J Sci Food Agric 85:1477–1484 (2005)
DOI: 10.1002/jsfa.2142

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.

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

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Contract/grant sponsor: Canada Fund for Innovation
Contract/grant sponsor: Atlantic Innovation Fund
Contract/grant sponsor: University of Moncton Research Fund
(*Received 28 March 2004; revised version received 11 September 2004; accepted 11 October 2004*)
Published online 4 March 2005

their actions as free-radical scavengers,[17,18] reducers,[19] metal chelators,[20] modulators of enzyme activity,[21] inhibitors of calcium influx into cells,[22] and regulators of cell messaging and gene expression.[23] 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.[18]

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.[24,25] Antioxidant capacity, anthocyanins and total phenolics were comparable between highbush blueberry wine and selected red wines.[26] Phenolic degradation has also been reported from incubation with bacteria from intestinal microflora and from the plant's rhizosphere.[27–29] 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.

EXPERIMENTAL

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 $1{:}10^6$ 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 cut-off 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 $500 \times g$ for 6 min in a IEC Centra MP4R centrifuge (International Equipment Company, Needham Heights, MA, USA), to remove non-homogenized particles, prefiltrated 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.[30] 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.[32] 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 A_{520nm} 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 ($r^2 > 0.800$) 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 A_{515nm} 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 °C 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

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   1 TGGAGAGTTT GATCCTGGCT CAGATTGAAC GCTGGCGGCA GGCCTAACAC
  51 ATGCAAGTCG AGCGGTAGCA CGGGAGAGCT TGCTCTCTGG GTGACGAGCG
 101 GCGGACGGGT GAGTAATGTC TGGGAAACTG CCTGATGGAG GGGGATAACT
 151 ACTGGAAACG GTAGCTAATA CCGCATGATG TCGCAAGACC AAAGTGGGGG
 201 ACCTTCGGGC CTCACGCCAT CGGATGTGCC CAGATGGGAT TAGCTAGTAG
 251 GTGGGGTAAT GGCTCACCTA GGCGACGATC CTAGCTGGTC TGAGAGGATG
 301 ACCAGCCACA CTGGAACTGA GACACGGTCC AGACTCCTAC GGGAGGCAGC
 351 AGTGGGGAAT ATTGCACAAT GGGCGCAAGC CTGATGCAGC CATGCCGCGT
 401 GTGTGAAGAA GGCCTTAGGG TTGTAAAGCA CTTTCAGCGA GGAGGAAGGC
 451 GTTGTAGTTA ATAGCTGCAA CGATTGACGT TACTCGCAGA AGAAGCACCG
 501 GCTAACTCCG TGCCAGCAGC CGCGGTAATA CGGAGGGTGC AAGCGTTAAT
 551 CGGAATTACT GGGCGTAAAG CGCACGCAGG CGGTTTGTTA AGTCAGATGT
 601 GAAATCCCCG AGCTTAACTT GGGAACTGCA TTTGAAACTG GCAAGCTAGA
 651 GTCTTGTAGA GGGGGGTAGA ATTCCAGGTG TAGCGGTGAA ATGCGTAGAG
 701 ATCTGGAGGA ATACCGGTGG CGAAGGCGGC CCCCTGGACA AAGACTGACG
 751 CTCAGGTGCG AAAGCGTGGG GAGCAAACAG GATTAGATAC CCTGGTAGTC
 801 CACGCTGTAA ACGATGTCGA CTTGGAGGTT GTGCCCTTGA GGCGTGGCTT
 851 CCGGAGCTAA CGCGTTAAGT CGACCGCCTG GGGAGTACGG CCGCAAGGTT
 901 AAAACTCAAA TGAATTGACG GGGGCCCGCA CAAGCGGTGG AGCATGTGGT
 951 TTAATTCGAT GCAACGCGAA GAACCTTACC TACTCTTGAC ATCCAGAGAA
1001 TTTGCTAGAG ATAGCTTAGT GCCTTCGGGA ACTCTGAGAC AGGTGCTGCA
1051 TGGCTGTCGT CAGCTCGTGT TGTGAAATGT TGGGTTAAGT CCCGCAACGA
1101 GCGCAACCCT TATCCTTTGT TGCCAGCACG TAAGGTGGGA ACTCAAAGGA
1151 GACTGCCGGT GATAAACCGG AGGAAGGTGG GGATGACGTC AAGTCATCAT
1201 GGCCCTTACG AGTAGGGCTA CACACGTGCT ACAATGGCGT ATACAAAGAG
1251 AAGCGAACTC GCGAGAGCAA GCGGACCTCA TAAAGTACGT CGTAGTCCGG
1301 ATTGGAGTCT GCAACTCGAC TCCATGAAGT CGGAATCGCT AGTAATCGTA
1351 GATCAGAATG CTACGGTGAA TACGTTCCCG GGCCTTGTAC ACACCGCCCG
1401 TCACACCATG GGAGTGGGTT GCAAAAGAAG TAGGTAGCTT AACCTTCGGG
1451 AGGGCGCTTA CCACTTTGTG ATTCATGACT GGGGTGAAGT CGTAACAAGG
1501 TAACCGTAGG GGAACCTGCG GTGGATCACC TCCTT
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Figure 1. 16S rRNA gene partial sequence (1500 nts) for the novel bacterium as determined by MIDI Laboratories.

