Mucin Utilisation and Host Interactions of the Novel Intestinal Microbe *Akkermansia muciniphila*

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Abstract

Mucins are the major organic components of the defence barrier, known as mucus, covering epithelial cells in many organs, including the entire gastrointestinal (GI) tract. Microbes that can associate with mucins benefit from this interaction since they can access nutrients. Mucin-degrading bacteria are therefore an important community that have not been extensively studied as the substrate itself, mucin, is a complex and high molecular weight glycoprotein.

The work presented here is focused on the identification and isolation of mucin-degrading bacteria from the GI tract, their degradation of mucin and their interaction with the host.

Mucin-degrading bacteria were analysed by combining molecular- and cultivation-based approaches. The faecal mucin-degrading bacterial community was found to be highly diverse and host-specific. A novel isolate, representing a novel genus, was cultured from the highest dilution of the enrichment of a single individual. This intestinal isolate, *Akkermansia muciniphila,* was found to grow to a limited extent on a very limited amount of substrates but grew efficiently on mucin. Phylogenetic analysis based on 16S rRNA sequences indicated that *A. muciniphila* belonged to the phylum Verrucomicrobia, which was not known to contain intestinal members. Moreover, 16S rRNA genes from *A. muciniphila* have been retrieved in several clone libraries derived from either faecal or biopsy samples from human and mice. In addition, *A. muciniphila* was found to be rather abundant in the GI tract. Based on fluorescent in situ hybridisation and quantitative PCR, *A. muciniphila* was found to represent an average of 10^9 cells / g faecal sample. A negative correlation between the concentration of faecal mucin and the number of *A. muciniphila* was observed, suggesting it to be involved in mucin degradation *in vivo*. Several specific enzymes, mostly glycosidases were found to be secreted during its growth on mucin that was degraded for a major part (85%). Hence, the specific impact of *A. muciniphila* on the host was investigated in germ-free mice and compared to that of the non mucin-degrading bacterium *L. plantarum*. Transcriptomic microarray analysis showed that both *A. muciniphila* and *L. plantarum* modulated a similar number of genes but that host response was found to be highly specific for each bacterium, depending on the anatomical location. Amongst the major responses, we could detect for *A. muciniphila* a regulation of the immune response, cell proliferation, cell adhesion and apoptosis, and for *L. plantarum* a regulation of the lipid metabolism.

Overall, this work has brought new insights into the mucin-degrading community of bacteria, and in particular the role of *A. muciniphila,* an abundant human mucin-degrading bacterium.

Keywords. Mucin, *A. muciniphila*, mucin degradation, molecular techniques, host response

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Preface

The work presented here is focused on the identification and isolation of mucin-degrading bacteria from the gastrointestinal tract, their degradation of mucin, and their interaction with the host.

Chapter 1 provides an introduction in the structure and properties of mucin, the known bacteria that degrade mucin, and the interactions between bacteria and the host.

Chapter 2 is a review on intestinal substrates used by the intestinal microbiota, including prebiotics and substrates secreted by the host including mucus.

Chapter 3 describes the monitoring of faecal bacterial communities able to grow on isolated mucin. Fingerprinting of 16S rRNA amplicons by denaturing gradient gel electrophoresis followed by cloning and sequencing of the amplicons, resulted in the identification of the major populations.

Chapter 4 details the isolation from human faeces of a novel bacterium, *Akkermansia muciniphila*, able to grow on mucin as both carbon and nitrogen source, and its subsequent characterisation by molecular, phylogenetic and physiological approaches.

Chapter 5 provides an overview of the repertoire of mucinolytic enzymes of *A. muciniphila* by using a combination of biochemical and physiological approaches.

Chapter 6 describes the validation of a specific probe targeting a part of the 16S rRNA gene of *A. muciniphila*, and its application in faecal samples for the quantification of *A. muciniphila* by using fluorescent *in situ* hybridisation combined with flow cytometry.

Chapter 7 aims to describe the relation of the number of cells of *A. muciniphila* and the amount of mucus produced by human volunteers that were subject to the consumption of fructooligosaccharides (FOS). A possible link between the FOS diet, mucin increase and *A. muciniphila* population dynamics was investigated.

Chapter 8 is addressing the impact on the host by *A. muciniphila*. Germ-free mice were mono-associated with *A. muciniphila* and the global transcriptional response was determined by high throughput microarrays representing the genome of the mouse.

Chapter 9 summarises the work of the thesis and provides perspectives for future research.

à ma famille,

CHAPTER 1

General Introduction

The human gastro-intestinal (GI) tract is essentially a tube, which starts from the mouth and ends at the anus. The lumen of the GI tract is continuous with the external environment and only separated from the inside by a single layer of intestinal cells that are covered by a mucus layer. The intestinal lumen is colonised since birth by diverse microbial communities – collectively known as the microbiota - that predominantly consist of bacteria. There is a growing interest in the interactions between the microbiota, the host cells and nutrients as they constitute the three major key players of the intestinal ecosystem that are involved in health and disease. Hence, their main attributes are described here with special attention for the microbiota, the intestinal mucins and their interactions.

The human gastrointestinal tract and its microbiota

Following food intake, the digestive process starts in the mouth during mastication and maceration, and the food components are then passed on to the stomach for further treatment by acids and digestive enzymes. The last phase of digestion takes place in the small and large intestine (Fig. 1.1). The small intestine, composed of three distinct anatomical and functional parts, duodenum, jejunum and ileum, plays a major role in absorption of nutrients via action of several enzymes converting carbohydrates into monosaccharides, lipids into fatty acids and glycerol, and proteins into amino acids. Part of the food components are metabolised and subsequently absorbed in the small intestine but the complex ones reach the colon in an intact form for further microbial fermentation. The colon is considered as the metabolically most active site from the GI tract. It starts from the caecum, continues to ascending, transverse and descending colon and finishes at the rectum where the luminal contents leave the body as faeces. From the upper part of the GI tract to the lower part, the pH increases gradually in parallel to concentration of bacteria (Fig. 1.1).

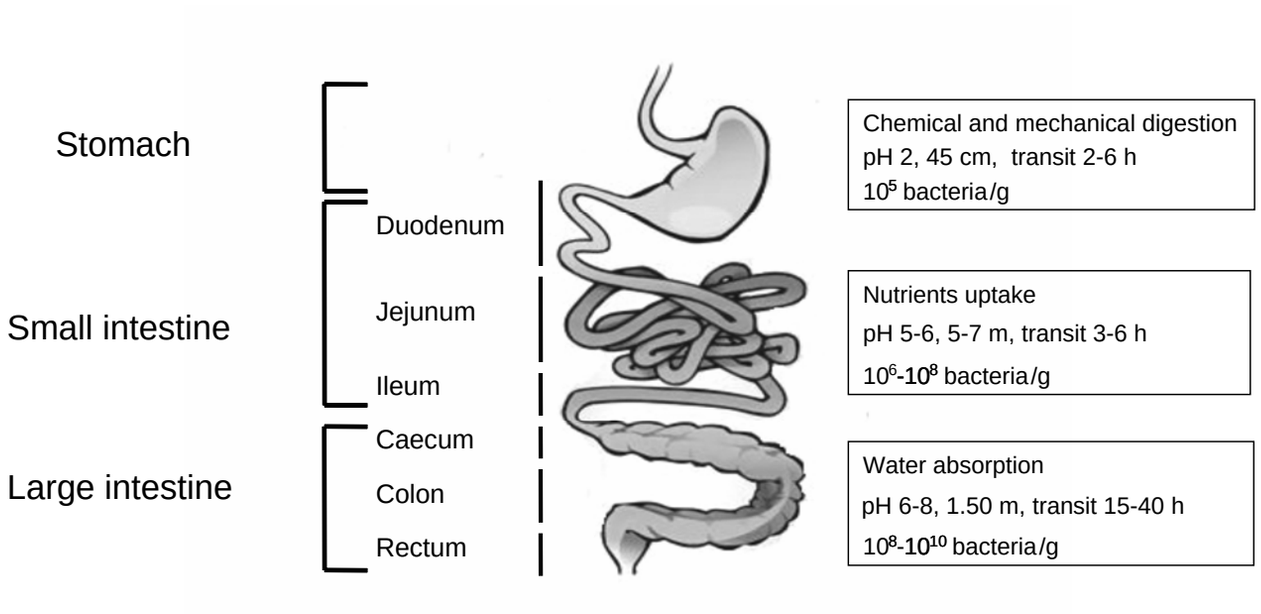

Figure 1.1. Schematic representation of the human GI tract. The role, pH, transit time of food and approximate concentration of bacteria of each region are indicated. Modified after [104]

In 1897, Louis Pasteur discovered that the microbiota is an essential and vital element for the host. Indeed, studies on germ-free animals, which by definition are devoid of any bacteria in any part of their body, showed that they had a short life [117]. This already showed the importance of bacteria on the animal physiology and their preponderant role in human body has

been well recognised, and even, collectively, the intestinal microbiota has even been referred as a virtual organ within an organ [208].

The adult human GI tract microbiota is composed of up to 10^{14} cells, which outnumber the total number of human body cells by a factor of 10, and the combined genome of all bacteria (microbiome) is expected to contain 100 times more genes than the human genome, with approximately two to four million genes [90]. The intestinal microbiota is highly complex and composed of mainly prokaryotes (predominantly bacteria and in some cases, archaea) [6] and their viruses [28], and a minority of eukaryotes (yeasts, protozoa). In the microbiota of a healthy adult individual, bacteria are present throughout the GI tract, but colonise the different regions in different numbers and composition (Fig. 1.1). The number of bacteria colonising the stomach is low due to the highly acid conditions (pH≤2) [21]. The small intestine contains a higher number of bacteria due to less hostile conditions than the stomach (pH 5-6), but, still remains low (10^5 to 10^7/ g) because of the fast flow of food (3-6 h transit time) that renders colonisation of bacteria difficult since they are rapidly washed. However, the terminal part of the small intestine (ileum) has a higher number of bacteria (10^8/g). In the large intestine, passage of nutrients is much slower (15-40 h), and this enables colonic bacteria to multiply and colonise the colon at a high level. A recent study has estimated the colonic microbiota to consist of more than 1000 different species of bacteria [227] which reach in total a concentration of one trillion (10^{12}) organisms per gram of faeces amounting to a total of 1.5 kg, and consisting of 60% of the total faecal mass [322].

16S ribosomal RNA based approaches

Till about ten years ago, our understanding of the GI tract ecosystem has been largely dependent on the cultivation and isolation of microbes. The GI tract microbiota is highly complex and the vast majority (99 %) of microbes are strict anaerobes [255]. The major drawbacks of the use of cultivation-based techniques are that they are slow, fastidious and relatively selective for the fast and easy-growing microbes. Moreover, a proportion of species have not been able to be yet cultivated, due to their specific growth conditions that are not known. The introduction of molecular approaches has revolutionised microbial ecology by overcoming the cultivation problem and the analysis of 16S ribosomal RNA (16S rRNA) sequences has greatly advanced our knowledge of the intestinal ecosystems. The molecular approaches showed that nine bacterial phyla are present in the human GI tract (Fig. 1.2), with a predominance of the Firmicutes, the Bacteroidetes and the Actinobacteria.

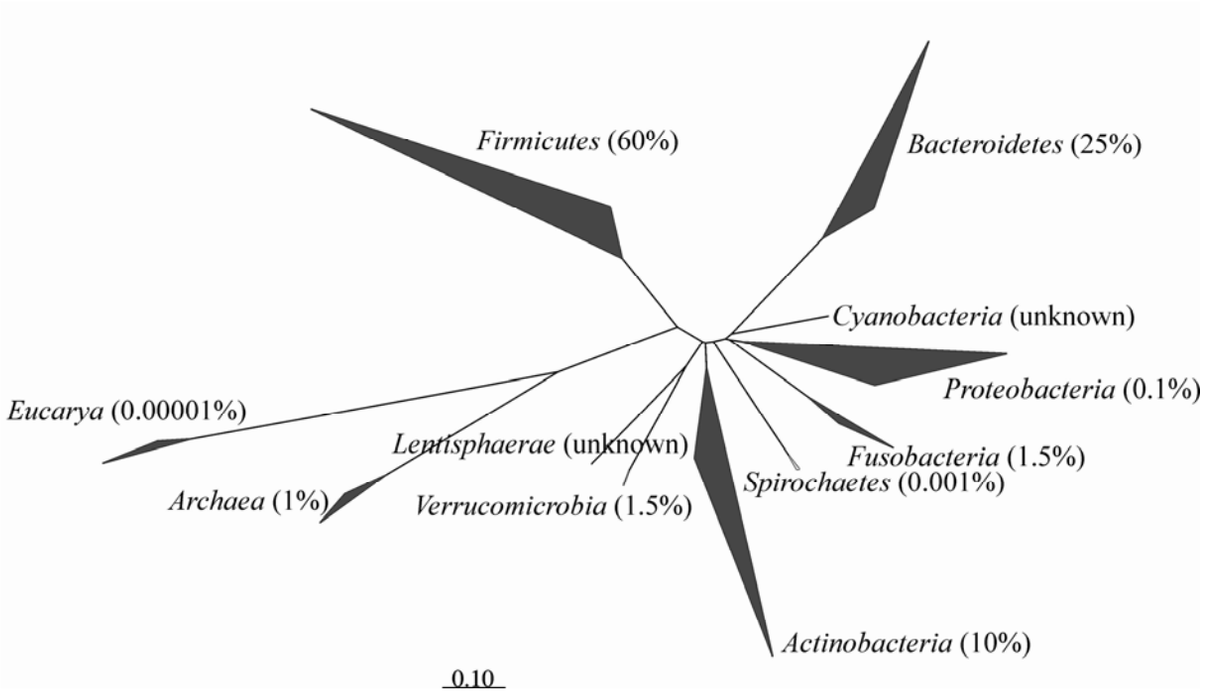

Figure 1.2. Phylogenetic tree showing the distribution of organisms and their average relative abundance in the human gut [227].

Several culture-independent techniques have been successfully employed on GI tract samples. These include cloning, sequencing as well as fingerprinting of 16S rRNA genes by DGGE/TGGE (Denaturing or Temperature Gradient Gel Electrophoresis) and Terminal-Restriction Fragment Length Polymorphism (T-RFLP) [76], which are semi-quantitative techniques that allow rapid profiling of total microbiota. Sequence analysis of cloned 16S rRNA genes derived from GI tract samples has shown that most of the sequences correspond to not yet cultivated organisms and it has been estimated that the cultivated bacteria from the GI tract represent only 20 to 50% of the total microbiota [75, 285, 319, 336], highlighting the huge gap between our culture abilities and the true diversity. Moreover, new phyla that were not known as GI tract inhabitants were discovered. The Verrucomicrobia phylum is an example of a bacterial group that was hardly detected by culturing, but frequently found in 16S rRNA clone libraries from a variety of ecosystems including soil, freshwater and GI tract. This phylum was discovered in 1997, as a new branch within the Bacteria domain and bacteria belonging to this phylum were found in faeces as well as mucosa from different locations in the intestinal tract [75, 108, 112, 313, 319]. Quantitative 16S rRNA gene techniques such as Fluorescence *In* Situ Hybridisation (FISH) combined with Flow Cytometry (FCM) and quantitative PCR (qPCR) have allowed an accurate and high throughput analysis of bacterial ecosystems. FISH-FCM has been successfully used in intestinal ecosystems for identification, quantification but also determination of viability of bacteria [18]. Alternatively, qPCR allows rapid, precise and continuous monitoring of nucleic

acids based on the detection of a fluorescent reporter molecule that increases with the accumulation of the targeted product during amplification cycles. qPCR has been applied in many studies using genus or species targeting primers and has contributed to monitor the composition and dynamics of the intestinal microbiota [69, 78, 96, 102, 103, 182, 216].

Recently, the DNA microarray technology, with its ability to detect and measure thousands of distinct DNA sequences simultaneously, allowed developing phylogenetic microarrays targeting 16S rDNA. These are powerful tools for the high-throughput and detailed studies of microbial communities, including those from human intestine [56, 333, 335]. A recent phylogenetic array, so-called Human Intestinal Tract Chip (HIT)-Chip has been developed and covers more than 1,000 bacteria reported in culture-dependent and culture-independent studies and employed successfully for monitoring the temporal variation of the microbiota in intestinal samples [227].

Microbial growth substrates

Carbohydrates are the major energy sources of human and intestinal bacteria. Some of these can not be degraded in the small intestine and reach the colon, where they are further metabolised. These so-called non-digestable oligosaccharides include fibres, resistant starch, and other sugar polymers, as well as specific oligosaccharides. Besides undigested food components, the microbiota is also exposed to other host-derived components, such as the glycosylated host proteins mucins, immunoglobulins A, trefoil factor or sloughed-off intestinal cell compounds [226, 248]. The major carbohydrates available for bacterial fermentation in the colon are depicted in Fig 1.3.

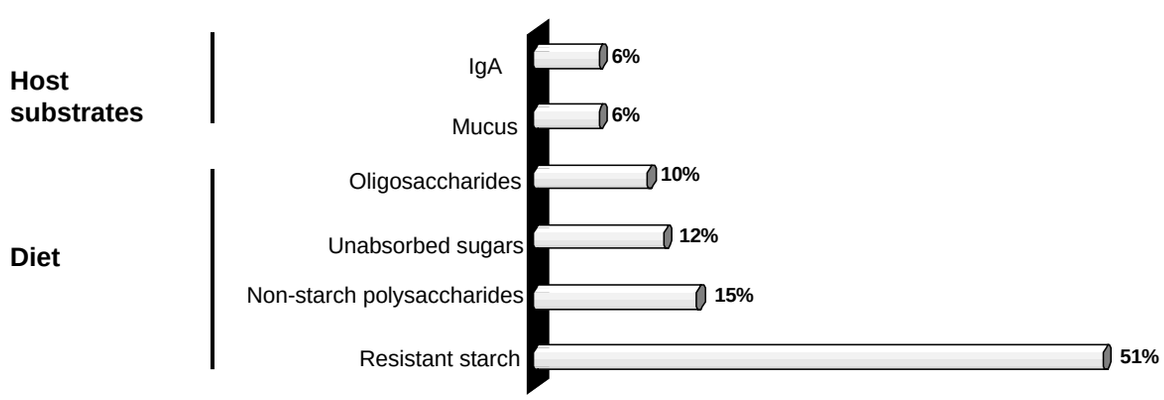

Figure 1.3. Proportion of carbohydrates that reach the colon per day. Adapted from [172] and [211]

Carbohydrate fermentation by bacteria results in a number of products including gases, such as hydrogen, methane and carbon dioxide, as well as short chain fatty acids (SCFA). The predominant SCFA are acetate, propionate and butyrate which ratio varies depending on the substrate and the microbial populations. For example, resistant starch fermentation yields high concentrations of butyrate, whereas pectin results in high concentrations of acetate. Butyrate is used mainly by the epithelial cells as an energy source and stimulates cell proliferation [320].

Mucus and mucins

Many human organs that are exposed to the external environment such as the respiratory, digestive and urinary tracts, are coated with a continuous layer of mucus. Mucus is a highly hydrated gel (± 95% water) that contains salts, lipids (e.g., fatty acids, phospholipids and cholesterol), proteins (e.g., lysozyme that serves a defensive purpose), immunoglobulins, defensins, growth factors, trefoil factors, and released epithelial cells. Human secrete a total of approximately 10 litres of mucus per day [194], which has many roles and can act as: 1) a lubricant since it facilitates the passage of food components; 2) a selective barrier by allowing passage of low molecular weight components, nutrients, gases, wastes through the cells; and 3) a protection system for the underlying cells against entrance of harmful substances (drugs, toxins, heavy metals), acid, luminal pepsin [2], mechanical damage, or pathogenic bacteria, viruses or parasites [1, 63, 80].

Mucus is constantly secreted, shed, and produced and its life time is short, varying from a few minutes to several hours [15]. In histological tissue sections, two different mucus layers have been observed [183]. The external layer, also named mobile or non-adherent layer, is largely soluble and constantly removed, and acts as a lubricant by expulsing aggressive agents (bacteria, viruses) trapped in this layer. The underlying internal layer, also termed adherent layer which is firmly adherent to the epithelial surfaces and not soluble in water, acts as a selective barrier [2]. Various methods have been employed to visualise and measure the thickness of the mucus layer. The use of unfixed fresh or snap-frozen tissues has provided more accurate results than the use of fixed samples. This can be ascribed to the use of fixatives, such as paraformaldehyde or glutaraldehyde followed by paraffin embedding, that result in shrinkage and dehydratation of the mucus layer, leading to underestimation of the thickness [3]. The use of unfixed tissues sections, followed by observation using an inverted microscope, allowed correct measurements of the thickness of mucus layer. The thickness of the mucus layer has been shown to vary from the stomach to the colon. In the stomach, the layer is the thickest and protects the stomach epithelium against the hostile acidic conditions. The thickness of the mucus layer follows a gradient from the

small intestine to the colon (Table 1.1). The mucus layer has been reported to be disturbed in case of GI disorders such as Crohn's disease (CD) or ulcerative colitis (UC). In case of UC, the mucus layer was found to be thinner in the inflammated part, while in CD, the mucus thickness was normal or even higher than usual [225]. Thus, the integrity of the mucus layer is crucial to ensure the protective layer observed in health.

Table 1.1. Thickness (Mean ± SD) in µm of the human mucus layer in healthy individuals and those suffering from inflammatory bowel disease, ulcerative colitis (UC) and Crohn's disease (CD) as observed in different studies (ND: Not determined).

Locations	Healthy mucosa	Disease		Reference
		UC	CD	
Gastric antrum	144 (±52)	ND	ND	[131]
Caecum	31.1 (±7.2)	ND	ND	[183]
Ascending colon	34.4 (±8.9)	ND	ND	[183]
	107 (±48)	90 (±79)	190 (±83)	[225]
Transverse colon	50.5 (±14)	ND	ND	[183]
	134 (±68)	43 (±45)	232 (±40)	[225]
Sigmoid colon	62 (±32.1)	ND	ND	[183]
Rectum	88.8 (±80.1)	ND	ND	[183]
	155 (±54)	60 (±86)	294 (±45)	[225]

Mucins are the main components of mucus and are synthesised and secreted in the GI tract by mucin-producing specialised cells of the epithelium (goblet cells) [279] or enterocytes (see below). Mucins are responsible for the viscosity and elastic gel-like properties of mucus and are characterised by a wide variety of carbohydrate chains (80% of the mucin content) attached to the polypeptide backbone (apomucin), and a high molecular weight (up to 40 MDa) [279].

The human genome contains a family of mucin genes, designated as *MUC* followed by a number that indicates their order of discovery [60]. This family has at least 19 members that differ considerably in size (Table 1.2). The smallest *MUC* gene encodes a few hundreds of amino acids, while the largest *MUC* gene encodes more than few thousands of amino acids. All *MUC* genes share common features: a large size of their respective mRNAs and the presence of extensive tandem repeats, rich in serine and threonine, in the encoded apomucin core that account for more than 60% of the size of the polypeptide. Each *MUC* gene codes for an exclusive number of amino acids per tandem repeat (Table 1.2).

Table 1.2. Mucin encoding genes (MUC) identified, their main anatomical sites of expression, their chromosome location, and the total amino acids (aa) of the produced protein and of the tandem repeat (TR), ND: Not determined

MUC genes	Main locations of expression	Chromo-some	Total aa	aa in the TR	Reference
Secreted					
MUC2	Small intestine, colon, cervix	11p15.5	5179	23	[98, 99]
MUC5AC	Stomach, small intestine, colon, cervix	11p15.5	1233	8	[101]
MUC5B	Submaxillaryglands, respiratory tissue	11p15.5	3570	29	[67]
MUC6	Stomach, small intestine	11p15.5	505	169	[297]
MUC7	Salivary glands	4q13-q21	377	23	[23]
MUC19	Salivary glands, respiratory tissue	12q12	701	ND	[46]
Membrane-bound					
MUC1	Breast, pancreas, small intestine, colon	1q21	1000-2000	20	[27, 83, 146, 158]
MUC3A	Small intestine, colon, gall bladder	7q22	213	17	[55, 222, 315]
MUC3B	Intestine	7q22	3312-7312	17	[207]
MUC4	Respiratory tissue, colon, cervix	3q29	4468- 8468	16	[193]
MUC8	Respiratory tissue	12q24	313	13 / 41	[265, 266]
MUC9	Fallopian tubes	1p13	678	15	[148]
MUC11	Small intestine, colon	7q22	957	28	[314]
MUC12	Colon	7q22	512 - 585	28	[314]
MUC13	Large intestine, trachea	3q13	1890	15	[316]
MUC15	Small intestine, colon, spleen	11p14.3	334	No TR	[214]
MUC16	Ocular surface	19p13.3	1890	156	[7, 324, 325]
MUC17	Colon, pancreas	7q22	4500	59	[97, 192]
MUC20	Kidney	3q29	555	19	[110]

The sequence of amino acids and number of tandem repeats are very polymorphic, depending on genetic background, and even for one *MUC* gene, the sequence of encoded tandem repeat can differ [269]. The central part of the peptide core is bordered at both ends with unique regions of amino acids, which sequence differs from the central region. The tandem repeats are decorated by carbohydrate chains consisting of a backbone and peripheral regions (Fig. 1.4). These are linked to the core protein via N-acetylglucosamine (GlcNac) that is coupled to the hydroxyl group of serine or threonine amino acid via a-O-glycosidic linkage. The backbone region consists of successive galactose and GlcNac residues. The peripheric region (with a size of 2-20 monosaccharides) is comprised of one or more of the major oligosaccharides N-

acetylgalactosamine (GalNac), fucose, galactose, and GlcNac [80]. The peripheral sugars of the oligosaccharide chains usually have an arrangement identical to those found in the ABO groups of the red blood cells (see Fig. 1.4) [43]. Further modification of the model structure of mucin as depicted in Fig.1.4 may occur via the addition of sialic acid or sulphate residues or other monosaccharides that all contribute to the specific function of mucin in the GI tract [38].

