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The effects of blueberry and strawberry serum metabolites on age-related oxidative and inflammatory signaling *in vitro*

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Abstract

Berry fruits contain a variety of bioactive polyphenolic compounds that exhibit potent antioxidant and anti-inflammatory activities. We have shown that consumption of freeze-dried whole berry powder, equivalent to 1 cup/day of blueberry (BB) or 2 cups/day of strawberry (SB), can differentially improve some aspects of cognition in healthy, older adults, compared to placebo-supplemented controls. We investigated whether fasting and postprandial serum from BB- or SB-supplemented older adults (60-75yo), taken at baseline or after 45 or 90 days of supplementation, would reduce the production of inflammatory and oxidative stress markers compared to serum from a placebo group, in LPS-stressed HAPI rat microglial cells, *in vitro*. Serum from both BB- and SB-supplemented participants reduced nitrite production, iNOS and COX-2 expression, and TNF-alpha release relative to serum from placebo controls ($p < 0.05$). Protection was greatest with serum from the 90-day time-point, suggesting that ongoing supplementation may provide the most health benefits. Serum was protective in both fasted and postprandial conditions, indicating that the effects are not only acute and that the meal did not challenge subjects' ability to regulate oxidative and inflammatory stress. These results suggest that berry metabolites, present in the circulating blood following ingestion, may be mediating the anti-inflammatory effects of dietary berry fruit.

Introduction

Increased susceptibility to effects of oxidative stress and inflammatory insults are thought to contribute to the decline in cognitive and motor performance observed in aging and neurodegenerative diseases[1-3]. Diet represents a modifiable lifestyle factor which can mitigate oxidative and inflammatory responses depending on its composition. Fruits contain an assortment of bioactive phytochemicals, and recent research has emphasized the potential health benefits of dietary berry fruit[4-6].

Blueberries (BB) and strawberries (SB) contain a variety of bioactive polyphenolic compounds, such as anthocyanins and flavonoids, which have strong antioxidant and anti-inflammatory activities[7]. Consumption of flavonoids in the form of whole foods can protect against cognitive decline observed during aging as well as other neurodegenerative conditions[8, 9].

Dietary interventions with BB have shown positive neurological outcomes in rodents and humans[10-13]. Aged rats consuming a BB-supplemented diet demonstrated enhanced motor performance and improved working memory compared to those consuming a control diet[11]. Additionally, consumption of freeze-dried whole BB powder, for 90 days, improved cognitive function, including executive function, in healthy older adults compared to placebo-supplemented controls[13]. In another study, consuming wild BB juice improved paired associate learning and word list recall in a sample of nine older adults with early memory changes[14]. Furthermore, cognitive improvements have also been observed in children consuming BB[15-16].

Dietary interventions with SB have also been associated with positive outcomes in rodents and humans[10-11, 17-19]. Aged rats consuming a SB-supplemented diet exhibited enhanced motor performance and improved cognition, specifically working memory, compared to those consuming a control diet[11]. Additionally, consumption of freeze-dried whole SB powder, for 90

days, improved learning and memory in healthy older adults compared to placebo-supplemented controls[19]. However, the mechanisms of action for berries' beneficial effects are not fully understood.

Cell models can provide tools for the assessment of the mechanisms behind the protective effects of various foods against oxidative stress and inflammation seen in aging[20]. The inflammatory response in the brain may be mediated by activated microglia leading to neuronal damage by cytotoxic molecules such as pro-inflammatory cytokines and other inflammatory enzymes[21]. Suppressing microglial activation and cytotoxicity may improve function in a diseased brain. In one study, BB extract inhibited the production of the inflammatory mediator nitric oxide (NO), and decreased the production of the cytokines interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), in lipopolysaccharide (LPS)-activated BV2 microglia[22].

However, it is important to note that the bioactive compounds in foods before consumption are different than those found in circulation following consumption; therefore, pre-treatment of cells with serum from humans or animals fed these foods may be a better model system than treating cells with extracts of the foods themselves. Furthermore, the consumption of berries may induce other factors in circulation that provide protection against oxidative stress and inflammation[23].

In this study, we investigated whether serum from BB- or SB-supplemented older adults would reduce the production of inflammatory stress signals, compared to serum from a placebo group, in LPS-stressed HAPI rat microglial cells, *in vitro*. Serum was collected at baseline (day 0) and at intervention days 45 and 90, in both fasting and postprandial conditions. Serum-exposed microglia were then examined for markers of inflammation including extracellular release of NO and TNF-α. NO is a free radical and secondary messenger involved in cellular

immune response and activation of apoptosis while TNF-α is a cytokine involved in inflammatory responses. Intracellular levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were also measured. Inducible nitric oxide synthase (iNOS) produces the inflammatory mediator nitric oxide (NO) while COX-2 is involved in the formation of prostanoids, which are inflammatory mediators.

