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Evaluation of the Prospects for the Use of Microalgae in Functional Bread Production

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Abstract: Microalgae are widely used to produce sorbitol, ethyl and methyl alcohols, acetone, organic acids, esters, pigments, chlorophylls, carotenoids, and other compounds. The purpose of this work was to study the potential of the Baltic Sea microalgae *Arthrospira platensis* and *Chlorella vulgaris*, as new ingredients for functional bread. Bread was baked with a dry mixture of *Arthrospira platensis* and *Chlorella vulgaris* microalgae (1:1). Gas chromatography, sequencing, chromametry, pH-metry, rheological methods, methods for determining the antioxidant ability of bread samples with microalgae for ferric reduction and removal of active free radicals were used for the research. When baking bread, the organoleptic, physicochemical properties, density, coefficients of elasticity, chewiness, stickiness, resistance to crumbling, the content of polyphenols and volatile compounds in the samples of bread with microalgae were controlled. It was found that with a higher content of microalgae in bread (3% of the flour weight on dry basis), the color of the crust and crumb decreased due to the degradation of the pigment during baking. The specific volume of a loaf with a large amount of microalgae (5%) was lower than the volume of loaves with the addition of 1% and 3% microalgae. It is shown that the moisture content increased with an increase in the amount of microalgae in bread. Replacing wheat flour with the amounts of microalgae of 1% and 3% did not affect the pH and water activity in bread. With the addition of 5% microalgae, bread became more sour, sticky, with a slight "fishy" smell. The addition of more microalgae led to an increase in polyphenols in bread samples, and, accordingly, to an increased antioxidant capacity. In total, 42 volatile compounds were found in bread with microalgae, which makes it possible to obtain functional bread. It is assumed that in the future, bread with microalgae will be competitive with ordinary wheat bread due to improved nutritional and biological value.

Keywords: *Arthrospira platensis*; *Chlorella vulgaris*; microalgae; bread; functional properties; antioxidant capacity

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1. Introduction

People have been using microalgae for food since ancient times [1]. The first documented data on the use of microalgae as food products date back to the XVI century. It is a historically documented fact that in 1521, biscuits made from dried spirulina layers were sold in Mexico City [1]. Microalgae can be used as industrial raw materials to produce all

kinds of organic compounds (sorbitol, ethyl and methyl alcohols, acetone, organic acids, esters, etc.) [2,3]. To date, the use of microalgae is a promising way to produce pigments such as chlorophylls, carotenes, etc. It can be especially noted that the pigments obtained in this way are not toxic. Chlorophyll obtained from microalgae is used in the cosmetic and food industries. In Japan, this pigment is used to color fish pastes, in Europe—oils and fats [4,5].

Microalgae *Arthrospira platensis*, *Chlorella vulgaris*, *Dunaliella salina* and others are common in the Baltic Sea. Cyanobacteria of the *Arthrospira* genera is a promising biological raw material, because in addition to easily digestible proteins, lipids, and polysaccharides, it has a unique combination of biologically active substances (BAS): vitamins A, C, E, B groups, carotenoids, polyunsaturated fatty acids, chlorophyll, etc. [6]. Cyanobacteria are used in many areas of the biotechnological industry: the production of biofuels, animal and poultry feed, raw materials for pharmaceuticals, as well as biofertilizers [7]. Cyanobacteria have shown their high effectiveness for the treatment and prevention of human diseases. The possibility of using *Arthrospira* microalgae for the biosynthesis of targeted secondary metabolites [8] is of particular interest. Spirulina was first discovered in the Baltic Sea by Vitkovsky in 1993 [9]. *A. platensis* of Baltic origin has a size of 3.0–5.0 μm and forms tightly twisted spirals. They slide due to typical oscillatory, helical movements on the surface. Cyanobacteria form opaque structures, usually blue-green in color, but pinkish-red filaments have also been observed in the Baltic Sea [10].

Spirulina is very rich in various chemical elements and organic substances. Spirulina has soft cell walls that consist of mucopolysaccharides, so it is easily digested in the intestine [11]. Spirulina has a large (among organisms using photosynthesis) content (60–70% dry weight) of easily digestible (85–90%) protein [12]. So, its dry mass contains up to 77% protein and all 20 amino acids, but leucine, valine, and isoleucine are most common. Spirulina lipids are mostly polyunsaturated fatty acids. The proportion of linoleic and γ-linolenic acids reaches 1/3 of each of all fatty acids (13.1–31.5% and 12.9–29.4%, respectively) [13]. Carbohydrates are mainly represented in the form of rhamnose and starch, which are sources of "fast" energy that cells easily absorb. Cyanobacteria biomass can be used for weight-reducing programs due to its unique composition (high protein content and low lipid and carbohydrate content) [14]. Spirulina contains a large number of trace elements such as K, Ca, Mg and P; it also contains Mn, Cu, Mb, Co, Ni, Z, and B. In addition, spirulina can store a large amount of these trace elements in the biomass contained in the form of chelate complexes (Cu, Z, Cr, Mn) and Se-containing amino acids and proteins. Vitamins, such as B-group (B2, B3, B6) and vitamin E, are also found in spirulina [15].

