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In Vitro Hypoglycemic Potential, Antioxidant and Prebiotic Activity after Simulated Digestion of Combined Blueberry Pomace and Chia Seed Extracts

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Abstract: This study aimed to evaluate the hypoglycemic potential, antioxidant activity and prebiotic activity of a hydroalcoholic extract of blueberry pomace (BP), an aqueous extract of chia seeds (CS) and a novel combination of BP–CS extracts (BCM) for further use as ingredient of functional food. Spectrometric and HPLC analyses were used to characterize the total phenolic and flavonoid content and composition of BP, while CS was analyzed for total carbohydrate content. Data showed that the BCM mixture exerted an inhibition of α-amylase activity, which was 1.36 times higher than that of BP and 1.25 higher than CS extract. The mixture also showed better scavenging activity of free DPPH radicals than individual extracts, and had an IC_{50} value of 603.12 µg/mL. *In vitro* testing indicated that both serum- and colon-reaching products of simulated intestinal digestion of BCM presented the capacity to protect Caco-2 intestinal cells against oxidative stress by inhibition of reactive oxygen species production. In addition, the colon-reaching product of BCM digestion had the capacity to significantly ($p < 0.05$) stimulate the growth of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus*, revealing a prebiotic potential. All these results indicated that improved biological activity of the novel combination of BP and CS extracts could be due to the synergistic action of constituents. The combination is recommended for further testing and the development of novel functional food for controlling type 2 diabetes and gastrointestinal conditions.

Keywords: berry polyphenols; chia polysaccharides; hypoglycemic activity; simulated digestion; reactive oxygen species; gut microbiota

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1. Introduction

The production of berry juice yields a series of by-products, such as seeds, peel and pulp, known as pomace [1–3]. This waste represents 20–30% of the initial biological material and it is often used for biogas production, to feed animals, or in agriculture as compost. However, pomace of different berries (strawberry, blueberry, raspberry, blackberry, cranberry) represents an undervalued natural source of bioactive compounds, such as phenolic acids, flavonoids, anthocyanins and proanthocyanidins, which could also be valorized for the development of novel functional food with beneficial effects on human health and significant activity against inflammation and redox imbalance, along with antitumor and antimicrobial properties [4–8].

Previous studies have shown that the fruits of blueberry shrubs (*Vaccinium myrtillus* L.) have long been used as a traditional medicine for treating diabetes and various associated cardiovascular complications, and for maintaining the integrity of blood vessels [9]. This action could be attributed to the complex composition of flavonols (quercetin, myricetin), which mainly occur in glycosylated form, and phenolic acids (abscisic acid), but also to anthocyanins, which represent the major component of phenolics and are responsible for amelioration of type 2 diabetes symptoms after oral administration [10]. In addition, the polyphenolic extract of blueberries could inhibit the activity of α-glucosidase and α-amylase [11,12], thus helping to control type 2 diabetes [13] and present high antioxidant potential [14]. Oral administration of phenolics in mice resulted in several beneficial actions, such as ameliorating the effects of a high-fat diet, preventing insulin resistance by modulation of redox signaling pathways [15] and down-regulation of pro-inflammatory cytokine secretion [16]. It has been reported that their action targeted the peroxisome proliferator-activated receptor gamma (PPAR-γ), similar to thiazolidinediones used as anti-diabetic drugs [17]. Blueberries also contain cinchonain I isomers of flavonolignans, and their oral administration can increase the level of plasma insulin, similar to the action of glibenclamide [18]. Shi et al. (2017) reported that blueberry anthocyanins exerted an *in vitro* activity of decreasing glucose production in H4IIE hepatocytes in variable proportions between 24 and 74% [19]. No studies were found on blueberry pomace.

Recent research directions have indicated that phenolic compounds could be valorized as a new generation of prebiotics, as they stimulate probiotic growth and inhibit pathogenic bacteria in a simulated intestinal environment, interfering with the process of microbial adhesion [20,21]. A polyphenolic extract from grape seeds demonstrated *in vivo* prebiotic properties, stimulating the metabolism and adhesion of microbiota colonizing the gut [22]. No studies were found on the prebiotic effect of phenolics extract obtained from blueberry pomace.

Besides phenolics, grain-derived polysaccharides have shown important and beneficial actions on patients with type 2 diabetes by inhibition of α-glucosidase and α-amylase, as key digestive enzymes involved in postprandial blood sugar levels [23,24]. Previous experiments have demonstrated the *in vitro* antioxidant, anti-inflammatory and antidiabetic activity of chia (*Salvia hispanica*) seeds [25–27]. Moreover, chia seeds consumption could improve the function of the digestive tract by stimulating the development of beneficial microorganisms [28]. Although chia seeds contain a significant proportion of dietary fibers [29], no reports were found on polysaccharidic extracts of chia seeds.

The hypothesis of this study took into consideration the fact that ingested phenolics present low bioavailability, poor solubility and instability [30,31], and recent studies have targeted a combination of phenolics with other natural bioactive compounds, such as polysaccharides or proteins, to optimize their benefits for human health [32,33].

In this context, the present study aimed to prepare, for the first time, a mixture of blueberry pomace and chia seed extracts and to investigate its biological properties in experimental models *in vitro*, before and after simulated gastrointestinal digestion, in terms of α-amylase inhibition, antioxidant and prebiotic capacity, comparatively to individual extracts, in order to demonstrate that its constituents could synergistically act to improve biological activity. In addition, the study proposed valorization of blueberry pomace from Romanian production, to provide a novel source of bioactive ingredients for functional food development with potential economic benefits.

