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Prebiotic and Immunomodulatory Properties of the Microalga *Chlorella vulgaris* and Its Synergistic Triglyceride-Lowering Effect with Bifidobacteria

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Abstract: The microalga *Chlorella* and strains of *Bifidobacterium* have been used in human or animal food supplements for decades because of their positive health effects. The presented study assessed different properties of *C. vulgaris* and its combination with bifidobacteria with the aim to develop new functional foods. The growth of four bifidobacteria strains in milk and whey supplemented with 1.0% (*w/v*) *C. vulgaris* and the immunomodulatory effects of aqueous *Chlorella* solutions (0.5%, 1.0%, and 3.0%) on human peripheral mononuclear cells were evaluated. Furthermore, synergistic effects on lipid metabolism of rats fed a high-fat diet with *Chlorella* and *B. animalis* subsp. *lactis* BB-12® were analysed. *Chlorella* had a positive growth-promoting effect on the tested bifidobacteria ($p < 0.05$), and significantly increased the secretion of inflammatory cytokines (tumor necrosis factor-α, interleukin-10, and interleukin-6), depending on the concentration of *Chlorella* ($p < 0.05$). After 8 weeks, significant synergistic effects of *Chlorella* and bifidobacteria on triglyceride levels in rat heart, liver, and serum were observed ($p < 0.05$). These results demonstrate that various combinations of *Chlorella* and bifidobacteria have significant potential for the development of new fermented products, dependent on the algal species, probiotic strain, application form, and concentrations for acceptable sensory quality for consumers.

Keywords: *Bifidobacterium*; *Chlorella*; cytokines; functional food; health; prebiotic; triglyceride

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1. Introduction

Chlorella vulgaris as a food supplement acts as a source of nutritionally-valuable substances, including proteins, carbohydrates, vitamins, pigments, antioxidants, and unsaturated fatty acids. *Chlorella* and *Arthrospira* (Spirulina), the most consumed microalgae, accumulate high-quality proteins with a balanced amino acid profile according to WHO recommendations for essential amino acids [1]. Commercial prebiotics are generally carbohydrate compounds that act as substrates for probiotic microorganisms. The most important carbohydrate in *Chlorella* cells, in terms of its usefulness for living organisms, is β-1,3-glucan, which is a branched polysaccharide of β-D-glucose units. β-1,3-glucan is also

a water-soluble fiber and readily fermentable in the colon; it represents an important part of the diet because of its enhancing effect on digestion [2]. Apart from β-1,3-glucan, almost all vitamins (A, B1, B2, B6, B12, C, E, biotin, pantothenate, etc.) are provided by microalgae most commonly used in food supplements. The availability of these vitamins likely supports the observed growth increase of bacteria. Microalgae are also rich in pigments, containing on a dry mass basis mainly chlorophyll (0.5–1.0%), carotenoids (0.1–0.2%), and phycobiliproteins [3]. The positive effects of microalgae on human health, such as prebiotic, immunomodulatory, anti-oxidative, anti-cancer, and hypocholesterolemic effects, have been reported in a variety of studies; however, the mechanism imparting the positive effect is strongly dependent on the specific microalgal strain and the content of bioactive substances [4–6].

The most common bacteria used in the food and feed industry are species of *Lactobacillus* and *Bifidobacterium*, which also represent probiotics and have the status "Generally Recognized As Safe" (GRAS) [7]. Fermented dairy products with probiotics are popular and available in markets worldwide [8]. *Bifidobacterium animalis* subsp. *lactis* is one of the preferred strains used by manufacturers because of its tolerance to acid conditions (low pH) and/or low molecular oxygen arising during fermentation [7,9]. An important qualitative parameter is the viability of lactic acid bacteria and probiotic microorganisms in the final products, including until the end of their shelf-life. A value for viable cells of 10^6/g or 10^6/mL in probiotic products is considered satisfactory for fulfilling the criterion of viable counts [9]. Bifidobacteria represent one of the first colonizing bacteria in the neonatal intestine and they are known to assist in the development of adaptive immunity, as well as modulate mucosal physiology [10]. Numerous studies have assessed the use of probiotics from genera *Bifidobacterium* in the prevention or treatment of many diseases, such as gastroenteritis caused by rotavirus, enteric diseases, diarrhea, lactose intolerance, allergies, and the reduction of serum cholesterol [10–12].

