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Binding of *Neisseria meningitidis* Pili to Berry Polyphenolic Fractions

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Blocking bacterial adhesion to host surfaces provides novel potential to control infections. The present study was directed to binding and inhibitory activity of different fresh berries and berry and fruit juices against *Neisseria meningitidis*. Berries and juices were fractionated according to their molecular size into three fractions. A microtiter well assay for binding of *N. meningitidis* pili to berry and juice fractions was constructed. In addition, adhesion inhibition to human epithelial cells (HEC-1B) was tested. The active fractions were then subfractionated by employing solid-phase extraction. Subfractions were characterized by RP-HPLC-DAD, and the pili binding was evaluated by using microtiter well binding assay. Binding and inhibitory activity were detected to bilberry, cranberry, lingonberry, and crowberry fractions, which contained anthocyanins or a mixture of proanthocyanidins and flavonols. Thus, the findings identify several previously unknown binding and inhibitory activities and may suggest *Vaccinium* berries and crowberry as promising sources against meningococcal adherence.

KEYWORDS: Meningococcus; berries; polyphenols; adhesion

INTRODUCTION

Most pathogenic bacteria adhere to host tissues by bacterial adhesins that bind to complementary carbohydrates on the host cell surface (*1*). Soluble carbohydrates have been shown to have protective activity against experimental infections induced by adhesin-carrying bacteria in animals (*2*−*4*). Among cultivated berries, cranberry (*Vaccinium macrocarpon* Ait.) is known to prevent the adhesion of *Escherichia coli* (*5*, *6*), *Helicobacter pylori* (*7*), and mutans streptococci (*8*). The antiadhesion activity of cranberry has been attributed to carbohydrates (*6*) as well as to high molecular weight material (*8*) and to polyphenols such as proanthocyanidins (*9*). Among other fruits, orange and pineapple juice (*6*), guava lectins (*10*), and blueberry proanthocyanidins (*11*) are known to have effect against the binding of *E. coli* in vitro and the polysaccharides from black currant seeds against *H. pylori* (*12*) in situ. Berries are known as rich sources of polyphenols, such as anthocyanins, proanthocyanidins, and flavonols (*13*).

Neisseria meningitidis is an important human pathogen causing severe infections such as meningitis or septicemia (*14*). It colonizes the nasopharynx of approximately 10% of healthy individuals (*15*). There is lack of an effective vaccine especially against group B serotype. Antimicrobial prophylaxis is used to prevent bacterial colonization (*14*). However, the use of antibiotics for prevention can lead to resistance (*16*) and to serious secondary infections as the normal microflora is disturbed (*17*).

Encapsulated *N. meningitidis* cells adhere to epithelial and endothelial cells by type IV pili (*18*). Human cell-surface receptor CD46 has been suggested as a potential receptor (*19*), but carbohydrate receptor structures have not been reported. Recently, we have reported that purified pili of *N. meningitidis* bind to bovine thyroglobulin and human salivary agglutinin, glycoproteins containing carbohydrate chains (*20*). This binding was prevented by purified bovine milk acidic and human milk neutral oligosaccharide fractions (*20*), which suggests carbohydrate recognition for *N. meningitidis* pili.

Because of increasing antibiotic resistance and the lack of effective vaccines, new prevention methods against bacterial diseases are needed. The strategy of preventing the first step of infection, by blocking the adhesion of pathogens to host surfaces, provides novel potential to control infectious diseases. The aim of the present study was to investigate several berries and fruits as potential sources of antiadhesives against *N. meningitidis* together with chemical characterization of the berry and fruit samples.

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MATERIALS AND METHODS

Berries and Juices. Wild bilberry (*Vaccinium myrtillus* L.), European cranberry (*Vaccinium oxycoccos* L.), lingonberry (*Vaccinium vitis-idaea* L.), and cloudberry (*Rubus chamaemorus* L.) and juice concentrates (referred as juices in the text) of wild bilberry, cranberry, lingonberry, and crowberry (*Empetrum nigrum* and *hermaphroditum* L.) (65 °Brix, content of soluble solids g/100 g of solution) were purchased from Kiantama Ltd. (Suomussalmi, Finland). Juice concentrates of apple (mix of cultivars) (70 °Brix), black currant (*Ribes nigrum* L.) (65 °Brix), pineapple (*Ananas comosus* L.) (60 °Brix), raspberry (*Rubus idaeus* L.) (65 °Brix), red grapefruit (*Citrus paradisi* L.) (63 °Brix), sour cherry (*Prunus cerasus* L.) (65 °Brix), and tomato (*Solanum lycopersicum* L.) (30 °Brix) were purchased from VIP-Juicemaker Ltd. (Kuopio, Finland). Berries and additive-free juice concentrates were stored at −20 °C.

Chemicals. Methanol and acetonitrile (VWR International Ltd., Leuven, Belgium) were of HPLC grade; hydrochloric acid (Riedel-de Haën, Seelze, Germany), ethyl acetate (Laboratory-Scan, Dublin, Ireland), and formic acid (Merck, Darmstadt, Germany) were of analytical grade. Phosphate buffer, pH 7.0, was from J. T. Baker (Deventer, The Netherlands), and water used was purified on a Millipore Milli-Q apparatus (Molsheim, France). Phenol was from Merck, concentrated sulfuric acid from Riedel-de Haën, and D-glucose from Sigma (St. Louis, MO). D-Biotinoyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester, streptavidin−POD conjugate, and ABTS substrate were from Roche Diagnostics (Mannheim, Germany). Hepes buffer, high-glucose DMEM, heat-inactivated FBS, L-glutamine, trypsin-EDTA, and sterile PBS used in cell culture were from Gibco (Paisley, U.K.), and Super Signal solution was from Pierce (Rockford, IL).