C. vulgaris are green eukaryotic microalgae of the *Chlorella* genus. Chlorella, as well as spirulina, grows in water and prefers warm, stagnant reservoirs. In the Baltic Sea, chlorella grows in the southern part [16]. Chlorella includes about 650 compounds, such as vitamins, fats, essential amino acids, macro- and microelements in an easily digestible form [17]. Each cell of chlorella is a spherical or oval cell with a chloroplast and a nucleus, covered with a shell; the cell sizes are from 2 to 10 μm. In the inner part of the cell there are also organelles, which together with the nucleus and chloroplast are enclosed in a well-protected thick fibrous cell wall [18]. In addition to protein, 20–30% fat, 10–20% carbohydrates, the biochemical composition includes up to 10% macro- and microelements, vitamins A, B, B6, B12, C, E, PP (a nicotinic acid), essential amino acids (tryptophan, valine, threonine, leucine), fatty acids and other BAS in an easily digestible form [19]. Various organic substances for the food, chemical and pharmaceutical industries are obtained from these microalgae. In addition, there are prospects for the use of algae for wastewater treatment, air regeneration and to increase soil fertility in agriculture. Under favorable conditions, *Chlorella* is able to increase its biomass by 32 times in one life cycle [20]. The carotene content in chlorella exceeds all vegetable feeds; it contains a lot of tocopherol, riboflavin and nicotinic acid, thiamine and pyridoxine: as much as in corn, barley, and oats. Vitamins B12 and D (not synthesized in green plants) are found in significant amounts in chlorella biomass [19,21].

Some studies have evaluated the potential of spirulina and chlorella to be used as ingredients in the production of milkshakes, vegetable soups, snacks, pasta, yoghurts and pastries, including bread and cookies [22]. García-Segovia et al. [23] reported that, although there were differences in color compared to the control, the textural properties of bread did not change after the inclusion of microalgae *Isochrysis galbana*, *Tetraselmis suecica*, *Scenedesmus almeriensis* or *Nannochloropsis gaditana* in a concentration of 1.5% (wt./wt.). However, there is limited information about the organoleptic properties of bread prepared using microalgae of spirulina and chlorella species [22].

In recent years, much global attention has been paid to the enrichment of bread with various useful substances that give it therapeutic and preventive properties. Increasing the production of dietary bakery products is an important reserve for improving the health of the nation. The therapeutic effect of dietary bakery products is provided either by introducing the necessary additional components into the recipe or by excluding the undesirable ones. The introduction of components that give bakery products therapeutic and preventive properties can effectively solve the problem of prevention and treatment of various diseases associated with the deficiency of certain substances.

The chemical composition of bread is not quite biologically complete. Bread proteins are poor in essential amino acids such as lysine and threonine. There are not enough calcium salts and vitamins in bread. An increase in digestibility and nutritional value leads to an increase in bread quality. To achieve this, the organoleptic, physicochemical, structural, and mechanical properties, nutritional, energy, and biological values, and other indicators that improve bread volume, porosity, appearance, taste, and aroma must be improved. These measures include mixing different flour batches, brewing some flour for specific bread types, activating pressed yeast, adding fat to the dough in the form of a water–fat emulsion, using the best dough preparation technique for this flour, and adding biologically active additives of both animal and plant origin. Marine microalgae, which are abundant year-round in the seas and have high nutritional, energy, and biological values, are receiving more and more attention in recent years [23]. That is why increasing the nutritional value of bread, giving it functional properties for disease prevention, and maintaining and strengthening the population's health is important.

C. vulgaris and *A. platensis* are promising and cost-effective raw materials for increasing the nutritional value of bakery products with microalgae, with a high content of biologically active substances and a wide distribution, including in the Baltic Sea. The authors' findings indicate the potential for using green microalgae of the genus *C. vulgaris* in the technology of producing bakery products from a mixture of rye and wheat flour to broaden the range of these products, improve crumb structure, and increase nutritional value [24]. The conducted studies [12–15] suggest that *A. platensis* can add additional nutritional and biologically active ingredients to bread. In light of this, this study aimed to examine the potential for incorporating a combination of the Baltic microalgae *A. platensis* and *C. vulgaris* as fresh ingredients in functional bread.

Based on the gap of knowledge about the organoleptic, physicochemical and biological properties of bread with added microalgae (spirulina and chlorella), the purpose of this work was to study the potential of microalgae *A. platensis* and *C. vulgaris* when used as new ingredients for the production of functional bread.

2. Materials and Methods

2.1. Characteristics of Microalgae

For this study, microalgae samples collected in the Baltic Bay (59°43′ n.l. 28°24′ e.l., Russia), on the Baltic Sea coast (54°42.4′0″ n.l., 20°30.4′0″ e.l., Russia) were used.

Microalgae were sampled with a box-shaped bottom sampler developed at the Institute for Biology of Inland Waters of the Russian Academy of Sciences (Borok, Russia), with a square bottom area of 160 × 160 mm in size and a maximum immersion depth of 440 mm in bottom sediments. Samples were taken at a depth of 400 mm. Immediately after transportation to the shore, test cores were taken using plastic tubes with an inner diameter

of 45 mm. The tubes were sealed at both ends and stored in an upright position at +4 °C. In the laboratory, the tubes were cut lengthwise and halved using two thin stainless-steel plates inserted into the cut. The halves of the tubes were then divided into transverse samples (slices) with a step of 5–10 mm. All samples were stored at –20 °C in the dark, in plastic bags with air access, from which microalgae samples were taken for research.

Further, pure microalgae cultures were isolated and microalgae strains that can actively accumulate biomass and target products (lipids, proteins, and carbohydrate–mineral complex) and are suitable for cultivation in laboratory conditions were identified. The collected microalgae were washed to remove impurities and cultivated in 500 mL Erlenmeyer laboratory flasks at +8 °C during nighttime and +12 °C during daytime, while illuminated with artificial light lamps (Deko-Light Plus G13 58 W 4000 K 162048, DV-Expert, Moscow, Russia) with an intensity of 2000–3000 lux, the light photoperiod was 12 h, pH = 5.5–6.5, cultivation duration 6–7 days. The cultivation was carried out on an orbital shaker Unimax 1010 (Heidolph, Schwabach, Germany) at 118 rpm. Before extraction, the samples were stored at T = 4 °C.

