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The Bioactivities of Lactic Acid-Fermented *Arthrospira platensis* and Its Application in Functional Beverages

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Abstract: The demand for functional beverages with clean labels is growing. *Arthrospira platensis* and fermented products offer bioactive compounds, including antimicrobials. This study aimed to produce food-grade extracts from lactic acid-fermented *A. platensis* and evaluate its antimicrobial activity, lipid-reducing and glucose uptake effects, and antioxidant properties. An in situ test was also conducted to assess antimicrobial activity in commercial soft drinks against *Escherichia coli*. *Arthrospira platensis* was fermented with five different QPS LAB strains: *Limosilactobacillus fermentum* UPCCO 1986, *Companilactobacillus farciminis* UPCCO 4841, *Levilactobacillus brevis* UPCCO 4873, *Lentilactobacillus diolivorans* UPCCO 5571, and *Latilactobacillus curvatus* UPCCO 6133, obtaining good results in aerobic and anaerobic conditions. The results have shown that the most versatile strain in fermenting biomass is *L. brevis* UPCCO 4873. Important in vitro antimicrobial activity was seen against *Salmonella enterica*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli*. The extracts that exerted the highest antimicrobial activity (4841AE/AN, 5571AE, and 6133AN) were assessed for the in situ antimicrobial activity against *E. coli* ATCC 11229. Overall, the antimicrobial activity of the extracts was concentration-dependent, with higher concentrations exhibiting bactericidal effects and lower concentrations displaying bacteriostatic effects. Extracts from fermented *A. platensis* have also significantly reduced the neutral lipid reservoirs, which were not observed without fermentations. The strongest lipid-reducing effect was obtained with *A. platensis* fermented with *Levilactobacillus brevis* UPCCO 4873. This work opens the possibility of developing bioactive extracts or natural preservatives from fermented microalgae to be used in novel functional beverages.

Keywords: natural antimicrobial; *Arthrospira platensis*; lactic acid fermentation; foodborne pathogenic bacteria; microbiological challenge test; lipid-reducing activity

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1. Introduction

In recent years, the growing consumer demand for clean-label, functional, safe, and high-quality products has driven the search for new natural antimicrobial agents that can enhance quality and safety while minimizing their impact on product composition [1]. In this context, the use of alga extracts in food formulations as antimicrobial agents is gaining increasing interest, as both microalgae and macroalgae contain bioactive compounds, such as polysaccharides, proteins, peptides, and phenolics, which exhibit antimicrobial properties effective against various foodborne pathogens [2,3]. These compounds inhibit bacterial, fungal, and viral growth, making algae a valuable ingredient in enhancing food safety and extending the shelf life of beverages, reducing the need for synthetic preservatives [4].

The interest in the use of microalgae in beverages is also emphasized by the recent literature as functional ingredients due to their wide spectrum of bioactive compounds, including proteins, lipids, carbohydrates, vitamins, and antioxidants [5]. Several studies discuss the potential of algae in food and beverage applications. *Arthrospira platensis*, mostly commercialized as *Spirulina*, is a cyanobacterium frequently used as a dietary supplement due to its rich nutritional profile such as high protein content (60%), offering all essential amino acids, carbohydrates, and polyunsaturated fatty acids, including omega-3 and omega-6 [6,7]. Microalgae like *A. platensis* and *Chlorella* have been used in beverage formulations [8] because of their highly favored protein content, as well as bioactive compounds like phycocyanins and chlorophylls, which offer antioxidant, anti-inflammatory, and even potential anti-cancer properties [9]. These microalgae have been incorporated into energy drinks, smoothies, and health drinks, often marketed for their "superfood" properties. For instance, *A. platensis*-enriched beverages have demonstrated higher energy and salt content but are favored for their high antioxidant properties and vibrant color, making them a popular choice in natural health products [10].

Several papers have highlighted the bioactive composition of microalgae and their prospects in the food industry, considering both their antimicrobial and health-promoting properties [11] which make them suitable for incorporation into functional foods and beverages [12]. Moreover, alga-derived bioactives not only contribute to the improvement of beverage formulations, but also align with the current trends of plant-based diets and sustainable sourcing [13]. Despite these advantages, the incorporation of algae into functional beverages faces several challenges. Sensory properties such as taste, color, and odor, as well as the functional stability of alga-derived bioactives during processing and storage, are critical factors that influence consumer acceptance [14]. Advances in food technology, such as microencapsulation and fermentation, have been explored to overcome these challenges and enhance the palatability and stability of alga-based beverages [15]. The fermentation of microalgae such as *A. platenisis* with lactic acid bacteria has been proposed to obtain cyanobacterial biomasses with more pleasant sensory properties for potential use in food formulations [16]. On the other hand, *A. platenisis* has been used to boost the growth of lactic acid bacteria to be used as starters in the production of novel functional fermented dairy foods [17].

Considering all these factors, this study aimed to improve the bioactivity and shelf life of soft drinks by incorporating ethanolic extracts from fermented *Arthrospira platensis*. To achieve this goal, five different LAB species were evaluated to determine which fermentation-derived ethanolic extract was most effective based on the following criteria: (i) antimicrobial activity against the most common foodborne pathogens, (ii) efficacy as a preservative in soft drinks against spoilage caused by *Escherichia coli*, (iii) lipid-reducing effect and glucose uptake (the zebrafish Nile red fat metabolism assay and 2-NDBG assay), and (iv) the antioxidant activity and quantification of phenolic content.

2. Materials and Methods

2.1. Arthrospira Platensis and Bacterial Strains Used for Fermentation

Dried *Arthrospira platensis* (composition: lipids 5%, carbohydrates 24%, fiber 5%, proteins 57%), generously provided by Bertolini Farm (Fidenza, PR, Italy), was utilized as a fermentation substrate. This biomass, commercially available as "Organic *Spirulina*" under the EU Organic Aquaculture Regulation (EC No. 834/07), is cultivated in raceway ponds and processed through dehydration before marketing. For fermentation, five different strains from the *Lactobacillaceae* family, part of the University of Parma Culture Collection (UPCCO, Parma, Italy), were used. These strains, previously isolated from various food matrices and identified through 16S rRNA sequencing (Table 1), were stored at −80 °C in de Man, Rogosa and Sharpe medium (MRS) (Oxoid, Basingstoke, UK), added with 12.5% (*v*/*v*) glycerol.

Table 1. Lactic acid bacteria strains used for the solid-state fermentation of *Arthrospira platensis*.

ID	Species	Optimal Growth Temperature
UPCCO 1986	*Limosilactobacillus fermentum*	42 °C
UPCCO 4841	*Companilactobacillus farciminis*	37 °C
UPCCO 4873	*Levilactobacillus brevis*	30 °C
UPCCO 5571	*Lentilactobacillus diolivorans*	37 °C
UPCCO 6133	*Latilactobacillus curvatus*	30 °C