Methods

Participants

Serum was collected from healthy, older men and women (60-75 years; BMI 18.5-29.9 kg/m^2) enrolled in one of two double-blind, 2-arm, controlled, 90-day feeding studies. Participants in the blueberry (BB) study group consumed 24g/day of lyophilized, cultivated blueberries (Tifblue variety; equivalent to 1 cup/day of blueberries; 12g powder in ~1 cup water taken with each morning and evening meal) (see Table S1 for phenolic composition of the BB powder). Participants in the strawberry (SB) study group consumed 24g/day of a lyophilized, standardized blend of cultivated strawberries (equivalent to 2 cups/day of strawberries; 12g powder in ~1 cup water taken each morning and evening meal) (see Table S2 for phenolic composition of the SB powder). Participants in the placebo groups consumed 24g of a seemingly identical, isocaloric placebo powder, matched to the respective berry group. Participants were instructed to abstain from consuming either berries or other berry products for the duration of the study but to otherwise maintain their usual diet. Serum was collected at baseline (day 0) and at intervention days 45 and 90, both fasting (overnight, pre-meal) and 2 hours postprandial (following a standard breakfast consisting of a corn muffin, butter, apple juice, a banana and coffee (~600 calories, 58g sugar, and 21g fat)). The day 0 breakfast did not include the berry supplement, but the day 45 and 90 standard breakfast contained either the berry or placebo drink (12g powder in ~1 cup water). Informed consent was obtained from all study participants, and these studies were approved by the Tufts University Institutional Review Board (clincaltrials.gov identifiers: NCT01888848 and NCT02051140).

Cell Culture

HAPI rat microglial cells (generously provided by Dr. Grace Sun, University of Missouri, Columbia, MO) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin, and 100ug/ml streptomycin at 37˚C in a humidified incubator under 5% CO_2. Cells were maintained in 100mm plates and then split into 12-well plates prior to treatment. Treatments were carried out in duplicate for each subject on the 12-well plates when the cells were approximately 75% confluent. For experiments, cells were incubated in serum-free DMEM and pre-treated with a concentration of 10% serum from individual subjects from each of the groups for 8 hours. Following pretreatment with the serum, the media was removed and the cells were washed once with serum-free DMEM without phenol red and were subsequently stimulated with lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO) at 100ng/ml overnight in DMEM without phenol red.

Nitrite Quantification

To assess the production of NO from LPS-treated HAPI cells, extracellular release of nitrite (NO_2^-) was measured by Greiss reagent (Invitrogen) according to manufacturer's instructions. Absorbance was read at 548nm and the concentration of nitrite was calculated with the linear equation derived from the standard curve generated by known concentrations of nitrite.

TNF-α ELISA

Quantification of tumor necrosis factor-alpha (TNF-α) in cell-conditioned media was performed with an enzyme-linked immunosorbent assay (ELISA, eBioscience, San Diego, CA)

according to manufacturer's instructions. TNF-α concentration for each sample was calculated from the linear equation derived from the standard curve of known concentrations of the cytokine.

Western Blots

Cells were washed in ice-cold PBS, resuspended and lysed by agitation in CelLytic-M Cell Lysis Reagent (Sigma), and centrifuged at 18,000 x g for 10 min at 4˚C to yield the resultant supernatant lysate. Western blots were performed as described previously by Poulose et al. (JAFC, 60, 1084-93, 2012), except that 10% polyacrylamide gels were used. Primary antibodies for iNOS (Millipore, Billerica, MA) and COX-2 (Santa Cruz, Dallas, TX) were used at 1:1000 dilutions for incubation overnight at 4˚C. Following ECL (enhanced chemiluminescence) development, the optical density of antibody-specific bands was analyzed by the VisionWorks LS image acquisition and analysis software (UVP, Upland, CA).

Statistical Analyses

All statistical analyses were performed using SYSTAT software (SPSS, Inc, Chicago, IL). Data are expressed as mean ± SEM. The data were analyzed by two-way analysis of variance (ANOVA) followed by post hoc testing with Fisher's LSD test to determine differences among groups. Results were considered statistically significant if the observed significance level was $p < 0.05$. Note that pretreatment with serum did not significantly affect cells in the absence of LPS in any of the endpoints assayed (data not shown).

