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MAGISTERSKÁ DIPLOMOVÁ PRÁCE  
Antiadhesivní účinky oligosacharidů kravské syrovátky  
proti *Neisseria meningitidis*

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MASTER OF SCIENCE THESIS  
Anti-adhesion activity of bovine whey oligosaccharides  
against *Neisseria meningitidis*

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I proclaim that I composed this Master of Science Thesis on my own and I only used literature and information sources listed in references.

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## ABBREVIATIONS

ABTS	2,2'- azino-di-(3-ethylbenzthiazoline sulfonate)
AECH	anion exchange chromatography
BBB	blood-brain barrier
BMO	bovine milk oligosaccharides
BWO	bovine whey oligosaccharides
CSF	cerebrospinal fluid
<i>E.</i>	<i>Escherichia</i>
FMS	fulminant meningococcal septicaemia
GSP	general secretory pathway
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMO	human milk oligosaccharides
<i>N.</i>	<i>Neisseria</i>
PBS	phosphate-buffered saline
Streptavidin-POD	streptavidin-linked H <sub>2</sub> O <sub>2</sub> oxidoreductase (horseradish peroxidase)
WAO	whey acidic oligosaccharides
WNO	whey neutral oligosaccharides

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ABBREVIATIONS

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## INTRODUCTION

*Neisseria meningitidis*, meningococcus, is a human-specific pathogen causing life-threatening septicaemia and meningitis. Meningococcal diseases are always very serious since they can be fatal within a few hours after first observed symptoms. Thus, fast diagnosis and immediate antibiotic treatment is important. Meningitis also remains an important cause of morbidity and mortality among children under 5 years of age and is especially prevalent in developing countries.

Unfortunately, present increase of antibiotics resistant bacterial strains makes it more difficult to decrease its incidence. Therefore, it is imperative nowadays to search for new means of treatment and prevention.

One of these challenges may be anti-adhesion therapy employing milk oligosaccharides and glycoconjugates. Oligosaccharides and glycoconjugates in human milk represent a defensive system on the newborn and have inhibitory activity against many pathogens, both Gram negative and Gram positive. Bovine milk oligosaccharides have been recently shown as effective inhibitors against *N. meningitidis*.

Thus, milk oligosaccharides could be used as novel antimicrobials. Furthermore, adding them to infant or adult food may therefore have benefits beyond the nutritional effect. However, such application has got high financial demands. It could be economically reasonable by employing whey, the largest by-product and “waste“ from dairy companies, as source of oligosaccharides.

Whey poses a major world wide disposal and pollution problem. Disposal of whey is released into the wastewater and cause serious pollution problems in rivers and the sea due to the high biochemical oxygen demand (BOD) of its dissolved organic components.

Upgrading the whey such a way, as a cheap source of oligosaccharides, could be one way to reduce the serious pollution problems and could help to prevent bacterial diseases e.g. such as caused by meningococcus.

This novel means of therapy of microbial diseases has a perspective future, but before it comes true, more studies are needed for the function of inhibition mechanism of these components that have anti-adhesion activity against different kinds of bacteria.

# 1 REVIEW OF LITERATURE

## 1.1 GENUS *NEISSERIA*

*Neisseria meningitidis* and *Neisseria gonorrhoeae* are two pathogenic members of the neisseriae family of Gram-negative bacteria. They belong to the same genus and therefore share a great deal of relatedness at the nucleotide level (Nassif and So, 1995). The homology of the DNA sequence of these related pathogens is 80 to 90% (Tinsley and Nassif, 1996). These two species cause diseases with very different characteristics (Nassif and So, 1995). *N. meningitidis* causes life-threatening diseases, septicaemia and meningococcal meningitis. *N. gonorrhoeae* causes localized inflammation of the urogenital tract, known as the venereal disease gonorrhoea. Both disease-causing bacteria are associated with remarkable morbidity and mortality for their hosts (Tønjum and Koomey, 1997). The reasons for this striking difference in the pathogenesis of these two microorganisms remain mostly unclear. Until now, the only major bacterial attribute implicated in this difference was the capsule, which is found in meningococcus but not gonococcus (Nassif and So, 1995).

### 1.1.1 MENINGOCOCCAL DISEASES

*N. meningitidis* is a common inhabitant of the mucosal membranes of the human nasopharynx, where it usually survives as a harmless commensal. Up to 5–10% of a population may be asymptomatic carriers. This carrier state may last for a few days to a few months. It provides the reservoir for *N. meningitidis* and enhances the immunity of the host. The meningococcus is spread among others by person-to-person contact through aerosol droplets or contacts with respiratory secretions from the asymptomatic carriers ([http://www.who.int/vaccine\\_research/diseases/soa\\_bacterial/en/index2.html](http://www.who.int/vaccine_research/diseases/soa_bacterial/en/index2.html), 14.4.06). The nasopharyngeal infection may lead at small minority of those who become infected to occult bacteraemia up to fulminant sepsis resulting in death only a few hours after the first symptoms occur. Invasive disease is

usually manifested in one of two ways: meningococemia or meningitidis (with or without meningococemia) (Singh and Arrieta, 2004).

### **Fulminant meningococcal septicaemia (FMS)**

The meningococci colonise the nasopharynx and they gain entry into the bloodstream, where it causes meningococemia (Nassif, 1999). FMS is very sudden disease and induces the mortality rate very high, between 20 to 80% (van Deuren et al., 2000). It is acute infection and death can occur 12 to 48 hours after first symptoms (Riedo et al., 1995). On the other side, FMS is the rarest form of meningococcal disease (van Deuren et al., 2000).

Patients are generally febrile, with headache, vomiting, malaise and often complain of myalgia (Singh and Arrieta, 2004). They may present with septic shock, multiorgan failures, hypotension, purpuric rash and disseminated intravascular coagulation (Riedo et al., 1995)

### **Meningococcal meningitidis**

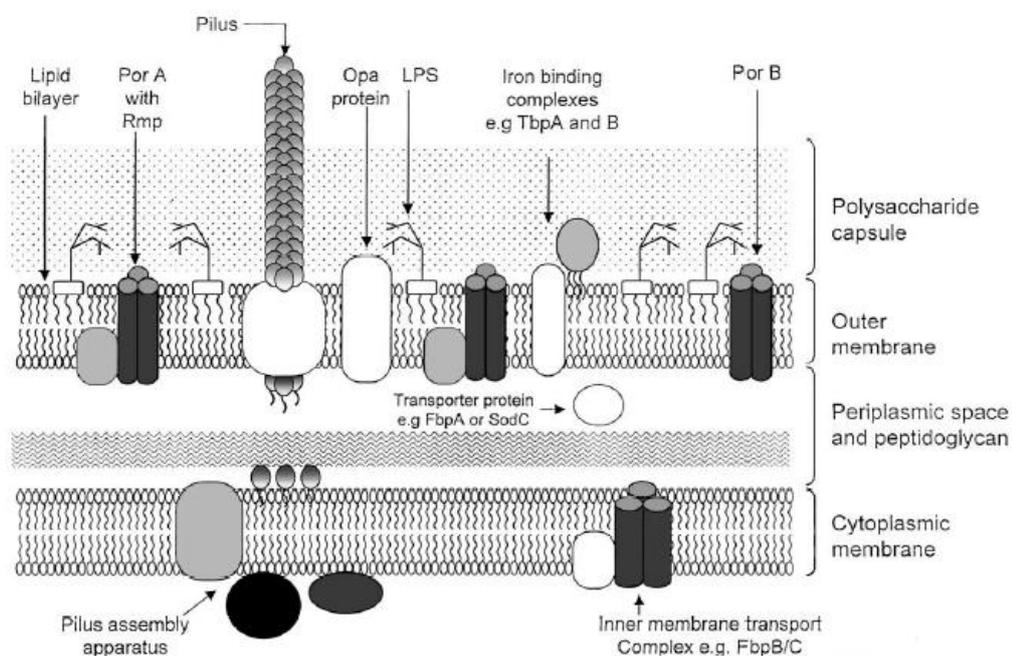
In small percentage of colonised people, meningococci progress from the bloodstream to the cerebrospinal fluid (CSF) to cause meningitis after crossing the blood-brain barrier (BBB) (Nassif, 1999).

Acute meningitidis is also fatal meningococcal disease if untreated (Pron et al., 1997) The mortality rate associated with meningococcal meningitidis is up to 5% as a consequence of rapidly progressive fatal complications (van Deuren et al., 2000; Stephenson, 1998). The classical symptoms for meningitides are acute high fever, stiff neck and headache (Singh and Arrieta, 2004). Permanent neurological deficits are unexceptional for 11-19% of patients surviving meningococcal disease. Sensorineural hearing loss, impaired vestibular function, neurological disability or losses of limbs are comparatively frequent after meningococcal disease (Singh and Arrieta, 2004; Naess et al., 1994).

### 1.1.2 *NEISSERIA MENINGITIDIS*

*N. meningitidis* is a Gram-negative diplococcus (Singh and Arrieta, 2004).

The meningococcus has both an outer and inner (cytoplasmic) cell membrane that are separated by a peptidoglycan cell wall (Fig. 1). The outer membrane is surrounded by a polysaccharide capsule that is essential for pathogenicity. The outer membrane contains numerous protein structures, which enable the organism to interact with and adhere to host cells, and which also act as transport proteins allowing control of the intracellular environment. The outer membrane also contains lipopolysaccharide (endotoxin), which helps to confer serum resistance and is involved in the pathogenesis of meningococcal disease. The outer membrane proteins together with the capsular polysaccharide form the principal surface antigens of the meningococcus (Morley and Pollard, 2002). Some important meningococcal surface structures are outlined in Tab. 1.



**Fig. 1** Surface structures of *N. meningitidis* (figure obtained from Morley and Pollard, 2002).

**Tab. 1** Some function of major surface structures of *N. meningitidis* (modified from Pollard and Frasch, 2001).

<b>Structure</b>	<b>Function</b>
Polysaccharide capsule	Anti-phagocytic, reduce immunogenicity, down regulates alternative complement pathway activation, antigenic mimicry
Lipopolysaccharide (LPS)	Antigenic mimicry, reduces C3 and C9 deposition and downregulates alternative complement pathway activation
Pili	Adhesion
PorA (class 1)	Cation porin
PorB (class 2/3)	Anion porin
Class 4, reduction modifiable protein (Rmp)	Unknown
Opa (class 5)	Adhesion, invasion
Opc (class 5c)	Invasion, adhesion
Transferrin binding protein 1 (Tbp1)	Transferrin binding
Transferrin binding protein 2 (Tbp2)	Transferrin binding
Ferric enterobactin A (FetA; previously designated FrpB)	Ferric-enterobactin receptor
Ferric binding protein (FbpA)	Iron-binding
Neisserial surface protein A (NspA)	Unknown

Meningococci have been classified using serological method based on antigenicity of the surface structure. Further classification depends on antigenic differences in their major outer membrane proteins PorB (serotype) and PorA (subtype) and the structure of the outer membrane lipopolysaccharide. They are grouped into 12 serogroups: A, B, C, 25E, H, I, K, L, W135, X, Y and Z. Strains belonging to groups A, B, C, Y, and W135 are pathogenic (Morley and Pollard, 2002). Group A strains have caused most of the major epidemics. Group B and C generally cause epidemic disease but on occasion cause an epidemic outbreak. Other serogroups less frequently cause disease but are quite common found in nasopharyngeal cultures (Segal and Pollard, 2005).

### 1.1.3 CAPSULAR POLYSACCHARIDE

The capsules of the pathogenic meningococcal serogroups differ in both chemical structure and antigenic properties. The serogroup A capsule is composed of *N*-acetylmannosamine-1-phosphate whereas those of serogroup B, C, Y, and W135 all contain sialic acid (*N*-acetylneuraminic acid). Serogroup B and C are homopolymers of sialic acid but critically differ in being linked  $\alpha$ 2-8 and  $\alpha$ 2-9. The serogroup C capsule is usually de-*O*-acetylated whereas the serogroup B capsule is not. These subtle differences in serogroup B capsule are sufficient to prevent it being an efficient immunogen in humans as  $\alpha$ 2-8 *N*-acetylneuraminic acid is structurally and antigenically identical to the polysialosyl glycopeptides of the human neural cell adhesion molecule in foetal and adult neural and external tissues. This molecular mimicry is believed to prevent recognition of the capsule through host self-tolerance mechanisms (Morley and Pollard, 2002).

### 1.1.4 TYPE IV PILUS

Pili are filamentous protein structures on the bacterial surface (Nassif and So, 1995). Type IV pili are 1-5  $\mu$ m long, 60-70 Å in diameter, mechanically strong polymeric fibres containing 500-2000 repeating identical subunits of the pilin (Pile), an 18-22 kDa polypeptide, forming right-handed helix structure (Parge et al., 1995; Merz and So, 2000; Nassif and So, 1995; Collins et al., 2005). The type IV pilus has five pilin subunits per turn of helix (Parge et al., 1995). Mechanical stability that the pili require is achieved by pilus central layer  $\beta$ -sheet hydrogen bonding (Forest and Tainer, 1997). The assembly of pilin into pili, as well as pilus disassembly, is controlled by a complex interacting apparatus of up to 30 proteins (GSP) (Collins et al., 2005). The type IV pili have various functions (see Tab. 2). They are retractile and this retraction process is responsible for twitching motility on solid and mucosal surfaces. The pili mediate cellular attachment to epithelial tissue receptors, and are involved in several other bacterial processes, including bacterial auto-agglutination, variation of target tissue specificity and natural competence for DNA uptake (Collins et al., 2005).

