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Production and Analysis of Beer Supplemented with *Chlorella vulgaris* Powder

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Abstract: The microalgae *Chlorella vulgaris* is a cheap source of nutrients and bioactive compounds, and thus is used in many interventional studies. This study evaluated the potential effects of *C. vulgaris* powder on fermentation parameters; sensory, phytochemical, and antioxidant activity; and the abundance of volatile organic compounds (VOCs) of treated versus control beers. A German Pilsner-style lager beer (GPB) was brewed and supplemented with *C. vulgaris* at various levels (3.3, 5, and 10 g/L) after primary fermentation. The apparent °Brix and pH was used to monitor the progress of fermentation. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydrogen peroxide (H_2O_2) was used to measure the antioxidant activity of beers. Addition of *C. vulgaris* increased the concentration of total polyphenols, total flavonoids, and antioxidant activity of treated beers (CGB) compared to the control (GPB). Treatment had no effects ($p > 0.05$) on higher alcohols such as 3-methyl-1-butanol, 2-hexanol, and phenylethyl alcohol. An increase in the concentration of *C. vulgaris* had no significant effects on sensory perception of enriched beers. The results showed that *C. vulgaris* could be used as a potential ingredient for designing functional beer with improved health benefits.

Keywords: *Chlorella vulgaris*; antioxidants; bioactive compounds; beer; supplement

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1. Introduction

Beer is an alcoholic drink obtained from either controlled or spontaneous fermentation of wort by yeast, which is readily consumed by diverse cultures, religions, and age groups. It is one of the most consumed alcoholic beverages in the world with a long cultural history that spans from different civilizations as early as the fourth millennium BC in Mesopotamia and ancient Egypt [1]. Beer is rich in carbohydrates, amino acids, minerals, vitamins, and bioactive compounds such as phenols, which serve as antioxidants [2]. Therefore, moderate consumption may improve the immune system, cardiovascular system, and cholesterol metabolism of consumers [3,4].

With growing demand for functional foods, a lot of research and resources are channeled toward investigating the physiological effects of high-value biological components from natural sources that improve the nutritional quality and taste of foods and beverages (including alcoholic beverages) [5].

Chlorella vulgaris is a specialized group of freshwater green microalgae, overflowing with carotenes, protein, fiber, vitamins, essential amino acids (including glutathione peptide), minerals, nucleic acids, polysaccharides, and chlorophyll [6]. *C. vulgaris* has been extensively used in many intervention studies to improve the nutritional content of foods due to its well-balanced chemical composition and potential biological effects (i.e., antioxidant, anti-diabetic, and anti-inflammatory effects) on human health [7,8]. Commercially, food industries have fortified beverages, snacks, and baked products with *C. vulgaris* to improve nutritional and sensory profiles [8]. Preclinical studies have further confirmed the antitumor [9], hepatoprotective, and antioxidant properties [10] of *C. vulgaris* on human health. A recent study showed the neuroprotective effects of a *C. vulgaris*-enriched

Brazilian alcoholic beverage on the brain cells of young-adult rats [7]. Furthermore, in Delft, The Netherlands, beer enriched with *C. vulgaris* powder has been reported [11] with no data supporting its biological effects. Others have recently demonstrated the potential utilization of starch-producing *Tetraselmis chui* in various compositions with malt in micro-brewing [12]. The authors of [12] recommended further study to assess the impact of algae supplementation on the final beer. Thus, this manuscript fills the knowledge gap in that regard.

This work aims to evaluate the effects of *C. vulgaris* supplementation on fermentation parameters (apparent gravity, percentage alcohol by volume, color, bitterness units, pH, total acidity, and free amino nitrogen); sensory, phytochemical, and antioxidant properties; and the abundance of volatile organic compounds (VOCs) of enriched versus control beers. This research is a preliminary follow-up to the series of studies aimed at investigating the potential utilization of *C. vulgaris* in designing beer with improved health benefits.

2. Materials and Methods

2.1. Materials and Chemicals

Food-grade *C. vulgaris* powder was sourced from Zhengzhou Sigma Chemical Co., Ltd., (Zhengzhou, China). Mangrove Jack's dried M54 California lager yeast was bought from Bevie Handcraft, Limited (Nelson, New Zealand). Chateau Pilsen 2rs (3 EBC), and Chateau Cara blond (20 EBC) malts were purchased from Castle Malting Limited (Beloeil, Belgium). T90 German Perle (α-acid 8%) and Hersbrucker (α-acid 3%) hops were gifted by Beersfan (Yekaterinburg, Russia). Ethanol (95%) was purchased from Rosbio (Saint Petersburg, Russia). Gallic acid (anhydrous), Folin–Ciocalteu solution, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma–Aldrich (Darmstadt, Germany). Sodium carbonate crystal (Na_2CO_3), sodium hydroxide (NaOH), aluminum nitrite $Al(NO_3)_3$, and sodium nitrite ($NaNO_2$) were sourced from Bashkir Soda Company (Ufa, Bashkortostan, Russia). Quercetin was purchased from Conscientia Industrial Co., Ltd. (Zhejiang, China). All chemicals used are of analytical grade.