2. Materials and Methods

2.1. Materials

Blueberries (*V. myrtillus* L.) were harvested from the Research Institute for Fruit Growing, Pitesti, Romania, in 2020. Blueberry pomace was obtained as a by-product during juice production by S.C. Santo Raphael srl, Bucharest, Romania. Chia (*Salvia hispanica* L.) seeds o Argentinian origin were purchased from BioSano, Piatra Neamt, Romania.

HPLC standards of gallic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, rutin, luteolin 7-glucoside, kaempferol 3-glucoside, myricetin, resveratrol and quercetin were acquired from Merck (Darmstadt, Germany). High purity, thermostable α–amylase from Bacillus sp. (3000 U/mL) (E.C. 3.2.1.1) was purchased from Megazyme (Bray, Ireland). HPLC-grade acetonitrile 99.9%, Folin–Ciocalteu reagent, 3,5-dinitrosalicylic acid (DNS), acarbose, 2,2-diphenyl-1-picrylhydrazyl (DPPH), pepsin from porcine gastric mucosa (E.C. 3.4.23.1), bile salts, trypsin from porcine pancreas (E.C. 3.4.21.4) and other chemical reagents of analytical purity were acquired from Sigma-Aldrich (Schnelldorf, Germany), unless otherwise specified. The Caco-2 human intestinal epithelial cell line from ECACC (Sigma-Aldrich, Schnelldorf, Germany) was used at passage 20. The Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), non-essential amino acids, glutamax and a mixture of penicillin–streptomycin–neomycin (PSN) were acquired from Sigma-Aldrich (Schnelldorf, Germany).

2.2. Preparation of Bioactive Extracts and Their Mixture

Blueberry pomace was incubated in a drying oven (Memmert, Schwabach, Germany), at 40 °C, for 3 days and ground to a fine powder using an electric mill (Mockmill, Groß-Umstadt, Germany). For extraction, the powder was incubated in ethanol/water 70:30 (*v*/*v*), in a ratio of 1:10 (*w*/*v*) under magnetic stirring, at room temperature, for 24 h. The extract was centrifuged at 9000× *g* for 20 min and the residue was extracted again. The reunited supernatants were evaporated in a rotary evaporator (Heidolph, Schwabach, Germany) to remove the solvent and, finally, lyophilized to obtain the blueberry pomace extract (BP), which was stored in a sealed container at −20 °C until further analyses.

Chia seeds were hydrated in distilled water in a ratio of 1:5 (*w*/*v*) and occasionally stirred at room temperature for 3 h. Then, polysaccharide extraction was performed in distilled water in a ratio of 1:10 (*w*/*v*) in a Soxhlet equipment operated at 100 °C for 1 h [34]. After filtration, the procedure was repeated using the remaining residue. In order to purify the extract, the reunited supernatants were mixed with chilled ethanol solution, in a ratio of 1:3 (*v*/*v*) and stored at 4 °C. The precipitate obtained by centrifugation at 9000× *g* for 20 min was washed with distilled water and lyophilized. The purified chia seeds extract (CS) was stored in a sealed container at −20 °C, until further analyses.

Several mixtures of BP and CS extracts were prepared to different weight ratios. The mixture in a weight ratio of 5:1 (BCM) presented the highest content of phenolics and antioxidant activity and was selected for further analyses.

2.3. Determination of Total Phenolic Content (TPC), Total Flavonoids Content (TFC) and Total Carbohydrate Content

TPC of the extracts was determined according to a Folin–Ciocalteu assay [35] with minor modifications. Briefly, the sample (150 µL) was added to Folin–Ciocalteu reagent (750 µL), vortexed and incubated at room temperature, in the dark, for 5 min. A solution of 12% (*w*/*w*) sodium carbonate (2 mL) and distilled water (12.1 mL) was added to the mixture, vortexed and incubated at room temperature, for 30 min. The optical density (OD) of the solution was measured at a wavelength of 765 nm (V-650 spectrometer, Jasco, Japan). A solution of 0.5 mg/mL gallic acid was used to build a standard curve and to calculate the results as gallic acid equivalents (GAE).

TFC was determined according to an aluminum chloride assay [35]. Briefly, methanol (1.5 mL) was combined with the sample (0.5 mL) and 10% (*w*/*w*) $AlCl_3$ solution (0.1 mL), vortexed and incubated for 3 min. Then, 1 M sodium acetate solution (0.1 mL) and distilled water (2.8 mL) were added, followed by vortexing and incubation at room temperature, for 30 min. The OD of the solution was measured at a wavelength of 415 nm (V-650 spectrometer, Jasco, Tokyo, Japan). The 0.5 mg/mL quercetin solution served to build a standard curve and calculate the results as quercetin equivalents (QE).

Total carbohydrate content was determined according to AOAC standard [36] by subtracting the content of protein, lipid and ash.

