DOI:10.1021/jf9035468

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Atheroprotective Effects of Bilberry Extracts in Apo E-Deficient Mice

Aurelie Mauray,[†] Dragan Milenkovic,[†] Catherine Besson,[†] Nelly Caccia,[†] Christine Morand,[†] Francoise Michel,[‡] Andrzej Mazur,[†] Augustin Scalbert,[†] and Catherine Felgines*[,§]

[†]INRA, Unité de Nutrition Humaine, UMR1019, Centre de Recherche de Clermont Ferrand/Theix, Saint Genès Champanelle, F-63122 France, [‡]Hôpital Lapeyronie, Laboratoire de Biochimie, Montpellier, F-34295 France, and [§]Université Clermont 1, UFR Pharmacie, Laboratoire de Pharmacognosie et Phytothérapie, Clermont-Ferrand, F-63001 France

Previous studies have demonstrated that the intake of berry foods was associated with a reduced risk of cardiovascular diseases. The aim of the present study was to evaluate the effects of two bilberry extracts, one rich in anthocyanins extracted from untreated bilberries (BE) and a second one extracted from yeast-fermented bilberries (FBE), on the development of atherosclerosis in apolipoprotein E-deficient mice (apo $E^{-/-}$). Apo $E^{-/-}$ mice received for 16 weeks a diet supplemented with 0.02% of either BE or FBE. Atherosclerotic plaque area was measured in the aortic sinus. Supplementation of the diet with both bilberry extracts led to a significant inhibition of plaque development, whereas no effect on oxidative stress parameters or lipid profiles could be observed, suggesting the implication of other mechanisms of action. In addition, a better protection was observed with FBE, suggesting that the fermentation generates new bioactive compounds more effective in attenuating progression of the atherosclerotic lesions.

KEYWORDS: Bilberry (*Vaccinium myrtillus*); anthocyanins; dietary supplementation; atherosclerosis; fermentation

INTRODUCTION

Atherosclerosis, a multifactorial disease resulting from genetic and environmental factors, is the first cause of cardiovascular disease (CVD) in industrialized countries. Mortality data from the American Heart Association statistics 2009 update show that CVD accounted for 1 of every 2.8 deaths in the United States (*1*). Development of nutritional strategies of prevention appears essential to reduce the risk of cardiovascular disease. Inverse association between consumption of fruits and vegetables and risk of cardiovascular disease was deduced from numerous epidemiological studies (*2*). Polyphenols, natural constituents of fruits and vegetables, have received considerable attention these past years because the consumption of polyphenol-rich foods or beverages has been associated with a significant reduction of mortality from coronary heart disease (CHD) (*3*).

Beneficial effects of berries have been reported in relation to CVD. Consumption of blueberries was associated with a reduced risk of CHD in a cohort of postmenopausal women from the Iowa Women's Health Study (*4*). In addition, favorable changes in platelet function, HDL cholesterol, and blood pressure were observed after intake of moderate amounts of various berries in middle-aged unmedicated subjects with cardiovascular risk factors (*5*). Most of the protective effects of berries are ascribed to their high content of phytochemicals. In particular, edible berries are rich sources of phenolic compounds, mainly anthocyanins but also hydroxycinnamic acids, flavonols, flavan-3-ols, and proanthocyanidins (or so-called condensed tannins) (*6*). Bilberry (*Vaccinium myrtillus* L.) is one of the richest dietary sources of anthocyanins, with an anthocyanin glycoside content of about 300−600 mg/100 g of fresh weight (*7*,*8*). A recent review reported a variety of therapeutic activities of berry anthocyanins, notably from bilberry, such as antioxidant potential, angiogenesis inhibition, anticancer properties, diabetes prevention, vision improvement, and finally neuro- and cardioprotective effects (*9*). Moreover, treatment with bilberry anthocyanoside oligomers was shown to induce better visual acuity and contrast sensitivity in myopia subjects (*10*). However, many investigations evaluating the health-promoting effects of polyphenol-rich bilberry extracts were conducted in vitro, and animal studies or clinical trials are still limited (*11*).

The aim of this study was thus to investigate the effects of dietary bilberry extracts on the development of atherosclerosis in apolipoprotein E-deficient mice (apo $E^{-/-}$), a widely used model of atherosclerosis (*12*). This model has been intensely used to demonstrate protective effects of various polyphenolic supplementations on the development of atherosclerotic lesions (*13*), but up to now, there were no data on the potential protective action of bilberry products. The aim of the present study was thus to evaluate the potential protective action of two bilberry extracts, one rich in anthocyanins extracted from untreated bilberries (BE) and a second one extracted from yeast-fermented bilberries

*Corresponding author (telephone +33-4-73-17-80-31; fax +33-4-73-28-28-49; e-mail catherine.felgines@u-clermont1.fr).

 Published on Web 11/06/2009

(FBE), on atherosclerosis in this model together with their effects on some oxidative stress markers and lipid parameters.

MATERIALS AND METHODS

Chemicals. Two commercially available extracts prepared from fresh bilberry (*V. myrtillus* L.), Antho 50 and Catocyanic Complex, were supplied by Ferlux Mediolanum SA (Cournon d'Auvergne, France). Antho 50 (BE) is a purified bilberry anthocyanin extract resulting from an alcoholic extraction followed by concentration and final chromatographic purification onto an adsorbent macro-cross-linked resin (*14*). Catocyanic Complex (FBE) is a polyphenol-rich fraction obtained from bilberry fermentation with wine yeast (*Saccharomyces cerevisiae*) and purified by precipitation with hot alcohol (*15*, *16*). Cyanidin 3-glucoside (C3G), gallic acid, and protocatechuic acid were purchased from Extrasynthèse (Genay, France).