Several researchers have tested the influence of additional algal biomass on yogurt, cheese, or fermented milk products [13–15]. The addition of algae, such as *C. vulgaris*, *Ch. regularis*, or *Arthrospira* has resulted in a positive prebiotic effect on the viability of lactic acid bacteria and also helped to improve the nutritional quality of fermented products. However, to develop new fermented foods enriched with probiotics and algae, it is necessary to first increase our understanding of the influence of individual algae or their components on the growth promotion of bacteria and their health effects on human cells. The prebiotic effect of *C. vulgaris* in vitro and its non-cytotoxic effects were described in our previous study [16]. In the presented study, we continued to evaluate the growth promoting potential of *C. vulgaris* on selected bifidobacteria in bovine milk and whey and also determined its immunomodulatory effect on human peripheral blood mononuclear cells (hPBMCs) isolated from healthy adult donors. The synergistic hypocholesterolemic effects of a combination of *Chlorella* and *B. animalis* subsp. *lactis* BB-12® in an animal model of Prague hereditary hypercholesterolemic rats with diet-induced hypercholesterolemia [17] were also evaluated. Our results demonstrate that Chlorella and bifidobacteria used in various combinations have significant synergistic potential for the development of new fermented products with positive health effects.

2. Materials and Methods

2.1. Microorganisms

The tested microorganism strains were selected from the Culture Collection of Dairy Microorganisms Laktoflora® (Tabor, Czech Republic) and the commercial strain *B. animalis* subsp. *lactis* BB-12® was obtained from Ch. Hansen (Hørsholm, Denmark) (Table 1). Bacterial strains were stored at −20 °C in Wilkins Chalgren anaerobic broth (Oxoid, Hampshire, UK) with 10% (*v/v*) glycerol. Before each analysis, cells were transferred twice in fresh Wilkins Chalgren anaerobic broth with *L*-cysteine hydrochloride (Merck, Darmstadt, Germany) and cultivated at 37 °C for 24 h in anaerobic jars. Heterotrophic *C. vulgaris* was obtained from the Microbiology Department of Academy of Sciences, Třeboň—ALGATECH

(Třeboň, Czech Republic). The tested *Chlorella* powder sample contained 50.3 g of protein, 8.1 g of lipid, and 15.2 g of carbohydrate per 100 g of algal biomass.

Table 1. Selected microorganisms.

Strain	Species	Origin
CCDM [1] 93	*Bifidobacterium animalis* subsp. *lactis*	Original culture
BB12®	*Bifidobacterium animalis* subsp. *lactis*	Original culture
CCDM 562	*Bifidobacterium breve*	GIT of child
CCDM 486	*Bifidobacterium breve*	Human feces

[1] CCDM, Culture Collection of Dairy Microorganisms; GIT, gastrointestinal tract.

2.2. Prebiotic Effect

To assess bacterial growth, milk and whey were supplemented with 1.0% (*w/v*) *Chlorella* powder and pasteurized at 85 °C for 10 min. Inoculated milk and whey without *Chlorella* served as negative controls. Prior to inoculation, bifidobacterial suspensions were prepared as described [18]. Inoculated samples were cultivated in anaerobic jars (Oxoid, Hampshire, UK) at 37 °C for 24 h. Counts of the tested strains were determined using 10-fold serial dilutions and plated onto MRS agar with *L*-cysteine hydrochloride (pH 6.2). The pH of the cultivated medium and the concentration of lactic and acetic acids were also measured. Concentrations of organic acids, as the main fermentation products of tested bifidobacteria, were determined by the isotachophoretic method using IONOSEP 2003 (RECMAN, Ostrava, Czech Republic). A mixture of 10 mmol/L HCl, 22 mmol/L 6-aminocaproic acid, and 0.1% hydroxyethylcellulose (Merck) was used as the leading electrolyte and 10 mmol/L caproic acid acted as the terminating electrolyte. The conditions of analysis were selected according to the manufacturer's instructions (RECMAN, Ostrava, Czech Republic). Results were obtained from three independent measurements.