2.2. Cyanobacterial Biomass Fermentation

The selected LAB strains were subcultured twice in an MRS broth (Oxoid) with a 3% (*v*/*v*) inoculum, and incubated for 16 h under aerobic conditions at their optimal growth temperatures (Table 1). Subsequently, the strains were inoculated into fresh a MRS broth (3% *v*/*v*) and incubated for 15 h at the optimal growth temperature to achieve a bacterial concentration of approximately 9 log CFU/mL. The revitalized cultures were centrifuged (Eppendorf Centrifuge 5810 R, Eppendorf, Hamburg, Germany) at 12,857× *g* for 10 min at 4 °C, and the bacterial cells were collected, washed twice with Ringer solution (Oxoid, Milan, Italy) to remove residual media, and then suspended in sterile bidistilled water. The *A. platensis* biomass was rehydrated with 75% *w*/*w* sterile water and inoculated with each LAB suspension to reach an approximate inoculum of 7 log CFU/mL in each sample. Fermentations were conducted under two conditions: aerobic and anaerobic. For anaerobic conditions, the inoculated biomass was vacuum-sealed using a food vacuum machine (ECO VAC 300S TB, Imballaggi Service Group S.r.l., Rome, Italy). LAB concentrations were measured immediately after inoculation (T0) and at 24 h (T1), 48 h (T2), and 72 h (T3) of fermentation. Serial dilutions of the samples were prepared in a Ringer solution (Oxoid), plated on an MRS agar (Oxoid), and incubated for 48 h at the optimal temperature of each strain. The pH of all samples was monitored before, during, and after each fermentation step using a Mettler Toledo pH meter (Greifensee, Switzerland). All fermentations were performed in duplicate, and triplicate analyses were conducted at each sampling time. After 72 h, the fermented biomasses were lyophilized for 48 h using a Lio-5P freeze dryer (5Pascal, Milan, Italy) and stored at −20 °C.

2.3. Extraction Process

To extract compounds with potential bioactivities such as organic acids, small peptides, and polyphenols, the extraction described by Martelli et al. (2020) [3] was performed. Before extraction, each freeze-dried lacto-fermented *A. platensis* sample was broken up using a porcelain mortar and pestle to enhance the extraction process. Then, 10 g of each lyophilized fermented biomass was extracted with 100 mL of ethanol/water (70:30 *v*/*v*) acidified with 1% formic acid (CH_2O_2). A double extraction was executed twice, alternating a shaking cycle in an HS 501 digital shaker (IKA) (IKA-Werke GmbH & Co, Staufen, Germany) at 200 strokes/minute with a sonication cycle in an Ultrasonic Cleaner sonicator (VWR, Radnor, PA, USA), each lasting 15 min. The sample was then centrifuged (Eppendorf 5800 Centrifuge, Model 5810R, Hamburg, Germany) at 12,857× *g* for 10 min at 10 °C. The solution was filtered on a paper filter to recover the solid part so as to proceed to the second extraction. The two extracts obtained were combined and dried under vacuum on a Strike 300 rotary evaporator (Steroglass, Perugia, Italy) at 4× *g* with a bath temperature of 40 °C.

2.4. Foodborne Pathogenic and Spoiling Bacteria

The antimicrobial activity of the extracts was tested for 4 of the main foodborne pathogenic bacteria: *Salmonella* spp. (*S. enterica* ATCC 14028), *Listeria monocytogenes* (LMG 13305), *Escherichia coli* (ATCC 25922), and *Staphylococcus aureus* (NCTC 9393). One spoiling strain of *Escherichia coli* (ATCC 11229) was also used. These strains belong to international collections: American Type Culture Collection (ATCC), Belgian Co-ordinated Collection of Microorganisms (BCCM/LMG), and National Collection of Type Cultures (NCTC). Bacteria were kept at −80 °C in a tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) supplemented

with 12.5% glycerol (*v*/*v*). Before use, they were revitalized twice by inoculum (3% *v*/*v*) in TSB (Oxoid) supplemented with 0.6% of yeast extract and then incubated for 16 h at 37 °C in aerobic conditions.

2.5. In Vitro Antimicrobial Activity

Agar well diffusion assays were performed to estimate the antimicrobial activity of the fermented microalgal extracts [3,18]. The pathogenic and spoiling bacteria were diluted until reaching a concentration of 8 log CFU/mL and spread on a tryptone soya agar (TSA) (Oxoid) using sterile swabs. After that, wells with a diameter of 7 mm were created using sterile tips in the agar and filled with 30 µL of each extract dissolved in sterile bidistilled water with a concentration of 250 mg/mL. The antimicrobial activity was calculated by measuring the diameter of the inhibition zone (mm) after 24 and 48 h of incubation at 37 °C in aerobic conditions. Sterile water was used as a negative control. The assays were performed in triplicate for each extract and strain, and average values ± standard deviations were reported.

2.6. In Situ Antimicrobial Activity Evaluation in Functional Beverages

The experiment was conducted to evaluate the use of fermented extracts in flavored water present on the market to prevent the growth of possible spoiling bacteria. These kinds of beverages are produced by using an aseptic filling. Aseptic fillings are quite expensive, and the use of this practice could be avoided by using natural extracts that could forbid the growth of pathogenic and spoiling microorganisms. A commercial non-alcoholic beverage formulated with birch sap was used (50 mL of birch sap contained in 500 mL) (ingredients: water, birch sap (10%), acidifier: citric acid, potassium phosphate, magnesium carbonate 12 mg; flavors, niacin 2,40 mg, vitamin B6 (0.21 mg), vitamin B12 (0.38 mg); sweetener: acesulfame K). The extracts that showed the highest in vitro antimicrobial activity (4841AN, 5571AE, and 6133AN) were selected to test the antimicrobial activity in situ through a product microbiological challenge test (MCT) conducted as described by Martelli et al. (2020) with some modifications [3]. Four different extract concentrations (1.71 mg/mL, 3.43 mg/mL, 6.87 mg/mL, and 13.75 mg/mL) were evaluated. The antimicrobial activity was tested for *Escherichia coli* ATCC 11229. Flavored water samples, added with different extracts at different concentrations, were contaminated with *E. coli* ATCC 11229 at a concentration of 6 log CFU/g in the product. The beverages were then mixed to evenly distribute the contamination. The *E. coli* concentration was monitored after 0, 4, 8, 24, 28, 32, 48, 52, 56, 72, 76, and 80 h of storage at room temperature (25 °C) by a plate count on a Chromocult agar (Oxoid, Basingstoke, UK) and the plates were incubated at 37 °C for 24 h. Analyses were performed in triplicate and the average values ± standard deviations were reported. A positive control consisting of *E. coli* inoculated in flavored water without any extracts was considered, as well as a negative control consisting of flavored water with extract addition (13.75 mg/mL) and no *E. coli*.

2.7. Zebrafish Nile Red Fat Metabolism Assay and 2-NDBG Assays

The zebrafish Nile red fat metabolism assay was employed to evaluate the lipid-reducing activity, while the 2-NDBG assay was used to assess the glucose uptake of all the obtained extracts [19]. According to the EC Directive 86/609/EEC, ethical committee approval was not required as these procedures do not qualify as animal experimentation. Prior to analysis, all extracts were dissolved in DMSO to achieve a concentration of 10 mg/mL. Briefly, zebrafish embryos were reared from day 1 post-fertilization (DPF) in an E3 medium with 0.01% methylene blue and with 200 µM 1-phenyl-2-thiourea (PTU) to inhibit pigmentation.

For the zebrafish Nile red fat metabolism assay, zebrafish larvae were exposed to the extracts at a final concentration of 25 µg/mL from 3 to 5 DPF in 96-well plates, with 3–4 larvae per well. Resveratrol (REV, 50 µM) was used as a positive control, while DMSO

(0.1%) served as the solvent control. Neutral lipids were stained overnight with Nile red at a concentration of 10 ng/mL.

For the 2-NDBG assay, zebrafish larvae were exposed to 25 µg/mL of fermented extracts from 3 to 4 DPF. After 24 h of exposure, wells designated for Emodin treatment (positive control) received 10 µM Emodin for 1 h. Then, 100 µL of the medium was removed from each well and replaced with 100 µL of 2-NBDG (200 µM in E3 medium), resulting in a final concentration of 100 µM per well. Larvae were exposed to 2-NBDG for 3 h to measure glucose uptake. After exposure, larvae were washed to eliminate excess fluorescent dye by removing 100 µL of medium from each well and replacing it with 100 µL of a washing solution consisting of an E3 medium with 5% ethanol. This washing step was repeated twice, followed by three additional washes with fresh E3 medium.

