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Functional Properties of *Chlorella vulgaris*, Colostrum, and *Bifidobacteria*, and Their Potential for Application in Functional Foods

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Abstract: The market for new functional foods and food supplements is rapidly evolving, with a current emphasis on using natural sources. Algae, probiotics, and colostrum are rich sources of nutrients and bioactive compounds with positive effects on human and animal health. To determine the potential for developing new functional foods combining these components, we evaluated their synergistic effects. We assessed the growth of selected bifidobacteria in a medium supplemented with *Chlorella vulgaris* and its immunomodulatory and cytotoxic effects on the human peripheral mononuclear cells and colon cancer cell lines Caco-2 and HT29. The hypocholesterolemic effects of *Chlorella* powder and bovine colostrum fermented by *Bifidobacterium animalis* subsp. *lactis* BB12® on lipid metabolism in rats fed a high-fat diet were also determined. *Chlorella* addition promoted *Bifidobacteria* growth, with significantly increased inflammatory cytokine (TNF-α and IL-6) levels following 1.0% (*w*/*v*) *Chlorella* stimulation. Rats fed diets containing fermented colostrum with 0.5% (*w*/*v*) added *Chlorella* powder exhibited significantly decreased triglyceride, very low-density lipoprotein, and alanine and aspartate aminotransferase levels, compared to those of the control group. These results support that *C. vulgaris* is not cytotoxic in intestinal cell models and affords prebiotic and immunomodulatory effects, as well as synergistic triglyceride-lowering effects with bovine colostrum and *B. animalis* subsp. *lactis* BB-12.

Keywords: microalgae; *Chlorella vulgaris*; *Bifidobacterium*; colostrum; functional food; probiotics

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Citation: Hyrslova, I.; Krausova, G.; Smolova, J.; Stankova, B.; Branyik, T.; Malinska, H.; Huttl, M.; Kana, A.; Curda, L.; Doskocil, I. Functional Properties of *Chlorella vulgaris*, Colostrum, and *Bifidobacteria*, and Their Potential for Application in Functional Foods. *Appl. Sci.* **2021**, *11*, 5264. https://doi.org/10.3390/app11115264

Academic Editors: Wojciech Kolanowski and Alessandra Durazzo

Received: 7 April 2021
Accepted: 2 June 2021
Published: 6 June 2021

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1. Introduction

The continuing increases in the global population, expected to reach 9.5 billion by 2050, will necessitate the identification of novel food sources [1]. One option would be to exploit the properties of microalgae since they can be produced on a large scale at a relatively low cost [1–3]. Additionally, they represent a rich source of essential nutrients and bioactive compounds that exhibit prebiotic, immunomodulatory, antioxidative, anticancer, and hypocholesterolemic effects in animal and human models [4–8]. *Chlorella vulgaris*, a blue-green microalga, constitutes an important source of beneficial nutrients such as carotenoids

(e.g., β-carotene, lutein, and zeaxanthin), pigments, proteins with well-balanced amino acid profiles, polyunsaturated fatty acids, minerals, and vitamins (e.g., A, B12, E, and K). Moreover, it is among the few microalgae designated as having Generally Recognized as Safe (GRAS) status by the US Food and Drug Administration and is approved by the European Food Safety Authority [9]. Accordingly, different forms of *Chlorella* biomass have been used in a wide range of products, such as functional foods, pigments, yogurt, cheese, and bakery products [1,10,11]. In turn, the combination of *Chlorella* with probiotic strains in dietary supplements or functional foods will permit the creation of a new segment of products.

Probiotics are defined as live microorganisms that, when administered in adequate amounts, exert health benefits to the host, such as regulating the immune system, preventing diarrhea, allergies, and atopic diseases, and reducing hypertension, obesity, or high serum cholesterol [12–14]. Strains from the genera *Lactobacillus* and *Bifidobacterium* have been widely used as probiotics and are preferred by manufacturers because of their health benefits and ability to survive technological processes (e.g., low pH and molecular oxygen). Both genera also produce antimicrobial and bioactive compounds (e.g., exopolysaccharides, bacteriocins, organic acids, and hydrogen peroxide) that compete with gastrointestinal and food spoilage pathogens [14,15]. Probiotic strains may also have utility for the preservation of colostrum to improve the nutritional quality of food supplements for animals and humans [16]. Colostrum, the first milk produced by mammals immediately after birth, exhibits a specific nutritive composition and is a rich source of proteins, fats, immunoglobulins, minerals, and bioactive compounds such as lactoferrin, lysozyme, lactoperoxidases, and growth factors. The benefits of colostrum on human or animal health have been extensively studied, demonstrating the positive influence of colostrum consumption on the immune system, intestinal microflora balance, and tissue regeneration [17–19]. For example, we have previously demonstrated the positive growth-promoting effects of bovine, goat, and human colostrum on lactic acid bacteria [20,21]. In turn, the interaction of probiotics with intestinal microflora offers the potential to positively affect obesity, the global rise of which in adults and children increases the risk of developing cardiovascular diseases, type 2 diabetes, and pediatric metabolic syndrome [22].