Prior to testing, the presence of heavy metals, toxic residues, and pathogenic microorganisms in microalgae was determined. Samples with the determined content of heavy metals, toxic impurities, and pathogenic microorganisms were either rejected and not used in the experiment, or were purified using Millipore membrane filters (Merck KGaA, Darmstadt, Germany) with the following characteristics: diameter 47 mm; GSWP, pore size 0–22 μm; HAWP, pore size 0.45 μm; DAWP, pore size 0.8 μm; RAWP, pore size 1–2 μm). Antibiotics were not used to eliminate pathogenic microflora.

Taxonomic identification of microalgae was carried out by capillary sequencing of variable loci of mitochondrial cytochrome oxidase subunit 1 (COI) genes [18], plastid elongation factor Tu (tufA) gene, as well as transcribed spacer ITS1 + 5.8S + ITS2.

DNA was isolated from microalgae biomass by phenol-chloroform extraction [19]. Before isolation, samples of green microalgae were treated with a solution of 1 M KOH in a ratio of 3:1 to a dry sample and incubated for 10 min at 94 °C. Gene amplification was carried out using specific primers (Table 1) and Tersus polymerase (Eurogen, Moscow, Russia) in the amount of 100 μL according to the manufacturer's protocol. The cycling parameters are presented in Table 2.

Table 1. Sequences of primers for the identification of microalgae.

Gene	Primer F	Primer R	Segment
ITS1 + 5.8 S + ITS2	5′-GTCGCTCCTACCGA TTGGGTGTG-3′	5′-TCCCTTTTCGCTC GCCGTTACTA-3′	Green algae
Tu (tufA)	5′-GGNGCNGCNCAAAT GGAYGG-3′	5′-CCTTCNCGAATMGCRA AWCGC-3′	Green algae

Table 2. Cycling parameters during amplification of variable genes.

Stages	Stage	Incubation Temperature, °C	Time, s
1	Pre-denaturation	95	60
2 *	Denaturation	95	30
3 *	Annealing	55–60	30
4 *	Elongation	72	60
5	Final elongation	72	300

* Repeat 2–4 stages 29 times.

The amplification results were visualized by horizontal 1% agarose gel electrophoresis. To purify the amplicons, a commercial Cleanup Mini kit was used (Eurogen, Moscow, Russia). The concentration of purified amplicons was measured using a Qubit 2.0 fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA).

Preparation of libraries for capillary sequencing included molecular cloning of amplicons using the commercial Quick-TA kit (Eurogen, Moscow, Russia) into the pAL2-T vector in the ratio (insert:vector) 3:1 in a volume of 10 µL per reaction. Chemically competent *E. coli* DH5 alfa cells were used for cloning.

Competent cells were manipulated on ice. Stored at a temperature of −80 °C, tubes with cell suspension were thawed on ice for 30 min. After that, 50 µL of cells was added to 5 µL of ligating liquid and incubated on ice for 30 min, followed by heating at 42 °C for 90 s. Then the cells were transferred to ice and incubated for 2 min. 200 µL of LB liquid nutrient medium was added to the test tubes and placed in an orbital shaker for 120 min at 80 rpm. Incubation was carried out at a temperature of 37 °C. The resulting cell suspensions with a volume of 200 µL were applied to a dense LB nutrient medium (1.5% agar, 1% trypton, 1% NaCl, 0.5% yeast extract). Ampicillin at a concentration of 50 µg/L and X-gal + IPTG were used as a selective marker. Petri dishes were cultured for 8 h at 37 °C. The transformation analysis was performed using blue–white screening.

White colonies containing inserted vectors were subjected to PCR screening using standard primers for the pAL2-T M13 Forward vector (5-GTTGTAAAACGACGGCCAGTG-3) and M13 Reverse (5 AGCGGATAACAATTTCACACAGGA-3). Screening was performed using a reaction mixture qPCR-mix HS (Eurogen, Moscow, Russia),15 µL in volume. The results of PCR screening were analyzed using horizontal agarose gel electrophoresis.

Inoculates that showed a positive result were used to obtain microalgae cultures in laboratory conditions and develop sufficient amounts of plasmid biomass. The suspension of one colony was transferred to 5 mL of LB medium with ampicillin and incubated to an optical density of OD 600 equal to 0.6–0.8 at 37 °C. Plasmids were isolated using a commercial Plasmid Miniprep kit (Eurogen, Moscow, Russia), the concentration of plasmids was measured using a Qubit 2.0 fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA).

The sequencing reaction was performed with M13 Forward and M13 Reverse primers using the BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA). The sequence reaction was carried out in two repetitions for a forward and reverse primer for each sample. The volume of the reaction mixture was 10 µL: 3 µL of buffer, 1 µL of terminator, 100–200 ng of plasmid DNA and H_2O mQ to the final volume. Library amplification was performed on the Thermal Cycler C1000 Touch device (Bio-Rad Laboratories LLC, Moscow, Russia). The cycling parameters are presented in Table 3.

Table 3. Parameters of sequencing reaction cycling.

Stages	Stage	Incubation Temperature, °C	Time, s
1	Pre-denaturation	96	60
2 *	Denaturation	96	10
3 *	Annealing	50	4
4 *	Elongation	72	240
5	Final elongation	72	300

* Repeat 2–4 stages 29 times.