2.4. HPLC Analysis

HPLC analysis was conducted on a HPLC system (Agilent 1200, Agilent, Santa Clara, CA, USA) consisting of quaternary pump, degasser, thermostatted autosampler and diode-array detector. The BP sample was filtered through regenerated cellulose membranes of 0.22 µm porosity, and aliquots (10 µL) were injected on a reverse phase column (Zorbax XDB-C18, 4.6 i.d. × 150 mm, 5 µm) (Agilent, Santa Clara, CA, USA). A mobile phase of 2 mM sodium acetate buffer, pH 3.05 (solvent A) and acetonitrile (solvent B) was used. The elution was carried out at a flow rate of 0.5 mL/min using a linear gradient: 0–30 min, 2–20% B; 30–40 min, 20–30% B; 40–50 min, 30% B; and 50–60 min, 30–2% B [37]. The identification and quantification of compounds was carried out using peak area integration, reported to that of standard phenolic acids and flavonoids, using Chemstation software.

2.5. Determination of α-Amylase Activity Inhibition

The inhibition of α-amylase was analyzed according to Apostolidis et al. [38] with minor modifications. The sample (20 µL) was mixed with 2 U/mL α-amylase (10 µL) and 100 mM phosphate buffer, pH 6.8 (50 µL), in the wells of a 96-well microplate. After incubation at 37 °C for 20 min, 1% starch solution (20 µL) was mixed and the plate was maintained at 37 °C for 30 min. Finally, DNS solution (100 µL) was mixed and the reaction took place at 100 °C. After 10 min, the OD was measured at a wavelength of 540 nm (Spectrostar Nano microplate reader, BMG Labtech, Ortenberg, Germany). A mixture containing distilled water in place of the sample served as control. A solution of acarbose, known as an α-amylase inhibitor, served as a positive control. The inhibition of α-amylase was calculated using the following equation:

$$\text{Inhibition of } \alpha\text{-amylase } (\%) = (OD_{control} - OD_{sample}/OD_{control}) \times 100 \quad (1)$$

The inhibitory concentration of 50% α-amylase activity (IC_{50}, mg/mL) was determined from the regression curve using Microsoft Excel 2018 software.

2.6. Determination of Free Radical Scavenging Capacity

The free DPPH radical scavenging capacity of samples was analyzed as previously described [35]. Briefly, mixtures of different concentrations (10–500 µg/mL) of sample in 0.25 mM DPPH methanolic solution and 0.1 M Tris-HCl buffer, pH 7.4, were prepared. They were incubated at room temperature, in the dark, for 30 min. Measurement of OD was carried out at 517 nm (V-650 spectrometer, Jasco, Tokyo, Japan). A blank was obtained by replacing the sample with the same volume of buffer. The following equation was used:

$$\text{Free DPPH radical inhibition } (\%) = (OD_{blank} - OD_{sample})/OD_{blank} \times 100 \quad (2)$$

The antioxidant agent Trolox, an analogue of vitamin E, was used as a positive control. IC_{50} was calculated from the nonlinear regression curve of DPPH inhibition vs. concentration plot using Microsoft Excel 2018 software.

2.7. In Vitro Simulated Gastrointestinal Digestion

A model of simulated gastrointestinal digestion was carried out in two successive steps as previously described [39], with minor modifications. First, 2.5 mL sample was mixed with 20 mg/mL pepsin solution in 5 M HCl, pH 2, supplemented with 0.9% NaCl (simulated gastric juice), in a shaking water bath fixed at 90 g and a temperature of 37 °C for 2 h. Then, the post-gastric digestion product (PG) was cooled on ice and stored at −20 °C. In the second phase, an aliquot of PG sample was further digested in a beaker with simulated intestinal medium, consisting of 25 mg/mL trypsin and 30 mg/mL bile salts dissolved in 0.1 M $NaHCO_3$ solution, pH 7.5. A dialysis bag (molecular weight cutoff of 12 kDa) was filled with a solution of 0.1 M $NaHCO_3$, pH 7.5, and placed into the beaker to gradually increase the pH, mimicking the gastrointestinal transition. The incubation took place at 37 °C, in the dark, for 2 h, with continuous stirring. A solution was obtained in the

dialysis bag containing the digestion products that can pass the intestine into the serum (PS), while the beaker solution contained the products that go into the colon (PC) following digestion. The samples were immediately frozen, until further analyses. For cell culture experiments, all samples (PG, PS, PC) were sterile filtered through 0.45 µm membranes.

2.8. In Vitro Cytocompatibility Testing by Neutral Red Assay

Human Caco-2 intestinal epithelial cells were seeded in the wells of a 96-well culture plate at a density of 1×10^5 cells/mL and cultivated in DMEM supplemented with 20% FBS, 1% glutamax, 1% non-essential amino acids and 1% PSN antibiotic mixture. The plate was incubated in the standard conditions of humidified atmosphere of 5% CO_2/95% air at 37 °C. After cell adhesion, the culture medium was replaced by digested samples in different concentrations (100–1500 µg/mL) in fresh medium and the plate was maintained in standard conditions for 24 h.