2.2. Brewing and Addition of Chlorella vulgaris Powder

The production of *C. vulgaris*-enriched beer is based on the recipe for German Pilsner beer (GPB). Before commencing brewing, all equipment was washed and sanitized with ethanol (95%). Wort production was carried out as previously described with slight modifications [13–16]. A single infusion method of mashing was used in which milled Chateau Pilsen 2rs (6 kg) and Chateau Cara blond (0.5 kg) malts were measured into a mash tun with filtered water (25 L) and the temperature raised to 64 °C for 60 min. The temperature was then increased to 72 °C for 20 min and mashed out at 78 °C for 5 min to stop further enzymatic activity. Using a false bottom, lautering was performed with 80 °C water (15 L). Before the actual lautering was performed, a brief period of recirculation of turbid runoff was carried out until a clear wort was obtained. The obtained wort was boiled for 90 min followed by the addition of Perle (30 g) and Hersbrucker (30 g) hop pellets at 60 and 15 min, respectively. The boiled wort was cooled to 20 ± 2 °C with an immersed copper wort chiller connected to running cold water, and the wort was transferred to a fermentation vessel (20 L) pitched with California M54 lager yeast (10 g). The vessel was equipped with an airlock containing approximately 5 mL of 75% ethanol and fermented for 7 days at 21 °C (Figure 1).

After 7 days of fermentation, the ferments were divided into 4 lots (5 L each) and 3.3 g/L (CGB1), 5 g/L (CGB2), and 10 g/L (CGB3) of *Chlorella* were added to 3 of them and allowed to ferment for 2 days at 20 °C. The ferment without *Chlorella* served as the control (GPB). The green beers obtained were transferred into amber polyethylene terephthalate (PET) bottles (700 mL) with dextrose sugar (3 g) and allowed to carbonate at 22 ± 1 °C for 3 days. The temperature was dropped to 4 °C for 1 week for maturation.