The reaction mixture was purified using a commercial X-Terminator kit (Applied Biosystems, Foster City, CA, USA). Capillary sequencing was performed on a 3500 DNA Analyzer device (Applied Biosystems, Foster City, CA, USA).

Sequencing results were processed in 4 Peaks (Cedar Sahin, 2020, Istanbul, Turkey) and CLC Genomics Workbench (Illumina, 2020, Oxford, UK) programs. Using 4 Peaks, two repetitions were aligned on top of each other for visual identification of unread nucleotides in the samples.

Further processing in the CLC Genomics Workbench program included trimming the 5′ and 3′ ends of the sequences and assembly, which resulted in the coupling of the analyzed contigs. The sequences were analyzed using the local BLAST algorithm using the nr/nt, 18S rRNA and ITS databases.

2.2. Preparation of Microalgae Bread Samples

Microalgae samples were dried in a drying cabinet with natural convection ED 53 (Millab LLC, St. Petersburg, Russia) to a constant mass, ground into powder on a Pulverisette 14 rotary mill (Millab LLC, St. Petersburg, Russia). A 1:1 mixture of chlorella and spirulina was used to add to the bread. For research, bread samples were baked using a straight dough method. Estimated in preliminary tests, the levels of substitution of flour with a dry mixture of microalgae biomass during bread baking varied at 1% (0.5% spirulina and 0.5% chlorella), 3% (1.5% spirulina and 1.5% chlorella), and 5% (2.5% spirulina and 2.5% chlorella) (weight/weight). In a preliminary experiment, it was discovered that adding less than 1% of microalgae had no effect on the change in the properties of bread and that such samples were similar to the control ones (without the addition of microalgae), but adding more than 5% of microalgae caused a deterioration of the properties of bread (a fishy smell, an unattractive greenish color, and increased stickiness). It was difficult to determine the adequate taste of bread due to these defects.

A mixture of microalgae was produced by cultivating separate samples of *A. platensis* and *C. vulgaris*, two microalgae isolated from the Baltic Sea, in a Biostat A MO UniVessel Glass bioreactor (Sartorius Stedim, Göttingen, Germany), then drying them for 12 h at 30–40 °C to a moisture content of 5–6% in an oven with natural convection E 28 (Millab, Moscow, Russia). Next, the microalgae were ground on a P14 laboratory rotary mill (Millab, Moscow, Russia) into a powder with a size of 10–50 μm. When baking bread, a mixture of *A. platensis* and *C. vulgaris* microalgae (1:1) was used instead of high grade wheat flour.

According to the findings of the experiments (not presented in this paper), adding one of the types of microalgae (either chlorella or spirulina) did not result in bread samples with a porous, light texture, optimal organoleptic, physicochemical, structural mechanical properties, and, most importantly, increased antioxidant activity. A significant decrease in the amount of polyphenols responsible for the antioxidant properties of bread was observed in bread samples containing only one type of microalgae. The addition of a mixture of *A. platensis* and *C. vulgaris* (1:1) to bread samples resulted in the highest amount of volatile substances (alcohols, aldehydes, ketones, ethers, volatile sulfur compounds, terpenes, acids, and hydrocarbons) and polyphenols, resulting in the presence of high antioxidant activity in the samples.

With the straight dough method, filtered water, "Voronezhskie" yeast (SAF-Neva LLC, Voronezh, Russia) salt and sugar were added to the bowl, according to the formulation for 100 kg of flour (Makfa, St. Petersburg, Russia), presented in Table 4, were mixed, flour and dry biomass of microalgae were added. The mixture was mixed until smooth. The dough was fermented for 30 min, then was formed into loaves. The dough was divided into pieces taking into account the oven loss and drying loss. The formed dough pieces were laid on the trays and sent for proofing. The duration of proofing was 50 min. The rested test pieces were baked for 25 min at a temperature of 210 °C in the bakery oven EShP-ZKP (LLC "Klyon", St. Petersburg, Russia). After baking, the loaves were placed on trays and, if necessary, thrown out.

Table 4. The formulation of wheat flour bread with microalgae.

Component, kg	Formulation			
	1	2	3	4
Baking wheat flour, top-grade	21.0	20.0	20.0	20.0
Baking wheat flour, first-grade	72.0	72.0	70.0	68.0
Pressed yeast	2.5	2.5	2.5	2.5
Salt	1.5	1.5	1.5	1.5
Sugar	3.0	3.0	3.0	3.0
Dry biomass of microalgae	0.0	1.0	3.0	5.0
Total:	100.0	100.0	100.0	100.0

1—control sample, without added microalgae; 2—bread with a microalgae content of 1.0%; 3—bread with a microalgae content of 3.0%; 4—bread with a microalgae content of 5.0%.

2.3. Organoleptic Analysis of Bread with Microalgae

The following organoleptic properties of bread samples were determined: appearance (shape, surface, color), crumb condition (mixing, doneness), volume yield of bread, taste and smell according to the methods described in [25].

2.4. Determination of Physico-Chemical Properties of Bread with Microalgae

The bread color values were determined using a Minolta CR-200 chromameter (Minolta INC., Tokyo, Japan) and a standard D65 light source determined by the International Commission on Illumination and corresponding to the average midday light in Europe. The color was calculated in three repetitions, as described in [26] and was determined on the 1st day of baking.

