL of diluted sulphuric acid (1:6.4, H_2SO_4:H_2O), 100 mL of 54 g L^{-1} ascorbic acid solution, and 50 mL of 1.36 g L^{-1} potassium antimony tartrate solution. We allowed at least 10 min for colour development and measured the absorbance at 880 nm using a reagent blank to zero the spectrophotometer. The reagent blank was made using 50 mL of distilled water carried through the digestion and subsequent steps. Finally, the samples' absorbances were checked against the calibration curve phosphate standard, and the concentrations were determined. The determination

of heavy metals was performed by Standard Methods for Examination of Water and Wastewater [33]. The metal analyses (Zn, Cu, Cd, Pb, Ni, and Hg) were carried out by means of atomic absorption spectrophotometry (Perkin Elmer 3110, Waltham, MA, USA). Each wastewater sample was filtered through a 0.45-micron nylon filter and acidified to a pH of 4–5 with HCl. Afterwards, 35 mL of Methyl isobutyl ketone (MIBK) and 7 mL of 1% (*w/v*) ammonium pyrrolidine dithiocarbamate (APDC) were added to 750 mL of the filtered solution, and each sample was equilibrated for 30 min on a mechanical shaker, and the organic layer was separated in a separatory funnel. The concentration of the heavy metals (Zn, Cu, Cd, Pb, Ni, and Hg) was determined by reading the concentrations of the elements of interest directly versus appropriate standards and a reagent blank. Wastewater was analysed for heavy metals only at the beginning of removal experiments because, in both cases (MW1 and MW2), the contents were below the detectable limits. COD analysis was performed using specific test kits (Nanocolor CSB 40 and Nanocolor CSB 1500), and BOD_5 was monitored using the Velp Respirometric Sensor BOD_5 (Monza-Brianza, Italy). For BOD_5 analysis, all samples were saturated with oxygen using an air pump, and after 5 days of incubation in the dark, the final dissolved oxygen level was taken directly from the sensor, and the difference between the final and initial levels was recorded. Each analysis was replicated in triplicate. To evaluate the nutrient removal ability by microalgae, Total Kjeldahl Nitrogen (TKN), Total Phosphorus (TP), Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD_5), pH, and Electrical Conductivity (EC) were determined according to the standard methods recommended by the American Public Health Association [33]. For these parameters, removal quantity (RQ, $mg \cdot L^{-1}$) and removal efficiency (RE, %) were calculated using the following equations [34]:

$$RQ = x_0 - x_i$$

$$RE\ (\%) = \left(\frac{x_0 - x_i}{x_0}\right) \times 100$$

Where $x_0$and $x_i$are defined as the mean values of nutrient concentrations at initial time t_0 and final time t_i, respectively.

2.3. Microalgae Strains and Cultivation Conditions

The microalgae tested in the present study were *Chlorella vulgaris* ACUF863 and *Scenedesmus quadricauda* ACUF581, which were kindly provided by the Algal Collection Federico II of Naples (Italy). In addition, a strain of *Klebsormidium* sp. K39, belonging to the Di3A microbial culture

collection and previously isolated from the same phytoremediation pond [35], was used. All strains were cultured in sterilized standard Bold Basal Medium (BBM) or BBM agar medium.

Microalgae cultivation was carried out in axenic conditions in 2 L Erlenmeyer flasks maintained at 25 ± 1 °C in a climate chamber under a light intensity of 100 µmol photons·m^{-2} s^{-1} with a light source (PHILIPS SON-T AGRO 400, Eindhoven, the Netherlands), and a photoperiod of 16 h on/off, according to the best microalgae growth conditions. The cultures were bubbled with air with immersion water pumps [31]. The microalgae species used in the described experiments were inoculated at their logarithmic growth phase.

2.4. Evaluation of Bacterial Removal Efficiency

In order to evaluate the *Escherichia coli* removal efficiency of the tested microalgal treatments, microbiological analyses were performed following the membrane filtration method [36]. In detail, 100 mL of sample were treated on membrane filters (0.45 µm pores, Cellulose, Merck, Darmstadt, Germany), and the filters were then poured into RAPID' *E. coli* 2 Agar plates (Bio-Rad, Milan, Italy). Plates were incubated at 37 °C for 24 h. The analyses were performed in triplicate, and results were expressed as mean log10 colony-forming units (CFU) per unit of volume.

2.5. Experimental Set-Up

The experimental set-up consisted of eight lab-scale open photobioreactors (Table 2), each with a 4 L capacity, illuminated for a 12 h photoperiod by an LED lamp (100 µmol photons·m^{-2} s^{-1}), in order to simulate the nearest natural environmental conditions. Each reactor was filled with 3 L of wastewater [Wastewater 1 (MW 1) and Wastewater 2 (MW 2)] collected from the Imhoff tank of the phytoremediation system at the farmhouse, as above described (Figure 1).

Table 2. Design criteria and conditions adopted in each photobioreactor used in the experimental trials.

PBRs	Substrate	Microalgae Species	Microalgae Biomass ($g\ L^{-1}$)	Inoculum Size (n. Cells $10^9\ L^{-1}$)
1	MW 1	0	-	-
2	MW 1	*C. vulgaris*	0.42	1.6
3	MW 1	*S. quadricauda*	0.44	2.2
4	MW 1	*Klebsormidium* sp. K39	0.45	1.8
5	MW 2	0	-	-
6	MW 2	*C. vulgaris*	0.42	1.6
7	MW 2	*S. quadricauda*	0.44	2.2
8	MW 2	*Klebsormidium* sp. K39	0.45	1.8

Each microalga, grown in BBM, was collected by centrifugation at 4000 rpm for 10 min when it reached the logarithmic growth phase. Pellets were washed with deionized water and centrifuged a second time at the same conditions, then were suspended in a small quantity of wastewater, and, finally, inoculated in the reactors [37]. The photobioreactors were inoculated with *C. vulgaris*, *S. quadricauda*, and the autochthonous *Klebsormidium* sp. K39 strains at an initial cell concentration, as determined by cell count in the Burker counting chamber (Blaubrand), of 100 $mg \cdot L^{-1}$, equal to 1.6, 2.2, and 1.8×10^9 $cells \cdot L^{-1}$, respectively. For each microalga, the cell dry weight and the size of the inoculums were found to be 0.42, 0.44, and 0.45 g (fresh weight), respectively. The wastewater samples that were not inoculated were routinely used as controls. The microalgae were thus fed in the reactors exclusively with the wastewaters as they are, without nutrient addition or dilution, considering that the effluents can supply all inorganic nutrients required for microalgae growth [38]. Samples of 50 mL were then collected after 2, 5, 10, 30, 45, and 60 days from each photobioreactor in order to evaluate the remediation ability of the tested microalgae, determining the concentrations of Total Kjeldahl Nitrogen (TKN), Total Phosphorus (TP), Chemical Oxygen Demand (COD), Biological Oxygen Demad (BOD_5), pH, and Electrical Conductivity (EC) (as previously described). In order to monitor microbiological parameters, samples were collected at 0, 2, 5, 7, 9, 15, 30, 45, and 60 days after inoculum and immediately processed for *E. coli* detection and microalgae counting (as previously described). All experiments were carried out in triplicate.

2.6. Determination of Microalgal Growth

The microalgal growth was determined as cell number by Burker's counting chamber (Blaubrand), as fresh weight, measuring the weight (mg) of fresh biomass per litre and as dry weight, measuring the weight (mg) of dry biomass per litre, obtained oven-dried at 60 °C until a constant weight was reached. The daily productivity (g L^{-1}·d) was calculated according to the following formula [39]:

$$Daily\ productivity = \frac{CDW_i - CDW_0}{t_i - t_0}$$

where CDW and CDW_0 are the final and initial concentrations of cell dry weight and t_i and t_0 are the final and initial time. Moreover, at the end of the experimental test, the samples containing the microalgae were centrifuged at 2500 rpm for 10 min, and the pellet was oven-dried at 60 °C until constant weight and weighed to measure the total biomass [40].

2.7. Statistical Analysis

The collected data were subjected to a two-way analysis of variance (ANOVA) based on a factorial combination (specie × time). Since the laboratory assays were performed in triplicate, F and *p* values were calculated to evaluate whether the effects of single factors such as as specie, time, and the interaction specie × time were significant. In post-hoc analyses, the means were compared using Fischer's protected least significant difference (LSD) test ($p \leq 0.05$). The calculations were carried out on Excel version 2019 (Microsoft Corporation, Redmond, WA, USA) and Minitab (version 16.1.1, Minitab Inc., State College, PA, USA).

3. Results

3.1. Dynamics of Microalgae Population

The microalgae strains were cultivated in wastewater for 60 days, and the growth performances, in terms of cell density, are reported in Figure 2. The lag phase, or time necessary for their adaptation to wastewater conditions, was found to be quite short in both case studies (48 h), and in this period the main parameters monitored were not significantly reduced.

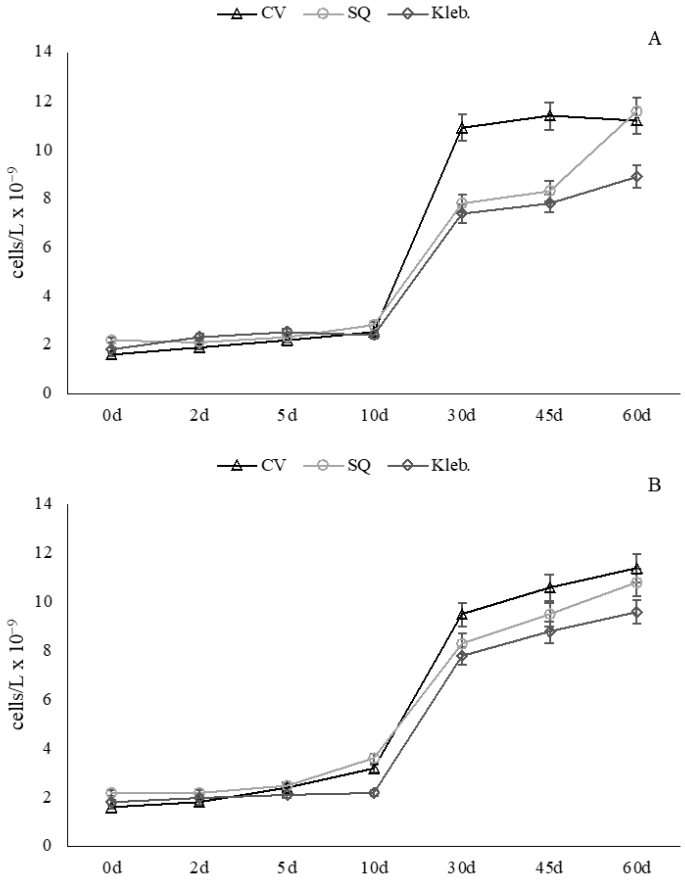


Figure 2. Microalgal growth performance in (**A**) Wastewater 1 (MW 1) and (**B**) Wastewater 2 (MW 2). CV: *Chlorella vulgaris*, SQ: *Scenedesmus quadricauda*, Kleb: *Klebsormidium* sp. K39.

However, the effect of a single factor (species) was found to be not significant for any of the parameters monitored in both trials (Tables 3 and 4).

Table 3. Effects of single factors in ANOVA relative to the daily productivity, the fresh weight of biomass collected, and the dry weight of biomass collected in MW1.

Factor	Daily Productivity		Fresh Weight of Biomass Collected		Dry Weight of Biomass Collected	
	F	***p* Value**	**F**	***p* Value**	**F**	***p* Value**
Species	0.95	0.437	1.23	0.356	0.43	0.667

Table 4. Effects of single factors in ANOVA relative to the daily productivity, the fresh weight of biomass collected, and the dry weight of biomass collected in MW2.

Factor	Daily Productivity		Fresh Weight of Biomass Collected		Dry Weight of Biomass Collected	
	F	***p* Value**	**F**	***p* Value**	**F**	***p* Value**
Species	0.21	0.813	0.18	0.84	1.58	0.281

In the first case study (MW 1), microalgae quickly adapted to the conditions, as shown by the growth curves (Figure 2A). In details, the *C. vulgaris* strain reached the stationary phase earlier (30 days)

compared to the other species, whereas at the end of the trial (60 days), a similar number of cells to those obtained using *S. quadricauda* were counted. As regards *Klebsormidium* sp. K39, a cell number always lower than other species was recorded, although daily productivity and microalgae biomasses collected were similar to those of *C. vulgaris* and *S. quadricauda* (Tables 3 and 5). Furthermore, in Table 5, in which the daily productivity and the microalgae biomasses collected at the end of the trials are reported, it is relevant to point out that no differences in terms of cell density growth or daily productivity were observed.

Table 5. Microalgae daily productivity and biomasses collected at the end of the trial (60 days).

Microalgae Species	Daily Productivity ($g\ L^{-1} \cdot d^{-1}$)		Fresh Weight of Biomass Collected ($g\ L^{-1}$)		Dry Weight of Biomass Collected ($g\ L^{-1}$)	
	MW 1	MW 2	MW 1	MW 2	MW 1	MW 2
C. vulgaris	0.017 ± 0.003	0.016 ± 0.001	5.5 ± 0.4	5.4 ± 0.4	1.10 ± 0.2	1.08 ± 0.06
S. quadricauda	0.015 ± 0.002	0.016 ± 0.002	5.3 ± 0.3	5.3 ± 0.3	1.00 ± 0.1	1.07 ± 0.04
Klebsormidium sp. K39	0.018 ± 0.03	0.015 ± 0.003	5.8 ± 0.3	5.2 ± 0.5	1.08 ± 0.09	0.98 ± 0.11

In the second case study (MW 2), although water samples exhibited a higher nutrient concentration, the three microalgae showed a similar behaviour of adapting to the culturing conditions, as shown by the growth curves reported in Figure 2B. However, the differences in cell numbers among species were less evident, and no significant differences in microalgae growth were detected (Table 4). The daily productivity of the strains was 0.017, 0.015, and 0.018 $g\ L^{-1} \cdot d^{-1}$ for C. *vulgaris*, *S. quadricauda,* and *Klebsormidium* sp. K39, respectively (Table 5).

3.2. Nutrient Removal

Removal pollutant indices were calculated to evaluate the performance of microalgae treatments. As regards the pH values of the wastewaters, they continued to increase from the lag phase through the microalgae growth phase, as shown in Figures 3A (MW1) and 4A (MW2), while EC values showed a decreasing tendency (Figures 3B and 4B), according to nutrient consumption.

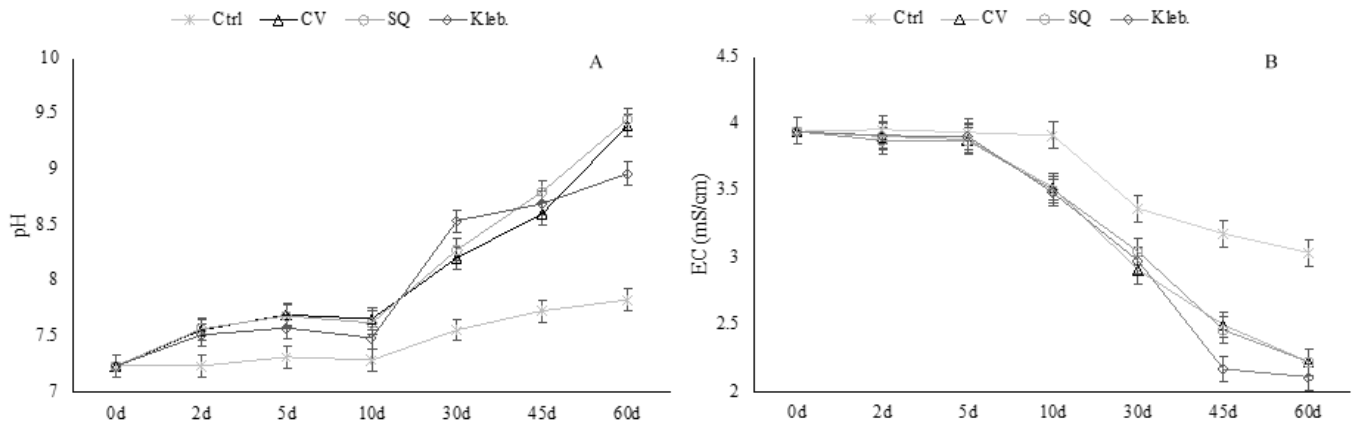


Figure 3. pH **(A)** and EC ($mS \cdot cm^{-1}$) **(B)** values measured at each sampling (MW1).

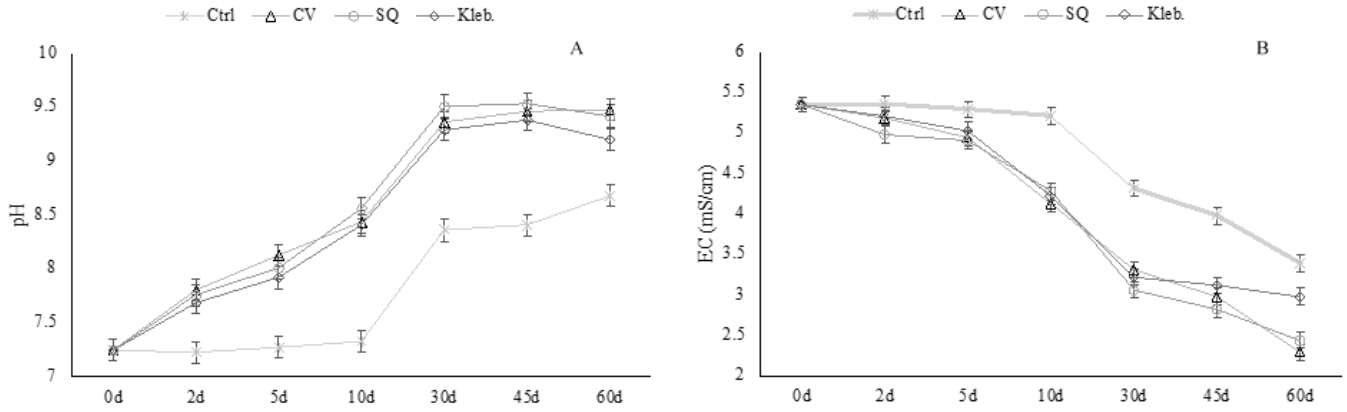


Figure 4. pH **(A)** and EC ($mS \cdot cm^{-1}$) **(B)** values measured at each sampling (MW2).