Chemical Composition of Bilberry Extracts. The total polyphenol content of both extracts was determined according to the Folin−Ciocalteu assay using gallic acid as a standard. Both extracts (250 mg) were dissolved in 5 mL of acetone/water (70:30) containing 1.2 M aqueous HCl. These latter solutions were then diluted in water (1000- and 200-fold for, respectively, BE and FBE solutions), and a mixture of sample solutions or standard (150 μL) with Folin−Ciocalteu's reagent (750 μL) and Na_2CO_3 solution (75 g L^{-1}; 600 μL) was incubated 5 min at 50 °C. After the mixture had been cooled for 5 min in ice, the absorbance was measured at 760 nm in an ultramicroplate reader EL_X 808 (Bio-Tek Instruments, Winooski, VT). Total polyphenols were expressed as grams of gallic acid equivalents per 100 g of extract.

Total red pigments were quantified by colorimetry. The same extract solutions as described above were used and then diluted in 1 M aqueous HCl (2000- and 100-fold for, respectively, BE and FBE solutions). Wavelengths as the absorption maxima were 515, 505, and 511 nm for, respectively, BE and FBE solutions and C3G (used as a standard). Total red pigments were expressed as grams of C3G equivalents per 100 g of extract.

Quantification of individual anthocyanin glycosides in bilberry extracts was also carried out by high-performance liquid chromatography (HPLC) using a photodiode array detector (DAD200, Perkin-Elmer) as previously described (*17*). Anthocyanin content was expressed as grams of C3G equivalents per 100 g of extract. Protocatechuic acid was determined in BE and FBE extract by HPLC, detected at 280 nm and quantified using the corresponding standard.

Animals and Diets. Pairs of homozygous apo E-deficient mice were purchased from Jackson Laboratories (Charles River Laboratories, L'Arbresle, France) and interbred to obtain the males used for the present study. Seven-week-old mice (n = 45), weighing ~ 20 g, were individually housed in wire-bottom cages in a temperature-controlled room (22 ± 0.8 °C) with a 12 h light−dark cycle and a relative humidity of 55 ± 10% and had free access to food and water. All animals were maintained and handled according to the recommendations of the Institutional Ethics Committee of the INRA, in accordance with decree 87-848. They were all fed a standard breeding diet A03 (Safe, Epinay-sur-Orge, France) for 1 week before the beginning of the experiment. After this 1 week adaptation period, the mice were randomly divided into three groups (n = 15 per group) and fed ad libitum for 16 weeks either a semipurified control diet (UPAE, INRA Jouy-en-Josas, France) or the same control diet supplemented with 0.02% (w/w) of bilberry extracts: either anthocyanin-rich bilberry extract (BE, 0.2 g kg^{-1} of diet) or fermented bilberry extract (FBE, 0.2 g kg^{-1} of diet). These experimental diets were isoenergetic, and their detailed compositions are given in **Table 1**. At the end of the experimental period, mice were anesthetised in the morning using sodium pentobarbital (40 mg kg^{-1} of body weight). Blood was collected from the abdominal aorta into heparinized tubes. Plasma was prepared by centrifugation at 12000*g* for 2 min, and samples were stored at −20 °C. The organs were washed with physiological saline solution maintained at 37 °C by direct injection in the heart left ventricle. The heart and liver were freeze clamped and stored at − 80 °C.

Quantitative Assessment of Atherosclerotic Lesions. Atherosclerotic lesion size was calculated by measuring lipid deposition in the aortic sinus (*18*). The peripheral fat of the upper aorta was removed, and the thoracic and abdominal aorta was discarded. The heart with the aortic arch was dissected under a stereomicroscope and frozen in liquid N_2 in a cutting embedding medium for serial cryosectioning covering 400 μm of the aorta root. The heart was cut in a cryotome (Cryo-Star HM 560, Microm GmbH, Germany) at −20 °C. Five sections of 10 μm in thickness were harvested per slide, and sections at 200, 300, 400, 500, 600, 700, and 800 μm distance from the cusps were evaluated for fatty streak lesions after staining with Oil red O and counterstaining with hematoxylin. For each randomized heart, one section per slide was evaluated for Oil red O staining area by capturing images directly from a color camera (Sony XC-71P CCD RGB, Kenmore, WA) attached to an Olympus light microscope (Reichert-Jung Polyvar, Vienna, Austria). Images were displayed on an RGB monitor using AxioVision release 4.6.3 software (Zeiss, France). Image analysis was carried out using the ImageJ free software (http://rsb.info.nih.gov/ij/). To reduce errors induced by sectioning angle, results were expressed as the percentage of the cross-sectional vessel area stained with Oil red O.

Table 1. Composition of the Experimental Diets[a]

component	control diet (g kg^{-1} of diet)	supplemented diet (g kg^{-1} of diet)
wheat starch	629	629
casein	200	200
corn oil	70	70
Alphacell	50	50
mineral mix AIN-93G	35	35
vitamin mix AIN-93G	10	10
L-cysteine	3	3
choline bitartrate	2.50	2.50
tert-butyl hydroquinone	0.14	0.14
bilberry extract[b]	0	0.2

[a] Apo $E^{-/-}$ mice received each diet in a solid shape (pellets). [b] Anthocyanin-rich bilberry extract (BE) or fermented bilberry extract (FBE).