The weight and dimensions for 10 loaves of bread were averaged. The volume of a loaf was calculated using the method of the Register of Accredited International Methods AACC 10–05.01 (final approval on 30 October 1975; re-approval on 3 November 1999). The moisture content of the crumb was determined by the method of the Register of Accredited International Methods AACC 44–15.02. (final approval on 30 October 1975; re-approval on 3 November 1999). The water activity a_w of all samples was determined using the Aqualab Pawkit water activity meter (Decagon Devices Inc., Washington, DC, USA). The measurements were carried out in three repetitions. The pH of 1 g of bread sample added to 10 g of distilled water was determined in three repetitions using a Basic 20 pH meter (Crison Instruments S.A., Barcelona, Spain). Porosity of the crumb (P) was calculated by the formula:

$$P = 100\ (V - V_1)/V,$$

where V is the volume of the cut-out crumb; V_1 is the volume of the nonporous crumb pressed to failure.

Texture characteristics were evaluated using a TA.XTplusC analyzer (Stable Micro Systems Ltd., Surrey, UK) connected to Exponent v.5.0.6.0 software (OIC Group, Inc, version 5.6.x+, 2022, Peoria, IL, USA). The bread texture profile was analyzed as described [27] using an aluminum compression probe P/20. The hardness of bread was determined using a knife blade with a slit probe (HDP/BS), as described in [22]. Ten samples of each formulation were taken, the values were averaged.

2.5. Determination of the Total Phenol Content in Bread with Microalgae

The total phenol content in the bread was determined by the Folin–Ciocalteu method in accordance with the protocol described in [28]. The extraction time was 2 h at room temperature. The amount of phenols was determined three times, and the results were expressed in mg of gallic acid equivalents per 100 g of dry weight.

2.6. Determination of Antioxidant Activity of Bread with Microalgae

To determine the antioxidant capacity of bread, the same extract as for the determination of phenolic compounds was used as well as the Ferric Reducing Antioxidant Power Assay (FRAP) and the removal of active free radicals DPPH method. The subsequent analysis of antioxidant activity was carried out according to [29]. The analysis was carried out in three repetitions, and the results were expressed in mg of ascorbic acid equivalent per 100 g of dry weight.

2.7. Determination of Volatile Compounds in Bread with Microalgae

Extraction and determination of volatile compounds released by the bread was carried out using gas chromatography with mass spectrometry (GC-MS) in accordance with the conditions described in [30]. To do this, 1 g (±0.005 g) of crushed bread sample was weighed into 20 mL vials and mixed with 10 mL of 20% (weight/volume) sodium chloride at pH 3.0. The vials were immersed in a water bath at 60 °C for 60 min. SPME fiber (65 mm PDMS/DVB; Supelco Co., Burlington, MA, USA) was used, which after extraction was injected for thermal desorption into the injector port for 10 min. GC-MS analyses were per-

formed using a 6890 N gas chromatograph–mass spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an HPFFAP column (50 m × 0.2 mm; 0.33 microns); the column was purchased from Agilent Technologies Inc., Santa Clara, CA, USA.

The temperature of the injector and detector was 240 °C. The mass spectra were obtained by the electron impact method at 70 eV. The scanning mode was used in the range of m/z 20–350 to detect all compounds. The preliminary identification of volatile compounds was carried out and the obtained mass spectral data were compared to the NIST62 mass spectrum databases.

2.8. Statistical analysis

The results were expressed as an average value ± standard deviation (SD). The differences between the samples were analyzed using variance analysis (ANOVA) with JMP 13 (SAS Institute Inc., version 13+, 2021, Cary, NC, USA). In the case where significant differences were present, Tukey's pairwise comparison was performed in order to determine where the differences in the sample occurred ($p < 0.05$).

3. Results

3.1. Organoleptic Properties of Bread with Microalgae

Through the identification of microalgae by sequencing, it was found that the algae isolated from the Baltic Sea belonged to the different species. The composition of microalgae collected and identified by sequencing is presented in Table 5.

Table 5. Composition of microalgae in samples collected from the Baltic Sea.

Microalgae	Content in the Sample, %
Chlorella vulgaris	36.4 ± 1.1
Chlorella fusca	12.0 ± 0.3
Arthrospira platensis	21.4 ± 0.6
Pleurochrysis carterae	15.3 ± 0.5
Dunaliella salina	8.6 ± 0.4
Ankistrodesmus acicularis Korsch	6.3 ± 0.4

The microalgae samples (Table 5) collected in the Baltic Sea contained the largest amount of *C. vulgaris* (36.4%) and *A. platensis* (21.4%). The amounts of other microalgae (*P. carterae, D. salina, A. acicularis Korsch*, and *C. fusca*) were 1.5–2 times less (15.3%, 8.6%, 6.3%, and 12.0%, respectively).

The results of the study of the organoleptic properties of bread with microalgae are presented in Table 6. According to organoleptic parameters (Table 6), the best samples of bread were samples with the addition of a mixture of microalgae in the amount of 3%.

Table 6. Organoleptic properties of bread with microalgae.

Values	Samples			
	1	2	3	4
	Appearance			
Form	Corresponds to the bread form	Corresponds to the bread form	Corresponds to the bread form	Corresponds to the bread form
Surface	Smooth	Smooth	Smooth	Smooth
Color	Corresponds to wheat bread	Yellow	Greenish	Green
	Crumb condition			
Mixing	Without lumps and traces of under-mixing	Without lumps and traces of undermixing	Without lumps and traces of undermixing	A small number of lumps and traces of undermixing
Doneness		Well-done, elastic	Well-done, elastic	Poorly done, inelastic
Taste and smell		Characteristic of this type of bread, without foreign taste and smell	Characteristic of this type of bread, without foreign taste and smell	Characteristic of this type of bread, with a slight "fishy" smell, with a sour taste

1—control sample, without added microalgae; 2—bread with a microalgae content of 1.0%; 3—bread with a microalgae content of 3.0%; 4—bread with a microalgae content of 5.0%.

