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Inhibition of adhesion of *Neisseria meningitidis* to human epithelial cells by berry juice polyphenolic fractions

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Short title: Inhibition of meningococcal attachment

ABSTRACT

Adhesion of pathogens to host tissues is the requirement for the initiation of the majority of infectious diseases. We recently showed that the binding of *Neisseria meningitidis* pili to immobilised human epithelial cells is inhibited by molecular size fractions (10–100 kDa) of berry juices. Additionally, the isolated meningococcal pili bound to polyphenolic fractions of berry juices. In the present study we investigated the antiadhesive effects of berry juice polyphenolics against living meningococcal bacteria in a human epithelial cell culture model. The ability of bilberry, cranberry, crowberry, and lingonberry juice polyphenolic fractions to inhibit the attachment of *N. meningitidis* bacteria to HEC-1B human epithelial cells in a cell culture model was examined. Antibacterial effect of the fractions was tested using microtiter broth microdilution assay. The most effective adhesion inhibition of 75 % was achieved with cranberry juice polyphenolic fraction followed by crowberry (63%), bilberry (63%), and lingonberry (57%) juice polyphenolic fractions. Bacterial survival rates after incubation with the fractions varied between 75–100 %. The present results suggest berry juice polyphenols as inhibitors of adherence of *N. meningitidis*. Thus the binding of meningococci to berry juice polyphenols might be protective for the host against the infection.

Keywords: berry polyphenols; meningococci; epithelial cells; antiadhesion; antibacterial

INTRODUCTION

Meningitis caused by *Neisseria meningitidis* is a serious public health problem worldwide. The bacteria are transmitted from person to person through droplets of respiratory or throat secretions. *N. meningitidis* primarily infects human mucosal cell surfaces and colonises the nasopharyngeal epithelium. In Europe and the United States point-prevalence nasopharyngeal carriage rates have been estimated to range from 10 to 35% in young adults (Caugant and Maiden, 2009). There is no vaccine against the serogroup B which together with serogroup C accounts for a large majority of cases in Europe and in the Americas (Schwartz *et al*., 1989). Clinically the most important meningococcal phenotype is encapsulated (Carbonnelle *et al*., 2009). In encapsulated strains meningococcal adhesion to epithelial and endothelial cells occurs through type IV pili (Nassif *et al*., 1999; Pujol *et al*., 1997). The major neisserial pilus subunit, the pilin (Nassif and So, 1995) and the tip-located PilC1 protein (Nassif *et al*., 1994) are the potential adhesins.

One strategy to avoid bacterial infections could be antiadhesive agents which may prevent the attachment of infection causing bacteria to human cells. Unattached bacteria can be swept away by natural cleansing mechanisms of the host. Berries could be used as antiadhesives against binding of pathogenic bacteria to human epithelia. Cranberry polyphenols have been shown to be capable to inhibit the attachment of *Escherichia coli* (Gupta *et al*., 2007), *Helicobacter pylori* (Shmuely *et al*., 2004) and *Streptococcus mutans* (Weiss *et al*., 2004).

In our previous studies we have shown that *N. meningitidis* pili, *Streptococcus pneumoniae* bacterial cells and *Streptococcus agalactiae* bacteria bind to berry molecular size fractions especially from *Vaccinium* species and they inhibit

hemagglutination caused by *Streptococcus suis* (Toivanen *et al*., 2009; Toivanen *et al*., 2010). Our results (Toivanen *et al*., 2009) showed that berry juice molecular size fractions of 10–100 kDa inhibited the binding of isolated *N. meningitidis* pili to membrane-bound epithelial cells in a dot assay. Additionally, meningococcal pili bound to bilberry, cranberry, crowberry and lingonberry juice polyphenolic fractions containing anthocyanins and proanthocyanidins. The aim of the present study was to investigate the ability of these polyphenolic fractions to inhibit the adherence of whole bacterial cells to human epithelial cells. Antimicrobial effect of the fractions against *N. meningitidis* was also examined.

MATERIALS AND METHODS

Berry juices. Bilberry (*Vaccinium myrtillus* L.), cranberry (*Vaccinium oxycoccos* L.), lingonberry (*Vaccinium vitis-idaea* L.) and crowberry (*Empetrum nigrum* & *E. hermaphroditum* L.) (65 °Brix, content of soluble solids g/100 g of solution) juice concentrates were purchased from Kiantama Ltd (Suomussalmi, Finland). Juice concentrates contained no additives.

Chemicals. Methanol (VWR International Ltd., Leuven, Belgium) was of HPLC grade and ethyl acetate (Laboratory-Scan, Dublin, Ireland) was of analytical grade. Water was purified on a Millipore Milli-Q apparatus (Molshein, France). High-glucose DMEM, FBS, L-glutamine, trypsin-EDTA and sterile PBS were from Gibco (Paisley, U.K.) and ampicillin was from Sigma (St. Louis, MO).

