

Blueberries: Genotype-dependent variation in antioxidant, free-radical scavenging, and prebiotic activities

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It is well known that the dietary intake of blueberries has a positive and profound impact on human health, performance, and disease. The total phenolic contents (TPC), antioxidant and free radical-scavenging properties of aqueous extracts prepared from the berries of two rabbiteye (*Vaccinium ashei*) and two highbush (*V. corymbosum*) genotypes of blueberry, were studied *in vitro*. The results showed that for the highbush genotypes, extracts from the 'Jersey' cultivar had higher TPC than extracts from 'Dixi' berries. Among the rabbiteye genotypes, 'Tifblue' had higher TPC than 'Ono'. Antioxidant and radical-scavenging activities among the rabbiteye and highbush genotypes were in accordance with the results of TPC for all genotypes studied which indicates that the antioxidant activity is related to the TPC.

An animal model was used to assess the effects of orally administered aqueous berry extracts on the proliferation of lactobacilli and bifidobacterial species and some undesirable bacteria in the caeca of rats. Gavaging rats with aqueous extracts from inulin, 'Ono', 'Tifblue' and 'Jersey' genotypes but not 'Dixi' 3 times weekly for 4 weeks resulted in a significant increase in the numbers of bifidobacteria while the extracts from all the genotypes but not from inulin were able to significantly increase the numbers of lactobacilli in comparison with the control group gavaged with water. A significant decrease in the numbers of bacteroides and clostridia was observed in the caeca of rats gavaged with the blueberry genotypes when compared with the water-gavaged control rats. Moreover, rats gavaged with inulin, 'Ono', 'Tifblue', 'Jersey' and 'Dixi' showed 15.1%, 10.5%, 21.9%, 22.9% and 5.6% reduction in the activity of β -glucuronidase and 17.6%, 27.2%, 49%, 25.2% and 15.8% increase in the activity of β -glucosidase when compared to the control group gavaged with water, respectively. In conclusion, significant differences were found in antioxidant/antiradical activities and TPC among various genotypes of blueberries. Furthermore, we hypothesize that blueberry extracts could positively modify the bacterial profile by increasing the numbers of beneficial bacteria and thereby improve gut health.

Keywords: blueberry extract; antioxidant activity, total phenolic content, prebiotic activity; lactobacilli; bifidobacteria; antimicrobial activity.

Introduction

Many epidemiological studies have shown an inverse association between fruit consumption and reduction of the risk of various lifestyle diseases such as obesity, cardiovascular diseases, diabetes, Parkinson's disease, Alzheimer's disease and various types of cancers [1, 2]. Many studies have suggested that the phytochemical content of vegetables and fruits, such as polyphenols, vitamins and carotenoids, that have shown potent antioxidant/free radical scavenging activities are behind those health-promoting properties [3-4].

The human colon is a mobile bioreactor that harbours a large and complex microflora and it is now generally accepted that the composition of the human intestinal microbiota has an important role in health and disease [5]. Positive modification of the human intestinal microbiota can be achieved by: inclusion in the diet of a significant proportion of beneficial bacteria, mainly *Bifidobacterium* and *Lactobacillus* species, with the expectation that they will be able to colonise the intestinal tract (probiotics); administration of non-digestible food ingredients (prebiotics) such as fructo-oligosaccharides that beneficially affect the host by selectively stimulating the growth of desirable probiotic bacteria; or administration of symbiotics, which are probiotics and prebiotics used in combination [6-7].

Unlike probiotics, which are living microorganisms, prebiotics are generally carbohydrates that bypass the small intestine. Prebiotics are often especially intended to stimulate the growth of bifidobacteria because of the presumed beneficial role of bifidobacteria on gastrointestinal health [8]. Inulin, a fructo-oligomer isolated from chicory roots, has been shown to stimulate the growth of bifidobacteria in several well-designed *in vitro* studies using either pure bacterial cultures or undefined inocula of gastrointestinal origin [9]. It has also been demonstrated that dietary consumption of non-digestible fructo-oligosaccharides (FOS) has a positive impact on the cell numbers of bifidobacteria in humans [10]. Currently available prebiotics, such as FOS and inulin, can aid in the survival and proliferation of probiotic bacteria, but are limited by some side effects [11]. Therefore, there remains a need for alternative prebiotics with fewer or no side effects that could either be incorporated in a probiotic food matrix, or used as a stand-alone prebiotic to enhance proliferation of lactic acid bacteria naturally present in the intestine.

