Nutrition, Metabolism & Cardiovascular Diseases (2012) 22, 127–132

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journal homepage: www.elsevier.com/locate/nmcd

Nutrition, Metabolism & Cardiovascular Diseases

The temporal effect of a wild blueberry (*Vaccinium angustifolium*)-enriched diet on vasomotor tone in the Sprague-Dawley rat

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Received 15 December 2009; received in revised form 28 April 2010; accepted 14 May 2010

KEYWORDS
Wild blueberries;
Diet duration;
Biosensors;
Vasomotor tone;
Vasoconstriction

Abstract *Background and aims:* We have previously reported that wild blueberry (*Vaccinium angustifolium*)—enriched diets (WB) attenuate aortic adrenergic response through endothelial-mediated pathways. The duration of dietary intervention necessary to induce the positive changes on vasomotor tone has not been studied to date. Thus, our objective was to investigate the temporal effect of WB consumption on vascular function and reactivity in Sprague-Dawley (SD) rat aorta after 4 and 7 weeks of dietary treatment.
Methods and results: Forty male SD rats were randomly assigned to a control (AIN-93) (C) or a WB diet for 4 or 7 weeks. Vascular ring studies were conducted in 3-mm isolated rat aortic rings to investigate vasoconstriction induced by six doses of the α_1-adrenergic agonist, L-phenylephrine (Phe, 10^{-8}–3×10^{-6} M) alone or in the presence of the NOS inhibitor, L-N^G-monomethyl-arginine (L-NMMA, 10^{-4} M). The maximum force of contraction (F_{max}) and vessel sensitivity (pD_2) were determined. Analysis of variance revealed no significant differences on F_{max} after 4 weeks of the WB diet but only a significant increase in pD_2 in the absence of L-NMMA. Seven week WB consumption significantly attenuated contraction in response to L-Phe and resulted in lower pD_2. Inhibition of NOS induced a significant increase in the constrictor response in both diet groups at both time periods, with the WB group fed for 7 weeks having the greater response.
Conclusion: Thus wild blueberries incorporated into the diet at 8% w/w positively affect vascular smooth muscle contractility and sensitivity but these effects are evident only after 7 weeks of WB consumption.

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doi:10.1016/j.numecd.2010.05.004

Introduction

Cardiovascular disease (CVD) represents a major cause of death in developed countries [1]. Epidemiological studies suggest that consumption of polyphenol-rich foods can reduce CVD risk [2,3] by increasing nitric oxide (NO) production and bioavailability, thus improving endothelial function and vascular tone. Nitric oxide inhibits vascular smooth muscle contraction and growth, platelet aggregation, and leukocyte adhesion [4] and thus is critical in the maintenance of a functional endothelium. Endothelial dysfunction, a hallmark of atherosclerosis, diabetes and hypertension, is characterized by impaired NO signaling, attenuation of vasorelaxation pathways and deregulation of vascular tone [5].

The bioactivity of polyphenols, specifically anthocyanins present in fruits and vegetables is associated with maintaining or correcting endothelial function [6–10]. *In vitro* studies investigated the effects of anthocyanin-enriched fruit extracts on the ability to produce endothelium-dependent relaxation in aortic rings [7]. A mixture of bilberry anthocyanins, induced vasorelaxant activity via the NO pathway [8]. Nakamura et al. [9] reported that a black-currant concentrate produced vasorelaxation of rat aorta. Similar *ex vivo* effects have also been demonstrated after addition of red or white wine in human coronary arteries and rat aortic rings [10].

Blueberries and other berries have been recognized to protect against chronic diseases including glaucoma, fibrocystic breast disease, cancer, cerebrovascular conditions, diabetes, atherosclerosis, and CVD [11].

However, the majority of studies have been conducted *in vitro* either in cell cultures or with isolated blueberry bioactive compounds or fruit extracts added to tissue baths [12]. The potential benefits of wild blueberry diets (WB) on endothelial function have been documented *ex vivo* in our laboratory [13–15]. We were the first to report that WB-enriched diets (*Vaccinium angustifolium*) reduced the α_1-adrenergic receptor-agonist-mediated contraction thus improving vascular tone in young normotensive Sprague-Dawley (SD) rats through an endothelium-dependent pathway after 13 weeks of WB consumption [13]. Similar findings were documented after 7 weeks on a WB diet [14]. Furthermore, we have reported that wild blueberries alter acetylcholine (Ach)-mediated vasorelaxation in young SD and spontaneously hypertensive rat (SHR) aorta, most likely by modulating the cyclooxygenase (COX) pathway in the latter [14,15]. Thus, the observed cardiovascular benefit of wild blueberries could potentially aid in the maintenance of arterial health and the prevention of a dysfunctional endothelium.