Determination of Cholesterol and Triglyceride Levels in Plasma and Liver. Plasma total cholesterol and triacylglycerol (TAG) concentrations (mM) were enzymatically measured using the Cholesterol RTU and TG PAP 150 kits from BioMerieux (Marcy-l'Etoile, France) (*19*). Liver samples were homogenized in NaCl (9 g L^{-1}) with a Polytron homogenizer PT-MR2100 (Kinermatica AG, Littau/Luzern, Switzerland), and lipids were extracted by chloroform−methanol (2:1, v/v) under overnight agitation. The chloroform phase was recovered after centrifugation and evaporated under dry air. TAG from the lipid residue were saponified with 0.5 M KOH−ethanol at 70 °C for 30 min followed by the addition of 0.15 M $MgSO_4$ to neutralize the mixture. After centrifugation (2000*g*, 5 min), supernatants were collected. Cholesterol in the lipid residue was dissolved with isopropanol. Total cholesterol and TAG levels (mg g^{-1}) were determined by enzymatic assays as previously described above. Absorbance at 492 nm was measured in an ultramicroplate reader EL_X 808 (Bio-Tek Instruments).

Determination of Plasma Antioxidant Capacity. The plasma antioxidant capacity was determined using the oxygen radical absorption capacity (ORAC) assay (*20*), which measures the ability of antioxidant compounds in a sample to scavenge peroxyl radicals generated from 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37 °C using fluorescein. The assay was carried out in black-walled 96-well plates, and plasma samples were diluted 600-fold in phosphate buffer (75 mM, pH 7.4). Trolox, a water-soluble analogue of vitamin E, was used as a control standard. Twenty-five microliters of sample, standard, or blank (phosphate buffer) was mixed with 25 μL of AAPH at room temperature. The plate was placed in an ultramicroplate reader EL_X 808 (Bio-Tek Instruments) maintained at 37 °C, and fluorescein (150 μL) was then immediately and automatically added. Fluorescence was measured for 1 h using an excitation λ = 485 ± 20 nm and an emission λ = 530 ± 20 nm. The final results were calculated using the difference of areas under the fluorescein decay curve between the blank and each sample and were expressed as micromoles of Trolox equivalents (TE) per liter (μmol of TE L^{-1}).

Determination of Lipid Peroxidation in the Liver. The susceptibility of liver to peroxidation was determined on 1 g of wet tissue homogenized on ice in 150 mM KCl (9 mL) using a Polytron homogenizer. Susceptibility to oxidation was measured by induction of lipid peroxidation

Table 2. Composition of Bilberry Extract (BE) and Fermented Bilberry Extract (FBE)

	bilberry extracts	
	BE	FBE
total polyphenols (g of GAE[a]/100 g of extract)	62	12
total red pigments (g of C3GE[b]/100 g of extract)	61	4.1
anthocyanins (g of C3GE[b]/100 g of extract)	52	0.2

[a] Gallic acid equivalents. [b] Cyanidin 3-glucoside equivalents.

with $FeSO_4$ (0.2 mM) and ascorbate (5 mM) for 30 min at 37 °C, using 1,1,3,3-tetraethoxypropane as a standard. Thiobarbituric acid-reactive substances (TBARS) were measured at 532 nm, using an ultramicroplate reader EL_X 808 (Bio-Tek Instruments) (*21*).

Determination of F_2-Isoprostane Levels in the Aorta. Frozen tissues were treated with 5000 pg of 4(*RS*)-F4t-NeuroPas an IS, diluted with 9 mL of acidified water (pH 3), and subjected to two extractions using successively a Sep-Pak Vac RC C18 and Vac RC NH2 cartridges (500 mg). The C18 cartridge was preconditioned with 5 mL of methanol and 5 mL of acidified water (pH 3). After washes with 10 mL of acidified water (pH 3) and 10 mL of acetonitrile/water (15:85; v/v), elution of the compounds was performed with 4 mL of hexane/ethyl acetate/propan-2-ol (30:65:5; v/v). The eluate was then applied to the NH_2 cartridge, which was preconditioned with 5 mL of hexane. The cartridge was washed successively with 5 mL of hexane/ethyl acetate (30:70; v/v) and 5 mL of acetonitrile. The isoprostanes were then eluted with 5 mL of ethyl acetate/methanol/glacial acetic acid (10:85:5; v/v). After purification, the solvents were evaporated under nitrogen, samples were dried under nitrogen and then derivatized and subjected to gas chromatography−mass spectrometry analysis as previously described (*22*).

Statistical Calculations. All values are given as means±SEM. A two-way ANOVA (mice diets and aortic sections) coupled with the Student−Newman−Keuls multiple-comparison test was used to analyze the lesion areas. Other data were analyzed by one-way ANOVA coupled with the Student−Newman−Keuls multiple-comparison test to compare the effect of supplemented diets with bilberry extracts versus the control diet. Values of $P < 0.05$ were considered to be significant.

RESULTS

Characterization of Bilberry Extracts. Anthocyanins were analyzed by HPLC. The BE extract contains 52% anthocyanins, whereas almost no native anthocyanins were detected in FBE (**Table 2**; **Figure 1**). Fifteen different native anthocyanins, derived from 5 aglycones (cyanidin, delphinidin, malvidin, peonidin, and petunidin), were identified in BE (**Figure 1**). FBE showed a large peak highly retained on the column with a wavelength absorption maximum similar to those found with native anthocyanins (508 nm), suggesting that anthocyanins in FBE are largely condensed into complex polymers. Protocatechuic acid, a known product of anthocyanin degradation (*23*), was also detected in trace amounts (0.05% w/w) in FBE.

Phenolic pigments in FBE appear to be different from native anthocyanins and absorb at a slightly shifted wavelength (505 nm) as compared to the nonfermented BE (515 nm). The contents of total polyphenols, as estimated by the Folin assay, and red pigments, estimated by colorimetry, also appear to be lower in FBE than in the nonfermented BE.

Body Weight. After 16 weeks of supplementation, no differences in body weight gain and final body weight were observed between bilberry extract fed mice and control ones (data not shown).