Blueberries (*Vaccinium* spp.) are rich sources of bioactive compounds, such as phenolics and organic acids, which have antimicrobial activities against human pathogens [12-13]. Several components in blueberry fruit including anthocyanins [14] have been shown to have antioxidant activity. Recent research conducted by Lau et al. [15] has shown that nutritional antioxidants, such as the polyphenols found in blueberries, can reverse age-related declines in

neuronal signal transduction as well as cognitive and motor deficits. Other studies have demonstrated for the first time that polyphenolic compounds are able to cross the blood-brain barrier and localize in various brain regions important for learning and memory [16]. Kraft et al. [17] reported that lowbush blueberries contain a range of compounds such as phytosterols, phenolic acids, flavan-3-ols, anthocyanins, and oligomeric proanthocyanidins that protect against the initiation, promotion, and progression stages of carcinogenesis and that different compounds are effective against each of these stages. Wang et al. [18] reported that rats pretreated with blueberry, spinach, and spirulina diets for 4 weeks had reduced cerebral infarction after ischemia and reperfusion and they concluded that these diets have neuroprotective effects against transient focal ischemia. Recently, Molan et al. [19] studied the satiating effects of aqueous extracts from two blueberry genotypes ('Maru' and 'Centurion') and found that extracts from both genotypes had a satiating influence on experimental rats, as evidenced by their ability to decrease food intake by 8.6% and 6.2%, respectively. In addition, body weight gain of rats gavaged with extracts from 'Maru' and 'Centurion' genotypes decreased by 9.2% and 5.3% relative to the rats in the control group, respectively. The authors concluded that the reduction in food intake when compared with control rats preloaded with the same volume of water suggests that the decrease in food intake was mainly a consequence of a satiating effect, rather than a stomach distension effect.

In this chapter we present the results of some studies which have been conducted to explore the antioxidant properties of water-soluble extracts from the berries of 4 different blueberry genotypes and the total phenolic compounds (TPC). Moreover, the influence of extracts from these genotypes and from inulin on the numbers of bifidobacteria, lactobacilli, bacteroides, and clostridial numbers in the caecal contents of rats gavaged orally with these extracts was evaluated using fluorescence in situ hybridization (FISH) molecular method. In addition, the effect of these extracts on the activity of selected metabolic indexes (β -glucuronidase and β -glucosidase enzymes) in rats was also investigated.

Materials and methods

Chemicals and standards

2,4,6-Tripyridyl-s-triazine (TPTZ), sodium acetate, ferric chloride, gallic acid, Folin-Ciocalteu's phenol reagent and ferrous sulfate were purchased from Sigma (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW 1765, Australia).

Preparation of extracts

Blueberry fruits used in this study were grown at commercial farms in Hamilton, New Zealand. All berries were picked at the commercially ripe stage. The berries were maintained in polyethylene bags at -20 °C until extract preparation. Crude aqueous extracts from two rabbiteye blueberry (*Vaccinium ashei*) genotypes ('Ono' and 'Tifblue') and two highbush (*V. corymbosum*) genotypes ('Dixi' and 'Jersey') were prepared by weighing frozen fruits (100 g), mixing with 100 ml of distilled water and then milling with a commercial mini-processor (Braun Miniprimer MR300, Germany).

The crushed berries were centrifuged (3000g, 15 min) and the clear supernatant fluid was collected and used within 1 h of collection. Inulin powder (Sigma, Australia) was dissolved in sterile distilled water. For rat trial, all the extracts were prepared on the same day of gavaging.

Determination of total phenolic content (TPC)

The total phenolic content (TPC) was quantified according to the method of Molan et al. [19]. Briefly, an aliquot of 12.5 μ l of water-soluble extract was mixed with 250 μ l of 2% sodium carbonate solution in 96-well microplates and allowed to react for 5 minutes at room temperature. Then 12.5 μ l of Folin-Ciocalteu phenol reagent (50 %) was added and allowed to stand for 30 minutes at room temperature before the absorbance of the reaction mixture was read at 650 nm using a plate reader. Calibration was achieved with an aqueous gallic acid solution (100-1000 μ g/ml). The TPC of the extract was expressed as mg gallic acid equivalent (GAE) per gram of blueberry fruits and all determinations were performed in triplicate in two separate experiments.