3.2. Physicochemical Properties of Bread with Microalgae

The physicochemical properties of bread samples with microalgae are presented in Table 7. In terms of physicochemical properties, all samples (Table 7) did not contradict the generally accepted standards for wheat bread.

Table 7. Physicochemical properties of bread samples with microalgae.

Value	Samples			
	1	2	3	4
Weight, g	49.4 ± 0.2	47.8 ± 0.1	47.1 ± 0.1	50.2 ± 0.2
Maximum height, mm	58.1 ± 0.2	56.6 ± 0.2	55.1 ± 0.2	52.1 ± 0.2
Specific crumb volume, mL/g	3.5 ± 0.01	3.5 ± 0.01	3.7 ± 0.01	3.2 ± 0.01
Crumb density, g/mL	0.27 ± 0.0008	0.29 ± 0.0009	0.27 ± 0.0008	0.34 ± 0.0010
Crumb moisture content, %	28.0 ± 0.08	29.8 ± 0.09	32.4 ± 0.10	39.2 ± 0.10
Crumb porosity, %	71.8 ± 0.2	69.7 ± 0.2	68.4 ± 0.2	55.6 ± 0.2
pH	6.6 ± 0.02	6.6 ± 0.02	6.6 ± 0.02	6.2 ± 0.02
a_w	0.975 ± 0.003	0.953 ± 0.003	0.938 ± 0.003	0.918 ± 0.003

1—control sample, without added microalgae; 2—bread with a microalgae content of 1.0%; 3—bread with a microalgae content of 3.0%; 4—bread with a microalgae content of 5.0%, a_w—water activity. Data presented as a mean ± SD (n = 3). The results shown in the table are obtained on the 1st day after baking.

The results of studying the volume yield of bread with the addition of various amounts of microalgae are shown in Figure 1. The data (Figure 1) indicate that bread samples with the addition of 3% microalgae had the highest volumetric yield.

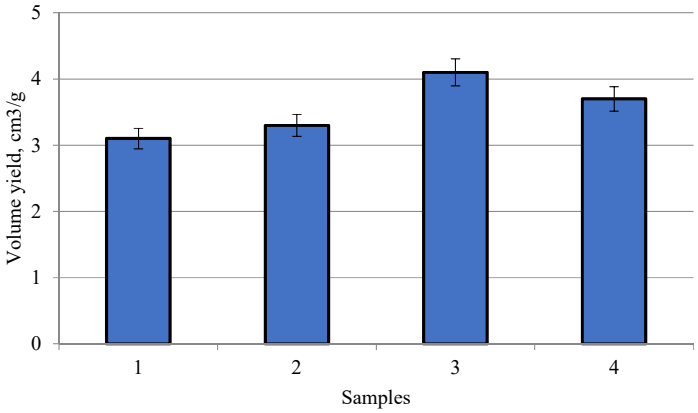

Figure 1. The results of studying the volume yield of bread with the addition of various amounts of microalgae: 1—control sample (no added microalgae); 2—bread with a microalgae content of 1.0%; 3—bread with a microalgae content of 3.0%; 4—bread with a microalgae content of 5.0%.

The texture of bread samples with microalgae are presented in Table 8. Bread samples (Table 8) with 3% and 5% microalgae mixture additions had slightly better textural properties.

Table 8. Texture of bread samples with microalgae.

Values	Samples			
	1	2	3	4
Density, kg/m^3	0.88 ± 0.27a	0.90 ± 0.27a	0.93 ± 0.28a	0.98 ± 0.29a
Elasticity coefficient	0.28 ± 0.08a	0.28 ± 0.08a	0.28 ± 0.08a	0.24 ± 0.07a
Chewiness coefficient	1.9 ± 0.6a	2.0 ± 0.6a	2.0 ± 0.06a	2.0 ± 0.06a
Stickiness coefficient	2.1 ± 0.6a	2.1 ± 0.6a	2.1 ± 0.06a	2.6 ± 0.8b
Crumpling resistance coefficient	0.33 ± 0.1a	0.33 ± 0.1a	0.33 ± 0.1a	0.25 ± 0.08a

1—control sample, without added microalgae; 2—bread with a microalgae content of 1.0%; 3—bread with a microalgae content of 3.0%; 4—bread with a microalgae content of 5.0%. Values in a row followed by the same letter do not differ significantly ($p > 0.05$) as assessed by the post hoc test. Data presented as a mean ± SD (n = 3).

3.3. Polyphenol Content and Antioxidant Activity of Bread with Microalgae

The content of polyphenols and antioxidant activity of bread with microalgae are presented in Table 9.

Table 9. Polyphenol content and antioxidant activity of bread with microalgae.

Amount of Microalgae, %	Polyphenol Content, mg/100 g Dry Weight	FRAP, mg/100 g Dry Weight	DPPH, mg/100 g Dry Weight
0.0	80.5 ± 25.2a	0.836 ± 0.27a	0.779 ± 0.26a
1.0	85.2 ± 25.6a	0.894 ± 0.27a	0.874 ± 0.26a
3.0	92.4 ± 27.7b	0.998 ± 0.30a	0.999 ± 0.30ab
5.0	93.4 ± 28.0b	1.000 ± 0.30a	1.100 ± 0.33b

All values in columns followed by the same letter do not differ significantly ($p > 0.05$). Data presented as a mean ± SD ($n = 3$).

