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Marko Toivanen

Antiadhesive Molecules in Milk and Berries against Respiratory Pathogens

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MARKO TOIVANEN

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Author's address: School of Pharmacy
University of Eastern Finland
P.O.Box 1627
FI-70211 Kuopio
Finland
Tel. +358 40 355 2962
Fax. +358 17 16 2424
E-mail: marko.toivanen@uef.fi

Supervisors: Docent Carina Tikkanen-Kaukanen, PhD
Institute of Public Health and Clinical Nutrition
University of Eastern Finland
Kuopio, Finland

Professor Seppo Lapinjoki, PhD
School of Pharmacy
University of Eastern Finland
Kuopio, Finland

Professor Seppo Auriola, PhD
School of Pharmacy
University of Eastern Finland
Kuopio, Finland

Reviewers: Docent Anni Virolainen-Julkunen, MD, PhD
Department of Infectious Disease Surveillance and Control
National Institute of Health and Welfare
Helsinki, Finland

Professor Heikki Vuorela, PhD
Division of Pharmaceutical Biology
University of Helsinki
Helsinki, Finland

Opponent: Docent Kirsi-Marja Oksman-Caldentey, PhD
VTT Technical Research Centre
Espoo, Finland

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ABSTRACT

Bacterial attachment to host mucosal tissues is the essential first step in microbial colonization and pathogenesis. The strategy behind antiadhesive agents is to block the adhesion of pathogen to the host cells. Since this reduces the reservoir of bacteria in the human population, it may diminish the frequency of carriers and ultimately reduce the prevalence of bacterial infections. Thus, antiadhesives hold the potential for preventing infectious diseases. This is important since new methods are needed due to shortcomings of effective vaccines and increasing antibiotic resistance.

In the present study, the binding and inhibitory activity of milk, berries and juices were investigated against meningitis- and respiratory infection-associated bacteria. The binding of *Neisseria meningitidis* pili to bovine thyroglobulin was clearly inhibited by human milk neutral and bovine milk acidic oligosaccharides in a microtiter well assay. In cell culture experiments and the microtiter well assay, inhibitory and binding activity with *N. meningitidis* were detected for those bilberry, cranberry, lingonberry, and crowberry fractions which contained anthocyanins or a mixture of proanthocyanidins and flavonols. *Streptococcus pneumoniae* cells bound most extensively to the low-molecular size fraction of cranberry juice and *Streptococcus agalactiae* cells to the high-molecular size fraction of cranberry. Hemagglutination induced by *Streptococcus suis* was most effectively inhibited by the middle-molecular size fraction of cranberry.

In conclusion, it has been possible to identify several previously unknown binding and inhibitory sources which impair bacterial adherence; their activities depend on both the natural component and the bacterial species. These results indicate that neutral human milk oligosaccharides and acidic bovine milk oligosaccharides possess potential value in the development of functional foods or drugs against meningococcal infections and furthermore, berry material, especially from the *Vaccinium* species, may be able to inhibit the attachment of *N. meningitidis*, *S. pneumoniae*, *S. agalactiae*, and *S. suis* to epithelial cells. However, clinical trials will be needed to confirm these *in vitro* results.

National Library of Medicine Classification: QW 52, QU 75, QU 220, QU 83, WB 430, WB 428, WC 245, WC 217

Medical Subject Headings: Bacterial Adhesion; Fimbriae, Bacterial; Milk; Oligosaccharides; Fruit; Vaccinium myrtillus; Vaccinium macrocarpon; Vaccinium vitis-idaea; Flavonoids; Anthocyanins; Proanthocyanidins; Flavonols; Meningococcal Infections; Neisseria; Pneumococcal Infections; Streptococcus; Hemagglutination; Cells, Cultured; In Vitro

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TIIVISTELMÄ

Monet infektiotaudit saavat alkunsa bakteerien sitoutumisella isännän limakalvoille. Tätä sitoutumista voidaan estää antiadhesiivisilla aineilla. Bakteerien sitoutumisen estäminen vähentää myös kantajien määrää väestössä ja bakteeri-infektioiden yleisyyttä. Näin antiadhesiivisilla aineilla voisi olla mahdollista ehkäistä infektiosairauksia. Tämä on tärkeää, koska tehokkaiden rokotteiden puuttuessa ja antibioottiresistenssin lisääntyessä tarvitaan uusia menetelmiä infektioiden ehkäisyyn ja hoitoon.

Tässä tutkimuksessa selvitettiin maidon, marjojen ja mehujen sitoutumis- ja inhibitioaktiivisuutta aivokalvontulehdusta ja hengitystieinfektioita aiheuttavia bakteereita kohtaan. *Neisseria meningitidis* -bakteerin pilusten sitoutuminen tyroglobuliiniin estyi selvästi äidinmaidon neutraaleilla ja lehmänmaidon happamilla oligosakkarideilla kuoppalevykokeissa. Mustikan, karpalon, puolukan ja variksenmarjan fraktioilla, jotka sisälsivät antosyaaneja tai seoksen proantosyanidiineja ja flavonoleja, todettiin sitoutumis- ja inhibitiovaikutusta *N. meningitidis* -bakteeria kohtaan sekä kuoppalevykokeissa että soluviljelmässä. *Streptococcus pneumoniae* -bakteeri sitoutui eniten karpalomehun pienimolekyylikokofraktioon ja *Streptococcus agalactiae* -bakteeri karpalon suurimolekyylikokofraktioon. *Streptococcus suis* -bakteerin aikaansaamaa hemagglutinaatiota esti tehokkaimmin karpalon keskimolekyylikokofraktio.

Tutkimuksessa saatiin selville useita aiemmin tuntemattomia lähteitä molekyyleille, jotka vaikuttavat bakteerien sitoutumiseen riippuen sekä luonnonainekomponentista että bakteerilajista. Nämä tulokset viittaavat siihen, että äidinmaidon neutraaleilla ja lehmänmaidon happamilla oligosakkarideilla voisi olla merkitystä funktionaalisten elintarvikkeiden tai lääkkeiden kehityksessä meningokokki-infektioita vastaan. Lisäksi marjapohjainen aines, etenkin *Vaccinium*-suvun marjoista, saattaa kyetä estämään *N. meningitidis*, *S. pneumoniae*, *S. agalactiae* ja *S. suis* – bakteerien tarttumista limakalvosoluihin. Tarvitaan kuitenkin kliinisiä kokeita varmentamaan näiden *in vitro* –kokeiden tulokset.

Yleinen suomalainen asiasanasto: bakteerit; sitoutuminen; maito; oligosakkaridit; marjat; mehut; mustikka; karpalo; puolukka; variksenmarja; polyfenolit; flavonoidit; antosyaanit; flavonolit; meningokokki; pneumokokki; streptokokit

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Kuopio, January 2011 Marko Toivanen

List of original publications

The present thesis is based on the following original publications, referred to in the text by Roman numerals **I-IV**:

I Hakkarainen J, Toivanen M, Leinonen A, Frängsmyr L, Strömberg N, Lapinjoki S, Nassif X, Tikkanen-Kaukanen C. Human and bovine milk oligosaccharides inhibit *Neisseria meningitidis* pili attachment in vitro. Journal of Nutrition 135: 2445-2448, 2005

II Toivanen M, Ryynänen A, Huttunen S, Duricová J, Riihinen K, Törrönen R, Lapinjoki S, Tikkanen-Kaukanen C. Binding of *Neisseria meningitidis* pili to berry polyphenolic fractions. Journal of Agricultural and Food Chemistry 57: 3120-3127, 2009

III Toivanen M, Huttunen S, Duricová J, Soininen P, Laatikainen R, Loimaranta V, Haataja S, Finne J, Lapinjoki S, Tikkanen-Kaukanen C. Screening of binding activity of *Streptococcus pneumoniae*, *Streptococcus agalactiae* and *Streptococcus suis* to berries and juices. Phytotherapy Research 24: S95-S101, 2010

IV Toivanen M, Huttunen S, Lapinjoki S, Tikkanen-Kaukanen C. Inhibition of adhesion of *Neisseria meningitidis* to human epithelial cells by berry juice polyphenolic fractions. Phytotherapy Research 2011 (Epub ahead of print, doi: 10.1002/ptr.3349)

Contents

Abbreviations

AC	Anthocyanin
Alp	α-like protein
App	Adhesion and penetration protein
BMO	Bovine milk oligosaccharides
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
ChoP	Phosphorylcholine
CpbA	Choline-binding protein
CSF	Cerebrospinal fluid
DAD	Diode array detection
ECM	Extracellular matrix
EHEC	Enterohemorrhagic *E.coli*
FBA	Fructose-1,6-bisphosphate aldolase
FBPS	Fibronectin-fibrinogen-binding protein
FLAV	Flavonols
Fuc	L-fucose
Gal	D-galactose
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBS	Group B streptococcus
Glc	D-glucose
GlcNAc	*N*-acetylglucosamine
HMO	Human milk oligosaccharides
HPLC	High-performance liquid chromatography
HrpA	Haemagglutin/heamolysin-related protein A
kDa	kilodalton
Lmb	Laminin-binding protein
LPXTG	Leu-Pro-X-Thr-Gly-motif
MC	Meningococci

MspA	Meningococcal serine protease A
NadA	Neisserial adhesin A
N-CAM	Neural cell-adhesion molecule
Neu5Ac	*N*-acetyl neuraminic acid (NANA)
Neu5Gc	*N*-glycolylneuraminic acid
NDM	Non-dialysable material
NhhA	*Neisseria* hia homolog A
NMR	Nuclear magnetic resonance
NspA	Neisserial surface protein A
OMP	Outer membrane proteins
PA(C)	Proanthocyanidin
PAFr	Platelet-activating-factor receptor
PavA/B	Pneumococcal adherence and virulence factor A/B
6PGD	6-phosphogluconate dehydrogenase
pIgR	Polymeric immunoglobulin receptor
PsaA	Pneumococcal surface antigen A
RP	Reversed-phase
ScpB	Streptococcal C5a peptidase
Spb1	Surface protein of group B streptococcus 1
Srr-1	Serine-rich repeat protein 1
SrtA	Sortase A
UV	Ultraviolet
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infections

1 Introduction

Respiratory illnesses are one of the leading reasons for seeking medical care. These diseases can evoke both human suffering and considerable financial losses (Waterer and Wunderink 2009). Though upper respiratory infections are very frequent but seldom life-threatening, meningitis and lower respiratory infections, such as pneumonia, are responsible for serious diseases that can lead to acute respiratory failure and death. *Neisseria meningitidis* is a causative agent of sepsis and meningitis (Nassif 2000). Meningococcal meningitis remains a major threat to global health, accounting for an estimated annual 500 000 cases worldwide, with at least 50 000 deaths and as many cases of neurological disability (Pollard 2004). *Streptococcus pneumoniae* is the leading cause of acute bacterial otitis media but responsible also for less frequent, severe infections like meningitis, septicemia, and pneumonia. It is a major cause of morbidity and mortality among children all over the world, particularly in the developing countries (Bogaert et al. 2004). *Streptococcus agalactiae* is one of the most important infectious causes of neonatal morbidity and mortality causing meningitis, pneumonia and septicemia in the newborns and their mothers (Schuchat 2001). *Streptococcus suis* is a porcine pathogen, but has also recently been described as a causative agent behind severe meningitis- and septicemia-associated epidemics in humans (Yu et al. 2006). Only a proportion of these infections are preventable by vaccination, and antibiotic resistance compicates the treatment of some of these infections. Therefore, there is a clear need to develop novel means to prevent and treat these infections for instance by antiadhesive agents.

Most bacterial infections begin with molecular ligand-receptor interactions that occur between the bacterial adhesins located on the surface of the pathogen and the glycoconjugates of host mucosal cell receptors (Ofek et al. 2003). Adherence initiates colonization of oral, nasorespiratory, and genitourinary tracts by bacteria and enhances resistance against host cleansing mechanisms, such as urine flow through the urinary tract or airflow through the lungs. The adhering bacteria are also able to penetrate into host tissues. If one were able to interfere with this interaction by using antiadhesive agents, the risk of infections would diminish. Moreover, bacterial spread between humans and the carrier rate would also be affected, contributing to lower prevalence of bacterial infections.

Milk has evolved to nourish mammalian offspring. Evolution has led to the appearance in milk of components that promote health and survival, such as proteins, peptides, lipids, and antibodies

(German et al. 2002). Certain milk oligosaccharides and glycoconjugates have structures which resemble those found in epithelial cells which are believed to be receptors for pathogens (Gopal and Gill 2000). The presence of these structures in milk has evolved most likely to protect infants from diseases. Several studies have revealed the antiadhesive effect of milk, i.e. that oligosaccharides and glycoconjugates can act as decoys to prevent binding of pathogens to epithelial cells (Coppa et al. 2006, Morrow et al. 2004, Newburg et al. 2004, Martín-Sosa et al. 2002). However, there is lack of information about their effects against *N. meningitidis*.

Plants have also developed defense mechanisms against microbes in the course of Darwinian selection. In addition to antimicrobial peptides, essential oils, and terpenoids, phenolic secondary metabolites can combat pathogens (Cowan 1999). Plant phenolics may also cross-react with human pathogens, for example, cranberries have been traditionally used to protect women from urinary tract infections (UTI). In the American cranberry (*Vaccinium macrocarpon* Ait.), secondary metabolites such as proanthocyanidins, as well as the high-molecular-weight fraction and fructose have been claimed to evoke an antiadhesive effect against *Escherichia coli in vitro* (Di Martino et al. 2006, Foo et al. 2000a, Zafriri et al. 1989). The therapeutic effect of cranberry against UTI has also been demonstrated *in vivo* (Ferrara et al. 2009, Bailey et al. 2007, Stothers 2002). American cranberry has been extensively studied also against *Helicobacter pylori* (Zhang et al. 2005, Shmuely et al. 2004, Burger et al. 2000) and oral bacteria (Koo et al. 2010, Yamanaka-Okada et al. 2008, Weiss et al. 2004). However, the close relatives, European cranberry (*Vaccinium oxycoccos* L.) and lingonberry (*Vaccinium vitis-idaea* L.), have not been evaluated for their antiadhesion activity except for one clinical trial which showed some positive effects against UTI caused by *E. coli* (Kontiokari et al. 2001).

The main objectives of the present study were to investigate the antiadhesive effects of human and bovine milk oligosaccharides against *N. meningitidis* and to screen several berries and juices made from berries and fruits as possible sources of antiadhesive agents to help combat serious respiratory- and meningitis-associated pathogens i.e. *N. meningitidis*, *S. pneumoniae*, *S. agalactiae*, and *S. suis*.

2 Review of the literature

2.1 ANTIADHESION

2.1.1 General

Bacterial attachment to host tissues is the initial infection step. This occurs via a ligand-receptor interaction where bacterial adhesin molecules on the surface of the pathogen bind to complementary carbohydrates on the host cell surface receptors (Ofek et al. 2003) (**Figure 1A**). These initial interactions between the microbe and the host cell are essential for extracellular colonization and internalization. Normally the oligosaccharide units of host cell surface glycoproteins and glycolipids are present to bind to other cells and to function as receptors for hormones and other humoral effectors but many pathogens have specific adhesins that recognize and bind to host cells through these glycoconjugates (Soto and Hultgren 1999, Newburg 1997). The binding of the pathogen activates the expression of new genes in the microbe that are important in the pathogenic process. The binding can also switch on the activation of the innate host defense systems (Soto and Hultgren 1999).

Mucosal epithelia represent the first line of defence against pathogens so bacterial attachment requires effective adhesion strategies (Virji 2009). Ciliated and non-ciliated epithelial cells with overlying mucus layers that are wafted around by the movement of cilia create an unstable environment for bacterial adhesion. In addition, the net negative charge of the host cell surface creates a charge barrier against the negative charge present on most bacterial surfaces.