Influence of Bilberry Extracts on Atherosclerotic Plaque Formation. Atherosclerotic lesions were visible in the aortic sinus of 24-week-old apo E-deficient mice fed the control diet or the bilberry extract-supplemented diets. Mice fed the diets supplemented with the bilberry extracts showed a significant reduction of lipid deposits. The lesion area was decreased by 15% ($p < 0.05$) in the BE group and by 36% ($p < 0.0001$) in the FBE group compared to the control group. Additionally, mice fed the FBE-supplemented diet showed a 25% higher reduction of the lesion area compared to the BE group ($p < 0.01$) (**Figure 2**).

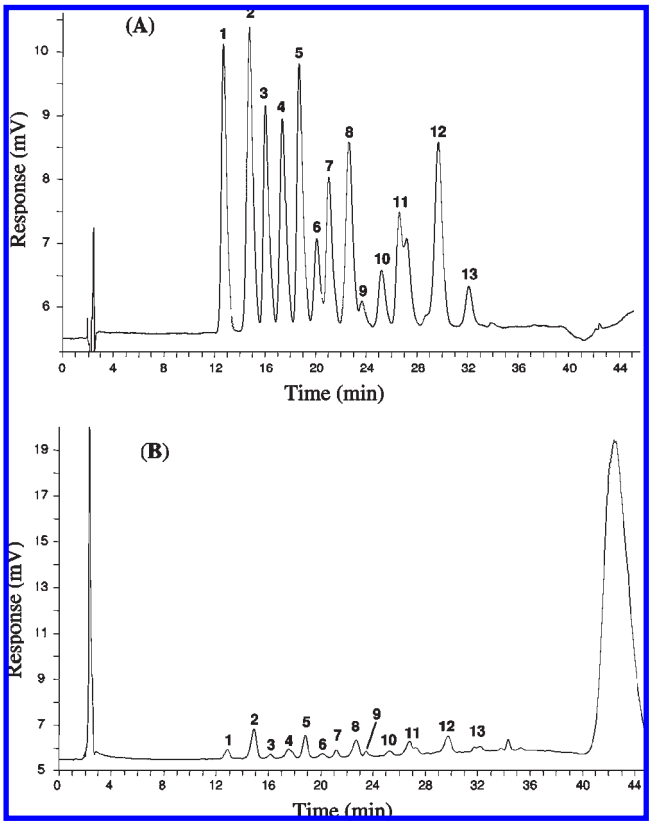

Figure 1. HPLC chromatogram of the bilberry (**A**) and fermented bilberry (**B**) extracts. The following quantities of extracts were injected: bilberry extract, 1.6 μg; fermented bilberry extract, 100 μg. Detection was at 524 nm. Peaks: 1, Dp 3-gal; 2, Dp 3-glc; 3, Cy 3-gal; 4, Dp 3-ara; 5, Cy 3-glc; 6, Pt 3-gal; 7, Cy 3-ara; 8, Pt 3-glc; 9, Pn 3-gal; 10, Pt 3-ara; 11, Pn 3-glc + Mv 3-gal; 12, Pn 3-ara + Mv 3-glc; 13, Mv 3-ara. Cy, cyanidin; Dp, delphinidin; Mv, malvidin; Pn, peonidin; Pt, petunidin; ara, arabinoside; gal, galactoside; glu, glucoside.

Influence of Bilberry Extracts on Oxidative Stress Parameters. The intake of bilberry extracts during 16 weeks did not affect the plasma antioxidant capacity. The concentration of F_2-isoprostanes in the aorta remained unchanged over the 16-week period for all dietary groups, and there was no significant difference between groups. TBARS concentrations in the liver were not more influenced by BE and FBE supplementation (**Table 3**).

Influence of Bilberry Extracts on Lipid Profile. As expected, all animals were hypercholesterolemic at the end of the experimental period, with a high plasma level of total cholesterol compared to the wild C57Bl/6J mice (1.8 mmol L^{-1}, in Mouse Phenome Database; Jackson Laboratory, Bar Harbor, ME; http://phenome.jax.org/pub-cgi/phenome/mpdcgi?rtn=strains/details&strainid=7). However, no significant differences in TAG and total cholesterol levels in either plasma or liver were observed between dietary groups (**Table 4**).

DISCUSSION

This work shows antiatherogenic effects of two bilberry extracts in apo $E^{-/-}$ mice after 16 weeks of dietary supplementation (0.02% in the diet). Bilberry extract (BE) is a purified anthocyanin-rich extract; thus, the supplementation of the diet with 0.02% of this extract (i.e., around 0.01% of anthocyanin

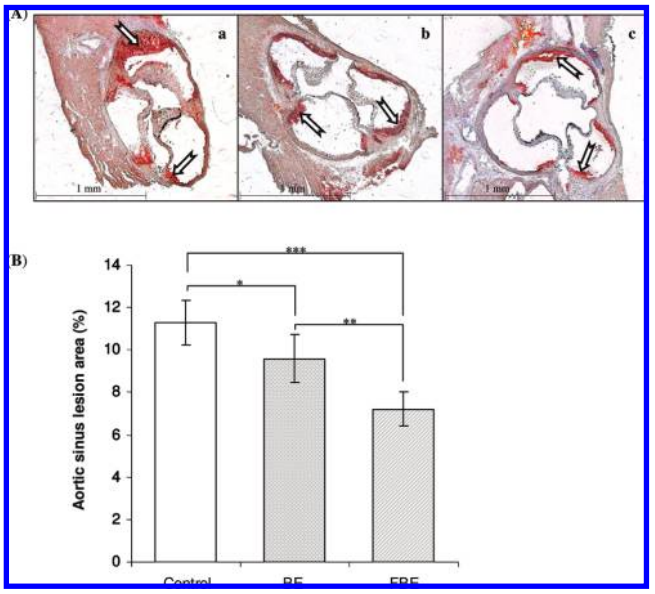

Figure 2. Effect of a dietary supplementation of an anthocyanin-rich bilberry extract (BE) or a fermented bilberry extract (FBE) on the development of atherosclerotic lesions in apo E-deficient mice: (**A**) histological sections of aortic sinus stained by Oil red O [(a) control, (b) BE, (c) FBE; arrows indicate fat deposition in aortic sinus]; (**B**) mean atherosclerotic plaque area (values are means ± SEM, $n = 15$; ∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.0001$).