Preparation of Berry and Juice Fractions (FI, FII, and FIII). Fresh berries and the juice concentrates were fractionated into three different fractions according to their molecular size, that is, <10 kDa fraction (referred to as FI), 10−100 kDa fraction (FII), and >100 kDa fraction (FIII). Thawed berries were crushed and diluted with water (1:1, v/v). After centrifugation (8000*g* at 4 °C for 10 min), the berry juice was filtered through a gauze and filter paper. Fifteen milliliters of the filtered juice was loaded onto a 100 kDa cutoff Biomax Ultrafree-15 centrifugal filter device (Millipore Corp., Bedford, MA) and centrifuged (2000*g* at 4 °C) to a volume of 1.5 mL. The retentate (fraction FIII) was frozen (−20 °C). The filtrate was loaded onto a 10 kDa cutoff Biomax Ultrafree-15 centrifugal filter device (Millipore Corp.) and centrifuged as described above to a volume of 1.5 mL. Both the retentate (fraction FII) and the filtrate (fraction FI) were frozen (−20 °C).

The berry and fruit juice concentrates were diluted with water (1:4 and 1:3−1:5, v/v, respectively) and fractionated as described above. The content of soluble solids of the fractions was analyzed as °Brix value using an ATAGO NAR-1T laboratory refractometer (Tokyo, Japan).

Phenol−Sulfuric Assay for Total Sugars. Content of reducing sugars in fresh berry and juice fractions was determined by a colorimetric method (*21*). Briefly, to 50 μL of diluted fractions were added 150 μL of water, 200 μL of 5% phenol, and 1000 μL of concentrated sulfuric acid. After 30 min of incubation at 37 °C, the absorbance values were measured at 490 nm. D-Glucose was used as standard.

Spectrophotometric Analysis of Total Proanthocyanidins and Anthocyanins. Total proanthocyanidin contents were determined for the fractionated fresh berry and juice samples with a modification of the HCl−butanol method in which HCl catalyzes depolymerization of colorless proanthocyanidins in butanol (in the modification, in methanol) to yield red-colored anthocyanidins (*22*, *23*). The fractions were diluted with water to the concentration of 10 mg/mL according to °Brix value, and 12.5 or 25 μL of the dilution was mixed into acidified methanol (5% HCl in methanol) to the total volume of 5 mL. Absorbance values (A_0) were measured at 520 nm against acidified methanol. The samples were incubated at 70 °C for 3 h, the absorbances (A) were measured, and the values $\Delta A = A - A_0$ were calculated. Semiquantification was done against a lingonberry-derived mixture of oligomeric proanthocyanidins (*24*) by constructing a calibration curve of change of absorbance versus proanthocyanidin concentration, with the concentration range of 0.5−8 μg/mL ($R^2 > 0.999$).

The estimation of total anthocyanins was done for the A_0 of each fraction against a mixture of six anthocyanidin glycosides, which represented 3-*O*-glucosides of cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin (Polyphenols Laboratories AS, Sandnes, Norway). The anthocyanin mixture was dissolved into pure methanol and diluted with acidified methanol (5% HCl in methanol). Absorbance values were measured at 520 nm against acidified methanol (*25*). A calibration curve of absorbance versus anthocyanin concentration, with the concentration range of 0.10−10.0 μg/mL, was constructed ($R^2 > 0.999$).

Solid-Phase Extraction (SPE) Subfractionation of Berry Juice Fractions. To subfractionate the polyphenolic compounds for further identification and activity testing, the method previously described by Sun et al. (*26*) was used with modifications. The fraction of berry juice (3 mL) was mixed with commercial phosphate buffer with pH 7.0 (1:1, v/v) and passed through a C-18 SPE cartridge of 3 cm^3 capacity filled with 500 mg of adsorbent (Waters Corp., Milford, MA) previously conditioned with 15 mL of methanol and equilibrated with 5 mL of pH 7.0 phosphate buffer. After removal of the phenolic acids with 5 mL of diluted phosphate buffer (1:8, v/v, with water) and washing with 5 mL of water, the cartridge was dried under vacuum for 30 s. Then the elution with 5 mL of ethyl acetate (ethyl acetate subfraction) was performed and the cartridge was dried again under vacuum for 30 s before the next elution with 3 mL of water (water subfraction). After that, the elutions with 3 mL of 10% and 3 mL of 20% aqueous methanol were discarded and the one with 3 mL of 60% aqueous methanol (methanol subfraction) was collected. Solvents in the subfractions were removed using rotary evaporation with a water bath at 30 °C. Finally, the solids were reconstituted with water to the concentration of 0.5 or 1.0 mg/mL.