The aim of the present study was therefore to extend information about functional properties of *Chlorella vulgaris* and its possible synergic effects with probiotics (*Bifidobacteria*) and colostrum that contribute to the development of new functional food or food supplements for humans or animals with the content of mentioned components. All are rich and affordable sources of nutritive and bioactive compounds. On the market, it is possible to buy individually or in combination with lyophilized probiotics (*Lactobacilli*, *Bifidobacteria*), but there is a lack of information about their synergic/antagonistic effects. Just a few studies were published about the functional properties of aqueous chlorella extracts. First, we determined the cytotoxic, immunomodulatory, and prebiotic effects of *Chlorella*. The immunomodulatory and prebiotic effects of bovine colostrum were described in our previous studies [20,21]. In addition, since the increased incidence of obesity at the population level is predominantly caused by a sedentary lifestyle, stress, or overabundant food intake, we also evaluated the synergic hypocholesterolemic effects of a combination of all three components in an animal model of Prague hereditary hypercholesterolemic rats (PHHC) with diet-induced hypercholesterolemia [23].

2. Materials and Methods

2.1. Microorganisms

Three tested bifidobacteria (*Bifidobacterium animalis* subsp. *lactis* CCDM 93, *Bifidobacterium breve* CCDM 486, and CCDM 562) were selected from the Culture Collection of Dairy Microorganisms Laktoflora® (Tabor, Czech Republic), and one commercial strain *Bifidobacterium animalis* subsp. *lactis* BB-12® was obtained from Ch. Hansen (Hørsholm, Denmark). Prior to each analysis, bacterial cells were transferred twice in fresh De Man–Rogosa–Sharpe (MRS, pH 6.2) broth (Merck, Darmstadt, Germany) with *L*-cysteine hydrochloride

(Merck) and cultivated in anaerobic conditions at 37 °C for 18 h. Heterotrophic *C. vulgaris* was obtained from the Microbiology Department of the Academy of Sciences, Třeboň–ALGATECH (Třeboň, Czech Republic).

2.2. Colostrum

Lyophilized skimmed bovine colostrum, obtained during the early stage of lactation with 30% immunoglobulin content, was provided by Ingredia Ltd. (Frydek-Mistek, Czech Republic). For testing, a 10% (*w*/*v*) reconstituted colostrum solution was prepared.

2.3. Immunomodulation Assay

The immunomodulatory effect of an aqueous solution of *C. vulgaris* was evaluated using Luminex multiplex assays for the simultaneous quantitative determination of multiple human cytokine concentrations in cell culture supernatants, serum, and plasma according to the previous study with slight modifications [20]. Briefly, eight samples of the blood from healthy adults, for the isolation of human peripheral blood mononuclear cells (hPBMCs) via Ficoll–Hypaque gradient separation, were ordered from the Blood Transfusion Center of General Faculty Hospital (Prague, Czech Republic). Following separation and purification, hPBMCs were adjusted to a final concentration of 10^7 cells mL^{-1}. Mononuclear cells (0.1 mL) were stimulated in an X-vivo medium (Cambrex, East Rutherford, NJ, USA) with 0.1 mL of 1.0% or 3.0% aqueous solution of *C. vulgaris* at 37 °C for 3 days. The total volume was 1 mL. The negative control was composed of unstimulated hPBMCs and the X-vivo medium. The levels of cytokines produced by stimulation of hPBMCs with different concentrations of *C. vulgaris* were determined using Fluorokine MAP Human Base Kit A (R&D Systems, Minneapolis, MN, USA) for interferon (IFN)-γ, interleukin (IL)-4, IL-6, tumor necrosis factor (TNF)-α, and IL-17 by multiplex analysis using a Luminex 200 Analyzer (Luminex Corp., Austin, TX, USA). The concentration of cytokines produced by hPBMCs was assessed using the Luminex IS 2.3. The results are values from three different measurements.