In the first case study, the effects of single factors, species, and time were always significant on all the parameters monitored, as was the interaction between them on TKN, TP, and COD parameters, except for the BOD_5 parameter (Table 6).

Table 6. Effects of single factors and their interaction in ANOVA—MW1.

Factor(s)	TKN		TP		COD		BOD_5	
	F	***p* Value**	**F**	***p* Value**	**F**	***p* Value**	**F**	***p* Value**
Species	2388.08	<0.0001	620.74	<0.0001	338.13	<0.0001	41.85	<0.0001
Time	2618.61	<0.0001	1337.53	<0.0001	16,601.52	<0.0001	1962.83	<0.0001
Species × time	214.24	<0.0001	174.96	<0.0001	17.87	<0.0001	2.53	0.08

The variations in total nitrogen, total phosphorous, chemical oxygen demand, and biological oxygen demand contents during the two experiments are depicted in Figure 5.

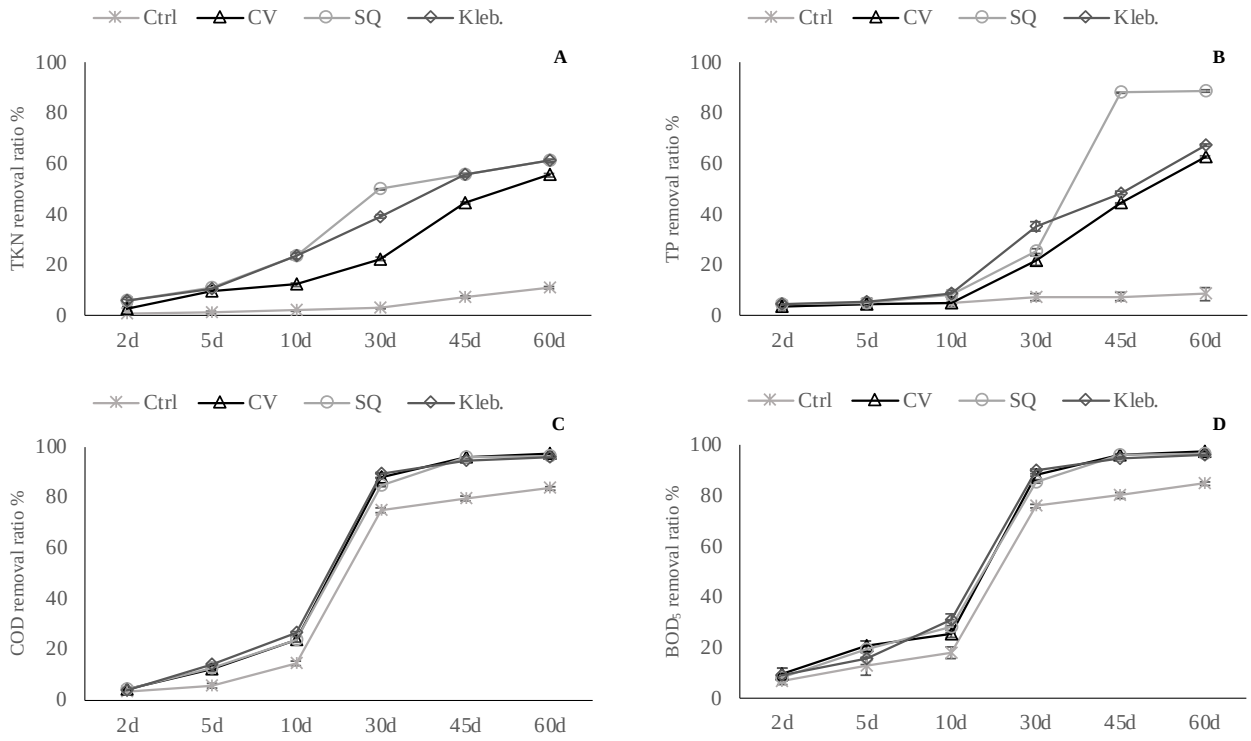


Figure 5. Removal percentage of monitored parameters at each sampling—MW 1 ((**A**)—TKN; (**B**)—TP; (**C**)—COD; (**D**)—BOD_5).

Post-hoc analyses to establish the ranking of effectiveness at each sampling are shown in Supplementary Materials Table S1. Based on these data, at each sampling, the microalgae significantly reduced all the parameters monitored with respect to the control in MW1. The pollutant concentration in all the tested wastewaters showed a different decrease during the first 2 days. The removal of pollutants gradually levelled off until the end of the experimental trial. At the end of the treatment, the maximum removal efficiency of *C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39 was 55.5, 61.0, and 61.2% for total nitrogen, 62.7, 88.7, and 67.2% for total phosphorous, and 97.3, 96.6, and 96.2% for COD, respectively. The maximum total nitrogen, total phosphorous, and COD removal efficiency from wastewater control were 11.1%, 8.5%, and 83.8%, respectively.

As regards the second case study, the performance evaluation of microalgae in contaminants degradation showed that the effects of species, time, and species × time were always significant versus all pollutant parameters monitored (Table 7).

Table 7. Effects of single factors and their interaction in ANOVA—MW2.

Factor(s)	TKN		TP		COD		BOD_5	
	F	*p* Value	F	*p* Value	F	*p* Value	F	*p* Value
Specie	9247.55	<0.0001	968.86	<0.0001	1492.78	<0.0001	164.25	<0.0001
Time	5798.76	<0.0001	2806.37	<0.0001	22,354.06	<0.0001	2353.97	<0.0001
Specie × time	416.51	<0.0001	229.33	<0.0001	109.62	<0.0001	13.12	<0.0001

The variations in total nitrogen, total phosphorous, COD, and BOD_5 contents during the two experiments are shown in Figure 6.

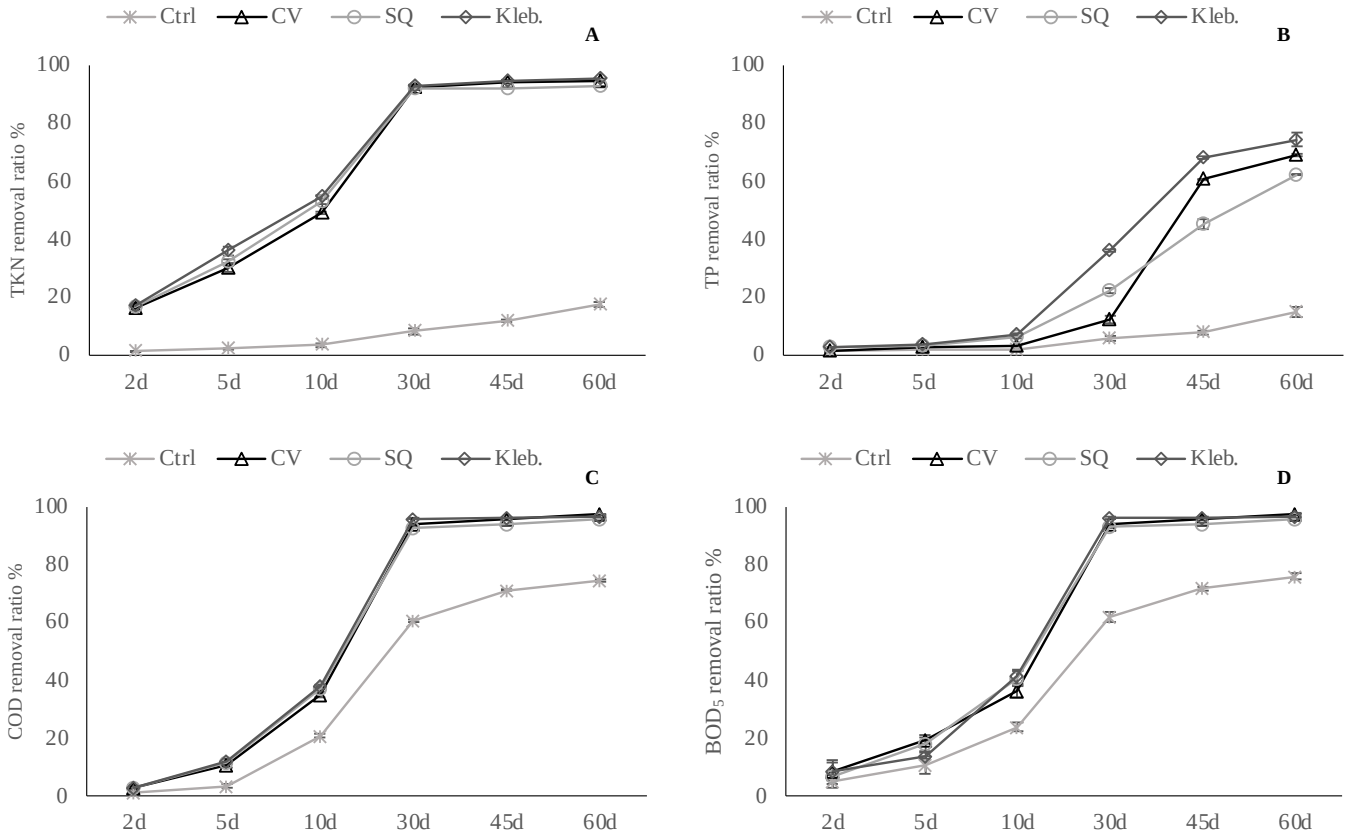


Figure 6. Removal percentage of monitored parameters at each sampling—MW 2 ((**A**)—TKN; (**B**)—TP; (**C**)—COD; (**D**)—BOD_5).

Post-hoc analyses to establish the ranking of effectiveness at each sampling are shown in Supplementary Materials Table S2. Post-hoc analysis of the data revealed a similar ranking of efficacy among the three tested microalgae, which gradually levelled off until the end of the experimental trial for all parameters monitored (Figure 6). In detail, at this sampling, each microalga significantly reduced the TKN variable with values between 92.7 and 95.5%. As well, concerning the removal of TP, COD, and BOD_5, *C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39 significantly reduced from 62.0 up to 74.3%, from 95.6 up to 97.3%, and from 95.4 up to 97.4%

compared to the starting values. As already seen in the above-mentioned trial, a decrease of the same parameters in the control (not-inoculate wastewater) was observed, and the maximum total nitrogen, total phosphorous, COD, and BOD_5 degradation were 16.9, 14.7, 74.5, and 75.0%, respectively.

3.3. *E. coli* Removal Efficiency

The cell density of *E. coli* detected in MW1 (panel A) and MW2 (panel B) water samples, un-inoculated (control) and inoculated with different microalgal cultures (*C. vulgaris* ACUF863, *S. quadricauda* ACUF581, *Klebsormidium* sp. K39) after 0, 2, 5, 7, 9, 15, 30, 45, and 60 days from the inoculum is reported in Figure 7. Overall, a significant decrease in cell density was observed in all tested samples except the controls. In particular, regarding MW1 samples (Figure 7, panel A), no significant difference was detected in the removal efficiency of the tested microalgae. In detail, 5 days after the inoculum, *S. quadricauda* ACUF581 and *C. vulgaris* ACUF863 induced a decrease of 3.14 and 3.28 unit Log in *E. coli* cell densities, whereas *Klebsormidium* sp. K39 induced a decrease of 2.74 unit Log. After 7 days, higher reductions were registered in microalgal treatments as 1.43 unit Log by *S. quadricauda* ACUF581 and *C. vulgaris* ACUF863 and 1.75 unit Log by *Klebsormidium* sp. K39, while *E. coli* in the control sample was at 6.1 Log CFU mL^{-1}. After 9 days, *E. coli* showed a cell density of 6.2 Log CFU mL^{-1} while in treated samples higher decreases, as 0.45, 0.50, and 0.55, were observed for *C. vulgaris* ACUF863, *S. quadricauda* ACUF581, and *Klebsormidium* sp. K39, respectively. In the same samples, no *E. coli* was detected after 15, 30, 45, and 60 days from the microalgal inoculum. A different trend was observed in controls, where *E. coli* was constantly increasing, reaching, at the end of the trial (60 days), a cell density of 3.80 Log CFU mL^{-1}. The bacterial removal results on MW2 samples were significant (Figure 7, panel B). In details, after 5 days from inoculum, in samples treated with *S. quadricauda* ACUF581 and *C. vulgaris* ACUF863, the target bacteria were reduced by 3.34 and 3.49 unit Log, respectively, whereas in samples treated with *Klebsormidium* sp. K39, the target bacteria were reduced by 3.15 unit Log. The reduction values were significantly different compared to the control sample, where the *E. coli* density was found to be 7.53 Log CFU mL^{-1}, while no significant differences were found among the treatments. After 7 days, more than 0.97, 1.03, and 1.06 unit Log CFU mL^{-1} of reduction were observed for *S. quadricauda* ACUF581, *Klebsormidium* sp. K39, and *C. vulgaris* ACUF863, respectively, when the target bacteria cell density in control samples showed a load of 7.85 Log CFU mL^{-1}. After 9 days, the target bacteria showed a cell density of 7.54 Log CFU mL^{-1}, while the treated samples registered a higher reduction, as 0.80, 0.84, and 0.85 for *C. vulgaris* ACUF863, *S. quadricauda* ACUF581, and *Klebsormidium* sp. K39, respectively. After days 15, 30, 45, and 60 days, *E. coli* was never detected

in any treated samples, while its density was found at a mean value of 4.1 Log CFU mL^{-1} in untreated samples at the end of the trial (60 days).

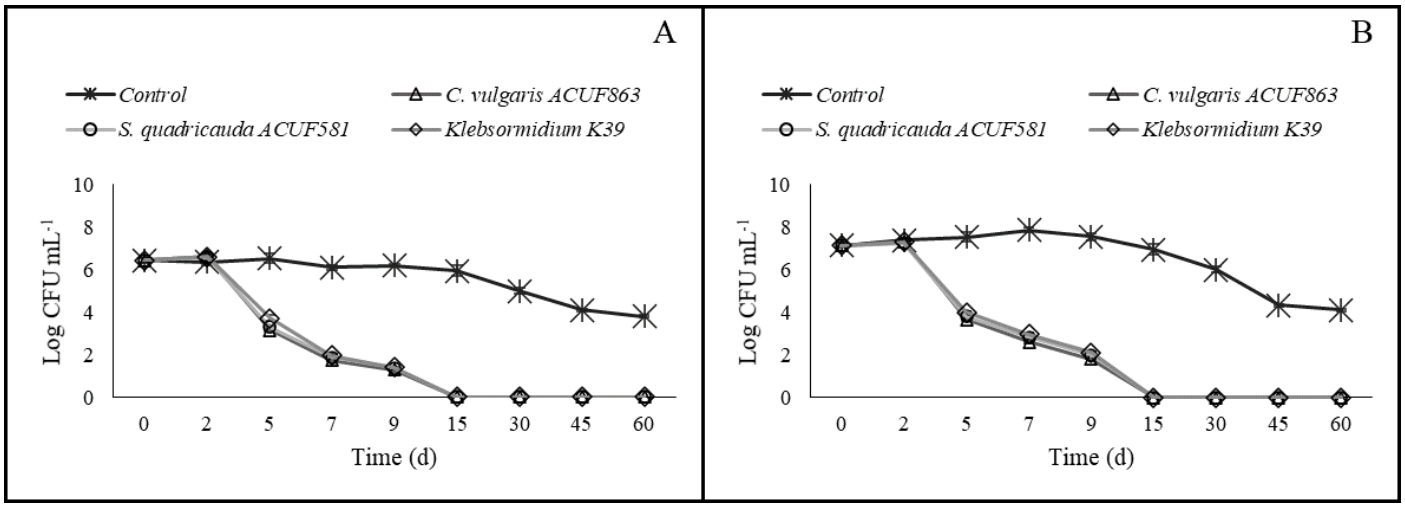


Figure 7. *E. coli* load detected (as Log cells mL^{-1}) detected in MW1 (**A**) and MW2 (**B**) samples, un-inoculated (control) and inoculated with different microalgal cultures (*C. vulgaris* ACUF863, *S. quadricauda* ACUF581, or *Klebsormidium* sp. K39) after 0, 2, 5, 7, 9, 15, 30, 45, and 60 days from the inoculum.

