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OPTIMIZATION OF MICRO- AND MACROALGAL BIOMASS PRETREATMENT FOR ITS CONVERSION TO HIGHER VALUE FEED MATERIALS

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CONTENTS

ABBREVIATIONS

ABTS•+	–	2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity
Ag	–	silver
Al	–	aluminium
As	–	arsenic
B	–	boron
Ba	–	barium
BA	–	biogenic amines
Be	–	beryllium
C16:0	–	methyl palmitate
C18:2	–	methyl linoleate
C18:3ɣ	–	gamma-linolenic acid methyl esters
Ca	–	calcium
CAD	–	cadaverine
Cd	–	cadmium
CFU	–	colony-forming units
ClaG	–	*Cladophora glomerata*
ClaR	–	*Cladophora rupestris*
Co	–	cobalt
Cr	–	chromium
Cs	–	caesium
Cu	–	copper
DIZ	–	diameter of inhibition zone
DM	–	dry matter
DPPH•-	–	2,2-diphenyl-1-picrylhydrazyl radical scavenging activity
FA	–	fatty acids
FAME	–	fatty acid methyl esters
Fe	–	iron
FRAP	–	ferric ion-reducing antioxidant power
Furc	–	*Furcellaria lumbricalis*
Ga	–	gallium
GABA	–	gamma-aminobutyric acid
GAE	–	gallic acid equivalents
Hg	–	mercury
HIS	–	histamine
HPLC	–	high-performance liquid chromatography
I	–	iodine
K	–	potassium
LAB	–	lactic acid bacteria
L-Glu	–	L-glutamic acid
Li	–	lithium
LSMU	–	Lithuanian University of Health Sciences
Mg	–	magnesium
Mn	–	manganese
Mo	–	molybdenum
Na	–	sodium

Ni – nickel
P – phosphorus
Pb – lead
PHE – phenylethylamine
PUT – putrescine
Rb – rubidium
Sb – stibium
Se – selenium
Si – silicon
SMF – submerged fermentation
Sn – tin
SPRM – spermine
SPRMD – spermidine
Sr – strontium
SSF – solid-state fermentation
TBC – total bacteria count
TCC – total carotenoids count
Ti – titanium
TPC – total phenolic compounds
TPTZ – 2,4,6-tri-2-pyridinyl-1,3,5-triazine
TRP – tryptamine
TYR – tyramine
Ul – *Ulva intestinalis*
V – vanadium
Zn – zinc

INTRODUCTION

The increasing demand for nutrients in the livestock industry has led to broader utilization of micro- and macroalgal biomass as natural sources of valuable compounds [1–3].

Macroalgae are natural components of the marine ecosystem, and their chemical composition makes them desirable for various industrial applications [4]. Currently, the major producers of algae are China (47.9%), Indonesia (38.7%), the Philippines (4.7%), and the Republic of Korea (4.5%), while the major producers of wild macroalgae are Chile, China, and Norway [5]. Marine macroalgae, in particular, are a good alternative to terrestrial biomass because they do not compete with plants for use as food or in feed preparation, and they do not require special resources to accumulate biomass [6].

Microalgae are becoming an important source of bioactive compounds for many industries; however, only a few varieties of microalgae have been generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) and can be used for food or feed: *Arthrospira platensis* (Spirulina), which is a member of the phylum Cyanobacteria; *Chlamydomonas reinhardtii*, *Auxenochlorella protothecoides*, *Chlorella vulgaris*, and *Dunaliella salina* (formerly *Dunaliella bardawil*), which are members of *Chlorophyta*; and *Euglena gracilis*, a member of *Euglenozoa* [7]. Spirulina is one of the most popular microalgae [8]. It contains a high protein content, valuable essential fatty acids (FA), minerals, pigments, carotenoids, and vitamins [9]. The prebiotic and antioxidant properties of Spirulina have also been widely reported [9–11]. There is scientific evidence attesting to Spirulina's antibacterial, antiviral, and immunomodulatory properties [12–16]. Moreover, Spirulina is a safe ingredient when grown under controlled conditions [10, 12–14, 17, 18].

The growing need for functional materials encourages increased consumption of algae by introducing these bioactive components into feed formulas [19]. One of the most popular ways to provide additional value to feed is lacto-fermentation. Despite the long history of feed fermentation technology, studies about fermented feed material produced from algae are scarce. This could be related to the specific chemical composition of algal carbohydrates, which contain mostly polysaccharides (alginate and fucoidan in brown algae, galactan in red algae, and cellulose and hemicelluloses in green algae), which are known to be unfavorable substrates for fermentation [20, 21]. Biotransformation with lactic acid bacteria (LAB) is a popular solution to degrade plant and cyanobacterial cell walls and to produce smaller

molecules with enhanced properties (immunomodulatory, antioxidant, antimicrobial, etc.) [22–25]. LAB show economic advantages at the industrial scale and are GRAS microorganisms [26].

Furthermore, biotechnological methods are evolving to align with sustainability standards [27, 28]. Solid-state fermentation (SSF) involves the growth of microorganisms and production of substances on solid substrates without the presence of free water. This approach is more cost-effective than the conventional technique of cultivating biomass in a nutrient-rich liquid medium [29]. Additionally, SSF is frequently recognized for its ability to lower overall expenses compared with liquid fermentation [30]. The minimal water usage in SSF significantly enhances economic efficiency, primarily due to the smaller size of bioreactors, decreased costs for downstream processing, and reduced expenses for sterilization, among other factors.

Algal biomass pretreatment (e.g., lacto-fermentation, ultrasonication, etc.) before extraction can lead to higher functionality of algae-based products (higher antioxidant activity and total phenolic compound (TPC) content, as well as stronger antimicrobial properties against a broader spectrum of pathogenic and opportunistic strains). The importance of algal biomass pretreatment can be explained by the composition of algal cells, which are protected by complex cell walls [31, 32]. It has been reported that the crucial step in obtaining bioactive compounds from micro- and macroalgal biomass is efficient cell disruption [33]. Some algal pretreatment technologies have been described in the literature, and the most effective mechanical and biological techniques have been mentioned [34, 35]. Despite the fact that physical pretreatment is a cost-intensive process, ultrasonication has been recommended as the most promising method for cell disintegration [32, 36, 37]. Ultrasound breaks down the cell structure and improves material transfer by enhancing extraction from microalgae [32, 38–40]. Moreover, biological pretreatment with fungi, bacteria, and/or their enzymes can be used to degrade lignin and hemicelluloses of algal cells [35, 41]. In addition to lignin breakdown, biological pretreatment produces other valuable compounds such as phenolic acids, benzoic acid, syringaldehyde, and others [42]. Despite the many desirable properties of biological pretreatment, it should be noted that nondesirable compounds can also be synthesized. For example, gamma-aminobutyric acid (GABA) is a desirable functional compound that can be obtained through the decarboxylation of L-glutamic acid (L-Glu) by the intracellular enzyme glutamate decarboxylase [43–45], and Spirulina [46–48] has a significant content of GABA precursors [49]. However, even though the functional value of Spirulina can be enhanced through biomass fermentation with selected LAB strains – such as *Lactobacillus* and other LAB cocci known for abundant GABA production, including *Levilactobacillus brevis*

[43, 50–56], *Lactobacillus buchneri* [57, 58], *Lactobacillus delbrueckii* subsp. *bulgaricus* [54, 59], *Lactobacillus fermentum* [60, 61], *Lactobacillus helveticus* [43, 62], *Lacticaseibacillus paracasei* [54, 63], and *Lactiplantibacillus plantarum* [43, 54, 63, 64], among others – the decarboxylation process can lead to the formation of undesirable compounds (e.g., biogenic amines (BA)). Most BA are classified as nondesirable compounds [65, 66]. The most toxic BA are tyramine (TYR) and histamine (HIS) [67, 68].

Finally, despite the fact that algal biomass represents a very promising material for feed preparation, its chemical composition, including both desirable and nondesirable compounds, should be evaluated. Moreover, the most appropriate solutions for algal biomass pretreatment should be identified to ensure their safety parameters and to increase their functional value.

The aim of the study

The aim of the study was to develop innovative technologies to modify micro- and macroalgal biomass into value-added feed raw materials.

Objectives of the study

1. To generate a data base of the micro- and macroelement profiles of the macroalgae *Ulva intestinalis*, *Cladophora rupestris*, and *Furcellaria lumbricalis*, and to evaluate the effectiveness of fermentation with *Lb. plantarum* LUHS135 on the microbial profile, and antimicrobial and antioxidant properties of *U. intestinalis*, *C. rupestris*, and *F. lumbricalis*.
2. To evaluate the characteristics (including the total carotenoid content (TCC), the chlorophyll *a* and *b* concentrations, the TPC content, the antioxidant and antimicrobial properties, and the transmission of trace elements from algal samples to extracts) of the extracts of the macroalgae *U. intestinalis*, *C. rupestris*, *F. lumbricalis*, and *Cladophora glomerata*, as well as the microalgae Spirulina and *C. vulgaris*.
3. To analyze the influence of fermentation and ultrasonication pretreatment on *U. intestinalis*, *C. rupestris*, *F. lumbricalis*, *C. glomerata*, and Spirulina extracts, as well as their combinations with *Lb. plantarum* LUHS135, on the functional (antimicrobial and antioxidant) properties of the extracts.
4. To evaluate the changes in Spirulina characteristics (including the BA content, the L-Glu and GABA concentrations, and the FA profile) during submerged fermentation (SMF) and SSF with *Lb. plantarum* No. 122, *Lb. casei* No. 210, *Lactobacillus curvatus* No. 51,

Lb. paracasei No. 244, *Lactobacillus coryniformi*s No. 71, *Pediococcus pentosaceus* No. 183, *Lactobacillus brevis* No. 173, *Pediococcus acidilactici* No. 29, *Leuconostoc mesenteroides* No. 225, and *Liquorilactobacillus uvarum* No. 245, and to analyze the antimicrobial activity of Spirulina samples with the lowest BA content and the highest GABA concentration.

The scientific novelty and practical usefulness

Algae grow extremely fast and are capable of converting large amounts of carbon dioxide into oxygen. Some species of algae can be grown on alternative substrates, and their biomass growth is very intense. For these reasons, algae represent a prospective source for animal feed. However, there are limited data regarding the detailed chemical composition and microbial profile of Baltic Sea macroalgae. Additionally, despite the fact that the data about Spirulina's properties have been reported in many scientific studies, there is missing information regarding the best pretreatment for Spirulina to increase the functionality of this valuable material. The scientific novelty of this study lies in (I) the creation of new data regarding the detailed chemical composition and microbial profile of Baltic Sea macroalgae and (II) the development of novel solutions for micro- and macroalgal biomass pretreatment to develop safe materials with greater value. The results have practical significance because they demonstrate a new approach for pretreating macro- and microalgae to convert them into higher value safe feed materials. This study represents a promising field associated with the safe and sustainable application of algae materials, including in the livestock sector, meeting the current need for more sustainable solutions in agriculture.

1. MATERIALS AND METHODS

1.1. Investigation venue

The experiments were conducted from 2020 to 2024 at the Lithuanian University of Health Sciences (LSMU) Institute of Animal Rearing Technologies; the Institute of Microbiology and Virology at Klaipėda University, the Marine Research Institute (Klaipėda, Lithuania); the Institute of Food Safety, Animal Health, and Environment – "BIOR" (Riga, Latvia); and the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry (Babtai, Lithuania).

1.2. Materials and principal schemes of the experiments

1.2.1. Baltic Sea macroalgae, microalgae, and lactic acid bacteria strains used for algal treatment

1.2.1.1. Macroalgae

U. intestinalis, *C. rupestris*, and *F. lumbricalis* samples were collected in September 2020 and May–June 2021 near Klaipeda, Lithuania. *U. intestinalis* was hand-picked from 10 different stones up to a depth of 1 m. *C. rupestris* and *F. lumbricalis* were collected from the water (~1 m depth) along the shore (about 100 m) a few days after a storm. *C. glomerata* was collected only in May–June 2021 from stones near the surface of the water. The algal samples were cleaned by carefully removing other entangled algae and macroscopic animals and then washing them twice – first with tap water and then with distilled water. After washing, the specimens were frozen at –80 °C. Subsequently, the samples were milled to 1-mm particles and stored in a sealed plastic bag in a freezer (–80 °C) for further analysis.

1.2.1.2. Microalgae

Spirulina samples were obtained from three different companies: (I) Sp1, Spirulina obtained from the University of Texas Biological Labs (Austin, Texas, USA) and cultivated following the manufacturer's guidelines); (II) Sp2, Spirulina obtained from Ltd. "Spila" (Vilnius, Lithuania, with an origin from Irvine, CA, USA) in powder form; and (III) Sp3, lyophilized Spirulina powder provided by Now Foods Company (Bloomingdale, IL, USA). *C. vulgaris* was acquired from the Biological Laboratories at the University of Texas (Austin, Texas, USA) and multiplied following the manufacturer's guidelines.

1.2.1.3. Lactic acid bacteria strains used for micro- and macroalgal fermentation

The LAB strains (*Lb. plantarum* No. 135, *Lb. plantarum* No. 122, *Lb. casei* No. 210, *Lb. curvatus* No. 51, *Lb. paracasei* No. 244, *Lb. coryniformis* No. 71, *P. pentosaceus* No. 183, *Lb. brevis* No. 173, *P. acidilactici* No. 29, *L. mesenteroides* No. 225, and *Lb. uvarum* No. 245) were acquired from the LSMU collection (Kaunas, Lithuania). Before the experiment, the LAB strains were grown in De Man, Rogosa, and Sharpe (MRS) broth culture medium (Biolife, Milano, Italy) at 30 °C under anaerobic conditions for 24 h.

1.2.1.4. Other materials used in experiments

Pathogenic and opportunistic bacterial strains (*Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Enterococcus durans*, *Bacillus pseudomycoides*, *Salmonella enterica*, *Aeromonas hydrophila*, *Aeromonas veronii*, *Acinetobacter baumannii*, *Acinetobacter johnsonii*, *Enterobacter cloacae*, *Cronobacter sakazakii*, *Kluyvera cryocrescens*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Pseudomonas aeruginosa*), used to test the antimicrobial properties of the algae samples, were acquired from the LSMU collection (Kaunas, Lithuania).

1.2.2. Optimization of micro- and macroalgal biomass pretreatment methods

For the first stage of the experiment, *U. intestinalis*, *C. rupestris*, and *F. lumbricalis* samples were subjected to SMF with *Lb. plantarum* LUHS135. Before and after pretreatment, the metagenomic profile, antimicrobial and antioxidant properties, and the micro- and macroelement contents of the samples were analyzed. The principal scheme of the experiment is provided in Fig. 1.2.2.1. The entire experiment is described in detail by Tolpeznikaite et al. [69].

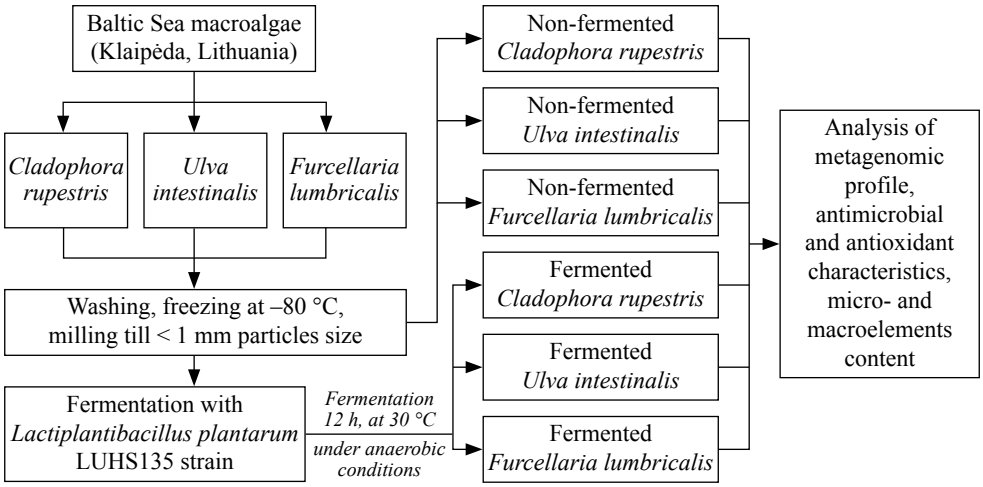


Fig. 1.2.2.1. *Principal scheme for the pretreatment and analysis of the macroalgae Ulva intestinalis, Cladophora rupestris, and Furcellaria lumbricalis*

For the second stage of the experiment, extracts from the macroalgae *U. intestinalis*, *C. rupestris*, *F. lumbricalis*, and *C. glomerata*, and the microalgae Spirulina and *C. vulgaris* were prepared. Following pretreatment, the TCC, the chlorophyll *a* and *b* concentrations, the TPC content, the antioxidant and antimicrobial characteristics, as well as the micro- and macroelement contents of the samples were analyzed. The principal scheme of the experiment is provided in Fig. 1.2.2.2.

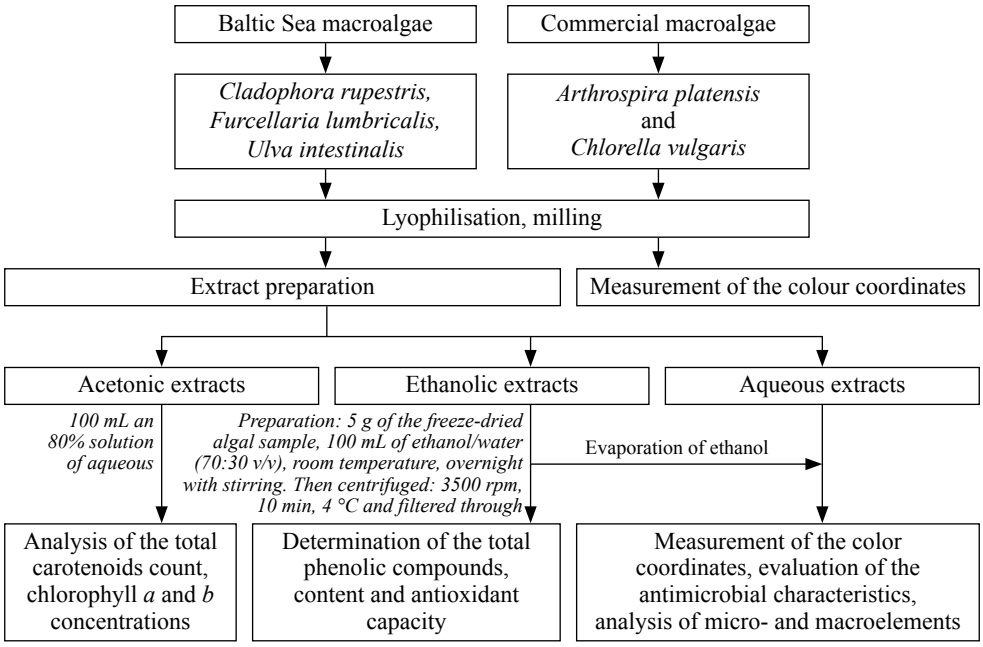


Fig. 1.2.2.2. *Principal scheme for the preparation and analysis of Ulva intestinalis, Cladophora rupestris, Furcellaria lumbricalis, Cladophora glomerata, Spirulina, and Chlorella vulgaris extracts*

For the third stage of the experiment, *U. intestinalis*, *C. rupestris*, *F. lumbricalis*, *C. glomerata*, and Spirulina were subjected to (I) SMF with *Lb. plantarum* LUHS135 and (II) ultrasonication. Extracts were prepared from both nontreated and fermented as well as ultrasonicated algae. Additionally, combinations of the extracts with *Lb. plantarum* LUHS135 were prepared and analyzed. Three groups of sample were obtained: (I) extracts and extract × *Lb. plantarum* LUHS135 combinations prepared from nonpretreated algae, (II) extracts and extract × *Lb. plantarum* LUHS135 combinations prepared from ultrasonicated algae, and (III) extracts and extract × *Lb. plantarum* LUHS135 combinations prepared from fermented algae. In each group, the pure extract and the extract and *Lb. plantarum* LUHS135 combinations were tested. Before and after pretreatment, the color coordinates, acidity parameters, and antimicrobial and antioxidant properties of the samples were analyzed. The principal scheme of the experiment is provided in Fig. 1.2.2.3. The entire experiment in details is described by Tolpeznikaite et al. [70].

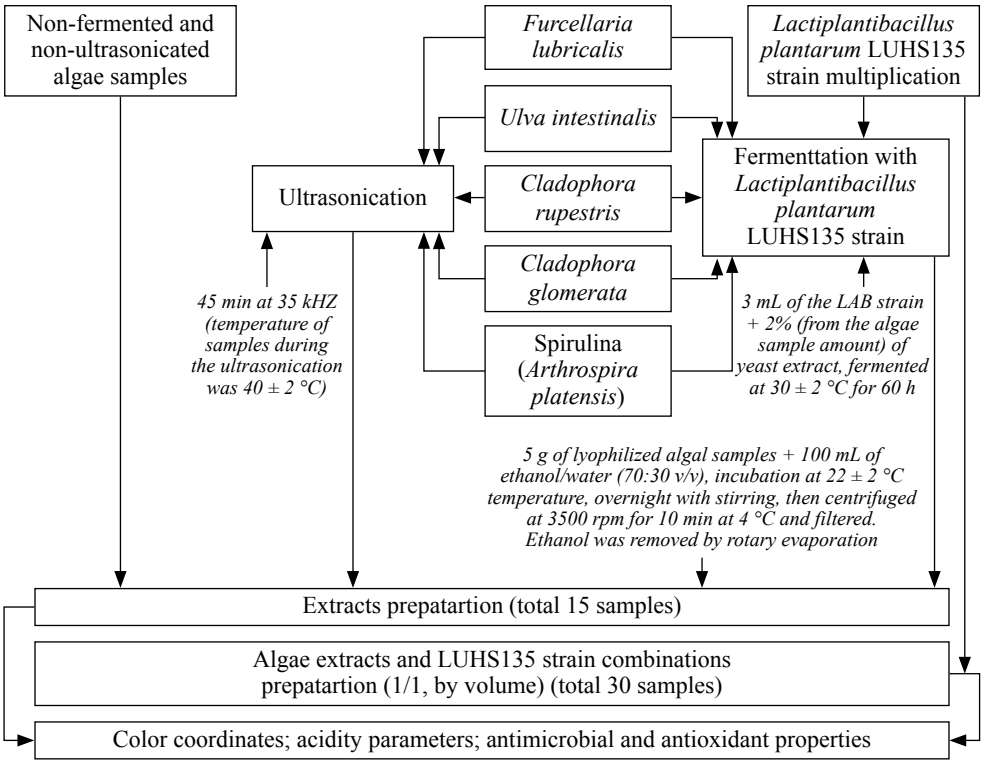


Fig. 1.2.2.3. *Principal scheme for the analysis of extracts from Ulva intestinalis, Cladophora rupestris, Furcellaria lumbricalis, Cladophora glomerata, and Spirulina, as well as their combinations with Lactiplantibacillus plantarum LUHS135*

For the fourth stage of the experiment, Spirulina samples were subjected to SMF and SSF with the following strains: *Lb. plantarum* No. 122, *Lb. casei* No. 210, *Lb. curvatus* No. 51, *Lb. paracasei* No. 244, *Lb. coryniformis* No. 71, *P. pentosaceus* No. 183, *Lb. brevis* No. 173, *P. acidilactici* No. 29, *L. mesenteroides* No. 225, and *Lb. uvarum* No. 245. Before and after pretreatment, the pH, color characteristics, BA content, L-Glu and GABA concentrations, and FA profile of the samples were analyzed. The principal scheme of the experiment is provided in Fig. 1.2.2.4. The entire experiment is described in detail by Tolpeznikaite et al. [71].

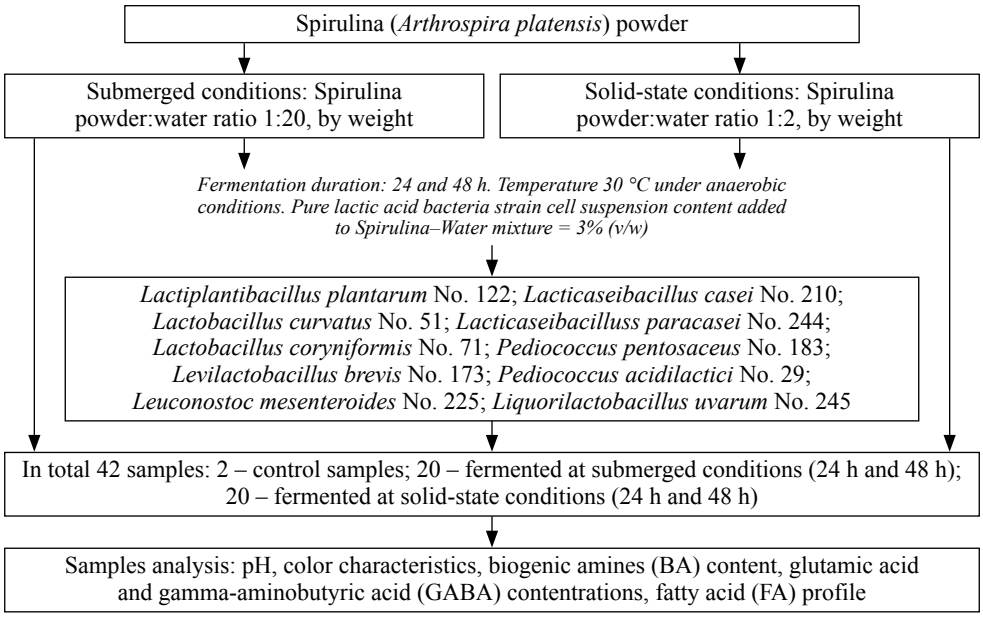


Fig. 1.2.2.4. *Principal scheme of the Spirulina solid-state fermentation (SSF) and submerged fermentation (SMF) experiment*

Finally, for the fifth stage of the experiment, Spirulina samples underwent SMF and SSF with *Lb. paracasei* No. 244, *Lb. brevis* No. 173, *L. mesenteroides* No. 225, and *Lb. uvarum* No. 245. Before and after pretreatment, the antimicrobial activity of the Spirulina samples with the lowest BA content and the highest GABA concentration was analyzed. The principal scheme of the experiment is provided in Fig. 1.2.2.5. The entire experiment is described in detail by Tolpeznikaite et al. [29].

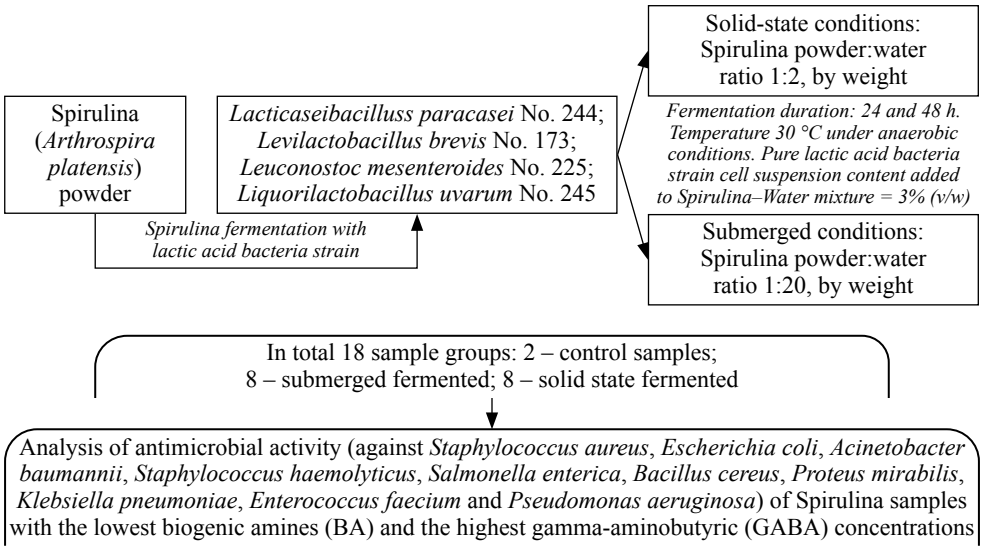


Fig. 1.2.2.5. *The principal scheme for evaluating the antimicrobial properties of non-pretreated and fermented Spirulina samples*

1.2.3. Analysis methods

1.2.3.1. Microbiological analysis methods

The LAB colony-forming unit (CFU) counts were determined according to the ISO 15214:1998 standard [72]. In brief, 10 g of sample was homogenized with 90 mL of aqueous saline (9 g L^{-1} NaCl solution). Serial dilutions of 10^{-4} to 10^{-8} with the same saline solution were prepared for inoculation. Petri dishes with 5 mm of sterile MRS agar (CM0361, Oxoid, Basingstoke, UK) were used for cultivation. The dishes were individually inoculated with the sample suspension using surface sowing and then incubated under anaerobic conditions at 30 °C for 72 h. The results are expressed as $\log_{10}$ of CFU per gram ($\log_{10}$ CFU g^{-1}) [73].

The total bacterial count (TBC) was determined using plate count agar (PCA, CM0325, Oxoid Ltd.). The bacteria were incubated under aerobic conditions at 32° C for 24–48 h. (CM0325, Oxoid, UK). The number of microorganisms was calculated and is expressed as $\log_{10}$ CFU per gram and/or milliliter ($\log_{10}$ CFU g^{-1} and/or CFU mL^{-1}).

1.2.3.2. Microbial profiling

Next-generation sequencing was performed based on 16S ribosomal RNA (rRNA) with the aim to explore the microbiome of the bacteria in algae before and after fermentation. Each sample (1 g) was kept in DNA/RNA

Shield (1:10, w/v) (R1100-250, Zymo Research, Irvine, CA, USA) at –70 °C before DNA extraction. DNA was extracted with a DNA MiniPrep kit (D6010, Zymo Research) [74]. The ZymoBIO- MICS Microbial Community Standard (D6300, Zymo Research) was used as a microbiome profiling quality control. The results were analyzed, and the taxonomic classification was visualized using an interactive online platform.

1.2.3.3. Evaluation of the antimicrobial activity

The antimicrobial activity against a variety of pathogenic and opportunistic bacterial strains (listed in Section 1.2.1.4) was evaluated with the agar well diffusion method and in liquid medium. In liquid medium, the antimicrobial activity was interpreted as (-) if the pathogens did not grow on the selective medium and (+) if they did grow on the selective medium. For the agar well diffusion assay, the antimicrobial activity against each tested bacterium was determined by measuring the diameter of the inhibition zone (DIZ, in mm). Both methods are described in detail by Tolpeznikaite et al. [70].

1.2.3.4. Evaluation of the dry matter content, pH, and color characteristics

The dry matter (DM) content of the algal samples was evaluated by drying the samples at 103 ± 2 °C until they reached a constant weight.

The pH of the algae was measured using a pH meter (Inolab 3, Hanna Instruments, Villafranca Padovana, Italy) by inserting the pH electrode into the algal mass.

The color characteristics were assessed at three different positions on the algal surface using a CIE L*a*b* system (CromaMeter CR-400, Konica Minolta, Tokyo, Japan). The results are expressed as the CIE color values L* (brightness/darkness), a* (redness/greenness), and b* (yellowness/blueness).

1.2.3.5. Determination of the total phenolic compound content and antioxidant capacity

The Folin–Ciocalteu reagent was used to determine the TPC content. [75]. The method was adapted from Ainsworth and Gillespie [76] and is described in detail by Tolpeznikaite et al. [70]. The data are expressed as gallic acid equivalents (GAE) 100 g^{-1} DM.

The 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging activity of the algal samples was determined based on the method described by Brand-Williams et al. [77], with modifications as described elsewhere [78]. The decrease in absorbance was measured at 515 nm using a Genesys-10 UV/VIS spectrophotometer (Thermo Spectronic, Rochester, NY, USA).

The 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical (ABTS•+) scavenging activity of the algae samples was also measured [79] as described by Urbonaviciene et al. [80]. Absorbance was read at 734 nm using a Genesys-10 UV/Vis spectrophotometer (Thermo Spectronic).

The ferric ion-reducing antioxidant power (FRAP) assay was performed according to the method described by Benzie and Strain [81], with some modifications [82]. The change in absorbance due to the reduction of the ferric-tripyridyltriazine (Fe III-TPTZ) complex by the antioxidants present in the samples was measured at 593 nm using a Genesys-10 UV/VIS spectrophotometer (Thermo Spectronic).

1.2.3.6. Analysis of micro- and macroelements

The micro- and macroelements in algal samples were analyzed with inductively coupled plasma mass spectrometry (ICP-MS) using a 7700x ICP-MS instrument (Agilent, Santa Clara, CA, USA) with the Mass Hunter Work Station software for ICP-MS, version B.01.03 [83].

1.2.3.7. Chlorophyll *a* and *b*, and total carotenoids content analysis

Freeze-dried algal samples (500 ± 2 mg) were removed and weighed accurately. The samples were carefully transferred into a ceramic mortar, and 1.5 mL of ultrapure water was added for rehydration. The mortar was covered with aluminum foil for 2 min. Then, the rehydrated sample was ground accurately with a pestle and 2 g of pure quartz sand. The pigments were extracted and transferred to a volumetric flask; the volume was adjusted to 100 mL with an 80% aqueous acetone solution. The homogenized mixture was centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was separated and analyzed immediately.

The acetonic solution mixture was analyzed for the TCC as well as chlorophyll *a* and *b* and their derivatives using a spectrophotometer based on the modified method reported by Dere et al. [84], Sakalauskaitė et al. [85], and Sumanta et al. [86]. These compounds were measured at a wavelength of 470 nm after subtracting the concentrations of chlorophyll *a* and *b*, which were determined at 649 and 665 nm, respectively, along with the corresponding absorption coefficients where carotenoids do not absorb [84, 86]. The TCC and the contents of chlorophyll *a* and *b* and their derivatives were determined spectrophotometrically. The absorption was measured using a Cintra 202 spectrophotometer (GBC Scientific Equipment Pty Ltd., Mulgrave, Victoria, Australia), and the results were analyzed using the Cintral ver. 2.2 program (GBC Scientific Equipment Pty Ltd.).

1.2.3.8. Evaluation of the L-glutamic acid and gamma-aminobutyric acid concentrations

Analysis was performed on a TSQ Quantiva MS/MS coupled to an Ultimate 3000 high-performance liquid chromatography (HPLC) instrument (Thermo Scientific, Waltham, MA, USA). Chromatographic separation was carried out on a Luna Omega Polar C18 (2.1 × 100 mm, 3.0 μm) column at 40°C using an injection volume of 5 μL. The methods are described in detail or cited by Tolpeznikaite et al. [71].

1.2.3.9. Analysis of biogenic amine concentrations

Sample preparation and determination of the BA, including tryptamine (TRP), phenylethylamine (PHE), putrescine (PUT), cadaverine (CAD), HIS, TYR, spermidine (SPRMD), and spermine (SPRM), in Spirulina samples were conducted by following the procedure reported by Ben-Gigirey et al. [87], with some modifications as described in detail or cited by Tolpeznikaite et al. [71]. The chromatographic analyses were carried out using a Varian ProStar HPLC system (Varian Corp., Palo Alto, CA, USA) with two ProStar 210 pumps, a ProStar 410 autosampler, a ProStar 325 UV/VIS detector, and the Galaxy software (Agilent) for data processing.

1.2.3.10. Analysis of fatty acid profile

Lipids were extracted using chloroform/methanol (2:1, v/v). Fatty acid methyl esters (FAME) were prepared according to Pérez-Palacios et al. [88]. The FA composition was determined using a GC-2010 Plus gas chromatograph (Shimadzu Europa GmbH, Duisburg, Germany) equipped with a GCMS-QP2010 mass spectrometer (Shimadzu Europa GmbH). Separation was carried out on a Stabilwax-MS column (30 m length, 0.25 mm ID, and 0.25 μm df) (Restek Corporation, Bellefonte, PA, USA).

1.2.3.11. Statistical analysis

The study was conducted in five different stages: (I) fermentation of macroalgae was performed once; (II) algal sample extracts were prepared in duplicate; (III) algal samples were prepared in duplicate; (IV) fermentation of the samples was performed in duplicate; and (V) fermentation of Spirulina samples was performed in duplicate. All analytical experiments, at all stages, were carried out in triplicate, except for the fifth stage, which was performed with six replicates. SPSS Statistics Version 15.0 for Windows (SPSS, Chicago, IL, USA) was used for statistical analysis. The means were calculated and compared using Duncan's multiple range test, with $p \leq 0.05$ considered to

indicate a significant difference. Pearson correlation analysis was used to assess the strength of the relationship between the variables. The influence of different factors on algal parameters was evaluated using one-way analysis of variance (ANOVA), with $p \leq 0.05$ considered to indicate a significant difference.

2. RESULTS AND DISCUSSION

2.1. Influence of fermentation on the characteristics of Baltic Sea macroalgae

The LAB and TB counts and pH of the nontreated and fermented algae are shown in Fig. 2.1.1. Fermentation with LUHS135 reduced the pH of the *C. rupestris* samples. However, the pH of the *U. intestinalis* and *F. lumbricalis* samples after fermentation for 12 h was not significantly different compared with the nonfermented samples. In all fermented algal sample groups, the LAB count was higher than 6.0 $\log_{10}$ CFU g^{-1}. Fermentation increased the TBC in the algal samples: on average, by 41.1% for *C. rupestris*, 19.8% for *U. intestinalis*, and 28.4% for *F. lumbricalis.*

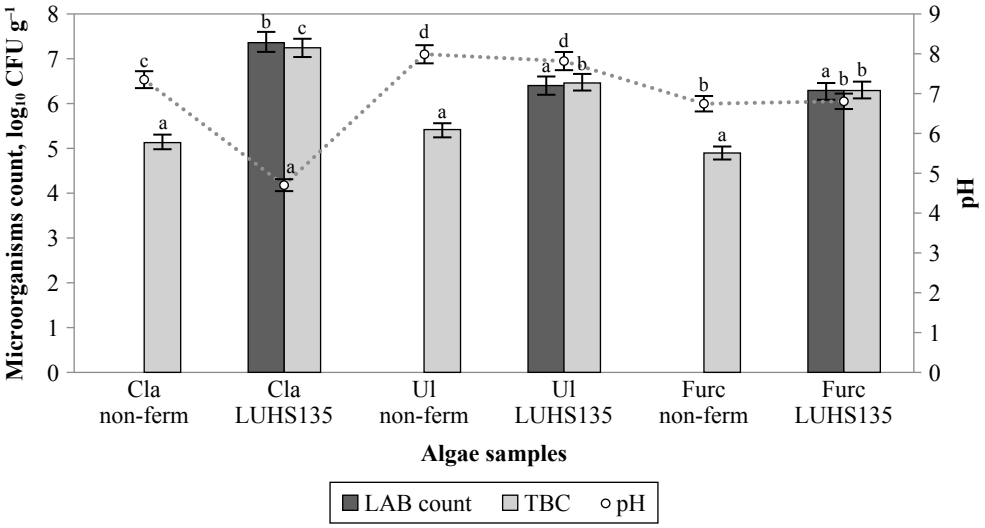
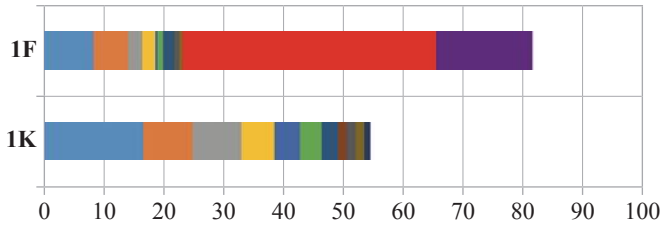


Fig. 2.1.1. *The microorganism counts and pH of the nontreated and fermented algae*

Cla non-ferm – non-fermented *Cladophora rupestris*; Cla LUHS135 – *C. rupestris* fermented with *Lactiplantibacillus plantarum* LUHS135; Ul non-ferm – non-fermented *Ulva intestinalis*; Ul LUHS135 – *U. intestinalis* fermented with *Lb. plantarum* LUHS135; Furc non-ferm – non-fermented *Furcellaria lumbricalis*; Furc LUHS135 – *F. lumbricalis* fermented with *Lb. plantarum* LUHS135; LAB – lactic acid bacteria; TBC – total bacteria count; CFU – colony-forming units). The data are presented as the mean (n = 3, replicates of analysis) ± standard error. For the same analytical parameters, means with different letters (a–d) are significantly different ($p \leq 0.05$).

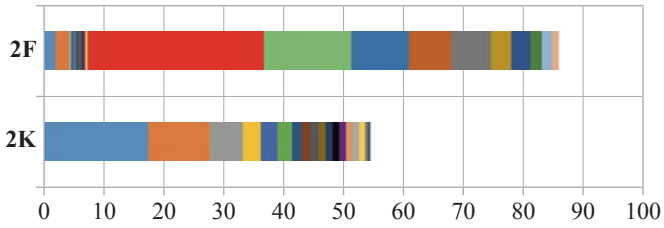
The main bacterial species in the nontreated *C. rupestris* samples were *Portibacter lacus*, *Actinobacterium* spp., *Lewinella cohaerens*, and *Acidimicrobium* spp., the prevalence of which was > 5% of the TBC (Fig. 2.1.2 A). In the fermented samples, *Lb. plantarum* was the most abundant species, accounting for 42.3% of all bacteria detected within the sample (Fig. 2.1.2 A). The bacteria that had the highest prevalence in the untreated samples, such as *P. lacus* and *Acidobacterium*, also remained high. However, the prevalence of *Escherichia vulneris*, which was low before treatment (0.02%), increased significantly after fermentation (15.9%; $p \leq 0.05$). The most prevalent microbiota in the untreated *U. intestinalis* samples comprised species from the class Alphaproteobacteria, *Luteolibacter* algae, and *Marivirga tractuosa*, representing > 5% of the TBC. After fermentation, the most prevalent species was *Lb. plantarum* (29.4%) (Fig. 2.1.2 B). *Terrisporobacter glycolicus* was the second most prevalent species (14.6%), and *Exiguobacterium mexicanum* was the third most prevalent species (9.6%) in the fermented samples. The most prevalent bacteria in the untreated *F. lumbricalis* samples were *P. lacus*, *Actinobacterium* spp., and *Actinomicrobium* spp., whereas in the fermented sample, two cultivable species – *Pseudomonas putida* (54.2%) and *E. vulneris* (24.3%) – presented the highest prevalence (Fig. 2.1.2 C). *Lb. plantarum* was only in third place, accounting for 8.6% of the TBC within the sample.



	1K	1F
Portibacter lacus	16.7	8.4
Actinobacterium spp.	8.3	5.8
Lewinella cohaerens	8.1	2.3
Unclassified *Acidimicrobium*	5.6	2.2
Lewinella nigricans	4.2	0.4
Alpha proteobacterium	3.6	1
Lacibacter cauensis	2.7	1.75
Phaeodactylibacter luteus	1.5	0.1
Maribacter lutimaris	1.5	0.7
Phaeodactylibacter xiamenesis	1.4	0.6
Caldilinea aerophila	1	0.01
Lactobacillus plantarum	0	42.3
Escherichia vulneris	0.02	15.9

Fig. 2.1.2 A. *The bacterial composition of the nontreated (1K) and fermented (1F) samples of Cladophora rupestris*

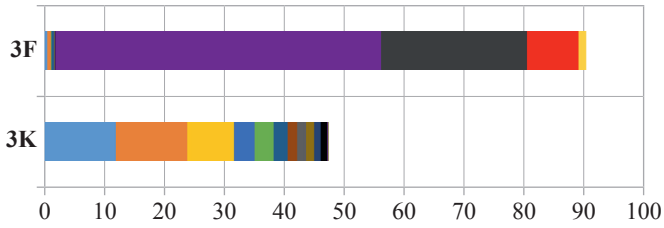
The species and amount (%) of each species are presented. Only those species with a prevalence of at least 1%, irrespective of the group (nontreated of fermented), are presented.



	2K	2F
Alpha proteobacterium	17.5	2
Luteolibacter algae	10.2	2.2
Marivirga tractuosa	5.6	0.2
Dokdonia donghaensis	3	0.13
Erythrobacter atlanticus	2.8	0.7
Portibacter lacus	2.4	0.16
Algibacter lectus	1.6	0.3
Actinobacterium spp.	1.4	0.11
Erythrobacter marinus	1.4	0.4
Ascidiaceihabitans donghaensis	1.2	0.12
Polaribacter irgensii	1.2	0.2
Lewinella agarilytica	1.1	0
Sulfitobacter donghicola	1.1	0.4
Sphingorhabdus wooponesis	1.1	0.12
Lewinella cohaerens	1.1	0.02
Marinosulfonomas methylotropha	1	0.3
Lactobacillus plantarum	0.01	29.4
Terrisporobacter glycolicus	0	14.6
Exiguobacterium mexicanum	0	9.6
Stenotrophomonas maltophilia	0.01	7.1
Pseudomonas putida	0.4	6.6
Pseudomonas stutzeri	0.04	3.4
Escherichia vulneris	0.4	3.2
Paraclostridium benzoelyticum	0	1.9
Pseudomonas geniculata	0.01	1.7
Romboutsia lituseburensis	0.01	1.1

Fig. 2.1.2 B. *The bacterial composition of the nontreated (2K) and fermented (2F) samples of Ulva intestinalis*

The species and amount (%) of each species are presented. Only those species with a prevalence of at least 1%, irrespective of the group (nontreated of fermented), are presented.



	3K	3F
Portibacter lacus	12	0.6
Actinobacterium spp.	11.9	0.6
Acidimicrobium spp.	7.8	0
Alpha proteobacterium	3.4	0
Lewinella cohaerens	3.2	0.1
Flavobacterium frigidarium	2.3	0.4
Lewinella nigricans	1.6	0.01
Maribacter lutimaris	1.5	0.05
Blastopirellula spp.	1.3	0.06
Psychrobacter piscatorii	1.1	0.07
Truepera radiovictrix	1.1	0.07
Pseudomonas putida	0.1	54.2
Escherichia vulneris	0.1	24.3
Lactobacillus plantarum	0	8.6
Pseudomonas syringae	0.01	1.2

Fig. 2.1.2 C. *The bacterial composition of the nontreated (3K) and fermented (3F) samples of Furcellaria lumbricalis*

The species and amount (%) of each species are presented. Only those species with a prevalence of at least 1%, irrespective of the group (nontreated of fermented), are presented.

The antimicrobial activity of the fermented and non-pretreated algae against pathogenic opportunistic microorganisms in liquid medium is shown in Table 2.1.1. All nonfermented algal samples inhibited *B. pseudomycoides* and *K. cryocrescens*; additionally, nonfermented *F. lumbricalis* inhibited *S. haemolyticus*, and nonfermented *C. rupestris* inhibited *P. aeruginosa*. In comparison to the fermented samples, *C. rupestris* inhibited 9 out of 13 tested pathogens. Fermented *U. intestinalis* and *F. lumbricalis* inhibited 6 out of the 13 tested pathogens.

Table 2.1.1. *Antimicrobial activity of the fermented and non-pretreated algal samples against pathogenic opportunistic microorganisms in liquid medium*

Algea samples	Experimental design: 0.5 mL of the diluted algae samples, 0.01 mL of the pathogenic and opportunistic bacterial strains													
	Pathogenic and opportunistic bacteria strains													
	Staphylococcus aureus	*Staphylococcus haemolyticus*	*Enterococcus durans*	*Bacillus pseudomycoides*	*Salmonella enterica*	*Acinetobacter baumanii*	*Acinetobacter johnsonii*	*Enterobacter cloaceae*	*Cronobacter sakazakii*	*Kluyvera cryocrescens*	*Klebsiella pneumoniae*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Total pathogens inhibited*
Cla non-ferm	+	+	+	–	+	+	+	+	+	–	+	+	–	3
Cla LUHS135	+	–	+	–	–	–	+	–	–	–	–	+	–	9
Ul non-ferm	+	+	+	–	+	+	+	+	+	–	+	+	+	2
Ul LUHS135	–	–	+	–	+	–	+	+	+	–	+	+	–	6
Furc non-ferm	+	–	+	–	+	+	+	+	+	–	+	+	+	3
Furc LUHS135	–	–	+	–	–	+	+	+	+	–	+	+	–	6
	Experimental design: 0.5 mL of the LAB, 0.01 mL of the pathogenic and opportunistic bacterial strains													
LAB	–	–	–	–	–	–	+	–	–	–	–	–	–	12
	Pathogen control													
Pathogen	+	+	+	+	+	+	+	+	+	+	+	+	+	–

Interpretation: (–) indicates that the pathogen did not grow on the selective culture medium, while (+) indicates that the pathogen grew on the selective culture medium (Cla non-ferm – non-fermented *Cladophora rupestris*; Cla LUHS135 – *C. rupestris* fermented with *Lactiplantibacillus plantarum* LUHS135; Ul non-ferm – non-fermented *Ulva intestinalis*; Ul LUHS135 – *U. intestinalis* fermented with *Lb. plantarum* LUHS135; Furc non-ferm – non-fermented *Furcellaria lumbricalis*; Furc LUHS135 – *F. lumbricalis* fermented with *Lb. plantarum* LUHS135; LAB – lactic acid bacteria).

The fermented and nontreated *C. rupestris* samples showed the highest antioxidant activity, averaging 58.0% (Fig. 2.1.3). However, fermentation reduced the TPC content in the *C. rupestris* and *F. lumbricalis* samples compared with the nonfermented samples, on average, by 1.8 and 1.3 times, respectively (Fig. 2.1.3).

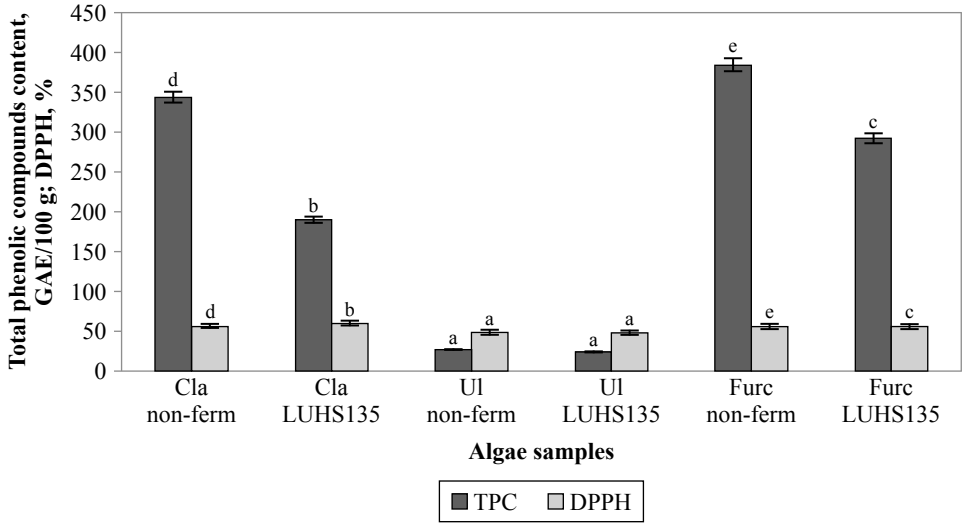


Fig. 2.1.3. *The total phenolic compound content (mg GAE 100 g^{-1}) and DPPH• antioxidant activity (%) of the different algal specimens before and after fermentation*

Cla non-ferm – non-fermented *Cladophora rupestris*; Cla LUHS135 – *C. rupestris* fermented with *Lactiplantibacillus plantarum* LUHS135; Ul non-ferm – non-fermented *Ulva intestinalis*; Ul LUHS135 – *U. intestinalis* fermented with *Lb. plantarum* LUHS135; Furc non-ferm – non-fermented *Furcellaria lumbricalis*; Furc LUHS135 – *F. lumbricalis* fermented with *Lb. plantarum* LUHS135; GAE – gallic acid equivalents; DPPH• – 1,1-diphenyl-2-picrylhydrazyl radical. The data are presented as the mean (n = 3, replicates of analysis) ± standard error. For the same analytical parameters, means with different letters (a–f) are significantly different ($p \leq 0.05$).

The concentration of micro- and macroelements in the algal samples is shown in Table 2.1.2. There were no significant difference in macroelements between the fermented and nontreated samples. However, on average, the sodium (Na) concentration was 3.5 times higher in the *U. intestinalis* samples compared with the *C. rupestris* and *F. lumbricalis* samples. The potassium (K) concentration was, on average, 8.3, 14.6, and 20.5 g kg^{-1} in the *U. intestinalis*, *C. rupestris*, and *F. lumbricalis* samples, respectively. In the *C. rupestris* and *F. lumbricalis* samples, the Na/K ratio was 3.0 and 3.7, respectively.

The *U. intestinalis* samples showed the highest magnesium (Mg) concentration, on average, 5.7 and 1.7 times higher than that in the *C. rupestris* and *F. lumbricalis* samples, respectively. The *C. rupestris* samples had the lowest calcium (Ca) concentration (on average, 2.4 times lower than that in the *U. intestinalis* and *F. lumbricalis* samples). Regarding the essential microelements, the *C. rupestris* samples had the highest manganese (Mn), cobalt (Co), nickel (Ni), selenium (Se), and iodine (I) concentrations (on average, 0.741 g kg^{-1}, and 1.54, 6.08, 0.304, and 205.5 mg kg^{-1}, respectively). In all algal samples analysed, the molybdenum (Mo), silver (Ag), stibium (Sb), caesium (Cs), titanium (Ti), and beryllium (Be) concentrations were < 0.25 mg kg^{-1}; the mercury (Hg) concentration was < 0.01 mg kg^{-1}; and the tin (Sn) concentration was < 0.01 mg kg^{-1}. However, the *C. rupestris* samples had the highest cadmium (Cd), barium (Ba), and lead (Pb) concentrations, on average 2.2, 1.3, and 1.9 times, respectively, higher than those in the *U. intestinalis* samples, and 1.7, 1.9, and 3.8 times, respectively, higher than in the *F. lumbricalis* sample.

Table 2.1.2. *The micro- and macroelements concentrations in the nontreated and fermented (with Lactobacillus plantarum LUHS135) algal samples*

Trace elements, DM	Algae samples					
	Cla non-ferm	**Cla LUHS135**	**Ul non-ferm**	**Ul LUHS135**	**Furc non-ferm**	**Furc LUHS135**
Macroelements, DM						
Na, g kg^{-1}	4.39 ± 0.43	5.46 ± 0.54	17.8 ± 1.7	19.1 ± 1.9	5.22 ± 0.52	5.91 ± 0.59
Mg, g kg^{-1}	2.79 ± 0.27	2.95 ± 0.29	16.1 ± 1.6	16.5 ± 1.6	9.50 ± 0.95	9.29 ± 0.92
K, g kg^{-1}	13.8 ± 1.3	15.4 ± 1.5	7.70 ± 0.77	8.94 ± 0.89	21.7 ± 2.1	19.3 ± 1.9
Ca, g kg^{-1}	4.51 ± 0.45	5.03 ± 0.50	11.8 ± 1.18	12.1 ± 1.2	11.1 ± 1.1	11.1 ± 1.1
Essential microelements, DM						
Cr, mg kg^{-1}	0.681 ± 0.068	0.634 ± 0.013	11.4 ± 1.1	13.1 ± 1.3	< 0.010	< 0.010
Mn, g kg^{-1}	0.701 ± 0.070	0.781 ± 0.078	0.128 ± 0.012	0.115 ± 0.011	0.091 ± 0.009	0.095 ± 0.009
Fe, g kg^{-1}	1.04 ± 0.10	1.31 ± 0.13	1.26 ± 0.12	1.13 ± 0.11	0.285 ± 0.028	0.318 ± 0.031
Co, mg kg^{-1}	1.38 ± 0.13	1.69 ± 0.16	0.392 ± 0.039	0.343 ± 0.034	0.376 ± 0.037	0.441 ± 0.044
Ni, mg kg^{-1}	5.98 ± 0.59	6.18 ± 0.61	1.60 ± 0.16	1.55 ± 0.10	3.66 ± 0.36	3.64 ± 0.36
Cu, mg kg^{-1}	9.88 ± 0.78	10.8 ± 1.0	15.1 ± 1.5	15.9 ± 1.5	10.5 ± 1.0	97.41 ± 0.84
Zn, mg kg^{-1}	16.5 ± 1.6	17.1 ± 1.7	38.7 ± 3.8	45.1 ± 4.5	26.5 ± 2.6	27.7 ± 2.7
Se, mg kg^{-1}	0.232 ± 0.023	0.284 ± 0.028	< 0.2	< 0.2	< 0.2	< 0.2
I, mg kg^{-1}	199 ± 19	212 ± 21	22.8 ± 2.2	20.5 ± 1.6	49.5 ± 4.9	56.1 ± 5.6
P, g kg^{-1}	0.984 ± 0.098	1.20 ± 0.12	1.93 ± 0.19	2.39 ± 0.23	1.56 ± 0.15	1.60 ± 0.16

Table 2.1.2. *Continued*

Trace elements, DM	Algae samples					
	Cla non-ferm	**Cla LUHS135**	**Ul non-ferm**	**Ul LUHS135**	**Furc non-ferm**	**Furc LUHS135**
Non-essential microelements, DM						
Ga, mg kg^{-1}	0.283 ± 0.028	0.332± 0.033	0.297± 0.029	0.284± 0.028	< 0.25	< 0.25
As, mg kg^{-1}	3.67 ± 0.36	3.91 ± 0.39	4.31 ± 0.43	3.89 ± 0.38	5.61 ± 0.56	5.15 ± 0.51
V, mg kg^{-1}	1.75 ± 0.17	2.20 ± 0.22	2.23 ± 0.22	2.14 ± 0.21	1.19 ± 0.11	1.20 ± 0.13
Rb, mg kg^{-1}	9.33 ± 0.93	10.4 ± 1.0	5.77 ± 0.57	6.09 ± 0.60	13.8 ± 1.3	12.9 ± 1.2
Sr, mg kg^{-1}	136 ± 13	144 ± 14	183 ± 18	199 ± 19	206 ± 20	171 ± 17
Mo, mg kg^{-1}	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25
Ag, mg kg^{-1}	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25
Sb, mg kg^{-1}	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25
Cs, mg kg^{-1}	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25
Ti, mg kg^{-1}	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25
Be, mg kg^{-1}	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25
Cd, mg kg^{-1}	0.085 ± 0.008	0.102 ± 0.011	0.043 ± 0.004	0.041 ± 0.004	0.061 ± 0.006	0.051 ± 0.005
Sn, mg kg^{-1}	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Ba, mg kg^{-1}	23.4 ± 2.3	27.6 ± 2.7	19.5 ± 1.9	19.8 ± 1.9	14.3 ± 1.4	13.2 ± 1.3
Hg, mg kg^{-1}	< 0.010	< 0.010	< 0.010	< 0.010	< 0.010	< 0.010
Pb, mg kg^{-1}	1.38 ± 0.13	1.44 ± 0.17	0.760 ± 0.076	0.706 ± 0.070	0.385 ± 0.038	0.356 ± 0.035
B, g kg^{-1}	3.33 ± 0.33	3.67 ± 0.37	13.2 ± 1.3	14.2 ± 1.4	2.99 ± 0.29	3.06 ± 0.30
Al, g kg^{-1}	0.913 ± 0.081	1.03 ± 0.10	0.814 ± 0.081	0.736 ± 0.073	0.151 ± 0.015	0.162 ± 0.019
Li, mg kg^{-1}	0.812 ± 0.081	0.876 ± 0.088	0.758 ± 0.079	0.674 ± 0.067	0.400 ± 0.040	0.409 ± 0.041

Cla non-ferm – non-fermented *Cladophora rupestris*; Cla LUHS135 – *Cladophora rupestris* fermented with *Lb. plantarum* LUHS135; Ul non-ferm – non-fermented *Ulva intestinalis*; Ul LUHS135 – *Ulva intestinalis* fermented with *Lb. plantarum* LUHS135; Furc non-ferm – non-fermented *Furcellaria*; Furc LUHS135 – *Furcellaria* fermented with *Lb. plantarum* LUHS135; DM – dry matter, %; Na – sodium; Mg – magnesium; K – potassium; Ca – calcium; Cr – chromium; Mn – manganese; Fe – iron; Co – cobalt; Ni – nickel; Cu – copper; Zn – zinc; Se – selenium; I – iodine; P – phosphorus; Ga – gallium; As – arsenic; V – vanadium; Rb – rubidium; Sr – strontium; Mo – molybdenum; Ag – silver; Sb – stibium; Cs – caesium; Ti – titanium; Be – beryllium; Cd – cadmium; Sn – tin; Ba – barium; Hg – mercury; Pb – lead; B – boron; Al – aluminium; Li – lithium. Data are represented as means (n = 3, replicates of analysis) ± SE.