2.4. Cytotoxic Effect

Caco-2 and HT29 cell lines derived from human adenocarcinoma (American Type Culture Collection, Rockville, MD, USA) were cultured in Eagle's minimal essential medium (EMEM, Sigma-Aldrich, Steinheim, Germany) supplemented with 10% fetal bovine serum (Merck, Darmstadt, Germany), 1.0% sodium bicarbonate (Merck, Darmstadt, Germany), 1.0% sodium pyruvate (Sigma-Aldrich, Steinheim, Germany), 5.0 mM glutamine (Sigma-Aldrich Steinheim, Germany), 1.0% MEM nonessential amino acids (Sigma-Aldrich, Steinheim, Germany), and 1.0% penicillin–streptomycin solution (10,000 units/mL penicillin and 10.0 mg/mL streptomycin, Sigma-Aldrich, Steinheim, Germany). Cultures were incubated at 37 °C with 5% CO_2, and media were replenished every 2–3 d, and passaged every 7 d. Cell passages 12–16 were used for the assay; viability was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich, Steinheim, Germany) cytotoxicity assay [24] with modifications. Briefly, Caco-2 and HT29 cells (1:9 ratio) were seeded in 96-well plates at a density of 2.5×10^3. After 24 h, the cells were treated with twofold serially diluted samples (16–512 μg/mL) for 72 h. Then, MTT reagent (1 mg/mL) in EMEM was added to each well and incubated for an additional 2 h at 37 °C with 5.0% CO_2. The medium with MTT was removed and the intracellular formazan product was dissolved in 100 μL dimethylsulfoxide. The absorbance was measured at 555 nm using a Tecan Infinite M200 spectrometer (Tecan Group, Männedorf, Switzerland), and the percentage of viability (IC_{50} and IC_{80} value) was calculated and compared to that of the untreated control. The results are values from three different measurements.

2.5. Prebiotic Assay

The bacterial growth was assessed according to the methods described [25], with some modifications. In brief, the following media were prepared with different concen-

trations of *C. vulgaris* as the sole carbon source. Basal medium [25] was supplemented with 1.0% and 3.0% (*w*/*v*) *Chlorella* biomass (pH 6.5). Medium without *Chlorella* biomass inoculated with *Bifidobacteria* served as the negative control (BS-basal medium) and Wilkins–Chalgren (WCH) anaerobic broth (Oxoid, Hampshire, UK) as the positive control. Following overnight growth, bifidobacteria cultures in the exponential growth phase were separated from the medium by centrifugation (6000× *g*, 7 min), washed with sterile saline solution, and then resuspended at a final concentration of 10^3–10^4 colony forming units (CFU) mL^{-1}. All media were inoculated with 1.0% (*v*/*v*) bacterial suspension and cultivated in anaerobic jars (Merck) at 37 °C for 24 h. The counts of tested strains at 24 h were determined using 10-fold serial dilutions and cultivated on MRS agar (Merck). To determine the concentrations of lactic and acetic acids, as the main fermentation products of *Bifidobacteria*, the isotachophoretic method was used, as previously described [21]. After cultivation, the samples were subjected to isotachophoretic separation using an EA 102 electrophoretic analyzer (Villa-Labeco, Spišská Nová Ves, Slovakia). A mixture of 10 mmol/L HCl, 22 mmol/L 6-aminocaproic acid and 0.1% hydroxyethylcellulose (MERCK) was used as a leading electrolyte, and 10 mmol/L caproic acid as a terminating electrolyte. The results are values from three different measurements.

2.6. Animal Model and Study Design

Male Prague hereditary hypercholesterolemic rats (PHHC), 8 weeks old with an average body weight of 181.4 ± 7.7 g, were obtained from Albert Weber-SEMED (Praha, Czech Republic). Animals were acclimatized to laboratory conditions for two weeks prior to the experiment and housed at room temperature (22–24 °C), humidity (55–60%) in a 12/12 h light–dark cycle with *ad libitum* access to water and food. The hypercholesterolemic diet (Albert Weber-SEMED, Praha, Czech Republic) was fortified with 2.0% (*v*/*w*) cholesterol to potentiate hypercholesterolemia. All experiments were performed in accordance with the Animal Protection Law of the Czech Republic (311/1997) in compliance with the European Community Council recommendations (86/609/ECC) for the use of laboratory animals and approved by the ethical committee of the Ministry of Education, Youth and Sports (jMSMT-46654/2015-8).

After adaptation, rats were randomly divided into four groups (10 animals per group) as follows: I, rats fed the hypercholesterolemic diet (control group); II, rats fed the hypercholesterolemic diet with 600 µL 0.5% (*w*/*v*) aqueous solution *Chlorella*; III, rats fed the hypercholesterolemic diet with a mix of 600 µL colostrum 10% (*w*/*v*) and 0.5% (*w*/*v*) *Chlorella* powder; and IV, rats fed the hypercholesterolemic diet with a mix of 600 µL colostrum 10% (*w*/*v*) and 0.5% (*w*/*v*) *Chlorella* powder fermented by *Bifidobacterium animalis* subsp. *lactis* BB-12® (10^7–10^8 CFU/mL). The experimental groups (II–IV) received the tested suspension by oral gavage daily for 4 and 8 weeks. Rats were euthanized by decapitation following light anesthetization (zoletil 5 mg/kg b.wt., Virbac, Carros, France) in the postprandial state. Tissue samples and serum aliquots were stored at −80 °C for analysis.