**Tab. 2** Function requiring type IV pili. (modified from Merz and So, 2000).

<i>N. meningitidis</i> and <i>N. gonorrhoeae</i>	References
Bacterial aggregation	Swanson et al., 1971
Adhesion to host cells	Nassif et al., 1993; Swanson, 1973
Twiching motility	Brossay et al., 1994; Swanson, 1978
Pilus retraction	AJ Merz, M So & MP Sheetz, submitted
Dispersal from aggregates and loss of pili	Pujol et al., 1999
Host cell response	
Cytosolic Ca <sup>2+</sup> fluxes	Källström et al., 1998
Exocytosis	P Ayala & M So, submitted
Cortical plaque formation	Merz et al., 1999
Cytotoxicity	Dunn et al., 1995; McGee et al., 1981
DNA transformation	Sparling, 1966; Wolfgang et al., 1998
Other bacterial species	
Type II secretion ( <i>P. aeruginosa</i> )	Lu et al., 1997
Bacterial aggregation ( <i>V. cholerae</i> )	Kirn et al., 2000
Complement resistance ( <i>V. cholerae</i> )	Chiang et al., 1995
Adhesion to host tissue ( <i>Azoarcus</i> sp.)	Dor et al., 1998
Virulence gene regulation in vivo ( <i>V. cholerae</i> )	Lee et al., 1999
Twiching/social gliding motility ( <i>M. xanthus</i> )	Wu et al., 1997
Dispersal from aggregates (enteropathogenic <i>E. coli</i> )	Bieber et al., 1998
Pilus bundle structural change (enteropathogenic <i>E. coli</i> )	Knutton et al., 1999
Biofilm formation ( <i>P. fluorescences</i> )	O'Toole & Kolter, 1998
Cytotoxicity ( <i>P. aeruginosa</i> )	Comolli et al., 1999
Horizontal genetic transfer	
DNA transformation ( <i>L. pneumophila</i> )	Stone & Kazik, 1999
Conjugation (IncII plasmids)	Yoshida et al., 1999
Bacteriophage infection ( <i>P. aeruginosa</i> )	Bradley, 1974
Bacteriophage assembly ( <i>V. cholerae</i> ΦVPI)	Karaolis et al., 1999

The meningococcal pili belong to the Type IV family of pilins, like pili of many Gram-negative bacterial pathogens including *N. gonorrhoeae*, *Vibrio cholerae*, enteropathogenic *Escherichia coli*, and *Pseudomonas aeruginosa*.

*N. meningitidis* produces two types of different pilin variants designated class I and class II. This antigenic variation is due to changes in the primary sequence of the PilE locus (Nassif, 1999).

### **Class I pilin**

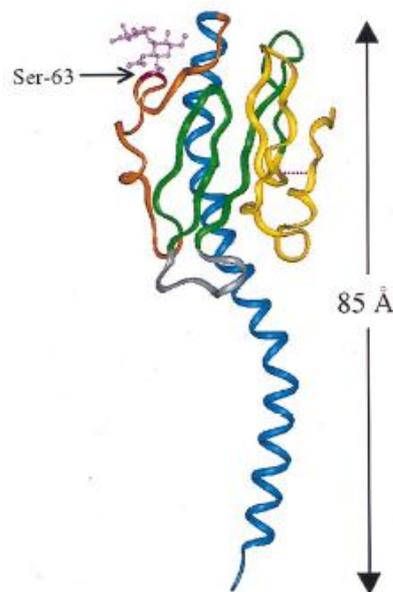
Class I pilins bind monoclonal antibody SM1 and are similar to those of *N. gonorrhoeae* and overall organisation of the PilE gene is identical in both species (Nassif, 1999; Nassif and So, 1995). There is a highly conserved 5' region encoding amino acids 1-53, which comprises the amino-terminal alpha helix of the protein. This conserved domain is followed by a semivariable region encoding amino acids 54-114. The remaining 3' portion of PilE, encoding the disulphide region and carboxy-terminal tail of pilin, is often referred to as the hypervariable region that exhibits the greatest primary sequence and antigenic diversity (Nassif, 1999; Merz and So, 2000).

### **Class II pilin**

The class II pilins do not bind monoclonal antibody SM1 (Nassif and So, 1995). It shares extensive amino acids identity with the amino-terminal conserved regions of class I meningococcal pilins, however, the sequence displays several unique features compared to the sequences of other neisserial pilin. The crystallographic analysis of pilin variant reveals a ladle-shaped molecule divided into several distinct structural regions: an amino-terminal hydrophobic alpha helix, a sugar loop carrying an *O*-linked disaccharide, a four stranded antiparallel beta-sheet demarcated by the conserved cystein residues and followed by a carboxy-terminal tail (Nassif, 1999).

### 1.1.5 PILIN GLYCOSYLATION

Pilin is synthesized as a precursor protein (pre-pilE) and undergoes characteristic processing by the PilD prepilin peptidase/transmethylase. The result is a mature pilin subunit with an  $\alpha$ -methylated phenylalanine residue at its N terminus (N-met-Phe). Neisserial pilin is further post-translationally modified by *O*-glycosylation and phosphorylation at sites mapping to the exposed surface of the fibre (Merz and So, 2000). Two types of sugar have been found. An *O*-linked *N*-acetyl-D-glucosamine- $\alpha$ 1,3-galactose (GlcNAc- $\alpha$ 1,3-Gal) and a trisaccharide, a digalactosyl 2,4-diacetoamido-2,4,6-trideoxyhexose. The pathways that lead to pilin glycosylation remain mostly unknown (Nassif, 1999).



**Fig. 2** Ribbon representation of pilin from *N. gonorrhoeae*. GlcNAc-1,3-Gal is covalently bound with *O*-linkage to Ser 63 (figure obtained from Soto and Hultgren, 1999). Similar modification occurs in class I meningococcal pili due to the homology to gonococcal pili (Virji, 1997).

### **1.1.6 ROLE OF PILIN ANTIGENIC VARIATION**

The type of pilin variant expressed by a strain is crucial in determining bacterial adhesiveness. Some variants are responsible for a high adhesive phenotype, whereas others result in a low adhesive phenotype (Nassif, 1999). Further studies of class I pilin find out that all high adhesive variants produced pilin SB, while all low adhesive variants produced either of two pilins SA and SC. These variants differed in only few amino acid residues and adhered with the same frequencies to both epithelial and endothelial cells. The high adhesive variants produced highly bundled pili and that bacteria expressing these variants had a tendency to clump. In contrast, pili of low adhesive variants did not form bundles. The ability to form bundles, and thereby increase bacterial aggregation, is responsible for modification of adhesiveness. Glycosylation of pilin may also play a role in modulating meningococcus interaction with host cells (Nassif and So, 1995). Loss of glycosylation favours agglutination of pili and the formation of bundles of pili, whereas glycosylation increases the amount of soluble, truncated monomers of pilin. In addition, the loss of glycosylation is responsible for a slight increase in pilus-mediated adhesion because of increase in piliation (Nassif, 1999).

### **1.1.7 THE PILC PROTEINS**

PilC1 and PilC2 are large proteins of 110 kDa with conventional signal sequence (Merz and So, 2000). These proteins have been found at the tip of the pilus and in the outer membrane where they are believed to be at the base of the fibre (Nassif, 1999). Genes involved in structural and phase variations of proteins are PilC1 and PilC2 loci (Merz and So, 2000). The PilC proteins are important in pilus-mediated adhesion and pilus biogenesis (Nassif, 1999). The PilC has a specific receptor binding ability. It has been shown to bind to the host epithelial receptors CD 46 (Collins, 2005). Recent publication has shown that human CD46 is pilus receptor for pathogenic *Neisseria* that mediates *N. meningitidis* passage through BBB and access to meninges (Johansson et al., 2003). Therefore, only piliated cells are capable of binding, and attachment of bacteria could be blocked by monoclonal antibodies against CD46 and

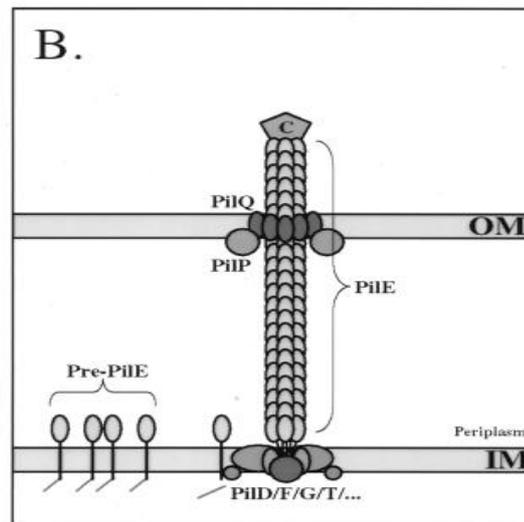
by recombinant CD46 protein produced in *E. coli* (Nassif, 1999). On the other side, mutation of the pilC1 gene results in a piliated, transformation-competent strain that is unable to adhere to epithelial cells whether the strain is producing high or low adhesion pilin (Merz and So, 2000; Nassif and So, 1995). PilC1 thus has an essential role in pilus-mediated cell adhesion that is not fulfilled by pilC2 (Merz and So, 2000). PilC proteins also play a role in the assembly process for filament and appear to antagonize pilus retraction (Collins, 2005; Merz and So, 2000).

### 1.1.8 ASSEMBLY OF PILI

The assembly of type 4 pili is through the general secretory pathway (GSP), which is widespread among Gram-negative bacteria, including *N. meningitidis* and *N. gonorrhoeae* (Shi and Sun, 2002). The formation requires the expression of several proteins, including the following: a prepilin peptidase that cleaves a short leader peptide from the subunits; an integral membrane protein located in the inner cytoplasmic membrane that may serve as a platform for fimbrial assembly; a hydrophilic nucleotide-binding protein located in the cytoplasm or associated with the cytoplasmic face of the inner membrane that may energize secretion by ATP hydrolysis; and an outer membrane component that forms a channel allowing the translocation of assembled pili through the outer membrane (Soto and Hultgren, 1999).

The pre-PilE precursor subunits are translocated into the periplasmic compartment by the general secretion apparatus. These molecules are retained in the inner membrane by their hydrophobic N-terminal segments, with their hydrophilic C-terminal domains oriented towards the periplasm. The PilD signal peptidase removes the positively charged leader sequence from the cytoplasmic side of the prepilin to generate mature PilE, which can then undergo assembly as subunits associate with their hydrophobic stems. PilF, PilG, and PilT are among the factors required for this assembly, although their functions are not well understood. The assembled pili are thought to be translocated across the outer membrane by a gated pore formed by a multimeric form of PilQ. A lipoprotein, PilP, appears to function in

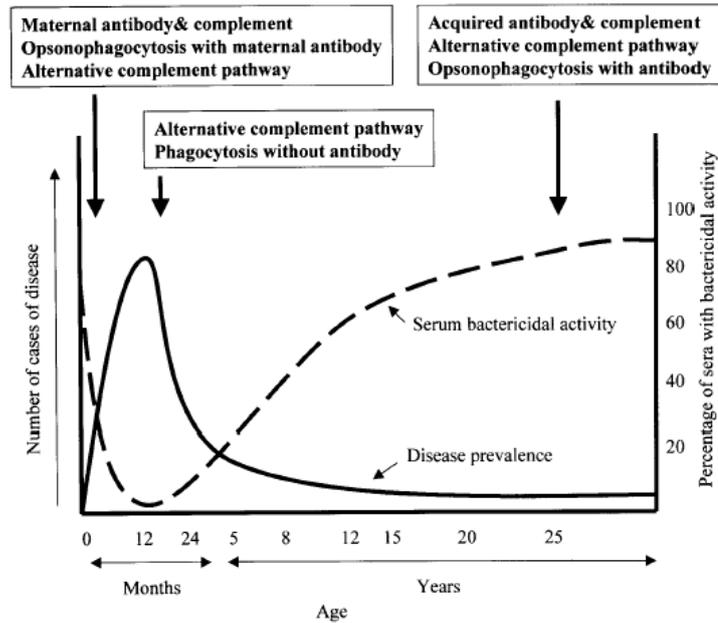
stabilizing the expression of PilQ as a multimer. The PilC adhesin appears to facilitate passage of the growing organelle through this pore, although the molecular basis for the role of PilC in this process is not well understood (Soto and Hultgren, 1999).