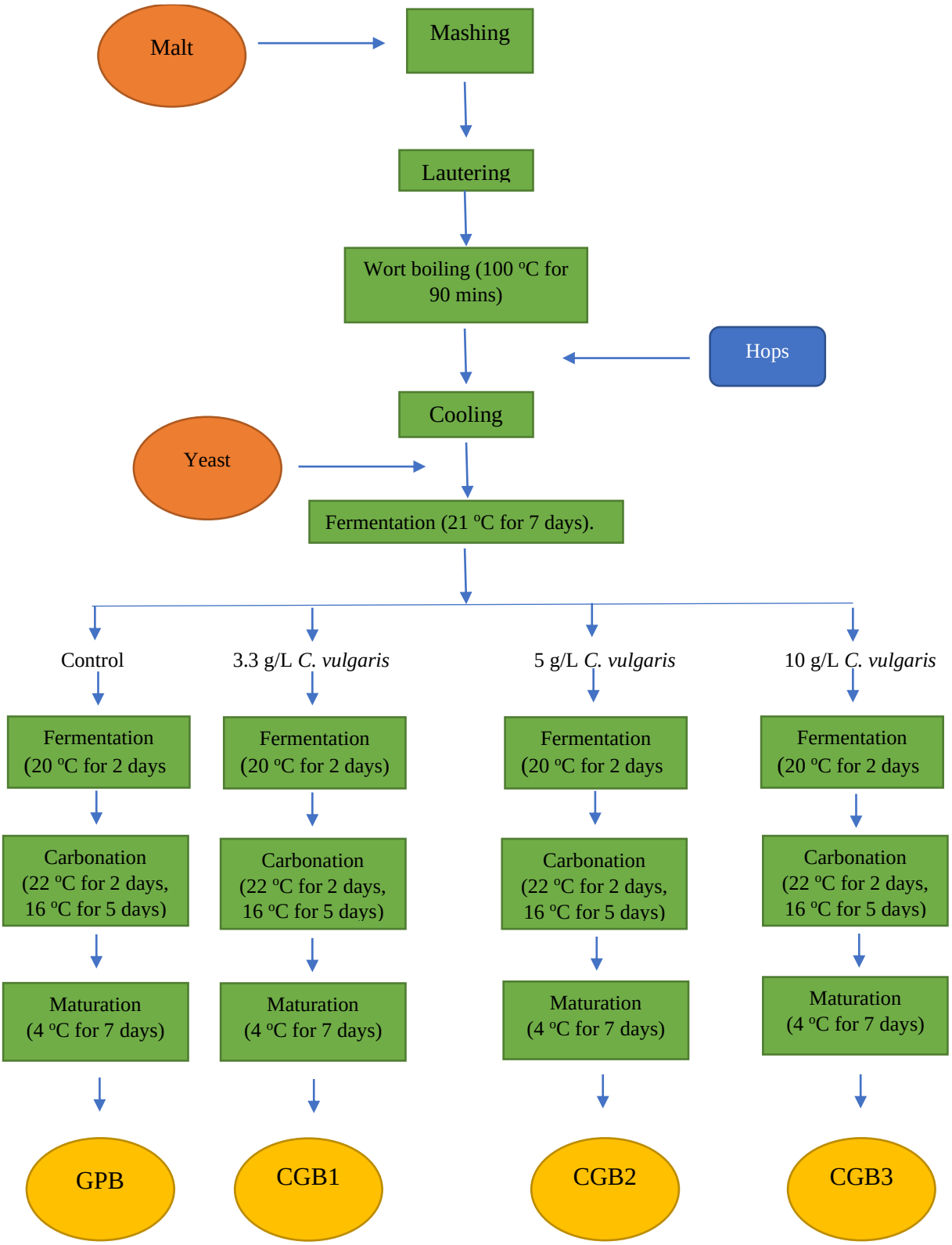

Figure 1. Flow chart of *Chlorella vulgaris*-enriched beer production.

2.3. Physicochemical Analyses

Before analysis, the beer samples were degassed in an ultrasonic bath (Dietikon, Switzerland) for 15 min (45 kHz, 180 W) and centrifuged at 2000× *g* for 10 min. The apparent Brix (°Bx) was measured using a refractometer RSG-100ATC (COMINHKPR124469,

Xindacheng, China). Alcohol by volume (ABV), titratable acidity (TA), pH, color, and international bitterness units (IBU) were measured according to ASBC methods [17]. Free amino nitrogen (FAN) was measured according to ASBC methods [17]. The supernatant (20 mL) of centrifuged beers was frozen for later analysis of VOCs.

2.4. Phytochemical Analysis

The total phenolic content of beers was determined according to the previously described method [18,19].

2.5. Antioxidant Analyses

2.5.1. In Vitro DPPH Antioxidant Activity by Electron Paramagnetic Resonance (EPR)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) in vitro antioxidant activity (AOA) was determined by electron paramagnetic resonance (EPR) as previously described [13–16]. Briefly, DPPH (1 mM) was dissolved in ethanol (60 mL), mixed by agitation, and stored in the dark at room temperature (28 °C) for 20 min. Without pretreatment, beer samples (10 µL) were pipetted into Eppendorf tubes containing DPPH (1 mL). Electron paramagnetic resonance spectra (EPR Elexys E-500) (Bruker Biospin, Karlsruhe, Germany) was measured every 30 s for 10 min. The ability of samples to scavenge DPPH radicals was quantified using Equation (1):

$$\text{AOA} = \frac{\text{ns1} - \text{ns2}}{\text{ns1}} \times \text{CDPPH} \quad (1)$$

where AOA is antioxidant activity (meq); CDPPH is the concentration of DPPH in an initial solution (M); ns1 is the initial number of paramagnetic centers of DPPH and ns2 is the number of paramagnetic centers of DPPH after interaction with the analytes (i.e., beers).

2.5.2. Hydrogen Peroxide (H_2O_2) Scavenging Activity

The hydrogen peroxide scavenging capacity of beer samples was measured according to the previously described method with a slight modification [20]. Briefly, H_2O_2 solution (43 mM) was prepared in a 1 M phosphate buffer (pH 7.4). An aliquot (3 mL) of diluted beer samples (50 times) was transferred into separate test tubes and H_2O_2 solution (1 mL) added. The reaction mixture was incubated for 10 min at room temperature. After incubation, the absorbance was measured using the Shimadzu UV-1800 spectrophotometer (Kyoto, Japan) at 230 nm against a blank solution (phosphate buffer only). The percentage of H_2O_2 scavenging of beer samples was estimated using the following formula:

$$\text{Percentage (\%) } H_2O_2 \text{ scavenging activity} = \left[\frac{\text{The absorbance of control (without beer)} - \text{Absorbance of sample}}{\text{The absorbance of control sample}}\right] \times 100 \quad (2)$$

2.6. Analysis of Volatile Organic Compounds

The VOCs were detected according to the previously described method [13,16]. Briefly, without pretreatment beer samples (1 µL) were manually injected into a gas chromatography–mass spectrometry (GC-MS, 7890B; Agilent Technologies, Santa Clara, CA, USA) coupled with mass spectrometer (Agilent Technologies, Beijing, China). The VOCs were separated on a capillary column, DB-5 ms (30 m × 0.25 mm, 0.25 µm, Agilent Technologies). Helium (99.9% purity) was used as a carrier gas at a flow rate of 1.5 mL/min. The column oven temperature was kept at 40 °C for 5 min, then increased to 190 °C, and kept for 5 min. The injection port, ion source, and quadrupole temperatures were set at 240 °C, 230 °C, and 150 °C, respectively. All mass spectra were acquired using the electron ionization (EI) mode at 70 eV. The mass range was between 30 and 300 m/z. The VOCs were identified by comparing mass spectra obtained from the Mass Spectral Library of the National Institute of Standards and Technology.