4. Discussion

Discharge of wastewater into water bodies represents a serious issue because the high concentrations of contaminants may pose a serious threat to ecosystem health. In this frame, one of the main reasons for removing nutrients from wastewater is to control eutrophication, which is due to the uncontrolled growth of algae or higher hydrophytes triggered by the addition of a nutrient surplus in the ecosystem [4,41]. In the present study, a sustainable and eco-friendly wastewater treatment was tested in order to support a circular system in which the microalgae play a key role, representing both the agent of the remediation and the final product of the process, which leads to a useful biomass suitable for several further purposes. The importance of low-cost biomass production is crucial because the economic and environmental drawbacks could be partly overcome using urban wastewater as a microalgae growth substrate [7,42]. Because of their ability to perform photoautotrophic, mixotrophic, or heterotrophic metabolism, microalgae represent a promising biological system for a variety of wastewaters. To achieve this aim, employing species able to remediate wastewater is crucial and guarantees a successful sustainable process, and the best candidate is represented by autochthonous microalgae, which are able to naturally grow in a specific wastewater. Furthermore,

microalgal systems are designed mainly to achieve high biomass productivity with minimum energy inputs because essential nutrients and a carbon source, required for an efficient cultivation process, are largely available in the effluent [43,44]. The identification process of several isolates recently affiliated with the genus *Klebsormidum* revealed that *Klebsormidum sp.* K39 lacks a proper grouping at the species level due to unclear species boundaries [45]. For this genus, the morphological traits as well as some features considered taxonomically relevant (showing variations depending on the age and the physiological conditions) result in a taxonomically and systematically complex taxon in which phylogenetic relationships are still poorly understood [45,46]. Despite *Klebsormidum sp.* K39 being subjected to molecular analyses for phylogenetic study, further studies are required to cluster this strain into a species, as Novis [47] had already shown, with the description of the *Klebsormidium acidophilum* species. It is relevant to highlight that the K*lebsormidum sp.* K39 strain used in the present study has been recently tested to evaluate its dynamic within an autochthonous microalgal pool in terms of *E. coli* removal efficiency [35]. Zooming in on microalgal yields obtained during the phycoremediation process, they were quite different from data reported in the literature due to the different composition of treated effluents [7,34]. In particular, Li et al. [34], cultivating five microalgae species, among them *C. vulgaris* and *S. quadricauda*, in post hydrothermal liquefaction wastewater, obtained a daily productivity of 0.031 and 0.0071 g $L^{-1} \cdot d^{-1}$, respectively. Regarding *Klebsormidium* sp., available data indicate a biomass production that may vary from about 0.010 g $L^{-1} \cdot d^{-1}$ in horticultural wastewater to about 0.035 g $L^{-1} \cdot d^{-1}$ in synthetic wastewater [37]. Although, the yields are quite different than optimal conditions, at the end of the present experimental tests, all the microalgae demonstrated a good growth aptitude in urban wastewaters with different pollutant contents, and this could be mainly related to their physiochemical and biochemical characteristics. Indeed, many studies report the remediation ability and biomass production of *C. vulgaris* and *S. quadricauda* using wastewater from various sources; they have proven abilities of removing nitrogen, phosphorus, and COD and shown their potentiality as a tertiary biotreatment step in the remediation process [7]. For instance, Baglieri et al. [31] investigated the feasibility of cultivating *C. vulgaris* and *S. quadricauda* in agricultural wastewater for inorganic nutrient removal, and the two species showed similar behavior, determining comparable remediation performance in terms of nitrogen (both about 99%) and phosporous (88 and 94%, respectively). On the contrary, limited studies on the cultivation of *Klebsormidium* sp. K39 in wastewater are still reported. Among *Klebsormidium species*, *Klebsormidium flaccidum* showed good feasibility for nutrient removal from municipal wastewater, being able to provide a complete removal of nitrogen and phosphorous [48]. Similarly, Liu and Vyverman [49] evaluated differences in the uptake of nutrients of *Klebsormidium* sp. from wastewater under varying nitrogen and phosphorous contents. The authors observed that the microalgae achieved

an approximately 99% phosphorous removal rate and a consistent nitrogen removal rate (about 99%) under almost any tested conditions. However, with a N/P ratio of 20, *Klebsormidium* sp. exhibited a lower nitrogen removal efficiency (76.4%). Overall, the daily productivity and the growth results confirm the suitability of urban wastewater as a substrate for cultivation of *Klebsormidium* sp. K39 and the absence of negative effects. Similarly, the three species showed quite comparable increases in terms of fresh and dry biomass produced. A good adaptability of *Klebsormidium* sp. K39 was also observed in a study under consideration [35]. In particular, *Klebsormidium* sp. K39, during a lab-scale wastewater treatment at lab scale using a microalgae pool, was the dominant microalgae at the end of the treatment. Results clearly showed that the initial concentrations of both tested wastewaters did not affect the final biomass accumulation or the daily productivity of the three microalgae species. This may be mainly due to the characteristics of the tested urban wastewater, a kind of effluent usually rich in nutrient compounds and characterized by low concentrations of toxic substances that may inhibit microalgae growth. In detail, in the first case study using MW 1, *S. quadricauda* showed the highest phosphorous removal rate (91.9%), followed by *Klebsormidium* sp. K39 (69.6%) and *C. vulgaris* (64.7%) of total phosphorous. In terms of nitrogen removal, no significant differences were detected between *S. quadricauda* and *Klebsormidium* sp. K39, which showed the highest removal efficiency (62.8 and 63.1%, respectively), while for *C. vulgaris,* a lower degradation rate was observed at each sampling time. In the control, the decrease of total nitrogen and total phosphorous due to naturally occurring abiotic degradation, was very low. Regarding the removal of COD and BOD_5, slight differences were observed among the tested strains, and both of these parameters always significantly decreased at any time in all treated samples. In the second case study, using MW 2, *C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39 induced a progressive reduction of measured parameters with increasing treatment time in total nitrogen, total phosphorous, COD, and BOD_5 to values below the reuse for irrigation in agriculture, according to law limits (Italian Ministerial Decree n. 185/2003) for irrigation use. A comparable bioremediation performance, in terms of total nitrogen, COD, and BOD_5, was recorded regardless of the microalgae species. Instead, the highest phosphorous removal rate was achieved by *S. quadricauda*. The highest amount of nutrient removal matched the biomass production; in fact, it is well known that the nutrient reduction is mainly related to the metabolic activity of microalgae cells [34]. In both case studies, the *E. coli* removal rates achieved with *C. vulgaris*, *S. quadricauda*, and *Klebsormidium* sp. K39 were in line with the values previously reported. Although pathogen removal mechanisms of microalgae have been related to different phenomena such as competition for nutrients, pH increases, and higher dissolved oxygen levels, for *E. coli* removal, adherence to the microalgal surface [50] is reported as the most likely mechanism [51,52]. In a study conducted in photobioreactors, *Chlorella sorokiniana* performed a *E. coli* removal

rate of 99.8% in anaerobically treated black water in photobioreactors [53]. Overall, as reported in a recent review, the *E. coli* removal rate is on average higher than 98% [54]. The results of the present study indicate that the two different levels of contaminants did not negatively affect the nutrient removal ratio or cell growth, in accordance with findings reported in several studies [27,34,37]. In these studies, the authors, starting from effluents with various nutrient concentrations, observed that the microalgae screened, including *C. vulgaris* and *S. quadricauda*, were able to reproduce similar performances in terms of both cell growth and nutrient uptake capacity. In Table 8, a summary of nutrient removal rates reported in various recent studies is provided, supporting and confirming the remediation capacity of the microalgae species tested in the current study.

Table 8. Removal rates by *C. vulgaris*, *S. quadricauda* and *Klebsormidium* sp. K39 in wastewaters.

Microalga Species	Wastewater Type	Starting Values (mg L^{-1})	Treatment Efficiency (%)	Reference
C. vulgaris	Municipal wastewater 1	N: 10 P: 3.2	N: 57 P: 65	Present study
C. vulgaris	Municipal wastewater 2	N: 50.7 P: 10.7	N: 95 P: 69	Present study
C. vulgaris	Agricultural wastewater	NH_4^+: 1.4 NO_3^-: 210.0 P: 4.0	NH_4^+: 99 NO_3^-: 83 P: 88	[33]
C. vulgaris	Synthetic effluent	NO_3^-: 20.2 PO_4^{3-}: 4.7	NO_3^-~50 PO_4^{3-} > 98	[55]
C. vulgaris	Municipal wastewater (25%)	NO_3^-: 8.2 PO_4^{3-}: 3.2	NO_3^-: 88 PO_4^{3-}: 91	[56]
C. vulgaris	Municipal wastewater (50%)	NO_3^-: 16.4 PO_4^{3-}: 6.3	NO_3^-: 79 PO_4^{3-}: 88	[56]
C. vulgaris	Municipal wastewater (75%)	NO_3^-: 24.6 PO_4^{3-}: 9.5	NO_3^-: 63 PO_4^{3-}: 85	[56]
C. vulgaris	Municipal wastewater (100%)	NO_3^-: 32.8 PO_4^{3-}: 12.6	NO_3^-: 54 PO_4^{3-}: 83	[56]
S. quadricauda	Municipal wastewater 1	N: 10.0 P: 3.2	N: 62 P: 92	Present study
S. quadricauda	Municipal wastewater 2	N: 50.7 P: 10.7	N: 93 P: 62	Present study
S. quadricauda	Agricultural wastewater	NH_4^+: 1.4 NO_3^-: 210 P: 4.0	NH_4^+: 99 NO_3^-: 83 P: 88	[33]
S. quadricauda	Sewage treatment works	N~30.0 P~3.0	N > 95 P > 90	[32]
Klebsormidium sp. K39	Municipal wastewater 1	N: 10 P: 3.2	N: 63 P: 69	Present study
Klebsormidium sp. K39	Municipal wastewater 2	N: 50.7 P: 10.7	N: 96 P: 74	Present study

5. Conclusions

The use of microalgae as wastewater remediation agents is becoming an interesting alternative to conventional treatments, offering two undeniable benefits, i.e., the wastewater remediation and the production of valuable biomass for multipurpose applications. Overall, our findings confirm that microalgae-based treatment offers potential for sustainable, eco-friendly, and resource-efficient solutions for wastewater remediation that may also be used for irrigation in agriculture, contributing to a more environmentally friendly approach to water management. Furthermore, it is noteworthy that this study represents the first investigation into the use of *Klebsormidium* sp. K39, according to the promising performances of other species of this genus for wastewater remediation treatment. Our findings demonstrate that this species exhibits high adaptability to various wastewater conditions and displays efficient nutrient removal capabilities. These results are promising because they suggest that indigenous species like *Klebsormidium* sp. K39 exhibit the potential to deliver similar decontamination performances as the extensively studied microalgae species. However, further studies, as well as a full-scale demonstration, are necessary to verify the practicality, efficiency, and cost-effectiveness of microalgae-based treatment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su151511644/s1. Table S1: Wastewater parameters (mg L^{-1}) along the experimental period in MW 1; Table S2: Wastewater parameters (mg L-1) along the experimental period in MW 2.

Author Contributions: Conceptualization, I.P. and A.B.; methodology, E.L.B. and P.S.O.; software, E.L.B.; validation, C.L.R., C.C., I.P., and A.B.; formal analysis, E.L.B., P.S.O., F.F., N.R., and R.S.; data curation, E.L.B.; writing—original draft preparation, E.L.B. and P.S.O.; writing—review and editing C.C., I.P., and A.B.; supervision, C.C., I.P., A.B., and C.L.R.; funding acquisition, C.C., I.P., A.B., and C.L.R. All authors have read and agreed to the published version of the manuscript.

Funding: This study has been partially supported by PON "RICERCA E INNOVAZIONE" 2014–2020, Azione II—Obiettivo Specifico 1b—Progetto "Miglioramento delle produzioni agroalimentari mediterranee in condizioni di carenza di risorse idriche—WATER4AGRIFOOD and partially funded by the European Union (NextGeneration EU) through the MUR-PNRR project Sustainable management of natural resources in agriculture: SAMOTHRACE (ECS00000022) and the Agritech National Research Center (PNRR—MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4—D.D. 1032 17/06/2022, CN00000022). This manuscript reflects only the authors' views and opinions; neither the European Union nor the European Commission can be considered responsible for them.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: This study was conducted within a Ph.D. research program in Agricultural, Food, and Environmental Science (cycle XXXVI) by Emanuele la Bella (Scientific Tutor: Ivana Puglisi and Andrea Baglieri) and Biotechnology (cycle XXXVI) by Paride Salvatore Occhipinti (Scientific Tutor: Cinzia Caggia and Cinzia Lucia Randazzo), who received a grant from the University of Catania. The authors sincerely thank Antonino Pollio, from the University of Naples, for kindly providing the strains *Chlorella vulgaris* ACUF863 and *Scenedesmus quadricauda* ACUF581.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Schewe, J.; Heinke, J.; Gerten, D.; Haddeland, I.; Arnell, N.W.; Clark, D.B.; Dankers, R.; Eisner, S.; Fekete, B.M.; Colón-González, F.J.; et al. Multimodel Assessment of Water Scarcity under Climate Change. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 3245–3250.
2. Arora, A.; Saxena, S. Cultivation of Azolla Microphylla Biomass on Secondary-Treated Delhi Municipal Effluents. *Biomass Bioenergy* **2005**, *29*, 60–64.
3. de-Bashan, L.E.; Bashan, Y. Immobilized Microalgae for Removing Pollutants: Review of Practical Aspects. *Bioresour. Technol.* **2010**, *101*, 1611–1627.
4. Chai, W.S.; Tan, W.G.; Halimatul Munawaroh, H.S.; Gupta, V.K.; Ho, S.-H.; Show, P.L. Multifaceted Roles of Microalgae in the Application of Wastewater Biotreatment: A Review. *Environ. Pollut.* **2021**, *269*, 116236.
5. Olguín, E.J. Phycoremediation: Key Issues for Cost-Effective Nutrient Removal Processes. *Biotechnol. Adv.* **2003**, *22*, 81–91.
6. Rizzo, L.; Malato, S.; Antakyali, D.; Beretsou, V.G.; Đolić, M.B.; Gernjak, W.; Heath, E.; Ivancev-Tumbas, I.; Karaolia, P.; Lado Ribeiro, A.R.; et al. Consolidated vs New Advanced Treatment Methods for the Removal of Contaminants of Emerging Concern from Urban Wastewater. *Sci. Total Environ.* **2019**, *655*, 986–1008.
7. La Bella, E.; Baglieri, A.; Fragalà, F.; Puglisi, I. Multipurpose Agricultural Reuse of Microalgae Biomasses Employed for the Treatment of Urban Wastewater. *Agronomy* **2022**, *12*, 234.
8. World Health Organization. *Guidelines for the Safe Use of Wasterwater Excreta and Greywater*; World Health Organization: Geneva, Switzerland, 2006.
9. Ofori, S.; Puškáčová, A.; Růžičková, I.; Wanner, J. Treated Wastewater Reuse for Irrigation: Pros and Cons. *Sci. Total Environ.* **2021**, *760*, 144026.
10. Łuczkiewicz, A.; Jankowska, K.; Fudala-Ksiazek, S.; Olańczuk-Neyman, K. Antimicrobial Resistance of Fecal Indicators in Municipal Wastewater Treatment Plant. *Water Res.* **2010**, *44*, 5089–5097.
11. Bouki, C.; Venieri, D.; Diamadopoulos, E. Detection and Fate of Antibiotic Resistant Bacteria in Wastewater Treatment Plants: A Review. *Ecotoxicol. Environ. Saf.* **2013**, *91*, 1–9.
12. Novo, A.; André, S.; Viana, P.; Nunes, O.C.; Manaia, C.M. Antibiotic Resistance, Antimicrobial Residues and Bacterial Community Composition in Urban Wastewater. *Water Res.* **2013**, *47*, 1875–1887.
13. Rizzo, L.; Manaia, C.; Merlin, C.; Schwartz, T.; Dagot, C.; Ploy, M.C.; Michael, I.; Fatta-Kassinos, D. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Sci. Total Environ.* **2013**, *447*, 345–360.
14. Ventura, D.; Consoli, S.; Barbagallo, S.; Marzo, A.; Vanella, D.; Licciardello, F.; Cirelli, G.L. How to Overcome Barriers for Wastewater Agricultural Reuse in Sicily (Italy)? *Water* **2019**, *11*, 335.
15. González-Fernández, C.; Sialve, B.; Bernet, N.; Steyer, J.-P. Impact of Microalgae Characteristics on Their Conversion to Biofuel. Part II: Focus on Biomethane Production. *Biofuels Bioprod. Biorefin.* **2012**, *6*, 205–218.
16. Puglisi, I.; Barone, V.; Fragalà, F.; Stevanato, P.; Baglieri, A.; Vitale, A. Effect of Microalgal Extracts from *Chlorella vulgaris* and *Scenedesmus quadricauda* on Germination of *Beta vulgaris* Seeds. *Plants* **2020**, *9*, 675.

