Increase of antioxidant capacity of the lowbush blueberry (Vaccinium angustifolium) during fermentation by a novel bacterium from the fruit microflora

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Abstract: Members of the genus Vaccinium, such as blueberry and cranberry, are known to be excellent sources of antioxidant phenolic compounds, for example anthocyanins, flavonols and phenolic acids. The fruit also provides a natural habitat for numerous microorganisms. Interaction between the fruit and the microflora might affect the antioxidant phenolic compounds. The aim of this study was to investigate the effects on phenolic content and antioxidant capacity of wild blueberry fermented by a newly identified bacterium isolated from blueberry-fruit surface microflora, Serratia vaccinii. Increase in the antioxidant capacity following fermentation of blueberries by the novel bacterium, as determined with the 2,2′-diphenyl-1-picrylhydrazyl method, was attributed not only to an increase in total phenolics, but also to a change in the phenolic profile, as demonstrated by the production of gallic acid and of a novel compound of phenolic or phenylpropanoic structure.

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Keywords: Vaccinium angustifolium; fruit surface microflora; HPLC; DPPH method; antioxidant; tannin degradation

INTRODUCTION

Lowbush ‘wild’ blueberry (Vaccinium angustifolium Aiton) is native to North America. Its production is localized in eastern Canada and the northeastern USA. Fields of wild blueberries are composed of many genetically and phenotypically different clones.1 Blueberries are known for their high concentration of phenolic compounds, including anthocyanins, and their high antioxidant capacity, as determined with the oxygen radical absorbing capacity (ORAC) assay.2 Members of the genus Vaccinium (Ericaceae), such as blueberry and cranberry, are excellent sources of flavonoids, such as anthocyanins, flavonols and proanthocyanidins.3

Variations in the anthocyanins, total phenolics and antioxidant capacity have been noticed between different fields of wild blueberries and between different years of blueberry production for a single field.4 In wild blueberries, as in any other small fruits, the synthesis of anthocyanins and other phenolic compounds can be influenced by various biotic and abiotic factors, including temperature, irradiation and pathogenic infection.5

Blueberries are known to possess great potential health benefits, mostly attributable to their high content of phenolic compounds. Fractions from wild blueberries rich in proanthocyanidins have been shown to be effective inhibitors of the promotion stage of chemically induced carcinogenesis by inducing the activity of quinone reductase enzyme.3,6 More recently, resveratrol, known for its cardioprotective and cancer chemopreventive activities, has been found in wild blueberries.7 Brain function enhancing activity has also been associated with blueberry consumption of polyphenolics.8,9 Juices of Vaccinium fruits have been shown to possess anti-adhesin activity, blocking the binding of bacteria to the urinary tract wall and preventing urinary tract infections.10 Moreover, blueberry juice could possibly inhibit gastric mucosa adhesion of Helicobacter pylori, reducing the incidence of gastric ulcers.11

More generally, phenolic compounds found in fruits and vegetables have been shown to possess anti-tumoral,12 anti-allergenic,13 anti-platelet,14 anti-ischemic,15 and anti-inflammatory activities.16 Physiological effects of flavonoids have been attributed to
their actions as free-radical scavengers, reducers, metal chelators, modulators of enzyme activity, inhibitors of calcium influx into cells, and regulators of cell messaging and gene expression. Phenolic compounds are able to scavenge free radicals by giving a hydrogen from a reactive hydroxyl group to form a phenoxyl radical which is stabilized by resonance. The capacity of phenolic compounds to give hydrogens depends on the redox properties of their hydroxyl groups and on their structural features, including degrees of hydroxylation and of glycosylation.

No previous data has been reported on blueberry fermentation by its normal microflora or on its effects on phenolic composition and antioxidant capacity. However, multiple chemical modifications, concerning phenolic profiles, have been reported during the process of wine fermentation. Antioxidant capacity, anthocyanins and total phenolics were comparable between highbush blueberry wine and selected red wines. Phenolic degradation has also been reported from incubation with bacteria from intestinal microflora and from the plant’s rhizosphere. These degradations were characterized by C-ring fission of flavonoids, including quercetin and catechin, resulting in the production of various phenolic acid structures.

The aim of this study was to investigate the effects on phenolic content and antioxidant capacity of wild blueberry fermented by a newly identified bacterium isolated from blueberry fruit microflora, Serratia vaccinii. The method relies on fermenting wild blueberries with predetermined conditions such as temperature, agitation and inoculum. The fermenting blueberry mixture was sampled at different times during the fermentation to observe the changes in phenolic content and antioxidant capacity by measuring radical scavenging activity.