The samples of bread with microalgae content of 3% and 5% had the highest antioxidant activity (Table 9). These samples contained approximately the same amount of polyphenols (92.4 mg/100 g dry weight and 93.4 mg/100 g dry weight). The lowest amount of polyphenols and the lowest antioxidant activity were observed for bread samples without added microalgae. This fact indicates a significant contribution of microalgae polyphenols to the antioxidant activity of bread. Polyphenols retained their effectiveness after heat treatment.

3.4. The Content of Volatile Compounds in Bread with Microalgae

The content of volatile compounds in bread samples with microalgae is presented in Table 10. The experimental results (Table 10) show that bread samples containing 3% and 5% microalgae contained a slightly higher concentration of biologically active substances (such as sulfur compounds, terpenes, acids, etc.). Bread samples without added microalgae contained the smallest amount of all the studied volatile compounds.

Table 10. The content of volatile compounds of bread with microalgae.

Compounds	Content, %			
	1	2	3	4
Alcohols	0.29 ± 0.1a	0.33 ± 0.1a	0.35 ± 0.12a	0.38 ± 0.11a
Aldehydes	0.001 ± 0.0006a	0.002 ± 0.0006a	0.002 ± 0.0006a	0.003 ± 0.0009a
Ketones	0.0006 ± 0.0002a	0.0007 ± 0.0002a	0.0009 ± 0.0003a	0.001 ± 0.0003a
Esters	0.0003 ± 0.0002a	0.0005 ± 0.0002a	0.0007 ± 0.0002a	0.0009 ± 0.0003a
Sulfur compounds	0.12 ± 0.05a	0.15 ± 0.05a	0.23 ± 0.07a	0.26 ± 0.08a
Terpenes	0.0001 ± 0.00003a	0.0001 ± 0.00003a	0.0001 ± 0.00003a	0.0001 ± 0.00003a
Acids	23.6 ± 8.82a	29.4 ± 8.82b	30.1 ± 9.03b	32.6 ± 9.78b
Hydrocarbons	0.0001 ± 0.00006a	0.0002 ± 0.0000 a	0.0002 ± 0.00006a	0.0003 ± 0.00003a

1—control sample, without added microalgae; 2—bread with a microalgae content of 1.0%; 3—bread with a microalgae content of 3.0%; 4—bread with a microalgae content of 5.0%. All values in rows followed by the same letter do not differ significantly ($p > 0.05$). Data presented as a mean ± SD ($n = 3$).

4. Discussion

In the course of the research, it was found that a higher content of microalgae led to a decrease in color in bread, both for the crust and crumb ($p < 0.05$). For the crust, the color value was 0.913, for the crumb—0.826. Similar results were reported earlier [31,32]. These results are also confirmed in studies with baked goods containing *S. platensis* [33], *C. vulgaris* [34] and *I. galbana* [35] and are explained by the pigment degradation during baking to the effect of saturation of the pigment above a certain microalgae concentration. No differences in the color of bread with different microalgae content were observed on the 3rd day after baking compared to freshly baked bread except for a decrease in the color values of the crust, which is probably due to moisture loss during storage.

In the current study, the specific volume of loaf samples with a large amount of microalgae (5%) was lower than the volume of loaves with the addition of 1% and 3% microalgae (Table 7). These results can be explained by the dilution of starch and gluten after replacing part of the flour with microalgae and a decrease in the number of fully hydrated starch granules caused by the fact that the added microalgae powder competes for water with starch. A decrease in the volume of the loaf obtained after the microalgae addition led to a higher density of bread with 5% microalgae compared to bread containing 1% and 3% microalgae.

The higher concentration of microalgae probably led to an increase in moisture content in the bread samples. The moisture content in the samples with the addition of 1% microalgae was lower than in the samples with the addition of 3% and 5% microalgae. A decrease in moisture was observed on the 3rd day after baking due to staling. Moisture loss during storage was 8.8%, 5.1% and 4.9% for bread with the addition of 1%, 3% and 5% microalgae, respectively.

Replacing wheat flour with different amounts of microalgae biomass did not affect the pH and a_w of bread with the addition of 1% and 3% (Table 7). However, with the addition of 5% microalgae, the pH shifted towards more acidic values. A decrease in a_w was observed in bread samples with the addition of 3% and 5% microalgae during storage, which is probably caused by a higher loss of moisture during storage. Similar values of a_w were also observed in bread enriched with biologically active ingredients [25].

A higher density of bread often correlates with increased hardness. However, no statistically significant differences were found in the current study. Similar results were observed after adding freeze-dried broccoli by-products to bread at a concentration of 2% [27]. The observed increase in hardness on the 3rd day compared to the values obtained on the day of baking may be due to the staling of bread and the loss of moisture. In addition, there were no significant differences in elasticity, cohesivity, stickiness, ease of chewing and stability between bread containing 1% and 3% microalgae, which suggests a comparable sensation in the mouth and similar preservation of textural properties after self-pressing, both on the 1st and 3rd day of baking. As for bread with the addition of 5% microalgae, the density and the coefficient of stickiness increased, while the coefficient of elasticity and the coefficient of resistance to crumbling decreased. The results were consistent with the results obtained in [23]. Similar results were observed earlier in other food products, where the addition of microalgae did not affect their functional properties [23,36].