For both assays, larvae were anesthetized with tricaine (MS-222, 0.03%) for 5 min prior to imaging using a fluorescence microscope (Thunder Imaging System, Leica Microsystems, Germany). The fluorescence intensity in individual zebrafish larva was quantified using Fiji [20]. Both assays were performed in duplicate.

2.8. Chemicals

Ethanol, methanol, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA), while bidistilled water was produced in-house by a Millipore Alpha Q purification system (Waters, Billerica, MA, USA). Sodium carbonate, gallic acid, and Trolox utilized were acquired from Sigma-Aldrich (St. Louis, MO, USA), while Folin–Ciocalteu's phenol reagent solution was obtained from VWR (Milano, Italy).

2.9. Total Phenolic Content Quantification and Antioxidant Activity Evaluation

The determination of total phenolic content was carried out following the protocols described by Martelli et al. (2020) [3]. The total phenolic content was quantified as mg gallic acid equivalents per gram (mg GAE/g), based on a calibration curve constructed by measuring the absorbance at 760 nm of five gallic acid solutions with concentrations of 0.01, 0.025, 0.05, 0.075, and 0.1 mg/g. Antioxidant activity was assessed using the DPPH radical scavenging activity assay [21]. The calibration curve was generated using Trolox as a reference, and the results are expressed as mg Trolox equivalents (TEAC) per gram (mg TEAC/g). The absorbance for these spectrophotometric analyses was measured using a JASCO V-530 spectrophotometer (Jasco Europe, Cremella, Italy).

2.10. Statistical Analysis

GraphPad Prism (Version 8, San Diego, CA, USA) was used for graph creation and statistical analyses. To evaluate significant differences in the fermentation ability of each LAB species on *Arthrospira platensis* biomass, a two-way ANOVA was conducted ($p < 0.05$). Similarly, a two-way ANOVA was used for the statistical analysis of the agar well diffusion assay results to evaluate the different efficacy of extracts on foodborne pathogenic bacteria ($p < 0.05$). Furthermore, a two-way ANOVA was performed to evaluate the extract efficacy during in situ analysis. For the zebrafish Nile red fat metabolism assay and the 2-NDBG assay, data normality was assessed using the Shapiro–Wilk normality test, and the homogeneity of variances was checked with Bartlett's test. If the data met the assumptions for an ANOVA, one-way ANOVAs with Dunnett's post hoc tests ($p < 0.05$) were applied to identify significantly different extracts compared to the solvent control group. If the data did not meet these assumptions, the Kruskal–Wallis test with Dunn's post hoc tests ($p < 0.05$) was used instead.

3. Results and Discussion

3.1. Fermentation Results

All selected LAB strains were capable of growing in the *A. platensis* biomass under aerobic conditions, as pictured in Table 2. Following inoculation, the strains entered an exponential growth phase within 24 h, achieving concentrations between 8 log CFU/g and

9 log CFU/g (Figure 1a). Specifically, *Limosilactobacillus fermentum* UPCCO 1986, *Levilactobacillus brevis* UPCCO 4873, and *Latilactobacillus curvatus* UPCCO 6133 showed an increase of ≥2 log CFU/g following the 72 h of fermentation, while *Companilactobacillus farciminis* UPCCO 4841 and *Lentilactobacillus diolivorans* UPCCO 5571 exhibited a smaller increase of 1.43 ± 0.30 log CFU/g and 1.37 ± 0.04 log CFU/g, respectively. Also under anaerobic conditions, all selected LAB strains proved the ability to grow and ferment the *A. platensis* biomass (Table 2). After 24 h, the strains entered an exponential growth phase. Strain UPCCO 1986, after 24 h, exhibited an increase higher than 2 log CFU/g. However, from 48 h onward, a decline in microbial load was observed for strain UPCCO 1986, which further decreased at 72 h to approximately 6.85 ± 0.52 log CFU/g (Δ (T0–T3) = 0.53 ± 0.75 log CFU/g). In contrast, the microbial loads of strains UPCCO 4841, UPCCO 4873, UPCCO 5571, and UPCCO 6133 remained stable at both 48 and 72 h (Figure 1b).

Table 2. Growth ability of different lactic acid bacteria (LAB) species/strains on *Arthrospira platensis* after 72 h of fermentation at the optimal growth temperature (37 °C and 30 °C). Values are reported as CFU/g ± standard deviation. The initial LAB concentration is indicated with T0, the LAB concentration after 3 days of fermentation is indicated with T3, and Δ (T0–T3) indicates the increment in the concentration during the 72 h of fermentation. Statistical differences in Δ (T0–T3) growth are expressed with letters.

	Aerobic			Anaerobic		
ID	**T0**	**T3**	**Δ (T0–T3)**	**T0**	**T3**	**Δ (T0–T3)**
UPCCO 1986	6.08 ± 0.22	8.87 ± 0.02	2.79 ± 0.20 ^{a}	6.35 ± 0.28	6.85 ± 0.52	0.53 ± 0.75 ^{c}
UPCCO 4841	7.14 ± 0.03	8.57 ± 0.27	1.43 ± 0.30 $_{ac}$	7.65 ± 0.01	8.83 ± 0.13	1.18 ± 0.11 ^{bc}
UPCCO 4873	6.98 ± 0.17	9.52 ± 0.12	2.54 ± 0.29 ^{a}	7.37 ± 0.18	9.50 ± 0.01	2.14 ± 0.16 ^{ab}
UPCCO 5571	7.45 ± 0.04	8.82 ± 0.01	1.37 ± 0.04 ^{ac}	7.24 ± 1.00	8.88 ± 0.13	1.64 ± 0.13 ^{ac}
UPCCO 6133	6.79 ± 0.04	8.87 ± 0.09	2.08 ± 0.06 ^{ab}	7.41 ± 0.45	8.87 ± 0.08	1.46 ± 0.54 ^{ac}