EXPIMENTAL

Bacterial cultures and media
The new bacterium was isolated from the surface of lowbush blueberries (Vaccinium angustifolium Aiton) by inoculation of a Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, MI, USA) with whole blueberry fruit, growth at 25 °C for 36 h, and selection on Tryptic Soy agar (TSA) by serial dilutions in 0.5% peptonized water. De Man, Rogosa and Sharpe (MRS) and Potato Dextrose agars (Becton Dickinson, Cockeysville MD, USA) were also used to determine the properties of the bacteria. Colonies selection for identification was made randomly from 1:10⁶ dilutions plated on TSA. Stock cultures were maintained at −70 °C in broth supplemented with 30% (v/v) glycerol.

Bacterial identification
Carbohydrate fermentation patterns were determined using API 50CH galleries as specified by the manufacturer (Bio Mérieux SA, Marcy-l’Étoile, France). API 20Strep and oxidation-fermentation (OF) medium (Bio Mérieux SA) were also used for bacterial characterization.

The partial sequence of the 16S rRNA gene (1500 nucleotides) was determined by MIDI Laboratories (MIDI Labs, Newark DE, USA) by standard procedures, and a phylogenetic analysis was performed based on this partial 16S rRNA gene sequence. The sequence data were compared to sequences in the Microseq™ microbial analysis software and database (PE Applied Biosystems, Foster City, CA, USA).

The 16S rRNA gene was PCR-amplified from genomic DNA isolated from colonies of the novel bacterium. The primers used for the amplification correspond to positions 5, 338, 357, 515, 531, 776, 1087, 1104, 1174, 1193 and 1540 in the E coli 16S rRNA gene. Amplification products were purified from excess primers and dNTPs using Microcon 100 (Millipore, Billerica, MA, USA) molecular weight cutoff membranes, and checked for quality and quantity by running a portion of the products on an agarose gel.

Cycle sequencing of the 16S rRNA amplification products was performed using AmpliTaq FS DNA polymerase and dRhodamine dye terminators (Applied Biosystems, Foster City, CA, USA). Excess dye-labeled terminators were removed from the sequencing reactions using a Sephadex G-50 spin column. The products were collected by centrifugation, dried under vacuum and frozen at −20 °C until ready to load. Samples were resuspended in a solution of formamide/blue dextran/EDTA and denatured prior to loading. The samples were electrophoresed on an ABI Prism 377 DNA Sequencer. Data was analyzed using PE Applied Biosystems DNA editing and assembly software.

Preparation of blueberry mixture
Fully matured wild blueberries (Vaccinium angustifolium Aiton) were mechanically harvested from selected areas of the Atlantic region and thoroughly mixed. The mixture was prepared by blending blueberries with an equivalent volume (1:1 v/v ratio) of Minimal Broth Davis (Difco Laboratories). The blueberry mixture was then centrifuged at 500 x g for 6 min in a IEC Centra MP4R centrifuge (International Equipment Company, Needham Heights, MA, USA), to remove non-homogenized particles, prefiltered under vacuum and sterilized by filtration through a 0.22-μm Express Millipore filter apparatus (Millipore, Billerica, MA, USA).

Fermentation
Fermentations were made with the novel bacterium, isolated from the blueberry fruit surface normal flora, by inoculation of 100 ml of sterilized blueberry juice with 2% (v/v) of novel bacterium-saturated TSB culture at (7.5 ± 0.3) log CFU ml⁻¹. For each flask that was inoculated, a control flask was prepared under the same conditions, but without inoculation. Instead, a quantity of 2% of total blueberry juice volume of sterilized TSB (Difco Laboratories) was
added to the mixture. The blueberry media were incubated in a Lab-Line low-temperature benchtop incubated shaker (Lab-Line Instruments, Inc, Melrose Park, IL, USA) at 22 °C, 120 rev min⁻¹, under aerobic conditions, for up to 7 days, in 250-ml flasks. A series of 15 fermentations were done in triplicate in order to assure reproducibility. A controlled fermentation without inoculation was made in a 2.5-l BIOFLO 3000 fermentor (New Brunswick Scientific, Edison, NJ, USA) by simulating pH variation with addition of 4 N acetic acid and 4 N NaOH.