Results

Nitric Oxide

Serum from BB- and SB-supplemented older adults attenuated LPS-induced NO production in HAPI microglial cells, at both fasting (PRE) and postprandial (POST) time points compared to placebo-supplemented individuals (Fig. 1). This attenuation was most evident after supplementation for 90 days in both diet groups at both PRE and POST time points compared to placebo-supplemented groups ($p<0.05$). Furthermore, serum from subjects consuming BB or SB for 90 days significantly reduced LPS-induced NO production compared to the serum collected at baseline (day 0) for both PRE and POST time points ($p<0.05$). Interestingly, serum from individuals consuming the SB placebo for 90 days significantly increased LPS-induced NO production at the PRE time point compared to baseline ($p<0.05$).

iNOS

Serum from BB- and SB-supplemented older adults was protective against LPS-induced iNOS expression in HAPI microglial cells, at both PRE and POST time points compared to placebo-supplemented individuals (Fig. 2). Lipopolysaccharide-induced iNOS expression was significantly reduced by serum from both diet groups, particularly at 90 days, both before and following the meal compared to placebo-supplemented groups ($p<0.05$). Additionally, serum from subjects consuming SB for 90 days significantly reduced LPS-induced iNOS expression compared to baseline (day 0) for both PRE and POST time points ($p<0.01$), and for 45 days for the POST time point ($p<0.05$). For BB, serum collected after 45 and 90 days significantly reduced iNOS expression only at the PRE time point compared to baseline ($p<0.01$).

TNF-α.

Serum from BB- and SB-supplemented older adults reduced the LPS-induced release of TNF-α in HAPI microglial cells, at both PRE and POST time points compared to the placebo-supplemented individuals; however, this effect was stronger in the BB-supplemented group (Fig. 3). LPS-induced TNF-*α* release was significantly reduced by serum from both diet groups at 90 days, before the meal for SB and before and after the meal for BB, compared to placebo-supplemented groups ($p<0.05$). Additionally, serum from subjects consuming BB for 45 and 90 days significantly reduced LPS-induced TNF-α release, compared to the serum collected at baseline, for both PRE and POST time points ($p<0.05$). Serum from the SB-supplemented group at PRE and POST time points also significantly reduced TNF-α release compared to serum collected at baseline ($p<0.05$); however, this effect was not observed at 45 days.

COX-2

Serum from BB- and SB-supplemented older adults attenuated LPS-induced expression of COX-2 in HAPI microglial cells, at both PRE and POST time points compared to placebo-supplemented individuals (Fig. 4). LPS-induced expression of COX-2 was significantly reduced by serum from the SB group at 90 days for both PRE and POST time points compared to the placebo-supplemented groups ($p<0.05$). However, serum from the BB group at 90 days significantly reduced expression of COX-2 only at the PRE time point ($p<0.01$). Furthermore, serum from subjects consuming BB and SB for 90 days significantly reduced LPS-induced expression of COX-2 compared to serum collected at baseline for only the PRE time point ($p<0.01$). Interestingly, serum from individuals consuming the strawberry placebo for 90 days significantly increased LPS-induced COX-2 expression production at the PRE and POST time

point compared to baseline ($p<0.05$).

Discussion

Previous work in our lab provided the first evidence for the anti-inflammatory potential of serum in a study using animals fed a walnut-supplemented diet[20]. Although walnut oil extract had earlier been shown to protect microglial cells from increases in inflammatory markers[24], the model developed by Fisher and colleagues[20] may provide a clearer picture as to the mechanisms behind the anti-inflammatory effects observed and the cognitive benefits seen *in vivo*. Serum circulating in the blood of animals contains different bioactive compounds than are found in the whole food prior to consumption. Microglia are not directly exposed to unmetabolized food extracts *in vivo* making it a less ideal system. A recent study using serum collected from rodents fed diets supplemented with BB also demonstrated anti-inflammatory potential *in vitro*[25]. In this study, BV-2 mouse microglial cells were treated with serum from mice fed either a high fat diet (HFD) or a HFD supplemented with BB. Serum from the mice fed the HFD significantly increased LPS-induced nitric oxide; however, serum from mice feed the HFD with BB produced less nitric oxide compared to serum collected from mice fed only the HFD.

The results of our present study showed that serum collected from older adults supplemented with freeze-dried BB powder, equivalent to 1 cup/day of fresh fruit, or SB powder, equivalent to 2 cups/day, reduced LPS-induced inflammatory signals in stressed HAPI microglia *in vitro*. Attenuation in inflammatory markers was observed after 45 days of supplementation; however, protection was greatest at the 90-day time point, suggesting that ongoing supplementation may provide the most health benefits. We also found that serum from BB- and SB-supplemented older adults showed protection in both fasted and postprandial conditions, suggesting that the high-fat meal did not challenge their ability to regulate oxidative and inflammatory stress and that the compounds in the berry fruit were still active in fasted state.