17. La Bella, E.; Baglieri, A.; Rovetto, E.I.; Stevanato, P.; Puglisi, I. Foliar Spray Application of *Chlorella vulgaris* Extract: Effect on the Growth of Lettuce Seedlings. *Agronomy* **2021**, *11*, 308.
18. Puglisi, I.; La Bella, E.; Rovetto, E.I.; Stevanato, P.; Fascella, G.; Baglieri, A. Morpho-Biometric and Biochemical Responses in Lettuce Seedlings Treated by Different Application Methods of *Chlorella vulgaris* Extract: Foliar Spray or Root Drench? *J. Appl. Phycol.* **2022**, *34*, 889–901.
19. Caporgno, M.P.; Mathys, A. Trends in Microalgae Incorporation Into Innovative Food Products with Potential Health Benefits. *Front. Nutr.* **2018**, *5*, 58.
20. Vaz, B.D.S.; Moreira, J.B.; Morais, M.G.D.; Costa, J.A.V. Microalgae as a New Source of Bioactive Compounds in Food Supplements. *Curr. Opin. Food. Sci.* **2016**, *7*, 73–77.
21. Vanni, A.; Anfossi, L.; Cignetti, A.; Baglieri, A.; Gennari, M. Degradation of Pyrimethanil in Soil: Influence of Light, Oxygen, and Microbial Activity. *J. Environ. Sci. Health B* **2006**, *41*, 67–80.
22. Rawat, I.; Ranjith Kumar, R.; Mutanda, T.; Bux, F. Dual Role of Microalgae: Phycoremediation of Domestic Wastewater and Biomass Production for Sustainable Biofuels Production. *Appl. Energy* **2011**, *88*, 3411–3424.
23. Cai, T.; Park, S.Y.; Li, Y. Nutrient Recovery from Wastewater Streams by Microalgae: Status and Prospects. *Renew. Sustain. Energy Rev.* **2013**, *19*, 360–369.
24. Colak, O.; Kaya, Z. A Study on the Possibilities of Biological Wastewater Treatment Using Algae. *Doga Biyolji Serisi* **1988**, *12*, 18–29.
25. Abdel-Raouf, N.; Al-Homaidan, A.A.; Ibraheem, I.B.M. Microalgae and Wastewater Treatment. *Saudi J. Biol. Sci.* **2012**, *19*, 257–275.
26. Sánchez-Zurano, A.; Lafarga, T.; Morales-Amaral, M.M.; Gómez-Serrano, C.; Fernández-Sevilla, J.M.; Acién-Fernández, F.G.; Molina-Grima, E. Wastewater Treatment Using *Scenedesmus almeriensis*: Effect of Operational Conditions on the Composition of the Microalgae-Bacteria Consortia. *J. Appl. Phycol.* **2021**, *33*, 3885–3897.
27. García, D.; Posadas, E.; Blanco, S.; Acién, G.; García-Encina, P.; Bolado, S.; Muñoz, R. Evaluation of the Dynamics of Microalgae Population Structure and Process Performance during Piggery Wastewater Treatment in Algal-Bacterial Photobioreactors. *Bioresour. Technol.* **2018**, *248*, 120–126.
28. Law, X.N.; Cheah, W.Y.; Chew, K.W.; Ibrahim, M.F.; Park, Y.-K.; Ho, S.-H.; Show, P.L. Microalgal-Based Biochar in Wastewater Remediation: Its Synthesis, Characterization and Applications. *Environ. Res.* **2022**, *204*, 111966.
29. Wang, L.; Min, M.; Li, Y.; Chen, P.; Chen, Y.; Liu, Y.; Wang, Y.; Ruan, R. Cultivation of Green Algae *Chlorella* sp. in Different Wastewaters from Municipal Wastewater Treatment Plant. *Appl. Biochem. Biotechnol.* **2010**, *162*, 1174–1186.
30. Wong, Y.K.; Yung, K.K.L.; Tsang, Y.F.; Xia, Y.; Wang, L.; Ho, K.C. *Scenedesmus quadricauda* for Nutrient Removal and Lipid Production in Wastewater. *Water Environ. Res.* **2015**, *87*, 2037–2044.
31. Baglieri, A.; Sidella, S.; Barone, V.; Fragalà, F.; Silkina, A.; Nègre, M.; Gennari, M. Cultivating *Chlorella vulgaris* and *Scenedesmus quadricauda* Microalgae to Degrade Inorganic Compounds and Pesticides in Water. *Environ. Sci. Pollut. Res.* **2016**, *23*, 18165–18174.
32. Ren, H.; Tuo, J.; Addy, M.M.; Zhang, R.; Lu, Q.; Anderson, E.; Chen, P.; Ruan, R. Cultivation of *Chlorella vulgaris* in a Pilot-Scale Photobioreactor Using Real Centrate Wastewater with Waste Glycerol for Improving Microalgae Biomass Production and Wastewater Nutrients Removal. *Bioresour. Technol.* **2017**, *245*, 1130–1138.
33. APHA. *Standard Methods for the Examination of Water and Wastewater*, 21st ed.; American Public Health Association/American Water Works Association/Water Environment Federation: Washington, DC, USA, 2005.
34. Li, Z.; Haifeng, L.; Zhang, Y.; Shanshan, M.; Baoming, L.; Zhidan, L.; Na, D.; Minsheng, L.; Buchun, S.; Jianwen, L. Microalgae Production for Bioenergy from Post Hydrothermal Liquefaction Wastewater: Effects of Strain, Nutrients Concentration and Inoculum Size on Microalgae Culture. *Int. J. Agric. Biol. Eng.* **2017**, *10*, 194–204.
35. Occhipinti, P.S.; Russo, N.; Foti, P.; Pino, A.; Randazzo, C.L.; Pollio, A.; Caggia, C. An indigenous microalgal pool from a constructed wetland as an alternative strategy for Esch-erichia coli removal in urban wastewater. *J. Environ. Manag.* **2023**, *submitted.*
36. APHA. *Standard Methods for the Examination of Water and Wastewater*, 23rd ed.; American Public Health Association: Washington DC, USA, 2017.
37. Liu, J.; Danneels, B.; Vanormelingen, P.; Vyverman, W. Nutrient Removal from Horticultural Wastewater by Benthic Filamentous Algae *Klebsormidium* sp., *Stigeoclonium* spp. and Their Communities: From Laboratory Flask to Outdoor Algal Turf Scrubber (ATS). *Water Res.* **2016**, *92*, 61–68.
38. Ruiz-Martinez, A.; Garcia, N.M.; Romero, I.; Seco, A.; Ferrer, J. Microalgae cultivation in wastewater: Nutrient removal from anaerobic membrane bioreactor effluent. *Bioresour. Technol.* **2012**, *126*, 247–253.
39. Pham, M.; Schideman, L.; Scott, J.; Rajagopalan, N.; Plewa, M.J. Chemical and Biological Characterization of Wastewater Generated from Hydrothermal Liquefaction of Spirulina. *Environ. Sci. Technol.* **2013**, *47*, 2131–2138.
40. Baglieri, A.; Nègre, M.; Trotta, F.; Bracco, P.; Gennari, M. Organo-Clays and Nanosponges for Acquifer Bioremediation: Adsorption and Degradation of Triclopyr. *J. Environ. Sci. Health B* **2013**, *48*, 784–792.
41. Hammouda, O.; Gaber, A.; Abdel-Raouf, N. Microalgae and Wastewater Treatment. *Ecotoxicol. Environ. Saf.* **1995**, *31*, 205–210.
42. Delrue, F.; Álvarez-Díaz, P.D.; Fon-Sing, S.; Fleury, G.; Sassi, J.-F. The Environmental Biorefinery: Using Microalgae to Remediate Wastewater, a Win-Win Paradigm. *Energies* **2016**, *9*, 132.

43. Nasr, M. Design considerations of algal systems for wastewater treatment. In *Application of Microalgae in Wastewater Treatment: Volume 1: Domestic and Industrial Wastewater Treatment*; Springer: Berlin/Heidelberg, Germany, 2019; pp. 411–426.
44. Nasr, M. Aquatic pollution and wastewater treatment system. In *Algae and Aquatic Macrophytes in Cities*; Elsevier: Amsterdam, The Netherlands, 2022; pp. 23–37.
45. Škaloud, P.; Rindi, F. Ecological Differentiation of Cryptic Species within an Asexual Protist Morphospecies: A Case Study of Filamentous Green Alga *Klebsormidium* (Streptophyta). *J. Eukaryot. Microbiol.* **2013**, *60*, 350–362.
46. Rindi, F.; Mikhailyuk, T.I.; Sluiman, H.J.; Friedl, T.; López-Bautista, J.M. Phylogenetic Relationships in Interfilum and *Klebsormidium* (Klebsormidiophyceae, Streptophyta). *Mol. Phylogenet. Evol.* **2011**, *58*, 218–231.
47. Novis, P.M. Taxonomy of *Klebsormidium* (Klebsormidiales, Charophyceae) in New Zealand Streams and the Significance of Low-PH Habitats. *Phycologia* **2006**, *45*, 293–301.
48. Umetani, I.; Sposób, M.; Tiron, O. Indigenous Green Microalgae for Wastewater Treatment: Nutrient Removal and Resource Recovery for Biofuels and Bioproducts. *Bioenergy Res.* **2023**. https://doi.org/10.1007/s12155-023-10611-9.
49. Liu, J.; Vyverman, W. Differences in Nutrient Uptake Capacity of the Benthic Filamentous Algae *Cladophora* sp., *Klebsormidium* sp. and *Pseudanabaena* sp. under Varying N/P Conditions. *Bioresour. Technol.* **2015**, *179*, 234–242.
50. Markou, G.; Wang, L.; Ye, J.; Unc, A. Using agro-industrial wastes for the cultivation of microalgae and duckweeds: Contamination risks and biomass safety concerns. *Biotechnol. Adv.* **2018**, *36*, 1238–1254.
51. Ansa, E.D.O.; Lubberding, H.J.; Ampofo, J.A.; Gijzen, H.J. The role of algae in the removal of *Escherichia coli* in a tropical eutrophic lake. *Ecol. Eng.* **2011**, *37*, 317–324.
52. Cho, K.H.; Wolny, J.; Kase, J.A.; Unno, T.; Pachepsky, Y. Interactions of *E. coli* with algae and aquatic vegetation in natural waters. *Water Res.* **2022**, *209*, 117952.
53. Slompo, N.D.M.; Quartaroli, L.; Fernandes, T.V.; Silva, G.H.R.D.; Daniel, L.A. Nutrient and Pathogen Removal from Anaerobically Treated Black Water by Microalgae. *J. Environ. Manag.* **2020**, *268*, 110693.
54. Amaro, H.M.; Salgado, E.M.; Nunes, O.C.; Pires, J.C.M.; Esteves, A.F. Microalgae Systems-Environmental Agents for Wastewater Treatment and Further Potential Biomass Valorisation. *J. Environ. Manag.* **2023**, *337*, 117678.
55. Kube, M.; Mohseni, A.; Fan, L.; Roddick, F. Impact of Alginate Selection for Wastewater Treatment by Immobilised *Chlorella vulgaris*. *Chem. Eng. J.* **2019**, *358*, 1601–1609.
56. Singh, D.V.; Upadhyay, A.K.; Singh, R.; Singh, D.P. Implication of Municipal Wastewater on Growth Kinetics, Biochemical Profile, and Defense System of *Chlorella vulgaris* and *Scenedesmus vacuolatus*. *Environ. Technol. Innov.* **2022**, *26*, 102334.

CHAPTER 5. POLY-EXTREMOPHILE MICROALGAE AND MIXOTROPHY FOR INDUSTRIAL WASTE WATER TREATMENT

Mixotrophic and heterotrophic growth of Galdieria sulphuraria using buttermilk as a carbon source

Paride Salvatore Occhipinti[1], Flavia Del Signore[3], Stefano Canziani[3], Cinzia Caggia[1,2], Valeria Mezzanotte[4], Narcís Ferrer-Ledo[3]

[1]Department of Agriculture, Food and Environment (Di3A), University of Catania, Via S. Sofia, 100, 95123 Catania, Italy.

[2]ProBioEtna srl, Spin off of University of Catania, via S. Sofia 100, 95123 Catania, Italy.

[3]Algreen B.V., Bronland 10, 6708 WH Wageningen, the Netherlands.

[4]DISAT, Università di Milano Bicocca, Piazza della Scienza,1, Milano, Italy.

*Correspondence: narcis@algreen.eu

This article has been published in *Journal of Applied Phycology*. 2023 1-13. https://doi.org/10.1007/s10811-023-03012-0

Abstract

The growth of the polyextremophile red microalga *Galdieria sulphuraria* ACUF 064 was evaluated under mixotrophy and heterotrophy in a 13L lab-scale stirred photobioreactor, using buttermilk as a carbon source. Beforehand, *G. sulphuraria* ACUF 064 growth on glucose, galactose and lactose was evaluated. No significant differences were observed in terms of growth when lactose, glucose or galactose was used as a carbon source. Overall, the biomass yield on carbon was 70% higher in mixotrophy than heterotrophy for galactose (2.0 g_x g_C^{-1}) and lactose (2.0 g_x g_C^{-1}), while it was similar for glucose (1.5 g_x g_C^{-1}). At the same time, the yield of biomass on nitrogen for cultures grown in lactose was the lowest in comparison to all the other tested substrates. This was also supported by a higher C-phycocyanin content, with 5.9% $w_{C\text{-}PC}/w_x$ as the highest value in mixotrophy. A preliminary experiment in flasks under mixotrophic conditions with different buttermilk dilutions revealed that a dilution ratio of 40% v/v of buttermilk (corresponding to 2.0 g_C L^{-1}) was optimal for biomass production. Finally, *G. sulphuraria* ACUF 064 was grown in the optimal buttermilk dilution ratio in a 13 L photobioreactor. The highest biomass productivity was also obtained in mixotrophy (0.55 g_x L^{-1} d^{-1}), corresponding to a carbon removal of 61%. Overall, lactose-containing substrates such as buttermilk hold promise as a substrate for the growth of *G. sulphuraria* while revalorizing an industrial effluent.

Keywords: Polyextremophile microalgae; *Galdieria sulphuraria;* mixotrophy; dairy effluent; phycocyanin.

1. Introduction

The dairy sector is among the most polluting agri-food industries due to its large water consumption and consequently, the large volumes of wastewater produced (Raghunath et al., 2016). Reducing waste or adding value to by-products from milk processing is a pivotal strategy to enhance industrial food-related sustainability (Olabi et al., 2015). Buttermilk is one of the most important by-products of dairy industries and it is made of the aqueous phase released during the churning of cream in butter processing. Between 6.5% and 7.0% of milk produced worldwide is used for the preparation of butter which results in about 3.2 million tons/year of buttermilk generated (Kumar et al., 2019). Europe is one of the largest contributors with an estimated production of buttermilk of 2 million tons/year (Ali, 2019). Buttermilk is generally classified as sweet (or cultured) buttermilk and whey buttermilk (Ali, 2019). The most important differences between whey buttermilk and cultured buttermilk are the absence of casein and the lower phospholipid content (Konrad et al., 2013). The chemical composition of sweet buttermilk is quite similar to that of skimmed milk for lactose (48.7–53.8% w/w) and protein (31.5–33.1% w/w) and presents a lower fat content (5.7–13.1% w/w) than buttermilk from cheese (Lambert et al., 2016; Sodini et al., 2006). Due to its bioactive properties, buttermilk is currently used in the formulation of different products for consumption. In cheese, the increase of sweet-cream buttermilk content significantly modifies its composition, resulting in a softer and moister curd. Cheese containing more than 25% of sweet-cream buttermilk would not be acceptable from a sensorial point of view. It can also be used as a powder in the food industry, which implies the application of energy-costly steps such as evaporation and spray-drying processes. Although several studies have reported different uses of buttermilk in several food productions, such as in yoghurt, bakery or cheese (Dewettinck et al., 2008; Vanderghem et al., 2010; Fuller et al., 2013; Gassi et al., 2016), biotechnological applications with buttermilk remain limited (Bahrami et al., 2015). Due to the large number of organic compounds, buttermilk represents a valuable carbon source useful in bioprocessing. Results obtained by Vigliengo and Reinheimer (2009) revealed that whey or buttermilk could be suitable medium ingredients for biomass production of thermophilic lactic acid bacteria or other microbes. Some microalgae obtain energy by using alternatively light or organic substrates, and their cultivation represents a valid strategy for the treatment of effluents (Jain et al., 2022). In this scenario, the red extremophile species belonging to the *Galdieria* genus are among the most suitable microalgae for the valorisation of buttermilk. *Galdieria sulphuraria* is a polyextremophile unicellular microalga able to grow at temperatures up to 56 °C and a pH between 0.5 and 4. Additionally, *G. sulphuraria* presents a versatile metabolism: its ability to grow autotrophically, heterotrophically or mixotrophically has been proven many years ago (Oesterhelt et

al., 1999). The mixotrophic metabolism consists of the combined use of an organic carbon source and light energy for chemoheterotrophic and photoautotrophic growth. In mixotrophy, it was observed that the biomass productivity was two-fold higher than in autotrophy (Curien et al., 2021; Abiusi et al., 2021, 2022a; Canelli et al., 2023), and pigment production was higher than in heterotrophy (Sloth et al., 2006). Mixotrophic growth has been proposed to overcome autotrophy limits for biomass productivity (Tredici, 2010). *G. sulphuraria* can use several carbon sources (up to 50 different) (Gross and Schnarrenberger, 1995), including lactose, showing a broad metabolic versatility (Tischendorf et al., 2007; Zimermann et al., 2020). Mixotrophic metabolism and versatility in organic carbon source assimilation make *G. sulphuraria* very interesting in bioremediation processes. On the other side, heterotrophy is a tool to overcome the costly light-based bioremediation processes using microalgae. The heterotrophic production results in higher biomass concentrations and higher growth rates, and therefore higher substrate removal rates (Barros et al., 2019). However, contamination by bacteria and fungi is a notable issue when the medium contains organic carbon. The cultivation of extremophilic microalgae is a strategy to limit the growth of competitors and predators, such as bacteria, improving the efficacy of microalgae-based treatment (Abiusi et al., 2022b). The main interest in *G. sulphuraria* lies in the production of the high-value blue phycobiliprotein C-phycocyanin (C-PC), allophycocyanin, and chlorophyll (Albertano et al., 2000). Phycocyanins are used as dyes in diagnostic histochemistry, as colourants in the cosmetics and food industry and, as therapeutic agents due to their antioxidant properties in the pharma sector (Pagels et al., 2019). Commercially, C-PC is produced by the cyanobacterium *Spirulina* (*Arthrospira*) *platensis* in phototrophic cultures, with all the problems derived from light efficiency use (Tredici et al. 2010). The cultivation of *G. sulphuraria* in mixotrophy on a large scale could overcome this bottleneck and it could be used as an alternative source of C-PC. Additionally, *G. sulphuraria* could potentially be used as an ingredient for human consumption due to its interesting chemical and nutritional characteristics (Graziani et al., 2013). In the last years, the cultivation of *G. sulphuraria* on several organic wastes from the food industry has been explored. *G. sulphuraria* 074G growth on food waste from restaurants and bakeries was evaluated (Sloth et al., 2017), as well as on granular starch derived from potatoes (Rahman et al., 2020). Zimermann et al. (2020) tested whether the carbon contained in whey permeate was used by *G. sulphuraria* SAG 107.79 for growth. Exploiting the same strain, Russo et al. (2021) assessed another milk by-product, called “scotta”, derived from second cheese whey. Corn stover hydrolysates have been recently supplied to *G. sulphuraria* UTEX 29.19 (Portillo et al., 2022), while digestate and hydrolyzed straw were investigated for the SAG 21.92 strain (Pleissner et al., 2021). With a few exceptions, the growth of *G. sulphuraria* on carbon sources containing disaccharides, such as lactose, had not been deeply explored before. The use of complex

carbohydrates (di-, tri, and polysaccharides) depends on the presence of hydrolytic enzymes. While polysaccharides are generally converted into simpler sugars in the extracellular environment, di- and trisaccharides can only be metabolized in the cytosol, therefore requiring specific carriers (Tischendorf et al., 2007). The present study aimed to assess the possibility of using buttermilk as a carbon source for both mixotrophic and heterotrophic cultivation of *G. sulphuraria* ACUF 064 to obtain high-value biomass and a treated effluent with a lowered organic load. First, the effect of lactose, glucose and galactose on growth and C-PC accumulation was assessed both under mixotrophy and heterotrophy. Then, the dilution ratio of buttermilk in the synthetic medium for biomass production was evaluated in lab-scale batch experiments. Finally, the growth of *G. sulphuraria* ACUF 064 and its phycocyanin content were evaluated both mixotrophically and heterotrophically in a 13 L photobioreactor.

2. Materials and methods

2.1. Microalgal strain, media and cultivation conditions

G. sulphuraria ACUF 064, kindly provided by "Federico II" Naples University, was cultivated photo-autotrophically in 250 mL Erlenmeyer flasks containing 100 mL of modified Allen's medium (Allen and Stanier, 1968). The medium used for flask and reactor cultivation contained the following components (mol L^{-1}): $12.2 \cdot 10^{-3}$ H_3PO_4, $80.0 \cdot 10^{-3}$ $(NH_4)_2SO_4$, $6.5 \cdot 10^{-3}$ $MgSO_4 \cdot 7H_2O$, $4.7 \cdot 10^{-4}$ $CaCl_2 \cdot 2H_2O$, $6.3 \cdot 10^{-4}$ FeNaEDTA, $0.2 \cdot 10^{-3}$ Na2EDTA·2 H_2O, $1.7 \cdot 10^{-3}$ NaCl, $8.1 \cdot 10^{-3}$ KCl, $8.0 \cdot 10^{-4}$ H_3BO_3, $8.1 \cdot 10^{-5}$ $MnCl_2 \cdot 4H_2O$, $8.2 \cdot 10^{-5}$ $ZnCl_2$, $3.2 \cdot 10^{-5}$ $CuSO_4 \cdot 5H_2O$, $1.7 \cdot 10^{-5}$ $Na_2MoO_4 \cdot 2H_2O$ and $1.7 \cdot 10^{-5}$ $CoCl_2 \cdot 6H_2O$. pH was adjusted to 1.6 with 2 M H_2SO_4. Axenic autotrophic stock cultures were incubated in 250 mL flasks containing 100 mL of culture, in an incubator (Multitron II, Infors HT, Switzerland) operated at 37 °C, 2% v/v CO_2, 60% of humidity, 125 rpm, under a photon flux density of 100 μmol m^{-2} s^{-1} and with a photoperiod 16:8 (day: night). These cultures were used for the experiments described below.