Initial bacterial adherence and subsequent infection may be prevented if adhesin-receptor interactions can be disrupted or inhibited. Antiadhesion therapy could be used not only for preventing disease but also for the treatment of infection, to detach bacteria which are already attached (Ofek et al. 2003). Antiadhesion therapy consists of two strategies. First, soluble adhesin analogs can bind to the receptor and competitively block adhesion of bacteria (**Figure 1B**). Antiadhesive agent of this type may be isolated adhesin molecules or synthetic or recombinant fragments (Kelly et al. 1999, Lee et al. 1992, Dale et al. 1994). However, careful consideration must be given to their toxicity, immunogenicity, induction of signalling and interference with the physiological functions of the receptor when they bind to host receptors. The second strategy is to

use soluble receptor analogs that are structurally similar to those of the glycoprotein or glycolipid receptors for the adhesin and which act through competitive inhibition (**Figure 1C**). Even non-carbohydrate receptor analogues, although not identical to the native carbohydrate, may bind efficiently to a microbial adhesin (Ofek and Sharon, 1990). If bacteria bind to the receptor analog they can be cleared by physiological cleansing mechanisms such as mucociliary action in the respiratory tract or urine flow in the urinary tract. Affinity of the antiadhesive agents for the bacterial adhesins can be increased by attaching many copies to a suitable carrier, creating a multivalent adhesin inhibitor. Several receptor analogs have been shown to prevent bacterial colonization or infection *in vivo* (Morrow et al. 2004, Ruiz-Palacios et al. 2003, Cywes et al. 2000, Bryan et al. 1999, Mysore et al. 1999, Idänpään-Heikkilä et al. 1997, Johnson et al. 1994, Mouricout et al. 1990, Aronson et al. 1979). Soluble carbohydrate receptor analogs are unlikely to be immunogenic or toxic since these carbohydrates are normal components of human body (Sharon and Ofek 2000).

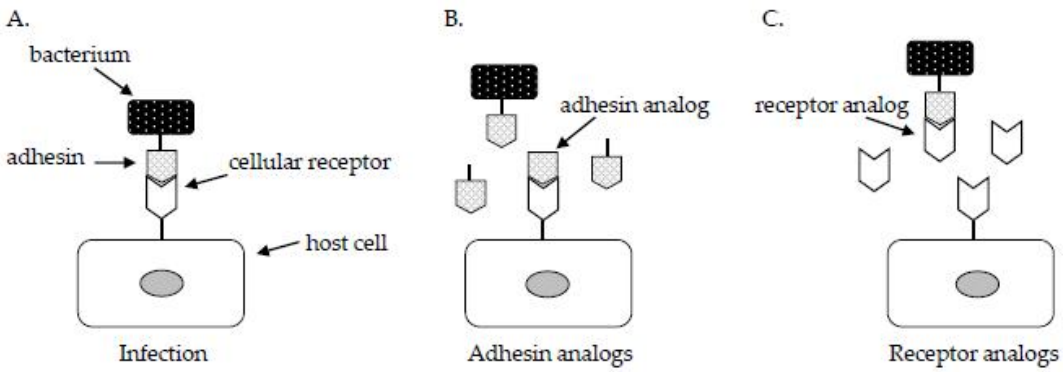

Figure 1. Schematic illustration of bacterial adherence to host cell (A) and action of antiadhesive agents inhibiting bacterial adherence: adhesin analogs (B) and receptor analogs (C).

Antiadhesion therapy has attracted attention as it is considered to be more gentle and ecologically sound compared to tradional antibiotic treatments (Karlsson 1998) and some of the antiadhesive agent candidates are naturally found in foods. Furthermore, although some resistance to antiadhesive agents may occur, the spread of resistant bacterial strains is likely to appear at a significantly lower rate compared to antibiotic-resistant strains (Ofek et al. 2003).

Carbohydrates having antiadhesion activity do not possess bactericidal activity and so the selection of resistant strain is unlikely (Sharon and Ofek 2000). Furthermore, if bacteria lose their affinity to an antiadhesive agent acting as receptor analogue, they will simultaneously lose their ability to bind to the native receptor on host cells.

However, antiadhesion therapy has also some limitations. Pathogenic bacteria often encode genes for more than one type of adhesin and express adhesins on either a random basis or by adapting to different environmental conditions via phase variation (Meyer and van Putten 1989). Therefore a mix of different antiadhesive agents that target several adhesins or a single agent that has a broad spectrum of antiadhesion activity may be necessary (Ofek et al. 2003). Delivery and maintenance of effective concentrations of inhibitor particularly at less accessible mucosal sites is also challenging.

In addition to bacteria, some viruses, fungi, and protozoa bind to oligosaccharide receptors (Olofsson and Bergström 2005, Calderone 1993, Pasvol 1984) and could become targets for antiadhesion therapy.

2.1.2 Milk

Evolutionary pressure has led to the appearance of components in milk that promote health and survival of newborn whose immune system is immature. Milk delivers a defensive capability to the newborns through immunoglobulins, oligosaccharides, and glycoconjugates (Newburg 1997). Human milk contains high concentrations of oligosaccharides and thus the nasopharyngeal and gastric mucosas of the suckling infant are effectively bathed in antiadhesive oligosaccharides.

Human milk is composed mainly of lactose, fat, oligosaccharides, and proteins, but also several other molecules are present in milk (Ebringer et al. 2008). The oligosaccharide fraction is the third largest solid component in milk and represents over 12 g/l in mature milk and even higher, 22 g/l, in colostrum (Newburg 1997). Oligosaccharides are composed of 3 to 10 carbohydrate residues, covalently linked through glycosidic bonds and their composition is very complex. Human milk oligosaccharides are composed of D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), L-fucose (Fuc), and, there are also acidic oligosaccharides, *N*-acetyl neuraminic acid

(sialic acid, Neu5Ac, NANA), (Boehm and Stahl 2003, Rivero-Urgell and Santamaria-Orleans 2001). The oligosaccharides are synthesized in breast, starting with lactose at the reducing end, and they have a core structure of polylactosamine with repetitive galactose and *N*-acetylglucosamine units attached to lactose via a *ß*-glycosidic linkage (Boehm and Stahl 2003). The neutral oligosaccharides are primarily fucosylated at the nonreducing terminus, while most of the acidic oligosaccharides contain a terminal sialic acid conferring a negative charge on the molecule. The lactose at their reducing end can be elongated at the non-reducing galactose (Gal) terminus by the attachment of *N*-acetylglucosamine (GlcNAc) via β-1-3 or β-1-4 linkages, and further addition of galactose (Gal) also by β-1-3 or β-1-4 linkages (Kunz et al. 2000). This results in a multiplicity of polylactosamine core structures. Further variations result from the attachment of fucose (Fuc) (by *α*-1-2, *α*-1-3, or *α*-1-4 linkages), and/or sialic acid (NANA) (by *α*-2-3, *α*-2-6, or *α*-2-8 linkages) to the core structures and to core elongation chains (Newburg et al. 2005). These processes all result in the extensive isomerism of oligosaccharides in human milk and it has been estimated to contain potentially several thousand of such oligosaccharides which could be present and more than 200 oligosaccharides have been found in human milk (Ninonuevo et al. 2006). Concentrations and types of human milk oligosaccharides vary between individuals and change during the course of lactation as the expression of specific glycosyltransferases varies according to the genotype of the mother and by the stage of lactation (Chaturvedi et al. 2001).

The glycoconjugates and oligosaccharides in bovine milk have not been investigated as extensively as those in human milk. Bovine milk has one order of magnitude less oligosaccharides, mostly they are present in colostrum. Their concentration in colostrum is approximately 0.7–1.2 g/l, i.e. 20-fold lower than the corresponding values in human colostrum (Veh et al. 1981).

Almost 40 oligosaccharides have been shown to be present in bovine milk (Tao et al. 2008). When compared to human milk oligosaccharides, the extent of fucosylation in bovine milk oligosaccharides is either totally absent or very low. Bovine milk oligosaccharides are significantly more acidic, with over 70% containing at least one sialic acid. Sialic acid residues include both *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc), the former type being significantly more abundant (Tao et al. 2008). Bovine milk has less diversity with fewer structures per composition compared to human milk oligosaccharides. Bovine milk oligosaccharides are generally composed of shorter oligomeric chains than those present in human milk and they

consist primarily of tri- and tetrasaccharides. Bovine milk oligosaccharides are built around two core disaccharides: lactose and lactose amines. A trisaccharide core composed of a Galβ(1–3) or β(1–6) linked to a lactose is also present, but, at very low abundance. This core structure can be either sialylated or branched with an additional lactose amine. The most abundant oligosaccharides are sialyllactose and sialyllactosamine (Tao et al. 2008).

The oligosaccharide fraction, fucocylated oligosaccharides, siallyllactose, gangliosides, mucin, mannosylated glycopeptides, macromolecule-associated glycans, and glycoproteins in milk have been shown to possess antiadhesive effects against both gram-negative and gram-positive bacteria (**Table 1**). Addition of oligosaccharides to infant food or formulas or adult food may have benefits beyond the nutritional effects, since it may reduce the risk of infections at mucosal sites. This kind of application could be economically feasible by employing milk whey, which is an abundant by-product in the manufacture of cheese (Manso and Lopez-Fandino 2004), as inexpensive source of oligosaccharides.

In addition to inhibition of bacterial attachment, milk glycans have been shown to inhibit the action of toxins secreted by various bacteria (Idota et al. 1995, Newburg et al. 1992, Newburg et al. 1990, Schengrund et al. 1989, Otnaess et al. 1983) as well as viruses such as HIV (Viveros-Rogel 2004, Newburg et al. 1995) and noroviruses (Jiang et al. 2004).

Table 1. Oligosaccharides and glycoconjugates in milk with antiadhesive effect against bacterial pathogens.

I *In vitro* studies		
Bacteria	**Inhibitor**	**Reference**
enterohemorrhagic *E.coli* (EHEC)	mannosylated glycopeptide	Ashkenazi et al. 1991
enteropathogenic *E.coli* (EPEC)	oligosaccharides	Coppa et al. 2006
	gangliosides	Idota and Kawakami 1995
	oligosaccharides	Cravioto et al. 1991
enterotoxigenic *E.coli* (ETEC)	oligosaccharides	Martín et al. 2002
	sialyloligosaccharide fraction	Martín-Sosa et al. 2002

uropathogenic *E.coli* (UPEC)	sialyloligosaccharide fraction	Martín-Sosa et al. 2002
E. coli (S-fimbriated)	sialyllactose	Stins et al. 1994
	mucin	Schröten et al. 1992
H. pylori	glycoproteins	Hirmo et al. 1998
	3′-siallyllactose	Simon et al. 1997
	fucose-containing carbohydrate part of κ-casein	Strömqvist et al. 1995
L. monocytogenes	oligosaccharides	Coppa et al. 2003
M. pneumoniae	sialyllactose	Roberts et al. 1989
P. aeruginosa	macromolecule-associated glycans	Lesman-Movshovich et al. 2003
	sialyllactose	Devaraj et al. 1994
S. fyris	oligosaccharides	Coppa et al. 2006
S. mutans	glycoproteins	Vacca-Smith et al. 1994
S. pneumoniae	sialyllactose	Barthelson et al. 1998
	oligosaccharides	Andersson et al. 1986
S. suis	sialyl oligosaccharides	Liukkonen et al. 1992
V. cholerae	oligosaccharides	Coppa et al. 2006

II *in vivo* studies

Disease	**Inhibitor**	**Reference**
C. jejuni diarrhea on infants	2′-linked fucosylated oligosaccharides	Morrow et al. 2004
C. jejuni diarrhea on mice	fucosylated oligosaccharides	Ruiz-Palacios et al. 2003
E. coli diarrhea on infants	2′-linked fucosylated oligosaccharides	Newburg et al. 2004
H. pylori infection on monkeys	siallyllactose	Mysore et al. 1999
H. pylori infection on mice	milk glycoconjugates	Wang et al. 2001

2.1.3 Berries

In plants, secondary metabolites have developed as one part of the defense mechanism during the course of Darwinian selection. These molecules are produced in response to environmental stress and microbial infections (Scalbert 1991). There is a wide spectrum of phenolic secondary metabolites typically found in berries and in addition to acting against plant pathogens they may also cross-react with human pathogens.

If one considers all berries then it seems that the antiadhesion activities of cranberries (*Vaccinium macrocarpon* Ait.) have been studied most intensively. This may be attributed to their traditional use for the prevention of urinary tract infections. Cranberry preparations have been shown to prevent the adhesion of *E. coli* to different cells, adhesion of *H. pylori* to human gastrointestinal cells, and adhesion or coaggregation of various oral bacteria *in vitro* (**Table 2**). With respect to the other fruits, orange and pineapple juice (Zafriri et al. 1989), guava lectins (Coutiño-Rodríguez et al. 2001), and blueberry proanthocyanidins (Schmidt et al. 2004) are known to prevent the binding of *E. coli in vitro* and the polysaccharides from black currant seeds were active against *H. pylori* (Lengsfeld et al. 2004) *in situ*. Recently, the adhesion of *S. pneumoniae* to human bronchial cells was shown to be inhibited by bilberry, cranberry, and crowberry juice fractions (Huttunen et al. 2011).

The high-molecular weight (>15 kDa) non-dialysable material (referred to as NDM) of cranberry juice has been shown to possess antiadhesion activity (**Table 2**). This fraction is highly soluble in water, devoid of proteins, carbohydrates, and acids and it contains 0.35% anthocyanins and 65.1% proanthocyanidins (Labrecque et al. 2006, Ofek et al. 1996).

In cranberries, two specific compounds have been shown to possess an antiadhesive effect. Fructose was reported to inhibit the adhesion of mannose sensitive type 1 fimbriated *E. coli* to uroepithelial cells (Zafriri et al. 1989). Proanthocyanidins could inhibit the adhesion of P-fimbriated *E. coli*, which adhere to oligosaccharide receptor sequences (α-Gal(1→4)β-Gal) (Källenius et al. 1980), to uroepithelial cells (Gupta et al. 2007, Foo et al. 2000a, Foo et al. 2000b, Howell et al. 1998). Proanthocyanidins, or condensed tannins which is their trivial name, are plant phenolic compounds that have a wide array of potential health benefits (Cos et al. 2004). Proanthocyanidins from different food sources account for the majority of flavonoids consumed in

the Western diet (Gu et al. 2004). One characteristic property of proanthocyanidins is their ability to bind proteins (Hagerman and Butler 1981).

Proanthocyanidins are composed of dimeric to polymeric chains of flavan-3-ols, such as cathechin or epicathechin (Harborne 1994). The universal B-type proanthocyanidins are present in common food sources of proanthocyanidins, such as apples and cocoa (Gu et al. 2003) and the structural units are linked through a single bond (**Figure 2**). The rare A-type proanthocyanidins are double-linked since there is a second ether bond between the A-ring of the lower unit and the C-2 ring of the upper unit (O7→C2) (**Figure 2**). Proanthocyanidins in cranberries consist predominantly of epicatechin units with at least one A-type linkage (Foo et al. 2000a, Foo et al. 2000b). The antiadhesion activity of cranberry has been associated with proanthocyanidins having at least two A-type linkages and the structures of A-linked dimers and trimers that prevent the adhesion of P-fimbriated *E. coli* to uroepithelial cells have been determined (Foo et al. 2000a, Foo et al. 2000b). A-type linkages have also been detected in plums, avocados, and peanuts (Gu et al. 2003) as well as in Finnish *Vaccinium* species, lingonberry, cranberry, bog whortleberry, and bilberry (Määttä-Riihinen et al. 2005).

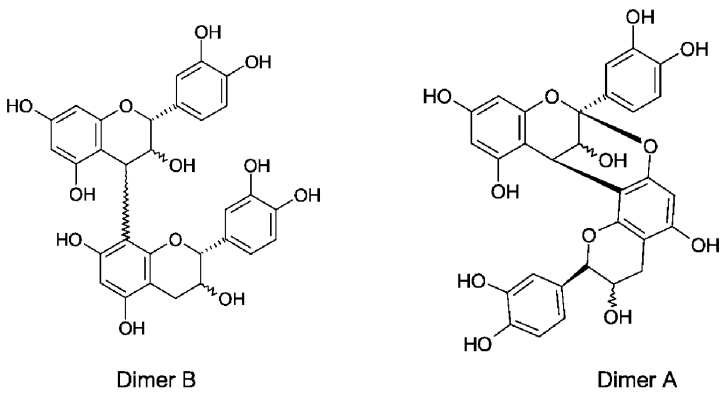

Figure 2. Structures of B-type proanthocyanidins (Dimer B) and A-type proanthocyanidins (Dimer A).

Cranberry may influence bacterial adhesion also by inducing conformational changes in the surface molecules of bacteria (Liu et al. 2006) and by reducing fimbrial expression at the genetic level as well as altering the shape of the bacteria (Ahuja et al. 1998). However, it is unclear how these morphological changes influence adhesion ability. In addition, cranberry NDM has been shown to inhibit enzyme activity which is involved in biofilm formation (Steinberg et al. 2004).

Table 2. Berry and fruit material with antiadhesive effect against bacterial pathogens *in vitro* and *ex vivo*.