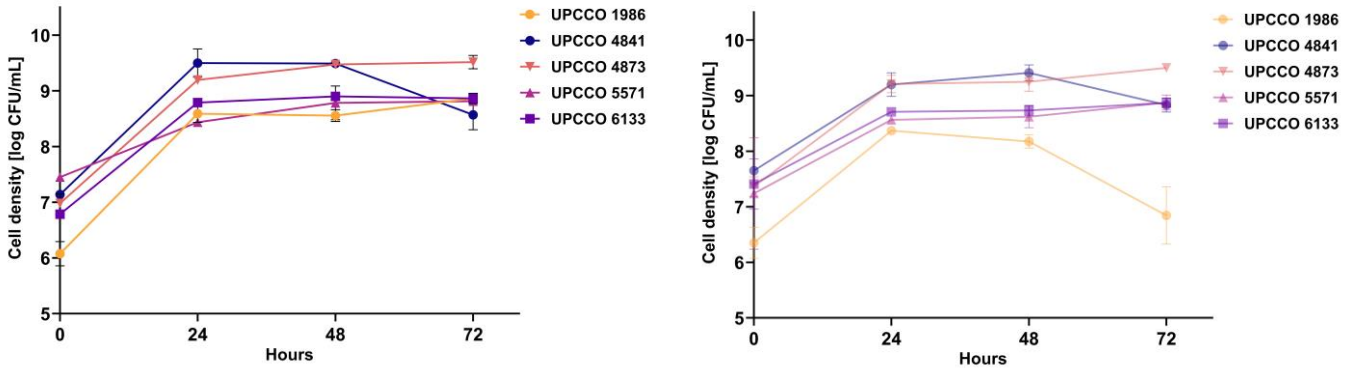
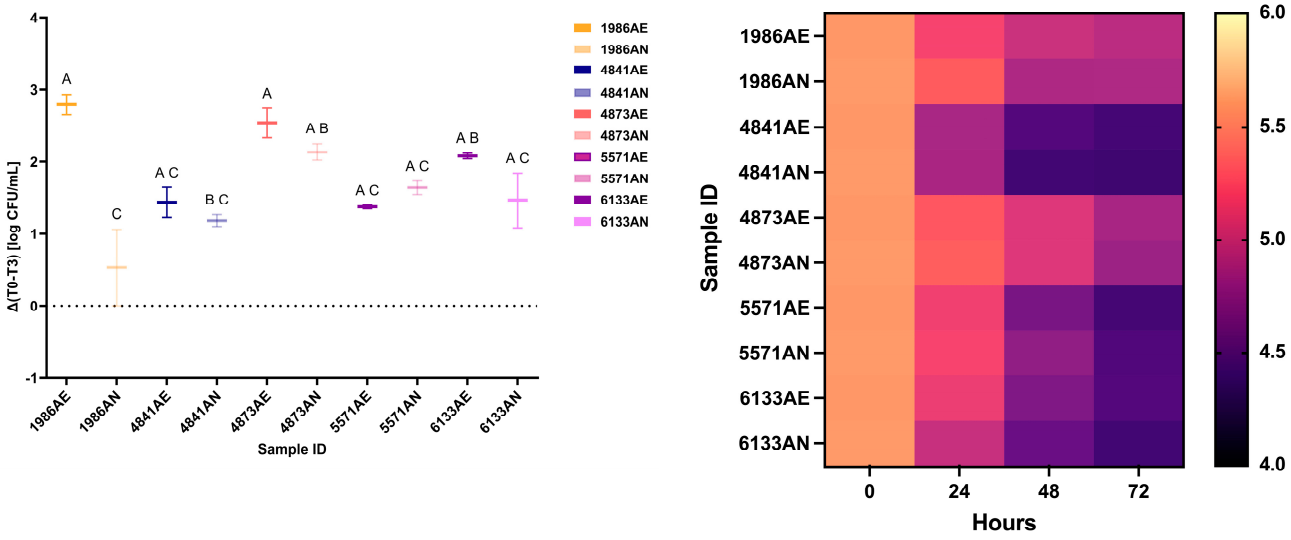


Figure 1. Growth curves of the five LAB strains during fermentations of *Arthrospira platensis*. The microbial concentration of each strain was evaluated just after the inoculum (0), after 24 h, 48 h, and 72 h of fermentation: (**a**) growth curves in aerobic conditions; (**b**) growth curves after incubation in anaerobic conditions. Different color lines indicate the lactic acid bacteria used for fermentation (*Limosilactobacillus fermentum* UPCCO 1986 (yellow), *Companilactobacillus farciminis* UPCCO 4841 (Blue), *Levilactobacillus brevis* UPCCO 4873 (orange), *Lentilactobacillus diolivorans* UPCCO 5571 (pink), and *Latilactobacillus curvatus* UPCCO 6133 (purple)).

Comparing the two types of incubation conditions, the statistical analysis showed few significant differences in the Δ (T0–T3) of the selected strains (Figure 2a). The only significant difference that was seen in the Δ (T0–T3) was *Limosilactobacillus fermentum* UPCCO 1986, which proved a significantly higher ability to grow in aerobic conditions (p = 0.0031). In other scientific papers, the solid-state fermentation of *A. platensis* proved

to be a valuable technique to ferment the biomass [16,22]. However, for the first time, the species *Limosilactobacillus fermentum*, *Companilactobacillus farciminis*, *Levilactobacillus brevis*, *Latilactobacillus curvatus*, and *Lentilactobacillus diolivorans* were used. The selected species proved a general incrementation of their concentration. The selection of new starters for novel fermented foods is a very promising technique given the evidence that fermented foods have on human health and the potential of the use of fermentation as a tool to valorize food products and food waste [23–25]. These results have shown that the most versatile strain in fermenting the *A. platensis* biomass, able to grow in both conditions, is *Levilactobacillus brevis* UPCCO 4873. A growing tendency has been seen in the use of microalgal biomasses to produce new food formulations or use microalgal biomasses to valorize new fermented foods [15,17,26]. Furthermore, given the composition rich in small peptides, various evidence underlining the great potential of *Arthrospira platensis* as a stimulant for lactic acid bacteria has been seen [17,27,28]. This activity has been observed in different species of LAB, such as that of *Lacticaseibacillus casei*, *Streptococcus thermophilus*, *Lacticaseibacillus acidophilus*, and *Bifidobacteria*.



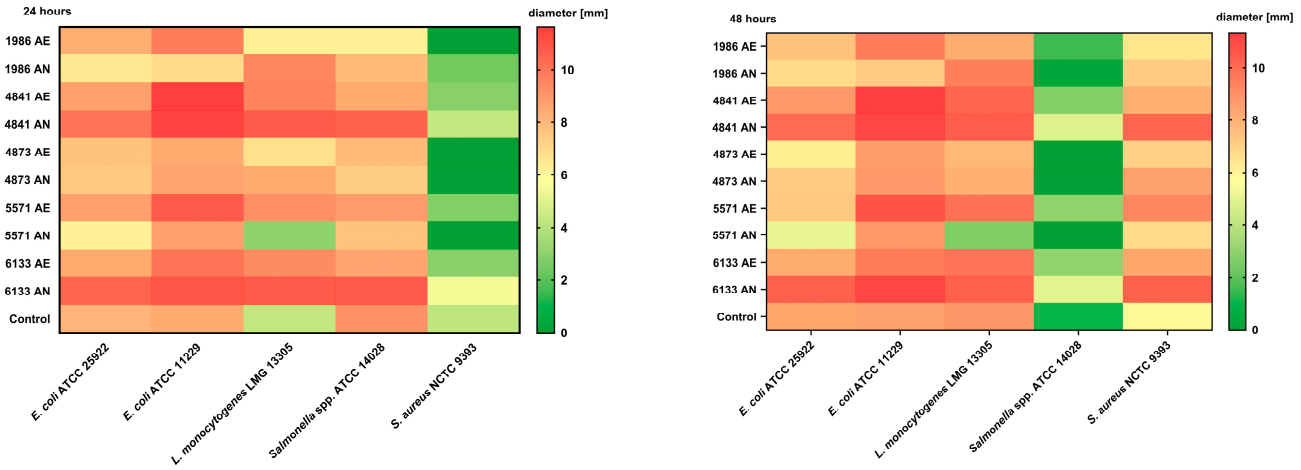
(**a**) (**b**)

Figure 2. (**a**): Boxplot picturing the Δ (T0–T3) of LAB strains growth in *Arthrospira platensis* samples. Comparisons between the strain's ability to ferment the biomass are expressed with letters. (**b**): Heatmap representing the pH changes during the three days of fermentation. Legend: AE = growth in aerobic conditions; AN = growth in anaerobic conditions.