HPLC Analysis of Subfractions. An amount of 200−300 μL of aqueous subfraction was mixed with pure methanol (1:1, v/v), and, prior to HPLC analysis, the samples were filtered through a 0.45 μm syringe filter (Pall Life Sciences, Ann Arbor, MI). A 20 μL injection of the samples was separated on a 150 × 4.6 mm i.d., 5 μm LiChroCart Purospher Star RP-18e column (Merck) with a 4 × 4 mm i.d. guard column using an HP 1100 series HPLC (Waldbronn Analytical Division, Waldbronn, Germany) equipped with a quarternary pump, an autosampler, and an online diode array detector linked to an HP ChemStation data handling system. The method utilized a binary gradient with mobile phases containing 0.1% v/v aqueous formic acid (mobile phase A) and acetonitrile/methanol 85:15 (mobile phase B). Eluting peaks were monitored at the wavelength range of 190−550 nm (2 nm step). The elution conditions were 10% B for the first 8 min, a linear gradient from 10 to 25% B for 8−12 min, 12−18 min with 25% B, 18−22 min with 25−40% B, 22−26 min with 40% B, 26−43 min with 40−90% B, 43−52 min with 90% B, and 52−57 min with 90−10% B followed by an isocratic elution for 3 min before the next injection. The flow rate of the mobile phase was 0.5 mL/min for 0−12 min, 0.4 mL/min for 18−43 min, and 0.5 mL/min for 52−57 min. Chromatographic peaks were identified on the basis of the online UV−visible spectra (*24*, *27*).

Purification of Bilberry Juice Anthocyanins. Two milliliters of bilberry juice concentrate (65 °Brix) was mixed with 3 mL of water. Then a rough liquid−liquid extraction with 5 mL of ethyl acetate was performed to remove most of the ethyl acetate soluble polyphenols, that is, mainly the flavonol glycosides and a part of the oligomeric proanthocyanidins, from the juice. After vigorous mixing for 1 min, the mixture was centrifuged at 3000*g* for 3 min, and the ethyl acetate phase was removed. The procedure was repeated, and the treated juice (aqueous phase) was filtered through a 0.45 μm syringe filter (Pall Life Sciences) before HPLC separation.

A 30 μL injection of the treated juice was separated on a 250 × 4.6 mm i.d., 5 μm LiChroCart Purospher Star RP-18e column (Merck) with a 4 × 4 mm i.d. guard column using a Gilson series HPLC (Gilson S.A., Villiers-le-Bel, France) equipped with a model 321 binary pump, a model 234 autosampler, a model 151 UV−vis detector, and a model 204 fraction collector linked to a Gilson UniPoint version 3.2 data handling system. The method utilized a binary gradient with mobile

Table 1. Contents of Sugars, Proanthocyanidins, and Anthocyanins in Fractionated Berries and Berry and Fruit Juices[a]

	sugars (mg/mL)			proanthocyanidins (mg/g)			anthocyanins (mg/g)		
product	FI	FII	FIII	FI	FII	FIII	FI	FII	FIII
Vaccinium myrtillus									
bilberries	43	35	42	tr	1	1	nd	1	nd
bilberry juice	178	160	**204**[b]	3	6	**7**	tr	14	**39**
Vaccinium oxycoccos									
cranberries	21	37	31	tr	3	2	nd	1	tr
cranberry juice	83	104	**174**	1	10	**30**	nd	1	**4**
Vaccinium vitis-idaea									
lingonberries	42	37	42	1	10	1	nd	1	tr
lingonberry juice	113	**187**	152	4	**22**	9	nd	**3**	1
Empetrum nigrum									
crowberry juice	136	177	**232**	2	14	**27**	1	16	**38**
Ribes nigrum									
black currant juice	96	20	27	1	3	2	1	tr	nd
Citrus paradisi									
red grapefruit juice	124	162	212	nd	nd	nd	nd	nd	nd
Prunus cerasus									
sour cherry juice	139	151	188	1	6	3	tr	2	1
Malus domestica									
apple juice	188	228	177	nd	nd	nd	nd	nd	nd
Rubus chamaemorus									
cloudberries	43	36	35	nd	nd	nd	tr	nd	nd
Ananas comosus									
pineapple juice	118	91	258	nd	nd	nd	nd	nd	nd
Rubus idaeus									
raspberry juice	127	153	166	1	4	2	tr	2	1
Solanum lycopersicum									
tomato juice	50	61	65	nd	nd	nd	nd	nd	nd

[a] Abbreviations: FI, <10 kDa fraction; FII, 10−100 kDa fraction; FIII, >100 kDa fraction; tr, traces; nd, not detected. [b] Juice fractions chosen for further analyses based on biological assay are marked in bold.

phases containing 8.5% v/v aqueous formic acid (mobile phase A) and acetonitrile/methanol 85:15 (mobile phase B). Eluting peaks were monitored at 520 nm. The elution conditions were 2% B for the first 13 min, a linear gradient from 2 to 10% B for 13−20 min, 20−40 min with 10−11% B, 40−53 min with 11% B, 53−65 min with 11−35% B, 65−68 min with 35% B, 68−71 min with 35−80% B, 71−73 min with 80% B, and 73−75 min with 80−2% B followed by an isocratic elution for 5 min before the next injection. The flow rate of the mobile phase was 0.85 mL/min for 0−12 min, 0.7 mL/min for 13−68 min, and 0.85 mL/min for 71−80 min. The parameters for the fraction collector were as follows: peak level, 15; peak width, 1; and peak sensitivity, 100. Positive peaks were collected during 25−41 min of the HPLC run. After several runs, the collected fractions containing anthocyanins were combined, and the sample was concentrated to a volume of 2.5 mL using a rotary evaporator with a water bath at 35 °C. The HPLC purity of the collected anthocyanin fraction was checked with the HPLC protocol described above for the subfractions.

Bacterial Strain and Culture Conditions. *N. meningitidis* serogroup C class I strain 8013 (X. Nassif, INSERM U570, Paris) (*18*) was cultured at 37 °C in CO_2 atmosphere for 18 h on GCB agar (Oxoid Ltd., Basingstoke, U.K.) containing Kellogg's supplement I and II (*28*).