**Fig. 3** Assembly of type IV pilus of *N. gonorrhoeae* via the general secretion pathway (obtained from Soto and Hultgren, 1999). OM - outer membrane, IM - inner membrane

### 1.1.9 IMMUNE MECHANISM AGAINST MENINGITIDIS

Defence against disease caused by *N. meningitidis* involves both innate and acquired immune mechanisms that recognize bacterial surface structures. The complexity of the immune response involves a central role of antibody and complement. The immune responses may vary and depending on the age of the child as well as the serogroup of the organism (Fig. 4). Serum bactericidal activity in the newborn results from transplacental transfer of maternal IgG directed against capsular polysaccharide and outer membrane antigens. Moreover, the environment of the nasopharynx in infancy does not favour colonization by pathogenic meningococci both because of innate immune mechanism, environmental factors and because of the presence of competing non-pathogenic *Neisseriae*. 50 % of newborns have anti-meningococcal bactericidal activity. However, immunity decreases rapidly after birth to reach a nadir between 6 and 12 months of age. Through infancy the level of maternally derived antibody falls, there is little or no specific humoral immunity, phagocyte function is poor and the terminal complement components are at relatively low level. Childhood exposure to non-pathogenic *Neisseriae* and other cross-reacting species in the nasopharynx and gastrointestinal tract lead to increasing levels of specific antibody. This specific antibody confers protection from disease through local immunity in the nasopharyngeal mucosa (antibody mediated opsonization and phagocytosis) and through opsonophagocytosis and complement-mediated bactericidal activity in the blood. Serum bactericidal activity steadily increases through childhood (50-80 % bactericidal activity by 12 years) so that by adulthood 65-85 % of individuals have bactericidal activity against *N. meningitidis* (Pollard and Frasch, 2001).



**Fig. 4** The age dependent prevalence of meningococcal disease in relation to population immunity. (figure obtained from Pollard and Frasch, 2001).

### 1.1.10 VACCINES AGAINST BACTERIAL MENINGITIS

Meningococcal disease remains one of the most feared infections due to its rapid progression and tendency to cause outbreaks and epidemics. Immunization against the causative organism, *N. meningitidis*, will further reduce the morbidity and mortality of the disease (Morley and Pollard, 2002).

An important virulence factor of *N. meningitidis* is its polysaccharide capsule since encapsulated form of meningococcus is protected against phagocytosis and capable to survive at bloodstream (Lindberg, 1999, Nassif, 1999). Purified polysaccharides were used to produce serogroup A and C polysaccharide vaccines. These vaccines have limited usefulness because of poor immunogenicity and the duration of the immunity. Conjugation of partially hydrolysed, size fractionated oligosaccharide derived from capsular polysaccharide with carrier proteins have resulted in protective serogroup A, C, Y, W135 glycoconjugate vaccines (Morley and Pollard, 2002).

A majority of endemic disease in industrialized countries is caused by serogroup B meningococci, and vaccines against this organism are much more difficult to develop. The B polysaccharide is not immunogenic because of its chemical identity to human neural surface antigens, and attempts to improve immunogenicity could

lead to the induction of autoantibodies that cross-react with glycosylated host antigens, most notably in foetal brain tissue (Segal and Pollard, 2005). To overcome this tolerance, Jennings et al. developed a chemically modified B polysaccharide that contains *N*-propionyl group in place of the *N*-acetyl groups conjugated to a carrier protein (Morley and Pollard, 2002; Segal and Pollard, 2005). At present outer-membrane vesicles vaccines have been used more widely than any other serogroup B vaccines (Morley and Pollard, 2002). Outer-membrane vesicles contain a number of proteins that are with PorA the most highly expressed and immunodominant antigens (Segal and Pollard, 2005).

### **1.1.11 INTERACTION WITH A HOST CELL**

Many bacterial infections begin with molecular interaction between the pathogen and the host cell (Soto and Hultgren, 1999). The adhesion of bacteria is a ligand-receptor interaction where the bacterial adhesin binds to the host cell receptor (Kunz and Rudloff, 1993). Bacterial adhesins are proteins present on the surface of the organisms that bind to complementary carbohydrate constituents of glycoproteins or glycolipids on the surface of host cell tissues (Sharon and Ofek, 2000). Normally, the oligosaccharide units of the glycoproteins and glycolipids on the host cell surface have a role in binding to the other cell and act as receptors to hormones and other humoral effectors (Newburg, 1997). The interaction between adhesin and receptor of the host cell is non-covalent, highly specific but weak. The strength of the pathogen-cell binding results from the large numbers of individual protein-carbohydrate interaction (Zopf and Roth, 1996). The bacterial surface adhesins are frequently in the form of fimbriae or pili and meningococcus initiate infection by attaching to host cell via surface-associated type IV pili (Sharon and Ofek, 2000; Merz and So, 2000). Receptor binding induces the activation of multistep adhesion cascade, followed by invasion of the host cell, intracellular persistence, transcytosis and exit. These activities are modulated by signals send from pathogen to host and host to pathogen at multiple stages of the adhesion cascade (Merz and So, 2000). The binding also activates the expression of new genes in microbe that are important in pathogenic process (Grifantini et al, 2002).

The interaction of *Neisseriae* with their host is a complex process. The integration of tools from cell biology, biochemistry, biophysics and genomics has opened the way to understand this process and the next few years will bring unprecedented insights into Neisseria-host interaction (Merz and So, 2000).

## 1.2 WHEY

Whey is a product of removal of the casein micelles from skim milk by clotting with rennet. Whey is mainly obtained from bovine milk and therefore the majority of components of milk are found in it. The composition of whey products varies according to the milk source, type of cheese, and manufacturing process. There are two basic types of whey: sweet whey with a pH of >5.6 and acid whey with a pH of <5.1. Sweet whey, derived from the manufacture of enzyme-produced cheeses such as Cheddar, Mozzarella or Swiss. Acid whey is obtained from the manufacture of cottage, cream, or ricotta cheese. Whey thus contains protein, lactose, minerals (e.g., calcium, phosphorus, magnesium, zinc), vitamins, and traces of milkfat (Tab. 3). Whey contains also bioactive components that may offer protection against infections and enhance immunity, protect against some cancers, positively affect cardiovascular health, and is advantageous for physically active individuals. These components include immunoglobulins (Igs), lactoferrin and its peptide derivative, lactoferricin, lactoperoxidase, glycomacropeptide, and sphingolipids (<http://www.nationaldairyCouncil.org/NationalDairyCouncil/Health/Digest/dcd74-6Page1.htm>, 23.11.2005).

Oligosaccharides also belong among bioactive components that are present in whey in minor. For a long time, oligosaccharides were not believed to have any biological significance (Newburg, 1997), but nowadays there are few reports on bovine milk oligosaccharides and several reports on human milk oligosaccharides and glycoconjugates having biological activity. Together, these reports and observations suggest employing whey from dairy companies as a source for milk oligosaccharides and their use in development of functional dietary supplements and drugs to combat infectious disease (Hakkarainen et al., 2005).

**Tab. 3** Composition of whey (<http://www.albalagh.net/halal/co14.shtml>, 23.11.2005).

	Fluid whey		Dried whey	
	Sweet	Acid	Sweet	Acid
Total solid	6.35 %	6.50 %	96.5 %	96 %
Moisture	93.7 %	93.50 %	3.5 %	4 %
Lipids	0.50 %	0.04 %	0.8 %	0.6 %
Proteins	0.80 %	0.75 %	13.1 %	12.5 %
Lactose	4.85 %	4.9 %	75 %	67.4 %
Ash	0.50 %	0.80 %	7.3 %	11.8 %
Lactic acid	0.05 %	0.40 %	0.2 %	4.2 %

### 1.2.1 WHEY PRODUCTION

Whey is a by-product of cheese making. Cheese making involves a number of main stages that are common to most types of cheese. The milk is pre-treated, possibly preripened after addition of a bacteria culture appropriate to the type of cheese, and mixed with rennet.

The main task of the starter culture is to convert the lactose present in the milk to lactic acid. Development of acid lowers the pH, which is important in assisting syneresis, the contraction of the curd accompanied by elimination of whey (<http://www.food-info.net/uk/dairy/cheese-production.htm>, 6.3.2006).

The enzyme rennet catalyzes the conversion of casein in milk to para-casein by removing a glycopeptide from the soluble casein. Para-casein further coagulates, in the presence of calcium ions to form white, creamy curd, leaving the whey supernatant (<http://www.glue.umd.edu/~nsw/ench485/lab1.htm>, 6.3.2006).

During the rest of the curd-making process the bacteria grow and form lactic acid, and the curd grains are subjected to mechanical treatment with stirring tools, while at the same time the curd is heated.

Growth of bacteria, mechanical treatment and heat treatment, together results in separation of whey from the curd grains (<http://www.food-info.net/uk/dairy/cheese-production.htm>, 6.3.2006).

## **Steps in cheese/whey making :**

### **Pasteurisation**

The majority of cheeses are made from pasteurised milk to create optimum conditions for production.

Pasteurisation must be sufficient to kill bacteria capable of affecting the quality of the cheese, e.g. *coliforms*. Regular pasteurisation at 72 – 73°C for 15 – 20 seconds is most commonly applied. However, spore-forming microorganisms survive pasteurisation, e.g. *Clostridium tyrobutyricum*. More intense heat treatment would seriously impair the general properties of the milk. Therefore, certain chemicals have been added to milk. The most commonly used chemical is sodium nitrate and hydrogen peroxide.

### **Starter cultures**

Three characteristics of starter cultures are of primary importance in cheese making: ability to produce lactic acid, ability to break down the protein and, when applicable, ability to produce carbon dioxide.

Two principal types of culture are used: *mesophilic* cultures with a temperature optimum between 20 and 40°C and *thermophilic* cultures which develop at up to 45°C.

The most frequently used cultures are *mixed strain* cultures, in which two or more strains of both *mesophilic* and *thermophilic* bacteria exist in symbiosis. These cultures not only produce lactic acid but also aroma components and CO<sub>2</sub>.

*Single-strain* cultures are mainly used where the object is to develop acid and contribute to protein degradation.

### **Rennet**

All cheese manufacture depends upon formation of curd by the action of rennet or similar enzymes. The only exception is fresh type of cheese such as cottage, in which the milk is clotted mainly by lactic acid and the acid whey is the result of this production.

The active principle in rennet is an enzyme *chymosine*, the extract from the stomachs of young calves. Nowadays, there are two main types of substituents for animal rennet: coagulating enzymes from plants and coagulating enzymes from micro-organisms.

(<http://www.food-info.net/uk/dairy/cheese-production.htm>, 6.3.2006)

**Tab. 4** Other additions to milk before making the curd (modified from <http://www.food-info.net/uk/dairy/cheese-production.htm>, 6.3.2006).

Addition	Function
Calcium chloride	Sufficient coagulation
Carbon dioxide	Lower the pH Shortening of the coagulation time
Disodium phosphate	Production of low-fat cheese
Saltpetre ( $\text{KNO}_3$ or $\text{NaNO}_3$ )	Counteract the bacteria
Coloring agents carotene chlorophyll orleana	Correct the seasonal colour of the milk fat

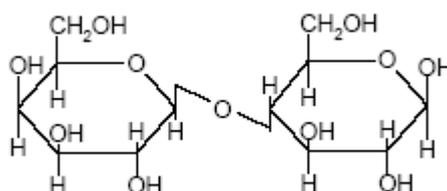


**Fig. 5** Decanting whey from the curds

([http://biology.clc.uc.edu/fankhauser/Cheese/Cheese\\_5\\_gallons/CHEESE\\_5gal\\_00.htm](http://biology.clc.uc.edu/fankhauser/Cheese/Cheese_5_gallons/CHEESE_5gal_00.htm), 3.5.2006).

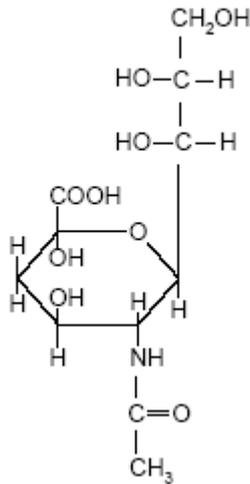
## 1.2.2 BOVINE OLIGOSACCHARIDES AND GLYCOCONJUGATES

Because whey is the watery portion of bovine milk, it is assumed that it contains bovine milk oligosaccharides (BMO) and glycoconjugates. The glycoconjugates and oligosaccharides in bovine milk have been less researched and therefore only little information is available about these molecules (Martín-Sosa et al., 2002). The fraction of the main carbohydrate compound in bovine milk, lactose, is more than 95 % of all bovine milk components and other carbohydrates are present only in traces (Nakamura et al., 2003).



**Fig. 6** Lactose, Gal(β1→4) Glc

Oligosaccharides contain between three and ten monosaccharide residues covalently linked through glycosidic bonds. They are divided into two broad classes, neutral and acidic. Neutral oligosaccharides do not contain any charged carbohydrate residues. However, acidic oligosaccharides contain one or more residues of *N*-acetylneuraminic acid (sialic acid) that is negatively charged in neutral solutions. Ten sialyl oligosaccharides and eight neutral oligosaccharides were identified in bovine milk and colostrum (Tab. 5) (Gopal and Gill, 2000). The most abundant were acidic oligosaccharides, 6-sialyllactosamine and 3-sialyllactose (Martín-Sosa et al., 2003).