The current knowledge about wild algae from Lithuania, as well as algal fermentation, is scarce. It has been reported that several LAB strains are suitable to ferment algae [89]. This study showed that to increase the effectiveness of fermentation, 2 out of 3 tested algae need pretreatment before fermentation, and the addition of fermentable sugars or enzymatic pretreatment before fermentation can be recommended. Nevertheless, LAB-rich

products are associated with numerous health benefits for animals [74, 90, 91]. As shown by metagenomic studies, precautions must be taken to avoid health risks. Algae should be pretreated or their extracts prepared to prevent microbial and chemical contamination. Extract preparation is particularly promising, as it not only enhances biosafety, but also yields various antimicrobial compounds. Algae are rich sources of bioactive molecules such as proteins, peptides, polysaccharides, polyphenols, polyunsaturated FA, and pigments [92], all of which possess antimicrobial properties [93]. A combination of separate algal compounds and LAB could be very promising: It may lead to a synergistic mechanism of action, resulting in a broader spectrum of pathogen inhibition. This study demonstrated that the tested macroalgae possess antimicrobial potential. It has been reported that LAB fermentation significantly impacts the phenolic profile and antioxidant activity of the substrate, as various phenolic acids are excreted during the process [94]. This study showed that the Baltic Sea macroalgae and their combination with *Lb. plantarum* LUHS135 could be a good source of some trace elements. However, monitoring the heavy metal concentrations in macroalgae-based products is recommended given the variable bio-absorption capacity of metals [95]. One of the major considerations for the use of algae as feed material is the need to quantify the levels of arsenic (As). Inorganic As is categorized as a class I carcinogen; arsenobetaine is nontoxic; and fat-soluble As compounds, arsenosugars, and other organoarsenicals are potentially toxic [96]. In algae, most of As is present in arsenosugars, typically ligated to glycerol, sulphonate, or phosphonate. These forms are metabolized in vivo to at least 12 different metabolites, the toxicity of which is unknown [97–100].

Finally, the algae and LAB showed a broader spectrum of pathogen inhibition. Moreover, the Baltic Sea macroalgae and their combination with *Lb. plantarum* LUHS135 could be a good source of some trace elements. However, additional extract preparation is warranted to increase biological and chemical safety, especially considering the Pb and As concentrations.

2.2. Characterization of micro- and macroalgal extract bioactive compounds and micro- and macroelement transition from algae to extract

The *C. rupestris* samples had the higher TCC content (1.26 mg g^{-1}); the TCC in the *U. intestinalis* and *F. lumbricalis* samples, was, on average, 1.6 and 6.3 times lower, respectively (Fig. 2.2.1). In the microalgae, *C. vulgaris* showed the highest TCC concentration (1.52 mg g^{-1}). However, the chlorophyll *a* and *b* concentrations in *C. vulgaris* were, on average, 89.6% and

55.0% lower, respectively, than those in Spirulina. There was a moderate negative correlation (r = −0.4644) between the chlorophyll *a* concentration and the TCC in the macroalgal samples. Moreover, there was a strong positive correlation (r = 0.7604) between the chlorophyll *a* and *b* concentrations. There was a very weak positive correlation (r = 0.1065) between the chlorophyll *b* concentration and the TCC in algae. In all of the tested macroalgal samples, chlorophyll *a* was the predominant form of chlorophyll. The chlorophyll *a* content in the *C. rupestris*, *F. lumbricalis*, and *U. intestinalis* samples was, on average, 9.0%, 63.3%, and 55.2%, respectively (Fig. 2.2.1).

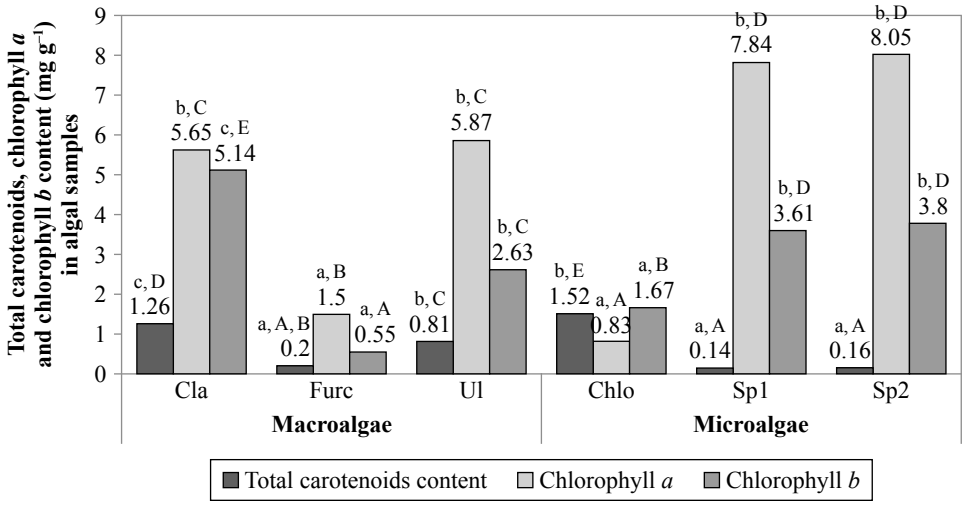


Fig. 2.2.1. *The total carotenoid content (TCC) and chlorophyll a and chlorophyll b concentrations (mg g^{-1}) in the algal samples*

Cla – *Cladophora rupestris*; Furc – *Furcellaria lumbricalis*; Ul – *Ulva intestinalis*; Chlo – *Chlorella vulgaris*; Sp1 – Spirulina (*Arthrospira platensis*) from the University of Texas; Sp2 – Spirulina (Ltd. "Spila")). The data are presented as the mean (n = 3, replicates of analysis) ± standard error. For the same analytical parameters, in the macro- and microalgal groups, means with different letters (a–c) are significantly different ($p \leq 0.05$). For the same analytical parameters, in all algal samples, means with different letters (A–E) are significantly different ($p \leq 0.05$).

The *C. rupestris* and *F. lumbricalis* extracts had the highest TPC content (on average, 352.6 mg GAE 100 g^{-1}). The TPC content in the *U. intestinalis* extract was, on average, 53.1% lower (Fig. 2.2.2 A). The *C. rupestris* extract had significantly higher DPPH• antioxidant activity (5.82%) than the *U. intestinalis* and *F. lumbricalis* extracts. The *U. intestinalis* and *F. lumbricalis* extracts showed, on average, 1.8- and 2.4-times lower DPPH• antioxidant activity, respectively, compared with the *C. rupestris* extract (Fig. 2.2.2 B). There was a weak positive correlation (r = 0.2419) between the TPC content

and the DPPH• antioxidant activity. The *C. rupestris* extract also exhibited significantly higher DPPH• antioxidant activity (5.82%) than the *U. intestinalis* and *F. lumbricalis* extracts. The DPPH• antioxidant activity of the macroalgae demonstrated a very strong positive correlation with the TCC (r = 0.9372). There were moderate to very strong positive correlations between the DPPH• antioxidant activity and the chlorophyll *a* and *b* concentrations (r = 0.6731 and r = 0.9771, respectively) in the macroalgal samples. Moreover, for the microalgal extracts, there was a moderate positive correlation (r = 0.5979) between the TPC content and the DPPH• antioxidant activity.

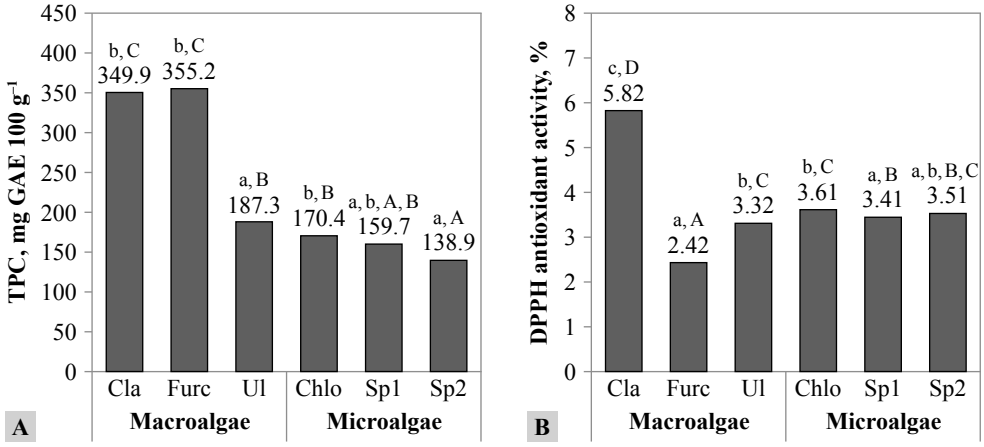


Fig. 2.2.2. (A) *The total phenolic compound (TPC) content (mg GAE 100 g^{-1}) and* ***(B)*** *the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH•) antioxidant activity (%) of the algal extracts*

Cla – *Cladophora rupestris*; Ul – *Ulva intestinalis*; Furc – *Furcellaria lumbricalis*; Chlo – *Chlorella vulgaris*; Sp1 – Spirulina (*Arthrospira platensis*) multiplied in the laboratory; Sp2 – Spirulina (Ltd. "Spila")); GAE – gallic acid equivalents. The data are presented as the mean (n = 3, replicates of analysis) ± standard error. For the same analytical parameters, in the macro- and microalgal groups, means with different letters (a–c) are significantly different ($p \leq 0.05$). For the same analytical parameters, in all algal samples, means with different letters (A–D) are significantly different ($p \leq 0.05$).

The results of the antimicrobial activity tests on algal extracts showed that, using the agar well diffusion method, the Spirulina extracts inhibited *S. haemolyticus* (average DIZ of 28.3 mm). Additionally, the *C. rupestris*, *F. lumbricalis*, and *U. intestinalis* extracts inhibited *B. subtilis*, with an average DIZ of 12.0, 8.0, and 17.0 mm, respectively. Moreover, the Spirulina extract inhibited *B. subtilis* (average DIZ of 10.1 mm), and the *U. intestinalis* extract inhibited *S. mutans* (average DIZ of 14.2 mm) (Fig. 2.2.3).

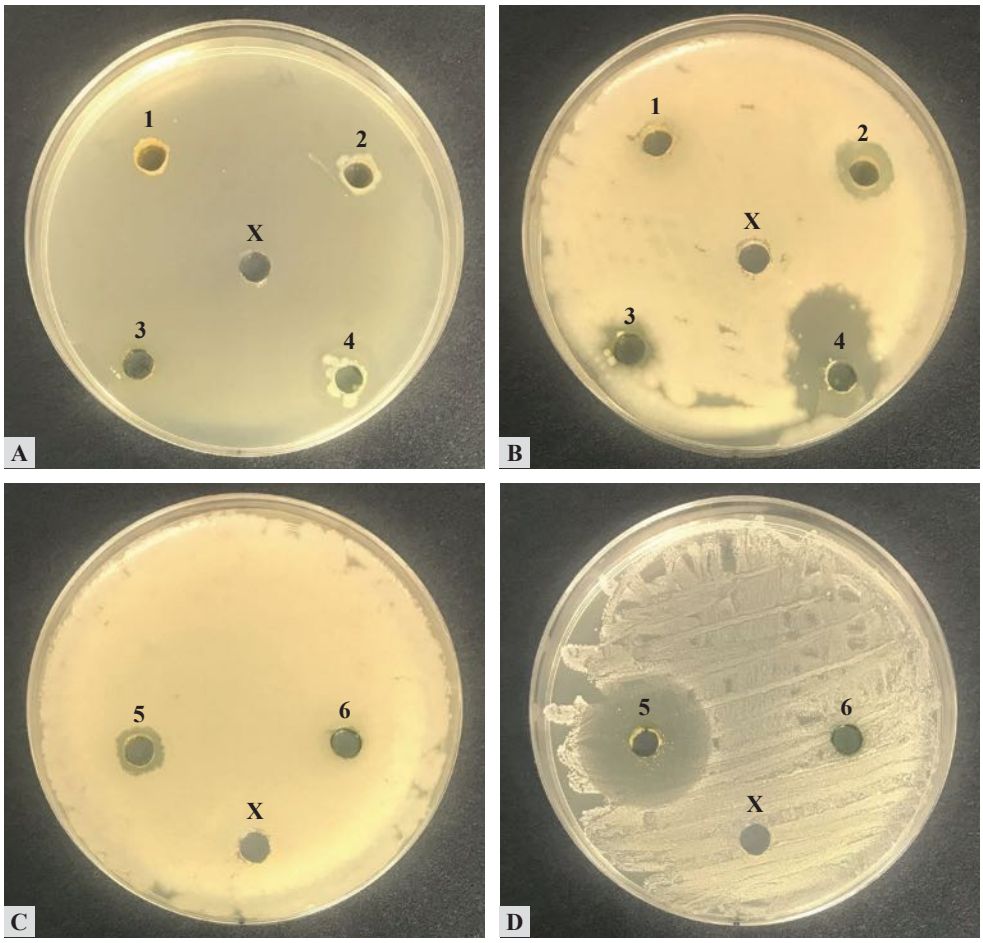


Fig. 2.2.3. *The antimicrobial activity of the algal extracts assessed by using the agar well diffusion method for* ***(A)*** *Streptococcus mutans,* ***(B)*** *Bacillus subtilis,* ***(C)*** *Bacillus subtilis, and* ***(D)*** *Staphylococcus haemolyticus*

1 – *Chlorella vulgaris*; 2 – *Furcellaria lumbricalis*; 3 – *Cladophora rupestris*; 4 – *Ulva intestinalis*; 5 – Spirulina (*Arthrospira platensis*) from University of Texas; 6 – Spirulina (Ltd. "Spila"); X – control (physiological solution).

The micro- and macroelement concentrations in the algal extracts are shown in Table 2.2.1. Regarding the macroelements in the microalgal extracts, the Sp2 extract had the highest Na concentration (458 mg kg^{-1} DM), the highest Mg concentration (on average, 3.3- and 4.4-times higher than that the Sp1 and *C. vulgaris* extracts, respectively), and the highest K concentration (950 mg kg^{-1} DM). In comparison, the *C. rupestris* extract had highest concentrations of the essential microelements Mn, iron (Fe), Co, and Ni (on

average, 7.41, 4.34, 0.064, and 0.301 mg kg^{-1}, respectively). The *U. intestinalis* extract showed the highest copper (Cu) concentration (on average, 0.202 mg kg^{-1}). In all analysed macroalgal extracts, the Se concentration was, on average, 0.002 mg kg^{-1}. In contrast to the previous study stage (as described in Section 2.1), zinc (Zn), I, and phosphorus (P) were not present in the macroalgal extracts. Most of the essential microelement concentrations in the extracts decreased compared with fresh macroalgal samples (as described in Section 2.1). Regarding the essential microelement concentrations in the microalgal extracts, the Sp2 extract had the highest concentrations of most of the determined essential microelements (chromium (Cr), Mn, Fe, Co, Ni, and Se). The *C. vulgaris* and Sp2 extracts showed the highest Cu concentration (on average, 0.074 mg kg^{-1}). Compared with the whole algal samples (as described in Section 2.1), gallium (Ga), Be, Sn, Hg, boron (B), Ti, and Cd did not remain in the extracts. Additionally, Mo was not detected in the *C. rupestris* extract, and Cs was not detected in the *C. rupestris* and *U. intestinalis* extracts. Most of the nonessential microelement concentrations decreased in the extracts compared with the whole macroalgal samples. Finally, considering the nonessential microelements in the microalgal extracts, there were similar tendencies to those of the essential microelements. The Sp3 extract had the highest As, rubidium (Rb), strontium (Sr), Mo, Ag, Sb, Ba, Al, and lithium (Li) concentrations (on average, 0.022, 0.181, 0.346, 0.164, 0.010, 0.098, 0.043, 1.15, and 0.070 mg kg^{-1}, respectively). Additionally, the Sp2 extract had, on average, 0.001 mg kg^{-1} Ti and Cd. In all of the tested microalgal extracts, the Pb concentration was, on average, 0.001 mg kg^{-1}, and Cs was not found. Vanadium (V) was detected in the *C. vulgaris* and Sp2 extracts (on average, 0.001 mg kg^{-1}).

Table 2.2.1. *The micro- and macroelement concentrations in the algal extracts*

Trace Element, DM	Algal Extract Samples					
	Macroalgae			Microalgae		
	Cla	Furc	Ul	Chlo	Sp1	Sp2
Macroelements, DM						
Na, mg kg^{-1}	220 ± 13 a, C	277 ± 11 b, D	517 ± 25 c, F	27.1 ± 1.3 a, A	136 ± 6 b, B	458 ± 18 c, E
Mg, mg kg^{-1}	140 ± 9 a, D	206 ± 14 b, E	259 ± 14 c, F	11.7 ± 0.9 a, A	15.5 ± 1.1 b, B	51.0 ± 3.2 c, C
K, mg kg^{-1}	975 ± 32 c, E	445 ± 21 b, D	336 ± 19 a, C	77 ± 4 a, A	110 ± 5 b, B	950 ± 29 c, E
Ca, mg kg^{-1}	40.2 ± 3.1 b, E	68.3 ± 4.2 c, F	18.8 ± 0.7 a, C	2.36 ± 0.14 a, A	7.17 ± 0.16 b, B	29.1 ± 1.7 c, D
Essential microelements, DM						
Cr, mg kg^{-1}	0.003 ± 0.001 a, A	0.042 ± 0.003 c, C	0.023 ± 0.002 b, B	0.002 ± 0.001 a, A	0.005 ± 0.002 a, A	0.056 ± 0.004 b, D
Mn, mg kg^{-1}	7.41 ± 0.52 c, F	1.10 ± 0.07 b, E	0.435 ± 0.021 a, D	0.028 ± 0.002 a, A	0.063 ± 0.004 b, B	0.333 ± 0.021 c, C
Fe, mg kg^{-1}	4.34 ± 0.31 c, E	2.66 ± 0.19 b, D	1.09 ± 0.08 a, A	2.25 ± 0.011 b, C	1.37 ± 0.009 a, B	4.73 ± 0.15 c, E
Co, mg kg^{-1}	0.064 ± 0.005 d, D	0.003 ± 0.001 a, A	0.006 ± 0.001 b, B	0.002 ± 0.001 a, A	0.002 ± 0.001 a, A	0.040 ± 0.003 c, C
Ni, mg kg^{-1}	0.301 ± 0.011 c, D	0.015 ± 0.002 b, B	0.005 ± 0.001 a, A	nd	nd	0.020 ± 0.001 C
Cu, mg kg^{-1}	0.101 ± 0.009 b, D	0.004 ± 0.001 a, A	0.202 ± 0.013 c, E	0.077 ± 0.003 b, C	0.020 ± 0.002 a, B	0.071 ± 0.005 b, C
Se, mg kg^{-1}	0.004 ± 0.002 a, A	0.001 ± 0.001 a, A	0.002 ± 0.001 a, A	nd	nd	0.004 ± 0.002 A
Non-essential microelements, DM						
As, mg kg^{-1}	0.334 ± 0.021 b, D	0.255 ± 0.016 a, C	0.437 ± 0.031 c, E	0.004 ± 0.002 a, A	0.002 ± 0.001 a, A	0.022 ± 0.002 b, B
V, mg kg^{-1}	0.006 ± 0.001 a, B	0.004 ± 0.001 a, A, B	0.004 ± 0.001 a, A, B	0.001 ± 0.001 a, A	nd	0.002 ± 0.001 a, A
Rb, mg kg^{-1}	0.507 ± 0.026 c, F	0.252 ± 0.017 b, E	0.140 ± 0.009 a, C	0.020 ± 0.001 b, B	0.010 ± 0.001 a, A	0.181 ± 0.012 c, D
Sr, mg kg^{-1}	0.398 ± 0.014 b, E	0.621 ± 0.035 c, F	0.245 ± 0.011 a, C	0.017 ± 0.002 a, A	0.060 ± 0.003 b, B	0.346 ± 0.019 c, D
Mo, mg kg^{-1}	nd	0.004 ± 0.001 a, A	0.007 ± 0.001 b, B	nd	0.004 ± 0.001 a, A	0.010 ± 0.001 b, C
Ag, mg kg^{-1}	0.018 ± 0.001 a, A	0.098 ± 0.007 c, C	0.029 ± 0.002 b, B	nd	0.034 ± 0.003 a, B	0.164 ± 0.013 b, D

Table 2.2.1. *Continued*

Trace Element, DM	Algal Extract Samples					
	Macroalgae			Microalgae		
	Cla	Furc	Ul	Chlo	Sp1	Sp2
Non-essential microelements, DM						
Sb, mg kg^{-1}	0.046 ± 0.003 b, C	0.005 ± 0.001 a, A	0.163 ± 0.007 c, E	nd	0.032 ± 0.003 a, B	0.098 ± 0.008 b, D
Cs, mg kg^{-1}	nd	0.001 ± 0.000	nd	nd	nd	nd
Ti, mg kg^{-1}	nd	nd	nd	nd	nd	0.001 ± 0.000
Cd, mg kg^{-1}	nd	nd	nd	nd	nd	0.001 ± 0.000
Ba, mg kg^{-1}	0.032 ± 0.002 b, C	0.033 ± 0.002 b, C	0.008 ± 0.001 a, B	0.001 ± 0.000 a, A	0.006 ± 0.001 b, B	0.043 ± 0.003 c, D
Pb, mg kg^{-1}	0.002 ± 0.001 a, A	0.003 ± 0.001 a, b, A, B	0.001 ± 0.000 a, A	0.001 ± 0.000 a, A	0.001 ± 0.000 a, A	0.002 ± 0.001 a, A
Al, mg kg^{-1}	0.343 ± 0.018 b, B	1.14 ± 0.01 c, C	0.111 ± 0.009 a, A	nd	nd	1.15 ± 0.009 C
Li, mg kg^{-1}	0.013 ± 0.002 a, B	0.023 ± 0.002 b, C	0.047 ± 0.003 c, D	0.002 ± 0.001 a, A	0.005 ± 0.002 a, A	0.070 ± 0.006 b, E

Cla – *Cladophora rupestris*; Ul – *Ulva intestinalis*; Furc – *Furcellaria lumbricalis*; Chlo – *Chlorella vulgaris*; Sp1 – Spirulina (*A. platensis*) obtained from the University of Texas; Sp2 – Spirulina (Ltd. „Spila"); DM – dry matter, %; Na – sodium; Mg – magnesium; K – potassium; Ca – calcium; Cr – chromium; Mn – manganese; Fe – iron; Co – cobalt; Ni – nickel; Cu – copper; Zn – zinc; Se – selenium; I – iodine; P – phosphorus; Ga – gallium; As – arsenic; V – vanadium; Rb – rubidium; Sr – strontium; Mo – molybdenum; Ag – silver; Sb – stibium; Cs – caesium; Ti – titanium; Be – beryllium; Cd – cadmium; Sn – tin; Ba – barium; Hg – mercury; Pb – lead; B – boron; Al – aluminium; Li – lithium. Data are represented as means (n = 3, replicates of analysis) ± SE. a–c for the same analytical parameters, in macro- and microalgae groups, means with different letters are significantly different ($p \leq 0.05$). A–F for the same analytical parameters, in all algal samples, means with different letters are significantly different ($p \leq 0.05$); nd – not determined.

It is important to note that the extraction method affects the concentration of pigments in the extract [101]; therefore, comparing results from different studies is very challenging. Carotenoids are natural plant pigments responsible for the vibrant colors found in various fruits, vegetables, and algae, including shades of red, green, yellow, and orange [80, 102]. Carotenoids have been applied in the feed industry to enhance animal health and to improve the quality of animal-derived products [103]. Furthermore, colored compounds frequently enhance the antioxidant properties of the product or extract; however, the specific antioxidant characteristics are determined based on the unique composition of phenolic compound profiles. Santoso et al. [104) and Wang et al. [105] noted that synergistic effects among the different substances that comprise the TPC content in algae should also be considered. Additionally, it has been reported that *U. intestinalis* exhibits antioxidant activity [106], and the antioxidant properties of its extracts vary depending on the solvent used: The antioxidant activity of the dichloromethane, ethanol, methanol, and hexane extracts was 87.54%, 31.9%, 22.6%, and 22.5%, respectively [107]. However, Farasat et al. [108] reported that methanolic extracts of *U. intestinalis* exhibited the highest DPPH• scavenging activity (48% inhibition) and had a lower IC50 of 2.32 mg mL^{-1}.

Researchers have reported that green, red, and brown algae exhibit various properties such as antifungal, antibacterial, cytostatic, antiviral, anthelmintic, and more [109–111], and algal extracts could inhibit bacteria, yeast, and fungi [112–116]. The differential inhibition of gram-positive but not gram-negative bacteria by the extracts may be linked to variations in permeability barriers [107]. Specifically, in gram-negative species, the outer membrane may not allow the tested compounds to pass [117].

One of the primary concerns regarding the safety of macroalgae is contamination with heavy metals, including Al, Cd, Pb, Rb, silicon (Si), Sr, and Sn [118, 119]. The concentration of toxic elements in microalgae can vary, and this variability may be linked to environmental contamination of the biomass's origin [120, 121]. Some microalgae take up toxic metals, and from this perspective, the chemical quality of the medium profoundly affects the presence of contaminants in microalgal biomass [122]. This study represents the first analysis of the micro- and macroelement levels in extracts from micro- and macroalgae. The results indicate that the extraction method employed is effective in reducing the concentration of toxic metals in these algae. However, it should be noted that some of the desirable microelement concentrations were also reduced during the extraction process, and only the final products, according to their specific composition, could be applied for feed preparation.

2.3. Effect of solid-state fermentation and ultrasonication processes on the antimicrobial and antioxidant properties of algae extracts

The pH changes of the algal samples are shown in Fig. 2.3.1. The pH of the nonfermented *C. rupestris*, *U. intestinalis* and Spirulina samples was > 7.0. The pH of the nonfermented *C. glomerata* and *F. lumbricalis* samples was, on average, 5.95 and 6.74, respectively. The *C. glomerata*, *C. rupestris*, and *U. intestinalis* samples after fermentation for 36 and 48 h had the lowest pH. Univariate ANOVA showed that the variety of algae is a significant factor in sample pH ($p = 0.017$). Based on these results, a fermentation duration of 36 h for algal pretreatment before extract preparation was selected.

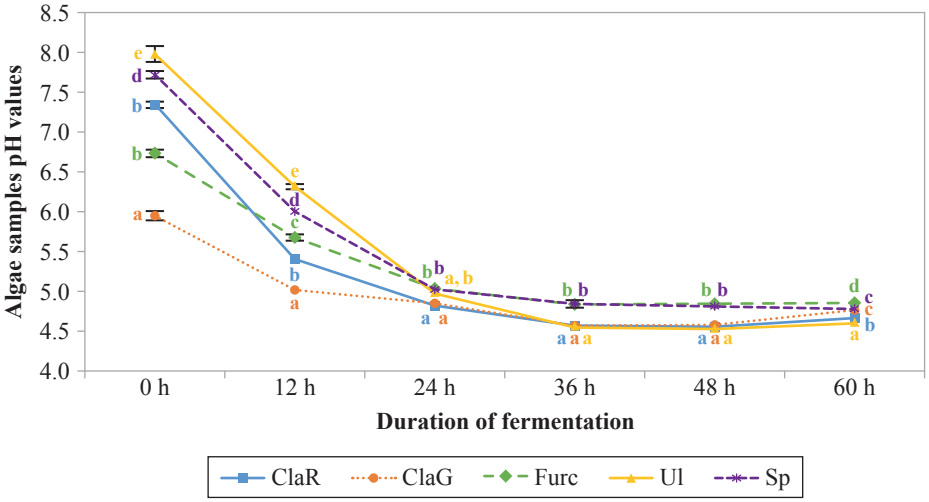


Fig. 2.3.1. *The pH of the nonfermented and fermented algal samples after 12, 24, 36, 48, and 60 h of fermentation*

ClaR – *Cladophora rupestris*; ClaG – *Cladophora glomerata*; Ul – *Ulva intestinalis*; Furc – *Furcellaria lumbricalis*; Sp – Spirulina (*Arthrospira platensis*). The data are presented as the mean (n = 3) ± standard error. Means with different letters (a–e) are significantly different ($p \leq 0.05$). The color of each letter coincides with the color of the sample in the graph.

The color coordinates and pH of the algal extracts and alga extract × *Lb. plantarum* LUHS135 combinations are shown in Table 2.3.1. When comparing all three groups of extracts (prepared from non-pretreated, ultrasonicated, and fermented algae), the $ClaG_{non}$, $ClaR_{ultr}$, and $ClaR_{fermLUHS135}$ samples showed the lowest L* coordinates (42.5, 41.3 and 49.5 NBS, respectively). The Ul_{non}, Ul_{ultr}, and Ul_{ferm} samples showed the most intense green (-a*) (–14.7, –13.7 and –6.86 NBS, respectively). The $ClaG_{non}$, Sp_{ultr}, and $ClaG_{ferm}$ samples showed the least intense yellow (b*) (24.8, 23.7 and 23.1 NBS, respectively). In all cases, the addition of *Lb. plantarum* LUHS135 reduced the pH of the algal extract × *Lb. plantarum* LUHS135 combinations; however, the highest pH was observed in the Sp_{non}, Sp_{ultr}, and $Furc_{ferm}$ (8.69, 7.67 and 5.59, respectively).

Table 2.3.1. *The color coordinates and pH of the algal extracts and the algal extract × Lactiplantibacillus plantarum LUHS135 combinations*

Extracts and extract × LUHS135 combination	Color coordinates, NBS			pH	Multivariate analysis of variance		
	L*	a*	b*		Factor	Dependent variable	p
Extracts and extracts × LUHS135 combinations prepared from non-pre-treated algae					Algae species	L*	0.0001
$Cla R_{non}$	64.6 ± 0.32 g	–13.8 ± 0.11 b	47.5 ± 0.36 g	6.77 ± 0.031 d		a*	0.0001
$Cla R_{nonLUHS135}$	61.1 ± 0.26 e	–1.40 ± 0.15 g	44.6 ± 0.33 e	3.95 ± 0.032 a		b*	0.0001
$Cla G_{non}$	42.5 ± 0.10 a	–1.75 ± 0.192 f	24.8 ± 0.18 a	5.92 ± 0.124 b		pH	0.712
$Cla G_{nonLUHS135}$	50.6 ± 0.12 b	2.61 ± 0.105 h	34.8 ± 0.39 c	3.96 ± 0.115 a	Pretreatment used before extracts preparation	L*	0.0001
$Furc_{non}$	79.2 ± 0.34 h	–3.57 ± 0.022 c	32.2 ± 0.16 b	6.19 ± 0.036 c		a*	0.0001
$Furc_{nonLUHS135}$	60.5 ± 0.25 d	10.4 ± 0.24 k	47.8 ± 0.25 g	3.92 ± 0.025 a		b*	0.0001
Ul_{non}	52.4 ± 0.32 c	–14.7 ± 0.16 a	41.3 ± 0.37 d	6.99 ± 0.092 e		pH	0.052
$Ul_{nonLUHS135}$	62.9 ± 0.13 f	–2.27 ± 0.031 e	45.8 ± 0.33 f	3.95 ± 0.071 a	Extract × LUHS135 combination interaction	L*	0.0001
Sp_{non}	59.9 ± 0.32 d	–3.40 ± 0.114 d	49.1 ± 0.31 h	8.69 ± 0.102 f		a*	0.0001
$Sp_{nonLUHS135}$	64.6 ± 0.10 g	4.04 ± 0.015 j	44.9 ± 0.12 e	3.94 ± 0.044 a		b*	0.0001
Extracts and extracts × LUHS135 combinations prepared from ultrasonicated algae						pH	0.0001
$Cla R_{ultr}$	41.3 ± 0.31 a	–1.55 ± 0.064 d	24.4 ± 0.21 b	5.82 ± 0.032 b	Algae species × pre-treatment interaction	L*	0.0001
$Cla R_{ultrLUHS135}$	45.0 ± 0.24 b	3.42 ± 0.121 j	29.2 ± 0.10 c	3.94 ± 0.091 a		a*	0.0001
$Cla G_{ultr}$	50.8 ± 0.37 c	–7.16 ± 0.092 b	33.5 ± 0.34 d	6.37 ± 0.034 d		b*	0.0001
$Cla G_{ultrLUHS135}$	59.8 ± 0.36 f	–0.65 ± 0.021 f	40.0 ± 0.32 e	3.93 ± 0.022 a		pH	0.058
$Furc_{ultr}$	71.8 ± 0.44 h	2.23 ± 0.105 g	52.6 ± 0.35 j	6.09 ± 0.093 c	Algae species × LUHS135 combination interaction	L*	0.0001
$Furc_{ultrLUHS135}$	65.1 ± 0.26 g	4.43 ± 0.113 k	45.7 ± 0.22 g	3.89 ± 0.031 a		a*	0.0001
Ul_{ultr}	55.4 ± 0.37 d	–13.7 ± 0.24 a	45.7 ± 0.34 g	7.01 ± 0.074 e		b*	0.0001
$Ul_{ultrLUHS135}$	57.1 ± 0.10 e	–1.26 ± 0.031 e	47.1 ± 0.12 h	3.92 ± 0.032 a		pH	0.362

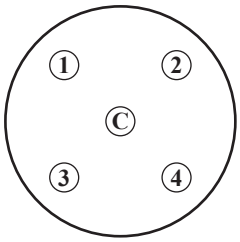
Table 2.3.1. *Continued*

Extracts and extract × LUHS135 combination	Color coordinates, NBS			pH	Multivariate analysis of variance		
	L*	a*	b*		Factor	Dependent variable	p
Extracts and extracts × LUHS135 combinations prepared from ultrasonicated algae					Pre-treatment × LUHS135 combination interaction	L*	0.0001
Sp_{ultr}	79.9 ± 0.41 j	–5.40 ± 0.154 c	23.7 ± 0.24 a	7.67 ± 0.107 f		a*	0.0001
$Sp_{ultrLUHS135}$	65.3 ± 0.31 g	5.17 ± 0.072 l	44.6 ± 0.27 f	3.92 ± 0.094 a		b*	0.0001
Extracts and extracts × LUHS135 combinations prepared from fermented algae						pH	0.031
$ClaR_{ferm}$	54.7 ± 0.25 b	–4.55 ± 0.094 b	33.5 ± 0.34 c	5.09 ± 0.064 b	Algae species × pre-treatment × LUHS135 combination interaction	L*	0.0001
$ClaR_{fermLUHS135}$	49.5 ± 0.37 a	3.33 ± 0.046 f	34.5 ± 0.22 d	4.02 ± 0.084 a		a*	0.0001
$ClaG_{ferm}$	63.2 ± 0.22 e	1.95 ± 0.164 d	23.1 ± 0.40 a	5.06 ± 0.040 b		b*	0.0001
$ClaG_{fermLUHS135}$	62.4 ± 0.24 d	7.75 ± 0.140 h	45.8 ± 0.41 g	4.07 ± 0.011 a		pH	0.004
$Furc_{ferm}$	65.6 ± 0.27 g	4.67 ± 0.021 g	43.8 ± 0.44 f	5.59 ± 0.064 c			
$Furc_{fermLUHS135}$	64.0 ± 0.38 f	8.31 ± 0.163 j	48.0 ± 0.31 h	4.06 ± 0.052 a			
Ul_{ferm}	76.8 ± 0.25 j	–6.86 ± 0.111 a	31.6 ± 0.22 b	4.95 ± 0.081 b			
$Ul_{fermLUHS135}$	56.8 ± 0.42 c	6.50 ± 0.202 h	41.7 ± 0.14 e	3.97 ± 0.094 a			
Sp_{ferm}	83.1 ± 0.14 k	–1.67 ± 0.174 c	31.5 ± 0.15 b	5.20 ± 0.107 b			
$Sp_{fermLUHS135}$	71.7 ± 0.21 h	3.08 ± 0.037 e	43.2 ± 0.38 f	3.98 ± 0.075 a			

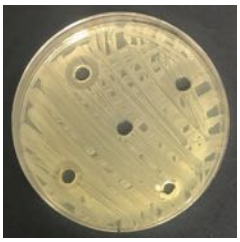
ClaR – *Cladophora rupestris*; ClaG – *Cladophora glomerata*; Ul – *Ulva intestinalis*; Furc – *Furcellaria lumbricalis*; Sp – Spirulina (*A. platensis*); non – extracts prepared from non-pre-treated algae; ultr – extracts prepared from ultrasonicated algae; ferm – extracts prepared from fermented algae; LUHS135 – extract × LUHS135 strain combination; L* – lightness; a* – redness; −a* – greenness; b* – yellowness; −b* – blueness; NBS – National Bureau of Standards units; data are represented as means (n = 3 replicates of analysis) ± SE. a–l indicate the same analytical parameters in different algae species groups. Means with different letters are significantly different ($p \leq 0.05$).

Multivariate ANOVA showed that the algal species ($p \leq 0.0001$), the interaction between the algal species and pretreatment before extraction ($p \leq 0.0001$), and the interaction between the algal species and *Lb. plantarum* LUHS135 combination ($p \leq 0.003$) were significant factors affecting the TPC content in the samples. The $ClaR_{nonLUHS135}$ and $Furc_{nonLUHS135}$ samples had the highest TPC content (on average, 13.28 mg GAE mL^{-1}). The non-pretreated *C. rupestris* and *F. lumbricalis* extract and *Lb. plantarum* LUHS135 combinations had the highest DPPH•, ABTS•+, and FRAP antioxidant activities, compared with the extracts without *Lb. plantarum* LUHS135. There was a moderate positive correlation between the TPC content and the ABTS•+ ($r = 0.300$, $p = 0.004$) and FRAP ($r = 0.247$, $p = 0.019$) antioxidant activities. However, there were no correlations between the DPPH• antioxidant activity and the TPC content in the samples.

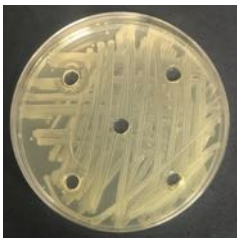
The antimicrobial properties of the non-pretreated, ultrasonicated, and fermented samples were compared by using the agar well diffusion method. Of the three groups, more non-pretreated samples inhibited at least one pathogen (Fig. 2.3.2). All tested samples in this group inhibited *Bacillus cereus*. Additionally, 3 out of 10 samples in this group ($ClaR_{non}$, $ClaR_{nonLUHS135}$, and $ClaG_{nonLUHS135}$) inhibited *Enterococcus faecium*, and 4 out of 10 samples ($ClaR_{nonLUHS135}$, $ClaG_{nonLUHS135}$, $Furc_{nonLUHS135,}$ and $Ul_{nonLUHS135}$) inhibited *S. aureus*. Despite the fact that the highest number of samples (of all tested samples) in the non-pretreated sample group inhibited at least one pathogen, the ultrasonicated sample group presented a broader spectrum of pathogen inhibition: $ClaR_{ultr}$, $ClaR_{ultrLUHS135}$, and $Sp_{ultrLUHS135}$ inhibited *B. cereus*; $ClaG_{ultrLUHS135}$ inhibited *E. faecium*; $ClaR_{ultrLUHS135}$, $ClaG_{ultrLUHS135}$, and $Sp_{ultrLUHS135}$ inhibited *S. aureus*; and $Furc_{ultrLUHS135}$ and $Ul_{ultrLUHS135}$ inhibited *S. mutans*).



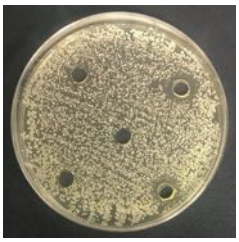
Experimental design:
1 – 2 – 3 – 4 – C (Control)



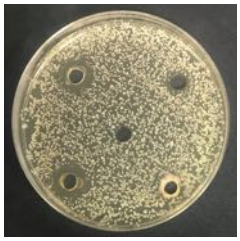
Streptococcus mutans
1. Sp+US+LUHS135-
2. ClaR++LUHS135-
3. ClaR+LAB+LUHS135-
4. ClaG+LUHS135-



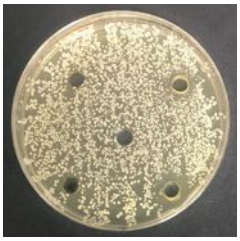
Streptococcus mutans
1. ClarG+LAB+LUHS135-
2. Furc+LAB-
3. Furc+LAB+LUHS135-
4. Ul+LAB



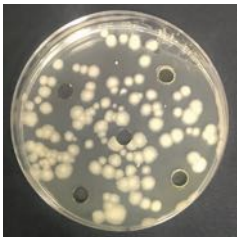
Staphylococcus aureus
1. Furc-
2. Furc+LUHS135-
3. Ul-
4. Ul+LUHS135



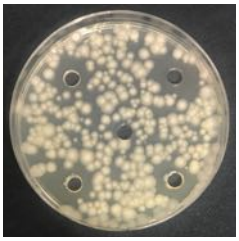
Staphylococcus aureus
1. Sp+US+LUHS135-
2. ClaR+LUHS135-
3. ClaR+LAB+LUHS135-
4. ClaG+LUHS135-



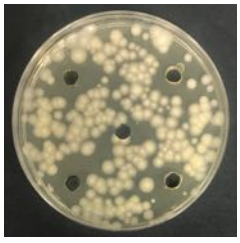
Staphylococcus aureus
1. Sp-
2. Sp+LUHS135-
3. ClaR+US-
4. ClaG+US+LUHS135



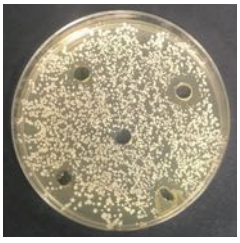
Bacillus cereus
1. Sp-
2. Sp+LUHS135-
3. ClaR+US-
4. ClaG+US+LUHS135



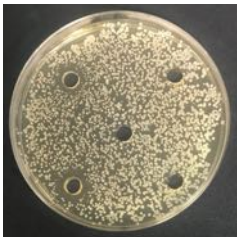
Bacillus cereus
1. ClaG+LAB+LUHS135-
2. Furc+LAB-
3. Furc+LAB+LUHS135-
4. Ul+LAB



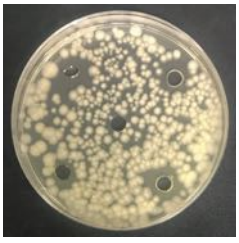
Bacillus cereus
1. ClaR-
2. ClaR+LUHS135-
3. ClaG-
4. ClaG+LUHS135



Staphylococcus aureus
1. ClaR-
2. ClaR+LUHS135-
3. ClaG-
4. ClaG+LUHS135



Staphylococcus aureus
1. ClaG+LAB+LUHS135-
2. Furc+LAB-
3. Furc+LAB+LUHS135-
4. Ul+LAB



Bacillus cereus
1. Furc-
2. Furc+LUHS135-
3. Ul-
4. Ul+LUHS135

Fig. 2.3.2. *Images of the inhibition zones of the algal extracts and the algal extract × Lactiplantibacillus plantarum LUHS135 combinations evaluated using the agar well diffusion method*

ClaR – *Cladophora rupestris*; ClaG – *Cladophora glomerata*; Ul – *Ulva intestinalis*; Furc – *Furcellaria lumbricalis*; Sp – Spirulina (*Arthrospira platensis*); US – algal biomass pretreated with ultrasound; LAB – algal biomass fermented with Lb. plantarum LUHS135 before extraction; LUHS135 – extract composition with Lb. plantarum LUHS135; C – control (physiological solution).

In liquid medium, 500 and 2000 µL of the algal extracts and the algal extract × *Lb. plantarum* LUHS135 combinations were tested against 10 µL of pathogen. When 500 µL was added to the liquid medium, Sp_{non} inhibited *B. cereus* growth; $ClaR_{ferm}$ inhibited *E. faecium* growth; $Sp_{ultrLUHS135}$, $ClaR_{ferm}$, and $Sp_{fermLUHS135}$ inhibited *S. aureus* growth; and Sp_{non} inhibited *S. mutans* growth. When adding 2000 µL to the liquid medium, in addition to the aforementioned inhibition, $ClaR_{non}$, Sp_{non}, $Sp_{nonLUHS135}$, and Ul_{ultr} inhibited *E. faecium* growth; $ClaR_{ultr}$, Ul_{ultr}, and Sp_{ferm} inhibited *S. mutans* growth; and $ClaR_{non}$, $ClaG_{non}$, Sp_{non}, and $ClaG_{ferm}$ inhibited *E. faecalis* growth.

The primary objective of the fermentation process is to lower the pH, typically aiming for a recommended pH of 4.6 for the fermented substrate. During fermentation, numerous compounds are produced as secondary metabolites by technological microorganisms [123, 124]. Additionally, bound phenolic compounds undergo bioconversion from their conjugated forms to their free forms, a phenomenon explained by their breakdown, the activities of fermentable substrate enzymes, as well as the activity of technological microorganisms [22]. This study demonstrated that yeast extract is an effective supplement for enhancing the efficiency of algae fermentation. During fermentation, the substrate undergoes acidification, and organic acids affect oxidation processes that may result in changes in color [125]. Colored compounds often contribute to enhanced antioxidant properties of the product or extract; however, the specific antioxidant characteristics are closely linked to the composition of the phenolic compound profile. Besides fermentation, ultrasonication can also induce color changes in compounds. Ultrasonic waves generate rapid compressions and expansions that disrupt substrate cells. Cavitation phenomena are responsible for reducing the diffusion boundary layer [126–130]. Ultrasonication has been reported to increase extraction efficiency [131, 132]. However, other published studies have shown that the use of ultrasound as a pretreatment contributed to significant changes in color [133]. From this perspective, it is essential to assess the alterations in the antioxidant properties of the treated samples because decreases in colored compounds could potentially lower antioxidant activity. Essentially, the DPPH• and ABTS•+ scavenging activities rely on the ability of antioxidants to donate a hydrogen atom or an electron to stabilize radicals, converting them into nonradical species [134, 135]. Our findings demonstrated that all ethanolic extracts were capable of donating a hydrogen atom or electron to both radicals. In general, algal extracts enriched with natural polyphenolics exhibit antioxidant properties [134, 136]. Algae are a good source of bioactive compounds, with some demonstrating broad-spectrum activities, including antimicrobial properties [137, 138]. Sirbu et al. [139] reported that the TPC content in algal extracts is related to their

antibacterial activity. In this study, there were moderate correlations between the ABTS•+ antioxidant activity and the *E. faecalis* DIZ and between the TPC content in the extracts and the *S. aureus* DIZ ($r = 0.388$, $p = 0.0001$; $r = 0.340$, $p = 0.001$, respectively). However, additional research is necessary to identify the specific compounds responsible for inhibiting these pathogens.

2.4. Changes in Spirulina's physicochemical properties during submerged and solid-state lacto-fermentation

The L-Glu and GABA concentrations of the nonfermented and fermented Spirulina samples are shown in Fig. 2.4.1 A. Compared with the L-Glu concentration in the SMF and control (I) samples, after SMF for 48 h, the L-Glu concentration in 7 out of 10 SMF samples was higher; the L-Glu concentration in the other 3 SMF samples was lower compared with the control (I). In comparison, most of the SSF samples (except after SSF for 24 and 48 h with *Lb. paracasei* No. 244), the L-Glu concentration increased after SSF, and fermentation (SMF or SSF) had a significant impact on the L-Glu concentration of Spirulina. In all cases (i.e., after 24 and 48 h of SMF), the GABA concentration consistently increased compared with the control (I), with the highest GABA concentration (286.5 mg kg^{-1}) found in the sample submitted to SMF for 48 h with *Lb. paracasei* No. 244. The same trend was observed for the SSF samples: After SSF for 24 and 48 h, the GABA concentration was higher than that in control (II), with the highest concentration (2395.9 mg kg^{-1}) found in the sample submitted to SSF for 48 h with *Lb. paracasei* No. 244.

The BA concentrations in nontreated and fermented Spirulina samples are shown in Fig. 2.4.1 B. PUT and SPRMD were the main BA found in the fermented Spirulina samples. There were higher PUT concentrations in the SSF samples compared with the SMF samples, with the highest PUT content in the samples submitted to SSF with *Lb. brevis* No. 173 (833.4 mg kg^{-1} after 24 h and 854.7 mg kg^{-1} after 48 h). All the analyzed factors and their interactions were significant regarding the PUT concentration in the Spirulina samples ($p \leq 0.0001$). SMF decreased the SPRMD concentration in the Spirulina samples – on average, from 3.3 to 4.9 times (for the samples submitted to SMF for 48 h with *Lb. paracasei* No. 244 and SMF for 24 h with *P. pentosaceus* No. 183, respectively). However, there were opposite trends for the SPRMD concentrations in the SSF samples. Additionally, all the analyzed factors and most of their interactions – except for the interaction between the duration of fermentation and the fermentation conditions (SMF or SST) – were significant for the SPRMD concentrations in the Spirulina samples

($p \leq 0.0001$). The Spirulina samples with the highest GABA concentrations also showed the highest BA concentrations.

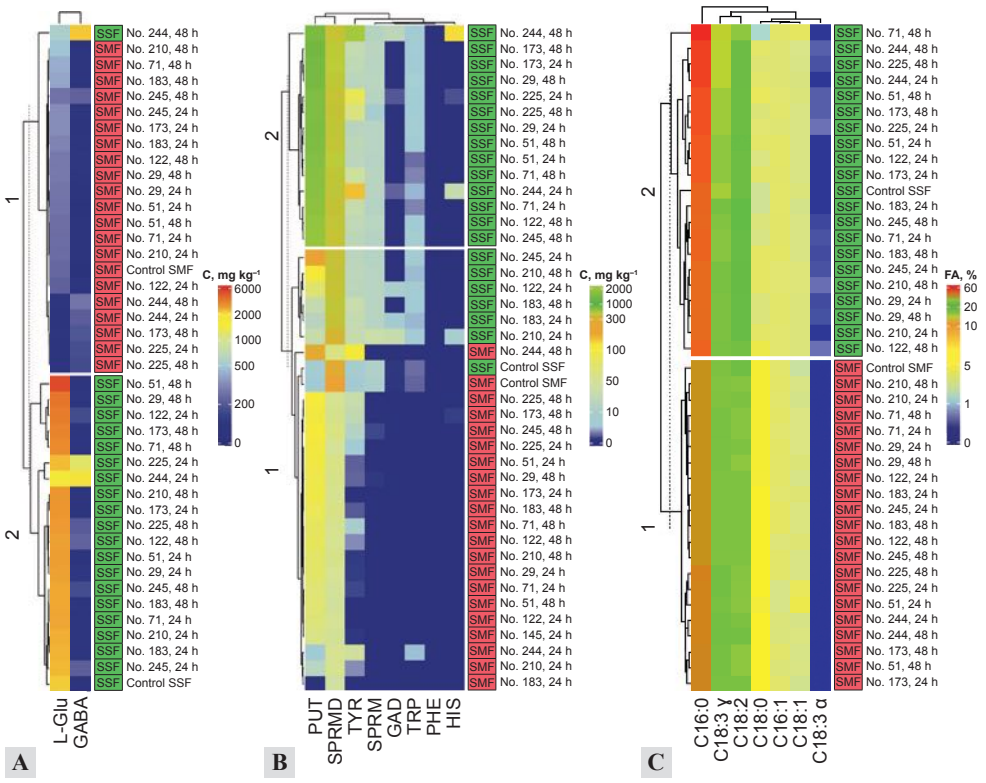


Fig. 2.4.1. (A) *Changes in the L-glutamic acid (L-Glu) and gamma-aminobutyric acid (GABA),* ***(B)*** *biogenic amine concentrations and the* ***(C)*** *fatty acid (FA) profile in the nontreated and fermented Spirulina samples*

PUT – putrescine; TRP – tryptamine; PHE – phenylethylamine; CAD – cadaverine; HIS – histamine; TYR – tyramine; SPRMD – spermidine; SPRM – spermine. C16:0 – methyl palmitate; C16:1 – methyl palmitoleate; C18:0 – methyl stearate; C18:1 cis – trans-cis, trans-9-oleic acid methyl ester; C18:2 – methyl linoleate; C18:3γ – gamma-linolenic acid methyl ester; C18:3α – alpha linolenic acid methyl ester. No. 122 – *Lactobacillus plantarum*; No. 210 – *Lacticaseibacillus casei*; No. 51 – *Lactobacillus curvatus*; No. 244 – *Lacticaseibacillus paracasei*; No. 71 – *Lactobacillus coryniformis*; No. 183 – *Pediococcus pentosaceus*; No. 173 – *Levilactobacillus brevis*; No. 29 – *Pediococcus acidilactici*; No. 225 – *Leuconostoc mesenteroides*; No. 245 – *Liquorilactobacillus uvarum*; SMF – submerged fermentation; SSF – solid state fermentation; C – concentration of L-glutamic acid and biogenic amines, mg kg^{-1}.

The main FA in the nontreated and fermented Spirulina samples were methyl palmitate (C16:0), methyl linoleate (C18:2), and gamma-linolenic (C18:3ɣ) acid methyl esters (Fig. 2.4.1 C). When investigating the C16:0 content in the nontreated and SMF samples, the concentration was higher in 18 out of 20 samples compared with the control (I) samples. Regarding the C18:2 content in the Spirulina samples, the contents were lower in all the SMF and SSF samples compared with the control (I) and control (II) samples, respectively. When comparing the C18:3ɣ content with the control, there were different trends in the SMF and SSF samples. In the case of SMF, the C18:3ɣ content increased in 6 out of 20 SMF samples, decreased in 8 out of 20 SMF samples, and remained similar to the control (I) in 6 out of 20 SMF samples. In the case of SSF, the C18:3ɣ content increased in 14 out of 20 SSF samples, decreased in 4 out of 20 SSF samples, and remained similar to the control (II) in 2 out of 20 SSF samples. Likewise, there were changes in the FA profile of the Spirulina samples throughout the fermentation processes.

The proximate composition of Spirulina is influenced by various factors, including the source of the cyanobacteria, the season of harvest, and the manufacturing process. The predominant amino acids in Spirulina are glutamic acid, followed by leucine and aspartic acid [140]. Specific bacterial genera are involved in the production of GABA [141]. It has been reported that LAB can induce the structural breakdown of cyanobacterial cell walls through hydrolysis, which converts complex compounds into simpler forms [142]. The present study showed that the fermentation conditions (SMF or SSF) are also a very significant factor, especially for the GABA content in Spirulina. In addition, TYR, HIS, PUT, CAD, SPRM, and SPRMD are mainly produced by microbial decarboxylation of amino acids [143, 144]. PUT is a precursor for the synthesis of SPRMD [144]. In addition to the individual toxicity of BA, Wang et al. [145] reported that the sum of primary, secondary, and tertiary BA is very important. PUT and CAD potentiate intoxication in the presence of other BA [146]. In conclusion, the samples with the highest GABA concentrations also showed the highest BA concentrations. This highlights the necessity of investigating both beneficial and potentially undesirable compounds in the final product, particularly when both are formed via amino acid decarboxylation pathways.

The lipid concentration of Spirulina can vary from ca. 5% to 10% (of the DM) [16]. Omega-6 FA constitute the majority of the total Spirulina FA [147, 148]. Furthermore, Spirulina contains a significant amount of palmitic acid (16:0), which represents more than 25% from the total fat content [16]. Polyunsaturated FA levels in Spirulina range from 1.5% to 2.0% of total fat [149], whereas the polyunsaturated FA content represents 30% of the total fat content [150]. Spirulina is the sole food source known to contain substantial

quantities of essential FA, notably γ-linolenic acid. Additionally, the FA profile of Spirulina heavily relies on the fermentation process; thus, by selecting the most appropriate pretreatment conditions, the FA profile may be changed.

This study demonstrated the importance of controlling not only the concentration of beneficial compounds in the final product but also undesirable substances. Both types of compounds are produced through similar metabolic pathways involving the decarboxylation of amino acids.

2.5. Relationship between the formation of bioactive compounds of proteinaceous origin in Spirulina

The concentrations of bioactive compounds of proteinaceous origin (the total BA content and the GABA and L-Glu concentrations) in the Spirulina samples are shown in Fig. 2.5.1. The SSF samples showed the highest GABA concentrations, specifically the samples submitted to SSF for 24 and 48 h with *Lb. paracasei* No. 244 (> 2000 mg kg^{-1}) and to SSF for 24 h with *L. mesenteroides* No. 225 (1264 mg kg^{-1}); the BA/GABA ratio was 0.72, 0.86 and 1.07, respectively. Overall, the BA/GABA ratio in the samples ranged from 0.5 (the sample submitted to SMF for 24 h with *Lb. paracasei* No. 244) to 62 (the samples submitted to SSF for 24 h with *Lb. brevis* No. 173).