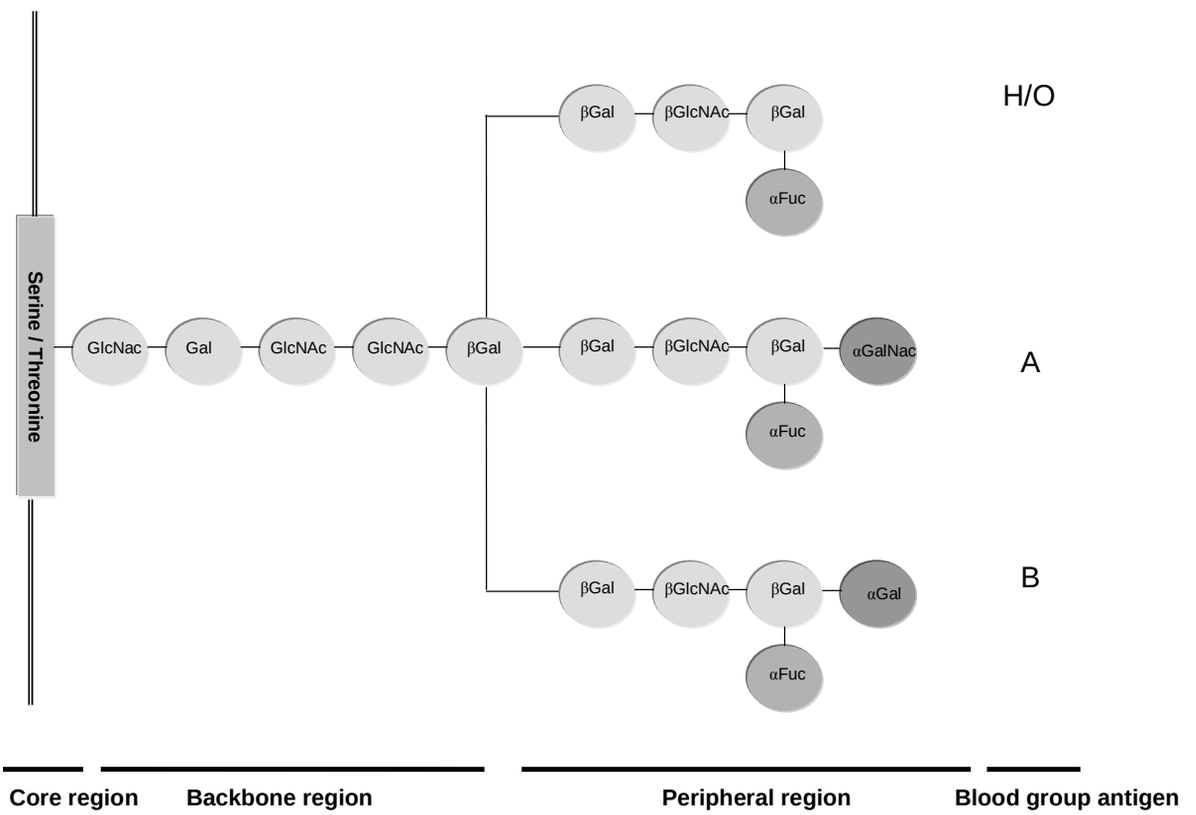

Figure 1.4. Composition of the peripheral structures of mucins – the peripheral regions ressembling the blood group antigens (H/O, A, B) are indicated. See text for a further explanation.

Based on their structure, mucins are usually subdivided into two classes. The secretory mucins are secreted by specialised cells, the goblet cells and characterised by their high molecular weight and size, and ability to form a viscous gel. The membrane-associated mucins, in contrary, are synthesised by enterocytes, and integrated into membranes. They are monomeric and do not form gels [52]. The secretory mucins are composed of cystein-rich regions, which allow the formation of disulfide bridges interconnecting the mucins. Mucins are released into the lumen by exocytosis, which has been shown to be dependent on several stimuli, including the concentration of intracellular calcium, protein kinases A and C, G proteins, and ATP [80]. In contrast, the membrane-associated mucins do not have the property to form a gel. They contain a large mucin-like extracellular subunit, a hydrophobic transmembrane domain (EGF-like domains)

and a short cytoplasmic carboxy terminal tail, which is involved in intracellular signalling. Up to now, membrane-bound mucins have been thought to play a role as cell surface receptors and sensors, which translate information about external conditions into cellular responses including proliferation, differentiation, apoptosis, or secretion of specialised cellular products [114].

Depending on the sugar composition, mucins can be differentiated histochemically as neutral or acidic mucins. Acidic mucins are composed of either sialic acids (sialomucins) or sulphate (sulphomucins) giving a strong negative charge to the mucin molecule. The ratio neutral/acidic differs along the GI tract. In the stomach and small intestine, mucins are more neutral and rather sialyted than sulphated. In contrast, the mucins in the colon are more sulphated. This additional sulphation has been proposed to provide an extra protection of the underlying epithelium against the high density of bacteria, because sulphation confers resistance to most bacterial mucin-degrading enzymes [31, 237]. The extensive glycosylation strongly affects the physical properties of mucins. In addition, this glycoslylation can mediate specific binding of immune cells, such as antibodies and leukocytes, pathogenic and commensal microbes, and may be important in inflammation and cancer metastasis [30, 114, 231, 234].

Mucus-associated bacteria

The mucus layer can be considered as the first line of defence between the bacteria in the lumen and the host cells, and may serve as the initiation point for host-microbe interactions [256]. Bacteria that are able to associate with mucus probably gain an advantage over the luminal or planktonic bacteria. Indeed, bacteria that colonise the mucus gel are less susceptible to elimination by the passage of luminal contents, and have increased access to carbon sources provided by the mucus layer, in comparison to luminal bacteria [224]. Intestinal mucus has a dual role as it protects the mucosa from certain microorganisms and provides an initial binding site, nutrient source, and matrix on which bacteria can proliferate (Fig. 1.5).

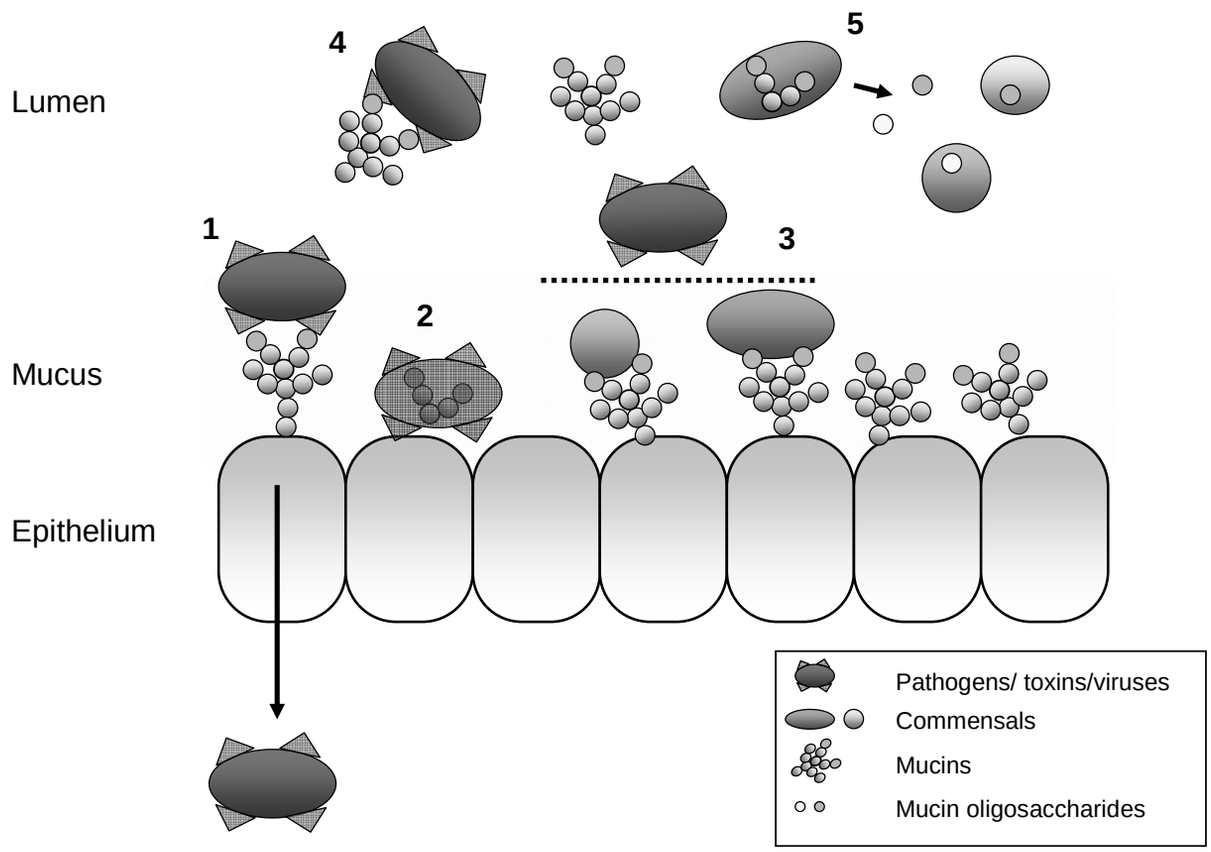

Figure 1.5. Schematic representation of the different associations of mucin glycoproteins with intestinal bacteria. Mucins can elicit entrance of pathogens into the epithelium by serving as a first adhering site before access to the cells (1). Moreover some pathogens can degrade mucin glycoproteins by producing specific mucin-degrading enzymes, which favour the disturbance of the protective mucus layer (2). On the other hand, mucins are a barrier against pathogens. Some commensals, and possibly probiotics, can bind to membrane-bound mucins and lead to antagonistic competition with pathogens for binding sites (3). Mucins can also act as soluble receptors that bind to pathogens and block bacterial adhesion to the cells (4). Finally, degradation of mucins can provide an ecologic advantage to some commensal bacteria as it generates nutrients (5).

It was shown in a model reactor with a mucus layer that the microbial community living inside the mucus matrix differed phylogenetically and metabolically from the luminal community [173]. This had also been found in human where mucosa-associated bacteria varied between individuals but were similar in different sites of the colon of one individual [154, 201, 336]. The mucosa-associated bacteria are assumed to be in close contact with the host, and therefore can have a major impact on host cells. However, a recent report based on FISH with a combination of a general 16S rRNA oligonucleotide probe targeting all bacteria, and specific probes targeting particular groups of bacteria, indicated that there was no specific population present in the mucus layer and that commensal bacteria are located in the lumen [304]. This is

contradictory to model, animal and human studies that showed bacteria to be embedded in the mucus layer by using FISH, light and scanning electronic microscopy (see Fig. 1.6) [75,79,80].

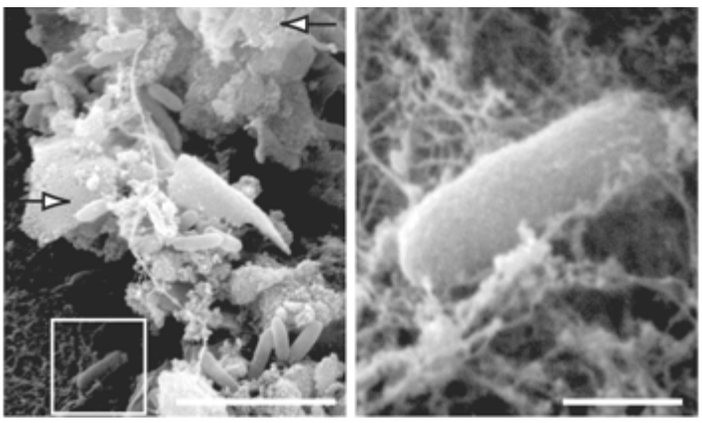

Figure 1.6. Scanning electronic micrograph showing a commensal intestinal bacterium, *Bacteroides thetaiotaomicron*, embedded in the mucus layer from the ileum of mono-associated mice. (Reprinted with permission of Dr. Justin Sonnenburg [278]).

Mucin binding

The carbohydrate structures found to decorate mucins are extremely diverse (see Fig. 1.4). Therefore, mucins offer an oasis of binding sites for bacterial (pathogens and commensals) adhesion. By offering binding sites similar to those of epithelial cells, mucin can prevent pathogen adhesion to the underlying epithelial cells. In addition, binding of commensal bacteria to mucin glycoproteins may prevent pathogen interaction with epithelial cells, and further translocation into the mucosa. For instance, mucins from human breast milk, from which MUC1 is the predominant one [267], bind to certain pathogenic microorganisms, such as rotavirus [326], *Campylobacter* [241], *Escherichia coli* (ETEC) [181], and thus interfere with their colonisation in the infant GI tract. The ability to adhere to mucins has been suggested to be one of the criteria for the selection of lactic acid bacteria with probiotic activity [142, 247]. However, as comparative studies on the efficiency of probiotic strain are scarce, the validation of this attribute has not been yet confirmed. The molecular mechanism by which binding to mucus may occur is receiving increasing attention. Several genomes of probiotic lactic acid bacteria contain genes with predicted mucus-binding activity [24]. One of them, the protein GroEL of *Lactobacillus johnsonni* was shown to bind to mucins and to aggregate *Helicobacter pylori* suggesting that the protein could facilitate clearance of the pathogen during mucus flushing [20]. The oligosaccharides acting as attachment sites on mucus can also promote the invasion of pathogens by providing them a first attachment site that facilitates further access to epithelial cells. In line with this possiblility, it was observed that the pathogen *H. pylori* binds to the secreted mucin MUC5AC [303].

Mucin degradation

Besides providing attachment sites to bacteria, mucus can be a platform for bacterial colonisation by providing an energy source. Mucus is an important source of carbon for the bacteria, mainly in the distal colon where the availability of carbohydrates is limited [226]. The oligosaccharide side chains account for more than 80% of the dry weight of mucin and hence constitute a significant endogenous carbon and energy source for intestinal bacteria able to cleave the linkages.

In a series of pioneering studies, Hoskins and co-workers have studied the adult faecal microbiota able to degrade mucin *in vitro*. They first quantified it using Most Probable Number (MPN) counting of bacteria grown in mucin-based medium after faecal inoculation [187]. This study revealed that 1% of the faecal microbiota was able to use mucin as carbon source, including the genera *Ruminococcus* and *Bifidobacterium*. It was shown that many bacteria are able to degrade mucin partially but that the complete degradation of mucin requires the action of several bacteria [317].

Mucin degradation is often regarded as an initial stage in pathogenesis, since it disturbs the first protection of the host mucosal surfaces. However, this may hold only for an excessive degree, as mucin degradation has been found to be a normal process of mucus turn over in the GI tract, starting a few months after birth [204], and that occurs via peristalsis, digestion by host and bacterial enzymes. Midtvedt *et al.* studied the establishment of the mucin-degrading microbiota from 30 Swedish children from birth to the age of two years old, based on agar gel electrophoresis of their faecal samples. They found that the establishment of mucin-degrading bacteria starts during the first months of life and is completed when the children are around two years old. Interestingly, a relation with diet was also observed: breast-fed babies showed a delay in the mucin degradation profile as compared with babies fed with formula milk. A potential explanation might be that mucins and the other mucin-like glycoproteins present in the breast milk compete with endogenous mucins as microbial substrates [186].

Mucin degradation has also been found to affect the host. The epithelium of germ-free rats differs morphologically from that of conventional raised animals: undegraded mucin is found in faeces, their goblet cells are smaller and less abundant [133], the mucus layer is approximately two times thicker [287] and the weight of the caecum can reach up to eight times that of conventional animals [184]. All those changes can be attributed to the accumulation of mucus, and the resulting retention of water, due to the absence of mucin-degrading bacteria [159]. Indeed, when faecal suspension or pure cultures of *Clostridium*, *Bacteroides* or *Peptococcus* were introduced into germ-free rats, the caecum showed a noticeable reduction to its normal weight [163, 272].

Mucin degradation is achieved by a combination of mainly saccharolytic and proteolytic enzymes from the host and bacteria. As dicussed above, the composition of mucin sugars, their glycosylation degree, linkage and terminal sugar differs along the GI tract, being more neutral in the upper part, while more acidic in the lower part. Mucin-degrading bacteria can adapt to the host mucins by producing specific enzymes that can degrade the blood group oligosaccharides [121-123]. Due to their high complexity and diversity, mucins can only be completely degraded by a panel of diverse enzymes, including proteases, glycosidases, sialidases and sulphatases [54, 123] (Fig. 1.7). Mucin-degrading enzymes start with an initial cleavage of the non-glycosylated regions of the peptide backbone (at the extremity of the peptide core) performed by proteolytic enzymes. Subsequently, the oligosaccharides chains are degraded by a panel of diverse glycosidases, finally followed by proteolytic degradation of the exposed protein core. Proteases are secreted by both host and bacteria while glycosidases are only secreted by bacteria. Glycosidases include mainly α-D-galactosidase, β-D-galactosidase, N-acetyl-β-D-galactosaminidase, β-fucosidase, α-L-fucosidase and N-acetyl-β-D-glucosaminidase. Sialidases (neuraminidases) and sulphatases act on the external side of the oligosaccharide chains (Fig. 1.7).

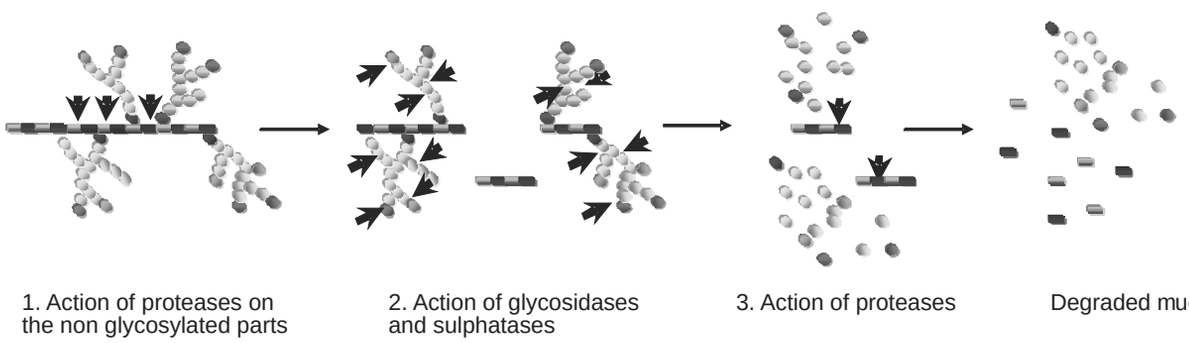

Figure 1.7. Schematic representation of the multi-step degradation of the mucin molecule and the action sites of mucin-degrading enzymes (black arrows).

In summary, mucin degradation requires the simultaneous presence of several microbial enzymes, mainly glycosidases, each having the specificity to degrade a specific glycoside linkage. Few intestinal bacteria produce all the enzymes necessary to degrade the completely mucin. Several bacteria belonging to the genera *Ruminococcus, Bacteroides, Bifidobacterium, Clostridium, Prevotella, Streptomyces* produce all one or more mucin-degrading enzymes (Table 1.3).

Table 1.3. Bacterial mucin-degrading enzymes identified in the human GI tract.

Organisms	Enzymes	Reference
Bacteroides fragilis	Neuraminidase, sulphatase, protease α- N-acetylgalactosaminidase, β-galactosidase, β -N-acetylglucosaminidase, α-fucosidases,	[19, 167, 169]
Bacteroides thetaiotaomicron	Sulphatase, neuraminidase α-fucosidase, β-galactosidase α- N-acetylgalactosaminidase β-N-acetylglucosaminidase	[322] [299]
Bacteroides vulgatus	Neuraminidase, α and β-galactosidases, α-fucosidase β-N-acetylglucosaminidase, α and β -N-acetylgalactosaminidase	[244]
Bifidobacterium sp	α-L-Fucosidase, α-N-acetylgalactosaminidase	[135]
Bifidobacterium bifidum	Galactosyl-N-acetylhexosamine	
Clostridium cocleatum	Neuraminidase, β-galactosidases, β -glucosidase, β-N-acetylglucosaminidase, α-N-acetylgalactosaminidase.	[25]
Clostridium septicum	β-Galactosidase,β-N-acetylglucosaminidase, glycosulphatase, neuraminidase	[171]
Prevotella sp RS2	Glycosulphatase	[321]
Ruminococcus torques	α-N-Acetylgalactosaminidase	[124]
Streptomyces sp	α-L-Fucosidase	[92]
Vibrio cholerae	Neuraminidase, endo-beta-N-acetylhexosaminidase, proteinases.	[283]

The ratio of production and degradation of mucin is usually stable in healthy individuals, but can be affected in pathogenesis. Mucin mRNA expression has been shown to be disturbed for several *MUC* genes in ileal and colonic mucosal biopsies from inflammatory bowel diseases patients such as UC and CD [49]. Changes in the properties of mucins and mucus have been noted in UC and CD, such as decrease of the length and sulphation and increase of sialylation of the oligosaccharide chain [268]. Moreover, the thickness of the mucus layer is altered in these IBD patients (Table 1.2), leading to a weakness of the protective barrier [225, 231, 262]. In UC, the mucus layer has been shown to be thinner, harbouring a reduced proportion of goblet cells, and an increased level of faecal mucin-degrading enzymes, including sulphatases, as compared to a healthy individual, leading all a dysfunction of the mucosal barrier. The rate of mucin turn over can be modified by bacterial colonisation [157, 174], SCFA [11], starvation [273], intake of fibres and other oligosaccharides [11, 38, 144, 254, 260, 294, 302].

The high degree of diversity of the mucin oligosaccharide chains along the GI tract with potential binding or degrading sites for bacteria could explain the site-specific colonisation of bacteria in the GI tract. Recent studies have shown that a fucose-utilising strain of the commensal *B. thetaiotaomicron* could regulate production of particular glycans, including fucose, and thus controlled the production of its own substrate [120].

Host-microbe interaction

As described above, mucosal surfaces play an important role in host-microbe interactions. The cross-talk between microbiota and its host has become an important field that initially focused on the host response to pathogens, as they impact human health [253]. In recent years, commensal-host interactions are receiving growing attention, due to the notion that commensals represent the majority of intestinal bacteria and must have an essential role in our physiology. However, very little is known about the interaction between commensals and their host, since the effects on the host are less clear to identify compared to pathogens. Notably, the development of new technologies such as microarrays has facilitated the study of the dialogue between host and commensal bacteria [229] as is discussed below.

Host models

In vitro models, such as cell lines [82], or more complex system such as gastrointestinal models have been used in host-microbe interaction studies to mimic the host side. For instance, the TNO gastro-Intestinal tract Models (TIM), which reflect the stomach and small intestine (TIM-1) [189]

and colon (TIM-2) [190], are dynamic computer-controlled *in vitro* systems that mimic the human physiological conditions in the stomach, small intestine and colon. These models have the advantage to be easy of manipulation, but have limited predictive value as they do not reflect the *in vivo* conditions.