2.3. Stimulation and Immunomodulation

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 a previous study with slight modifications [16]. In brief, eight samples of blood from healthy adults, for the isolation of 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. Mononuclear cells (0.1 mL) were stimulated in X-vivo medium (Cambrex, Whippany, NJ, USA) with 0.1 mL of a 0.5%, 1.0%, or 3.0% aqueous solution of *C. vulgaris* at 37 °C. The total volume was 1 mL. Unstimulated hPBMCs and X-vivo medium were used as the negative controls. Microplates with the samples were incubated for 3 d at 37 °C. Levels of cytokines produced by stimulation of hPBMCs with different concentrations of *Chlorella* were determined using the Fluorokine MAP Human Base Kit A (R&D Systems, Minneapolis, MN, USA) for interferon (IFN)-γ, interleukin (IL)-4, IL-10, IL-6, IL-17, and tumor necrosis factor (TNF)-α by multiplex analysis using a Luminex 200 Analyzer (Luminex Corp., Austin, TX, USA). The concentration of cytokines produced by hPBMCs was assessed using Luminex IS 2.3 (Luminex Corp.). Results were obtained from three independent measurements.

2.4. Experimental Animals and Diet

A total of 24 male Prague hereditary hypercholesterolemic rats with a body weight of 249 ± 16 g were obtained from Albert Weber-SEMED (Praha, Czech Republic). Animals were acclimatized to laboratory conditions for 2 weeks before the experiment by housing at room temperature (22–24 °C) and 55–60% humidity on a 12 h/12 h light-dark cycle with ad libitum access to water and food. The hypercholesterolemic diet was fortified with 2.0% (*w/w*) cholesterol. After acclimation, the rats were divided into a control group and

three test groups as follows: C, rats on a hypercholesterolemic diet; GI, rats on a hypercholesterolemic diet with 1.0% *Chlorella* powder (*w/w*), GII, rats on a hypercholesterolemic diet with lyophilized *B. animalis* subsp. *lactis* BB-12® (10^6 CFU/g per one g of pellet); and GIII, rats on a hypercholesterolemic diet with lyophilized *B. animalis* subsp. *lactis* BB-12® (10^6 CFU/g per one g of pellet) and 1.0% *Chlorella* powder (*w/w*). The experimental groups received their respective diets for 8 weeks. At the end of the experiment, rats were euthanized by decapitation after light anesthetization (zoletil, 5 mg/kg body weight) in the postprandial state. Blood was collected into tubes without anticoagulant addition. Aliquots of serum and heart, liver, and aorta tissue samples were stored at −80 °C until analysis. All experiments were performed in accordance with the Animal Protection Law of the Czech Republic (311/1997) in compliance with 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 of the Czech Republic (MSMT-2309/2018-2; 2/2/2018).

2.5. Biochemical Analysis

Lipid parameters were determined as previously described [16]. Briefly, total cholesterol and lipoprotein fractions—very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), intermediate-density lipoproteins (IDL), and high-density lipoproteins (HDL)—were assessed by enzymatic colorimetric methods (CHOD/PAP, direct homogeneous enzymatic-colorimetric reaction without precipitation, GPO/PAP; Lab Mark a.s., Prague, Czech Republic) using an automatic biochemical analyzer modular (Roche, Prague, Czech Republic). Subfractions of LDL were analyzed by 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 represent small dense LDL (sd-LDL). Concentrations of cholesterol in sd-LDL over 6 mg/dL or peak LDL particle diameter $\leq$ 26.8 nm were denoted phenotype pattern B with a predominance of sd-LDL [19]. To determine triglycerides and cholesterol in the tissues, samples were extracted in a chloroform/methanol mixture. The resulting pellet was dissolved in isopropyl alcohol, after which the triglyceride content was determined using an enzymatic assay (Erba-Lachema, Brno, Czech Republic).

2.6. Statistical Analysis

All statistical evaluations were performed using Microsoft Office Excel 2019 and Statistica 13.1CZ statistical software. Normality of data was checked using the Shapiro–Wilk test. One-way analysis of variance (ANOVA) at a significance level of α = 0.05, followed by Tukey's test, was applied to the comparison of tested biochemical parameters. ANOVA with a post hoc least significance difference test (LSD) for multiple comparisons was used to evaluate the results of prebiotic effects, considering statistical significance at the level of α = 0.05.

3. Results

3.1. Prebiotic Assay

Prebiotic activity was evaluated by determining the bacterial counts of four selected bifidobacterial strains, changes in pH, and production of lactic and acetic acids (Tables 2 and 3) after fermentation of whey and bovine milk with 1.0% (*w/v*) *Chlorella* powder supplementation. The production of acids was significantly increased in milk and whey enriched with *Chlorella* powder than not enriched ($p < 0.05$). The concentration of these acids was 5-fold higher in milk supplemented with *Chlorella* and 1.5-fold higher in whey supplemented with *Chlorella*. The pH values also correlated with increased concentrations of lactic and acetic acids produced by bifidobacteria (Table 3).