There is paucity of research though on the length of dietary intervention to induce the beneficial effects of wild blueberries on vasomotor tone, which is of special interest to scientists conducting research in the above areas.

Therefore the goal of the present study was to investigate the effect of a shorter duration (4 weeks) WB-enriched diet and compare it to the previously reported effective 7-week period on vascular contraction and reactivity in SD aortic rings, in an attempt to evaluate whether a shorter time of exposure is sufficient to obtain the beneficial effects of wild blueberries on vasomotor tone.

Methods

Animals and diets

The Animal Care and Use Committee of the University of Maine approved animal care and experimental procedures. Forty weanling male SD rats (Charles River, Portage, NY) were randomly assigned to four groups of 10 rats each. Twenty rats were placed on a control diet for 4 (C4), or 7 weeks, (C7), and twenty on a wild blueberry (WB) diet for 4 (WB4), or 7 weeks (WB7). The diets, composed of dextrose, egg white solids, vitamin mix, D-L-Methionine, biotin, corn oil (Harlan Teklad, Madison, WI) and mineral mix (MP Biomedicals, Solon OH), were prepared as previously described [13–16]. Wild blueberries, provided as a composite from Wyman's (Cherryfield, ME), freeze-dried and powdered with standard procedures (Futureceuticals, Monmence, IL), were incorporated at 8% w/w to the C diet substituting for dextrose [13–16]. Analysis of the wild blueberry powder demonstrated the presence of twenty one different ACNs. The main compounds were: Malvidin 3-galactoside (Mv-3-gal) and Peonidin-3-glucoside (Pn-3-glc) that represented about 13% of the total amount of ACNs (1.6 ± 0.2 mg/100 mg) [17].

The animals were individually housed in metal mesh-bottomed cages in an environmentally controlled room maintained at 22 °C with a 12:12 h light:dark cycle. Tap water and food were provided *ad libitum*. Animals were weighed weekly and food consumption was measured daily. Rats consumed about 16.2 ± 1.37 g/day of WB diet, providing the equivalent of approximately 20.7 mg/day of ACNs.

Drugs and chemicals

Salts for the physiological salt solution (PSS), acetylcholine chloride (Ach), L-Phenylephrine (L-Phe) and L-N^G-monomethyl-arginine (L-NMMA) were purchased from Sigma Chemical Co. (St. Louis, MO).

Aortic ring preparation

At the end of the diet period, animals were anesthetized with 95% CO_2/5% O_2, the thoracic aorta was extracted and aortic rings were prepared as previously described [13–16]. Briefly, extracted aortas were placed in a PSS solution (composition in mM: NaCl 118, KCl 4.7, CaCl 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 12.5 and dextrose 11.1) and the middle part of the aorta was divided into four rings (3 mm length). Aortic rings, suspended by two stainless steel triangles, were mounted in a 20 mL Radnoti tissue bath containing PSS aerated with 95% O_2/5% CO_2 (pH 7.45) and maintained at 37 °C by a heat circulator. One end of the ring-triangle complex was connected by a weightless wire to a biosensor (force transducer), while the other was attached to a fixed glass hook in the tissue bath. The isometric force developed by the aortic rings in response to the agonist was transmitted through the biosensor to a microprocessor-based Tissue Force Analyzer (model 410, Micro-Med, Louisville, KY). All aortic rings were mounted in tissue baths within 60 min after administration of anesthesia and were subjected to 1.5 g preload, maintained throughout the experiment.

Experimental design

A total of 160 aortic rings (40 rats, four rings per rat) were used. After preconditioning with one dose of Ach (10^{-8} M) and L-Phe (10^{-8} M), rings were randomly assigned to treatment with no inhibitor, or L-NMMA (10^{-4} M) (NOS I, II and III inhibitor) [13–15]. Cumulative concentrations of L-Phe (10^{-8}–3×10^{-6} M) were applied in the bath at 6-min intervals to generate a concentration-response curve. After the L-Phe curve, a single concentration of Ach (3×10^{-6} M) was utilized to confirm the ability of rings to relax more than 70%, used as an index of endothelial viability. A second L-Phe concentration-response curve was generated to validate the first curve [13–15].