Humans are the gold standard models to examine the impact of bacteria on our physiology, and have been employed in many clinical trials. However experimental and ethical limitations generally prevent the detailed analysis of physiological processes. The *in vivo* model that has provided the most information regarding the role of bacteria in host physiology has been the germ-free animal model. Germ-free animals have been studied since the derivation of a germ-free guinea pig in 1895. However, as a result of the nutritional insufficiency of early sterilised diets, perpetual rodent colonies were not developed until the late 1940s. Other animals have also been studied in the germ-free state, including calves, goats, chickens, dogs and pigs, and recently, zebrafish [230]. However, most studies have been performed with germ-free rodents (mice and rats) that are models for nutritional studies. One of the advantages of germ-free animals is that a single bacterium or a well-defined mixture of selected bacteria can be inoculated followed by subsequent analysis of both physiological and molecular responses of the host as well as the microbes [85, 91] (see also Table 1.4). The use of germ-fee animals has advanced our insight into major activities of single species of our microbiota, including the metabolic, immunologic and physiologic functions [202].

Microarray analysis

It is clear that the human host and the GI tract microbiota establish interactions with each other. However, the understanding of the details of these interactions remains a major challenge. The intestinal mucosal surfaces, exposed to a huge number of bacteria, have evolved a way to distinguish between commensal microbes and pathogens: i.e. tolerate the first [139] and fight the latter by inducing an inflammatory response [138, 184], in which the nuclear transcription factor NF-κB has been reported to play a fundamental role in mediating various immune responses [137, 176, 200] .

High throughput molecular tools such as DNA microarrays hybridisation have greatly facilitated the global analysis of bacterial and host gene responses, since it allows identification of the complete transcriptome at once (Fig. 1.8), [12, 162, 188, 229]. Since the publication of the first entire genome sequence from a free-living organism, *Haemophilus influenzae*, in 1995 [79], a multitude of other genomes have been, or are in the process of being sequenced. The availability of complete genome sequences of model organisms, human [311], mouse [10] and

microbial species, including pathogens and commensal intestinal bacteria, such as *Bacteroides thetaiotaomicron* [322], several lactic acid bacteria [177], have provided considerable opportunities to simultaneously monitor gene expression of the host and the microbe. This has resulted in a large body of literature that is summarised below (see Table 1.4).

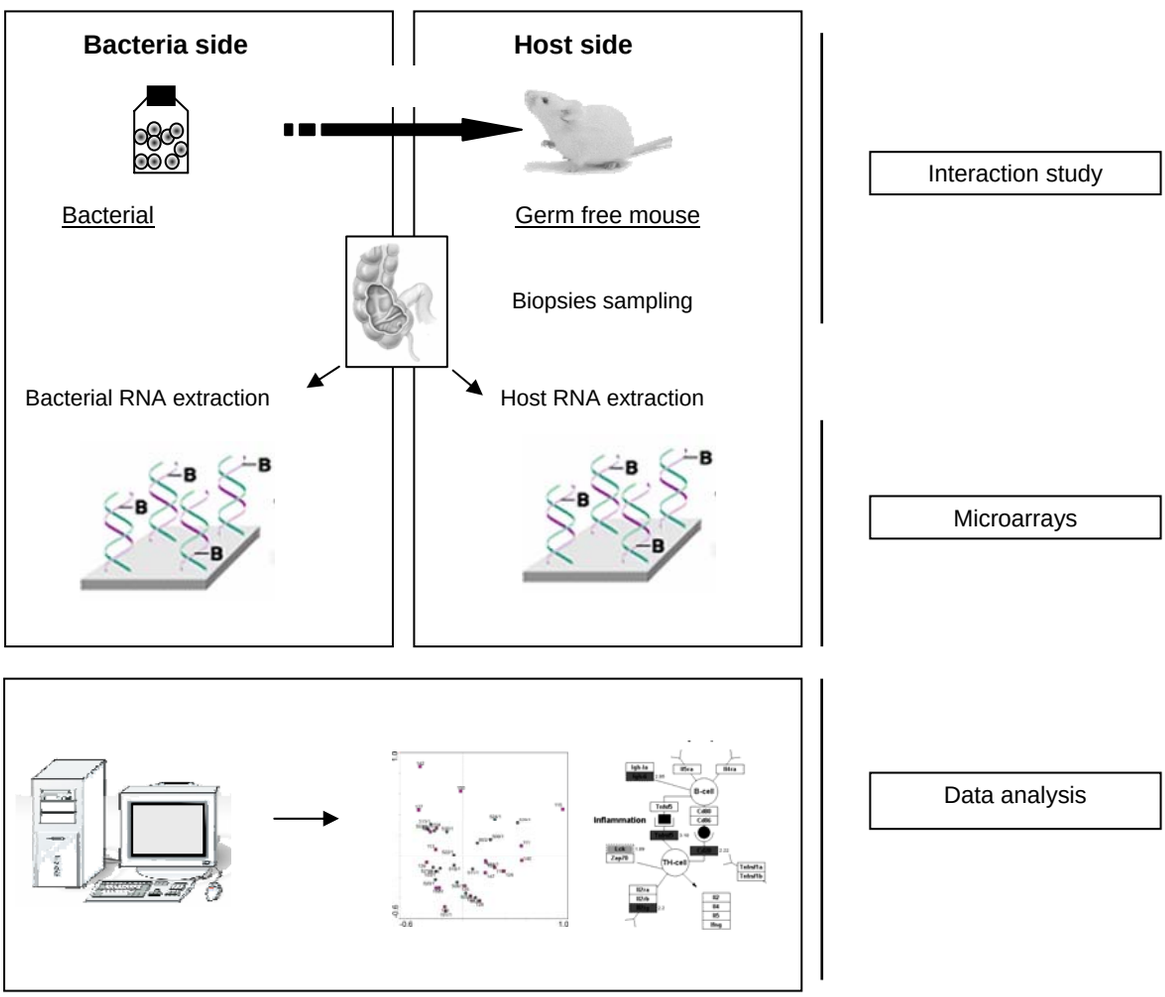

Figure 1.8. Schematic representation of a host-microbe interaction study and the analysis of the transcriptional responses with microarrays.

The pioneers of the field of host-microbe interactions were Gordon and co-workers who showed in a series of elegant studies using a germ-free mice model, that a common and predominant microbe, *Bacteroides thetaiotaomicron* is able to regulate diverse host functions including metabolism, immune system, angiogenesis [282], and fat storage [8]. Moreover, besides affecting host metabolism, *B. thetaiotaomicron* was able to adapt itself to varying host diets. Indeed, when mouse were fed with simple sugars or polysaccharides diets and inoculated with *B. thetaiotaomicron,* the microbe switched its metabolism to the host mucus as energy source when incubated in mice fed with simple sugars diet, indicating that B. *thetaiotaomicron* is flexible and changes its metabolism to adapt to a new environment [278].

Table 1.4. Overview of host-microbe interactions in which either host and/or microbial responses are investigated by whole-genome transcriptional profiling.

Microorganism	**Host model (location)**	**Host response**	**Bacteria response**	**Reference**
Animals models				
B. thetaiotaomicron VPI-5482	NMRI germ-free mice (Ileum and caecum)	Fat storage, angiogenesis, immune response	Carbohydrate metabolism	[8, 115, 116, 120, 278]
B. thetaiotaomicron (BT) ATCC 29148 *and* *Bifidobacterium longum (BL)* NCC 2705	NMRI germ-free mice C57/BL 6J (Caecum)	Innate immunity	*BT*: Carbohydrate, transport and metabolism *BL*: Energy production and conversion	[276]
B. thetaiotaomicron (BT) ATCC 29148 *and* *Bifidobacterium animalis* DN-173010	NMRI germ-free mice C57/BL 6J (Caecum)	ND	*BT:* Transcription and replication	[276]
B. thetaiotaomicron (BT) ATCC 29148 *and* *Lactobacillus casei (LC)* DN-114001	NMRI germ-free mice C57/BL 6J (Caecum)	ND	*BT:* Carbohydrate, transport and metabolism *LC*: Inorganic ion, transport and metabolism	[276]
B. thetaiotaomicron VPI-5482 *(BT)* and *M. smithii* (archeon)	NMRI germ-free mice (Caecum)	Increase of adipocity, energy storage	*BT :* DNA replication, protein production, carbohydrate metabolism	[251]

Table 1.4 (follow)

Microorganism	Host model (location)	Host response	Bacteria response	Reference
L. plantarum WCFS1	R-IVET mice	ND	Sugar, aminoacids, nucleotides, cofactors, vitamins, stress related functions	[32]
L. plantarum WCFS1	C57 Black-6 germ-free mice (Ileum, caecum, colon)	Ileum and colon: Immune response, Lipid metabolism	Caecum: Carbohydrate transport and metabolism cell surface proteins	Marco ML, Peters E, Personal communication
Human models				
Lactobacillus GG	Human (Duodenum)	Immune and inflammatory response, apoptosis, cell growth and differentiation, cell-cell signalling, cell adhesion, signal transcription and transduction	ND	[68]
L. plantarum WCFS1	Human (Duodenum)	Transcription, immune response	Transport and binding proteins, Cell envelope, protein synthesis	[59, 298]

In conclusion, the field of host-microbe interactions is rapidly expanding with the release of genome sequences, the technology of high throughput microarrays and the development of *in vivo* models on one hand, and the development of new approaches in human studies on the other hand. As shown in this introduction, interactions of intestinal bacteria with mucus and mucins are important to be studied since they are the first contacts between bacteria and host. For this reason we have focused on microbes utilising mucin, and analysed their diversity, and determined their interaction with the host cells and nutrients.

CHAPTER 2

Prebiotics and other Microbial Substrates for Gut Functionality

Arthur C. Ouwehand, Muriel Derrien, Willem M. de Vos, Kirsti Tiihonen and Nina Rautonen

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The intestinal microbiota is of great importance to our health and wellbeing. Modulation of the intestinal microbiota by exogenous and endogenous substrates can be expected to improve various physiological functions of our body, not just those in the intestine. Recently, new targets such as immune function and areas outside the colon have been considered to be influenced by the intestinal microbiota. Novel approaches might include the application of prebiotics in different combinations or the provision of nutrients to different bacterial groups and to different parts of the intestine.

Introduction

The intestinal microbiota plays a major role in our health and wellbeing. Its composition and, in particular, its activity might have effects extending beyond the intestine, as illustrated by diseases such as atopic eczema, rheumatoid arthritis and possibly even autism [22]. Strategies have therefore been sought to influence the intestinal microbiota.

Probiotics — live microorganisms that exert health benefits upon ingestion in certain numbers [95] — have long been used to modify the intestinal microbiota. More recently, prebiotics have been used; these are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/ or activity of one or a limited number of bacterial species already resident in the colon [88]. Carbohydrates are the preferred energy source of intestinal microbes; however, in their absence certain groups of microbes, such as clostridia, turn to protein fermentation, which produces harmful nitrogenous metabolites in the lumen (e.g. biogenic amines, indoles and ammonia). Chronic putrefaction can increase the risk of colon cancer through the production of both ammonia, which can lead to neoplastic growth of colon epithelium, and phenols, which can act as co-carcinogens [57, 274]. Although dietary fibre and prebiotics share many properties and in some cases are used interchangeably, they are not the same. Both are non-digestible, but prebiotics are fermented selectively in the colon whereas dietary fibre is fermented by a wide range of colonic microbes or might not be fermented at all.

In addition to prebiotics from food, the body itself produces a large quantity of ‘endogenous prebiotics’, mainly in the form of mucins. These mucins are rich in carbohydrates and are degraded by highly specialised members of the gastrointestinal microbiota. To assess the efficacy of prebiotics, for a long time only changes in the faecal microbiota composition that are induced by the consumption of a prebiotic have been studied. In the past, the mere bifidogenic effect (encouraging the growth of bifidobacteria) of a prebiotic was considered proof of its efficacy. However, it still remains to be proven that a high level of bifidobacteria indeed cures, prevents or reduces the risk of disease. *Bifidobacterium* levels are therefore not a sufficient biomarker for gut health. In an attempt to make a numerical comparison of their efficacy, Palframan et al. [213] devised a prebiotic index for in vitro prebiotic fermentations. This is a promising approach for the objective comparison of prebiotics. However, it currently only takes into account the levels of bifidobacteria, lactobacilli, clostridia and *Bacteroides*, and should be expanded to include microbial metabolites. This index could, in principle, also be used in vivo and more (potential) biomarkers could be included. The safety of a food is of prime importance to health and prebiotics are no exception to this. In general, prebiotics are considered to be safe for human consumption as many of them are constituents of the normal diet, albeit in lower

concentrations. The main side effects of prebiotic over consumption are flatulence, bloating and diarrhoea; the latter is often caused by an increase in osmotic load from the prebiotics or their fermentation products. Such symptoms usually disappear when prebiotic consumption is stopped. However, recent reports have suggested that under certain circumstances the prebiotics inulin and fructo-oligosaccharides (FOS) might enhance the colonisation and translocation of *Salmonella enterica* serovar Enteritidis in rats [292]. It has also been observed that inulin stimulates intestinal tumour formation in a mouse model for intestinal cancer [212], although this has not been substantiated by other studies [136]. Thus, although there is no direct reason for alarm — afterall inulin and FOS are naturally present in many foods and are not known to cause any adverse effects — these issues do deserve further attention and should also be investigated for other prebiotics. Below, the health benefits and physiological changes induced by selected prebiotics and endogenous substrates will be discussed. Also, potential new applications of prebiotics will be described, suggesting areas of prebiotic research.

Current and future prebiotics

Most of the currently available prebiotics are extracted from natural sources, some of which are subsequently modified. Most prebiotic research has been done with β(2–1) fructans, but prebiotic potential has also been shown for galacto-oligosaccharides, xylo-oligosaccharides, soyabean oligosaccharides, polyols and polydextrose. Modification of gut functionality by prebiotics and probiotics has been reviewed by Salminen *et al*. [246]. First generation prebiotics focused on the bifidogenic effect and fibre-like properties of prebiotics on bowel habit. Second-generation prebiotics focused on certain biomarkers in the field of lipid and mineral metabolism, immunology and colon diseases. The development of future prebiotics should have a more holistic approach to modulating microbial fermentation along the gastrointestinal tract as well as to their complementary properties. Complementary prebiotics could be mixtures of prebiotics that have different effects in different parts of the gastrointestinal tract [168]. In animal studies on the immune-modulating effects of prebiotics, increased immunoglobulin A (IgA) production has been shown, especially with prebiotic combinations [217]. This strengthens the notion that a single type of prebiotic cannot support all the beneficial bacteria in the gastrointestinal tract. Future prebiotics could therefore consist of mixtures of different prebiotics, supporting a beneficial balance of the microbial community rather than a specific genus. Considering the site of most prominent gastrointestinal diseases, such as colon cancer, a sustained fermentation throughout the whole colon is preferable. Fast-fermented prebiotics (often with a low degree of polymerisation) do not reach the distal colon, but second-generation prebiotics such as

polydextrose and certain types of inulin can provide beneficial effects, such as immune modulation, throughout the colon [223, 245]. The gradual fermentation of prebiotics might also diminish the osmotic disturbances. Fermentation of prebiotics has several effects on gut functionality. The effects of short-chain fatty acids (SCFAs) on colonic physiology are reviewed by Topping and Clifton [296]. They either directly or indirectly affect the proliferation of enterocytes, inflammation, colorectal carcinogenesis, mineral availability, colonisation by pathogens, enzyme activities and the production of nitrogenous metabolites (Fig. 2.1). The fermentation product butyrate is the principal energy source for epithelial cells [36] and is believed to protect against colon cancer by promoting cell differentiation [140]. Recent preclinical studies support the assumption that butyrate might be chemo preventive in carcinogenesis [257], although direct evidence for protection is still not available. In addition to butyrate, propionate can have anti-inflammatory effects on colon cancer cells [206]. The production of SCFAs and the following acidification of the colonic content affect mineral availability. The possible mechanisms involved in the stimulation of mineral absorption by prebiotics are summarised by Scholz-Ahrens et al. [261]. Lowered pH causes an increase in mineral availability, which consequently increases passive absorption down a chemical gradient [327]. Prebiotics might also stimulate active calcium absorption at the mucosa [209]. Most of these studies have been obtained in rats; knowledge of the effects of prebiotics on mineral metabolism in humans is limited. The available data about the effects of prebiotics on lipid metabolism are also limited. The type of SCFAs produced might modify the liver lipogenesis. In studies with rat hepatocytes, acetate acts as a lipogenic substratewhereas propionate inhibits lipogenesis [62]. Prebiotics might also decrease lipogenic enzyme activity in the liver [61]. It can also be assumed that cholesterol absorbed by the host can be decreased if microbes assimilate it or bind it to their cell walls. In addition to the health benefits in the colon, other parts of the gastrointestinal tract are considered to be potential prebiotic targets. Microbes are found throughout the gastrointestinal tract, starting from the oral cavity. Xylitol can be considered to be an oral prebiotic, as it inhibits specifically the actions of *Streptococcus mutans* but not other oral bacteria, thereby reducing the risk of plaque and dental caries [239]. Furthermore, prebiotics could be designed to promote the health of the small intestine. In the small intestine the microbial community is smaller in numbers and diversity than in the colon; different prebiotic compounds to those useful in the colon can thus be utilised. Also, the urogenital tract can be considered to be a new potential target for prebiotics as it harbours a diverse microbiota that is prone to disturbance. Different stages of life might require different kinds of prebiotics to enhance health.

The successful colonisation of the gastrointestinal tract in infants [180, 191] and the slowing down of immunosenescence in the elderly require different approaches [155].

Additional prebiotic functions

Although the main aim of prebiotics is to modulate the levels and activities of different groups of endogenous intestinal microbes, other functions can also be considered. Sugar residues exposed on the surface of cells (the glycocallix) are often used as receptors by many (potential) pathogenic microbes or their toxins. Attachment to such receptors is the first step in pathogenesis [33]. The use of prebiotics that could function as receptor analogues might provide a protection against such colonisation by pathogens. Although this approach has been pursued with many receptor analogues, the successes have been limited. Cranberry juice is one of the few positive examples, reducing *Escherichia coli* adhesion in vitro and the risk of urinary tract infection in vivo. It remains therefore to be determined whether this approach will be successful for specific prebiotics [145].

Recent results suggest that selected prebiotics are able to modulate the immune response. This is not surprising as the intestine is the largest immune organ of the body and the intestinal microbiota is a very potent immune modulator. Hence, modulating the composition and/or the activity of the microbiota can influence the immune system [259], as is most clearly illustrated in germ-free animals that have an immature and poorly developed immune system [203]. However, such effects are not always observed in humans [37]. This indicates subject to-subject variation; an optimal functioning immune system might not respond to prebiotics. Moreover, different prebiotics are likely to influence the microbiota, and thereby the immune system, in different ways. Another new observation is that prebiotics might influence the elimination of toxins. Polydextrose, indigestible dextrin and soyabean polysaccharides were recently observed to increase the urinary excretion of polychlorinated biphenyls [141]. The mechanism behind this is not clear, but it might be that a prebiotic-induced change in the microbiota composition or activity leads to an alteration in xenobiotic metabolism or could interfere with the enterohepathic cycling of xenobiotics. There are thus many new potential areas of application for prebiotics. The mechanisms behind these applications and their possible health benefits are, in most cases, mediated through the intestinal microbiota.

Microbial metabolism of secreted substrates in the intestinal tract

Many intestinal microbes have developed intimate interactions with the intestinal mucosa impacting a variety of major physiological functions.

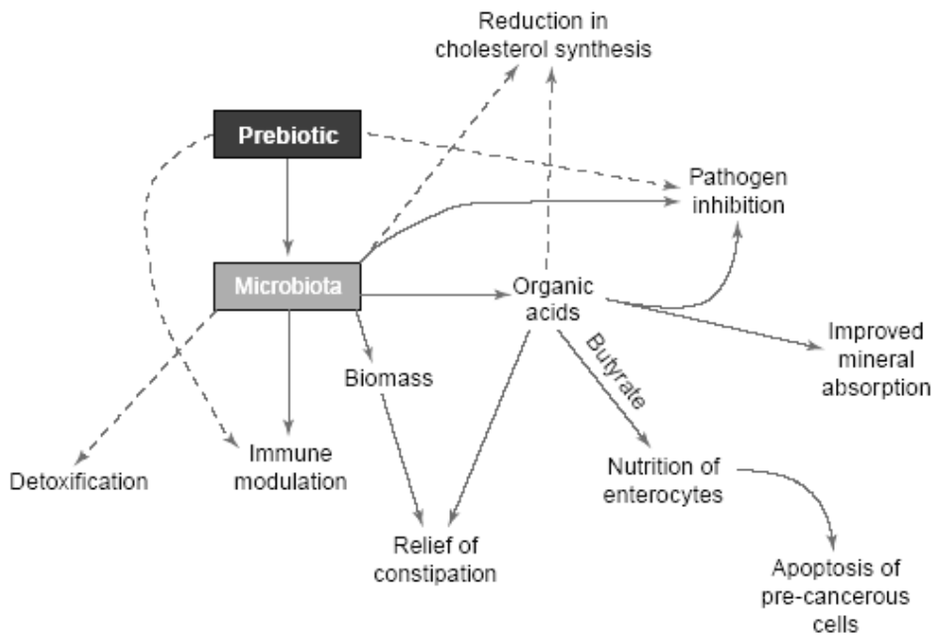

Figure 2.1. Schematic showing the possible mechanisms of prebiotic action. Solid lines indicate relatively well-established modes of action. Dotted lines indicate less well-established or speculative modes of prebiotic action.

In a series of elegant studies using germ-free mice it was shown that the intestinal isolate *Bacteroides thetaiotaomicron* was able to modulate the host response, affecting nutrient processing, immune function and a vast array of other responses. These included an increased production of angiogenin 4, which has antimicrobial activity, and hence this interaction contributes to the dynamics of the intestinal microbial community [118]. However, it is generally accepted that the intestinal microbial community is shaped and maintained by metabolic interactions. These metabolic interactions are mostly involved in the degradation of nutrients, in particular carbohydrates that are not digested or sequestered by the host. These have been reviewed recently by Hooper *et al.* [117]. In this section we focus on the microbial metabolic interactions that result from the degradation of components that are secreted by the intestine and the associated organs (Table 2.1). Although the details of these interactions are just beginning to emerge, the enormous quantity of these secreted compounds as well as their specific properties make them likely candidates to considerably affect the microbial ecosystem.

The mucosal layer

The first line of defense of the mucosa against the luminal contents is the mucus layer. The mucus is a dynamic structure that is modulated by different factors [63]. Goblet cells secrete trefoil factor peptides (TFFs) and high molecular weight (>10^6 Da) mucin to form a continuous slimy layer on the mucosal surface. The thickness of the mucus gel and the number of goblet cells vary according to the anatomical site and the activity of the mucosa. In the colon the thickness of the mucus layer gradually increases from the right to the left, reaching an average of 88 mm in the rectum with a maximum of 285 mm [183]. Mucus has specific viscous properties as it consists of mucins: heavily glycosylated and charged glycoproteins consisting of peptide repeats and coupled by disulfide bridges. In the colon, where the number of bacteria exceeds 10^{11} bacteria per gram of intestinal content, the mucin is more sulfated giving it a strong negative charge, which renders it less sensitive to degradation by bacterial enzymes. To date, 16 mucin (*MUC*) genes encoding for the peptide backbone have been described and 2 classes of mucins established. The membrane associated mucins (MUC1, 3, 4, 11–13 and 15–17) have been proposed to have a role in interacting with intracellular proteins by way of the C-terminal cytoplasmic tail. The gel-forming mucins (MUC2, 5AC, 5B and 6–9) are able to form polymers and are responsible for the viscosity of mucus. *MUC* gene expression is specific to the location in the body. In the intestine, MUC1–4 are expressed with MUC 2 being the major secreted mucin, whereas in the stomach MUC1, 5AC and 6 are expressed with a predominance of MUC5. As it is an abundant and accessible carbon and nitrogen source, mucus is degraded by a variety of intestinal bacteria. It has been estimated that approximately half of the carbon in the human intestine is salvaged from mucus. The continuous production of mucin by the host and its oligosaccharide-rich composition make the intestine a niche for bacteria; however, despite its constant secretion, the majority of intestinal bacteria are not able to utilise mucin. Hoskins and Boulding [123] showed that only 1% of the total cultivable intestinal microbiota was able to produce the panel of enzymes required to degrade the complex structure of mucin into free sugars. The isolated mucin degrading species are members of the genera *Bifidobacterium*, *Bacteroides* and *Ruminococcus*; more recently a human-specific mucin-degrading bacterium, *Akkermansia muciniphila*, has been isolated from adult faeces, which uses mucin as a nitrogen and carbon source [66]. Full degradation of mucin is a complex activity because of the structure of mucin itself. Even though some pure cultures are able to produce a panel of enzymes, it is accepted that it is a cooperative activity. Mucin-associated TFFs have been shown to increase the viscosity of the mucus and to promote its protective role [295]. TFFs have a cysteine-rich structure, which is able to form a three-loop domain (trefoil) that renders the molecule dense and

resistant to degradation by intestinal proteases. Hence, the degradation of TFFs has not been reported. However, recent studies have addressed the microbial production of TFFs to establish their crucial role as protectants of the epithelium. A new therapeutic approach to treat acute colitis (damaged epithelium) has been developed on the basis of a *Lactococcus lactis* strain that is able to produce murine TFFs. Mice with acute colitis were inoculated with this strain and TFF production was found to decrease the mortality of mice [308]. Although the turnover of TFFs appears to be limited, it is relevant to consider the degradation of mucin, as binding to mucin or its degradation are viewed as either a pathogenic or a probiotic criterion. From a positive point of view, the degradation of mucin releases nutrients that other bacteria unable to degrade mucin can then use. However, a possible negative effect is the damage to the protective layer. This strong contradiction demonstrates the lack of understanding of what these mucin-associated bacteria do. Pathogens as well as probiotics or commensals are able to modulate the mucin synthesis by regulating some of the *MUC* genes. A global increase in the synthesis and secretion of mucin by epithelial cells seems to be a common activity in response to the presence of bacteria (reviewed in [63]).