Bacteria	Disease	Berry material	Reference
E. coli (type 1 fimbriated)	urinary tract infections (UTI)	cranberry cocktail, cranberry, orange, and pineapple juice	Zafriri et al. 1989
E. coli (type P fimbriated)	UTI	blueberry oligomeric PACs	Schmidt et al. 2004
		cranberry juice cocktail	Howell and Foxman 2002
		cranberry PACs	Foo et al. 2000a/b
		cranberry PAC extract	Howell et al. 1998
		cranberry cocktail NDM	Zafriri et al. 1989
Enterohemorrhagic *E. coli* (EHEC)	gastrointestinal infections	guava lectin	Coutiño-Rodríguez et al. 2001
Uropathogenic *E. coli* (UPEC)	UTI	cranberry powder capsules	Howell et al. 2010
		cranberry juice	Di Martino et al. 2006
		cranberry and blueberry juice	Ofek et al. 1991
		cranberry juice cocktail	Sobota 1984
H. pylori	gastric ulcer	black currant seed high-molecular weight polysaccharides	Lengsfeld et al. 2004
		cranberry juice NDM	Shmuely et al. 2004
		cranberry juice NDM	Burger et al. 2000
Oral bacteria	dental decay	molecular size fractions of berry juices	Riihinen et al. 2011
		cranberry polyphenol fraction	Yamanaka-Okada et al. 2008
		cranberry juice	Yamanaka et al. 2004
		cranberry and blueberry juice NDM	Weiss et al. 2002
		cranberry juice NDM	Weiss et al. 1998
P. gingivalis	periodontitis	cranberry juice NDM	Labrecque et al. 2006
S. mutans	dental decay	cranberry PACs	Koo et al. 2010

		cranberry PACs and FLAVs	Duarte et al. 2006
		cranberry juice	Koo et al. 2006
S. pneumoniae	respiratory infections	bilberry, cranberry, and crowberry juice fractions	Huttunen et al. 2011
S. sorbinus	dental decay	cranberry juice NDM	Steinberg et al. 2005
		cranberry juice NDM	Steinberg et al. 2004
		cranberry NDM	Weiss et al. 2004

Clinical trials have shown the efficacy of cranberry in women with recurrent urinary tract infection (UTI) (**Table 3**), but not in other groups of patients such as people with spinal cord injuries or neuropathic bladder needing cathedrisation (Lee et al. 2007, Linsenmeyer et al. 2004, Waites et al. 2004, Schlager et al. 1999, Foda et al. 1995). Consumption of cranberry juice may not be acceptable over long periods of time as the drop-out rates have been quite high (Weites et al. 2004, Foda et al. 1995, Haverkorn 1994). For example the optimum dosage or method of administration (e.g. juice, tablets or capsules) remains unclear.

Table 3. Berry and fruit material with antiadhesive effect against bacterial pathogens *in vivo*.

Bacteria	**Disease**	**Berry material**	**Effect**	**Reference**
E. coli	urinary tract infections (UTI)	cranberry juice concentrate	prevention of recurrent symptomatic UTI	Ferrara et al. 2009
		cranberry extract capsules	no difference on risk to develop UTI between cranberry and antibiotic profylaxis	McMurdo et al. 2009
		concentrated cranberry extract capsules	prevention of recurrent UTI	Bailey et al. 2007
		cranberry tablets	no preventative effect on UTI	Lee et al. 2007
		cranberry juice	no effect on incidence of symptomatic UTI	McMurdo et al. 2005
		cranberry tablets	no preventative effect on UTI	Linsenmeyer et al. 2004

continues on page 14

continued from page 13

		concentrate cranberry juice capsules	no reduction in bacteriuria or pyuria	Waites et al. 2004
		cranberry juice and concentrated cranberry tablets	decrease in the incidence of symptomatic UTI	Stothers 2002
		cranberry-lingonberry juice	decrease in the incidence of recurrent UTI	Kontiokari et al. 2001
		cranberry juice	reduced biofilm formation	Reid et al. 2001
		cranberry concentrate	no effect on bacteriuria	Schlager et al. 1999
		cranberry capsules	prevention of recurrent UTI	Walker et al. 1997
		cranberry juice cocktail	no preventative effect on UTI	Foda et al. 1995
		cranberry juice cocktail	decrease in the incidence of recurrent UTI	Avorn et al. 1994
		cranberry juice	fewer occurrence of UTI	Havernkorn et al. 1994
H. pylori	gastric ulcer	cranberry juice	suppression of helicobacter infection	Zhang et al. 2005
		cranberry juice	clearance of helicobacter infection in mice	Xiao and Shi 2003
S. mutans	dental decay	cranberry PAC	reduced dental caries development in rats	Koo et al. 2010
		mouthwash with cranberry juice NDM	reduced salivary mutans counts	Weiss et al. 2004
		cranberry	reduced salivary mutans counts	Weiss et al. 2002
Respiratory pathogens and colonic bacteria	respiratory and enteric infections	cranberry juice	no effects on infection rates or nasopharyngeal carriage	Kontiokari et al. 2005

Only a few clinical trials for other bacterial infections have been conducted though there are some positive results for *H. pylori* and *S. mutans* (**Table 3**).

2.2 *Neisseria meningitidis*

2.2.1 *Neisseria meningitidis* as pathogen

Neisseriae are extracellular gram-negative diplococcal bacteria. *Neisseria meningitidis*, meningococcus, and *Neisseria gonorrhoeae*, gonococcus, are significant human pathogens but also the non-pathogenic species such as *Neisseria lactamica*, *Neisseria mucosa*, *Neisseria polysaccharea*, *Neisseria sicca*, *Neisseria subflava*, *Neisseria perflava, Neisseria flava* and *Neisseria flavescens* may cause opportunistic infections in immunosuppressed individuals (Feder et al. 1984).

Humans are the only carriers of *N. meningitidis* and bacteria may colonize the nasopharynx of up to 10% of healthy individuals without evoking any symptoms (Stephens 1999). The duration of carriage varies from transient of a few months to persistent, and it is likely that most individuals are colonized with meningococci at some period during their life. Since there are those asymptomatic carriers, bacteria can spread person to person in close contact through droplets of respiratory or throat secretions. In a small proportion of people, probably due to genetic predisposition, meningococci can invade the bloodstream and cause meningococcal septicemia. Fulminant meningococcal septicemia is a very acute infection with a mortality rate as high as 20–80% (van Deuren et al. 2000). Patients with fulminant meningococcal septicemia may develop septic shock, multiorgan failure, hypotension, purpuric rash (petechiae), and disseminated intravascular coagulation (Namork and Brandtzaeg 2002, Riedo et al. 1995). In this disease, the endotoxin (LPS) levels in plasma can be very high.

Bacteria are also able to cross the blood-brain-barrier. They can gain access to cerebrospinal fluid, CSF, invade the subarachnoid space of brain (Hardy et al. 2000, Nassif 2000), and cause meningococcal meningitis (Nassif 1999, Nassif and So 1995). The classical symptoms of meningitis are acute high fever, stiff neck, and headache (Riedo et al. 1995). A mortality rate of 5 to 15% is associated with meningococcal meningitis even if treated with antibiotics and intensive care (Riedo et al. 1995). Sensorineural hearing loss or impaired vestibular functions are comparatively frequent after meningococcal disease (Naess et al. 1994). Since acute meningococcal diseases can be fatal within a few hours after the appearance of the first symptoms in previously healthy subjects

(Namork and Brandtzaeg 2002, van Deuren et al. 2000, Riedo et al. 1995) rapid diagnosis and prompt antibiotic treatment are important.

Encapsulated *N. meningitidis* is clinically the most important meningococcal phenotype. During dissemination from the site of colonization, polysaccharide capsule expression is switched off and once in blood it is switched on again to protect the bacterium from phagocytosis and to allow the micro-organism to survive in the bloodstream (Nassif 1999). The outer membrane containing lipopolysaccharides and outer membrane proteins (OMP) lies beneath the capsule. There is a cytoplasmic membrane situated under the peptidoglycan cell wall. Based on the structure of capsular polysaccharide, meningococci are grouped into 13 serogroups: A, B, C, D, 29E, H, I, K, L, Y, W-135, X, and Z and more than 90% of meningococcal diseases are caused by serogroups A, B, C, W-135, X, and Y (Brigham and Sandora 2009).

Serogroups B and C are the most likely cause of meningococcal disease in the developed countries, especially in Europe. Serogroup B is the most common, accounting for about 2/3 of all meningococcal invasive diseases in the European Union and approximately 1/3 of disease in the United States (Pillai et al. 2005). In North America, serogroups Y and W-135 are responsible for most of the infections (Riordan 2010). Epidemic meningococcal disease in developing countries is mainly due to serogroup A. The largest and most frequently recurring outbreaks have occurred in the semi-arid area of sub-Saharan Africa, known as the African meningitis belt stretching from Senegal to Ethiopia (Greenwood 1999). Within the meningitis belt, meningococcal disease occurs in epidemic cycles which last between 5 to 10 years.

Due to the rapid progression of meningococcal disease, rapid diagnosis and immediate antibiotic treatment are crucial. In most cases, patients are treated with parenteral administration of β-lactam antibiotics (Chaudhuri et al. 2008). Chloramphenicol may be used for patients allergic to penicillins (Ferguson et al. 2002). However, decreased susceptibility or resistant strains have been reported for penicillin (van Deuren et al. 2000, Dillon et al. 1983), chloramphenicol (Galimand et al. 1998), ciprofloxacin (Shultz et al. 2000), rifampicin (Yagupsky et al. 1993), and tetracycline (Winterscheid et al. 1994).

Antibiotics are also used for prophylaxis for high-risk contacts to eradicate any nasopharyngeal carriage of *N. meningitidis*. One dose of ciprofloxacin, cetriaxone or azithromycin, or rifampicin for two days is recommended for household contacts, persons in day care, individuals with direct

exposure to the secretions of an index case, persons who have been frequently exposed to an index patient by eating or sleeping with the patient or health care professionals who have been exposed to mouth or throat secretions (Ruotsalainen et al. 2009).

Meningococcal diseases caused by serogroups A, C, Y, and W-135 can be readily prevented by monovalent or multivalent polysaccharide vaccines and conjugate vaccines. However, the polysaccharide vaccines which are based on high-molecular-weight capsular polysaccharides (Gotschlich et al. 1969) do not induce T cell-dependent immunity. They are poorly immunogenic in children less than two years old (Al-Mazrou et al. 2005) and fail to elicit an immunological memory. However conjugate vaccines using diphtheria or tetanus toxoid as a carrier of polysaccharide antigens can provide immunological protection also for infants and they have been shown to elicit significantly higher and more persistent serum bactericidal antibody responses than the polysaccharide vaccine (Snape et al. 2008).

No effective vaccine is available against serogroup B meningococci which are responsible for the majority of meningococcal diseases in the developed countries. The poor immunogenicity and its similarity to glycosylated antigens on human cells have complicated the development of an effective and safe vaccine. The polysaccharide capsule of serogroup B is a homolinear polymer of $\alpha(2\rightarrow8)$ linked *N*-acetylneuraminic acid (polyα2-8NeuAc) (Finne et al. 1983) and it has been found to be non-immunogenic (in men, rabbit or mice) even when conjugated to a protein carrier most likely due to its similarities to the polysialic acid units of the neural cell-adhesion molecule (N-CAM). Additionally, monoclonal IgG antibodies against the polysaccharide capsule of serogroup B cross-react with neural and extraneural tissues (Finne et al. 1987). Therefore, vaccination based on polysaccharide of *N. meningitidis* serogroup B may lead to autoimmune reactions and have serious unwanted effects (Finne et al. 1987). Several group B vaccine candidates, mainly based on bacterial outer membrane vesicles or proteins, have been investigated in clinical and preclinical trials as recently reviewed by Granoff (Granoff 2010). However, these vaccines seem to elicit only narrow strain-specific complement-mediated bactericidal activity directed against the strain which had been used to prepare the vaccine.

2.2.2 Adhesion mechanisms

Human specificity poses a problem if one wishes to develop animal tests to model the pathogenic mechanisms behind meningococcal diseases. Although the molecular mechanisms of the attachment of *N. meningitidis* to the cells are not yet fully understood, the major adhesins of *N. meningitidis* include type IV pili which provide specific interactions with non-ciliated cell of the nasopharyngeal epithelium in encapsulated strains (Nassif 1999, Pujol et al. 1997, Pinner et al. 1991) and the outer membrane opacity proteins, Opa and Opc (**Figure 3**). Type IV pili are widely found in gram-negative bacteria and they play an important role also in the movement of bacterium on solid surfaces referred to as twitching motility (Mattick 2002) These proteins are also involved in extracellular protein secretion (Lu et al. 1997), in the transfer of genetic material (Mattick 2002), and in bacteriophage adsorption (Soto and Hultgren 1999).

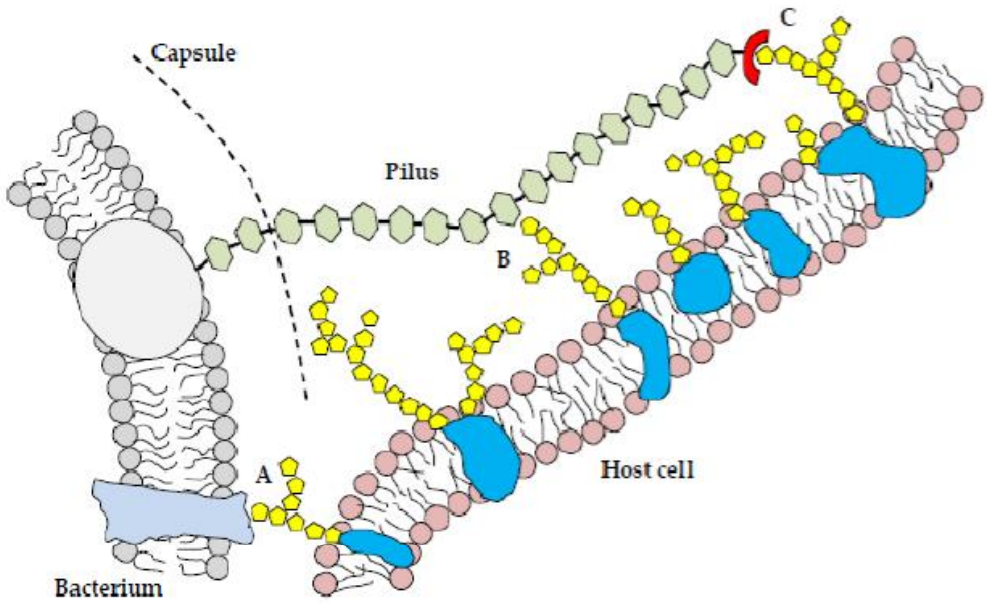

Figure 3. The possible attachment mechanisms of *N. meningitidis*: outer membrane proteins (A), pilin protein (B) or tip-located PilC protein (C) bind to the oligosaccharide structures of surface receptor of host cell. According to Nassif 1999.

Type IV pili are long and flexible filamentous, hair-like protein structures comprising of thousands of copies of a single subunit, pilin (Craig et al. 2006). *N. meningitidis* type IV pilus is 500–

6000 nm in length and approximately 4–6 nm in diameter (Stephens et al. 1985). The major structural subunit is a 17-21 kDa pilin protein (PilE) (Stephens et al. 1985). Genetic analyses have identified 15 proteins involved in the biogenesis, assembly, and disassembly of meningococcal pili (Carbonnelle et al. 2006). The mechanical stability that is an essential property of the type IV pili is achieved by pilus central layer β-sheet hydrogen bonding (Forest and Tainer 1997). Subunits are assembled such that only the hypervariable and sugar-binding regions are exposed and this kind of assembly of pilin subunits is considered to account for the antigenic variation of pilin (Soto and Hultgren 1999). This antigenic variation due to the amino acid changes in the hypervariable regions and phase variation, i.e. a reversible switching of gene expression on and off (Meyer and van Putten 1989), are devices to modulate surface antigenic make-up and they can occur very quickly to evade the host immune responses and control the expression of adhesins.

Pilin undergoes various post-translational modifications, such as glycosylation which potentially affect its cellular interactions (Virji 1997). The two common post-translational modifications of *N. meningitidis* pilin are the rather unusual O-linked trisaccharide Galβ1-4Galα1-3[2,4-diacetamido-2,4,6-trideoxyhexose] attached to Ser or Thr (Stimson et al. 1995) and α-glycerophosphate, a unique substituent at Ser^{93} (Stimson et al. 1996). Ser^{68} may also be modified with phosphoethanolamine or phosphorylcholine (Hegge et al. 2004, Weiser et al. 1998). These modifications lie within negative patches in the assembled pili and their variations are belived to modulate the adhesion properties of the pilus (Craig et al. 2006).

Certain pilin variants contribute to high-adhesive phenotype by enhancing interbacterial interactions via pilus bundling (Marceau et al. 1995). Groups of 8-10 pili may associate in a bundle and act as coordinated retractable units. The successive extension, binding and retraction of type IV pili enable bacteria to move by twitching motility and spread on the apical surface of the host cells.