2.2. Carbon source flask experiments

G. sulphuraria ACUF 064 cultures, containing either lactose, galactose or glucose (5 g_C L^{-1}), were grown mixotrophically and heterotrophically. In particular, a 10-day pre-adaptation period for each carbon source was conducted to adapt *G. sulphuraria* ACUF 064 from autotrophy to mixotrophy and heterotrophy. Mixotrophic flask experiments were carried out into 250 mL Erlenmeyer flasks containing 150 mL of modified Allen's medium. Cultures were inoculated at a 0.2 OD_{750} with the

pre-acclimated culture and incubated at the same conditions reported above. The same conditions were used for the heterotrophic experiment, but the flasks were wrapped in aluminium foil. For both experiments, OD_{750} was measured in samples taken after 0, 22, 27, 46, 70, 75 and 94 hours from inoculation. Dry weight ($g_x\ L^{-1}$) and cell count (cells mL^{-1}) determinations were performed in samples taken after 0, 22, 70 and 94 hours from inoculation. An aliquot of 15 mL was aseptically taken after 0, 46, 75 and 94 hours after inoculation and centrifuged at 4700 rpm for 10 min. The supernatant fractions were stored at −20 °C and used for total organic carbon (TOC) and total nitrogen (TN) determinations, while the pellet, washed with demineralized water, was cooled to −20 °C, lyophilized and stored. The C-phycocyanin content was measured on a lyophilized pellet obtained from the last sampling time for each flask. The specific growth rate (μ) was obtained after plotting the logarithm of the DW over time and fitting a linear equation to the data points. The slope of the linear function was the specific growth rate. The heterotrophic and mixotrophic biomass yield per carbon consumed ($Y_{x/C}$) was calculated as follows:

$$Y_{x/C} = \frac{C_{x(n+1)} - C_{xn}}{C_n - C_{n+1}} \quad (1)$$

where $C_n - C_{n+1}$ stands for the carbon concentration ($g_C\ L^{-1}$) at times t_n and t_{n+1}.
The biomass yield on nitrogen consumed ($Y_{x/N}$) was calculated as described above, considering the nitrogen concentrations ($g_N\ L^{-1}$). Experiments were performed in duplicate

2.3. Assessment of optimal buttermilk dilution for biomass production

Buttermilk samples, kindly provided by “Caseificio del Cigno SPA” located in Agnadello (CR) - Italy, were pre-treated as follows: frozen buttermilk samples were thawed and centrifuged at 4700 rpm, for 10 min at 7° C. After centrifugation, the liquid phase was grossly separated from the solid upper organic phase (mainly fat) and then immediately used for trials. Three different buttermilk dilutions were obtained by adding 20, 40 and 60% (v/v) of buttermilk into sterilized modified Allen’s medium. The nitrogen and phosphate concentrations in Allen’s medium were adjusted for each dilution based on the nitrogen and phosphate already present in buttermilk. This was done differently for the reactor experiments, where Allen’s medium was simply concentrated to the final working volume of the experiment. In the flask experiments, one culture containing lactose instead of buttermilk was used as a positive control, and one culture with buttermilk at 20% v/v and deprived of nitrogen in the synthetic medium was used to assess the carbon removal with minimal medium requirements. The test was performed in flasks and in mixotrophy for 7 days, in duplicate, to assess

the effect on G. sulphuraria ACUF 064 growth, in terms of biomass concentration (Cx) at the end of the trial, overall productivity (rx) and TOC concentration measured immediately after inoculation and at end of the trial. The overall productivity (rx) was calculated according to equation 2:

$$r_x = \frac{C_{x(n+1)} - C_{xn}}{t_{(n+1)} - t_n} \quad (2)$$

where Cn and C(n+1) are biomass concentration at times tn and tn+1.

The pH of the resulting medium was adjusted to 1.6 – 1.8 with 2 M H2SO4 and the cultivation was done as described in the previous section. A preliminary characterization of centrifuged buttermilk was carried out and is reported in Table 1S in the supplementary section. It is important to notice the wide variability detected of total carbon concentration, between 3 and 5 gC L-1.

2.4. Photobioreactor setup and operation

Experiments in mixo- and heterotrophic conditions were conducted in batch mode in a 13 L stirred tank bioreactor (NW200, Infors HT, Switzerland), controlled through Labfors 4 benchtop (Infors HT, Switzerland), for 8 days. A picture of the photobioreactor setup is reported in Figure 1. The bioreactor presents a cylindrical shape, with an inner diameter of 200 mm and a maximum height of 445 mm. The reactor was used at a working volume of 8 L. During mixotrophy, half of the lateral surface of the reactor was illuminated using a vertical light panel (ReaLight-24, Ontwikkelwekplaats WUR, NL) placed 8 cm far from the reactor. Incident light intensity on the reactor surface was calibrated by measuring 24 points equally distributed on the inner surface of the empty reactor with a light meter (LI-250A, LI-COR, USA). Light intensity was provided in a continuous mode starting from 100 to 200 $\mu mol_{ph}\ m^{-2}\ s^{-1}$ and was adjusted according to the biomass growth by keeping a constant specific light supply rate (q_{ph}) between 5.8 and 1.8 $\mu mol_{ph}\ g_x^{-1}\ s^{-1}$, as referenced in previous studies by Abiusi et al. (2021). During heterotrophic cultivation, the reactor was kept in the darkness.

The reactor was equipped with a dissolved oxygen (DO) sensor (InPro 6800 Series, Mettler Toledo, USA) and a pH probe (EasyFerm Bio HB K8 325, Hamilton, USA). The DO probe was calibrated at 0% and 100% DO. Zero-point oxygen calibration was performed by immersing the probe into a 15 mL tube containing 2-5 mg of $Na_2S_2O_4$ dissolved in deionised water. The 100% saturation point was performed leaving overnight the probe inside the reactor under maximal aeration (1 L min^{-1}). The pH probe was calibrated by using the two standard buffer solutions at pH 2 and pH 4 (VWR Chemicals, USA). The pH was continuously measured and controlled at 1.6 by automatic base addition (2 M NaOH) with a cascade loop. The temperature of the reactor, monitored constantly with a probe

inserted in the culture medium, was kept at 37 °C by the heat exchange between the surface of the culture vessel and a water jacket. The temperature of the water jacket was regulated by an external water bath. To prevent evaporation, the reactor was equipped with a condenser (4 °C). Stirring was controlled in a cascade loop where agitation changed from 100 to 250 rpm to keep a DO of 20%. Air enriched with 2% v/v carbon dioxide, sterilized by 0.2 μm pore size filter (Whatman® PolyVENT, PTFE filters,) was provided at a flow rate of 0.5 - 1 L min^{-1} (according to minimum DO of 20%) using mass flow controllers (Smart TMF 5850S, Brooks Instruments, USA). Both mixotrophic and heterotrophic experiments were performed as follows: once the empty reactor vessel was autoclaved at 121°C for 15 min, it was aseptically filled with medium filtered through a 0.22 μm pore size filter (Sartobran® Capsule 0.2μm, Sartorius, USA). Consequentially, the DO sensor was inserted in the reactor vessel and left overnight for 100% DO calibration. After calibration, buttermilk was added to reach a concentration of 2.0 g_C L^{-1}, corresponding to about 40 % of the total volume, and immediately inoculated with a fresh culture of *G. sulphuraria* ACUF 064 pre-adapted on lactose (mixotrophically or heterotrophically, according to the experimental set) to OD_{750} of 0.3. Daily sampling was done at the same time, except for the exponential phase, where multiple samplings were performed. Concurrently, 15 mL samples were treated and stored for TOC, TN and C-PC content determinations, as previously described. During the cultivation, the DO was monitored and its increase over the setpoint was used as a reference that substrate was depleted. At this point, the experiment was diluted and a new repetition was started. The heterotrophic and mixotrophic biomass yield per carbon unit $Y_{x/C}$ and per nitrogen unit $Y_{x/N}$ consumption, the specific growth rate during exponential growth (μ) and overall productivity (r_x) were calculated as reported above.

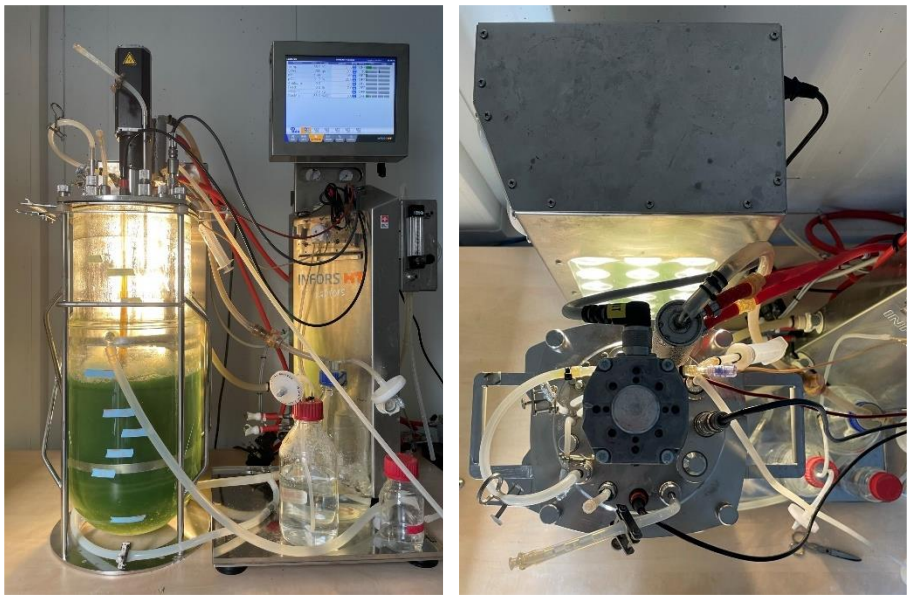


Figure 2. Image of the13 L photobioreactor operated during mixotrophic cultivation. Side view (sx) and top view (dx)

2.5. Offline analysis

Cell concentrations were determined by using a Coulter Multisizer III (Beckman Coulter Inc., USA) with a 50 µm aperture tube. Samples set at an OD_{750} of 0.3-0.8 were 100 times diluted in ISOTON II diluent and the number of cells was analysed in 1 mL in the range between 2 and 10 µm, corresponding to the range for *G. sulphuraria* cells. Dry weight concentration (DW) was calculated by measuring the weight difference between pre-weighted empty filters and filters containing biomass. Shortly, an aliquot of the culture (2−5 mL) was diluted into 25 mL of deionised water and filtered over a pre-weighted Whatman GF/F glass microfiber filter (diameter of 55 mm, pore size of 0.7 µm). Pre-weighted filters and filters with biomass were washed with deionised water (25 mL) and dried at 105 °C overnight, cooled down in a desiccator with silica for at least 2 h, and finally weighed on a scale (Cubis MCE225S-2S00-I, Sartorius Lab Instruments, Germany). DW measurements were performed in duplicate. The TOC and TN content in the supernatant was measured by using a TOC-L analyzer (Shimadzu, Japan). The supernatant was diluted in demineralized water to reach a carbon content of 100-1000 ppm and a nitrogen content of 10-100 ppm. The optical density was measured with a spectrophotometer (DR6000, Hach-Lange, USA) at 620 and 750 nm. The samples were diluted with modified Allen's medium until an OD_{750} of 0.2-0.8. The average absorption cross-section (a_x, $m^2\ g_x^{-1}$) in the PAR region (400–700 nm) of the spectrum was determined as described by de Mooij et al. 2015. Briefly, the absorbance was measured with a UV-VIS/double beam spectrophotometer

(Shimadzu, Japan) equipped with an integrating sphere (ISR-2600) and using cuvettes with an optical path of 2 mm. The absorbance from 740 to 750 nm was subtracted from the whole spectrum, and the average absorbance was normalised to the DW concentration of the sample. To monitor any possible contamination during cultivation, observations were done with a light microscope and captures were taken. In detail, 10 µL of undiluted culture samples taken before, during and after the reactor run were placed on a glass microscope slide and used for observation in a light microscope (DM 2500, Leica Microsystems, Germany) equipped with a camera (DFC450, Leica Microsystems, Germany) for image capturing, at 40X and 100X magnitude lens.

2.6. Phycocyanin extraction and quantification

Phycocyanin from *G. sulphuraria* ACUF 064 was quantitatively extracted by bead beating (Precellys 24, Bertin Technologies, France) 10 mg of lyophilised biomass as described by Abiusi et al. (2022a). The C-phycocyanin (C-PC) was calculated by measuring the absorbance at 620 nm and 652 nm of the supernatant and converting it into concentration using the Kursar and Alberte equation (Kursar & Alberte, 1983). The concentration of C-PC was then normalised to the DW of *G. sulphuraria*.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's HSD post hoc test for means separation were performed using the STATISTICA ETL software (version 10, StatSoft. inc., USA). The significance level was set at $p \leq 0.01$.

3. Results

3.1 Carbon sources flask experiments

Prior to the experiments with buttermilk, we evaluated the growth of *G. sulphuraria* ACUF 064 individually in glucose, galactose and lactose. The initial TOC and TN concentrations were estimated to be sufficient (5 $g_C\ L^{-1}$ and 2 $g_N\ L^{-1}$) to collect enough data during the exponential phase. In Figure 2, dry weight (DW) and TOC concentration at 0, 2, 3 and 4 days after inoculation in mixotrophy (panel A) and heterotrophy (panel B) are shown. In Figure 1S (supplementary section), TN concentration in mixotrophy (panel A) and heterotrophy (panel B) is reported. *G. sulphuraria* ACUF 064 registered the highest values of biomass dry weight in mixotrophy, being 8.4 $g_x\ L^{-1}$ on galactose and 8.0 $g_x\ L^{-1}$ on glucose. Growth on lactose 4 days after inoculation was lower leading to a biomass

dry weight concentration of 4.3 $g_x\,L^{-1}$ in mixotrophy. In heterotrophy, values of DW were 6.2 and 5.6 $g_x\,L^{-1}$ for glucose and galactose, respectively, being slightly lower than in mixotrophy. For lactose, the DW obtained was the lowest (3.6 $g_x\,L^{-1}$). Besides the DW, the specific growth rate (μ), the cell concentration, the biomass yield on nitrogen and carbon and the C-PC content were also monitored and the results are reported in Table 1. In mixotrophy, the μ estimated from DW measurements did not differ significantly between all the different substrates. From the cell counts, the highest concentration was observed on glucose ($3.03\cdot10^8$ cells·mL^{-1}), compared to galactose ($2.51\cdot10^8$ cells·mL^{-1}) and lactose ($2.21\cdot10^8$ cells·mL^{-1}). In heterotrophy, the highest μ were observed with glucose (1.06 d^{-1}) and galactose (1.02 d^{-1}), while the growth rate for lactose was significantly lower than in all the different conditions tested. Also in heterotrophy, the highest cell concentration was observed for the cultures grown on glucose ($1.97\cdot10^8$ cells·mL^{-1}) and in decreasing order, on galactose ($1.87\cdot10^8$ cells·mL^{-1}) and then on lactose ($1.43\cdot10^8$ cells·mL^{-1}). The cultures grown in heterotrophy showed a lower cell concentration than the cultures grown in mixotrophic conditions, most likely due to the additional growth from CO_2 assimilation. The measured carbon and nitrogen contents in the media at the beginning and the end of the experiment were used to estimate the yields of biomass. Considering that no carbon limitation occurred in any sample (as shown in Figure 2), the yields of biomass on carbon ($Y_{x/C}$) consumed were higher in mixotrophic conditions compared to heterotrophic conditions for each substrate. Under mixotrophy, the highest $Y_{x/C}$ were observed in lactose (1.9 $g_x\ g_C^{-1}$) and galactose (1.9 $g_x\ g_C^{-1}$), compared to glucose (1.6 $g_x\ g_C^{-1}$). An opposite trend was observed in heterotrophy, where the highest $Y_{x/C}$ observed for glucose was decreased by 13%, while the $Y_{x/C}$ for galactose and lactose were also reduced by 33% and 47%, respectively. Concerning the yield of biomass per nitrogen ($Y_{x/N}$), the highest yield under mixotrophy was observed for galactose (9.9 $g_x\ g_N^{-1}$), followed by glucose (8.2 $g_x\ g_N^{-1}$) and then lactose (7.6 $g_x\ g_N^{-1}$). The $Y_{x/N}$ in heterotrophy were generally higher (46% and 5% higher for glucose and lactose), except for galactose, where the yield decreased by 12%. Overall, the nitrogen used in cultures grown with glucose was more efficiently used to generate new biomass than in cultures grown with lactose. No nitrogen limitation occurred (Fig. 1S). The accumulation of C-PC differs depending on the substrate used. The C-PC content was highest when *G. sulphuraria* ACUF 064 was grown on lactose and under mixotrophic conditions, reaching a value of 5.9% $w_{C\text{-}PC}\ w_x^{-1}$. Instead, the C-PC content when *G. sulphuraria* ACUF 064 was grown on glucose (4.3 % $w_{C\text{-}PC}\ w_x^{-1}$) and galactose (3.7% $w_{C\text{-}PC}\ w_x^{-1}$) was 27% and 37% lower, respectively. The C-PC content was more than 2-fold reduced in heterotrophy compared to mixotrophy for all the different tested substrates. Still, the highest content in C-PC occurred after using lactose (2.3% $w_{C\text{-}PC}\ w_x^{-1}$), while equally decreased by 36% for both glucose and galactose. The results emphasise that *G. sulphuraria* ACUF 064, when cultivated on lactose, showed a significantly

higher C-PC content in mixotrophy than in heterotrophy if compared to cultivation on glucose or galactose.