**Fig. 7** *N*-acetylneuraminic acid, Neu5Ac

Quantitatively, acidic oligosaccharides are only present in bovine milk in trace amounts, as mentioned above; considerably higher concentrations are present in bovine colostrum. As milk production matures postparturition, the concentration of these oligosaccharides decline rapidly (Gopal and Gill, 2000). In spite of this, the concentration of total hexose increases (Nakamura et al., 2003).

Only a few percent of oligosaccharides are included in carbohydrate parts of glycoconjugates where the carbohydrate chains are attached covalently to the backbone of either proteins or lipids (Nakamura et al., 2003; Gopal and Gill, 2000). Bovine glycoconjugates comprise gangliosides, neutral glycolipids, glycoproteins, glycopeptides and mucins (Gopal and Gill, 2000).

**Tab. 5** Neutral and acidic oligosaccharides of bovine milk and colostrum (Gopal and Gill, 2000).

Structure	Name
<i>Neutral</i>	
Gal $\beta$ (1-3) Gal $\beta$ (1-4) Glc	3'-Galactosyl-lactose
Gal $\beta$ (1-6) Gal $\beta$ (1-4) Glc	6'-Galactosyl-lactose
Gal $\beta$ (1-4) Fuc $\alpha$ (1-3) GlcNAc	3-Fucosyl <i>N</i> -acetyl-lactosmine
Gal $\beta$ (1-4) GlcNAc	<i>N</i> -Acetyl-lactosamine
Gal NAc $\beta$ (1-4) Glc	<i>N</i> -Acetylgalactosaminy-glucose
Gal NAc $\alpha$ (1-3) Gal $\beta$ (1-4) Glc	<i>N</i> -Acetylgalctosyl-lactose
Gal $\beta$ (1-4) GlcNAc $\beta$ (1-6) Gal $\beta$ (1-3) [Gal $\beta$ (1-4) Glc]	Lacto- <i>N</i> -novopentaose
Gal $\alpha$ (1-3) Gal $\beta$ (1-4) Glc	$\alpha$ -3'-Galactosyl-lactose
<i>Acidic</i>	
NeuAc $\alpha$ (2-3) Gal $\beta$ (1-4) Glc	3-Sialyl-lactose
NeuAc $\alpha$ (2-6) Gal $\beta$ (1-4) Glc	6-Sialyl-lactose
NeuGl $\alpha$ (2-6) Gal $\beta$ (1-4) Glc	6-Glucolyneuraminy-lactose
NeuAc $\alpha$ (2-6) Gal $\beta$ (1-4) GlcNAc	6-Sialyl-lactosamine
NeuGl $\alpha$ (2-6) Gal $\beta$ (1-4) GlcNAc	6-Glucolyneuraminy-lactosamine
NeuAc $\alpha$ (2-3) Gal $\beta$ (1-3) Gal $\beta$ (1-4) Glc	3-Sialyl galactosyl-lactose
NeuAc $\alpha$ (2-8) NeuAc $\alpha$ (2-3) Gal $\beta$ (1-4) Glc	Disialyl lactose
NeuAc $\alpha$ (2-6) Gal $\beta$ (1-4) GlcNAc $\alpha$ -1-P	Sialyl-lactosamine-1-phosphate
NeuAc $\alpha$ (2-6) Gal $\beta$ (1-4) GlcNAc $\alpha$ -6-P	Sialyl-lactosamine-6-phosphate
NeuGl $\alpha$ (2-3) Gal $\beta$ (1-4) Glc	3-Glucolyneuraminy-lactose

### 1.2.3 BIOLOGICAL ACTIVITY OF OLIGOSACCHARIDES

Biological activity of oligosaccharides is due to their ability to act as competitive inhibitor or soluble receptor analogues of cell surface carbohydrates (Kunz and Rudolf, 1993; Gopal and Gill, 2000). The majority of infection diseases are initiated by adhesion of pathogenic organism to the host tissue. This adhesion is mediated by adhesins present on the surface of the infectious organism that bind to complementary glycoconjugates on the surface of the host tissue (Sharon and Ofek, 2000). Because the milk oligosaccharides are made by the same types of glycosyltransferases that are responsible for the synthesis of cell surface glycan moieties of the more complex glycoconjugates, such as glycolipids, glycoproteins, mucins and glycosaminoglycans, they could have structural homology to the cell surface glycoconjugates used as receptors by pathogens (Newburg, 2005). Milk

oligosaccharides thus act as protective components. Two types of mechanism may cause such protection. In one case, because of homology of protective milk oligosaccharide to a cell surface receptor, they may bind to a pathogen and inhibit its binding to its cell surface receptor. A different mechanism is that oligosaccharides may bind to the cell surface receptor and thereby inhibit the ability of the pathogen to affect the host cell (Newburg, 1997).

There are numerous reports about human milk oligosaccharides (HMO) and glycoconjugates that are capable to inhibit bacterial adhesion to epithelial cells (Tab. 6). HMO is acting as a soluble receptor analogues preventing especially gastrointestinal infections in infants (Kunz and Rudolff, 1993, Kunz et al., 2000). Compared with HMO, levels of oligosaccharides in bovine milk are very low. The chemical structure of the bovine milk oligosaccharides (BMO) and many of the glycoconjugates are similar to those in human milk. It is likely that BMO and glycoconjugates can be used as bioactive components (Gopal and Gill, 2000).

Neutral HMO and acidic BMO inhibited adhesion of *N. meningitidis* (Hakkarainen et al., 2005). *Campylobacter jejuni* is the most common cause of bacterial diarrhoea in infants. Binding of *Campylobacter* was inhibited by fucosylated oligosaccharides of human milk (Ruiz-Palacios et al., 2003). Enterotoxigenic *E. coli* causes diarrhoea in infants. The hemagglutination mediated by enterotoxigenic *E. coli* was strongly inhibited by HMO. The hemagglutination of uropathogenic *E. coli*, causes pyelonephritis and cystitis, was inhibited by sialylated HMO. Although BMO were less efficient at inhibition of enterotoxigenic *E. coli*, they were still quite good inhibitors of uropathogenic *E. coli* (Martín-Sosa et al., 2002). The attachment of *Haemophilus influenzae* was inhibited by human milk high-molecular-weight fraction (Andersson, 1986). The adhesion of *Helicobacter pylori* to epithelial cell was inhibited by sialyllactose (Simon et al., 1997). The glycoproteins and sialylated oligosaccharides of human milk may have inhibitory effect of adhesion of *Streptococcus pneumoniae* that causes, among other diseases, otitis media (Barthelson et al., 1998). Human milk high-molecular-weight fraction, low-molecular-weight fraction and free oligosaccharides were able to inhibit the attachment of *S. pneumoniae* (Andersson, 1986). Human milk mucin complex, a macromolecule, links with oligosaccharides, binds to *rotavirus* and thus causes its

inhibition (Shah, 2000). The oligosaccharide part of glycolipid GM1 was able to block the interaction of *Vibrio cholera* toxin (Schengrund et al., 1989).

**Tab. 6** Human milk glycoconjugates that inhibit pathogens (modified from Newburg et al., 2005; Shah, 2000).

<b>Glycoconjugate</b>	<b>Pathogen</b>
GM1 ganglioside	<i>Vibrio cholera</i>
	<i>Campylobacter jejuni</i>
GM2 ganglioside	Enteropathogenic <i>E. coli</i>
Gb3	Shiga toxin
Sulfatide	Human immunodeficiency virus
Chondroitin sulphate	Human immunodeficiency virus
Lactadherin	Rotavirus
Mannosylated glycopeptide	Enterohemorrhagic <i>E. coli</i>
Oligosaccharides	<i>Streptococcus pneumoniae</i>
	Enteropathogenic <i>E. coli</i>
	<i>Listeria monocytogenes</i>
Fucosylated oligosaccharides	<i>C. jejuni</i>
	<i>V. cholerae</i> stable toxin
Macromolecule-associated glycans	Noroviruses
	<i>Pseudomonas aeruginosa</i>
Sialyllactose	Cholera toxin
	<i>E. coli</i>
	<i>P. aeruginosa</i>
	<i>Aspergillus fumigatus</i> conidia
	Influenza virus
	Polyomavirus
	<i>Helicobacter pylori</i>
	<i>C. jejuni</i>
	<i>S. pneumoniae</i>

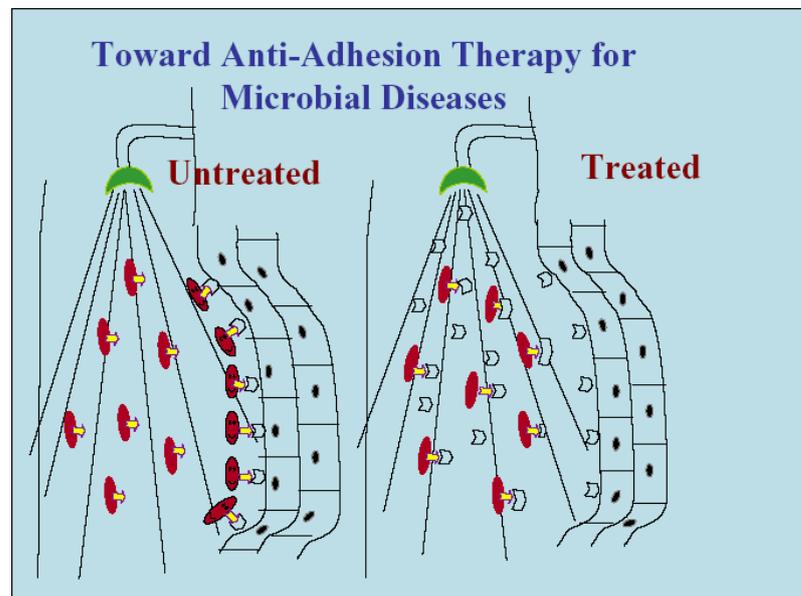
### 1.3 ANTI-ADHESION THERAPY

Anti-adhesion therapy prevents the attachment or adhesion of the bacteria to host tissue, or detach them from the tissue at the early stages of infection (Sharon and Ofek, 2000). This mechanism of inhibition is entirely distinct from that of the current antibiotics in use. The antimicrobials in use today inhibit metabolic processes of bacteria but leave the niche of the microbe intact. In contrast, anti-adhesion antimicrobials make the niche of the pathogen effectively unavailable (Newburg et al., 2005). Naturally occurring glycans (oligosaccharides and glycoconjugates) have this anti-adhesion activity and are ideal for this purpose (Sharon and Ofek, 2000; Newburg et al., 2005). Because of the alarming increase in antibiotic resistant pathogens, such approach may be mild and gentle and safer compared with present chemotherapy. Glycans are unlikely to be toxic and immunogenic and are normal constituents of cell surface or body fluids, especially of human milk. Moreover, because they are not bactericidal, selection of resistant strains is unlikely to occur, thus reducing considerably the spread of such strains in the environment (Sharon and Ofek, 2000).

However, the use of glycans as active ingredients in infant foods, or as novel antimicrobials for older children and adults is currently limited. One of these limitations is genetic differences in the expression of key cell surface glycans affecting the susceptibility of different populations to a given disease (Newburg, 2005). In addition, the presence of multiple adhesins of the pathogen is another impediment for use of glycans as anti-adhesion drugs. Therefore, more information of the specificities and properties of the bacterial adhesins and anti-adhesive components that block the adhesion are needed to overcome this problem (Ofek et al., 2003). This will allow the preparation of suitable cocktails of inhibitory glycans for the treatment of bacterial infections, instead of the single sugars in use until now (Sharon and Ofek, 2000). The low affinity of free saccharides for the adhesins is another block, which may be overcome by their attachment to polymeric carriers or presentations as dendrimers (Sharon and Ofek, 2000). Production of saccharides is still extremely costly and we are not still able to synthesize large and pure enough quantities (Sharon and Ofek, 2000; Newburg, 2005). The creation of genetically modified microbes that would produce specific saccharides in high yield

or employing the whey from dairy industry promise to lower this cost (Sharon and Ofek, 2000; Newburg, 2005). Another possibility is to develop suitable carbohydrate analogs that are more potent inhibitors of bacterial adhesion agents than the presently available saccharides (Sharon and Ofek, 2000).

This all gives more challenge for the anti-adhesion therapy. Anyhow, the anti-adhesion therapy could be used for prevention of disease but also for the treatment of infection (Ofek et al., 1996).



**Fig. 8** Mechanism of anti-adhesion therapy (<http://cranberries.de/81.0.html>, 3.5.2006).

## 2 AIMS OF THE STUDY

*In this study, I used in vitro test system, inhibition assays, to measure inhibitory activity of different fractions of bovine whey oligosaccharides against Neisseria meningitidis type IV pili.*

The goal of this study was to isolate whey oligosaccharide from bovine whey and separate them to neutral oligosaccharides (WNO) and acidic ones (WAO I and II) by analysing total hexose and sialic acid contents of fractions obtained from crude oligosaccharide fraction.