The acidification process during fermentation was evaluated through pH measurements. Figure 2 b illustrates the pH variations during the two different types of fermentation among the five fermented biomasses over 72 h, providing a visual representation of the acidification dynamics. During fermentation, the observed decrease in pH was associated with LAB growth and the consequent production of organic acids, primarily lactic acid. At the start of fermentation, all samples exhibited an initial pH of 5.56 ± 0.01. The pH after aerobic fermentation varied depending on the strain used, with *L. fermentum* UPCCO 1986 and *L. brevis* UPCCO 4873 showing less pronounced pH decreases compared to other strains (4.90 ± 0.06 and 4.82 ± 0.02, respectively). The other strains proved a higher acidification rate, acidifying the biomass below a pH of 4.5 (*C. farciminis* UPCCO 4841 = 4.41 ± 0.01, *L. curvatus* UPCCO 6133 = 4.46 ± 0.01, and *L. diolivorans* UPCCO 5571= 4.40 ± 0.02). After 72 h of anaerobic fermentation, as pictured during aerobic fermentation, strains UPCCO 1986 and UPCCO 4873 presented higher final pH values compared to strains UPCCO 4841, UPCCO 5571, and UPCCO 6133, indicating a lower acidification of the biomass also under anaerobic conditions.

3.2. Agar Well Diffusion Assay Method

The "agar well diffusion assay" to test the antimicrobial activity of extracts was performed as described by Martelli et al. (2020) [3]. The assay is based on the measurement of the inhibition halo on a bacterial cell layer after the application of antibiotic solutions or extracts into wells on the surface of agar plates previously inoculated with the bacterial strain to be tested. The diffusion of the solution through the agar medium inhibits bacterial growth near the source, creating clear zones devoid of bacterial colonies. The diameter of these zones increases proportionally with the concentration of the inhibiting solution [18]. The antimicrobial activity of the extracts was evaluated against four strains belonging to the major foodborne pathogens: Salmonella sp. (*S. enterica* ATCC 14028), *Listeria monocytogenes* (LMG 13305), *Escherichia coli* (ATCC 25922), and *Staphylococcus aureus* (NCTC 9393), as well as the spoilage organism *E. coli* ATCC 11229. The activity of the extracts was determined by measuring the inhibition zone diameter (mm) around the well after 24 and 48 h of incubation at 37 °C. Figure 3 illustrates the measurement of the inhibition halos after 24 and 48 h of incubation.



(**a**) (**b**)

Figure 3. Antimicrobial activity of alga extracts for *Escherichia coli* (ATCC 25922 and ATCC 11229), *Listeria monocytogenes* (LMG 13305), Salmonella sp. (*S. enterica* ATCC 14028), and *Staphylococcus aureus* (NCTC 9393) represented by a heatmap. A scale ranging from a minimum of 0 mm (green) to a maximum of 12 mm (red) was used to represent the size of the inhibition diameter calculated, as average values of triplicates after 24 h (**a**) and 48 h (**b**) of incubation at 37 °C. Legend: AE = growth in aerobic conditions; AN = growth in anaerobic conditions.

Overall, the ethanolic extracts of fermented *Arthrospira platensis* exhibited antimicrobial activity against the tested microorganisms. As can be seen, not all foodborne pathogenic bacteria react in the same way. Any extracts exerted a high effect against *Staphylococcus aureus* NCTC 9393 after 24 h, proving an inhibition halo smaller than 6 mm. However, an incrementation of the inhibition was seen after 48 h, with a maximum inhibition proved by 6133 AN ($p < 0.0001$), 4841 AN ($p < 0.0001$) (10 mm), and 5571AE ($p = 0.0062$) (8.75 mm). Overall, the strain that displayed a higher susceptibility to the extracts was the spoiling *E. coli* ATCC 11229. The most efficient extracts on this strain were 4841AE ($p = 0.001$) (12 mm) and 4841AN ($p = 0.031$) (11.5 mm) that were also maintained for 48 h. Extracts 5571AE and 6133AN also demonstrated good efficacy (10 mm). For *E. coli* ATCC 25922, a moderate inhibition was observed at 24 h for most extracts but no significant differences were seen compared to the unfermented extract ($p > 0.005$). Also, for *L. monocytogenes* LMG 13305, moderate inhibition was observed. Interestingly, the antimicrobial activity was significantly higher for all the extracts after fermentation, except for 5571 AN, compared to the control. In general, a higher efficacy was recorded for extracts 4841AN and 6133AN. The very low

antimicrobial activity of unfermented *A. platensis* extract is discordant with other works in which a strong activity was seen [3]. The reduction in bioactivity could be linked to the great variability in the composition of cyanobacterial biomass that is connected to the strain and also the cultivation conditions [29].

For *Salmonella enterica* ATCC 14028, variable efficacy was noted for all extracts at 24 h, with extracts 4841AN and 6133AN showing a slightly higher activity, but all extracts proved a significantly higher activity compared to the control ($p > 0.005$). A significant reduction in efficacy ($p < 0.0001$) for almost all the tested extracts (except for 1986 AE) was observed at 48 h, which could indicate the microorganism's ability to adapt or develop resistance.

The control, consisting of the ethanolic extract of unfermented and lyophilized biomass, exhibited antimicrobial activity against most of the tested microorganisms. Only a few extracts showed higher activity compared to the control. In particular, extracts 4841AN, 5571AE, and 6133AN proved good antimicrobial activity against *Escherichia coli* ATCC 11229.

As observed in the study by Martelli et al. (2020a) [3], the extracts obtained from *A. platensis* were effective against pathogenic microorganisms; however, a general loss of efficacy was recorded during the incubation period, with some extracts becoming inactive against the tested microorganisms. These results highlight how some lactic acid bacterial strains were able to increment the antimicrobial activity. This bioactivity enhancement is not new in the literature [18]. However, for the first time, the antimicrobial activity of fermented *A. platensis* was evaluated with good results. The antimicrobial activity of unfermented *Arthrospira platensis* has been observed in several studies [30–33].

The statistical analysis revealed no significant differences between the extracts fermented with the LAB strain under aerobic and anaerobic conditions.

3.3. Microbiological Challenge Test in Analcolic Beverage

Functional beverages are substrate-rich in nutrients and if not well-treated or packed, they can be contaminated by food-spoiling microorganisms such as yeasts, lactic acid bacteria (LAB), acetic acid bacteria, *Escherichia coli*, *Alicyclobacillus* spp., and filamentous fungi during their shelf life [34]. This microbial growth can lead to a deterioration in sensory quality, typically manifested by unpleasant tastes, odors, and visual changes, ultimately reducing the overall quality of the beverages.

This study aimed to evaluate whether ethanolic extracts obtained from lyophilized and fermented biomass of *Arthrospira platensis* could control the growth of the spoilage microorganism *E. coli* ATCC 11229 over a shelf-life period of three days in flavored water. The microorganism's growth via plate counts was performed after 0, 4, 8, 24, 28, 32, 48, 52, 56, 72, 76, and 80 h of incubation at room temperature (25 °C).

The tested beverage falls under the category of functional beverages, specifically a flavored water with birch aroma. The following three extracts that exerted the highest antimicrobial activity during the agar well diffusion analysis were selected for the study: 4841AN, 5571AE, and 6133AN (Figure 4).