The cell viability was assessed according to Neutral Red assay [40]. Briefly, cell culture media was replaced with 100 µL of 0.005% (*w*/*w*) Neutral Red solution and incubation was carried out at 37 °C, in the dark, for 3 h. After three washes in phosphate-buffered saline, cells were fixed and the stain was extracted from the viable cells by gentle shaking for 15 min. Untreated cells were used as a negative control. A positive control was represented by cells treated with 0.3 mM H_2O_2. The OD measured at a wavelength of 540 nm (Spectrostar Nano microplate reader, BMG Labtech, Ortenberg, Germany) was proportional to the cell viability, according to the following equation:

$$\text{Cell viability (\%)} = (OD_{sample}/OD_{control}) \times 100 \quad (3)$$

2.9. Determination of Intracellular Reactive Oxygen Species (ROS) Production by Flow Cytometry

The intracellular ROS production was determined in the same experimental model of adhered Caco-2 intestinal cells treated with 100 µg/mL digestion products, for 24 h, as described above. Then, the cells were oxidative-stressed by treatment with 0.05 mM t-butyl hydroperoxide (t-BHP) for 30 min. The intracellular ROS production was determined by using the cell permeant fluorogenic dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA) [41]. Briefly, a solution of 10 µM DCFH-DA was added to the cells and the plate was incubated for 30 min, to allow formation of DCF fluorescent product analyzed at a BD LSR II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Untreated cells were used as a negative control. Cells treated with 12 µM ascorbic acid, known as antioxidant agent, served as a positive control. The acquired histograms of fluorescence intensity served to calculate the percentage of ROS production using FACSDiva and FlowJo software.

2.10. Determination of the Prebiotic Effect

The prebiotic effect was evaluated in experimental models *in vitro* developed using microbial strains of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus*, as previously described [42] with minor modifications. Briefly, cultures of bacterial cells in MRS broth medium were incubated at 35 °C for 24 h. The working culture was adjusted at a concentration of 1×10^8 CFU/mL to 0.5 McFarland standard. Then, microbial suspensions were added to the wells of a 96-well microplate and cultivated in the presence of non-digestible products (PC) (100 µg/mL) by incubation at 35 °C for 24 h. Measurements of OD carried out at 600 nm (Sunrise microplate reader, Tecan, Grödig, Austria) were proportional to microbial growth. An untreated culture served as a control.

2.11. Statistical Analysis

Three independent experiments were carried out and the obtained data were presented as mean ± standard deviation (SD) ($n = 3$). The statistical analysis on each control-sample or sample–sample pair of interest was conducted according to two-tailed, paired Student's *t*-test (Microsoft Excel 2018 software, Redmond, WA, USA). Differences were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Characterization of Bioactive Extracts

BP and CS extracts were analyzed for TPC, TFC and total carbohydrate content. The results are presented in Table 1. Data showed that BP had 30.40% TPC and 1.89% TFC. A similar TPC value (28.5 g GAE/100 g dry weight) was previously reported for the blueberry pomace extract prepared in HCl-methanol solution at room temperature, and for the extract in H_2SO_4-methanol solution at 85 °C [43]. CS contained 70.78% carbohydrates, as calculated by subtracting the content of protein, lipids and ash.

Table 1. Total phenolic, flavonoid and carbohydrate content of blueberry pomace extract (BP) and chia seed extract (CS). The results are expressed as mean ± SD (n = 3).

Sample	Total Phenolic Content (g GAE/100 g Dry Weight)	Total Flavonoids Content (g QE/100 g Dry Weight)	Total Carbohydrates Content (g/100 g Dry Weight)
BP	30.40 ± 1.28	1.89 ± 0.07	-
CS	3.93 ± 0.15	0.72 ± 0.03	70.78 ± 4.41

HPLC analysis was carried out to provide the concentration of the main phenolics present in BP extract. The results are presented in Table 2. The data indicated that gallic and chlorogenic acid were the main identified phenolic acids, while the flavonoids rutin, quercetin and luteolin 7-glucoside prevailed over the other identified compounds. Resveratrol was quantified in BP extract at a level of 103.43 mg/100 g dry weight. A previous study identified a similar composition of acidified hydroalcoholic extracts in blueberry pomace [44].

Table 2. HPLC analysis of phenolic compounds in blueberry pomace extract. The results are expressed as mean ± SD (n = 3).

Compound	Quantity (mg/100 g Dry Weight)
Gallic acid	5043.27 ± 181.03
Chlorogenic acid	1509.41 ± 61.37
Caffeic acid	200.60 ± 9.45
p-Coumaric acid	251.85 ± 8.75
Ferulic acid	118.14 ± 5.18
Rutin	609.78 ± 23.26
Luteolin 7-glucoside	262.11 ± 12.91
Kaempferol 3-glucoside	161.46 ± 6.76
Myricetin	175.18 ± 7.22
Resveratrol	103.43 ± 4.69
Quercetin	316.46 ± 11.84

3.2. Inhibition of α-Amylase Activity

Previous studies have reported that phenolic compounds, such as quercetin, rutin, catechin, procyanidins and tannic acid, could inhibit α-amylase activity through hydrophobic interactions [43]. In addition, a diet with chia seeds could control postprandial glycemia and manage the obesity risk in diabetes [45]. For the first time, in the present study, the capacity of BCM mixture to inhibit α-amylase activity was comparatively assessed to both BP and CS individual extracts. The results are presented in Figure 1.

The inhibition of α-amylase activity varied in a dose-dependent manner. The mixture presented the highest inhibition of α-amylase activity, close to that of acarbose, a known inhibitor of α-amylase activity. The mixture had a higher value of inhibition than those of individual extracts, at each tested concentration. The calculated IC_{50} value of BCM was 0.69 mg/mL, while those of BP and CS were 1.28 and 1.07 mg/mL, respectively. Acarbose

had an IC_{50} value of 0.31 mg/mL. These data indicated that the compounds from BCM could exert a synergistic action that led to inhibition of α-amylase activity.