303 This result was not surprising based on findings from a recent study by Sandhu and colleagues
304 that quantified 3 anthocyanins/metabolites, 3 urolithin metabolites, and 15 phenolic acid
305 metabolites in the plasma of the same SB- and placebo-supplemented subjects used in the present
306 study[26]. They observed persistent concentrations of strawberry anthocyanins/metabolites,
307 urolithins, and phenolic acids in the fasting plasma on day 45 and 90. Additionally,
308 enhancements in anthocyanin/metabolite and phenolic acid concentrations were seen 2h
309 following the breakfast meal containing SB. Among the anthocyanins/metabolite, pelargonidin
310 glucuronide was present in the highest concentration at the 90-day postprandial time-point.
311 These results demonstrated that strawberry polyphenols are not only readily absorbed and
312 metabolized, but they can also persist in the circulation[26].
313 Sandhu and colleagues also examined the metabolic fate of BB polyphenols using
314 plasma collected from the same groups of subjects in which serum was obtained and used in the
315 present study[27]. Increased plasma concentrations of BB anthocyanins/glucuronide were observed
316 in the 2h postprandial BB samples. In addition, selective phenolic acids also increased after BB
317 consumption. Interestingly, chronic exposure of BB anthocyanins resulted in the accumulation of
318 hippuric acid compared to placebo. The results of this study showed that BB anthocyanins are
319 absorbed and metabolized producing different phenolic acid derivatives that may be contributing
320 to the anti-inflammatory effects observed in the present study.
321 Berries' beneficial effects on cognitive performance observed in animals and humans
322 may be due to a decrease in neuroinflammation. A recent study from our lab indicated that
323 cognitive performance was correlated with innate anti-inflammatory capability[28]. In this study,
324 aged rats were assessed for cognition in the radial arm water maze (RAWM) and then grouped
325 by performance (good, average, and poor performers). Rats were then fed either a control or 2%

BB diet for eight weeks and then retested. Latency in the RAWM was significantly reduced in the BB-fed poor performers and preserved in the BB-fed good performers. Serum was also collected from rats, pre-diet and post-diet, and used in an *in vitro* study with microglial cells. Pre-diet levels of LPS-induced nitrite and TNF-alpha were positively correlated with latency to the platform in the RAWM at baseline, with poor performers having the highest baseline levels of these markers. Post-diet, BB supplementation reduced LPS-induced nitrite and TNF-alpha in the poor performers.

In an additional study, aged rats were tested for balance, muscle strength, and coordination and then grouped into good, average, and poor performers based on an overall motor composite score[29]. Rats in each category were then fed a control diet or a raspberry-supplemented diet for 8 weeks and re-tested. Notably, rats with lower post-diet composite scores (indicating better motor performance) had higher levels of serum IL-1β. In addition, poor performers fed the raspberry-supplemented diet had a higher overall composite score, compared to the control-fed rats. The results from both these studies suggest that berry metabolites circulating in the blood may be responsible for the behavioral enhancements observed in animals, and these improvements may be due to a decrease in neuroinflammation.

Future research will explore the connections between cognitive function, serum levels of BB and SB metabolites, and inflammatory processes. It is likely that the anti-inflammatory effects observed *in vitro* are due to a synergy among the many bioactive compounds found in berries and their metabolites, rather than one single compound[30]. Exploring potential synergistic effects among these compounds will be crucial to determine the potential mechanisms behind the observed anti-inflammatory effects.