2.7. Biochemical Analysis

Plasma concentrations of total cholesterol and triglycerides (TAG) were measured using enzymatic–colorimetric methods (Boehringer, Mannheim, Germany). High-density lipoprotein cholesterol (HDL–C) was determined in the supernatant following the precipitation of lipoproteins B using phosphotungstic acid/Mg^{2+} (Merck, Darmstadt, Germany). Low-density lipoprotein cholesterol (LDL–C) levels were calculated according to the Friedewald formula. Subfractions of LDL were analyzed via high-performance discontinued gel electrophoresis using polyacrylamide gel tubes (Lipoprint® LDL System, Quantimetrix, Redondo Beach, CA, USA). LDL particles were separated into seven subfractions (LDL1–LDL7). The subfractions LDL1 and LDL2 represent large (buoyant) particles and LDL3–7 reflect small dense LDL (sd-LDL). Concentrations of cholesterol in sd-LDL over 6 mg/dL or peak LDL particle diameter ≤ 26.8 nm denoted phenotype pattern B with a predominance

of sd-LDL [26]. To determine TAG and cholesterol levels in the aorta, samples were extracted in a chloroform/methanol mixture. The resulting pellet was dissolved in isopropyl alcohol, after which the TAG content was determined using an enzymatic assay (Erba-Lachema, Brno, Czech Republic). The catalytic activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum were measured using commercial analytical methods according to the International Federation of Clinical Chemistry and Laboratory Medicine using an analyzer (Unicel DxC 880i; Beckman Coulter, Brea, CA, USA).

2.8. Statistical Analysis

Homoscedasticity and normality of data distribution were tested using the Levene and Shapiro–Wilk tests, respectively, as assumptions for the use of analysis of variance (ANOVA). Heteroscedasticity and small deviations from the normal distribution were observed in some cases, probably owing to small group sizes. Therefore, the nonparametric Kruskal–Wallis test, followed by Dunn's test, was used for data evaluation to prevent potential platykurtosis. For comparison of the two individual groups, the Mann–Whitney test was used. Statistica 13.1 (StatSoft, Inc., Tulsa, OK, USA) and Real Statistics Resource Pack (Release 7.2, Charles Zaiontz) were used for the tests. ANOVA with a post hoc least significance difference test (LSD) for multiple comparisons was used to evaluate the results of prebiotic and immunomodulatory effects, considering statistical significance at the level of $\alpha = 0.05$. Statistical analysis of cytotoxic effects was performed using MagellanTM (Tecan Group) and Microsoft Office Excel 2013 (Microsoft, Redmond, WA, USA), based on data from three different experiments.

3. Results and Discussion

Spray-dried biomass of microalgae generally contains 8–17% carbohydrates, representing the main products derived from photosynthesis and carbon fixation metabolism. The nondigestible oligosaccharides from microalgae can be isolated by hydrolytic processes in the food and feed industries. These oligosaccharides have been shown to selectively stimulate the growth and activity of bacteria primarily from the genera *Lactobacillus* and *Bifidobacterium*, thus contributing to the improvement of the host's health [1,27]. However, the chemical profile and metabolic pathways of carbohydrates vary between species and the composition and properties of the cell wall also depend on the growth stage and cultivation conditions [4,7,15,28]. In particular, the polysaccharide profile of the *C. vulgaris* cell wall contains rhamnose (45–54%) in combination with galactose (14–26%), xylose, mannose, arabinose, and glucose. The most important carbohydrate compound is β-1,3-glucan, a branched polysaccharide comprising units of β-D-glucose, which is very well fermentable in the colon. β-1,3-glucan is a water-soluble polysaccharide that has a positive effect on digestion and immunomodulatory activities [2,29].