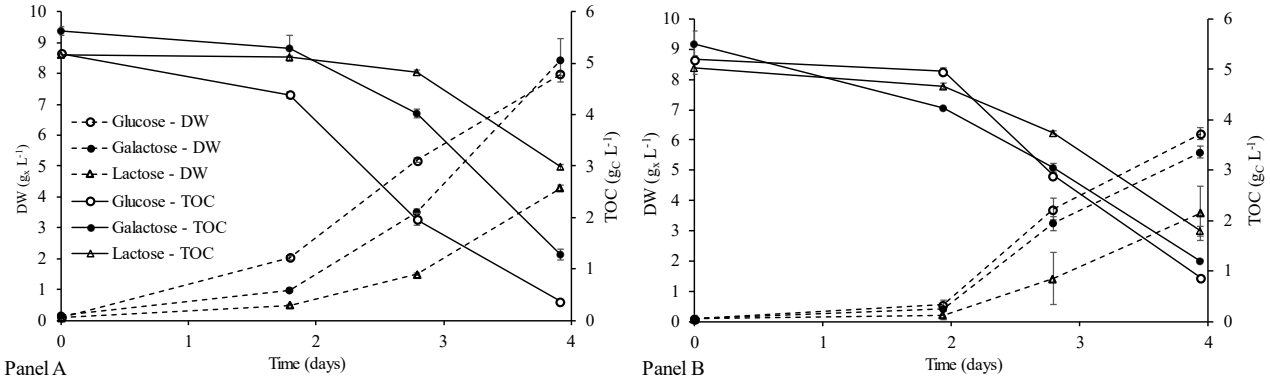


Figure 2. DW measurements (expressed as g_x L^{-1}) and TOC concentrations (expressed as g_C L^{-1}) of *G. sulphuraria* ACUF 064 cultivated in flask under mixotrophy (panel A) and heterotrophy (panel B) using glucose, galactose and lactose at a concentration of 5 g_C L^{-1}. Error bars indicate the standard deviation of replicates (n=2).

Table 1. Overview of the offline measurements after 4 days from inoculation (C_x, C-PC content and cell counts), process parameters (μ) and stoichiometric yields ($Y_{x/C}$, $Y_{x/N}$) of *G. sulphuraria* ACUF 064 grown in heterotrophic and mixotrophic conditions in the flask. Values are expressed as average ± standard deviation of replicates (n=2)

	Glucose		Galactose		Lactose	
	Heterotrophy	Mixotrophy	Heterotrophy	Mixotrophy	Heterotrophy	Mixotrophy
μ (d^{-1})	1.06 ± 0.01^{a}	1.12 ± 0.03^{a}	1.02 ± 0.01^{a}	1.03 ± 0.16^{a}	0.94 ± 0.02^{b}	1.02 ± 0.01^{a}
$Y_{x/C}$ (g_x g_C^{-1})	1.4 ± 0.2^{a}	1.6 ± 0.1^{a}	1.3 ± 0.1^{a}	1.9 ± 0.2^{a}	1.1 ± 0.3^{a}	1.9 ± 0.0^{a}
$Y_{x/N}$ (g_x g_N^{-1})	12.8 ± 0.2^{a}	8.2 ± 0.0^{d}	9.2 ± 0.5^{c}	9.9 ± 0.3^{b}	7.9 ± 0.6^{e}	7.6 ± 0.0^{f}
C-PC (% $w_{C\text{-}PC}$ w_x^{-1})	1.5 ± 0.2^{d}	4.3 ± 0.3^{b}	1.5 ± 0.1^{d}	3.7 ± 0.2^{b}	2.3 ± 0.1^{c}	5.9 ± 0.2^{a}
C_x (g_x L^{-1})	6.2 ± 0.2^{b}	8.0 ± 0.1^{a}	5.6 ± 0.2^{c}	8.4 ± 0.7^{a}	3.6 ± 0.9^{d}	4.3 ± 0.1^{d}
Cells count ($\cdot 10^8$ cells mL^{-1})	1.96 ± 0.07^{d}	3.03 ± 0.12^{a}	1.87 ± 0.15^{d}	2.51 ± 0.02^{b}	1.43 ± 0.12^{e}	2.21 ± 0.01^{c}

Among the rows, the same letter indicates no significant differences ($p > 0.01$).

3.2 Growth of *G. sulphuraria* on different buttermilk dilution ratios

Three different medium dilutions were obtained by adding buttermilk into modified Allen's medium at 20, 40 and 60% v/v in flasks, while one flask containing lactose was used as a positive control. Also, one flask with buttermilk at 20% v/v and deprived of nitrogen in the synthetic medium was used to assess the carbon removal with minimal medium requirements. The different dilutions were tested in mixotrophy to define a buttermilk dilution that promotes the highest biomass productivity for *G. sulphuraria*. The biomass concentration (C_x) and productivity (r_x) after 7 days of cultivation, the TOC concentration measured at the beginning and end of the experiment and the relative carbon removal are reported in Table 2. The TN concentration measured at the beginning and end of the experiment is reported in Figure 2S in the supplementary section. The TOC concentration in the supernatant, measured immediately after inoculation, reveals values of 1.7, 2.0 and 5.2 $g_C\ L^{-1}$ in cultures diluted with 20%, 40% and 60% v/v of buttermilk, respectively. The C_x measured 7 days after inoculation was 3.9 and 4.3 $g_x\ L^{-1}$ at 20% and 40% v/v dilution, while C_x was 1.6 $g_x\ L^{-1}$ at 60% v/v. The carbon concentration at 60% v/v was unexpectedly higher than the other dilutions, which might also explain the lack of growth. The highest biomass concentration was obtained with the control experiment with lactose (5.3 $g_x\ L^{-1}$) as well as the highest productivity (0.75 $g_x\ L^{-1}\ d^{-1}$), while the highest productivity in buttermilk was observed with a dilution of 20% (0.54 $g_x\ L^{-1}\ d^{-1}$). The biomass productivity decreased with an increasing ratio of buttermilk down to 0.17 $g_x\ L^{-1}\ d^{-1}$ at 60% v/v. When nitrogen was limited, the productivity decreased by 30% compared to the replete nitrogen experiment. Nevertheless, the productivity observed for 20% v/v buttermilk and with limited nitrogen was higher than the culture at 60% v/v. Similar to the productivity, the highest carbon removal observed was 69% and it was observed at 20% v/v, while it decreased down to 7% at a buttermilk dilution of 60% v/v. When nitrogen was limited, the carbon removal achieved in buttermilk was 40%. Despite the best result of carbon removal was observed at 20% v/v of buttermilk, a similar biomass productivity was obtained at 40% v/v. The use of this dilution ratio would be preferred since a higher amount of effluent would be treated.

Table 2. Summary of growth data of G. sulphuraria ACUF 064 in flasks at different buttermilk dilution ratios in mixotrophy. The TOC concentration at the inoculation time (Start) and after 7 days (End) and the carbon removal (%) are shown. The biomass concentration (C_x) and the productivity (r_x) are shown after 7 days. Values are expressed as average ± standard deviation (n=2).

	Buttermilk dilution ratio (% v/v)	TOC (g_C L^{-1}) Start	TOC (g_C L^{-1}) End	C removal (%)	C_x (g_x L^{-1})	r_x (g_x L^{-1} d^{-1})
Lactose	20	1.7 ± 0.1	1.0 ± 0.2	40 ± 10	5.3 ± 0.4	0.75 ± 0.00
Buttermilk	20	1.7 ± 0.0	0.5 ± 0.0	69 ± 1	3.9 ± 0.1	0.54 ± 0.01
	40	2.0 ± 0.0	1.5 ± 0.0	27 ± 2	4.3 ± 0.2	0.50 ± 0.02
	60	5.2 ± 0.1	5.1 ± 0.0	2 ± 1	1.6 ± 0.3	0.17 ± 0.04
	20 (N-)	1.5± 0.1	0.9 ± 0.2	40 ± 16	2.0 ± 0.1	0.39 ± 0.00

3.3 Growth of *G. sulphuraria* on buttermilk in stirred tank photobioreactor.

The mixotrophic and heterotrophic metabolism of *G. sulphuraria*, with buttermilk fed as a carbon source, was more extensively evaluated in a 13 L stirred tank reactor. In this system, cultivation was monitored and maintained at the ideal temperature, pH and light supply rate (in mixotrophy) for *G. sulphuraria* growth. Additionally, a buttermilk dilution ratio of 40% v/v was used, given the high biomass productivities observed at this concentration in the previous experiment. The biomass dry weight concentration (g_x L^{-1}) and TOC concentration (g_C L^{-1}) were measured daily and reported in Figure 3. After 6 days, the biomass dry weight concentration was highest in mixotrophy, reaching a final value of 1.22 g_x L^{-1}, compared to 0.78 g_x L^{-1} found in heterotrophy. After dilution and repetition of the cultivation, the highest concentrations for each cultivation type were achieved in lesser time (2 days). This is explained by a higher specific growth rate observed in the second cultivation repetition, which was 28% higher in mixotrophy compared to heterotrophy (Table 3). On the contrary, the specific growth rate was 20% lower in mixotrophy than heterotrophy in the first batch repetition. In the first batch repetition, the productivity was lower due to the adaptation of the inoculum to the new conditions as well as the low specific growth rates observed. In the second batch repetition, productivities were more than 2-fold and 4-fold higher in mixotrophy and heterotrophy, respectively, being the highest productivity observed in mixotrophy (0.55 g_x L^{-1} d^{-1}). Regarding carbon consumption, the highest removal rate was observed in the first batch repetition in mixotrophy (68%), achieving a similar result in the second. In heterotrophy, the carbon removal was 38% in the first batch repetition and it was improved in the second batch repetition (55%). The $Y_{x/C}$ remained constant in each repetition, being 45% higher in mixotrophy compared to heterotrophy (Table 3). The absorption spectrum was also monitored over the culture to gain information on the pigmentation changes. At the end of the first repetition, the a_x was higher in mixotrophy, being 76.6 m^2 kg^{-1} in the first repetition and 96.5 m^2 kg^{-1} in the second repetition (Table 3). The a_x in heterotrophy was lower (58.7 and 41.8 m^2 kg^{-1} in the first and second repetition, respectively) due to the dispensable use of photosynthetic activity and consequently, the size reduction of photosynthetic membranes. The a_x was relatively constant during the second repetition in heterotrophy, while it seemed to increase in mixotrophy probably due to an increased need for photosynthetic activity.The C-PC content, as expected, was higher in mixotrophy than in heterotrophy, reaching 2.18 and 1.19 % $w_{C\text{-}PC}$ w_x^{-1}, at the end of the first and second repetitions. In heterotrophy, the C-PC content was lower (0.58 and 0.52 % $w_{C\text{-}PC}$ w_x^{-1} in the first and second repetition, respectively), in line with the changes in a_x. The mismatch between an a_x and C-PC content in the second repetition of the mixotrophic experiments is explained by an increased ratio of carotenoids over chlorophyll and phycocyanin (Fig. 3S)

During the cultivation, light microscopy observations were done to evaluate the presence of contaminants. After the end of the second repetition, an outbreak of fungal populations became visible in the culture (Fig. 4). The fungal contamination was observed independently of the trophic strategy, coincidentally 8 days after the start of the cultivation. The observation of hyphae at the end of the second repetition is likely derived from the growth of spores that were originally present in buttermilk. In a different heterotrophic batch experiment, the presence of fungal contamination was detected after 8 days, in particular after the start of a new batch repetition. In this case, the presence of filamentous fungi led to an overestimation of the DW. (Fig. 4S)

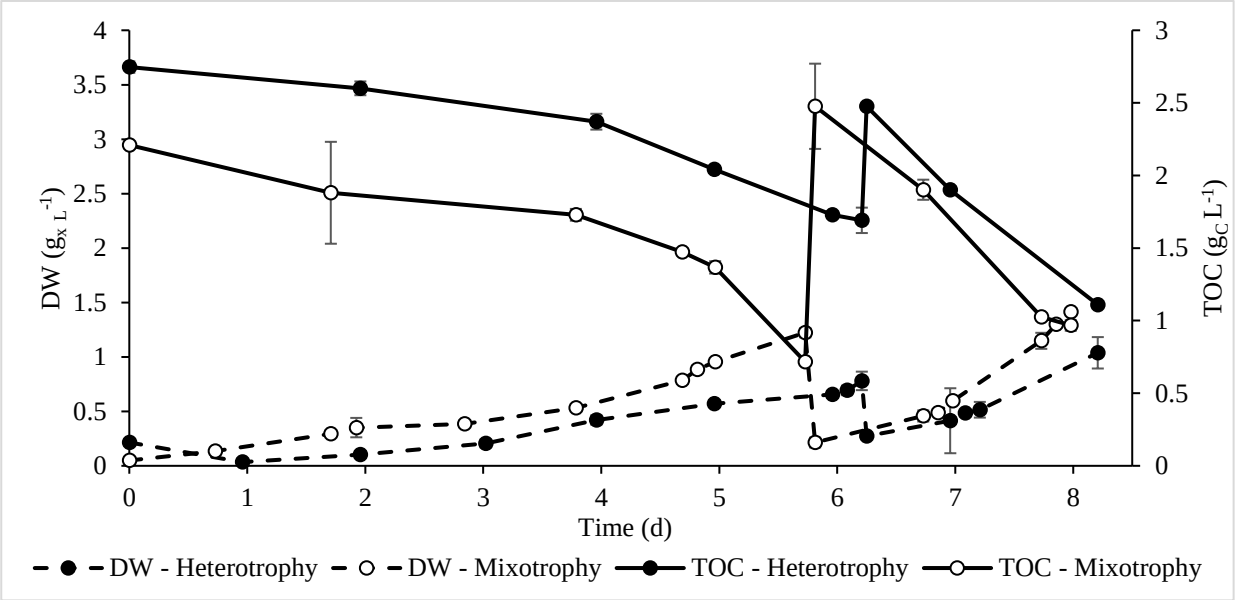


Figure 3. Heterotrophic and mixotrophic *G. sulphuraria* ACUF 064 growth expressed as DW (g_x L^{-1}) and TOC concentration (g_C L^{-1}) in a 13 L stirred tank reactor, using 40% v/v buttermilk as carbon source. Error bars indicate the standard deviation of technical replicates.

Table 3. Summary of the offline measurements (C_x, C-PC and a_x), process parameters (r_x and μ) and stoichiometric yield ($Y_{x/C}$ and C removal) of *G. sulphuraria* ACUF 064 growth in heterotrophic and mixotrophic conditions in a stirred tank reactor containing 40% v/v of buttermilk as carbon source.

	Trial	C_x	μ	r_x	$Y_{x/C}$	C removal	C-PC	a_x
		$g_x\ L^{-1}$	d^{-1}	$g_x\ L^{-1}\ d^{-1}$	$g_x\ g_C^{-1}$	%	% $w_{C\text{-}PC}\ w_x^{-1}$	$m^2\ kg^{-1}$
Mixotrophy	1	1.22 ± 0.04	0.35	0.20 ± 0.03	0.79 ± 0.04	68 ± 0	2.18 ± 0.31	76.6
	2	1.42 ± 0.02	0.89	0.55 ± 0.10	0.80 ± 0.15	61 ± 14	1.19 ± 0.00	96.5
Heterotrophy	1	0.78 ± 0.08	0.44	0.09 ± 0.00	0.54 ± 0.10	38 ± 4	0.58 ± 0.11	58.7
	2	1.04 ± 0.14	0.69	0.39 ± 0.02	0.56 ± 0.11	55 ± 0	0.57 ± 0.17	41.8

Values are expressed as averages ± standard deviation of replicates. (n=2).

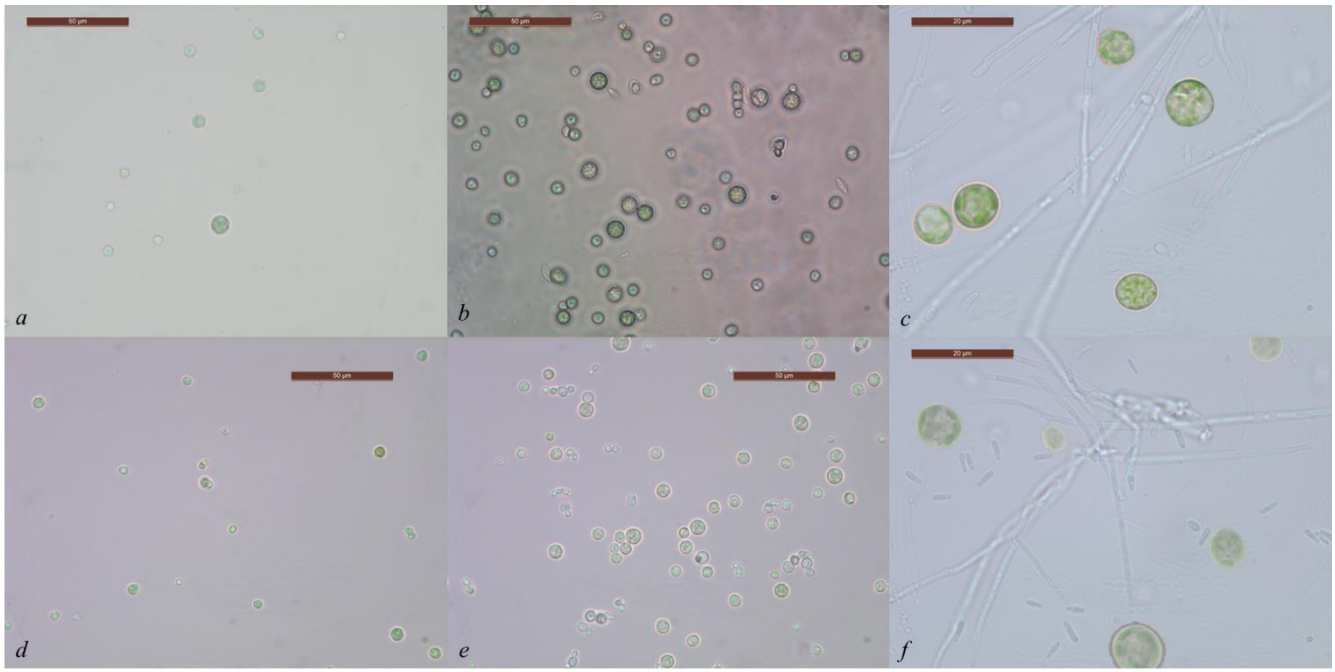


Figure 4. Microscope images of *G. sulphuraria* ACUF 064 during cultivation in the photobioreactor in mixotrophy or heterotrophy with buttermilk. Pictures at 40X magnitude of mixotrophic cultivation: at day 0 (panel *a*) and day 8th (panel *b*); in *panel c,* Image with fungal contamination was observed after the second batch repetition in mixotrophy (100X magnitude). Pictures at 40X magnitude of heterotrophic cultivation: at day 0 (panel *d*) and day 8th (panel *e*); in *panel f*, Image with fungal contamination observed after the second batch repetition in heterotrophy (100X magnitude).

