

ORIGINAL ARTICLE

## Antibacterial activity of berry juices, an *in vitro* study

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### Abstract

**Objective.** The aim of the present study was to evaluate *in vitro* antibacterial activities of blackcurrant and sea buckthorn juices on bacteria associated with gingival inflammation. **Materials and methods.** The growth of selected bacteria (*Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus sanguinis*, *Streptococcus gordonii*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*) was studied *in vitro* on agar plates. The content of phenols in the different extracts was measured with HPLC-ESI-MS. **Results.** The spectrometric analysis identified that the highest level of the single phenols studied was found for ferulic acid (113 µg/ml) in blackcurrant juice. Sea buckthorn contained low levels of selected phenols. Total bacterial inhibition for all bacterial species studied was found at 20% berry juice concentration with pH varying between 4.1–5.4. **Conclusions.** The present study identified that *in vitro* bacterial growth on agar plates was inhibited by blackcurrant and sea buckthorn juices and that low juice pH explains bacterial *in vitro* growth. This may have clinical implications in biofilm development, reducing the risks for both tooth decay and gingivitis.

**Key Words:** berries, microbiology, pH, polyphenols, periodontal disease

### Introduction

Wild and cultivated berries are rich in bioactive compounds that may be used for the prevention and/or treatment of different diseases. In adults, diets that have a high proportion of fruit and vegetables and with a low content of meat are associated with a highly diverse gut microbiota [1]. Data suggest that berry juice polyphenols can inhibit the adherence of bacteria to epithelial cells, suggesting that berry juice polyphenols might be protective for the host against infections [2].

The oral microbiota is diverse, including more than 600 bacterial species and with distinct sub-sets predominating at different habitats [3]. The infectious poly-microbial infectious etiology of periodontal diseases including gingivitis (a reversible inflammation) and periodontitis (a condition resulting in alveolar bone loss and tooth loss) are well established.

The establishment of a biofilm at tooth surfaces (dental plaque) is critical in the development of gingivitis. From a clinical perspective, gingivitis presents with swollen tissues and increased redness, but with

no loss of attachment between root surfaces and connective tissues and bone. Caries is an endogenous infection of the calcified tissues of the teeth and is a result of their demineralization by organic acids produced by bacteria that ferment dietary carbohydrates resulting in tooth decay, the other dominant oral disease in humans. The bacteria in both gingivitis and tooth decay development include mainly early colonizing aerobic bacteria with *streptococci* and *actinomyces* species as the dominating bacterial species. Whereas bacteria associated with periodontitis prefer a neutral or slightly basic pH, the microorganisms associated with the early development of gingivitis and tooth decay require and prefer a pH in the range of 4–5 [4,5]. There is extensive evidence that control of gingivitis is important in preventing the development of periodontitis. Nevertheless, there is a need for new information on the human microbiome needed to change our current concepts concerning the prevention, diagnosis and treatment of periodontal diseases [6].

The control of exaggerated inflammatory responses by modulation of the immune response may be

achievable through a natural process, including the intake of food rich in anti-inflammatory properties and an important method to manage periodontitis [7]. Antioxidant, anti-inflammatory and antibacterial effects of plant extracts have been documented. In the human diet, berries are particularly rich in polyphenols comprising phenolic acids, flavonoids (anthocyanins, flavonols and flavanols), condensed tannins (proanthocyanidins), hydrolysable tannins (ellagitannins and gallotannins), stilbenes and lignins. The main flavanols are catechins with well-known anti-inflammatory properties [8]. Chlorogenic acid, ferulic acid and protocatechuic acid belong to the group phenolic acids, which in cell and animal studies also have shown anti-inflammatory properties [9–11]. Quercetin is present in many fruits and vegetables and is the main flavonol in our diet [12]. Berry polyphenol composition is highly variable both qualitatively and quantitatively. Some of the compounds are ubiquitous, whereas others are restricted to specific families or species. Within berry species, large variations may occur, particularly because of genetic factors (cultivar), environmental conditions, growth or maturation stages of the berries.