**Phenolic compounds**

The samples were analyzed for total phenolics using the standard Folin–Ciocalteu method. Gallic acid (Acros, New Jersey, NJ, USA) was used as a calibration standard. The prepared samples were read by a µQuant Microplate Reader (Biotek Instruments Inc, Winooski, VT, USA) set at a wavelength of 700 nm. Antioxidant capacity was tested using the DPPH method, and high-pressure liquid chromatography (HPLC) profile of phenolic compounds was done using a reverse-phase technique based on Kalt et al.³¹

**Determination of radical scavenging activity**

A modified DPPH method, based on the use of the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), was employed for the determination of radical scavenging activity. Two hundred microlitres of DPPH in 80% methanol (150 µM) was added to 22 µl of each of the test samples, which were diluted at different concentrations (0–500 µM) in 80% methanol. Each mixture was then shaken and incubated at room temperature in the dark. The decrease in absorbance of DPPH was measured at 30, 180 and 360 min of incubation at 520 nm in a µQuant Microplate Reader (Biotek Instruments Inc). Methanol was used as a blank solution. DPPH solution (200 µl) in ethanol (22 µl) served as the control. To verify whether the increase in radical scavenging activity is attributable to the increase in total phenolics or to changes in the phenolic profiles during fermentation, a series of samples were analyzed following an adjustment of their total phenolics concentrations to the initial values before fermentation. All tests were performed in triplicate.

A plot of A₅₂₀nm versus concentration of sample in the final solution was made for each time interval. The radical scavenging activity was defined by the initial slope value (r² > 0.800) in µM of DPPH µM⁻¹ of blueberry sample in gallic acid equivalents (GAE). The concentration of DPPH was initially determined from the calibration curve equation given by Brand-Williams et al.,³³ where A₅₁₅nm was equal to 12,509 × concentration of DPPH in M −0.00258.

**Quantification of the unknown compound by HPLC**

The method used for phenolic compound separation on HPLC was derived from Kalt et al.³¹ A 50-µl injection of sample was separated on a Zorbax SB-C18 Rapid Resolution 4.6 mm ID × 150 mm, 3.5 µm column associated to a ODS-Hypersil (C-18) guard column 2.1 mm ID × 20 mm, 5 µm (Agilent Technologies Inc, Mississauga, ON, Canada) using an Agilent HPLC 1100 series system equipped with a quaternary pump and a diode array detector. Compounds were separated with 5% formic acid in water (solvent B) and methanol (solvent D), using a gradient elution program; 0–10.24 min, 14–17% D; 10.24–35.28 min, 17–23% D; 35.28–64.59 min, 23–47% D; 64.59–66.59 min, 47–14% D; 66.59–70 min, 14% D. Flow rate was 1.0 ml min⁻¹. Column temperature was maintained at 30 ºC during separation. Data were collected at 270 nm for phenolic acids, 520 nm for anthocyanins, 340 nm for flavones, 320 nm for stilbenes and catechins, and 365 nm for flavonols, with 800 nm set as reference. Samples were filtered through a 0.22-µm Millex-GP filter unit (Millipore) prior to injection. System was equilibrated for 15 min at the initial gradient before each injection. HPLC quantification of the unknown phenolic acid was made with gallic acid as reference, by comparison between peak heights. Chlorogenic acid, p-coumaric acid, sinapic acid and gallic acid were quantified using authentic standards (Sigma–Aldrich Canada Ltd, Ontario, Canada).

**Statistical analysis**

Data represent the mean of three replicate analyses tested. Results were processed for statistical significance using single factor ANOVA and Student’s t test. Differences at p < 0.05 were considered to be significant.

**RESULTS**

**Identification of the novel bacterium**

After several screening experiments, consisting of fermentations with the fruit’s microflora, one isolate was selected for further study. The new bacterium isolated from lowbush blueberries was a Gram-negative, catalase positive, facultatively anaerobic coccobacillus.

From the results obtained from API 50CH, the bacterium fermented D-glucose, D-fructose, D-mannose, arbutin, esculin, salicin, saccharose and D-raffinose. Furthermore, API 20Strep results showed that, under some conditions, the bacterium could ferment mannitol, lactose and trehalose. The bacterium also showed positive results for acetoin production, hippurate hydrolysis, pyrrolidonyl arylamidase, α-galactosidase, β-galactosidase, alkaline phosphatase and leucine arylamidase activity. Results from OF medium indicated that the novel bacterium has a fermentative metabolism.