In the present study, we utilized *Chlorella* powder containing 50.3 g proteins, 8.1 g lipids, and 15.2 g carbohydrates per 100 g, as specified by the manufacturer. Growth-promoting effects of two concentrations of *Chlorella* powder (1.0 and 3.0% *w*/*v*) were observed on four *Bifidobacteria* strains. Following cultivation, the prebiotic effects were evaluated based on lactic and acetic acid production (Table 1), pH, and cell count. Production of both acids significantly increased in three strains (*Bifidobacterium breve* CCDM 562, *Bifidobacterium animalis* subsp. *Lactis* BB-12®, and CCDM 93), compared to that in the control ($p < 0.05$). This increase was dependent on the concentration of *Chlorella* added. The strains *B. breve* CCDM 486 and CCDM 562 are isolates from human GIT and feces, unlike *B. animalis* subs. *lactis* BB-12 and CCDM 93, which are commercial cultures used in the dairy industry. Human isolates from the GIT are known to be more sensitive to the laboratory in vitro cultivation than commercially used strains, in which also their technological properties are considered. This factor could have an influence on the production of acids by *B. breve* CCDM 486 and pH values after 24 h of this strain also correlated with decreased concentration of acids.

Table 1. Production of lactic and acetic acids and pH values following 24 h cultivation and.

Tested Strains	Concentration of Acids (mg/L)/pH	Control (WCH Broth)	BS + *Chlorella* 1% (*w*/*v*)	BS + *Chlorella* 3% (*w*/*v*)
CCDM 93	Lactic acid	218 ± 10^{A}	862 ± 40^{B}	1422 ± 70^{C}
	Acetic acid	1388 ± 70^{A}	1526 ± 20^{B}	1953 ± 100^{C}
	pH	4.47 ± 0.03^{A}	4.80 ± 0.03^{C}	4.64 ± 0.03^{B}
BB-12	Lactic acid	170 ± 10^{A}	865 ± 40^{B}	1455 ± 70^{C}
	Acetic acid	1349 ± 65^{A}	1522 ± 80^{B}	2007 ± 100^{C}
	pH	4.47 ± 0.03^{A}	4.80 ± 0.00^{C}	4.65 ± 0.00^{B}
CCDM 486	Lactic acid	158 ± 10^{A}	208 ± 10^{B}	753 ± 35^{C}
	Acetic acid	1268 ± 60^{B}	606 ± 30^{A}	1271 ± 65^{B}
	pH	4.57 ± 0.01^{B}	5.37 ± 0.01^{C}	4.44 ± 0.00^{A}
CCDM 562	Lactic acid	378 ± 20^{A}	908 ± 45^{B}	1507 ± 75^{C}
	Acetic acid	1514 ± 75^{A}	1720 ± 85^{B}	2120 ± 105^{C}
	pH	4.39 ± 0.03^{A}	4.71 ± 0.00^{C}	4.60 ± 0.00^{B}

Values represent the means of triplicate measurements ± standard deviation (n = 3); data in a column with different superscripts ($^{A–C}$) differ significantly ($p < 0.05$).

Similar conclusions were obtained in previous studies evaluating the prebiotic or positive growth effects of *Chlorella*. Behestipour et al. (2012) demonstrated that the addition of 0.5 or 1.0% (*w*/*v*) of *C. vulgaris* and *Arthrospira platensis* in yogurt enriched with probiotics *Lactobacillus acidophilus*, and *Bifidobacterium animalis* subsp. *lactis* BB-12® [15] significantly increased the viability of both probiotic strains at the end of fermentation compared to the control yogurt. A study by Cantú-Bernal (2020) supported the improved viability of *Bifidobacterium longum* and *Lbc. plantarum* in dairy products with the addition of *Chlorella sorokianiana* [10]. Additionally, the results indicated an antiviral effect of cells against rotavirus following treatment with probiotics and/or microalgae [10].

The immunostimulatory activities of microalgae are attributed to their polysaccharides [7,30]. In this study, we evaluated the ability of *C. vulgaris* to stimulate cytokine secretion by hPBMCs isolated from eight healthy donors using multiplex analysis. The immunomodulatory effect was assessed based on the production of cytokines (IL-4, IL-6, IL-17, TNF-α, and IFN-γ) following stimulation with 3.0% and 1.0% (*w*/*v*) aqueous solution of *Chlorella* (Table 2). The results showed a significant increase in the secretion of the inflammatory cytokines TNF-α and IL-6 following stimulation with 1.0% (*w*/*v*) *Chlorella* ($p < 0.05$). In comparison, in a study by Ewart et al. (2007) [31], PBMCs from healthy donors stimulated with three concentrations of aqueous *Chlorella pyrenoidosa* extract (1, 10, and 100 µg/mL) for 24 h exhibited increased concentrations of the regulatory cytokines IL-10, TNF-α, and IFN-γ, whereas the latter did not significantly change in our study. In particular, the production of Th1 cytokines (TNF-α) can facilitate protection against various intracellular microorganisms and tumors. In turn, the anti-inflammatory IL-6 cytokine, which inhibits TNF-α, is produced in response to increased TNF-α levels to maintain the Th1/Th2 balance [32,33]. In our study, the concentration of observed cytokines was the highest after using a lower amount of *Chlorella*. Similar results have been observed by Sibi and Rabina (2016) [33], who tested the inhibition of inflammatory mediators and cytokines by fractions of *Chlorella* using n-hexane, ethanol, and water, as extract solvent [33]. This trend could have caused by decreasing viability of mononuclear cells after incubation with various concentrations of aqueous *Chlorella* solution.