Currently, polyphenols derived from algae are one of the main ingredients of functional nutrition for the prevention of cardiovascular diseases and diabetes mellitus [37]. The addition of microalgae led to an increase in polyphenols in both samples of bread with microalgae and, as expected, to an increased antioxidant capacity (Table 9). Several studies report high antioxidant activity of microalgae biomass due to the high content of phenols and carotenoids [38]. There was a positive correlation between the amount of phenolic compounds and their antioxidant capacity. The antioxidant capacity of bread with microalgae was 0.894 for bread with the addition of 1% microalgae, 0.998 for bread with the addition of 3% microalgae and 1.000 for bread with the addition of 5% microalgae (FRAP method) and 0.874 for bread with the addition of 1% microalgae, 0.999 for bread with the addition of 3% microalgae and 1.100 for bread with the addition of 5% microalgae (DPPH method). Other studies have also reported an increased content of polyphenols and a higher antioxidant capacity after the addition of microalgae to pasta [36] or broccoli to bread [26].

The amount of polyphenols (Table 9) in enzymatic digestive extracts obtained after imitation of gastrointestinal digestion is higher in bread with the addition of 5% microalgae than in bread with the addition of 1% and 3% microalgae. This is probably caused by a higher release of polyphenols under the action of digestive enzymes. A longer extraction time may also partially explain these results. Not only the content of phenols, but also the antioxidant capacity of enzymatic digestive extracts of bread with the addition of 5% microalgae were higher than the ones of bread with the addition of 1% and 3% microalgae.

The observed increase in antioxidant capacity with an increase in the content of microalgae in bread may be associated with a higher content of phenols, as well as with the creation of bioactive peptides with antioxidant capacity, as shown in studies [39,40], in which microalgae were sources of bioactive peptides. When digesting bread with microalgae, the bioavailability of biologically active substances with beneficial nutritional properties increases [41].

Cavonius et al. [42] suggested that cell destruction, and, to a lesser extent, increased lipid bioavailability, improves the availability of proteins derived from *C. vulgaris*. The bioavailability of other antioxidant compounds found in microalgae, such as carotenoids, depends on the type of food product and its processing conditions [43].

The inclusion of microalgae in the bread formulation in the concentrations studied in this research did not affect the bread appearance estimate. Lafarga et al. [27] reported a high visual estimate of the acceptability of green bread prepared using broccoli leaves.

There were no differences in the estimates of the texture and taste of bread with the addition of 1% microalgae and 3% microalgae, however, in samples of bread with 5% microalgae, a note of unpleasant "fishy" taste was observed. Trimethylamine, which is synthesized from trimethylamine oxide (trimethylamine oxide) by enzymatic reactions, is responsible for the fishy smell. Treating the dough with acidic ingredients such as lemon, vinegar, or tomatoes can cause trimethylamine to bind to water and become less volatile, resulting in less fishy smell. The established fact requires further research on the possibility of increasing the proportion of microalgae in bread without losing organoleptic properties. The aroma of bread with 5% microalgae was more pronounced. Eight volatile compounds were found in bread with microalgae (Table 10), including alcohols (5), aldehydes (12), ketones (4), esters (11), sulfur compounds (2), acids (1), terpenes (4) and hydrocarbons (3).

The most common alcohols were 3-methyl-1-butanol, 1-octen-3-ol and 2-ethyl-1-hexanol, which are released during the dough fermentation [44]. In addition to identifying aromatic compounds such as 1-octen-3-one, 6-methyl-5-hepten-2-one and 2-nonanone, in the ketone group, 2-undecanone was isolated, which was the most common ketone in this study. Groups of esters, acids, sulfides, terpenes and hydrocarbons completed the list of identified compounds. The presence of these compounds was reported in a study using GC–MS analyses of bread with microalgae [45]. The most significant aldehyde in bread with microalgae was (Z)-2-heptenal, a breakdown product of linoleic acid [45].

The basis for the creation of fortified foods is the modification of traditional products by introducing physiologically functional ingredients into their composition. It is advisable to regulate the chemical composition of products by using various types of traditional raw materials for baking, which are used in significant quantities, and new types of raw materials, including additives of vegetable and animal origin, which allow changing the chemical composition of products. In this regard, the use of marine objects, algae in particular, for bread enrichment has recently been gaining relevance.

Marine microalgae are used as food flavors, colorants, and nutrients. In the baking industry, algae are increasingly important as improvers and functional ingredients. Their use is designed to improve the properties of the dough, improve the quality of finished products, extend shelf life, and increase biological value. Marine microalgae contain almost all the microelements necessary for humans, so preparations and food products with them can serve as an additional and in some cases, the main source of the necessary functional substances. Thus, the use of algae in baking is a promising area, as it allows creating a new group of functional products with specified characteristics, as well as with increased nutritional and biological value. Wheat flour of the highest grade is most often used for baking bread. Unfortunately, its chemical composition is rather poor, therefore, increasing the nutritional value of bread by introducing additional ingredients into the composition of wheat flour seems relevant.

5. Conclusions

One of the tasks of the State policy in the field of healthy nutrition aimed at the formation and preservation of human health, reducing the risk of foodborne diseases, is the creation of foods enriched with essential components, functional products, as well as dietary (therapeutic and preventive) foods. The active introduction of microalgae containing physiologically significant amounts of micronutrients into the nutrition structure will make it possible to effectively adjust the nutritional status of the population.

The results showed that samples of bread with microalgae contain a large amount of bioavailable polyphenols, volatile substances compared with control samples of bread baked without added microalgae. Such bread exhibits a high antioxidant capacity, which suggests the possibility of obtaining more "healthy" products. Their use will also allow food manufacturers to expand the range of bakery products using the functional ingredient. The organoleptic evaluation showed that the samples of bread with microalgae were competitive with the samples of traditional wheat bread and had improved nutritional value.

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