Table 2. Production of lactic acid and acetic acid after 24 h cultivation.

Strain	Acid Production (mg/L)	Milk	Milk + 1.0% *Chlorella*	Whey	Whey + 1.0% *Chlorella*
CCDM 93	lactic acid	388 ± 20^{A}	1754 ± 85^{B}	1661 ± 80^{B}	2437 ± 120^{C}
	acetic acid	1105 ± 50^{A}	2354 ± 110^{D}	1309 ± 65^{B}	2058 ± 58^{C}
BB-12®	lactic acid	208 ± 15^{A}	1876 ± 90^{C}	1193 ± 60^{B}	1937 ± 55^{C}
	acetic acid	512 ± 25^{A}	2246 ± 110^{C}	1812 ± 90^{B}	2588 ± 130^{D}
CCDM 486	lactic acid	259 ± 15^{A}	1891 ± 90^{C}	1524 ± 75^{B}	2013 ± 100^{C}
	acetic acid	477 ± 25^{A}	2384 ± 110^{D}	1747 ± 85^{B}	2124 ± 105^{C}
CCDM 562	lactic acid	719 ± 35^{B}	2200 ± 110^{D}	431 ± 20^{A}	1768 ± 90^{C}
	acetic acid	836 ± 39^{B}	2272 ± 110^{D}	368 ± 40^{A}	1734 ± 85^{C}

Values are the means of triplicate measurements ± standard deviation (SD). ^{A,B,C,D} Data in the lines with different superscripts differ ($p < 0.05$).

Table 3. pH and cell counts after 24 h cultivation.

Tested Parameter	Strain	Milk	Milk + 1.0% *Chlorella*	Whey	Whey + 1.0% *Chlorella*
pH	CCDM 93	5.50 ± 0.03^{C}	4.85 ± 0.01^{B}	4.79 ± 0.01^{B}	4.15 ± 0.02^{A}
	BB12	5.90 ± 0.03^{D}	4.89 ± 0.00^{C}	4.57 ± 0.00^{B}	4.38 ± 0.12^{A}
	CCDM 486	5.90 ± 0.06^{D}	4.87 ± 0.07^{C}	4.50 ± 0.16^{B}	4.24 ± 0.04^{A}
	CCDM 562	5.13 ± 0.35^{B}	4.78 ± 0.06^{AB}	4.86 ± 0.64^{AB}	4.21 ± 0.12^{A}
cell counts (CFU/mL)	CCDM 93	6.24 ± 0.76^{A}	8.34 ± 1.26^{B}	7.39 ± 0.12^{AB}	7.54 ± 0.55^{AB}
	BB12	7.90 ± 1.01^{A}	8.37 ± 0.19^{A}	8.37 ± 0.34^{A}	8.35 ± 0.35^{A}
	CCDM 486	8.59 ± 1.01^{A}	8.31 ± 0.08^{A}	8.26 ± 0.06^{A}	8.38 ± 0.27^{A}
	CCDM 562	8.16 ± 0.61^{A}	7.76 ± 0.33^{A}	7.02 ± 1.02^{A}	7.31 ± 0.53^{A}

Values are the means of triplicate measurements ± SD. ^{A,B,C,D} Data in the lines with different superscripts differ ($p < 0.05$).

3.2. Immunomodulatory Effect

The immunomodulatory effects of three different aqueous solutions of Chlorella (0.5, 1.0, and 3.0% *w/v*) on hPBMCs were compared based on the production of pro-inflammatory and regulatory cytokines. Levels of selected ILs (IL-4, IL-10, IL-17, IL-6), TNF-α, and INF-γ were determined using multiplex analysis. In contrast with other studies where the levels of tested cytokines are usually evaluated by the method of flow cytometry or ELISPOT assay [20,21]. Our results showed that the production of all tested cytokines was significantly different ($p < 0.05$) after stimulation with the lowest concentration of Chlorella (0.5% *w/v*) compared with the other Chlorella concentrations (Table 4). The production of TNF-α, IL-10, and IL-6 by hPBMCs was dependent on the concentration of Chlorella added.

Table 4. Cytokine production by mononuclear cells.