Measurement of physiological parameters

The contraction force developed to cumulative L-Phe concentrations in the absence or presence of L-NMMA, was determined for each ring from digitized data recorded by an integrator software program (DMSI-410 version 1.01, MicroMed, Louisville, KY). The maximum force of contraction (F_{max}, g) was determined as the highest value of each L-Phe curve and was used to evaluate the effect of the diet on aorta contractility. Concentration-response curves were fitted by nonlinear regression and the effective concentration of the agonist required to obtain 50% of maximum response (EC_{50}) and vessel sensitivity to the α_1-adrenergic receptor response (pD_2,-$\log_{10}$ EC_{50}), were determined for each ring [13–15].

Statistics

Statistical analysis was conducted with Sigmastat Statistical Program Package version 2.0 (SPSS Inc., Chicago, IL). Results are expressed as mean ± standard error of the mean (SEM). The effect of diet on body weight and food consumption was determined by a Student *t*-test. Vasoconstriction force at each concentration was tested with a Student Neuman-Keuls for multiple comparisons to determine the effect of diet and inhibitor treatments.

Equal number of rank-ordered observations of F_{max} and pD_2 values, with or without L-NMMA, were compared using a two-way Analysis of Variance (ANOVA) to determine the possible effect of diet and time duration. A value of $p \leq 0.05$ was considered statistically significant.

Results

Growth rate

Animals in all diet groups gained weight with no significant differences between groups [Body Weight: 253.10 ± 4.92 g (C4) and 259.00 ± 35.18 g (WB4); 363.20 ± 7.50 g (C7) and 355.50 ± 10.17 g (WB7)]. Food intake was similar in all diet groups as previously observed [13–16].

Endothelium-dependent vasoconstriction

The effects of diet at 4 and 7 week duration on the maximum force of contraction of the arterial rings before and after the application of the NOS inhibitor are presented on Table 1 and Fig. 1A and B.

The four week WB consumption did not significantly affect the F_{max} developed by arterial rings in response to the α_1-adrenergic agonist, L-Phe (WB4: 0.89 ± 0.03 g vs C4: 0.90 ± 0.03 g) (Table 1). While the F_{max} developed by the aortic rings in response to L-Phe, after 7 weeks of WB consumption was significantly lower (0.52 ± 0.03 g) than the C7 group (0.74 ± 0.03 g) (Table 1). Fig. 1A presents the L-Phe concentration-response curves without the NOS inhibitor in rats fed for 4 and 7 weeks. A significant decrease in the maximum force of contraction in response to different concentrations of L-Phe was observed above 3×10^{-7} M in the WB7 group compared to C7 but not in 4 week diet duration group except at L-Phe of 10^{-7} M.

Treatment with L-NMMA to inhibit basal NOS synthesis, resulted in significant increases in F_{max} in all diet groups as expected (Table 1). However, while the responses were similar between C and WB groups at 4 weeks, differences emerged after 7 weeks of WB consumption. In fact, after addition of L-NMMA, the WB7 group developed a significantly higher F_{max} with respect to the C7 group (1.97 ± 0.03 g vs 1.82 ± 0.03 g respectively). Fig. 1B presents the L-Phe concentration-response curves in presence of L-NMMA in rats fed for 4 and 7 weeks. The maximum force of contraction generated to L-Phe at concentrations 3×10^{-7} M to 3×10^{-6} M was significantly higher in the WB7 group compared to C7 only after 7 weeks of WB consumption. No significant differences were observed in the F_{max} at each L-Phe concentration applied in the 4 week diet group except at L-Phe of 10^{-7} M.

The effect of time duration on F_{max} in response to L-Phe of the C and WB diet group aortic rings is also presented on Table 1. Significant decreases in F_{max} were documented both in the C (C4: 0.90 ± 0.03 vs. C7: 0.74 ± 0.03; $p \leq 0.05$) and in the WB groups (WB4: 0.89 ± 0.03 vs. WB7:0.52 ± 0.03; $p \leq 0.05$) and varied depending on the duration of the corresponding diets.