Isolated and biotin labelled type IV pili of *N. meningitidis* and different acidic and neutral fraction of gradually decreasing concentrations of whey oligosaccharides were used in vitro assays. The ability of whey oligosaccharides to prevent *N. meningitidis* bacterial adhesion was studied by preincubation with isolated oligosaccharides and meningococcal pili. The study was carried out with inhibition assays on microtiter plates with immobilized bovine thyroglobulin as reference binding glycoprotein of meningococcal pili and preincubated mixtures of pili with oligosaccharides. Inhibitory activity was determined by colorimetric reaction with streptavidin-POD conjugate with HRP activity and measuring of absorbance.

### 3 MATERIALS

#### 3.1 WHEY

Whey was obtained from Lieksan Laatuherkut oy, Lieksa, Finland.

#### 3.2 BACTERIAL STRAIN

*Neisseria meningitidis* serogroup C class I strain 8013

#### 3.3 CHEMICALS

ABTS tablets	Roche Diagnostic, Germany, 1112422
Blue Dextran	Pharmacia Fine Chemicals AB, Sweden
CuSO <sub>4</sub> .5H <sub>2</sub> O	Riedel-de Haën, Germany, 31293
D-Biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimide ester	Roche Diagnostic, Germany, 1008960
DEAE Sephadex A-50	Sigma Aldrich, Germany, 065 K 1500
Dimethylsulfoxide	Boehringer Mannheim, Germany, 1418165
Dry milk powder, fat free	Valio, Finland
Etax A, 96.1% vol-% ethanol	Primalco Rajamäki, Finland
Glycerol p.A.	Merck, Darmstad, Germany
Hepes buffer	Sigma Chemicals Co., USA
Hydrochloric acid, 36-38%	J.T.Baker B.V., Holland
α-Lactose	Sigma Chemicals Co., USA
Phenol	Riedel-de Haën, Germany, 35517
Periodic acid	Sigma Chemicals Co., USA, P-7875

Resorcinol	Sigma Chemicals Co., USA, R-5645
Sephadex G-25	Pharmacia LKB, Sweden, 17-0033-01
Sodium Chloride	J.T.Baker B.V., Holland
Streptavidin-POD conjugate	Roche Diagnostic, Germany, 1089153
Sulphuric acid, 95-97%	Riedel-de Haën, Germany, 84720 307 43
tert-Butanol	Merck, Schuchardt, Germany
TRIS Ultra Pure	ICN Biomedicals, Inc., USA, 9070E
Tween 20	Fluca Chemika, 93773

### 3.4 SOLUTIONS

ABTS substrate for HRP	5 ml concentrated substrate solution, 50 ml H <sub>2</sub> O and one ABTS tablet
BHI-solution	37.0 g brain-heart infusion in 1 liter of sterile H <sub>2</sub> O
Blocking buffer	5 % dry milk powder in 0.05 % Tween 20 in 1 x PBS, pH 7.4
Kellogg's supplement I	400 g D-glucose, 10 g L-glutamine 20 mg cocarboxylase ad 1 liter H <sub>2</sub> O
Kellogg's supplement II	1.25 g Fe(NO <sub>3</sub> ) <sub>3</sub> ·9H <sub>2</sub> O ad 250 ml H <sub>2</sub> O
0.5 M NaCl in 50mM TRIS-HCl buffer	2.922 g NaCl in 100 ml 50mM TRIS-HCl buffer, pH 8.7
10 x PBS buffer	1.36 M NaCl, 26.8 mM KCl, 123.6 mM Na <sub>2</sub> HPO <sub>4</sub> ·H <sub>2</sub> O, 14.7 mM KH <sub>2</sub> PO <sub>4</sub> , pH 7.4
50mM TRIS-HCl buffer	6.057 g TRIS Ultra Pure ad 1 liter H <sub>2</sub> O, pH 8.7
Washing buffer	0.05 % Tween 20 in 1 x PBS, pH 7.4

### 3.5 GLYCOPROTEINS

Albumin, bovine serum	Sigma Chemical Co., USA, A-4161
Asialofetuin, fetal calf serum	Sigma Chemical Co., USA, A-4781
Ovalbumin: Albumin, chicken egg	Sigma Chemical Co., USA, A-5503
Thyroglobulin, bovine	Sigma Chemical Co., USA, T-1001

### 3.6 EQUIPMENT

AECH column	Varian bound elut reservoir, 2 x 6 cm
Agitator	Vortex-2 genie, Scientific Industrie, USA
Centrifugal filter device	Amicon Ultra Cut-off 100kDa, Millipore, USA
Centrifuges	Superspeed centrifuge Sorvall RC-5B Sorvall TC-6
Cuvettes	10 x 4 x 45 mm, Sarstedt, Germany
Fraction collector	7000 Ultrorac, LKB Broma, Sweden RediFrac, Amersham Biosciences AB, USA
Freeze dryer (lyophilizer)	ModulyoD, ThermoSavant, USA
Gel chromatographic column	1.6 x 102 cm
Microtiter plates	Falcon flexible plate, Becton Dickinson Labware, USA
Microtiter plate measuring device	Victor <sup>2</sup> multilabel counter, Wallac, Finland
pH meter	ORION 420A, USA
Pump	Econo pump EP-1, Bio-Rad, USA Pump P-1, Pharmacia Biotech AB, USA
Rotavapor	Büchi rotavapor R-114, Switzerland Vacuumbrand CVC <sup>II</sup> MD 4C, Germany Büchi water bath B-480, Switzerland
Spectrophotometer	Hitachi U-1100
Water bath	Memmert, Germany Eppendorf Thermomixer 5436

## **4 METHODS**

### **4.1 ISOLATION OF OLIGOSACCHARIDES**

The method for isolation of oligosaccharides from whey was modified from Kobata's method (Kobata, 1972). 250 ml of whey was defatted by centrifugation at 3 800 rpm at +4°C for 15 minutes. Solidified lipids were removed by filtration through a glass wool. Ethanol (96.1 vol-%) was added to the filtrate to a final concentration of 68 % and left overnight at +4°C to precipitated the most of proteins and lactose. Precipitate was removed by centrifugation at 15 000 rpm at 0°C for 15 minutes and washed twice with 50 ml of 67 % ethanol at 0°C. The supernatant solutions and washings were combined and concentrated under reduced pressure by rotary evaporation. The residue was dissolved in 10 ml of H<sub>2</sub>O and insoluble material was removed by centrifugation at 3 800 rpm at +4°C for 15 minutes. The supernatant was divided into 5 ml aliquots and stored at -20°C.

### **4.2 GEL CHROMATOGRAPHY AND FRACTIONATION**

The method for gel chromatography of oligosaccharides was modified from Kobata's method (Kobata, 1972). Chromatography was performed on a column (102 x 1.6 cm) of Sephadex G-25 that was washed with H<sub>2</sub>O overnight. The crude oligosaccharide solution was loaded onto the column and eluted with H<sub>2</sub>O. After the void volume (73 ml) that was determined by using Blue Dextran solution passed through, the effluent was collected in 3.5 ml fractions. Flow rate of eluent was 0.7 ml/min.

### **4.3 ANALYSIS OF TOTAL HEXOSE AND SIALIC ACIDS**

#### **4.3.1 TOTAL HEXOSE**

Phenol-sulphuric method for content of total hexose was performed by the method of Kobata (Kobata, 1972). Aliquots (25 µl) from every odd fraction was diluted with 100 µl H<sub>2</sub>O and treated with 100 µl of 5 % aqueous phenol and 500 µl of concentrated sulphuric acid.

The tubes were incubated in water bath at 37°C for 30 minutes. The absorbances were measured at 490 nm using spectrophotometer.

#### **4.3.2 SIALIC ACIDS**

Periodate-resorcinol method for assay of sialic acids was performed by the method of Jourdian et al. (1971). Aliquot (250 µl) from every odd fraction was taken and 50 µl of 0.04 M periodic acid solution was added. The tubes were mixed and incubated on ice for 20 minutes. 625 µl of the 0.6 % resorcinol reagent (6 % resorcinol reagent stock diluted 1:10) was added, mixed and incubated on ice for 5 minutes. After this tubes were heated to +95°C and incubated for 15 minutes, cooled in tap water, and 625 µl of tert-butyl alcohol was added.

The tubes were placed in a 37°C water bath for 3 minutes to stabilize color. The absorbances were measured at 630 nm using spectrophotometer.

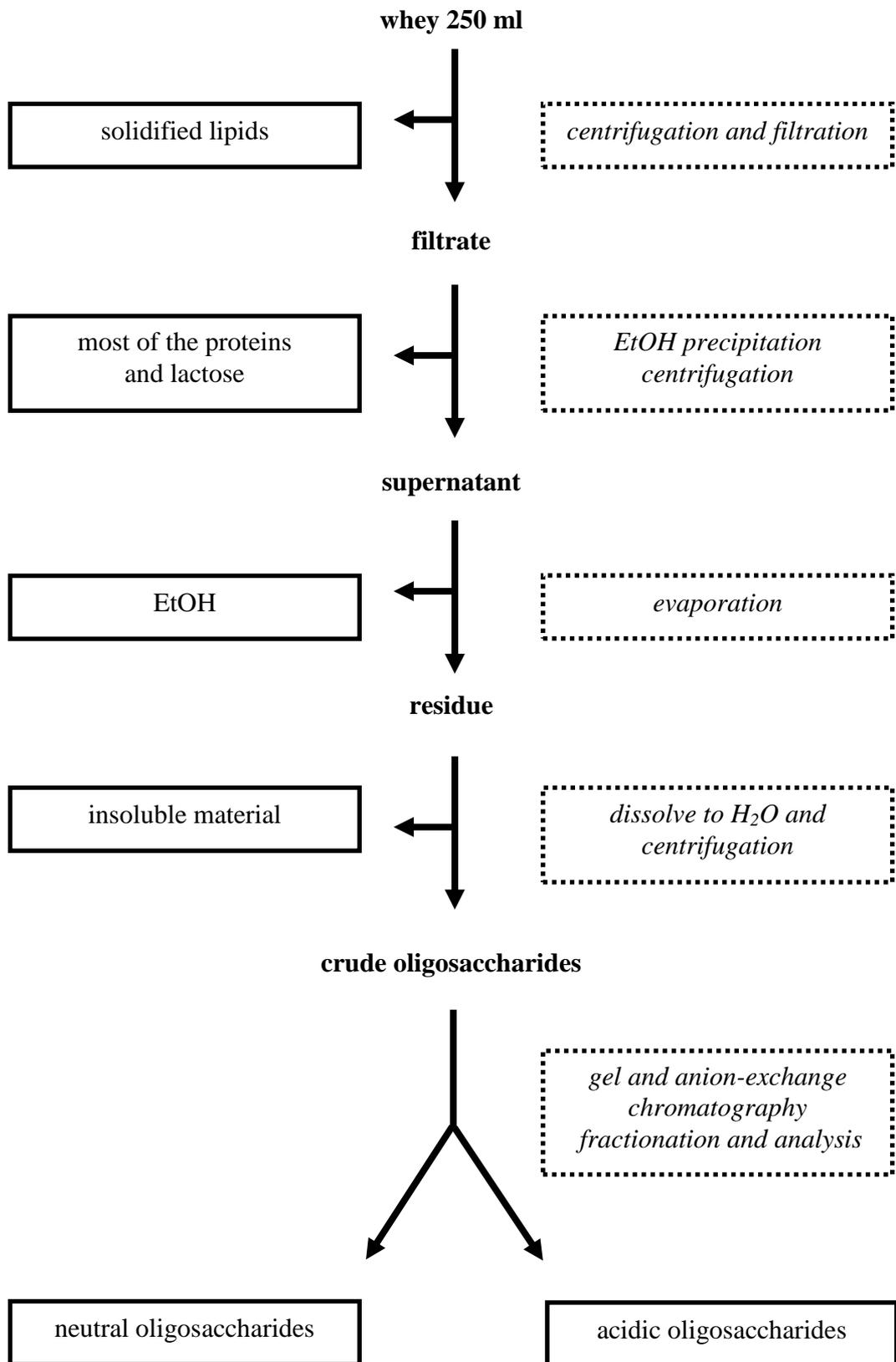
After the analysis of total hexose and sialic acids, the oligosaccharide eluent was pooled into the 3 fractions and lyophilized. The whey oligosaccharides fraction were acidic (WAO I, WAO II) and neutral (WNO) oligosaccharides.

#### **4.4 ANION EXCHANGE CHROMATOGRAPHY (AECH) OF WNO**

The fraction of WNO, which was positive by the periodate-resorcinol method, as well as with the phenol-sulphuric acid method, was later subjected to anion exchange chromatography. The method was modified from Urashima et al. (2004). 500 mg of WNO fraction was dissolved in 2 ml of 50 mM TRIS-HCl (pH 8.7) buffer and loaded onto the column of DEAE-Sephadex A-50 (2 x 6 cm) equilibrated with the same buffer. The unabsorbed components were eluted with 150 ml of the buffer and the adsorbed components were then eluted with 200 ml of linear gradient from 0 to 0.5 M NaCl in the buffer. Elution was done at flow rate of 25 ml/hour and 5 ml fractions were collected. Aliquot (50  $\mu$ l) of each fraction was analysed using phenol-sulphuric acid method. Peak fractions were pooled and lyophilized.