Evaluation of antioxidant activity

The antioxidant capacity of blueberry extracts was determined using the FRAP assay, a colorimetric assay that measures the ability of the tested sample to reduce the intense blue ferric tripyridyltriazine complex to its ferrous form, thereby changing its absorbance [19]. Briefly, an aliquot of 8.5 μ l of extract was added to 275 μ l of diluted FRAP reagent using a microplate and the plates were incubated at 37 °C for 30 minutes before measuring the absorbance at 395 nm using a plate reader. A standard curve was prepared using different concentrations (200-2000 μ mol/L) of FeSO₄·7H₂O. The antioxidant capacity based on the ability to reduce ferric ions of the extract was expressed as

micromole FeSO₄ equivalents per litre of aqueous extracts. All solutions were used on the day of preparation and all determinations were performed in triplicate.

Evaluation of free radical scavenging activity using DPPH discoloration assay

The DPPH assay detects scavenging of free radicals by the tested compound through the scavenging activity of the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical. This assay was performed using a previously described method [19] with some minor modifications. Briefly, 25 µL of blueberry extract was allowed to react with 250 µL of 0.2 mM DPPH in 95% ethanol in a 96-well microplate. The plate was then incubated at room temperature (21 °C) for 30 minutes in the dark after which the absorbance was measured at 550 nm using a microplate reader.

The antiradical activity was calculated as a percentage of DPPH decolouration relative to a negative control using the following equation:

Antiradical activity (%) = (absorbance of control incubation – absorbance of the blueberry extract/ absorbance of control incubation) x 100.

The degree of discoloration indicates the free-radical scavenging efficiency of the extract.

Assessment of prebiotic effects of blueberry extracts

Sixty female Sprague Dawley rats aged ten weeks were housed individually in hanging wire-mesh stainless steel cages in a room with a temperature of 22 ± 1 °C and a 12-h light:dark cycle. The animal protocol was approved by Massey University Animal Ethics Committee.

The rats were divided into 6 equal groups (n = 10). Rats in the first group (negative control) were gavaged 3 times weekly for 4 weeks with 4 ml of distilled water/kg body weight whereas those in the second group were gavaged with 1 ml of inulin/kg (positive control). Rats in the third, fourth, fifth and sixth groups were gavaged with 4 ml of extract/kg prepared from 'Ono', 'Tifblue', 'Jersey' and 'Dixi' cultivar 3 times weekly for 4 weeks. The diet was standardized across all treatments and was formulated to meet the nutrient requirements of growing rats (AIN-93G).

The dose was administered to the rat via gavage (at the back of the throat) using a very soft silicon rubber tube attached to a 3-ml syringe. The amount of the dose given to each rat was individually calculated based on body weight, at a rate of 4 ml (diluted extract)/kg body weight. This rate was selected based on the assumption that 60 kg adult person could reasonably consume a 120 ml volume of concentrated juice, which is equivalent to 2 ml/kg body weight, which can be translated to 4 ml of diluted (50%) extract per kg body weight in the rat.

At the end of the trial, rats were euthanized under CO₂ and the caeca were removed, labelled and stored at -80 °C until use. The numbers of caecal lactobacilli and bifidobacteria were counted using fluorescent *in situ* hybridization (FISH) method.

Fluorescence in situ hybridization analysis of microbiota in rat caecal contents

Bacterial measurement was undertaken at the end of the experiment using fluorescent in situ hybridization (FISH) method. The probes used in the study were specific for *Lactobacillus spp.*, *Bifidobacterium spp.*, *Clostridium spp.*, and *Bacteroides spp.* These were commercially synthesized and labelled with the fluorescent dye Cy3 (GeneWorks, Australia). The procedure described by Dinoto et al. [20] was followed with some modifications [21].

Bacterial enzyme activities

The β-glucuronidase and β-glucosidase activities were determined aerobically according to the method of Goldin et al. [22] and as described by Molan et al. [21] with some modifications.