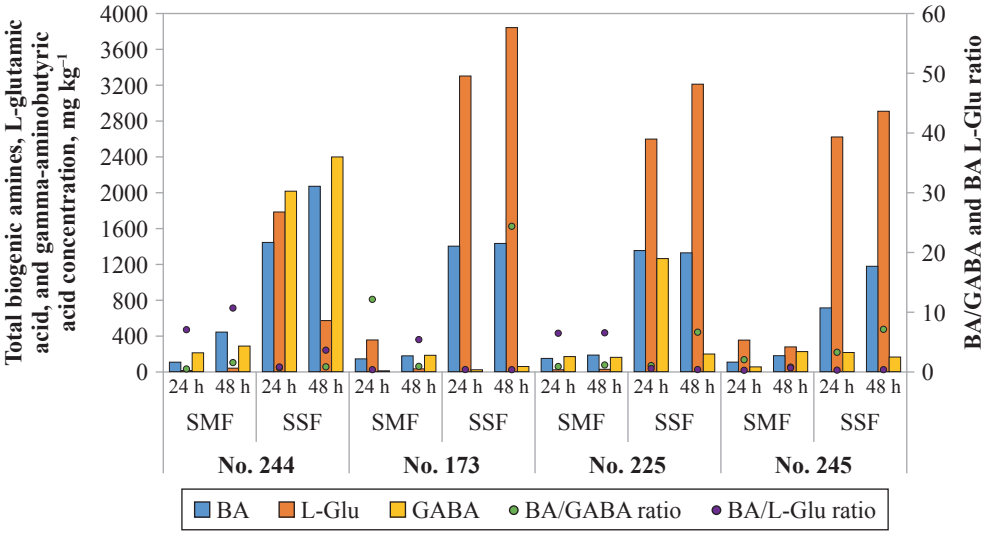


Fig. 2.5.1. *The total biogenic amine (BA) content and the L-glutamic acid (L-Glu) and gamma-aminobutyric acid (GABA) concentrations (mg kg^{-1}) in Spirulina samples, as well as the BA/GABA and BA/L-Glu ratios*

No. 244 – fermented with *Lacticaseibacillus paracasei* No. 244; No. 173 – fermented with *Levilactobacillus brevis* No. 173; No. 225 – *Leuconostoc mesenteroides* No. 225; No. 245 – fermented with *Liquorilactobacillus uvarum* No. 245.

The GABA content in the Spirulina samples exhibited significant correlations with the PUT, CAD, HIS, TYR, SPRMD, and SPRM concentrations (Table 2.5.1). The BA/L-Glu ratio in the Spirulina samples varied from 0.31 for the sample submitted to SMF for 24 h SMF with *Lb. uvarum* No. 245 to 10.7 for the sample submitted to SMF for 48 h with *Lb. paracasei* No. 244. Moreover, the L-Glu concentration in the Spirulina samples showed positive moderate correlations with the TRP, PUT, SPRMD, and SPRM concentrations. The viable LAB count in the Spirulina samples exhibited weak negative correlations with the CAD and SPRM concentrations. Although the viable LAB count was a significant factor in the formation of GABA and L-Glu, there were no significant correlations between these factors. Furthermore, the GABA concentration in the Spirulina samples exhibited a weak positive correlation with the *S. aureus* DIZ. Despite these results, there were no correlations between the pH of the samples and the other analyzed parameters.

Table 2.5.1. *Pearson correlations and their significance between the analyzed Spirulina parameters*

		pH	TRP	PUT	CAD	HIS	TYR	SPRMD	SPRM	GABA	L-Glu	DIZ	LAB
pH	r	1	−0.086	−0.197	0.187	0.098	0.053	−0.169	−0.123	0.107	−0.029	0.023	−0.128
	p		0.534	0.153	0.175	0.480	0.704	0.222	0.377	0.442	0.833	0.872	0.355
TRP	r	−0.086	1	**0.722** **	0.255	**0.289** *	**0.314** *	**0.877** **	**0.842** **	0.256	**0.541****	−0.147	−0.098
	p	0.534		**0.0001**	0.062	**0.034**	**0.021**	**0.0001**	**0.0001**	0.061	**0.0001**	0.304	0.482
PUT	r	−0.197	**0.722** **	1	0.259	**0.377** **	**0.447** **	**0.872** **	**0.747** **	**0.396** **	**0.519** **	0.011	0.073
	p	0.153	**0.0001**		0.059	**0.005**	**0.001**	**0.0001**	**0.0001**	**0.003**	**0.0001**	0.938	0.600
CAD	r	0.187	0.255	0.259	1	**0.923** **	**0.894** **	0.248	**0.314** *	**0.531** **	−0.124	−0.142	**−0.282** *
	p	0.175	0.062	0.059		**0.0001**	**0.0001**	0.070	**0.021**	**0.0001**	0.372	0.322	**0.039**
HIS	r	0.098	**0.289** *	**0.377** **	**0.923** **	1	**0.977** **	**0.297** *	**0.306** *	**0.630** **	−0.085	−0.029	−0.100
	p	0.480	**0.034**	**0.005**	**0.0001**		**0.0001**	**0.029**	**0.024**	**0.0001**	0.539	0.840	0.472
TYR	r	0.053	**0.314** *	**0.447** **	**0.894** **	**0.977** **	1	**0.325** *	**0.310** *	**0.656** **	−0.065	0.050	-.0045
	p	0.704	**0.021**	**0.001**	**0.0001**	**0.0001**		**0.016**	**0.023**	**0.0001**	0.640	0.727	0.747
SPRMD	r	−0.169	**0.877** **	**0.872** **	0.248	**0.297** *	**0.325** *	1	**0.941** **	**0.322** *	**0.627** **	−0.125	−0.181
	p	0.222	**0.0001**	**0.0001**	0.070	**0.029**	**0.016**		**0.0001**	**0.018**	**0.0001**	0.383	0.191
SPRM	r	−0.123	**0.842** **	**0.747** **	**0.314** *	**0.30 6***	**0.310** *	**0.941** **	1	**0.317** *	**0.572** **	−0.133	**−0.347** *
	p	0.377	**0.0001**	**0.0001**	**0.021**	**0.024**	**0.023**	**0.0001**		**0.019**	**0.0001**	0.351	**0.010**
GABA	r	0.107	0.256	**0.396** **	**0.531** **	**0.630** **	**0.656** **	**0.322** *	**0.317** *	1	0.163	**0.337** *	−0.055
	p	0.442	0.061	**0.003**	**0.0001**	**0.0001**	**0.0001**	**0.018**	**0.019**		0.240	**0.016**	0.691
L-Glu	r	−0.029	**0.541** **	**0.519** **	−0.124	−0.085	−0.065	**0.627** **	**0.572** **	0.163	1	−0.099	0.007
	p	0.833	**0.0001**	**0.0001**	0.372	0.539	0.640	**0.0001**	**0.0001**	0.240		0.489	0.960
DIZ	r	0.023	−0.147	0.011	−0.142	−0.029	0.050	−0.125	−0.133	**0.337** *	−0.099	1	0.027
	p	0.872	0.304	0.938	0.322	0.840	0.727	0.383	0.351	**0.016**	0.489		0.853
LAB count	r	−0.128	−0.098	0.073	**−0.282** *	−0.100	−0.045	−0.181	**−0.347** *	−0.055	0.007	0.027	1
	p	0.355	0.482	0.600	**0.039**	0.472	0.747	0.191	**0.010**	0.691	0.960	0.853	

** Correlation is significant at the 0.01 level (2-tailed); * correlation is significant at the 0.05 level (2-tailed); r – Pearson correlation; p – significance (2-tailed); LAB – lactic acid bacteria strain used for fermentation; TRP – tryptamine; PHE – phenylethylamine; PUT – putrescine; CAD – cadaverine; HIS – histamine; TYR – tyramine; SPRMD – spermidine; SPRM – spermine; GABA – gamma-aminobutyric acid; L-Glu – L-glutamic acid; DIZ – diameter of inhibition zone against *Staphylococcus aureus*.

Unlike chemical synthesis, biological production of GABA using technological microorganisms is safer and more environmentally friendly [151–153]. GABA can be formed during protein metabolism, typically through enzymatic conversion from L-Glu by glutamate decarboxylase [43]. The parameters for the GABA production process can be easily regulated [154]. In technological LAB strains, glucose metabolism generates various metabolites, including GABA [155]. LAB, as economically viable technological microorganisms, are the most studied for GABA production [154]. However, several factors, such as temperature, pH, the duration of the process, and others, can significantly influence the GABA content [156). In addition to desirable compounds, LAB are also involved in the formation of BA [157]. Most of the BA are classified as undesirable compounds, with the exception of beta-phenylethylamine (β-PEA), which is associated with neurotransmitter functions [65, 66]. This neurotransmitter regulates the release and response of dopamine, norepinephrine, acetylcholine, and GABA [158].

This study demonstrated that during the fermentation of Spirulina with LAB, high concentrations of desirable compounds are produced. However, nondesirable compounds such as BA are also formed due to their similar mechanisms of synthesis. Therefore, their potential presence in high concentrations in the end products must be carefully considered.

CONCLUSIONS

1. The data collected regarding the micro- and macroelement profiles of *U. intestinalis*, *C. rupestris*, and *F. lumbricalis*, as well as their characteristics before and after fermentation, has revealed the following:
   1.1. Despite high concentrations of desirable trace elements, the tested macroalgae showed high contamination with undesirable ones: the As content in the *U. intestinalis*, *C. rupestris*, and *F. lumbricalis* samples, was, on average, 4.1, 3.79, and 5.38 mg kg^{-1}, respectively; the Pb content was, on average, 0.733, 1.41, and 0.371 mg kg^{-1}, respectively; the Rb content was, on average, 5.93, 9.86, and 13.35 mg kg^{-1}, respectively, the Sr content was, on average, 191, 140, and 189 mg kg^{-1}, respectively; and the Al content was, on average, 0.971 g kg^{-1}. Based on these results, the macroalgae need to be decontaminated before their use for feed preparation.
   1.2. Wild algae obtained from the Baltic Sea can be contaminated with potentially harmful microorganisms (*E. vulneris*, *Actinobacterium* spp., *P. putida*, and *P. lacus*) that remain after fermentation. Hence, to ensure the biosafety of this material, biological decontamination is needed.
   1.3. The algae and LAB combination showed a broader spectrum of pathogen inhibition (the fermented samples inhibited 6–9 of the 13 tested pathogens, while the nonfermented samples inhibited 2–3 of the 13 tested pathogens).
   1.4. The fermented and nonfermented *C. rupestris* samples showed highest antioxidant activity (on average, 58.0%); however, fermentation reduced the TPC content of the *C. rupestris* and *F. lumbricalis* samples (on average, by 45 and 24%, respectively).

2. The preparation of extracts is a suitable technological approach to extract functional compounds from algal biomass and to ensure the chemical and biological safety of alga-based materials.
   2.1. The *C. rupestris* and *C. vulgaris* extracts had the highest TCC (on average, 1.26 and 1.52 mg g^{-1}, respectively). The Spirulina extracts had the highest chlorophyll *a* content (on average, 7.95 mg g^{-1}). Finally, the *C. rupestris* extract had the highest chlorophyll *b* content (on average, 5.14 mg g^{-1}).
   2.2. The *C. rupestris* and *F. lumbricalis* extracts showed the highest TPC content (on average, 349.9 and 355.2 mg GAE 100 g^{-1}, respectively). The *C. rupestris* extract had the highest DPPH• antioxidant activity (on average, 5.82%).

2.3. The Sp1 and *U. intestinalis* extracts had the broadest antimicrobial activity: the Sp1 extract inhibited *S. haemolyticus* and *B. subtilis* (with an average DIZ of 28.3 and 10.1 mm, respectively), and the *U. intestinalis* extract inhibited *B. subtilis* and *S. mutans* (with an average DIZ of 17.0 and 14.2 mm, respectively).

2.4. The nondesirable trace elements Ga, Be, Sn, Hg, B, Ti, and Cd did not remain in the extracts; Mo was not detected in the *C. rupestris* extract; Cs was not detected in the *C. rupestris* and *U. intestinalis* extracts; the As content in the *U. intestinalis*, *C. rupestris*, and *F. lumbricalis* extracts decreased by, on average, 90%, 91%, and 96%, respectively; the Pb content in the *U. intestinalis*, *C. rupestris*, and *F. lumbricalis* extracts decreased by, on average, 99.8%, 99.2%, and 99.9%, respectively; and the Sr content in the *U. intestinalis*, *C. rupestris*, and *F. lumbricalis* extracts decreased by, on average, 99.7%, 99.9%, and 99.7%, respectively.

3. Both tested pretreatments, fermentation and ultrasonication, were significant factors for the extract characteristics.

3.1. Fermentation of *U. intestinalis*, *C. rupestris*, *F. lumbricalis*, *C. glomerata*, and Spirulina before extract preparation increased the TPC content in the *U. intestinalis*, *C. rupestris*, *C. glomerata* and Spirulina extracts, on average, by 3.13, 5.44, 1.28, and 2.52 times, respectively; the DPPH• antioxidant activity of the *C. rupestris* extract (on average, 1.60 times); the ABTS•+ antioxidant activity of the *C. rupestris* extract (on average, 4.86 times); and the FRAP antioxidant activity of the *U. intestinalis*, *C. rupestris*, and *F. lumbricalis* extracts, on average, 4.10, 3.56, and 1.47 times, respectively. Although the antimicrobial properties of most extracts prepared from fermented algae were weaker than those of extracts from nonfermented algae, the *F. lumbricalis* extract prepared from fermented material inhibited *S. aureus* more effectively (with an average DIZ of 13.3 mm).

3.2. Ultrasonication of *U. intestinalis*, *C. rupestris*, *F. lumbricalis*, *C. glomerata*, and Spirulina before extract preparation increased their TPC content (on average, 1.61 and 4.91 times for the *U. intestinalis* and *C. rupestris* extracts, respectively); the DPPH• antioxidant activity (on average, 2.05 and 1.60 times for the *U. intestinalis* and *C. rupestris* extracts, respectively); the ABTS•+ antioxidant activity (on average, 3.37 times for the *C. rupestris* extract); the FRAP antioxidant activity (on average, 14.81 and 1.19 times for the *C. ru-*

pestris, and *F. lumbricalis* extracts, respectively); and the antimicrobial properties (after ultrasonication, the *C. rupestris* extract had a larger DIZ against *B. cereus* (on average, 18.2 ± 0.5 mm)).

3.3. A combination of extracts with *Lb. plantarum* LUHS135 led to a broader antimicrobial properties of alga-based material. Specifically, the extracts prepared from non-pretreated algae in combination with *Lb. plantarum* LUHS135 inhibited 1–3 of the 7 tested pathogens, extracts prepared from fermented algae in combination with *Lb. plantarum* LUHS135 inhibited 1–2 of the 7 tested pathogens, and extracts prepared from ultrasonicated algae in combination with *Lb. plantarum* LUHS135 inhibited 1–2 of 7 tested pathogens.

4. Fermentation with selected LAB strains is a suitable technological method for improving the functional value of Spirulina; however, BA formation should be controlled.

4.1. The lowest total BA content was found in the samples submitted to SMF for 24 h with *P. pentosaceus* No. 183 (only SPRMD, on average, 41.3 mg kg^{-1}).

4.2. The highest L-Glu (5506.4 mg kg^{-1}) and GABA (2396 mg kg^{-1}) concentrations occurred in the samples submitted to SSF for 48 h with *Lb. curvatus* No. 51 and *Lb. paracasei* No. 244, respectively. The BA/GABA and BA/L-Glu ratios in the Spirulina samples ranged from 0.5 to 62 and from 0.31 to 10.7, respectively.

4.3. The main FA in the nontreated and fermented Spirulina samples were C16:0, C18:2, and C18:3ɣ. The Spirulina samples with the lowest BA and the highest GABA concentrations (*Lb. paracasei* No. 244: SMF for 24 and 48 h, and SSF for 24 and 48 h; *Lb. brevis* No. 173: SMF for 48 h, and SSF for 24 and 48 h; *L. mesenteroides* No. 225: SMF for 48 h, and SSF for 24 and 48 h; and *Lb. uvarum* No. 245: SSF for 24 and 48 h) inhibited *S. aureus* (on average, a DIZ of 9.1–16.3 mm).

RECOMMENDATIONS

1. Based on the micro- and macroelement profiles of *U. intestinalis*, *C. rupestris*, and *F. lumbricalis* as well as their characteristics before and after fermentation, algal extract preparation is a suitable technological approach to obtain functional compounds from algal biomass and to ensure chemical and biological safety of alga-based material.
2. Fermentation of algal biomass is recommended because the synergistic mechanism of the algae and LAB combinations inhibits a broader range of pathogens.
3. Fermentation and ultrasonication are suitable pretreatments that can be recommended to modify algal biomass before extract preparation, thus altering the characteristics of the resulting alga-based material.
4. Combining extracts with *Lb. plantarum* LUHS135 can be recommended to prepare materials with broader antimicrobial activity.
5. Fermentation with selected LAB strains – *Lb. paracasei* No. 244, *Lb. brevis* No. 173, *L. mesenteroides* No. 225, and *Lb. uvarum* No. 245 – can be recommended to increase the functional value of Spirulina (fermentation leads to a higher GABA concentration and broader antimicrobial activity).

SUMMARY IN LITHUANIAN

SANTRUMPOS

ABTS•+	–	2,2-azino-bis(3-etilbenzotiazolin-6-sulfonrūgštis) radikalų-katijonų surišimo aktyvumas
Ag	–	sidabras
Al	–	aliuminis
As	–	arsenas
B	–	boras
Ba	–	baris
BA	–	biogeniniai aminai
Be	–	berilis
C16:0	–	palmitino rūgštis
C18:2	–	metillinoleatas
C18:3ɣ	–	gamma-linoleno rūgšties metilo esteris
Ca	–	kalcis
CAD	–	kadaverinas
Cd	–	kadmis
ClaG	–	*Cladophora glomerata*;
ClaR	–	*Cladophora rupestris*;
Co	–	kobaltas
Cr	–	chromas
Cs	–	cezis
Cu	–	varis
DIZ	–	slopinimo zonos skersmuo
DPPH•	–	2,2-difenil-1-pikrilhidrazilo radikalo surišimo aktyvumas
FAME	–	riebalų rūgščių metilo esteriai
Fe	–	geležis
FRAP	–	geležies jonų redukcijos antioksidantinė galia
Furc	–	*Furcellaria lumbricalis*
Ga	–	galis
GABA	–	gamma-aminosviesto rūgštis
GAE	–	galio rūgšties ekvivalentas
Hg	–	gyvsidabris
HIS	–	histaminas
HPLC	–	aukštos kokybės skysčių chromatografija
I	–	jodas
K	–	kalis
KSV	–	kolonijas sudarančių vienetų skaičius
L-Glu	–	L-glutamo rūgštis
Li	–	litis
LSMU	–	Lietuvos sveikatos mokslų universitetas
Mg	–	magnis
Mn	–	manganas
Mo	–	molibdenas
Na	–	natris
Ni	–	nikelis

P – fosforas
Pb – švinas
PHE – feniletilaminas
PRB – pieno rūgšties bakterijos
PUT – putrescinas
Rb – rubidis
RR – riebalų rūgštys
Sb – stibis
Se – selenas
Si – silicis
SM – sausos sedžiagos
SMF – skystos fazės fermentacija
Sn – alavas
SPRM – sperminas
SPRMD – spermidinas
Sr – stroncis
SSF – kietafazė kermentacija
TBC – bendras bakterijų skaičius
TCC – bendras karotinoidų kiekis
Ti – titanas
TPC – bendras fenolinių junginių kiekis
TPTZ – 2,4,6-tri-2-pyridinyl-1,3,5-triazinas
TRP – triptaminas
TYR – tiraminas
Ul – *Ulva intestinalis*
V – vanadis
Zn – cinkas

ĮVADAS

Didėjantis maistinių medžiagų poreikis gyvulininkystės pramonėje paskatino mikro- ir makrodumblių biomasės, kaip alternatyvių pašarinių žaliavų, mokslinių tyrimų plėtrą [1–3].

Makrodumbliai yra natūralūs jūrų ekosistemos komponentai, dėl savo išskirtinės cheminės sudėties, naudojami įvairiose pramonės srityse [4]. Daugiausiai dumblių biomasės užaugina ir perdirba Kinija (47,9 proc.), Indonezija (38,7 proc.), Filipinai (4,7 proc.) ir Korėjos Respublika (4,5 proc.), o pagrindiniai laukinių makrodumblių produkcijos tiekėjai yra Čilė, Kinija ir Norvegija [5]. Jūrų makrodumbliai yra gera alternatyva sausumoje auginamai biomasei, nes jie nekonkuruoja dėl žemės ploto su augalais, kurie auginami maisto ir pašarų pramonės reikmėms, o biomasės augimui nereikia specialių išteklių [6].

Mikrodumbliai tampa svarbiu biologiškai aktyvių junginių šaltiniu daugelyje pramonės šakų, tačiau, pagal Maisto ir vaistų administraciją (FDA) tik kelios mikrodumblių rūšys yra pripažintos saugiomis (GRAS) ir gali būti

naudojamos maisto ar pašaro gamybai: *Arthrospira platensis* (Spirulina) – cianobakterijos, *Chlamydomonas reinhardtii, Auxenochlorella protothecoides, Chlorella vulgaris, Dunaliella salina* (anksčiau *Dunaliella bardawil*) – *Chlorophyta* ir *Euglena gracilis – Euglenozoa* [7]. Spirulina yra viena iš populiariausių mikrodumblių rūšių [8]. Šis mikrodumblis pasižymi dideliu baltymų, vertingų nepakeičiamųjų riebalų rūgščių (RR), mineralų, pigmentų ir vitaminų kiekiu [9]. Paskelbtose mokslinėse publikacijose gausu informacijos apie Spirulinos prebiotines, antioksidacines [9–11], antibakterines, antivirusines ir imunomoduliuojančias savybes [12–16]. Kontroliuojamomis sąlygomis auginama Spirulina yra saugus ingredientas maisto bei pašarų pramonei [10, 12–14, 17, 18].

Didėjantis funkcionaliųjų medžiagų poreikis gyvulininkystėje, skatina didinti dumblių vartojimą, įtraukiant šiuos biologiškai aktyvius komponentus į pašarų formules [19]. Kitas būdas, padidinti pašarinių žaliavų funcionalumą ir suteikti joms papildomos pridėtinės vertės yra fermentacija pieno rūgšties bakterijomis. Nors pašarų fermentacijos technologijos plačiai aprašytos ir naudojamos pramoniniu mastu, tyrimų apie fermentuotą pašarinę medžiagą, pagamintą iš dumblių, yra nedaug. Dumblių angliavandenių cheminė sudėtis skiriasi nuo tradicinių pašarų pramonėje naudojamų žaliavų pagrindinių sudėties komponentų (dumbliuose vyrauja polisacharidai, pvz., ruduosiuose dumbliuose – alginatas ir fukoidanas, raudonuosiuose – galaktanas, žaliuosiuose – celiuliozė ir hemiceliuliozės), todėl fermentacijai parenkami mikroorganizmai ir sąlygos turi būti specifiškai pritaikomi [20, 21]. Biotransformacija pieno rūgšties bakterijomis (PRB) yra plačiai taikomas būdas augalų ir melsvadumblių ląstelių sienelių komponentų degradavimui į mažesnės molekulinės masės junginius, pasižyminčius imunomoduliuojančiomis, antioksidacinėmis, antimikrobinėmis ir kt. pageidautinomis savybėmis [22–25]. Fermentacija PRB pramoniniu mastu yra ekonomiškai efektyvi strategija [26].

Tačiau, ir taip efektyvių biotechnologinių procesų modeliavimui, tvaresnių koncepcijų integracija, vis dar labai aktuali [27, 28]. Kietafazė fermentacija (SSF) apibūdinama, kaip mikrobų augimas ir substrato biokonversija ant kietų jo dalelių, esant mažiausiai galimai vandens koncentracijai, kuri pakankama technologinių mikroorganizmų gyvybingumui palaikyti. Ši technologija yra daug ekonomiškesnė, palyginus su tradiciniu biomasės apdorojimu skystoje fazėje [29, 30]. Mažas vandens kiekis ir apdorojamos masės tūris, leidžia padidinti proceso efektyvumą, nes naudojami mažesni bioreaktoriai, reikalingos mažesnės sterilizavimo talpos, naudojama mažiau vandens ir kt. išteklių.

Taip pat, koncentruojant dumblių veikliuosius junginius, biomasės apdorojimas (pvz., fermentacija, apdorojimas ultragarsu ir kt.) prieš ekstrahavimą,

gali padidinti ekstraktų funkcionalumą (pagerinti antioksidacines ir antimikrobines savybes, padidinti bendrą fenolių junginių (TPC) kiekį ekstraktuose). Pirminis dumblių biomasės apdorojimas, prieš ekstraktų gamybą, labai svarbus technologinis etapas, nes padidina bioaktyvių molekulių pralaidumą per sudėtingas biomasės ląstelių sieneles [31–33]. Literatūroje aprašomi galimi dumblių biomasės pirminio apdorojimo mechaniniai ir biologiniai metodai [34, 35]. Nepaisant to, kad pirminis fizinis apdorojimas yra brangus, tačiau, apdorojimas ultragarsu yra rekomenduotas, kaip perspektyviausias ląstelių ardymo metodas [32, 36, 37]. Apdorojimas ultragarsu pažeidžia ląstelių struktūrą ir pagerina veikliųjų medžiagų pernašą į ekstraktą [32, 38–40]. Biologinis apdorojimas mikrogrybais, bakterijomis ir (arba) jų fermentais gali būti naudojamas dumblių ląstelių sienelių ligninui ir hemiceliuliozėms skaidyti [35, 41]. Biologinis pirminis apdorojimas papildomai sukuria pridėtinę vertę, nes jo metu susidaro kiti, nebūdingi biomasei, junginiai: fenolio rūgštys, benzenkarboksirūgštis, siringaldehidas ir kt. [42]. Gama-aminosviesto rūgštis (GABA) – pageidaujamas funkcionalusis junginys, kuris susintetinamas iš L-glutamino, dekarboksilinanat glutamato dekarboksilazės fermentu [43–45]. Spirulina [46–48] turi daug GABA pirmtakų [49], tačiau, nepaisant daugelio pageidaujamų pirminio biologinio apdorojimo savybių, reikia kontroliuoti šio proceso metu susidarančius nepageidaujamus junginius, pvz., nepaisant to, kad Spirulinos funkcionalioji vertė gali būti padidinta, taikant biomasės apdorojimui fermentaciją pasirinktomis PRB padermėmis (*Lactobacillus* padermės, įskaitant *Levilactobacillus brevis* [43, 50–56], *Lactobacillus buchneri* [57, 58], *Lactobacillus delbrueckii* subsp. *bulgaricus* [54, 59], *Lactobacillus fermentum* [60, 61], *Lactobacillus helveticus* [43, 62], *Lacticaseibacillus paracasei* [54, 63], *Lactiplantibacillus plantarum* [43, 54, 63, 64] ir pan.), dekarboksilinimo proceso metu gali susidaryti ir nepageidaujami junginiai (pvz., biogeniniai aminai (BA)) [65, 66]. Toksiškiausi BA yra tiraminas (TYR) ir histaminas (HIS) [67, 68].

Nors dumblių biomasė yra labai perspektyvi žaliava pašarų pramonei, reikėtų detaliai ištirti jos cheminę sudėtį, įskaitant tiek pageidaujamus, tiek ir nepageidaujamus junginius bei pasirinkti tinkamiausius sprendimus pirminiam dumblių biomasės apdorojimui, taikomam prieš veikliųjų junginių koncentravimą ekstraktuose.

Darbo tikslas

Sukurti inovatyvias, mikro- ir makrodumblių biomasės modifikavimo į pridėtinės vertės žaliavas, technologijas.

Darbo uždaviniai:

1. Išanalizuoti *Ulva intestinalis, Cladophora rupestris* ir *Furcellaria lumbricalis* makrodumblių mikro- ir makroelementų profilį bei įvertinti fermentacijos *Lb. plantarum* LUHS135 paderme įtaką *U. intestinalis, C. rupestris* ir *F. lumbricalis* mikrobiniam profiliui bei antimikrobinėms ir antioksidacinėms savybėms.
2. Įvertinti makrodumblių *U. intestinalis, C. rupestris, F. lumbricalis* ir *Cladophora glomerata* bei mikrodumblių Spirulina ir *C. vulgaris* ekstraktų savybes (įskaitant bendrą karotinoidų kiekį (TCC), chlorofilo *a* ir *b* koncentracijas, TPC kiekį, antioksidacines ir antimikrobines savybes bei mikro- ir makroelementų perėjimo iš dumblių biomasės į ekstraktą efektyvumą).
3. Išanalizuoti pirminio *U. intestinalis, C. rupestris, F. lumbricalis, C. glomerata* ir Spirulina dumblių biologinio apdorojimo ir apdorojimo ultragarsu įtaką dumblių ekstraktų bei jų kompozicijų su LUHS135 paderme antimikrobinėms ir antioksidacinėms savybėms.
4. Įvertinti Spirulinos sudėties pokyčius (įskaitant BA kiekį, L-glutamo rūgšties ir GABA koncentracijas, RR profilį) tradicinės (SMF) ir SSF fermentacijos *Lb. plantarum* No. 122; *Lacticaseibacillus casei* No. 210; *Lactobacillus curvatus* No. 51; *Lb. paracasei* No. 244; *Lactobacillus coryniformis* No. 71; *Pediococcus pentosaceus* No. 183; *Lb. brevis* No. 173; *Pediococcus acidilactici* No. 29; *Leuconostoc mesenteroides* No. 225; *Liquorilactobacillus uvarum* No. 245 padermėmis metu ir išanalizuoti Spirulinos mėginių, kuriuose mažiausia BA ir didžiausia GABA koncentracija, antimikrobinį aktyvumą.

Darbo naujumas ir praktinė reikšmė

Dumbliai yra greičiausiai augantys organizmai, galintys konvertuoti didelius anglies dioksido kiekius į deguonį. Kai kurios dumblių rūšys gali būti efektyviai auginamos ant alternatyvių substratų. Dėl šių savybių, dumblių įtraukimas į gyvūnų mitybos racionus galėtų būti itin perspektyvus. Tačiau duomenų apie Baltijos jūros makrodumblių detalią cheminę sudėtį ir mikrobų profilį iki šiol yra nedaug. Priešinga situacija stebima apie tyrimus mikrodumblių srityje. Tačiau, nors mokslinių tyrimų rezultatų apie Spirulinos savybes ir panaudojimo galimybes publikuota nemažai, iki šiol, informacijos apie šių mikrodumblių pirminio apdorojimo sprendimus, siekiant padidinti šios vertingos žaliavos funkcionalumą, nėra daug. Šio darbo mokslinis naujumas yra (I) sukurti naujas žinias apie Baltijos jūros makrodumblių detalią cheminę sudėtį ir mikrobų profilį, taip pat (II) rasti naujus sprendimus mikro- ir makrodumblių biomasės pirminiam apdorojimui, siekiant juos konvertuoti į

aukštesnės pridėtinės vertės žaliavas pašarų sektoriui. Gautų rezultatų praktinė nauda yra labai reikšminga ir siejama su šių alternatyvių maistinių medžiagų išteklių tolimesnio taikymo pašarinių žaliavų gamyboje perspektyvomis. Analizuojama perspektyvi ir atitinkanti šiandienos poreikius žaliava, ieškant tvaresnių sprendimų žemės ūkiui, susijusių su saugiu ir tvariu dumblių panaudojimu, įskaitant ir gyvulininkystės sektorių.

1. MEDŽIAGOS IR METODAI

1.1. Tyrimų vieta ir laikas

Eksperimentai atlikti 2020–2024 metais Lietuvos sveikatos mokslų universiteto (LSMU) Gyvūnų auginimo technologijų institute bei Mikrobiologijos ir virusologijos institute; Klaipėdos universiteto Jūros tyrimų institute (Klaipėda, Lietuva); Maisto saugos, gyvūnų sveikatingumo ir aplinkosaugos institute – BIOR (Ryga, Latvija); Lietuvos agrarinių ir miškų mokslų centro Sodininkystės ir daržininkystės institute (Babtai, Lietuva).

1.2. Tyrimo medžiagos ir pagrindinės schemos

1.2.1. Baltijos jūros makrodumbliai, mikrodumbliai ir dumblių apdorojimui naudotos pieno rūgšties bakterijos

1.2.1.1. Makrodumbliai

U. intestinalis, C. rupestris ir *F. lumbricalis* mėginiai buvo surinkti iš Baltijos jūros 2020 m. rugsėjį ir 2021 m. gegužės–birželio mėn. netoli Klaipėdos miesto (Lietuva). *U. intestinalis* mėginiai buvo renkami rankiniu būdu nuo 10 skirtingų akmenų, esančių iki 1 m gylyje. *C. rupestris* ir *F. lumbricalis* mėginiai buvo renkami netoli kranto (~1 m gylyje, ~100 m atstumu nuo kranto) praėjus kelioms dienoms po audros. *C. glomerata* mėginiai buvo surinkti 2021 m. gegužės–birželio mėn. nuo akmenų. Dumbliai buvo išvalyti, atskiriant nuo kitos rūšies dumblių ir makroskopinių gyvūnų, kruopščiai nuplauti du kartus po tekančio vandens srove bei, perplovus distiliuotu vandeniu, užšaldyti –80 °C temperatūroje ir sumalti iki 1 mm skersmens dalelių. Taip paruošti mėginiai laikomi sandariame plastikiniame maišelyje, šaldiklyje (–80 °C).

1.2.1.2. Mikrodumbliai

Spirulinos mėginiai buvo įsigyti iš trijų skirtingų gamintojų: (I) iš Teksaso universiteto biologinės laboratorijos (Ostinas, Teksasas, JAV) buvo pagausinti, pagal gamintojo pateiktas instrukcijas (Sp1); (II) iš UAB „Spila“ (Vilnius, Lietuva, kilmė Irvinas, CA, JAV) miltelių pavidalo (Sp2); (III) iš

įmonės „Now Foods Company“ (Blomingdeilas, IL, JAV) liofilizuoti milteliai. Mikrodumbliai *C. vulgaris* buvo įsigyti iš Teksaso universiteto biologinės laboratorijos (Ostinas, Teksasas, JAV) buvo pagausinti, pagal gamintojo pateiktas instrukcijas.

1.2.1.3. Mikro- ir makrodumblių apdorojimui naudotos pieno rūgšties bakterijų padermės

PRB padermės (*Lb. plantarum* No. 135; *Lb. plantarum* No. 122; *Lb. casei* No. 210*; Lb. curvatus* No. 51; *Lb. paracasei* No. 244; *Lb. coryniformis* No. 71; *P. pentosaceus* No. 183; *Lb. brevis* No. 173; *P. acidilactici* No. 29; *L. mesenteroides* No. 225; *Lb. uvarum* No. 245) buvo gautos iš LSMU kolekcijos (Kaunas, Lietuva). Prieš eksperimentą PRB padermės buvo pagausintos De Man, Rogosa ir Sharpe (MRS) sultinyje (Biolife, Milanas, Italija) 30 °C temperatūroje anaerobinėmis sąlygomis 24 valandas.

1.2.1.4. Kitos tyrimams naudotos medžiagos

Patogeninės oportunistinės bakterijų padermės (*Staphylococcus aureus, Staphylococcus haemolyticus, Enterococcus durans, Bacillus pseudomycoides, Salmonella enterica, Aeromonas hydrophila, A. veronii, Acinetobacter baumannii, A. johnsonii, Enterobacter cloacae, Cronobacter sakazakii, Kluyvera cryocrescens, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa*), naudotos dumblių mėginių antimikrobinių savybių vertinimui, buvo gautos iš LSMU kolekcijos (Kaunas, Lietuva).

1.2.2. Mikro- ir makrodumblių biomasės pirminio apdorojimo metodai

Pirmajame eksperimento etape *U. intestinalis*, *C. rupestris* ir *F. lumbricalis* dumblių mėginiai buvo fermentuoti tradiciniu būdu, skystoje fazėje (SMF) *Lb. plantarum* LUHS135. Prieš ir po SMF buvo ištirtas biomasės metagenominis profilis, antimikrobinės ir antioksidacinės savybės, mikro- ir makroelementų kiekis. Principinė eksperimento schema pateikta 1.2.2.1 pav. Visas eksperimentas išsamiai aprašytas Tolpežnikaitė ir kt. [69].

Antrajame eksperimento etape buvo paruošti makrodumlių *U. intestinalis, C. rupestris, F. lumbricalis* ir *C. glomerata* bei mikrodumblių Spirulina ir *C. vulgaris* ekstraktai. Ekstraktuose analizuotas TCC, chlorofilo *a* ir *b* koncentracijos, TPC kiekis, įvertintos antioksidacinės ir antimikrobinės savybės, mikro- ir makroelementų kiekis ekstraktuose. Principinė eksperimento schema pateikta 1.2.2.2 pav.

Trečiajame eksperimento etape dumbliai *U. intestinalis, C. rupestris, F. lumbricalis*, *C. glomerata* ir Spirulina buvo apdoroti (I) SMF *Lb. plantarum* LUHS135 ir (II) ultragarsu. Iš neapdorotų ir fermentuotų bei ultragarsu

apdorotų mėginių buvo paruošti ekstraktai. Taip pat, paruoštos ekstraktų ir LUHS135 kompozicijos. Gautos trys mėginių grupės: (I) ekstraktai ir ekstraktų × LUHS135 kompozicijos, paruoštų iš neapdorotų dumblių, (II) ekstraktai ir ekstraktų × LUHS135 kompozicijos, paruoštų iš ultragarsu apdorotų dumblių ir (III) ekstraktai ir ekstraktų × LUHS135 kompozicijos, paruoštų iš fermentuotų dumblių. Buvo analizuojamos mėginių spalvų koordinatės, rūgštingumo rodikliai, antimikrobinės ir antioksidacinės savybės. Principinė eksperimento schema pateikta 1.2.2.3 pav. Eksperimentas išsamiai aprašytas Tolpežnikaitė ir kt. [70].

Ketvirtajame eksperimento etape, Spirulinos mėginiai buvo SMF ir SSF *Lb. plantarum* No. 122; *Lb. casei* No. 210; *Lb. curvatus* No. 51; *Lb. paracasei* No. 244; *Lb. coryniformis* No. 71; *P. pentosaceus* No. 183; *Lb. brevis* No. 173; *P. acidilactici* No. 29; *L. mesenteroides* No. 225; *Lb. uvarum* No. 245 padermėmis. Prieš ir po apdorojimo analizuoti šie mėginių rodikliai: pH, spalvų charakteristikos, BA kiekis, L-glutamo rūgšties ir GABA koncentracijos, RR profilis. Principinė eksperimento schema pateikta 1.2.2.4 pav. Eksperimentas išsamiai aprašytas Tolpežnikaitė ir kt. [71].

Penktajame eksperimento etape buvo tirtas Spirulinos mėginių (SMF ir SSF *Lb. paracasei* No. 244, *Lb. brevis* No. 173, *L. mesenteroides* No. 225 ir *Lb. uvarum* No. 245), kuriuose nustatyta mažiausia BA ir dižiausia GABA koncentracija, antimikrobinis aktyvumas. Principinė eksperimento schema pateikta 1.2.2.5 pav. Eksperimentas išsamiai aprašytas Tolpežnikaitė ir kt. [29].

1.2.3. Analizės metodai

1.2.3.1. Mikrobiologinės analizės metodai

PRB kolonijas sudarančių vienetų skaičius (KSV) g^{-1} nustatytas pagal ISO 15214:1998 standartą [72]. Jo nustatymui buvo sumaišyta 10 g mėginio ir 90 ml fiziologinio tirpalo (9 g l^{-1} NaCl tirpalo). Iš suspensijos paruošti nuo 10^{-4} iki 10^{-8} skiedimai, naudojant fiziologinį tirpalą ir pasėti ant 5 mm storio sterilus MRS (Man, Rogosa, Sharpe) agaro (CM0361, Oxoid, Basingstoke, JK). Lėkštelėse pasėti mėginio skiedimai buvo inkubuojami anaerobinėmis sąlygomis 30 °C temperatūroje 72 val. Visi rezultatai buvo išreikšti KSV $\log_{10}$ grame ($\log_{10}$ KSV g^{-1}) [73].

Bendras bakterijų skaičius (TBC) buvo nustatytas naudojant selektyų agarą Petri lėkštelėse (PCA, CM0325, Oxoid Ltd.). Bakterijos buvo inkubuotos aerobinėmis sąlygomis 32 °C temperatūroje 24–48 valandas (CM0325, Oxoid, JK). Mikroorganizmų skaičius buvo apskaičiuotas ir išreikštas $\log_{10}$ KSV grame ir (arba) mililitre ($\log_{10}$ KSV g^{-1} ir (arba) KSV ml^{-1}).

1.2.3.2. Mikrobinio profilio analizė

Mikrobinio profilio analizė atlikta naudojant 16S rRNR geno sekoskaitą, kurios metu buvo siekiama ištirti bakterijų mikrobiomą dumblių mėginiuose prieš ir po fermentacijos. Analizei atlikti buvo naudojamas 1g tiriamosios dumblių medžiagos, DNR buvo išskirta naudojant DNR MiniPrep rinkinį (D6010, Zymo Research, JAV) [74]. ZymoBIO-MICS Mikrobinio profilio standartas (D6300, Zymo Research Corporation, Irvine, CA, JAV) buvo naudojamas kaip mikrobiomų profiliavimo kokybės kontrolė. Rezultatai buvo išanalizuoti ir taksonominė klasifikacija vizualiai pateikta naudojant interaktyvią internetinę platformą.

1.2.3.3. Antimikrobinių savybių analizė

Antimikrobinis aktyvumas nustatytas, vertinant patogeninių ir oportunistinių bakterijų padermių (paminėtų 1.2.1.4 skyriuje) slopinimo efektyvumą, taikant tyrimo skystoje terpėje bei difuzijos į agarą metodus. Antimikrobinio aktyvumo skystoje terpėje rezultatai interpretuojami kaip (-), jei patogenai selektyvioje terpėje neaugo, ir (+) – jei patogenai selektyvioje terpėje augo. Antimikrobinis aktyvumas, vertinant difuzijos į agarą metodu, buvo nustatytas vertinant patogenų slopinimo zonos skersmenį (mm) (DIZ). Abu metodus išsamiai aprašo Tolpežnikaitė ir kt. [70].

1.2.3.4. Sausųjų medžiagų kiekio, pH ir spalvų koordinačių nustatymo analizė

Sausųjų medžiagų kiekis (SM) dumblių mėginiuose buvo įvertintas išdžiovinus mėginius 103 ± 2 °C iki pastovios masės ir apskaičiavus masės nuostolius procentine išraiška.

Dumblių mėginių pH vertės buvo išmatuotos pH-metru (Inolab 3, Hanna Instruments, Italija).

Spalvos koordinatės buvo nustatytos naudojant CIE L*a*b* sistemą (CromaMeter CR-400, Konica Minolta, Japonija) trijose skirtingose mėginio paviršiaus vietose. Rezultatai buvo išreikšti CIE spalvų koordinatėmis L* (šviesumas/tamsumas), a* (nuo raudonos iki žalios spalvos) ir b* (nuo geltonos iki mėlynos spalvos).

1.2.3.5. Bendro fenolinių junginių kiekio ir antioksidacinių savybių nustatymo analizė

TPC kiekiui nustatyti buvo naudojamas Folin-Ciocalteu reagentas [75]. TPC kiekis nustatytas remiantis Ainsworth ir Gillespie [76] aprašyta metodika. Išsamiai tyrimą aprašo Tolpežnikaitė ir kt. [70]. Duomenys buvo išreikšti galo rūgšties ekvivalentais (GAE) 100 g^{-1} SM.

Dumblių mėginių 2,2-difenil-1-pikrilhidrazilo radikalo surišimo aktyvumas (DPPH•) buvo nustatytas Brand-Williams ir kt. [77] metodu su kituose moksliniuose šaltiniuose aprašytais pakeitimais [78], naudojant Genesys-10 UV/VIS spektrofotometrą (Thermo Spectronic, Rochester, NY, JAV).

Taip pat buvo vertinamas 2,2-azino-bis(3-etilbenzotiazolin-6-sulfonrūgšties) radikalų-katijonų surišimo aktyvumas (ABTS•+) [79], su Urbonavičienė ir kt. [80] aprašytomis modifikacijomis. Absorbcija buvo matuojama esant 734 nm, naudojant Genesys-10 UV/Vis spektrofotometrą (Thermo Spectronic, Rochester, NY, JAV).

Geležies jonų redukcijos antioksidantinė galia (FRAP) buvo nustatyta Benzie ir Strain [81] metodu su modifikacijomis [82]. Absorbcija buvo matuojama esant 593 nm, naudojant Genesys-10 UV/VIS spektrofotometrą (Thermo Spectronic, Rochester, NY, JAV).

1.2.3.6. Mikro- ir makroelementų analizė

Mikro- ir makroelementų analizė dumblių mėginiuose buvo atlikta induktyviai susietos plazmos masės spektrometrija (ICP-MS), naudojant Agilent 7700x ICP-MS instrumentą su Mass Hunter Work Station programine įranga, skirta ICP-MS, versija B.01.03 [83].

1.2.3.7. Chlorofilo *a* ir *b*, ir bendro karotinoidų kiekio nustatymo analizė

Liofilizuoti 500 ± 2 mg dumblių mėginiai pasveriami ir perkeliami į keraminį grūstuvą, kuriame, siekiant mėginį rehidratuoti, įpilama 1,5 ml vandens. Palaukus 2 min. rehidratuotas mėginys sumalamas grūstuvu su 2 g gryno kvarcinio smėlio. Pigmentai buvo išekstrahuoti ir perkelti į matavimo kolbą, o tūris vandeninio acetono tirpalu buvo papildytas iki 100 ml 80%. Homogenizuotas mėginio mišinys centrifuguotas 10 000 aps./min. greičiu 15 minučių 4 °C temperatūroje.

Acetoninio tirpalo mišinys buvo ištirtas, siekiant įvertinti TCC, chlorofilą *a* ir *b* bei jų darinius spektrofotometru metodu, kuris buvo modifikuotas pagal Dere ir kt. [84], Sakalauskaitė ir kt. [85] ir Sumanta ir kt. [86] aprašytas metodikas. Šie junginiai buvo nustatyti esant 470 nm bangos ilgiui, atėmus chlorofilo *a* ir *b* koncentracijas, naudojant atitinkamai 649 ir 665 nm bangos ilgius ir atitinkamus sugerties koeficientus [84, 86]. TCC, chlorofilo *a* ir *b* bei jų darinių kiekis nustatytas spektrofotometrijos metodu, absorbcija matuota spektrofotometru Cintra 202 (GBC Scientific Equipment Pty Ltd., Mulgrave Victoria, Australia), rezultatai analizuoti naudojant Cintral 2.2 versijos programa (GBC Scientific Equipment Pty Ltd., Mulgrave Victoria, Australija).

1.2.3.8. L-glutamo rūgšties ir gama-aminosviesto rūgšties koncentracijos įvertinimas

Analizė buvo atlikta naudojant TSQ Quantiva MS/MS kartu su Thermo Scientific Ultimate 3000 HPLC (Thermo Scientific, Waltham, MA, JAV). Chromatografinis atskyrimas buvo atliktas Luna Omega Polar C18 (2,1 × 100 mm, 3,0 µm) kolonėlėje 40 °C temperatūroje, naudojant 5 µl įpurškimo tūrį. Metodas išsamiai aprašytas Tolpežnikaitė ir kt. [71].

1.2.3.9. Biogeninių aminų kiekio nustatymo analizė

BA ekstrahavimas ir nustatymas Spirulinos mėginiuose buvo atliktas pagal Ben-Gigirey ir kt. [87] aprašyta metodika, su modifikacijomis. Analizuoti BA: triptaminas (TRP), feniletilaminas (PHE), putrescinas (PUT), kadaverinas (CAD), HIS, TYR, spermidinas (SPRMD) ir sperminas (SPRM)). Chromatografinė analizė atlikta „Varian ProStar“ efektyvaus slėgio skysčių chromatografine sistema (Varian Corp., Palo Alto, CA, JAV) su dviem „ProStar 210” siurbliais, „ProStar 410” automatiniu mėginių ėmimo įrenginiu, „ProStar 325 UV/VIS” detektoriumi ir „Galaxy” programine įranga (Agilent, Santa Clara, CA, JAV) duomenų apdorojimui. BA atskirti buvo naudojama Discovery ® HS C18 kolonėlė (150 × 4,6 mm, 5 µm; SupelcoTM Analytical, Bellefonte, Pensilvanija, JAV).

1.2.3.10. Riebalų rūgščių kiekio analizė

Lipidų ekstrakcija RR analizei atlikta chloroformo/metanolio mišiniu (2:1 v/v), o RR metilo esteriai (FAME) paruošti pagal Pérez-Palacios ir kt. [88] metodiką. Dumblių mėginių RR sudėtis nustatyta naudojant dujų chromatografą GC-2010 Plus (Shimadzu Europa GmbH, Duisburgas, Vokietija), su integruotu masės spektrometru GCMS-QP2010 (Shimadzu Europa GmbH, Duisburgas, Vokietija). Atskyrimas atliktas Stabilwax-MS kolonėlėje (30 m ilgio, 0,25 mmID ir 0,25 µm df) (Restek Corporation, Bellefonte, PA, JAV). Nešančiosios dujos – helis, jų srautas – 0,91 ml/min. FAME buvo identifikuoti lyginant FAME smailių sulaikymo kolonėlėje laiką su FAME standartu (Merck & Co., Inc., Kenilworth, NJ, JAV).

1.2.3.11. Statistinė analizė

Atliekant tyrimą, eksperimentai buvo suplanuoti penkiais etapais: (I) pirmojo etapo metu buvo atlikta makrodumblių fermentacija, fermentacija kartota vieną kartą; (II) antrojo etapo metu dumblių mėginių ekstraktų paruošimas, atliktas su dviem pakartojamumais; (III) dumblių mėginių ekstraktų paruošimas, atliktas su dviem pakartojamumais; (IV) mėginių fermentacija, atlikta fermentuojant 2 paralelinius mėginius; (V) Spirulinos mėginių fermentacija, atlikta su dviem pakartojamumais. Visų fizikinių cheminių rodiklių analizė kartota 3 kartus (n = 3), išskyrus paskutinį, penktąjį etapą, kuriame

(n = 6). Vidutinės rezultatų vertės apskaičiuotos, naudojant statistinį paketą SPSS Windows (ver.15.0, SPSS, Čikaga, IL, JAV). Siekiant įvertinti technologinių veiksnių ir jų sąveikos įtaką analizuotiems rodikliams buvo atlika daugiafaktorinė dispersinė analizė (ANOVA), taip pat buvo taikomas *Duncan's* testas, o tiesinis ryšys tarp dviejų kiekybinių kintamųjų vertinamas naudojant Pirsono koreliaciją. Rezultatai patikimi (p), kai $p \leq 0,05$.

2. TYRIMO REZULTATAI IR JŲ APTARIMAS

2.1. Fermentacijos įtaka Baltijos jūros makrodumblių savybėms

Neapdorotų ir fermentuotų makrodumblių pH ir mikrooganizmų skaičiaus rezultatai pavaizduoti 2.1.1 pav. Fermentacija su LUHS135 sumažino *C. rupestris* mėginių pH, tačiau *U. intestinalis* ir *F. lumbricalis* pH vertės po 12 val. fermentacijos reikšmingai nepakito. Visose, 12 val. fermentuotose dumblių mėginių grupėse PRB skaičius buvo didesnis nei 6,0 $\log_{10}$ KSV g^{-1}. Fermentacija padidino TBC skaičių dumblių mėginiuose: *C. rupestris* – vidutiniškai 41,1 proc., *U. intestinalis* – vidutiniškai 19,8 proc., o *F. lumbricalis* – vidutiniškai 28,4 proc.

Nefermentuotuose *C. rupestris* mėginiuose dominavo *Portibacter lacus, Actinobacterium* spp., *Lewinella cohaerens* ir *Acidimicrobium* spp., o jų paplitimas viršijo 5 proc. visų bakterijų (2.1.2 pav. A). Fermentuotuose mėginiuose didžiausias paplitimas nustatytas *Lb. plantarum* (42,3 proc. visų aptiktų bakterijų) (2.1.2 pav. A). Po fermentacijos, bakterijų, kurių paplitimas buvo didžiausias neapdorotuose mėginiuose (*Portibacter lacus* ir *Acidobacterium*), paplitimas išliko didelis; tačiau *Escherichia vulneris*, kurios paplitimas prieš fermentaciją buvo mažas (0,02 proc.), po fermentacijos, reikšmingai padidėjo (15,9 proc.; $p \leq 0,05$). Labiausiai paplitusi mikrobiota neapdorotuose *U. intestinalis* mėginiuose buvo *Alphaproteobacteria, Luteolibacter algae* ir *Marivirga tractuosa*, kurių paplitimas viršijo 5 proc. TBC, o po fermentacijos labiausiai paplitusi rūšis buvo *Lb. plantarum* (29,4 proc.) (2.1.2 pav. B). *Terrisporobacter glycolicus* buvo antra pagal paplitimą bakterijų rūšis fermentuotuose dumbliuose (14,6 proc.), o *Exiguobacterium mexicanum* – trečioji, pagal paplitimą (9,6 proc.). Labiausiai paplitusios rūšys neapdorotuose *F. lumbricalis* mėginiuose buvo *Portibacter lacus, Actinobacterium* spp. ir *Actinomicrobium* spp., o fermentuotuose mėginiuose didžiausias paplitimas nustatytas *Pseudomonas putida* (54,2 proc.) ir *Escherichia vulneris* (24,3 proc.) (2.1.2 pav. C). *Lb. plantarum* buvo tik trečioje, pagal paplitimą, vietoje (8,6 proc.).

Fermentuotų ir neapdorotų dumblių antimikrobinio aktyvumo prieš patogeninius oportunistinius mikroorganizmus skystoje terpėje rezultatai pateikti

2.1.1 lentelėje. Visi nefermentuoti dumblių mėginiai slopino *Bacillus pseudomycoides* ir *Kluyvera cryocrescens*; taip pat, nefermentuoti *F. lumbricalis* mėginiai slopino *Staphylococcus haemolyticus*, o nefermentuoti *C. rupestris – Pseudomonas aeruginosa.* Fermentuoti *C. rupestris* mėginiai slopino 9 iš 13 testuotų patogenų, o fermentuoti *U. intestinalis* ir *F. lumbricalis* slopino 6 iš 13 testuotų patogenų.

Didžiausiu antioksidaciniu aktyvumu pasižymėjo *C. rupestris* mėginiai (fermentuoti ir neapdoroti), vidutiniškai 58,0 proc. (2.1.3 pav.). Tačiau fermentacija sumažino TPC kiekį *C. rupestris* ir *F. lumbricalis* mėginiuose, palyginti su nefermentuotais, atitinkamai, 1,8 ir 1,3 karto (2.1.3 pav.).

Mikro- ir makroelementų koncentracija dumblių mėginiuose pateikta 2.1.2 lentelėje. Lyginant makroelementus fermentuotuose ir neapdorotuose mėginiuose, reikšmingų skirtumų nenustatyta. Lyginant makroelementų koncentracijas, *U. intestinalis* nustatyta vidutiniškai 3,5 karto didesnė natrio (Na) koncentracija, nei *C. rupestris* ir *F. lumbricalis*. Kalio (K) koncentracija *U. intestinalis, C. rupestris* ir *F. lumbricalis* dumbliuose nustatyta 8,3; 14,6 ir 20,5 g kg^{-1}, vidutiniškai. Na/K santykis *C. rupestris* ir *F. lumbricalis* nustatytas, atitinkamai, 3,0 ir 3,7. Didžiausia magnio (Mg) koncentracija nustatyta *U. intestinalis* mėginiuose (vidutiniškai, 5,7 ir 1,7 kartų, atitinkamai, didesnė nei *C. rupestris* ir *F. lumbricalis*). Mažiausias kalcio (Ca) kiekis nustatytas *C. rupestris* mėginiuose (vidutiniškai 2,4 karto mažesnis nei *U. intestinalis* ir *F. lumbricalis*). Lyginant mikroelementų koncentracijas skirtingose dumblių rūšyse, didžiausia mangano (Mn), kobalto (Co), nikelio (Ni), seleno (Se), jodo (I) koncentracija nustatyta *C. rupestris* mėginiuose (atitinkamai, vidutiniškai 0,741 g kg^{-1} ir 1,54; 6,08; 0,304 ir 205,5 mg kg^{-1}). Visuose tirtuose dumblių mėginiuose molibdeno (Mo), sidabro (Ag), stibio (Sb), cezio (Cs), titano (Ti) ir berilio (Be) koncentracija buvo < 0,25 mg kg^{-1}; gyvsidabrio (Hg) < 0,01 mg kg^{-1}, alavo (Sn) < 0,01mg kg^{-1}. Didžiausia kadmio (Cd), bario (Ba) ir švino (Pb) koncentracija nustatyta *C. rupestris* mėginiuose, atitinkamai, 2,2; 1,3 ir 1,9 karto didesnė nei *U. intestinalis* mėginiuose bei, atitinkamai, 1,7; 1,9 ir 3,8 karto didesnė nei *F. lumbricalis* mėginiuose.

Paskelbtų duomenų apie Lietuvoje Baltijos jūroje išgaunamus laukinius dumblius bei jų fermentaciją yra nedaug. Publikuota, kad keletas PRB padermių yra tinkamos dumblių fermentacijai [89]. Šis tyrimas parodė, kad norint padidinti fermentacijos efektyvumą, 2 iš 3 tirtų dumblių reikia taikyti papildomus technologinius sprendimus. Pvz., pridėti fermentuojamųjų sacharidų arba atlikti fermentinį išankstinį apdorojimą, siekiant konvertuoti nefermentuojamus cukrus. Nors fermentacija nesumažino 2 iš 3 tirtų dumblių mėginių pH, tačiau didesnis nei 6,0 $\log_{10}$ KSV g^{-1} PRB buvimas žaliavoje yra siejamas su palankiu poveikiu gyvūnų sveikatingumo rodikliams [74, 90, 91]. Kita vertus, metagenominės analizės rezultatai parodė, kad dumblių

fermentacija neužtikrina potencialiai pavojingų bakterijų slopinimo, todėl, ekstraktų ruošimas būtų perspektyvus technologinis sprendimas šios žaliavos perdirbimui. Dumbliai yra daugelio biologiškai aktyvių molekulių: baltymų ir peptidų, polisacharidų, polifenolių, polinesočiųjų RR ir pigmentų šaltinis [92]. Pastarieji junginiai gali pasižymėti antimikrobinėmis savybėmis [93]. Šis tyrimas parodė, kad makrodumbliai turi antimikrobinį potencialą. Taip pat, dumblių ekstraktų ir PRB kompozicija gali būti labai perspektyvi, nes sinerginis veikimo mechanizmas, slopinantis patogenus, gali lemti platesnį patogenų slopinimo spektrą. Publikuota, kad PRB fermentacija turi reikšmingą įtaką substrato fenolinių junginių profiliui bei antioksidaciniam aktyvumui, nes proceso metu išsiskiria įvairios fenolinės rūgštys bei kiti, antioksidaciniu aktyvumu pasižymintys junginiai [94]. Kiti vertingi dumblių komponentai, tai mikro- ir makroelementai. Nustatėme, kad Baltijos jūros makrodumbliai ir jų kompozicijos su LUHS135 gali būti geras kai kurių deficitinių mikroelementų šaltinis. Tačiau makrodumbliai gali būti užteršti ir sunkiaisiais metalais [95]. Vienas iš svarbiausių saugos rodiklių, norint dumblius naudoti pašarų pramonėje, tai arseno (As) kiekis juose. Neorganinis As priskiriamas I klasės kancerogenų kategorijai, arsenobetainas – netoksiškas, o riebaluose tirpus arsenas, arsenocukrai ir kiti organiniai arseniniai junginiai – potencialiai toksiški [96]. Dumbliuose didžioji dalis As yra arseno kompleksų pavidale, dažniausiai su gliceroliu, sulfonatu arba fosfonatu, kurie *in vivo* metabolizuojami į mažiausiai 12 skirtingų metabolitų, kurių toksiškumas nėra žinomas [97–100]. Apibendrinant, galima teigti, kad sinerginis dumblių ir PRB kompozicijos mechanizmas yra veiksmingas, nes slopino platesnį patogenų spektrą. Taip pat, Baltijos jūros makrodumbliai ir jų kompozicija su LUHS135 gali būti geras kai kurių deficitinių mikroelementų šaltinis. Tačiau saugiam jų naudojimui buvo pasiūlyta ruošti ekstraktus, siekiant sukoncentruoti dumblių veikliuosius komponentus bei užtikrinti žaliavos biologinę ir cheminę saugą, ypač atkreiptinas dėmesys į Pb ir As koncentracijų galimą sumažinimą.