There are no data on oral effects from dietary intake of blackcurrant and sea buckthorn. Studies of medicinal plants such as *Caesalpinia bonducella*, *Gardenia gummifera* and *Acacia arabica* have showed remarkable antibacterial potential against several bacterial species including *P. aeruginosa* and *S. aureus* [13]. Methanol extracts from juniper berries appear to have effects on bacteria [14]. Antimicrobial properties of berries have been reported [15]. Streptococci species colonize the oral cavity early in life [16] and participate in the early development of dental biofilms [17]. There are several different species of the Streptococcus family. *Streptococcus sanguinis* occurs normally in the mouth, where it modifies the environment to make it less hospitable for *S. mutans* and *S. gordonii*, which are commonly found in the human oral cavity and in dental plaque. *S. mutans* and *S. gordonii* ferment sugars. The acidity developed contributes to tooth decay. These bacteria are adapted to growth at pH <5. Consumption of polyphenol-rich berries formulated in a juice may reduce bacterial growth and gingival inflammation and tooth decay. *In vitro* data with regards to the effects of the concentration of berries on bacteria associated with tooth decay and periodontitis have not been studied.

The aim of the present study was to evaluate *in vitro* antibacterial activities of blackcurrant and sea buckthorn juices on selected bacteria that are commonly present in the oral cavity and associated with gingivitis. The objectives were to focus on the potential antibacterial effects by selected phenols and berry juice concentrations at pH concentrations <5.5.

## Materials and methods

### Preparation of berry juices

Blackcurrant and sea buckthorn berries were purchased from a local supplier. Frozen berries from each species were thawed and pressed. The prepared juices were filled in containers and disinfected by heating until the temperature reached 80°C.

### Analysis of phenols by HPLC-ESI-MS

The content of major single phenols in the different extracts was measured on a Perkin Elmer Sciex (Waltham, Massachusetts, USA) API 150EX Single Quadrupole mass spectrometer (with a Turbo Ion Spray interface) according to a modified method from that described by Salminen et al. [18]. The HPLC system consisted of two Perkin Elmer pumps connected to a Perkin Elmer autosampler (Serie 200). The compounds were separated using a Phenomenex Synergi 4 $\mu$  Hydro-RP 80A, 250  $\times$  4.6 mm column and a C<sub>18</sub> pre-column. The mobile phase consisted of 0.4% formic acid (Buffer A) and acetonitrile (Buffer B). The eluent was run at a flow rate of 1.0 mL/min and the gradient elution was as follows: 0% B (0–2.5 min), 30% B (2.5–10 min), 40% B (10–12.5 min), 60% B (12.5–18 min) and 0% B (18–23.5 min). The injection volume was for all samples 4  $\mu$ L. The eluent was split to 0.3 mL/min before being introduced to the ESI-MS system. The electrospray chamber was operated at 4.0 kV in the negative ion mode and mass ions obtained by acquiring data in peak jump and scan mode (90–1000 amu). Catechin and epicatechin was detected at m/z 289 (M-H)<sup>-</sup>, chlorogenic acid 353 (M-H)<sup>-</sup>, protocatechuic acid 153 (M-H)<sup>-</sup>, quercetin 301 (M-H)<sup>-</sup> and ferulic acid 193 (M-H)<sup>-</sup>. The general conditions for the quantitative HPLC-ESI-MS analyses were the following: nebulizer gas 9.0 L/min, curtain gas 12.0 L/min and dry gas temperature 300°C.

### Bacterial strains

Seven aerobic strains were selected, including *Streptococcus mitis* (CCUG 31611), *Streptococcus mutans* (CCUG 6519), *Streptococcus sanguinis* (CCUG 17826), *Streptococcus gordonii* (CCUG 25608), *Staphylococcus aureus* (CCUG 17621), *Staphylococcus epidermidis* (CCUG 39508) and *Pseudomonas aeruginosa* (CCUG 551). The strains were grown in Brain Heart Infusion broth (Oxoid Ltd, Basingstoke, Hampshire) at 37°C, 150 rpm in ambient air. The bacteria were frozen in 20% glycerol and kept at -80°C until used for study purposes.

### Antibacterial activity of berry juices

Bacterial growth was studied on agar plates containing different berry juice concentrations. As control

reference, bacterial growth was studied on brain heart infusion (BHI) agar. The inhibitory effects of the berry juice treated agar plates were calculated as the proportional growth relative to bacterial growth of species studied on the control non-treated agar plates.