Isolation and Biotin Labeling of Meningococcal Pili. Isolation and biotin labeling of the meningococcal pili were carried out as described before (*20*). Briefly, five plates of the cultivated *N. meningitidis* were suspended in 10 mmol/L Hepes buffer at neutral pH. After vigorous mixing and centrifugation (8000*g* at 4 °C for 20 min), the supernatant was loaded onto a 100 kDa cutoff Biomax Ultrafree-15 centrifugal filter device and centrifuged (1000*g* at 4 °C). The concentrated solution was washed twice with 15 mL of 10 mmol/L Hepes and concentrated by centrifugation to a volume of 1 mL as described above.

Biotin labeling of the isolated pili was performed in PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 12.4 mmol/L Na_2HPO_4, 1.5 mmol/L KH_2PO_4, pH 7.4) by using D-biotinoyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester according to the instructions of the manufacturer. The biotin-labeled pili were stored at 4 °C.

Microtiter Well Binding Assay. To evaluate the binding activity of meningococcal pili to berry and fruit molecules, a microtiter well binding assay previously developed (*20*) was here modified and used as follows. Aliquots of 100 μL of fresh berry or juice fraction (fractions FII and FIII were diluted 1:10 with water back to the original concentration to achieve concentrations comparable to the fraction FI), serially diluted subfractions or dry milk powder solution [5% (w/v) dry milk powder, 0.05% (v/v) Tween 20 in PBS, pH 7.4] were incubated in Falcon flexible plate polyvinylchloride microtiter plate wells (Becton Dickinson Labware, NJ) at 4 °C overnight. The wells were washed five times with the washing buffer [0.05% (v/v) Tween 20 in PBS, pH 7.4]. The nonspecific binding sites were saturated by incubating 250 μL of the dry milk powder solution in the wells for 60 min at room temperature. The wells were washed five times as above. One hundred microliters of biotin-labeled pili (corresponding to isolated pili from about 10^8 CFU of *N. meningitidis*) diluted 1:4 in PBS, pH 7.4, were added to the wells. The plates were incubated for 60 min at room temperature and washed five times as above. One hundred microliters of streptavidin−POD conjugate in the dry milk powder solution (diluted 1:4000) was added and incubated in the wells for 60 min at room temperature. After five washes with the washing buffer, an aliquot of 100 μL of the ABTS substrate was added. The absorbances were measured at 405 nm. Five percent dry milk powder solution was used as a control background (*20*), and the binding activity to milk powder was subtracted from the absorbance values and multiplied by 100. Thus achieved values describe the binding of pili solely to fresh berry or juice molecules bound to the microtiter plate wells. All of the assays were carried out in triplicates or duplicates.

Cell Culture of HEC-1B Cells. HEC-1B human epithelial cells were obtained from X. Nassif (INSERM U570, Paris). They were cultured on cell culture dishes in high-glucose DMEM supplemented with 10% heat-inactivated FBS and 4 mol/L L-glutamine at 37 °C with 5% CO_2. Cells from passages 5−20 were harvested by using trypsin−EDTA solution diluted 1:10 in sterile PBS, pH 7.4, and centrifuged at 400*g* for 4 min at room temperature. Cells were washed twice with 10 mL of ice-cold sterile PBS, pH 7.4, to remove trypsin and contaminating proteins from FBS. Cells were centrifuged as previously described, the supernatant was discarded, and the cells were stored at −20 °C until use.

Dot Binding and Binding Inhibition Assay. HEC-1B cells have been used as a model for *N. meningitidis* pili adhesion to epithelial cells (*18*). Inhibition of the binding of the isolated native meningococcal pili by berry and fruit juice fractions was tested in a dot binding assay. HEC-1B cells from one confluently grown cell culture plate prepared as described above were suspended in PBS (200 μL) and diluted 1:10 with PBS, pH 7.4. Two-microliter dots of cell suspension were pipetted on a Protran nitrocellulose membrane (Schleicher & Schuell BioScience, Germany) and allowed to dry. Nonspecific binding sites were saturated by incubating the membrane in dry milk powder solution [5% (w/v) dry milk powder, 0.05% (v/v) Tween 20 in PBS, pH 7.4] for 2 h at room temperature. Previously prepared FII fractions from juice were diluted in water (1:2). Biotinylated meningococcal pili were diluted 1:10 in PBS, pH 7.4. The diluted FII fractions and the labeled diluted pili were mixed 1:1, and the solution was incubated for 1 h at room temperature. The mixtures (1.5 mL) were loaded on a 100 kDa cutoff Biomax Ultrafree-15 centrifugal filter devise and washed with PBS by centrifugation (1000*g* at 4 °C). Finally, the pili−FII aggregates were diluted to 1.5 mL with the dry milk powder solution. The pili−FII aggregates or pili (positive control) in the dry milk powder solution diluted 1:20 were added on the membrane and incubated for 90 min at room temperature. The membrane was washed three times with PBS, pH 7.4. Streptavidin−POD conjugate (diluted 1:4000) in the dry milk powder solution was added to the membrane and incubated for 1 h at room temperature. The membrane was washed as above and developed with Super Signal solution. The membrane was exposed to Hyperfilm (Amersham Pharmacia Biotech, U.K.) for 1 min, after which the film was developed.

Statistics. Statistical analysis was performed using GraphPad Prims 4.03 for Windows. One-way ANOVA followed by Tukey's multiple-comparison test was performed to compare the absorbances of bacterial binding between fractions and control milk powder. Differences in binding to fractions and control milk powder were considered to be significant at P values of <0.05.