Table 2. Cytokine levels following *Chlorella* stimulation.

	Cytokine Levels (pg/mL)				
Chlorella Concentration	**TNF-α**	**IL-17**	**IL-6**	**IL-4**	**IFN-γ**
1% (*w*/*v*)	15.4 ± 9.1^{B}	0.7 ± 0.3^{A}	143.6 ± 96.8^{B}	2.7 ± 0.0^{A}	0.7 ± 0.3^{A}
3% (*w*/*v*)	1.3 ± 0.7^{A}	0.9 ± 1.0^{A}	37.9 ± 11.8^{A}	3.2 ± 0.4^{B}	1.0 ± 0.5^{A}
0% (Control)	0.0 ± 0.7^{A}	2.0 ± 0.0^{B}	7.3 ± 2.9^{A}	4.9 ± 1.3^{B}	3.5 ± 1.1^{B}

Values represent the means ± SD, $n = 8$. Data in each row with different superscripts (^{A,B}) differ significantly ($p < 0.05$). TNF: tumor necrosis factor, INF: interferon, IL: interleukin.

Cytotoxic effects were determined using a mixed coculture of Caco-2/HT29 cells (ratio 9:1). According to Taylor et al. (2014) [34], extracts at IC50 > 90 µg/mL are considered nontoxic. Similar to our previous studies evaluating plant toxicity [35,36], we evaluated the effects of *Chlorella* up to a concentration of 512 µg/mL; a similar method is commonly used in microbiological testing [37]. We observed that the IC50 was higher than 512 µg/mL and therefore concluded that the tested concentration of *C. vulgaris* is safe. In comparison, it has been stated that polysaccharides isolated from *Chlorella* show inhibitory activity on the HCT8 colorectal carcinoma cell line, corresponding to 3.14 mg/mL isolated exopolysaccharides at a yield of 364 mg/L depending on *Chlorella* species [38].

The hypocholesterolemic effects of *Chlorella* or its components have been demonstrated in numerous studies in animals (rats and mice) fed a high-fat diet or in humans [39–42]; however, the mechanisms by which *C. vulgaris* and algae influence lipid metabolism remain unclear. Daily administration of *Chlorella* supplement (5 g/day) in mildly hypercholesterolemic adults significantly reduced serum lipid risk factors (TAG and total cholesterol); this effect was related to carotenoid (particularly lutein) concentrations, which limit the intestinal absorption of TAG [43]. The effect of *Chlorella* powder and the indigestible fraction isolated from *Chlorella* on the hepatic metabolism of cholesterol has also been evaluated, based on the enzymatic activity and mRNA levels of cytochrome P450 7A1 (CYP7A1), the rate-influencing enzyme in the main biosynthesis pathway that produces bile acids, and HMG-CoA reductase in male Wistar rats [40]. The *Chlorella* fractions both increased *Cyp7a1* mRNA levels in the liver and influenced the concentration of cholesterol in the serum and liver.

In addition to water-soluble fibers, antioxidants such as chlorophyllin, and carotenoids, *Chlorella* also contain phospholipids and arginine, which can help to prevent or treat obesity, hypercholesterolemia, and cardiovascular and gastrointestinal diseases [1,8,44,45]. In the present study, we therefore also sought to test the influence of *C. vulgaris* in combination with bovine colostrum and the probiotic strain *B. animalis* subsp. *lactis* BB-12® on lipid metabolism in hypercholesterolemic male rats. In addition, the concentrations of lipid parameters, such as TAG, total cholesterol (TC), lipoproteins (LDL, IDL, HDL, VLDL), and aminotransferases (ALT and AST) were determined in serum and tissues at the midpoint and end of the experiment (8 weeks). Group IV showed significantly decreased TAG levels in the serum and aorta ($p < 0.05$) after 8 weeks (Table 3), concordant with decreased VLDL concentration (Table 4). These effects may be related to the inhibition of hepatic fatty acid synthesis and TAG production leading to a limitation in the levels of VLDL [42]. Lee et al. (2008) [8] also demonstrated the positive influence of *C. vulgaris*-containing diets (5.0 and 10.0%) on lipid metabolism in Wistar rats fed a high-fat diet, in which serum total lipid and liver TAG levels were significantly lower in both tested groups than in the control group after nine weeks. However, the concentrations of AST and ALT did not differ among groups [8], whereas in the present study, the serum AST and ALT levels were significantly decreased at the end of the experiment in all test groups ($p < 0.05$; Table 5).