Table 3. Oxidative Stress Parameters in Apo $E^{-/-}$ Mice Fed a Diet Supplemented with an Anthocyanin-Rich Bilberry Extract (BE) or a Fermented Bilberry Extract (FBE) for 16 Weeks[a]

	group		
	control	BE	FBE
plasma ($n = 15$)			
ORAC (μmol of TE L^{-1})[b]	7024 ± 339	7262 ± 266	6864 ± 396
aorta ($n = 10$)			
F_2-isoprostanes (ng g^{-1})	8.9 ± 1.9	8.2 ± 2.0	8.8 ± 2.1
liver ($n = 15$)			
TBARS (nmol g^{-1})	147 ± 10	133 ± 9	123 ± 8

[a] Values are means ± SEM. [b] Data are expressed as micromoles of Trolox equivalents (TE) per liter.

Table 4. Plasma and Hepatic Lipid Concentrations in Apo $E^{-/-}$ Mice Fed a Diet Supplemented with an Anthocyanin-Rich Bilberry Extract (BE) or a Fermented Bilberry Extract (FBE) for 16 Weeks[a]

	group		
	control	BE	FBE
plasma			
triacylglycerol (mM)	0.81 ± 0.12	0.92 ± 0.09	0.76 ± 0.09
total cholesterol (mM)	5.78 ± 0.47	6.96 ± 0.44	6.22 ± 0.70
liver			
triacylglycerol (mg g^{-1})	24.3 ± 1.5	33.0 ± 3.5	31.6 ± 3.6
total cholesterol (mg g^{-1})	2.68 ± 0.15	2.77 ± 0.22	2.77 ± 0.17

[a] Values are means ± SEM; $n = 15$.

glycosides) may correspond to an equivalent human intake of about 30 mg of anthocyanidins per day, when expressed on the basis of diet content (for a human food intake estimate of 500 g of dry weight). Large differences in average anthocyanidin intake have been reported according to the population considered: 2.9 mg day^{-1} in Australia (*24*), 7.6 mg day^{-1} in Belgium (*25*), or 12.5 mg day^{-1} in the United States (*26*). The supplementation level applied here is close to the highest intake level observed in Finland, estimated at 47 mg day^{-1} (*27*).

The FBE extract obtained after bilberry fermentation contains complex pigments absorbing at a wavelength close to those of native anthocyanins. The reduced ratio of total red pigments over total polyphenols (**Table 2**), the lack of native anthocyanins in the HPLC chromatograms (**Figure 1**), and the presence of protocatechuic acid in the FBE extract indicate a degradation of the bilberry anthocyanins. Native anthocyanins appear to be largely rearranged into complex polymers as suggested by the large peak of pigments strongly retained on the HPLC column (**Figure 1B**). A similar extensive degradation of anthocyanins during fermentation has been reported for other fruits such as grape (*28*) and blackberry (*29*). Thus, the polyphenols contained in the FBE extract can be compared to those formed during the fermentation of berry wines. For the production of berry wines, pressed juice is made from the berries and then fermented similarly to red grape juice in the winemaking process (*30*). During fermentation, anthocyanins contained in bilberry undergo condensation with other phenolic compounds present in the fruit (*16*) as has been described in red wine (*31*) and in black olives (*32*). In red wine, the anthocyanin contents decrease during alcoholic fermentation due to adsorption to yeast cell walls and the formation of more complex pigments such as pyranoanthocyanins (vitisins) and ethyl-linked anthocyanin−flavanol (particularly catechins and procyanidins) polymers, both formed by the reaction of anthocyanins with the yeast- derived pyruvic acid and acetaldehyde (*33*, *34*). Anthocyanin−4-vinylphenol adducts have also been identified in red wine. They are formed during fermentation by condensation of the anthocyanins with 4-vinylphenols, themselves resulting from the yeast-catalyzed decarboxylation of hydroxycinnamic acids (*p*-coumaric acid) or by direct reaction between anthocyanins and hydroxycinnamic acids (*35*). Another source of pyranoanthocyanins, from fermented cherry juice, has also been recently reported (*36*). FBE could contain anthocyanin-derived polymeric pigments close to that found in red wine during fermentation.

After a 16-week supplementation, a significant reduction of atherosclerotic plaques was observed in both BE and FBE groups as compared to the control one (−15 and −36%, respectively). Similar effects on the same animal model were recently observed with an anthocyanin extract from purple sweet potato (*37*). Our results also show that the in vivo atheroprotective effect of bilberry extracts varies with the type of bilberry extract. The fermented bilberry extract more effectively attenuates atherosclerotic plaque progression than the nonfermented one. Some biological activities of fermented products have been compared to nonfermented ones in in vitro studies. The fermented juices from blueberry (*38*) or bokbunja (*39*) showed better antioxidant activity than their respective unfermented compounds. Fermented blueberry juice was also shown to stimulate glucose uptake in myotubes and adipocytes, whereas unfermented juice had no effect on glucose transport (*40*). Some extracts obtained from fermented foods rich in flavanoids were also shown to limit atherosclerosis in animal models. Extracts from natto or other fermented soybean foods prevented the progression of atherosclerosis in cholesterol-fed rabbits, improved oxidative stress markers in cholesterol-fed rabbits or rats (*41*, *42*), and inhibited intimal thickening in rats (*43*). No comparison with the nonfermented soybean extracts was carried out. The active compounds responsible for the improved antiatherogenic effects of FBE are not yet identified. Anthocyanin-derived polymeric pigments in FBE might attenuate formation of atherosclerotic lesions more effectively than native anthocyanins as found in BE.