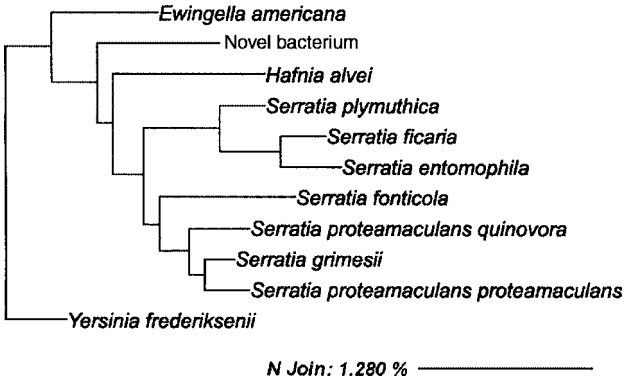

Figure 2. Phylogenetic analysis based on the partial 16S rDNA fragment determined by MIDI Laboratories.

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

Days of fermentation	Total phenolic content (mg of GAE kg^{-1})		
	Control	Fermentation	pH simulation
0	1682.5 ± 35.5	1587.2 ± 48.8	1052.7 ± 19.9
1	1251.3 ± 278.7	3640.3 ± 201.1	989.8 ± 44.8
2	1541.9 ± 119.1	3746.6 ± 146.4	907.1 ± 26.9
3	1507.5 ± 37.7	3479.4 ± 120.0	1026.3 ± 121.9
4	1321.6 ± 163.6	3690.3 ± 144.7	985.3 ± 9.3
5	1155.9 ± 269.1	4105.9 ± 334.4	993.6 ± 29.4
7	1470.0 ± 44.6	3926.3 ± 194.3	1014.4 ± 38.9

day 7. Total soluble solids, measured in °Brix units, decreased from day 1 (6.40 ± 0.05) to day 7 (3.87 ± 0.11) of fermentation. A decrease in pH value from day 1 (4.73 ± 0.01) to day 3 (3.31 ± 0.05), followed by a gradual increase on day 7 (5.09 ± 0.17), was also noticeable. Controlled fermentation simulating pH variation, but without inoculation, showed no significant increase in total phenolics ($p > 0.05$) (Table 1), indicating that increase in total phenolics during fermentation was not attributed to pH variation.

Radical scavenging activity

Samples collected from blueberry fermentation with the novel bacterium were studied for radical scavenging activity (RSA), using the DPPH method (Fig 3). Increase in RSA was not only associated with an increase in total phenolics during fermentation, but also with a change in the phenolic profile, resulting in the production of phenolic compounds with better antioxidant capacity, as shown in Fig 3a and b. However, during the first 3 days of fermentation, increase in RSA for days 1 and 2 was followed by a decrease for day 3 (Fig 3c and d). This loss in RSA on day 3 of the fermentation can be attributed to an alteration of the phenolic profile, but not to a loss in total phenolics (Fig 3c and d).