The present results may also be derived from the synergistic effect of the three tested components. Probiotics can influence the mechanisms of cholesterol reduction by several means, such as deconjugation of bile via bile salt hydrolase activity, assimilation and incor-

poration of cholesterol into the cell membrane, production of short-chain fatty acids from oligosaccharides, and coprecipitation of cholesterol with deconjugated bile [46,47]. Leptin and insulin-like growth factor-1, which is present in bovine colostrum, can also influence the levels of cholesterol and TAG because they are associated with the fat breakdown in muscle [48].

Table 3. TAG accumulation in tissues.

Tested Parameters	Times	Group of Rats			
		GI (control)	GII	GIII	GIV
TAG aorta (μmol/g)	week 4	0.5 ± 0.1^{A}	0.9 ± 0.3^{AB}	1.1 ± 0.0^{B}	1.3 ± 0.2^{B}
	week 8	1.4 ± 0.2^{AB}	1.6 ± 0.4^{B}	1.6 ± 0.4^{B}	0.9 ± 0.3^{A}
TAG serum (mmol/L)	week 4	2.9 ± 0.8^{AB}	2.6 ± 0.5^{A}	3.9 ± 0.6^{B}	2.5 ± 0.5^{A}
	week 8	2.3 ± 0.6^{B}	2.1 ± 0.2^{B}	1.6 ± 0.5^{A}	1.6 ± 0.4^{A}

TAG: triglyceride. Values represent the means ± SD, $n = 6$. Data in each column with different superscripts (^{A,B}) differ significantly ($p < 0.05$). GII, *Chlorella* 0.5% (*w*/*v*); GIII, *Chlorella* 0.5% + colostrum 10% (*w*/*v*); GIV, *Chlorella* 0.5% + colostrum 10% (*w*/*v*), and *B. lactis* BB-12®.

Table 4. Serum lipid concentrations as determined using the Lipoprint® LDL System.

Tested Parameters	Time	Group of Rats			
		GI (control)	GII	GIII	GIV
VLDL (mg/dL)	week 4	88.0 ± 16.8^{A}	130.5 ± 22.5^{B}	142.0 ± 27.1^{B}	118.0 ± 23.4^{AB}
	week 8	153.0 ± 59.8^{A}	163.5 ± 21.2^{A}	95.0 ± 86.9^{A}	91.0 ± 37.3^{B}
IDL-C (mg/dL)	week 4	63.0 ± 9.4^{AB}	77.5 ± 25.9^{A}	49.0 ± 5.2^{BC}	43.0 ± 12.3^{C}
	week 8	89.0 ± 22.4^{AB}	65.0 ± 15.1^{A}	122.0 ± 30.4^{B}	160.0 ± 33.7^{B}
IDL-B (mg/dL)	week 4	27.0 ± 4.8^{A}	50.5 ± 10.4^{B}	24.0 ± 5.9^{C}	19.0 ± 7.5^{C}
	week 8	35.0 ± 19.9^{A}	35.0 ± 11.2^{A}	73.0 ± 10.3^{B}	78.0 ± 21.4^{B}
IDL-A (mg/dL)	week 4	5.0 ± 1.2^{A}	12.0 ± 2.5^{B}	6.0 ± 1.8^{A}	3.0 ± 3.3^{A}
	week 8	13.0 ± 6.05^{A}	10.5 ± 3.20^{A}	23.0 ± 4.31^{B}	29.0 ± 5.54^{B}
HDL (mg/dL)	week 4	51.0 ± 6.4^{A}	68.0 ± 13.2^{B}	67.0 ± 10.9^{AB}	78.5 ± 19.1^{B}
	week 8	73.0 ± 12.7^{A}	81.5 ± 8.4^{AB}	71.0 ± 11.3^{A}	92.0 ± 10.6^{B}

VLDL: very low-density lipoprotein; IDL: intermediate-density lipoprotein; HDL: high-density lipoprotein. Values represent the means ± SD, $n = 6$. Data in each column with different superscripts ($^{A–C}$) differ significantly ($p < 0.05$). GII, *Chlorella* 0.5% (*w*/*v*); GIII, *Chlorella* 0.5% + colostrum 10% (*w*/*v*); GIV, *Chlorella* 0.5% + colostrum 10% (*w*/*v*), and *B. lactis* BB-12®.