Table 2.1. Secreted substances from the human host, their properties, production sites and secretion levels.

Compound	Properties	Produced by	Human secretion[a]	Reference
Bile	Deoxycholate salts	Hepatocytes	1 liter	[14]
Mucus	Cystein-rich glycoprotein	Goblet cells	3-5 g[b]	[248]
TFF	Cystein-rich peptide	Goblet cells	ND	
sIgA	Glycoprotein-Imunoglobulin	Plasma cells	3-5 g	[51]
SC	Proteolytic fragment of pIgR	Epithelial cells	Similar as IgA	[51]

[a] Secretion per day; [b] level secreted in the colon; ND: Not determined; TFF: Trefoil Factor Peptide

Immunoglobulin A and bile

Other secretions such as IgA and bile are also degraded and/or hydrolysed by the intestinal microbiota. In general, however, this does not lead to the release of energy, but to protection. A major percentage of the faecal microbes is coated with IgA [306]; this coating prevents the induction of immune reactions against commensal residents. However, in patients with inflammatory bowel disease (IBD) a higher proportion of IgA-coated bacteria is found, which might be explained as a disturbance to the microbiota in these patients [305]. One other major intestinal secretion is the bile (reviewed in [14]). Bile is a natural antimicrobial that is synthesised in the liver and composed of lipids (e.g. cholesterol and phospholipids), bile acids, bile salts and pigments. Mucus and IgA are also secreted in the bile. Cholic acid and chenodesoxycholic acid

are the most abundant primary biles acids, derived from cholesterol catabolism in the liver. They are conjugated with either glycine or taurine to give the bile salts glycocholate, glycochenodesoxycholate, taurocholate and taurochenodesoxycholate. The main conversion of bile by bacteria is deconjugation. The released amino acids can be used as substrates for endogenous bacteria. A *Clostridium* strain was isolated from rat faeces and shown to use taurine as a nutrient [127].

Conclusions

The field of prebiotic research is still young, yet the progress made in elucidating the beneficial health effects of specific prebiotics is significant. To obtain consistent evidence about the health effects of prebiotics, long-term, double-blind randomised placebo-controlled human intervention studies in different populations are needed. This will require the development of methods that are suitable for large-scale human studies, as well as the understanding of the individual and intra-individual variation of biomarkers. Furthermore, as with probiotics, prebiotics are likely to have substance-specific effects. Generalisation between prebiotics is not acceptable. For the development of new prebiotics it might also be important to understand the functioning of endogenous substrates and to supplement or mimic them.

The potential future directions for prebiotics are numerous and only a small number of possibilities have been exploited, both in terms of anatomical and microbial targets as well as prebiotic substrates and target groups. Sustainable fermentation and additional properties, unrelated to fermentation, should be explored.

CHAPTER 3

Monitoring of the Bacterial Mucin-Degrading Consortium from the Human Intestinal Tract

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Mucins are glycoproteins that are produced in large quantities in the human intestinal tract and have functions in protection, lubrication and hydration. Moreover, they constitute an important host-produced substrate for intestinal bacteria. There is limited insight into the diversity of mucin-degrading bacteria in humans. Cultivation approaches allowed the isolation of several pure cultures able to grow when mucins are supplied. Molecular approaches based on the 16S rRNA gene allow the analysis of bacterial diversity without cultivation. In this study the molecular diversity of faecal mucin-degrading bacteria was analysed using denaturing gradient gel electrophoresis (DGGE) of the amplified 16S rRNA genes, followed by their cloning and sequencing. The DGGE analysis revealed the presence of an enormous bacterial diversity after growth of faecal inocula in media where mucin is the only source of carbon. The cloning and sequence analysis of a total of 26 predominant amplicons revealed that most of them corresponded to uncultivated species (69%). 19 out of 26 of the sequenced 16S rRNA clones, were related to sequences from *Ruminococcus* and *Clostridium* and the remaining clones belonged to *Verrucomicrobium, Eubacterium, Desulfovibrio,* and *Escherichia*. We conclude that *Ruminococcus* and *Clostridium* are the major mucin-degrading bacteria but that the consortium is composed of different groups of intestinal bacteria.

Introduction

The gastrointestinal tract (GI tract) is covered with a mucus layer comprised of high molecular weight glycoproteins, termed mucins, that can serve as a barrier to protecting the underlying epithelium from pathogens attachment. They also serve as a source of nutrients for commensal bacteria. The constant availability of these host glycans provides a major growth factor for colonisation of intestinal microorganisms [240]. However, excessive degradation of mucin may be considered as a virulence factor, since loss of the protective mucus layer may expose gut cells more to pathogens [244, 329]. Under normal circumstances, mucin-degrading bacteria live in mutual coexistence with host cells and the rate of degradation is balanced with the rate of synthesis by goblet cells. However, a disturbance of the mucus layer has been shown in cases of chronic inflammatory bowel disease (IBD) such as Crohn's disease and Ulcerative Colitis [225] where the origin and the activity of bacteria are still unclear. A link between IBD and intestinal sulphate-reducing bacteria is suspected but not fully confirmed [63]. Improved knowledge of the microbiota, the gastrointestinal tract and its mucus would help in understanding the role of these microorganisms in health and disease.

Based on cultivation techniques and using samples from healthy adults, it has been estimated that 1% of the cultivable colonic microbiota is able to degrade host mucin using specific enzymes [187] and the responsible bacteria were identified as strains of *Ruminococcus torques*, *Ruminococcus gnavus*, *Bifidobacterium bifidum* or *Clostridium* species.

The analysis of the faecal microbiota has been for long dependent on cultivation approaches. The major inconvenient of this technique is that many intestinal bacteria are not able to grow on laboratory classical media and that when they can be cultivated, their identification remains fastidious. Molecular tools based on the 16S rRNA gene provide a good alternative to it, since it encompasses the need to cultivate microbes. The 16S rRNA strategies have been widely used in the intestinal field and brought new information about the diversity of the intestinal microbiota. Amongst them, denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are molecular fingerprinting tools that have been used frequently in intestinal microbial ecology. When combined with sequencing of 16S rRNA clones, they allow the identification of members of the microbial community.

In our study, we used a strategy combining both culture-based approaches and -independent methods in order to describe the microbiota able to utilise mucin or a part of it, as measured by growth in a medium containing mucin as sole carbon source. The enrichments were analysed by denaturing DGGE of PCR-amplified 16S ribosomal RNA (rRNA) sequences, followed by identification of the major bands by cloning and sequencing.

Material and methods

Enrichment of mucin-degrading bacteria from faeces. 0.5 g of faeces from six healthy adult volunteers was diluted into 9 ml of sterile anaerobic Ringers solution containing 0.5 g/l of cystein. This suspension was thoroughly mixed and serially 10-fold diluted in Ringers solution. Each dilution (1 ml) was inoculated into 9 ml of a bicarbonate-buffered medium, containing 0.25% (vol/vol) of commercial hog gastric mucin (Type III, Sigma, St. Louis, Mo, USA), as previously described [66].

DNA extraction from enriched cultures and faecal samples. DNA was isolated from the faecal samples (0.2 g) and enriched cultures (5 ml) as previously described [331].

PCR amplification for DGGE. Primers 968-GC-F and 1401-R were used to amplify the V6 to V8 regions of the bacterial 16S rRNA genes (Table 3.1). PCRs were performed with a *Taq* DNA polymerase kit from Life Technologies (Gaithersburg, Md.). Each 50-µl PCR mixture contained each deoxynucleoside triphosphate at a concentration of 10 mM, 1.25 U of *Taq* polymerase, 10 µmol of each primer, and 1 µl of a DNA solution. The samples were amplified with a PE Applied Biosystems GenAmp PCR system 9700 (Foster City, Calif., USA) using the following program: pre-denaturation at 94°C for 5 min, 35 cycles of denaturation 94°C for 30 sec, annealing temperature of 56°C for 20 sec, extension at 68°C for 40 sec, and final extension at 68°C for 7 min. The integrity of the nucleic acids was checked visually after gel electrophoresis on a 1.2% agarose gel containing ethidium bromide.

DGGE of PCR amplicons. PCR fragments, were separated by DGGE by using the specifications of Muyzer *et al*. [197] and the Decode system (Bio-Rad Laboratories, Hercules, Calif., USA), with the following modifications. Polyacrylamide gels (dimensions, 200 by 200 by 1 mm) consisted of 8% (vol/vol) polyacrylamide (ratio of acrylamide-bisacrylamide, 37.5:1) and 0.5x Tris-acetate-EDTA (pH 8.0) (TAE) buffer; 100% denaturing acrylamide was defined as 7 M urea and 40% formamide. The gels were poured from the top by using a gradient maker and a pump (Econopump; Bio-Rad Laboratories) set at a speed of 5 ml per min. We used 30 to 60% gradients to separate products amplified with universal primers. Before polymerisation of the denaturing gel (gradient volume, 28 ml), a 7.5-ml stacking gel without denaturing chemicals was added, and the appropriate comb was subsequently inserted. Electrophoresis was performed in 0.5x TAE buffer first for 5 min at 200 V and then for 16 h at 85 V at a constant temperature of 60°C. The gels were stained with $AgNO_3$ as described by Sanguinetti *et al.* [252], scanned at 400 dpi, and

analysed using the Bionumerics software package version 3.0 (Applied MathS, Kortrijk, Belgium). The similarity between the DGGE profiles was determined by calculating similarity indices of the densiometric curves of the profiles compared using the Pearson product-moment correlation [105, 331]. The UPGMA algorithm was used as implemented in the analysis software for the construction of dendrograms.

Table 3.1. Oligonucleotides primers used in this study.

Primers	Sequence (5'-3')	Temp (°C)	Reference
968- GC F	CGCCCGGGGCGCGCCCCGGGCGGGGCGGG GGCACGGGGGGAACGCGAAGAACCTTAC	56	[205]
1401R	CGG TGT GTA CAA GAC CC	56	[205]
8F	CACGGATCCAGAGTTTGAT[C/T][A/C]TGGCTCAG	52	[205]
1510R	GTGAAGCTTACGG[C/T]TACCTTGTTACGACTT	52	[205]
T7	TAATACGACTCACTATAGG	44	Promega
SP6	GATTTAGGTGACACTATAG	44	Promega

Cloning and sequencing of the PCR-amplified products. Primers 8F and 1510R were used for construction of the clone libraries for bacterial 16S rDNA (Table 3.1). The following PCR program was used: 94°C for 5 min, 35 cycles consisting of 94°C for 1 min 30 s, 52°C for 30 s, and 68°C for 1 min, and finally 68°C for 7 min. The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and were cloned into *Escherichia coli* JM109 by using the pGEM-T vector system (Promega, Madison, Wis.). Colonies of ampicillin-resistant transformants were added to 20 µl of Tris-EDTA buffer and boiled for 15 min to lyse the cells. A PCR was performed by using the cell lysates as the template and pGEM-T-specific primers T7 and SP6 to check the sizes of the inserts. The following program was used: 94°C for 3 min, 35 cycles of 94°C for 30 s, 44°C for 30 s, and 68°C for 1 min 30 s, and finally 68°C for 7 min. The migration profiles of the amplified V6 to V8 regions of the clones were compared to the DGGE profile obtained with the same faecal sample in order to identify the origin of a band. Plasmids were purified with a QIAprep Spin miniprep kit (Qiagen, Hilden, Germany) and were used for sequence analysis (Westburg, Leusden, The Netherlands). Similarity searches with sequences in the GenBank database were performed by using the BLAST algorithm at the NCBI database. Alignment and further phylogenetic analysis of the sequences was done using ARB software package [166] and the tree was constructed using the neighbour joining method.

Nucleotide sequence accession numbers. The sequences of the enriched cultures clones obtained from the six faecal samples (26 sequences that were 410 to 1510 bases long) have been deposited in the GenBank database under accession numbers AY451995-AY452019.

Results and discussion

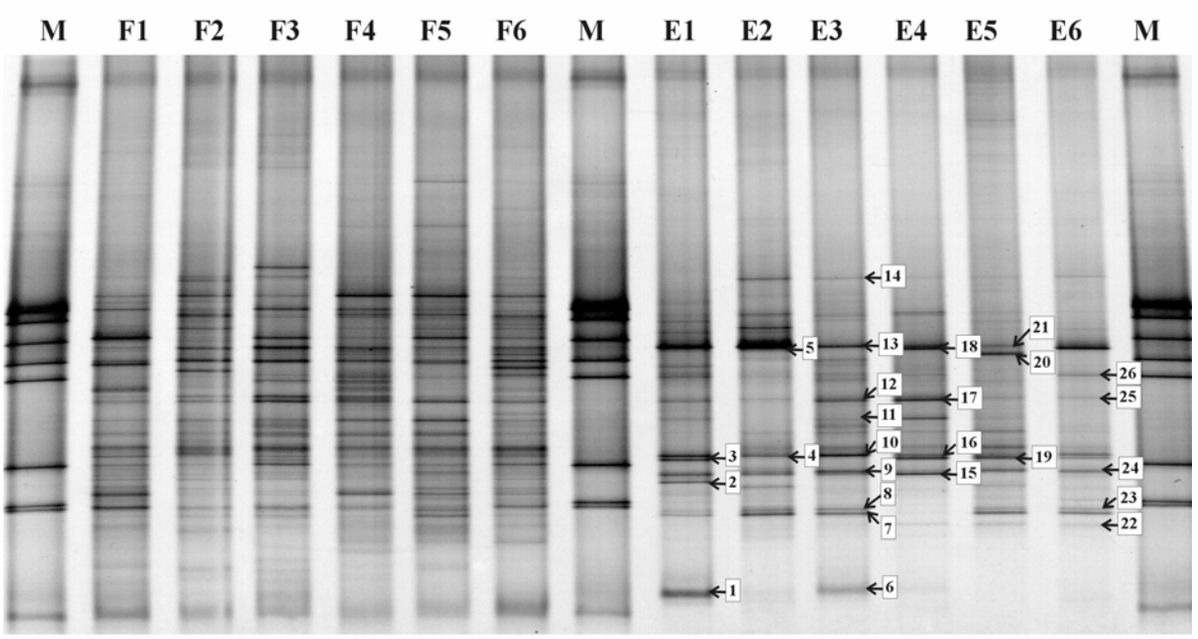
In this study we have determined the diversity of bacterial communities degrading mucin using a molecular, culture-independent approach based on the 16S rRNA gene.

PCR-DGGE

Fresh faecal samples were collected from six healthy volunteers and inoculated in a basal medium containing hog gastric mucin as sole carbon source, and serially diluted until dilution 10^{-3}. DNA was extracted from both faeces and the enriched cultures, and V6 to V8 regions of the 16S rRNA genes from the faeces and enrichment cultures were analysed by DGGE (Fig. 3.1A).

The number of DGGE bands was calculated for all samples. There was a significant difference in bands ($p<0.05$) between the faeces group (28 ± 4.8) and the enrichment cultures group (15.5 ± 2.0). In order to determine the changes between the bacterial faecal community and mucin-associated bacteria enriched in the specific medium, a similarity index was calculated based on the Pearson correlation coefficient. Then, cluster analysis was performed using the UPGMA algorithm, and dendogram generated. The analysis revealed that faecal samples clustered separately from the enriched cultures (27.5 ± 11.1 % of similarity). Profiles of the enrichment cultures showed a large diversity of mucin-degrading bacteria consortium that differed between the persons. However, we could observe that some dominant bands in the profiles seemed to be shared between individuals. The cluster analysis indicated that when analysed separately, the faeces group had an average similarity index of 52.7 ± 3.5% and the enriched cultures group, 57.4 ± 9.2 % (Fig. 3.1B). Thus, the mucin-degrading or associated bacterial community is diverse and individual-specific.

A



B

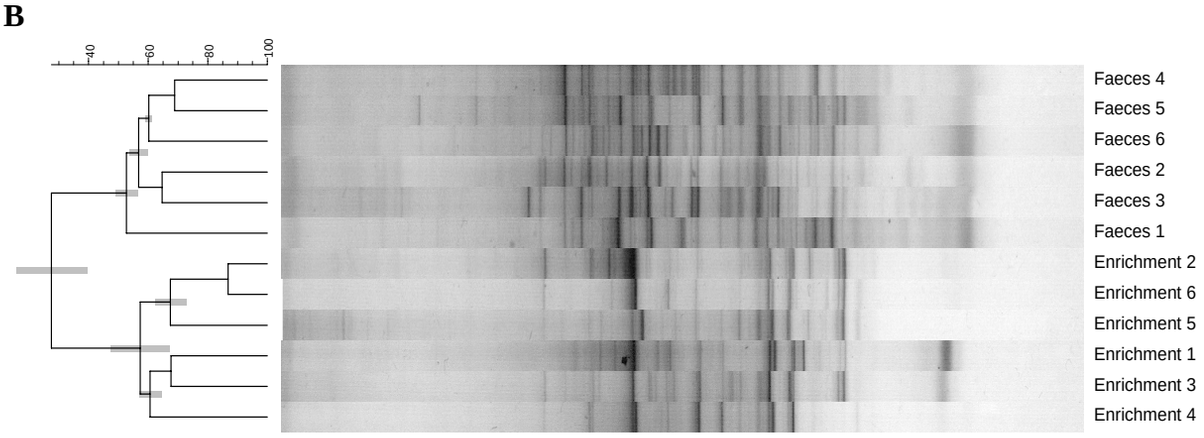


Figure 3.1. (A) Silver-stained DGGE gel showing profiles which represent the dominant communities of faeces from six individuals (F) and enrichment (E) cultures on mucin medium. M represents the DGGE marker. The bands identified from the 16S rRNA gene clone libraries are numbered. The origin of the bands is presented in Table 3.2. (B) UPGMA dendrogram illustrating the correlation between the different DGGE profiles. The grey bars represent the error bars.

Phylogenetic analysis of dominant bands in the DGGE patterns

A clone library of each enrichment culture was constructed from the 16S rRNA gene. In total, 26 clones, representing the major DGGE bands from the six enrichment cultures were sequenced and a phylogenetic tree was constructed (Fig. 3.2). Amongst the 26 sequenced clones, 18 (69%) were related to bacterial sequences with a similarity lower than 98% suggesting that they have not hitherto been cultivated (Table 3.2). This revealed that the majority of the sequences obtained from the clone libraries originated from strict anaerobic Gram-positive bacteria, and to a lesser extent from Gram-negative bacteria.

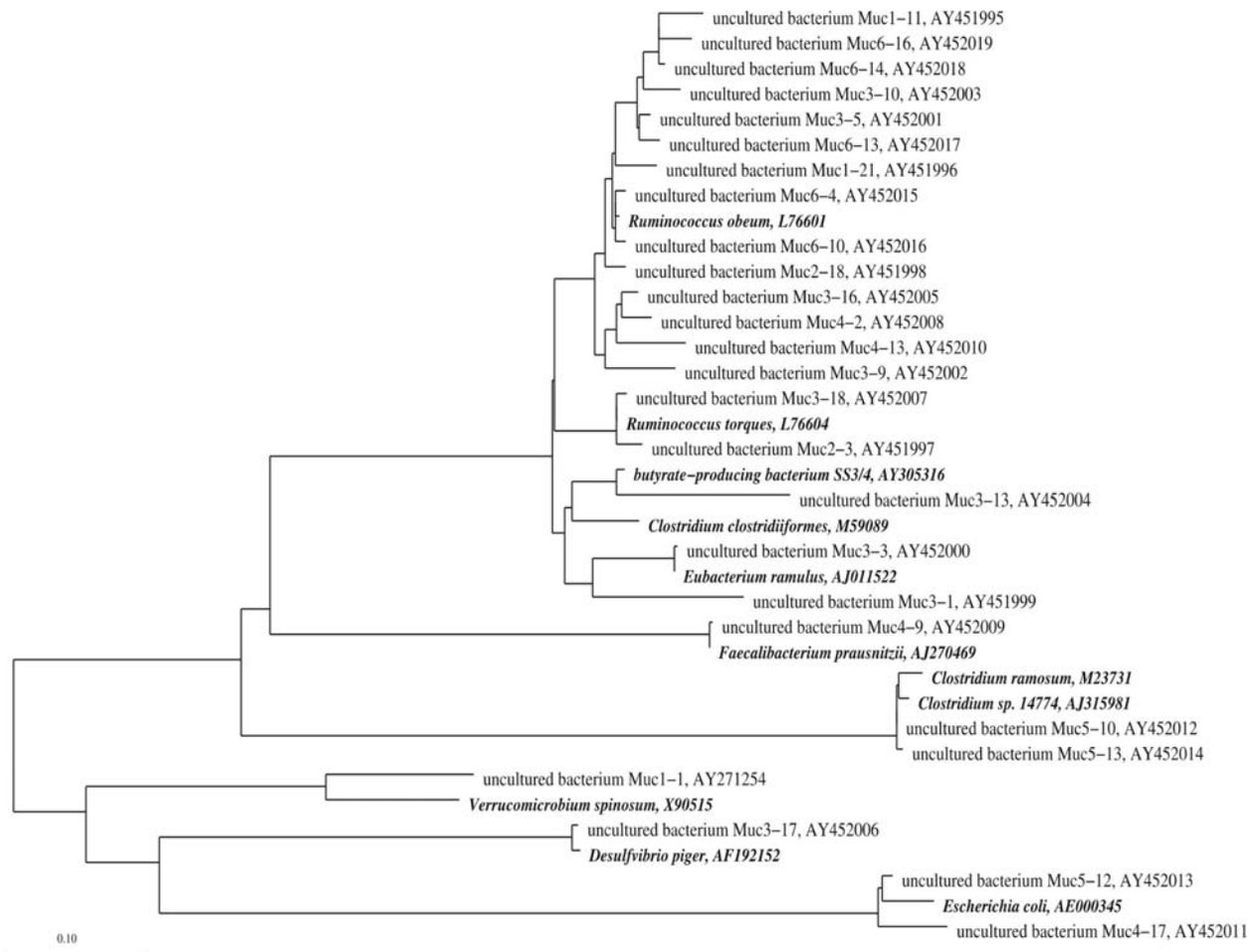


Figure 3.2. Phylogenetic tree derived from the 16S rRNA sequence data. Bar represents 10% sequence divergence. Analyses were performed with the ARB software package [166].

Table 3.2. Identification of dominant bands from the enrichment cultures from Fig. 3.1. Listed are the closest relatives of the clones corresponding to the bands, their level of identity with the closest relative, class, clone designation, and accession number.