Data analysis

Caecal concentrations of bacteria were expressed as log number of bacterial cells/g caecal content wet weight. Logarithmically-transformed data were analysed by one way analysis (ANOVA) of variance using SAS system with the level of significance set at P < 0.05.

Results and Discussion

Total phenolic contents and antioxidant activity

Significant differences were found in antioxidant activity and total phenolic contents (TPC) among various genotypes of blueberries. Among highbush genotypes, 'Jersey' showed significantly higher TPC (P < 0.0001) and antioxidant activity (as measured by FRAP assay; P < 0.0001) than 'Dixi' cultivar (Fig.1). Among the rabbiteye genotypes, 'Tifblue' showed higher TPC (P < 0.0001) and higher FRAP values (P < 0.0001) than 'Ono'. In general, rabbiteye

genotypes had significantly higher TPC ($P < 0.01$) and higher FRAP values ($P < 0.0001$) than highbush ones. Genotype specific variations in TPC have been previously assessed [4, 23]. Ehlenfeldt and Prior [24] studied the chemical composition of 87 highbush blueberry genotypes and varieties and estimated 1.78 mg/g as a mean TPC for these genotypes. Recently, Skupien [25] studied the chemical composition of four highbush blueberry genotypes and reported that the TPC ranged from 1.94-3.07 mg/g fresh fruits. Many factors, such as the cultivars, variety, cultivation, region, environmental conditions, ripeness, harvesting time, storage time and condition, determine the content of polyphenolic compounds [2, 4, 19].

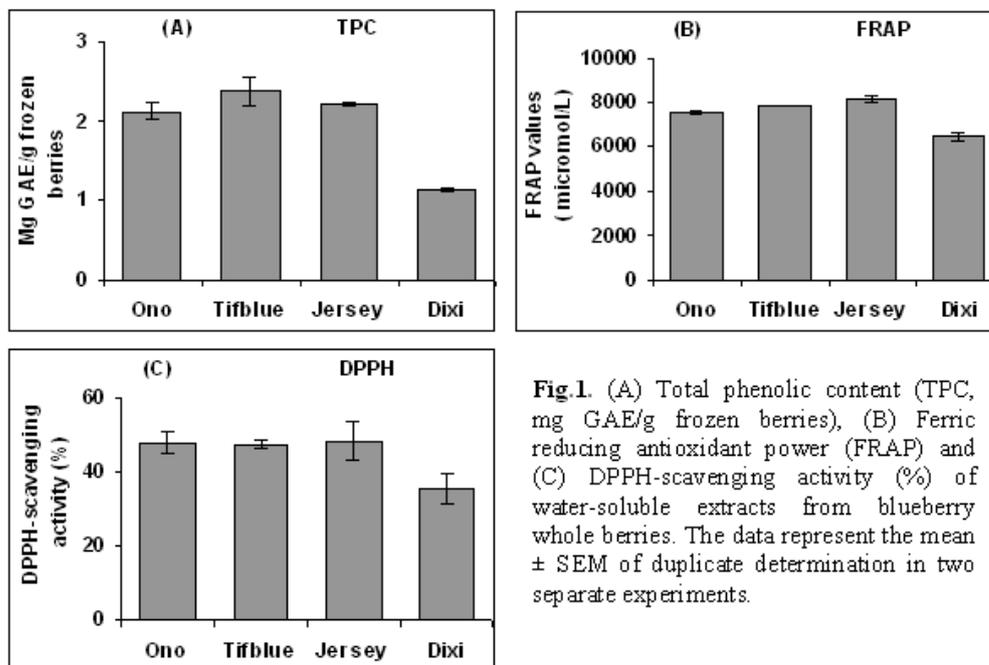


Fig.1. (A) Total phenolic content (TPC, mg GAE/g frozen berries), (B) Ferric reducing antioxidant power (FRAP) and (C) DPPH-scavenging activity (%) of water-soluble extracts from blueberry whole berries. The data represent the mean \pm SEM of duplicate determination in two separate experiments.

In other studies, significant differences in antioxidant activity among blueberry cultivars [4, 26] were observed.