RESULTS AND DISCUSSION

Sugar, Proanthocyanidin, and Anthocyanin Contents. The highest sugar concentrations were analyzed in apple juice fraction FII and in pineapple, crowberry, red grapefruit, and bilberry juice fractions FIII (**Table 1**). The highest proanthocyanidin concentrations were found in lingonberry juice fraction FII and cranberry and crowberry juice fractions FIII (**Table 1**). Bilberry and crowberry juice fractions FIII contained the highest amount of anthocyanins (**Table 1**).

Binding Activity of Pili to Fresh Berry and Juice Fractions. The binding of *N. meningitidis* pili to fractions of different molecular size prepared from various fresh berries and juices was screened (**Table 2**). The most active binding was found to the fractions of high molecular size of *Vaccinium* species (FII, FIII), especially to lingonberry juice fractions (**Table 2**). However, there also was remarkable binding activity to cranberry, bilberry, and crowberry juice fractions of high molecular size (FIII) and black currant juice fraction FII. In addition to berry juice fractions, *N. meningitidis* bound to all fractions (FI−FIII) of fresh bilberry and had binding activity to fresh cranberry fraction FII. Berry juice fractions having high binding activity had also high content of proanthocyanidins and/or anthocyanins (**Table 1**). In addition, there was no binding activity to any of the fractions derived from cloudberries or apple, pineapple, raspberry, or tomato juice (**Table 2**). These fractions had high sugar content, but none of the prepared fractions from apple, pineapple, and tomato juice contained detectable amounts of proanthocyanidins or anthocyanins. Cloudberry fraction FI contained only trace amounts of anthocyanins, and raspberry juice contained small amounts of proanthocyanidins or anthocyanins compared to juices of *Vaccinium* species (**Table 1**).

Table 2. Binding Activity of Pili to Berry and Juice Fractions and °Brix Values of the Fractions[a]

berry fraction	°Brix	activity[b]	berry fraction	°Brix	activity[b]
Vaccinium myrtillus					
bilberries			bilberry juice		
FI	3.8	14 ± 2*	FI	17.9	8 ± 2
FII	4.2	18 ± 1*	FII	18.0	13 ± 1
FIII	3.8	13 ± 2*	FIII	18.0	32 ± 2*
Vaccinium oxycoccos					
cranberries			cranberry juice		
FI	3.5	7 ± 1*	FI	17.3	5 ± 1
FII	5.0	11 ± 1*	FII	17.2	14 ± 2*
FIII	4.2	nd	FIII	23.4	42 ± 7*
Vaccinium vitis-idaea					
lingonberries			lingonberry juice		
FI	5.7	12 ± 1	FI	16.8	10 ± 3
FII	10.1	12 ± 2	FII	22.0	61 ± 8*
FIII	7.0	3 ± 0	FIII	19.1	38 ± 4*
Empetrum nigrum			*Ribes nigrum*		
crowberry juice			black currant juice		
FI	16.1	nd	FI	17.2	6 ± 1
FII	11.0	5 ± 0	FII	23.9	17 ± 2*
FIII	11.0	24 ± 2*	FIII	19.2	8 ± 0
Citrus paradisi			*Prunus cerasus*		
red grapefruit juice			sour cherry juice		
FI	16.2	nd	FI	19.3	nd
FII	14.0	nd	FII	14.0	8 ± 0
FIII	16.3	14 ± 2	FIII	17.1	3 ± 0
Malus domestica			*Rubus chamaemorus*		
apple juice			cloudberries		
FI	22.0	nd	FI	3.2	nd
FII	23.1	nd	FII	3.8	nd
FIII	23.1	nd	FIII	4.0	nd
Ananas comosus			*Rubus idaeus*		
pineapple juice			raspberry juice		
FI	15.4	nd	FI	20.2	nd
FII	8.1	nd	FII	20.0	nd
FIII	17.1	nd	FIII	20.3	nd
Solanum lycopersicum					
tomato juice					
FI	8.4	nd			
FII	8.8	nd			
FIII	9.4	nd			

[a] Abbreviations: FI, <10 kDa fraction; FII, 10−100 kDa fraction; FIII, >100 kDa fraction; nd, not detected. Mean ± SD (n = 9). [b] Values marked with * are significantly different (P < 0.05) from binding to dry milk powder.

Previous studies with cultivated cranberry (*V. macrocarpon* Ait.) have indicated that for various bacteria the high molecular weight, nondialyzable material and proanthocyanidins are effective in inhibiting bacterial adhesion and coaggregation (*7*−*9*, *29*). In the present study, berry proanthocyanidins, as well as anthocyanins, were present in the fractions of high molecular size to which *N. meningitidis* pili showed high binding activity. Differences in binding activities between fresh berry and berry juice fractions may result from manufacturing process (e.g., heating, evaporation, enzyme treatment) and higher °Brix values (which were used for determination of the differences in concentrations of soluble solids) in juice fractions derived from commercial berry juice concentrates.