Isolation of berry juice polyphenols. Berry juice polyphenols were isolated as described before (Toivanen *et al*., 2009). Briefly, berry juice concentrates were fractionated into three fractions according to their molecular size, i.e. <10 kDa fraction (referred as FI), 10–100 kDa fraction (FII) and >100 kDa fraction (FIII) using centrifugal filtering devices (Millipore Corp., Bedford, MA). The fractions FIII of the bilberry, cranberry and crowberry juices and the fraction FII of the lingonberry juice were further subfractionated using solid-phase extraction with C-18 SPE cartridge (Waters Corp., Milford, MA). Subfractions were eluted with ethyl acetate, water and methanol. The eluted subfractions were analysed using RP-HPLC-DAD. Subfractions (here polyphenolic fractions), which were eluted with water and contained anthocyanins and proanthocyanidins (Toivanen *et al*., 2009) were used in the inhibition experiments of the cell culture studies.

Bacterial strain and culture conditions. *Neisseria meningitidis* serogroup C class I strain 8013 (Nassif *et al*., 1993) (X. Nassif, INSERM U570, Paris) was cultured at 37 °C in CO_2 atmosphere for 18 h on GCB agar (Oxoid Ltd., Basingstoke, England) containing Kellogg's supplement I and II (Kellogg *et al*., 1963.). After three washings with PBS (2000 × g at 4 °C for 10 min) the absorbance of the bacterial suspension was adjusted to A_{600} = 0,770 and the bacteria were suspended to DMEM and diluted serially to 1:1000.

Cell culture. Adhesion of *N. meningitidis* has been tested using HEC-1B human epithelial cells as a model (Nassif *et al*., 1994). HEC-1B human epithelial cell line was obtained from X. Nassif (INSERM U570, Paris) and used between passages 11 and 28.

Cells were cultured in cell culture dishes (Costar®, Corning Inc., Corning, NY) by using 10 mL of high-glucose DMEM supplemented with 10% heat-inactivated FBS and 4 mM L-glutamine without addition of antibiotics at 37 °C in a humidified 5% CO_2/ 95% air incubator. Medium was changed 2–3 times a week and cells were passaged once or twice a week at 1:2–1:4 ratio using 1 mL of trypsin-EDTA solution. For adhesion-inhibition assays HEC-1B cell monolayers were prepared in six-well tissue culture plates (Costar®, Corning Inc., Corning, NY). 10^5 cells were inoculated per well to obtain confluence and were cultured overnight prior to the adhesion-inhibition assay.

Adhesion-inhibition assay. Berry juice polyphenolic fractions were diluted with cell culture medium to different concentrations between 0.5–50.0 µg/mL. The diluted polyphenolic fractions (1.5 mL) were added on the overnight cultured HEC-1B cells and incubated over the cells for one hour at 37 °C in a humidified 5% CO_2/ 95% air incubator. The control wells were prepared by adding culture medium without polyphenolic fractions. Bacterial suspension (1.5 mL) was added to the wells (approx. 10 CFU per one HEC-1B cell) and the wells were incubated for one hour at 37 °C. Non-adherent bacteria were removed by washing the wells twice with 3 mL of PBS. The cells were detached with trypsin-EDTA solution (0.5 mL) from the bottom of the wells. The detached cells were diluted 1:10 with PBS and 100 µL of the diluted cell samples was plated on the GCB agar plates. The plated bacteria were cultured overnight at 37 °C in CO_2 atmosphere. The amount of the attached bacteria to the epithelial cells was determined by counting the bacterial colonies. The inhibitory activity of the berry juice polyphenolic fractions was calculated as follows:

$$Inhibition\% = \frac{CFU_{control} - CFU_{sample}}{CFU_{control}} x100$$, where CFU is a colony forming unit

Antibacterial activity assay. Microtiter broth microdilution assay (Amsterdam, 1996) was chosen for testing the antibacterial activity of cranberry, bilberry, lingonberry and crowberry juice polyphenolic fractions against *N. meningitidis* because it has been described most suitable for plant extracts and phenolic compounds (King *et al*., 2008). Bacterial suspension and berry juice polyphenolic fractions in GC broth (1.5% of proteose peptone #3, 23 mM K_2HPO_4, 7 mM KH_2PO_4, 85 mM NaCl, Kellogg's supplements I and II) were mixed together using the same amount of bacteria per berry polyphenolic fraction as in the adhesion-inhibition assay. The mixtures were incubated in wells of the microtiter plate strips (Nunc™, Brand Product, Roskilde, Denmark) at 37 °C in CO_2 atmosphere for 2 hours. In control wells bacterial suspension was incubated with GC broth without adding berry juice polyphenolic fractions or by adding ampicillin (100 µg/mL). The antibacterial activity of the samples was measured by plating the incubation mixtures on GCB agar plates. The surviving colony forming units were counted next day. All the tests were made in duplicate.