	Cytokines (pg/mL)					
Chlorella Concentration (*w/v*)	TNF-α	IL-17	IL-10	IL-6	IL-4	IFN-γ
0.5%	187.57 ± 62.02^{C}	1.16 ± 0.09^{B}	12.73 ± 7.58^{D}	4976.43 ± 781.21^{D}	2.68 ± 0.10^{B}	2.34 ± 0.37^{B}
1.0%	23.85 ± 8.98^{B}	$0.65 \pm 0.10^{A,B}$	0.74 ± 0.11^{B}	202.18 ± 103.13^{C}	2.68 ± 0.00^{A}	0.82 ± 0.21^{A}
3.0%	1.85 ± 0.67^{A}	0.65 ± 0.33^{A}	0.11 ± 0.07^{A}	55.13 ± 18.91^{B}	3.22 ± 0.13^{A}	1.01 ± 0.17^{A}
control	0.93 ± 0.91^{A}	0.00 ± 0.90^{A}	1.66 ± 0.33^{C}	7.7 ± 3.1^{A}	5.35 ± 2.70^{A}	3.66 ± 1.00^{A}

Values are the means of triplicate measurements ± SD. ^{A,B,C,D} Data in the column with different superscripts differ ($p < 0.05$).

3.3. Serum Lipid Profile

Serum total cholesterol (TC), triacylglycerides, VLDL, LDL, IDL, and HDL levels were evaluated in a rat model after feeding a high-cholesterol diet supplemented with Chlorella powder and/or B. animalis subsp. lactis BB-12 for 8 weeks (Table 5). In the group fed the diet with B. animalis subsp. lactis BB-12® (GII), all tested serum lipid parameters, apart from the HDL concentration, significantly increased ($p < 0.05$) in comparison with all the other diet groups. Values among the rest of the diet groups and the control group did not statistically differ. Nevertheless, levels of triglycerides in liver and heart tissues, as well as serum, significantly decreased ($p < 0.05$) in rats fed a diet fortified with Chlorella powder plus B. animalis subsp. lactis BB-12® (GIII), in contrast to the control and the rest of the groups tested (Table 6). Thus, a positive synergistic effect of Chlorella and B. animalis subsp. lactis BB-12 was observed.

Table 5. Serum lipid profiles in a rat model after feeding a high-cholesterol diet supplemented with *Chlorella* powder and/or *B. animalis* subsp. *lactis* BB-12 for 8 weeks.

Tested Group	VLDL (mg/dL)	IDL-C (mg/dL)	IDL-B (mg/dL)	IDL-A (mg/dL)	HDL (mg/dL)	TC (mg/dL)	LDL (mg/dL)
C	74.8 ± 6.5^{A}	29.8 ± 5.2^{A}	16.5 ± 3.8^{A}	3.7 ± 0.8^{A}	52.2 ± 18.5^{A}	184.5 ± 13.5^{A}	55.7 ± 10.7^{A}
GI	76.0 ± 18.9^{A}	33.8 ± 6.6^{A}	23.7 ± 5.7^{A}	6.3 ± 2.0^{B}	71.7 ± 26.0^{A}	219.6 ± 13.4^{B}	69.5 ± 11.4^{A}
GII	106.0 ± 13.1^{B}	43.2 ± 5.9^{B}	26.7 ± 5.7^{B}	7.0 ± 2.2^{B}	79.8 ± 10.4^{A}	272.6 ± 20.3^{C}	85.7 ± 5.3^{B}
GIII	86.5 ± 7.7^{A}	36.8 ± 4.6^{A}	22.2 ± 4.0^{A}	5.3 ± 1.4^{A}	67.7 ± 23.7^{A}	224.7 ± 13.3^{B}	69.7 ± 11.8^{A}

VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TC, total cholesterol; IDL, intermediate-density lipoprotein; C, control; GI, *Chlorella*; GII, *B. animalis* subsp. *lactis* BB-12®; GIII, *Chlorella* + *B. animalis* subsp. *lactis* BB-12®. Values are the mean ± SD, $n = 6$. ^{A,B,C} Data in the column with different superscripts differ ($p < 0.05$).

Table 6. Accumulation of triglycerides in tissues.