The plates containing the juice were prepared by dissolving brain heart infusion (BHI) agar in distilled water and autoclaved. Different volumes of berry juice were added to the agar, mixed thoroughly and poured into petri dishes such that studies could be performed with a negative control and with 10% or 20% concentrations of the different berry juices. The pH of the different concentrations of the berry juices together with the growth media was measured with an electronic pH-meter. Viable counts were used to investigate bacterial growth. Following growth of the bacteria, the bacteria were diluted 5-fold and a 100 µl sample was added and spread over the surface of the agar plates containing the different concentrations of berry juice or control (dH<sub>2</sub>O). The plates were incubated aerobically at 37°C for 2 days. Following incubation the minimum inhibitory concentration was determined as the lowest concentration of berry juice that completely inhibited visible growth of the bacteria. Each experiment was performed with 10 replicates and the number of colonies growing on the plates was photographed and counted.

#### Statistical analysis

Descriptive statistics were used with mean values and standard deviations as appropriate.

## Results

#### Analysis of single phenols in blackcurrant or sea buckthorn juice

The juices were analyzed for content of single phenolic compounds using mass spectrometry. The content of catechin, epicatechin, chlorogenic acid, protocatechuic acid, quercetin and ferulic acid is presented in Table I. The present study identified that specifically ferulic acid (113.1 µg/ml) and chlorogenic (9.4 µg/ml) acid could be identified in the blackcurrant juice. In contrast, the sea buckthorn juice contained only minor traces of chlorogenic acid (0.9 µg/ml), protocatechuic acid (1.0 µg/ml) and quercetin (0.1 µg/ml).

#### Bacterial growth on agar plates prepared with blackcurrant or sea buckthorn

In a minimum inhibitory concentration assay at pH 7.0, both berry juices were assayed for their antibacterial activities. Neither *S. sanguinis*, *S. gordonii* nor *S. mitis* grew on sea buckthorn agar at any

Table I. Content of major single phenols in different berry juices.

Polyphenol	Blackcurrant	Sea buckthorn
Catechin	3.6	0.0
Epicatechin	2.0	0.0
Chlorogenic acid	9.4	0.9
Protocatechuic acid	0.7	1.0
Quercetin	0.7	0.1
Ferulic acid	113.1	0.0

Data are presented as µg/ml extract.

concentration. Only *S. sanguinis* could be detected in blackcurrant juice at a juice concentration of 10% and with an average inhibition of 75% relative to the growth of *S. sanguinis*.

The data demonstrated that, at pH 7.0, *S. mutans* could be detected in the growth medium with sea buckthorn at 10% and 20% concentrations. At pH 5.4, *S. mutans* could be identified at the 10% sea buckthorn concentration. No growth of *S. mutans* could be detected at pH 4.4 for any concentration of sea buckthorn studied.

Bacterial growth of *S. aureus*, *S. epidermidis*, *S. mutans* and *P. aeruginosa* could be detected at a 10% concentration of blackcurrant or sea buckthorn (Table II). Growth was, however, completely inhibited at a 20% or higher berry juice concentration for the blackcurrant containing growth medium. Growth of *S. epidermidis* was almost inhibited on 10% blackcurrant, but marginally inhibited on 10% sea buckthorn. In contrast, *S. aureus* grew 1.5-times more on 10% sea buckthorn agar in comparison to the control agar. The study data identified similar antibacterial inhibitory effects by blackcurrant and sea buckthorn.

## Discussion

Blackcurrant is rich in ferulic and chlorogenic acids. Ferulic acid has been identified as one of several phenols with antibacterial activities against  $\beta$

Table II. Proportional growth of bacteria in berry juice in relation to bacterial growth on control agar plates (a value >1.0 means enhancement of bacterial growth while a value <1.0 means inhibitory effect). Bacterial count (mean values  $\pm$  SD) on berry juice agar plate.