Figure 4a illustrates the behavior of *E. coli* ATCC 11229 in the presence of different concentrations of extract 4841AN. The growth of *E. coli* ATCC 11229 within the functional beverage remains stable at approximately 6 Log CFU/mL during the first 24 h, indicating a lag phase as the microorganism adapts to the new environment. After 24 h, differences in growth were observed depending on the concentration of the extract tested. At a concentration of 13.75 mg/mL, a decrease of 2 logarithmic units was observed, reaching a concentration of 4.2 ± 0.07 Log CFU/mL, with a further decrease to 2.00 ± 0.00 log CFU/mL (detection limit) by 80 h, showing a reduction of 4.84 log CFU/mL. At a concentration of 6.87 mg/mL, a decrease of 2 logarithmic units was observed after 48 h, followed by an additional decrease of 1 logarithmic unit at 56 h, resulting in a final concentration of 3.19 ± 0.26 Log CFU/mL and a microbial concentration reduction of 3.56 log CFU/mL. At lower concentrations (3.43 mg/mL and 1.71 mg/mL), no significant reduction in *E. coli* load was observed, with microbial counts remaining around 6 log CFU/mL up to 80 h (5.99 ± 0.05 and 6.44 ± 0.30, respectively). In the control sample (functional beverage with

E. coli inoculum without extract), the strain grew and multiplied, reaching concentrations of 7.95 ± 0.15 log CFU/mL, with a concentration increase of 1.11 log CFU/mL. Figure 4b shows the growth trend of *E. coli* in the selected functional beverage enriched with 5571AE. Like the previous extract, *E. coli* concentrations remained stable at the inoculum level during the first 24 h, with differences in growth observed after 24 h due to varying extract concentrations. At a concentration of 13.75 mg/mL, a gradual decrease in *E. coli* load was observed, reaching 3.28 ± 0.03 log CFU/mL after 48 h. This is followed by a stationary phase of 24 h before a further decline to the detection limit (2.00 ± 0.00 log CFU/mL), with a decrease of 4.59 log CFU/mL. At 6.87 mg/mL, the *E. coli* load remained nearly unchanged until 56 h post-inoculation. A rapid decrease in the concentration below the detection limit (2.00 ± 0.00 log CFU/mL) was observed at 72 h, remaining constant until 80 h, with a decrease of 4.51 log CFU/mL. As with extract 4841AN, no significant reduction in *E. coli* load was observed at concentrations of 3.43 mg/mL and 1.71 mg/mL, with microbial counts remaining at the inoculum level up to 80 h (6.42 ± 0.02 and 6.70 ± 0.06, respectively).

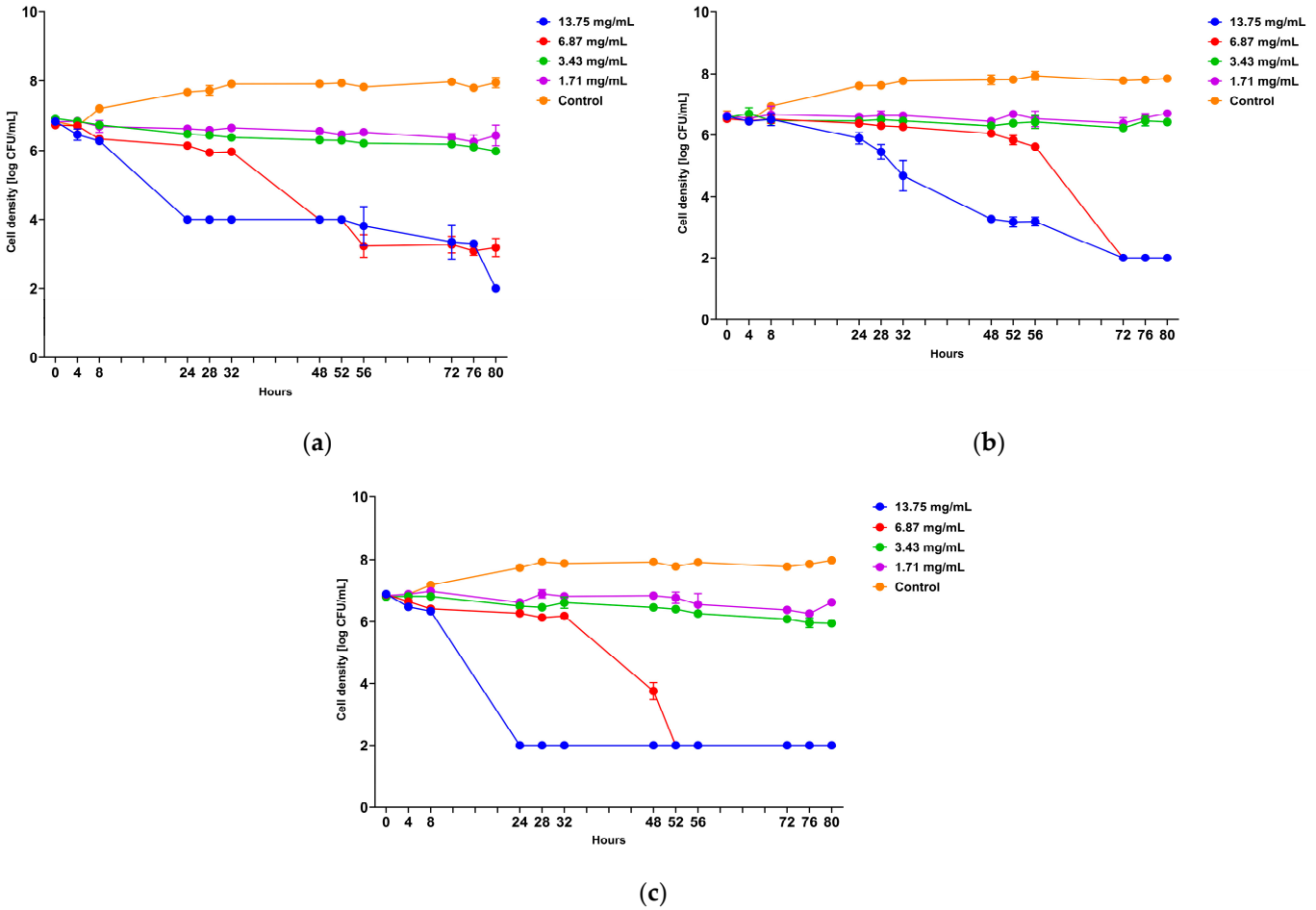


Figure 4. Behavior of *Escherichia coli* ATCC 11229 during 80 h of shelf life in flavored water with different concentrations of *A. platensis* extracts: 13.75 mg/mL (blue), 6.87 mg/mL (red), 3.43 mg/mL (green), 1.71 mg/mL (purple), and 0 (control) (orange). (**a**) 4841AN; (**b**) 5571AE; (**c**) 6133AN.

In Figure 4c, the *in situ* activity of extract 6133AN is displayed. During the first 24 h, *E. coli* concentrations remained stable, indicating a lag phase due to adaptation to new environmental conditions. After 24 h, differences in growth were observed depending on the extract concentrations. At a concentration of 13.75 mg/mL, a 4-logarithmic unit decrease was observed 24 h post-inoculation, reaching the detection limit of 2.00 ± 0.00 log CFU/mL and remaining constant throughout the study, with a decrease of 4.89 log CFU/mL. At 6.87 mg/mL, *E. coli* concentrations remained constant until 32 h, followed by a decline to

below 2.00 ± 0.00 log CFU/mL by 52 h, with the levels remaining unchanged until 80 h. At concentrations of 3.43 mg/mL and 1.71 mg/mL, *E. coli* did not show any reduction, with counts remaining stable throughout the experiment.