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

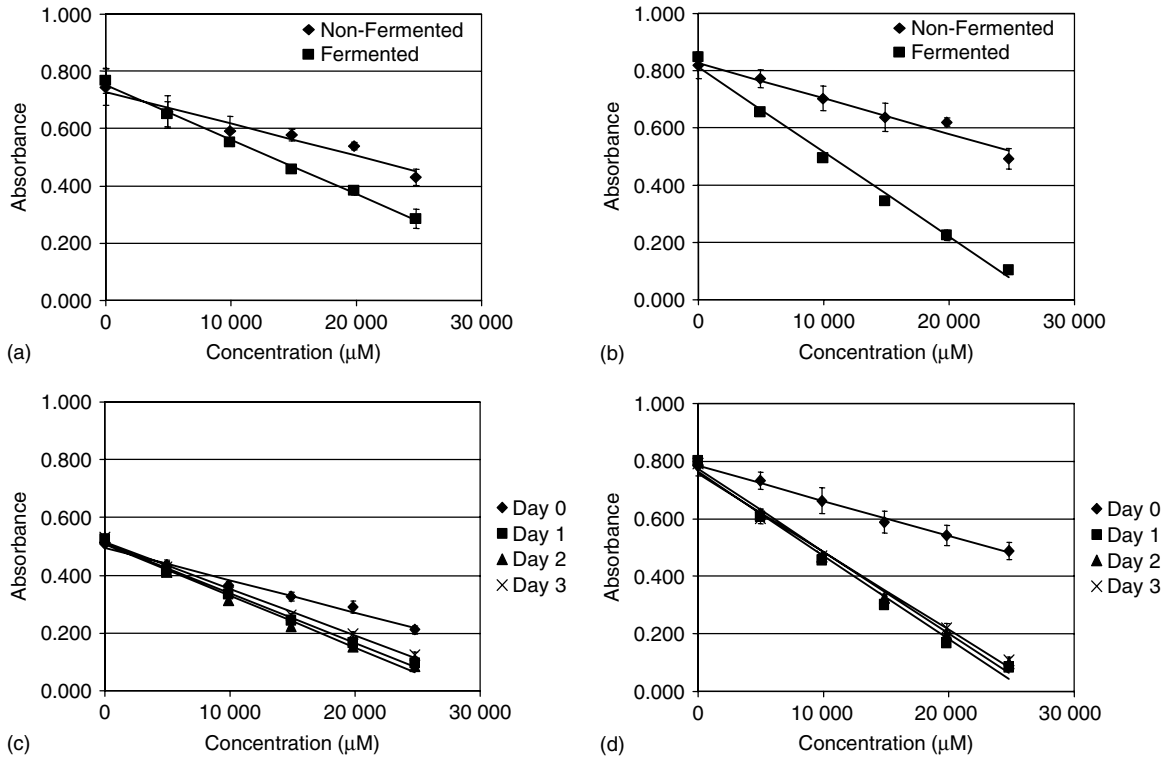

Figure 3. Radical scavenging activity (RSA) of wild blueberry fermented by novel bacterium, using the DPPH method. Each plot indicates the absorbance in relation to various concentrations of blueberry sample in gallic acid equivalents. The steeper the plot, the greater is the RSA. (a) Difference in RSA between fermented and non-fermented blueberry samples adjusted at the same total phenolic concentrations after 3 days of fermentation with the novel bacterium. (b) Difference in RSA between fermented and non-fermented blueberry samples with different total phenolic concentrations after 3 days of fermentation with the novel bacterium. (c) Increase in RSA of fermented blueberry samples adjusted at the same total phenolic concentrations for 3 days of fermentation with the novel bacterium. (d) Increase in RSA of fermented blueberry samples with increase in total phenolic concentrations for 3 days of fermentation with the novel bacterium.