In addition to pilin, *N. meningitidis* pili consist of 110 kDa pilus associated protein, PilC, located at the tip of the pili (Rudel et al. 1995). Most pathogenic *Neisseria* express two PilC variants, PilC1 and PilC2, which are independently expressed from separate loci and distinctly regulated (Taha et al. 1998, Jonsson et al. 1995). Both PilC proteins play a role in piliation as both $PilC1^{-}/PilC2^{+}$ and $PilC1^{+}/PilC2^{-}$ strains are piliated and a null mutation to both *pilC* genes can abolish piliation (Nassif et al. 1994). PilC proteins also play a crucial role in adherence as major regulators of

meningococcal adhesion and pilus retraction (Morand et al. 2004). PilC protein has been postulated to carry the cell binding domain as inhibition of adhesion was achieved using purified PilC molecules (Rudel et al. 1995). However, non-adhesive non-piliated isolates of serogroup B strain meningococci with high PilC expression and piliated adhesive isolates with barely detectable PilC expression have been described as well (Virji et al. 1995). The most recent results show that both PilC1 and PilC2 are capable of mediating adhesion independently (Morand et al. 2009). PilC1 enables meningococcal attachment to many epithelial or endothelial cells whereas PilC2 selectively mediates adhesion to a more restricted range of cell types. They also elicit different structural and signalling responses in the host cell. The independent regulation of both PilC variants in wild type bacteria could enable meningococci to sequentially modulate the host cell response in a controlled manner depending on the cellular diversity of each ecological niche.

CD46 is a transmembrane glycoprotein which regulates complement activation. CD46 has been proposed to be involved in binding of type IV pili of pathogenic *Neisseriae* to host cells (Källström et al. 1997) and mediating *N. meningitidis* passage through blood-brain barrier assisting the bacterium gain access to the meninges (Johansson et al. 2003). However, these observations are not supported by some reports (Kirchner et al. 2005, Tobiason and Seifert 2001) suggesting that other cellular components in addition to CD46 are likely to be responsible for the interactions between pili and human barrier cells.

N. meningitidis is capable of modulating host cell signalling machinery through pili (Opitz et al. 2009). Pili-mediated adhesion induces cytoskeleton re-arrangements as well as modification of global intracellular signalling networks (Lambotin et al. 2005, Hoffmann et al. 2001). This exploitation of the host-cell signalling pathways could be pivotal in promoting intimate attachment to the cell surface, avoiding bacterial detachment under flow conditions in the nasopharynx as well as in the blood (Mikaty et al. 2009, Mairey et al. 2006). Signalling leads to the formation of microvilli-like structures, cortical plaques, at the site of bacterial attachment (Merz et al. 1999) and it facilitates the invasion process. Extension and cellular attachment followed by retraction or disassembly of the pili may decrease the distance between the bacterial and eukaryotic membranes, thereby enabling more intimate cellular interactions via integral outer membrane adhesins.

In addition to pili which mediate adhesion in encapsulated strains, several other meningococcal adhesins have been described (**Table 4**). The most widely studied are the meningococcal integral outer membrane opacity proteins, Opa and Opc, which further aid pili-facilitated adhesion to host cells. These proteins are basic in nature and that may enable better targeting of the host receptors as the net negative charge of the host cell surface creates a charge barrier against the negative charge present on most bacterial surfaces. Both of these adhesins are also known to bind to host glycans and proteins through specific interactions that are not entirely dependent on electrostatic charge. Capsule decreases Opa- and Opc-mediated adhesion and invasion which may be due to the physical masking of adhesins, or to modifications of charge or hydrophobicity. The inaccessibility of these adhesins in the meningococcal cell surface may become important during the later stages of the infection as strains isolated from the blood or the CSF are usually encapsulated.

Opa proteins are transmembrane molecules forming eight-stranded β-barrel structures with two hypervariable, one semivariable and one invariant surface-exposed loops (Malorny et al. 1998). Several complete copies of *opa* genes have been found in *N. meningitidis*. Each *opa* gene is expressed independently giving rise to Opa antigenic variation (Hobbs et al. 1998, Aho et al. 1991), generating an enormous number of Opa variants. However, only specific Opa variants seem to be prevalent in *N. meningitidis* isolates (Callaghan et al. 2006).

Opc (OpcA) is encoded by a single gene and is relatively invariant in structure. It is a 10-stranded β-barrel protein with five surface exposed loops (Zhu et al. 1999, Olyhoek et al. 1991, Achtman et al. 1988). The levels of Opc expressed may be modulated by a transcriptional control mechanism (Sarkari et al. 1994). The expression of Opc has been speculated to be associated with the ability of *N. meningitidis* to cause meningitis (Unkmeir et al. 2002).

Both Opa and Opc bind to two common negatively charged molecules, heparan sulphate proteoglycans (HSPG) and sialic acids (Moore et al. 2005, de Vries et al. 1998) but they also show a degree of receptor specificity towards pyranose saccharides and maltose, lactose, and sialic acid-containing oligosaccharides (Moore et al. 2005). Opa can also interact with carcinoembryonic antigen-related cell adhesion molecules (CEACAM) which belongs to the immunoglobulin superfamily (Virji 1996). One of the responses to inflammatory cytokines is the up-regulation of CEACAM1 and this may increase Opa-mediated binding and promote bacterial invasion of human

epithelial cells (Griffiths et al. 2007). Opc can also bind to vitronectin and fibronectin, which are proteins present in the extracellular matrix (Unkmeir et al. 2002, Virji et al. 1994).

The recent genome sequencing of *N. meningiditis* has revealed several additional minor adhesins and related proteins (**Table 4**). They are generally expressed at low levels *in vitro* and are several orders of magnitude less effective in mediating interactions with target cells compared with pili or the opacity proteins. Nonetheless, they may be important *in vivo* as they may operate simultaneously to increase the avidity of bacterial binding to the host cell surface.

Autotransporter proteins include NhhA (*Neisseria* hia homologue A) and App (adhesion and penetration protein). The capsule has no interfering effect on adhesion mediated by NhhA or App (Scarselli et al. 2006, Serruto et al. 2003). NhhA-mediated adhesion has been described to epithelial cells, heparan sulphate proteoglycans, and laminin (Scarselli et al. 2006). App is believed to aid bacterial colonization by contributing to the initial adherence to eukaryotic cells or by helping to achieve a more intimate contact between the meningococcus and the cell surface (Serruto et al. 2003).

Outer membrane-localized FBA (fructose-1,6-bisphosphate aldolase) is a highly conserved protein that is required for optimal adhesion of meningococci to human cells (Tunio et al. 2010).

HrpA/HrpB (haemagglutin/haemolysin-related proteins) is a two partner secretion system found in all meningococcal strains which favour bacterial adhesion to some epithelial cell lines (Schmitt et al. 2007) and which are essential for intracellular survival of *N. meningitidis* (Talà et al. 2008).

MspA (meningococcal serine protease A) mediates adhesion to both epithelial and endothelial cells (Turner et al. 2006) and it is expressed by several virulent strains.

NadA (neisserial adhesin A) has an N-terminal globular region that interacts with an unidentified protein receptor on epithelial cells (Capecchi et al. 2005). It is expressed in several hyper-virulent lineages and its expression is phase variant (Comanducci et al. 2004).

NspA (neisserial surface protein A) is a highly conserved basic β-barrel protein with four surface exposed loops. It is a homolog of Opa proteins but not phase variable (Vandeputte-Rutten et al. 2003). These potential binding sites for hydrophobic ligands such as lipids are still to be characterized in detail.

Table 4. Adhesins of *Neisseria meningitidis*.

Adhesin	Mass	Receptor	Reference
Pilus adhesins:			
Pilin (PilE)	17–21 kDa	CD46?	Nassif et al. 1994, Källström et al. 1997
PilC	110 kDa	CD46?	Taha et al. 1998, Källström et al. 1997
Non-pilus adhesins:			
Opa	24–35 kDa	CEACAM1, HSPG, sialic acid	Aho et al. 1991, Moore et al. 2005, de Vries et al. 1998, Virji 1996
Opc	24–35 kDa	HSPG, sialic acid, vitronectin, fibronectin	Olyhoek et al. 1991, Moore et al. 2005, Unkmeir et al. 2002, de Vries et al. 1998, Virji et al. 1994
NhhA (*Neisseria* hia homolog A)	57 kDa	Laminin, HSPG	Scarselli et al. 2006
App (adhesion and penetration protein)	160 kDa	uncharacterized protein	Serruto et al. 2003
FBA (fructose-1,6-bisphosphate aldolase)	38 kDa	unknown	Tunio et al. 2010
HrpA (haemagglutin/heamolysin-related protein A)	180 kDa	unknown	Schmitt et al. 2007 Talà et al. 2008
MspA (meningococcal serine protease A)	157 kDa	unknown	Turner et al. 2006
NadA (neisserial adhesin A)	38 kDa	uncharacterized protein	Capecchi et al. 2005 Comanducci et al. 2004
NspA (neisserial surface protein A)	18 kDa	uncharacterized lipid	Vandeputte-Rutten et al. 2003

2.3 Streptococci

2.3.1 General

Streptococci are gram-positive bacteria of which over 100 species have been characterized so far (Nobbs et al. 2009). Different streptococcal species adhere selectively to different sites in the human body, this being based on the expression of various adhesins. The diseases caused by streptococci range from middle ear infections, pharyngitis, and dental caries to meningitis and necrotizing fasciitis (Nobbs et al. 2009). However, many streptococcal species are members of the commensal microflora, present on mucosal surfaces and generally causing no harm.

Streptococcal adherence and colonization are complex multilevel processes. Gram-positive bacteria, like streptococci, have a more extensive and complex surface proteome than gram-negative bacteria. This enables multiple interactions with different host components. Due to the diverse adhesin-receptor interactions with their different affinities, it has been difficult to characterize involved the adhesins in these processes. Antibodies generated to specific surface proteins may only have a minor effect on streptococcal adherence and single-gene knockouts may reveal very little about mechanisms of adhesion.

It has been postulated that multiple streptococcal adhesins with differing affinities for the eukaryotic cells act in two steps (Hasty et al. 1992). The first interaction is relatively weak and reversible and it is mediated by components of the cell wall. The second interaction involves proteins and this leads to a firm adhesion of bacteria to eukaryotic cells.

Several streptococcal surface proteins are known to be involved in binding to various extracellular matrix (ECM) proteins (Nobbs et al. 2009). These proteins attach bacteria to the ECM, which acts as a bridge between streptococci and host cells. The ECM is a stable macromolecular structure underlying epithelial and endothelial cells and surrounding connective tissue cells (Westerlund and Korhonen 1993). Its composition varies in the different organs, but the main components are fibronectin, collagen, elastin, laminin, and glycosaminoglycans. Many of these proteins can potentially serve as surface receptors for bacterial binding to host cells via their adhesins (Westerlund and Korhonen 1993). The most widely described interaction is the binding with fibronectin (Joh et al. 1999), which is a large dimeric glycoprotein present in the ECM in a fibrillar form. All streptococci express fibronectin binding proteins (Nobbs et al. 2009).

2.3.2 *Streptococcus pneumoniae*

Streptococcus pneumoniae, pneumococcus, is a facultative anaerobic diplococcal human pathogen which causes severe infections such as meningitis, septicemia, pneumonia as well as the less severe but more frequent otitis media, sinusitis or recurrent bronchitis (Bogaert et al. 2004).

Pneumococcus is transmitted through respiratory droplets between individuals. From the nasopharynx, pneumococci gain access to the sinuses and middle ear cavity or to the lungs. Invasive pneumococcal disease occurs after the bacteria pass into the blood and/or the blood-brain-barrier.

Pneumococcus is a leading cause of morbidity and mortality among children worldwide, particularly in the developing countries. Pneumococcal infections are considered as only second to malaria in importance in the World Health Organization´s list of serious infectious diseases. It is estimated that 10.6 million children less than 5 years suffer pneumococcal disease every year and these infections kill about one million children in the world annually (Bogaert et al. 2004, Black et al. 2003).

Like many other species of streptococci, *S. pneumoniae* is also part of the commensal microflora found on mucosal surfaces. The bacterium is frequently carried asymptomatically in the nasopharynx of small children, and even as high carriage rates as 60% have been described although the carriage rate varies greatly for instance with age, seasonality, and geography (Bogaert et al. 2004). The highest frequency of pneumococcal colonization is found in crowded communities such as in hospitals and day-care centres with young children being considered to be the most important vector for horizontal spread of pneumococcal strains within the community (Principi et al. 1999, Reichler et al. 1996). Therefore, prevention of nasopharyngeal colonization especially in children is an important part of any strategy to prevent pneumococcal disease.

The encapsulated strains of *S. pneumoniae* are virulent as they are able to invade lungs and they are not easily removed by phagocytosis (Jonsson et al. 1985). The capsular polysaccharides on the surface of pneumococcus are highly heterogenous and 40 serogroups consisting of over 90 capsular serotypes have been described (Joloba et al. 2010). The most common serogroups worldwide are 6, 14, 19, and 23 (Hausdorff et al. 2000); 20 serotypes are responsible for more than 80 % of invasive pneumococcal disease (Lynch and Zhanel 2009). The cell wall under the capsule consists of polysaccharides and teichoic acid.

The two types of currently licensed vaccines (Bridy-Pappas et al. 2005) are the pneumococcal polysaccharide vaccine which is based on purified capsular polysaccharides, and the pneumococcal conjugate vaccine in which the capsular polysaccharides are chemically conjugated to a protein carrier. The 23-valent pneumococcal vaccine contains 23 serotypes that comprise approximately 90 % of the most frequent isolates. Since September 2010, the 10-valent conjugate vaccine has been included in the national vaccination program of children in Finland. Surface protein based newer vaccine approaches have been developed and animal and clinical trials have been promising (Hamel et al. 2004, Briles et al. 2000).

Penicillins have been the main antibiotics used for the treatment of pneumococcal infections. However, the development of antibiotic resistance has complicated the treatment of pneumococcal infections. Resistance to essential antimicrobials such as the penicillins and other β-lactam antibiotics, fluoroquinolones, tetracyclines, trimethoprim, cephalosporins, and macrolides is a serious and rapidly spreading problem worldwide (Whitney et al. 2000). High-dose β-lactam antibiotics (penicillins, second or third generation cephalosporins) are used to overcome resistance mechanism (Yu et al. 2003).

Different pneumococcal strains vary in the composition, expression, and exposure of their surface-associated proteins and this has been shown to associate with variation in colonization and invasion capabilities between the strains. Reversible phenotypic variation within pneumococcal strains has a role in host interactions; the transparent phase variants have been demonstrated to show increased colonisation compared to the opaque variants (Weiser et al. 1994). The variation is associated with lower expression of capsule polysaccharides and higher expression of certain cell-surface proteins and carbohydrate-containing cell-wall structures (Weiser and Kapoor 1999, Weiser et al. 1996).

The capsule of *Streptococcus pneumoniae* is crucial during colonization, invasion, and dissemination from the respiratory tract. Surface proteins contribute to the hydrophobic and electrostatic surface characteristics of pneumococci and might facilitate adherence to host cells partly through non-specific, physicochemical interactions (Swiatlo et al. 2002). Pneumococcal neuraminidase improves pneumococcal colonisation by cleaving *N*-acetylneuraminic acid (sialic acid) from mucin and decreasing the viscosity of the mucus (Tong et al. 2000). It also cleaves glycolipids, glycoproteins, and oligosaccharides, and exposes the *N*-acetyl-glycosamine receptors

on the non-inflamed resting host epithelial cells which can interact with pneumococcal surface-associated proteins leading to asymptomatic colonization (Tong et al. 2000). Glycoconjugates containing the disaccharide units Galβ1-4GlcNAcβ1-3Gal, GalNAcβ1-4Gal, and GalNAcβ1-3Gal have been proposed as pneumococcal cell receptors, which exist on normal resting respiratory epithelial cells, type 2 lung cells, and endothelial cells (Tuomanen 1997, Cundell et al. 1995, Andersson et al. 1983). The conversion of asymptomatic colonization to invasive disease may be due to the local generation of inflammatory factors such as interleukin 1 and tumour necrosis factor, as seen in the presence of viral infections (Tuomanen 1997). The inflammatory cascade may change the type and number of receptors on target epithelial and endothelial cells.

Pneumococcal cell-wall phosphorylcholine (ChoP) (**Table 5**) has been shown to bind to the platelet-activating-factor receptor (PAFr) that is upregulated by cytokine stimulation during the inflammatory response and viral infections, and this might explain the increased occurrence of pneumococcal pneumonia following viral infection (Cundell et al. 1995). The adherence of pneumococci to lung epithelial cells increases in the presence of human IgA. Pneumococcal IgA1 protease cleaves IgA, which results in neutralisation of the surface charge and increases the physical proximity of ChoP to the PAFr (Weiser et al. 2003, Ring et al. 1998). This binding process promotes the transcellular migration of pneumococci through respiratory epithelium and vascular endothelium, resulting in invasion of living bacteria (McCullers and Rehg 2002, Cundell et al. 1995).