2.2. Mikro- ir makrodumblių ekstraktų bioaktyvūs komponentai ir jų charakteristikos bei mikro- ir makroelementų perėjimas iš dumblių biomasės į ekstraktą

Palyginus TCC makrodumblių mėginiuose, didžiausias TCC nustatytas *C. rupestris* (1,26 mg g^{-1}). *U. intestinalis* ir *F. lumbricalis* TCC buvo, atitinkamai, 1,6 ir 6,3 karto mažesnis (2.2.1 pav.). Lyginant TCC mikrodumbliuose, didžiausias TCC nustatytas *C. vulgaris* (1,52 mg g^{-1}). Tačiau chlorofilo *a* ir *b* koncentracijos *C. vulgaris* nustatytos, atitinkamai, 89,6 proc. ir 55,0 proc. mažesnės nei Spirulina mėginiuose. Nustatyta vidutinio stiprumo neigiama

koreliacija tarp chlorofilo *a* ir TCC dumblių mėginiuose (r = –0,4644); stipri teigiama koreliacija tarp chlorofilo *a* ir *b* koncentracijų (r = 0,7604); labai silpna teigiama koreliacija tarp chlorofilo *b* ir TCC dumbliuose (r = 0,1065). Visuose tirtuose makrodumblių mėginiuose vyravo chlorofilo *a* forma (palyginti su chlorofilu *b*, chlorofilo *a* kiekis *C. rupestris, F. lumbricalis* ir *U. intestinalis* nustatytas vidutiniškai 9,0 proc., 63,3 proc. ir 55,2 proc., atitinkamai, didesnis) (2.2.1 pav.).

Didžiausias TPC kiekis nustatytas *C. rupestris* ir *F. lumbricalis* (vidutiniškai 352,6 mg GAE 100 g^{-1}). TPC kiekis *U. intestinalis* ekstraktuose nustatytas, vidutiniškai, 53,1 proc. mažesnis (2.2.2 pav. A). *C. rupestris* ekstrakto DPPH• antioksidacinis aktyvumas nustatytas 5,82 proc. didesnis, nei *U. intestinalis* ir *F. lumbricalis* ekstraktų. *U. intestinalis* ir *F. lumbricalis* ekstraktai pasižymėjo 1,8 ir 2,4 karto, atitinkamai, mažesniu DPPH• antioksidaciniu aktyvumu, nei *C. rupestris* ekstraktas (2.2.2 pav. B). Nustatyta silpna teigiama koreliacija tarp TPC kiekio ir DPPH• antioksidacinio aktyvumo (r = 0,2419), tačiau *C. rupestris* ekstrakto DPPH• antioksidacinis aktyvumas nustatytas 5,82 proc. didesnis, nei *U. intestinalis* ir *F. lumbricalis* ekstraktų. Tarp makrodumblių DPPH• antioksidacinio aktyvumo ir TCC nustatyta labai stipri teigiama koreliacija (r = 0,9372). Vidutinio stiprumo ir labai stipri teigiama koreliacija nustatyta tarp DPPH• antioksidacinio aktyvumo ir chlorofilo *a* bei *b* kiekio makrodumblių mėginiuose (atitinkamai, r = 0,6731 ir r = 0,9771). Nustatyta vidutinio stiprumo teigiama koreliacija tarp TPC kiekio mikrodumblių ekstraktuose ir jų DPPH• antioksidacinio aktyvumo (r = 0,5979).

Įvertinus antimikrobinį aktyvumą, nustatyta, kad Spirulinos ekstraktai slopino *Staphylococcus haemolyticus* (DIZ 28,3 mm) (2.2.3 pav.). Visi makrodumblių ekstraktai (*C. rupestris, F. lumbricalis* ir *U. intestinalis*) slopino *B. subtilis* (atitinkamai, DIZ 12,0; 8,0 ir 17,0 mm). Taip pat, Spirulina ekstraktai slopino *B. subtilis* (DIZ 10,1 mm), o *U. intestinalis* ekstraktai slopino *S. mutans* (DIZ 14,2 mm).

Mikro- ir makroelementų koncentracijos dumblių ekstraktuose pateiktos 2.2.1 lentelėje. Vertinant makroelementus mikrodumblių ekstraktuose, didžiausia Na koncentracija nustatyta Spirulinos (UAB „Spila“) ekstraktuose (Sp2) (458 mg kg^{-1} SM). Sp2 ekstraktai pasižymėjo didžiausia Mg koncentracija (atitinkamai, 3,3 ir 4,4 karto didesne, nei Spirulinos Sp1ir *C. vulgaris* ekstraktų mėginių). Didžiausia K koncentracija nustatyta Sp2 ekstraktuose (950 mg kg^{-1} SM). Lyginant mikroelementų koncentracijas makrodumblių mėginiuose, didžiausia Mn, geležies (Fe), Co ir Ni koncentracijos nustatyta *C. rupestris* ekstraktuose (atitinkamai, 7,41; 4,34; 0,064 ir 0,301 mg kg^{-1}). Didžiausia vario (Cu) koncentracija nustatyta *U. intestinalis* ekstraktuose (vidutiniškai 0,202 mg kg^{-1}). Visuose tirtuose makrodumblių ekstraktuose Se

koncentracija nustatyta, vidutiniškai, 0,002 mg kg^{-1}. Priešingai nei makrodumbliuose, jų ekstraktuose nenustatyta cinko (Zn), I ir fosforo (P). Daugumos pagrindinių mikroelementų koncentracijos ekstraktuose nustatytos mažesnės, nei makrodumblių mėginiuose. Lyginant mikroelementų koncentracijas mikrodumblių ekstraktuose, didžiausios daugumos nepakeičiamųjų mikroelementų (chromo (Cr), Mn, Fe, Co, Ni ir Se) koncentracijos nustatytos Sp2 ekstraktuose. Didžiausia Cu koncentracija nustatyta *C. vulgaris* ir Sp2 ekstraktuose (vidutiniškai 0,074 mg kg^{-1}). Lyginant dumblių ekstraktus su dumbliais, galio (Ga), Be, Sn, Hg, boro (B), Ti ir Cd ekstraktuose nenustatyta, o Mo nenustatyta *C. rupestris* ekstraktuose, taip pat *C. rupestris* ir *U. intestinalis* ekstraktuose nenustatyta Cs. Ekstraktuose nepageidaujamų mikroelementų koncentracija nustatyta mažesnė, lyginant su jų kiekiu dumbliuose. Lyginant nepageidaujamus mikroelementus mikrodumblių ekstraktuose, nustatyta panašios tendencijos, kaip ir pageidaujamų mikroelementų, o didžiausia koncentracija As, rubidžio (Rb), stroncio (Sr), Mo, Ag, Sb, Ba, Al ir ličio (Li) nustatyta Sp2 ekstraktuose (atitinkamai 0,022; 0,181; 0,346; 0,164; 0,010; 0,098; 0,043; 1,15 ir 0,070 mg kg^{-1}). Taip pat, Sp2 ekstraktuose nustatyta Ti ir Cd (0,001 mg kg^{-1}). Visuose mikrodumblių ekstraktuose Pb koncentracija buvo vidutiniškai 0,001 mg kg^{-1}, o Cs mikrodumblių mėginiuose neaptikta. Vanadžio (V) nustatyta *C. vulgaris* ir Sp2 ekstraktuose (vidutiniškai 0,001 mg kg^{-1}).

Pažymėtina, kad ekstrahavimo būdas turi įtakos pigmentų koncentracijai ekstrakte [101]; dėl šios priežasties skirtingų tyrimų rezultatai gali skirtis. Karotinoidai yra natūralūs augalų pigmentai, atsakingi už vaisių, daržovių ir dumblių raudonos, žalios, geltonos ir oranžinės spalvos intensyvumą [80, 102]. Karotinoidai naudojami pašarų pramonėje, siekiant pagerinti gyvūnų sveikatą ir gyvūninės kilmės produktų kokybę [103]. Be to, spalvoti junginiai, daugeliu atvejų, padidina produkto ir (ar) ekstrakto antioksidacinį aktyvumą; tačiau specifinės antioksidacinės savybės yra susijusios su specifine fenolinių junginių profilio sudėtimi. Santoso ir kt. [104] ir Wang ir kt. [105] publikavo, kad svarbu atsižvelgti ir į skirtingų medžiagų sinerginį poveikį TPC kiekiui dumbliuose. Yra duomenų, kad *U. intestinalis* pasižymi antioksidaciniu aktyvumu [106], o šio dumblio ekstraktų antioksidacinės savybės priklauso nuo naudojamo ekstrahento, nustatyta, kad ekstrahuojant dichlormetanu, etanoliu, metanoliu ir heksanu ekstraktų antioksidacinis aktyvumas buvo 87,54; 31,9; 22,6 ir 22,5 proc. [107]. Tačiau publikuoti duomenys yra prieštaringi. Farasat ir kt. [108] publikavo, kad *U. intestinalis* metanolinių ekstraktų DPPH• yra didžiausias (48 proc. slopinimas), o IC50 reikšmė mažesnė – 2,32 mg ml^{-1}.

Publikuota, kad ne tik žalieji, raudonieji ir rudieji dumbliai pasižymi antigrybinėmis, antibakterinėmis, citostatinėmis, antivirusinėmis, antihelmintinėmis ir kt. [109–111] savybėmis, bet ir dumblių ekstraktai gali slopinti

bakterijas, mieles ir grybus [112–116]. Ekstraktų gebėjimas slopinti gramteigiamas, o ne gramneigiamas bakterijas gali būti susijęs su bakterijų sienelių pralaidumo skirtumais [107], nes gramneigiamų bakterijų rūšių išorinė ląstelės sienelės membrana nepraleidžia kai kurių, gramteigiamas bakterijas slopinančių, junginių [117].

Vienas iš pagrindinių iššūkių, susijusių su makrodumblių saugumu, yra jų užterštumas sunkiaisiais metalais (Al, Cd, Pb, Rb, siliciu (Si), Sr ir Sn) [118, 119]. Toksiškų elementų kiekis mikrodumbliuose gali skirtis ir tai gali būti susiję su aplinkos, kurioje biomasė užaugo, užterštumu [120, 121]. Kai kurie mikrodumbliai absorbuoja toksiškus metalus, todėl terpės, turinčios tiesioginį sąlytį su mikrodumbliais, tarša yra svarbus veiksnys mikrodumblių biomasės saugai [122]. Iki šiol, duomenų apie mikro- ir makroelementų perėjimą iš dumblių į ekstraktus nebuvo publikuota. Šiame darbe taikytas ekstrahavimo metodas sumažino toksiškų metalų koncentraciją mikro- ir makrodumbliuose. Tačiau, ekstrakcija sumažino ir kai kurių pageidaujų mikroelementų kiekį ekstrakte.

2.3. Dumblių kietafazės fermentacijos ir apdorojimo ultragarsu poveikis jų ekstraktų antimikrobinėms ir antioksidacinėms savybėms

Dumblių mėginių pH dinamika fermentacijos metu pavaizduota 2.3.1 pav. Nefermentuotų *C. rupestris, U. intestinalis* ir Spirulina mėginių pH vertės nustatytos didesnės nei 7,0, o nefermentuotų *C. glomerata* ir *F. lumbricalis* mėginių, atitinkamai, 5,95 ir 6,74. Mažiausia pH vertė nustatyta *C. glomerata, C. rupestris* ir *U. intestinalis* po 36 ir 48 val. fermentacijos. Dumblių rūšis buvo reikšmingas veiksnys mėginių pH ($p = 0,017$). Pagal gautus, pagrindinio fermentacijos efektyvumo rodiklio rezultatus, dumblių apdorojimui prieš ekstrakciją, pasirinkta 36 val. fermentacijos trukmė.

Dumblių ekstraktų ir jų kompozicijų su LUHS135 spalvų koordinatės ir pH vertės pateiktos 2.3.1 lentelėje. Lyginant visas tris ekstraktų grupes (paruoštus iš neapdorotų, ultragarsu apdorotų ir fermentuotų dumblių), mažiausia L* koordinatės reikšmė nustatyta $ClaG_{non}$, $ClaR_{ultr}$ ir $ClaR_{fermLUHS135}$ (atitinkamai 42,5; 41,3 ir 49,5 NBS). Intensyviausia žalumo koordinatė (-a*) nustatyta Ul_{non}, Ul_{ultr} ir Ul_{ferm} (atitinkamai, –14,7; –13;7 ir –6,86 NBS). Mažiausia geltonumo koordinatė (b*) nustatyta $ClaG_{non}$, Sp_{ultr} ir $ClaG_{ferm}$ (atitinkamai, 24,8; 23,7 ir 23,1 NBS). Visais atvejais, kompozicijų su LUHS135 pH vertės nustatytos mažesnės, nei ekstraktų, o didžiausios pH vertės nustatytos Sp_{non}, Sp_{ulr} ir $Furc_{ferm}$ (atitinkamai, 8,69; 7,67 ir 5,59).

Dumblių rūšis ($p \leq 0{,}0001$), dumblių rūšies ir pirminio apdorojimo prieš ekstrahavimą sąveika ($p \leq 0{,}0001$) ir dumblių rūšies ir LUHS135 kompozicijos sąveika ($p \leq 0{,}003$) buvo reikšmingos TPC kiekiui mėginiuose. Didžiausias TPC kiekis nustatytas $ClaR_{nonLUHS135}$ ir $Furc_{nonLUHS135}$ mėginiuose (vidutiniškai apie 13,28 mg GAE ml^{-1}). Didžiausias DPPH•, ABTS•+ ir FRAP antioksidacinis aktyvumas nustatytas prieš ekstrakciją neapdorotų *C. rupestris* ir *F. lumbricalis* ekstraktų kompozicijų su LUHS135, palyginus su tų pačių rūšių dumblių ekstraktais be LUHS135. Nustatyta vidutinio stiprumo teigiama koreliacija tarp TPC koncentracijos mėginiuose ir ABTS•+ ($r = 0{,}300$, $p = 0{,}004$) bei FRAP ($r = 0{,}247$, $p = 0{,}019$), tačiau tarp mėginių DPPH• antioksidacinio aktyvumo ir TPC kiekio reikšmingų koreliacijų nenustatyta.

Lyginant mėginių antimikrobines savybes, prieš ekstrakciją papildomai neapdorotų mėginių ekstraktų antimikrobinis aktyvumas buvo didžiausias (2.3.2 pav.). Visi šios grupės mėginiai slopino *Bacillus cereus*. Taip pat, 3 iš 10 šios grupės mėginių ($ClaR_{non}$, $ClaR_{nonLUHS135}$ ir $ClaG_{nonLUHS135}$) slopino *Enterococcus faecium*, o 4 iš 10 šios grupės mėginių ($ClaR_{nonLUHS135}$, $ClaG_{nonLUHS135}$, $Furc_{nonLUHS135}$ ir $Ul_{nonLUHS135}$) slopino *Staphylococcus aureus*. Nepaisant to, kad didžiausias mėginių skaičius (iš visų tirtų mėginių) slopino bent vieną patogeną prieš ekstrakciją papildomai neapdorotų mėginių ekstraktų grupėje, ultragarsu apdorotų mėginių grupė pasižymėjo platesniu patogenų slopinimo spektru (*Bacillus cereus* slopino $ClaR_{ultr}$, $ClaR_{ultrLUHS135}$ ir $Sp_{ultrLUHS135}$ ekstraktai, *Enterococcus faecium* slopino $ClaG_{ultrLUHS135}$, *Staphylococcus aureus* slopino $ClaR_{ultrLUHS135}$, $ClaG_{ultrLUHS135}$ ir $Sp_{ultrLUHS135}$ ir *Stretococcus mutans* slopino $Furc_{ultrLUHS135}$ ir $Ul_{ultrLUHS135}$).

Skystoje terpėje, ištyrus 500 ir 2000 µl dumblių ekstraktų bei jų kompozicijų su LUHS135 koncentracijų poveikį 10 µl patogeno koncentracijai, nustatyta, kad esant 500 µl koncentracijai skystoje terpėje, Sp_{non} slopina *Bacillus cereus*, $ClaR_{ferm}$ slopina *Enterococcus faecium*, $Sp_{ultrLUHS135}$, $ClaR_{ferm}$ ir $Sp_{fermLUHS135}$ slopina *Staphylococcus aureus*, o Sp_{non} slopina *Streptococcus mutans*. Padidinus dumblių ekstraktų ir jų kompozicijų su LUHS135 koncentracijas iki 2000 µl, be jau minėtų patogenų, $ClaR_{non}$, Sp_{non}, $Sp_{nonLUHS135}$ ir Ul_{ultr} slopino *Enterococcus faecium*; $ClaR_{ultr}$, Ul_{ultr}, ir Sp_{ferm} slopino *Streptococcus mutans*, o $ClaR_{non}$, $ClaG_{non}$, Sp_{non} ir $ClaG_{ferm}$ slopino *Enterococcus faecalis*.

Vienas iš pagrindinių fermentacijos proceso efektyvumo rodiklių yra pH vertė, kuri fermentuotų substratų yra, vidutiniškai, 4,6. Fermentacijos metu, susidaro įvairūs junginiai, kurie sintetinami, kaip antriniai technologinių mikroorganizmų metabolitai [123, 124]. Vyksta ir atvirkštiniai procesai, t. y., fenoliniai junginiai iš konjuguotų formų pereina į laisvas formas, dėl mikroorganizmų veiklos bei paties substrato fermentinių aktyvumų pokyčių [22].

Šis tyrimas parodė, kad mielių ekstraktas yra tinkamas priedas dumblių fermentacijos efektyvumui padidinti. Fermentacijos metu substratas rūgštėja, o organinės rūgštys turi įtakos oksidacijos procesams, dėl kurių gali pakisti spalva [125]. Daugeliu atvejų spalvoti junginiai padidina produkto ir (arba) ekstrakto antioksidacines savybes; tačiau specifinės antioksidacinės savybės yra susijusios su specifine fenolinių junginių profilio sudėtimi. Apdorojimas ultragarsu taip pat gali sukelti spalvų pokyčius, nes ultragarso bangos sukelia greitą susitraukimą ir išsiplėtimą, kurie suardo substrato ląsteles, o kavitacija atsakinga už difuzijos ribinio sluoksnio sumažėjimą [126–130]. Publikuota, kad ultragarsas padidina ekstrakcijos efektyvumą [131, 132]. Tačiau kituose darbuose skelbiama, kad ultragarsas turi reikšmingos įtakos substrato spalvų pokyčiams [133]. Spalvoti junginiai, dažnu atveju, pasižymi antioksidacinėm savybėm, todėl labai svarbu įvertinti ultragarsu apdorotų mėginių antioksidacinių savybių pokyčius, nes sumažėjus spalvotų junginių kiekiui, gali sumažėti antioksidacinis aktyvumas. Iš esmės DPPH• ir ABTS•+ veikimas yra pagrįstas antioksidantų gebėjimu paaukoti vandenilio atomą arba elektroną, kad stabilizuotų radikalus, paverčiant juos neradikaline forma [134, 135]. Mūsų rezultatai atspindėjo visų paruoštų etanolinių ekstraktų gebėjimą paaukoti vandenilio atomą arba elektroną abiem radikalams. Dumblių ekstraktai, kuriuose gausu polifenolinių junginių, pasižymi antioksidaciniu aktyvumu [134, 136]. Dumbliai yra geras biologiškai aktyvių junginių šaltinis, o kai kurie iš jų turi platų veikimo spektrą, įskaitant antimikrobinį [137, 138]. Sirbu ir kt. [139] pranešė, kad TPC kiekis dumblių ekstraktuose yra susijęs su jų antibakteriniu aktyvumu. Mes nustatėme vidutinio stiprumo teigiamas koreliacijas tarp ABTS•+ ir *E. faecalis* DIZ bei tarp TPC kiekio ekstraktuose ir *S. aureus* DIZ (atitinkamai, $r = 0{,}388$, $p = 0{,}0001$; $r = 0{,}340$, $p = 0{,}001$). Tačiau reikia atlikti tolesnius tyrimus, siekiant įvertinti, kurie junginiai yra atsakingi už šių patogenų slopinimą.

2.4. Spirulinos fizikinių cheminių savybių pokyčiai skystos fazės ir kietafazės laktofermentacijos metu

L-glutamo rūgšties (L-Glu) ir GABA koncentracijos nefermentuotuose ir fermentuotuose Spirulinos mėginiuose pavaizduotos 2.4.1 pav. A. Palyginus L-Glu koncentraciją SMF ir kontroliniuose (I) mėginiuose, po 48 val. SMF, 7 iš 10 SMF mėginių L-Glu koncentracija nustatyta didesnė, tačiau 3 iš 10 SMF mėginių – mažesnė, lyginant su kontroline grupe (I). Lyginant SSF mėginius, daugeliu atvejų (išskyrus 24 ir 48 val. SSF *Lb. paracasei* Nr. 244 mėginiais) L-Glu koncentracija po SSF padidėjo, o fermentacijos sąlygos (SMF arba SSF) buvo reikšmingas veiksnys L- Glu koncentracijai Spiruli-

noje. Visais atvejais (t. y., po 24 ir 48 val. SMF) GABA koncentracija nuosekliai didėjo, lyginant su GABA kiekiu kontroliniuose (I) mėginiuose, o didžiausia GABA koncentracija (286.5 mg kg^{-1}) nustatyta po 48 val. SMF *Lb. paracasei* No. 244. Ta pati tendencija nustatyta ir SSF mėginiuose, t. y., po 24 ir 48 val. SSF, GABA koncentracija nustatyta didesnė nei kontroliniuose (II) mėginiuose, o didžiausia GABA koncentracija (2395.9 mg kg^{-1}) nustatyta po 48 val. SSF *Lb. paracasei* No. 244.

BA kiekis neapdorotuose ir fermentuotuose Spirulinos mėginiuose pavaizduotas 2.4.1 pav. B. Dominuojantys BA fermentuotoje Spirulinoje buvo PUT ir SPRMD. Didesnės PUT koncentracijos nustatytos SSF mėginiuose, lyginant su SMF, o didžiausias PUT kiekis nustatytas SSF *Lb. brevis* Nr. 173 mėginiuose (833,4 mg kg^{-1} po 24 val. ir 854,7 mg kg^{-1} po 48 val. fermentacijos). Visi analizuoti veiksniai ir jų sąveika buvo statistiškai reikšmingi PUT koncentracijai Spirulinos mėginiuose ($p \leq 0{,}0001$). Nustatyta, kad SMF sumažino SPRMD koncentraciją Spirulinos mėginiuose, vidutiniškai, nuo 3,3 iki 4,9 karto (atitinkamai, po 48 val. SMF *Lb. paracasei* Nr. 244 ir po 24 val. SMF *P. pentosaceus* Nr. 183). Tačiau SSF mėginiuose nustatytos priešingos SPRMD koncentracijos kitimo tendencijos. Visi analizuoti veiksniai ir dauguma jų sąveikų, išskyrus fermentacijos trukmės ir fermentacijos sąlygų (SMF arba SSF) sąveiką, buvo reikšmingi SPRMD koncentracijai Spirulinos mėginiuose ($p \leq 0{,}0001$). Spirulinos mėginiuose, kuriuose nustatyta didžiausia GABA koncentracija, nustatytas ir didžiausias BA kiekis.

Pagrindinės RR neapdorotuose ir fermentuotuose Spirulinos mėginiuose buvo palmitino rūgštis (C16:0), metillinoleatas (C18:2) ir gama-linoleno (C18:3ɣ) rūgšties metilo esteris (2.4.1 pav. C). Lyginant C16:0 kiekį neapdorotuose ir SMF mėginiuose, C16:0 kiekis 18 fermentuotų mėginių buvo didesnis (iš 20 mėginių), nei kontroliniuose (I) mėginiuose. Vertinant C18:2 kiekį Spirulinos mėginiuose, visose tiriamosiose grupėse (SMF ir SSF) nustatytas mažesnis kiekis, nei atitinkamose kontrolinėse (I ir II kontrolinėse). Lyginant C18:3ɣ kiekį tiriamosiose grupėse su kontroline, nustatytos skirtingos C18:3ɣ kiekio kitimo tendencijos SMF ir SSF mėginiuose. SMF mėginiuose C18:3ɣ kiekis: padidėjo 6 iš 20 mėginių; sumažėjo 8 iš 20 SMF mėginių; nesiskyrė nuo kiekio kontroliniuose (I) mėginiuose – 6 iš 20 SMF mėginių. SSF mėginiuose C18:3ɣ kiekis: padidėjo 14 iš 20 mėginių; sumažėjo 4 iš 20 mėginių; nesiskyrė nuo kiekio kontroliniuose (II) mėginiuose – 2 iš 20 SSF mėginių. Spirulinos RR profilio pokyčiai buvo nustatyti visuose fermentacijos etapuose.

Spirulinos sudėtis yra tiesiogiai susijusi su daugybe veiksnių, tokių kaip melsvadumblių gavybos šaltinis, metų sezonas, gamybos technologija ir kt. Iš aminorūgščių Spirulinoje dominuoja glutamo rūgštis, taip pat leucinas ir asparto rūgštis [140]. GABA sintezėje dalyvauja specifinės bakterijų gentys

[141]. Publikuota, kad PRB hidrolizuoja cianobakterijų ląstelių sieneles, dėl to susidaryti sudėtingi junginiai [142]. Mes nustatėme, kad fermentacijos sąlygos (SMF arba SSF) yra statistiškai reikšmingas veiksnys GABA sintezei Spirulinos mėginiuose. Tačiau kiti junginiai, pvz. BA (TYR, HIS, PUT, CAD, SPRM ir SPRMD) taip pat susidaro dekarboksilinant aminorūgštis [143, 144]. PUT yra SPRMD sintezės pirmtakas [144]. Wang ir kt. [145] publikavo, kad pirminių, antrinių ir tretinių BA suma yra labai svarbi jų toksiškumui. PUT ir CAD sustiprina intoksikaciją, esant kitiems BA [146]. Mes nustatėme, kad mėginiuose, kuriuose susidaro didžiausia GABA koncentracija, taip pat susidaro didžiausias BA kiekis. Todėl labai svarbu tirti ir nepageidaujamų junginių koncentraciją galutiniame produkte, ypač kai pageidaujamų ir nepageidaujamų junginių sintezės mechanizmai yra panašūs.

Spirulinos lipidų koncentracija gali skirtis nuo 5 iki 10 proc. [16]. Omega-6 sudaro didžiąją dalį viso Spirulinos RR profilio [147, 148]. Spirulinoje yra daug palmitino rūgšties (16:0), kuri sudaro daugiau nei 25 proc. viso RR kiekio [16]. PUFA kiekis Spirulinoje varijuoja nuo 1,5 iki 2,0 proc., viso RR kiekio [149], o PUFA – 30 proc. viso RR kiekio [150]. Spirulina yra vienintelis maistinių medžiagų šaltinis, kuriame yra toks didelis kiekis nepakeičiamųjų RR, ypač γ-linoleno rūgšties. Mes nustatėme, kad Spirulinos mėginių RR profilis priklauso nuo fermentacijos proceso, todėl modeliuojant technologinius rodiklius, galima pasiekti pageidaujamus RR profilio pokyčius.

2.5. Ryšys tarp biologiškai aktyvių baltyminės kilmės junginių susidarymo Spirulinoje

Baltyminės kilmės bioaktyvių junginių (suminio BA kiekio, GABA, L-Glu) koncentracijos Spirulinos mėginiuose pavaizduotas 2.5.1 pav. Didžiausia GABA koncentracija nustatyta 24 ir 48 val. SSF Nr. 244 mėginiuose (didesnis nei 2000 mg kg^{-1}) ir 24 val. SSF Nr. 225 mėginiuose (1264 mg kg^{-1}). BA/GABA santykis mėginiuose nustatytas, atitinkamai, 0,72; 0,86 ir 1,07. BA/GABA santykis mėginiuose varijavo nuo 0,5 iki 62 (atitinkamai, 24 h SMF Nr. 244 mėginyje ir 24 val. SSF Nr. 173 mėginyje). Nustatytos reikšmingos koreliacijos tarp GABA kiekio Spirulinos mėginiuose ir PUT, CAD, HIS, TYR, SPRMD ir SPRM koncentracijos (2.5.1 lentelė). BA/L-Glu santykis Spirulinos mėginiuose varijavo nuo 0,31 iki 10,7 (atitinkamai, 24 h SMF Nr. 245 mėginyje ir 48 val. SMF Nr. 244 mėginyje). Taip pat, vidutinio stiprumo teigiama koreliacija nustatyta tarp L-Glu koncentracijos ir TRP, PUT, SPRMD ir SPRM kiekio Spirulinos mėginiuose. Silpna neigiama koreliacija nustatyta tarp PRB skaičiaus Spirulinoje ir CAD bei SPRM koncentracijos mėginiuose. PRB skaičius buvo reikšmingas veiksnys GABA ir L-Glu koncentracijai Spirulinoje, tačiau koreliacijos tarp jų nebuvo nustatyta. Silpna

teigiama koreliacija nustatyta tarp GABA koncentracijos mėginiuose ir *Staphylococcus aureus* DIZ. Tačiau koreliacijos tarp mėginių pH ir kitų analizuotų rodiklių nenustatyta.

Priešingai nei cheminės sintezės metu, biologinė GABA gamyba, naudojant technologinius mikroorganizmus, yra saugesnė ir ekologiškesnė [151–153]. GABA gali susidaryti baltymų apykaitos metu. Paprastai GABA fermentacijos metodu gaminama iš L-Glu, naudojant glutamato dekarboksilazę [43]. GABA gamybos proceso parametrus galima lengvai valdyti [154]. Technologinėse PRB padermėse gliukozės metabolizmas susijęs su daugybės kitų metabolite sinteze, taip pat ir GABA [155]. PRB, ekonomiškai perspektyvūs technologiniai mikroorganizmai, yra labiausiai analizuojami, siekiant panaudoti juos GABA sintezei [154]. Tačiau daugelis veiksnių (temperatūra, pH, proceso trukmė ir kt.) turi įtakos GABA išeigai [156]. Be pageidaujamų junginių, PRB dalyvauja ir BA susidarymo procesuose [157]. Dauguma BA yra klasifikuojami kaip nepageidaujami junginiai, išskyrus beta-feniletilaminą (β-PEA), kuris priskiriamas neurotransmiteriams [65, 66]. Šis neurotransmiteris keičia dopamino, norepinefrino, acetilcholino ir GABA išsiskyrimą ir atsaką į juos [158].

Šis tyrimas parodė, kad nors fermentuojant Spiruliną susidaro didelė koncentracija pageidaujamų junginių, nepageidaujami junginiai, tokie kaip BA, taip pat susidaro dėl panašių jų sintezės mechanizmų. Todėl svarbi nepageidaujamų junginių kontrolė galutiniuose produktuose.

IŠVADOS

1. Sukurta duomenų bazė apie *U. intestinalis, C. rupestris* ir *F. lumbricalis* makrodumblių mikro- ir makroelementų profilį bei šių dumblių pokyčius prieš ir po fermentacijos parodė, kad:
   1.1. Nepaisant didelės pageidaujamų mikroelementų koncentracijos, makrodumbliuose yra didelis kiekis nepageidaujamų mikroelementų: As kiekis *U. intestinalis, C. rupestris* ir *F. lumbricalis*, nustatytas vidutiniškai 4,1; 3,79 ir 5,38 mg kg^{-1}, Pb – 0,733; 1,41 ir 0,371 mg kg^{-1}, Rb – 5,93; 9,86 ir 13,35 mg kg^{-1}, Sr – 191; 140 ir 189 mg kg^{-1}, Al kiekis *C. glomerata* – 0,971 g kg^{-1}; rezultatai rodo, kad būtina taikyti technologijas šių toksiškų junginių koncentracijos mažinimui dumbliuose.
   1.2. Laukiniai Baltijos jūros dumbliai gali būti užteršti potencialiai patogeniškais mikroorganizmais (*Escherichia vulneris, Actinobacterium* spp., *Pseudomonas putida* ir *Portibacter lacus*), o fermentacija nėra

efektyvi technologija jų slopinimui, todėl, siekiant užtikrinti analizuotos medžiagos biosaugą, būtinas technologinis sprendimas juos debakterizuoti.

1.3. Sinerginis dumblių ir PRB kompozicijos veikimas pasižymi platesniu patogenų slopinimo spektru (fermentuoti mėginiai slopino nuo 6 iki 9, o nefermentuoti – nuo 2 iki 3 iš 13 testuotų patogenų).

1.4. Didžiausiu antioksidaciniu aktyvumu pasižymėjo fermentuoti ir nefermentuoti *C. rupestris* mėginiai (vidutiniškai, 58,0 proc.), tačiau fermentacija sumažino TPC kiekį *C. rupestris* ir *F. lumbricalis* mėginiuose (atitinkamai, 45 ir 24 proc.).

2. Ekstraktų paruošimas yra tinkamas technologinis metodas, leidžiantis iš dumblių biomasės išskirti funkcionaliuosius junginius ir užtikrinti cheminę bei biologinę dumblių pagrindu pagamintų žaliavų (ekstraktų) saugą:

2.1. Didžiausias TCC kiekis nustatytas *C. rupestris* ir *C. vulgaris* ekstraktuose (atitinkamai, 1,26 ir 1,52 mg g^{-1}); didžiausia chlorofilo *a* koncentracija nustatyta Spirulinos ekstraktuose (vidutiniškai, 7,95 mg g^{-1}); didžiausia chlorofilo *b* koncentracija nustatyta *C. rupestris* ekstraktuose (vidutiniškai, 5,14 mg g^{-1}).

2.2. Didžiausias TPC kiekis nustatytas *C. rupestris* ir *F. lumbricalis* ekstraktuose (atitinkamai, 349,9 ir 355,2 mg GAE 100 g^{-1}); didžiausias DPPH• antioksidacinis aktyvumas nustatytas *C. rupestris* ekstraktuose (vidutiniškai 5,82 proc.).

2.3. Plačiausią antimikrobinio aktyvumo spektrą parodė Spirulinos (Sp1) ir *U. intestinalis* ekstraktai: Spirulinos ekstraktai slopino *S. haemolyticus* ir *B. subtilis* (DIZ, atitinkamai, 28,3 ir 10,1 mm), *U. intestinalis* ekstraktai slopino *B. subtilis* ir *S. mutans* (DIZ, atitinkamai, 17,0 ir 14,2 mm).

2.4. Nepageidaujamų mikroelementų Ga, Be, Sn, Hg, B, Ti ir Cd ekstraktuose nenustatyta, Mo nenustatyta *C. rupestris* ekstraktuose, Cs nenustatyta *C. rupestris* ir *U. intestinalis* ekstraktuose; As perėjimas iš *U. intestinalis, C. rupestris* ir *F. lumbricalis* biomasės į ekstraktus buvo atitinkamai 90; 91 ir 96 proc., mažesnis; Pb kiekis *U. intestinalis, C. rupestris* ir *F. lumbricalis* ekstraktuose sumažėjo, atitinkamai, 99,8; 99,2 ir 99,9 proc.; Sr kiekis *U. intestinalis, C. rupestris* ir *F. lumbricalis* ekstraktuose sumažėjo, atitinkamai, 99,7; 99,9 ir 99,7 proc.

3. Dumblių apdorojimas fermentacija ir ultragarsu, prieš ekstraktų gamybą yra reikšmingi veiksniai ekstraktų savybėms:
   3.1. *U. intestinalis, C. rupestris, F. lumbricalis, C. glomerata* ir Spirulina fermentacija prieš ekstrakciją padidina *U. intestinalis, C. rupestris, C. glomerata* ir Spirulina ekstraktuose TPC kiekį, atitinkamai, 3,13; 5,44; 1,28 ir 2,52 karto; DPPH• antioksidacinį aktyvumą *C. rupestris* ektraktuose (vidutiniškai 1,60 karto); ABTS•+ antioksidacinį aktyvumą *C. rupestris* ektraktuose (vidutiniškai 4,86 karto); FRAP antioksidacinį aktyvumą *U. intestinalis, C. rupestris* ir *F. lumbricalis* ekstraktuose, atitinkamai 4,10; 3,56 ir 1,47 karto; nors daugumos ekstraktų, paruoštų iš fermentuotų dumblių, antimikrobinės savybės buvo silpnesnės, nei ekstraktų, paruoštų iš nefermentuotų dumblių, *F. lumbricalis* ekstraktas, pagamintas iš fermentuotos žaliavos, efektyviau slopino *Staphylococcus aureus* (DIZ vidutiniškai, 13,3 mm).
   3.2. *U. intestinalis, C. rupestris, F. lumbricalis, C. glomerata* ir Spirulina dumblių biomasės apdorojimas ultragarsu prieš ekstrakciją, padidino *U. intestinalis* ir *C. rupestris* ekstraktų TPC kiekį (atitinkamai, 1,61 ir 4,91 karto); *U. intestinalis* ir *C. rupestris* DPPH• antioksidacinį aktyvumą (atitinkamai, 2,05 ir 1,60 karto); *C. rupestris* ABTS•+ antioksidacinį aktyvumą (3,37 karto); *C. rupestris* ir *F. lumbricalis* FRAP antioksidacinį aktyvumą (atitinkamai 14,81 ir 1,19 karto); *C. rupestris* ekstraktai, išgauti iš ultragarsu apdorotų dumblių, efektyviau slopino *Bacillus cereus* (DIZ vidutiniškai 18,2 mm).
   3.3. Ekstraktų kompozicija su LUHS135 lėmė žaliavos platesnį antimikrobinių savybių spektrą: ekstraktai, paruošti iš neapdorotų dumblių ir LUHS135 slopino nuo 1 iki 3 testuotų patogenų; ekstraktai, pagaminti iš fermentuotų dumblių su LUHS135, slopino nuo 1 iki 2; ekstraktai, pagaminti iš ultragarsu apdorotų dumblių su LUHS135, slopino nuo 1 iki 2 iš 7 testuotų patogenų.

4. Fermentacija parinktomis PRB padermėmis yra tinkamas metodas Spirulinos funkcionaliosios vertės padidinimui, tačiau BA koncentracija galutiniame produkte turi būti kontroliuojama:
   4.1. Mažiausias suminis BA kiekis nustatytas 24 val. SMF Nr. 183 mėginių grupėje (nustatytas tik SPRMD, 41,3 mg kg^{-1}).
   4.2. Didžiausia L-Glu (5506,4 mg kg^{-1}) ir GABA (2396 mg kg^{-1}) koncentracija, nustatyta 48 val. SSF Nr. 51 ir Nr. 244, atitinkamai, mėginiuose. BA/GABA ir BA/L-Glu santykis Spirulinos mėginiuose buvo, atitinkamai, nuo 0,5 iki 62 ir nuo 0,31 iki 10,7.
   4.3. Pagrindinės RR neapdorotuose ir fermentuotuose Spirulinos mėginiuose buvo C16:0, C18:2 ir C18:3ɣ.

4.4. Spirulinos mėginiai su mažiausia BA ir didžiausia GABA koncentracija (fermentuoti Nr. 244: 24 val. ir 48 val. SMF, 24 val. ir 48 val. SSF; fermentuoti Nr. 173: 48 val. SMF, 24 val. ir 48 val. SSF; fermentuoti Nr. 225: 48 val. SMF, 24 val. ir 48 val. SSF, fermentuoti Nr. 245: 24 val. ir 48 val. SSF) slopino *Staphylococcus aureus* (nustatytos DIZ, varijavo nuo 9,1 iki 16,23 mm).

REKOMENDACIJOS

1. Pagal sukurtos duomenų bazės apie *U. intestinalis, C. rupestris* ir *F. lumbricalis* makrodumblių mikro- ir makroelementų profilį bei jų savybes prieš ir po fermentacijos rezultatus rekomenduojame dumblių naudojimą ekstraktų gamybai, nes ekstraktų paruošimas yra tinkamas technologinis metodas, leidžiantis iš dumblių biomasės išskirti funkcionaliuosius junginius ir užtikrinti dumblių pagrindu pagamintos žaliavos cheminę ir biologinę saugą.
2. Rekomenduojama fermentuoti dumblių biomasę, nes sinerginis dumblių ir PRB kompozicijos mechanizmas pasižymi platesniu patogenų slopinimo spektru.
3. Fermentacija ir apdorojimas ultragarsu yra tinkami metodai dumblių apdorojimui, prieš tikslinių savybių ekstraktų gamybą.
4. Ekstraktų kompozicija su LUHS135 rekomenduojama platesnio spektro antimikrobinių savybių žaliavų gamybai.
5. Norint padidinti Spirulinos funkcionaliąją vertę, rekomenduojama fermentacija su pasirinktomis PRB padermėmis: *Lb. paracasei* Nr. 244, *Lb. brevis* Nr. 173, *L. mesenteroides* Nr. 225 ir *Lb. uvarum* Nr. 245 (fermentacija padidina GABA koncentraciją ir lemia platesnį žaliavos antimikrobinio aktyvumo spektrą).

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LIST OF PUBLICATIONS

This PhD thesis is based on the following publications:

1. **Tolpežnikaitė, Ernesta**; Bartkevics, Vadims; Skrastina, Anna; Pavlenko, Romans; Mockus, Ernestas; Zokaitytė, Eglė; Starkutė, Vytautė; Klupšaitė, Dovilė; Ruibys, Romas; Rocha, João Miguel; Santini, Antonello; Bartkienė, Elena. Changes in Spirulina's physical and chemical properties during submerged and solid-state lacto-fermentation // Toxins. Basel: MDPI, 2023, vol. 15, no. 1, p. 1–19, ISSN 2072-6651, 2072-6651. doi:10.3390/toxins15010075. Link: <10.3390/toxins15010075> <https://hdl.handle.net/20.500.12512/116596> <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9862786/>. Science Citation Index Expanded; PubMed; MEDLINE (PubMed); Scopus. [S1] [Field of science: A003] [Citav. rodiklis: 4.2, bendr. cit. rod.: 4.7, kvartilis: Q1 (2022. InCites JCR SCIE)]

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2. **Tolpežnikaitė, Ernesta**; Bartkevics, Vadims; Skrastina, Anna; Pavlenko, Romans; Ružauskas, Modestas; Starkutė, Vytautė; Zokaitytė, Eglė; Klupšaitė, Dovilė; Ruibys, Romas; Rocha, João Miguel; Bartkienė, Elena. Submerged and solid-state fermentation of Spirulina with lactic acid bacteria strains: antimicrobial properties and the formation of bioactive compounds of protein origin // Biology. Basel: MDPI, 2023, vol. 12, no. 2, p. 1–23, ISSN 2079-7737, 2079-7737. doi:10.3390/biology12020248. Link: <10.3390/biology12020248> <https://hdl.handle.net/20.500.12512/116822> <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9952912/>. Science Citation Index Expanded; Scopus; PubMed; PubMed Central. [S1] [Field of science: A003] [Citav. rodiklis: 4.2, bendr. cit. rod.: 4.5, kvartilis: Q2 (2022. InCites JCR SCIE)]

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3. **Tolpežnikaitė, Ernesta**; Starkutė, Vytautė; Zokaitytė, Eglė; Ružauskas, Modestas; Pilkaitytė, Renata; Viškelis, Pranas; Urbonavičienė, Dalia; Ruibys, Romas; Rocha, João M; Bartkienė, Elena. Effect of solid-state fermentation and ultrasonication processes on antimicrobial and antioxidant properties of algae extracts // Frontiers in Nutrition. Lausanne, Switzerland: Frontiers Media SA, 2022, vol. 9, p. 1–19, ISSN 2296-861X. doi:10.3389/fnut.2022.990274. Link: <10.3389/fnut.2022.990274> <https://hdl.handle.net/20.500.12512/115519> <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9453264/>. Science Citation Index Expanded; PubMed. [S1a] [Field of science: A003, N010, A001] [Citav. rodiklis: 5, bendr. cit. rod.: 5.5, kvartilis: Q2 (2022. InCites JCR SCIE)]

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4. **Tolpežnikaitė, Ernesta**; Ružauskas, Modestas; Pilkaitytė, Renata; Bartkevics, Vadims; Zavistanavičiūtė, Paulina; Starkutė, Vytautė; Lėlė, Vita; Zokaitytė, Eglė; Mozūrienė, Erika; Ruibys, Romas; Klupšaitė, Dovilė; Santini, Antonello; Bartkienė, Elena. Influence of fermentation on the characteristics of Baltic Sea macroalgae, including microbial profile and trace element content // Food Control. Oxon: Elsevier Science LTD, 2021, vol. 129, p. 1–12, ISSN 0956-7135, 1873-7129. doi:10.1016/j.foodcont.2021.108235. Link: <https://hdl.handle.net/20.500.12512/110340> <https://doi.org/10.1016/j.foodcont.2021.108235> <10.1016/j.foodcont.2021.108235>. Science Citation Index Expanded; Biological Abstracts; BIOSIS Previews|; Scopus. [S1] [Field of science: A003] [Citav. rodiklis: 6.652, bendr. cit. rod.: 5.238, kvartilis: Q1 (2021. InCites JCR SCIE)]

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International scientific conferences:

1. Bartkienė, Elena; **Tolpežnikaitė, Ernesta**; Bartkevics, Vadims; Skrastina, Anna; Pavlenko, Romans; Ružauskas, Modestas; Starkutė, Vytautė; Zokaitytė, Eglė; Klupšaitė, Dovilė; Ruibys, Romas; Rocha, João Miguel. The changes in bioactive compounds during the fermentation of Spirulina // 26th Congress of SCTM with International Participation: Book of Abstracts: September 20–23, 2023, Ohrid, N. Macedonia/ Society of Chemists and Technologists of Macedonia, p. 142, ISBN 978-9989-760-19-8.

Link: <https://congress.sctm.mk/event/1/attachments/1/554/26th%20C ongress%20of %20SCTM_Book%20of%20abstrakts.pdf> <https://hdl.handle.net/20.500.12512/238680>. [T1e] [Field of science: A002, A003]

2. Klementavičiūtė, Jolita; **Tolpežnikaitė, Ernesta**; Starkutė, Vytautė; Ružauskas, Modestas; Pilkaitytė, Renata; Viškelis, Pranas; Urbonavičienė, Dalia; Bartkienė, Elena. Enhancing total phenolic content and antibacterial potential of algae biomass through ultrasonication and solid-state fermentation // 16th World Congress on Polyphenols Applications: September 28–29, 2023, Malta and Online: Abstracts Book, p. 91. Link: <https://hdl.handle.net/20.500.12512/238775>. [T2] [Field of science: A003]

3. Bartkienė, Elena; **Tolpežnikaitė, Ernesta**; Skrastina, Anna; Pavlenko, Romans; Mockus, Ernestas; Zokaitytė, Eglė; Starkutė, Vytautė; Klupšaitė, Dovilė; Ruibys, Romas; Bartkevics, Vadims. Spirulina (Arthrospira platensis) bio-conversion: desirable and undesirable changes // Tagungsband: 21. BOKU-Symposium Tierernährung Fütterungsstrategien in Zeiten knapper Ressourcen: April 20, 2023, Wien/Institut für Tierernährung, Tierische Lebensmittel und Ernährungsphysiologie (TTE). Department für Agrarbiotechnologie, IFA-Tulln. Universität für Bodenkultur Wien; Herausgebers: Pedro H S Ferzola, Christiane Schwarz, Martin Gierus. Wien: Institut für Tierernährung, Tierische Lebensmittel und Ernährungsphysiologie (TTE), 2023. ISBN 9783900397067, p. 209–212: pav, ISBN 9783900397067. Prieiga per internetą: [P1d] [M.kr.: A003]

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5. **Tolpežnikaitė, Ernesta**; Ružauskas, Modestas; Pilkaitytė, Renata; Bartkevics, Vadims; Zavistanavičiūtė, Paulina; Starkutė, Vytautė; Lėlė, Vita; Zokaitytė, Eglė; Mozūrienė, Erika; Ruibys, Romas; Klupšaitė, Dovilė; Bartkienė, Elena. Baltic Sea macro-algae characteristics including microbial profile and trace elements content // The Vital Nature Sign: 15th International Scientific Conference The Vital Nature Sign: May 20–21, 2021, Kaunas, Lithuania: abstract book/[Editors: Audrius Maruška, Nicola Tiso, Vilma Kaškonienė, Mantas Stankevičius]. Kaunas: Vytautas Magnus University, 2020, p. 33, ISSN 2335-8653, 2335-8718. Link: <https://hdl.handle.net/20.500.12512/111032>. [T1e] [Field of science: A003]

National scientific conferences:

1. **Tolpežnikaitė, Ernesta**; Starkutė, Vytautė; Zokaitytė, Eglė; Ružauskas, Modestas; Pilkaitytė, Renata; Viškelis, Pranas; Urbonavičienė, Dalia; Bartkienė, Elena. Effect of biological and physical treatments on antimicrobial and antioxidant properties of macro- and microalgae extracts // 11-osios jaunųjų mokslininkų konferencijos Jaunieji mokslininkai – žemės ūkio pažangai: pranešimų tezės: [2022 m. lapkričio 10 d.]/

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2. **Tolpežnikaitė, Ernesta**; Bartkevics, Vadims; Ružauskas, Modestas; Pilkaitytė, Renata; Viškelis, Pranas; Urbonavičienė, Dalia; Zavistanavičiūtė, Paulina; Zokaitytė, Eglė; Starkutė, Vytautė; Bartkienė, Elena. Baltijos jūros makrodumblių cheminė ir biologinė sauga = Chemical and bio-safety of the Baltic Sea macroalgae // 10-oji jaunųjų mokslininkų konferencija Jaunieji mokslininkai – žemės ūkio pažangai: pranešimų tezės: [2021 m. lapkričio 18 d.]/Lietuvos mokslų akademijos Žemės ūkio ir miškų mokslų skyrius; [Leidinį sudarė Reda Daukšienė]. Vilnius: Lietuvos mokslų akademija, 2021. ISBN 9789986080831, p. 37, ISBN 9789986080831, 9789986080848. Link: <https://hdl.handle.net/20.500.12512/112305>. [T2] [Field of science: A002, A003]

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Influence of fermentation on the characteristics of Baltic Sea macroalgae, including microbial profile and trace element content

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ABSTRACT

The aim of this study was to evaluate the possibility of fermenting Baltic Sea macroalgae (*Ulva intestinalis*, *Cladophora rupestris*, and *Furcellaria lumbricalis*) using *Lactobacillus plantarum* strain LUHS135, which possesses antimicrobial properties, without additional pre-treatment with a fermentable substrate. To evaluate the effectiveness of the treatment, analysis of the microbial profile, antimicrobial and antioxidant characteristics, and trace element concentrations was performed. Fermentation with strain LUHS135 reduced the pH of *C. rupestris* < 4.5; however, that of *U. intestinalis* and *F. lumbricalis* remained unchanged after 12 h of fermentation. Metagenomic analysis showed that the algae can be contaminated with potentially harmful microorganisms which remain after fermentation despite the use of a technological microorganism possessing antimicrobial properties. However, the synergic mechanism of the algae and LUHS135 combination showed a broader spectrum of pathogen inhibition. Also, the health claims associated with algal products must be based on sufficient evidence of algal chemical safety indicators, for which heavy metals (especially lead and arsenic) are very important. Finally, the use of fermentable sugars (to reduce the pH of fermented samples and to prolong shelf life) and application of ultrasound treatment before fermentation could be further tested as a pre-pre-treatment method to control the fermentation process. Also, preparation of extracts of the antimicrobial compounds could be a possible way of reducing heavy metal concentrations and of using this prospective raw material at industrial scale.

1. Introduction

Macroalgae are natural components of the marine ecosystem, which have a chemical composition making them desirable for possible application in various industries (El-Shenody et al., 2019). Nowadays, the major producers of algae are China (47.9%), Indonesia (38.7%), the Philippines (4.7%), and the Republic of Korea (4.5%), and the major producers of wild macroalgae are Chile, China, and Norway (FAO, 2018). The most common macroalgae which can be used for food/feed purposes in the Baltic Sea region are *Ulva intestinalis*, *Cladophora rupestris*, and *Furcellaria lumbricalis*.

The macroalgae of the genus *Ulva* represent a promising material for

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functional foods, and their sulphated polysaccharides are promising for medical applications (Rahimi, Tabarsa, & Rezaei, 2016). *Ulva* spp. contain 10–26% crude protein (Fleurence, 1999) and 42.1–48.4% of the total amino acid content of all the essential amino acids (Wong & Cheung, 2000); they also contain vitamins and minerals (García-Casal, Pereira, Leets, Ramírez, & Quiroga, 2007; Ortiz Viedma et al., 2006). *Ulva* possesses many health-promoting effects, because of its bioactive components which include dietary fibre and its component ulvan (Kidgell, Magnusson, de Nys, & Glasson, 2019). Also, it is important to process *Ulva* spp. because they have high productivity across diverse geo-climatic conditions and can result in the formation of problematic 'green tides' (Largo, Sembrano, Hiraoka, & Ohno, 2004).

Algae of the genus *Cladophora* are rich in phytochemical compounds that have health benefits in both humans and animals (Munir, Qureshi, Bibi, & Khan, 2019). Secondary metabolites of *Cladophora* species possess anti-diabetic, antioxidant, anti-parasitic, anti-hypertensive, anti-cancer, and cytotoxic activity (Surayot et al., 2016). *Cladophora* is popular in the food/feed industries and medicine (Fang, Liu, & Xu, 2006). This algal genus contains minerals and essential amino acids, the content of which is higher than that in *Spirulina* and *Porphyra* (Fang et al., 2006), saturated and polyunsaturated fatty acids (Stabili et al., 2014), and desirable bioactive compounds (Peerapornpisal, Amornled-pison, Rujjanawate, Ruangrit, & Kanjanapothi, 2006). *Cladophora* extracts possess desirable antimicrobial properties (Cavallo et al., 2013).

F. lumbricalis is a source of furcellaran (Tuvikene et al., 2010). The total phenolic compound content and antioxidant activity of *F. lumbricalis* are remarkable (Zubia, 2009). *F. lumbricalis* is a common species in the North Atlantic, as well as in the Baltic Sea (Weinberger, Paalme, & Wikström, 2020). The total biomass of the red algal community varies yearly (on average, 150 000 tons by wet weight), *F. lumbricalis* accounting for 60–73% (Weinberger et al., 2020).

The growing need for functional products encourages increased consumption of macroalgae by introducing these bioactive components into food/feed formulas (Cofrades, Benedí, Garcimartin, Sánchez-Muniz, & Jimenez-Colmenero, 2017). One of the most popular ways to provide additional value to food/feed is lacto-fermentation. Despite the long history of food/feed fermentation technology, studies about fermented food/feed produced from algae are scarce. This could be related to the specific chemical composition of algal carbohydrates, containing mostly polysaccharides (brown algae – alginate and fucoidan, red algae – galactan, green algae – cellulose and hemicellulose), which are known to be unfavourable substrates for fermentation (Uchida & Murata, 2002, 2004).

In this study, the main idea was to use the lactic acid bacterial (LAB) strain *Lactobacillus plantarum* LUHS135, which possesses desirable antimicrobial properties, and to create combinations of LAB and macroalgal bioproducts possessing a synergic antimicrobial effect against pathogenic and oportunistic strains and which could be recommended for human and/or animal nutrition. Our previous studies showed that *L. plantarum* LUHS135 inhibits a broad spectrum of various pathogenic and opportunistic strains (Bartkiene, Bartkevics et al., 2018; Bartkiene et al., 2019; Lele et al., 2018), as well as fungi (Bartkiene et al., 2020). Also, in combination with other LAB strains, LUHS135 increases the lactobacillus population and reduces opportunistic and pathogenic strains in the microbiota of piglet faeces *in vivo* (Vadopalas et al., 2020). Fermentation leads to a higher nutritional value and better bioaccessibility of various foods, because it transforms primary substrates into more digestible lower molecular weight compounds (Hur, Lee, Kim, Choi, & Kim, 2014).

However, first of all, in this study the main attention was focused on the biochemical and chemical safety of Baltic Sea algae (*U. intestinalis*, *C. rupestris*, and *F. lumbricalis*). Also, we hypothesized that the use of strain LUHS135 possessing antimicrobial properties for algal fermentation could lead to controlled fermentation without sterilizing the substrate. Despite a very limited number of outbreaks having been linked to macroalgal biosafety (Park et al., 2015), macroalgae are contaminated with bacteria, some of which can be potentially toxic for humans and/or animals (Duinker et al., 2016). For this reason, metagenomic studies of macroalgae were performed, and microorganism communities determined before and after fermentation.

Another challenge which can be associated with the use of algae for food/feed purposes is their contamination with heavy metals. There are no studies published about this safety indicator of the Baltic Sea macroalgae from Lithuania. However, published data on the contamination of Norwegian macroalgae with heavy metals and microbial pathogenic strains confirm the importance of these studies, before possible application of the macroalgae at industrial scale (Duinker et al., 2016, 2020). Current efforts to catalogue information about Baltic Sea macroalgae will aid the industry in targeting attention on resources until now used inefficiently.

The aim of this study was to evaluate the possibility of fermenting Baltic Sea macroalgae (*U. intestinalis*, *C. rupestris*, and *F. lumbricalis*) using the antimicrobial *L. plantarum* strain LUHS135, without additional pre-treatment with a fermentable substrate. To evaluate the effectiveness of the treatment, analysis of the microbial profile, antimicrobial and antioxidant characteristics, and trace element concentrations was performed.