Conflicts of interest

There are no conflicts of interest to declare

Acknowledgements

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Figures

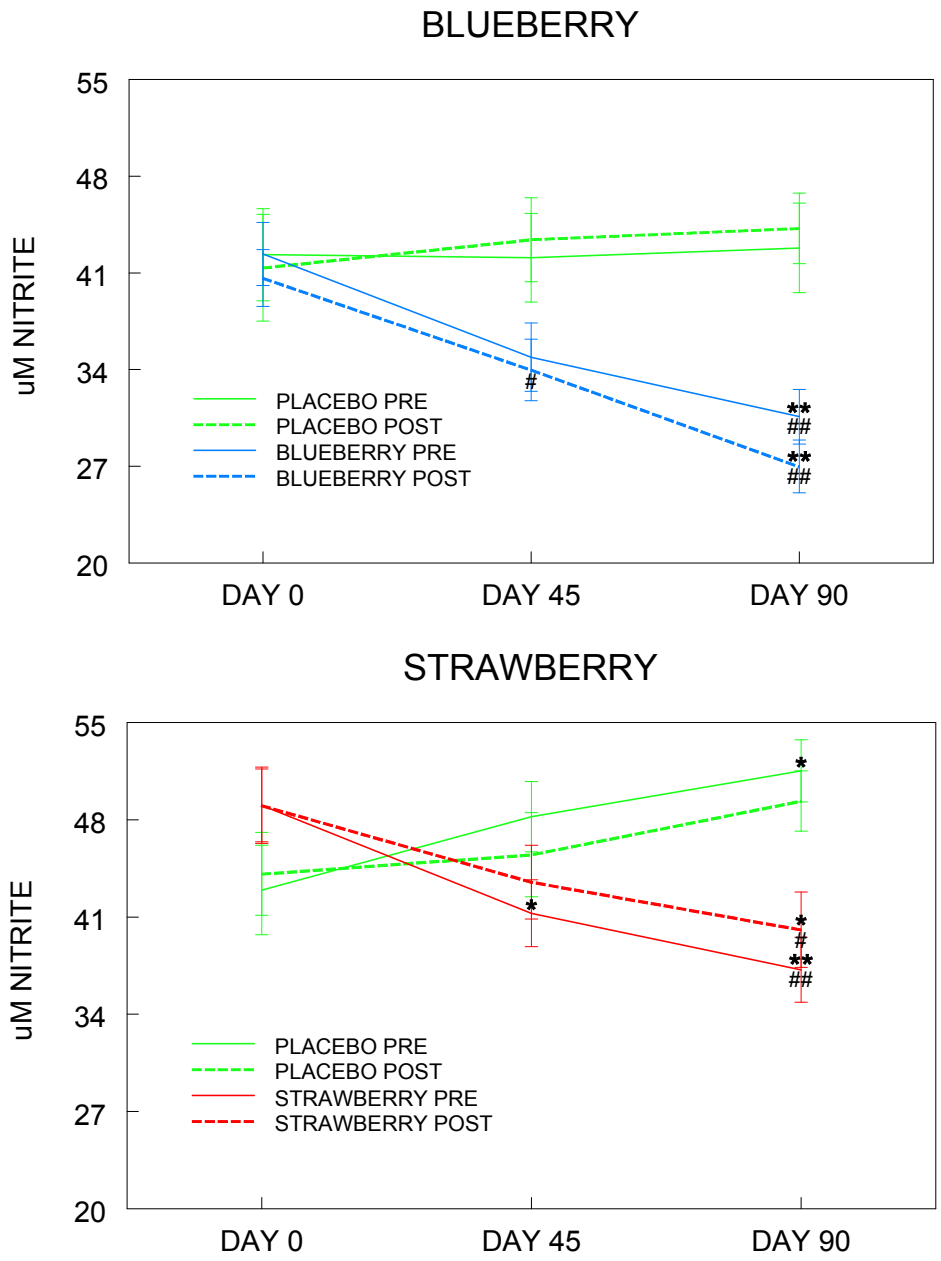

Figure 1. Serum from BB- and SB-supplemented older adults significantly attenuated LPS-induced NO production in HAPI microglial cells, at both fasting (PRE) and postprandial (POST) time points, compared to placebo-supplemented individuals. Data are represented as mean ± SEM. Asterisk (*) indicates significant difference from baseline (* $p < 0.05$, ** $p < 0.01$); pound (#) indicates significant difference between diet groups at the same time point (# $p < 0.05$, ## $p < 0.01$).