4. Discussion

4.1. Carbon sources flask experiment

The carbon source flask experiment carried out in this study shows that the specific growth rate (μ) of *G. sulphuraria* ACUF 064 on lactose resembled the one observed on glucose or galactose both under mixotrophy and heterotrophy. Despite the known variability among species from the same genus, and even within strains, *G. sulphuraria* ACUF 064 showed a similar specific growth rate on glucose and galactose, as reported in previous literature for other strains. Overall, the heterotrophic μ registered in this study was similar to that observed for *G. sulphuraria* 74G when sucrose (disaccharide), glucose and galactose were the carbon sources used (Gross & Schnarrenberger, 1995). The μ observed during the heterotrophic experiment for *G. sulphuraria* ACUF 064 were similar to those reported by Schmidt et al. (2005) for *G. sulphuraria* 74G. In their study, μ values of 1.10 d^{-1}, 1.08 d^{-1}, and 1.01 d^{-1} for glucose, fructose and sucrose, respectively, were obtained. Sloth et al. (2006) reported for *G. sulphuraria* 74G on glucose, fructose or glycerol similar growth rates. The μ was 1.2 d^{-1} in all three cases, independently of the light intensity tested (0 and 117 μmol photons m^{-2} s^{-1}). Also, no significant differences in μ were reported when comparing heterotrophic and mixotrophic growth with xylose as a carbon source (0.97 d^{-1} and 1.10 d^{-1}, respectively) for *G. sulphuraria* UTEX2919 (Portillo et al. 2022). When glucose was used, they registered a μ of 1.46 d^{-1} in heterotrophy and 1.49 d^{-1} in mixotrophy, a growth rate significantly higher than the ones obtained in this study. The C-PC content measured in mixotrophy for each carbon source was far from the 10% $w_{C\text{-}PC}$ w_x^{-1} obtained in a previous study with the same strain (Abiusi et al., 2022a). This divergence is explained by differences in the cultivation conditions. Experiments by Abiusi et al. (2022a) were performed in chemostat in a stirred reactor and at a low growth rate. Interestingly, the C-PC content we observed both in heterotrophic conditions on glucose and galactose were similar to those registered by Graverholt and Eriksen (2007) with *G. sulphuraria* 74G in batch with sufficient glucose (1.6 % $w_{C\text{-}PC}$ w_x^{-1}), while the content was higher (2.7% $w_{C\text{-}PC}$ w_x^{-1}) in fed-batch cultures where glucose supply was limited. The C-PC content of *G. sulphuraria* ACUF 064 grown with lactose (2.34% $w_{C\text{-}PC}$ w_x^{-1}) was comparable to the results registered when glucose was depleted or limited. As explained in previous studies, phycocyanin accumulation can be triggered under glucose limitation (Graverholt and Eriksen, 2007; Portillo et al., 2022; Sloth et al., 2006; Sørensen et al., 2013). Furthermore, Sloth et al., (2006) reported that the substrate structure could also affect the biomass yield and the C-PC content. *G. sulphuraria* 74G showed a higher biomass yield on substrate, as well as a higher specific C-PC content in mixotrophy with glycerol (triol with three carbon atoms) than with glucose (sugar

with six carbon atoms). Hence, in line with these observations, and due to the structural complexity of lactose (sugar with twelve carbon atoms), the specific carbon uptake of *G. sulphuraria* ACUF 064 was lower for lactose, resulting in a lower growth rate and a higher C-PC content. These observations are supported by the biomass yield on carbon or nitrogen unit. The low $Y_{x/C}$ and the high TOC concentration at the end of the experiment, indicate a low efficiency of *G. sulphuraria* ACUF 064 in converting lactose into biomass, compared to simpler sugars such as glucose and galactose. The high $Y_{x/C}$ observed in mixotrophy compared to heterotrophy is explained by the additional use of light and carbon assimilation for biomass synthesis. At the same time, the low $Y_{x/N}$ values coupled with high $Y_{x/C}$ values indicate that more nitrogen was consumed per gram of carbon consumed. This implies that more nitrogen is accumulated either in the form of chlorophylls, phycobiliproteins or proteins. The more than two-fold increase of C-PC content in mixotrophy in comparison to heterotrophy might indicate that nitrogen is channelled in the form of C-PC. The use of certain carbon sources in heterotrophy was shown to exert a downregulation of the genes involved in photosynthesis and pigment synthesis. As a matter of fact, a different response in the transcription of genes involved in pigment synthesis was observed when glycerol or glucose was used (Perez Saura et al., 2022). In other studies, it was hypothesised that glucose and fructose repress C-PC synthesis in more extent than glycerol (Sloth et al. 2006). A similar conclusion was reached when xylose was used as a carbon source instead of glucose since a higher C-PC content was reached. The high C-PC content observed with lactose could suggest that lactose induces a similar effect such as glycerol or xylose. The phycocyanin content in lactose-fed cultures in heterotrophy disagrees with the results of previous studies by Tischendorf et al. (2007) and Zimermann et al. (2020), who observed an almost complete loss of pigmentation when the strain was grown in the same conditions. Finally, a direct comparison was made with data from Zimermann et al. (2020) between two strains in heterotrophic conditions fed on lactose. In particular, in our heterotrophic flask experiment, the specific growth rate of *G. sulphuraria* ACUF 064 was lower on lactose than on glucose but the pigmentation was greater, while in the case of *G. sulphuraria* SAG 107.79 the opposite results were obtained.

4.2. Growth of *G. sulphuraria* on different buttermilk dilution ratios

G. sulphuraria was previously grown on a lactose-rich medium such as whey permeate (Zimmerman et al., 2020), but it is the first time that specifically buttermilk was used as a carbon source for growth. In this study, it was shown that a high concentration of effluent could be toxic for the growth of *G. sulphuraria* (Zimmerman et al., 2020). Therefore, the effect of different buttermilk dilution ratios on *G. sulphuraria* ACUF 064 growth was pre-tested in flasks, showing also a decrease in productivity

with an increasing ratio of buttermilk on synthetic medium. The highest productivity was achieved in the positive control, with a medium containing only lactose and free of organic molecules. The lowest productivity achieved at 60% v/v might infer an inhibitory effect by specific compounds contained in buttermilk. A similar result was obtained in growth experiments of *G. sulphuraria* on whey permeate (Zimmerman et al., 2020), where dilutions higher than 20% v/v resulted in a longer adaptation phase. Growth was inexistent at dilutions ratios higher than 30% v/v. The removal of nitrogen from synthetic medium limited the growth of *G. sulphuraria*, but not the carbon removal. Overall, the low biomass productivity and carbon removal observed at 60% v/v dilution might indicate the presence of inhibitory compounds for *G. sulphuraria* in buttermilk. Hancock et al. (2002) reported that the antibacterial activity exhibited by buttermilk proteins such as whey proteins and caseins could affect negatively the microalgal growth or affect positively by limiting bacterial contamination. Further investigation would be necessary to confirm whether there are toxic molecules that hamper the growth of *G. sulphuraria*.

4.3. Growth of *G. sulphuraria* on buttermilk in stirred tank photobioreactor.

Mixotrophic and heterotrophic experiments were performed on a lab-scale photobioreactor (13L photobioreactor) with the aim to assess the growth performance of *G. sulphuraria* and carbon removal in buttermilk. In contrast to flask experiments, parameters such as light supply rate during mixotrophy or the oxygen concentration during heterotrophy were monitored and controlled to an optimal setpoint. In that way, the carbon contained in buttermilk was expected to be the only limiting component for growth. A dilution ratio of 40% v/v was used since it was shown a high biomass productivity in flasks experiments, as described above. Compared to autotrophy, heterotrophy is known to lead to high biomass production (Barros et al., 2019). Mixotrophy involves the simultaneous assimilation of sugar and the fixation of carbon for growth. The maximal carbon removal obtained in the photobioreactor was 68% in mixotrophy. Similar results were obtained in the flasks at a dilution of 20% v/v. Overall, these results indicate that about 30% of the carbon present in buttermilk cannot be easily assimilated by *G. sulphuraria* and needs to be removed by different means. Mixotrophy was the best strategy for buttermilk since it led to a higher specific growth rate, biomass productivity and biomass yield on carbon. The higher yields observed in mixotrophy compared to heterotrophy are influenced by the photoautotrophic growth and their extent. The yields of biomass on carbon found for buttermilk were still lower compared to the yields found for lactose. This might be explained by an increased occurrence of futile cycles or overflow metabolism when buttermilk is used where energy is spilt in other metabolic processes than growth. The growth rates found in the photobioreactor in the second repetition were similar to those measured for *G. sulphuraria* 74G, in

heterotrophy, fed on food and bakery waste (Sloth et al., 2017). The authors found μ values of 0.69 and 0.60 d^{-1} in restaurant and bakery waste, respectively. In that case, both substrates had been subjected to a hydrolyzation pre-treatment to convert complex sugars into monomers, predominantly glucose. In a recent study, Pleissner et al., (2021), using *G. sulphuraria* SAG21.92, found a μ of 1.0 d^{-1} in heterotrophy in the presence of 100% (v/v) of hydrolysed digestate supplemented with glucose in excess (50 g L^{-1}). In experiments carried out by Rahman et al., (2020), instead, *G. sulphuraria* 74G was grown in the presence of corn and potato starch where lower growth rates were found compared to our study (0.3 – 0.4 d^{-1}). The lower growth rate observed with buttermilk in the first repetition reinforces the fact that cultures needed a long adaptation phase to hydrolyse the different carbon molecules in buttermilk at the expense of an additional metabolic effort. The use of acidic and high-temperature conditions is usually regarded as an advantage over microbial competitors such as bacteria. Nevertheless, fungal optimal growth temperatures are found above 30 °C and optimal pH conditions range between 3 and 8 (Laezza et al., 2022). In our trials with the photobioreactor, fungal contamination became visible at the end of the second repetition, while no evidence was observed during the first repetition. Abiotic factors such as mechanical stirring or aeration could have influenced the growth of fungi since they were not previously observed in our flask tests. However, the time of cultivation was lower than in the photobioreactor, which might explain why fungi were not observed. The co-cultivation of microalgae and fungi is a relatively new concept which has been poorly researched. It is still not clear whether the interaction between them could imply an improvement in the growth of microalgae. A recent study reported a mutual growth benefit between *G. sulphuraria* and the fungus *Penicillium citrinum* at conditions optimal for the growth of *G. sulphuraria* (Salvatore et al., 2023). Therefore, further studies on the symbiotic relationship between microalgae and fungi are needed for the remediation and valorisation of industrial effluents.

5. Conclusion

The results of the present study revealed that buttermilk, when adequately diluted, can be used as a substrate for biomass production. This is beneficial in environmental terms, providing a solution for treatment and avoiding disposal, allowing for the recovery of material. *G. sulphuraria* ACUF 064 proved to be able to use lactose as a carbon source, despite its complexity compared to simpler sugars such as glucose and galactose. Even though the growth of *G. sulphuraria* on buttermilk was slower than on optimal substrates, the obtained biomass still accumulated C-PC. This confirms the potential of microalgal cultivation to recover buttermilk or lactose-enriched effluents to support the *G. sulphuraria* ACUF 064 growth while obtaining a valuable product. For such purposes, mixotrophy

proved to be the most suitable cultivation mode. Nevertheless, considering the complexity and heterogeneity of buttermilk, extensive investigations are needed to evaluate whether any organic compounds (and at which concentrations) have a hampering effect on the growth of *G. sulphuraria*. In addition, further research is needed to improve the biomass production and phycocyanin content through optimization of light supply, pre-treatment of buttermilk or the use of other operational strategies such as chemostats or the adoption of fed-batch approaches. Finally, further research is required to understand the interactions between *G. sulphuraria* and other microbes such as fungi and how they affect the growth of *G. sulphuraria*.

Abbreviations

DO Dissolved oxygen concentration (% air saturation)
TN Total Nitrogen ($g_N \cdot L^{-1}$)
TOC Total Organic Carbon ($g_C \cdot L^{-1}$)
OD_{750} Optical density at 750 nm
C_x biomass dry weight concentration ($g_x \cdot L{-}1$)
$Y_{x/C}$ Biomass yield per carbon unit ($g_x\ g_C^{-1}$)
$Y_{x/N}$ Biomass yield per nitrogen unit ($g_x\ g_N^{-1}$)
μ Specific growth rate (d^{-1})
r_x Volumetric biomass production rate ($g_x \cdot L^{-1} \cdot d^{-1}$)
a_x Average- specific optical cross section ($m^2 \cdot kg^{-1}$)

Subscript

x Biomass

C Carbon

N Nitrogen

Authorship contribution statement

Conceptualization, V.M., N.F.L., S.C.; Methodology, N.F.L., F.D.S.; Formal analysis, P.S.O., F.D.S. and N.F.L.; Investigation, P.S.O., F.D.S. and N.F.L.; Resources, S.C.; Data curation, P.S.O. and N.F.L.; writing—original draft preparation, P.S.O.; Writing—review and editing, V.M., N.F.L., S.C. and C.C.; Visualization, P.S.O. and N.F.L.; Supervision, N.F.L., S.C. and C.C.; Project administration, V.M., S.C.; Funding acquisition, S.C. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

We would like to thank Michele Locatelli from Caseificio del Cigno S.P.A. for the supply of buttermilk. And our most sincere thanks to Prof. Antonino Pollio from the University of Naples Federico II for his guidance and fruitful discussions on *Galdieria* cultivation.

Conflict of interests

We declare that the authors have no competing interests, as defined by Springer, or other interests that might be perceived to influence the results and/or discussion reported in this manuscript.

Data availability statement

The raw data generated during this research is available by the corresponding author upon request without reservation.

Funding statement

The contents of this work were financially supported by Algreen B.V.

References

Abiusi F, Trompetter E, Hoenink H, et al (2021) Autotrophic and mixotrophic biomass production of the acidophilic *Galdieria sulphuraria* ACUF 064. Algal Res 60:. https://doi.org/10.1016/j.algal.2021.102513

Abiusi F, Moñino Fernández P, Canziani S, et al (2022a) Mixotrophic cultivation of *Galdieria sulphuraria* for C-phycocyanin and protein production. Algal Res 61:. https://doi.org/10.1016/j.algal.2021.102603

Abiusi F, Trompetter E, Pollio A, et al (2022b) Acid Tolerant and Acidophilic Microalgae: An Underexplored World of Biotechnological Opportunities. Front Microbiol 13:. https://doi.org/10.3389/fmicb.2022.820907

Albertano P, Ciniglia C, Pinto G, Pollio A (2000) The taxonomic position of *Cyanidium, Cyanidioschyzon* and *Galdieria*: an update. https://doi.org/10.1023/A:1004031123806

Ali AH (2019) Current knowledge of buttermilk: Composition, applications in the food industry, nutritional and beneficial health characteristics. Int J Dairy Technol 72:169–182. https://doi.org/10.1111/1471-0307.12572

Bahrami M, Ahmadi D, Beigmohammadi F, Hosseini F (2015) Mixing sweet cream buttermilk with whole milk to produce cream cheese. Irish Journal of Agricultural and Food Research 54:73–78. https://doi.org/10.1515/ijafr-2015-0008

Barros A, Pereira H, Campos J, et al (2019) Heterotrophy as a tool to overcome the long and costly autotrophic scale-up process for large-scale production of microalgae. Sci Rep 9:. https://doi.org/10.1038/s41598-019-50206-z

Canelli G, Abiusi F, Vidal Garcia A, et al (2023) Amino acid profile and protein bioaccessibility of two *Galdieria sulphuraria* strains cultivated autotrophically and mixotrophically in pilot-scale photobioreactors. Innovative Food Science & Emerging Technologies 84:103287. https://doi.org/10.1016/j.ifset.2023.103287

Curien G, Lyska D, Guglielmino E, et al (2021) Mixotrophic growth of the extremophile *Galdieria sulphuraria* reveals the flexibility of its carbon assimilation metabolism. New Phytologist 231:326–338. https://doi.org/10.1111/nph.17359

de Freitas Mascarello A, Isabel Pinto G, Souza de Araújo I, et al (2019) Technological and Biological Properties of Buttermilk: A Minireview. In: Whey - Biological Properties and Alternative Uses. IntechOpen. DOI: 10.5772/intechopen.80921

de Mooij T, Janssen M, Cerezo-Chinarro O, et al (2015) Antenna size reduction as a strategy to increase biomass productivity: A great potential not yet realized. J Appl Phycol 27:1063–1077. https://doi.org/10.1007/s10811-014-0427-y

Dewettinck K, Rombaut R, Thienpont N, et al (2008) Nutritional and technological aspects of milk fat globule membrane material. Int Dairy J 18:436–457. https://doi.org/10.1016/j.idairyj.2007.10.014

Di Caprio F (2020) Methods to quantify biological contaminants in microalgae cultures. Algal Res 49. https://doi.org/10.1016/j.algal.2020.101943

Fuller KL, Kuhlenschmidt TB, Kuhlenschmidt MS, et al (2013) Milk fat globule membrane isolated from buttermilk or whey cream and their lipid components inhibit the infectivity of rotavirus in vitro. J Dairy Sci 96:3488–3497. https://doi.org/10.3168/jds.2012-6122

Gassi JY, Blot M, Beaucher E, et al (2016) Preparation and characterisation of a milk polar lipids enriched ingredient from fresh industrial liquid butter serum: Combination of physico-chemical modifications and technological treatments. Int Dairy J 52:26–34. https://doi.org/10.1016/j.idairyj.2015.08.012

Graverholt, O. S., & Eriksen, N. T. (2007). Heterotrophic high-cell-density fed-batch and continuous-flow cultures of *Galdieria sulphuraria* and production of phycocyanin. Applied Microbiology and Biotechnology, 77(1), 69–75. https://doi.org/10.1007/s00253-007-1150-2

Graziani G, Schiavo S, Nicolai MA, et al (2013) Microalgae as human food: Chemical and nutritional characteristics of the thermo-acidophilic microalga *Galdieria sulphuraria*. Food Funct 4:144–152. https://doi.org/10.1039/c2fo30198a