2.7. Sensory Evaluation

The sensory evaluation of beer enriched with *C. vulgaris* and of the control beer was carried out according to the descriptive free sorting technique [15,16]. The panelists

(10 members, 5 male and 5 female, from the Department of Technology for Organic Synthesis Technology, Ural Federal University, Yekaterinburg, Russia) were trained in the evaluation of sensory attributes using conventional beer purchased from the supermarket. Four of the panelists were certified wine connoisseurs from Beersfan, a local brewery. The parameters evaluated were the flavor, transparency, mouthfeel, bitterness, alcohol strength, color, and overall acceptance of each beer using the 9-point hedonic scale (like extremely = 9, like very much = 8, like moderately = 7, like slightly = 6, neither like nor dislike = 5, dislike slightly = 4, dislike moderately = 3, dislike very much = 2, dislike = 1). The sensory evaluation was conducted in duplicate.

2.8. Statistical Analysis

Data obtained from triplicate measurements were subjected to a one-way analysis of variance (ANOVA) using Minitab® 21.0 (Minitab Ltd., Coventry, UK) software. Turkey's test was used to identify the differences between means ($p < 0.05$). Results were presented as means ± standard deviation (SD).

3. Results

3.1. Physicochemical Analysis

As fermentation progressed, a decrease in gravity (°Bx) was observed from 12.8 to ~6 °Bx during the 7 days before splitting, and *C. vulgaris* was added at various levels (except to the control). The control ferment (GPB) had significantly ($p < 0.05$) lower final °Bx compared to CGB1, CGB3, and CGB3 (Table 1). Furthermore, the °Bx values of treated ferments (CGB1, CGB2) were significantly different ($p < 0.05$) from the CGB3 ferments (Table 1). With regards to alcohol by volume (%), the treated ferments, i.e., CGB1 (3.51 ± 0.20% ABV), CGB2 (3.51 ± 0.03% ABV), and CGB3 (3.72 ± 0.12% ABV) were significantly different compared the control ferment GPB (3.40 ± 0.03% ABV), which corresponded with the °Bx. The addition of *C. vulgaris* had no effects ($p > 0.05$) on pH and titratable acidity. Regarding color, ferments supplemented with *C. vulgaris* were significantly different ($p < 0.05$) from untreated ferments, which correlated with the levels of *C. vulgaris* supplemented and the color unit measured. For example, ferments supplemented with 10 g/L *C. vulgaris* (CGB3) had high EBC units (9.2) compared to CGB2 (5 g/L), CGB1 (3.3 g/L), and the control ferments (GPB) (Table 1). The bitterness unit of GPB (20.17 IBU) was statistically different ($p < 0.05$) from the treated ferments. The decrease in FAN concentration was from 136.2 ± 7.4 mg/L (wort) to 114.84 ± 5.35 (CGB1), 131.21±6.93 (CGB2), 162.5 ± 22.5 (CGB3), and 116.84 ± 2.88 (CPB). All ferments were statistically different ($p < 0.05$) from wort with regards to FAN content.

Table 1. Physicochemical properties of *Chlorella* beers.

Samples	pH	FG (°Bx)	ABV (%)	Bitterness (BU)	Color (EBC)	TA (%)	FAN
CGB1	$3.98^{a} \pm 0.03$	$5.80^{b} \pm 0.10$	$3.51^{b} \pm 0.20$	$17.50^{c} \pm 0.10$	$7.7^{c} \pm 0.01$	$0.22^{a} \pm 0.01$	$114.84^{b} \pm 5.35$
CGB2	$4.03^{a} \pm 0.03$	$5.80^{b} \pm 0.40$	$3.51^{b} \pm 0.03$	$18.60^{b} \pm 0.10$	$8.5^{b} \pm 0.30$	$0.22^{a} \pm 0.03$	$131.21^{b} \pm 6.93$
CGB3	$4.05^{a} \pm 0.03$	$5.86^{a} \pm 0.06$	$3.72^{a} \pm 0.12$	$11.43^{d} \pm 0.42$	$9.2^{a} \pm 0.08$	$0.23^{a} \pm 0.01$	$162.51^{b} \pm 22.5$
GPB	$4.03^{a} \pm 0.05$	$5.40^{c} \pm 0.02$	$3.49^{b} \pm 0.03$	$20.17^{a} \pm 0.29$	$7.1^{d} \pm 0.03$	$0.21^{a} \pm 0.02$	$116.84^{b} \pm 2.88$
Wort	_	_	_	_	_	_	$396.23^{a} \pm 75$

The results represent the means ± SD of the measurements made in triplicate. Means with different letters in each column denote significant differences ($p < 0.05$), where CGB, *Chlorella* German beer (CGB1 = 3.3 g/L *Chlorella*, CGB2 = 5 g/L *Chlorella*, CGB3 = 10 g/L *Chlorella*); GPB, German pilsner green beer; °Bx, °Brix; EBC unit, European Brewery Convention unit; %ABV percentage alcohol by volume.

3.2. Phytochemical Composition and Antioxidant Activity of Beer Samples

The total flavonoid content of *C. vulgaris*-enriched beer ranged between 201.96 to 242.7 mg QE/L. Ferments supplemented with 10 g/L *C. vulgaris* (CGB3) were statistically different ($p < 0.05$) compared to control ferments (GPB) (Table 2). Similarly, 10 g/L *C. vulgaris* (CGB3) significantly ($p < 0.05$) enhanced the total phenolic contents of CGB3 compared to CGB1 and control ferments (Table 2).