The neutral peak fraction was dissolved in 15 ml of H<sub>2</sub>O and passed through a Sephadex G-25 column (102 x 1.6 cm) to remove the salt under the same condition as those described previously. An aliquot of each fraction was analysed for content of total hexose and sialic acids as described above. Peak fractions were pooled in to WNO<sub>I</sub>, WNO<sub>II</sub> and lyophilized.

#### 4.5 DIAGRAM OF ISOLATION OF OLIGOSACCHARIDES



#### **4.6 CULTIVATION OF *NEISSERIA MENINGITIDIS***

Strain 8013 *N. meningitidis* serogroup C class 1 was cultivated on GC medium plates containing Kellogg's supplement I and II. Bacterium stock (code: 2C4.3W) was stored at -80°C and transferred to -20°C before cultivation. The bacterial stuff was spread under laminar flow to the plates that were prewarmed for 2 hours at room temperature. Plates were incubated in anaerobic conditions at +37°C in 5 % CO<sub>2</sub> atmosphere for 18 hours.

#### **4.7 ISOLATION OF PILI**

Isolation of pili was carried out at 0°C under laminar flow. Hepes buffer 1 mol/l was diluted with sterile water 1:100 to get 10 mmol/l. Harvested bacterial colonies of five plates were suspended in 20 ml of sterile Hepes buffer solution (10 mmol/l). The tubes were vortexed vigorously for exactly 30 seconds and then centrifuged at 8 000 rpm at +4°C for 20 minutes (Sorvall RC-5B). An aliquot of 16 ml of supernatant was loaded onto the 100 kD cut-off filter device and centrifuged at 2 300 rpm (Sorvall TC) at +4°C to a volume of 1 ml. Concentrated solution was washed 2 times with 15 ml of Hepes buffer (10 mmol/l) as above. Isolated pili were stored at +4°C.

#### **4.8 BIOTIN LABELLING OF PILI**

2.2 mg of D-biotinoyl- $\epsilon$ -aminocaproic acid-N-hydroxysuccinimide ester was dissolved in 110  $\mu$ l of dimethylsulfoxide (DMSO). Isolated pili were diluted 1:4 with 1 x phosphate-buffered saline (1 x PBS: 10 x PBS diluted 1:10) to get 1 ml solution. An aliquot of 50  $\mu$ l of biotinylation reagent was mixed with 1 ml of diluted pili and incubated for 2 hours with gently mixing on the rocking platform at room temperature. After incubation the mixture was loaded onto the 100 kD cut-off centrifugal filter device, filled with 1 x PBS and centrifuged at 2 300 rpm (Sorvall TC) at +4°C to a volume of 1 ml. Concentrated solution was washed 3 times with PBS as above. Biotin labelled pili were stored at +4°C.

## 4.9 INHIBITION AND BINDING ASSAYS

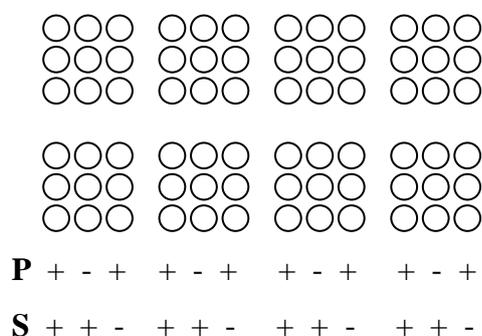
### 4.9.1 INHIBITION ASSAY WITH GRADUALLY DECREASING OLIGOSACCHARIDE CONTENT

#### Assay consists of following steps:

- 1) Immobilization of glycoprotein to a microtiter plate
- 2) Blocking of additional binding sites by blocking buffer
- 3) Addition of reaction mixture (pili-oligosaccharides)
- 4) Addition of streptavidin-POD conjugate (streptavidin binds strongly to biotinylated pili)
- 5) Addition of ABTS substrate (enzyme reaction and development of colour) and measurement of absorbance

Thyroglobulin was diluted with 1 x PBS to get concentration 0.1 mg/ml. An aliquot of 100 µl of thyroglobulin (0.1mg/ml) per well was immobilized to polyvinylchloride microtiter plate by incubation overnight at +4°C. After incubation the wells were washed five times with washing buffer and dried. Additional binding sites were blocked by 250 µl of blocking buffer per well and incubated at room temperature for 60 minutes. The wells were washed five times with washing buffer and dried. Inhibition of binding of *N.meningitidis* was performed by preincubation of biotin labelled pili with different concentrations of whey oligosaccharides. The lyophilized fractions of WNO, WAO I and WAO II were dissolved in 1 x PBS to get solution of 40 mg/ml. Following dilution series were made by dilution of an aliquot of this solution 1:1 with 1 x PBS. The concentration of oligosaccharides were 40; 20; 10; 5; 2.5; 1.25; 0.625; 0.3125; 0.156; 0.078; 0.039 and 0.020 mg/ml. Biotin labelled pili were diluted 1:4 with 1 x PBS. Diluted pili were mixed with different oligosaccharides concentration of different fraction 1:1 and preincubated for 60 minutes with gently mixing on the rocking platform at room temperature. Pili diluted 1:8 with 1 x PBS were incubated at the same condition as mixture of pili-oligosaccharide and used as control. Preincubated solutions were pipetted to microtiter plate 100 µl per well according to pipetting scheme (Fig. 9) and incubated

for 60 minutes at room temperature. 1 x PBS was used as control. The wells were washed five times with washing buffer and dried. Streptavidin-POD conjugate was diluted 1:4 000 with blocking buffer and pipetted 100 µl per well according to pipetting scheme (Fig. 9) and incubated for 60 minutes at room temperature. Blocking buffer was used as a control. The wells were washed five times with washing buffer and dried. An aliquot of 100 µl of ABTS-substrate was added per well and absorbances were measured at 405 nm (Victor<sup>2</sup> multilable counter) at duplicate.



**Fig. 9** Pipetting scheme of microtiter plate of inhibition assay. Each 9 wells are meant for different concentration of oligosaccharides and control pili.

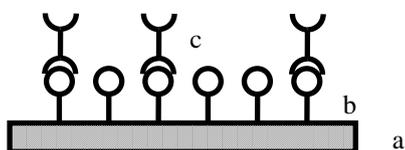
P + means wells containing pili

S + means wells containing streptavidin-PODconjugate

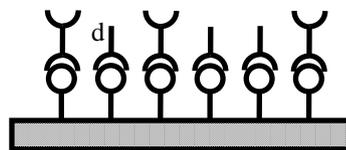
P - means control wells with 1 x PBS

S - means control wells with blocking buffer

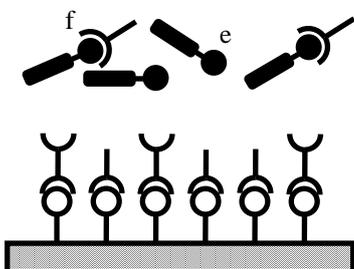
## 4.9.2 DIAGRAM OF INHIBITION ASSAY



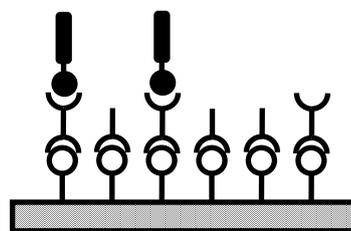
**Fig. A** Immobilization of glycoprotein to a microtiter plate.



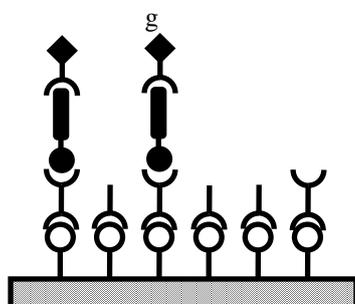
**Fig. B** Blocking of additional binding sites by blocking buffer.



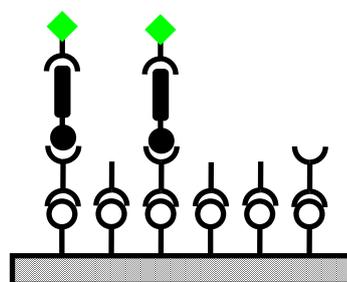
**Fig. C** Addition of reaction mixture (pili-oligosaccharides).



**Fig. D** Binding of non-inhibited pili.



**Fig. E** Binding of streptavidin-POD conjugate to biotinylated pili.



**Fig. F** Enzyme reaction and development of colour by addition of ABTS substrate.

### Legend:

a – PVC microtiter plate  
 b – binding sites of MP  
 c – glycoprotein  
 d – blocking buffer

e - pili  
 f – pili inhibited by oligosaccharides  
 g – streptavidin-POD conjugate

### **4.9.3 INHIBITION ASSAY WITH WASHED AND NON-WASHED WAO II**

Thyroglobulin was diluted with 1 x PBS to get concentration 0.1 mg/ml. An aliquot of 100 µl of thyroglobulin (0.1mg/ml) per well was immobilized to polyvinylchloride microtiter plate by incubation overnight at +4°C. After incubation the wells were washed five times with washing buffer and dried. Additional binding sites were blocked by 250 µl of blocking buffer per well and incubated at room temperature for 60 minutes. The wells were washed five times with washing buffer and dried. Biotin labelled pili were diluted 1:4 with 1 x PBS. Lyophilized WAO II were dissolved in 1 x PBS to get concentration 10 mg/ml. Diluted pili and oligosaccharide solution were mixed 1:1 at duplicate and preincubated for 60 minutes with gently mixing on the rocking platform at room temperature. Pili diluted 1:8 with 1 x PBS were incubated at the same condition as mixture of pili-oligosaccharide and used as control. After incubation one of the mixtures was loaded onto the 100 kD cut-off centrifugal filter device, filled with 1 x PBS and centrifuged at 2 300 rpm (Sorvall TC) at +4°C to as small volume as possible. Concentrated solution was washed 1 time more with 1 x PBS as above. Blocking buffer was added after washing to get the same volume as before centrifugation and washing. Washed, non-washed and control pili were pipetted to microtiter plate 100 µl per well according to pipetting scheme and incubated for 60 minutes at room temperature. Blocking buffer was used as control. The wells were washed five times with washing buffer and dried. Streptavidin-POD conjugate was diluted 1:4 000 with blocking buffer and pipetted 100 µl per well according to pipetting scheme and incubated for 60 minutes at room temperature. Blocking buffer was again used as a control. The wells were washed five times with washing buffer and dried. An aliquot of 100 µl of ABTS-substrate was added per well and absorbances were measured at 405 nm (Victor<sup>2</sup> multilable counter) at duplicate.

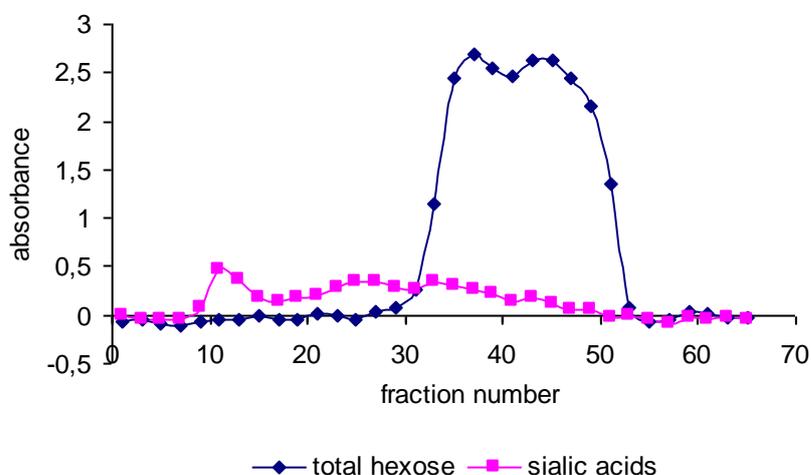
#### **4.9.4 BINDING ASSAY TO REFERENCE GLYCOPROTEINS AND BLOCKING BUFFER**

Binding assay was used for investigating of binding of pili to bovine albumin, asialofetuin, ovalbumin, thyroglobulin and blocking buffer. Each glycoprotein was dissolved in 1 x PBS to get final concentration 0.1 mg/ml. An aliquot of 100 µl of bovine albumin, asialofetuin, ovalbumin, thyroglobulin (0.1mg/ml) and blocking buffer per well was immobilized to polyvinylchloride microtiter plate by incubation overnight at +4°C. After incubation the wells were washed five times with washing buffer and dried. Additional binding sites were blocked by 250 µl of blocking buffer per well and incubated at room temperature for 60 minutes. The wells were washed five times with washing buffer and dried. Binding of *N. meningitidis* was performed by biotin labelled pili diluted 1:4 with 1 x PBS. Diluted pili were pipetted to microtiter plate 100 µl per well according to pipetting scheme and incubated for 60 minutes at room temperature. 1 x PBS was used as a control. The wells were washed five times with washing buffer and dried. Streptavidin-POD conjugate was diluted 1:4 000 with blocking buffer and pipetted 100 µl per well according to pipetting scheme and incubated for 60 minutes at room temperature. As control was used blocking buffer. The wells were washed five times with washing buffer and dried. An aliquot of 100 µl of ABTS-substrate was added per well and absorbances were measured at 405 nm (Victor<sup>2</sup> multilable counter) at duplicate.