2. Materials and methods

2.1. Sampling of U. intestinalis, C. rupestris, and F. lumbricalis

Samples of U. intestinalis, C. rupestris, and F. lumbricalis were taken in September of 2020 near Klaipeda city (Lithuania). Specimens of U. intestinalis were hand-picked from 10 different stones up to 1 m depth. Specimens of C. rupestris and F. lumbricalis were collected from the water (~1 m depth) along the shore (about 100 m) a few days after a storm. The algae were cleaned by removing entangled other algae and macroscopic animals by washing them carefully twice: with tap water and then with distilled water. In total, appr. 500 g wet weight of each species was collected. After washing, the specimens were frozen at −80 °C. The frozen samples were milled using a blender to pass through a 1 mm screen and stored in a sealed plastic bag in a freezer (−80 °C) for further analysis.

2.2. Fermentation of algal samples with L. plantarum LUHS135 strain

The L. *plantarum* LUHS135 strain was incubated and multiplied in De Man, Rogosa, and Sharpe (MRS) broth culture medium (Biolife, Italy) at 30 °C under anaerobic conditions. A total of 3 mL of LAB multiplied in MRS (cell concentration, on average, 9.0 $\log_{10}$ CFU mL^{-1}) was inoculated to 100 g of milled algal media, followed by anaerobic fermentation in a modified carbon dioxide atmosphere in a chamber incubator (Memmert GmbH + Co. KG, Schwabach, Germany) for 12 h at 30 °C.

Finally, six samples of algae were tested: non-fermented (control) *U. intestinalis*, *C. rupestris*, and *F. lumbricalis*, and the three algae fermented with LUHS135 strain.

2.3. Microbiological analysis, dry matter content, and acidity evaluation

Microbiological analyses (LAB and total bacterial count) using algal samples were performed immediately after 12 h of fermentation in treated samples; in non-fermented samples, algae were defrosted at room temperature and immediately used for further analysis.

To evaluate the LAB count, an algal sample (10 g) was homogenized with 90 mL of sterile NaCl solution (9 g L^{-1}). Serial dilutions of 10^{-4}–10^{-8} with NaCl solution were used for sample preparation. Sterile MRS agar (Oxoid code CM0361, Oxoid Ltd.) was used for bacterial growth on Petri plates. After inoculation with the sample suspension, the plates were incubated under anaerobic conditions at 30 °C for 72 h. The number of total bacteria was determined using plate count agar (PCA, CM0325, Oxoid Ltd.). The number of microorganisms was calculated

and expressed as $\log_{10}$ of colony-forming units per gram and (or) millilitre (CFU g^{-1} and/or CFU mL^{-1}). All results were expressed as the mean values of three determinations.

The dry matter content of non-fermented *U. intestinalis*, *C. rupestris*, and *F. lumbricalis* samples was determined after oven-drying the samples at 103 ± 2 °C till constant weight was obtained. The pH of algae was evaluated with a pH meter (Inolab 3, Hanna Instruments, Italy) by inserting the pH electrode into the algae mass. Algae mass preparation is described in section 2.1.

2.4. Microbial profiling

Next generation sequencing was performed according to 16S rRNA genes with the aim to explore the microbiome of the bacteria in algae before and after fermentation. The material of each sample (1 g) was kept in DNA/RNA Shield 1:10 (R1100-250, Zymo Research, USA) at −70 °C before DNA extraction. DNA was extracted with a faecal DNA MiniPrep kit (D6010, Zymo Research, USA). Library preparation, metagenomic sequencing, and taxonomic characterization of reads were performed as described previously (Vadopalas et al., 2020). ZymoBIOMICS Microbial Community Standard (D6300, Zymo Research Corporation, Irvine, CA, USA) was used as a microbiome profiling quality control. The results were analysed and taxonomic classification was visualized using an interactive online platform.

2.5. Evaluation of the antimicrobial activity of the algal samples

The antimicrobial activity of all the initial non-fermented and fermented (12 h) algae was assessed against a variety of 15 pathogenic and opportunistic bacterial strains previously isolated from clinical material from animals and humans (*Staphylococcus aureus*, *S. haemolyticus*, *Enterococcus durans*, *Bacillus pseudomycoides*, *Salmonella enterica*, *Aeromonas hydrophila*, *A. veronii*, *Acinetobacter baumannii*, *A. johnsonii*, *Enterobacter cloacae*, *Cronobacter sakazakii*, *Kluyvera cryocrescens*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*). To evaluate the antimicrobial activity of algae, algal samples were diluted in a 1:3 (*v/v*) ratio with sterile NaCl solution (9 g L^{-1}). Then, to 0.5 mL of diluted algal samples, 0.01 mL of the pathogenic and opportunistic bacterial strains (suspensions of 0.5 McFarland turbidity standard of each pathogenic bacteria) was added, mixed, and incubated at 35 °C for 24 h. After incubation, the viability of pathogenic and opportunistic bacterial strains in algal samples was assessed by plating them on selective (*Aeromonas hydrophila* and *A. veronii* on Aeromonas medium base (Thermo Fisher, Hampshire, United Kingdom), *Staphylococcus aureus* and *S. haemolyticus* on mannitol salt agar (Thermo Fisher, Hampshire, United Kingdom), *Enterococcus durans* on Slanetz and Bartley medium (Thermo Fisher, Hampshire, United Kingdom), *Salmonella enterica*, *Enterobacter cloacae*, *Cronobacter sakazakii*, *Kluyvera cryocrescens*, *Klebsiella pneumoniae*, and *Escherichia coli* on MacConkey agar (Sigma-Aldrich, Saint Louis, United States)) and non-selective (*Acinetobacter baumannii*, *A. johnsonii*, *B. pseudomycoides*, and *P. aeruginosa* on Mueller Hinton Agar (Thermo Fisher, Hampshire, United Kingdom)) culture media. The results were interpreted as negative if no growth of tested strains was observed and as positive if the pathogens grew (visible colonies) on media. Experiments were performed in triplicate.

2.6. Evaluation of the total content of phenolic compounds, antioxidant activity, and colour coordinates of the algal samples

First of all, extracts of the non-fermented and fermented milled algal media were prepared. Milled algal media sample (10 g) was transferred to dark-coloured flasks and mixed with 200 mL of methanol and stored at room temperature. After 24 h, infusions were filtered through Whatman No. 1 filter paper and the residue was re-extracted with an equal volume of methanol. Combined supernatants were evaporated to dryness under vacuum at 40 °C using a rotary evaporator. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4 ± 1 °C. The total content of phenolic compounds (TPC) was determined using the Folin–Ciocalteu method (Singleton & Rossi, 1965). The ability of the macroalgal extract to scavenge DPPH free radicals was assessed by the standard method (Zhu, Lian, Guo, Peng, & Zhou, 2011).

The colour characteristics were evaluated at three different positions on the surface using a CIE L*a*b* system (CromaMeter CR-400, Konica Minolta, Japan). The results were expressed as the CIE colour values L* (brightness/darkness), a* (redness/greenness), and b* (yellowness/blueness). Fermented and untreated milled (description of the sample pre-preparation is given in section 2.1.) algal samples were placed in a 1 cm-high cylindrical Petri dish and the colour coordinates were measured on the flattened surface of the sample. Before each measurement, the instrument was calibrated with a light trap and white colour standard. After each measurement, the chroma meter glass was cleaned with distilled water, and moisture was removed with a cotton cloth.

2.7. Analysis of micro- and macroelements in algal samples

Analysis of micro- and macroelements in algal samples was performed by inductively coupled plasma mass spectrometry (ICP-MS) according to a published method (Bartkiene et al., 2016), using an Agilent 7700x ICP-MS instrument with Mass Hunter Work Station software for ICP-MS, version B.01.03.

2.8. Statistical analysis

Fermentation of macroalgae was performed once, while all analytical experiments were carried out in triplicate. Data were subjected to analysis of variance (ANOVA) using the statistical package SPSS for Windows (Ver.15.0, SPSS, Chicago, Illinois, USA). The calculated mean values were compared using Duncan's multiple range test with significance defined at $p \leq 0.05$. To evaluate the influence of fermentation on algal parameters, statistical analysis was performed using the one-way ANOVA.

3. Results and discussion

3.1. Dry matter content, microbial count, and pH of the fermented and untreated algal samples

The dry matter content of the non-fermented *U. intestinalis*, *C. rupestris*, and *F. lumbricalis* samples was 17.95 ± 0.65, 34.42 ± 1.10, and 32.57 ± 1.04, respectively, and that of the fermented samples was 23.69 ± 0.69, 36.04 ± 1.05, and 33.65 ± 0.97%, respectively.

LAB and total bacteria (TBC) counts, as well as the pH of algal samples, are shown in Fig. 1. Fermentation with L. *plantarum* LUHS135 strain significantly reduced the pH of *C. rupestris* (to < 4.5); however, that of *U. intestinalis* and *F. lumbricalis* remained unchanged (on average, 7.90 and 6.78, respectively) after 12 h of fermentation. It should be pointed out that, during food acidification, it is very important to drop the pH of the fermentable substrate below 4.6 within 24 h. This study showed that to increase the effectiveness of fermentation, two of the three tested algae need pre-treatment before fermentation, and the addition of fermentable sugars or enzymatic treatment before fermentation is recommended. Finally, further research is needed to increase the acidification rates of *U. intestinalis* and *F. lumbricalis* during the fermentation process.

In non-fermented algal samples, no LAB were found; however, after 12 h of fermentation the LAB count was higher than 6.0 $\log_{10}$ CFU g^{-1} in all samples. Comparing non-fermented samples, the TBC in algal samples was, on average, 5.14 $\log_{10}$ CFU g^{-1}, and fermentation lead to an increase in TBC: in *C. rupestris* samples by 41.1%, in *U. intestinalis* samples by 19.8%, and in *F. lumbricalis* samples by 28.4%. Moderate negative correlations were found between algal samples' pH and LAB, as well as TBC ($r = -0.497$, $p = 0.036$ and $r = -0.5909$, $p = 0.011$,

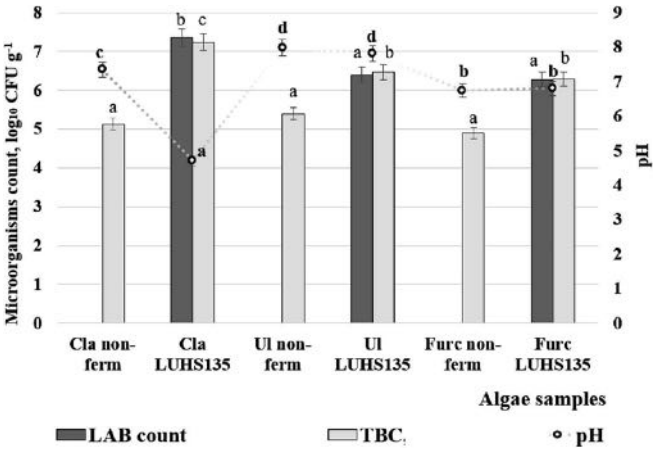
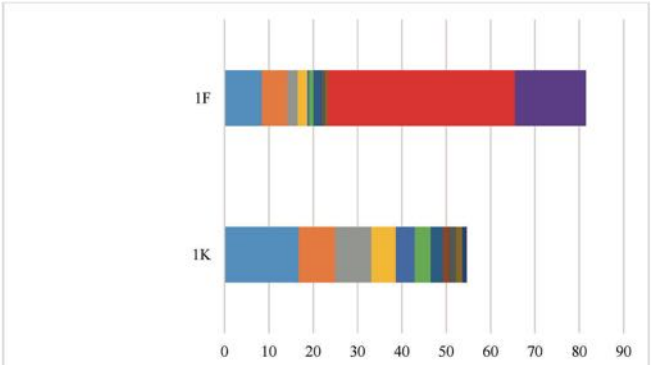


Fig. 1. Microbial and pH characteristics of untreated and fermented algae (Cla non-ferm – non-fermented *Cladophora rupestris*; Cla LUHS135 – *C. rupestris* fermented with *L. plantarum* LUHS135; Ul non-ferm – non-fermented *Ulva intestinalis*; Ul LUHS135 – *U. intestinalis* fermented with *L. plantarum* LUHS135; Furc non-ferm – non-fermented *Furcellaria lumbricalis*; Furc LUHS135 – *F. lumbricalis* fermented with *L. plantarum* LUHS135; LAB – lactic acid bacteria; TBC – total bacterial count; CFU – colony-forming units. Data are represented as means (n = 3, replicates of analysis) ± SD. [a–d] For the same analytical parameters, means with different letters are significantly different ($p \leq 0.05$)).



	1K	1F
Portibacter lacus	16.7	8.4
Actinobacterium spp.	8.3	5.8
Lewinella cohaerens	8.1	2.3
unclassified Acidimicrobium	5.6	2.2
Lewinella nigricans	4.2	0.4
alpha proteobacterium	3.6	1
Lacibacter cauensis	2.7	1.75
Phaeodactylibacter luteus	1.5	0.1
Maribacter lutimaris	1.5	0.7
Phaeodactylibacter xiamenensis	1.4	0.6
Caldilinea aerophila	1	0.01
Lactobacillus plantarum	0	42.3
Escherichia vulneris	0.02	15.9

Fig. 2. Bacterial composition in untreated (1K) and fermented (1F) samples of *Cladophora rupestris*. Species and amount (%) of each species are presented. Only those species with a prevalence of at least 1% irrespective of the group (untreated or fermented) are presented.

respectively). This indicates that there is a significant effect of algal species and fermentation, as well as interaction of these factors, on LAB, TBC, and pH ($p \leq 0.05$).

The current knowledge about wild algae from Lithuania, as well as algal fermentation, is scarce. Several LAB strains are suitable for algal fermentation (Uchida, Murata, & Ishikawa, 2007). Fungal fermentation (using *Monascus purpureus* and *M. kaoliang*) of the seaweeds *Saccharina japonica* and *Undaria pinnatifida* increases their functionality: it increases the concentration of phenolics and flavonoids, and provides anti-diabetic, antioxidant, and anti-lipase features (Suraiya et al., 2018). In this study, a viable LAB count higher than 6.0 $\log_{10}$ CFU g^{-1} remained in the above-mentioned samples. LAB-rich products are associated with numerous health benefits, of which antimicrobial, mycotoxin detoxification, and microbiota modulation properties are very important (Bartkiene et al., 2020; Bartkiene, Zavistanaviciute, et al., 2018; Vadopalas et al., 2020). For this reason, during the second step, we tested the hypothesis that the use of LUHS135, which possesses antimicrobial properties, for treatment of algae could lead to controlled fermentation without sterilizing the algal substrate, as the current overall knowledge about the microbiomes of wild algae is scarce.

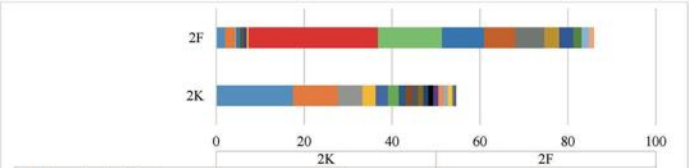
3.2. Microbial profiles of algal samples

The number of reads after metagenomic sequencing in all the samples was similar and varied from 12 134 to 13 678. The number of bacterial species in wild untreated algal samples varied from about 120 to 150 with a prevalence of least 10 reads.

The main bacterial species in the untreated *C. rupestris* were *Portibacter lacus*, *Actinobacterium* spp., *Lewinella cohaerens*, and *Acidimicrobium* spp., the prevalence of which was above 5% of the total bacterial load (Fig. 2). In the fermented samples, *Lactobacillus plantarum* was the most abundant species, accounting for 42.3% of all bacteria detected within the sample (Fig. 2). The amounts of bacteria which had the highest prevalence in untreated samples, such as *Portibacter lacus* and *Acidobacterium*, also remained high; however, the number of *Escherichia vulneris*, the prevalence of which before treatment was low (0.02%), increased significantly after fermentation (15.9%; $p \leq 0.05$).

The most prevalent microbiota in the untreated *U. intestinalis* sample were species from the class Alphaproteobacteria, *Luteolibacter algae*, and *Marivirga tractuosa*, the prevalence of which was above 5% of the total bacterial count, while after fermentation the most prevalent species was *Lactobacillus plantarum* (29.4%) (Fig. 3). Two bacterial species which were not detected in the untreated sample were detected in large quantities in the fermented sample: *Terrisporobacter glycolicus* was the second most prevalent species (14.6%) and *Exiguobacterium mexicanum* was the third most prevalent species (9.6%) (Fig. 3).

The most prevalent bacteria in untreated *F. lumbricalis* samples were *Portibacter lacus*, *Actinobacterium* spp., and *Actinomicrobium* spp. whereas

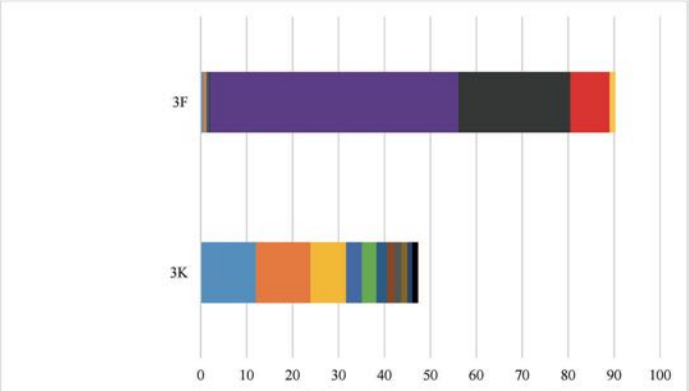


	2K	2F
alpha proteobacterium	17.5	2
Luteolibacter algae	10.2	2.2
Marivirga tractuosa	5.6	0.2
Dokdonia donghaensis	3	0.13
Erythrobacter atlanticus	2.8	0.7
Portibacter lacus	2.4	0.16
Algibacter lectus	1.6	0.3
Actinobacterium spp.	1.4	0.11
Erythrobacter marinus	1.4	0.4
Ascidiaceihabitans donghaensis	1.2	0.12
Polaribacter irgensii	1.2	0.2
Lewinella agarilytica	1.1	0
Sulfitobacter donghicola	1.1	0.4
Sphingorhabdus wooponensis	1.1	0.12
Lewinella cohaerens	1.1	0.02
Marinosulfonomonas methylotropha	1	0.3
Lactobacillus plantarum	0.01	29.4
Terrisporobacter glycolicus	0	14.6
Exiguobacterium mexicanum	0	9.6
Stenotrophomonas maltophilia	0.01	7.1
Pseudomonas putida	0.4	6.6
Pseudomonas stutzeri	0.04	3.4
Escherichia vulneris	0.4	3.2
Paraclostridium benzoelyticum	0	1.9
Pseudomonas geniculata	0.01	1.7
Romboutsia lituseburensis	0.01	1.1

Fig. 3. Bacterial composition in untreated (2K) and fermented (2F) samples of *Ulva intestinalis*. Species and amount (%) of each species are presented. Only those species with a prevalence of at least 1% irrespective of the group (untreated or fermented) are presented.

in the fermented sample the highest prevalence was observed for two cultivable species – *Pseudomonas putida* (54.2%) and *Escherichia vulneris* (24.3%) (Fig. 4). *L. plantarum* was only in third place, accounting for 8.6% of the TBC within the sample. Both *P. putida* and *E. vulneris* were also detected in the untreated sample with low prevalence (0.1%). Current knowledge about the microbiomes of wild algae is scarce. Hence we investigated the microbiomes of macroalgae from the Baltic Sea and have detected 120–150 bacterial species with a prevalence of at least 10 reads in the small amount taken for metagenomic analysis. The data demonstrate that the thallus of algae is a complex of living organisms and should be treated as a micro-ecological system.

The most prevalent species of bacteria included *Portibacter lacus* and *Luteolibacter algae*, as well as different species of *Actinobacterium*, *Acidimicrobium*, and *Lewinella*. *P. lacus* was first described by Yoon, Matsuo, Kasai, and Yokota in 2012 and was isolated from saline lake water in Japan. *L. algae* was first described by the same group of researchers in 2008 and was isolated from a marine environment (Yoon et al., 2008). Actinobacteria, which possesses the characteristics of both bacteria and fungi, are widely distributed in both terrestrial and aquatic ecosystems. They are considered as biotechnologically valuable bacteria that are exploited for their production of secondary metabolites (Anandan, Dharumadurai, & Manogaran, 2016). *Acidimicrobium* is a genus within the phylum Actinobacteria, the species of which live in different environments and are known as ferrous iron oxidizers, while species of the genus *Lewinella* are known as chemoorganotrophic aerobic Gram-negative bacteria which are important for the breakdown of complex organic compounds in the environment (McIlroy, 2014). The composition of bacteria detected in macroalgae consisted of the above-mentioned bacteria and others found in a natural salt water environment. Fermentation of algae changed the overall microbial profiles in such a way that the relative abundance of natural microbiota decreased because *Lactobacillus*, as a fermentative microorganism, was added to the samples and started to multiply. Analysis of the algal microbiomes after fermentation revealed that, regardless of ensuring of favourable growing conditions for *L. plantarum*, some other culturable species of bacteria started to multiply in the samples during fermentation. In the sample of *U. intestinalis*, two bacterial species – *Terrisporobacter glycolicus* and *Exiguobacterium mexicanum* – that were not detected in the untreated samples were present in large quantities, whereas in the fermented *F. lumbricalis* sample, *Pseudomonas putida* and *Escherichia vulneris*, which were detected in untreated samples as well, were also able to multiply during fermentation and therefore increased the pH of the fermented products. The occurrence of *T. glycolicus* and *E. mexicanum* in fermented samples might be explained by the ability of the single bacteria that were present in small quantities (and therefore were absent in part of the untreated samples) to multiply fast in



	3K	3F
Portibacter lacus	12	0.6
Actinobacterium spp.	11.9	0.6
Acidimicrobium spp.	7.8	0
alpha proteobacterium	3.4	0
Lewinella cohaerens	3.2	0.1
Flavobacterium frigidarium	2.3	0.4
Lewinella nigricans	1.6	0.01
Maribacter lutimaris	1.5	0.05
Blastopirellula spp.	1.3	0.06
Psychrobacter piscatorii	1.1	0.07
Truepera radiovictrix	1.1	0.07
Pseudomonas putida	0.1	54.2
Escherichia vulneris	0.1	24.3
Lactobacillus plantarum	0	8.6
Pseudomonas syringae	0.01	1.2

Fig. 4. Bacterial composition in untreated (3K) and fermented (3F) samples of *Furcellaria lumbricalis*. Species and amount (%) of each species are presented. Only those species with a prevalence of at least 1% irrespective of the group (untreated or fermented) are presented.

favourable conditions. *T. glycolicus* is a strict anaerobe and *E. mexicanum* is a facultative anaerobe; therefore, the environment for lactobacillus cultivation was suitable for proliferation of those species. *E. mexicanum* was originally isolated from shrimp (López-Cortés, Schumann, Pukall, & Stackebrandt, 2006); however, bacteria of this genus, similarly to *T. glycolicus*, are known to cause human infections (Chen et al., 2017; Cheng, Domingo, Lévesque, & Yansouni, 2016). The presence of potential pathogens and live bacteria that are able to multiply in algae during fermentation suggests the necessity of inactivating microorganisms before fermentation, as the sample without overgrowth by external microorganisms demonstrated much better results and a lower pH. For this reason, application of ultrasound treatment before fermentation could be further tested as a pre-pre-treatment method to control the fermentation process.

3.3. Antimicrobial activity of non-fermented and fermented algal samples

The antimicrobial activity of the fermented and untreated algal samples against pathogenic opportunistic microorganisms in liquid medium is shown in Table 1. Comparing non-fermented samples, *C. rupestris*, *U. intestinalis*, and *F. lumbricalis* inhibited 3, 2, and 3 pathogens, respectively, of the 13 tested. All non-fermented algal specimens inhibited *Bacillus pseudomycoides* and *Kluyvera cryocrescens*; in addition, non-fermented *F. lumbricalis* inhibited *Staphylococcus haemolyticus*, and non-fermented *C. rupestris* inhibited *Pseudomonas aeruginosa*. Comparing fermented algal samples, the broadest spectrum of pathogen inhibition was found for the *C. rupestris* samples, which inhibited 9 out of 13 tested pathogens. Fermented *U. intestinalis* and *F. lumbricalis* inhibited 6 pathogens out of the 13 tested. Information about the antimicrobial activity of Baltic Sea algae (not extracts) is scarce. However, as the metagenomic study of the microbiomes of the tested macroalgae showed, to avoid health risk, algae must be pre-treated, or extracts prepared, by separating different potential antimicrobial compounds. Preparation of extracts would be very perspective, because different antimicrobial compounds can be obtained. Algae are a source of many bioactive molecules: proteins and peptides, polysaccharides, polyphenols, polyunsaturated fatty acids, and pigments (Silva et al., 2020), which possess antimicrobial properties (Abu-Ghannam & Rajauria, 2013). The most studied algal antimicrobial compounds are polyphenols (Bhowmick, Mazumdar, Moulick, & Adam, 2020; Pérez, Falqué, & Domínguez, 2016). The antimicrobial characteristics of algal polyphenols are associated with their ability to alter membrane permeability, inhibit enzymes and different metabolic pathways, bind to surface molecules, and other mechanisms, which are related to the number of hydroxyl groups and also the degree of polymerization (Abu-Ghannam & Rajauria, 2013; Bhowmick et al., 2020). Algal proteins and peptides have an amphiphilic nature and can interact with polar and non-polar sites of bacterial cell membranes, causing disruption of the membrane and cellular rupture (Bhowmick et al., 2020). The antimicrobial activity of macroalgal polysaccharides depends on their molecular weight, charge density, sulphated content in the case of sulphated polysaccharides, and structural and conformational characteristics (Pérez et al., 2016). Macroalgal polysaccharides interact with the glyco-receptors of the bacterial cell wall and nucleic acids, as well as polysaccharides of the bacteria membrane, which leads to disruption of the cell membranes (Pérez et al., 2016). Also, algal lipids and fatty acids can inhibit the electron transport chain and oxidative phosphorylation in bacterial cell membranes, which leads to peroxidation and auto-oxidation, and cellular lysis (Kasanah, Amelia, Mukminin, Triyanto, & Isnansetyo, 2019). Finally, the combination of separate algal compounds and LAB could be very promising, because a synergic mechanism of action to inhibit pathogens leads to a broader spectrum of pathogen inhibition, and this study showed that the tested macroalgae have some antimicrobial potential.

3.4. Antioxidant characteristics and colour coordinates of algal samples

The antioxidant characteristics and colour coordinates of algal samples are shown in Fig. 5a and b, respectively. The antioxidant activity of the algal samples depended on the species of algae; however, fermentation was not a significant factor in antioxidant activity compared with non-fermented samples. The highest antioxidant activity was shown by *C. rupestris* samples (fermented and untreated): on average, 58.0%. The antioxidant activity of *U. intestinalis* was, on average, 21.3% and 16.0% lower than that of *C. rupestris* and *F. lumbricalis*, respectively. In contrast to its effect on antioxidant activity, fermentation reduced the TPC content in *C. rupestris* and *F. lumbricalis* samples, compared with non-fermented ones, on average by 1.8 and 1.3 times, respectively. However, a strong positive correlation was found between antioxidant activity and TPC content ($r = 0.622$, $p = 0.06$). Significant losses of flavonoids during fermentation may be due to the degradation of phenolic compounds (Adebo, Njobeh, & Kayitesi, 2018). In addition, oxidation of diffused phenolic compounds may also lead to reduced TPC (Othman, Roblain, Chammen, Thonart, & Hamdi, 2009). In contrast, enzymes (amylases and proteases produced by the starter culture) can hydrolyse phenolic conjugates with one or more bound sugars into free polyphenols (Taylor & Duodu, 2015). In this study, correlations of algal colour coordinates a* and b* with TPC, as well with antioxidant activity, were found ($r = -0.869$, $p = 0.0001$; $r = -0.870$, $p = 0.0001$ and $r = -0.594$, $p = 0.009$; $r = -0.641$, $p = 0.004$, respectively). Also, by reducing samples' pH, their antioxidant activity increased (a negative correlation between antioxidant activity and pH was found $r = -0.641$, $p = -0.04$, respectively). LAB fermentation has a significant impact on the phenolic profile, as well as on antioxidant activity, because, during the process, various phenolic acids are excreted

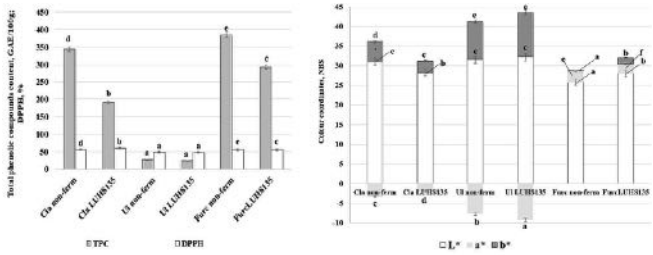


Fig. 5. a –Total phenolic compound content (mg GAE 100 g^{-1}) and DPPH antioxidant activity (%) of the different specimen algae before and after fermentation; **b** – colour coordinates (L*, a*, b*) of the different specimen algae before and after fermentation (Cla non-ferm – non-fermented *Cladophora rupestris*; Cla LUHS135 – *C. rupestris* fermented with *L. plantarum* LUHS135; Ul non-ferm – non-fermented *Ulva intestinalis*; Ul LUHS135 – *U. intestinalis* fermented with *L. plantarum* LUHS135; Furc non-ferm – non-fermented *Furcellaria lumbricalis*; Furc LUHS135 – *F. lumbricalis* fermented with *L. plantarum* LUHS135; L* lightness; a* redness or −a* greenness; b* yellowness or −b* blueness; NBS – National Bureau of Standards units. Data are represented as means (n = 3, replicates of analysis) ± SD. $^{a-f}$ For the same analytical parameters, means with different letters are significantly different ($p \leq 0.05$)).

Table 1
Antimicrobial activity of fermented and untreated algal samples against pathogenic opportunistic microorganisms in liquid medium.

Algal sample	Experimental design: 0.5 mL of diluted algal sample, 0.01 mL of pathogenic and opportunistic bacterial strains													
	Pathogenic and opportunistic bacterial strains													
	Staphylococcus aureus	*Staphylococcus haemolyticus*	*Enterococcus durans*	*Bacillus pseudomycoides*	*Salmonella enterica*	*Acinetobacter baumannii*	*Acinetobacter johnsonii*	*Enterobacter cloacae*	*Cronobacter sakazakii*	*Kluyvera cryocrescens*	*Klebsiella pneumoniae*	*Escherichia coli*	*Pseudomonas aeruginosa*	Total pathogens inhibited
Cla non-ferm	+	+	+	–	+	+	+	+	+	–	+	+	–	3
Cla LUHS135	+	–	+	–	–	–	+	–	–	–	–	+	–	9
Ul non-ferm	+	+	+	–	+	+	+	+	+	–	+	+	+	2
Ul LUHS135	–	–	+	–	+	–	+	+	+	–	+	+	–	6
Furc non-ferm	+	–	+	–	+	+	+	+	+	–	+	+	+	3
Furc LUHS135	–	–	+	–	–	–	+	+	+	–	+	+	–	6
Experimental design: 0.5 mL of LAB, 0.01 mL of pathogenic and opportunistic bacterial strains														
LAB	–	–	–	–	–	–	+	–	–	–	–	–	–	12
Pathogen control														
Pathogen	+	+	+	+	+	+	+	+	+	+	+	+	+	-

Results interpreted as negative (–) if the pathogens did not grow on the selective culture medium and as positive (+) if the pathogens grew on the selective culture medium. Cla non-ferm – non-fermented *Cladophora rupestris*; Cla LUHS135 – *C. rupestris* fermented with *L. plantarum* LUHS135; Ul non-ferm – non-fermented *Ulva intestinalis*; Ul LUHS135 – *U. intestinalis* fermented with *L. plantarum* LUHS135; Furc non-ferm – non-fermented *Furcellaria lumbricalis*; Furc LUHS135 – *F. lumbricalis* fermented with *L. plantarum* LUHS135; LAB – lactic acid bacteria.

to the fermentable substrate (Li et al., 2021).

3.5. Micro- and macroelement concentrations in algal samples

The concentrations of micro- and macroelements in untreated algal samples and those fermented with strain LUHS135 are shown in Table 2. Comparing macroelements in fermented and untreated algal samples, no significant differences were observed. Comparing the macroelement concentrations in samples of different algal species, on average, a 3.5 times higher sodium (Na) concentration was found in *U. intestinalis* samples compared with *C. rupestris* and *F. lumbricalis*. The concentration of potassium (K) in algal samples was, on average, 8.3, 14.6, and 20.5 g kg^{-1} in *U. intestinalis*, *C. rupestris*, and *F. lumbricalis*, respectively. In *C. rupestris* and *F. lumbricalis* samples, the Na/K ratio was 3.0 and 3.7, respectively. It is recommended that Na intake does not exceed 3.5 g day^{-1} (World Health Organization, 2012a, 2012b). *U. intestinalis* had the highest magnesium (Mg) concentration (on average, 5.7 and 1.7 higher than that in *C. rupestris* and *F. lumbricalis*, respectively). Macroalgae are a good source of Mg, but the bioaccessibility of Mg varies between species, e.g., the Mg content of *U. pertusa*, *Laminaria japonica*, and *Gloiopeltis furcata* is 10.47 mg kg^{-1} (41.8% bioaccessible), 6.55 mg kg^{-1} (60.8% bioaccessible), and 8.18 mg kg^{-1} (72.5% bioaccessible), respectively, under simulated gastrointestinal conditions (Nakamura, Yokota, & Matsui, 2012). The lowest content of calcium (Ca) was found in *C. rupestris* samples (on average 2.4 times lower than that in *U. intestinalis* and *F. lumbricalis*). Despite Ca playing a very important role in the body, its insufficiency is a public concern these days (Waheed et al., 2019). Comparing the concentrations of essential microelements in different algal species, the highest concentrations of manganese (Mn), cobalt (Co), nickel (Ni), selenium (Se), and iodine (I) were found in *C. rupestris* samples (on average, 0.741 g kg^{-1}, and 1.54, 6.08, 0.304, and 205.5 mg kg^{-1}, respectively). The dietary intake of Se recommended by the WHO is 40 μg day^{-1} for men and 30 μg day^{-1} for women, based on the Se intake needed to achieve two-thirds of the maximum activity of erythrocyte glutathione peroxidase, with lower limits of safe intake for the population calculated from basal metabolic rate (Thompson & Raizada, 2018). An adequate intake of Mn of 3 mg day^{-1} has been proposed by the European Food Safety Authority (EFSA) for adults, including pregnant and lactating women (EFSA, 2013). According to the EFSA's, 2009 report, no data are available in the open literature on the potential carcinogenicity of Co in humans or in experimental animals. The WHO has reported that the Ni concentrations corresponding to an excess lifetime risk of 1: 10 000, 1: 100 000 and 1: 1 000 000 are about 250, 25, and 2.5 ng $(m^3)^{-1}$, respectively (WHO Regional Office for Europe, 2000). The recommended mean population intake for I is 100–150 μg day^{-1} (World Health Organization, 1996). Also, the EFSA has concluded that the adequate intake (AI) for I for adult men and women can be set at 150 μg day^{-1}, and for lactating women the same AI is proposed as for pregnant women (200 μg day^{-1}) (EFSA, 2017). The US Food and Nutrition Board of the Institute of Medicine has estimated a tolerable upper intake of I of 1100 μg day^{-1} (Institute of Medicine, Food and Nutrition Board, 2001).

The I content in seaweeds ranges from 0.06 mg 100 g^{-1} of dry weight (*U. lactuca*) to 624.5 mg 100 g^{-1} of dry weight (*Laminaria digitata*), but many published reports do not quantify I (Cherry, O'Hara, Magee, McSorley, & Allsopp, 2019). The concentration of iron (Fe) in *C. rupestris* and *U. intestinalis* samples was, on average 3.9 times higher than that in *F. lumbricalis*. Algae may be a good source of Fe; for example, *Sargassum* spp. contain 156.9 mg 100 g^{-1} of dry weight of Fe (García-Casal et al., 2007). *U. intestinalis* had the highest concentrations of chromium (Cr), copper (Cu), zinc (Zn), and phosphorus (P) (on average 12.25, 15.5, and 41.9 mg kg^{-1}, and 2.16 g kg^{-1}, respectively). The Panel on Dietetic Products, Nutrition and Allergies (NDA) considered that there is no evidence of beneficial effects associated with Cr intake in healthy subjects, and it was concluded that the setting of an AI for Cr is not appropriate (EFSA, 2014). For Cu, for adults, the proposed AI is 1.6 mg

Table 2
Micro- and macroelement concentrations in untreated algal samples and those fermented with *L. plantarum* strain LUHS135.

Trace element, d.m.	Algal sample					
	Cla non-ferm	Cla LUHS135	Ul non-ferm	Ul LUHS135	Furc non-ferm	Furc LUHS135
Macroelements, g kg^{-1} d m.						
Na	4.39 ± 0.43	5.46 ± 0.54	17.8 ± 1.7	19.1 ± 1.9	5.22 ± 0.52	5.91 ± 0.59
Mg	2.79 ± 0.27	2.95 ± 0.29	16.1 ± 1.6	16.5 ± 1.6	9.50 ± 0.95	9.29 ± 0.92
K	13.8 ± 1.3	15.4 ± 1.5	7.70 ± 0.77	8.94 ± 0.89	21.7 ± 2.1	19.3 ± 1.9
Ca	4.51 ± 0.45	5.03 ± 0.50	11.8 ± 1.18	12.1 ± 1.2	11.1 ± 1.1	11.1 ± 1.1
Essential microelements, d.m.						
Cr, mg kg^{-1}	0.681 ± 0.068	0.634 ± 0.013	11.4 ± 1.1	13.1 ± 1.3	<0.010	<0.010
Mn, g kg^{-1}	0.701 ± 0.070	0.781 ± 0.078	0.128 ± 0.012	0.115 ± 0.011	0.091 ± 0.009	0.095 ± 0.009
Fe, g kg^{-1}	1.04 ± 0.10	1.31 ± 0.13	1.26 ± 0.12	1.13 ± 0.11	0.285 ± 0.028	0.318 ± 0.031
Co, mg kg^{-1}	1.38 ± 0.13	1.69 ± 0.16	0.392 ± 0.039	0.343 ± 0.034	0.376 ± 0.037	0.441 ± 0.044
Ni, mg kg^{-1}	5.98 ± 0.59	6.18 ± 0.61	1.60 ± 0.16	1.55 ± 0.10	3.66 ± 0.36	3.64 ± 0.36
Cu, mg kg^{-1}	9.88 ± 0.78	10.8 ± 1.0	15.1 ± 1.5	15.9 ± 1.5	10.5 ± 1.0	97.41 ± 0.84
Zn, mg kg^{-1}	16.5 ± 1.6	17.1 ± 1.7	38.7 ± 3.8	45.1 ± 4.5	26.5 ± 2.6	27.7 ± 2.7
Se, mg kg^{-1}	0.232 ± 0.023	0.284 ± 0.028	<0.2	<0.2	<0.2	<0.2
I, mg kg^{-1}	199 ± 19	212 ± 21	22.8 ± 2.2	20.5 ± 1.6	49.5 ± 4.9	56.1 ± 5.6
P, g kg^{-1}	0.984 ± 0.098	1.20 ± 0.12	1.93 ± 0.19	2.39 ± 0.23	1.56 ± 0.15	1.60 ± 0.16
Non-essential microelements, d.m.						
Ga, mg kg^{-1}	0.283 ± 0.028	0.332 ± 0.033	0.297 ± 0.029	0.284 ± 0.028	<0.25	<0.25
As, mg kg^{-1}	3.67 ± 0.36	3.91 ± 0.39	4.31 ± 0.43	3.89 ± 0.38	5.61 ± 0.56	5.15 ± 0.51
V, mg kg^{-1}	1.75 ± 0.17	2.20 ± 0.22	2.23 ± 0.22	2.14 ± 0.21	1.19 ± 0.11	1.20 ± 0.13
Rb, mg kg^{-1}	9.33 ± 0.93	10.4 ± 1.0	5.77 ± 0.57	6.09 ± 0.60	13.8 ± 1.3	12.9 ± 1.2
Sr, mg kg^{-1}	136 ± 13	144 ± 14	183 ± 18	199 ± 19	206 ± 20	171 ± 17
Mo, mg kg^{-1}	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Ag, mg kg^{-1}	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Sb, mg kg^{-1}	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Cs, mg kg^{-1}	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Ti, mg kg^{-1}	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Be, mg kg^{-1}	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Cd, mg kg^{-1}	0.085 ± 0.008	0.102 ± 0.011	0.043 ± 0.004	0.041 ± 0.004	0.061 ± 0.006	0.051 ± 0.005
Sn, mg kg^{-1}	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Ba, mg kg^{-1}	23.4 ± 2.3	27.6 ± 2.7	19.5 ± 1.9	19.8 ± 1.9	14.3 ± 1.4	13.2 ± 1.3
Hg, mg kg^{-1}	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Pb, mg kg^{-1}	1.38 ± 0.13	1.44 ± 0.17	0.760 ± 0.076	0.706 ± 0.070	0.385 ± 0.038	0.356 ± 0.035
B, g kg^{-1}	3.33 ± 0.33	3.67 ± 0.37	13.2 ± 1.3	14.2 ± 1.4	2.99 ± 0.29	3.06 ± 0.30
Al, g kg^{-1}	0.913 ± 0.081	1.03 ± 0.10	0.814 ± 0.081	0.736 ± 0.073	0.151 ± 0.015	0.162 ± 0.019
Li, mg kg^{-1}	0.812 ± 0.081	0.876 ± 0.088	0.758 ± 0.079	0.674 ± 0.067	0.400 ± 0.040	0.409 ± 0.041

Cla non-ferm – non-fermented *Cladophora rupestris*; Cla LUHS135 – *C. rupestris* fermented with *L. plantarum* LUHS135; Ul non-ferm – non-fermented *Ulva intestinalis*; Ul LUHS135 – *U. intestinalis* fermented with *L. plantarum* LUHS135; Furc non-ferm – non-fermented *Furcellaria lumbricalis*; Furc LUHS135 – *F. lumbricalis* fermented with *L. plantarum* LUHS135. Data are represented as means (n = 3, replicates of analysis) ± SD; d.m.: dry mass.

day^{-1} for men and 1.3 mg day^{-1} for women (EFSA, 2014a). The European population reference intake for zinc for adult males and females is 9.5 and 7.0 mg day^{-1}, respectively and estimated average requirements are 7.3 and 5.5 mg day^{-1} for males and females, respectively (European Food Safety Authority, 2006). In the US, the guidelines recommend daily intake of 11 and 8 mg day^{-1} for men and women, respectively (Institute of Medicine (US) Panel on Micronutrients, 2001). To ensure that very few individuals in a population have a Zn intake of 60 mg or higher, the WHO recommended that the adult population's mean intake should not exceed 45 mg if a 20% variation in intake is assumed (WHO, 1996). For P, habitual dietary intake in European countries is estimated to be on average around 1000–1500 mg day^{-1}, ranging up to about 2600 mg day^{-1} (European Food Safety Authority, 2006).

In all algal samples analysed, the concentrations of molybdenum (Mo), silver (Ag), stibium (Sb), caesium (Cs), titanium (Ti), and beryllium (Be) were <0.25 mg kg^{-1}; the concentration of mercury (Hg) was <0.01 mg kg^{-1}, and that of tin (Sn) was <0.01 mg kg^{-1}. However, the highest concentrations of cadmium (Cd), barium (Ba), and lead (Pb) were found in *C. rupestris* samples, on average 2.2, 1.3, and 1.9 times higher than those in *U. intestinalis* samples, and 1.7, 1.9, and 3.8 times higher than in *F. lumbricalis*. *F. lumbricalis* samples had the lowest vanadium (V), aluminium (Al), and lithium (Li) concentrations, which were, on average 1.7 times lower than those in *C. rupestris* and *U. intestinalis* samples, respectively. According to an EFSA report, V has not been shown to be essential for humans, and the intake of V from normal food is estimated to be in the order of 10–20 μg day^{-1} (European Food Safety Authority, 2006).

The gallium (Ga) concentration in *F. lumbricalis* samples was <0.25 mg kg^{-1} and in *C. rupestris* and *U. intestinalis* samples, it was, on average, 0.299 mg kg^{-1}. In contrast to these findings, a 1.4 times higher arsenic (As) concentration was found in *C. rupestris* than in *F. lumbricalis* and *U. intestinalis* samples. *C. rupestris* had the highest concentration of rubidium (Rb) (on average, 9.87 mg kg^{-1}); in *U. intestinalis* and *F. lumbricalis* samples it was, on average, 55.6% and 26.1% lower, respectively. *U. intestinalis* samples had, on average, a 4.2 times higher boron (B) concentration than that in *C. rupestris* and *F. lumbricalis* samples. Despite macroalgae being a good source of desirable macro- and microelements, one of the main concerns associated with the safety seaweed of is its exposure to heavy metals such as, Al, Cd, Pb, Rb, Si, Sr, and Sn (Desideri et al., 2016). The contamination of algae with heavy metals depends on their growing areas and shows little risk to human health (Phaneuf, Côté, Dumas, Ferron, & LeBlanc, 1999). However, regular consumption of algae may lead to a risk of heavy metal toxicity in humans due to accumulation of these pollutants in the body (Burger, Gochfeld, Jeitner, Donio, & Pittfield, 2012; Caliceti, Argese, Sfriso, & Pavoni, 2002). Monitoring of the heavy metal concentrations in macroalgae-based products is recommended, because of the different bio-absorption capacity of metals (Hwang, Park, Park, Choi, & Kim, 2010). One of the major considerations for the use of algae as food/feed is the need to quantify the levels of As. Inorganic As is categorized as a class I carcinogen, arsenobetaine as nontoxic, and fat-soluble arsenic, arsenosugars, and other organoarsenicals as potentially toxic (Feldmann & Krupp, 2011). In algae, most of the As is present in arsenosugar form, typically ligated to glycerol, sulphonate, or phosphonate, which *in vivo*

are metabolized to at least 12 different metabolites whose toxicity is unknown (Andrewes et al., 2004; Filipkowska et al., 2008; Francesconi, Tanggaar, McKenzie, & Goessler, 2002; Hulle et al., 2004). There are set maximum levels for As in some foodstuffs, which vary from 0.10 to 0.3 mg kg^{-1}; however, the maximum level for As in macroalgae is not regulated (Commission Regulations, 2006). In 2004, 2005, and 2006, the highest metal concentrations determined at Sopot beach (Baltic Sea) were: Cd – 1.41 μg g^{-1}, Pb – 10.50 μg g^{-1}, Ni – 5.13 μg g^{-1}, Zn – 223 μg g^{-1}, Cu – 19.50 μg g^{-1}, and Cr – 4.38 μg g^{-1} (Filipkowska et al., 2008). Also, the content of Cd, Cu, Ni, Pb, Zn, Mn, K, Na, Ca, and Mg in the green alga *Cladophora* sp. from coastal and lagoonal waters of the southern Baltic was reported by Żbikowski, Szefer, and Latała (2007). They found that algae from the southern Baltic contained more Na and K while the anthropogenic impact of Cu, Pb, and Zn was observed in the case of *Cladophora* sp. and *Enteromorpha* sp. from the Gulf of Gdansk in the vicinity of Gdynia. They also suggested that *Cladophora* sp. and *Enteromorpha* sp. could be used as biomonitors of Pb, Cu, and Zn content in the Baltic Sea because of their ability to accumulate metal contaminants from seawater (Zbikowski et al., 2007). The European food legislation sets maximum permitted levels (MPL) for contaminants in foodstuffs (Commission Regulations, 2006). For Cd and Pb, there are MPL for vegetables, but macroalgae are excluded. For Cd, Pb, and Hg, there are MPL (3.0, 3.0, and 0.1 mg kg^{-1} wet weight, respectively) for food supplements (including algae). For Cd, it is specified that the MPL applies to 'food supplements consisting exclusively or mainly of dried seaweed, products derived from seaweed, or of dried bivalve molluscs'. The European feed legislation sets MPL for a range of undesirable substances in feed ingredients (12% moisture content) (Commission Directive 2002/32/EC, 2002, p. 32). The MPL are set for the level of total As, not inorganic arsenic (below 2 mg kg^{-1}). For Cd and Pb, the current MPL in fish feed are 1 and 5 mg kg^{-1}, respectively, and for feed materials of vegetable origin are 1 and 10 mg kg^{-1}, respectively. The current MPL for Hg in fish feed and marine feed ingredients are 0.1 and 0.2 mg kg^{-1}, respectively. Finally, the health and/or functionality claims associated with macroalgal products must be based on sufficient evidence about safety parameters, for which micro- and macroelements are very important.

4. Conclusions

Metagenomic sequencing analysis shows that macroalgae naturally living in Baltic Sea can be treated as complex living organisms harbouring multiple species of various bacteria which might change the technological fermentation process by multiplying unwanted bacteria in the fermentable substrate. Therefore, inactivation of microorganisms before fermentation is necessary. Fermentation with L. *plantarum* strain LUHS135 reduced the pH of *C. rupestris* to <4.5; however, to increase the effectiveness of *U. intestinalis* and *F. lumbricalis* fermentation, the addition of fermentable sugars is recommended, as their pH remained unchanged after fermentation. The highest antioxidant activity was shown by *C. rupestris* (on average 58.0%); however, fermentation reduced the TPC content in *C. rupestris* and *F. lumbricalis* samples. The synergic mechanism of the algae and LAB combination showed a broader spectrum of pathogen inhibition, and the Baltic Sea macroalgae and their combination with strain LUHS135 could be a good source of some trace elements; however, further research is needed to increase their biochemical and chemical safety, especially taking into the consideration the concentrations of Pb and As. Finally, the use of fermentable sugars (to reduce the pH of fermented samples and to prolong shelf life) and application of ultrasound treatment before fermentation could be further tested as a pre-pre-treatment method to control the fermentation process. Also, the preparation of extracts of the antimicrobial compounds could be a possible way to reduce heavy metal concentration and to use this prospective raw material at industrial scale.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare no conflict of interest whatsoever to declare.

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Effect of solid-state fermentation and ultrasonication processes on antimicrobial and antioxidant properties of algae extracts

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Algal biomass (AB) is prospective source of valuable compounds, however, Baltic Sea macroalgae have some challenges, because of their high microbial and chemical contamination. These problems can be solved, by using appropriate technologies for AG pre-treatment. The aim of this study was to evaluate the influence of two pre-treatments, solid-state fermentation with the *Lactiplantibacillus plantarum* LUHS135 and ultrasonication, on the antioxidant and antimicrobial characteristics of macro- (*Cladophora rupestris, Cladophora glomerata, Furcellaria lumbricalis, Ulva intestinalis*) and Spirulina (*Arthrospira platensis*) extracts. Also, combinations of extracts and LUHS135 were developed and their characteristics were evaluated. The total phenolic compound content was determined from the calibration curve and expressed in mg of gallic acid equivalents; antioxidant activity was measured by a Trolox equivalent antioxidant capacity assay using the DPPH$^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl), ABTS$^{\bullet+}$ 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), FRAP (Ferric Reducing Ability of Plasma) discoloration methods. Antimicrobial activity was measured by using agar well diffusion assay and in a liquid medium. The highest DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ was shown by *C.rupestris* and *F.lumbricalis* extract × LUHS135 combinations, the highest

FRAP - by non-pretreated *C.rupestris* and *F.lumbricalis* extract × LUHS135 combinations. Ultrasonicated samples inhibited four out of seven tested pathogens. Finally, the tested pre-treatments showed good perspectives and can be recommended for AB valorization.

KEYWORDS

solid-state fermentation, ultrasonication, algae, extracts, antimicrobial properties, antioxidant properties, lactic acid bacteria

Introduction

Algal biomass can be converted into a wide range of functional products (1). Despite, that they are a valuable source of functional compounds, our previous studies showed that the application of Baltic Sea macroalgae have some challenges because of their high microbial and chemical contamination (2). However, algae safety parameters could be improved by applying ethanolic extraction, which is a suitable technology for pathogen decontamination and reduces the toxic metal concentration in algae extracts (3). In addition to improvements in algae products' safety parameters, it would be very beneficial to increase extraction efficiency. Therefore, in this study, two methods for algae pre-treatment were tested before extraction: (I) solid-state fermentation (SSF) with a selected lactic acid bacteria (LAB) strain and (II) ultrasonication. We hypothesized that algae biomass pre-treatment before extraction can lead to better properties of the extracts (higher antioxidant activity and total phenolic compound (TPC) content, as well as stronger antimicrobial properties against a broader spectrum of pathogenic and opportunistic strains). In addition, to increase the antimicrobial and antioxidant activity of the prepared extracts, combinations of algae extracts and a pure *Lactiplantibacillus plantarum* LUHS135 strain were developed. Our previous studies showed that the above-mentioned strain inhibits various pathogenic and opportunistic microorganisms and is suitable for fermentation of various substrates (4–7). The importance of algae biomass pre-treatment before extraction can be explained by algae cell composition, which is protected by complex cell walls (8, 9). It has been reported that the crucial step in obtaining bioactive compounds from micro- and macroalgal biomass is to achieve efficient cell disruption (10). Some algae pre-treatment technologies are described in the literature, and the most effective mechanical and biological techniques were mentioned (11, 12). Despite the fact that physical pre-treatment was found to be a cost-intensive process, ultrasonication was recommended as the most promising method for cell disintegration (9, 13, 14). Ultrasound breaks the cell structure and improves material transfer by enhancing the extraction from microalgae (9, 15–17). Also, biological pre-treatment with fungi, bacteria and/or their enzymes can be used to degrade lignin and hemicelluloses of algae cells (12, 18). There are numerous studies on algae pre-treatment using biological tools (19–21). In addition to the breakdown of lignin, biological pre-treatment generates other valuable compounds such as phenolic acids, benzoic acid, syringaldehyde, etc. (22). Other major advantages of biological pre-treatment are low energy consumption, simple operating conditions and equipment, no requirement for recycling the chemicals after pre-treatment, etc. (23–25). Solid state fermentation (SSF) process is based on the microorganisms grown on solid or semi-solid substrates or supports, and is more effective than the liquid phase submerged fermentation (26). We hypothesized that algae biomass SSF can lead to the deeper algae cells breakdown, which will lead to better properties of the extracts.

The aim of this study was to evaluate the influence of two pre-treatments, solid-state fermentation (SSF) with the *Lactiplantibacillus plantarum* LUHS135 strain and ultrasonication (for 45 min at 35 kHz), on the antioxidant and antimicrobial characteristics of macroalgae (*Cladophora rupestris, Cladophora glomerata, Furcellaria lumbricalis* and *Ulva intestinalis*) and microalgae [Spirulina (*Arthrospira platensis*)] extracts. In addition, combinations of algae extracts and the pure LUHS135 strain were developed and their antioxidant and antimicrobial characteristics were evaluated.

Materials and methods

Algae samples and lactic acid bacteria strain used in experiments

Samples of macroalgae (*Furcellaria lumbricalis, Ulva intestinalis, Cladophora rupestris* and *Cladophora glomerata*) were collected in May–June of 2021 on the Lithuanian coast. *Ulva intestinalis* and *C. glomerata* samples were taken from stones near the surface, while *F. lumbricalis* and *C. rupestris* samples were taken after a storm along the shore. The collected samples were cleaned three times in distilled water to remove sand and macroscopic invertebrates. Microalgae Spirulina (*Arthrospira platensis*) was purchased from the University of Texas Biological Labs (Austin, Texas, United States),

multiplied according to instructions given by producer and used in experiments.

Before the experiments, all algal samples were lyophilized using a freeze-dryer FD8512S (ilShin® Europe, Ede, The Netherlands) and ground into a powder (particle size < 0.2 mm) using a knife mill GM200 (Retsch, Düsseldorf, Germany). Freeze-dried samples were maintained at room temperature in a dark place until they were used.

The *Lactiplantibacillus plantarum* LUHS135 strain (LUHS135) was obtained from the Lithuanian University of Health Sciences collection (Kaunas, Lithuania). The characteristics of the LAB strain used, including the inhibition of strains of pathogenic and opportunistic bacteria, and fungi are described by Bartkiene et al. (4). In addition, our previous studies showed that fermentation of feed with LUHS135 had a positive influence *in vivo* on piglets' health parameters (27–29). The above-mentioned LAB strains were stored at−80°C in a Microbank system (Pro-Lab Diagnostics, United Kingdom) and propagated in de Man–Rogosa–Sharpe (MRS) broth (CM 0359, Oxoid Ltd, Hampshire, United Kingdom) at 30 ± 3°C for 48 h before their use for algae fermentation.

Fermentation and ultrasonication of algal samples

The LUHS135 strain was multiplied as described in Algae samples and lactic acid bacteria strain used in experiments and used for algae powder (AP) fermentation. A total of 3 mL of the LAB strain multiplied in MRS (cell concentration, on average, 9.0 $\log_{10}$ CFU mL^{-1}) was inoculated to 100 g of AP media (for 100 g of AP, 60 mL of water was used) and fermented at 30 ± 2°C for 60 h. Control samples for pH analysis were prepared without the addition of LAB. Our previous studies showed that pure algae samples are not suitable substrates for effective LAB growth (2), thus 2% (from the algae sample amount) of yeast extract was added (ThermoFisher, Kandel, Germany), with the purpose of improving the growth of the LUHS135. Anaerobic conditions were attained by incubating the fermentable substrate in anaerobic jars (Oxoid, Basingstoke, Hampshire, United Kingdom), with GasPak Plus™ (BBL, Cockeysville MD, United States). Samples for pH analysis were taken after 12, 24, 36, 48 and 60 h of fermentation.

Algae samples were ultrasonicated before extract preparation for 45 min at 35 kHz (temperature of samples during the ultrasonication was 40 ± 2°C) using ultrasonic bath (Bandelin Sonorex, Bandelin electronic GmbH & Co. KG, Berlin, Germany).

Both fermented and ultrasonicated algae samples were lyophilised and used for extract preparation.

Extracts and extract × l*actiplantibacillus plantarum* LUHS135 strain combinations preparation

Five grams of the lyophilized algal samples (non-pretreated, fermented and ultrasonicated, for a total of 15 samples) were extracted with 100 mL of ethanol/water (70:30 v/v) (30) by incubation at room temperature (22 ± 2°C) overnight with stirring (Vibramax 100, Heidolph, Nuremberg, Germany). Then, extracts were centrifuged at 3,500 rpm for 10 min at 4°C and filtered through Whatman No. 4 filter paper. Ethanol was removed by rotary evaporation in the extract. The concentrate and the supernatant of the extract were lyophilized and weighted.