Table 2. Phytochemical and antioxidant activities of beers.

Samples	Total Flavonoid Content (mg QE/L)	Total Polyphenol Content (mg GAE/L)	EPR Antioxidant Activity (10^{-2} M-eqv)	H_2O_2 Scavenging Activity (%)
CGB 1	201.96 ^{ab} ± 13.98	257.81 ^{b} ± 15.20	4.39 ^{c} ± 0.20	88.86 ^{c} ± 0.15
CGB 2	200.1 ^{ab} ± 9.10	328 ^{ab} ± 10.60	4.64 ^{b} ± 0.03	89.18 ^{b} ± 0.14
CGB 3	242.7 ^{a} ± 16.04	442.02 ^{a} ± 15.20	4.66 ^{a} ± 0.01	89.98 ^{a} ± 0.04
GPB	185.3 ^{b} ± 25.70	257.8 ^{b} ± 30.40	3.79 ^{d} ± 0.20	88.86 ^{c} ± 0.13

The results represent the means ± SD of the measurements made in triplicate. Means with different letters in each column denote significant differences ($p < 0.05$), where CGB, *Chlorella* German beer (CGB1 = 3.3 g/L *Chlorella*, CGB2 = 5 g/L *Chlorella*, CGB3 =10 g/L *Chlorella*); GPB, German pilsner green beer; EPR, electron paramagnetic resonance; H_2O_2, hydrogen peroxide.

Treatment significantly ($p < 0.05$) increased the DPPH AOA of ferments compared to control ferments. Ferments supplemented with 10 g/L *C. vulgaris* (CGB3) showed the highest AOA (4.66×10^{-2} M-eqv) compared to CGB2 (4.64×10^{-2} M-eqv) and GPB (3.79×10^{-2} M-eqv) (Table 2). Similarly, 10 g/L (CGB3) and 5 g/L (CGB2) *C. vulgaris* significantly ($p < 0.05$) increased the H_2O_2 scavenging activity compared to CGB1 and GPB ferments.

3.3. Volatile Composition of Beer Samples

A total of 28 VOCs were selected based on the National Institute of Standards and Technology library match factor (M) and probability (>80%), as well as their contribution to beer flavor [21] (Table 3). The selected VOCs in the treated and control beers included nine higher alcohols (HAs), nine ketones, four esters, three organic acids, two vicinal diketones (VDKs), and one phenol. Treatment had no effects ($p > 0.05$) on the abundance of important HAs such as 3-methyl-1-butanol, phenylethyl alcohol, and 2-hexanol. CGB3 had a significant abundance ($p < 0.05$) of ethanol compared to CGB2. Similarly, the control beers had a significant abundance ($p < 0.05$) of 2-furanmethanol compared to treated samples.

Table 3. Volatile compounds identified in beers.