A positive correlation between the TPC and the antioxidant activity (FRAP values) in all blueberry genotypes was observed (data not shown), confirming the findings previously observed for several genotypes of berries [4, 26]. Recently, Castrejon et al. [27] reported that phenolic compounds other than anthocyanins in highbush blueberry fruits have contributed positively to the total antioxidant activity and suggested to implement that in future breeding programs.

The ability of the extracts from different genotypes to donate hydrogen was evaluated using the stable free radical DPPH. In the presence of hydrogen donors, DPPH is reduced and a stable free radical is formed from the scavenger. It is well known that free radicals are the major cause of various chronic and degenerative diseases such as aging, coronary heart disease, inflammation, stroke, diabetes mellitus and cancer and can cause cellular injuries and initiate peroxidation of polyunsaturated fatty acids in biological membranes [28].

Among highbush genotypes, 'Jersey' showed significantly higher ($P < 0.0001$) free radical scavenging activity in the DPPH assay (55%) than 'Dixi' cultivar (36%). Among the rabbiteye genotypes, 'Tifblue' had the highest free radical scavenging activity (56%) while 'Ono' showed the lowest (50%). No significant differences ($P > 0.05$) were observed between the four genotypes. In general, rabbiteye genotype had significantly higher ($P < 0.05$) free radical scavenging activity than highbush genotype. The presence of variable quantities of diverse phytochemicals, other than polyphenolic compounds with potent antioxidant activities in fruits including blueberries may be behind the differences in the degree of correlation between the total phenolic contents and antioxidant/antiradical activities in different blueberry genotypes.

Prebiotic and antimicrobial activities

The probe technology can provide information on bacterial populations at the genus, group, and even species levels in terms of identification and enumeration. Fluorescence in situ hybridization is a powerful method for the enumeration of bacteria in complex habitats such as the gut. Most notably, it does not require cultivation of the target organisms. This method was used in this study to enumerate the good and bad bacteria in caecal contents of rats gavaged with blueberry extracts and inulin.

Gavaging rats with aqueous extracts from inulin and from 'Ono', 'Tifblue' and 'Jersey' genotypes but not 'Dixi' 3 times weekly for 4 weeks resulted in a significant increase in the numbers of bifidobacteria ($P < 0.01-0.0001$) while the extracts from all the genotypes but not from inulin were able to significantly increase the numbers of lactobacilli ($P < 0.05-0.001$) in comparison with the control group gavaged with water (Fig. 2).

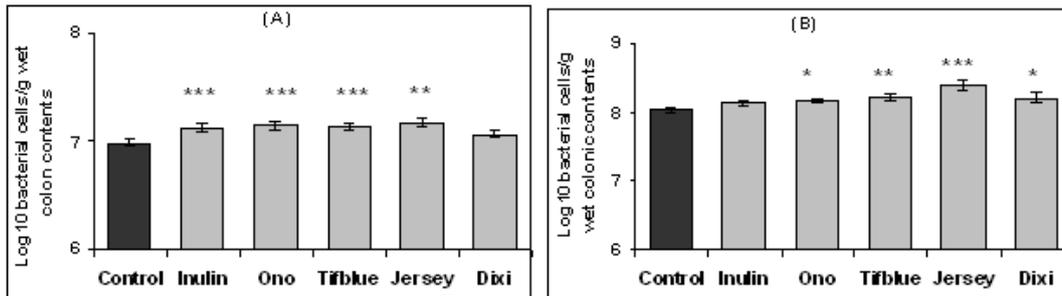


Fig. 2. Enumeration of *Bifidobacterium* species and *Lactobacillus* species (Log₁₀ cells/ml) from the six groups of rat cecal samples hybridized with genus-specific oligonucleotide probes (Bif164 and Lab158, respectively) in FISH analysis. The rat groups were gavaged with water (negative control), inulin (positive control) or with aqueous blueberry extracts from Ono, Tifblue, Jersey or Dixi three times weekly for 4 consecutive weeks. The data are expressed as means ± standard errors of the means (n = 10 rats/group). * P ≤ 0.05; ** P ≤ 0.01; P < 0.0001 by analysis of variance versus the negative control group.