CbpA (also referred to as PspC or SpsA), one of the choline-binding proteins on the pneumococcal cell-surface, is involved in the adhesion of bacteria to the nasopharynx (Rosenow et al. 1997). CbpA directly interacts with the polymeric immunoglobulin receptor (pIgR) in a human- and cell-specific manner and mediates translocation of pneumococci across mucosal respiratory epithelial cells (Brock et al. 2002, Hammerschmidt et al. 2000, Zhang et al. 2000). CbpA gene expression is upregulated in pneumococci during attachment to nasopharyngeal epithelial cells (Orihuela et al. 2004). CbpA has increased affinity for immobilised sialic acid and lacto-N-neotetraose in cytokine-activated human cells (Rosenow et al. 1997). There are also other choline-binding proteins, CbpD, CbpE, CbpG, LytB, and LytC, which promote colonization of the nasopharynx (Gosink et al. 2000).

Pneumococcal adherence and virulence factor A (PavA), a protein present on the surface of pneumococcus, has been shown to bind to the immobilized form of the adhesive glycoprotein

fibronectin (van der Flier et al. 1995). PavA is also essential for fibronectin-independent adherence of pneumococci (Pracht et al. 2005). Instead of acting directly as an adhesin, PavA has been proposed to be involved in modulating other unidentified virulence determinants of pneumococci. However, another surface-exposed pneumococcal adherence and virulence factor, PavB, which interacts with fibronectin and plasminogen, was recently postulated as being a pneumococcal adhesin since it contributes to pneumococcal colonization and infections of the respiratory airways (Jench et al. 2010).

It has been suggested that pneumococcal surface antigen A (PsaA), a metal-binding lipoprotein, functions directly as an adhesin (Zhang et al. 2000). PsaA is upregulated in pneumococci which are attaching to nasopharyngeal cells as shown in microarray analysis (Orihuela et al. 2004) and it binds to nasopharyngeal cells through an interaction with E-cadherin, a transmembrane glycoprotein involved in calcium-dependent associations between cells (Anderton et al. 2007).

Sortase A (SrtA) is a membrane-anchored transpeptidase which anchors surface proteins containing the LPXTG (Leu-Pro-X-Thr-Gly) motif to the bacterial cell wall peptidoglycan (Paterson and Mitchell 2004). *S. pneumoniae* SrtA has been claimed to play a role in attachment to pharyngeal cells (Kharat and Tomasz 2003) and in nasopharyngeal colonization (Paterson and Mitchell 2006).

Pili in gram-positive bacteria have been discovered only recently and investigations are still in their infancy. Gram-positive pili are extended polymers assembled from covalently cross-linked pilin subunits and tethered to the cell wall peptidoglycan (Telford et al. 2006). Pneumococci encode at least two types of pili that play a role in the initial host cell contact to the respiratory tract. Pili of *S. pneumoniae* serotype 4 are approximately 6 nm wide flexible filaments that can be over 1 μm long and they consist of three structural proteins (Hilleringmann et al. 2009). RrgB is the major pilin subunit, RrgA and RrgC are present at the distal and the proximal ends respectively. RrgA mediates the adhesion to host cells and RrgC has a putative role of cell wall anchoring. In addition to pilus-mediated adherence, RrgA mediates adhesion even in the absence of polymeric pilus production (Nelson et al. 2007). The second pilus found in *S. pneumoniae* is composed of polymers of the major pilus protein PitB which have been shown to mediate adhesion to eukaryotic cells. Sortase SrtG1 and the signal peptidase-related protein SipA are necessary for assembly and polymerization of the pilus (Bagnoli et al. 2008).

Table 5. Adherence molecules of *Streptococcus pneumoniae*.

Adherence molecule	Target	Receptor	Reference
ChoP (phosphorylcholine)	epithelial cells	PAFr	Cundell et al. 1995
CpbA (choline-binding protein)	nasopharynx	choline, pIgR, sialic acid, lacto-N-neotetraose	Zhang et al. 2000 Rosenow et al. 1997
PavA (pneumococcal adherence and virulence factor A)		fibronectin	van der Flier et al. 1995
PavB (pneumococcal adherence and virulence factor B	respiratory epithelia	fibronectin plasminogen	Jench et al. 2010
PsaA (pneumococcal surface antigen A)	nasopharynx	E-cadherin	Anderton et al. 2007 Orihuela et al. 2004
SrtA (sortase A)	pharyngeal cells	unknown	Kharat and Tomasz 2003
Pilus	respiratory epithelia	unknown	Hilleringmann et al. 2009 Bagnoli et al. 2008

2.3.3 *Streptococcus agalactiae*

Streptococcus agalactiae, also named as group B streptococcus or GBS, is an aerobic gram-positive diplococcus that causes meningitis, pneumonia, and septicemia in newborns and their mothers (Schuchat 2001). It is one of the most important infectious causes of neonatal morbidity and mortality and the main cause of neonatal sepsis, pneumonia, and meningitis in Western Europe and the United States, and an emerging pathogen in immunocompromised adults (Schuchat 2001). *S. agalactiae* is also a significant cause of mastitis in dairy cattle being responsible for financial losses for the farmers and the dairy industry (Jain 1979).

S. agalactiae is a member of the gastrointestinal normal flora in some humans (Mhalu 1976) and it can spread to secondary sites such as the vagina and rectum of a pregnant woman and thus

cause infections of the amniotic cavity. Rectovaginal GBS colonization has been reported to occur in about 30% of women of childbearing age (Bliss et al. 2002, Votava et al. 2001). Infants are colonised through aspiration of contaminated amniotic fluid or acquisition of the organism during delivery and the bacteria can spread systemically to cause sepsis. This micro-organism can invade both alveolar and epithelial cells (Gibson et al. 1993, Rubens et al. 1992).

Invasive GBS disease also has been frequently reported in adults with diabetes, neurological impairment, breast cancer, and cirrhosis (Jackson et al. 1995). Its manifestations in adults include soft tissue infections, bone and joint infections, pneumonia, or more infrequently endocarditis and meningitis (Edwards and Baker 2005). Adults over 65 years of age are at the highest risk of death from invasive GBS disease (Farley 2001).

There are currently nine known serotypes (Ia, Ib, II–VIII) of which the serotype III is particularly important because it causes the majority of infections in neonates (Schuchat 1998). Five GBS types (Ia, Ib, II, III and V) account for 96% of cases of neonatal and 88% of cases of GBS invasive disease in adults (Phares et al. 2008).

One major difficulty in developing vaccines against *S. agalactiae* is the variety of serotypes which seems to have distinct geographical distributions (Johri et al. 2006). In addition, administration of the vaccine to pregnant women may be difficult because of the fear of causing birth defects. Polysaccharide-protein conjugate vaccines have been developed for each of the major disease-causing group B streptococcus capsular types (Ia, Ib, II, III, and V), and their safety and immunogenicity have been evaluated in healthy nonpregnant adults and also in pregnant women (Baker et al. 2007, Baker et al. 2003a, Baker et al. 2003b, Baker et al. 1999).

During the 1990s, the increased use of intrapartum antibiotic prophylaxis led to a notable reduction in the incidence rate of early-onset disease which occurs before seven days of life (Schrag et al. 2000). Intravenous penicillin is the first-line antibiotic for intrapartum prophylaxis as well as for the treatment of infection (Schuchat 2001). Clindamycin and erythromycin are recommended for individuals who are allergic to penicillin, however, up to 32% of isolated strains have been shown to be resistant to these agents (Phares et al. 2008).

Adherence to the host pulmonary epithelium is the first step in GBS pathogenesis. The polysaccharide capsule is the major virulence factor and the size of the capsule is subject to phase variation (Sellin et al. 1995). Since the interactions of *S. agalactiae* with host cells are complex, the specific molecular interactions that are responsible for the adhesion and colonization of *S. agalactiae* are still not fully understood.

The presence of Lmb, laminin-binding protein (**Table 6**), a surface-exposed lipoprotein ubiquitous in all serotypes of *S. agalactiae*, has been shown to be a prerequisite for adherence of GBS to human laminin, which is the major component of the basement membrane (Timpl 1989). This binding is claimed to be essential for the bacterial colonization of damaged epithelium (Spellerberg et al. 1999).

S. agalactiae surface proteins belonging to the Alp (α-like protein) family have been postulated to bind human host cell glycosaminoglycans (Baron et al. 2004).

The FbsA protein promotes the binding of *S. agalactiae* to human fibrinogen, and fibrinogen-binding epitopes within FbsA are involved in the adherence of *S. agalactiae* to epithelial cells (Schubert et al. 2004).

Streptococcal C5a peptidase (ScpB), which is a surface-localized serine protease that inactivates human C5a, a component of the human complement system, was reported to bind to fibronectin and human epithelial cells (Beckmann et al. 2002, Cheng et al. 2002).

Spb1, surface protein of group B streptococcus 1, a protein expressed on the surface of *S. agalactiae*, has been shown to contribute to the ability of *S. agalactiae* to adhere to epithelial cells (Adderson et al. 2003).

Streptococcus agalactiae also interacts with keratin. Srr-1, the serine-rich repeat protein, is localized on the surface of *S. agalactiae* cells. This protein was shown to be involved in adherence to epithelial cells and there may also be an interaction with human keratin 4, a protein present also in human saliva (Samen et al. 2007). Bacteria also bind to cytokeratin 8 (Tamura et al. 2000). It was suggested that adherence of *S. agalactiae* to cytokeratin may be important for the maintenance of colonization at sites of keratinized epithelium, such as the vagina, or for adherence to damaged epithelial cells at other sites.

S. agalactiae pilus is composed of three structural subunit proteins: PilA (pilus associated adhesin located at intervals along the pilus backbone), PilB (major pilus component), PilC (minor pilus associated component mainly localized at the base of the pilus) (Dramsi et al. 2006). Recent

results indicate that the pilus structure is necessary for optimal display of the PilA subunit at the bacterial surface (Konto-Ghiorghi et al. 2009). However, PilA remains a functional adhesin even in the absence of a pilus fiber. The von Willebrand adhesion domain of PilA is essential for its adhesive function and it recognizes some specific, yet unidentified, ligand on epithelial cells.

Table 6. Adherence molecules of *Streptococcus agalactiae*.

Adherence molecule	Target	Receptor	Reference
Lmb (laminin-binding protein)	epithelial cells	laminin	Spellerberg et al. 1999
Alp (α-like protein) family	epithelial cells	glycosaminoglycans	Baron et al. 2004
FbsA	epithelial cells	fibrinogen	Schubert et al. 2004
ScpB (streptococcal C5a peptidase)	epithelial cells	fibronectin	Beckmann et al. 2002
Spb1 (surface protein of group B streptococcus 1)	epithelial cells	unknown	Adderson et al. 2003
Srr-1 (serine-rich repeat protein 1)	epithelial cells	keratins	Samen et al. 2007 Tamura et al. 2000
Pilus	epithelial cells	unknown	Konto-Ghiorghi et al. 2009

2.3.4 *Streptococcus suis*

Streptococcus suis is a gram-positive facultative anaerobic pathogen which causes a wide variety of infections in pigs such as meningitis, bronchopneumonia, pericarditis, arthritis, polyserositis, septicemia, and abortion (Sanford and Tilker 1982). These bacteria can also be isolated from other animals, such as ruminants, cats, dogs, and horses, and this strain is believed to be a commensal in the intestinal flora (Devriese et al. 1992, Devriese et al. 1990, Hommez et al. 1988). *S. suis* can also infect humans who are in contact with pigs or raw pork and cause meningitis (Agass et al. 1977),

septicemia (Büngener and Bialek 1989), arthritis (Cheng et al. 1987) or endocarditis (Trottier et al. 1991) in these individuals.

S. suis can colonize tonsils (van Leengoed et al. 1987), upper respiratory tract (Brisebois et al. 1990) or nose (Flores et al. 1993), and transmission occurs via the respiratory route (Williams et al. 1973). The highest carrier rates occur in piglets between four and ten weeks of age, and it may persist in the tonsils of carrier pigs for over one year and thus these carriers are infectious to other pigs for a long time and they are a significant factor in the transmission of the disease (Clifton-Hadley et al. 1984).

Thirty-five capsular serotypes (types 1–34 and 1/2) have been identified, but only a limited number are responsible for infections in pigs, including serotypes 1–9 and 14 (Gottschalk et al. 2007). Serotype 2 is prevalent and most commonly associated with clinical disease (Wisselink et al. 2000).

The prevalence of morbidity and mortality from *S. suis* vary between herds. Morbidity can range from less than 1% to more than 50%, although it rarely exceeds 5% (Guise et al. 1985, Windsor 1977). With prompt and appropriate treatment, mortality in swine herds is usually low, 0–5% (Windsor 1977) but without treatment, mortality approaches 20% (Guise et al. 1985).

S. suis has frequently caused diseases all over the world; the most recent and largest recorded epidemics ocurred in China in 2005 with 647 pig deaths and 215 reported cases of human disease, 39 of which were fatal (Yu et al. 2006).

Various types of vaccines have been developed for pigs, with varying protective efficacies. Inactivated autogenous vaccine generated from virulent strains isolated from sick pigs is commonly used in the pig industry (Haesebrouck et al. 2004). Due to the lack of safety and efficacy data each new batch of an autogenous vaccine needs to be tried out on animals and critically assessed before it can be released on a larger scale. Although the use of inactivated autogenous vaccine is empirical, it can protect healthy pigs from *S. suis* infection and prevent the spread of this disease in herds during outbreaks of *S. suis* infection. However, because of the large number of capsular types, overall success with commercial vaccines may be difficult to achieve until specific virulence factors contributing to the pathogenicity of the organisms are better characterized. At present, there is no *S. suis* vaccine for humans.

The treatment of choice for *Streptococcus suis* infections is penicillin (Gottschalk et al. 1991). However, penicillin-resistant strains have been isolated (Gottschalk et al. 1991). *S. suis* is also sensitive to ampicillin, amoxicillin, ceftriaxone, and cephalosporin but strains highly resistant to these antibiotics have also been reported (Aarestrup et al. 1998). Tiamulin has been used as prophylaxis, and its addition to drinking water or feed can decrease the incidence of *S. suis* type 2 infection (Chengappa et al. 1990). However, in spite of treatment with antimicrobial agents, total recovery of affected pigs is rarely achieved, and many die or suffer from deafness, blindness or purulent arthritis (Windsor 1977). Prophylactic antibiotic treatment may select for resistant strains, making the treatment of subsequent outbreaks much more difficult. Furthermore, most antimicrobial therapies will not eliminate the *S. suis* carrier state in pigs (Spicer 2002).

Streptococcus suis is able to bind to endothelial and epithelial cells of porcine and human origin (Benga et al. 2005, Lalonde et al. 2000). However, the specific mechanisms involved in these interactions remain unknown. In addition, *S. suis* can use ECM proteins to potentiate its virulence and it is able to adhere to fibronectin, fibrin, vitronectin, and laminin and different types of collagens (Esgleas et al. 2005).

A fibronectin-fibrinogen-binding protein (FBPS) with binding capacity for these two host proteins (**Table 7**) has been described for *S. suis* (de Greeff et al. 2002). However, the role of FBPS in pathogenesis is not totally understood and it was proposed that FBPS may have a role in *S. suis* colonization of various organs but not of the tonsils (de Greeff et al. 2002).

Enolase, a multifunctional glycolytic enzyme, can be localized also in bacterial cell surface and it has been shown to play a critical role in the adherence of *S. suis* type 2 to epithelial cells (Feng et al. 2009). Enolase binds to fibronectin (Esgleas et al. 2008).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *S. suis* is able to bind to different host proteins such as plasminogen and albumin (Jobin et al. 2004, Quessy et al. 1997). The GAPDH protein of *S. suis* seems to be involved in the first steps of the bacterial adhesion to host cells as observed in an adherence assay with porcine tracheal rings (Brassard et al. 2004).

The *S. suis* cell wall surface protein, 6-phosphogluconate dehydrogenase (6PGD), has also been shown to be involved in adhesion of *S. suis* type 2 to host epithelial cells (Tan et al. 2008).

Streptococcus suis, like *S. pneumoniae*, possesses sortases. Sortase A (SrtA) in *S. suis* plays an important role in bacterial colonization of host cells and adhesion to ECM proteins, fibronectin and collagen type I (Vanier et al. 2008).