Peak#	Compounds	Abundance (TIC) × 10^6				*p*-Value
		CGB1	CGB2	CGB3	GPB	
1	Ethanol	2360.8 ^{ab} ± 57.3	2256.5 ^{b} ± 60.1	2570.9 ^{a} ± 42.9	2452.1 ^{ab} ± 58.3	0.02
2	3-Methyl-1-butanol	9.21 ^{a} ± 0.54	9.12 ^{a} ± 0.16	10.47 ^{a} ± 0.10	9.86 ^{a} ± 0.31	0.06
3	1-Hydroxy-2-propanone	34.64 ^{a} ± 1.77	13.79^{c} ± 0.76	24.51 ^{b} ± 2.11	22.94 ^{b} ± 1.50	0.01
4	Acetic acid	54.53 ^{b} ± 0.71	38.56 ^{c} ± 1.4	53.20 ^{b} ± 1.22	60.40 ^{a} ± 1.15	0.01
5	Ethyl acetate	114.33 ^{b} ± 1.25	67.20 ^{c} ± 5.41	111.05 ^{b} ± 1.83	121.57 ^{a} ± 1.25	0.01
6	2,3-Butanediol	13.70 ^{ab} ± 0.70	11.85 ^{b} ± 0.92	15.30 ^{ab} ± 0.4	16.20 ^{a} ± 1.37	0.03
7	(R-(R*,R*))-2,3-butanediol	3.89 ^{ab} ± 0.41	3.46 ^{b} ± 0.53	5.73 ^{a} ± 0.71	4.94 ^{ab} ± 0.42	0.04
8	2-Hexanol	2.27 ^{a} ± 0.55	3.32 ^{a} ± 0.81	3.62 ^{a} ± 0.87	3.27 ^{a} ± 0.65	0.40
9	Isomaltol	1.61 ^{a} ± 0.52	0.0 ^{b} ± 0.00	0.96 ^{ab} ± 0.08	1.77 ^{a} ± 0.51	0.03
10	2-Furanmethanol	110.38 ^{b} ± 1.63	84.55 ^{c} ± 1.01	109.73 ^{b} ± 2.13	126.47 ^{a} ± 6.72	0.01
11	1,2-Cyclopentanedione	6.96 ^{ab} ± 0.10	5.08 ^{b} ± 0.33	7.710 ^{a} ± 0.71	8.46 ^{a} ± 0.93	0.02
12	2(5H)-furanone	3.75 ^{a} ± 0.69	2.76 ^{a} ± 0.33	3.63 ^{a} ± 0.67	4.28 ^{a} ± 0.86	0.29
13	2-Cyclohexen-1-ol	n.d.	4.95 ^{b} ± 0.40	7.69 ^{a} ± 0.62	n.d.	0.01
14	6-Oxabicyclo (3.1.0)hexan-3-one	7.35 ^{ab} ± 0.29	5.85 ^{b} ± 0.32	n.d.	8.09 ^{a} ± 0.64	0.01
15	Phenylethyl alcohol	7.46 ^{a} ± 0.49	5.81 ^{a} ± 0.81	7.18 ^{a} ± 0.57	7.80 ^{a} ± 0.37	0.09
16	Maltol	37.79 ^{bc} ± 0.60	35.54 ^{c} ± 0.95	41.18 ^{ab} ± 0.38	43.26 ^{a} ± 1.31	0.01
17	2H-pyran-2,6(3H)-dione	1.72 ^{ab} ± 0.45	n.d.	2.31 ^{a} ± 0.39	3.21 ^{a} ± 0.10	0.02
18	Dihydroxyacetone	12.90 ^{bc} ± 0.28	10.88 ^{c} ± 0.50	16.38 ^{a} ± 0.75	14.80 ^{ab} ± 0.83	0.01
19	Cyclopropyl carbinol	28.37 ^{a} ± 1.64	16.30 ^{b} ± 1.29	30.91 ^{a} ± 1.12	33.77 ^{a} ± 2.04	0.01
20	(S)-(+)-2′,3′-Dideoxyribonolactone	22.04 ^{ab} ± 0.43	14.58 ^{c} ± 1.24	19.29 ^{b} ± 1.34	23.215 ^{a} ± 0.43	0.01
21	2-Hydroxy-gamma-butyrolactone	7.30 ^{a} ± 0.93	4.48 ^{a} ± 1.03	8.03 ^{a} ± 1.35	6.03 ^{a} ± 0.79	0.09
22	1,2,3-Propanetriol-1-acetate	5.13 ^{a} ± 1.53	2.25 ^{a} ± 0.75	4.02 ^{a} ± 0.13	2.91 ^{a} ± 0.37	0.10
23	Ethyl caprylate	8.59 ^{ab} ± 0.89	6.38 ^{b} ± 0.73	8.02 ^{ab} ± 0.28	10.37 ^{a} ± 1.22	0.40
24	Glycerin	248.50 ^{ab} ± 9.81	203.51 ^{b} ± 6.05	291.27 ^{a} ± 14.09	267.70 ^{a} ± 14.7	0.01
25	2-Furancarboxylic acid	2.13 ^{a} ± 0.26	n.d.	n.d.	3.09 ^{a} ± 0.00	0.01
26	2-Methylbutyl isobutyrate	13.69 ^{bc} ± 0.79	11.10 ^{c} ± 0.93	15.35 ^{ab} ± 0.74	17.83 ^{a} ± 0.71	0.01

Table 3. *Cont.*

Peak#	Compounds	Abundance (TIC) × 10^6				*p*-Value
		CGB1	CGB2	CGB3	GPB	
27	Dihydro-4-hydroxy-2-(3H)-furanone	10.46 ^{ab} ± 1.9	8.64 ^{b} ± 0.30	12.82 ^{ab} ± 0.59	14.96 ^{a} ± 1.32	0.02
28	Catechol	6.20 ^{a} ± 1.11	2.08 ^{b} ± 1.07	4.06 ^{ab} ± 0.87	7.42 ^{a} ± 1.00	0.02

The results represent the means ± SD of the measurements made in triplicate. Means with different letters in each row denote significant differences ($p < 0.05$), where TIC, total ion chromatogram; n.d., not detected; GPB, German pilsner green beer; CGB, *Chlorella* German beer (CGB1 = 3.3 g/L *Chlorella*, CGB2 = 5 g/L *Chlorella*, CGB3 =10 g/L *Chlorella*).

Regarding volatile esters, CGB2 had the least ($p < 0.05$) abundance of ethyl acetate compared to CGB1, CGB3, and GPB. In contrast, the abundance of ethyl caprylate was significantly ($p < 0.05$) greater in GPB compared to CGB2. 2-methylbutyl isobutyrate followed a similar trend as observed for ethyl caprylate.

The control ferment (GPB) had a greater abundance of acetic acid ($p < 0.05$) compared to CGB2. Furthermore, treatment had no effects ($p > 0.05$) on the abundance of 2-furancarboxylic acid.