For the preparation of extract × LUHS135 strain combinations, it was propagated in MRS broth (CM 0359, Oxoid Ltd, Hampshire, United Kingdom) at 30 ± 3°C for 48 h, and a pure LUHS135 strain was used (LUHS135 strain/algae extract; 50/50, by volume). The principal scheme of the experiment is given in Figure 1. Three groups of samples were prepared: (I) extracts and extracts × LUHS135 combinations prepared from non-pre-treated algae, (II) extracts and extracts × LUHS135 combinations prepared from ultrasonicated algae and (III) extracts and extracts × LUHS135 combinations prepared from fermented algae. In every group pure extract as well as extract combinations with the LUHS strain were tested (ClaR = *Cladophora rupestris*; ClaG = *Cladophora glomerata*; Ul = *Ulva intestinalis*; Furc = *Furcellaria lumbricalis*; Sp = Spirulina (*Arthrospira platensis*); non = extracts prepared from non-pre-treated algae; ultr = extracts prepared from ultrasonicated algae; ferm = extracts prepared from fermented algae; LUHS135 = extract × LUHS135 strain combination). There were 30 samples total: Group (I): $ClaR_{non}$, $ClaR_{nonLUHS135}$, $ClaG_{non}$, $ClaG_{nonLUHS135}$, $Furc_{non}$, $Furc_{nonLUHS135}$, Ul_{non}, $Ul_{nonLUHS135}$, Sp_{non} and $Sp_{nonLUHS135}$; Group (II): $ClaR_{ultr}$, $ClaR_{ultrLUHS135}$, $ClaG_{ultr}$, $ClaG_{ultrLUHS135}$, $Furc_{ultr}$, $Furc_{ultrLUHS135}$, Ul_{ultr}, $Ul_{ultrLUHS135}$, Sp_{ultr} and $Sp_{ultrLUHS135}$ and Group (III): $ClaR_{ferm}$, $ClaR_{fermLUHS135}$, $ClaG_{ferm}$, $ClaG_{fermLUHS135}$, $Furc_{ferm}$, $Furc_{fermLUHS135}$, Ul_{ferm}, $Ul_{fermLUHS135}$, Sp_{ferm} and $Sp_{fermLUHS135}$.

Analysis of algae color characteristics and pH

The color coordinates of the algae extracts and their combinations with the LUHS135 strain were evaluated using a CIE L*a*b* system (CromaMeter CR-400, Konica Minolta, Marunouchi, Tokyo, Japan) (3). The pH of samples was evaluated with an "inoLab pH Level 3" pH meter (Hanna Instruments, Weilheim, Germany).

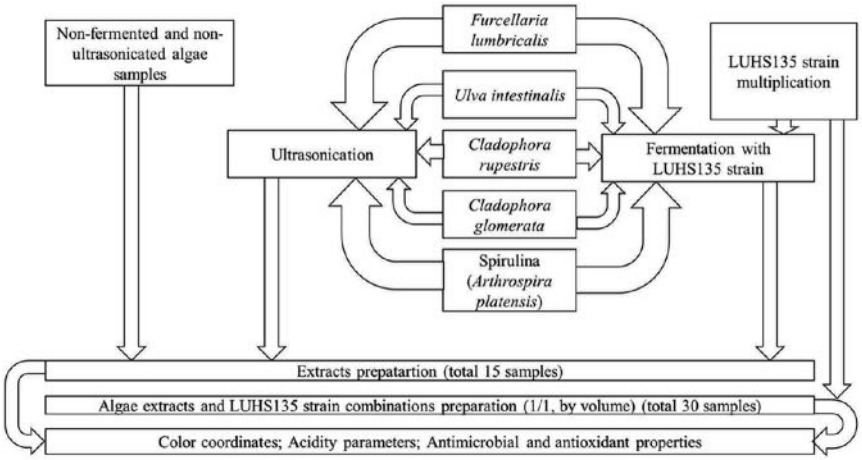

FIGURE 1
The principal scheme of the experiment.

Determination of the total phenolic compound content

The total phenolic compound (TPC) content in the extracts was determined according to the Folin–Ciocalteu method (31) with slight modifications (32). Samples (1.0 mL) were introduced into test cuvettes followed by 5.0 mL 10% (1/10, v/v) of Folin–Ciocalteu's reagent by diluting a stock solution with ultra-pure distilled water and 4.0 mL of Na_2CO_3 (7.5%). The system was then placed at ambient temperature for 1 h. The absorbance was measured at 765 nm using a Genesys-10 UV/VIS spectrophotometer (Thermo Spectronic, Rochester, NY, United States). The concentration of TPC was determined from the calibration curve and expressed in mg of gallic acid equivalents (GAE) in ml of extracts.

Determination of the antioxidant capacity of algae extracts

The antioxidant activity of algae extracts was measured by DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and FRAP discoloration methods. Calculation of all antioxidant activity assays was carried out using Trolox calibration curves and expressed as μmol of the Trolox equivalent (TE) per one gram of ml of extract (μmol TE/ml).

DPPH$^{\bullet}$ activity

The DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl hydrate free radical) scavenging capacity of the algal extracts was determined by the method of Brand-Williams et al. (33) with slight modifications (34). Twenty microliters of extract were allowed to react with 2 mL of DPPH$^{\bullet}$ ethanolic solution (2 mL, 6×10^{-5} M) by mixing in a cuvette with a 1 cm path length for 30 min in the dark. The decrease in absorbance was measured at 515 nm using a Genesys-10 UV/VIS spectrophotometer (Thermo Spectronic, Rochester, NY, United States).

ABTS$^{\bullet+}$ activity

The radical scavenging activity of extracts was also measured by ABTS$^{\bullet+}$ (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical cation assay (35) as described by Urbonaviciene et al. (32). ABTS$^{\bullet+}$ solution (2 mM) was prepared by dissolving 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt in 50 mL of phosphate-buffered saline (PBS) obtained by dissolving 8.18 g NaCl, 0.27 g KH_2PO_4, 1.42 g Na_2HPO_4 and 0.15 g KCl in 1 L of pure water. The pH of the prepared solution was adjusted to 7.4 using NaOH. Then the $K_2S_2O_8$ solution (70 mM) was prepared in pure water. Briefly, 2 mL of ABTS$^{\bullet+}$ radical solution was mixed with 20 μL extract also in a 1 cm path length cuvette. The reaction mixture was kept at ambient temperature in the dark for 30 min, and the absorbance was read at 734 nm using a Genesys-10 UV/Vis spectrophotometer (Thermo Spectronic, Rochester, NY,

United States). Trolox was used as a standard. A duplicate determination was made from each extract.

FRAP activity

The ferric reducing antioxidant power (FRAP) assay was carried out by the method of Benzie and Strain (36) with some modifications (37). For the FRAP assay, 0.3 M of sodium acetate buffer (pH 3.6) was prepared by dissolving 3.1 g of sodium acetate and 16 mL of acetic acid in 1,000 mL of distilled water; a 10 mM TPTZ solution was prepared by dissolving 0.031 g of TPTZ in 10 mL of 40 mM HCl; and a 20 mM ferric solution was prepared by dissolving 0.054 g of $FeCl_3 \cdot 6H_2O$ in 10 mL of distilled water. Working FRAP reagent was prepared by freshly mixing acetate buffer, TPTZ and ferric solutions at a ratio of 10:1:1. Two milliliters of freshly prepared FRAP working solution and 20 μL of extract were mixed and incubated for 30 min at ambient temperature. The change in absorbance due to the reduction of the ferric-tripyridyltriazine (Fe III-TPTZ) complex by the antioxidants present in the samples was measured at 593 nm using a Genesys-10 UV/VIS spectrophotometer.

Evaluation of the antimicrobial activity of algal extract samples

The algal extracts as well as algal extract × LUHS135 strain combination antimicrobial properties were evaluated by testing their abilities to inhibit the following pathogenic and opportunistic strains: *Salmonella enterica*, *Bacillus cereus*, *Enterococcus faecium*, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus mutans* and *Enterococcus faecalis*. Antimicrobial properties of the samples were evaluated by using the agar well diffusion method and in a liquid medium.

For the agar well diffusion assay, suspensions of 0.5 McFarland standard of each pathogenic bacterial strain were inoculated onto the surface of cooled Mueller–Hinton agar (Oxoid, Basingstoke, UK) using sterile cotton swabs. Wells 6 mm in diameter were punched in the agar and filled with 50 μL of the algal extract. The antimicrobial activities against the tested bacteria were established by measuring the inhibition zone diameters (mm). The experiments were repeated three times, and the average diameter of the inhibition zones in mm was calculated.

To evaluate the antimicrobial activity of the algal extracts and algal extracts × LUHS135 combinations in liquid medium, the algal samples were diluted 1:3 (v/v) with physiological solution. Then we added 10 μL of the pathogenic and opportunistic bacterial strains, cultured in a selective medium, to the different concentrations of samples (500 and 2,000 μL) and incubated them at 35°C for 24 h. After incubation, the viable pathogenic and opportunistic bacterial strains in algal extract and/or in algal extracts × LUHS135 combination were controlled by plating them on selective medium. The results were interpreted as (–) if the pathogens did not grow on the selective medium and (+) if the pathogens grew on the selective medium. Experiments were performed in triplicate.

Statistical analysis

Extract preparation of algal samples was performed in duplicate, while all analytical experiments were carried out in triplicate. The calculated mean values, using the statistical package SPSS for Windows (Ver.15.0, SPSS, Chicago, IL, United States), were compared using Duncan's multiple range test with significance defined at $p \leq 0.05$. A linear Pearson's correlation was used to quantify the strength of the relationship between the variables. The results were recognized as statistically significant at $p \leq 0.05$.

Results and Discussion

Selection of algae fermentation duration before extract preparation according to changes in their pH

The changes in pH values during algae fermentation are shown in Figure 2. In comparison to the non-fermented samples, a pH higher than 7.0 was established for *Cladophora rupestris*, *Ulva intestinalis* and Spirulina samples (7.35, 7.98 and 7.72, respectively). Non-fermented *Cladophora glomerata* and *Furcellaria lumbricalis* samples had average pH values of 5.95 and 6.74, respectively. The most intensive fermentation and reductions of pH values was found from 0–12 h and from 12–24 h of fermentation. From 0–12 h and from 12–24 h of fermentation the pH values of *Cladophora rupestris, Cladophora glomerata, Ulva intestinalis, Furcellaria lumbricalis* and Spirulina samples reduced by an average of 1.36 and 1.12, 1.18 and 1.04, 1.17 and 1.13, 1.26 and 1.27 and 1.28 and 1.19 times, respectively.

Although fermentation during the period from 24–36 h was not as intensive as fermentation in previous studies, after 36 h of fermentation significantly lower pH values for all of the tested algae samples were found when compared with samples fermented for 24 h. However, after 48 h of fermentation significant differences between the algae pH values were not found, and after 72 h of fermentation some of the algae sample pH values started to increase. Univariate analyses of variance showed that the variety of algae is a significant factor in sample pH ($p = 0.017$). However, the duration of fermentation and interaction with analyzed factors did not significantly affect the pH of the samples. According to these

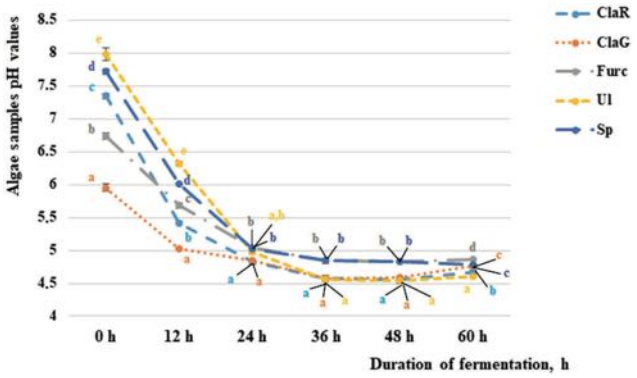

FIGURE 2
The pH of the non-fermented and fermented algae samples after 12, 24, 36, and 60 h of fermentation [ClaR - *Cladophora rupestris*; ClaG - *Cladophora glomerata*; Ul - *Ulva intestinalis*; Furc - *Furcellari lumbricalis*; Sp - Spirulina (*Arthrospira platensis*)]. Data are represented as means ($n = 3$) ± SE. Means with different letters (a–e) are significantly different ($p \leq 0.05$). The color of the letters coincides with the color of the sample in the graph.

results, a fermentation duration of 36 h for extract preparation was selected.

Literature on algae fermentation is scarce; however, our previous studies showed that fermentation of the LUHS135 strain (duration of fermentation 12 h) significantly reduced the pH of *C. rupestris*. However, the pH of other tested algae samples (*U. intestinalis* and *F. lumbricalis*) remained unchanged (2). One of the main goals of the fermentation process is to drop the pH, and on average, the recommended pH for fermented food is 4.6. A decrease in pH is an indicator of an effective process; however, changes to the fermentable substrate can be caused by many factors, i.e., the technological microorganism's (used for fermentation) characteristics, nutrient source in fermentable media, duration of fermentation, humidity of the substrate, etc. It has been reported that the moisture content of the substrate has a significant influence on pH and, in most cases, lower pH values and higher total titratable acidity were obtained for peas in solid state fermentation conditions (38). The practice of LAB-based food, as well as feed fermentations, happened accidentally in the beginning, but soon spread due to its many benefits including nutrition, safety and flavor (38, 39). Overall, during the fermentation process many compounds are obtained as secondary metabolites of technological microorganisms (40, 41). Also, bound phenolic compounds are bio-converted from their conjugated forms to their free forms, and this is explained by their breakdown, activities of the fermentable substrate enzymes, as well as activity of technological microorganisms (42). Finally, this study showed that yeast extract is a suitable supplement for increasing algae samples fermentation effectiveness.

Color coordinates and pH of algae extracts and algae extracts × LUHS135 combinations

Color coordinates (L^* = lightness; a^* = redness; $-a^*$ = greenness; b^* = yellowness; $-b^*$ = blueness) and pH of the algae extracts and algae extracts × LUHS135 combinations are shown in Table 1. When comparing all three groups of extracts (non-pre-treated, ultrasonicated and fermented before extraction), the lowest L^* coordinates were from $ClaG_{non}$, $ClaR_{ultr}$ and $ClaR_{fermLUHS135}$ samples (42.5, 41.3 and 49.5 NBS, respectively). The most intensive greenness ($-a^*$) was from Ul_{non}, Ul_{ultr} and Ul_{ferm} samples (-14.7,−13.7 and−6.86 NBS, respectively). The lowest yellowness (b^*) was from $ClaG_{non}$, Sp_{ultr} and $ClaG_{ferm}$ samples (24.8, 23.7 and 23.1 NBS, respectively).

When comparing all of the samples, all of the analyzed factors as well as their interactions had significant effects on all color coordinates; however, algae species, pre-treatment used before extract preparation, extract × LUHS135 combination interaction, algae species × pre-treatment interaction and the algae species × LUHS135 combination interaction did not have significant effects on pH of samples (Table 1). In contrast, the pre-treatment × LUHS135 combination interaction, as well as the algae species × pre-treatment × LUHS135 combination interaction, showed a significant influence on sample acidity ($p = 0.031$ and $p = 0.004$, respectively). Also, a weak, negative correlation between the sample pH and a^* coordinate was found ($r = -0.289$, $p = 0.006$) (Table 2). In all cases, the addition

TABLE 1 Color coordinates (L*, lightness; a*, redness; −a*, greenness; b*, yellowness; −b*, blueness) and pH of the algae extracts and algae extracts × LUHS135 combinations.

Extracts and extract × LUHS135 combination	Color coordinates, NBS			pH	Multivariate analysis of variance		
	L*	a*	b*		Factor	Depen-dent variable	*p*
Extracts and extracts × LUHS135 combinations prepared from non-pre-treated algae					Algae species	L*	0.0001
$ClaR_{non}$	64.6 ± 0.32^{g}	-13.8 ± 0.11^{b}	47.5 ± 0.36^{g}	6.77 ± 0.031^{d}		a*	0.0001
$ClaR_{nonLUHS135}$	61.1 ± 0.26^{e}	-1.40 ± 0.15^{g}	44.6 ± 0.33^{e}	3.95 ± 0.032^{a}		b*	0.0001
$ClaG_{non}$	42.5 ± 0.10^{a}	-1.75 ± 0.192^{f}	24.8 ± 0.18^{a}	5.92 ± 0.124^{b}		pH	0.712
$ClaG_{nonLUHS135}$	50.6 ± 0.12^{b}	2.61 ± 0.105^{h}	34.8 ± 0.39^{c}	3.96 ± 0.115^{a}	Pretreatment used before	L*	0.0001
$Furc_{non}$	79.2 ± 0.34^{h}	-3.57 ± 0.022^{c}	32.2 ± 0.16^{b}	6.19 ± 0.036^{c}	extracts preparation	a*	0.0001
$Furc_{nonLUHS135}$	60.5 ± 0.25^{d}	10.4 ± 0.24^{k}	47.8 ± 0.25^{g}	$3.92 \pm 0 .025^{a}$		b*	0.0001
Ul_{non}	52.4 ± 0.32^{c}	-14.7 ± 0.16^{a}	41.3 ± 0.37^{d}	6.99 ± 0.092^{e}		pH	0.052
$Ul_{nonLUHS135}$	62.9 ± 0.13^{f}	-2.27 ± 0.031^{e}	45.8 ± 0.33^{f}	3.95 ± 0.071^{a}	Extract × LUHS135	L*	0.0001
Sp_{non}	59.9 ± 0.32^{d}	-3.40 ± 0.114^{d}	49.1 ± 0.31^{h}	8.69 ± 0.102^{f}	combination interaction	a*	0.0001
$Sp_{nonLUHS135}$	64.6 ± 0.10^{g}	4.04 ± 0.015^{j}	44.9 ± 0.12^{e}	3.94 ± 0.044^{a}		b*	0.0001
Extracts and extracts × LUHS135 combinations prepared from ultrasonicated algae						pH	0.0001
$ClaR_{ultr}$	41.3 ± 0.31^{a}	-1.55 ± 0.064^{d}	24.4 ± 0.21^{b}	5.82 ± 0.032^{b}	Algae species ×	L*	0.0001
$ClaR_{ultrLUHS135}$	45.0 ± 0.24^{b}	3.42 ± 0.121^{j}	29.2 ± 0.10^{c}	3.94 ± 0.091^{a}	pre-treatment interaction	a*	0.0001
$ClaG_{ultr}$	50.8 ± 0.37^{c}	-7.16 ± 0.092^{b}	33.5 ± 0.34^{d}	6.37 ± 0.034^{d}		b*	0.0001
$ClaG_{ultrLUHS135}$	59.8 ± 0.36^{f}	-0.65 ± 0.021^{f}	40.0 ± 0.32^{e}	3.93 ± 0.022^{a}		pH	0.058
$Furc_{ultr}$	71.8 ± 0.44^{h}	2.23 ± 0.105^{g}	52.6 ± 0.35^{j}	6.09 ± 0.093^{c}	Algae species × LUHS135	L*	0.0001
$Furc_{ultrLUHS135}$	65.1 ± 0.26^{g}	4.43 ± 0.113^{k}	45.7 ± 0.22^{g}	3.89 ± 0.031^{a}	combination interaction	a*	0.0001
Ul_{ultr}	55.4 ± 0.37^{d}	-13.7 ± 0.24^{a}	45.7 ± 0.34^{g}	7.01 ± 0.074^{e}		b*	0.0001
$Ul_{ultrLUHS135}$	57.1 ± 0.10^{e}	-1.26 ± 0.031^{e}	47.1 ± 0.12^{h}	3.92 ± 0.032^{a}		pH	0.362
Sp_{ultr}	79.9 ± 0.41^{j}	-5.40 ± 0.154^{c}	23.7 ± 0.24^{a}	7.67 ± 0.107^{f}	Pre-treatment × LUHS135	L*	0.0001
$Sp_{ultrLUHS135}$	65.3 ± 0.31^{g}	5.17 ± 0.072^{l}	44.6 ± 0.27^{f}	3.92 ± 0.094^{a}	combination interaction	a*	0.0001
Extracts and extracts × LUHS135 combinations prepared from fermented algae						b*	0.0001
$ClaR_{ferm}$	54.7 ± 0.25^{b}	-4.55 ± 0.094^{b}	33.5 ± 0.34^{c}	5.09 ± 0.064^{b}		pH	0.031
$ClaR_{fermLUHS135}$	49.5 ± 0.37^{a}	3.33 ± 0.046^{f}	34.5 ± 0.22^{d}	4.02 ± 0.084^{a}	Algae species ×	L*	0.0001
$ClaG_{ferm}$	63.2 ± 0.22^{e}	1.95 ± 0.164^{d}	23.1 ± 0.40^{a}	5.06 ± 0.040^{b}	pre-treatment × LUHS135	a*	0.0001
$ClaG_{fermLUHS135}$	62.4 ± 0.24^{d}	7.75 ± 0.140^{h}	45.8 ± 0.41^{g}	4.07 ± 0.011^{a}	combination interaction	b*	0.0001
$Furc_{ferm}$	65.6 ± 0.27^{g}	4.67 ± 0.021^{g}	43.8 ± 0.44^{f}	5.59 ± 0.064^{c}		pH	0.004
$Furc_{fermLUHS135}$	64.0 ± 0.38^{f}	8.31 ± 0.163^{j}	48.0 ± 0.31^{h}	4.06 ± 0.052^{a}			
Ul_{ferm}	76.8 ± 0.25^{j}	-6.86 ± 0.111^{a}	31.6 ± 0.22^{b}	4.95 ± 0.081^{b}			
$Ul_{fermLUHS135}$	56.8 ± 0.42^{c}	6.50 ± 0.202^{h}	41.7 ± 0.14^{e}	3.97 ± 0.094^{a}			
Sp_{ferm}	83.1 ± 0.14^{k}	-1.67 ± 0.174^{c}	31.5 ± 0.15^{b}	5.20 ± 0.107^{b}			
$Sp_{fermLUHS135}$	71.7 ± 0.21^{h}	3.08 ± 0.037^{e}	43.2 ± 0.38^{f}	3.98 ± 0.075^{a}			

ClaR, Cladophora rupestris; ClaG, Cladophora glomerata; Ul, Ulva intestinalis; Furc, Furcellaria lumbricalis; Sp, Spirulina (Arthrospira platensis); non, extracts prepared from non-pre-treated algae; ultr, extracts prepared from ultrasonicated algae; ferm, extracts prepared from fermented algae; LUHS135, extract × LUHS135 strain combination; L*, ightness; a*, redness; −a*, greenness; b*, yellowness; −b*, blueness; NBS, National Bureau of Standards units; data are represented as means ($n = 3$ replicates of analysis) ± SE. a–l indicate the same analytical parameters in different algae species groups. Means with different letters are significantly different ($p \leq 0.05$).

of the LUHS135 multiplied strain reduces the algae extracts × LUHS135 combinations until an average pH of 3.96; however, the highest pH was for Sp_{non}, Sp_{ultr} and $Furc_{ferm}$ samples (8.69, 7.67 and 5.59, respectively).

The color changes can be explained by the fact that during fermentation, the substrate is acidified, and organic acids have an influence on oxidation processes which can lead to color changes (38). In many cases, colored compounds

TABLE 2 Correlations between the color coordinates (L^* = lightness; a^* = redness; $-a^*$ = greenness; b^* = yellowness; $-b^*$ = blueness) and pH of the algae extracts and algae extracts × LUHS135 combinations.

Parameters	Pearson correlation (r) and significance (*p*)	Parameters			
		L*	a*	b*	pH
L*	r	1	−0.157	0.452**	0.135
	p		0.140	0.0001	0.205
a*	r	−0.157	1	−0.065	−0.289**
	p	0.140		0.546	0.006
b*	r	0.452**	−0.065	1	0.093
	p	0.0001	0.546		0.386
pH	r	0.135	−0.289**	0.093	1
	p	0.205	0.006	0.386	

**Correlation (r) is significant (p) at the 0.01 level (2-tailed).

lead to higher antioxidant properties of the product and/or extract; however, specific antioxidant properties are related to specific phenolic compound profile composition (3). However, oxidation of diffused phenolic compounds can also occur (43). In addition to fermentation, ultrasonication could cause color changes in compounds. Ultrasonic waves cause rapid compressions and expansions and destroy substrate cells, and the phenomenon of cavitation is responsible for a reduction of the diffusion boundary layer (44–48). It has been reported that ultrasonication increases extraction efficiency (49, 50). However, other published studies showed that the use of ultrasound as a pre-treatment for carrots contributed to significant changes in their color (51). From this point of view, it is very important to evaluate the changes of the antioxidant properties of the treated samples because reductions in colored compounds could lead to lower antioxidant activity. For this reason, during the second stage of the experiment, antioxidant activities and total phenolic compound content were analyzed.

The total phenolic compound and antioxidant activity of algae extracts and algae extracts × LUHS135 combinations

The aim of this study was to evaluate Also, combinations of extracts and LUHS135 were developed and their characteristics were evaluated. The total phenolic compounds content was determined from the calibration curve and expressed in mg of gallic acid equivalents; antioxidant activity was measured by a Trolox equivalent antioxidant capacity assay using the DPPH$^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl), ABTS$^{\bullet+}$ 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), FRAP (Ferric Reducing Ability of Plasma) discoloration methods.

The total phenolic compounds (TPC) content of the algae extracts and the influence of two pre-treatments, solid-state fermentation with the *Lactiplantibacillus plantarum* LUHS135 and ultrasonication is given in Table 3. In comparison, the TPC content multivariate analysis of variance showed that algae species ($p \leq 0.0001$), algae × pre-treatment before extraction interaction ($p \leq 0.0001$) and algae species × LUHS135 combination interaction ($p \leq 0.003$) had significant effects on TPC content in samples. The lowest TPC content in the non-pre-treated samples group was found in $ClaR_{non}$, Ul_{non} and Sp_{non} samples (on average 1.18 mg GAE/mL), and the highest was found in $ClaR_{nonLUHS135}$ and $Furc_{nonLUHS135}$ samples (on average 13.28 mg GAE/mL). In comparison, for extracts and extracts × LUHS135 combinations prepared from ultrasonicated algae, the lowest TPC content was found in Sp_{ultr} samples (0.51 mg GAE/mL), and the highest TPC content was in $ClaR_{ultrLUHS135}$, $ClaG_{ultrLUHS135}$ and $Furc_{ultrLUHS135}$ samples (on average 12.23 mg GAE/mL). Similar tendencies were established in the fermented samples group, and the lowest TPC content was found in Sp_{ferm} samples (2.77 mg GAE/mL) while the highest was in $ClaR_{fermLUHS135}$ and $Furc_{fermLUHS135}$ samples (on average 12.76 mg GAE/mL).

The antioxidant properties of two pre-treatments, solid-state fermentation with the *Lactiplantibacillus plantarum* LUHS135 and ultrasonication, on of macro- (*Cladophora rupestris, Cladophora glomerata, Furcellaria lumbricalis, Ulva intestinalis*) and Spirulina (*Arthrospira platensis*) extracts were estimated and compared by DPPH$^{\bullet}$, ABTS$^{\bullet+}$, and FRAP methods. In a comparison of the 2,2-diphenyl-1-picryhydrazyl (DPPH$^{\bullet}$) radical scavenging activity of all three groups of samples (non-pre-treated, ultrasonicated and fermented), multivariate analysis of variance showed that all of the analyzed factors and their interactions had significant effect on the DPPH$^{\bullet}$ radical scavenging activity of the samples (factors: algae species and pre-treatment before extraction (fermentation and/or ultrasonication), LUHS135 combination, algae species × LUHS135 combination interaction, algae extract × pre-treatment before extraction interaction, pre-treatment before extraction × LUHS135 combination interaction and the algae species × LUHS135 combination × pre-treatment before extraction interaction, $p \leq 0.0001$). In comparison to the non-pre-treated (before extraction) samples group, the lowest DPPH$^{\bullet}$ radical scavenging activity was found in $ClaR_{non}$, Ul_{non} and Sp_{non} samples (on average, 0.188 μmol TE/mL), and the highest DPPH$^{\bullet}$ radical scavenging activity was shown in $ClaR_{nonLUHS135}$ and $Furc_{nonLUHS135}$ samples (on average 1.86 μmol TE/mL). In extracts and extracts × LUHS135 combinations prepared from ultrasonicated algae, the lowest DPPH$^{\bullet}$ radical scavenging activity was found in Sp_{ultr} (0.078 μmol TE/mL); however, $ClaR_{ultrLUHS135}$, $ClaG_{ultrLUHS135}$ and $Furc_{ultrLUHS135}$ samples showed an average of 14.4 times higher DPPH$^{\bullet}$ radical scavenging activity. Similar to the ultrasonicated group samples, in fermented samples we found

TABLE 3 Antioxidant activities and total phenolic compound content of algae extracts and algae extracts × LUHS135 combinations.

Extracts and extract × LUHS135 combination	DPPH•, μmol TE/mL	ABTS•+, μmol TE/mL	FRAP, μmol TE/mL	TPC, mg GAE/mL
Extracts and extracts × LUHS135 combinations prepared from non-pre-treated algae				
$ClaR_{non}$	0.180±0.017[a]	0.704±0.032[a]	0.077±0.006[b]	1.30±0.095[a]
$ClaR_{nonLUHS135}$	1.87±0.141[f]	4.60±0.092[f,g]	2.19±0.210[h]	12.8±0.032[f]
$ClaG_{non}$	0.245±0.028[b]	2.70±0.071[d]	0.360±0.034[c]	5.50±0.158[b]
$ClaG_{nonLUHS135}$	0.676±0.046[c]	4.44±0.110[f]	0.728±0.063[e]	11.7±0.140[e]
$Furc_{non}$	1.52±0.104[e]	3.68±0.101[e]	0.869±0.047[f]	9.76±0.086[c]
$Furc_{nonLUHS135}$	1.84±0.093[f]	4.65±0.152[f,g]	2.37±0.235[h]	13.77±0.160[f]
Ul_{non}	0.197±0.013[a]	2.20±0.076[b]	0.063±0.005[a]	1.15±0.073[a]
$Ul_{nonLUHS135}$	0.834±0.079[d]	4.26±0.095[f]	1.21±0.114[g]	11.27±0.079[d,e]
Sp_{non}	0.187±0.017[a]	2.44±0.084[c]	0.051±0.004[a]	1.10±0.081[a]
$Sp_{nonLUHS135}$	0.661±0.056[c]	4.41±0.141[f]	0.603±0.037[d]	10.83±0.011[d]
Extracts and extracts × LUHS135 combinations prepared from ultrasonicated algae				
$ClaR_{ultr}$	0.288±0.037[b]	2.37±0.110[d]	1.14±0.072[f]	6.38±0.284[d]
$ClaR_{ultrLUHS135}$	1.09±0.093[f]	4.45±0.312[g]	0.932±0.064[e]	12.26±0.546[f]
$ClaG_{ultr}$	0.259±0.035[b]	1.55±0.091[c]	0.117±0.009[c]	5.06±0.216[c]
$ClaG_{ultrLUHS135}$	1.02±0.104[f]	4.52±0.234[g]	0.540±0.047[d]	12.19±0.631[f]
$Furc_{ultr}$	0.704±0.078[d]	2.27±0.155[d]	1.03±0.084[f]	6.13±0.277[d]
$Furc_{ultrLUHS135}$	1.26±0.088[f]	4.67±0.191[g]	1.68±0.086[g]	12.23±0.495[f]
Ul_{ultr}	0.403±0.039[c]	1.33±0.084[b]	0.058±0.006[b]	1.85±0.115[b]
$Ul_{ultrLUHS135}$	0.762±0.066[d]	4.26±0.255[f]	1.23±0.121[f]	11.16±0.558[e]
Sp_{ultr}	0.078±0.010[a]	0.223±0.027[a]	0.031±0.013[a]	0.51±0.045[a]
$Sp_{ultrLUHS135}$	0.877±0.049[e]	3.91±0.214[e]	1.34±0.114[f]	11.11±0.533[e]
Extracts and extracts × LUHS135 combinations prepared from fermented algae				
$ClaR^{ferm}$	0.288±0.029[c]	3.42±0.212[c]	0.274±0.026[b]	7.07±0.234[c]
$ClaR^{fermLUHS135}$	1.63±0.052[f]	5.04±0.321[f]	1.82±0.154[f]	12.70±0.540 [f]
$ClaG^{ferm}$	0.202±0.025[b]	2.21±0.044[b]	0.227±0.021[b]	7.06±0.304c
$ClaG^{fermLUHS135}$	0.819±0.078[d]	4.43±0.103[e]	1.10±0.112[c]	11.43±0.482[e]
$Furc^{ferm}$	1.11±0.130[e]	3.86±0.094[d]	1.28±0.123[c]	9.53±0.270[d]
$Furc^{fermLUHS135}$	1.45±0.132[f]	5.36±0.332[f]	1.68±0.142[e]	12.81±0.499[f]
Ul^{ferm}	0.202±0.012[b]	2.11±0.073[b]	0.258±0.027[b]	3.60±0.245[b]
$Ul^{fermLUHS135}$	0.891±0.055[d]	4.52±0.081[e]	1.17±0.140[d]	11.24±0.334[e]
Sp^{ferm}	0.140±0.008[a]	1.29±0.050[a]	0.054±0.006[a]	2.77±0.142[a]
$Sp^{fermLUHS135}$	1.12±0.111[e]	4.79±0.131[e]	1.34±0.121[d]	11.75±0.422[e]

ClaR, Cladophora rupestris; ClaG, Cladophora glomerata; Ul, Ulva intestinalis; Furc, Furcellaria lumbricalis; Sp, Spirulina (Arthrospira platensis); non, extracts prepared from non-pre-treated algae; ultr, extracts prepared from ultrasonicated algae; ferm, extracts prepared from fermented algae; LUHS135, extract × LUHS135 strain combination; DPPH•, 1,1-diphenyl-2-picrylhydrazyl; ABTS•+, 2,2 -azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); FRAP, Ferric Reducing Ability of Plasma; TPC, total phenolic compounds content; GAE, gallic acid equivalents TE, Trolox equivalent; data are represented as means (n = 3 replicates of analysis) ± SE. a–h indicate the same analytical parameters for different algae species groups, and means with different letters are significantly different ($p \leq 0.05$).

the lowest DPPH• radical scavenging activity in Sp_{ferm} samples (0.140 μmol TE/mL) and the highest in $ClaR_{fermLUHS135}$ and $Furc_{fermLUHS135}$ samples (on average 1.54 μmol TE/mL). Also, DPPH• radical scavenging activity showed a weak positive correlation with samples' a* coordinates ($r = 0.231$, $p = 0.028$). The -a* and -b* coordinates are related to chlorophyll's (-a and -b) greenish lipid-soluble pigments and causes the typical coloration of green algae (52, 53). However, carotenoids with a higher number of conjugated double bonds show red color and possess antioxidant properties (54). Other colored algae compounds with antioxidant properties are astaxanthin (52, 55–59) and canthaxanthin (β,β-carotene-4,4′-dione), which belongs to xanthophylls, and is widely used as a feed additive as an antioxidant (60–64).

2, 2′-azino-bis ethylbenzthiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) radical cation scavenging of the samples showed similar tendencies to DPPH• and FRAP, and a multivariate analysis of variance showed that all of the analyzed factors and

TABLE 4 Antimicrobial activity of the algae extracts and algae extracts × LUHS135 combinations evaluated using the agar well–diffusion method.

Extracts and extract × LUHS135 combination	Pathogenic and opportunistic bacteria strain						
	Salmonella enterica	*Bacillus cereus*	*Enterococcus faecium*	*Staphylococcus aureus*	*Escherichia coli*	*Streptococcus mutans*	*Enterococcus faecalis*
	Diameter of the Inhibition zone, mm						
Extracts and extracts × LUHS135 combinations prepared from non-pre-treated algae							
$ClaR_{non}$	nd	16.1 ± 1.3^{d}	15.3 ± 0.2^{c}	nd	nd	nd	nd
$ClaR_{nonLUHS135}$	nd	$12.3 \pm .2^{b}$	11.5 ± 0.2^{b}	12.4 ± 0.4^{c}	nd	nd	nd
$ClaG_{non}$	nd	15.2 ± 0.6^{d}	Nd	Nd	nd	nd	nd
$ClaG_{nonLUHS135}$	nd	16.0 ± 0.3^{d}	8.0 ± 0.1^{a}	11.5 ± 0.3^{b}	nd	nd	nd
$Furc_{non}$	nd	13.4 ± 0.5^{c}	nd	nd	nd	nd	nd
$Furc_{nonLUHS135}$	nd	11.2 ± 0.1^{a}	nd	12.3 ± 0.1^{c}	nd	nd	nd
Ul_{non}	nd	12.3 ± 0.3^{b}	nd	Nd	nd	nd	nd
$Ul_{nonLUHS135}$	nd	16.1 ± 0.2^{d}	nd	8.0 ± 0.2^{a}	nd	nd	nd
Sp_{non}	nd	12.4 ± 0.2^{b}	nd	Nd	nd	nd	nd
$Sp_{nonLUHS135}$	nd	16.4 ± 0.3^{d}	nd	Nd	nd	nd	nd
Extracts and extracts × LUHS135 combinations prepared from ultrasonicated algae							
$ClaR_{ultr}$	nd	18.2 ± 0.5^{b}	nd	Nd	nd	nd	nd
$ClaR_{ultrLUHS135}$	nd	16.4 ± 0.2^{a}	nd	14 ± 0.5^{b}	nd	nd	nd
$ClaG_{ultr}$	nd	nd	nd	nd	nd	nd	nd
$ClaG_{ultrLUHS135}$	nd	nd	12.6 ± 0.4	8.0 ± 0.1^{a}	nd	nd	nd
$Furc_{ultr}$	nd	nd	nd	Nd	nd	nd	nd
$Furc_{ultrLUHS135}$	nd	nd	nd	Nd	nd	8.0 ± 0.2^{a}	nd
Ul_{ultr}	nd	nd	nd	nd	nd	Nd	nd
$Ul_{ultrLUHS135}$	nd	nd	nd	nd	nd	12 ± 0.3^{b}	nd
Sp_{ultr}	nd	nd	nd	nd	nd	Nd	nd
$Sp_{ultrLUHS135}$	nd	18.1 ± 0.5^{b}	nd	14.6 ± 0.6b	nd	Nd	nd
Extracts and extracts × LUHS135 combinations prepared from fermented algae							
$ClaR_{ferm}$	nd	16.3 ± 0.6^{c}	nd	Nd	nd	Nd	nd
$ClaR_{fermLUHS135}$	nd	Nd	nd	15.4 ± 0.3^{c}	nd	Nd	nd
$ClaG_{ferm}$	nd	Nd	nd	nd	nd	Nd	nd
$ClaG_{fermLUHS135}$	nd	14.2 ± 0.2^{b}	nd	12.1 ± 0.1^{a}	nd	Nd	nd
$Furc_{ferm}$	nd	13.4 ± 0.4^{a}	nd	13.3 ± 0.2^{b}	nd	Nd	nd
$Furc_{fermLUHS135}$	nd	13.1 ± 0.1^{a}	nd	nd	nd	Nd	nd
Ul_{ferm}	nd	nd	nd	nd	nd	Nd	nd
$Ul_{fermLUHS135}$	nd	nd	nd	nd	nd	Nd	nd
Sp_{ferm}	nd	nd	nd	nd	nd	Nd	nd
$Sp_{fermLUHS135}$	nd	nd	nd	nd	nd	Nd	nd

ClaR, Cladophora rupestris; ClaG, Cladophora glomerata; Ul, Ulva intestinalis; Furc, Furcellaria lumbricalis; Sp, Spirulina (Arthrospira platensis); non, extracts prepared from non-pre-treated algae; ultr, extracts prepared from ultrasonicated algae; ferm, extracts prepared from fermented algae; LUHS135, extract × LUHS135 strain combination; nd, not determined; data are represented as means (n = 3 replicates of analysis) ± SE. a–d indicate the same analytical parameters in different algae species group, and means with different letters are significantly different ($p \leq 0.05$).

their interactions had significant effects on sample $ABTS^{\bullet+}$ (algae species $p \leq 0.0001$, pre-treatment before extraction $p \leq 0.0001$, LUHS135 combination $p \leq 0.0001$, algae species × LUHS135 combination interaction $p = 0.015$, algae extract × pre-treatment before extraction interaction $p \leq 0.0001$, pre-treatment before extraction × LUHS135 combination interaction $p \leq 0.0001$, algae species × LUHS135 combination × pre-treatment before extraction interaction $p \leq 0.0001$). In comparison, in the non-pre-treated before extraction sample group, the lowest $ABTS^{\bullet+}$ was in $ClaR_{non}$ samples (0.704 μmol TE/mL) and the highest was in $ClaR_{nonLUHS135}$ and $Furc_{nonLUHS135}$ samples (on average 4.63 μmol TE/mL).

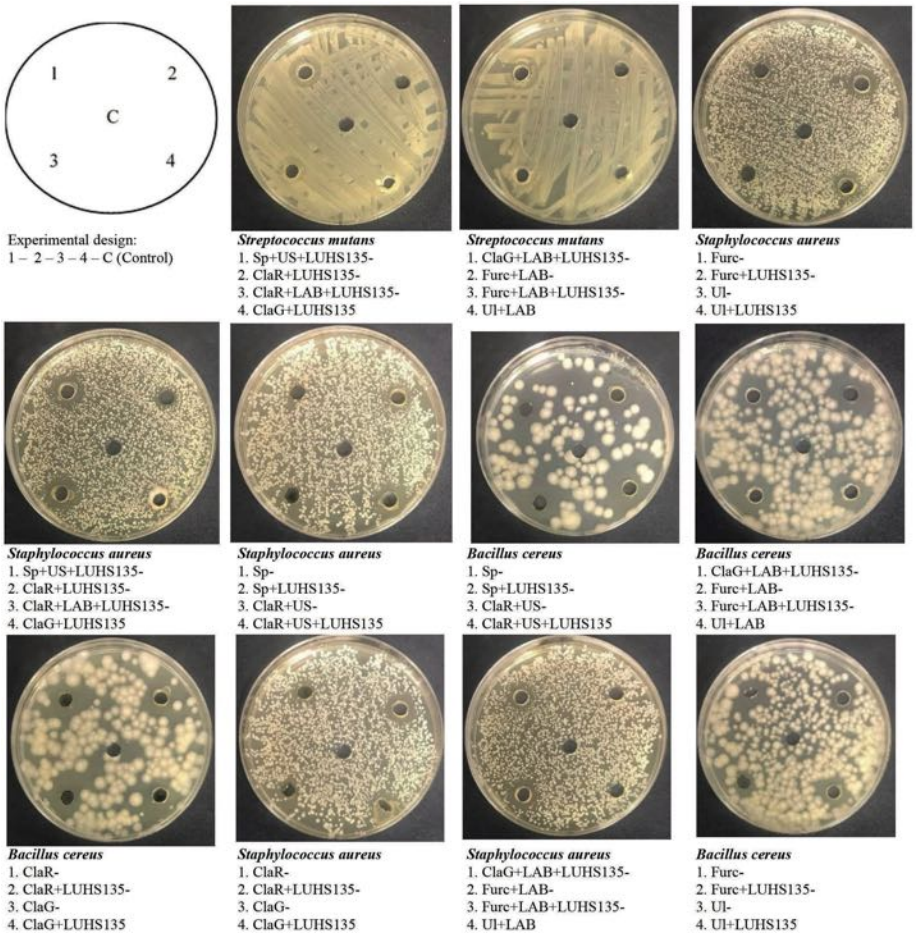

FIGURE 3
Images of the inhibition zones of the algae extracts and algae extracts × LUHS135 combinations evaluated using the agar well diffusion method [ClaR, Cladophora rupestris; ClaG, Cladophora glomerata; UI, Ulva intestinalis; Furc, Furcellaria lumbricalis; Sp, Spirulina (Arthrospira platensis)]; US, algae biomass pre-treated with ultrasound; LAB, algae biomas fermented with LUHS135 strain before extraction; LUHS135, extract composition with LUHS135 strain; C,control (physiological solution)].

The highest $ABTS^{\bullet+}$ in the ultrasonicated group was from $ClaR_{ultrLUHS135}$, $ClaG_{ultrLUHS135}$ and $Furc_{ultrLUHS135}$ samples (on average 4.55 μmol TE/mL) and the lowest was from Sp_{ultr} samples (0.223 μmol TE/mL). Similar tendencies were found in the fermented samples group: the lowest $ABTS^{\bullet+}$ was from Sp_{ferm} samples (1.29 μmol TE/mL) and the highest was from $ClaR_{fermLUHS135}$ and $Furc_{fermLUHS135}$ (on average 5.20 μmol TE/mL). $ABTS^{\bullet+}$ showed a weak, positive correlation with samples' a* coordinates ($r = 0.303$, $p = 0.004$).

The ferric reducing antioxidant power (FRAP), which shows the ability of an antioxidant in reducing Fe(III) into Fe(II), demonstrated that all of the analyzed factors and their interactions had significant effects on the FRAP of the samples ($p \leq 0.0001$). In comparison to the group that was not pre-treated before extraction, the lowest FRAP was established in

Ul_{non} and Sp_{non} samples (on average 0.057 μmol TE/mL) and the highest FRAP was found in $ClaR_{nonLUHS135}$ and $Furc_{nonLUHS135}$ samples (on average 2.28 μmol TE/mL). In comparison to the ultrasonicated sample group, the lowest FRAP was found in Sp_{ultr} samples (0.031 μmol TE/mL) and the highest in $Furc_{ultrLUHS135}$ samples (1.68 μmol TE/mL). In the fermented samples group, the lowest FRAP was in Sp_{ferm} samples (0.054 μmol TE/mL) and the highest was in $ClaR_{fermLUHS135}$ (1.82 μmol TE/mL). FRAP showed a moderate negative correlation with the b* coordinates of samples ($r = 0.509$, $p = 0.0001$). Phycobilin pigments are found in cyanobacteria and in the chloroplasts of red algae (52, 65). Liutein has a strong antioxidant effect (66). The main colored compounds in microalgae are fucoxanthin, lutein and β-carotene, and they also are described as good antioxidants (58, 59, 67–69). Zeaxanthin is a xanthophyll family carotenoid (70) and possesses antioxidant properties as well (71–75).

In essence, the radical scavenging activities of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ are based on the ability of antioxidants to donate a hydrogen atom or an electron to stabilize radicals by converting them to the non-radical species (76, 77). Our results reflected the ability of all prepared ethanolic extracts to donate a hydrogen atom or electron to both radicals. In general, algal extracts rich in natural polyphenolics can function as antioxidants (76, 78).

In this study, several methods based on different principles were used to determine the *in vitro* antioxidant activity of algae extracts. Other studies have reported that the FRAP method should be used in combination with other methods because it cannot measure all antioxidants of complex compounds (79, 80). Antioxidant properties of food and/or feed are desirable characteristics because antioxidants reduce oxidation processes (81). Also, it has been reported that both scavenging and antioxidant activities are related to TPC content (82). We found that TPC content in samples showed a moderate positive correlation with samples' a* coordinates ($r = 0.592$, $p = 0.0001$), a negative weak correlation with samples' pH ($r = -0.294$, $p = 0.005$) and a moderate positive correlation with samples' $ABTS^{\bullet+}$ ($r = 0.300$, $p = 0.004$) and FRAP ($r = 0.247$, $p = 0.019$). However, a correlation between the $DPPH^{\bullet}$ and TPC content was not found. It was previously reported that in ethanolic extracts the correlation between TPC content and total antioxidant capacity is high, but the correlation with FRAP assay is minimal, and the correlation between the total antioxidant capacity and TPC content is positive and very significant in ethanolic extracts, whereas it is negative in methanolic ones (83). However, in the free form, phenolic compounds have a better bio-accessibility because of released free aglycones and increased antioxidative activity (84, 85), and fermentation could decrease free phenolic compound content in samples because they may bind with other molecules present in the fermentable matrix, i.e., might be hydrolysed and/or be degraded by microbial enzymes (42, 84). According to Li et al. (86), LAB fermentation has a significant impact on the phenolic profile, as well as on antioxidant activity, because during the process, various phenolic acids could be excreted to the fermentable matrix (86). It was reported that *Furcellaria* extracts, in comparison with *Cladophora* and *Ulva sp*., had the highest antioxidant activity of all the macroalgae alcoholic extracts tested (87). It has also been shown that the ethanolic extract of green and red seaweeds exhibit a high scavenging activity and a higher $DPPH^{\bullet}$ of brown and green seaweeds in comparison with red (83, 88–90). The lower correlation between FRAP values and TPC content in extracts shows that the phenolic compounds are not involved in the antioxidant activity through this pathway, but there might be some effects involving other active compounds (83). The current study showed that the combinations of extracts and LUHS135 could improve antioxidant properties of the substrate.

Antimicrobial characteristics of the algal extracts

Antimicrobial activity of the algae extracts and algae extracts × LUHS135 combinations were evaluated using the agar well–diffusion method. The results are shown in Table 4 and Figure 3. In a comparison of all three groups (non-pre-treated, ultrasonicated and fermented), the highest number of samples (of all tested samples) that showed antimicrobial properties against at least one pathogen was found in the non-pre-treated samples group. All of the tested samples in this group showed inhibition properties against *Bacillus cereus* (the highest diameter of inhibition zones (DIZ), on average 16.0 mm, was found by $ClaR_{non}$, $ClaG_{non}$, $ClaG_{nonLUHS135}$, $Ul_{nonLUHS135}$ and $Sp_{nonLUHS135samples}$). Also, 3 out of 10 samples of this group showed inhibition properties against *Enterococcus faecium* ($ClaR_{non}$, $ClaR_{nonLUHS135}$ and $Cla_{GnonLUHS135}$, with DIZ of 15.3, 11.5 and 8.0 mm, respectively) and 4 out of 10 samples of this group showed inhibition properties against *Staphylococcus aureus* ($ClaR_{nonLUHS135}$, $ClaG_{nonLUHS135}$, $Furc_{nonLUHS135and}$ $Ul_{nonLUHS135}$, with DIZ of 12.4, 11.5, 12.3 and 8.0 mm, respectively). Despite the fact that the highest number of samples (of all tested samples) showed antimicrobial properties against at least one pathogen in the non-pre-treated samples group, a broader spectrum of pathogen inhibition was found in the ultrasonicated sample group (inhibition properties against *Bacillus cereus* showed in $ClaR_{ultr}$, $ClaR_{ultrLUHS135}$ and $Sp_{ultrLUHS135}$ samples, inhibition properties against *Enterococcus faecium* showed in $ClaG_{ultrLUHS135}$, inhibition properties against *Staphylococcus aureus* showed in $ClaR_{ultrLUHS135}$, $ClaG_{ultrLUHS135}$ and $Sp_{ultrLUHS135}$ and inhibition properties against *Stretococcus mutans* showed in $Furc_{ultrLUHS135}$ and $Ul_{ultrLUHS135}$ samples). In the comparison of extract samples prepared from fermented algae, $ClaR_{ferm}$, $ClaR_{fermLUHS135}$ and $Furc_{fermLUHS135}$ showed inhibition properties against one out of seven tested pathogens

TABLE 5 Antimicrobial activity of the algae extracts and algae extracts × LUHS135 combinations evaluated in liquid medium by testing concentration of algae extract and/or algae extract × LUHS135 combination at a concentration of 500 μL and pathogen concentration at 10 μL.

Extracts and extract × LUHS135 combination	Pathogenic and opportunistic bacteria strains						
	Salmonella enterica	*Bacillus cereus*	*Enterococcus faecium*	*Staphylococcus aureus*	*Escherichia coli*	*Streptococcus mutans*	*Enterococcus faecalis*
Inhibition zone, mm							
Extracts and extracts × LUHS135 combinations prepared from non-pre-treated algae							
Concentration of algae extract 500 μL, concentration of pathogen 10 μL							
$ClaR_{non}$	+	+	+	+	+	+	+
$ClaR_{nonLUHS135}$	+	+	+	+	+	+	+
$ClaG_{non}$	+	+	+	+	+	+	+
$ClaG_{nonLUHS135}$	+	+	+	+	+	+	+
$Furc_{non}$	+	+	+	+	+	+	+
$Furc_{nonLUHS135}$	+	+	+	+	+	+	+
Ul_{non}	+	+	+	+	+	+	+
$Ul_{nonLUHS135}$	+	+	+	+	+	+	+
Sp_{non}	+	-	+	+	+	-	+
$Sp_{nonLUHS135}$	+	+	+	+	+	+	+
Extracts and extracts × LUHS135 combinations prepared from ultrasonicated algae							
Concentration of algae extract 500 μL, concentration of pathogen 10 μL							
$ClaR_{ultr}$	+	+	+	+	+	+	+
$ClaR_{ultrLUHS135}$	+	+	+	+	+	+	+
$ClaG_{ultr}$	+	+	+	+	+	+	+
$ClaG_{ultrLUHS135}$	+	+	+	+	+	+	+
$Furc_{ultr}$	+	+	+	+	+	+	+
$Furc_{ultrLUHS135}$	+	+	+	+	+	+	+
Ul_{ultr}	+	+	+	+	+	+	+
$Ul_{ultrLUHS135}$	+	+	+	+	+	+	+
Sp_{ultr}	+	+	+	+	+	+	+
$Sp_{ultrLUHS135}$	+	+	+	-	+	+	+
Extracts and extracts × LUHS135 combinations prepared from fermented algae							
Concentration of algae extract 500 μL, concentration of pathogen 10 μL							
$ClaR_{ferm}$	+	+	-	-	+	+	+
$ClaR_{fermLUHS135}$	+	+	+	+	+	+	+
$ClaG_{ferm}$	+	+	+	+	+	+	+
$ClaG_{fermLUHS135}$	+	+	+	+	+	+	+
$Furc_{ferm}$	+	+	+	+	+	+	+
$Furc_{fermLUHS135}$	+	+	+	+	+	+	+
Ul_{ferm}	+	+	+	+	+	+	+
$Ul_{fermLUHS135}$	+	+	+	+	+	+	+
Sp_{ferm}	+	+	+	+	+	+	+
$Sp_{fermLUHS135}$	+	+	+	-	+	+	+
Pathogen control							
Pathogen	+	+	+	+	+	+	+

Interpretation of results: negative (–) means the pathogens did not grow on the selective culture medium; positive (+) means the pathogens grew on the selective culture medium; ClaR, Cladophora rupestris; ClaG, Cladophora glomerata; Ul, Ulva intestinalis; Furc, Furcellaria lumbricalis; Sp, Spirulina (Arthrospira platensis); non, extracts prepared from non-pre-treated algae; ultr, extracts prepared from ultrasonicated algae; ferm, extracts prepared from fermented algae; LUHS135, extract × LUHS135 strain combination.

TABLE 6 Antimicrobial activity of the algae extracts and algae extracts × LUHS135 combinations evaluated in liquid medium by testing the concentration of algae extract and/or algae extract × LUHS135 combination at a concentration of 2,000 μL and pathogen concentration at 10 μL.

Extracts and extract × LUHS135 combination	Pathogenic and opportunistic bacteria strains						
	Salmonella enterica	*Bacillus cereus*	*Enterococcus faecium*	*Staphylococcus aureus*	*Escherichia coli*	*Streptococcus mutans*	*Enterococcus faecalis*
Inhibition zone, mm							
Extracts and extracts × LUHS135 combinations prepared from non-pre-treated algae							
Concentration of algae extract 2,000 μL, concentration of pathogen 10 μL							
$ClaR_{non}$	+	+	-	+	+	+	-
$ClaR_{nonLUHS135}$	+	+	+	+	+	+	+
$ClaG_{non}$	+	+	+	+	+	+	-
$ClaG_{nonLUHS135}$	+	+	+	+	+	+	+
$Furc_{non}$	+	+	+	+	+	+	+
$Furc_{nonLUHS135}$	+	+	+	+	+	+	+
Ul_{non}	+	+	-	+	+	+	+
$Ul_{nonLUHS135}$	+	+	+	+	+	+	+
Sp_{non}	+	-	-	+	+	-	-
$Sp_{nonLUHS135}$	+	+	-	+	+	+	+
Concentration of algae extract 2,000 μL, concentration of pathogen 10 μL							
$ClaR_{ultr}$	+	+	+	+	+	-	+
$ClaR_{ultrLUHS135}$	+	+	+	+	+	+	+
$ClaG_{ultr}$	+	+	+	+	+	+	+
$ClaG_{ultrLUHS135}$	+	+	+	+	+	+	+
$Furc_{ultr}$	+	+	+	+	+	+	+
$Furc_{ultrLUHS135}$	+	+	+	+	+	+	+
Ul_{ultr}	+	+	-	+	+	-	+
$Ul_{ultrLUHS135}$	+	+	+	+	+	+	+
Sp_{ultr}	+	+	+	+	+	+	+
$Sp_{ultrLUHS135}$	+	+	+	-	+	+	+
Extracts and extracts × LUHS135 combinations prepared from fermented algae							
Concentration of algae extract 2,000 μL, concentration of pathogen 10 μL							
$ClaR_{ferm}$	+	+	-	-	+	+	+
$ClaR_{fermLUHS135}$	+	+	+	+	+	+	+
$ClaG_{ferm}$	+	+	+	+	+	+	-
$ClaG_{fermLUHS135}$	+	+	+	+	+	+	+
$Furc_{ferm}$	+	+	+	+	+	+	+
$Furc_{fermLUHS135}$	+	+	+	+	+	+	+
Ul_{ferm}	+	+	+	+	+	+	+
$Ul_{fermLUHS135}$	+	+	+	+	+	+	+
Sp_{ferm}	+	+	+	+	+	-	+
$Sp_{fermLUHS135}$	+	+	+	-	+	+	+
Pathogen control							
Pathogen	+	+	+	+	+	+	+

Interpretation of results: negative (–) means the pathogens did not grow on the selective culture medium; positive (+) means the pathogens grew on the selective culture medium; ClaR, Cladophora rupestris; ClaG, Cladophora glomerata; Ul, Ulva intestinalis; Furc, Furcellaria lumbricalis; Sp, Spirulina (Arthrospira platensis); non, extracts prepared from non-pre-treated algae; ultr, extracts prepared from ultrasonicated algae; ferm, extracts prepared from fermented algae; LUHS135, extract × LUHS135 strain combination.

[$ClaR_{ferm}$ and $Furc_{fermLUHS135}$ inhibited *Bacillus cereus* (DIZ 16.3 and 13.1 mm, respectively) and $Furc_{fermLUHS135}$ inhibited *Staphylococcus aureus* (DIZ 16.3 mm)]. Also, $ClaG_{fermLUHS135}$ and $Furc_{ferm}$ samples showed inhibition properties against both *Bacillus cereus* and *Staphylococcus aureus* strains (DIZ against *Bacillus cereus* 14.2 mm and 13.4 mm, respectively, and DIZ against *Staphylococcus aureus* 12.1 mm and 13.3 mm, respectively).