Galactose-(α1-4)-galactose terminating oligosaccharides have been shown to be optimal receptors for *S. suis* and one galabiose-binding adhesin protein has been described (Tikkanen et al. 1995, Tikkanen et al. 1996). Two adhesin variants have been described, one which can be inhibited by galactose and *N*-acetylgalactosamine (type P_N) and the other blockable by galactose only (type P_O) (Haataja et al. 1994).

Recent results have revealed the presence of at least four discrete gene clusters in *S. suis* encoding putative pili, and highly virulent invasive *S. suis* serotype 2 isolates express pili from this cluster. However, it was postulated that pili might be redundant for the critical steps of the *S. suis* pathogenesis of infection (Fittipaldi et al. 2010).

Table 7. Adherence molecules of *Streptococcus suis*.

Adhesin molecule	Target	Receptor	Reference
FBPS (fibronectin-fibrinogen-binding protein)		fibronectin fibrinogen	de Greeff et al. 2002
Enolase	epithelial cells	fibronectin	Esgleas et al. 2008
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	tracheal cells	albumin plasminogen	Jobin et al. 2004 Quessy et al. 1997
6PGD (6-phosphogluconate dehydrogenase)	epithelial cells	unknown	Tan et al. 2008
SrtA (sortase A)		collagen fibronectin	Vanier et al. 2008
Galα1-4Gal –specific adhesins		Galα1-4Gal terminating oligosaccharides	Haataja 1994
Pilus		unknown	Fittipaldi et al. 2010

3 Aims of the study

The general aim of the present study was to investigate milk, berries, and juices as sources of antiadhesive agents against respiratory and meningitis-associated pathogens. The specific aims were:

1. To develop an assay for studying the binding activities of *Neisseria meningitidis* pili to glycoproteins, to isolate oligosaccharides from milk and to investigate their ability to inhibit the binding of *Neisseria meningitidis* pili to glycoproteins. **(I)**

2. To prepare molecular-size fractions from several berries and berry and fruit juices and analyze their sugar and polyphenol contents. **(II, III)**

3. To screen the binding activity of *Neisseria meningitidis* pili to berry and berry and fruit juice fractions and to subfractionate the most active fractions. To study the antiadhesive effect of selected berry or fruit material against meningococcal binding to human epithelial cells under cell culture conditions. **(II**, **IV)**

4. To screen the binding activity of *Streptococcus pneumoniae* and *Streptococcus agalactiae* to berry and berry and fruit juice fractions. **(III)**

5. To screen the hemagglutination inhibition activity of *Streptococcus suis* by berry fractions. **(III)**

4 Experimental

4.1 Chemicals

Details of commercially available chemicals are given in the original articles (**I-IV**). Bovine thyroglobulin and chicken ovalbumin were purchased from Sigma Chemical (St. Louis, USA) and agglutinin was purified from human parotid saliva as described in **I**.

4.2 Bacteria

4.2.1 Bacterial strains and culture

The bacterial strains and their pathogenic characteristics used in the present studies are described in **Table 8**. Strains were maintained in 15% glycerol in Brain Heart Infusion at –80 °C. For the experiments the bacteria were cultivated overnight as described in detail in the original publications.

Table 8. Bacterial strains and their pathogenic characteristics.

Bacterial strain	Origin	Pathogenic characteristics	Studies
I Human pathogens:			
Neisseria meningitidis C I 8013	isolated from blood	meningitis, septicemia	**I, II, IV**
Streptococcus pneumoniae SB 53845	isolated from lung	acute otitis media, meningitis, pneumonia, sinusitis	**III**
Streptococcus agalactiae B 133 IIIR	isolated from wound	neonatal meningitis	**III**
II Human and animal pathogen:			
Streptococcus suis 836	isolated from lung	septicemia, meningitis	**III**

4.2.2 Isolation and biotinylation of *Neisseria meningitidis* pili (I-II)

Isolation and biotin labelling of the meningococcal pili was carried out at 0 °C. Briefly, five plates of the cultivated *N. meningitidis* were suspended in 10 mM 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 cut-off Biomax Ultrafree-15 centrifugal filter device and centrifuged (1000 × g at 4 °C). The concentrated solution was washed twice with 15 ml of 10 mM Hepes and concentrated by centrifugation to a volume of 1 ml as described above.

Biotin labelling of the isolated pili was performed in PBS (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.

4.2.3 Isolation and biotinylation of *Streptococci* (III)

S. pneumoniae and *S. agalactiae* were harvested from ten or three plates, respectively, and suspended in 40 ml of PBS (pH 7.4) at 0 °C. The suspension was centrifuged at 2000 × *g* at 4 °C for 10 min and washed three times with cold PBS (pH 7.4). The density of the bacterial suspension was standardised to an absorbance value 0.6 at A_{600}.

For biotin labeling the bacteria were centrifuged (2000 × *g* at 4 °C for 10 min) and suspended in 2 ml of PBS (pH 7.4). Biotin labeling of the isolated bacteria was performed in PBS (pH 7.4) using D-biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimide ester (Roche Diagnostics, Germany) according to the instructions of the manufacturer. The biotin-labeled bacteria were suspended in 10 ml of PBS (pH 7.4) and stored at 4 °C.

4.3 HEC-1B cells (II, IV)

The human epithelial cell line, HEC-1B, was kindly provided by Prof. X. Nassif (INSERM U570, France) and used between passages 5 and 28. The cells were cultured on cell culture dishes (Costar®, Corning Inc., USA) in high-glucose DMEM supplemented with 10% heat-inactivated FBS and 4 mM L-glutamine at 37 °C in humidified 5% CO_2/ 95% air incubator. In the dot assay (**II**), cells from one confluently grown plate were harvested using trypsin-EDTA, washed and suspended in PBS. In the adhesion inhibition assay (**IV**), HEC-1B monolayers were prepared in six-well tissue

culture plates (Costar®, Corning Inc., USA) by inoculating 10^5 cells per well and cultivating overnight to obtain confluence.

4.4 Milk oligosaccharides

4.4.1 Isolation of milk oligosaccharide fractions (I)

Human milk was obtained from Kuopio University Hospital, Centre for Human Milk, Kuopio, Finland. Pasteurized low-fat bovine milk was purchased from local supermarket. Acidic and neutral oligosaccharide fractions were isolated from milk at 4 °C using the method described by Kobata (1972). Briefly, milk was defatted by centrifugation and filtered through glass wool. Ethanol was added to obtain a final ethanol concentration of 68% and the mixture was incubated overnight to precipitate proteins and lactose. After centrifugation and washings of the precipitate, the supernatants were concentrated using a rotary evaporator. Next, the syrup was diluted with water and centrifuged to separate the insoluble material. The fractionation was done using a Sephadex G-25 column (102 × 1.6 cm) and water elution.

4.4.2 Analysis of total hexose and sialic acids in milk fractions (I)

Total hexose content of milk fractions was determined using the phenol-sulphuric acid method (Kobata 1972). Briefly, 50 µl of fractions were diluted with distilled water and 200 µl of 5% aqueous phenol and 1000 µl concentrated sulphuric acid were added. After incubation (37 °C, 30 min), the absorbances were measured at 490 nm.

Periodate-resorcinol method for sialic acids (Jourdian et al. 1971) was used to determine acidic oligosaccharides in milk fractions. In brief, 250 µl of fractions were mixed with 50 µl of 0.04 M periodic acid and incubated on ice for 20 min. Subsequently, 625 µl of 0.6% resorcinol reagent was added, incubated on ice for 5 minutes and at 95 °C for 15 min. After cooling to room temperature, the absorbances were measured at 630 nm.

After the analysis of total content of hexose and sialic acids in the fractions, they were pooled into neutral and acidic oligosaccharide fractions, respectively, and freeze-dried (ModulyoD, ThermoSavant, USA).

4.5 Berries and juices

The berries and juices (as concentrates) investigated in the present studies are described in **Table 9**. All berries and juices were purchased from Finnish suppliers. Wild berries were harvested at maturity in northern Finland as also were the berries used in the production of bilberry, cranberry, lingonberry and crowberry juice. Apple (mix of cultivars), black currant, raspberry, sour cherry and tomato juice were of European Union origin. Pineapple and red grape juice were imported from outside the European Union. Berries and juices were received frozen and they were used as such without any additives.

Table 9. Berries and juices investigated in studies **II-IV**.

Latin name	Trivial name	°Brix	Studies
Vaccinum myrtillus L.	bilberry, wild	-	**II, III**
	bilberry juice	65	**II, III, IV**
Vaccinium oxycoccos L.	cranberry, wild	-	**II, III**
	cranberry juice	65	**II, III, IV**
Vaccinium vitis-idaea L.	lingonberry, wild	-	**II, III**
	lingonberry juice	65	**II, III, IV**
Empetrum nigrum and *hermaphroditum* L.	crowberry juice	65	**II, III, IV**
Rubus chamaemorus L.	cloudberry, wild	-	**II, III**
Rubus idaeus L.	raspberry juice	65	**II, III**
Ribes nigrum L.	black currant juice	65	**II, III**
Ananas comosus L.	pineapple juice	60	**II, III**
Citrus paradise L.	red grapefruit juice	63	**II, III**
Malus domestica L.	apple juice	70	**II, III**
Prunus cerasus L.	sour cherry juice	65	**II, III**
Solanum lycopersicum L.	tomato juice	30	**II, III**

4.6 Fractionation and subfractionation of berries and juices (II-IV)

Berries and the juice concentrates were fractionated into three different fractions according to their molecular size, i.e. <10 kDa fraction (referred as FI), 10–100 kDa fraction (FII), and >100 kDa fraction (FIII) (**II, III**). Briefly, thawed berries were crushed and diluted with water (1:1). After removing skins and seeds by centrifugation (8000 × g at 4 ºC for 10 min), the berry juice was filtered through a gauze and filter paper. The filtered juice (15 ml) was loaded onto a 100 kDa cut-off centrifugal filter device (Biomax Ultrafree-15, Millipore Corp., USA) and centrifuged (2000 × g at 4 ºC) to a volume of 1.5 ml. The retentate was fraction FIII. The filtrate was loaded onto a 10 kDa cut-off centrifugal filter device (Biomax Ultrafree-15, Millipore Corp., USA) and centrifuged as described above to a volume of 1.5 ml to obtain fraction FII (the retentate) and fraction FI (the filtrate). The berry and fruit juice concentrates were diluted with water (1:4 and 1:3 – 1:5, respectively) and fractionated as described above. The concentrations of the fractions were analyzed by determining the content of soluble solids (°Brix value) in a refractometer (ATAGO NAR-1T, Japan). The fractions were stored frozen at −20 °C before analyses.

The polyphenolic compounds were subfractionated for further analysis and activity testing, with solid-phase extraction being employed (**II**) by modifying the method previously described by Sun et al. (2006). The molecular size fraction mixed 1:1 with phosphate buffer was passed through a C-18 SPE cartridge (Waters Corp., USA). Diluted phosphate buffer was used to remove phenolic acids before the elution of molecules was done using ethyl acetate, water and methanol. Solvents were removed using rotary evaporator and the solids were reconstituted with water before analyses.

4.7 Purification of anthocyanins (II)

Anthocyanins were purified from bilberry juice concentrate using ethyl acetate extraction to remove flavonol glycosides and oligomeric proanthocyanidins from the juice followed by HPLC separation as described in detail in **II**.

4.8 Determination of sugar, proanthocyanidin and anthocyanin content in berries and juices (II)

A colorimetric method using phenol and sulphuric acid (Dubois et al. 1956) was used to determine the content of reducing sugars in molecular size fractions of berries and juices.

Total proanthocyanidin contents of berry and juice molecular size fractions were determined using the HCl–butanol method (Porter et al. 1986, Waterman and Mole 1994) with modifications. HCl catalyses depolymerization of colorless proanthocyanidins in butanol (here: in methanol) to yield red-colored anthocyanidins. Semiquantification was done against a lingonberry-derived mixture of oligomeric proanthocyanidins (Määttä-Riihinen et al. 2005).

In the estimation of total anthocyanins, berry and juice molecular size fractions were diluted and mixed with acidified methanol. The absorbance values were measured at 520 nm against acidified methanol (Strack and Wray 1989). A mixture of six anthocyanin glycosides was used to construct a calibration curve.

4.9 Nuclear magnetic resonance spectroscopy (NMR) (III)

For NMR spectroscopy the molecular size fractions were freeze-dried, dissolved into D_2O and lyophilized again. Finally, the samples were dissolved to D_2O and 50 mM TSP in D_2O was added to the samples. Routine proton, diffusion edited and T_2-filtered NMR spectra of molecular size fractions were measured using a Bruker Avance DRX 500 spectrometer (Bruker BioSpin GmbH, Germany) operating at 500.13 MHz equipped with a 5 mm TXI probe head as described in detail in **III**.

4.10 Analysis of polyphenolic subfractions by RP-HPLC (II)

Polyphenolic subfractions were analyzed using RP-HPLC with on-line diode array detector (**II**). In the HPLC analysis, the subfractions were mixed 1:1 with methanol and filtered through a 0.45 μm syringe filter before injection into the HPLC. The HPLC-DAD apparatus consisted of a Hewlett-Packard instrument with a 1100 series quaternary pump, an autosampler, and an on-line diode array detector linked to an HP-ChemStation data handling system. HPLC separation of compounds was achieved on a LiChroCART Purospher Star RP-18e column (150× 4.6 mm i.d., 5

μm) (Merck) protected with a guard column of the same material (4 × 4 mm). 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 in 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 on-line UV−visible spectra (Määttä-Riihinen et al. 2005, Bartolomé et al. 1996).

4.11 Solid phase binding and binding inhibition assays (I-III)

A microtiter plate method was developed for the testing of the inhibitory effect of milk oligosaccharides on meningococcal binding (**I**). First, the binding of purified pili to glycoproteins coated in microtiter wells was studied. Aliquots of 100 μl of bovine thyroglobulin (100 μg/ml), chicken ovalbumin (100 μg/ml), human salivary agglutinin (1 μg/ml) and 5 % dry milk powder solution [5% (w/v), 0.05% (v/v) Tween 20 in PBS, pH 7.4] were incubated overnight in polyvinylchloride microtiter plate wells (Falcon Flexible Plate, Becton Dickinson Labware) at 4 °C. After the non-specific binding sites were saturated with the dry milk powder solution biotin-labeled pili corresponding to isolated pili from 1:8 plate were further diluted 1:8 with PBS and 100 μl of the diluted pili was added to the wells and incubated for one hour at room temperature. Streptavidin–POD conjugate (diluted 1:4000) and ABTS-substrate were used for detection and the absorbances were measured at 405 nm (Victor^2 1420 Multilabel counter, Wallac, Finland). The wells were washed five times with the washing buffer [0.05% (v/v) Tween 20 in PBS, pH 7.4] after each incubation step.

The pili binding inhibition assay was performed by preincubating the biotin-labeled pili (diluted 1:4 with PBS) with 0.01 – 40 mg/ml milk oligosaccharide fractions (mixed 1:1) for 60 min at room temperature with gentle rocking on the rocking platform. Control biotin-labeled pili were diluted 1:8 with PBS and incubated identically. After preincubation, 100 μL of the 1:1 mixed solutions and control biotin-labeled pili were added to the glycoprotein-coated microtiter plate

wells and the binding assay procedure was carried out as described above. The inhibitory activity of milk oligosaccharides was calculated using **Equation 1**.

$$Inhibition\% = \frac{A_{405(PILI)} - A_{405(PILI+SAMPLE)}}{A_{405(PILI)}} x100\% \qquad \textbf{Equation 1}$$

To screen the binding activity of *N. meningitidis* pili, *S. pneumoniae* and *S. agalactiae* bacteria to molecular size fractions of berries and juices and the binding activity of meningococcal pili to polyphenolic subfractions, the microtiter well binding assay described above was modified as follows (**II, III**). Aliquots of 100 µl of berry and juice samples and 5% dry milk powder solution as control were incubated in polyvinylchloride microtiter plate wells (Falcon Flexible Plate, Becton Dickinson Labware, NJ, USA) at 4 °C overnight. After the non-specific binding sites were saturated with 5% dry milk powder solution, 100 µl of biotin-labeled meningococcal pili diluted 1:4 in PBS or 10^6 CFU of biotin-labeled bacteria were incubated on the berry fractions in the wells for one hour at room temperature or two hours at 37 °C, respectively. Streptavidin–POD conjugate (diluted 1:4000) and ABTS-substrate were used for detection and the absorbances were measured at 405 nm (Victor² 1420 Multilabel counter, Wallac, Finland). The wells were washed five times with the washing buffer [0.05% (v/v) Tween 20 in PBS, pH 7.4] after each incubation step. All the assays were carried out in triplicate. The binding activity was calculated using **Equation 2**.

$$\text{Binding activity} = (A_{405(\text{sample})} - A_{405(\text{control})}) \times 100 \qquad \textbf{Equation 2}$$

4.12 Binding of *Neisseria meningitidis* pili to epithelial cell dots (II)

Adhesion of *N. meningitidis* has been studied using human epithelial cell line HEC-1B as a model (Nassif et al. 1994). The ability of selected juice FII fractions to inhibit the adhesion of *N. meningitidis* pili to HEC-1B cells was tested in a dot assay which was developed in study **II**. HEC-1B cells derived from one plate were suspended in 200 µl of PBS and diluted 1:10 with PBS. Two-microliter dots of cell suspension were pipetted onto a nitrocellulose membrane (Protran, Schleicher & Schuell BioScience, Germany) and allowed to dry. Nonspecific binding sites were saturated by incubating the membrane in 5% dry milk powder solution for two hours at room

temperature. The diluted FII fractions (1:2, in water) and the biotin-labeled diluted pili (1:10, in PBS) were mixed 1:1, and the solution was incubated for one hour at room temperature. The mixtures (1.5 ml) were loaded on a 100 kDa cutoff Biomax Ultrafree-15 centrifugal filter device 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 onto the membrane and incubated for 90 min at room temperature. Streptavidin−POD conjugate (diluted 1:4000) in the dry milk powder solution was added to the membrane and incubated for one hour at room temperature. After each incubation step, the membrane was washed three times with PBS. The membrane was treated with Super Signal solution (Pierce, USA) and exposed to Hyperfilm (Amersham Pharmacia Biotech, U.K.) for 1 min, after which the film was developed.