Vessel sensitivity

Table 2 presents and compares the effects of diet on vessel sensitivity (pD_2) after 4 and 7 weeks of WB consumption.

Table 1 Maximum force of vasoconstriction (F_{max}) to L-Phe with and without the addition of NOS inhibitor, L-NMMA.

Diet duration	PSS	L-NMMA
4 Weeks		
Control	0.90 ± 0.03	1.84 ± 0.01[b]
Wild blueberry	0.89 ± 0.03	1.84 ± 0.01[b]
7 Weeks		
Control	0.73 ± 0.03[c]	1.82 ± 0.03[b]
Wild blueberry	0.52 ± 0.03[a,d]	1.96 ± 0.03[a,b,d]

Values represent mean ± SEM. Comparisons were conducted among diet groups in the same treatment (in each column), between the two different treatments (in each row) and diet duration. Significant differences are shown ($p \leq 0.05$).

[a] Significantly different from Control.
[b] Significantly different with or without L-NMMA.
[c] Significantly different from 4 weeks (Control).
[d] Significantly different from 4 weeks (Wild Blueberry).

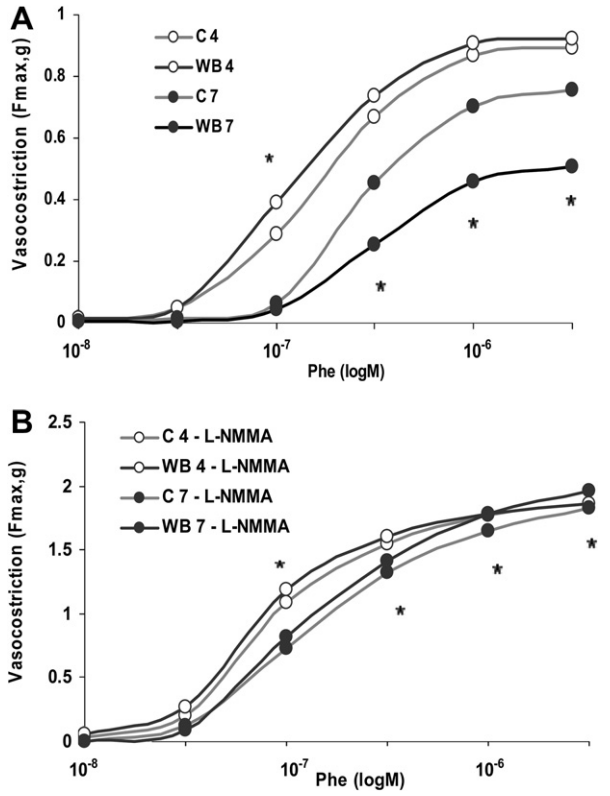

Figure 1 Contractile responses of aortic rings from control (C) and wild blueberry (WB) diets fed for 4 and 7 weeks. The graph represents the developed tension in response to cumulative doses of L-Phe ($n = 10$ rats per group) in the absence (A) and presence (B) of the NOS inhibitor, L-NMMA. *Statistically significant difference at $p \leq 0.05$.

The mean pD_2 value significantly increased in the aortic rings of animals on the WB4 diet (WB4: 7.51 ± 0.01 vs C4: 7.60 ± 0.01, $p \leq 0.05$). A significant decrease in vessel sensitivity to the agonist was observed in both diet groups (C4 and WB4) after adding L-NMMA.

The 7-week WB diet was able to decrease the pD_2 value when compared to the C7 group without the inhibitor (6.50 ± 0.03 vs 6.60 ± 0.07 for the WB group and C group respectively; $p \leq 0.05$) (Table 2). Thus, wild blueberries added to the diet of SD rats for 7 weeks were able to correct the high vessel reactivity (pD_2) observed in the C7 group in response to the α_1-adrenergic agonist, L-Phe. Addition of L-NMMA, resulted in significant increases in vessel sensitivity in both C7 and WB7 which was also found to be significantly lower in the WB7 diet group (WB7: 6.89 ± 0.04 vs C7: 6.91 ± 0.04, $p \leq 0.05$).