Species	%	Phylum/16S based group	Bp	Clones	GenBank Accession
Verrucomicrobium spinosum	92	Verrucomicrobia	1433	1-1	AY271254
Ruminococcus obeum	99	*Clostridium* cluster XIVa	522	1-21	AY451995
Ruminococcus obeum	95	*Clostridium* cluster XIVa	551	1-11	AY451996
Ruminococcus torques	96	*Clostridium* cluster XIVa	438	2-3	AY451997
Ruminococcus obeum	96	*Clostridium* cluster XIVa	534	2-18	AY451998
Clostridium clostriiformes	95	*Clostridium* cluster XIVa	978	3-1	AY451999
Eubacterium ramulus	99	*Clostridium* cluster XIVa	1414	3-3	AY452000
Ruminococcus obeum	96	*Clostridium* cluster XIVa	1457	3-5	AY452001
Ruminococcus obeum	92	*Clostridium* cluster XIVa	1457	3-9	AY452002
Ruminococcus obeum	95	*Clostridium* cluster XIVa	1457	3-10	AY452003
Butyrate-producing bacterium SS3/4	96	*Clostridium* cluster XIVa	1004	3-13	AY452004
Ruminococcus obeum	94	*Clostridium* cluster XIVa	1457	3-16	AY452005
Desulfovibrio piger	99	Delta-Proteobacteria	1505	3-17	AY452006
Ruminococcus torques	99	*Clostridium* cluster XIVa	1418	3-18	AY452007
Ruminococcus obeum	99	*Clostridium* cluster XIVa	410	4-2	AY452008
Faecalibacterium prausnitzii	96	*Clostridium* cluster IV	543	4-9	AY452009
Ruminococcus productus	93	*Clostridium* cluster XIVa	472	4-13	AY452010
Escherichia coli	96	Gamma-Proteobacteria	502	4-17	AY452011
Clostridiun ramosum	98	*Clostridium* cluster XVIII	534	5-10	AY452012
Escherichia coli	99	Gamma-Proteobacteria	533	5-12	AY452013
Clostridiun ramosum	97	*Clostridium* cluster XVIII	546	5-13	AY452014
Ruminococcus obeum	98	*Clostridium* cluster XIVa	538	6-4	AY452015
Ruminococcus obeum	97	*Clostridium* cluster XIVa	520	6-10	AY452016
Ruminococcus obeum	97	*Clostridium* cluster XIVa	512	6-11	AY452017
Ruminococcus obeum	95	*Clostridium* cluster XIVa	524	6-14	AY452018
Ruminococcus obeum	96	*Clostridium* cluster XIVa	506	6-16	AY452019

Among the 26 bacterial 16S rRNA clones sequenced from six human faecal samples inoculated in a mucin based medium, 19 (73%) were related to the *Clostridium* cluster XIVa (*Clostridium coccoides* group). The remaining clones belonged to the *Clostridium* cluster IV (*Clostridium leptum* group) and XVIII, *Proteobacteria* and *Verrucomicrobia*.

In the gut, mucin is an important source of energy for bacteria since it is composed of both amino acids and carbohydrates. Its complexity renders its degradation by bacteria challenging. Indeed, several specific enzymes such as glycosidases, proteases, sialidases and sulphatases are necessary to fulfil complete degradation of mucin [1]. Most intestinal bacteria obtain readily absorbable nutrients from the host diet. However, bacteria can benefit from mucin in distinct ways: either directly by degrading it when they possess the appropriate enzymes to do so or indirectly by taking advantage of the end products from this degradation. Therefore, mucin-degrading bacteria play an important ecological role since they provide nutrients for other bacteria. This could explain why genera that are not known to be able to degrade mucin, such as *Escherichia*, *Eubacterium, Fusobacterium* or *Verrucomicrobium* were also observed in the consortia, or that have not been yet cultivated.

This study demonstrated that a complex community is associated with the degradation of mucin in the GI tract, from which a major part has not yet been isolated. These new members of this community, their role in mucin degradation and host health remain to be fully elucidated.

Acknowledgements

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CHAPTER 4

Akkermansia muciniphila, gen. nov., sp. nov., a Human Intestinal Mucin-Degrading Bacterium

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The diversity of mucin-degrading bacteria in the human intestine was investigated by combining culture and 16S rRNA-dependent approaches. A dominant bacterium, strain Muc^T, was isolated by dilution to extinction of faeces in anaerobic medium containing gastric mucin as the sole carbon and nitrogen source. A pure culture was obtained by using the anaerobic soft agar technique. Strain Muc^T was a Gram-negative, strictly anaerobic, non-motile, non-spore forming, oval-shaped bacterium that could grow singly and in pairs. When grown on mucin medium the cells produced a capsule and were found to aggregate. Strain Muc^T could grow on a limited number of sugars, including N-acetylglucosamine, N-acetylgalactosamine and glucose but only when a protein source was provided and with a lower growth rate and final density than on mucin. The G+C content of the DNA from strain Muc^T was 47.6 mol%. 16S rRNA gene sequence analysis revealed that the isolate was part of the division Verrucomicrobia. The closest described relative of strain Muc^T was Verrucomicrobium spinosum showing 92% sequence similarity. Remarkably, the 16S rRNA gene sequence of strain Muc^T showed 99% similarity to three uncultured colonic bacteria. According to the data obtained in this work, this bacterium belongs to a new genus in subdivision 1 of the Verrucomicobia and the name *Akkermansia muciniphila* gen. nov., sp. nov. is proposed. The type strain is Muc^T. (=ATCC BAA-835^T =CIP 107961^T).

Introduction

The human gastrointestinal tract (GI-tract) harbours diverse and abundant microbiota, which have effects on the health and disease of the host, due to their close association [116]. A mucus layer covers the GI-tract providing a protective barrier for the underlying epithelium against pathogenic microorganisms as well as chemical, physical or enzymatic damage. Mucus is a viscous gel mainly composed of high molecular weight glycoproteins, termed mucins. Mucins have a large molecular weight and are composed of a peptide core rich in serine and threonine residues, that is decorated by oligosaccharides linked via O- or N-glycosidic bonds. The oligosaccharides are composed of one or more four primary sugars, N-acetylglucosamine, N-acetylgalactosamine, galactose and fucose and are terminated by sialic acids or sulphate groups. This mucus layer is considered to be an ecological niche for the intestinal microbiota. However, the association of the microbiota with the mucus is not well understood. Mucus can serve as a barrier to protect the underlying epithelium from the attachment of pathogens and also serves as a source of nutrients for commensal bacteria. Degradation of mucin is regarded as a pathogenicity factor since loss of the protective mucus layer may expose GI-tract cells more to pathogens [243, 329]. However, mucin also constitutes a carbon and energy source for intestinal microbiota. It has been estimated that 1% of the colonic microbiota is able to degrade host mucin using enzymes (glycosidases, sulphatases) that can degrade the oligosaccharide chains [123]. Despite the apparent low percentage of mucin-degrading bacteria, they provide nutrients for other resident bacteria which can use the monosaccharides or amino acids released from the degradation of the mucin structure. Based on their capacity to grow on mucin-containing media, isolates belonging to the genera *Ruminococcus, Bacteroides, Bifidobacterium* and *Clostridium* were demonstrated to degrade mucin [249]. By measuring the release of reducing sugars monomers from the mucin polymer, it was observed that only mixed cultures of faecal bacteria were able to degrade mucin for more than 90%, whereas pure cultures of *Bacteroides fragilis, Bifidobacterium longum* and *Clostridium perfringens* showed partial degradation [318]. It is therefore likely that, in vivo, a cooperative process is required to achieve an efficient degradation of the complex structure of mucin.

The introduction of high resolution molecular techniques has improved analyses of complex microbial ecosystems. The most important advance has been the use of 16S ribosomal RNA gene (16S rRNA) as a molecular fingerprint to analyse microbial diversity. These molecular approaches have indicated that cultivation has filtered our view of the intestinal microbiota [309]. As a consequence, a substantial proportion of the microbiota has not yet been cultured nor described [285, 330] and this may be due mainly to the lack of appropriate cultivation techniques.

However, new, alternative, and improved cultivation approaches are continuously being developed and recently a variety of new species and genera have been cultured from the GI-tract: *Roseburia intestinalis* sp. nov. [73], *Campylobacter hominis* sp. nov. [151], *Ruminococcus luti* sp. nov. [271], *Anaerostipes caccae* gen. nov., sp. nov. [263], *Dorea longicatena* gen. nov., sp. nov. [291] and *Victivallis vadensis* gen. nov, sp. nov. [334].

In the present study, mucin-degrading bacteria from human faeces were enriched using an MPN approach in which the medium contained mucin as the sole carbon and energy source. The enrichments were analysed by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene sequences. A single DGGE type dominated all the positive MPN enrichments. The organism corresponding to the dominant DGGE type was isolated and characterised, and represents a yet novel intestinal bacterium, strain Muc^T, that is able to use gastric mucin in pure culture.

Material and methods

A faecal sample from a healthy adult volunteer was freshly collected in a polyethylene bag and 0.5 g was diluted into 9 ml of sterile anaerobic Ringers solution containing 0.5 g/l of cystein. This suspension was thoroughly mixed and serially 10-fold diluted in Ringers. Each dilution (1 ml) was inoculated in triplicate into 9 ml of a bicarbonate-buffered medium. This basal medium contained (per liter): 0.4 g KH_2PO_4; 0.53 g Na_2HPO_4; 0.3 g NH_4Cl; 0.3 g NaCl; 0.1 g $MgCl_2$.6H2O; 0.11 g $CaCl_2$; 1 ml alkaline trace element solution; 1 ml acid trace element solution; 1 ml vitamin solution; 0.5 mg resazurin; 4 g $NaHCO_3$; 0.25 g Na_2S.7-9H_2O. The trace elements and vitamins were as described previously [281]. All compounds were autoclaved except the vitamins, which were filter-sterilised. This basal medium was supplemented with 0.7% (vol/vol) of clarified, sterile rumen fluid and 0.25% (vol/vol) of commercial hog gastric mucin (Type III, Sigma), purified by ethanol precipitation as described previously [187]. This medium is further referred to as mucin medium. Unless indicated, incubations were done in serum bottles sealed with butyl rubber stoppers and at 37°C under anaerobic conditions provided by a gas phase of 182 kPa (1.8 atm) N_2/CO_2 (80:20, vol/vol).

Enrichments were done in 30-ml serum bottles with 10 ml liquid volume. Negative controls comprised one series of mucin media that was not inoculated and another series that was inoculated but not supplemented with mucin. Mucin-degrading bacteria were quantified using the most probable number (MPN) technique (n=3). The soft agar technique was used in order to isolate a pure culture as follows: the highest dilution where growth was observed was serially diluted in phosphate buffer (pH 7) until 10^{-9} dilution and the 10^{-6} to 10^{-9} dilutions were re-

inoculated into the same medium containing 0.75% agar (agar noble, Difco). Single colonies were picked, grown in mucin medium and re-inoculated in soft agar mucin medium. This step was repeated until purity.

Generation times were determined in mucin medium and growth was analysed in triplicate by measuring absorbance at 600 nm. The optimum pH and temperature were measured in triplicate in Brain Heart Infusion (BHI, Difco), supplemented with 1 mM Na_2S. The optimum temperature was tested from 4 to 45°C with 5°C intervals, and the optimum pH was measured over a range from pH 5 to 9 with 0.5 pH unit intervals (adjusted with HCl or NaOH), at 37°C. Cultures were incubated for at least one month.

Potential substrates for growth were tested at a concentration of 10 mM in the same liquid basal medium, or in basal medium supplemented with peptone, tryptone, casitone and yeast extract at a concentration of 0.5 or 2 g/l. The cultures were incubated for up to four weeks. Human gastric mucin isolated from HT-29 MTX human intestinal cell lines and this mucin was added to the basal liquid medium at a concentration of 0.05%. Rich media BHI and Columbia broth (Difco), and Wilkens-Chalgren broth (WC broth, Oxoid, 16 g/l), were also tested as growth substrates. To test the origin of the nitrogen source, the solution containing NH_4Cl was not added to the mucin medium.

Cell morphology, motility and spore formation were investigated using phase-contrast microscopy. The Gram reaction was assessed using Gram-staining as previously described [219]. To test for the presence of a capsule, an India ink suspension was used.

For the transmission electronic microscopy of strain Muc^T, cells were fixed with 0.25% glutaraldehyde. Negative staining was performed on 400 copper-mesh grids with glow-discharged parladion carbon-support film. Micrographs were recorded at a magnification of 40,000x on a Jeol 1010 electron microscope operating at 80 kV. For the scanning electronic microscopy, droplets of strain Muc^T were put on poly-L-lysine coated Nuclepore Polycarbonate membrane (Costar). These membranes were fixed 1 hour in 4% glutaraldehyde in growing medium. The specimens were dehydrated in a graded series of ethanol and critical point dried with carbon dioxide. The samples were glued on a sample holder by carbon adhesive tabs. Samples were sputter coated with 10 nm Platinum in a dedicated preparation chamber (CT 1500 HF) and analysed with a field emission scanning electron microscope (JEOL 6300 F) at 5kV.
The G+C content of the DNA of strain Muc^T was determined at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) by high performance liquid chromatography (HPLC) [185].

To monitor the dynamics of the human faecal mucin-degrading population, DGGE analysis of 16S rRNA gene amplicons was performed. DNA was extracted from the faecal sample, enrichment cultures and a pure culture isolated from the highest dilution. DNA isolation and the amplification of the V6 to V8 regions of the 16S rRNA gene from these samples were performed as previously described [330]. PCR fragments were separated by DGGE consisting of 8% (vol/vol) polyacrylamide (ratio of acrylamide-bisacrylamide, 37.5:1) and 0.5x Tris-acetate-EDTA (pH 8.0) (TAE) buffer; 100% denaturing acrylamide was defined as 7 M urea and 40% formamide. Gradients of 38 to 48% were used to separate products amplified with universal primers. After migration of the PCR products at 85V during 16 hours, the gels were stained with $AgNO_3$ as described previously [252].

PCR on the 16S rRNA gene of strain Muc^T was performed with universal primers 11f and 1510r [21]. The following PCR program was used: 94°C for 5 min, 40 cycles consisting of 94°C for 1 min 30 s, 48°C for 30 s, and 68°C for 1 min 30, and finally 68°C for 7 min. The PCR products were purified and concentrated with the Qiaquick PCR purification kit (Quiagen) according to the manufacturer's instructions. The purified 16S rRNA gene product was sequenced on both strands using infrared Dye 41 labelled primers 7f, 342r, 805f, 1100r, 1510r [147], and 968f [205]. One extra primer Muc1 (5' GGA AAC CCT GAT GGT GCG 3') which targets a 339-bp-specific region of the 16S rRNA gene sequence of strain Muc^T was designed to obtain unambiguous results. The sequences were automatically analysed on a LI-COR DNA sequencer 4000L, and corrected manually. Pairwise sequence alignment was performed with the DNASTAR program. The 16S rRNA gene sequence was compared to sequences from GenBank using the program BLASTN 2.0 available through the National Centre for Biotechnology Information (NCBI) internet site. The ARB software package was used to align the clone sequences and the 16S rRNA gene sequences of their nearest relatives [284]. A phylogenetic tree was constructed with ARB using the neighbour-joining method. The distance matrix used in the neighbour-joining method included stretches of sequence corresponding to *E. coli* positions 63-1491. The GenBank accession number for the 16S rRNA gene sequence of strain Muc^T is AY271254.

Substrates and fermentation product concentration were determined in the culture before and after growth using HPLC and gas chromatography (GC) methods as described previously [281].

Results and discussion

The use of serial dilution in an anaerobic medium containing mucin as energy source lead to the isolation of the predominant mucin-degrading bacterium from a human faecal sample. As in all MPN studies, the low dilution cultures presumably gave rise to the fastest growing organisms under the given culture conditions, whereas the high dilution cultures supported growth of the numerically dominant organisms. In our study, a single band generated by PCR-DGGE (Fig. 4.1) dominated all dilutions, indicating that there was one predominant mucin-degrading bacterium and that this was also the fastest-growing mucin-degrading organism. The MPN of mucin-degrading organisms present in this faecal sample was estimated at 8.3 (± 0.3) 10^9/g of faeces. No growth was observed in the uninoculated mucin medium, indicating the sterility of the mucin, nor in the medium inoculated with faecal dilutions but not supplemented with mucin. This indicates that growth occurred solely due to the utilisation of mucin by the faecal bacteria.

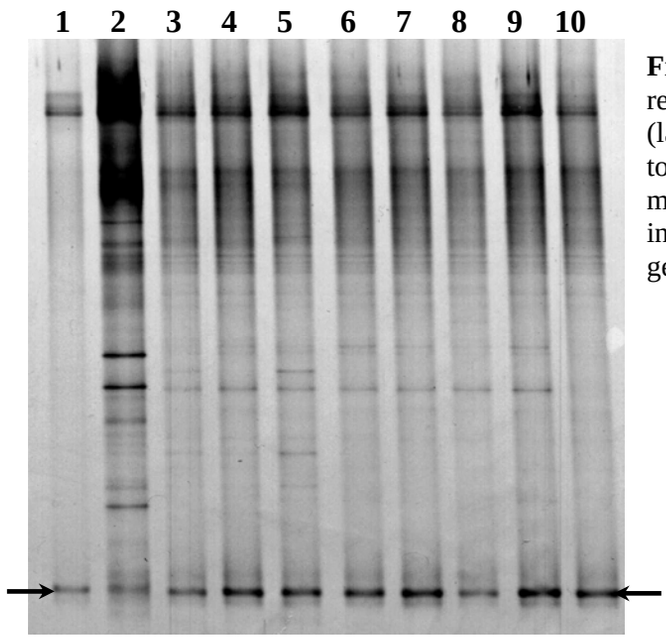

Figure 4.1. DGGE profiles of the V6 to V8 regions of 16S rRNA gene from strain Muc^T (lane 1), faecal sample (lane 2), and dilution 10^{-2} to 10^{-9} of the enrichment of faecal bacteria in mucin medium (lanes 3 to 10). The arrowheads indicate the migration position of the 16S rRNA gene amplicon of strain Muc^T.

Microscopic analysis of the enrichment dilutions revealed that an oval-shaped organism was predominant in the first dilutions of the enrichment. The 16S rRNA gene amplicons from all the dilutions of the enrichment where growth was observed (10^{-2} to 10^{-9}) were analysed by DGGE (Fig. 4.1, lanes 3-10). In the first dilutions, many bands were detected with a predominance of one type. This band became more intense with increasing dilution, and in the highest dilution where growth occurred (10^{-9}), this band was almost unique in the profile. The faecal sample profile showed a band at the same position, suggesting that the enriched micro-organism containing this 16S rRNA gene is the same as the one present in the faecal sample, and represents at least 1% of the total intestinal bacterial community [197]. The mucin-degrading bacterium,

whose 16S rRNA gene corresponded to the major DGGE band present in the enrichment (lane 10), was cultured from the highest dilution with growth (10^{-9}) using the soft agar technique. After 6 days, the most predominant colony type, white, was grown in the mucin medium. White colonies were picked, diluted in the mucin medium and transferred into soft agar mucin medium. This purification step was repeated twice. Finally, a single type of white colony appeared. Phase-contrast microscopy revealed only one morphotype, and the DGGE profile of the 16S rRNA gene amplicon of the strain isolated showed the presence of a unique band corresponding to the major band present in the enrichment (Fig. 4.1, lane 1). The pure culture was designated strain Muc^T.

An almost complete 16S rRNA gene sequence of strain Muc^T was determined (1433 bp). The most similar 16S rRNA gene sequences were 99% identical to strain Muc^T, and each derived from studies of uncultured colonic bacteria: HuCA18, HuCC13, [112] and L10-6 [250]. The cultured bacterium most closely related to strain Muc^T was *Verrucomicrobium spinosum*, and this was only distantly related (92%). A phylogenetic dendogram based on 16S rRNA gene sequences was constructed which revealed that strain Muc^T is related to the genera *Prosthecobacter* and *Verrucomicrobium* that are members of the order Verrucomicrobiales; thus Muc^T belongs to the division Verrucomicrobia and the class Verrucomicrobiae (Fig. 4.2). The majority of the members of this new division are only clones, and the few cultivated bacteria comprise the single-genus *Verrucomicrobium spinosum* [258] after which the division was named, four species of the genus *Prosthecobacter* (*P. debontii, P. dejongeii, P. fusiformis, P. vanneervenii*) [109, 280], *Opitutus terrae* [47] and other ultramicrobacteria [130] and the recently isolated *Victivallis vadensis*, the first member of the Verrucomicrobia division to be isolated from the GI-tract [334]. Members of the 'Verrucomicrobium' group of bacteria have also been identified in low numbers in human faecal derived 16S rRNA gene libraries [112, 285, 319]. The division Verrucomicrobia is composed of 5 subdivisions [125] and the genera *Prosthecobacter* and *Verrucomicrobium* are part of the subdivision 1. Bacteria from these two genera were isolated from freshwater habitats and are both Gram-negative, aerobic, and heavily fimbriated. Cells of *Verrucomicrobium* have many prosthecae, whereas cells of *Prosthecobacter* have only a single prosthecae. Strain Muc^T shares some of the common characteristics: it is Gram-negative, and can grow without vitamins. However, strain Muc^T is distinct among the members of subdivision 1 in that it is strictly anaerobic and cells are oval-shaped in contrast to the other members (See Table 4.1). On the basis of the phylogenetic analysis, strain Muc^T does not belong to the *Verrucomicrobium* nor *Prosthecobacter* cluster and should be considered as a separate phylogenetic branch. We therefore propose that strain Muc^T is a new genus belonging to the subdivision 1 of the Verrucomicrobia.

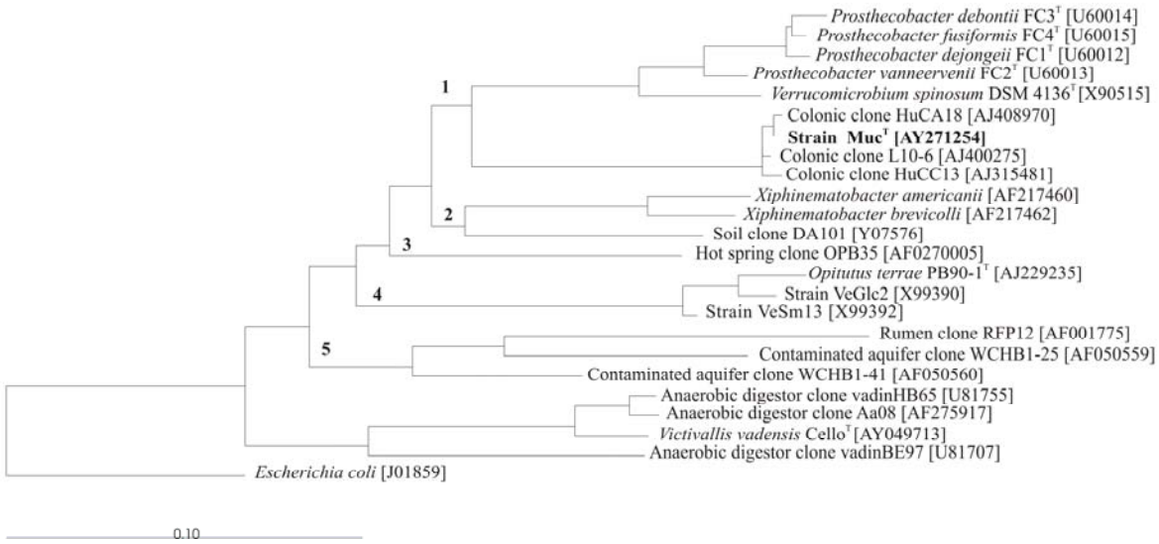

Figure 4.2. Phylogenetic tree showing the position of strain Muc^T among selected clones or strains belonging to the Verrucomicrobia division. The tree, which was rooted by using *Escherichia coli* as the outgroup, was generated by the neighbour-joining method. The numbers before the interior branch points indicate the five major lineages within the division Verrucomicrobia as proposed by Hugenholtz *et al.*, (1998). The bar represents 10% sequence divergence

Strain Muc^T is an obligate chemo-organotroph. No growth was detected on the basal medium supplemented with vitamins and purged with H_2/CO_2 (80:20). Rumen fluid and vitamins were not required for growth on mucin, and for further characterisation of the strain, they were not added anymore in the mucin medium. Growth was not observed in mucin medium in the absence of a reducing agent as indicated by the pink colour of the medium, demonstrating the strict anaerobic nature of strain Muc^T. The isolate could grow between 20°C and 40°C with an optimum at 37°C. The optimum pH for growth was 6.5. No growth was found below pH 5.5 and above 8. The doubling time of the strain was approximately 1.5 hour in mucin medium.

No growth was observed on glucose, cellobiose, lactose, galactose, xylose, fucose, rhamnose, maltose, succinate, acetate, fumarate, butyrate, lactate, casitone (0.5%), casamino acids (0.5%), tryptone (0.5%), peptone (0.5%), yeast extract (0.5%), proline, glycine, aspartate, serine, threonine, glutamate, alanine, N-acetylglucosamine, N-acetylgalactosamine after 4 weeks of incubation. Gastric mucin isolated from human intestinal cell lines and adapted on 10^{-5} M methotrexate (HT-29 MTX) to produce a high amount of mucin [156], resulted in growth of strain Muc^T to the same density as with hog gastric mucin. Strain Muc^T could also grow on rich media, Columbia and BHI but with a final optical density half that of the mucin medium. No growth was observed on the rich WC anaerobe broth. When 2 g/l of peptone, yeast extract, tryptone and casitone, were added to the basal medium, growth was observed only when the

sugars N-acetylglucosamine, N-acetylgalactosamine and glucose were added although bacterial growth was less than a quarter of that on mucin medium. When the solution containing the nitrogen source was not added to the basal medium supplemented with mucin, strain Muc^T could grow to the same density, indicating that the isolate obtained both the carbon and nitrogen source. Strain Muc^T showed no production of H_2 but acetate, propionate and ethanol were formed from mucin fermentation. No sulphides were produced. Sulphates were released during the fermentation of mucin (0.71 mM) demonstrating a sulphatase activity of strain Muc^T. We presume that the limited ability of strain Muc^T to grow on the many substrates tested may be due to the complex structure of the mucin, which is composed of both oligosaccharides and amino acids, and that strain Muc^T requires a combination of all these components to reach a high density. It is likely that strain Muc^T produces one or more appropriate glycosidases to degrade the N-acetylgalactosamine and N-acetylglucosamine components from mucin, which might be exposed in the terminal part, and to use them as growth substrates. The G+C content of the DNA of the isolate Muc^T was 47.6 mol%.