Very few studies have evaluated biological activities of phenolic compounds resulting from fermentation. Synthesized pyranoanthocyanins have been shown to scavenge superoxide anion radicals but to a lesser extent than their corresponding anthocyanin precursor (*44*). Other mechanisms could be thus implied in atheroprotective effects of anthocyanin-derived polymeric pigments, or other compounds introduced by the fermentation process, such as organic acids observed in wine (*45*), could also contribute to these beneficial effects.

The plasma antioxidant capacity, measured by the ORAC assay, was not modified by the bilberry extracts. Similarly, diet supplementation with an anthocyanin-rich extract from black rice did not affect the total serum antioxidant ability of apo $E^{-/-}$ mice after 20 weeks (*46*). Levels of aortic F_2-isoprostanes and hepatic TBARS, markers of lipid oxidation, were not more affected by the bilberry extract supplementation. In agreement with these results, it has been reported that dealcoholized red wine did also reduce aortic lipid deposition in apo $E^{-/-}$ mice without affecting lipid peroxidation (*47*). All of these data support the hypothesis that the antiatherogenic effects of bilberry extracts are independent of their antioxidant capacity in this animal model. In addition, none of the bilberry extracts modified the total cholesterol and triacylglycerol levels in both plasma and liver. These results are similar to those observed in previous experiments with purple sweet potato anthocyanins (*37*), tea catechins (*48*), or apple polyphenols (*49*). Thus, neither direct antioxidant activity nor modification of lipid profile seems to explain the observed cardioprotective effects of bilberry extracts. Other properties of these extracts and their constituents seem to be involved. Several in vivo studies showed some anti-inflammatory properties of anthocyanins (*37*, *46*) or red wine polyphenols (*50*), which could help to explain the protective effect observed here. These hypotheses are new leads of investigation for understanding notably the antiatherogenic properties of bilberry extracts, which should be explored in more detail.

In summary, the present study reveals that dietary supplementation with bilberry extracts clearly limits the development of atherosclerotic lesions in a model of apo $E^{-/-}$ mice. In this animal model, the protective effect of bilberry extracts is independent of modifications of plasma antioxidant status, biomarkers of oxidative stress, and lipid profile but varies with the type of extract. Indeed, the FBE extract exerts more effective antiatherogenic activity in vivo than the anthocyanin-rich BE extract, suggesting that fermentation generates some new compounds with improved health-promoting properties as compared to the anthocyanin-rich standardized extract. More studies will be necessary to identify the compounds responsible for these effects and to investigate possible mechanisms underlying such effects.

LITERATURE CITED

(1) Lloyd-Jones, D.; Adams, R.; Carnethon, M.; De Simone, G.; Ferguson, T. B.; Flegal, K.; Ford, E.; Furie, K.; Go, A.; Greenlund, K.; Haase, N.; Hailpern, S.; Ho, M.; Howard, V.; Kissela, B.; Kittner, S.; Lackland, D.; Lisabeth, L.; Marelli, A.; McDermott, M.; Meigs, J.; Mozaffarian, D.; Nichol, G.; O'Donnell, C.; Roger, V.; Rosamond, W.; Sacco, R.; Sorlie, P.; Stafford, R.; Steinberger, J.; Thom, T.; Wasserthiel-Smoller, S.; Wong, N.; Wylie-Rosett, J.; Hong, Y. Heart Disease and Stroke Statistics − 2009 Update. A report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* **2009**, *119*, 480–486.

(2) Joshipura, K. J.; Hu, F. B.; Manson, J. E.; Stampfer, M. J.; Rimm, E. B.; Speizer, F. E.; Colditz, G.; Ascherio, A.; Rosner, B.; Spiegelman, D.; Willett, W. C. The effect of fruit and vegetable intake on risk for coronary heart disease. *Ann. Intern. Med.* **2001**, *134*, 1106–1114.

(3) Manach, C.; Mazur, A.; Scalbert, A. Polyphenols and prevention of cardiovascular diseases. *Curr. Opin. Lipidol.* **2005**, *16*, 77–84.

(4) Mink, P. J.; Scrafford, C. G.; Barraj, L. M.; Harnack, L.; Hong, C. P.; Nettleton, J. A.; Jacobs, D. R., Jr. Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. *Am. J. Clin. Nutr.* **2007**, *85*, 895–909.

(5) Erlund, I.; Koli, R.; Alfthan, G.; Marniemi, J.; Puukka, P.; Mustonen, P.; Mattila, P.; Jula, A. Favorable effects of berry consumption on platelet function, blood pressure, and HDL cholesterol. *Am. J. Clin. Nutr.* **2008**, *87*, 323–331.

(6) Maatta-Riihinen, K. R.; Kamal-Eldin, A.; Mattila, P. H.; Gonzalez-Paramas, A. M.; Torronen, A. R. Distribution and contents of phenolic compounds in eighteen Scandinavian berry species. *J. Agric. Food Chem.* **2004**, *52*, 4477–4486.

(7) Nyman, N. A.; Kumpulainen, J. T. Determination of anthocyanidins in berries and red wine by high-performance liquid chromatography. *J. Agric. Food Chem.* **2001**, *49*, 4183–4187.

(8) Latti, A. K.; Riihinen, K. R.; Kainulainen, P. S. Analysis of anthocyanin variation in wild populations of bilberry (*Vaccinium myrtillus* L.) in Finland. *J. Agric. Food Chem.* **2008**, *56*, 190–196.