The results of antimicrobial activity of the algae extracts and algae extracts × LUHS135 combinations evaluated in liquid medium by testing concentrations of algae extract and/or the algae extract × LUHS135 combination in 500 and 2000 μL concentrations and pathogen concentration of 10 μL are shown in Tables 5, 6, respectively. We found that at a concentration of 500 μL in liquid medium, Sp_{non} samples inhibited *Bacillus cereus* growth, $ClaR_{ferm}$ samples inhibited *Enterococcus faecium* growth, $Sp_{ultrLUHS135}$, $ClaR_{ferm}$ and $Sp_{fermLUHS135}$ samples inhibited *Staphylococcus aureus* growth and Sp_{non} samples inhibited *Streptococcus mutans* growth. By increasing algae extract and algae extracts × LUHS135 combinations concentrations to 2000 μL, in addition to the mentioned antimicrobial properties, *Enterococcus faecium* was also inhibited by $ClaR_{non}$, Sp_{non}, $Sp_{nonLUHS135}$ and Ul_{ultr} samples, *Streptococcus mutans* was inhibited by $ClaR_{ultr}$, Ul_{ultr} and Sp_{ferm} samples and *Enterococcus faecalis* was inhibited by $ClaR_{non}$, $ClaG_{non}$, Sp_{non} and $ClaG_{ferm}$ samples.

Algae are a good source of bioactive compounds, and some of them possess broad spectrum activities, including antimicrobial activities (3, 91, 92). *Bacillus cereus* is a facultative aerobic spore-forming bacterium (93, 94), and is a well-known foodborne pathogen that is able to grow in the intestinal tracts of insects and mammals (94). *Ulva* species inhibit the growth of some Gram-positive pathogens (*Bacillus cereus* and *Staphylococcus aureus*) at $\leq$500 μg/mL concentration (95). Gram-positive bacteria are more susceptible to algae extracts, in comparison with Gram-negative bacteria, which is explained by extracts' compositions (high concentration of phenolic compounds, terpenoids, alkaloids, etc.), which damage the cellular wall. In contrast, the external membrane of Gram-negative bacteria acts as a barrier, preventing any substance from passing through (96). Among the predominant human pathogens, *Staphylococcus aureus* is the foremost cause of gastroenteritis (94, 97). *Cladophora rupestris* inhibits *S. aureus* growth (with DIZ 16.3 mm) (98, 99). Also, ethanolic extracts of *Cladophora* sp. possess stronger antibacterial activity against *S. aureus* in comparison with *Ulva sp.* extracts (96). However, different compositions of extrahent can lead to different properties of the extracts (96). In red seaweeds, including *F. lumbricalis*, strong inhibition properties against *S. aureus* were also reported (99–102), and it is thought that red types of seaweed are very promising agents against *S. aureus* (99). Also, Gram-positive bacteria (*B. cereus* and *S. aureus*) showed higher sensitivity to Spirulina extracts in comparison with Gram-negative ones (103). Elshouny et al. (104) reported, that Spirulina possesses antimicrobial activity against not only *S. aureus*, but also inhibits *E. coli* and *Salmonella* spp. growth. Mohammed et al. (105) reported, that Gram-positive strains are more sensitive to *Cladophora*, *Spirulina platensis* and *S. glomerata* extracts than Gram-negative ones, and the highest inhibitory efficacy was found to be against *S. aureus* (105). Another pathogenic and opportunistic strain, *E. faecium*, is a significant opportunistic human pathogen with a broad host range (106). *Enterococcus faecium* causes big problems because of its broad resistance to antimicrobials (106). From this point of view, natural antimicrobials, which could be used for opportunistic pathogenic strain inhibition, become very important. *Streptococcus mutans* can cause dental decay (107, 108), and some *S. mutans* proteins contribute to the pathogenesis of *S. mutans* by promoting adherence to dental plaque (107, 109–112). Also, Sirbu et al. (113) reported that TPC in algae extracts is related with their antibacterial activity. In this study we established that there are moderate correlations between $ABTS^{\bullet+}$ and *E. faecalis* DIZ and between the TPC content in extracts and *S. aureus* DIZ ($r = 0.388$, $p = 0.0001$; $r = 0.340$, $p = 0.001$, respectively). However, further research is needed to evaluate which compounds are responsible for the inhibition of these pathogens.

Conclusions

This study confirmed, that the species of algae is significant factor on samples pH ($p = 0.017$) and 2% of yeast extract leads to more effective fermentation of algal biomass, as after 36 h of SSF, significant lower algae pH values were obtained. The highest $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and FRAP antioxidant properties were shown by non-pretreated *Cladophora rupestris* and *Furcellaria lumbricalis* extract combinations with LUHS135, in comparison with extracts without LUHS135. A moderate positive correlation of TPC with samples $ABTS^{\bullet+}$ ($r = 0.300$, $p = 0.004$) and FRAP ($r = 0.247$, $p = 0.019$) was established, however, between samples $DPPH^{\bullet}$ and TPC content correlations were not found. Despite, that in the non-pre-treated samples group the highest number of samples showed antimicrobial properties at least against one pathogen, a broader spectrum of pathogens inhibition showed ultrasonicated samples group (inhibited 4 out of 7 tested pathogens). Finally, despite, that the extract combinations with LUHS135 strain showed prospective results, further research is needed to evaluate, which compounds are responsible for antioxidant properties of the extracts, as well as pathogens inhibition.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

Conceptualization: EB, PV, and MR. Methodology: EB, PV, DU, and MR. Software, validation, writing—original draft, and preparation: ET and EB. Formal analysis: VS, EZ, DU, and RR. Investigation: EB, ET, and MR. Resources, supervision, and project administration: EB. Data curation: ET. Writing—review and editing: EB, PV, MR, RP, and JR. Visualization: ET, VS, and EZ. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Changes in Spirulina's Physical and Chemical Properties during Submerged and Solid-State Lacto-Fermentation

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Abstract: The aim of this study was to select a lactic acid bacteria (LAB) strain for bio-conversion of Spirulina, a cyanobacteria ("blue-green algae"), into an ingredient with a high concentration of gamma-aminobutyric acid (GABA) for human and animal nutrition. For this purpose, ten different LAB strains and two different fermentation conditions (SMF (submerged) and SSF (solid state fermentation)) were tested. In addition, the concentrations of fatty acids (FA) and biogenic amines (BA) in Spirulina samples were evaluated. It was established that Spirulina is a suitable substrate for fermentation, and the lowest pH value (4.10) was obtained in the 48 h SSF with *Levilactobacillus brevis*. The main FA in Spirulina were methyl palmitate, methyl linoleate and gamma-linolenic acid methyl ester. Fermentation conditions were a key factor toward glutamic acid concentration in Spirulina, and the highest concentration of GABA (2395.9 mg/kg) was found in 48 h SSF with *Lacticaseibacillus paracasei* samples. However, a significant correlation was found between BA and GABA concentrations, and the main BA in fermented Spirulina samples were putrescine and spermidine. Finally, the samples in which the highest GABA concentrations were found also displayed the highest content of BA. For this reason, not only the concentration of functional compounds in the end-product must be controlled, but also non-desirable substances, because both of these compounds are produced through similar metabolic pathways of the decarboxylation of amino acids.

Keywords: Spirulina; fermentation; lactic acid bacteria; gamma-aminobutyric acid; biogenic amines

Key Contribution: In this study, lactic acid bacteria strains were used for bio-conversion of Spirulina into an ingredient with a high concentration of gamma-aminobutyric acid (GABA) for human and animal nutrition. For this purpose, ten different LAB strains and two different fermentation conditions were tested. In addition, the concentrations of fatty acids and biogenic amines in Spirulina samples were evaluated. The main finding, which is highlighted in this study, is that the samples in which the highest GABA concentrations were found also displayed the highest content of BA. For this reason, not only the concentration of functional compounds in the end-product must be controlled, but also non-desirable substances, because both of

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these compounds are produced through similar metabolic pathways of the decarboxylation of amino acids.

1. Introduction

Currently, a healthy lifestyle is very popular, and the practice of balanced diets—including the consumption of functional compounds—is of great interest to both humans and animals. Gamma-aminobutyric acid (GABA) is a functional compound that can be obtained through the decarboxylation of L-glutamate by the glutamate decarboxylase intracellular enzyme [1]. It has been confirmed that GABA can be synthetized by some microorganisms, including yeasts, fungi and bacteria [2–4]. However, to ensure efficient synthesis of GABA, a selection of the substrate is required, which should contain its precursors, as well as the appropriate microorganisms for the decarboxylation enzymatic process. Spirulina, which belong to the *Cyanobacteria* class (cyanobacteria) [5–7] have a significant content of GABA precursors [8]. In addition, these prokaryotic microalgae are commonly used as a functional food and feed material [5,9], because of their wide range of pharmacological activities [10], including amelioration of heavy metals and pesticide toxicity [11].

In this study, we hypothesized that the functional value of Spirulina (*Arthrospira platensis*) can be increased, by using biomass fermentation with selected lactic acid bacteria (LAB) strains. It was reported that the genus *Lactobacillus* and other cocci LAB have abundant GABA-producing species, including *Levilactobacillus brevis* [4,12–18], *Lactobacillus buchneri* [19,20], *Lactobacillus delbrueckii* subsp. *bulgaricus* [16,21], *Lactobacillus fermentum* [22,23], *Lactobacillus helveticus* [4,24], *Lacticaseibacillus paracasei* [16,25] and *Lactiplantibacillus plantarum* [4,16,25,26], among others. In this study, *Lactiplantibacillus plantarum* No. 122, *Lacticaseibacillus casei* No. 210, *Lactobacillus curvatus* No. 51, *Lacticaseibacillus paracasei* No. 244, *Lactobacillus coryniformis* No. 71, *Pediococcus pentosaceus* No. 183, *Levilactobacillus brevis* No. 173, *Pediococcus acidilactici* No. 29, *Leuconostoc mesenteroides* No. 225 and *Liquorilactobacillus uvarum* No. 245 strains were tested for Spirulina bioconversion. These LAB strains showed previously desirable antimicrobial and antifungal properties [27]. In addition to antimicrobial properties, some of the LAB can degrade mycotoxins [28], as well as display probiotic traits [29,30]. Nevertheless, and as expected, the metabolic activities of LAB are strongly correlated with environmental and processing factors, including the chemical composition of the substrates, moisture content, fermentation temperature and time, pH, buffer capacity, etc.

Currently, the industry is turning to more sustainable technologies. For this reason, biotechnological processes are also changing to meet sustainability requirements. The fermentation process can be performed in liquid as well as in solid state conditions. Solid-state fermentation (SSF) is a microbial process occurring mostly on the surface of solid materials that have the property to absorb or contain water, with or without soluble nutrients [31,32]. Moreover, SSF is often known to reduce global costs in comparison to liquid fermentation [33]. The low water volume in SSF has a large impact on the economy of the process mainly due to smaller bioreactor size, reduced downstream processing, lower sterilization costs, etc. Furthermore, many SSF processes focus on the utilization of cheap agri-industrial byproducts as culture media [32,34]. Submerged fermentation (SMF) is a very well-known methodology in the scientific literature, while SSF occupies a very small but emerging space in biotechnology [35]. In this study, we also hypothesized that the same microorganisms can be used in both fermentation techniques, but the results will differ due to the enormous differences in processing conditions.

Taking into consideration that LAB can excrete decarboxylases, the decarboxylation process can lead to the formation of desirable (e.g., GABA formation) and/or undesirable (e.g., biogenic amines (BA)) metabolites. Most of the BA are classified as non-desirable compounds, except for beta-phenylethylamine (β-PEA), which is attributed to neurotrans-

mitters [36,37]. Beta-phenylethylamine is a well-known and widespread endogenous neuroactive trace amine found throughout the central nervous system in humans [37]. This neurotransmitter modifies the release and the response to dopamine, norepinephrine, acetylcholine and GABA [38].

Moreover, during fermentation, various changes can be obtained, including that which concerns the bioconversion of lipids as well as once LAB can perform FA isomerization, hydration, dehydration and saturation in fermentable substrates [39].

The aim of this study was to select the most appropriate LAB strains for the bioconversion of Spirulina into an ingredient with a high concentration of GABA to be potentially used in human and animal nutrition. For this purpose, various LAB strains (*Lactiplantibacillus plantarum* No. 122; *Lacticaseibacillus casei* No. 210; *Lactobacillus curvatus* No. 51; *Lacticaseibacillus paracasei* No. 244; *Lactobacillus coryniformis* No. 71; *Pediococcus pentosaceus* No. 183; *Levilactobacillus brevis* No. 173; *Pediococcus acidilactici* No. 29; *Leuconostoc mesenteroides* No. 225; *Liquorilactobacillus uvarum* No. 245) and fermentation conditions (SMF and SSF) were investigated. Taking into consideration the complexity of the changes occurring during the fermentation processes, FA and BA profiles of Spirulina samples were evaluated.

2. Results and Discussion

2.1. pH Values and Color Coordinates (L, a* and b*) in the Spirulina Samples*

The pH and color coordinates of non-treated and fermented Spirulina samples are given in Supplementary File S1 (Table S1), Table 1 and Figure 1.

Table 1. Influence of the analyzed factors and their interaction on the color coordinates (L*, a* and b*) and pH values of Spirulina samples.

Factors and Their Interaction	Dependent Variable	*p*
Lactic acid bacteria strain used for fermentation	L*	0.403
	a*	**0.0001**
	b*	0.377
	pH	0.791
Duration of fermentation	L*	0.317
	a*	**0.0001**
	b*	0.807
	pH	0.898
Conditions of fermentation (submerged or solid state)	L*	0.438
	a*	**0.0001**
	b*	0.286
	pH	**0.042**
Lactic acid bacteria strain used for fermentation × Duration of fermentation	L*	0.422
	a*	**0.0001**
	b*	0.448
	pH	0.719
Lactic acid bacteria strain used for fermentation × Conditions of fermentation (submerged or solid state)	L*	0.398
	a*	**0.0001**
	b*	0.112
	pH	0.439
Duration of fermentation × Conditions of fermentation (submerged or solid state)	L*	0.307
	a*	**0.012**
	b*	0.313
	pH	0.665
Lactic acid bacteria strain used for fermentation × Duration of fermentation × Conditions of fermentation (submerged or solid state)	L*	0.393
	a*	**0.0001**
	b*	0.197
	pH	0.486

L*—lightness; a*—redness or −a*—greenness; b*—yellowness; −b*—blueness; influence of factor or factors interaction is recognized as statistically significant when $p \leq 0.05$. Significant influence of the analyzed factors or their interactions are marked in bold letters.

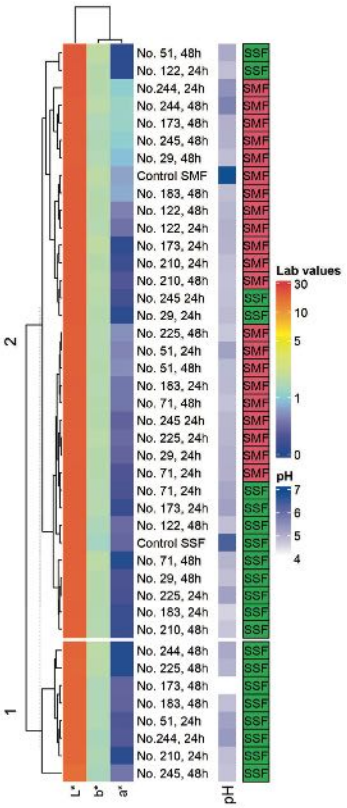

Figure 1. Changes in pH values and color coordinates (L*, a* and b*) in non-treated and fermented Spirulina samples. Note: No. 122—*Lactiplantibacillus plantarum*; No. 210—*Lacticaseibacillus casei*; No. 51—*Lactobacillus curvatus*; No. 244—*Lacticaseibacillus paracasei*; No. 71—*Lactobacillus coryniformis*; No. 183—*Pediococcus pentosaceus*; No. 173—*Levilactobacillus brevis*; No. 29—*Pediococcus acidilactici*; No. 225—*Leuconostoc mesenteroides*; No. 245—*Liquorilactobacillus uvarum*; SMF—submerged fermentation; SSF—solid state fermentation; L*, a and b* coordinates for the color in the CIE L*a*b* system.

When comparing pH values in SMF samples after 24 h, the highest pH (on average, 5.19 and 5.44) was found in samples fermented with *Lactobacillus curvatus* No. 51 and *Lacticaseibacillus paracasei* No. 244; however, most of the pH values were lower than 4.98. After 48 h of SMF, the lowest pH values were attained with samples fermented with *Leuconostoc mesenteroides* No. 225 (4.69). In two (out of ten) SMF samples, the pH values increased after 48 h of fermentation (viz., samples fermented with *Lacticaseibacillus paracasei* No. 244 and *Liquorilactobacillus uvarum* No. 245 strains). However, the same trends were not found in the above mentioned strains when employing SSF.

In 24 h SSF, the pH values of 4 out of 10 analyzed samples were lower in comparison with SMF samples at the same time of fermentation (SSF with *Lactiplantibacillus plantarum* No. 122, *Lacticaseibacillus paracasei* No. 244, *Pediococcus acidilactici* No. 29 and *Liquorilactobacillus uvarum* No. 245). Furthermore, the pH values of two samples were statistically

similar in SSF and SMF (viz., fermentation with *Lacticaseibacillus casei* No. 210 and with *Lactobacillus curvatus* No. 51). In the remaining 4 out of 10 analyzed 24 h SSF samples, the pH values were significantly higher in comparison with 24 h SMF samples (i.e., SSF with *Lactobacillus coryniformis* No. 71, *Pediococcus pentosaceus* No. 183, *Levilactobacillus brevis* No. 173, and *Leuconostoc mesenteroides* No. 225).

Among SSF samples after 48 h of fermentation, the lowest pH (4.10) was reached with *Levilactobacillus brevis* No. 173 strain. The increase in acidity is an indicator of the fermentation process and can be affected by many environmental and processing factors, such as LAB strains, carbon sources, type of fermentation, etc. In this respect, SSF are more effective in achieving lower pH values of the fermentable substrate when compared to SMF [40]. It was reported that the greatest changes during the fermentation of Spirulina with the *Lactiplantibacillus plantarum* strain are observed in the first 24 h (pH decreased from 7.3 to 5.1 and remained at the same value after 48 h) [41]. Moreover, Bao et al. [42] reported that the pH values of all fermented Spirulina samples were similar, and significant pH decreases to 4.3–5.3 were observed within the first 12 h. However, the acidification rate was the fastest when fermenting with *Lactiplantibacillus. plantarum* B7 strain [42]. Our study showed that the fermentation conditions (i.e., SMF or SSF) is a key factor on the final pH of the Spirulina samples ($p = 0.042$) (Table 1); in addition, after 48 h of fermentation, most of the pH values decreased in comparison with 24 h of fermentation.

Comparing the L* coordinates in SMF, in most of the cases, SMF samples showed similar L* values as the control (I) samples—except for 24 h SMF with *Lacticaseibacillus paracasei* No. 244, in which L* coordinate was 2.6% higher, and for 24 h SMF with *Pediococcus acidilactici* No. 29 and *Leuconostoc mesenteroides* No. 225, in which L* coordinate was 3.0% lower. Different trends of the L* coordinate were found in SSF samples after 24 and 48 h of fermentation. L* coordinate values were the same as that of the control (II) in 7 out of 20 samples after 24 and 48 h of SSF, chiefly 48 h SSF with *Lactiplantibacillus plantarum* No. 122, 48 h SSF with *Lacticaseibacillus casei* No. 210, 24 and 48 h SSF with *Lactobacillus coryniformis* No. 71, 24 h SSF with *Pediococcus pentosaceus* No. 183, 24 h SSF with *Levilactobacillus brevis* No. 173, and 48 h SSF with *Pediococcus acidilactici* No. 29). Furthermore, L* coordinates were higher than that of the control (II) in 4 out of 20 samples after 24 and 48 h SSF, chiefly 24 h SSF with *Lactiplantibacillus plantarum* No. 122, 48 h SSF with *Lactobacillus curvatus* No. 51, 24 h SSF with *Pediococcus acidilactici* No. 29, and 24 h SSF with *Liquorilactobacillus uvarum* No. 245. Finally, L* coordinates were lower than that of the control (II) in 9 out of 20 samples after 24 and 48 h SSF, viz. 24 h SSF with *Lacticaseibacillus casei* No. 210, 24 h SSF with *Lactobacillus curvatus* No. 51, 24 and 48 h SSF with *Lacticaseibacillus paracasei* No. 244, 48 h SSF with *Pediococcus pentosaceus* No. 183, 48 h SSF with *Levilactobacillus brevis* No. 173, 24 and 48 h SSF with *Leuconostoc mesenteroides* No. 225, and 48 h SSF with *Liquorilactobacillus uvarum* No. 245.

Analysis of between-subject effects unveiled that the analyzed factors and their interactions were not significant concerning the L* coordinates of the samples (Table 1). Additionally, pH and L* coordinate values between samples presented a weak negative correlation ($r = -0.277$, $p = 0.002$). In contrast to L* coordinates, the LAB strain used for fermentation, duration of fermentation, SMF or SSF conditions, as well as all the interaction of these factors, were statistically significant with respect to a* coordinate values of Spirulina samples (Table 1).

Comparing the a* coordinate of SMF samples with the control (I), lower values were found in 14 out of 20 samples. A similar trend occurred in most of the SSF (with 24 and 48 h fermentation) samples (17 out of 20 samples) in comparison with the control (II). Regarding b* coordinate, most of the SMF samples presented lower values in comparison with the control (I), except for 24 h SMF with *Levilactobacillus brevis* No. 173. Opposite trends were found in b* coordinates of SSF samples, where in all cases, they were higher in comparison with the control (II). The color changes may occur because of the acidification of the substrate medium. Organic acids influence oxidation processes in fermentable substrates, leading to color changes [40]. Spirulina contains different-colored compounds, including

carotenoids and C-phycocyanin [43]. In addition, β-cryptoxanthin and zeaxanthin are present in small amounts in Spirulina [44]. It was reported that the phycocyanin molecule is sensitive to environmental conditions, including pH [45,46]. As predicted, it was reported that the L* value of Spirulina has a significant correlation with pigment content [43]. Finally, our study showed that all the analyzed factors and their interactions were significant to the a* coordinate of Spirulina, and these findings led us to conclude that during the fermentation process, changes in Spirulina pigments occur.

2.2. L-Glutamic Acid (L-Glu) and Gamma-Aminobutyric Acid (GABA) Concentration in the Spirulina Samples

L-Glutamic acid and gamma-aminobutyric acid concentrations of the non-treated (non-fermented) and fermented Spirulina samples are given in Supplementary File S1 (Table S2), Table 2 and Figure 2.

Table 2. Influence of the analyzed factors and their interactions on L-glutamic acid (L-Glu) and gamma-aminobutyric acid (GABA) concentration in Spirulina samples.

Factors and Their Interaction	Dependent Variable	*p*
Lactic acid bacteria strain used for fermentation	GABA	**0.0001**
	L-Glutamic acid	0.641
Duration of fermentation	GABA	0.987
	L-Glutamic acid	0.328
Conditions of fermentation (submerged or solid state)	GABA	**0.020**
	L-Glutamic acid	**0.0001**
Lactic acid bacteria strain used for fermentation × Duration of fermentation	GABA	0.813
	L-Glutamic acid	0.942
Lactic acid bacteria strain used for fermentation × Conditions of fermentation (submerged or solid state)	GABA	**0.0001**
	L-Glutamic acid	0.740
Duration of fermentation × Conditions of fermentation (submerged or solid state)	GABA	0.499
	L-Glutamic acid	0.358
Lactic acid bacteria strain used for fermentation × Duration of fermentation × Conditions of fermentation (submerged or solid state)	GABA	0.893
	L-Glutamic acid	0.957

Gamma-aminobutyric acid (GABA); influence of factor or factors interaction is recognized as statistically significant when $p \leq 0.05$. Significant influences of the analyzed factors or their interactions are marked in bold letters.

From the comparison of glutamic acid concentration between 24 h SMF samples and control (I) samples, one may conclude that glutamic acid concentration: was lower in 3 out of 10 samples (in 24 h SMF with *Lactiplantibacillus plantarum* No. 122, *Lacticaseibacillus paracasei* No. 244, and *Leuconostoc mesenteroides* No. 225, by 8.5, 93.8 and 90.3%, respectively); was higher in 6 out of 10 samples (in 24 h SMF with *Lactobacillus curvatus* No. 51, *Lactobacillus coryniformis* No. 71, *Pediococcus pentosaceus* No. 183, *Pediococcus pentosaceus* No. 183, *Pediococcus acidilactici* No. 29, and *Liquorilactobacillus uvarum* No. 245, by 16.9, 7.72, 36.7, 47.2, 18.8 and 46.8%, respectively); and 1 out of 10 samples of glutamic acid concentration was similar (in 24 h SMF with *Lacticaseibacillus casei* No. 210). However, after 48 h of SMF, glutamic acid concentration was found to be higher in 7 out of 10 samples, and it was lower in 3 out of 10 samples when compared to the control (I).

When analyzing glutamic acid concentration SSF samples with the control (II), in most of the cases (except for in 24 and 48 h SSF with *Lacticaseibacillus paracasei* No. 244 samples), glutamic acid concentration increased, and the conditions of fermentation (SMF or SSF) proved to be a statistically significant factor on the glutamic acid concentration in Spirulina samples (Table 2).

Regarding GABA concentration in SMF samples, in all the cases (i.e., after 24 and 48 h of SMF), the values increased consistently in comparison with the control (I), and the highest GABA concentration (286.5 mg/kg) was found in 48 h SMF with *Lacticaseibacillus paracasei* No. 244. The same trend was established in SSF samples, i.e., after 24 and 48 h of

SSF, GABA concentration was higher than in the control (II), and the highest concentration of GABA (2395.9 mg/kg) was found in 48 h SSF with *Lacticaseibacillus paracasei* No. 244.

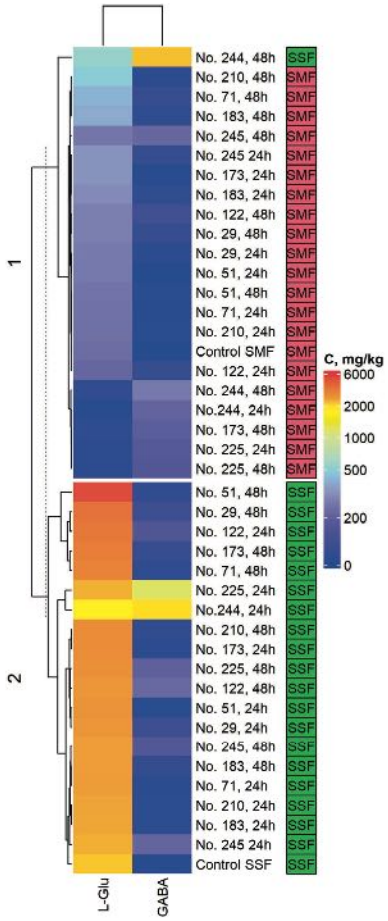

Figure 2. Changes in L-glutamic acid (L-Glu) and gamma-aminobutyric acid (GABA) concentrations in non-treated and fermented Spirulina samples. Note: No. 122—*Lactiplantibacillus plantarum*; No. 210—*Lacticaseibacillus casei*; No. 51—*Lactobacillus curvatus*; No. 244—*Lacticaseibacillus paracasei*; No. 71—*Lactobacillus coryniformis*; No. 183—*Pediococcus pentosaceus*; No. 173—*Levilactobacillus brevis*; No. 29—*Pediococcus acidilactici*; No. 225—*Leuconostoc mesenteroides*; No. 245—*Liquorilactobacillus uvarum*; SMF—submerged fermentation; SSF—solid state fermentation; C—concentration in mg/kg.

Despite that correlations between glutamic acid and GABA concentrations were not found, it was determined that the LAB strain used for fermentation, the conditions of fermentation (submerged or solid state) as well as the interactions between LAB strain used for fermentation and the conditions of fermentation (submerged or solid state) significantly affected GABA concentration in Spirulina samples. It was reported that LAB strains may

produce glutamic acid [47]. Even though the main aim of this study was to evaluate the chemical changes in Spirulina biomass, it can be hypothesized that the correlation between glutamic acid and GABA was not found due to the characteristics of the studied LAB, for which the metabolic pathways include not only the decarboxylation of amino acids but also the production of glutamic acid. Nevertheless, further studies are needed to confirm this hypothesis.

The most common amino acids in *Spirulina* spp. are glutamic acid followed by leucine and aspartic acid [48]. Specific bacterial genera are involved in the production of GABA [49]. It was reported that LAB may induce the structural breakdown of cyanobacterial cell walls via hydrolysis, leading to the conversion of complex compounds [50]. Most of the glutamic acid and GABA-producing microorganisms are LAB, including species from the genera *Lactococcus*, *Lactobacillus*, *Enterococcus* and *Streptococcus* [51]. However, the production of glutamic acid and GABA can vary in relation with microorganism characteristics, and it is species-dependent [52]. In addition to these findings, our study showed that fermentation conditions (SMF or SSF) are also a very statistically significant factor, especially for GABA content in Spirulina samples.

2.3. Biogenic Amine (BA) Content in the Spirulina Samples

Biogenic amine (BA) contents in non-treated and fermented Spirulina samples are given in Supplementary File S1 (Tables S3 and S4), Table 3 and Figures 3 and 4.

Table 3. Correlations between biogenic amines and gamma-aminobutyric acid (GABA) and L-glutamic acid (L-Glu) concentrations.

Correlations	Correlation (r)	Significance (p)	Correlation (r)	Significance (p)
	with GABA		with L-Glutamic acid	
TRP	0.215 *	0.016	0.541 **	0.0001
PUT	0.309 **	0.0001	0.486 **	0.0001
CAD	0.298 **	0.001	−0.076	0.401
HIS	0.648 **	0.0001	−0.073	0.414
TYR	0.681 **	0.0001	−0.014	0.879
SPRMD	0.211 *	0.018	0.627 **	0.0001
SPRM	0.172	0.054	0.528 **	0.0001

TRP—tryptamine; PUT—putrescine; CAD—cadaverine; HIS—histamine; TYR—tyramine; SPRMD—spermidine; SPRM—spermine. ** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

Phenylethylamine was not found in Spirulina samples. Furthermore, cadaverine (CAD) and histamine (HIS) were detected in 8 and 5 (out of 42) samples, respectively. However, concentrations of CAD and HIS in 48 h SSF with *Lacticaseibacillus paracasei* No. 244 were 24.4 and 137.9 mg/kg, respectively. In addition, concentrations of CAD higher than 30 mg/kg were found in 24 h SSF with *Lacticaseibacillus casei* No. 210; nevertheless, CAD was not detected after 48 h of SSF in the same samples. All the analyzed factors and their interactions were statistically significant regarding the concentration of CAD and HIS in Spirulina samples ($p \leq 0.0001$) (Table S4).

With respect to tryptamine (TRP) concentration, it was not found in most of the SMF samples (except for in 24 h SMF *Lacticaseibacillus paracasei* No. 244). Still, TRP was found in all the SSF samples, and its content ranged from 2.66 mg/kg (in 24 h SSF with *Lactobacillus curvatus* No. 51 samples) to 7.53 mg/kg (in 48 h SSF with *Liquorilactobacillus uvarum* No. 245 samples).

Putrescine (PUT) and spermidine (SPRMD) were the main BA found in fermented Spirulina samples. Concerning PUT, higher PUT concentrations were found in SSF samples in most of the cases, in comparison with SMF ones, and the highest PUT content was obtained in SSF with *Levilactobacillus brevis* No. 173 samples (833.4 mg/kg after 24 h and 854.7 mg/kg after 48 h). The lowest PUT concentration was found in samples fermented with *Pediococcus pentosaceus* No. 183, viz., in 24 h SMF and 24 h SSF, PUT concentrations

were 0 and 20.8 mg/kg, respectively; in 48 h SMF and 48 h SSF, PUT concentrations were 86.6 and 32.4 mg/kg, respectively. All the analyzed factors and their interactions were statistically significant regarding the concentration of PUT in Spirulina samples ($p \leq 0.0001$) (Table S4).

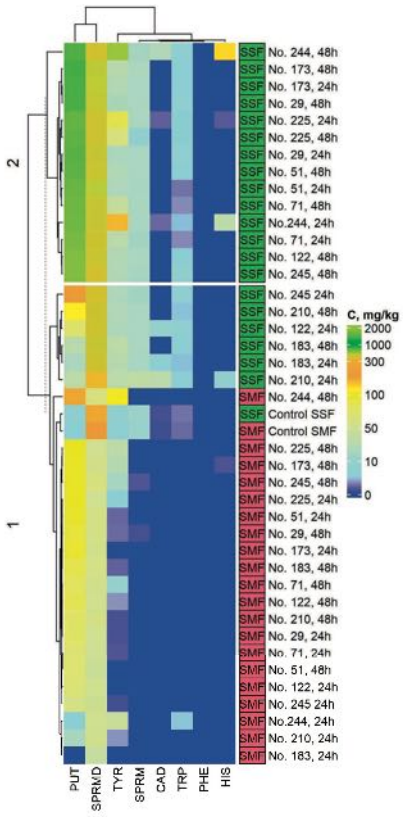

Figure 3. Changes in biogenic amine (BA) concentrations in non-treated and fermented Spirulina samples. Note PUT – putrescine; TRP—tryptamine; PHE—phenylethylamine; CAD—cadaverine; HIS—histamine; TYR—tyramine; SPRMD—spermidine; SPRM—spermine; No. 122—*Lactiplantibacillus plantarum*; No. 210—*Lacticaseibacillus casei*; No. 51—*Lactobacillus curvatus*; No. 244—*Lacticaseibacillus paracasei*; No. 71—*Lactobacillus coryniformis*; No. 183—*Pediococcus pentosaceus*; No. 173—*Levilactobacillus brevis*; No. 29—*Pediococcus acidilactici*; No. 225—*Leuconostoc mesenteroides*; No. 245—*Liquorilactobacillus uvarum*; SMF—submerged fermentation; SSF—solid state fermentation; C—concentration in mg/kg.

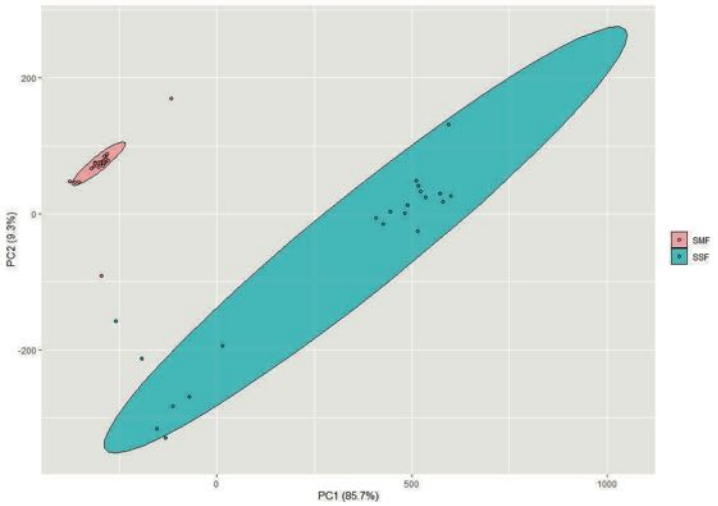

Figure 4. Biogenic amines distribution in submerged (SMF) and solid-state fermented (SSF) samples. Projection of the variables (biogenic amines) in the principal components 1 (PC1) and 2 (PC2), obtained by principal components analysis (PCA). The percentage of variability accounted for by PC1 and PC2 is 85.7% and 9.3%, respectively.

In regard to SPRMD in SMF samples, it was found that SMF decreased SPRMD concentration in Spirulina samples, on average from 3.3 to 4.9 times (in 48 h SMF with *Lacticaseibacillus paracasei* No. 244 and in 24 h SMF with *Pediococcus pentosaceus* No. 183 samples, respectively). However, opposite trends of the SPRMD concentration were found in SSF samples. Furthermore SPRMD concentrations were, on average, 7.7 times higher in SSF than in SMF samples. Additionally, all the analyzed factors and most of their interactions—except for the interaction between duration of fermentation and conditions of fermentation (submerged or solid state)—were statistically significant on SPRMD concentration in Spirulina samples ($p \leq 0.0001$) (Table S4), and the highest SPRMD concentration was obtained in 24 h SSF with *Lactobacillus curvatus* No. 51 and 24 and 48 h SSF with *Levilactobacillus brevis* No. 173 (on average, 519.9 mg/kg).

When observing the results of tyramine (TYR), the concentration in most of the SMF samples was lower than in the control (I), except in SMF with *Lacticaseibacillus paracasei* No. 244, *Levilactobacillus brevis* No. 173 and in *Leuconostoc mesenteroides* No. 225 and *Liquorilactobacillus uvarum* No. 245. Conversely, opposite trends were perceived in SSF samples, i.e., all the SSF samples exhibited higher TYR concentration when compared to the control (II). The highest TYR concentration was found in SSF samples with *Lacticaseibacillus paracasei* No. 244 (TYR concentration was 167.7 mg/kg after 24 h and 609.4 mg/kg after 48 h SSF). Furthermore, all the analyzed factors and their interactions were statistically significant regarding TYR concentration in Spirulina samples ($p \leq 0.0001$) (Table S4).

The comparison of SPRM in Spirulina samples with control (I) samples led us to conclude that SMF reduced its concentration. However, in SSF samples, SPRM concentration was similar or slightly higher in comparison to the control (II) samples (except for 48 h SSF with *Leuconostoc mesenteroides* No. 225 samples). All the analyzed factors and their interactions were statistically significant regarding their effect on SPRM concentration in Spirulina samples ($p \leq 0.05$) (Table S4).

Changes in eating habits and looking for functional compounds are often associated with the incorporation of new non-traditional food ingredients into the main diet. However, functional properties in most of these so-called "super foods" are causing concern in terms of food safety issues. For this reason, this study includes not only the evaluation of GABA but also of BA concentrations in fermented Spirulina samples due to the fact that both compounds are formed through the carboxylation of amino acids. Table 3 tabulates the correlations between BA and GABA and glutamic acid concentrations in Spirulina samples. More specifically, from these results, it was possible to unfold statistically significant correlations between TRP, PUT, CAD, HIS, TYR and SPRMD and GABA, as well as between TRP, PUT, SPRMD and SPRM and glutamic acid. TYR, HIS, PUT, CAD, SPRM and SPRMD are mainly produced by microbial decarboxylation of amino acids [53,54]. PUT is a precursor for the synthesis of SPRMD [54]. Likewise, PUT and CAD can be metabolized from ornithine and lysine, respectively [55]. TYR is associated with constricting of vascular system, and HIS is known as a vasodilator [56]. In addition to the individual toxicity of BA, Wang et al. [57] reported that the sum of primary, secondary and tertiary biogenic amines is very important. TYR causes migraines, and PUT and CAD potentiate intoxication in the presence of other BA [58]. Finally, the samples in which the highest GABA concentrations were found also presented the highest content of BA. This shows that it is important to simultaneously study the presence of functional and non-desirable compounds in the end-product, especially when both compounds (as in this case) are produced through decarboxylation pathways of amino acids. Figure 4 presents the principal component analysis (PCA) of the first two principal components (PC) and makes apparent the existence of two clusters formed by the SMF and SSF samples, respectively, and thus the existence of statistically significant differences between both type of fermentations. Our previous studies showed that during the SSF, microorganisms show more efficient capacity to excrete enzymes and to degrade fermentable substrates [59].

2.4. Fatty Acid (FA) Profile in the Spirulina Samples

Fatty acid (FA) content in non-treated and fermented Spirulina samples is given in Supplementary File S1 (Tables S5–S7) and Figure 5.

The main FA in non-treated and fermented Spirulina samples were methyl palmitate (C16:0), methyl linoleate (C18:2) and gamma-linolenic acid methyl ester (C18:3$_{\gamma}$). When investigating C16:0 content in non-treated and SMF samples, the concentration was higher in 18 out of 20 samples than that in the control (I) samples. Only in the cases of 24 and 48 h SMF with *Lacticaseibacillus casei* No. 210 samples was the concentration of C16:0 similar to the control (I) (on average, 42.4% from total fat content). The same investigation of C16:0 content but in the SSF samples led to discovering different trends, and the highest C16:0 concentration was obtained in 48 h SSF with *Lactobacillus coryniformis* No. 71 samples (on average, 60.8% from total fat content). Tests between subjects showed that the LAB strain used for fermentation, the interaction LAB strain used for fermentation $*$ duration of fermentation and the interaction LAB strain used for fermentation $*$ conditions of fermentation (submerged or solid state) were statistically different in terms of C16:0 concentration in Spirulina samples (Supplementary File S1, Table S6).

Regarding the content of C18:2 in Spirulina samples, the values were lower in all the SMF and SSF samples in comparison with the control (I) and control (II) samples, respectively. Tests between subjects showed that the C18:2 content in Spirulina samples was significantly affected in the following cases: LAB strain used for fermentation ($p = 0.003$); conditions of fermentation (SMF or SSF) ($p = 0.038$); LAB strain used for fermentation $*$ duration of fermentation ($p \leq 0.0001$); LAB strain used for fermentation $*$ conditions of fermentation (SMF or SSF) ($p \leq 0.001$); and LAB strain used for fermentation $*$ duration of fermentation $*$ conditions of fermentation (SMF or SSF) ($p \leq 0.0001$) (Supplementary File S1, Table S6).

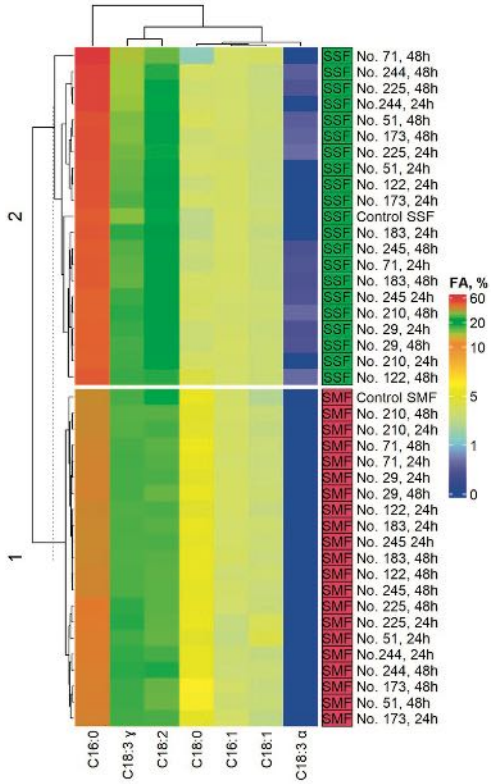

Figure 5. Changes in fatty acid (FA) profile in non-treated and fermented Spirulina samples. Note: C16:0—methyl palmitate; C16:1—methyl palmitoleate; C18:0—methyl stearate; C18:1 *cis, trans—cis, trans*-9- oleic acid methyl ester; C18:2—methyl linoleate; C18:3γ—gamma- linolenic acid methyl ester; C18:3α—alfa linolenic acid methyl ester; No. 122—*Lactiplantibacillus plantarum*; No. 210—*Lacticaseibacillus casei*; No. 51—*Lactobacillus curvatus*; No. 244—*Lacticaseibacillus paracasei*; No. 71—*Lactobacillus coryniformis*; No. 183—*Pediococcus pentosaceus*; No. 173—*Levilactobacillus brevis*; No. 29—*Pediococcus acidilactici*; No. 225—*Leuconostoc mesenteroides*; No. 245—*Liquorilactobacillus uvarum*; SMF—submerged fermentation; SSF—solid state fermentation; % from total fat content.

When comparing the content of C18:3γ with the control, different trends in SMF and SSF samples were perceived. In the case of SMF, C18:3γ content increased in 6 out of 20 SMF samples; C18:3γ content decreased in 8 out of 20 SMF samples; and C18:3γ content remained similar to the control (I) in 6 out of 20 SMF samples. In the case of SSF, C18:3γ content increased in 14 out of 20 SSF samples; C18:3γ content decreased in 4 out of 20 SSF samples; and C18:3γ content remained similar to the control (II) in 2 out of 20 SSF samples. Tests between subjects showed that the C18:3γ content in Spirulina samples was significantly affected in the following cases: LAB strain used for fermentation ($p = 0.004$); LAB strain used for fermentation $*$ duration of fermentation ($p \leq 0.0001$); LAB strain used for fermentation $*$ conditions of fermentation (SMF or SSF) ($p \leq 0.001$); and LAB strain

used for fermentation ∗ duration of fermentation ∗ conditions of fermentation (SMF or SSF) ($p \leq 0.0001$) (Supplementary File S1, Table S6).

Alfa-linolenic acid methyl ester (C18:3α) was only found in SSF samples, and its content ranged from 0.399 (in 48 h SSF with *Liquorilactobacillus uvarum* No. 245 samples) to 0.618% of total fat content (in 24 h SSF with *Leuconostoc mesenteroides* No. 225 samples). Tests between subjects showed that all the analyzed factors were significant regarding the concentration of C18:3α in Spirulina samples ($p \leq 0.0001$) (Supplementary File S1, Table S6).

Methyl palmitoleate (C16:1), methyl stearate (C18:0), and *cis, trans*-9-oleic acid methyl ester (C18:1 *cis, trans*) contents in Spirulina samples were lower than 5% from the total fat content. In addition, the analyzed factors and their interaction proved to not be significant on C16:1 content in Spirulina samples. On the other hand, the LAB strain used for fermentation, the conditions of fermentation (SMF or SSF), and the interaction LAB strain used for fermentation ∗ conditions of fermentation (SMF or SSF) were significant regarding C16:1 content in Spirulina samples. Likewise, the interaction LAB strain used for fermentation ∗ duration of fermentation was significant on C18:1 *cis, trans* in Spirulina samples.

The proximate composition of spirulina is related to numerous factors such as the source of the cyanobacteria, the season of the year, as well as to the manufacturing technology. The lipid concentration of *Arthrospira platensis* can vary from ca. 5 to 10% (of the dry weight) [60]. Long-chain FA are predominant compounds in Spirulina (mainly palmitic acid and gamma-linoleic acid) [61,62]. However, other studies reported higher contents of palmitic (46%), oleic (8%) and linoleic (12%) acids in Spirulina and lower contents of gamma-linoleic acid (20%) and stearic acid (1%) [63]. One of the most significant polyunsaturated FA is gamma-linoleic acid [62,64]. In addition to the FA profile of non-treated Spirulina, it was reported that 6 days of SSF with the fungus *Aspergillus niger*, *Spirulina* spp. attained the highest concentration of linoleic acid (60.63%, from total fat content), which was significantly higher than that obtained by SSF with *Lactiplantibacillus plantarum* (16.93%). However, the contents of elaidic, α-linoleic, stearic and palmitic acids of *Spirulina* spp. were higher in SSF with *Lactiplantibacillus plantarum*. The desirable changes in FA profile were explained by reduction of the substrate concentration during the fermentation process, because the nutrients were used for microbial growth and secondary metabolite production [65].

Omega-6 constitutes the majority of the total Spirulina FA [66,67]. Furthermore, Spirulina contains a significant amount of palmitic acid (16:0), which represents more than 25% from the total fat content [60]. PUFA levels in Spirulina ranged from 1.5 to 2.0% of total fat [68], whereas PUFA content represented 30% of the total fat content [69]. Another study reported that the FA profile of Spirulina contains sapienic acid (2.25 mg/100 g), linoleic acid (16.7%) and γ-linolenic acid (14%) [70]. According to Liestianty et al. [71], the FA of Spirulina encompasses myristic, heptadecanoic, stearic, oleic, palmitoleic, omega-3, omega-6, linoleic and palmitic acids. According to Al-Dhabi and Valan Arasu [70], myristic, stearic and eicosadienoic acids were the predominant saturated FA in Spirulina. Spirulina is the only food source that contains large amounts of essential FA, especially γ-linolenic acid. Finally, the FA profile of Spirulina samples is highly dependent on the fermentation process; thus, by selecting the most appropriate pre-treatment conditions desirable, changes in the FA profiles may be achieved.

3. Conclusions

All the tested LAB strains were suitable for Spirulina fermentation, and the lowest pH value (4.10) was obtained after 48 h of SSF with *Levilactobacillus brevis* No. 173. Changes in the pigments of Spirulina occurred during the fermentation process, and all the analyzed factors and their interactions were significant regarding the color's Spirulina a* coordinate. The main FA in non-treated and fermented Spirulina samples were methyl palmitate (C16:0), methyl linoleate (C18:2) and gamma-linolenic (C18:3ɣ) acid methyl esters. Likewise, changes in the FA profile of the Spirulina were detected throughout the fermentation

processes. Moreover, fermentation increased glutamic acid and GABA concentrations in Spirulina samples, and the highest GABA concentration was found in 48 h SMF with *Lacticaseibacillus paracasei* No. 244 (286.5 mg/kg) and in 48 h SSF with *Lacticaseibacillus paracasei* No. 244 (2395.9 mg/kg). Furthermore, putrescine (PUT) and spermidine (SPRMD) were the main BA in fermented Spirulina samples. In addition, significant correlations were found between BA concentration and GABA and glutamic acid. Spirulina samples where the highest GABA concentrations were found also showed the highest content of BA. Such correlation underlines the importance to study not only functional compounds but also potentially undesirable substances simultaneously, especially when they are involved in similar decarboxylation pathways of the amino acids.

4. Materials and Methods

4.1. Spirulina and Lactic Acid Bacteria Strains Used in Experiments and Fermentation Conditions

Lyophilized Spirulina powder (*Arthrospira platensis*) (content per 100 g: sodium 1.1 g, total carbohydrates 30.3 g, proteins 60.6 g, calcium 151.5 mg, potassium 1.7 mg, iron 48.5 mg) was provided by Now Foods Company (Bloomingdale, IL, USA).

The LAB strains (Lactiplantibacillus plantarum No. 122; Lacticaseibacillus casei No. 210; Lactobacillus curvatus No. 51; Lacticaseibacillus paracasei No. 244; Lactobacillus coryniformis No. 71; Pediococcus pentosaceus No. 183; Levilactobacillus brevis No. 173; Pediococcus acidilactici No. 29; Leuconostoc mesenteroides No. 225; Liquorilactobacillus uvarum No. 245) were acquired from the Lithuanian University of Health Sciences collection (Kaunas, Lithuania). Before the experiment, LAB strains were incubated and multiplied in De Man, Rogosa, and Sharpe (MRS) broth culture medium (Biolife, Milano, Italy) at 30 °C under anaerobic conditions for 24 h. A total of 3 mL of fresh LAB grown in MRS broth (average cell concentration of 9.0 $\log_{10}$ CFU/mL) was inoculated in 100 mL of Spirulina media (for SMF, Spirulina powder was mixed with sterilized water, in a ratio of 1:20 *w*/*w*, whereas for the SSF Spirulina/water, the ratio was 1:2 *w*/*w*)—thus giving rise to 3% (*v*/*w*) of purified LAB strain per Spirulina–water mixture.

Afterward, the algae samples were fermented under anaerobic conditions in a chamber incubator (Memmert GmbH Co. KG, Schwabach, Germany) for 24 and 48 h, at 30 °C. Non-fermented samples (mixed with sterilized water in appropriate proportions for SMF and SSF) were analyzed as a control. Before and after fermentation, the pH, color coordinates, glutamic acid, GABA, BA and FA concentrations of the samples were analyzed. The experimental design is schematized in Figure 6.

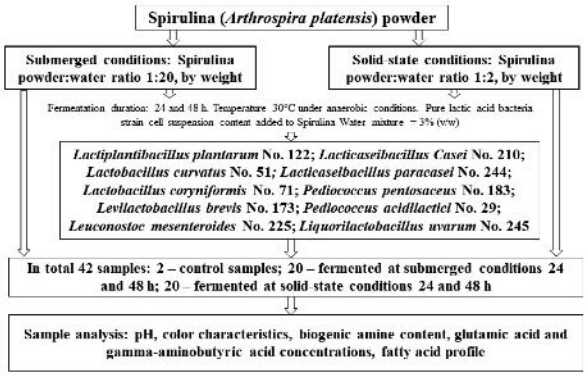

Figure 6. Schematic representation of the experimental design in this research study.

4.2. Analysis of pH and Color Coordinates (L, a* and b*) in the Spirulina Samples*

The pH of Spirulina samples was evaluated with a pH meter (Inolab 3, Hanna Instruments, Venet, Italy) by inserting the pH electrode into the algae samples. The color coordinates of the Spirulina samples were evaluated on the surface using the CIE L*a*b* system (CromaMeter CR-400, Konica Minolta, Marunouchi, Tokyo, Japan) [72].

4.3. Evaluation of L*-Glutamic Acid (L-Glu) and Gamma-Aminobutyric Acid (GABA) Concentration in Spirulina Samples*

First, 0.5 g of seaweed samples was extracted in 50 mL of Milli-Q water for 10 min using an overhead shaker. The samples were incubated for 30 min at 60 °C in a water bath. Then, the tubes were cooled down and centrifuged at 4500 rpm for 10 min. A 1 mL aliquot of the supernatant was transferred into 15 mL polypropylene test tubes and diluted with 9 mL of Milli-Q water. Finally, samples were filtered and transferred to a 2 mL autosampler vial. Analysis was performed on a TSQ Quantiva MS/MS coupled to Thermo Scientific Ultimate 3000 HPLC instrument (Thermo Scientific, Waltham, MA, USA). Chromatographic separation was carried out on a Luna Omega Polar C18 (2.1 × 100 mm, 3.0 µm) column at 40 °C using an injection volume of 5 µL. The mobile phase consisted of a 0,5 mM ammonium acetate solution in Milli-Q water (eluent A) and methanol (eluent B). A flow rate of 0.2 mL/min was used. The following gradient conditions were applied: 0.00 min, 1% B (99% A); 1.00 min, 1.0% B (99% A); 6.00 min, 99% B (1% A); 7.50 min, 99% B (1% A); 8.00 min, 1% B (99% A); 10.00 min, 1% B (99% A). LC-MS interface conditions for the ionization of GABA and L-Glu in the positive ESI mode were as follows: needle voltage + 4500 V; sheath gas 60 Arb; aux gas 25 Arb; sweep gas 5 Arb; ion transfer tube temperature 200 °C; vaporizer temperature 350 °C. The main fragments were identified using the selected reaction monitoring (SRM), with the following ionic transitions: GABA (m/z 104 > m/z 45.151, CE 25.72 V; m/z 104 > 69.165, CE 15.92 V; m/z 104 > m/z 87.36, CE 10.66 V); L-Glu (m/z 148 > m/z 56.05, CE 30 V; m/z 148 > m/z 84, CE 30 V). Method recovery ranged from 59% to 112% for GABA and from 58% to 152% for L-Glu. Method repeatability ranged from 5% to 23% for GABA and from 1% to 20% for L-Glu. The results were obtained in some rounds of experiments on different days.

4.4. Analysis of Biogenic Amine (BA) Concentration in the Spirulina Samples

Sample preparation and determination of the BAs, including tryptamine (TRP), phenylethylamine (PHE), putrescine (PUT), cadaverine (CAD), histamine (HIS), tyramine (TYR), spermidine (SPRMD) and spermine (SPRM), in Spirulina samples was conducted by following the procedure reported by Ben-Gigirey et al. [73] with some modifications. Briefly, the standard BA solutions were prepared by dissolving known amounts of each BA (including internal standard) in 20 mL of deionized water. The extraction of BA in samples (5 g) was performed by using 0.4 mol/L perchloric acid. The derivatization of sample extracts and standards was performed using dansyl chloride solution (10 mg/mL) as a reagent. The chromatographic analyses were carried out using a Varian ProStar HPLC system (Varian Corp., Palo Alto, CA, USA) with two ProStar 210 pumps, a ProStar 410 auto-sampler, a ProStar 325 UV/VIS Detector and Galaxy software (Agilent, Santa Clara, CA, USA) for data processing. For the separation of amines, a Discovery ® HS C18 column (150 × 4.6 mm, 5 µm; SupelcoTM Analytical, Bellefonte, PA, USA) was used. The eluents were ammonium acetate (A) and acetonitrile (B), and the elution program consisted of a gradient system with a 0.8 mL/min flow-rate. The detection wavelength was set to 254 nm, the oven temperature was 40 °C, and samples were injected in 20 µL aliquots. The target compounds were identified based on their retention times in comparison to their corresponding standards.

4.5. Analysis of Fatty Acid (FA) Profile in the Spirulina Samples

The extraction of lipids for fatty acids (FA) analysis was performed with chloroform/methanol (2:1 v/v), and FA methyl esters (FAME) were prepared according to Pérez-

Palacios et al. [74]. The fatty acid composition of the Spirulina samples was identified using a gas chromatograph GC-2010 Plus (Shimadzu Europa GmbH, Duisburg, Germany) equipped with Mass Spectrometer GCMS-QP2010 (Shimadzu Europa GmbH, Duisburg, Germany). Separation was carried out on a Stabilwax-MS column (30 m length, 0.25 mmID, and 0.25 μm df) (Restek Corporation, Bellefonte, PA, USA). Oven temperature program started at 50 °C, then increased at a rate of 8 °C/min to 220 °C, held for 1 min at 220 °C, increased again at a rate of 20 °C/min to 240 °C and, finally, held throughout 10 min. The injector temperature was 240 °C, interface −240 °C, and ion source 240 °C. The carrier gas was helium at a flow-rate of 0.91 mL/min. The individual FAME peaks were identified by comparing their retention times with FAME standards (Merck & Co., Inc., Kenilworth, NJ, USA).

4.6. Statistical Analysis

Fermentation of the samples was performed in duplicate, and all analytical experiments were carried out in triplicate. To evaluate a potential influence of different factors (SMF or SMF conditions, duration of fermentation, type of LAB strain used for fermentation) and their interaction on Spirulina sample characteristics, the mean of values was calculated, using the statistical package SPSS for Windows (v28.0.1.0 (142), SPSS, Chicago, IL, USA), and was compared using Duncan's multiple range test with significance defined at $p \leq 0.05$. A linear Pearson's correlation was used to quantify the strength of the relationship between the variables. The results were recognized as statistically significant at $p \leq 0.05$.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/toxins15010075/s1, Supplementary tables with experimental results. Table S1. Changes in the pH values and color coordinates (L*, a* and b*) in the Spirulina samples. Table S2. L-Glutamic acid (L-Glu) and gamma-aminobutyric acid (GABA) concentration in the spirulina samples. Table S3. Biogenic amine (BA) content (mg/kg) in the Spirulina samples. Table S4. Influence of the analyzed factors and their interaction on biogenic amine (BA) concentration in the Spirulina samples. Table S5. Fatty acid (FA) profile in the Spirulina samples. Table S6. Influence of the analyzed factors and their interaction on fatty acid (FA) content in the Spirulina samples. Table S7. Classification of fatty acids (FA) in the Spirulina samples.