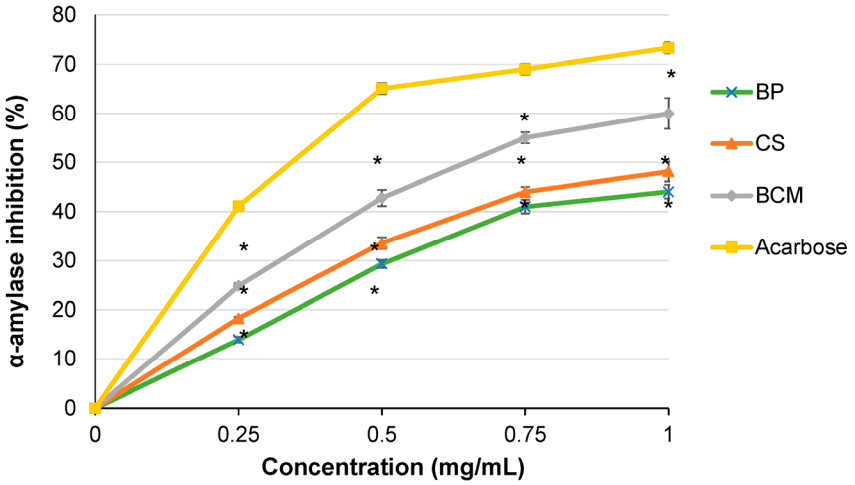

Figure 1. Inhibition of α-amylase activity in the presence of blueberry pomace extract (BP), chia seed extract (CS) and their mixture (BCM). Acarbose served as control. * $p < 0.05$, compared to control.

Similar activity of different methanolic and aqueous extracts of fruit pomace derived from grapes, lemon, orange and pineapple was previously reported in the range of 8–28% α-amylase inhibition [1]. Phenolic compounds, such as gallic and chlorogenic acid, acted as potent antidiabetic agents by inhibiting α-amylase and α-glucosidase activity *in vitro* and *in vivo* [46,47]. It was observed that these antioxidant phenolic compounds were found in significant quantities in BP extract and BCM mixture prepared in the present study. In addition, previous *in silico* studies using a docking program found novel phenolic inhibitors of α-amylase, such as corilagin, baicalein, quinoline and β-sitosterol, and identified the amino acids from the active situs of this enzyme involved in hydrophobic and/or hydrogen-bond interactions [48]. The mechanism of inhibition was described as a noncompetitive polyphenol-enzyme interaction and, in the case of catechins, good correlation between the affinity towards the enzyme and the inhibitory potential was found [49]. It was also found that phenolics represented a supportive treatment of type 2 diabetes and metabolic syndrome [50].

3.3. Free Radical Scavenging Activity

The results of free DPPH radical scavenging activity of BCM and individual extracts of BP and CS are presented in Figure 2. These data show that BCM mixture presented higher antioxidant activity than that of individual extracts. Accordingly, a lower IC_{50} value (606.12 μg/mL) was calculated for BCM mixture compared to that of BP (681.97 μg/mL). CS had a significantly higher IC_{50} value (2248.57 μg/mL), indicating low antioxidant activity, and so was not tested further in cell culture experiments. The IC_{50} value of Trolox, used as antioxidant control, was 127.02 μg/mL.

The capacity of BCM mixture to scavenge free DPPH radicals could be due to the high phenolic content of BP, according to a previous study reporting high correlation coefficient [43]. This activity was also influenced by the structural properties of phenolic constituents, such as the number and position of hydroxyl groups or other substituents [51]. In addition, it has been shown that polysaccharides had a pronounced interaction with fruit polyphenols through covalent, hydrogen and hydrophobic bonds [52], which might increase the antioxidant capacity of such combinations [53].

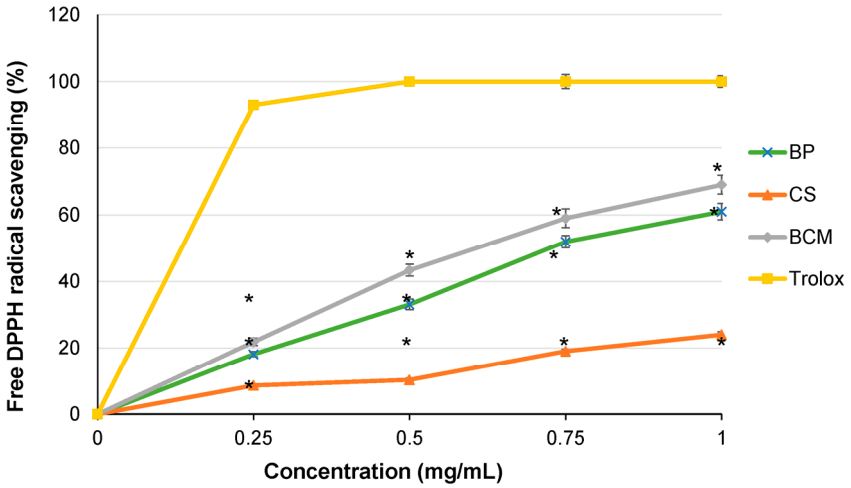

Figure 2. Free DPPH radical scavenging activity of blueberry pomace extract (BP), chia seed extract (CS) and their mixture (BCM). Trolox served as control. * $p < 0.05$, compared to control.