(9) Zafra-Stone, S.; Yasmin, T.; Bagchi, M.; Chatterjee, A.; Vinson, J. A.; Bagchi, D. Berry anthocyanins as novel antioxidants in human health and disease prevention. *Mol. Nutr. Food Res.* **2007**, *51*, 675–683.

(10) Lee, J.; Lee, H. K.; Kim, C. Y.; Hong, Y. J.; Choe, C. M.; You, T. W.; Seong, G. J. Purified high-dose anthocyanoside oligomer administration improves nocturnal vision and clinical symptoms in myopia subjects. *Br. J. Nutr.* **2005**, *93*, 895–899.

(11) Hooper, L.; Kroon, P. A.; Rimm, E. B.; Cohn, J. S.; Harvey, I.; Le Cornu, K. A.; Ryder, J. J.; Hall, W. L.; Cassidy, A. Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials. *Am. J. Clin. Nutr.* **2008**, *88*, 38–50.

(12) Kolovou, G.; Anagnostopoulou, K.; Mikhailidis, D. P.; Cokkinos, D. V. Apolipoprotein E knockout models. *Curr. Pharm. Des.* **2008**, *14*, 338–351.

(13) Auclair, S.; Milenkovic, D.; Besson, C.; Chauvet, S.; Gueux, E.; Morand, C.; Mazur, A.; Scalbert, A. Catechin reduces atherosclerotic lesion development in apo E-deficient mice: a transcriptomic study. *Atherosclerosis* **2009**, *204*, E21–27.

(14) Soulier, C.; Dufour, D. U.S. Patent 6,461,648 B2, 2002.

(15) Pourrat, H. FR Patent 1300869, 1961.

(16) Azar, M.; Verette, E.; Brun, S. Comparative study of fresh and fermented bilberry juices − state and modification of the coloring pigments. *J. Food Sci.* **1990**, *55*, 164–166.

(17) Talavera, S.; Felgines, C.; Texier, O.; Besson, C.; Mazur, A.; Lamaison, J. L.; Remesy, C. Bioavailability of a bilberry anthocyanin extract and its impact on plasma antioxidant capacity in rats. *J. Sci. Food Agric.* **2006**, *86*, 90–97.

(18) Rault-Nania, M. H.; Gueux, E.; Demougeot, C.; Demigne, C.; Rock, E.; Mazur, A. Inulin attenuates atherosclerosis in apolipoprotein E-deficient mice. *Br. J. Nutr.* **2006**, *96*, 840–844.

(19) Mazur, A.; Remesy, C.; Gueux, E.; Levrat, M. A.; Demigne, C. Effects of diets rich in fermentable carbohydrates on plasma lipoprotein levels and on lipoprotein catabolism in rats. *J. Nutr.* **1990**, *120*, 1037–1045.

(20) Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Prior, R. L. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *J. Agric. Food Chem.* **2002**, *50*, 4437–4444.

(21) Ohkawa, H.; Ohishi, N.; Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **1979**, *95*, 351–358.

(22) Mas, E.; Michel, F.; Guy, A.; Bultel, V.; Falquet, Y.; Chardon, P.; Rossi, J. C.; Cristol, J. P.; Durand, T. Quantification of urinary F2-isoprostanes with 4(RS)-F4t-neuroprostane as an internal standard using gas chromatography−mass spectrometry. Application to polytraumatized patients. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2008**, *872*, 133–140.

(23) Sadilova, E.; Stintzing, F. C.; Carle, R. Thermal degradation of acylated and non acylated anthocyanins. *J. Food Sci.* **2006**, *71*, C504–512.