A number of studies have shown that increasing the numbers of lactic acid bacteria (lactobacilli and bifidobacteria) in the colon reduces the formation of ammonia, skatole, harmful amines and other procarcinogens in the large intestine and the carcinogenic load on the intestine of humans [29-30]. Moreover, it has been found that modification of the gut microflora may interfere with the process of carcinogenesis and this opens up the possibility for dietary modification of colon cancer risk. Probiotics and prebiotics, which modify the microflora by increasing numbers of lactobacilli and/or bifidobacteria in the colon, have been a particular focus of attention in this regard [29].

The bifidogenic activity of inulin found in the present study matches other studies performed in rats and humans. Many human intervention studies had previously found a bifidogenic effect of inulin [31]. This gives a strong indication of the validity of the results obtained in this study.

Gavaging rats with blueberry extracts or inulin also resulted in a significant reduction (P < 0.01-0.0001) in the numbers of some pathogenic bacteria (such as *Clostridium spp* and *Bacteroides spp.*) (Fig. 3). To our knowledge, only two previous studies [12-13], have evaluated the antimicrobial effects of berry extracts on Gram-positive and Gram-negative bacteria. Lactobacilli and bifidobacteria species were tested as examples of Gram-positive bacteria. The authors concluded that the growth of *Lactobacillus* strains was not inhibited by any of the berry extracts at low concentrations (1 mg per ml). However, when five times higher concentrations of raspberry and blueberry extracts were used, growth of the selected *Lactobacillus* strains was inhibited by raspberry extract and slightly inhibited by blueberry extract. *Bifidobacterium lactis* was slightly inhibited by raspberry, strawberry and cloudberry extracts at low concentrations (1 mg per ml).

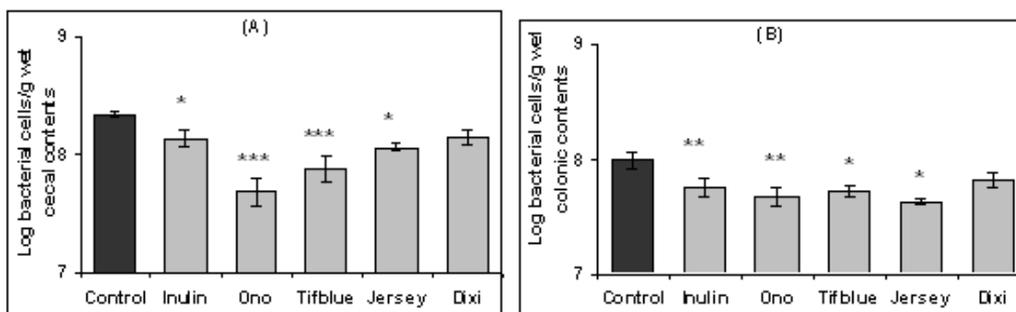


Fig. 3 Enumeration of bacteroides (A) and clostridia (B) [Log₁₀ cells/g of wet caecal contents] from the six groups of rat caecal samples hybridized with genus-specific probes in FISH analysis. The rat groups were gavaged with water (negative control), inulin (positive control) or with aqueous blueberry extracts from Ono, Tifblue, Jersey or Dixi three times weekly for 4 weeks. Data represent means ± SEM (n = 10 rats/group). * P ≤ 0.05; ** P ≤ 0.01; ***P < 0.0001 by ANOVA versus the negative control group.

Bacterial enzymes

The results have provided the first evidence that consumption of blueberry extracts can beneficially affect faecal parameters related to colon cancer risk. Under the present experimental conditions, rats gavaged with inulin, 'Ono', 'Tifblue', 'Jersey' and 'Dixi' showed 15.1%, 10.5%, 21.9%, 22.9% and 5.6% reduction in the activity of β-glucuronidase when compared to the control group gavaged with water, respectively (Fig.4A). It has been reported that certain bacteria (such as *Escherichia coli*, *Clostridium perfringens* and *Bacteroides* species) have high β-glucuronidase activity [32]. Moreover, this result may explain the significant increase in the numbers of bifidobacteria and lactobacilli in the same caecal samples. Results from some studies with probiotics have shown that increasing the proportion of the

fecal flora represented by bifidobacteria is associated with lower activity of reductive enzymes including caecal β -glucuronidase, and inhibition of the development of Azoxymethane (AOM)-induced colon carcinogenesis [33].