4.13 Hemagglutination and hemagglutination inhibition (III)

The slide hemagglutination assay for *S. suis* (**III**) was done as described by Korhonen and Finne (1985). Erythrocytes obtained from healthy adults were treated with sialidase and used at 4% concentration. In the hemagglutination inhibition assay, the molecular size fractions of berries were used and the minimum inhibitory concentration was defined as the lowest dilution achieving full inhibition of hemagglutination. *Escherichia coli* PapGII was used as control.

4.14 Adhesion inhibition assay in cell culture (IV)

The inhibitory effect of polyphenolic subfractions on the adhesion of living encapsulated meningococcal cells to epithelial cells (HEC-1B) was studied in a human epithelial cell culture model (**IV**). The subfractions were diluted with cell culture medium and added to HEC-1B cells which had been cultured overnight. The incubation with the subfractions was 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 the subfractions. The bacterial suspension was added to the wells (approx. 10 CFU per one HEC-1B cell) and they were incubated for one hour at 37 °C. Non-adherent bacteria were removed by washing the wells twice with PBS. The cells were detached with trypsin-EDTA solution from the bottom of the wells and diluted with PBS. Aliquots of 100 µl of cell samples were

plated on the GCB agar plates. The plates were cultured overnight at 37 °C in a 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 subfractions was calculated using **Equation 3**.

$$Inhibition\ \% = \frac{CFU_{control} - CFU_{sample}}{CFU_{control}}\ x\ 100\% \qquad \textbf{Equation 3}$$

where CFU is a colony forming unit

4.15 Antibacterial activity assay (IV)

Antibacterial activity of polyphenolic subfractions studied in the cell culture experiment was tested against *N. meningitidis* using the microtiter broth microdilution assay (**IV**). Bacterial suspension was mixed with berry juice subfractions, CG broth or ampicillin (100 μg/ml) and incubated in wells of the microtiter plate (Nunc™, Brand Product, Denmark) at 37 °C in a CO_2 atmosphere for two hours. An aliquot of 100 μl of the diluted mixtures was plated on GCB agar plates and the plates were cultured overnight at 37 °C in a CO_2 atmosphere. The surviving colony forming units were counted on the next day and the survival of *N. meningitidis* was calculated using **Equation 4**.

$$Bacterial\ survival\ \% = \frac{CFU_{sample}}{CFU_{control}}\ x\ 100\% \qquad \textbf{Equation 4}$$

where CFU is a colony forming unit

4.16 Statistical analysis (I-IV)

A 2-tailed paired Student's *t* test from Microsoft Excel was used to compare the differences of the means (**I**). GraphPad Prism 4.03 for Windows was used for one-way ANOVA followed by Tukey's multiple comparison test to compare the pili or bacterial binding between the fractions and the control (**II**, **III**) or one-way ANOVA followed by Dunnett's multiple comparison test to compare the amount of bacterial colonies between the samples and the control (**IV**).

5 Results and discussion

5.1 Milk (I)

Neisseria meningitidis pili bound to bovine thyroglobulin and human salivary agglutinin in the microtiter well binding assay (**I**: Figure 1A). Binding to chicken ovalbumin was not significant and did not differ from background (dry milk powder as control).

In the inhibition experiments, fixed concentrations (20 mg/ml) of acidic BMO inhibited pili binding to bovine thyroglobulin by 85–90% and binding to human salivary agglutinin by 46% (**I**: Figure 1). At this concentration (20 mg/ml), 84% inhibition was achieved with neutral HMO, 61% inhibition with acidic HMO and only 18% inhibition with neutral BMO to bovine thyroglobulin (**I**: Figure 1B). The high inhibitory activity of pili binding by acidic BMO and neutral HMO was confirmed when serial dilutions of these saccharides still exhibited 50% inhibition at concentrations of 1–2 mg/ml (**I**: Figure 2).

Endothelial and epithelial carbohydrate receptors for *N. meningitidis* remain unknown. In order to characterize the interaction between *N. meningitidis* and carbohydrates, an assay for the binding of biotinylated pili to glycoproteins coated on microtiter plate wells was developed. Since type IV pili mediate adhesion in encapsulated *N. meningitidis* strains (Nassif 2000), isolated meningococcal pili were used in the assays. Both chicken ovalbumin and bovine thyroglobulin are well-characterized glycoproteins which are commonly used to characterize carbohydrate receptors for microorganisms (Haataja et al. 1993, Hytönen et al. 2000). Human salivary agglutinin, a complex of the scavenger receptor cysteine-rich protein gp-340 and sIgA (Prakobphol et al. 2000), was selected, because it aggregates and adheres to a wide range of commensal and pathogenic microorganisms (Loimaranta et al. 2005) and contains domains for multiple host innate or immune defenses. Saliva also represents the first line of defense. Moreover, immobilized and fluid bovine thyroglobulin bind *N. meningitidis* wild-type cells, and fluid bovine thyroglobulin inhibits their hemagglutination by human erythrocytes (unpublished data). Purified pili do not hemagglutinate human erythrocytes, and a low-avidity pili binding to glycoproteins was anticipated.

The carbohydrate components of bovine thyroglobulin are composed of nearly the same monosaccharide residues (Vali et al. 2000, Arima et al. 1972) as human milk oligosaccharides (Kunz and Rudloff 1993). Bovine thyroglobulin contains N-linked complex and hybrid

(oligomannosidic) glycans (Dorland et al. 1984), and human salivary agglutinin O- or N-linked (poly)lactosamine and hybrid oligosaccharides (Loimaranta et al. 2005, Oho et al. 1998), serving as potential receptors. *N. meningitidis* pili did not bind to ovalbumin, which mainly consist of high-mannose–type glycans (Harvey et al. 2000). Therefore it was speculated that the receptor structures possibly did not contain mannose, and binding to bovine thyroglobulin could thus be mediated by a complex type of bovine thyroglobulin carbohydrate chains. Evidence for carbohydrate receptors for *N. meningitidis* also emerge from the ability of milk oligosaccharides to inhibit pili binding. These observations offer preliminary information on the carbohydrate composition of the possible receptor structure.

Human milk contains 5.0–13 mg/ml oligosaccharides, in colostrum, the values can be as high as 22 mg/ml; interference with adhesion may therefore occur at a physiologically feasible level (Gopal and Gill 2000, Kunz et al. 2000, Newburg 1997). Human milk contains diverse and complex neutral and acidic oligosaccharide structures (Brand Miller and McVeagh 1999), whereas bovine milk (Gopal and Gill 2000) is less complex and contains mainly sialylated oligosaccharides. The receptor epitope on bovine thyroglobulin may be an internal core rather than a terminal sialic acid sequence because both neutral HMO and acidic BMO inhibited pili binding effectively.

Two other bacteria of genus *Neisseria*, *N. subflava* and *N. gonorrhoeae* use sialyl (Nyberg et al. 1990) and neutral (Strömberg et al. 1988) oligosaccharides, respectively, for adhesion *in vitro*. However, the corresponding adhesins are not known. At present it is not known whether meningococcal pili binding to bovine thyroglobulin or human salivary agglutinin involves tip-located PilC1 (Rudel et al. 1995) or pilin subunit proteins (Nassif et al. 1994) differentially, in particular because acidic BMO almost completely inhibited binding to bovine thyroglobulin but inhibited only partially binding to human salivary agglutinin.

5.2 Analysis of sugars, proanthocyanidins and anthocyanins in berry and juice fractions

5.2.1 Molecular size fractions (II, III)

Sugar concentrations of the molecular size fractions of berries and juices varied from 20 mg/ml (black currant juice FII) to 258 mg/ml (pineapple juice FIII) (**II**: Table 1). The highest proanthocyanidin concentrations were found in juice fractions FIII of cranberry (30 mg/g) and crowberry (27 mg/g) and in lingonberry juice fraction FII (22 mg/g) (**II**: Table 1). Bilberry and

crowberry juice fractions FIII contained the highest amount of anthocyanins, 39 mg/g and 38 mg/g, respectively (**II**: Table 1). Fractions of cloudberries or red grapefruit, apple, pineapple, and tomato juice did not contain detectable amounts of proanthocyanidins or anthocyanins or contained only trace amounts.

In general, berries contain sugars 4-11 g/ 100 g and in imported fruits sugar content is 20-25 % higher (National Institute for Health and Welfare 2009, Viljakainen et al. 2002). Strongly coloured berries contain 300-800 mg anthocyanins/ 100 g fresh fruit (Määttä-Riihinen et al. 2004a, Määttä-Riihinen et al. 2004b). Several berries and fruits included in the present study are known to contain proanthocyanidins (Hellström et al. 2009, Gu et al. 2004, Määttä-Riihinen et al. 2004b), whereas pineapple, red grapefruit, and tomato contain no detectable amounts of these compounds (Hellström et al. 2009, Gu et al. 2003).

Based on NMR spectroscopy, the main component (>90%) in the molecular size fractions was glucose and it was used in the scaling of the standard proton NMR spectra (**III**: Figure 2A, D and G). The intensities of the signals in the edited spectra reflect the relative amounts of high (**III**: Figure 2B, E, H) and low (**III**: Figure 2C, F, I) molecular components in the fractions. NMR analysis revealed that only low-molecular-weight molecules were present in fractions FI (**III**: Figure 2A–C). The high-molecular-size fractions (FII and FIII) contained high-molecular-weight compounds but also lower molecular weight compounds were present (**III**: Figure 2D–I). In addition to glucose, some fractions contained benzoic acid and its derivatives (signals at around 8.1 ppm and 7.5–7.7 ppm, e.g. lingonberry juice, **III**: Figure 2A and C). No other common low molecular weight compounds could be identified from the spectra. The signals below 7 ppm at low aromatic and double bond regions indicate that fractions FII and FIII contain polyphenolic compounds (e.g. lingonberry, **III**: Figure 2D). The broad signal at 6–7.5 ppm in the diffusion edited spectra for the FII and FIII fractions (e.g. bilberry juice, **III**: Figure 2H) arises from polyphenol macromolecular complexes, including proanthocyanidins and, possibly, polyhydroxy flavonoids which do not have signals above 7.5 ppm. The profile of the signal (**III**: Figure 2E and H) resembles that of malvin (Santos et al. 1993), a diglucoside of malvidin, an anthocyanin found also in some berries.

5.2.2 SPE subfractions (II)

Four berry juice fractions showing high activities in meningococcal pili binding experiments were chosen to be subjected to solid-phase extraction. 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 (**II**: Table 1). Solid phase extraction was used to subfractionate the polyphenols and RP-HPLC with UV-DAD was used to analyze the polyphenolic subfractions.

In the ethyl acetate subfraction, the peaks refer to mainly flavonol glycosides and proanthocyanidins (**II**: Figure 2). 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 (Sun et al. 2006). In the present study, anthocyanins were detected in water and methanol subfractions (eluted after ethyl acetate), especially in crowberry and bilberry juice FIII (**II**: Figure 2), as expected. Polymeric proanthocyanidins and other high-molecular-weight constituents cannot be separated by RP-HPLC, but polymeric proanthocyanidins may cause a drift in chromatographic baseline (Rohr et al. 2000). The chromatograms of water and methanol subfractions of cranberry and crowberry juices show a drift in the baseline around 25–30 min, and a drift is also seen in the methanol subfraction of bilberry (**II**: Figure 2). These findings together with those previously reported data (Sun et al. 2006, Rohr et al. 2000) support the presence of polymeric proanthocyanidins or other polymerized structures at least, in the cranberry, bilberry, and crowberry juice fractions.

5.3 Biological activity of berry and juice fractions against *Neisseria meningitidis*

5.3.1 Binding activity of pili (II)

Biological activity of molecular size fractions was first measured by screening the binding activity of *N. meningitidis* pili to the fractions using solid phase assay. The most active binding of meningococcal pili was found to lingonberry juice fractions FII and FIII and to the high-molecular-size juice fractions of other *Vaccinium* species (**II**: Table 2). Significant binding activity was found also to high-molecular-size fraction of crowberry juice (FIII) and black currant juice (FII). In

addition to berry juice fractions, *N. meningitidis* pili bound to all of the fresh bilberry fractions (FI–FIII) as well as to fresh cranberry fractions FII and FI.

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 (Foo et al. 2000a, Foo et al. 2000b, Shmuely et al. 2004, Steinberg et al. 2004, Weiss et al. 2004). 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 (**II**: Table 1, Table 2). In addition, there was no binding activity to any of the fractions derived from cloudberries or apple, pineapple, raspberry, or tomato juice (**II**: Table 2). Apple, pineapple, and raspberry fractions had high sugar contents, but none of the apple, pineapple, and tomato juice fractions 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 (**II**: Table 1). As reported earlier, the levels of high-molecular-weight proanthocyanidins in raspberries are smaller than in berries of *Vaccinium* species (Gu et al. 2004). Furthermore, in raspberries and cloudberries, the main phenolic compound is ellagitannins (Määttä-Riihinen et al. 2004b). As also seen in NMR spectra, there were no detectable amounts of polyphenols in raspberries or cloudberries (**III**: Figure 2). The NMR spectra of fractions FIII, which were those most active in meningococcal pili binding, display the strongest phenolic and also polyphenolic signals (cranberry, lingonberry, bilberry, and crowberry juice, **III**: Figure 2G and H).

Differences were seen in binding activities between fresh berry and berry juice fractions. They may result from the manufacturing process of commercial juice concentrates, e.g. these may include heating, evaporation, and enzyme treatment. Oxidation or thermal degradation from particular processing techniques can alter the composition of phenolic compounds and induce high levels of structural variation, which may positively or negatively impact on bioactivity (Koponen et al. 2008, Landbo and Meyer 2004). Additionally, °Brix values in juice fractions derived from commercial berry juice concentrates were higher than in fractions prepared from fresh berries.

Based on the screening results, four berry juice molecular size fractions with high binding activity were selected for further evaluation. After solid-phase extraction, the binding activities of

N. meningitidis pili over a range of concentrations of the polyphenolic subfractions were studied in microtiter well binding assay. In general, dose-dependent increase in binding activity to a constant level was seen and significant binding was detected at very low concentrations of the subfractions **(II:** Figure 3; **Table 10**). Binding was associated with different subfractions (ethyl acetate, water, or methanol) depending on the species of the studied berry. This points to differences in the profiles of active components between the berries as also seen in the chromatographic profiles of the berry juice subfractions (**II**: Figure 2).

For both bilberry and crowberry juice, the pili binding was highest with the components of the water subfraction (**II:** Figure 3), which contained anthocyanins and especially in the case of crowberry, the polymeric proanthocyanidins. The binding activity reached a higher level with crowberry subfractions and also significant binding was seen at almost 10 times lower concentrations to crowberry than to bilberry juice in the case of water subfraction (**Table 10**).

For cranberry, the highest binding activity was achieved with anthocyanins and proanthocyanidins in the methanol subfraction (**II:** Figure 3). With ethyl acetate subfraction the binding was significant down to lower concentrations (**Table 10**). However, the highest achievable binding activity level remained considerably lower compared with that which could be attained with methanol and water subfrations (**II:** Figure 3).

For lingonberry, both the highest binding activity and the lowest concentration with significant binding was detected in the ethyl acetate subfraction containing flavonol glycosides and proanthocyanidins (Table 10; **II:** Figure 3). The highest binding activity level was lower compared with other berry juices and it was achieved with a much lower concentration of subfraction (**Table 10**).