The effect of time duration on the pD_2 value of the C and WB diet groups is also presented on Table 2. Significant decreases in pD_2 were documented both in the C diet groups (C4: 7.51 ± 0.01 vs. C7: 6.60 ± 0.07; $p \leq 0.05$) and in the WB groups (WB4: 7.60 ± 0.01 vs. WB7: 6.50 ± 0.03; $p \leq 0.05$) depending on the length of time on the corresponding diets.

Table 2 Vessel sensitivity (pD_2) to L-Phe with or without the addition of the NOS inhibitor, L-NMMA.

Diet duration	PSS	L-NMMA
4 Weeks		
Control	7.51 ± 0.01	7.32 ± 0.04[b]
Wild blueberry	7.60 ± 0.01[a]	7.32 ± 0.04[b]
7 Weeks		
Control	6.60 ± 0.07[c]	6.91 ± 0.04[b,c]
Wild blueberry	6.50 ± 0.03[a,d]	6.89 ± 0.04[b,d]

Values represent mean ± SEM. Comparisons were conducted among diet groups in the same treatment (in each column), between the two different treatments (in each row) and diet duration. Significant differences are shown ($p \leq 0.05$).
[a] Significantly different from Control.
[b] Significantly different before and after L-NMMA.
[c] Significantly different from 4 weeks (Control).
[d] Significantly different from 4 weeks (Wild Blueberry).

Discussion

The effect of oxidative stress on endothelial dysfunction has resulted in a plethora of investigations to evaluate the effects of antioxidants and their ability to improve endothelial function.

In fact, the effect of anthocyanins to scavenge reactive oxygen species that reduce NO bioavailability leading to the development of endothelial dysfunction, has been studied in several *in vitro* experiments [18—23] where extracts or single bioactive compounds were added in tissue baths. Studies with wine [18], pomegranate anthocyanins [20], wild strawberry leaf extract [21], grape seed extract [22] and grape polyphenols [23], induced NO-dependent vasodilation in isolated aortic rings with the involvement of the NO-cGMP system.

Dietary *ex vivo* studies in our laboratory were the first to demonstrate the beneficial effects of WB-enriched diets on vasomotor tone and endothelial function in both normotensive and hypertensive animal models fed at different time durations [13—15].

There is paucity of research though on the length of dietary treatment for the onset of the observed effects of wild blueberries on vasomotor tone. Thus, the aim of the present study was to investigate the effect of a short (4 week) WB consumption on vascular contraction with or without NOS inhibition in SD rat aortic rings and compare it to the already reported beneficial effect of a 7-week WB diet [15]. We documented that a 4-week consumption of wild blueberries did not significantly affect maximum vessel contraction in response to the α_1-adrenergic agonist, L-Phe, but only influenced vessel reactivity (pD_2). However, after 7 weeks of WB consumption, significant reductions in maximum vessel contraction and vessel sensitivity were observed.

The principal cause of the changes in SD aortic contractility observed in response to the WB diet may be the temporal effect of diet exposure. Several animal studies have demonstrated that a long-term exposure to bioactive compound treatment could improve endothelial function [19—23]. Long-term treatment with vitamin C (26—28 weeks) restored aortic NOS activity and improved endothelial function in apoE-deficient mice [24]. After 12 weeks of a diet rich in pomace olive oil, endothelial dysfunction improved in SHR aorta due to

enhanced eNOS expression [25]. An 8-week supplementation with coenzyme Q (Q10) increased endothelium-dependent vasodilatation in senescent Wistar rats [26].

In human studies, the consumption of green and black tea for 4 weeks did not alter blood pressure in normotensive individuals [27]. In another dietary intervention study, vascular function was not modified in subjects with established coronary artery disease that consumed flavonol-rich chocolate bars and cocoa beverages for 6 weeks [28]. Therefore, longer dietary treatments may be needed to alter vascular function and biomarkers associated with disease risk.

In the present study, addition of the NOS inhibitor, L-NMMA, significantly increased F_{max} in both groups (4 and 7 weeks) attributed to the impairment in NOS synthesis, which reduces the contractile effects produced by the α_1-adrenergic agonist, and promotes vasodilation. A comparison among diet groups C7 vs. WB7 demonstrated a significant difference in F_{max} after the NO pathway was inhibited i.e. a higher force of contraction in response to L-Phe was detected in the WB7group compared to the C7 group suggesting that a WB-enriched diet acts on the NO pathway to promote basal NO-mediated vasorelaxation.