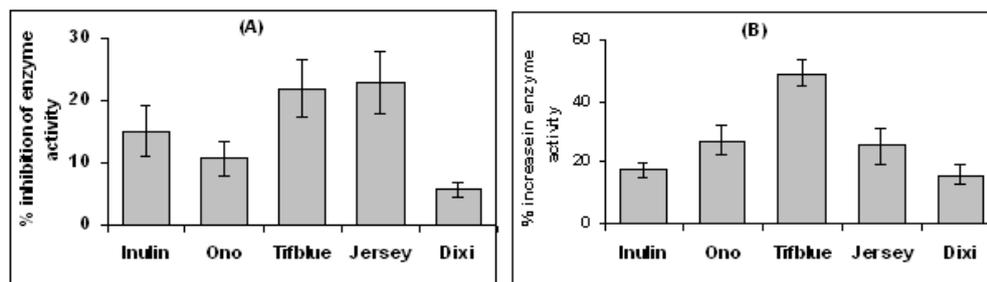


Fig. 4. (A) % reduction in the activity of β -glucuronidase and (B) % increase in the activity of β -glucosidase in caecal contents of rats gavaged with inulin (positive control) or with aqueous blueberry extracts from Ono, Tiffblue, Jersey or Dixi three times weekly for 4 consecutive weeks, relative to the control group gavaged with water. The data are expressed as means \pm standard errors of the means (n = 10 rats/group).

The importance of this finding is generated from the fact that the bacterial enzyme β -glucuronidase is considered to be one of the enzymes that increases risk for colorectal cancer [34]. It has been suggested that β -glucuronidase is a key enzyme in the activation of the procarcinogen 1, 2-dimethylhydrazine (DMH) into its toxic carcinogen [35]. Therefore, β -glucuronidase has assumed significance as colon cancer risk marker and its inhibitors have been suggested to have anticarcinogenic properties because they will increase clearance of glucuronidated carcinogens [36]. Accordingly, lower activity of this enzyme can be considered beneficial in terms of the risk of colon cancer. Some studies have shown that certain tumours and some bacteria have high β -glucuronidase activity [37], and that such tumours or bacterial infections may be treated by means of β -glucuronidase inhibitors which are toxic to the tumour cell or bacterial cell. This may suggest that blueberry extracts are good candidates for cancer prevention.

Relative to the control group, the level of β -glucosidase (an enzyme generated mainly by lactobacilli and bifidobacteria) was increased significantly ($P < 0.05$) in the caecal contents of the rats gavaged with either blueberry extracts or inulin when compared with the control group (Fig. 4B).

The increment in the activity of β -glucosidase probably due to the stimulation of the growth of lactic acid bacteria, which have high levels of β -glucosidase activity in comparison with the other members of the gut microflora [38]. Previous studies have shown that oligosaccharides have the ability to increase the activity of this enzyme and this has been attributed to their ability to stimulate the growth of lactic acid bacteria [38-39]. Marteau et al. [40] found an increase in faecal β -glucosidase activity in subjects consuming milk fermented with *Lactobacillus acidophilus* and *Bifidobacterium bifidum*.

In addition, the pH of the caecal contents collected from rats gavaged with blueberry extracts and inulin was significantly lower ($P < 0.0001$) than that of the control group (data not shown). The lower pH in the caecal samples of rats given blueberry extracts, compared to the controls, may explain the capacity to promote the growth of lactic acid bacteria. Again, the results of the present study have provided the first evidence that consumption of blueberry extracts decreases the caecal pH which can be considered beneficial in terms of the risk of colon cancer. An association between high colonic (or stool) pH and increased risk of colon cancer has been reported, suggesting that colonic neoplasia might be related to a reduction in the fermentation of dietary polysaccharides to organic acids in the large bowel [41].

Conclusions

This study has identified extracts from some blueberry genotypes as good prebiotic agents that can significantly promote the growth of friendly bacteria such as bifidobacteria and lactobacilli, and lower the numbers of undesirable bacteria such as bacteroides and clostridia in the cecum of rats. This study also showed that blueberry extracts can decrease the activity of β -glucuronidase and increase the activity of β -glucosidase, which could be perceived as potentially beneficial for the host. These promising results need to be confirmed in human studies involving both healthy volunteers and subjects with clinical conditions that have a supposed microbial aetiology.

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