Inhibition of the Binding of Meningococcal Pili to Epithelial Cells. *N. meningitidis* pili bound to HEC-1B cells in a dot binding assay developed in the present study (**Figure 1**). On the basis of the binding activity results (**Table 2**), FII juice fractions were chosen for the binding inhibition study. In general, the fractions that showed binding activity also inhibited the pili binding to epithelial cells. Bilberry, cranberry, lingonberry, crowberry, and black currant juice FII fractions inhibited the binding of meningococcal pili to HEC-1B cells totally (**Figure 1**). FII fraction of raspberry juice inhibited the binding of neisserial pili to the HEC-1B cells to some extent, but tomato,

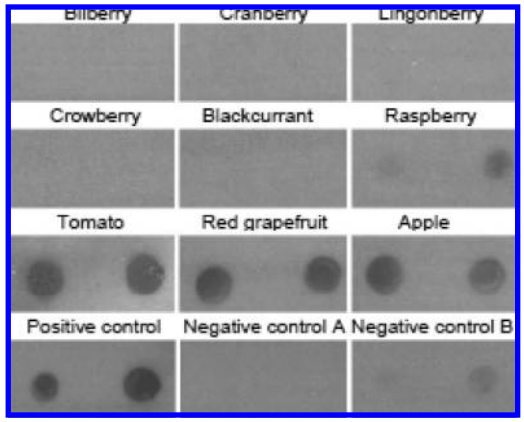

Figure 1. Inhibition of binding of meningococcal pili to HEC-1B cells by berry and fruit juice fractions (FII). Negative control A did not contain streptavidin−POD, and negative control B did not contain pili.

red grapefruit, or apple juice did not inhibit the pili binding at all (**Figure 1**). Raspberry juice FII fraction contained small amounts of proanthocyanidins and anthocyanins compared to juice fractions of *Vaccinium* species, and tomato, red grapefruit, and apple juice had neither proanthocyanidins nor anthocyanins present (**Table 1**). Similarly as described above in the binding activity studies, the fractions having high inhibitory activity had also high content of proanthocyanidins and/or anthocyanins (**Tables 1** and **2**; **Figure 1**).

Our previous studies suggest carbohydrate recognition for *N. meningitidis*. Binding of *N. meningitidis* pili to glycoproteins is prevented by milk oligosaccharide fractions (*20*). Here we have shown that the studied berry juice FII fractions, with a composition of proanthocyanidins and anthocyanins, of bilberry, cranberry, lingonberry, crowberry, and black currant inhibited meningococcal pili binding to the epithelial cell line HEC-1B totally, whereas the samples with no or only low levels of proanthocyanidins and/or anthocyanins did not have the anti-adhesive effect. However, the present study does not rule out a possible role of carbohydrates as receptors for *N. meningitidis*. Carbohydrate-recognizing adhesins may be blocked by berry fraction compounds such as proanthocyanidins. Thus, the results achieved are consistent with studies on *E. coli*, in which cranberry juice proanthocyanidins have been identified as inhibitors of mannose-resistant P-fimbriated Gal-Gal-recognizing *E. coli* (*9*).

SPE Subfractionation and Chemical Characterization of Subfractions. Four berry juice fractions (bilberry FIII, cranberry FIII, crowberry FIII, lingonberry FII) showing the highest activities in neisserial pili binding experiments were chosen for further studies. Cranberry juice FIII and lingonberry juice FII had high levels of proanthocyanidins and low levels of anthocyanins, bilberry juice FIII had a high level of anthocyanins and a low level of proanthocyanidins, and crowberry juice FIII had high levels of both (**Table 1**). SPE with a C-18 column was used in polyphenol subfractionation, and the subfractions were analyzed by RP-HPLC with UV-DAD. In ethyl acetate subfraction the peaks refer to mainly flavonol glycosides and proanthocyanidins (**Figure 2**). Spectrophotometric semiquantification estimated high proanthocyanidin quantities in lingonberry juice FII and cranberry and crowberry juices FIII. However, proanthocyanidins were not seen in the chromatograms in expected amounts. According to the literature, monomeric flavanols as well as oligomeric proanthocyanins can be eluted with ethyl acetate, leaving anthocyanins, polymeric proanthocyanidins, and other pigmented complexes fixed on the column (*26*). In the present study anthocyanins were detected in water and methanol subfractions (eluted after ethyl acetate),