Table 10. Binding of meningococcal pili to berry juice polyphenolic subfractions.

		Value of the highest binding	Conc. of the lowest significant binding Subfraction		
		(conc., subfraction)	Ethyl acetate	Water	Methanol
Bilberry FIII	41	(50 μg/ml, H_2O)	6.25 μg/ml	3.125 μg/ml	12.5 μg/ml
Cranberry FIII	51	(50 μg/ml, MeOH)	1.56 μg/ml	12.5 μg/ml	3.125 μg/ml
Crowberry FIII	52	(50 μg/ml, H_2O)	0.781 μg/ml	0.39 μg/ml	3.125 μg/ml
Lingonberry FII	37	(3.125 μg/ml, EtAc)	0.195 μg/ml	0.78 μg/ml	50 μg/ml

Since HPLC analysis showed that anthocyanins were abundant in water subfractions, a pure anthocyanin fraction from the bilberry juice concentrate was isolated using a semipreparative HPLC system. The binding activity testing of meningococcal pili to purified anthocyanins displayed lower binding compared to the bilberry juice subfractions and it was not so clearly dose-dependent (**II**: Figure 3E). This indicates that the binding effect of meningococcal pili 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 (Kennedy et al. 2001). Proanthocyanidins can also associate with pectin (Kennedy et al. 2001), polysaccharides (Matthews et al. 1997), and proteins (Hagerman and Butler 1980), and the association potential is significantly affected by the degree of their polymerization (Schmidt et al. 2004). It is also known that berry polyphenols can work synergistically (Sasaki et al. 2004; Seeram et al. 2004).

5.3.2 Adhesion inhibition (II, IV)

A dot binding assay was developed in the present study in order to study the inhibitory effect of selected molecular size fractions. *N. meningitidis* pili bound to HEC-1B epithelial cells (**II**: Figure 1). In general, the FII juice fractions that showed binding activity in the microtiter well assay also inhibited the pili binding to epithelial cells. Bilberry, cranberry, lingonberry, crowberry, and black currant juice FII fractions containing proanthocyanidins and anthocyanins totally inhibited the binding of meningococcal pili to HEC-1B cells (**II**: Figure 1). The FII fraction of raspberry juice, which contained small amounts of proanthocyanidins and anthocyanins compared to juice fractions of *Vaccinium* species and crowberry, inhibited the binding of meningococcal pili to the HEC-1B cells to some extent. Tomato, red grapefruit, or apple juice did not have the antiadhesive effect (**II**: Figure 1) and they had neither proanthocyanidins nor anthocyanins present (**II**: Table 1).

An adhesion inhibition assay based on human epithelial cell line HEC-1B and living encapsulated meningococcal cells was employed in order to test the inhibitory activity of berry juice polyphenolic subfractions eluted with water and mainly containing anthocyanins on whole bacterial cells. Significant antiadhesion activity was achieved with relatively low concentrations of polyphenolic fractions: 1 μg/ml of cranberry (74 % inhibition) and lingonberry (57 % inhibition),

and 5 μg/ml of bilberry (41 % inhibition) and crowberry juice subfraction (47 % inhibition) (**IV**: Figure 1). None of the studied berry juice polyphenolic fractions achieved total inhibition for the attachment of meningococci and the highest inhibition achieved was 75% with the cranberry juice polyphenolic fraction at 5 μg/ml. Although different methodologies were used in the experiments, the effective concentrations of the inhibition were at the same level as the concentrations in the microtiter well assay studies, where the isolated pili bound significantly to 0.39–12.5 μg/ml of these fractions (**Table 11**; **II**, Figure 3).

Table 11. Inhibitory effects of juice polyphenolic subfractions (eluted with water) in cell culture experiments and their concentrations giving significant pili binding in microtiter well assay studies.

	Inhibition %	Conc.of max. inhibition	Conc. of significant inhibition	Conc. of significant pili binding
Bilberry	27–63%	10 μg/ml	5–50 μg/ml	3.125 μg/ml
Cranberry	60–75%	5 μg/ml	1–50 μg/ml	12.5 μg/ml
Crowberry	37–63%	50 μg/ml	5–50 μg/ml	0.39 μg/ml
Lingonberry	48–57%	1–5 μg/ml	1–50 μg/ml	0.78 μg/ml

Encapsulated *N. meningitidis* has complex adhesion mechanisms and it interacts with host cells in a multistep process (Nassif 1999, Pujol et al. 1997). Meningococcus carries two potential adhesins in its type IV pili, the tip-located PilC (Rudel et al. 1995) and pilin (PilE) subunit proteins (Nassif et al. 1994). Although pili have an important role in colonization and infection in encapsulated *N. meningitidis* strains (Nassif 2000), the interactions of whole bacterial cells with epithelial cells are much more complicated than interactions of purified pili as meningococci can affect cellular signalling of host cells (Morand et al. 2009). Moreover, the concept of two different binding specificities located in two different components of the pilus is complicated by the fact that PilE can undergo antigenic variation (Meyer and van Putten 1989), which may influence the 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 PilC mediated adhesion.

Studies with milk pointed to carbohydrate recognition for *N. meningitidis* as the binding of meningococcal pili to glycoproteins was prevented by milk oligosaccharide fractions (**I**: Figure 1). However, results with berries do 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 concordant with studies on mannose-resistant P-fimbriated Gal-Gal-recognizing *E. coli*. The adherence of these bacteria to human erythrocytes and latex beads coated with synthetic P receptor analog was inhibited by the proanthocyanidins present in cranberry juice (Foo et al. 2000a, Foo et al. 2000b).

These positive *in vitro* results need to be confirmed in clinical trials, as under laboratory conditions one fails to mimic the host-generated molecular signals and adaptation to changes in environmental conditions which bacteria are able to utilize in their colonization.

5.3.3 Antibacterial activity (IV)

In the present study with polyphenolic subfractions eluted with water, the meningococcal 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 (**IV**: 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.

The adhesion inhibition of cranberry juice polyphenolic fraction at 1 μg/ml and at 50 μg/ml was 74% and 60%, respectively. Therefore, the adhesion inhibition effect of cranberry juice polyphenolic fraction can partly result from the killing effect of the fraction. For the other samples, the inhibitory effect achieved did not result from an antibacterial effect but rather from inhibition of bacterial attachment.

Berries and their phenolics have been previously shown to possess antimicrobial activity against human pathogenic bacteria such as *Campylobacter*, *Clostridium*, *Helicobacter*, *Salmonella*, and *Staphylococcus* (Nohynek et al. 2006, Puupponen-Pimiä et al. 2005, Rauha et al. 2000). However, cranberry NDM and juice concentrate, anthocyanin or proanthocyanidins-rich fractions of cranberry had no antibacterial activity against *P. gingivalis* (Labrecque et al. 2006) or *E. coli*, *P. aeruginosa*, and *S. mutans* (Ahuja et al. 1998).

5.4 Binding activity of *Streptococcus pneumoniae* to berry and juice fractions (III)

Screening results for *S. pneumoniae* differed from the results with other bacteria as it bound significantly only to the low-molecular-size fraction FI of cranberry and bilberry juices (**III**: Table 1). Non-significant binding was observed for *Vaccinium* species and crowberry juice. Bacteria did not bind to black currant, tomato, pineapple, red grapefruit, apple, sour cherry or raspberry juice fractions or fractions of lingonberries and cloudberries.

Results of screening do not refer to proanthocyanidins or anthocyanins as binding molecules for *S. pneumoniae*. Active fractions did not contain any considerable amounts of proanthocyanidins or anthocyanins compared to non-active fractions. In addition, inspection of the NMR spectra revealed that low-molecular-size fractions do not contain polyphenolic compounds. However, the activity of *S. pneumoniae* detected only in FI juice fractions may be related to lower molecular weight phenolic compounds. They are visible in the spectra of bilberry and cranberry juices and bilberry FI fractions (Fig. 2A, C, aromatic signals below 7 ppm) in relatively small amounts. This may point to an altered configuration in juices and highly specific components for *S. pneumoniae*.

5.5 Binding activity of *Streptococcus agalactiae* to berry and juice fractions (III)

Screening of binding activity of *Streptococcus agalactiae* to molecular size fractions showed that the binding activity of *S. agalactiae* was also mainly directed towards the high-molecular-size berry and juice fractions of *Vaccinium* species (**III**: Table 2). The most active binding was towards cranberry fraction FIII and to all fresh lingonberry fractions. *S. agalactiae* bacterial cells could bind also to fractions FII and FIII of bilberry juice and FII fraction of fresh cranberries and to all fractions of cranberry juice. In addition to the binding to the berries and juices of *Vaccinium* species, *S. agalactiae* bound significantly to sour cherry juice fraction FII and fresh cloudberry fraction FIII. No binding was detected for tomato, pineapple, red grapefruit, apple or raspberry juice fractions which all contained at best only small amounts (or even the complete absence) of proanthocyanidins or anthocyanins. On the other hand, fractions of crowberry and lingonberry juice were not active towards *S. agalactiae* although they contained high amounts of proanthocyanidins and/or anthocyanins (**II**, Table 1).

According to NMR spectra, the biologically active fractions for *S. agalactiae* most probably contain proanthocyanidins and/or other phenolic compounds as they emitted signals in the

aromatic regions (e.g. cranberry and lingonberry, **III** Figure 2D, 6.5–7.2 ppm). Some of the active fractions had polyphenol macromolecular complexes in the NMR spectra (e.g. bilberry and cranberry juices, **III** Fig. 2H). The highest binding activity was achieved with the cranberry fraction FIII but in the NMR spectra, no signals for any polyphenol macromolecular complexes were seen. This indicates that the polyphenol macromolecular complexes are not responsible for the binding activity present in cranberries, but may be for bilberry juice FIII.

In conclusion, differences in phenolic profiles between different berries and processing conditions in manufacturing of juice concentrates seem to be responsible for the variation in the binding activity of *S. agalactiae*.

5.6 Hemagglutination inhibition of *Streptococcus suis* by berry fractions (III)

Both *S. suis* and *E. coli* are known to bind galabiose (Haataja et al. 1993, Strömberg et al. 1990). *Streptococcus suis* 836 hemagglutinates human neuraminidase treated erythrocytes (Tikkanen et al. 1996, Kurl et al. 1989) and the hemagglutination inhibition assay has been successfully employed with *S. suis* to analyze the molecular interactions of the binding ligand (Haataja et al. 1993). Fractions prepared from fresh berries were tested with *S. suis*. Both cranberries and lingonberries inhibited the hemagglutination induced by *S. suis* or *E. coli* (**III**: Table 2). Cranberry fractions FII and FIII had the highest inhibitory power followed by lingonberry fractions FII and FIII. Cranberry fractions FI and FII and lingonberry fraction FII also were able to inhibit *E. coli* induced hemagglutination. Neither bilberry nor cloudberry fractions inhibited *S. suis* or *E. coli* hemagglutination, though cloudberry fractions FII and FIII impaired the hemagglutination induced by *E. coli*.

This ability to inhibit hemagglutination may indicate that there is a galactose containing inhibitor in berry fractions and, in fact, galactose is one of the most common sugar moieties in flavonoids (Määttä-Riihinen et al. 2004a). Alternatively, inhibition may result from unspecific receptor mimicking (Das and Devaraj 2006) blocking the galabiose-recognizing adhesin in *S. suis* and *E. coli*. The results are thus consistent with previous studies on *E. coli* in which proanthocyanidins from cranberry juice could inhibit mannose-resistant P-fimbriated Gal-Gal-recognizing *E. coli* (Foo et al. 2000a) while fructose inhibited mannose-sensitive type 1 fimbriated *E. coli* (Zafriri et al. 1989). The NMR revealed that in the biologically highest active fractions

against *S. suis* (cranberry and lingonberry FII) there were polyphenol macromolecular complexes (**III**: Fig. 2E). These complexes were not present in the cranberry and lingonberry fractions FIII, which evoked weaker hemagglutination inhibition as compared with the FII fractions of the berry. Furthermore, the measured proanthocyanidin and anthocyanin concentrations (**II**: Table 1) were higher in those fractions inhibiting the hemagglutination by *S. suis*.

6 Summary and conclusions

Evolutionary pressure has led to the appearance of certain molecules in milk and berries which can combat pathogens. Oligosaccharides, glycoconjugates, and phenolic secondary metabolites work as antiadhesive agents. These molecules offer an alternative method to control infectious diseases which lack of effective vaccines and antibiotics. Since these molecules can inhibit the initial attachment of pathogen to the human body, this could minimize the spread of bacteria from person to person. In this way, the prevalence of bacterial infections can be reduced by affecting the carrier status. In the present study, human and bovine milk as well as berries and juices were investigated as sources of preventive antiadhesive material against serious meningitis- and respiratory infection-associated pathogens.

In this study, neutral human milk oligosaccharides and acidic bovine milk oligosaccharides showed antiadhesive activity against binding of *Neisseria meningitidis* to bovine thyroglobulin which was used as model glycoprotein. In addition, a novel interaction of meningococci and human salivary agglutinin was described. The binding of *N. meningitidis* to salivary agglutinin was inhibited by up to 50% by acidic bovine milk oligosaccharides. This binding and inhibition constitute an interesting target for further studies, especially when considering the feasibility of using bovine milk oligosaccharides as protective food additive against meningococci. Additional studies including desialylation of acidic bovine milk oligosaccharides as well as purification and analysis of individual binding oligosaccharides are also justified to characterize the receptor active sequences for *N. meningitidis* pili. Milk carbohydrates do not have bactericidal activity and thus may not increase antibiotic resistance by selection of resistant strains of bacteria (Sharon and Ofek 2000). In conclusion, the present observation with *N. meningitidis* together with previous reports (Ruiz-Palacios et al. 2003, Martín et al. 2002, Martín-Sosa et al. 2002, Kunz et al. 2000, Barthelson et al. 1998, Simon et al. 1997, Kunz and Rudloff 1993, Schengrund and Ringler 1989, Andersson et al. 1986, Kolstø Otnæss et al. 1983) indicate that milk oligosaccharides have potential use as preventive antiadhesive agents.

In addition to the milk oligosaccharides, the study revealed novel interactions between berry-based material and *N. meningitidis*, *Streptococcus pneumoniae*, *Streptococcus agalactiae*, and *Streptococcus suis*. Berries and juices manufactured from *Vaccinium* species proved to be the most

effective in bacterial binding. *S. pneumoniae* bound to low-molecular-size fractions whilst all other bacteria studied, *N. meningitidis*, *S. agalactiae*, and *S. suis*, underwent significant interactions with high-molecular-size fractions. Further investigations were focused on *N. meningitidis*. The inhibitory effect of berry material was localized to phenolic compounds. The results of cell culture experiments and antimicrobial studies showed that the inhibitory effect of berry juice polyphenols was due to antiadhesive effect and partly from an antimicrobial effect in the case of cranberry juice polyphenols. Berry molecules may provide multiple binding sites for *N. meningitidis* pili adhesins and thus inhibit adhesion.

Applications of the results could be in the development of preventive antiadhesive drugs or nutritional products. The populations most at risk for developing a fatal respiratory disease are the very young, the elderly, and the immunocompromised. Molecular size fractions as well as polyphenolic water extracts could be easily utilized by the food industry. Novel preventive and protective antiadhesion agents may have significance also in the developing countries, where needle-based vaccination and the preservation of vaccines are difficult. Clinical trials will be needed to prove the effect of berry juice components on the carrier rate in healthy people to control of spread of infectious diseases. Further purification of active molecules will be needed to determine if the antiadhesive effect can be associated to a single molecule or the effect needs the synergic activities of several berry molecules.

On the basis of results from the present study it can be concluded that:

1. Acidic bovine milk oligosaccharides and neutral human milk oligosaccharides have anti-adhesion potential against meningococcal infections.

2. Berry polyphenols could possibly be used as antiadhesive agents against *Neisseria meningitidis*.

3. Low-molecular-size fractions of *Vaccinium* species may have potential in inhibiting the attachment of *Streptococcus pneumoniae*.

4. High-molecular-size fractions of *Vaccinium* species may have binding inhibitory potential against *Streptococcus agalactiae* and *Streptococcus suis*.

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Marko Toivanen

Antiadhesive Molecules in Milk and Berries against Respiratory Pathogens

The aim of this doctoral thesis was to evaluate potential natural antiadhesive agents against meningitis- and respiratory infection-associated pathogens. The first part describes the antiadhesive ability of human and bovine milk against *Neisseria meningitidis*. In the second part several berries and juices were investigated against *N. meningitidis, Streptococcus pneumoniae, Streptococcus agalactiae,* and *Streptococcus suis*.

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