3.4. In Vitro Cell Cytocompatibility

A two-step gastrointestinal digestion was performed on the BCM mixture, and PG, PS and PC products were obtained. The results of *in vitro* cytocompatibility testing on different concentrations of these products in a Caco-2 intestinal cell line are presented in Figure 3. The values of cell viability varied in a dose-dependent manner. The PG–BCM had good cytocompatibility in concentrations ranging from 100–1000 µg/mL; the cell viability values were close to those of control cells (100%) at a concentration of 100 µg/mL, and higher than 80% at concentrations between 400 and 1000 µg/mL. At 1500 µg/mL, the cell viability was 73.6%, indicating moderate cytocompatibility. The PS digestion product of BCM was also cytocompatible up to 1000 µg/mL (cell viability > 80%) and a value of 71.6% was recorded at 1500 µg/mL. The PC digestion product of BCM was cytocompatible and had values of cell viability higher than 80%, at all tested concentrations. The digestion products of BP had a similar degree of cytocompatibility to that of the BCM mixture.

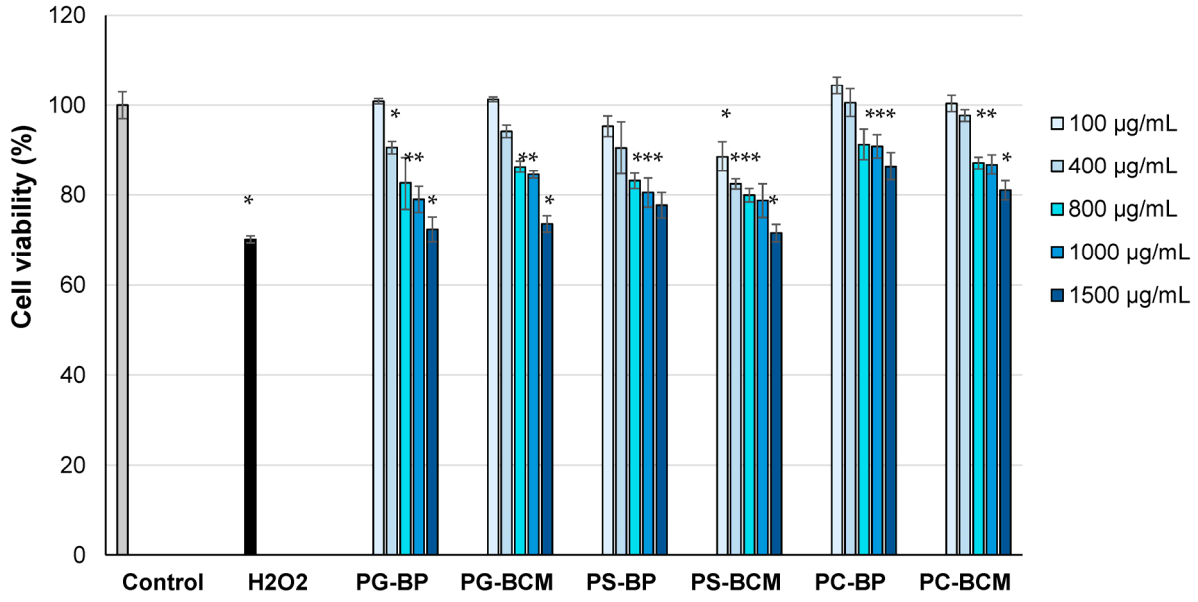

Figure 3. The cell viability of Caco-2 cells treated with post-gastric (PG) and post-intestinal (PS, PC) digestion products of blueberry pomace extract (BP) and BCM mixture for 24 h, assessed by Neutral Red assay. The results are expressed as mean ± SD ($n = 3$). * $p < 0.05$, compared to the untreated cells (control).

Based on these data, the cytocompatible concentration of 100 µg/mL digestion products was selected for further experiments in Caco-2 cells.

3.5. Effect on Intracellular ROS Production

An experimental model *in vitro* was used to assess the effect of PS and PC digestion products of the BCM mixture on the modulation of intracellular ROS production in an oxidative-stressed Caco-2 intestinal cell culture. The histograms acquired by flow cytometry are presented in Figure 4 and the calculated percentages of ROS production are given in Table 3. The results indicate that all tested samples had the capacity to significantly ($p < 0.05$) decrease the ROS level, compared to the oxidative-stressed cells. It is worth emphasizing that, in the case of cells treated with the PC digestion product of BCM, the ROS level was diminished at a similar value to that of cells treated with ascorbic acid, a known antioxidant agent. An antioxidant capacity was also observed in the case of cells treated with PS–BCM, but the levels of ROS production were slightly higher compared to that of the PC–BCM digestion product. These results confirm that the compounds from the BCM mixture could maintain their antioxidant activity after gastrointestinal digestion. The digestion products of BP presented antioxidant capacity, but to a lower extent than those of BCM.