A two-way ANOVA was performed for the concentration of 13.75 mg/mL for all three extracts, underlining the significance of the interactions between the time and type of extract. It was found that there were no significant differences among the extracts during the first 8 h of incubation. After 24 h, the three samples exhibited distinct behaviors: extract 6133AN proved a significantly higher reduction in *E. coli* concentration compared to the other two extracts ($p < 0.0001$); extract 5571AE required more time, reducing *E. coli* levels gradually and only reaching undetectable levels after 72 h; extract 4841AN significantly reduced *E. coli* counts to approximately 4 log CFU/mL after 24 h, maintaining this level until 76 h and finally reaching below 2 log CFU/mL after 80 h. Overall, the antimicrobial activity of the extracts was concentration-dependent, with higher concentrations exhibiting bactericidal effects and lower concentrations displaying bacteriostatic effects. The statistical analysis indicates faster *E. coli* inhibition in the presence of the extract 6133AN, proving bactericidal activity already after 24 h of incubation. This confirms the antibacterial activity of extracts 4841AN, 5571AE, and 6133AN. Given their growth-inhibitory effects, these extracts could have potential applications as food additives, particularly as preservatives. Not many articles are present in the literature that aim to assess the antimicrobial activity in situ of *A. platensis* extracts through a microbiological challenge test [3], and none have ever assessed it in flavored waters. Also, for the first time, the antimicrobial activity of extracts obtained from lacto-fermented *A. platensis* was assessed. Other strategies can be used to limit the presence of spoiling microorganisms in beverages and fruit juices [35,36], but the use of natural flavors that can guarantee a clean label most accepted by consumers [37,38].

3.4. Lipid-Reducing Effect of Extracts and 2-NDBG

All the extracts obtained, fermented and unfermented *A. platensis*, were screened for lipid-reducing effects using the zebrafish Nile red fat metabolism assay. The obtained results are presented in Figure 5a. No activity was noticed in unfermented *A. platensis.* Many studies have assessed the lipid-reducing activity of *cyanobacteria*, obtaining positive results [39,40]. The variability in the presence of bioactive compounds in these cyanobacteria can be explained by the variable composition that is strictly correlated to the strain and cultivation type [29,41]. Only one of the tested extracts has produced a significant difference from the solvent control group (DMSO), underlining possible use as a bioactive product (4873AE). This extract was obtained by fermenting the biomass in aerobic conditions with the strain *Levilactobacillus brevis* UPCCO 4873. This is the first time that this species of microorganisms was used to ferment *A. platensis* biomass and assess the lipid-reducing effect of this extract. Other data are present in the literature that have evaluated the lipid-reducing effect of fermented *A. platensis,* but different strains were used [22]. A strong lipid-reducing activity was observed after fermentation with *Lactobacillus delbrueckii bulgaricus* UPCCO 1932. The results suggest that the use of QPS LAB strains is a promising technique to enhance the bioactivities of microalgal biomasses. Further study should be conducted to enlarge the screening and unravel the mechanisms of action of these extracts. The metabolite profile performed by Martelli et al. [22] suggested that the fermentation has changed the composition in n clusters of fatty acids and fatty amides. The supplementation of PUFA was already related to the amelioration of obesity and other comorbidities [42–44]. Lactic acid fermentation is a biotechnological process with very well-known beneficial effect to health [45,46]. Some evidence that lipid accumulation is reduced when some classes of microorganisms are colonizers of the human gut has been produced in several research papers [47–49]. Any of the extracts produced have significantly impacted the glucose uptake in zebrafish larvae (Figure 5b). Further study and a wider screening will be performed to study the possible effect of fermented microalga extracts on glucose uptake.

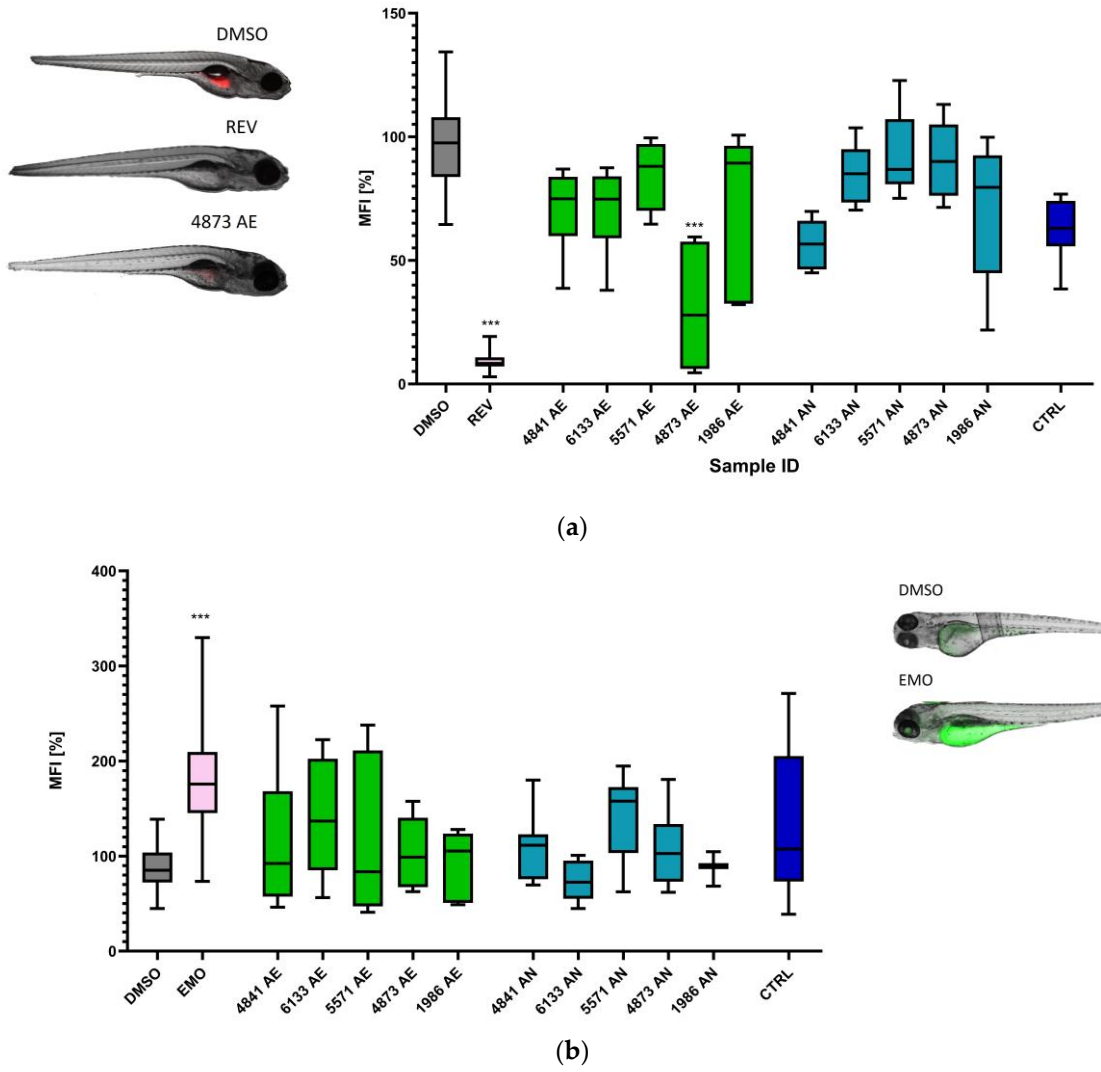


Figure 5. (**a**) Reduction in lipid accumulation evaluated in zebrafish larvae. Data are presented as the mean fluorescence intensity (MFI) relative to the solvent control (DMSO). Representative images of five DPF zebrafish larvae. Solvent control, 0.1% dimethyl sulfoxide (DMSO); positive control, 50 µM REV (resveratrol) and exposure to extract 4873 AE at 25 µg/mL. (**b**) Results of the 2-NDBG assay. Data are presented as the mean fluorescence intensity (MFI) relative to the solvent control (DMSO). Representative images of five DPF zebrafish larvae. Solvent control, 0.1% dimethyl sulfoxide (DMSO); positive control and 50 µM EMO (Emodine). Data in both graphs are presented as box–whisker plots from two independent assays (n = 6–9) for each extract. Significant differences compared to the solvent control were analyzed by a one-way ANOVA and Dunnett's post hoc test, and are shown as asterisks (*** = $p < 0.001$). In grey: solvent control (DMSO); in pink: positive control (REV) (Figure 5a) and (EMO) (Figure 5b); in green: extracts obtained from aerobically fermented *A. platensis*; in light blue: extracts obtained from anaerobically fermented *A. platensis*; in purple: unfermented *A. platensis*.