Treatment had significant effects ($p < 0.05$) on the abundance of most selected volatile ketones except for 2(5H)-furanone and 2-hydroxy-gamma-butyrolactone. The abundance of 1-hydroxy-2-propanone was significantly ($p < 0.05$) greater in CGB3 compared to CGB1 and CGB2. Regarding 1,2-cyclopentanedione, GPB and CGB3 had a greater abundance compared to CGB2. GPB had a greater abundance of 6-oxabicyclo (3.1.0) hexan-3-one compared to CGB2. Furthermore, CGB3 had a significant ($p < 0.05$) abundance of dihydroxyacetone compared to CGB2. For (S)-(+)-2′,3′-dideoxyribonolactone, GPB had a significant ($p < 0.05$) abundance compared to CGB2 and CGB3. Similarly, GPB had a significant ($p < 0.05$) abundance of dihy-dro-4-hydroxy-2-(3H)-furanone compared to CGB2.

The selected volatile VDKs included 2,3-butanediol and (R-(R*,R*))-2,3-butanediol, and treatment had significant effects ($p < 0.05$) on their abundances. For example, GPB had a greater abundance of 2,3-butanediol compared to CGB2. Likewise, CGB3 had a significant ($p < 0.05$) abundance of (R-(R*,R*))-2,3-butanediol compared to CGB2.

Regarding volatile phenolic, GPB had a significant ($p < 0.05$) abundance of catechol compared to CGB2.

3.4. Sensory Analysis

The mean sensory attributes of *C. vulgaris*-enriched beer, including the control samples, are presented in Table 4. The addition of *C. vulgaris* had no effects on the mean scores of any of the sensory attributes assessed ($p > 0.05$). However, GPB showed a slightly higher mean score in terms of flavor (7.8 ± 0.78) while sharing a similar score with CGB1 for color (7.5 ± 0.972) and bitterness (7.1 ± 1.6 for GPB and 7.1 ± 1.5 for CGB1). The subtle flavor that was recorded correlated with the overall acceptability of GPB and CGB1, which recorded mean scores of 7.2 ± 1.40 and 7.5 ± 0.7, respectively. GPB and CGB1 had mean scores of 7.2 ± 1.03 and 7.3 ± 0.95, respectively, for mouthfeel; however, only CGB1 had subtle foam retention (7.7 ± 0.68) with GPB 6.8 ± 1.55. Regarding clarity, GPB had a mean score of 7 ± 1.63, which was ascribed to less turbidity. Increased levels of *C. vulgaris* decreased mean scores, with CGB3 recording the lowest mean score for flavor (6.9 ± 1.10), clarity (6.1 ± 1.85), color (7 ± 1.41), alcohol strength (6.4 ± 1.51), mouthfeel (6.6 ± 0.84), and general acceptability (6.6 ± 1.40). Most of the sensory variables corroborated the results of the physicochemical analysis presented in Table 1, except for the alcohol strength.

Table 4. Scores of sensory properties of beers.

Samples	Foam	Flavor	Clarity	Bitterness	Color	Alcohol Strength	Mouthfeel	Overall Acceptability
CGB1	7.7 ^{a} ± 0.68	7.7 ^{a} ± 0.82	6.7 ^{a} ± 1.64	7.1 ^{a} ± 1.52	7.5 ^{a} ± 0.97	6.5 ^{a} ± 1.96	7.3 ^{a} ± 0.95	7.5 ^{a} ± 0.71
CGB2	7.3 ^{a} ± 1.25	7.2 ^{a} ± 1.23	6.3 ^{a} ± 1.70	6.4 ^{a} ± 1.58	7 ^{a} ± 1.33	6.8 ^{a} ± 1.81	7 ^{a} ± 1.23	6.7 ^{a} ± 1.70
CGB3	6.9 ^{a} ± 1.20	6.9 ^{a} ± 1.10	6.1 ^{a} ± 1.85	6.7 ^{a} ± 1.57	7 ^{a} ± 1.41	6.4 ^{a} ± 1.51	6.6 ^{a} ± 0.84	6.6 ^{a} ± 1.43
GPB	6.8 ^{a} ± 1.55	7.8 ^{a} ± 0.79	7 ^{a} ± 1.63	7.1 ^{a} ± 1.60	7.5^{a} ± 0.97	6.7 ^{a} ± 1.95	7.2 ^{a} ± 1.03	7.2 ^{a} ± 1.40

The results represent the means ± SD of the measurements made in triplicate. Means with different letters in each column denote significant differences ($p < 0.05$), where CGB, *Chlorella* German beer (CGB1 = 3.3 g/L *Chlorella*, CGB2 = 5 g/L *Chlorella*, CGB3 = 10 g/L *Chlorella*); GPB, German pilsner green beer.