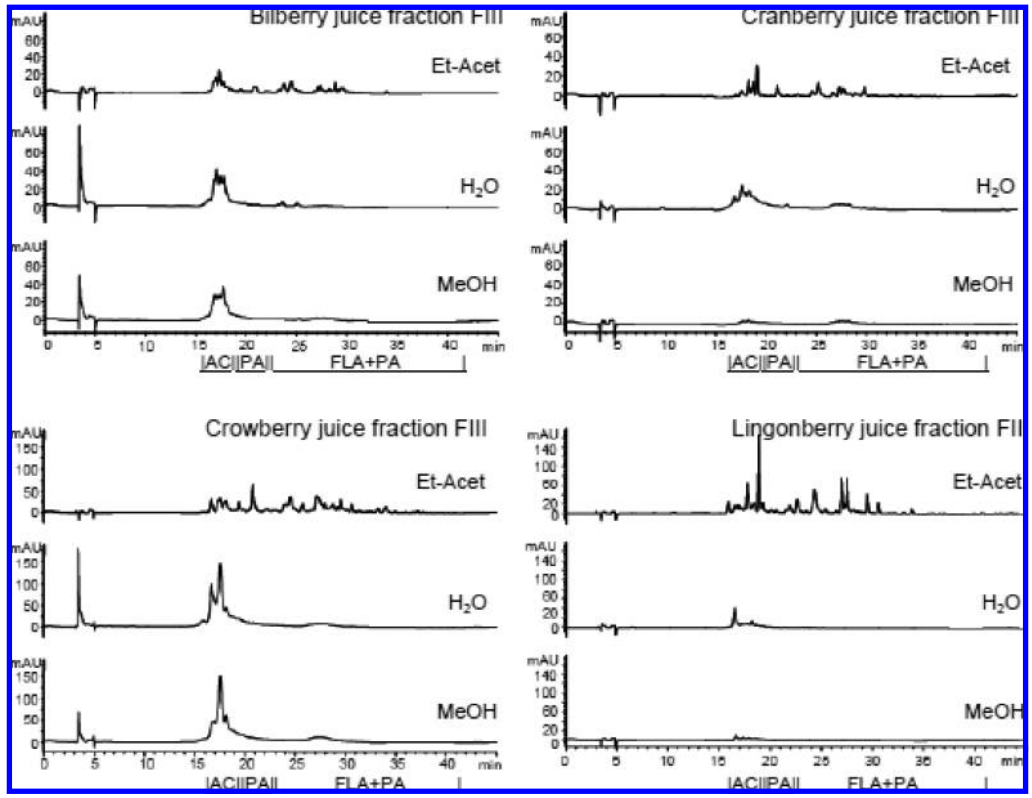

Figure 2. HPLC chromatograms of subfractions of bilberry juice fraction FIII, cranberry juice fraction FIII, crowberry juice fraction FIII, and lingonberry juice fraction FII at 280 nm. Subfractions were eluted from the C-18 SPE column with ethyl acetate (Et-Acet), water (H_2O), and 60% aqueous methanol (MeOH).

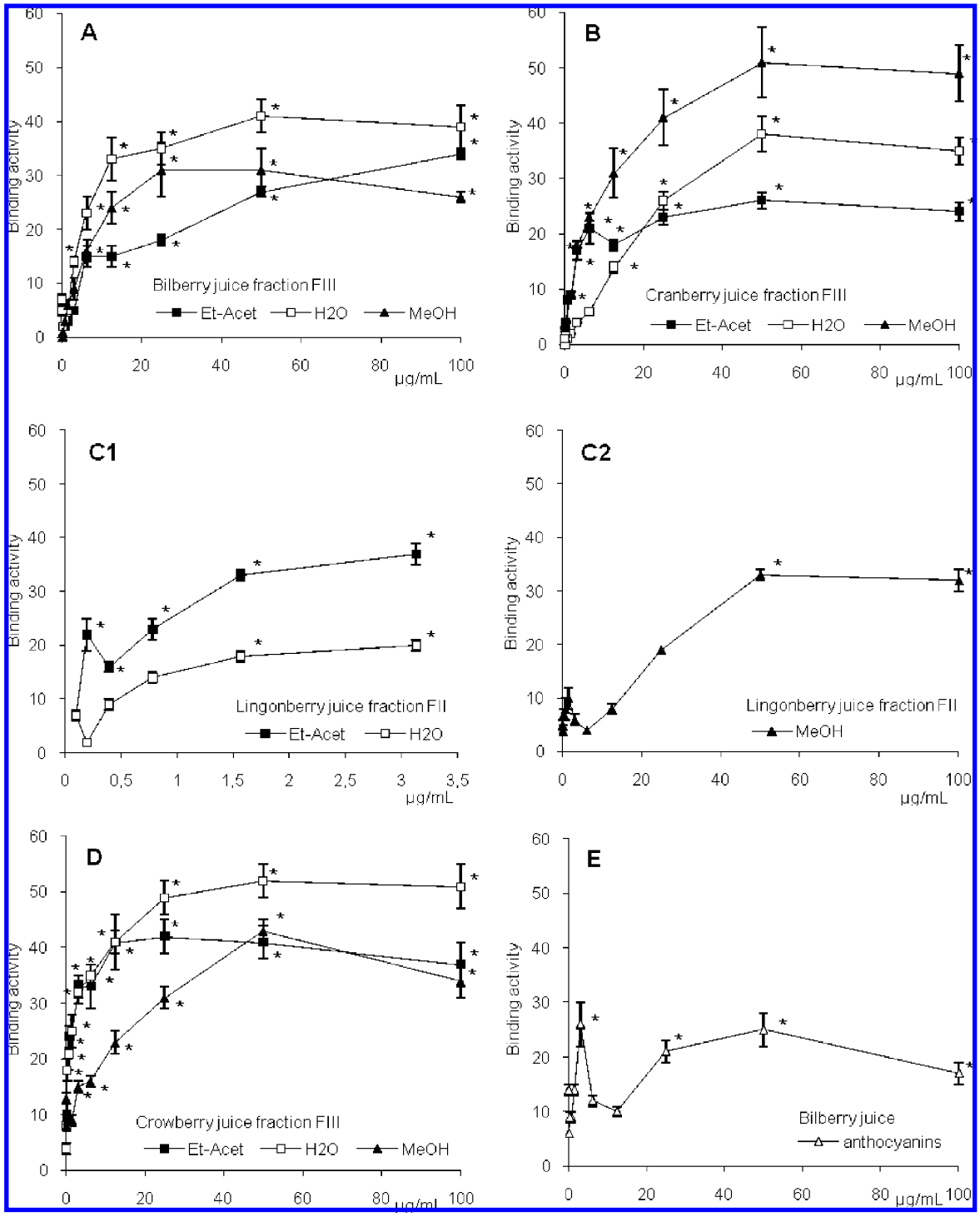

Figure 3. Binding activity (mean ± SD, $n = 3-9$) of neisserial pili to subfractions: (**A**) binding activity to subfractions (ethyl acetate, water, methanol) from bilberry juice fraction FIII (fraction >100 kDa); (**B**) subfractions (ethyl acetate, water, methanol) from cranberry juice fraction FIII (fraction >100 kDa); (**C1**) ethyl acetate subfraction and water subfraction; (**C2**) methanol subfraction from lingonberry juice fraction FII (fraction 10−100 kDa); (**D**) subfractions (ethyl acetate, water, methanol) from crowberry juice fraction FIII (fraction >100 kDa). (**E**) Binding activity of pili to anthocyanins purified from bilberry juice. Significant difference ($P < 0.05$) in pili binding to SPE fractions and to dry milk powder is marked with *.