Bacteria	Blackcurrant		Sea buckthorn	
	10% pH 4.7	20% pH 4.1	10% pH 5.4	20% pH 4.4
<i>S. aureus</i>	0.97 $\pm$ 0.85	0.00	2.53 $\pm$ 1.49	0.00
<i>S. epidermidis</i>	0.06 $\pm$ 0.06	0.00	0.83 $\pm$ 0.20	0.00
<i>S. mutans</i>	0.84 $\pm$ 0.28	0.00	0.95 $\pm$ 0.28	0.00
<i>P. aeruginosa</i>	0.79 $\pm$ 0.29	0.00	1.00 $\pm$ 0.0	0.00

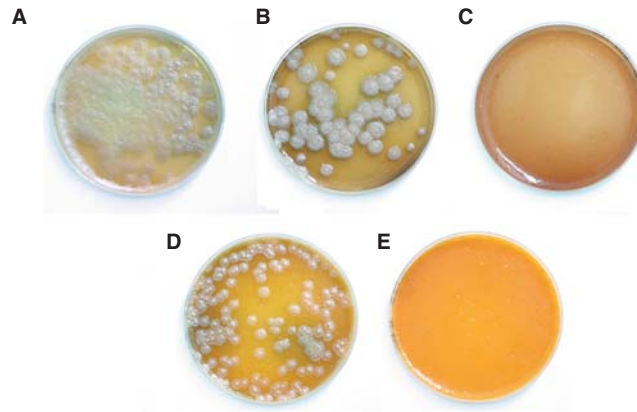


Figure 1. Bacterial growth of *P. aeruginosa* on control agar plate (A), blackcurrant 10% (B) and 20% (C) and sea buckthorn 10% (D) and 20% (E) agar plate. Bacterial growth was completely inhibited at 20% juice concentration and pH of 4.1 (blackcurrant) and 4.4 (sea buckthorn).

hemolytic *Streptococci* and *Escherichia coli* [19]. Data have shown that ferulic acid in LPS stimulated whole blood is a prominent active inhibitor of pro-inflammatory cytokines including interleukin 6 and TNF- $\alpha$  [20]. In addition, naturally occurring polyphenolic compounds such as chlorogenic acid may serve as potent anti-inflammatory agents and as alternatives to conventional chemotherapeutics [21]. Thus, the presence of ferulic acid and chlorogenic acid found in blackcurrant juice in the present study may, therefore, differ in the control of bacterial growth compared to control of bacterial growth with sea buckthorn incorporated in the growth medium.

Data suggest that aqueous and hydroalcoholic extracts of sea buckthorn leaves have marked antibacterial activities against *S. aureus* [22]. With sea buckthorn juice at a pH of 4.4, all species studied were inhibited. *S. aureus* was able to grow on sea buckthorn juice at pH 5.4 at a rate 1.5-times in comparison to the control. The inhibition of bacterial growth for *S. epidermidis* was limited in sea buckthorn juice at pH 5.4, *P. aeruginosa* was not inhibited in sea buckthorn juice at pH 5.4 at 10% concentration. The inhibition of growth of *P. aeruginosa* is exemplified in Figures 1A–E, demonstrating the ability of a 20% concentration of blackcurrant or sea buckthorn at pH 4.4 to inhibit growth of *P. aeruginosa*. This suggests that pH may be of importance in the control of bacterial growth in a berry juice. It is well established that *S. mutans* is tolerant to acidic pH and also that *S. aureus* can manage pH levels below 5.0. In contrast, the optimal pH for *P. aeruginosa* is 7.3. Thus, the present data for bacterial inhibition on both blackcurrant and sea buckthorn juices can not be fully explained by the lower pH at a 20% berry concentration. Further studies are necessary to explore the impact of pH and polyphenols on bacterial growth.

*P. aeruginosa* is generally considered as a key microorganism in the early development of biofilm in a fluid environment. *P. aeruginosa* has also been identified as

an important pathogen in periodontitis [23,24]. Our experiments suggest that a juice containing either blackcurrant or sea buckthorn could limit bacterial colonization on tooth surfaces and biofilm development. Thus consuming such a juice preparation may reduce the risks for both tooth decay and gingivitis. Our data suggest that the growth of *P. aeruginosa*, a key pathogen in early biofilm development and antibiotic resistance is also impacted by blackcurrant and sea buckthorn. The observation is consistent with what has been reported by others [22].

## Conclusion

The present study identified that *in vitro* bacterial growth of selected streptococci and staphylococci species, *E. coli* and *P. aeruginosa* on agar plates incubated with either blackcurrant or sea buckthorn juice was inhibited at a juice concentration  $\geq 20\%$ . The highest level of polyphenols studied was found for ferulic acid in blackcurrant juice.

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