A **B**

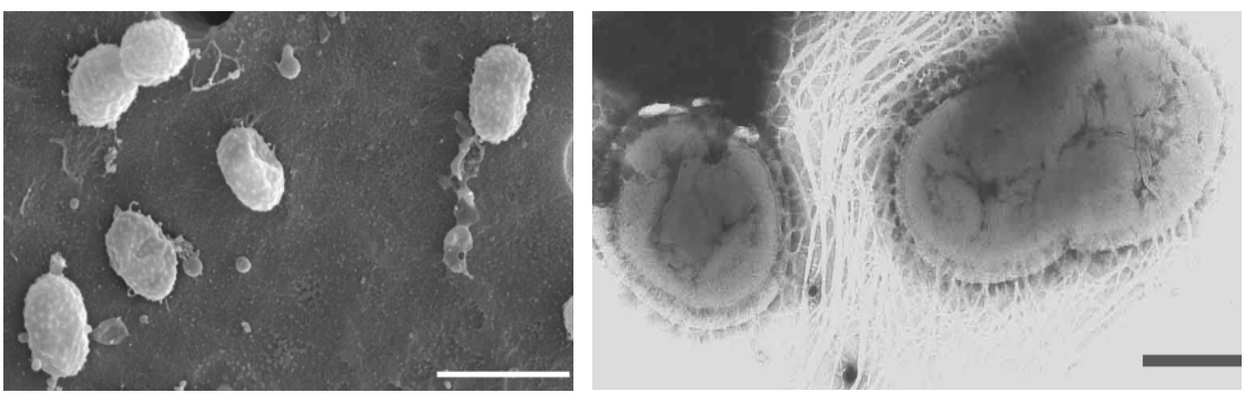

Figure 4.3. Electron microscopic images of strain Muc^T. (A) Scanning electron microscopic image. Bar, 1 µm. (B). Transmission electronic microscopic image of a negatively stained preparation. Note the thickened but extensive capsule fibres of the cells. Bar, 0.5 µm.

Cells of strain Muc^T were oval-shaped (Fig. 4.3A), showing a different size depending on the medium. In mucin medium, strain Muc^T was 640 nm in diameter and 690 nm in length and in BHI, strain Muc^T was 830 nm in diameter and 1µm in length. Cells stained Gram-negative. Flagella were not seen on negatively stained electron micrograph preparations. Formation of spores by the strain was never observed. In mucin medium, the organism could grow as single cells or in pairs, rarely in chains and often formed aggregates in which a translucent layer of material was observed between organisms. In BHI and Columbia media, this material was rarely if ever observed and growth occurred as single cells, in pairs and rarely in groups. In basal medium, supplemented with N-acetylglucosamine or N-acetylgalactosamine together with some

sources of proteins (a combination of yeast extract, peptone, tryptone, casitone), cells appeared mainly single and sometimes in pairs.

Cells of strain Muc^T grown in mucin medium could exclude Indian ink, characteristic of capsule-possessing bacteria. Electronic microscopy revealed the existence of filamentous structures on cells grown in mucin medium (Fig. 4.3B). We assume that these filaments are capsular polymers that are used to connect cells together. Since this aggregation is mainly observed in mucin medium, this capsule may aid in adhesion and colonisation of mucin-secreting epithelia in the GI-tract. On soft agar medium, colonies of strain Muc^T appeared white, with a size of 0.7 mm of diameter.

Table 4.1. Characteristics that differentiate the genus *Akkermansia* from other genera of the subdivision 1 of the Verrucomicrobia division. Data taken from Schlesner *et al.* (1987), Hedlund *et al.* (1997), Staley *et al.* (1976). +, positive; -, negative; w, weakly positive; ND, Not determined.

	Strain Muc^T	***Verrucomicrobium*†**	***Prosthecobacter*‡**
Cell morphology	Oval-shaped	Fusiform rod-shaped	Fusiform rod-shaped
Cell size (μm)	0.6-1	0.8-1.0 × 1.0-3.8	0.5 × 2-8
Tolererance to oxygen	-	+	+
Temperature range (°C)	20-40	26-34	1-40
Motility	-	-	-
Requirement of vitamins	-	-	-
Prosthecate	-	+	+
Fimbriae	-	+	+
Capsule	+	ND	ND
Growth on[#]			
Sugars			
Glucose	w[§]	+	+
Galactose	-	+	+
Fructose	-	+	+/- [*]
Cellobiose	-	+	+
N-acetylglucosamine	w[§]	+	+/- [*]
N-acetylgalactosamine	w[§]	ND	ND
Mucin	+	ND	ND
Amino acids	-	-	-
Other oraganic acids	-	-	-
Sensitivity to ampicillin	+	+	+
DNA G+C content (mol%)	47.6	57.9-59.3	54.6-60.1

[†] based on *Verrucomicrobium spinosum*
[‡] based on *P. debontii, P. dejongeii, P. fusiformis, P. vanneervenii*
[#] growth was determined by measuring OD_{600} in basal medium supplemented with the appropriate substrate (10mM final concentration; see Methods)
[*] depend on species.
[§] when a protein source is provided (peptone, yeast extract, tryptone, casitone at final concentration of 2% g/l each).

Based on morphological, physiological and phylogenetic features of the isolate Muc^T, we propose the description of a new genus, *Akkermansia*, with the type species *Akkermansia muciniphila* gen. nov., sp. nov. of which the type strain is strain Muc^T.

Description of *Akkermansia* gen. nov.

Akkermansia (Ak.ker.man' si.a. N.L. fem n. akkermansia, derived from Antoon Akkermans, name of a Dutch microbiologist recognised for his contribution to microbial ecology).

Cells are oval-shaped, non-motile, and stain Gram-negative. Strictly anaerobic. Chemo-organotrophic. Mucolytic in pure culture. Type species: *Akkermansia muciniphila*.

Description of *Akkermansia muciniphila* sp. nov.

Akkermansia muciniphila (mu.ci.ni' phi.la. N.L. neut. n. mucinum mucin, Gr. adj philos, friendly to, N.L. fem. adj. Muciniphila mucin-loving).

Cells are oval-shaped, non-motile, and stain Gram-negative. The long axis of single cells is between 0.6 µm and 1 µm, depending on the substrate used. Growth occurs as single cells, in pairs, short chains, and in aggregates. Growth occurs between 20°C and 40°C with an optimum at 37°C. The pH optimum for growth was 6.5 with a range of 5.5 to 8. Strictly anaerobic. Able to grow on gastric mucin, BHI and Columbia media, and on N-acetylglucosamine, N-acetylgalactosamine and glucose when these three sugars are in the presence of 2 g/l of peptone, yeast extract, casitone and tryptone. Cellobiose, lactose, galactose, xylose, fucose, rhamnose, maltose, succinate, acetate, fumarate, butyrate, lactate, casitone, casamino acids, tryptone, peptone, yeast extract, proline, glycine, aspartate, serine, threonine, glutamate do not support growth of the strain. Capable of using mucin as carbon, energy and nitrogen source. Able to release sulphate in a free form from mucin fermentation. In mucin medium, cells are covered with filaments. Growth occurs without vitamins. Colonies appear white, with a diameter of 0.7 mm in soft agar mucin medium. DNA G+C content of the type strain is 47.6 mol%. Isolated from human faecal sample.

The type strain: Muc^T (=ATCC BAA-835^T =CIP 107961^T).

Habitat: intestinal tract of the human.

Acknowledgements

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HT-29 MTX mucin. We thank Erwin G. Zoetendal for discussion and for critically reading the manuscript and Hauke Smidt for the help in the phylogenetic analysis. We thank Professor H. G. Trüper for his help regarding the Latin nomenclature. This work has been carried out with the financial support of the European Community specific RTD programme “Quality of Life and Management of Living Resources” research project EU & Microfunction (QKL1-2001-00135). C.P. was supported by the Research Council for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organisation for Scientific Research (NWO).

CHAPTER 5

Identification of the Mucin-Degrading Enzymes from the Intestinal Bacterium *Akkermansia muciniphila*

Muriel Derrien, Tongbin Song, Jolan de Groot, Erwin Zoetendal, Willem M de Vos and Raymond Schipper

The human gastrointestinal (GI) tract is coated by a viscous mucus layer that besides water, is mainly composed of mucins. Mucins are highly complex glycoproteins, composed of monosaccharides sequences interconnected by different linkages and their complete degradation requires a set of specific enzymes, including various glycosidases, proteases, sulphatases and sialidases. In this study, we focus on the enzymes of *Akkermansia muciniphila*, a bacterium isolated from human faeces that can use mucin as sole carbon and nitrogen source. *A. muciniphila* could use up to 85% of the total mucin and the identification of enzymes responsible for this mucin degradation was performed on extracellular and intracellular fractions using specific substrates. Sulphatase and several glycosidases (α-D-galactosidase, β-D-galactosidase, β-D-fucosidase, N-acetyl-β-D-glucosaminidase, N-acetyl-α-D-galactosaminidase, N-acetyl-β-D-galactosaminidase, β-D-glucosidase, α-L-fucosidase, β -D-mannosidase), were identified in these fractions. In conclusion, *Akkermansia muciniphila* is highly adapted on mucin utilisation and can produce a broad range of enzymes necessary for the almost complete degradation of mucin.

Introduction

Mucins are the major organic components of the mucus layer, which not only protect the epithelium of the gastrointestinal (GI), respiratory and urinary tracts against various damages but are also involved in other functions, such as growth, foetal development, epithelial renewal, differentiation and integrity, carcinogenesis, and metastasis [157]. Mucins are large glycoproteins with complex structures, in which oligosaccharides account for up to 80% of their dry weight. A variety of glycans are attached to the peptide core via O-glycosidic linkage to serine and threonine residues, and these O-glycan branches contain primarly N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), fucose, galactose, and sialic acid [264]. The oligosaccharide chains are responsible for many intrinsic physical properties of the mucus gel, including water retention and the resulting viscous properties of the mucus and protection against proteolysis. The addition of sulphate and sialic acids on the terminal side of mucins provide an extra charge to the molecule, which results in a higher viscosity of the mucus, resulting in a better protection against bacterial and host enzymes [31, 234]. In the GI tract, mucins differ in size, glycosylation, tissue distribution, adjusted to the environment conditions, such as pH and bacterial concentration [234, 235].

Since mucins are glycoproteins, they can serve as source of carbon and nitrogen for the residential bacteria that can produce proteolytic and glycolytic enzymes. In the human GI tract, mucin-degrading enzymes from the genera *Bacteroides, Bifidobacterium, Clostridium, Prevotella, Ruminococcus, Streptomyces* and *Vibrio* have been identified [54, 64, 92, 121, 124, 149, 167, 171, 299, 321]. However, none of these bacteria have been described to be able to degrade mucin completely.

In a previous study, we isolated a mucin degrader, *Akkermansia muciniphila,* from human faeces. In contrast to the other described mucin-degrading bacteria, its growth is almost fully dependent on utilisation of mucin [66]. In this study, we analysed the enzyme repertoire required for mucin degradation of *A. muciniphila* using purified pig gastric mucin as substrate.

Material and Methods

Mucin purification. 10 g crude pig gastric mucin, (PGM, type III, Sigma) was suspended in 0.02 M phosphate buffer (pH 7.8) containing 0.1 M NaCl and a few drops of toluene; the solution was readjusted to pH 7.2 with 2 M NaOH. After centrifugation (10,000 X g, 30 min 4°C), the resulting supernatant was taken and cooled down to 4°C. Prechilled ethanol was added to a final concentration of 60% (v/v). After centrifugation (10,000 X g, 30 min, 4°C), the resulting

precipitate was dissolved in 0.1 M NaCl and precipitated again with ethanol to a final concentration of 60% (v/v) for two more times. The final purified mucin pellet was washed once with 100% ethanol and then dissolved and dialyzed against distilled water for 16 h at 4°C with three changes. The resulting solution containing the purified Pig Gastric Mucin (pPGM) was freeze-dried and used for further analysis.

Analysis of mucin. *Carbohydrates.* The carbohydrate content of pPGM was analysed using high-performance anion-exchange chromatography (HPAEC) A Dionex ICS 300 HPLC system equipped with a Dionex CarboPac PA-1 (4 mm ID x 250 mm) column in combination with a CarboPac PA-1 guard column (2 mm ID x 250 mm). A flow rate of 0.3 ml/min was used with an isocratic elution (neutral and amino sugars) and linear gradients of sodium acetate (NaOAc) and NaOH (acid sugars, oligosaccharides); 0–25 min, 15 mM NaOH; 25-25.5 min, 15-100 mM NaOH; 25.5-65 min 0-0.4 M NaOAc in 100 mM NaOH; 65-70 min 1 M NaOAc in 100 mM NaOH; 70-75 min 100 mM NaOH; 75 – 90 min 15 mM NaOH. pPGM was hydrolysed in 2 M trifluoroacetic acid (TFA) for 1 hour at 121°C prior to analysis by HPAEC. *Protein.* Protein content (Nx6.25) of pPGM was determined by the Dumas combustion method using a NA 2000 Nitrogen and Protein analyzer (Interscience) and after correction for the nitrogen present in the amino sugars. Protein content in solutions was determined with the Pierce BCA^{TM} protein assay. *Sulphate.* Sulphate was quantified by measuring the formation of the intensely colored barium rhodizonate complex with free sulphate [270]. *Sialic acid.* N-acetylneuraminic acid was determined by the acetic ninhydrin reaction according to Yao *et al.* [323].

Bacterial strain, culture conditions, and mucin fermentation. *A. muciniphila* Muc^{T} (=ATCC BAA- 835) was cultured in 1L of an anaerobic basal medium, pH 7.0 [218] containing pPGM (0.25% [wt/vol]) as sole carbon and nitrogen source at 37°C for 24 h. This medium is further referred to as mucin medium. The growth was followed by counting viable cells on soft agar mucin medium as described previously [66].

Preparation of extracts. Culture samples (10 ml) were collected during late log and stationary phases, and cells of *A. muciniphila* were harvested by centrifugation (10,000 X g, 10 min, 4°C). This supernanant was concentrated using Centriprep YM-10 (Millipore) and contained the extra cellular proteins and/or loosely-associated outer membrane proteins. Cells were washed twice with phosphate buffer (20 mM, pH 6.8), followed by resuspension in 20 ml of the same buffer. Subsequently, cells were disrupted by sonic treatment (15 min, duty cycle 30%; Sonifier 250,

Branson Ultrasonics, Danbury, Conn., USA). This crude extract was centrifuged at 10,000 X g for 30 min to remove non-disrupted cells and the resulting supernatant was centrifuged at 22,000 X g for 30 min to pellet cell debris. The supernatant contained the intracellular proteins.

Mucin degradation. Mucin degradation during growth of *A. muciniphila* was measured as percent decrease in mucin hexose compared to the non-inoculated medium. Ethanol (60% final) was added to the non-inoculated bottle and each supernatant at 4°C. The precipitate was centrifuged and dissolved in 0.1 M NaCl and assayed for hexose content by the method of Dubois [72].

Enzyme activity assays. Enzymes activities of *A. muciniphila* were investigated in the supernatants and cell extracts. The activity of glycosidases was dermined by the hydrolysis of p-nitrophenol (*p*-NP) linked substrates (Sigma-Aldrich, St. Louis, MO, USA) at 37°C after 10 min incubation. The assay mixture consisted of 50 mM potassium phosphate buffer (pH 7), 1mg/ml *p*-NP solution and 40 µl of extracts. The reaction was stopped by adding an equal volume of 0.5 M Glycine/NaOH buffer pH 9.0, containing 2 mM EDTA. The color formation was measured spectrophotometrically at 445 nm. One unit of glycosidase activity was defined as 1 µmol of *p*-NP liberated per min per mg protein at 37°C. The molar extinction coefficient of p-nitrophenol under these assay conditions was 13,700 M^{-1} cm^{-1}. The optimal pH was determined in a pH range of 4.5 to 9.5 with 20 mM phosphate buffers at 37°C while the optimal temperature was measured in a 50 mM Phosphate buffer at pH 7. Sialidase activity was detected fluorimetrically using 4'-umbelliferyl-N-acetyl-α-D-neuraminic acid (1mg/ml) in 50 mM phosphate buffer (pH 7.0) according to Potier *et al*. [221]. The sulphatase activity was measured by quantifying the release of free sulphate from pPGM at 37°C according to Silvestry *et al* [270]. The protease activity was determined using Azocoll (Merck) as a chromotogenic substrate [45] since collagen has a similar structure to mucin.

Gel electrophoresis of the supernatant of pPGM grown cultures of *A. muciniphila*. Native gel electrophoresis and Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) were performed using a PhastSystem (Pharmacia LKB Biotechnology, Uppsala, Sweden) according to the instructions of the supplier by using a 12.5% polyacrylamide gel (Pharmacia LKB Biotechnology). Carbohydrates were stained with periodic acid-Schiff reagents (PAS) [134].

Results

Purification and composition of mucin

Commercial PGM was purified by repeated ethanol precipitation. The purification of the crude PGM reached a recovery of 45%. Most of the crude PGM contained aggregates from mucin, which could not be dissolved in the phosphate buffer, while the purified PGM dissolved easily in water. The pPGM was composed mainly of neutral oligosaccharides (galactose, fucose and glucose) and the amino monosaccharides N-acetylgalactosamine (GalNac) and N-acetylglucosamine (GlcNac). The latter accounted for 53.6% of the purified mucin with the following ratios: GlcNac 1; galactose 0.94; GalNac 0.6; fucose 0.4; glucose 0.03; sialic acids 0.02. Sulphate and protein accounted for 8 % and 21% of the purified mucin, respectively. This composition was in agreement with other studies [160, 236].

Assessment of mucin degradation *in vitro* by *A. muciniphila*

Growth of *A. muciniphila* in a mucin-based medium, containing mucin as sole carbon and nitrogen source, was followed in time by quantifying the increase in optical density and viable counts. Mucin degradation was studied in the culture supernatants by ethanol precipitation of the native mucin (Fig. 5.1A). High molecular weight glycoproteins were visualised on SDS-PAGE followed by PAS staining (Fig. 5.1B). Based on ethanol precipitation, mucin decreased in parallel to the increase of bacterial counts. At the end of the growth, *A. muciniphila* used 85% of the mucin (Fig. 5.1A).

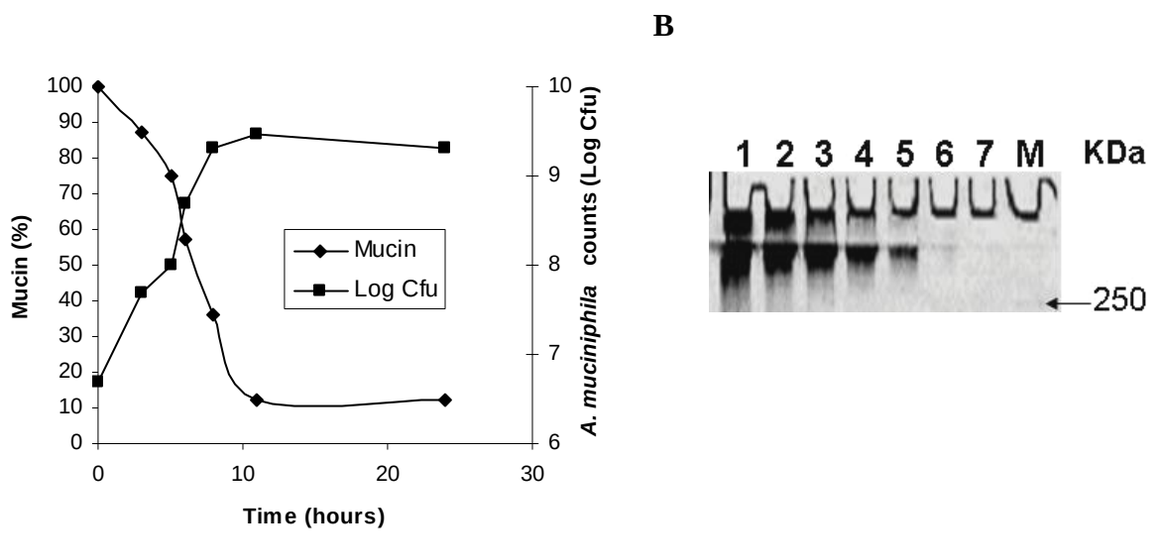

Figure 5.1. Evidence of mucin degradation by *A. muciniphila*. (A) Monitoring of *A. muciniphila* growth in mucin medium and amount of mucin in the supernatant culture after ethanol precipitation and measurement by the Dubois method (B) SDS-PAGE of supernatant samples separated on 12.5% gels and stained with PAS at the respective time points as in (A). Lanes 1, 0 h; 2, 3.5h; 3, 4.5h; 4, 6h; 5, 7.5h; 6, 10.5h; 7, 24h; M, Marker.

According to the PAS staining (Fig. 5.1B), the high molecular weight glycoproteins decreased from the inoculation point till 10 h incubation, after which time point they were not detected anymore. This was consistent with the Dubois determination (Fig. 5.1A).

HPAEC of culture supernatants was conducted to identify the formation of sugar from mucin by *A. muciniphila* in time (Fig. 5.2). Based on the HPAEC patterns, mucin was not fully degraded after 24 h of *A. muciniphila* incubation. Nevertheless, the profile of mucin changed notably during the bacterial incubation. Peaks corresponding to fucose, galactose and sialic acids increased along with incubation time and were absent at 24 h incubation, suggesting their release from the mucin molecule, and subsequent consumption by *A. muciniphila*. Unknown peaks corresponding to oligo- and polysaccharides showed an apparent change during *A. muciniphila* growth.

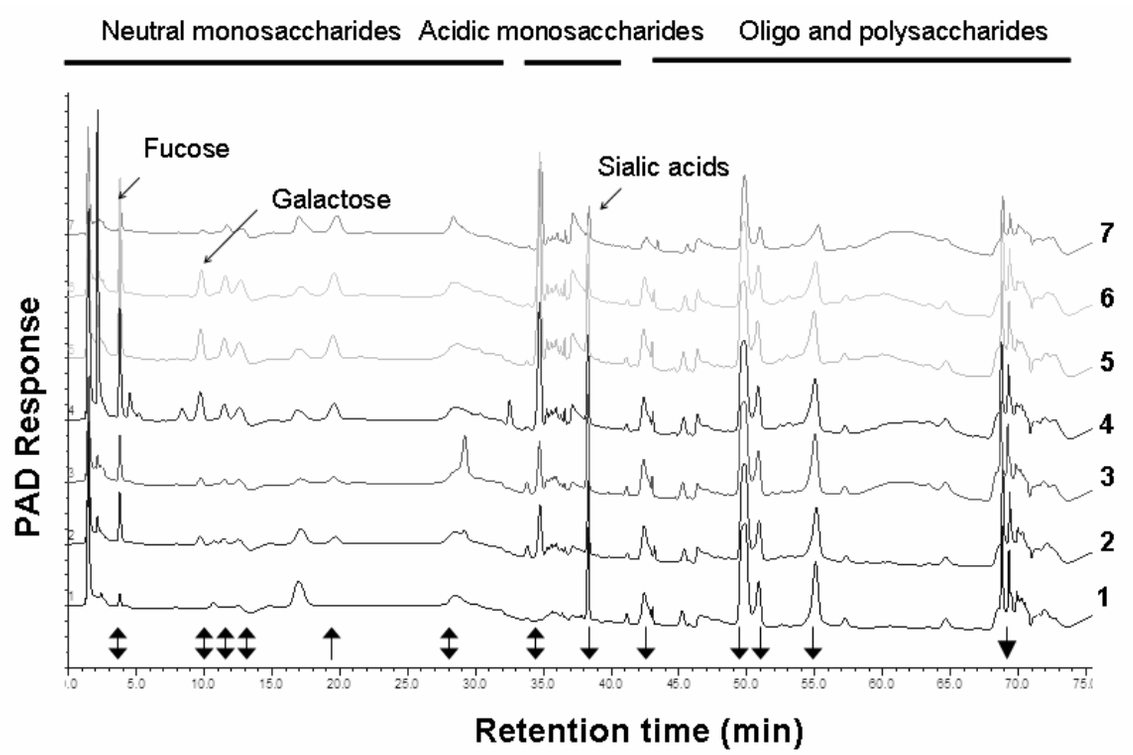

Figure 5.2. HPAEC elution pattern of mucin degradation products before (1) and after fermentation by *A. muciniphila* (2 to 7) corresponding to the time points of Fig. 5.1B. PAD: Pulsed amperometric detection. The different arrows show the modification of the mucin fractions after *A. muciniphila* incubation (↕ : increase and decrease, ↑: increase and ↓: decrease).