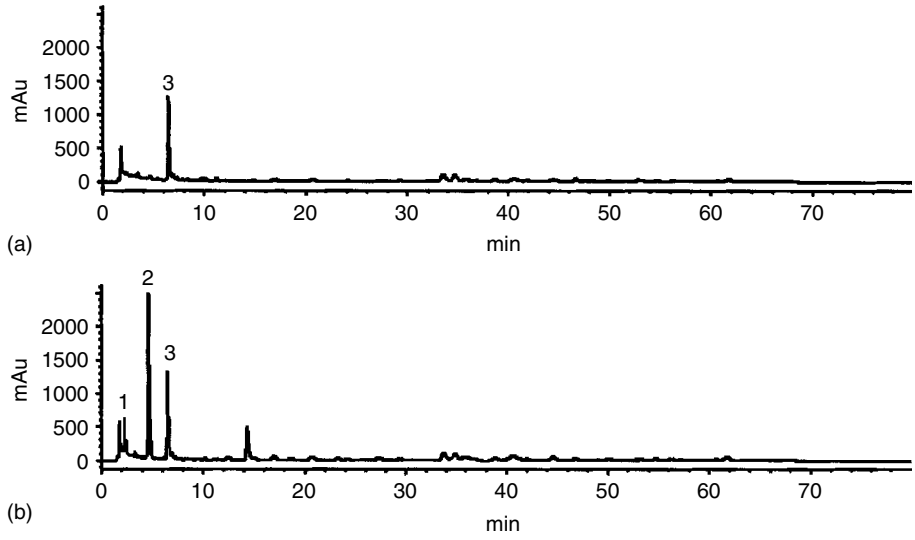

Figure 4. HPLC phenolic profiles for non-fermented blueberry after 3 days of incubation at 22 °C (**a**), and for fermented blueberry with novel bacterium after 3 days of incubation at 22 °C (**b**). Detection is made at a wavelength of 270 nm. Peaks of interest are: (1) gallic acid, (2) unknown phenolic acid, (3) chlorogenic acid.

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 $(64.6 \pm 0.5)\,\mathrm{mg\,kg^{-1}}$ FW on day 3 of blueberry fermentation (peak 1, Fig 4). The content of chlorogenic acid after 3 days of blueberry fermentation was $(852.7 \pm 2.8)\,\mathrm{mg\,kg^{-1}}$ 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 $\mathrm{kg^{-1}}$ 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.[34] 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.[35] 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.[36] 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.[28,37] Microorganisms found in the rhizosphere that are capable of flavonoid degradation include *Rhizobia*, *Agrobacterium*, *Pseudomonas*, *Bacillus* and *Rhodococcus* spp.[37] 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.[32,38] The most detailed study concerning the antioxidant potential of phenolic compounds from vegetables has been completed by this method,[39] and results have been confirmed by nuclear magnetic resonance.[40] 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.[41] Because *p*-coumaric acid is a precursor 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 deglycosylation in order to provide energy for bacterial growth. As determined by HPLC at 520 nm, blueberry fermentation resulted in the partial preservation of anthocyanin content of blueberry samples, as only $24.1 \pm 2.2\%$ of total anthocyanins were lost following blueberry fermentation by the novel bacterium compared to a net loss of $68.8 \pm 1.3\%$ of total anthocyanins in control, after 7 days of fermentation. Increase in anthocyanins stability in fermented blueberry could be attributed to acidification of the media, as shown in blueberry products.[42] As shown by HPLC at 365 nm, no major changes in flavonols contents were observed during the process of blueberry fermentation.

UV spectra for the unknown compound confirmed that it could not be of a flavonoid structure, such as anthocyanin or flavonol, because of the absence of absorbance in the 520 nm and 365 nm regions respectively. It is concluded that the unknown compound obtained from fermentation of blueberry with the novel bacterium could be of a phenolic or phenylpropanoic acid structure.

In summary, the results of this study indicate that a novel bacterium related to the Enterobacteriaceae and isolated from the normal flora of blueberry is able to enhance the increase in total phenolics after fermentation, and consequently affect the antioxidant activity of the fruit. The increase in the radical scavenging activity may be related not only to the increase in total phenolics, but also to a change in the phenolic profile, as demonstrated by the production of gallic acid and a novel compound. Production of gallic acid during fermentation could be indicative of hydrolyzable tannin degradation by the novel bacterium. From the UV profile and retention time of the novel compound isolated from HPLC, it is concluded that it could be of phenolic or phenylpropanoic acid structure, possibly conjugated with a sugar moiety. Further analyses by mass spectrometry are necessary, and in progress, to characterize and confirm the identity of this novel compound.

ACKNOWLEDGEMENTS

This project was financially supported by the Canada Fund for Innovation, the Atlantic Innovation Fund, and University of Moncton Research Fund.

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