3.5. Evaluation of Polyphenolic and Antioxidant Content

The total phenolic content of the various fermented *A. platensis* extracts, along with the unfermented extract, is presented in Figure 6a. The quantification of total phenolic compounds (TPCs) in the analyzed extracts was performed according to the protocol outlined by Martelli et al. (2020a) [3], with results expressed as gallic acid equivalents (GAE) per gram of sample.

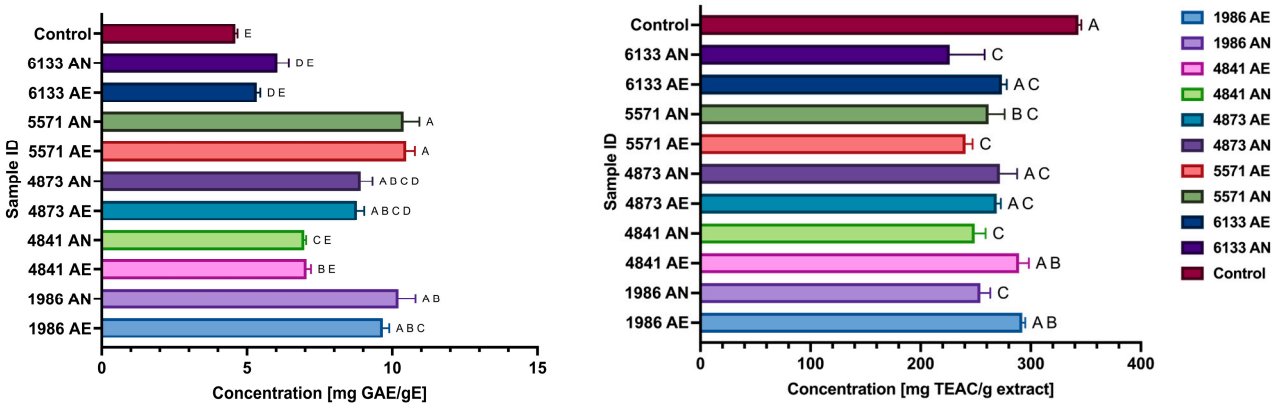


Figure 6. (**a**) Total phenolic content of the fermented *A. platensis* extracts quantified with the Folin–Ciocalteau method. Comparisons between the TPCs of different samples are expressed with letters; (**b**) antioxidant activity of fermented *A. platensis* quantified with a DPPH assay. Comparisons between the total antioxidant activity of different samples are expressed with letters.

The TPC values exhibited variability across the different samples, indicating a large range of phenolic content. Extract 5571AE demonstrated the highest concentration of phenolic compounds, at 10.48 ± 0.30 mg GAE/g, followed by extract 5571AN at 10.39 ± 0.55 mg GAE/g and extract 1986AN at 10.22 ± 0.58 mg GAE/g. Extracts fermented with *Latilactobacillus curvatus* UPCCO 6133 and *Companilactobacillus farciminis* UPCCO 4841 showed the lowest TPC concentrations compared to other extracts, and did not exhibit statistically significant differences from the unfermented extract. Additionally, no significant differences were observed between extracts fermented under aerobic and anaerobic conditions, indicating that the presence of oxygen does not substantially influence TPC levels. Statistical analysis identified significant differences ($p < 0.05$) in TPC concentrations in six out of ten fermented extracts compared to the unfermented control. This finding aligns with previous research, which has demonstrated an increase in TPC concentration in the lactic-acid-fermented *A. platensis* extract [50,51]. In this study, the antioxidant activity of extracts from both fermented and unfermented *A. platensis* was evaluated using the DPPH radical scavenging activity assay. This assay is widely employed to assess the ability of compounds to donate hydrogen atoms, neutralizing free radicals, and thus providing a measure of antioxidant capacity. The results of this analysis are presented in Figure 6b, illustrating the comparative antioxidant activities across the different extracts and the unfermented *A. platensis*. Interestingly, almost all extracts (except extract 4873AN) obtained from *A. platensis* fermented under anaerobic conditions exhibited a significant decrease ($p < 0.05$) in antioxidant activity compared to the unfermented counterpart. Specifically, the percentage of inhibition was reduced by approximately 20%, indicating a significant loss of free radical-scavenging compounds following fermentation. This reduction suggests that the fermentation process under anaerobic conditions may negatively affect the compounds responsible for antioxidant activity, such as phycocyanins or other potentially antioxidant components, or lead to their degradation. This result may be influenced by the extraction method employed. Stunda-Zujeva et al. (2023) demonstrated that the ethanol extracts of *A. platensis* contain lower concentrations of phycocyanin compared to aqueous extracts, potentially affecting antioxidant activity [52]. Additionally, fermentation time is a critical factor in the release and stability of antioxidant compounds. Marco Castro et al. (2019) reported that while lactic acid fermentation enhances the nutraceutical profile of *A. platensis*, a reduction in total antioxidant capacity occurs after 36 h of fermentation, likely due to the degradation of antioxidants that are sensitive to oxygen exposure and thermal conditions [50]. The quantification of total phenolic content (TPC) in the fermented *Arthrospira platensis* extract revealed an increase in phenolic compounds for most of the extracts analyzed. However,

DPPH scavenging assay results indicated a reduction in the antioxidant capacity of the *A. platensis* extract following fermentation. These findings agree with what was studied by Rumpf et al. (2023) in which no positive correlations between the two assays were seen [53]. The authors suggest using different methods to measure the antioxidant capacity, as it is such a complex property.

4. Conclusions

This study proved the great potential that the supplementation of natural extracts obtained from lacto-fermented *Arthrospira platensis* could play in the formulation of functional beverages, enhancing the bioactivities and shelf life of soft drinks. All five tested QPS LAB species effectively fermented the cyanobacterial biomass, producing ethanolic extracts with notable in vitro antimicrobial activity against common foodborne pathogens and efficacy in preserving soft drinks from *Escherichia coli*. Promising bioactivities, such as lipid-reducing effects and antioxidant activity, were seen, suggesting the efficacy of fermentation in modifying ingredients by enhancing bioactivities that could be used as functional ingredients to be added in beverages. Different LAB starters proved different abilities in modifying the composition of bioactive compounds. *Companilactobacillus farciminis* UPCCO 4841, *Lentilactobacillus diolivorans* UPCCO 5571, and *Latilactobacillus curvatus* UPCCO 6133 enhanced the antimicrobial properties of *A. platensis*, suggesting a role for these extracts as natural preservatives for clean-label beverages. Meanwhile, *Levilactobacillus brevis* UPCCO 4873 showed the ability to significantly increment the composition of *Arthrospira platensis* with lipid-reducing compounds, supporting the potential applications in health-oriented food supplements and functional beverages. In this context, a synergic effect of the extracts could play a crucial role in producing functional beverages with a long shelf life. Future studies should assess the consumer acceptance of these extracts in soft drinks, particularly evaluating taste and color through panel testing.

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