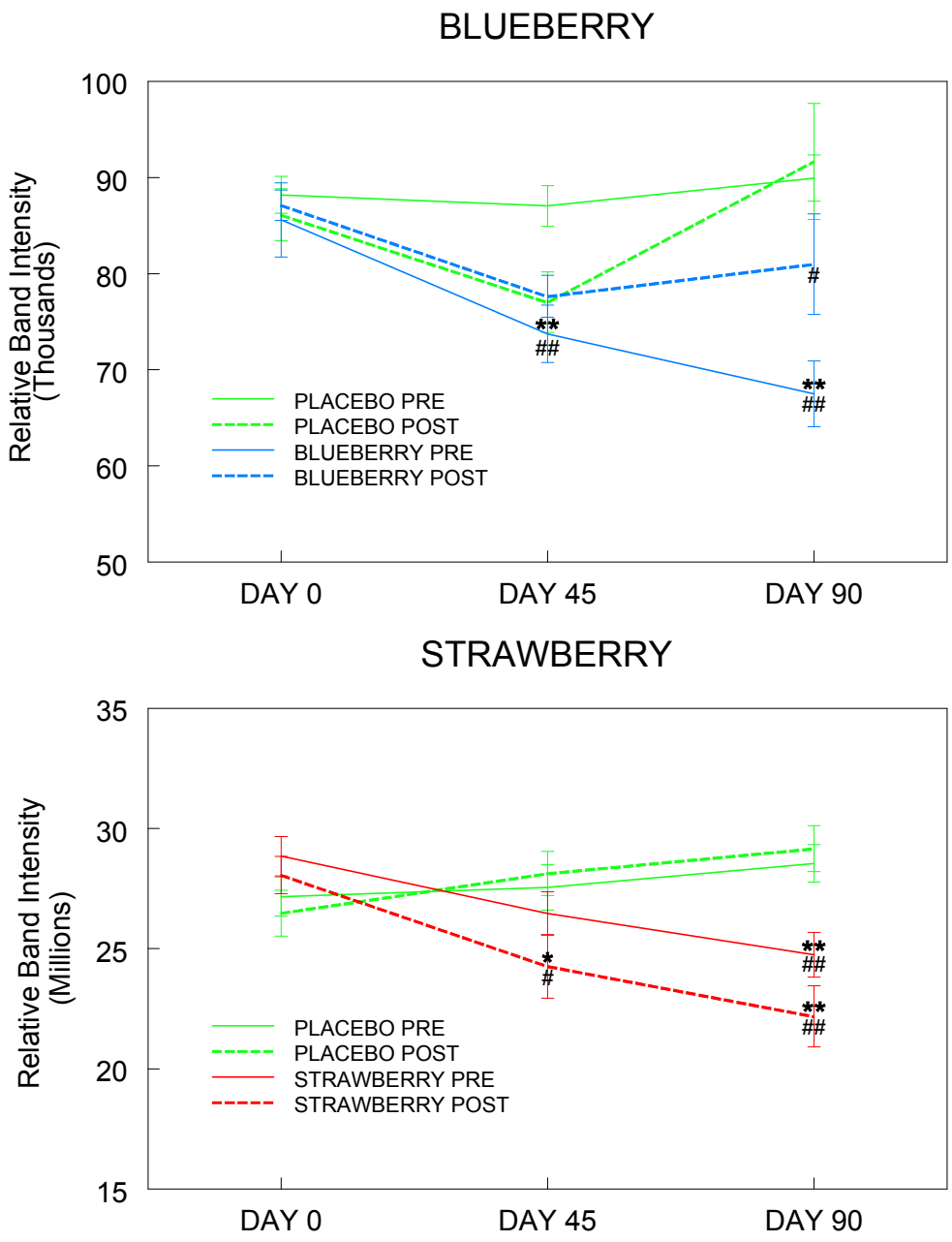

Figure 2. Serum from BB- and SB-supplemented older adults significantly reduced LPS-induced expression of iNOS in HAPI microglial cells, at both fasting (PRE) and postprandial (POST) time points, compared to placebo-supplemented individuals. Data are represented as mean ± SEM. Asterisk (*) indicates significant difference from baseline (* $p < 0.05$, ** $p < 0.01$); pound (#) indicates significant difference between diet groups at the same time point (# $p < 0.05$, ## $p < 0.01$).