especially in crowberry and bilberry juice FIII (**Figure 2**), as expected. RP-HPLC cannot separate polymeric proanthocyanidins and other high molecular weight constituents, but polymeric proanthocyanidins may give a drift in chromatographic baseline (*30*). The chromatograms of water and methanol subfractions of cranberry and crowberry juices show a drift in baseline around 25−30 min, and a drift is also seen in the methanol subfraction of bilberry (**Figure 2**). These findings together with previously reported (*26, 30*) data support the presence of polymeric proanthocyanidins or other polymerized structures in, at least, cranberry, bilberry, and crowberry juice fractions. Still, further studies are needed for structure elucidation.

Binding Activity of Pili to the Juice Subfractions. Figure 3 shows the binding activities of *N. meningitidis* pili over a range of concentrations of the subfractionated berry juices (bilberry FIII, cranberry FIII, crowberry FIII, lingonberry FII). In general, dose-dependent increase in binding activity to a constant level was seen. *N. meningitidis* pili binding was

associated with not only one of the subfractions (ethyl acetate, water, or methanol) but with different subfractions depending on the species of the studied berry. This indicates that there were differences in the profiles of active components between the berries. Differences could also be seen in the chromatographic profiles of the berry juice subfractions (**Figure 2**). For both bilberry and crowberry juice the pili binding was highest to components of the water subfraction. However, significant binding was seen at about 10 times lower concentrations to crowberry than to bilberry juice.

Pili binding to bilberry juice was highest to the water subfraction, reaching a constant binding activity level of 40 at 50 μg/mL (**Figure 3A**). Binding to water subfraction was significant down to 3.125 μg/mL, to ethyl acetate subfraction to 6.25 μg/mL, and to methanol subfraction to 12.5 μg/mL. The highest pili binding with cranberry juice was achieved using methanol subfraction; binding activity of 50 was seen at 50 μg/mL (**Figure 3B**). Significant binding to methanol subfraction was detected down to 3.125 μg/mL, to ethyl acetate subfraction to 1.56 μg/mL, and to water subfraction to 12.5 μg/mL. Pili binding activity, however, reached a significantly lower level (value of 23 at 25 μg/mL) with ethyl acetate subfraction compared to methanol subfraction. Ethyl acetate subfraction reached the highest binding activity among the lingonberry juice subfractions (**Figure 3C1**). Binding activity was significant even at 0.195 μg/mL, and it achieved the level of 35 at 3.125 μg/mL. Binding to water subfraction was significant down to 0.78 μg/mL and to methanol subfraction to 50.0 μg/mL (**Figure 3C1**,**3C2**). In crowberry juice the highest pili binding was detected in the water subfraction, reaching a constant binding activity level of 50 at 25 μg/mL (**Figure 3D**). Pili binding to water subfraction was significant even at 0.39 μg/mL, to ethyl acetate subfraction at 0.781 μg/mL, and to methanol subfraction at 3.125 μg/mL.

According to the HPLC results anthocyanins were abundant in water subfractions of bilberry and crowberry juices. To isolate pure anthocyanin fraction from the bilberry juice concentrate for the binding activity testing, a semipreparative HPLC system was used. A pure anthocyanin fraction free of other polyphenols was obtained (HPLC purity data not shown). In the neisserial pili binding experiments there was a trend related to increasing anthocyanin concentration up to a binding activity of 25 at a concentration of 50 μg/mL, although binding activity was highest to 3.125 μg/mL and decreased between the concentrations of 6.25 and 12.5 μg/mL (**Figure 3E**). This binding was lower compared to the binding to the bilberry juice subfractions. This indicates that the binding effect of meningococcus to berries is not solely induced by anthocyanins but also by other polyphenols or other associated compounds. It has been shown that anthocyanins can coexist in the proanthocyanidin fraction (*31*). It is also known that proanthocyanidins can associate with pectin (*31*), polysaccharides (*32*), and proteins (*33*), and the association potential is significantly affected by the degree of their polymerization (*11*). It has also been shown that berry polyphenols work synergistically (*34*).

In conclusion, our findings reveal novel sources of inhibitory compounds against *N. meningitidis*. *N. meningitidis* pili possessed remarkable binding activity to the berry juice fractions and the subfractions containing polyphenols, especially prepared from the *Vaccinium* species and crowberries. Applications of the results could be in the development of antiadhesive drugs and nutritional products. Fractions could be easily utilized by the food industry, because solvents other than water are not needed. Novel preventive and protecting antiadhesion agents may have significance also in developing countries, where needle-based vaccination and preservation of vaccines is difficult. Further analysis of structure elucidation of the active components will be needed, as will cell inhibition assays with whole bacterial cells to confirm the inhibitory antiadhesive effect of the studied berry juice fractions. Clinical trials will be needed to substantiate the possible effect of berry juice components on healthy carriers and blocking the attachment of bacteria to human nasopharynx.

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