Measurements of glycosidase activity and optimal conditions

Since different sugars were used during mucin fermentation, the activity of nine glycosidases was assayed on the supernatant and cells fractions. For this, the corresponding *p*-NP substrates for oligosaccharides commonly present on the mucin molecule were used. Among all the glycosidases tested, most of them exhibited activity either extracellularly or intracellularly (Table

5.1). The N-acetyl-β-D-glucosaminidase and β-D-galactosidase from the intracellular fraction showed a particularly high activity. β-D-fucosidase, N-acetyl- β-D-glucosaminidase, N-acetyl-α-D-galactosaminidase, N-acetyl- β-D-galactosaminidase were found to be entirely intracellular. α-D-galactosidase and β-D-galactosidase were both found extracellularly and intracellularly.

Table 5.1. Activity of mucin-degrading enzymes of *A. muciniphila* grown in mucin-based medium for 24h. (ND: no activity detectable)

Enzyme assay	Enzyme activity (μmol/mg protein/min)	
	Intracellular	Extracellular
Glycosidases		
α-D-galactosidase	9.7	5.8
β-D-galactosidase	60.0	6.5
α-L-fucosidase	ND	4.0
β-D-fucosidase	3.3	ND
N-acetyl-α-D-galactosaminidase	23.3	ND
N-acetyl-β-D-galactosaminidase	25.0	ND
N-acetyl-β-D-glucosaminidase	216.7	ND
β-D-glucosidase	ND	5.8
α-D-mannosidase	ND	3.2
Sialidase	ND	ND
Sulphatase	11.8*	ND

*nmol release sulphate /h.

The effect of pH and temperature on the activity of four predominant active glycosidases associated with *A. muciniphila* cells, i.e. β-D-galactosidase, N-acetyl-β-D-galactosaminidase, N-acetyl-α-D-galactosaminidase and N-acetyl-β-D-glucosaminidase, was investigated using the specific *p*-NP-glycopyranosides as substrates (Table 5.2). All four glycosidases had their highest activity between pH 6.8 and 8.8. N-acetyl-α-D-galactosaminidase and N-acetyl-β-D-galactosaminidase had a broad optimal pH range of activity while β-D-galactosidase and N-acetyl-β-D-glucosaminidase had a narrow range. All enzymes showed a broad temperature range of activity, except the N-acetyl-β-D-glucosaminidase which exhibited the most specific

conditions for optimal activity. Notably, its optimal temperature was higher (45°C) than that of the others.

Table 5.2. Optimal pH and temperature of β-D-galactosidase, N-acetyl-α-D-galactosaminidase, N-acetyl-β-D-galactosaminidase and N-acetyl-β-D-glucosaminidase

Enzymes	Optimal pH	Optimal temperature (°C)
β-D-galactosidase	8	28 - 38
N-acetyl-α-D-galactosaminidase	6.8 - 8.8	31 - 37
N-acetyl-β-D-galactosaminidase	6.8 - 7.4	37 - 40
N-acetyl-β-D-glucosaminidase	7.3	45

The dynamics of production of these predominant intracellular glycosidases during growth of *A. muciniphila* on mucin was investigated. The activity of all glycosidases increased exponentially until the end of log phase. Notably, they were all active at the same time with no preferential order of action (data not shown).

Other enzymes

Besides glycosidases, the activity of other enzymes involved in mucin degradation, such as proteases, sialidases and sulphatases was determined. Protease and sialidase were not detectable in any fraction while the activity of the sulphatase was found only in the intracellular fraction (Table 5.1).

Discussion

In this study, the production of mucin-degrading enzymes from *A. muciniphila* was examined during its growth in a mucin-based medium. A purified PGM was used as a substrate since it has been shown to be structurally similar to human mucin [167].

Based on HPAEC patterns and PAS staining of the supernatant, 85% of the mucin molecule was found to be degraded by *A. muciniphila*. This high capacity to degrade mucin has not been observed in any other described mucin-degrading bacteria.

The breakdown of mucin is usually initiated by proteases on the non-glycosylated parts, followed by glycosidases which cleave first the α-glycosidic linkages present on terminal residues, such as N-acetyl-α-D-galactosamine, α-galactose and α-fucose, and then on the β-linked internal monomers, resulting by the appearance of the peptide backbone, accessible to proteases activity ([63] Fig. 5.3).

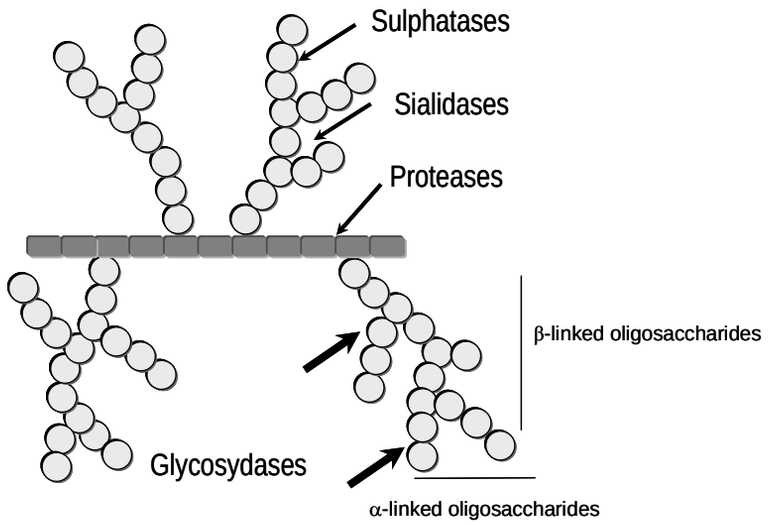

Figure 5.3. Schematic representation of the action sites of mucin-degrading enzymes. The rectangles represent the peptide backbone and the circles the oligosaccharide chains.

During *A. muciniphila* growth, enzymes acting on peripherical side of mucin, i.e. α-D-galactosidase, α-L-fucosidase and N-acetyl-α-D-galactosaminidase, and the core molecule, i.e. β-D-galactosidase, β-D-fucosidase, N-acetyl-β-D-glucosaminidase, N-acetyl-β-D-galactosaminidase, β-D-glucosidase, and β-D-mannosidase were active suggesting that degradation occurred in both peripherical and inside molecules.

Besides glycosidases, the activity of other enzymes involved in mucin degradation, such as sialidases, sulphatases and proteases were investigated. During the growth of *A. muciniphila*, protease activity was not detectable in the supernatant and cells. This suggests that they were not produced, inhibited or could not access to the peptide core of mucin due to oligosaccharides chains. In a previous study, *A. muciniphila* was found not to grow on the major amino acids present in mucin, i.e. threonine, proline and cysteine [66] suggesting that *A. muciniphila* preferentially uses sugars. Moreover, sialic acid and sulphate confer an additional protection to the mucin molecule against bacterial glycosidases or by host proteases [238]. Sialidase was neither found in the extracellular nor intracellular fractions, while sulphatase activity was only detected in the intracellular fraction. This is in agreement with the previous study that showed that sulphates were released during the fermentation of mucin by *A. muciniphila* [66]. Other intestinal commensals such as *Bacteroides fragilis* and *B. thetaiotaomicron* were also found to be producers of mucin sulphatase [299].

In the human GI tract, mucin degradation is generally accepted as a multi-step and cooperative event between bacteria. Studies on germ-free rats showed that their caecal mucin concentration is much higher compared to conventional rats, and that complete degradation of the caecal mucin could be only obtained when a faecal microbiota was inoculated [41]. Our results

indicate that *A. muciniphila* degrade the majority (85%) of mucin, and exhibited a variety of extracellular and intracellular glycosidases. Nevertheless, the released monosaccharides (fucose, galactose, GalNac, GlcNac) could not be used alone by *A. muciniphila* alone [66] suggesting that *A. muciniphila* could obtain its growth from either a combination of several monosaccharides or from oligomerised sugars. This is in agreement with a study that showed that *B. fragilis*, a common intestinal bacterium that grows on a variety of carbon sources including mucin, could only use 15% of mucin, obtaining its growth from specific mucin oligosaccharides consumption [236]. The proposed consortium of bacteria involving in mucin degradation included members of *Ruminococcus, Bifidobacterium*, *Bacteroides* that could produce extracellular and cell-associated glycosidases to degrade mucin [121, 299].

In conclusion, our results indicate that *A. muciniphila* provided mainly glycosidases and sulphatase. The ability of *A. muciniphila* to produce these enzymes suggests that it plays a role in the intestinal microenvironment. The genome sequence of *A. muciniphila* which is currently being determined, combined with detailed characterisation of mucin-degrading enzymes will provide insight into the specific role of these mucin-degrading enzymes in the human GI tract.

Acknowldegements

Dr. Caroline Plugge is acknowledged for critical reading of the manuscript.

CHAPTER 6

The Mucin-Degrader *Akkermansia muciniphila* Is an Important Member of the Human Intestinal Tract

Muriel Derrien, Kaouther Ben-Amor and Willem M. de Vos

Recently, we isolated an obligate mucin-utilising anaerobe from human faeces, *Akkermansia muciniphila*, belonging to the widely spread group of the Verrucomicrobia. In this present study, a 16S rRNA-targeted probe was designed and validated in order to determine the presence and numbers of *A. muciniphila* in the human intestinal tract using fluorescent in situ hybridisation and flow cytometry (FISH-FCM). A set of reference strains from the human gastro-intestinal tract was used as negative control. The newly developed probe hybridise specifically to *A. muciniphila* under optimised conditions 20%. Flow cytometric and microscopic analysis revealed that a group of morphologically similar bacteria in faeces did hybridise with this probe, while the eubacterial probe EUB-338 did not hybridise efficiently to the 16S rRNA of *A. muciniphila*. Quantification by FISH-FCM revealed that *A. muciniphila*. was present in all 12 samples out of the 13 samples analysed (92%) and accounted for 1.26 ± 0.85% of the total faecal cells representing up to 8.24 ± 7.35 x 10^8 bacteria/g wet faeces (n=12). The FISH-FCM method used in this study showed that *A. muciniphila* is a numerically important bacterial group and is a common bacterial member of the human gastro-intestinal tract. Its significance as an important member of the gastrointestinal tract microbial ecosystem has been hindered so far because its physiological requirement, small size and inability to hybridise to the general eubacterial probe.

Introduction

The human intestinal mucosal surfaces are exposed to a enormous amount of bacteria with densities approaching 10^{14} organisms and representing approximately 1,000 species [227]. With the advent of molecular tools based on the 16S rRNA gene analysis, it has been shown that the majority of the intestinal bacteria has not yet been obtained in culture [335]. Moreover, the extensive use of molecular based-methods revealed that the gastrointestinal tract contains a series of novel bacterial species (each representing up to 1 % of the total population) [125, 228] and that each individual harbours a unique dominant intestinal bacterial community. This intestinal microbiota has evolved in a way that had developed an alliance with its host. This symbiotic relation is of particular relevance at the protective mucus layer that covers the epithelium and represents the anatomical site where the host first encounters intestinal bacteria.

Mucus consists of water containing mucins [1] which are high molecular weight glycoproteins, and other host-encoded products such as trefoil factor peptides [215], antibacterial proteins (IgA, lysosyme), and other factors. Mucus offers numerous ecologic advantages to intestinal bacteria as mucins represent a direct source of nutrients for intestinal bacteria, and especially in the colon, where carbon sources are more limited [249].

Various interactions between intestinal bacteria and mucus have been reported. Early studies showed the presence of a bacterial population within this matrix where nutrients are permanently available [173, 224, 336]. Bacteria embedded in the mucus matrix can use this direct and available source of nutrients. Indeed, mucins are high molecular weight glycoproteins that can be used as carbon and nitrogen source. Many bacteria are able to ferment mucins, partially or completely and have the benefit not to depend on host diet [187]. Studies with germ-free mice revealed that a commensal microorganism, *Bacteroides thetaiotaomicron* was able to switch to utilisation of host mucins when polysaccharide availability from the diet is reduced [278]. However, the relation of mucin and bacteria has always been controversial. Many studies report the potential involvement of mucin-degrading bacteria in pathogenesis, such as inflammatory bowel disease [39]. Indeed, the mucosa from IBD patients has shown to be physically disturbed [53], harbouring a reduced bacterial diversity [210] but a higher number of mucosal-associated bacteria compared to controls [262] [286], suggesting that the mucosa can not display its protective role anymore. Eventhough the intestinal microbiota is believed to play a role in the aetiology of IBD, this has not been clearly demonstrated. In contrast, mucin-degrading bacteria benefit from an ecological advantage on luminal bacteria and can provide nutrients to them from mucin-degradation or end-products [224].

By incorporating mucin in a medium as major carbon and energy source, we were able to isolate a novel bacterium, *Akkermansia muciniphia* Muc^T [66]. It belongs to the Verrucomicrobia phylum, a recently discovered division of Bacteria [109], whose members are hardly detected by culturing, but frequently found in 16S rRNA clone libraries from a variety of ecosystems, including intestine, natural freshwater and soil microbial communities [125, 129, 228]. Molecular tools based on 16S rRNA gene have been successfully employed to characterise the gut microbiota [285, 330]. Amongst several techniques, fluorescent *in situ* hybridisation (FISH) and quantitative PCR have become robust methods for direct and cultivation-independent detection, identification and quantification of bacterial cells in a complex ecosystem. During the last decade, FISH application in microbial ecology has provided much information on the composition of diverse ecosystems [106, 198]. FISH combined with microscopic analysis has for long been the standard technique [107]. Recently, the use of FISH coupled with flow cytometry (FISH-FCM) has developed into a promising tool for the high-resolution and high-throughput identification and accurate enumeration of microorganisms in complex ecosystems [5, 17, 220, 310]. Although less frequently used, FISH-FCM has been increasingly employed to study human faecal microbiota from healthy adults [16, 70, 152, 153, 232, 233, 332], inflammatory bowel disease (IBD) patients [275] and infants [34, 77]. A large panel of oligonucleotide probes specific for various genera of the GI tract has been designed and validated [18]. Yet, the wide probe set does not cover all bacteria. Therefore, the design of additional probe targeting newly cultivated genera is still required.

In this study, we designed, validated and applied a specific probe targeting the 16S rRNA-gene sequence of *Akkermansia muciniphila* in faecal samples of healthy subjects to determine the prevalence and the proportion of the novel isolate.

Material and methods

Reference strains, growth conditions and fixation. The bacterial strains used in this study for FISH enumeration are listed in Table 6.1. Strains were obtained from the American Type Culture Collection (ATCC; Manassas, Va., USA), Deutsche Sammlung von Mikroorganismen und Zellcultures (DSMZ; Braunschweig, Germany), Culture Collection, University of Göteborg (CCUG; Göteborg, Sweden) and laboratory stock cultures at the Rowett Research Institute (RRI; Aberdeen, United Kingdom). *A. muciniphila* was cultivated in a mucin-based medium as described previously [66]. *Lachnopsira multiparus, Streptococcus bovis, Veilonella parvula* were grown on ruminal-fluid-containing medium M2 at 39°C [111].

Table 6.1. Reference strains used for the validation of the MUC-1437 probe.

Strain	Origin	Strain	Origin
Akkermansia muciniphila	ATCC BAA-835	*Faecalibacterium prausnitzii*	RRI
Atopobium minutum	DSM 20586	*Lachnospira multiparus*	RRI
Bacteroides distasonis	DSM 20701	*Lactobacillus acidophilus*	ATCC 4356
Bacteroides fragilis	DSM 2151	*Lactobacillus amylovorus*	DSM 20531
Bacteroides pyogenes	CCUG 15419	*Lactobacillus rhamnosus*	ATCC 53103
Bacteroides tectus	CCUG 25929	*Lactobacillus plantarum*	NCIMB 8826
Bacteroides thetaiotaomicron	RRI	*Lactobacillus reuteri*	DSM 20016
Bacteroides uniformis	DSM 6597	*Megasphaera elsdenii*	NCIMB 8927
Bifidobacterium lactis	DSM 10140	*Mitsuokella multiacidus*	RRI
Bifidobacterium bifidum	DSM 20082	*Peptostreptococcus anaerobius*	ATCC 27337
Bifidobacterium adolescentis	DSM 20083	*Pepstostreptococcus micros*	DSM 20468
Bifidobacterium breve	DSM 20091	*Prevotella albensis*	DSM 11370
Bifidobacterium longum	DSM 20090	*Prevotella brevis*	ATCC 19188
Clostridium aminophilum	ATCC 49906	*Prevotella bryantii*	DSM 11371
Clostridium sticklandii	ATCC 12662	*Prevotella ruminocola*	ATCC 19189
Clostridium perfringens	DSM 756	*Roseburia intestinalis*	RRI
Clostridium sporogenes	DSM 1664	*Ruminococcus albus*	RRI
Colinsella aerofaciens	DSM 3979	*Ruminococcus bromii*	RRI
Coprococcus sp.	RRI	*Ruminococcus flavefasciens*	RRI
Enterococcus faecalis	RRI	*Ruminococcus hansenii*	DSM 20583
Escherichia coli	NCTC 12900	*Ruminococcus productus*	DSM 2950
Eubacterium biforme	DSM 3989	*Streptococcus bovis*	RRI
Eubacterium cylindroides	RRI	*Veilonella parvula*	RRI
Eubacterium rectale	RRI	*Victivallis vadensis*	DSM 14823
Eubacterium ruminantium	RRI		

All remaining strains were grown under anaerobic conditions at 37°C in the appropriate medium. Exponentially grown cells were centrifuged (5,000 X g for 10 min) and washed twice with 0.2-µm-pore-size-filtered phosphate-buffered saline (PBS; per liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 2.24 g KH_2PO_4; pH 7.2). The pellet was finally resuspended in 1:3 PBS/ 4 % paraformaldehyde (PFA) and incubated at 4°C for 4 hours. Following fixation, cells were

centrifuged, washed twice in PBS and resuspended in 50% ethanol-PBS and stored at -20°C until FISH analysis. PFA-fixed cells of *Bacteroides thetaiotaomicron, Coprococcus sp, Enterococcus faecalis, Eubacterium cylindroides, Eubacterium rectale, Faecalibacterium prautnitzii, Roseburia intestinalis, Ruminococcus albus, Ruminocccus bromi* and *Ruminococcus flavefasciens* were kindly provided by Sylvia Duncan, Rowett Research Institute, Aberdeen.

Probe design. Sequences of the 16S rRNA genes of *A. muciniphila*, its closest relatives (Table 6.2) and sequences of 96 intestinal 16S rRNA gene clones were aligned using CLUSTAL-X and checked for regions of conserved and variable sequences. Based on this alignment, a 16S rRNA oligonucleotide probe specific to *A. muciniphila* was designed. A region corresponding to the nucleotide 1437 – 1456 of *Escherichia coli* was chosen. The newly designed probe was checked against the SSU-rRNA database of the Ribosomal Data Project II software package (RDP II) using the Probe Match function [50] and similarity search sequence in NCBI was performed with BLAST (www.ncbi.nlm.nih.gov/BLAST). The probe was named S-St-Muc-1437-a- A- 20 (MUC-1437) based on the nomenclature of the Oligonucleotide Probe Database [4]. The oligonucleotide probe sequence has been deposited at probeBase [165].

Table 6.2. Aligned sequences of the oligonucleotide probe and the 16S rRNA gene sequence of the closest relatives of *A. muciniphila* in the Verrucomicrobia division

Probe and targets	Sequence
S-St-Muc-1437-a- A- 20	3′ TAGACTTCGGTTGGCGTTCC 5′
Akkermansia muciniphila	5′ ATCTGAAGCCAACCGCAAGG 3′
Clones HuCA18, HuCC13	5′ •••••••••••••••••••• 3′
Verrucomicrobium spinosum	5′ GC•C•C-•••G••••••••• 3′
Prosthecobacter dejongeii	5′ GCG••••-•T•••••••••• `3′
Prosthecobacter vanneervenii	5′ GCG••••-•T••••••••••` 3′
Prosthecobacter debontii	5′ GCG••••-•T••••••••••` 3′
Prosthecobacter fusiformis	5′ GCG••C-•••••••••••••` 3′

Faecal samples fixation and processing. Fresh faecal samples were collected from 13 healthy adults (8 females and 5 males, age 26-40 years). These volunteers had not been subjected to any feeding trial, specific diet, or antibiotic treatment for the last year. Samples were processed immediately after collection. To extract faecal microbial cells, 0.5 g of faeces was homogenised in 4.5 ml of PBS containing 5 to 10 glass beads of 3-µm diameter. After centrifugation at 700 X g for 1 min, 1 ml of supernatant was added to 3 ml of PFA 4% and fixed for 4 hours at 4°C. After

fixation, cells were centrifuged, washed twice in PBS, resuspended in 50% ethanol-PBS and stored at -20°C until FISH analysis. All oligonucleotide probes (Table 6.3) were purchased from Thermo Electron (Ulm, Germany) and were double-labelled at both 5' and 3'-ends with Cy5, Cy3 or FITC. The EUB-338 probe was used as the positive control [6] and the NON-EUB as a negative control to monitor the non-specific binding [312]. The nucleic acid stain TOTO-1 iodide was purchased from Invitrogen (Leiden, The Netherlands) and used for total cell counts. TOTO-1 is a membrane impermeant nucleic acid with a very high fluorescence enhancement and quantum yield upon binding to the nucleic acids of a cell. When excited with a blue light (488 nm), the stained cells fluoresce yellow-green and give a positive single in both FL1 and FL2 detectors of the FCM.

Fluorescent *in situ* hybridisation of reference strains. The optimisation of the hybridisation conditions of MUC-1437 with *A. muciniphila* was carried out by increasing the concentration of formamide in the hybridisation buffer. The 50 reference strains were tested for non-specificity at the different formamide concentration in parallel with *A. muciniphila*. 50 µl of fixed cells of each pure culture were harvested and resuspended in 20 µl of hybridisation buffer (0.9 M NaCl, 0.02 M Tris/HCl pH 8, 0.1% SDS) and different amounts of formamide (0 to 60%) to which 2 µl of the Cy5-labelled MUC-1437, EUB-338 or NON-EUB probes were added to a final concentration of 3 ng/µl in independent reactions. The samples were incubated at 50°C in the dark overnight. This was followed by a 20-min-step washing at 50°C in 980 µl of prewarmed washing buffer. To achieve the same stringency used in the hybridisation step, the washing buffer as already described [126]. Cells were finally pelleted (13,000 X g, 5 min), resuspended in 1 ml of ice–cold PBS and stored on the ice and dark before analysis by FCM. Each experiment was done in triplicate.

Table 6.3. Oligonucleotide probes sequence and hybridisation conditions used in this study.

Probe	Probe sequence	Target	Hybridisation conditions		Reference
			Temperature (ºC)	Formamide (%)	
EUB-338	GCTGCCTCCCGTAGGAGT	Most bacteria	50	0	4
NON-EUB	ACATCCTACGGGAGGC	None	50	0	47
MUC-1437	CCTTGCGGTTGGCTTCAGAT	*Akkermansia*	50	20	This study

Fluorescent *in situ* hybridisation of faecal samples. Fixed faecal samples (100 µl) were hybridised in a 50 µl of 20% (vol/vol) formamide buffer (0.9 M NaCl, 0.02 M Tris/HCl pH 8, 0.1% (wt/vol) SDS) to which 5 µl the MUC-1437-Cy5 labelled probe were added. After hybridisation, cells were washed in 950 µl of a low salt washing buffer (0.02 M Tris/HCl pH 8, 0.225 M NaCl and 5 mM EDTA) at 50°C for 20 min. For total cell enumeration, samples were incubated in the presence of 1nM of the membrane-impermeant nucleic acid stain TOTO®-1 iodide (Molecular Probes, Leiden, The Netherlands) as described previously [16].