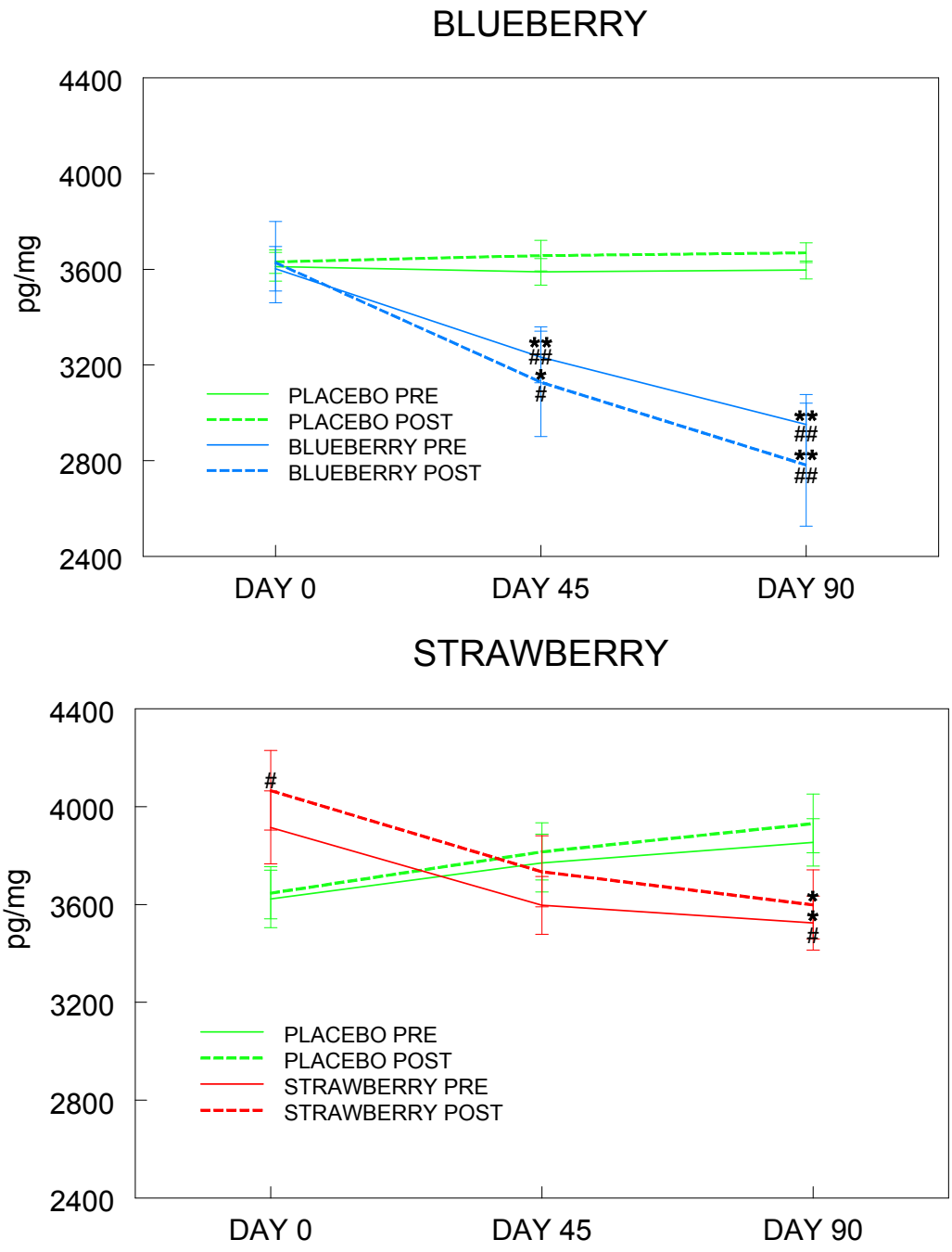

Figure 3. Serum from BB- and SB-supplemented older adults significantly reduced the LPS-induced release of the inflammatory cytokine TNF-α in HAPI microglial cells, at both fasting (PRE) and postprandial (POST) time points, compared to placebo-supplemented individuals.. Data are represented as mean ± SEM. Asterisk (*) indicates significant difference from baseline (* $p < 0.05$, ** $p < 0.01$); pound (#) indicates significant difference between diet groups at the same time point (# $p < 0.05$, ## $p < 0.01$).