Tested Group	Tissues			
	Liver (μmol/g)	Aorta (μmol/g)	Heart (μmol/g)	Serum (mmol/L)
C	5.4 ± 0.3^{A}	1.5 ± 0.4^{A}	3.1 ± 0.3^{A}	1.0 ± 0.3^{A}
GI	5.4 ± 0.3^{A}	1.4 ± 0.2^{A}	3.1 ± 0.3^{A}	1.4 ± 0.2^{B}
GII	5.3 ± 0.4^{A}	1.1 ± 0.2^{A}	2.9 ± 1.0^{A}	1.2 ± 0.2^{A}
GIII	4.8 ± 0.3^{B}	1.1 ± 0.3^{A}	1.9 ± 0.4^{B}	0.7 ± 0.1^{C}

C, control; GI, Chlorella; GII, *B. animalis* subsp. *lactis* BB12; GIII, *Chlorella* + *B. animalis* subsp. *lactis* BB12. Values are the mean ± SD, $n = 6$. ^{A,B,C}. Data in the column with different superscripts differ ($p < 0.05$).

4. Discussion

In the present study, we first investigated the growth-promoting effect of *C. vulgaris* on four *Bifidobacterium* strains in bovine milk and whey. Fermentation ability was evaluated based on the production of metabolic compounds, such as acetic acid and lactic acid, which are important metabolic products produced by bifidobacteria. Levels of both acids were significantly increased in milk and whey supplemented with *Chlorella* vs. without. The prebiotic or growth-promoting effects of *C. vulgaris* biomass on probiotics or intestinal bacteria has also been described in several other studies. For example, Pulz and Gross [22] reported that, compared to the control, the growth rate of lactobacilli (*Lbc. acidophilus*) increased up to 10-fold with the addition of algal biomass. The cell wall of *Chlorella* contains a wide range of oligo- and polysaccharides, which can serve as an energy source for bacteria. Their fermentability may be influenced by factors such as the chain length of individual carbohydrates, their structure (e.g., branching, glycosidic linkage, type of monosaccharide moieties, etc.), and cultivation conditions. The enzymatic reactions used by each bacterium may play a key role. Another study by Scieszka and Klewicki [23] evaluated the protective effect of *C. vulgaris* on the survival of four *L. brevis* strains under adverse environmental conditions in the human gastrointestinal tract, such as low pH and phenolic or bile salts. The addition of the alga had a positive effect on the increased

survival of tested lactobacilli; nevertheless, the protective effect at low pH was strain-specific and dependent on the features of individual strains. Cantú-Bernal et al. [16] reported enhanced viability and antiviral effects of *B. longum* and *L. plantarum* against rotavirus in combination with *C. sorokiniana* in dairy products. Beheshtipour et al. [8] observed the influence of *C. vulgaris* and *Arthrospira platensis* (Spirulina) addition on the viability of selected probiotic microorganisms in yogurt. Their results showed that the addition of the alga stimulated growth and maintained the viability of *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12®, both at the end of fermentation and during cold storage for 28 d. Sciezska and Klewicka [24] tested the influence of *C. vulgaris* (0.1% and 1.5% (*w/v*)) on the growth and metabolic activity of four *L. brevis* strains isolated from vegetable silage. Selected lactobacilli showed a relatively high production of *L*-lactic acid and lower *D*-lactic acid after cultivation in medium with *Chlorella*. Changes in enzymatic activity were also observed, for instance, *L. brevis* LOCK 0980 demonstrated higher enzymatic activity of valine arylamidase, α-galactosidase, and α-glucosidase when cultivated with *Chlorella*.

Chlorella polysaccharides have also shown strong immunomodulatory activities, primarily aqueous soluble polysaccharides. This activity has been reported in mice, human blood cells, and human clinical trials [5,25–27].TNF-α is an important initiator of the inflammatory response and mediator, together with IL-1β and IL-6, which are produced by activated monocytes and macrophages that influence cell proliferation stimulation in various types of cells [21]. Anti-inflammatory IL-6, which inhibits TNF-α, is produced in response to increased TNF-α levels, helping to maintain the Th1/Th2 balance [28]. Our results showed that the three tested aqueous solutions of *C. vulgaris* (0.5, 1.0, and 3.0% *w/v*) influenced production of TNF, IL-6, and IL-10. The mononuclear cells produced the highest amounts of these cytokines after stimulation with the lowest concentration of *Chlorella* tested. This trend could have been caused by the decreased viability of the mononuclear cells after incubation with various concentrations of aqueous *Chlorella* solutions. The inhibition of inflammatory mediators and cytokines by aqueous extracts of *C. vulgaris* was first described by Sibi and Rabina [28]. They determined the in vitro anti-inflammatory activities of different solvent fractions (*n*-hexane, chloroform, ethanol, and water) from *Chlorella*; however, the effect of the aqueous extract was not significant. Notably, they reported that the concentrations of observed parameters were the highest after using the lowest concentration of all tested solvent fractions of *Chlorella*, similar to our study. Ewart et al. [21] stimulated the production of cytokines by using three different concentrations of aqueous *C. pyrenoidosa* extract (1, 10, and 100 μg/mL) for 24 h. Levels of IL-10, TNF-α, and IFN-γ were markedly increased compared to that of the control; however, in our study, the levels of INF-γ did not significantly increase.