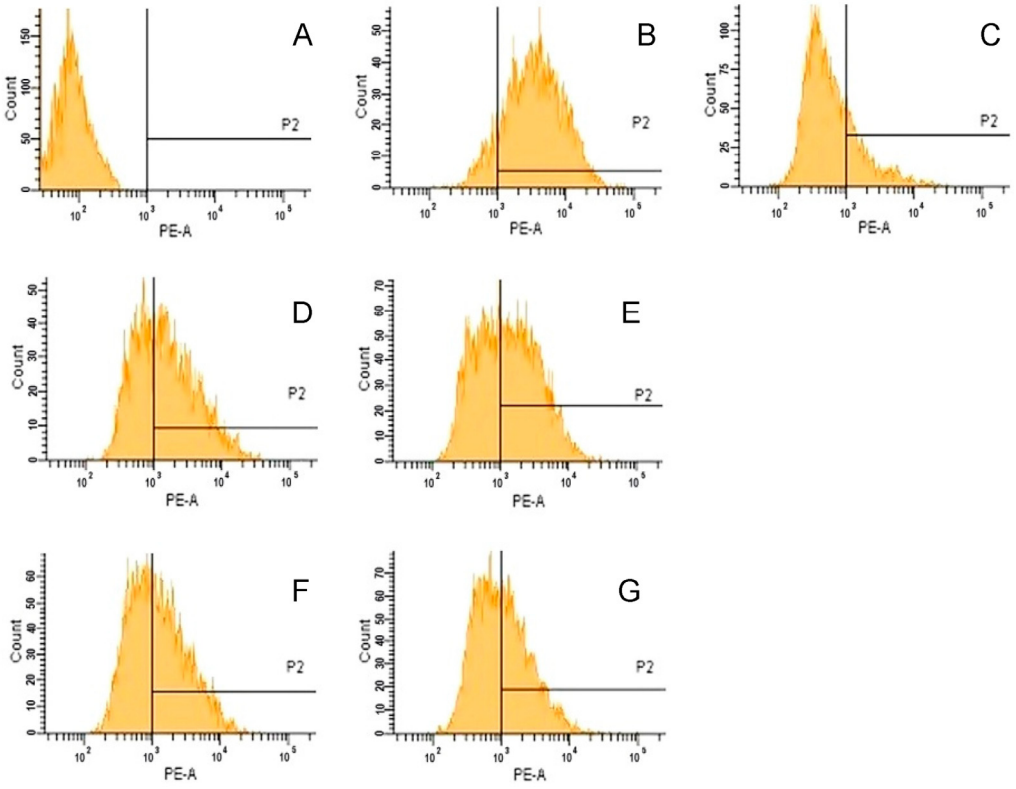

Figure 4. Flow cytometry analysis of oxidative-stressed Caco-2 cells (**B**) pretreated with 100 µg/mL of intestinal digestion products PS-BP (**D**), PS-BCM (**E**), PC-BP (**F**) and PC-BCM (**G**), for 24 h. Untreated cells (**A**) and cells treated with 12 µM ascorbic acid (**C**) served as a control.

The results were in accordance with a previous study on polyphenolic extracts of blueberry pomace showing their capacity to inhibit ROS generation in H_2O_2-treated keratinocytes [54]. Moreover, pre-treatment of cells with blueberry phenolic extract could protect them from H_2O_2-induced oxidative stress through the p-38 mitogen-activated protein kinase metabolic pathway [55]. In a similar study on blackberry phenolic extracts, their anti-ROS activity in cultured cells was reported after simulated gastrointestinal digestion, a process known to affect their stability and bioavailability *in vivo* [56]. Previous research reported the beneficial effect of grape polyphenols administration in mice for lowering the intestinal ROS level [57].

Table 3. Quantification of ROS production in oxidative stressed Caco-2 cells (B) treated with 100 µg/mL of intestinal digestion products PS-BP (D), PS-BCM (E), PC-BP (F) and PC-BCM (G), performed by flow cytometry. Untreated cells (A) and cells treated with 12 µM ascorbic acid (C) served as a control. The results are expressed as mean ± SD (n = 3). * $p < 0.05$, compared to the oxidative stressed group; # $p < 0.05$, compared to ascorbic acid-treated group.

Histogram	Sample	ROS Production (%)
B	Oxidative-stressed group	100.00 ± 4.03
C	PS-BP	46.70 ± 2.24 *,#
D	PS-BCM	41.43 ± 1.56 *,#
E	PC-BP	36.41 ± 2.13 *,#
F	PC-BCM	34.86 ± 1.06 *
G	Ascorbic acid (control)	30.16 ± 2.12 *

3.6. Prebiotic Effect

The results of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* growth in the presence of digestion products reaching the colon are shown in Figure 5. These data indicated that cell growth of both *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* bacterial strains was superior in the presence of the PC-BCM digestion product compared to that of untreated control. Thus, the sample has induced a stimulating effect on the growth of each tested strain of *Lactobacillus*, after 24 h of treatment. Additionally, significantly ($p < 0.05$) higher values were recorded in the cells treated with PC-BCM compared to those treated with PC-BP. These results demonstrated that the BCM mixture had a better capacity to up-regulate the growth of lactic acid bacteria strains. In addition, they suggested a synergistic action of bioactive compounds from the BCM mixture to stimulate probiotics and gut health.