Partial sequence of the 16S rRNA gene for the novel bacterium (shown in Fig 1) was compared with sequences in the Microseq microbial analysis software and database. The top ten alignment matches are
Figure 1. 16S rRNA gene partial sequence (1500 nts) for the novel bacterium as determined by MIDI Laboratories.

Figure 2. Phylogenetic analysis based on the partial 16S rDNA fragment determined by MIDI Laboratories.

presented in a percentage genetic difference format (Fig 2). A low percentage indicates a close match. Also provided for the novel bacterium is a neighbor-joining phylogenetic tree, generated using the top ten alignment matches.

Genetic relationships are expressed in the form of percentage genetic differences (%GD). A species level match may be assigned if the %GD between the unknown and the closest match is less than the approximate average %GD between species within that particular genetic family, which is usually 1%. A genus level match will be assigned when the sequence does not meet the requirements for a species level match, but still clusters within the branching of a well defined genus. For the novel bacterium, its closest match was *Serratia proteamaculans quinovora*, and the %GD determined was 1.82%, which is greater than the average %GD of 1.28% between species within the family of Enterobacteriaceae. From these results, the identification of the novel bacterium at the species level could not be confirmed as being *Serratia proteamaculans quinovora*. However, even though the novel bacterium could not be identified as being a *Serratia* from its 16S rRNA gene sequence, results from its biochemical profile and physical properties indicate that it could still belong to the genus *Serratia*, and that it could be considered as a new bacterium that had not yet been discovered. Since the new bacterium has been isolated from the surface of wild blueberry, *Vaccinium angustifolium* Ait, and shows some close resemblance with bacteria of the genus *Serratia*, the name proposed for this novel organism is *Serratia vaccinii*. In order to facilitate further analysis, the novel bacterium has been deposited under Accession Number 160 103, at the International Depositary Authority of Canada.

Fermentation with the novel bacterium

A significant increase in the total phenolic content of wild blueberry juice occurred after only 1 day of incubation (p < 0.05), indicating that the novel bacterium plays an important role in the increase of total phenolics (Table 1). Microbial counts increased from day zero (5.80 ± 0.31 log CFU ml⁻¹) to day one (9.11 ± 0.16 log CFU ml⁻¹) of fermentation, and then no significant decreases were observed until
Table 1. Total phenolic content (mg of gallic acid equivalent (GAE) kg⁻¹ of fresh weight (FW)), as determined by the Folin–Ciocalteu method, during fermentation of wild blueberry by the novel bacterium

<table>
<thead>
<tr>
<th>Days of fermentation</th>
<th>Total phenolic content (mg of GAE kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1682.5 ± 35.5</td>
</tr>
<tr>
<td>1</td>
<td>1251.3 ± 278.7</td>
</tr>
<tr>
<td>2</td>
<td>1541.9 ± 119.1</td>
</tr>
<tr>
<td>3</td>
<td>1507.5 ± 37.7</td>
</tr>
<tr>
<td>4</td>
<td>1321.6 ± 163.6</td>
</tr>
<tr>
<td>5</td>
<td>1155.9 ± 269.1</td>
</tr>
<tr>
<td>7</td>
<td>1470.0 ± 44.6</td>
</tr>
</tbody>
</table>

...continued...

Quantification and characterization of the unknown compound by HPLC

HPLC was used to characterize the unknown compound obtained from fermented blueberry sample with the novel bacterium. Quercetin could not be detected from phenolic profiles of fermented and non-fermented blueberry samples by HPLC spiking. However, rutin could be detected at a concentration of (12.2 ± 0.2) mg kg⁻¹ of fresh weight (FW) in non-fermented blueberry samples after 3 days of incubation. Significant decrease in rutin content to (10.9 ± 0.3) mg kg⁻¹ FW was observed in fermented...
blueberry samples with the same incubation time ($p < 0.05$). Production of gallic acid was observed during blueberry fermentation, as shown by HPLC spiking. Gallic acid went from a non-detectable concentration on day 0 to a concentration varying from (26.7 ± 0.9) to (64.6 ± 0.5) mg kg$^{-1}$ FW on day 3 of blueberry fermentation (peak 1, Fig 4). The content of chlorogenic acid after 3 days of blueberry fermentation was (852.7 ± 2.8) mg kg$^{-1}$ FW, with no significant difference between fermented and non-fermented blueberry ($p < 0.05$) (peak 3, Fig 4). No concentrations of $p$-coumaric acid and sinapic acid were detected in any of the fermented or non-fermented blueberry samples. Peak 2, at a concentration of (642.0 ± 1.3) mg of gallic acid equivalents kg$^{-1}$ FW after 3 days of incubation (Fig 4b), is produced only after fermentation of blueberry with the novel bacterium. No significant variation of concentration of the new compound was observed between day 1 and day 5 of fermentation ($p < 0.05$). The UV spectrum of peak 2, is characterized by a strong absorbance at 200 nm, followed by a plateau from 215 to 235 nm, and a second strong absorbance at 280 nm.