In this regard, recently a putative mechanism involved in the process of vasorelaxation has been attributed to red wine polyphenols and in particular to delphinidin, an anthocyanin that is also present in wild blueberries. This mechanism involves a direct interaction with a specific molecular target (Estrogen Receptor alpha) capable of stimulating vasorelaxation in response to an increase of NO production [29].

Cumulative L-Phe concentrations (10^{-8}—3×10^{-6} M), produced a concentration-dependent contraction of the aortic rings in all groups investigated (4 and 7 weeks independent of diet) as expected. However, only WB powder incorporated to the diet for 7 weeks attenuated vasoconstriction in comparison to C7 diet, evident starting at L-Phe 3×10^{-7} onward. Consequently, the time of exposure to the diet seems to play a crucial role on vasomotor tone of the isolated arteries under stimulation with vasoconstrictor agents.

The effect of age on vascular tone should also be considered when conducting research on vascular function. Even though our animals were three weeks apart in age (7 vs 10 week old) and can be classified as juvenile and/or borderline adult, (7—8 weeks juvenile, 11—12 weeks adult) [30], we observed significant differences both in F_{max} and pD_2 between 4- and 7-week diet duration in all diet groups. It has been documented in rat aorta, that physical development from juvenile to borderline adult stage is related to a reduction in maximum contractile responses induced by adrenoreceptor agonists, although this tendency is not uniform among different species and arterial beds [31,32]. Thus the significant reduction in F_{max} observed with the 7-week vs the 4-week diet duration in both diet groups (C and WB), coupled with the downward shift of the dose-response curve in the 7-week group, may be explained by the above studies [31,32]. Additionally, during animal growth and development, rapid increases in tissue mass are accompanied not only by extensive growth of the arteriolar, venular, and capillary beds but also by increases in microvascular pressure and tissue blood flow [31]. In light of these phenomena, it is reasonable to expect changes in the aortic lumen, the functional endothelium and the vascular smooth muscle. Our results seem to support the hypothesis that the differences in adrenoreceptor sensitivity and maximum force of contraction, could also arise from a reduced aortic lumen since rats consuming the diets for 4 weeks were at the juvenile stage of development (7 weeks of age).

Vessel reactivity (pD_2), serves as an indicator of receptor-agonist interactions and sensitivity to each fraction of agonist added to each tissue bath. After 4 weeks of diet duration, pD_2 in response to L-Phe significantly increased in the WB group, while after 7 weeks of WB consumption we observed a significant decrease in vessel reactivity. Additionally, pD_2 was significantly attenuated in response to L-Phe after 7 weeks of diet duration independent of diet group (C or WB). The differential effect of diet duration upon vessel sensitivity may be explained by the documented age-related changes in postsynaptic α_1-adrenoreceptor mechanisms in rat aorta [33]. Vessel sensitivity in response to L-Phe increases between 6 and 10 weeks of age but decreases after 10 weeks [33]. The mechanism is related to changes in receptor density or receptor reserve and not to the affinity of drugs to the α_1-adrenoreceptor [31]. The WB diet was capable in reducing vessel sensitivity after 7 weeks suggesting that the ability of the wild blueberries to affect vascular tone may involve interaction between agonist membrane receptors and blueberry components. This may be related to structural changes in molecules that modify receptor conformation or activation, such as WB diet-induced changes in GAG sulfation which are involved in cell receptor function [16].

Thus wild blueberries incorporated in the diet at 8% for a period of 4 weeks do not alter aortic contractility but affect membrane sensitivity in response to the α_1-adrenergic agonist. A period longer than 4 weeks is needed to detect the beneficial dietary effect of wild blueberries on vascular function by improving vascular tone and the responsiveness of arteries to factors that increase vessel contractility.

Thus, the duration of exposure to the diet is crucial for wild blueberries and their bioactive components to exert their beneficial effects. Additionally, results from this research emphasize the need for temporal studies to detect the possible effects of functional foods in relation to disease prevention, critical for researchers working in the area of dietary interventions.

Acknowledgements

The authors would like to thank FutureCeuticals (Momence, IL.) for contributing the wild blueberry powder. This work was supported by the Cariplo Foundation (Italy) and the Wild Blueberry Association of North America (USA). This is a Maine Agriculture and Forestry Experiment Station scientific contribution (#3090).

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