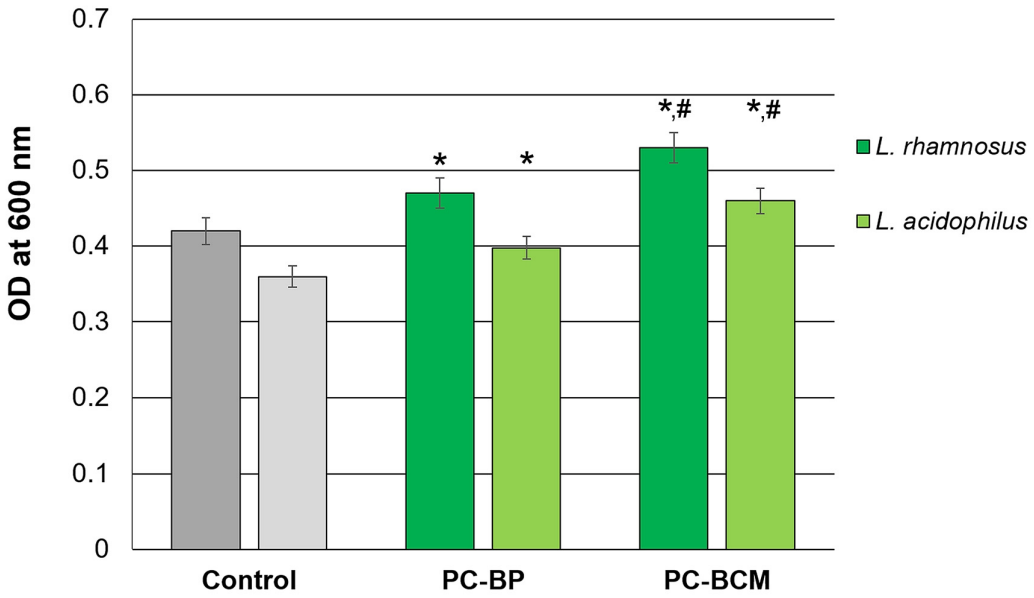

Figure 5. Growth of *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* cultivated in the presence of 100 µg/mL of digestion products reaching the colon (PC) of blueberry pomace extract (BP) and BCM mixture, after 24 h of treatment. The results are expressed as mean ± SD (n = 3). * $p < 0.05$, compared to untreated control; # $p < 0.05$, compared to PC-BP.

Several *in vitro* and *in vivo* studies have indicated the bioactivity of berry polyphenols in balancing between beneficial *Bacteroidetes* and *Firmicutes* through a decrease in short-chain fatty acid production [58]. Blueberry anthocyanin extracts presented prebiotic activity [59], while consumption of a phenolic extract has modulated the gut microbiota of mice, increasing the relative abundance of *Bifidobacteria* and revealing anti-obesity ef-

fects [60]. Phenolic extracts of elderberry skin were also reported to stimulate the growth of the probiotic strain of *Lactobacillus rhamnosus*, and are recommended for obtaining functional, pro-health food [42]. The combination of phenolics and probiotic strains induced significant beneficial effects on gut motility and microbiota in an in vivo model on *Drosophila* and in a simulated model of the human gastrointestinal tract, compared to that of each component, providing novel solutions for chronic metabolic diseases [61]. Moreover, chia seeds have high nutritional and therapeutic potential, in particular for gut microbiota due to their chemical composition rich in soluble fiber polysaccharides. Still, there is scarce scientific literature on the prebiotic effect of chia seed polysaccharides. A recent study has reported that chia mucilage protected the probiotics encapsulated in a food film and stimulated their survival increase [62]. Similar studies have reported that oligosaccharidic fractions extracted from grape seeds exerted prebiotic activity towards *Lactobacillus acidophilus*, improving *in vitro* bacterial growth [22].

4. Conclusions

For the first time, we proposed a combination of phenolics extract from blueberry pomace and a polysaccharidic extract from chia seeds with optimal biological activity. *In vitro* results indicated that this formulation exhibited higher inhibition of α-amylase and free DPPH radical scavenging compared to that of individual extracts. After simulated gastrointestinal digestion of the BCM mixture, all products were cytocompatible in human Caco-2 intestinal cells. The products reaching the colon decreased the intracellular ROS production to a similar extent to ascorbic acid, and stimulated lactic acid bacteria growth in a higher proportion than individual extracts.

All these data demonstrated that bioactive constituents from BCM in combination could synergistically act to improve the properties of individual extracts. Further studies could test this new valuable natural composition for applications in nutraceutical formulation for maintaining the functions of the digestive tract, in particular through hypoglycemic, antioxidant and prebiotic effect using *in vivo* models.

Author Contributions: E.M., O.C. and B.-S.N.-P. designed the study and had major contributions in writing the manuscript; E.M., T.C., E.U. and A.-M.G. performed extraction and biochemical experiments; E.M., T.C., E.U., A.-M.G. and T.N.-P. analyzed and interpreted the data; A.I. and V.C. performed cell culture experiments, data processing and interpretation; A.-M.S.-G. conducted flow cytometry experiments, and analyzed and interpreted the data; A.-M.P. conducted microbial experiments, analyzed and interpreted the data; O.C., B.-S.N.-P., A.I., V.C. and T.N.-P. were involved in writing and editing the manuscript; O.C., B.-S.N.-P. and T.N.-P. were involved in supervision. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

BP: blueberry pomace extract; CS: chia seed extract; BCM: mixture of BP and CS extracts in a ratio of 5:1 (*w*/*w*); DPPH: 2,2-diphenyl-1-picrylhydrazyl; DNS: 3,5-dinitrosalicylic acid; DMEM: Dulbecco's Modified Eagle Medium; FBS: fetal bovine serum; PSN: penicillin–streptomycin–neomycin mixture; TPC: total phenolic content; TFC: total flavonoids content; OD: optical density; GAE: gallic acid equivalents; QE: quercetin equivalents; PG: gastric digestion product; PS: intestinal digestion product that pass into the serum; PC: intestinal digestion product that go into the colon; t-BHP: tert-butyl hydroperoxide; ROS: reactive oxygen species; DCFH-DA: 2′,7′-dichlorofluorescein diacetate; SD: standard deviation.

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