Recently, the prevalence of obesity, diabetes, inflammatory bowel syndrome, cancer, and cardiovascular diseases (CVD) have rapidly increased worldwide [29]. Oxidative stress and hypercholesterolemia are risk factors for the development of CVD and are closely related to these diseases [30,31]. Therefore, new strategies have been developed to inhibit the growing incidence or prevention of these diseases using natural sources. Several studies have tested the effect of *Chlorella* or other microalgae on lipid metabolism in different animal models, such as rats, mice or broilers [30,32–36] Shibata et al. (2001) assessed the hypocholesterolemic effects of the indigestible fraction (5.7% *w/w*) of *Chlorella* powder (12.7% *w/w*) compared with the digestible fraction, and reported opposite conclusions. Their results proved the positive influence of the *Chlorella* fraction on decreasing serum cholesterol levels, but no effect on the levels of serum triglycerides and phospholipids. Lee et al. [30] investigated the effect of *C. vulgaris* on lipid metabolism in Wistar rats fed a high-fat diet containing 5.0% or 10.0% (*w/w*) *Chlorella* powder. In this case, serum total lipid and liver triglyceride concentrations were also significantly lower than those in the control group. Chovancikova and Simek [32] examined a mouse model fed a high-fat diet supplemented with 1.0% (*w/w*) *C. vulgaris*. After 10 weeks, the levels of TC and triglycerides in the serum and liver were significantly inhibited.

In the present study, the addition of *Chlorella* and *B. animalis subsp. lactis* BB-12 significantly decreased the concentration of triglycerides in the serum, liver, and heart of the treated rats, in contrast to the control and other tested groups (Table 6). This effect could be associated with inhibition of hepatic fatty acid synthesis and triglyceride production; thus, the output of VLDL was limited [32]. Bifidobacteria also show bile salt hydrolase activity and produce extracellular polysaccharides, which are relevant to the industrial production of human food or medicine [37,38]. Bile salt hydrolase activity, together with binding of cholesterol to the probiotic cellular surface and incorporation into the cell membrane, contributes to the cholesterol-lowering mechanisms of probiotics [39] Zanotti et al. [40] showed that daily supplementation with bifidobacteria modifies the fecal microbiota of mice toward those bacteria involved in the metabolism of cholesterol. Although the effect of the cholesterol-lowering activity of probiotics in human trials does not show consistent results, there are some successful human trials for bifidobacteria [41]. Lee et al. [42] suggested that the lipid-lowering effect of probiotics may be limited to populations with high total cholesterol and LDL-C levels.

5. Conclusions

In summary, the results demonstrated the prebiotic and immunomodulatory effect of the Chlorella powder and a synergic effect of *Chlorella* and *B. animalis* subsp. *lactis* BB-12 combination to decrease the level of triglycerides in the serum, liver, and heart of the treated rats. Consequently, the incorporation and/or combination of *Chlorella* together with bifidobacteria to functional food or fermented dairy products may have a positive effect on the viability of bifidobacteria and their properties, thus influencing human or animal health. Nevertheless, the limitations of this study include the lack of detailed analysis of the chemical composition of the tested alga relative to the content and influence of specific saccharides, lipids, or other functional compounds and their bioactive effects. Therefore, the isolation of individual fractions and determination of their functional properties will be the main aim of future studies. The mechanisms of the hypocholesterolemic effect of microalgae are also not fully understood, and other in vivo and in vitro studies are needed to elucidate this. Notably, the positive effects of algae and bacterial combinations seem to be dependent on the selected bacterial strains, the type of algae, their functional properties, and their synergistic effects.

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