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Submerged and Solid-State Fermentation of Spirulina with Lactic Acid Bacteria Strains: Antimicrobial Properties and the Formation of Bioactive Compounds of Protein Origin

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Simple Summary: Spirulina (Arthrospira platensis) is an edible blue-green alga that shows many desirable physiological activities in humans and animals. In this study, we hypothesized that the Spirulina composition can be improved (by increasing the gamma-aminobutyric acid concentration) during biotreatment with selected lactic acid bacteria (LAB) strains. Fermentation is the most popular and typically economically effective solution in the food and feed industry and used as biotechnology for the bioconversion of materials to higher-added-value products. However, in addition to desirable compounds, LAB are involved in the processes of biogenic amine formation. This study showed that most of the fermented Spirulina samples possess exceptional antimicrobial activity against Staphylococcus. However, the ratios of biogenic amine/gamma-aminobutyric acid and biogenic amine/L-glutamic acid ranged from 0.5 to 62 and from 0.31 to 10.7, respectively. It was concluded that the formation of non-desirable compounds (biogenic amines) must also be considered due to the similar mechanism of their synthesis as well as the possibility of obtaining high concentrations in the end products.

Abstract: The aim of this study was to investigate the changes in bioactive compounds (L-glutamic acid (L-Glu), gamma-aminobutyric acid (GABA) and biogenic amines (BAs)) during the submerged (SMF) and solid-state (SSF) fermentation of Spirulina with lactobacilli strains (*Lacticaseibacillus paracasei* No. 244; *Levilactobacillus* brevis No. 173; *Leuconostoc mesenteroides* No. 225; *Liquorilactobacillus uvarum* No. 245). The antimicrobial properties of the untreated and fermented Spirulina against a variety of pathogenic and opportunistic strains were tested. The highest concentrations of L-Glu (3841 mg/kg) and GABA (2396 mg/kg) were found after 48 h of SSF with No. 173 and No. 244 strains, respectively. The LAB strain used for biotreatment and the process conditions, as well as the interaction of these factors, had statistically significant effects on the GABA concentration in Spirulina ($p \leq 0.001$, $p = 0.019$ and $p = 0.011$, respectively). In all cases, the SSF of Spirulina had a higher total BA content than SMF. Most of the fermented Spirulina showed exceptional antimicrobial activity against *Staphylococcus aureus* but not against the other pathogenic bacteria. The ratios of BA/GABA and

BA/L-Glu ranged from 0.5 to 62 and from 0.31 to 10.7, respectively. The GABA content was correlated with putrescine, cadaverine, histamine, tyramine, spermidine and spermine contents. The L-glutamic acid concentration showed positive moderate correlations with tryptamine, putrescine, spermidine and spermine. To summarize, while high concentrations of desirable compounds are formed during fermentation, the formation of non-desirable compounds (BAs) must also be considered due to the similar mechanism of their synthesis as well as the possibility of obtaining high concentrations in the end products.

Keywords: spirulina; L-glutamic acid; gamma-aminobutyric acid; biogenic amines; fermentation; lactic acid bacteria (LAB)

1. Introduction

Arthrospira platensis is an edible blue-green alga that shows beneficial activities in humans and animals [1]. Spirulina is cultivated worldwide as a fundamental ingredient in many nutraceutical formulations [2]. This alga contains high protein content, which includes all essential amino acids. Additionally, it contains valuable essential fatty acids, minerals, pigments, carotenoids and vitamins [3]. The probiotic and antioxidant properties of Spirulina have been widely reported [3–5]; therefore, Spirulina is used as a nutritional supplement in the human diet, as well as for animal nutrition, just to prevent gut dysbiosis and pathogen colonization [3]. The United States Food and Drug Administration (FDA) granted Spirulina the "Generally Recognized as Safe (GRAS)" status [4]. Moreover, Spirulina is a safe ingredient when grown under controlled conditions [4,6–10]. There is scientific evidence attesting to Spirulina's hypolipemic, antihypertensive, antidiabetic, neuroprotective, antianemic, anticarcinogenic, hepatoprotective, antibacterial, antiviral and immunomodulatory properties [7,9–12]. In this study, we hypothesized that the Spirulina composition can be improved during biotreatment with selected lactic acid bacteria (LAB) strains. Fermentation is the most popular and typically economically effective solution in the food and feed industry and is used as biotechnology for the bioconversion of materials to higher-added-value products. Solid-state fermentation (SSF) consists of microbial growth and product formation on solid particles in the absence of water. This technology is more economical compared with the traditional method of biomass cultivation in a liquid medium containing nutrients.

Biotreatment/biotransformation with LAB is a popular solution to degrade plant and cyanobacterial cell walls and to produce smaller molecules with enhanced (immunomodulatory, antioxidant, antimicrobial, etc.) properties [13–15]. Additionally, via peptide bond hydrolysis, LAB proteases yield bioactive peptides with multiple health benefits [16].

Recently, the production of amino acids via a sustainable microbial approach (fermentation or enzymatic treatment) has gained interest [17,18]. However, the use of genetically modified microorganisms has been a major concern in the food and feed sectors [19]. This has led to the search for new (bio)technological starters. It was reported that wild-type LAB have the potential for the synthesis of various amino acids [20,21]. Lactic acid bacteria show economic advantages at the industrial scale and are generally recognized as safe microorganisms [17]. However, LAB multiplication in an environment that contains inorganic nitrogen is poor. Additionally, they often require an exogenous supply of nutrients (peptides and amino acids) to ensure their viability [22]. Many studies have concluded that the proteolytic system of LAB is important in the utilization of both proteins and peptides, and this enzymatic system activity can be designed by modeling the environmental and growth conditions [20,21,23,24].

Another compound that can be formed during protein metabolism is gamma-aminobutyric acid (GABA). Usually, GABA is enzymatically produced from L-glutamic acid (L-Glu) by glutamate decarboxylase [20]. This compound (GABA) has multiple physiological functions [20,24–26]. It was reported that many types of microorganisms can synthesize

GABA [27,28], and LAB are very good candidates for GABA production. Additionally, LAB can excrete various antimicrobial compounds to the fermentable substrate/medium [29], simply by improving the multifunctional properties of the fermentable substrate. Although many LAB strains have been identified as good GABA producers, this process is strain-specific. Additionally, the specific processing conditions are important in this synthesis. Therefore, optimizing the technological conditions has become a very important approach for effective GABA synthesis.

In addition to desirable compounds, LAB are involved in the processes of biogenic amine (BA) formation. Biogenic amines are involved in several pathogenic syndromes [30]. However, their toxicity is related to the type of BA and the individual sensitivity of the person [31]. The most toxic BAs are tyramine (TYR) and histamine (HIS) [32,33]. However, the presence of 2-phenylethylamine (PHE), putrescine (PUT), cadaverine (CAD), agmatine (AGM), spermine (SPRM) and spermidine (SPRD) can lead to toxicity, because these BAs can potentiate the effects of histamine and tyramine toxicity [34]. Finally, high concentrations of BAs can have toxicological consequences for both humans and animals.

The aim of this study was to investigate the changes in bioactive compounds of proteinaceous origin (L-glutamic acid, GABA and BAs) in the submerged (SMF) and solid-state (SSF) fermentation of Spirulina with lactobacilli strains (*Lacticaseibacillus paracasei* No. 244; *Levilactobacillus brevis* No. 173; *Liquorilactobacillus uvarum* No. 245) and *Leuconostoc mesenteroides* No. 225. Taking into consideration that these strains previously showed a broad spectrum of antimicrobial activities, the antimicrobial properties of untreated (non-fermented) and fermented Spirulina against a variety of pathogenic and opportunistic strains (*Staphylococcus aureus*, *Escherichia coli*, *Acinetobacter baumannii*, *Staphylococcus haemolyticus*, *Salmonella enterica*, *Bacillus cereus*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Enterococcus faecium* and *Pseudomonas aeruginosa*) were tested.

2. Materials and Methods

2.1. Spirulina, Microorganisms and Algae Fermentation Conditions

Lyophilized Spirulina (*Arthrospira platensis*) powder (in 100 g: total carbohydrates 30.3 g, proteins 60.6 g, Na 1.1 g, Ca 151.5 mg, K 1.7 mg, Fe 48.5 mg) was purchased from Now Foods Company (Bloomingdale, IL, USA).

Characteristics of the used LAB strains (*Lacticaseibacillus paracasei* No. 244; *Levilactobacillus brevis* No. 173; *Leuconostoc mesenteroides* No. 225; *Liquorilactobacillus uvarum* No. 245) are reported by Bartkiene et al. [29].

The experimental design used in the current study is schematized in Figure 1.

For SMF, Spirulina powder was mixed with sterilized water in a ratio of 1:20 w/w, and for SSF, the Spirulina/water ratio was 1:2 w/w. The LAB strains were multiplied in MRS (De Man, Rogosa, and Sharpe) broth culture medium (Biolife, Milano, Italy) at 30 °C under anaerobic conditions for 24 h. A total of 3 mL of multiplied LAB [9.0 $\log_{10}$ CFU/mL] was inoculated in 100 mL of Spirulina. Afterward, the Spirulina samples were fermented under anaerobic conditions in a chamber incubator (Memmert GmbH Co. KG, Schwabach, Germany) for 24 and 48 h at 30 °C. Non-fermented samples were analyzed as controls. Before the analysis, non-fermented Spirulina was mixed with sterilized water in appropriate proportions for SMF and SSF conditions.

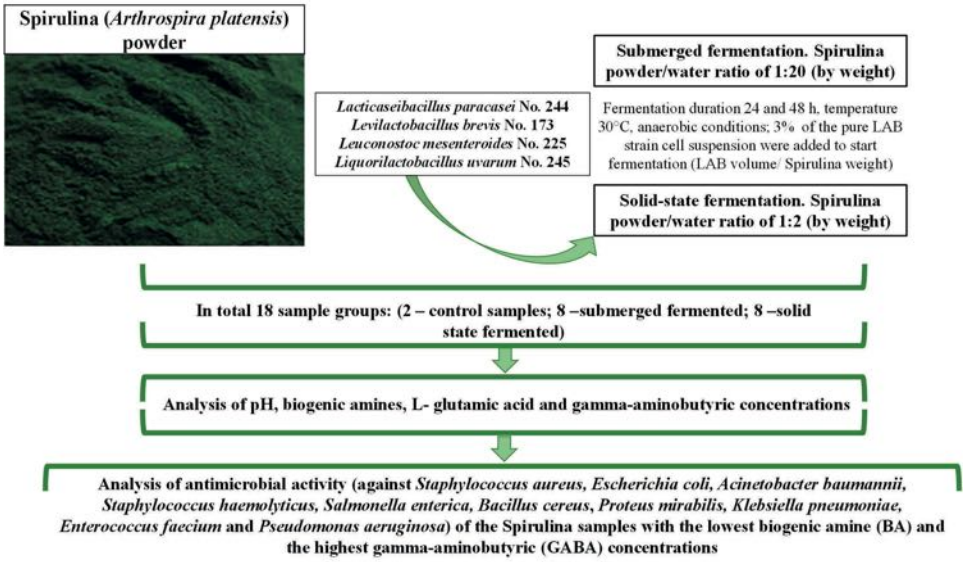

Figure 1. Experimental design.

2.2. Evaluation of pH and Lactic acid Bacteria (LAB) Counts in the Spirulina Samples

The pH of Spirulina samples was evaluated with a pH meter (Inolab 3, Hanna Instruments, Villafranca Padovana, Italy) by inserting the pH electrode into the algal samples.

For the evaluation of LAB counts ($\log_{10}$ CFU/mL) in Spirulina samples, MRS agar (CM0361, Basingstoke, UK) was used.

2.3. Evaluation of the Concentration of L-Glutamic Acid (L-Glu) and Gamma-Aminobutyric (GABA) Acid in Spirulina Samples

The evaluation of the concentrations of L-Glu and GABA acid in Spirulina samples was performed on a TSQ Quantiva MS/MS coupled to a Thermo Scientific Ultimate 3000 HPLC instrument (Thermo Scientific, Waltham, MA, USA). Analysis is given in detail in Supplementary File S1.

2.4. Evaluation of the Concentration of Biogenic Amines (BAs) in Spirulina Samples

The determination of the BAs in Spirulina was conducted using the method of Ben-Gigirey et al. (1998) [35], with some modifications (described in Supplementary File S2).

2.5. Evaluation of the Antimicrobial Activity of Spirulina Samples

All algal samples were assessed for their antimicrobial activities against a variety of pathogenic and opportunistic wild bacterial strains previously isolated from humans and animals in the Lithuanian University of Health Sciences (*Staphylococcus aureus, Escherichia coli, Acinetobacter baumannii, Staphylococcus haemolyticus, Salmonella enterica, Bacillus cereus, Proteus mirabilis, Klebsiella pneumoniae, Enterococcus faecium* and *Pseudomonas aeruginosa*) by using the agar well-diffusion method.

For the agar well-diffusion assay, suspensions of 0.5 McFarland standard of each pathogenic bacterial strain were inoculated onto the surface of cooled Mueller–Hinton agar (Oxoid, Basingstoke, UK) using sterile cotton swabs. Wells with 6 mm diameters were punched in the agar and filled with 50 μL of the Spirulina samples (mixture of Spirulina

powder and sterilized water). The antimicrobial activities against the tested bacteria were established by measuring the inhibition zone diameters (mm). The experiments were repeated three times, and the average diameter of the inhibition zones was calculated.

2.6. Statistical Analysis

The biotreatment of Spirulina was performed in duplicate, and all analytical experiments were carried out in triplicate. To evaluate the potential influences of different factors (SMF or SMF conditions, duration of fermentation, type of LAB strain used for fermentation) and their interactions on sample characteristics, data were compared using Duncan's multiple range test with significance defined at $p \leq 0.05$ using the IBM SPSS Statistics for Windows, v28.0.1.0 (142) (SPSS, Chicago, IL, USA). Pearson linear correlation was used to quantify the strength of the relationship between the variables. The results were recognized as statistically significant at a significance level of $p \leq 0.05$.

3. Results

3.1. Effectiveness of Submerged (SMF) and Solid-State (SSF) Fermentation of Spirulina

The average pH values of non-fermented samples, i.e., control (I) (Spirulina powder–water mixture (1:20 w/w)) and control (II) (Spirulina powder–water mixture (1:2 w/w)), were 6.85 and 6.33, respectively. The pH and viable LAB counts in fermented samples (SMF and SSF) of Spirulina are shown in Figure 2. Among all fermented Spirulina samples, the lowest pH was obtained in samples of 48 h SSF with the No. 173 strain (4.10); however, the highest viable LAB counts were obtained in samples of 24 and 48 h SMF with the No. 225 strain and 48 h SMF and SSF with the No. 245 strain (on average, 9.44 $\log_{10}$ CFU/g).

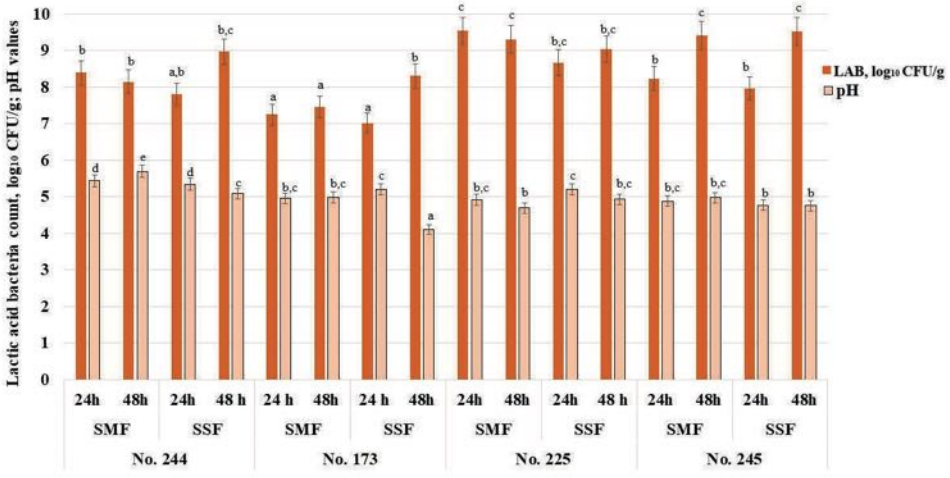

Figure 2. Spirulina pH and viable lactic acid bacteria counts ($\log_{10}$ CFU/g). No. 244—fermented with *Lacticaseibacillus paracasei* No. 244 strain; No. 173—fermented with *Levilactobacillus brevis* No. 173 strain; No. 225—*Leuconostoc mesenteroides* No. 225 strain; No. 245—fermented with *Liquorilactobacillus uvarum* No. 245 strain; LAB—lactic acid bacteria; CFU—colony-forming units; SMF—submerged fermentation; SSF—solid-state fermentation. Data are represented as means ($n = 6$) ± standard errors. $^{a–e}$ Mean values denoted with different letters indicate significantly different values between the columns ($p \leq 0.05$).

Comparing the results of 24 h fermentation with the same strain and the two types of fermentation (SMF and SSF samples), significant differences in pH values were not established. Significant differences in the viable LAB counts in samples after 24 h of fermentation were also not found. The highest viable LAB counts in Spirulina samples were obtained in SMF and SSF with No. 225 strain (on average, 9.28 $\log_{10}$ CFU/g). In the comparison of SMF and SSF samples after 48 h, significant differences in viable LAB counts between groups fermented with No. 244, No. 255 and No. 245 were not found, and viable LAB counts in these samples were, on average, 8.55, 9.17 and 9.47 $\log_{10}$ CFU/g, respectively. However, samples fermented with No. 173, after 48 h of SSF, showed higher LAB numbers (on average, 11.4% higher in comparison with SMF samples).

Additionally, the pH values of the Spirulina samples did not differ after 48 h of fermentation with the same LAB strain under different fermentation conditions (SMF or SSF). The effects of the analyzed factors and their interactions on sample pH and LAB count were not significant (Supplementary File S3, Table S1).

The high viable LAB count and the low pH of the medium are among the most important characteristics of fermented products [36,37]. The fermentation process is strongly influenced by the concentration of fermentable sugars in the substrate [37,38]. The main fermentable carbohydrates in Spirulina are glucose, ribose, galactose, xylose and mannose [39]. Therefore, Spirulina is a suitable material for LAB fermentation, without the need for additional carbon source enrichment [40]. It was reported that the initial pH of *Spirulin*a is, on average, 6.2, and the pH may decline in fermented Spirulina to values as low as 2.9–3.1 because of the organic acids produced during fermentation by LAB, in addition to other metabolites [41–43]. In our study, we obtained slightly higher values of pH, as mentioned previously, but it must be emphasized that acidification rates and LAB growth are strain-dependent [44]. The selection of the most appropriate technological starter strain is a critical step in the development of fermented products [44,45]. *Lactiplantibacillus plantarum*, *Lacticaseibacillus casei*, *Lacticaseibacillus rhamnosus* and *Bacillus* strains are popular starters for Spirulina fermentation [40–43,46] because of their probiotic properties and good technological characteristics for fermenting Spirulina [36,45,47]. The LAB strains used in this study previously showed a good capacity to ferment sugars found in Spirulina [29]. The high numbers of LAB in fermented Spirulina are desirable since they give the product additional probiotic properties [44]. This study showed that the analyzed factors and their interactions did not have statistically significant effects on the viable LAB counts or pH values of Spirulina samples. Finally, according to the results obtained, all of the used LAB strains showed a good capacity to ferment Spirulina without any enrichment with an additional carbohydrate source.

3.2. Evaluation of the Concentration of L-Glutamic (L-Glu) and Gamma-Aminobutyric (GABA) Acids in Spirulina Samples

L-Glutamic acid and gamma-aminobutyric acid concentrations in Spirulina samples are presented in Table 1. Comparing all samples, the highest concentration of L-glutamic acid was found in 48 h SSF samples with the No. 173 strain (3841 mg/kg), and this concentration was, on average, 40.2% higher than that found in control (II) samples. Comparing the L-glutamic concentration in 24 h SMF samples with control (I), different tendencies were found: in two sample groups, the L-glutamic acid concentration increased (47.1% on average in SMF samples with No. 244 and No. 225 strains); in contrast, in another two samples, a decrease was observed (93.8% on average in SMF samples with the No. 173 strain, and 90.3% on average in SMF samples with the No. 245 strain). After 48 h of SMF in samples fermented with No. 244 and No. 255, the L-glutamic acid concentration increased by 176 and 22.2% on average, respectively, in comparison with samples after 24 h of fermentation. However, in samples fermented with No. 173 and No. 245 strains, a decrease in the L-glutamic acid concentration was established (10.8 and 1.3 times on average, respectively).

Table 1. Concentrations of L-glutamic (L-Glu) and gamma-aminobutyric (GABA) acids in the Spirulina samples.

Spirulina Samples	Fermentation		L-Glutamic Acid, mg/kg	Gamma-Aminobutyric Acid, mg/kg
	Duration, h	Conditions		
Control (I)	-		242 ± 14.8^{f}	2.01 ± 0.18^{a}
Control (II)	-		2296 ± 59.3^{k}	17.2 ± 0.20^{c}
Lacticaseibacillus paracasei No. 244	24 h	SMF	15.1 ± 1.30^{a}	213 ± 15.0^{l}
	48 h		41.7 ± 3.20^{e}	287 ± 21.3^{h}
	24 h	SSF	1784 ± 24.3^{j}	2016 ± 46.5^{j}
	48 h		572 ± 21.2^{i}	2396 ± 38.6^{k}
Levilactobacillus brevis No. 173	24 h	SMF	357 ± 30.6^{h}	12.0 ± 0.10^{b}
	48 h		33.2 ± 2.10^{d}	187 ± 10.3^{g}
	24 h	SSF	3302 ± 44.9^{n}	22.6 ± 1.32^{d}
	48 h		3841 ± 37.5^{o}	58.8 ± 4.30^{e}
Leuconostoc mesenteroides No. 225	24 h	SMF	23.4 ± 2.14^{b}	$170 \pm 11.2^{f,g}$
	48 h		28.6 ± 2.12^{c}	162 ± 12.1^{f}
	24 h	SSF	2597 ± 51.8^{l}	1264 ± 47.5^{i}
	48 h		3209 ± 43.0^{n}	$200 \pm 15.0^{g,h}$
Liquorilactobacillus uvarum No. 245	24 h	SMF	356 ± 15.7^{h}	53.6 ± 3.1^{e}
	48 h		280 ± 11.0^{g}	225 ± 11.2^{l}
	24 h	SSF	2621 ± 58.4^{l}	217 ± 8.9^{l}
	48 h		2908 ± 60.1^{m}	165 ± 10.1^{f}

Control (I)—Spirulina powder and water mixture, 1:20 *w*/*w*; control (II)—Spirulina powder and water mixture, 1:2 *w*/*w*; SSF—solid-state fermentation; SMF—submerged fermentation. Data are represented as means ($n = 6$) ± standard errors. $^{a–o}$ Mean values denoted with different letters indicate significantly different values between the lines ($p \leq 0.05$).

Comparing the L-glutamic acid concentration in 24 h SSF samples with control (II), it was revealed that in three out of four sample groups, the L-glutamic content was higher (in SSF samples with No. 173, No. 225 and No. 245 strains: on average, 43.8, 13.1 and 14.2% higher, respectively), and it was on average 22.3% lower in SSF samples with the No. 244 strain. Additionally, when increasing the duration of fermentation, the same trends were seen. Specifically, in the comparison of L-glutamic acid concentrations after 24 and 48 h SSF with No. 173, No. 225 and No. 245 strains, the L-glutamic acid concentration was on average 16.3, 23.6 and 11.0% higher, respectively, and it was on average 67.9% lower in 48 h SSF samples with the No. 244 strain.

In the comparison of SMF and SSF sample groups, after 24 h of fermentation, higher L-glutamic acid concentrations were found in SSF samples in all cases. The same tendencies were established after 48 h of fermentation.

L-Glutamic acid is a very important brain neurotransmitter largely produced through microbial fermentation [48]. Various microorganisms have the capacity to excrete L-glutamic acid. LAB strains, which are very common microbiological starter cultures in food and feed fermentation [49,50], have a gene responsible for glutamic acid production [51]. Therefore, fermentation with LAB is the most appropriate process for glutamic acid production, because it is considered safe and eco-friendly. However, appropriate LAB strains with desirable metabolic capacities should be selected. Additionally, fermentation conditions constitute a key factor. The current research study showed that the fermentation condition (SMF or SSF) was a significant factor in the L-glutamic acid concentration in Spirulina samples ($p \leq 0.0001$) (Supplementary File S3, Table S2). It was reported that glucose is the

most appropriate carbon source for glutamic acid production [52], and Spirulina is good source of this sugar [39]. This could be the reason for the observed production of glutamic acid during Spirulina fermentation. It was stated that glutamic acid can be produced by bacteria from glucose via the Krebs cycle [53,54]. Therefore, the presence of glucose in the fermentable substrate is a very important factor for glutamic acid production by LAB. From the above, one may conclude that not only are the fermentation conditions and the type of microbial starters important factors for glutamic acid production, but the use of a substrate rich in glucose is also crucial. Additionally, the pH of the fermentable substrate is important for glutamic acid production [55]. It was reported that the maximum glutamic acid production can be obtained at a lower pH value (4.5) [56]. However, in our study, higher pH values were obtained, and this can be hypothetically explained by the fact that some of the LAB strains can excrete ammonia in an acidic environment, thus contributing to the survival of the microorganisms through pH neutralization [57]. It is important to emphasize that ammonia can reduce glutamic acid production [56]. Yet, it was reported that the production of glutamic acid is mostly dependent on the activity of bacterial cytoplasmic glutamate dehydrogenase [58]. Finally, further investigations are needed to discover the mechanisms involved in glutamic acid production by the LAB strains used in this experiment.

In the comparison of all sample groups, the highest concentration of GABA was found in 48 h SSF samples with the No. 244 strain (on average, 139 times higher than in control (II) samples). Comparing the GABA concentration in the 24 h SMF samples with control (I), in all cases, the GABA concentration in fermented samples increased (in samples with No. 244, No. 173, No. 225 and No. 245, it increased on average by 106, 6, 84.6 and 26.8 times, respectively). Additionally, in most cases, after 48 h of SMF, the GABA concentration increased, except in 48 h SMF samples with the No. 225 strain.

When comparing the GABA concentration in 24 h SSF samples with control (II), we found the same tendencies as those in SMF samples. However, the group of SSF samples with the No. 173 strain showed the lowest GABA content increase—viz., on average 31.4% higher in comparison with control (II).

In the comparison of the GABA concentration after 24 and 48 h of SSF, in sample groups fermented with No. 244 and No. 173 strains, the GABA content increased (on average by 18.8 and 61.6%, respectively), and, in contrast, in sample groups fermented with No. 255 and No. 245 strains, the GABA content decreased (on average by 84.2 and 3.6%, respectively). Moreover, comparing SMF and SSF sample groups after 24 h of fermentation, in all cases, higher GABA concentrations were found in SSF samples. However, after 48 h, different tendencies were observed: in two samples, after 48 h of SSF, the GABA content increased in comparison with SMF samples (in SSF with No. 173 and No. 245 strains), whereas in two samples, the GABA content decreased (in SSF with No. 244 and No. 225 strains).

In contrast to chemical synthesis, biological GABA production using technological microorganisms is safer and more eco-friendly [59–61]. There are many LAB species that possess the capacity to produce GABA [28,62–76], although the GABA production effectiveness of different LAB strains varies greatly [20]. These tendencies can be seen in our current study as well.

The parameters for the GABA production process can be easily controlled [77]. In technological LAB strains, glucose metabolism produces numerous metabolites, one of which is GABA [78]. However, during this process, GABA can be degraded by γ-aminobutyric acid aminotransferase and semialdehyde dehydrogenase [79]. It was reported that GABA-producing strains were isolated from common fermented food and beverages [80–87]. In this study, LAB were isolated from spontaneous bread sourdough, and some of them showed the potential to produce GABA. Lactic acid bacteria, as economically viable technological microorganisms, are the most studied for GABA production [77]. However, a number of factors (temperature, pH, duration of the process, etc.) can significantly affect the GABA content. It was reported that the optimal temperature for GABA synthesis is 30 °C [87]. However, in another study, the optimal temperature for GABA synthesis

was established to be 37 °C [88]. Regarding the optimal pH value, it was found to be 3.5–5 for GABA production by *Lev. brevis* [61], whereas in another study [86], the optimal pH for GABA production by *Enterococcus faecium* was set at 7.74. The influence of pH on GABA production is explained by the optimal pH values for the activity of glutamic acid decarboxylase (GAD) (pH 4.5). In fact, this enzyme in LAB is only active under acidic conditions, and when pH is above 5, GAD loses its activity [8,22]. Note that the optimal pH for fermentation by different LAB strains varies [61,86]. Our study showed that the LAB strain used for fermentation and the fermentation conditions (SMF or SSF), as well as the interaction between these factors, had statistically significant effects on the GABA concentration in Spirulina samples ($p \leq 0.001$, $p = 0.019$ and $p = 0.011$, respectively). Additionally, taking into consideration that LAB possess not only glutamic acid decarboxylase but also other decarboxylases, BA formation was analyzed in fermented Spirulina samples, because these compounds are usually non-desirable in food, although they can be applied in the pharmaceutical industry.

3.3. Evaluation of the Concentrations of Biogenic Amines (BA) in Spirulina Samples

The biogenic amine (BA) concentrations in Spirulina samples are shown in Figure 3, and all of the tested BAs are given in Tables S1 and S2 in Supplementary File S4. Phenylethylamine was not found in Spirulina samples. Cadaverine was found in 5 samples (control (I), control (II), 24 and 48 h SSF with the No. 244 strain, and 24 h SSF with the No. 225 strain) and histamine was detected in 4 samples (24 and 48 h SSF with the No. 244 strain, 48 h SMF with the No. 173 strain, and 24 h SSF with the No. 225 strain) out of 18 samples (2 controls and 16 fermented samples) (Supplementary File S4, Tables S1 and S2).

Cadaverine is formed during the direct decarboxylation of L-lysine through the diaminopimelic acid route in bacteria [89,90]. The direct decarboxylation of L-lysine is catalyzed by lysine decarboxylase in microbial starter cultures [91]. Cadaverine possesses multiple bioactivities [91] and plays a key role in cell survival under acidic conditions [92,93]. Due to its broad functional properties, cadaverine has a huge potential to be applied in agriculture, as well as in medicine [91].

Histamine has been confirmed as cytotoxic [94], and its synergistic effect with tyramine was also recognized [95]. The maximum legal limits of histamine have been established in fish and fish products (200–400 mg/kg, established by the European Union (EU) Commission (EC) Directives 2073/2005 [96], and 50 mg/kg, established by the US Food and Drug Administration (FDA) [97]). During fermentation, histamine is produced by certain LAB, which possess histidine decarboxylase activities [98,99]. The decarboxylation of amino acids is a proton-consuming reaction that may provide acid resistance to some microorganisms [100,101]. These findings suggest that pH is involved in amino acid decarboxylation via enzymatic activity or gene expression [102]. Histamine accumulation is also influenced by other factors, such as temperature, salt concentration, etc. [103,104].

Tryptamine was found in nine out of the sixteen analyzed fermented samples, and its content was below 10 mg/kg of the sample (Figure 3a). However, in all cases, tryptamine was formed in SSF samples (after 24 and 48 h of fermentation). All analyzed factors and their interactions had statistically significant effects on tryptamine formation in Spirulina samples (Supplementary File S3, Table S3). Tryptamines are medicinally important molecules that serve as precursors to clinically used indole alkaloid natural products [105]. Tryptamine is produced in a single step via tryptophan decarboxylation [99]. The European Food Safety Authority (EFSA) recognizes tryptamine as a potentially harmful BA in foods [106]. At high concentrations, tryptamine can accumulate in fish sauces, certain fish and fish products, dairy products and certain fermented meat products, such as fermented sausages [106]. However, regarding tryptamine accumulation in fermented Spirulina, the data are scarce. Dietary tryptamine can have harmful effects on humans [106–108]. Tryptamine can increase the toxicity of histamine [99,109]. The EFSA panel on Biological Hazards (BIOHAZ) highlighted that the lack of knowledge prevents any reliable quantitative or qualitative risk assessment of tryptamine in foods. However, taking into

consideration the toxic effect of tyramine, its control in the end product is needed, especially when desirable and non-desirable compound formation is based on the same technological process like it is fermentation with LAB.

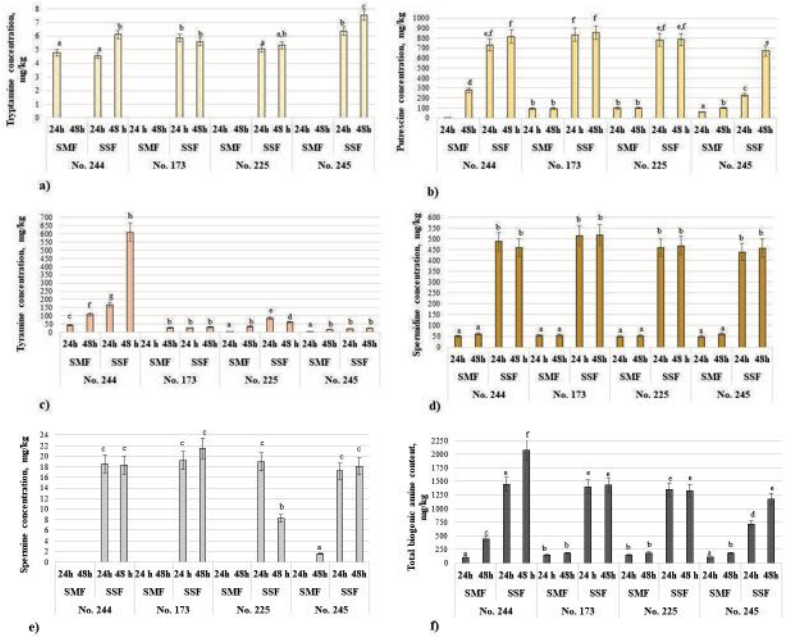

Figure 3. Biogenic amine (BA) content (mg/kg) in Spiulina samples: (**a**) tryptamine, (**b**) putrescine, (**c**) tyramine, (**d**) spermidine, (**e**) spermine, (**f**) total biogenic amine content; No. 244—fermented with *Lacticaseibacillus paracasei* No. 244 strain; No. 173—fermented with *Levilactobacillus brevis* No. 173 strain; No. 225—*Leuconostoc mesenteroides* No. 225 strain; No. 245—fermented with *Liquorilactobacillus uvarum* No. 245 strain; LAB—lactic acid bacteria; SMF—submerged fermentation; SSF—solid-state fermentation; $^{a-h}$ mean values denoted with different letters indicate significantly different values between the columns ($p \leq 0.05$); data are represented as means ($n = 6$) ± standard errors.

In all cases, fermentation increased the putrescine concentration in Spirulina samples, and when comparing all of the samples, the highest putrescine content was found in the 48 h SSF sample with the No. 173 strain (855 mg/kg) (Figure 3b). Contrasting SMF and SSF samples, in all cases, a higher putrescine concentration was found in SSF samples (in the comparison of 24 and 48 h SMF and SSF with the No. 244 strain, 125 and 2.96 times higher on average, respectively; in the comparison of 24 and 48 h SMF and SSF with the No. 173 strain, 9.02 and 8.93 times higher on average, respectively; in the comparison of 24 and 48 h SMF and SSF with the No. 225 strain, 8.11 and 7.85 times higher on average, respectively; and in the comparison of 24 and 48 h SMF and SSF with the No. 245 strain, 3.95 and 6.71 times higher on average, respectively). Moreover, all analyzed factors and their interactions had statistically significant effects on putrescine formation in Spirulina samples (Supplementary File S3, Table S3). Putrescine is synthesized via ornithine decarboxylation or agmatine deamination [110]. Additionally, it is known that putrescine is able to enhance

the toxicological effects of histamine [111]. From another point of view, putrescine is an essential BA to all living organisms and tissues [112,113].

In most cases, fermentation increased the tyramine content in Spirulina samples, except in 24 h SMF samples with No. 173 (in this sample, tyramine was absent) and No. 245 (in this sample, tyramine content was, on average, 7.86 times lower in comparison with control (I) samples) (Figure 3c, Tables S1 and S2 in Supplementary File S4). Additionally, in most cases, higher tyramine content was formed in SSF samples in comparison with SMF ones (except 48 h SSF samples with No. 173 and No. 245). All analyzed factors and their interactions had statistically significant effects on tyramine formation in Spirulina samples (Supplementary File S2, Table S3).

Both histamine and tyramine are the most toxic BAs. Furthermore, tyramine is the most abundant BA in fermented foods [114]. The European Food Safety Authority reported that 600 mg/kg tyramine in foods exerts toxic effects on health [106]. Tyramine is generated via the decarboxylation of tyrosine. As in other BAs, this reaction can be influenced by multiple factors, including bacterial activity, the pH of the substrate medium and the salt concentration [115]. The process of fermentation provides particularly high concentrations of tyramine in human nutrition [115]. Tyramine is involved in many physiological processes. However, at high concentrations, it exerts toxic effects, and thus, reliable data about the tyramine content in food and feed are also required. Tyramine-producing bacteria are very popular starters for food and fermentation [116–120]. Currently, commercial starter cultures are evaluated for their capability to generate BAs [106]. On the other hand, technological starters can produce or degrade BAs in fermentable substrate media [121]. The low pH of the substrate enhances tyramine production in a variety of LAB [116,122]. The decarboxylation of amino acids is a cellular mechanism, and the optimal activities of microbial decarboxylases are at acidic pH [123–125].

Comparing the spermidine concentrations in Spirulina samples, in all cases, SMF reduced and SSF increased this BA in Spirulina samples (Supplementary File S4, Tables S1 and S2). Concerning the spermidine concentrations in 24 and 48 h SMF and SSF samples, in all cases, higher spermidine content was found in SSF samples (in the comparison of 24 and 48 h SMF and SSF with the No. 244 strain, 9.50 and 7.44 times higher on average, respectively; in the comparison of 24 and 48 h SMF and SSF with the No. 173 strain, 9.58 and 9.53 times higher on average, respectively; in the comparison of 24 and 48 h SMF and SSF with the No. 225 strain, 9.26 and 8.95 times higher on average, respectively; and in the comparison of 24 and 48 h SMF and SSF with the No. 245 strain, 8.82 and 7.48 times higher on average, respectively) (Figure 3d). The LAB used for fermentation and the fermentation conditions (SMF or SSF), as well as the interaction between factors (LAB × SMF-SSF and LAB × duration of fermentation × SMF-SSF), had statistically significant effects on the spermidine concentration in Spirulina samples (Supplementary File S3, Table S3).

Observing the spermine content in Spirulina samples, in most of the SMF samples, spermine was not formed (except in 48 h SMF samples with the No. 245 strain), and this BA content in SSF samples ranged on average between 8.21 mg/kg (in 48 h SSF samples with the No. 225 strain) and 17.5 mg/kg (in the remaining SSF samples) (Figure 3e). Moreover, the LAB used for fermentation and the fermentation conditions (SMF or SSF), as well as the interaction between factors (LAB × SMF-SSF and LAB × duration of fermentation × SMF-SSF), had statistically significant effects on the spermine concentration in Spirulina samples (Supplementary File S3, Table S3).

Spermidine and spermine have been implicated in the protection against several age-related diseases. Still, increasing their concentrations in the diet is linked to improved health and reduced overall mortality [126]. It is admittedly important for the concentrations of spermidine and spermine in foodstuffs to maintain these BAs at optimal levels in the body [127–129]. Spermidine has general antiaging effects [130–140]. Although the contents of polyamines in various types of foods have been reported [127,141–164], there is no information about spermidine and spermine concentrations in Spirulina products.

Spermidine-rich foods are wheat germ, soybeans, select mushrooms, and various nuts and seeds [148]. Thus, the results of our study may be very important for the data basis for the spermidine and spermine contents in Spirulina products.

In all of the studied cases, higher total BA content was found in SSF Spirulina samples (after 24 and 48 h) than in SMF (in the comparison of 24 and 48 h SMF and SSF with the No. 244 strain, 13.5 and 4.65 times higher on average, respectively; in the comparison of 24 and 48 h SMF and SSF with the No. 173 strain, 9.60 and 7.99 times higher on average, respectively; in the comparison of 24 and 48 h SMF and SSF with the No. 225 strain, 8.95 and 7.10 times higher on average, respectively; and in the comparison of 24 and 48 h SMF and SSF with the No. 245 strain, 6.62 and 6.54 times higher on average, respectively) (Figure 3f).

3.4. Antimicrobial Activity of Spirulina Samples

From all of the tested opportunistic and pathogenic strains, fermented Spirulina samples showed exceptional antimicrobial activity against *Staphylococcus aureus* (Table 2). Comparing the diameters of inhibition zones (DIZs) of SMF and SSF samples, in all cases, higher antimicrobial activity was obtained in SSF samples; specifically, the diameters of inhibition zones ranged from 9.2 mm (24 and 48 h SSF samples with the No. 245 strain) to 16.0 mm on average (for the rest of the SSF samples). The LAB strain used for fermentation, as well as the interactions LAB × duration of fermentation, duration of fermentation × SMF-SSF, and LAB × duration of fermentation × SMF-SSF, had statistically significant effects on the diameter of the inhibition zone caused by Spirulina samples against *Staphylococcus aureus* (Supplementary File S3, Table S4).

Table 2. Diameters (in mm) of the inhibition zones (DIZs) of Spirulina samples.

Spirulina Samples	Fermentation		Pathogenic and Opportunistic Bacterial Strain									
	Dura-tion, h	Condi-tions	*Staphylococcus aureus*	*Escherichia coli*	*Acinetobacter baumannii*	*Staphylococcus haemolyticus*	*Salmonella enterica*	*Bacillus cereus*	*Proteus mirabilis*	*Klebsiella pneumoniae*	*Enterococcus faecium*	*Pseudomonas aeruginosa*
			Diameter of the Inhibition Zone (DIZ), mm									
Control (I)	-		0	0	0	0	0	0	0	0	0	0
Control (II)	-		0	0	0	0	0	0	0	0	0	0
No. 244	24 h	SMF	12.0 ± 0.3^{c}	0	0	0	0	0	0	0	0	0
	48 h		10.1 ± 0.1^{b}	0	0	0	0	0	0	0	0	0
	24 h	SSF	16.3 ± 0.2^{e}	0	0	0	0	0	0	0	0	0
	48 h		15.9 ± 0.3^{e}	0	0	0	0	0	0	0	0	0
No. 173	24 h	SMF	0	0	0	0	0	0	0	0	0	0
	48 h		13.1 ± 0.1^{d}	0	0	0	0	0	0	0	0	0
	24 h	SSF	15.9 ± 0.3^{e}	0	0	0	0	0	0	0	0	0
	48 h		16.1 ± 0.2^{e}	0	0	0	0	0	0	0	0	0
No. 225	24 h	SMF	0	0	0	0	0	0	0	0	0	0
	48 h		$12.2. \pm 0.3^{c}$	0	0	0	0	0	0	0	0	0
	24 h	SSF	16.0 ± 0.4^{e}	0	0	0	0	0	0	0	0	0
	48 h		15.8 ± 0.3^{e}	0	0	0	0	0	0	0	0	0
No. 245	24 h	SMF	0	0	0	0	0	0	0	0	0	0
	48 h		0	0	0	0	0	0	0	0	0	0
	24 h	SSF	9.1 ± 0.3^{a}	0	0	0	0	0	0	0	0	0
	48 h		9.3 ± 0.4^{b}	0	0	0	0	0	0	0	0	0

No. 244—fermented with *Lacticaseibacillus paracasei* No. 244 strain; No. 173—fermented with *Levilactobacillus brevis* No. 173 strain; No. 225—*Leuconostoc mesenteroides* No. 225 strain; No. 245—fermented with *Liquorilactobacillus uvarum* No. 245 strain; LAB—lactic acid bacteria; SMF—submerged fermentation; SSF—solid-state fermentation; control (I)—Spirulina powder diluted with distilled water (1:20 *w*/*w*) without fermentation; control (II)—Spirulina powder diluted with distilled water (1:2 *w*/*w*) without fermentation; SSF—solid-state fermentation; SMF—submerged fermentation; data are represented as means ($n = 6$) ± standard errors. $^{a–e}$ Mean values denoted with different letters indicate significantly different values between the samples ($p \leq 0.05$).

Data reported by some authors demonstrated that Spirulina polyphenols, alpha-linolenic acid, C-phycocyanin and the combination of lauric and palmitoleic acids show antimicrobial properties against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Aspergillus flavus* and *Aspergillus niger* [165]. Moreover, there are published data regarding the antimicrobial properties of Spirulina methanolic extract against both Gram-positive and Gram-negative pathogens [166]. Additionally, it was reported that the essential oil of *Spirulina platensis* inhibits *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922), with the most potent effects against *Bacillus antharcis*, *Staphylococcus epidermidis* and *E. coli*, whereas *Salmonella enteritidis* and *P. aeruginosa* (ATCC 27853) were less sensitive to Spirulina essential oils [167]. The antimicrobial activity of the essential oil of Spirulina was explained by the presence of heptadecane, which has a strong antimicrobial effect [168]. The different results of these studies may be explained by the use of different bacterial strains for testing (reference strains vs. wild isolates) or different testing conditions. It seems that the antimicrobial effect of Spirulina compounds is much better expressed against Gram-positive bacteria than Gram-negative bacteria. The results obtained in this study demonstrate a selective antimicrobial effect against *S. aureus*. More interestingly, there was no inhibitory effect on another *Staphylococcus* species—*S. haemolyticus*. Such data suggest that there might be a specific target in *S. aureus* that is affected by Spirulina. However, to confirm such data, further experiments are necessary using more strains of *S. aureus* as well as other *Staphylococcus* species. To date, only a few studies about the antibacterial activity of Spirulina extracts or essential oils have been reported, whereas results about the antimicrobial characteristics of fermented Spirulina are presented in this study for the first time. Other studies are necessary for a better understanding of the antimicrobial properties and mechanisms of fermented Spirulina products.

3.5. Relationship between the Formation of Bioactive Compounds of Proteinaceous Origin in Spirulina

The concentrations of bioactive compounds of proteinaceous origin (BA, GABA, L-Glu) in Spirulina samples are given in Figure 4.

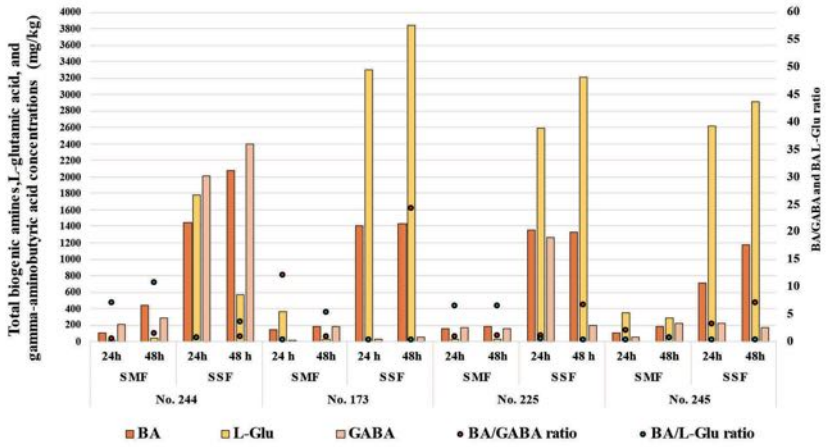

Figure 4. Total biogenic amine (BA), L-glutamic acid (L-Glu) and gamma-aminobutyric acid (GABA) contents (mg/kg) in Spirulina samples and BA/GABA and BA/L-Glu ratios (No. 244—fermented with *Lacticaseibacillus paracasei* No. 244 strain; No. 173—fermented with *Levilactobacillus brevis* No. 173 strain; No. 225—*Leuconostoc mesenteroides* No. 225 strain; No. 245—fermented with *Liquorilactobacillus uvarum* No. 245 strain).

Comparing all of the samples, the highest concentrations of GABA were obtained in the SSF samples (24 and 48 h) with the No. 244 strain (higher than 2000 mg/kg) and 24 h SSF samples with the No. 225 strain (1264 mg/kg). Additionally, the BA/GABA ratios in these samples were 0.72, 0.86 and 1.07, respectively. Overall, the BA/GABA ratios in the samples ranged from 0.5 to 62 (in 24 h SMF sample with No. 244 and in 24 h SSF sample with No. 173, respectively). Moreover, the GABA content in Spirulina samples showed significant statistical correlations with putrescine, cadaverine, histamine, tyramine, spermidine and spermine contents (Table 3). The BA/L-Glu ratio in Spirulina samples varied between 0.31 and 10.7 (in 24 h SMF sample with No. 245 and in 48 h SMF sample with No. 244, respectively), and the L-glutamic acid concentration in Spirulina samples showed positive moderate correlations with tryptamine, putrescine, spermidine and spermine (Table 3). Viable lactic acid bacteria counts in Spirulina samples showed weak negative correlations with cadaverine and spermine contents (Table 3). Although viable LAB counts were a significant factor in GABA and L-glutamic acid formation, correlations between them were not established. Furthermore, the GABA concentration in samples showed a weak positive correlation with the diameter of the inhibition zone against *Staphylococcus aureus*. Notwithstanding these results, in this study, correlations between the pH of the samples and the other analyzed parameters were not found.

Table 3. Pearson correlations and their significance between the analyzed Spirulina parameters.

		pH	TRP	PUT	CAD	HIS	TYR	SPRMD	SPRM	GABA	LGlu	DIZ	LAB
pH	r	1	−0.086	−0.197	0.187	0.098	0.053	−0.169	−0.123	0.107	−0.029	0.023	−0.128
	p		0.534	0.153	0.175	0.480	0.704	0.222	0.377	0.442	0.833	0.872	0.355
TRP	r	−0.086	1	**0.722 ****	0.255	**0.289 ***	**0.314 ***	**0.877 ****	**0.842 ****	0.256	**0.541****	−0.147	−0.098
	p	0.534		**0.0001**	0.062	**0.034**	**0.021**	**0.0001**	**0.0001**	0.061	**0.0001**	0.304	0.482
PUT	r	−0.197	**0.722 ****	1	0.259	**0.377 ****	**0.447 ****	**0.872 ****	**0.747 ****	**0.396 ****	**0.519 ****	0.011	0.073
	p	0.153	**0.0001**		0.059	**0.005**	**0.001**	**0.0001**	**0.0001**	**0.003**	**0.0001**	0.938	0.600
CAD	r	0.187	0.255	0.259	1	**0.923 ****	**0.894 ****	0.248	**0.314 ***	**0.531 ****	−0.124	−0.142	**−0.282 ***
	p	0.175	0.062	0.059		**0.0001**	**0.0001**	0.070	**0.021**	**0.0001**	0.372	0.322	**0.039**
HIS	r	0.098	**0.289 ***	**0.377 ****	**0.923 ****	1	**0.977 ****	**0.297 ***	**0.306 ***	**0.630 ****	−0.085	−0.029	−0.100
	p	0.480	**0.034**	**0.005**	**0.0001**		**0.0001**	**0.029**	**0.024**	**0.0001**	0.539	0.840	0.472
TYR	r	0.053	**0.314 ***	**0.447 ****	**0.894 ****	**0.977 ****	1	**0.325 ***	**0.310 ***	**0.656 ****	−0.065	0.050	−0.0045
	p	0.704	**0.021**	**0.001**	**0.0001**	**0.0001**		**0.016**	**0.023**	**0.0001**	0.640	0.727	0.747
SPRMD	r	−0.169	**0.877 ****	**0.872 ****	**0.248**	**0.297 ***	**0.325 ***	1	**0.941 ****	**0.322 ***	**0.627 ****	−0.125	−0.181
	p	0.222	**0.0001**	**0.0001**	**0.070**	**0.029**	**0.016**		**0.0001**	**0.018**	**0.0001**	0.383	0.191
SPRM	r	−0.123	**0.842 ****	**0.747 ****	**0.314 ***	**0.30 6***	**0.310 ***	**0.941 ****	1	**0.317 ***	**0.572 ****	−0.133	**−0.347 ***
	p	0.377	**0.0001**	**0.0001**	**0.021**	**0.024**	**0.023**	**0.0001**		**0.019**	**0.0001**	0.351	**0.010**
GABA	r	0.107	0.256	**0.396 ****	**0.531 ****	**0.630 ****	**0.656 ****	**0.322 ***	**0.317 ***	1	0.163	**0.337 ***	−0.055
	p	0.442	0.061	**0.003**	**0.0001**	**0.0001**	**0.0001**	**0.018**	**0.019**		0.240	**0.016**	0.691
LGlu	r	−0.029	**0.541 ****	**0.519 ****	−0.124	−0.085	−0.065	**0.627 ****	**0.572 ****	0.163	1	−0.099	0.007
	p	0.833	**0.0001**	**0.0001**	0.372	0.539	0.640	**0.0001**	**0.0001**	0.240		0.489	0.960
DIZ	r	0.023	−0.147	0.011	−0.142	−0.029	0.050	−0.125	−0.133	**0.337 ***	−0.099	1	0.027
	p	0.872	0.304	0.938	0.322	0.840	0.727	0.383	0.351	**0.016**	0.489		0.853
LAB count	r	−0.128	−0.098	0.073	**−0.282 ***	−0.100	−0.045	−0.181	**−0.347 ***	−0.055	0.007	0.027	1
	p	0.355	0.482	0.600	**0.039**	0.472	0.747	0.191	**0.010**	0.691	0.960	0.853	

** Correlation is significant at the 0.01 level (2-tailed); * correlation is significant at the 0.05 level (2-tailed); r—Pearson correlation; *p*—significance (2-tailed); LAB—lactic acid bacteria strain used for fermentation; TRP—tryptamine; PHE—phenylethylamine; PUT—putrescine; CAD—cadaverine; HIS—histamine; TYR—tyramine; SPRMD—spermidine; SPRM—spermine; GABA—gamma-aminobutyric acid; LGlu—L-glutamic acid; DIZ—diameter of inhibition zone against *Staphylococcus aureus*.

This study showed that although during the fermentation of Spirulina with LAB, high concentrations of desirable compounds are formed, non-desirable compounds such as BAs are also formed as a result of their similar mechanisms of synthesis, and thus, their eventual presence in high concentrations in the end products must be taken into consideration.

4. Conclusions

Spirulina is a suitable substrate for fermentation, and the lowest pH value was obtained in 48 h SSF with the No. 173 Spirulina strain (4.10). The highest viable counts of LAB were acquired in 24 and 48 h SMF samples with the No. 225 strain and 48 h SMF and SSF samples with the No. 245 strain (on average, 9.44 $\log_{10}$ CFU/g). The selected LAB strains in this study were shown to possess the capacity to produce L-glutamic acid and GABA in Spirulina biomass (the highest concentration of L-glutamic acid was found in 48 h SSF samples with the No. 173 strain, and the highest concentration of GABA was detected in 48 h SSF samples with the No. 244 strain). In all cases, higher total BA content was found in SSF Spirulina samples when compared with SMF ones. Additionally, fermented Spirulina showed exceptional antimicrobial activity against *Staphylococcus aureus, but not the other tested pathogens*. The biogenic amine/gamma-aminobutyric acid ratio in Spirulina samples ranged from 0.5 to 62, and the BA/L-Glu ratio ranged from 0.31 to 10.7. L-Glutamic acid and GABA contents in Spirulina samples showed significant correlations with some of the identified BAs. Finally, this study showed that, although during fermentation, high concentrations of desirable compounds are formed, non-desirable compounds are also likely to be formed and must be monitored in the end products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biology12020248/s1: Supplementary File S1. Analysis of L-glutamic acid (L-Glu) and gamma-aminobutyric (GABA) acid; Supplementary File S2. Evaluation of the concentration of biogenic amines (BAs) in Spirulina samples; Supplementary File S3. Influence of the analyzed factors (lactic acid bacteria (LAB) used for fermentation; duration of fermentation; fermentation conditions) and their interaction on analyzed parameters: Table S1. Influence of the analyzed factors (lactic acid bacteria (LAB) used for fermentation; duration of fermentation; fermentation conditions) and their interaction on viable lactic acid bacteria counts and pH of Spirulina samples. Table S2. Influence of the analyzed factors (lactic acid bacteria (LAB) used for fermentation; duration of fermentation; fermentation conditions) and their interaction on the concentrations of L-glutamic (L-Glu) and gamma-aminobutyric (GABA) acids in Spirulina samples. Table S3. Influence of the analyzed factors (lactic acid bacteria (LAB) used for fermentation; duration of fermentation; fermentation conditions) and their interaction on biogenic amine (BA) content in Spirulina. Table S4. Influence of the analyzed factors (lactic acid bacteria used for fermentation; duration of fermentation; fermentation conditions) and their interaction on diameter of inhibition zone (DIZ) by Spirulina against *Staphylococcus aureus*. Supplementary File S4. Biogenic amine content: Table S1. Biogenic amine (BA) concentrations in control Spirulina samples. Table S2. Biogenic amine (BA) concentrations in Spirulina samples.

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Abbreviations

Acinetobacter baumannii (Ac. baumannii), Agmatine (AGM), Arthrospira platensis (A. platensis), Aspergillus flavus (As. flavus), Aspergillus niger (A. niger), Bacillus antharcis (B. antharcis), Bacillus cereus (B. cereus), Bacillus subtilis (B. subtilis), biogenic amines (BA), cadaverine (CAD), colony-forming units (CFU), De Man, Rogosa and Sharpe (MRS), diameter of the inhibition zone (DIZ), Enterococcus faecium (Ec. faecium), Escherichia coli (E. coli), European Commission (EC), European Food Safety Authority (EFSA), European Union (EU), Food and Drug Administration (FDA), gamma-aminobutyric acid (GABA), Generally Recognized as Safe (GRAS), glutamic acid decarboxylase (GAD), histamine (HIS), Klebsiella pneumoniae (K. pneumoniae), lactic acid bacteria (LAB), Lacticaseibacillus paracasei (Lc. paracasei), Leuconostoc mesenteroides (L. mesenteroides), Levilactobacillus brevis (Lev. brevis), L-glutamic acid (L-Glu), Liquorilactobacillus uvarum (Liq. uvarum), phenylethylamine (PHE), Proteus mirabilis (P. mirabilis), Pseudomonas aeruginosa (Ps. aeruginosa), putrescine (PUT), Salmonella enterica (S. enterica), Salmonella enteritidis (S. enteritidis), selected reaction monitoring (SRM), solid-state fermentation (SSF), spermidine (SPRMD), spermine (SPRM), standard error (SE), Staphylococcus aureus (St. aureus), Staphylococcus epidermidis (St. epidermidis), Staphylococcus haemolyticus (St. haemolyticus), submerged fermentation (SMF), tryptamine (TRP), tyramine (TYR).

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