4. Discussion

During fermentation, brewing yeasts convert fermentable sugars in the wort into volatile esters, alcohols, acids, ketones, aldehydes, and carbon dioxide (CO_2) [21,22], resulting in the decrease in gravity and progressive increase in ABV (%) observed. A recent report showed that *Tetraselmis chui* SAG 8-6 biomass had no inhibitory effects on fermentable sugar release during micro-mashing [12]. The significantly ($p < 0.05$) higher °Bx observed in the final enriched beer might be ascribed to difference in yeast fermentability of each treatment. In addition, *Tetraselmis chui* SAG 8-6 had varying amounts of glucose and maltose [12] and thus *C. vulgaris* may have influenced °Bx values when supplemented in ferments. In addition, the presence of starch-degrading enzymes was reported [12]; such enzymes may degrade dextrin and other higher molecular sugars, thus altering the °Bx value.

The cell wall of *C. vulgaris* is made up of ~80% carbohydrates and thus may serve as a source of fermentable sugars (i.e., glucose, etc.) [23–25]. The addition of *C. vulgaris* after the primary fermentation may have potentially increased the sugar content, which corresponded to higher ABV (%) in the treated samples compared to the control ferments. The current study contradicts previous findings in which ferments supplemented with higher algal content had lower alcohol (% volume by volume (v/v)) compared to control ferments [12]. The addition of *C. vulgaris* decreased bitterness, which may be attributed to the additional sugars from supplemented *C. vulgaris*, which ranged from 13–19% [5,26,27]. The greenish color observed in the supplemented beers originated from *C. vulgaris*, and differed from the pale yellow of GPB. Previous reports showed high green pigment composition (~5.5% chlorophyll) [26,27] in *C. vulgaris*, which may have dissolved due to the fermenting alcohol and thus tainted the beer's hue.

FAN is a nitrogenous compound that yeast assimilates during fermentation and is important for yeast's synthesis of cellular proteins and other cell compounds [28], thus the observed decrease. A typical wort constitutes about 70% FAN produced during malting, whereas it constitutes 20–30% during mashing [28]. The FAN content in wort is an important indicator for assessing the formation of total vicinal diketones, esters, and HAs during fermentation [29]. The high protein content of *Chlorella* [5] may have contributed to elevated FAN content, hence the higher FAN in CGB2 and CGB3 compared to the control ferments.

C. vulgaris is a rich source of phenols and flavonoids, with compositions up to 220 mg/g GAE and 547.023 mg/g RE, respectively [30–32]. The addition of *C. vulgaris* increased the concentrations of TPC and TFC in treated beers compared to the control beers. A recent report demonstrated that ethanolic extract of *C. vulgaris* cultivated on banana stem compost (BCM) and Bold's basal media (BBM) both showed higher DPPH radical scavenging activity (97.9 ± 0.1% and 81.29 ± 0.088%, respectively) compared to *C. vulgaris* cultivated on aquaculture wastewater supplemented with 1.0 g/L NPK (ANM; 77.07 ± 1.657%) [32]. Others have reported low DPPH radical scavenging activity (19.5%) of methanolic extract (1 mg/mL) of *C. vulgaris* [30]. Flavonols, flavanones, flavones, phenolic acids, etc., have been reported to enhance the antioxidant activity of plants and microalgae [33] and these compounds are markedly pronounced in *Chlorella*. Moreover, phenols also contribute to the taste and aroma of beer [34]. These phenols characterize beer's flavors due to their chemical transformation properties, and yeast possesses an inherent potential

to bio-transform phenolic acids into volatile phenols such as 4-vinylguaiacol, 4-vinylphenol, 4-ethylguaiacol, and 4-ethylphenol [34].

VOCs consist of organic acids, esters, carbonyls, ketones, and sulfur-containing compounds produced by yeast metabolism during beer fermentation [16,35,36]. Their concentration is influenced by the type of raw material used, mashing protocol, yeast strain, and fermentation conditions [16,21,22]. VOCs are important because they influence consumer perception and acceptance of beer [13,22]. Most of the identified VOCs are similar to those previously reported in the literature [13,37,38]. However, the addition of *C. vulgaris* had negative effects on the abundance of VOCs (i.e., HAs and esters). The primary VOCs detected in *Chlorella* extract were alcohols, ketones, aldehydes, esters, acids, terpenes, and furans [39–42]. Specifically, 1-penten-3-ol (16000 $\pm$ 1800 µg/g), cis-2-penten-1-ol (7000 $\pm$ 550 µg/g), 3-hexen-1-ol (6100 $\pm$ 900 µg/g), 1-penten-3-one (4800 $\pm$ 260 µg/g), and 3-pentanone (4600 $\pm$ 700 µg/g) have been documented [40], and thus may have masked or tainted the synthesized VOCs in treated samples. Moreover, yeast metabolism after primary fermentation is low and thus could not metabolize the supplemented *C. vulgaris*.

5. Conclusions

The potential for utilization of *C. vulgaris* as an ingredient to design functional beer with enhanced health benefits has been demonstrated. The addition of *C. vulgaris* at various levels improved the phytochemical and antioxidant potential of the treated samples compared to the controls. Furthermore, treatment had no effect on the abundance of most VOCs. The sensory analysis of the treated and control samples showed no difference ($p > 0.05$). Further research is required to fully understand the right amount of *C. vulgaris* to supplement, either during mashing or after primary fermentation, without influencing the sensory perception of the resulting beer.

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