Statistics. Statistical analysis was performed using GraphPad Prism 4.03 for Windows. One-way ANOVA followed by Dunnett's Multiple Comparison Test was performed to compare the amount of bacterial colonies between the control and the samples in the adhesion-inhibition assay. Inhibition of adhesion was considered significant at P values of <0.05.

RESULTS

Adhesion-inhibition assay. Inhibitory activity of the bilberry juice polyphenolic fraction was significant between the concentrations of 5–50 µg/mL and peaked at the concentration of 10 µg/mL with the inhibition of 63% (Fig. 1a). The cranberry juice polyphenolic fraction inhibited significantly meningococcal adherence to epithelial cells, the inhibitory percent was 60–75% over the studied range of 1–50 µg/mL (Fig. 1b). Crowberry juice polyphenolic fraction showed significant inhibition from 47% to 63% between the concentrations of 5 µg/mL and 50 µg/mL, respectively (Fig. 1c). Significant inhibition against meningococcal attachment with 56–57% inhibition over the studied range was seen with the lingonberry juice polyphenolic fraction, except 48% inhibition at 25 µg/mL (Fig. 1d).

Antibacterial activity assay. Survival rates of *N. meningitidis* after incubation with three different concentrations (1 µg/mL, 25 µg/mL and 50 µg/mL) of the berry juice polyphenolic fractions are shown in Table 1. The lowest survival rates were detected with the cranberry juice polyphenolic fraction (75–90% survival). Only slight or no antibacterial activity was seen when incubating meningococci with the polyphenolic fractions of the other berry juices. Bacterial survival with ampicillin control (100 µg/mL) was 45%.

DISCUSSION

Inhibition of the bacterial attachment to human cells may offer a way to avoid infections (Ofek *et al*., 2003). In the present study we showed bilberry, cranberry, crowberry and lingonberry juice polyphenolic fractions containing anthocyanins and proanthocyanidins (Toivanen *et al*., 2009) acting as antiadhesives. These fractions inhibited the attachment

of *N. meningitidis* to epithelial cells in vitro. The results were generated using an adhesion assay based on human epithelial cell line HEC-1B and living encapsulated meningococcal cells. The significant antiadhesion activity was achieved with relatively low concentrations, 1 µg/mL of cranberry and lingonberry, and 5 µg/mL of bilberry and crowberry juice polyphenolic fractions. Although the methodologies used in the experiments differ, the effective concentrations of the inhibition were on the same level as the concentrations in our previous microtiter well assay studies, where the isolated pili bound significantly to 0.39–12.5 µg/mL of these fractions (Toivanen *et al*., 2009). None of the studied berry juice polyphenolic fractions gave total inhibition for the attachment of meningococci and the highest inhibition was achieved up to 75% with the cranberry juice polyphenolic fraction with the concentration of 5 µg/mL. In the binding assays with pili constant binding activity level was achieved at 50 µg/mL of polyphenolic fractions except 1.56 µg/mL of lingonberry juice polyphenolic fraction (Toivanen *et al*., 2009).

The important role of type IV pili is in colonisation and infection in encapsulated *N. meningitidis* strains (Nassif 2000). *N. meningitidis* has complex adhesion mechanisms and carries 2 potential adhesins in its type IV pili, the tip-located PilC1 (Rudel *et al*., 1995) and pilin (PilE) subunit proteins (Nassif *et al*., 1994). The mechanisms of PilE and PilC1 in epithelial cell binding are complicated, when encapsulated *N. meningitidis* interact with host cells in a multistep process (Nassif *et al*., 1999; Pujol *et al*., 1997). The concept of two different binding specificities located in two different components of the pilus is complicated by the fact that PilE undergoes antigenic variation (Meyer and van Putten 1989), which influences epithelial cell-specific adherence (Virji *et al*., 1993). Some pilin variants are more efficient than others in enhancing bacterial

interactions and forming large bundles with enhancement of adhesiveness (Marceau *et al*., 1995). The inhibition of the adhesion achieved in the present study by the berry juice polyphenolic fractions may result either from pilin and/or PilC1 mediated adhesion.