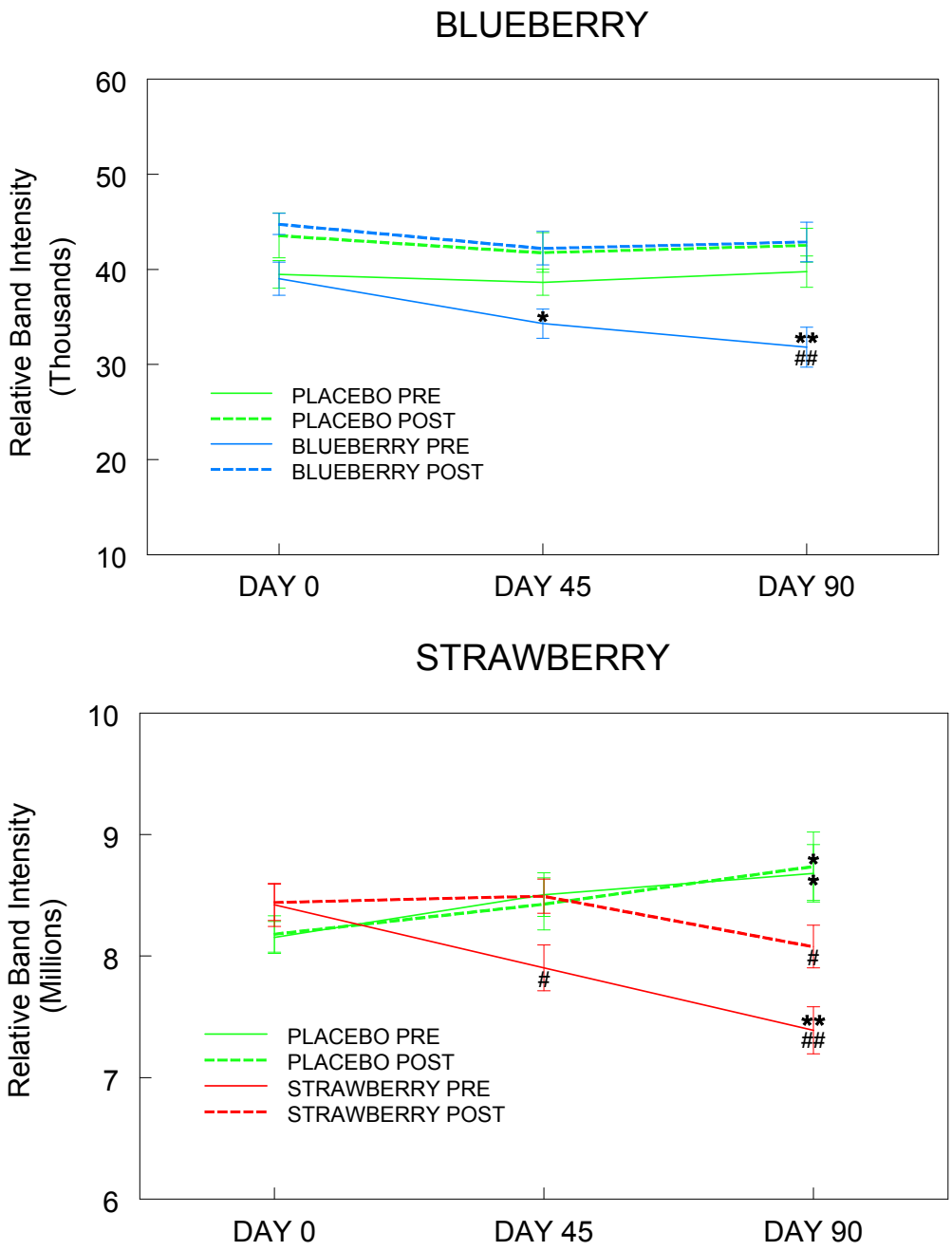

Figure 4. Serum from BB- and SB-supplemented older adults significantly reduced LPS-induced expression of COX-2 in HAPI microglial cells, at both fasting (PRE) and postprandial (POST) time points, compared to placebo-supplemented individuals. Data are represented as mean ± SEM. Asterisk (*) indicates significant difference from baseline (* $p < 0.05$, ** $p < 0.01$); pound (#) indicates significant difference between diet groups at the same time point (# $p < 0.05$, ## $p < 0.01$)

Electronic Supplementary Information

Table S1. Phenolic composition of the blueberry powder used in the blueberry supplementation study.

Phenolic compounds	Blueberry powder (mg/100 g of dry powder)
3-Chlorogenic acid	137.0
cyanidin-3-arabinoside	28.2
Cyanidin-3-galactoside	65.1
Cyanidin-3-glucoside	60.9
cyanidin-3-xyloside	1.5
Delphinidin-3-arabinoside	39.7
Delphinidin-3-galactoside	31.4
Delphinidin-3-glucoside	26.3
Delphinidin-3-xyloside	1.8
Malvidin-3-arabinoside	59.5
Malvidin-3-galactoside	71.4
Malvidin-3-glucoside	44.0
Malvidin-3-xyloside	2.3
Peonidin-3-arabinoside	6.5
Peonidin-3-galactoside	58.1
Peonidin-3-glucoside	14.9
Peonidin-3-xyloside	1.1
Petunidin-3-arabinoside	26.2
Petunidin-3-galactoside	43.2
Petunidin-3-glucoside	30.3
Petunidin-3-xyloside	1.9

Phenolic compounds analyzed by Amandeep Sandhu at Institute for Food, Safety, and Health at Illinois Institute of Technology, Bedford Park, IL using liquid chromatography–mass spectrometry[31,32]

Table S2. Phenolic composition of the strawberry powder used in the strawberry supplementation study.

Phenolic compounds[31,32]	Strawberry powder (mg/100 g of dry powder)
Gallic acid	0.40
3,4-dihydrobenzoic acid	0.16
Procyanidin B1	30.62
(+)-catechin	25.04
Cyanidin-3-glucoside	11.63
Syringic acid	0.02
Pelargonidin-3-glucoside	396.90
p-coumaric acid	0.26
2-hydroxycinnamic acid	0.20
Rutin (quercetin-rutinoside)	3.35
Ellagic acid	12.59
Isoquercetin (quercetin-glucoside)	6.62
Sinapic acid	0.40
Tiliroside (kaempferol-3-glucoside-6”-p-coumaroyl)	0.74
Quercetin	1.46
Kaempferol	0.36

Phenolic compounds analyzed by Jack Cappozzo at Institute for Food, Safety, and Health at Illinois Institute of Technology, Bedford Park, IL using liquid chromatography–mass spectrometry[31,32]