Gross W, Schnarrenberger C (1995) Heterotrophic Growth of Two Strains of the Acido-Thermophilic Red Alga *Galdieria sulphuraria*. https://doi.org/10.1093/oxfordjournals.pcp.a078803

Hancock JT, Salisbury V, Ovejero-Boglione MC, et al (2002) Antimicrobial Properties of Milk: Dependence on Presence of Xanthine Oxidase and Nitrite. Antimicrob Agents Chemother 46:3308–3310. https://doi.org/10.1128/AAC.46.10.3308-3310.2002

Holzmüller W, Kulozik U (2016) Quantification of MFGM proteins in buttermilk and butter serum by means of a stain free SDS-PAGE method. Journal of Food Composition and Analysis 49:102–109. https://doi.org/10.1016/j.jfca.2016.04.003

Jain M, Khan SA, Sharma K, et al (2022) Current perspective of innovative strategies for bioremediation of organic pollutants from wastewater. Bioresour Technol 344:126305. https://doi.org/10.1016/j.biortech.2021.126305

Konrad G, Kleinschmidt T, Lorenz C (2013). Ultrafiltration of whey buttermilk to obtain a phospholipid concentrate. International Dairy Journal, 30(1), 39-44. https://doi.org/10.1016/j.idairyj.2012.11.007

Kumar A, Thakur A, Panesar PS (2019) A comparative study on experimental and response surface optimization of lactic acid synergistic extraction using green emulsion liquid membrane. Sep Purif Technol 211:54–62. https://doi.org/10.1016/j.seppur.2018.09.048

Laezza C, Salbitani G, & Carfagna S (2022) Fungal contamination in microalgal cultivation: Biological and biotechnological aspects of fungi-microalgae interaction. Journal of Fungi, 8(10), 1099. https://doi.org/10.3390/jof8101099

Lambert S, Leconte N, Blot M, et al (2016) The lipid content and microstructure of industrial whole buttermilk and butter serum affect the efficiency of skimming. Food Research International 83:121–130. https://doi.org/10.1016/j.foodres.2016.03.002

Olabi A, Jinjarak S, Jiménez-Flores R, et al (2015) Compositional and sensory differences of products of sweet-cream and whey buttermilk produced by microfiltration, diafiltration, and supercritical CO21. J Dairy Sci 98:3590–3598. https://doi.org/10.3168/jds.2014-9141

Oesterhelt C, Schnarrenberger C, & Gross W (1999). Characterization of a sugar/polyol uptake system in the red alga *Galdieria sulphuraria*. European Journal of Phycology, 34(3), 271-277. https://doi.org/10.1080/09670269910001736322

Pagels F, Guedes AC, Amaro HM, et al (2019) Phycobiliproteins from cyanobacteria: Chemistry and biotechnological applications. Biotechnol Adv 37:422–443. https://doi.org/10.1016/j.biotechadv.2019.02.010

Perez Saura P, Chabi M, Corato A, Cardol P, & Remacle C (2022). Cell adaptation of the extremophilic red microalga *Galdieria sulphuraria* to the availability of carbon sources. Frontiers in Plant Science, 13. DOI: 10.3389/fpls.2022.978246

Pleissner D, Lindner AV, Händel N (2021) Heterotrophic cultivation of *Galdieria sulphuraria* under non-sterile conditions in digestate and hydrolyzed straw. Bioresour Technol 337: https://doi.org/10.1016/j.biortech.2021.125477

Portillo FVL, Sierra-Ibarra E, Vera-Estrella R, et al (2022) Growth and phycocyanin production with *Galdieria sulphuraria* UTEX 2919 using xylose, glucose, and corn stover hydrolysates under heterotrophy and mixotrophy. Algal Res 65: https://doi.org/10.1016/j.algal.2022.102752

Raghunath BV, Punnagaiarasi A, Rajarajan G, Irshad A, Elango A, & Mahesh Kumar G (2016). Impact of dairy effluent on the environment—a review. Integrated Waste Management in India: Status and Future Prospects for Environmental Sustainability, 239-249. https://doi.org/10.1007/978-3-319-27228-3_22

Rahman DY, Sarian FD, van der Maarel MJEC (2020) Biomass and phycocyanin content of heterotrophic *Galdieria sulphuraria* 074G under maltodextrin and granular starches–feeding conditions. J Appl Phycol 32:51–57. https://doi.org/10.1007/s10811-019-01957-9

Russo GL, Langellotti AL, Oliviero M, Baselice M, Sacchi R, & Masi P (2021). Valorization of second cheese whey through cultivation of extremophile microalga *Galdieria sulphuraria*. AIMS Environ. Sci, 8, 435-448. DOI: 10.3934/environsci.2021028

Salvatore MM, Carraturo F, Salbitani G, Rosati L, De Risi A, Andolfi A, ... & Carfagna S (2023) Biological and metabolic effects of the association between the microalga *Galdieria sulphuraria* and the fungus *Penicillium citrinum*. Scientific Reports, 13(1), 1789. https://doi.org/10.1038/s41598-023-27827-6

Sloth JK, Jensen HC, Pleissner D, Eriksen NT (2017) Growth and phycocyanin synthesis in the heterotrophic microalga *Galdieria sulphuraria* on substrates made of food waste from restaurants and bakeries. Bioresour Technol 238:296–305. https://doi.org/10.1016/j.biortech.2017.04.043

Sloth JK, Wiebe MG, Eriksen NT (2006) Accumulation of phycocyanin in heterotrophic and mixotrophic cultures of the acidophilic red alga *Galdieria sulphuraria*. Enzyme Microb Technol 38:168–175. https://doi.org/10.1016/j.enzmictec.2005.05.010

Sodini I, Morin P, Olabi A, Jiménez-Flores R (2006) Compositional and functional properties of buttermilk: A comparison between sweet, sour, and whey buttermilk. J Dairy Sci 89:525–536. https://doi.org/10.3168/jds.S0022-0302(06)72115-4

Tischendorf G, Oesterhelt C, Hoffmann S, et al (2007) Ultrastructure and enzyme complement of proplastids from heterotrophically grown cells of the red alga *Galdieria sulphuraria*. Eur J Phycol 42:243–251. https://doi.org/10.1080/09670260701437642

Tredici MR (2010) Photobiology of microalgae mass cultures: Understanding the tools for the next green revolution. Biofuels 1:143–162. https://doi.org/10.4155/bfs.09.10

Vanderghem C, Bodson P, Danthine S, Paquot M, Deroanne C, & Blecker, C. (2010). Milk fat globule membrane and buttermilks: from composition to valorization. *Base.* https://popups.uliege.be/1780-4507/index.php?id=5828.

Vigliengo E, Reinheimer J (2009) Use of whey and buttermilk-based media to obtain biomass of thermophilic LAB. Int J Dairy Technol 62:431–437. https://doi.org/10.1111/j.1471-0307.2009.00508.x

Zimermann JD arc F, Sydney EB, Cerri ML, et al (2020) Growth kinetics, phenolic compounds profile and pigments analysis of *Galdieria sulphuraria* cultivated in whey permeate in shake-flasks and stirred-tank bioreactor. Journal of Water Process Engineering 38:. https://doi.org/10.1016/j.jwpe.2020.101598

GENERAL RESULTS AND DISCUSSION

The research activities carried out during the PhD course focused on different stratefies to reclaim wastewater for crop irrigation or as a source of high value products, with a special focus on microalgae application.

Chapter I includes two papers: ***"Olive mill wastewater fermented with microbial pools as a new potential functional beverage"*** and ***"Phenols recovered from olive mill wastewater as a natural booster to fortify blood orange juice".***

In the first study, the aim was to enhance the nutraceutical value of OMWW through a driven fermentation using selected microbial pools. In particular, strains such as *L. plantarum*, *C. boidinii* and *W. anomalus* were selected to drive the fermentation process. Results showed that in all OMWW samples subjected to microfiltration, fermentation stopped after 21 days. Furthermore, in all inoculated samples a significant increase in phenols and organic acids were detected. Moreover, biological assays highlighted that fermentation determines an increase in the antioxidant and anti–inflammatory activity of OMWW. Lastly, an increase in the active permeability on Caco-2 cell line was also revealed. The results confirmid that fermented OMWW can be proposed as a new beverage and/or functional ingredient with nutraceutical effects. In the second paper, the aim was to use a tangential membrane filtration system, present at the company, to recover phenols from olive mill wastewater. The concentrates obtained through ultrafiltration and reverse osmosis were characterised for their physico-chemical traits, antioxidant activity and antimicrobial effects. In addition, the obtained permeates were evaluated for irrigation purposes. The best concentrate was subsequently added, in different ratios, into a commercial blood orange juice. The juice fortified with the addition of the concentrate, up to a ratio of 2:250 v/v, showed no off-flavour and off-odour compared to control. Furthermore, after 60 days of refrigerated storage, the fortified juice still presents a hydroxytyrosol content in accordance with the EFSA-recommended daily intake. The results obtained may be useful at industrial level for the production of orange juice fortified with a concentrate of natural antioxidants as a 'clean label' ingredient.

Chapter 2 includes a study: "***Treated urban wastewater for lettuce (Lactuca sativa var. Canasta) and tomato (Lycopersicon esculentum, var. Rio) crop irrigation***". The lettuce and tomato samples irrigated with wastewater treated by a phyto-remediation system or a phyto-remediation system

coupled with UV showed no significant differences, in terms of microbiological traits, compared to crops irrigated with conventional water. Same results were obtained for soil samples differently irrigated. The DGGE profiles revealed as the irrigation with reclaimed and conventional water did not affect the composition of microbial community. However, it is important to point out that although the removal efficacy of tested systems was considerable, indicators, as *E. coli, Clostridium* spores and sulphobacteria sporigene were detected in out let water samples but not vegetable or soil samples. The study concluded that vegetable crops irrigated with reclaimed water distributed by different drip lines are compliant with the UE regulation, for the absence of bacteria indicators.

Chapter 3 reports a review paper titled: ***"Current challenges of microalgae applications: exploiting the potential of non-conventional mi-croalgae species",*** that gives a clear overview about the different biotechnological applications of microalgae. In the same review points of strengths, as well as of weakness, of each single application field, as food, feed, nutraceutical, cosmetic, biofuel industry and wastewater treatment were highlithed. Moreover, the state of art of cultivation system has been deeply reported, highlighting both the limitations and obstacle to overcome to establish a production system at industrial scale. Limitation related to harvesting methods, high value compounds extraction systems, high cost of cultivation process, contamination issues as well as the immature market are the main and common weakness found in several application fields.

Chapter 4 presents two cases study where through a microalgae-based wastewater treatment was possible to obtain treated wastewater for irrigation purposes. In the first study, "***An indigenous microalgal pool from a constructed wetland as an alternative strategy for Escherichia coli removal in urban wastewater***", a wastewater treatment based on an autochthonous Microalgal Pool (MP) was compared to treatments based on *C. vulgaris* and *S. quadricauda* for remediation of wastewater from an agritourism structure. The autochthonous MP was characterized as mainly composed by four species belonging to green algae (Chlorophyceae), and highlighted a 100% *E. coli* removal efficiency as *C. vulgaris* and *S. quadricauda*, lowering the bacterial density to values compliant with the EU regulation limits. Furthermore, the autochthonous MP showed interesting adaptation and compositional stability, although a slight variation in the microalgal composition, as species ratio between initial and final samplings were observed. In the study, "***Comparative phycoremediation performance of three microalgae species in two different magnitudes of pollutants in wastewater from farmhouse***", the wastewater remediation was evaluated in chemical and microbiological terms, considering also the production of valuable biomass. Overall, the findings confirmed that microalgae-based treatment offers potential for sustainable, eco-friendly, and resource-efficient solutions for

wastewater remediation that may also be used for irrigation in agriculture, contributing to a more environmentally friendly approach to water management. More specifically, comparing removal efficiency among different microalgae species: *Chlorella vulgaris* (CV) and *Scenedesmus quadricauda* (SQ), with an autochthonous strain of *Klebsormidium* sp. K39 (Kleb), directly isolated from effluents of the same system, it was obtained removal efficiencies in the range of 57–63% for total nitrogen, 65–92% for total phosphorous, 94–95% for COD, and 100% for *E. coli*. The treated waters showed values of pollutants suitable for irrigation use, in accordance with environmental and national and EU regulation limits. Last, despite in both case studies, the lab experiments showed interesting results, remains fundamental to design a system economically sustainable in terms of larger scale.

Chapter 5 is based on a paper already published "***Mixotrophic and heterotrophic growth of Galdieria sulphuraria using buttermilk as a carbon source***" reporting the use of the poly-extremophile microalga *Galdieria sulphuraria*. The results revealed that buttermilk can be used as a substrate for G. sulphuraria biomass production. Even though the growth of *G. sulphuraria* on buttermilk was slower than on optimal substrates, the obtained biomass still accumulated phycocyanin. In this case, further research is needed to improve the biomass production and phycocyanin content through optimization of cultivation parameters.

POSTER PRESENTATION

1. ISWEE 2021. 2nd International Symposium on Water, Ecology and Environment. ***Phycoremediation as strategy for secondary urban wastewater treatment.*** **Paride S. Occhipinti**, Emanuele La Bella, Ferdinando Fragalà, Ivana Puglisi, Andrea Baglieri, Giovanna B. Grillo, Cinzia L. Randazzo, Cinzia Caggia. (ID: ISWEE-MS-1448)
2. Microbial Diversity 2021, Advances in Microbial Diversity. ***Microalgae-based system as an advanced strategy for urban wastewater treatment.*** **Paride S. Occhipinti**, Emanuele La Bella, Ferdinando Fragalà, Ivana Puglisi, Andrea Baglieri, Giovanna B. Grillo, Cinzia L. Randazzo, Cinzia Caggia.
3. CONVEGNO AISAM 2022 - Prof. Mario Tredici: ***Acidophilic microalgae for the treatment and valorization of buttermilk.*** Vidal A., Amenta S., **Occhipinti P.S**.., Ferrer-Ledo N., Canziani S., Mantovani M., Passalacqua E., Ficara E., Parati K., Mezzanotte V. Napoli, 16-18 ottobre 2022
4. ALGAEUROPE 2022. ***Bioremediation of buttermilk by using the acidophilic microalgae Euglena gracilis and Galdieria sulphuraria.*** **Occhipinti P.S.**, Ferrer Ledo N., Mezzanotte V., Caggia C., Canziani S. Roma, 13-15 dicembre 2022
5. Microbial Diversity 2023, Advances in Microbial Diversity. ***Phycoremediation: An indigenous microalga from a constructed wetland as a strategy for urban wastewater.*** **Paride S. Occhipinti**,_E. La Bella, I. Puglisi, F. Fragalà, C. L. Randazzo, A. Baglieri, C. Caggia.
6. 9th International Conference of MIKROBIOKOSMOS. ***Olive mill wastewater as a resource of biologically active phenols for food and beverages.*** Foti P., Vaccalluzzo A., Romeo F. V., Pino A., Russo N., Randazzo C. L., **Occhipinti P.S.**, Caggia C. Atene, 16-18 dicembre 2021.
7. XIII Convegno Nazionale sulla Biodiversità. ***Valorizzazione di acque di vegetazione olearie attraverso fermentazioni guidate.*** Foti P., **Occhipinti P.S** , Romeo F.V., Russo N., Finocchiaro M., Randazzo C.L., Caggia C. 7-9 settembre 2021

OTHER ACTIVITIES

Traineeship Abroad

During the second year of PhD course, a period of 9 months (from 12/05/2022 to 16/12/2022) was spent at R&D division of Algreen B.V., spin-off of Wageningen University and research (WUR), The Netherlands, to carry out a research activity under the supervision of Dr. Narcis Ferrer Ledo PhD and Dr. Stefano Canziani CEO.

The main focus of the activities can be summarized in:

1. Experiments in flask to test the *Galdieria sulphuraria* ACUF064 in mixotrophic and heterotrophic conditions, using glucose, galactose and lactose as carbon sources

2. Experiments in flask to test the strain *Galdieria sulphuraria* ACUF064 in mixotrophic and heterotrophic conditions, using buttermilk as carbon source

3. Experiments in 13L stirred tank reactor to test *Galdieria sulphuraria* ACUF064 growth using glucose and buttermilk as carbon source, mixotrophically, in batch conditions.

The obtained results were published as original paper in Journal of Applied Phycology, 2023. https://doi.org/10.1007/s10811-023-03012-0. ***Mixotrophic and heterotrophic growth of Galdieria sulphuraria using buttermilk as a carbon source***

Traineeship at other Institutions

During the first year of PhD course, a period of 2 months (from 18/01/2021 to 24/02/2021) was spent at Algae Biology Laboratory, Department of Biology of the Federico II University of Naples, under the supervision of Prof. Antonino Pollio.
During such a period the main activities focused on:

- techniques for isolating strains of cyanobacteria and microalgae.
- techniques for maintaining strains of cyanobacteria and microalgae.
- techniques for growing strains of cyanobacteria and microalgae

Attended Courses

1. ***Management and interpretation of complex biological data: theoretical foundations and use of analysis software***. Excel (advanced) and R. (32h) University of Catania. Prof. Mario di Guardo.

2. ***CRISPR/Cas genome editing in plants***. (10h) University of Catania. Prof.ssa Stefania Maria Bennici

3. ***Identification methods of algae.*** BIO/02 - 6 CFU - 2° Semester. University of Catania. Prof.ssa Giuseppina Alongi

Teaching Activities

CO-TUTOR of MASTER'S THESIS

1. **Title of thesis:** *"Wastewater treatment and microalgae role in E. coli removal efficiency"* (Academic year 2021). **Student**: Gloria Sciuto. **Relators:** Chiar.ma Prof.ssa Cinzia Caggia and Dott. Paride Salvatore Occhipinti

2. **Title of thesis:** *"Effetto del trattamento di acque reflue sul microbiota di ortive attraverso analisi microbiologiche coltivazione-indipendente"* (Academic year 2022). **Student**: Morena Zingale. **Relators:** Chiar.ma Prof.ssa Cinzia Caggia and Dott. Paride Salvatore Occhipinti

Progetto Alternanza scuola-lavoro (PCTO-2022). Presentazione attività di dottorato ed analisi microbiologiche delle acque. «F. Morvillo» Catania. 29/03/2021 and 10/02/2022.