(24) Johannot, L.; Somerset, S. M. Age-related variations in flavonoid intake and sources in the Australian population. *Public Health Nutr.* **2006**, *9*, 1045–1054.
(25) Mullie, P.; Clarys, P.; Deriemaeker, P.; Hebbelinck, M. Estimation of daily human intake of food flavonoids. *Plant Foods Hum. Nutr.* **2007**, *62*, 93–98.
(26) Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *J. Agric. Food Chem.* **2006**, *54*, 4069–4075.
(27) Ovaskainen, M. L.; Torronen, R.; Koponen, J. M.; Sinkko, H.; Hellstrom, J.; Reinivuo, H.; Mattila, P. Dietary intake and major food sources of polyphenols in Finnish adults. *J. Nutr.* **2008**, *138*, 562–566.
(28) Aron, P. M.; Kennedy, J. A. Compositional investigation of phenolic polymers isolated from *Vitis vinifera* L. Cv. Pinot Noir during fermentation. *J. Agric. Food Chem.* **2007**, *55*, 5670–5680.
(29) Rommel, A.; Wrolstad, R. E.; Heatherbell, D. A. Blackberry juice and wine: processing and storage effects on anthocyanin composition, color and appearance. *J. Food Sci.* **1992**, *57*, 385–391.
(30) Heinonen, I. M.; Lehtonen, P. J.; Hopia, A. I. Antioxidant activity of berry and fruit wines and liquors. *J. Agric. Food Chem.* **1998**, *46*, 25–31.
(31) Sarni-Manchado, P.; Cheynier, V. Composés phénoliques des boissons fermentées. In *Les Polyphénols en Agroalimentaire*; Tec&Doc Lavoisier; Paris, France, 2006; pp 212−264.
(32) Romero, C.; Brenes, M.; Garcia, P.; Garcia, A.; Garrido, A. Polyphenol changes during fermentation of naturally black olives. *J. Agric. Food Chem.* **2004**, *52*, 1973–1979.
(33) Medina, K.; Boido, E.; Dellacassa, E.; Carrau, F. Yeast interactions with anthocyanins during red wine fermentation. *Am. J. Enol. Vitic.* **2005**, *56*, 104–109.
(34) Morata, A.; Calderon, F.; Gonzalez, M. C.; Gomez-Cordoves, M. C.; Suarez, J. A. Formation of the highly stable pyranoanihocyanins (vitisins A and B) in red wines by the addition of pyruvic acid and acetaldehyde. *Food Chem.* **2007**, *100*, 1144–1152.
(35) Monagas, M.; Gómez-Cordovés, C.; Bartolomé, B. Evaluation of different *Saccharomyces cerevisiae* strains for red winemaking. Influence on the anthocyanin, pyranoanthocyanin and non-anthocyanin phenolic content and colour characteristics of wines. *Food chem.* **2007**, *104*, 814–823.
(36) Rentzsch, M.; Quast, P.; Hillebrand, S.; Winterhalter, P. Isolation and identification of 5-carboxy-pyranoanthocyanins in beverages from cherry (*Prunus cerasus* L.). *Innovative Food Sci. Emerg. Technol.* **2007**, *8*, 333–338.
(37) Miyazaki, K.; Makino, K.; Iwadate, E.; Deguchi, Y.; Ishikawa, F. Anthocyanins from purple sweet potato *Ipomoea batatas* cultivar ayamurasaki suppress the development of atherosclerotic lesions and both enhancements of oxidative stress and soluble vascular cell adhesion molecule-1 in apolipoprotein E-deficient mice. *J. Agric. Food Chem.* **2008**, *56*, 11485–11492.
(38) Vuong, T.; Martin, L.; Matar, C. Antioxidant activity of fermented blueberry juices and their effects on nitric oxide and tumor necrosis factor-α production in macrophages 264.7 γ NO(−) cell line. *J. Food Biochem.* **2006**, *30*, 249–268.
(39) Ju, H. K.; Cho, E. J.; Jang, M. H.; Lee, Y. Y.; Hong, S. S.; Park, J. H.; Kwon, S. W. Characterization of increased phenolic compounds from fermented Bokbunja (*Rubus coreanus* Miq.) and related antioxidant activity. *J. Pharm. Biomed. Anal.* **2009**, *49*, 820–827.
(40) Vuong, T.; Martineau, L. C.; Ramassamy, C.; Matar, C.; Haddad, P. S. Fermented Canadian lowbush blueberry juice stimulates glucose uptake and AMP-activated protein kinase in insulin-sensitive cultured muscle cells and adipocytes. *Can. J. Physiol. Pharmacol.* **2007**, *85*, 956–965.
(41) Iwai, K.; Nakaya, N.; Kawasaki, Y.; Matsue, H. Antioxidative functions of natto, a kind of fermented soybeans: effect on LDL oxidation and lipid metabolism in cholesterol-fed rats. *J. Agric. Food Chem.* **2002**, *50*, 3597–3601.
(42) Yamakoshi, J.; Piskula, M. K.; Izumi, T.; Tobe, K.; Saito, M.; Kataoka, S.; Obata, A.; Kikuchi, M. Isoflavone aglycone-rich extract without soy protein attenuates atherosclerosis development in cholesterol-fed rabbits. *J. Nutr.* **2000**, *130*, 1887–1893.
(43) Suzuki, Y.; Kondo, K.; Ichise, H.; Tsukamoto, Y.; Urano, T.; Umemura, K. Dietary supplementation with fermented soybeans suppresses intimal thickening. *Nutrition* **2003**, *19*, 261–264.
(44) Garcia-Alonso, M.; Rimbach, G.; Sasai, M.; Nakahara, M.; Matsugo, S.; Uchida, Y.; Rivas-Gonzalo, J. C.; De Pascual-Teresa, S. Electron spin resonance spectroscopy studies on the free radical scavenging activity of wine anthocyanins and pyranoanthocyanins. *Mol. Nutr. Food Res.* **2005**, *49*, 1112–1119.
(45) Volschenk, H.; van Vuuren, H. J. J.; Viljoen-Bloom, M. Malic acid in wine: origin, function and metabolism during vinification. *S. Afr. J. Enol. Vitic.* **2006**, *27*, 123–136.
(46) Xia, X.; Ling, W.; Ma, J.; Xia, M.; Hou, M.; Wang, Q.; Zhu, H.; Tang, Z. An anthocyanin-rich extract from black rice enhances atherosclerotic plaque stabilization in apolipoprotein E-deficient mice. *J. Nutr.* **2006**, *136*, 2220–2225.
(47) Waddington, E.; Puddey, I. B.; Croft, K. D. Red wine polyphenolic compounds inhibit atherosclerosis in apolipoprotein E-deficient mice independently of effects on lipid peroxidation. *Am. J. Clin. Nutr.* **2004**, *79*, 54–61.
(48) Miura, Y.; Chiba, T.; Tomita, I.; Koizumi, H.; Miura, S.; Umegaki, K.; Hara, Y.; Ikeda, M.; Tomita, T. Tea catechins prevent the development of atherosclerosis in apoprotein E-deficient mice. *J. Nutr.* **2001**, *131*, 27–32.
(49) Auclair, S.; Silberberg, M.; Gueux, E.; Morand, C.; Mazur, A.; Milenkovic, D.; Scalbert, A. Apple polyphenols and fibers attenuate atherosclerosis in apolipoprotein E-deficient mice. *J. Agric. Food Chem.* **2008**, *56*, 5558–5563.
(50) Rasmussen, S. E.; Frederiksen, H.; Struntze Krogholm, K.; Poulsen, L. Dietary proanthocyanidins: occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. *Mol. Nutr. Food Res.* **2005**, *49*, 159–174.

Received for review April 30, 2009. Revised manuscript received October 19, 2009. Accepted October 22, 2009. This work has been supported by FERLUX S.A.-Mediolanum Laboratory, France.