## 5 RESULTS

### 5.1 GEL CHROMATOGRAPHY

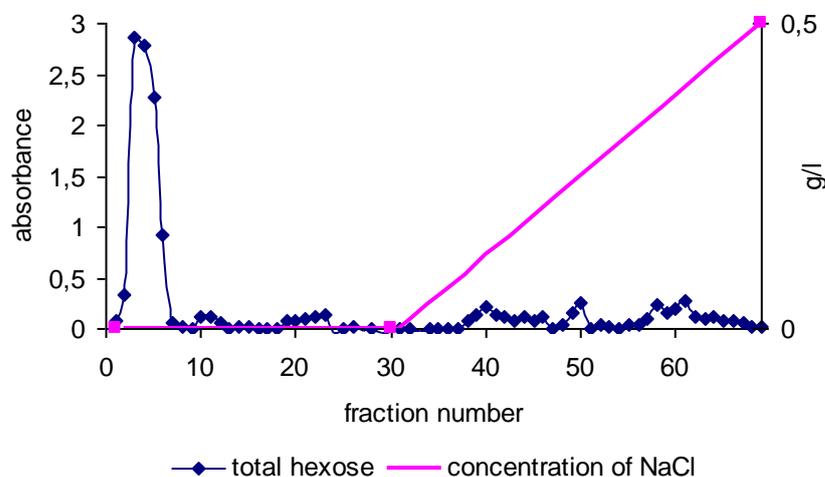
The crude whey bovine oligosaccharides were filtrated on a Sephadex G-25 column. Collected fraction were analysed for total hexose using the phenol-sulphuric acid method and for sialic acid with the periodate-resorcinol method (Fig. 10). The determination was made from an aliquot of every odd fraction. The sialic acid fractions were eluted immediately after the void volume. Sialic oligosaccharides were eluted mainly into two peak fractions, from tube 7 to 17 (WAO I) and from tube 18 to 31 (WAOII). The total content of neutral oligosaccharides (WNO) was eluted in fraction 32 to 53.



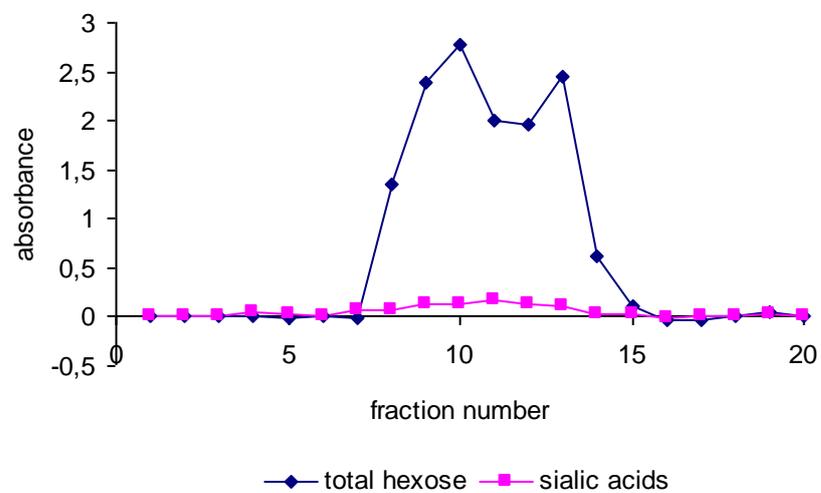
**Fig. 10** Gel chromatography of bovine whey oligosaccharide on Sephadex G-25 (1.6 x 102 cm). Elution was done with water at flow rate 0.7 ml/min and fractions of 3.5 ml were collected. An aliquot of every odd fraction was analysed for total hexose with phenol-sulphuric acid method at 490 nm and for sialic acids with periodate-resorcinol method at 630 nm. The fractions were pooled into three fractions WAO I (7–17), WAO II (18–31) and WNO (32–53).

## 5.2 ANION EXCHANGE CHROMATOGRAPHY OF WNO

A part of WNO fraction, containing both neutral and acidic oligosaccharides, was further subjected to AECH with DEAE Sephadex A-50 and separated into several peak fractions by the analysis of each fraction for total hexose with the phenol-sulphuric acid method. The peak fraction designated as  $WNO_{AE}$  was assumed to be neutrally charged and the other fractions designated as  $WAO_{AE\ I-V}$  were assumed to be negatively charged as they eluted at retarded positions relative to the neutral oligosaccharides (Fig. 11).  $WNO_{AE}$  fraction was further more purified by gel filtration on Sephadex G-25 column. Every fraction was analysed for total hexose and similarly for sialic acid to detect whether they were removed completely (Fig. 12). However, presence of infinitesimal amount of sialic oligosaccharides shows AECH was not thorough. It could be caused either by exceeding the available capacity of DEAE Sephadex A-50 or by pH of TRIS-HCl buffer.



**Fig. 11** Anion exchange chromatography of bovine whey neutral oligosaccharide on DEAE Sephadex A-50 (2 x 6 cm). Column was equilibrated with 50 mM TRIS-HCl buffer (pH 8.7). The unadsorbed components were eluted with 150 ml of the buffer and adsorbed components were then eluted with linear gradient of 0-0.5 M NaCl in the buffer. Elution was done at flow rate of 25 ml/hour and fractions of 5 ml were collected. An aliquot of every odd fraction was analysed for total hexose with the phenol-sulphuric acid method at 490 nm. The fractions were pooled into  $WNO_{AE}$  (1-7) and  $WAO_{AE\ I-V}$  (up from 10).



**Fig. 12** Gel chromatography of bovine whey neutral oligosaccharide (WNO<sub>AE</sub>) on Sephadex G-25 (1.6 x 102 cm). Elution was done with water at flow rate 0.7 ml/min and fractions of 3.5 ml were collected. An aliquot of every fraction was analysed for total hexose with phenol-sulphuric acid method at 490 nm and for sialic acids with periodate-resorcinol method at 630 nm. The fractions were pooled into WNO<sub>I</sub> (7-12) and WNO<sub>II</sub> (13-16).

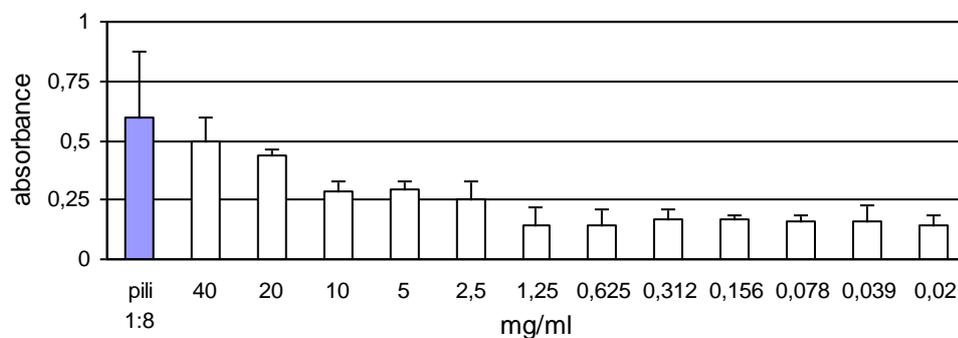
## 5.3 INHIBITION AND BINDING ASSAYS

### 5.3.1 INHIBITION ASSAY WITH GRADUALLY DECREASING OLIGOSACCHARIDE CONTENT

#### Inhibition with WNO

The binding of pili to thyroglobulin was inhibited by a series of 12 decreasing concentrations of WNO. The results represent binding of pili after inhibition and are compared to the binding of pili not inhibited by WNO.

Fig. 13 represents the data of carried assays and shows the trend that with decreasing concentration of oligosaccharides the binding of pili after their inhibition also decrease. The downfall trend of binding of pili was clear at concentrations from 40 to 2.5 mg/ml. At the concentration of 1.25 mg/ml the binding reached its minimum. Further decreasing of content of WNO did not have any efficacy on the binding.

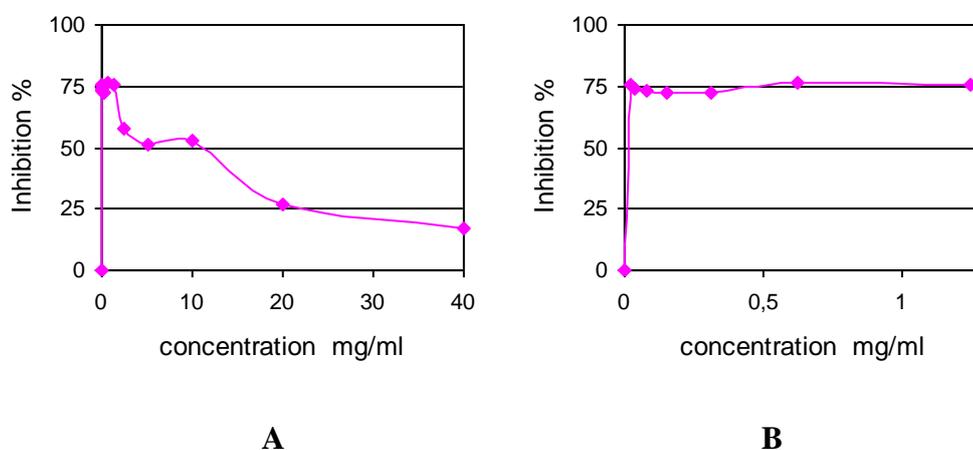


**Fig. 13** Inhibition of binding of meningococcal pili to thyroglobulin with decreasing concentration of WNO. The columns represent the means and bars indicate SD, n=6.

The percent inhibition of binding of pili to thyroglobulin with gradually decreasing oligosaccharide content was calculated by the following equation:

$$\left(1 - \frac{\text{inhibition of binding of pili (average of triplicate A405)}}{\text{binding of pili (average of triplicate A405)}}\right) \times 100 \%$$

Fig. 14 presents the data as percent of inhibition of binding of pili to thyroglobulin. WNO produced about 75 % inhibition at the concentration from 0.02 to 1.25 mg/ml. This plateau phase is more distinctly displayed in the Fig. 14/B, which shows an expanded part of plot A in the concentration range of 0.02-1.25 mg/ml. At the concentration higher than 1.25 mg/ml inhibition decreased nearly to 50 % and only 25 % inhibition was reached at the concentration of 20 mg/ml.

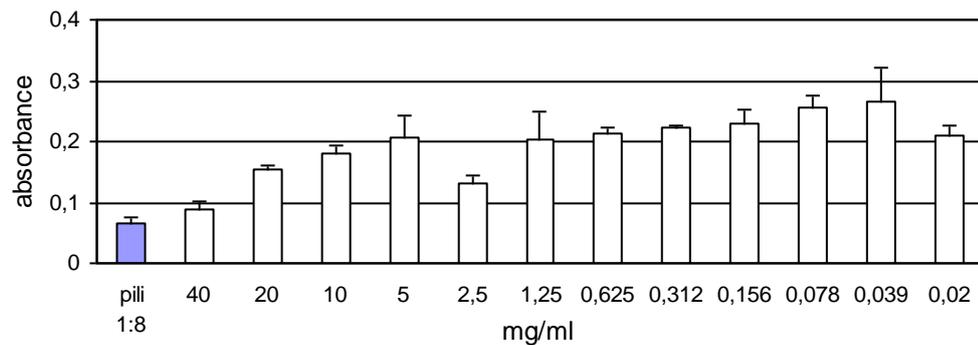


**Fig. 14** Inhibition of binding of meningococcal pili to thyroglobulin with decreasing concentration of WNO. Data are meant as percent inhibition of binding of pili to thyroglobulin, n=6. Fig. A represents decreasing trend of inhibition with increasing concentration of WNO. Fig. B represents plateau at concentration from 0.02 to 1.25 mg/ml.

## Inhibition with WAO I

The binding of pili to thyroglobulin was inhibited by a series of 12 decreasing concentrations of WAO I. The results represent the binding of pili after inhibition and are compared to the binding of pili not inhibited by WAO I.

WAO I produced no inhibition of binding and even the binding after inhibition with oligosaccharides was higher with lower concentrations (Fig. 15). At the concentration of 2.5 mg/ml there was fall of binding but it was still higher than binding of control pili. The binding of control pili, which were not inhibited by WAO I, to thyroglobulin, was very low.

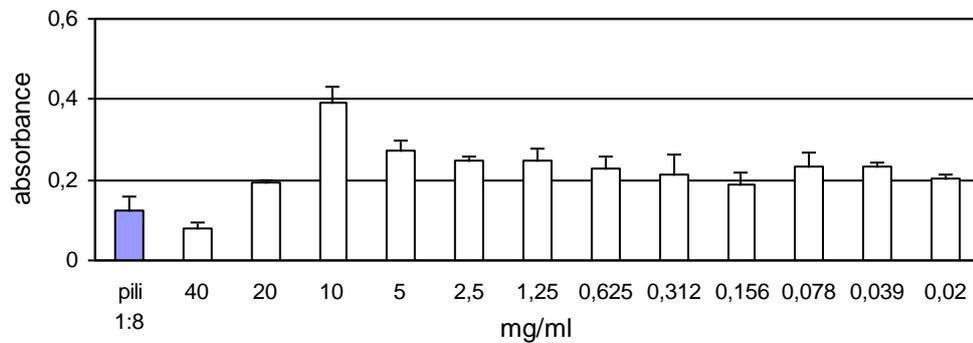


**Fig. 15** Inhibition of binding of meningococcal pili to thyroglobulin with decreasing concentration of WAO I. The columns represent the means and bars indicate SD, n=3.