Berries and their phenolics have been shown to inhibit the growth of human pathogenic bacteria (Puupponen-Pimiä *et al*., 2005). In the present study the bacterial survival rates were above 90% for all samples with the exception that cranberry juice polyphenolic fraction at 1 µg/mL induced 85% survival and at 50 µg/mL induced 75% survival (Table 1). At these concentrations adhesion inhibition was 74% and 60%, respectively. Thus, the inhibitory effect of cranberry juice polyphenolic fraction can partly result from the antibacterial effect of the fraction. For the other samples the inhibitory effect achieved did not result from antibacterial effect but from the inhibition of bacterial attachment.

Antiadhesive agents do not act by killing the pathogens so the spread of bacterial strains resistant to antiadhesive agents will likely occur at a significantly lower rate compared to antibiotic-resistant strains (Ofek et al. 2003). Small proportion of the bacteria may be mutants resistant to antiadhesive agent. In only a few people the mutant propagate, while in majority of cases bacteria bind to antiadhesive agents and are swept away, and hence slowing down the spread of resistant strains.

Bacteria may also start to express new adhesins which bind to different receptors. Therefore a single agent that has a broad spectrum of antiadhesion activity or a mix of different antiadhesive agents that target several adhesins may be necessary.

Our results indicate that it could be possible to use berry juice polyphenols as antiadhesives against *N. meningitidis*. Molecules may provide multiple binding sites for

N. meningitidis pili adhesins and thus inhibit adhesion. Investigated polyphenolic water extracts could be utilized easily. However, further purification of polyphenols will be needed to find out if the antiadhesive effect can be related to a single molecule or the effect needs synergic work of several berry molecules. Clinical trials will be necessary to prove the effect of berry juice polyphenols against meningococcal attachment to human nasopharynx and possible decrease in carrier rate.

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CONFLICT OF INTEREST

None to declare.

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Table 1. Survival of *Neisseria meningitidis* after incubation with berry juice polyphenolic fractions.

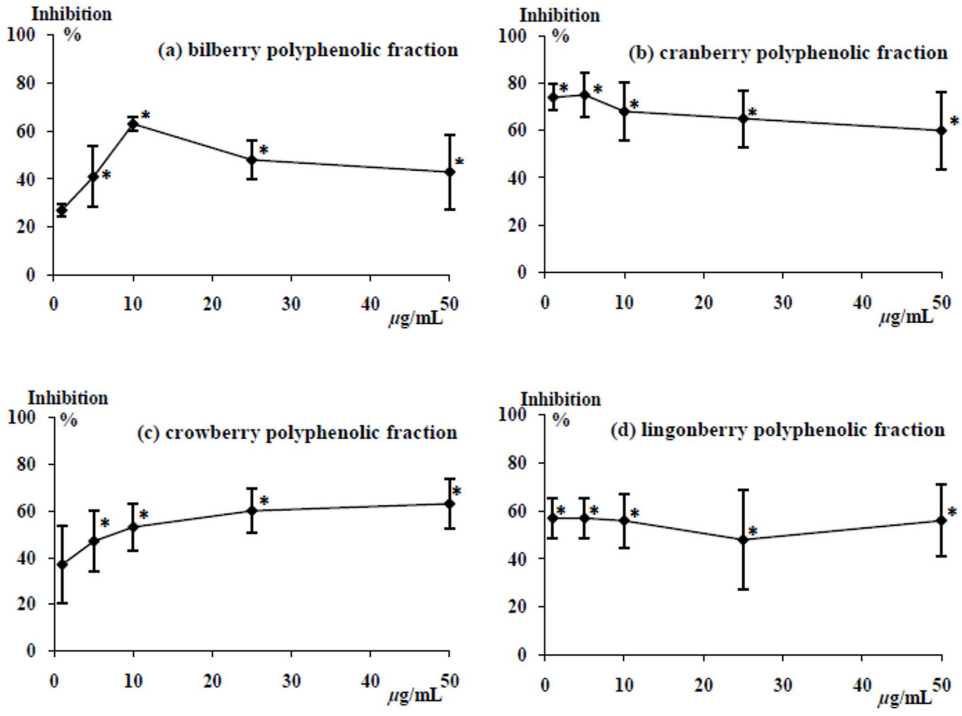
Concentration of polyphenols (µg/mL)	Bilberry juice	Cranberry juice	Crowberry juice	Lingonberry juice
1	90%	85%	≥100%	≥100%
25	100%	90%	100%	100%
50	100%	75%	100%	≥100%

Ampicillin (100 µg/mL): 45%

FIGURE LEGEND

FIGURE 1. Inhibition of the attachment of *Neisseria meningitidis* to HEC-1B cells by bilberry (a), cranberry (b), crowberry (c), and lingonberry (d) juice polyphenolic fractions. The values represent the mean and standard deviation calculated from at least three experiments. Significant difference ($P < 0.05$) in inhibition compared to control is marked with *.

Toivanen M, FIGURE 1



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