DISCUSSION

This research dealt with a novel approach to increase the antioxidant phenolic content of wild blueberries. This approach consists of using fermentation in the presence of a novel bacterium isolated from the blueberry fruit surface to enhance phenolic release and/or production from blueberries. Numerous mechanisms could be responsible for the increase of antioxidant phenolic compounds. It is possible that this bacterium, by its enzymatic activity, contributes to the deglycosylation of pre-existing glycosylated phenolic compounds. It should be noted that the non-glycosylated phenolic compounds have a better antioxidant capacity than their glycosylated counterparts. The increase in phenolic content might also be attributed to the ability of the bacterium to produce new phenolic compounds by biosynthesis. Indeed, certain bacteria are able to synthesize aromatic compounds with antioxidant capacity from acetic and malonic acid precursors by the polyketide pathway. Partial microbial degradation of tannins is another way by which the concentration of antioxidant phenolic compounds could be increased. Numerous bacteria pertaining to the Enterobacteriaceae family, possibly including our novel bacterium, are capable of such degradation.

It is well known that certain bacteria from the intestinal microflora, including bacteria of genera *Lactobacillus*, *Bacillus*, *Staphylococcus* and *Klebsiella*, are able to degrade tannins in simple phenolics in order to use degradation products for energy. Gallic acid and sugar can be obtained from hydrolyzable tannin degradation, following the action of a specific enzyme, called tannase. Gallic acid can then be used during oxidative degradation to produce simple organic acids further metabolized in the citric acid cycle. Such degradation of hydrolyzable tannins could explain gallic acid accumulation following blueberry fermentation by the novel bacterium. The novel bacterium, *Serratia vaccinii*, pertaining to the Enterobacteriaceae, could increase the total phenolic content of fermented blueberry by degradation of tannins to more bioactive phenolic or phenylpropanoic acids associated with a sugar moiety. Flavonoid degradation under aerobic conditions can also be done by bacteria isolated from the rhizosphere. Microorganisms found in the rhizosphere that are capable of flavonoid degradation include *Rhizobia, Agrobacterium, Pseudomonas, Bacillus* and *Rhodococcus* spp. These findings confirm the possibility of identifying certain bacteria from the plant environment, such as *Serratia vaccinii*, which may
be capable of degrading tannins in order to produce bioactive phenolic compounds.

The DPPH method has been used in order to study the activity of different antioxidant compounds. The most detailed study concerning the antioxidant potential of phenolic compounds from vegetables has been completed by this method, and results have been confirmed by nuclear magnetic resonance. From results obtained in our study, it was concluded that increase in antioxidant capacity during fermentation was attributed not only to increase in total phenolics, but also to a change in the phenolic profile, as shown by HPLC detection of gallic acid and of a novel phenolic compound. Furthermore, total soluble phenolics, as determined by the Folin–Ciocalteu method, are strongly correlated to the radical scavenging activity, as determined by the DPPH method ($r = 0.9972$).

Production of gallic acid was observed during blueberry fermentation. This increase in gallic acid content could be attributed to hydrolyzable tannin degradation during the fermentation process. Presence of sinapic acid was studied in order to view its retention time (RT) (26.7 min) on HPLC. However, sinapic acid was not detected in either fermented or non-fermented blueberry samples. From these results, it was concluded that the novel compound (RT of 4.5 min) was more polar than sinapic acid, p-coumaric acid (RT of 15.7 min), and chlorogenic acid (RT of 7.9 min). Chlorogenic acid is usually recognized as a major contributor to the antioxidant activity of blueberries, due to its high concentration in this fruit. Because p-coumaric acid is a predecessor of the flavonoid biosynthetic pathway and of the phenolic acids produced via the phenylpropanoid pathway, its presence during fermentation could have been an indicator that the novel compound is the result of biosynthesis, instead of degradation. Significant decrease of rutin content after blueberry fermentation by the novel bacterium could be attributed to its presence during fermentation, as shown by HPLC detection of gallic acid and a novel compound.

In vitro antiproliferative activity of blueberry fruits, species.