Table 5. LDL and TC (as determined using the Lipoprint® LDL System) and enzyme aminotransferases.

Tested Parameters	Time	Groups of Rats			
		GI (control)	GII	GIII	GIV
Total LDL (mg/dL)	week 4	109.0 ± 20.0^{A}	140.5 ± 32.0^{B}	88.0 ± 15.2^{A}	78.5 ± 20.0^{A}
	week 8	182.0 ± 58.1^{A}	119.5 ± 28.8^{A}	249.0 ± 33.0^{B}	303.0 ± 54.6^{B}
TC (mg/dL)	week 4	341.0 ± 20.0^{AB}	381.0 ± 47.1^{A}	295.2 ± 73.0^{B}	329.8 ± 27.9^{AB}
	week 8	413.4 ± 106.1^{AB}	388.4 ± 32.3^{A}	473.9 ± 111.3^{B}	500.8 ± 50.9^{B}
ALT (μkat/L)	week 4	1.66 ± 0.1^{AB}	1.77 ± 0.2^{A}	1.73 ± 0.1^{A}	1.53 ± 0.1^{B}
	week 8	1.90 ± 0.3^{A}	1.65 ± 0.2^{AB}	1.48 ± 0.2^{B}	1.39 ± 0.2^{B}
AST (μkat/L)	week 4	3.88 ± 0.2^{A}	4.36 ± 0.5^{A}	4.33 ± 0.4^{A}	4.14 ± 0.5^{A}
	week 8	4.15 ± 0.4^{A}	4.02 ± 0.9^{AB}	4.00 ± 0.8^{AB}	3.30 ± 0.7^{B}

LDL: low-density lipoprotein, TC: total cholesterol, ALT: alanine aminotransferase, AST: aspartate aminotransferase. Values represent the means ± SD, $n = 6$. Data in each column with different superscripts (^{A,B}) differ significantly ($p < 0.05$). GII, *Chlorella* 0.5% (*w*/*v*); GIII, *Chlorella* 0.5% + colostrum 10% (*w*/*v*); GIV, *Chlorella* 0.5% + colostrum 10% (*w*/*v*), and *B. lactis* BB-12®.

4. Conclusions

In summary, this study was designed to test the functional properties of *C. vulgaris* and assess the potential of its combination with probiotics and bovine colostrum for developing new dietary supplements or functional foods and thus create a new segment of products. The results demonstrated the prebiotic and immunomodulatory effects of the *Chlorella* powder and its cholesterol-lowering effects in the tested combinations. In addition, the nontoxicity of *Chlorella* toward mammalian cells was confirmed. The limitations of this study relate to missing information regarding the influence of the cultivation conditions of *C. vulgaris* or a more detailed analysis of the chemical composition of the tested algae as the content of saccharides or other functional compounds with immunomodulatory, prebiotic, or hypocholesterolemic effects can be modified by cultivation. The acquired results appear to depend on the functional abilities of the selected probiotic strains or lactic acid bacteria and the defined content of bioactive compounds in the colostrum. Although the results are promising, additional studies are needed to characterize further the synergic effects of algae, probiotics, and/or colostrum, e.g., identification/characterization of bioactive compounds of *Chlorella vulgaris* and their methods of extraction, as well as growth promotion effect on other probiotics (*Lactobacilli*), effective dose, etc. Nevertheless, the application of natural resources such as algae, probiotics, and colostrum that offer health benefits for humans and animals in supplements or foods holds considerable potential owing to their ready availability, low cost, and high nutrient qualities.

Author Contributions: Conceptualization, I.H., G.K., and J.S.; methodology, I.H., I.D., B.S., and M.H.; formal analysis, I.H., J.S., B.S., H.M., M.H., and I.D.; investigation, A.K., G.K., and I.H.; resources, I.H.; data curation, I.H., A.K., and B.S.; writing—original draft preparation, I.H., A.K., and J.S.; writing—review and editing, T.B., L.C., and M.H.; supervision, I.H., G.K., and A.K.; project administration, I.H.; funding acquisition, I.H. and G.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Agriculture of the Czech Republic, Institutional support, No. MZE-RO1421 and No. QK1910300. The METROFOOD-CZ research infrastructure project (MEYS Grant No.: LM2018100) includes access to its facilities.

Institutional Review Board Statement: This study was conducted in compliance with the laws of the Czech Republic (311/1997 column.) and the European Community Council recommendations (86/609/EEC) regarding the protection of animals used for experimental and other scientific purposes. This experimental study was approved by the committee of the Ministry of Education, Youth, and Sports of the Czech Republic, approval No. MSMT-46654/2015-8.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are presented in the paper.

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

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