## Inhibition with WAO II

The binding of pili to thyroglobulin was inhibited by a series of 12 decreasing concentrations of WAO II. The results represent the binding of pili after inhibition and are compared to the binding of pili not inhibited by WAO II.

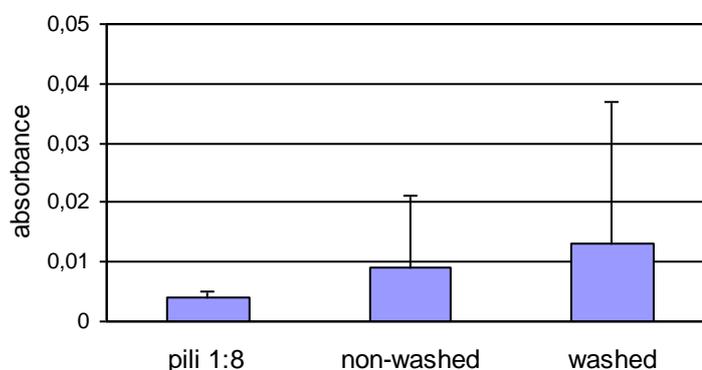
WAO II produced no inhibition of binding and even the binding after inhibition was higher except at the concentration of 40 mg/ml (Fig. 16). The highest binding was at the concentration of 10 mg/ml. The binding of control pili, which were not inhibited by WAO II, to thyroglobulin, was very low.



**Fig. 16** Inhibition of binding of meningococcal pili to thyroglobulin with decreasing concentration of WAO II. The columns represent the means and bars indicate SD, n=3.

### 5.3.2 INHIBITION ASSAY WITH WASHED AND NON-WASHED WAO II

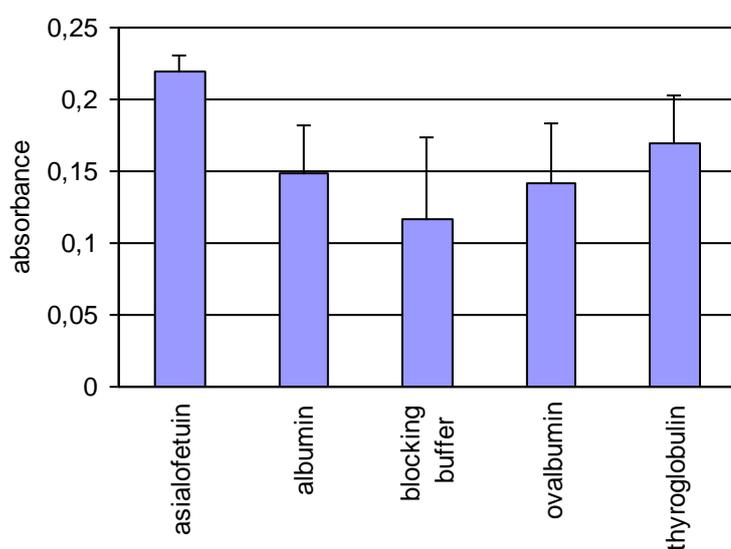
WAO increased the binding of pili to thyroglobulin. The following assay was used to investigate whether the oligosaccharide present in the preincubation mixture could anyhow induce the binding of pili to thyroglobulin. The inhibition was performed with WAO II at the concentration of 10 mg/ml because the binding of pili after inhibition at this concentration was the highest. After the preincubation one of the mixtures was washed to eliminate free oligosaccharides that did not inhibit pili. The efficacy of washing and non-washing on the binding was compared with control pili. After the background reduction, the results show there was almost any binding of pili to thyroglobulin. Washed or non-washed preincubation mixture did not have any significant effect and the variation of the results was high (Fig. 17).



**Fig. 17** Inhibition of binding of meningococcal pili to thyroglobulin with WAO II at concentration of 10 mg/ml considering the influence of washing on binding. The columns represent the means and bars indicate SD, n=3.

### 5.3.3 BINDING ASSAY TO REFERENCE GLYCOPROTEINS AND BLOCKING BUFFER

The binding of pili to different reference glycoproteins and to blocking buffer was also investigated because of low binding of control pili to thyroglobulin in the inhibition assays of WAO. The strongest binding of pili assigned asialofetuin (Fig. 18). Second best binding was observed with thyroglobulin. The ability of binding to bovine albumin and ovalbumin was almost on the same level. The lowest binding was observed to blocking buffer.



**Fig. 18** Binding of meningococcal pili to reference glycoproteins and blocking buffer. The columns represent the means and bars indicate SD, n=3.

## 6 DISCUSSION

In the present study, the anti-adhesion activity of bovine whey oligosaccharides against *N. meningitidis* was investigated. An inhibition assay based on attachment of isolated meningococcal pili to thyroglobulin after their inhibition by bovine whey oligosaccharides was used to study isolated WNO and WAO.

The rationale for this test system is both the important role of type IV pili in colonization and infection by encapsulated *N. meningitidis* strains and structural homology of oligosaccharides to the cell glycoconjugates used as a receptors by pathogens (Hakkarainen et al., 2005; Newburg, 2005). Thyroglobulin was used as a reference glycoprotein based on previous binding studies with meningococcal pili to reference glycoproteins (Hakkarainen et al., 2005).

It has been reported that bovine milk oligosaccharides (BMO) inhibit adhesion of *N. meningitidis*. The binding of neisserial pili to thyroglobulin was strongly inhibited by acidic BMO whereas the inhibition by the neutral BMO was not so pronounced. Inhibition was distinctly dose-dependent and with decreasing content of oligosaccharides the inhibition was lower (Hakkarainen et al., 2005). Therefore, it was expected the oligosaccharides derived from bovine whey would have had the same ability.

However, the inhibition activity of WNO had a different tendency than that of BMO. Interestingly, with decreasing content of oligosaccharide the inhibition increased to specific concentration and after this persisted on the same level. High concentration of carbohydrates having no inhibitory effect, present in major, may mask the activity of carbohydrates or components with inhibition effect, which may be present in minor. Gradual decreasing of content of WNO may enable the bioactive components with inhibition effect express their activity. The plateau effect at concentration of 0.02-1.25 mg/ml could be due to the very specific structure of these bioactives and their high anti-adhesion tendency even at minimal concentration.

WAO I and WAO II demonstrated no inhibition. An important fact of both assays was very low binding of neisserial pili, especially those, which were used for generating the anti-adhesion activity. These assays were not repeated because of low yields of acidic oligosaccharides from isolation.

Assay focused on the examination of increasing binding ability of WAO did not give any results because the isolated pili did not have any binding activity in these experiments. This effect could be due to the low binding ability of used pili or dry milk powder, which may prevent attachment of pili to thyroglobulin. Blocking buffer was prepared from dry milk powder and its components are known to bind to pili to some extent (Hakkarainen et al., 2005) and it was also shown in present study (Fig. 18).

Assay focused on the binding ability of used pili revealed various binding effect. Neisserial pili bound well to thyroglobulin and asialofetuin. The binding to bovine albumin, ovalbumin and blocking buffer had slightly lower efficacy. These differences may be a consequence of different glycosylation of glycoproteins. Furthermore, the low binding ability of used neisserial pili could be also caused due to the pilin antigenic variation or mutation. The tip-located PilC1 and pilin subunit proteins (PilE) are two potential adhesins of *N. meningitidis* (Nassif et al., 1994; Rudel et al, 1995). Mutation of the pilC1 gene results in a pili unable to adhere to receptors whether the strain is producing high or low PilE (Merz and So, 2000; Nassif and So, 1995). At present, however, we do not know whether pilus binding to glycoconjugates involves PilC1 or PilE (Hakkarainen et al., 2005).

Acquired results do not give any definite conclusion about anti-adhesion activity of BWO, furthermore they have not confirmed the suggestion that BWO would have had the same anti-adhesion potential as BMO. This striking difference between BWO and BMO may be due to their sources.

Investigated oligosaccharides were derived from acidic whey (pH 4.2). Before the whey is obtained, milk is treated with heating, starter bacterial cultures, rennet and other additions. These all may anyhow influence the composition and properties of whey and its components. For example heat coagulation of whey proteins during the separation process might lead to partial hydrolysis of glycoconjugates, liberation and degradation of glycosidic residues (Li and Mine, 2004). Also the lowering of pH may lead to degradation or neutralization of bioactive components. On the other side, some bioactive components could be produced during whey making, e.g glycomacropeptide. Paradoxically, this glycomacropeptide could be destruct during its isolation from whey, especially its sialic sugar chain (Li and Mine, 2004). Thus, the method used for isolation of oligosaccharides may also affect character and quantity of obtained components. Furthermore, purification and analysis of

individual components of BWO is also necessary, because derived fractions may not only contain oligosaccharides.

The results of this work give a reason for more studies about biological activity of bovine whey oligosaccharides and their glycoconjugates. Further research should target on isolation, purification and analysis of BWO and glycoconjugates, and likewise more inhibition assays should be performed to assign their anti-adhesion activity. It may be also reasonable to examine whey from different dairy companies because of variation in whey production and different sources of milk.

## **7 CONCLUSION**

This study shows the inhibition of binding of neisserial pili with the bovine whey oligosaccharides is not as clear as that with BMO. The effect of WNO at low concentration seem to be promising; however, more complex studies are necessary for investigating biological activity of oligosaccharides and glycoconjugates derived from bovine whey before it could be utilized as a cheap source of anti-infective compounds for anti-adhesion therapy.

## RESUME

*Neisseria meningitidis*, je lidský patogen způsobující život ohrožující onemocnění. Tato onemocnění jsou velice závažná a mohou být smrtelná již za několika hodin po manifestaci prvních příznaků. Proto je nezbytně nutná rychlá diagnóza a okamžité nasazení antibiotik.

Avšak díky zvýšenému výskytu rezistentních bakteriálních kmenů se stává léčba i prevence velmi obtížná, a jsou tedy hledány nové způsoby terapie.

Jedním z nových přístupů může být antiadhesivní terapie využívající glykanů, které zabraňují adhezi bakterií na hostitelské tkáně. Oligosacharidy a glykokonjugáty izolované z kravského a mateřského mléka prokázaly antiadhesivní účinky proti mnoho patogenům, stejně tak i proti *N. meningitidis*. Tyto komponenty by mohly být tedy využity jako bioaktivní přísada do potravin, a snižovat tak incidenci bakteriálních onemocnění.

Toto využití je však velmi ekonomicky náročné, a jsou tedy hledány jiné zdroje, které by mohly snížit náklady. Takový zdroj představuje syrovátka vznikající jako odpadní produkt při výrobě sýrů.

Cílem této práce tedy bylo izolovat oligosacharidy z kravské syrovátky, separovat je na neutrální a acidické a otestovat jejich antiadhesivní účinky.

Pro separaci izolovaných oligosacharidů bylo užito gelové a iontově výměnné chromatografie. Schopnost oligosacharidů zabránit adhezi *N. meningitidis* byla studována inhibičním testem využívajícího preinkubaci izolovaných a biotinem značených neisserialních pili s izolovanými oligosacharidy, jejichž koncentrace byla postupně snižována.

Testování bylo prováděno na mikrotitračních destičkách s imobilizovaným thyroglobulinem jako referenčním vazebným glykoproteinem neisserialních pili a preinkubovanou směsí oligosacharidů s pili. Inhibiční aktivita byla určena enzymatickou reakcí se streptavidin-POD konjugátem a měřením absorbance.

Bylo předpokládáno, že oligosacharidy izolované ze syrovátky budou mít stejné účinky jako oligosacharidy kravského mléka. Acidické oligosacharidy však neprokázaly žádnou inhibici. Neopomenutelným faktem při jejich testování byla

malá vazebná aktivita použitých pili. Neutrální oligosacharidy vykazovaly inhibici pouze při nízkých koncentracích.

Tento rozdíl v antiadhesivní aktivitě od oligosacharidů izolovaných z mléka může být způsobem jejich původem, tedy syrovátkou. Předtím, než je z mléka separována syrovátka, je ošetřeno varem, bakteriálními kulturami, syřidlem a jinými přísadami. To vše může ovlivnit vlastnosti a složení komponent syrovátky, což se může odrazit i v jejich biologické aktivitě.

Výsledky této práce nedávají jednoznačné závěry o antiadhesivní aktivitě oligosacharidů syrovátky, přestože neutrální oligosacharidy vykazaly inhibiční efekt, který je dobrým příslibem do budoucna. Předtím než bude syrovátka využívána jako nové antiinfektivum, je nutné provést více komplexních studií antiadhesivní aktivity jejich oligosacharidů a glykokonjugátů.

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