Flow cytometryic analysis. To quantify hybridised cells, samples were analysed using a FACScalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif., USA) equipped with an air-cooled argon ion laser providing 15mW at 488 nm combined with a 670 nm red-iode laser with the standard filter set-up. All parameters were collected at logarithmic scale and control samples were used for the instrument settings. Theses controls consisted of unlabelled faecal cells and / or cells hybridised with the NON-EUB probe, cells stained with TOTO-1 (FL1/FL2), cells hybridised with EUB-338-Cy5 (FL4). CaliBRITE beads were used to check the instrument sensitivity over time. Cells were discriminated using a double threshold set on both side scatter SSC (98) and forward scatter FSC (162), with FSC set on E01 and SSC on 400V. All samples were sonicated (2 x 60 sec) and thoroughly vortexed prior to FCM analysis. A dual dot plot FSC versus SSC in combination with one parameter histogram representing the TOTO-1 fluorescence (FL1) was used to back gate faecal cells and distinguish them from the background. For total cell enumeration, samples were incubated in the presence of 1 nM TOTO-1 for 5 min at room temperature. Unlabelled beads of 6.0-µm diameter provided from the Bacteria Counting Kit (Molecular Probes BV, Leiden, The Netherlands) were added to each sample stained with TOTO-1 at a final concentration of 10^6 beads/ml and served as an internal standard to calibrate the sample volume. The ratio of TOTO-1 stained cells to the number of beads was used to calculate the absolute total cell counts. The relative abundance (proportion) of *A. muciniphila*-like bacteria was estimated as the ratio of the number of cells hybridised with the Cy5-labelled MUC-1437 to the number of TOTO-1 stained cells. Analysis was performed using WinMDI version 2.8 software (http://facs.scripps.edu/software.html) or CellQuest Pro program (Becton Dickinson Immunocytometry Systems, san Jose, Calif., USA).

Fluorescent *in situ* hybridisation and image analysis. Aliquot (2 µl) of fixed cells of faeces were spotted on glass slides and dried for 10 min at 46°C. The cells were then dehydrated on the slide in an increasing ethanol series, 50, 80 and 96% (v/v) ethanol during 3 min 10 µl of a 20% formamide hybridisation buffer were spotted on the cells with 1 µl of Cy3-labelled MUC-1437

probe (30 ng/µl) and FITC-labelled EUB-338 probe (50 ng/µl) and incubated at 50°C for 1.5 h. Following hybridisation, the cells were washed and then incubated in 50 ml of preheated low salt washing buffer (50°C) for 10 min. Following washing, the slides were rinsed with cold distilled water (4°C), air dried with compressed air and embedded with Vectashield (Vector Laboratories, Burlingame, Calif., USA). Digital images of the slides were analysed using Qwin image analysis software (Leica Microsystems, Rijswijk, The Netherlands).

Results

Design and validation of MUC 1437 probe for FISH-FCM

A specific 16S rRNA-targeted oligonucleotide probe, MUC-1437, targeting part of the hypervariable region V9 of the 16S rRNA gene sequence of *A. muciniphila*, was designed by aligning a panel of 96 sequences from intestinal microorganisms and its closest relatives in the Verrucomicrobia division from RDP II databases. To validate the newly designed probe, 50 intestinal reference strains were selected based on their phylogenetic position and used (Table 6.2). Cells from exponentially-grown pure cultures were fixed and hybridised to the Cy5 labelled-MUC-1437 probe, using formamide as denaturant. First, a typical curve of the hybridisation of the MUC-1437-Cy5 probe to its target was performed on a function of the amounts of formamide (0 to 60%). The curve showed that the presence of more than 30% formamide strongly decreased the hybridisation signal of MUC-1437-Cy5 to the background level observed with similarly labelled NON-EUB probe (data not shown). Hence, up to 30% formamide was included in hybridisation buffers in further experiments. Subsequently, 50 fixed intestinal strains were hybridised to MUC-1437-Cy5 probe in the presence of 0 to 30% formamide. At 0% formamide, cells from *B. thetaiotaomicron, R. flavefasciens, R. hansenii, R. productus, R. torques* and *V. vadensis* showed cross-reactivity with the MUC-1437-Cy5 probe. Discrimination between these strains and *A. muciniphila* was achieved by increasing the concentration of formamide up to 20% and under these conditions, none of the non-target organisms showed some cross-hybridisation with the MUC-1437-Cy5 probe. Hence, these conditions were considered as specific to *A. muciniphila* in further FISH analysis of faecal samples. Remarkably, when cells were hybridised to the general eubacterial probe EUB-338, the percentage of hybridised cells was 35%, intermediate between those of the NON-EUB and MUC-1437 signals (Fig. 6.1).

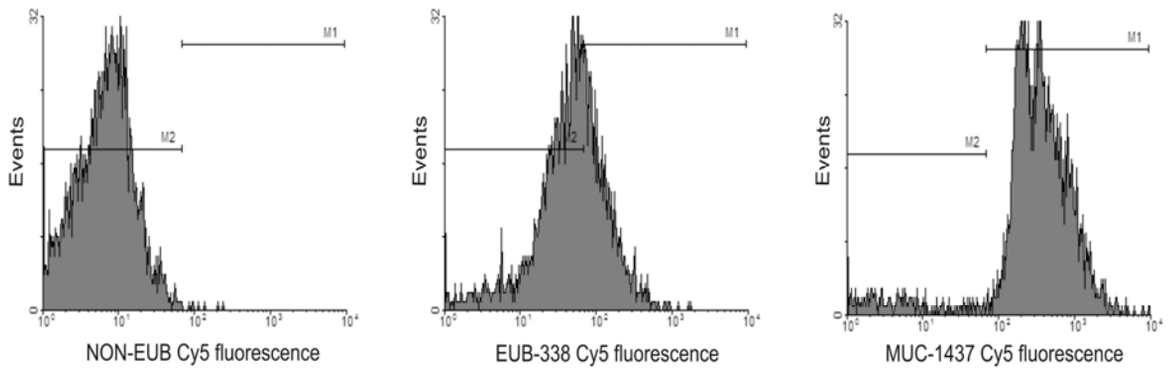

Figure 6.1. Flow cytometry histograms obtained by FISH analysis of the pure culture of *A. muciniphila*, hybridised with Cy5-labeled NON-EUB (A), EUB-338 (B) and MUC-1437 (C). M1 and M2 represent the population hybridised with MUC-1437 and non-hybridised respectively.

Quantification of *A. muciniphila* in faeces from healthy individuals using FISH-FCM

Thirteen faecal samples from healthy adult volunteers were tested for the presence of *A. muciniphila*. The hybridisation was checked using epifluorescence microscopy. A microcolony consisting of a single type of cells, small and oval was detected, corresponding to *A. muciniphila* (data not shown). Due to its small size (less than 1 µm diameter), FCM analysis was found to be more reliable and convenient than microscopic analysis. The cells detected with MUC-1437 were counted with bead-based method using FCM and reported to the total faecal bacteria determined with the TOTO-1 dye.

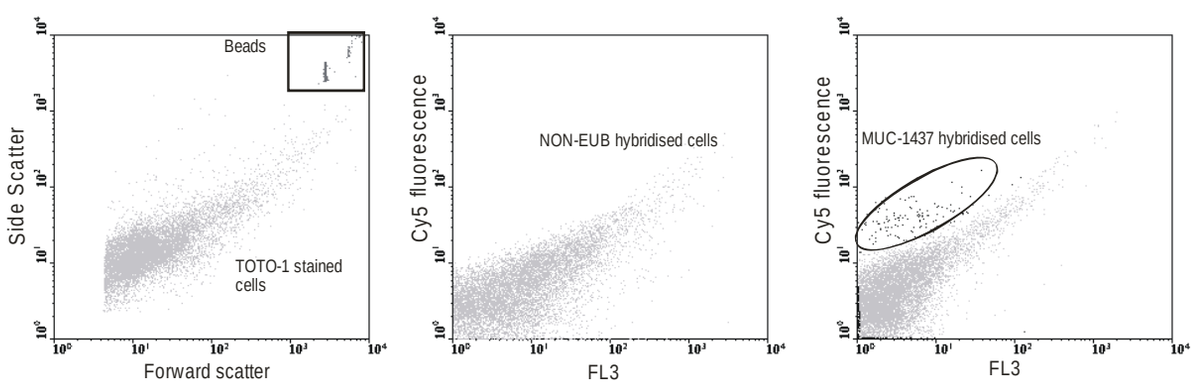

Figure 6.2. Flow cytometry dot-plots obtained by FISH analysis of a faecal sample, stained with TOTO-1 dye and unlabelled beads (A), hybridised with Cy5-labelled NON-EUB (B) and hybridised with Cy5-labelled MUC-1437 (C).

Cytometric dot plots graphs obtained after staining with TOTO-1 and hybridisation with the Cy-5 labelled-NON-EUB and MUC-1437 are presented in Fig. 6.2 (A to C). *A. muciniphila* cells (Fig.

6.2C) are represented in the FL4 signal (Cy-5 fluorescence) as the black population separated from the non-hybridised cells (grey).

The prevalence of *A. muciniphila* in the 13 samples was 92%, as it was clearly detected in 12 of the samples. For these 12 subjects, dot plots analysis based on the bead methods gave a mean of 1.26 % ± 0.85 of total faecal cells, corresponding to $8.24 \pm 7.35 \times 10^8$ cells g (wet weight)$^{-1}$. The amount of *A. muciniphila* cells ranged between the detection limit (<0.1%) and 3.08% of the total faecal cells (Table 6.4).

Table 6.4. Flow cytometry counts of total faecal and *A. muciniphila* cells per gram of weight faeces in 13 human faecal samples, obtained by FISH with TOTO-1 stain and MUC-1437 probe.

Volunteers	**Age (gender)**	**Mean cell count / g faeces (standard deviation)**		**% *A. muciniphila* cells [(3)]**
		Total cells [(1)]	**MUC-1437 Count [(2)]**	
1	26 (F)	3.08 (0.41) x 10^{10}	4.22 (0.42) x 10^8	1.37 (0.11)
2	40 (F)	5.30 (1.06) x 10^{10}	4.96 (0.27) x 10^8	0.94 (0.09)
3	26 (F)	4.94 (2.43) x 10^{10}	7.18 (0.45) x 10^8	1.45 (0.21)
4	33 (M)	7.78 (5.65) x 10^{10}	1.85 (0.47) x 10^8	0.24 (0.08)
5	26 (F)	5.24 (1.27) x 10^{10}	3.07 (0.08) x 10^8	0.59 (0.16)
6	26 (F)	8.06 (0.14) x 10^{10}	nd	nd
7	27 (F)	4.09 (3.52) x 10^{10}	1.26 (0.33) x 10^9	3.08 (0.34)
8	26 (F)	9.46 (2.18) x 10^{10}	5.22 (0.12) x 10^8	0.55 (0.43)
9	29 (M)	8.78 (6.61) x 10^{10}	5.77 (1.20) x 10^8	0.66 (0.23)
10	27 (M)	3.62 (0.82) x 10^{10}	6.20 (2.32) x 10^8	1.71 (1.08)
11	29 (M)	1.22 (1.09) x 10^{10}	1.60 (0.88) x 10^9	1.31 (0.13)
12	31 (M)	10.9 (2.54) x 10^{10}	2.78 (0.11) x 10^9	2.55 (0.51)
13	31 (F)	5.94 (1.15) x 10^{10}	4.03 (3.07) x 10^8	0.68 (0.28)
Mean[(4)]		**6.03 (2.69) x 10^{10}**	**8.24 (7.35) x 10^8**	**1.26 (0.85)**

[1)] Number of faecal cells/g wet faeces cells determined by TOTO-1 staining, [(2)] Number of cells hybridised by the probe Muc1437 with Cy5, [(3)] % based on TOTO-1 counts, [(4)] n=12, nd is not detected (under detection limit)

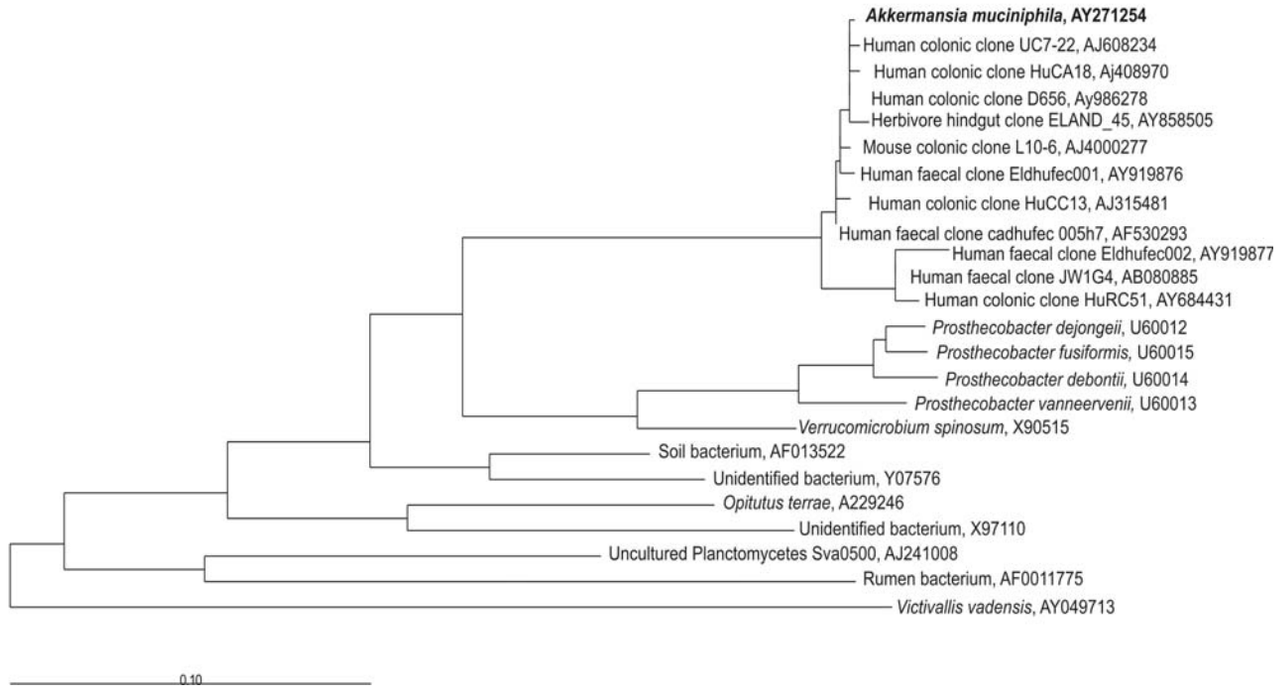

Figure 6.3. Phylogenetic tree showing the position of *A. muciniphila* and the clones sequences in the Verrucomicrobia phylum. The bar represents 10% sequence divergence.

Discussion

In our study, we present an oligonucleotide probe for use in FISH for detection and quantification of an obligate mucin-degrader *A. muciniphila* in human faecal samples. FISH analysis was done by a FCM approach, since it was more suitable and reliable tool for *A. muciniphila* quantification due to its small size. In the stool samples, cells of this species were present in 12 out the 13 samples and accounted for more than 1% of the total faecal cells population (n=12) (1.26 ± 0.85 %) corresponding to 8.24 ± 7.35 x 10^8 bacteria/g wet faeces as revealed by FISH analysis. Our data are in agreement with the results obtained from clone libraries generated from either faeces or biopsies samples. Indeed, up to now, eight 16S rRNA sequences showed sequence similarity ≥98% and three have <98% with the 16S rRNA gene sequence of *A. muciniphila* (Fig. 6.3), indicating that they probably represent other species in the genus *Akkermansia*. These sequences have been derived from human clone libraries from healthy individuals [75, 108, 112, 178, 285] (Saunier *et al*., unpublished) and biopsies of IBD patients [178], (Hutson and Collins, unpublished), and even animals such as herbivore [199] and mouse [250]. More precisely, the recent study of Wang *et al*. reported that the16S rRNA gene sequence of *A. muciniphila* was detected in the distal ileum, ascending colon and rectum with levels of 5, 6 and 9% of the clone libraries from a healthy 54-year-old female respectively [313]. In a larger scale study from

Eckburg *et al*. [75] win which 13,355 prokaryotic ribosomal RNA gene sequences were examined from various colonic mucosal sites and faeces of three healthy subjects, *A. muciniphila* was detected in every site from one subject, from the ascending colon, descending colon, and stool of another subject but was not detected in appreciable numbers in the third subject. The sequence was also detected in clone libraries derived from patients with Crohn's disease, and four healthy matching individuals. In one of the four Crohn's disease patients, 14.5% of the retrieved sequences corresponded to the one of *A. muciniphila*, while it accounted 0.35% in a healthy individual [178]. Recently, a similar FISH-FCM study conducted on faeces from 10 IBD patients, using the MUC-1437 probe, revealed that using the same approach that we did, FISH-FCM, *A. muciniphila* was detected in all the faeces (Rochet V, personal communication) at level of 8.84 ± 7.75% of eubacterial cells. Notably it was found in one sample at 27%. The mucus layer from of IBD patients, has several times been reported as affected, regarding glycosylation of mucins [128] and thickness of the mucus layer [225], suggesting a disbalance between mucin synthesis and degradation. This disbalance in mucin turnover could explain the higher number of *A. muciniphila*, which metabolism is largely dependent on mucin utilisation [66], observed in these samples.

These data validate our analysis, showing that *A. muciniphila* is an important member of the human gut microbiota. Interestingly we also found that the 16S rRNA sequence of *A. muciniphila* contains two mismatches with the eubacterial probe (EUB-338), commonly used as positive control and *A. muciniphila* cells showed only a weak signal after EUB-338 hybridisation. With the increase of availability of 16S rRNA sequences, it was shown that EUB-338 does not cover all bacterial phyla, such as the Planctomycetales and Verrucomicrobia. Therefore, two additional probes, EUB-338-II and EUB-338-III, targeting these two groups respectively were designed. When combined, these three probes allow detection of all the phyla of the Bacteria domain [58]. The EUB-338-III is 100% complementary only to members of the divisions 1 to 4 of the Verrucomicrobia phylum [307]. Nevertheless, in intestinal studies, it has not been often employed, suggesting that *A. muciniphila* has been omitted from the analysis. In FISH studies, a panel of probes is available and has been widely employed to characterise the intestinal microbiota. However, a proportion remains undetected [81, 107]. Recently, two studies described the composition of the colonic microbiota of northern Europeans, using FISH-FCM, using a wide array of oligonucleotide probes (14 and 18 respectively). Each of the following group, *Bacteroides putredinis, Bacteroides vulgatus, Enterobacteria, Lactobacillus-Streptococcus, Bifidobacterium, Atopobium* was present at the level of ≤1% of the total bacteria [152, 196]. Even though a large number of probes was used, more than 24% of the human faecal microbiota could

not be still identified, revealing the phylogenetic gap between the cultivated and non-cultivated species.

In conclusion, our study shows for the first time a validation and application of a novel phylogenetic probe targeting an intestinal member of Verrucomicrobia, showing the predominance of *A. muciniphila* in faecal samples. The results obtained are from healthy individuals and we did not investigate the presence of *A. muciniphila* in biopsies from healthy individuals and IBD patients. Future investigations of its localisation in biopsies will allow the understanding of its role in health and disease.

Acknowldegements

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CHAPTER 7

Mucin Secretion and Fructooligosaccharides in the Intestine: the Role of the Mucin-Degrading Bacterium *Akkermansia muciniphila*

Muriel Derrien, Sandra Ten Bruggencate, Willem M. de Vos and Erwin G. Zoetendal

Mucins are important components of the mucus layer that contribute to protecting the epithelium against pathogens and physical damage while providing lubrication for the passage of the intestinal contents. A previous placebo-controlled intervention study demonstrated a significant increase of faecal mucin excretion after consumption of a diet rich in fructooligosaccharides (FOS). Since FOS has a clear impact on the faecal mucin content, we studied the relation between mucin content and FOS on *Akkermansia muciniphila*, a predominant and common species in the human gastrointestinal (GI) tract which was found to be an obligate mucin degrader. This was realised by monitoring *A. muciniphila* population dynamics in faecal samples of 29 volunteers who participated in a FOS trial. Quantitative PCR of 16S rRNA genes demonstrated a correlation between decreasing *A. muciniphila* numbers and faecal mucin excretion. However, this correlation was only significant after the FOS consumption ($p<0.05$). Although the number of *A. muciniphila* cells tends to be lower after FOS consumption, the difference between placebo and FOS groups was not significant ($p=0.16$). Remarkably, the individual temporal variation in *A. muciniphila* numbers tends to be lower when FOS was taken after the placebo. However, the FOS consumption and *A. muciniphila* numbers were not linked, indicating that the *A. muciniphila* numbers and mucin content in faeces are affected by distinct, yet unknown host-factors. In conclusion, this study suggests *A. muciniphila* to have a role in the mucin degradation in the GI tract and indicates that FOS consumption has an indirect effect on the *A. muciniphila* population dynamics, probably by stimulating other bacterial groups.

Introduction

Mucins are the major component of the mucus layer, which covers various organs including the entire gastrointestinal (GI) tract. This coating protects the epithelial cells against several injuries including those due to bacteria, viruses, chemical and toxins, which can be present in the intestinal lumen. Hence, mucus is an essential barrier component, which is involved in the homeostasis of the GI tract. Moreover, the constant availability of intestinal mucus makes mucin an ideal niche and substrate for GI tract bacteria [157]. Indeed, many bacteria belonging to the genera *Ruminococcus*, *Bifidobacterium*, *Peptostreptococcus*, *Clostridium*, have been found to grow on mucin as sole carbon source in pure culture [42, 121, 171]. Recently, *A. muciniphila*, an obligate mucin-degrader was isolated from the faeces of a healthy adult human on a mucin based medium [66]. This bacterium was shown to be a predominant human GI tract member representing in average between 0.1 and 3% of the faecal microbiota (Chapter 6).

Studies with rodents indicated that mucin degradation is a normal process of mucus turn over in the GI tract. In contrast to conventional rats, it was shown in germ-free rats that undegraded mucin is present in faeces, due to the absence of mucolytic bacteria. Moreover, administration of antibiotics to rats resulted in a decrease in caecal mucin degradation [41]. These observations indicate that the microbiota plays a major role in mucin degradation. It has been reported that changes in mucin synthesis [302] or excretion [38, 144, 254, 260, 294] can be induced after dietary fibre intake in rats. Several of these dietary fibres are also commonly known as prebiotics. Prebiotics are defined as a non-digestible food ingredient which beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improving host health [87, 88]. Prebiotics are resistant to enzymatic digestion in the small intestine, and therefore, reach the colon in an intact form. Subsequently, they can be metabolised by colonic bacteria and stimulate the growth of saccharolytic bacteria notably those belonging to the genera *Bifidobacterium* and *Lactobacillus* [86]. The most commonly used prebiotics used in human nutrition are oligosaccharides, including inulins and their derivatives that consist of fructooligosaccharides (FOS) [170]. These are naturally present in artichokes, onions, chicory, garlic, leeks, and, to a lesser extent, in cereals [89]. A recent study investigated the effect of FOS on intestinal barrier function in human volunteers and demonstrated that FOS consumption increased the number of bifidobacteria and lactobacilli. In addition, FOS consumption doubled faecal mucin excretion, increased the lactate concentration, and lowered the pH [293]. Increase of mucin excretion could be an indicator of an increase of mucin synthesis, a decrease of mucin degradation in the lower GI tract, or both.

The capacity of *A. muciniphila* to digest the complex mucin structure can be an ecological advantage over microbes lacking the ability to utilise mucin. Since *A. muciniphila* appeared to be specialised in mucin utilisation, we investigated its population dynamics during the FOS trial that affected mucin excretion. For this purpose, quantitative PCR (qPCR) of 16S ribosomal RNA (rRNA) genes was used as molecular tool, since it has been successfully applied in quantification of lactobacilli, bifidobacteria, *Bacteroides* and desulfovibrios in faeces [69, 78, 96, 102, 103, 182, 216]. A pair of specific primers targeting the 16S ribosomal RNA (16S rRNA) was designed, validated and applied on faeces during the placebo and FOS treatment in order to quantify the population of *A. muciniphila*, monitor its dynamics in the GI tract in relation to faecal mucin excretion and FOS consumption.

Material and Methods

Growth conditions. *A. muciniphila* was cultivated under anaerobic conditions in 50 ml of a mucin based medium as previously [66]. Bacterial enumeration was performed by fluorescent *in situ* hybridisation as previously described (Chapter 6). Growth of *A. muciniphila* on FOS was determined in the basal medium supplemented with 1% of FOS (purity 93%, Raftilose P95, Orafti, Tienen, Belgium) alone or with a protein source (per litre: 2 g of peptone, tryptone, yeast extract and casitone).

FOS intervention study and faecal sample collection. To study the relation between *A. muciniphila* numbers, faecal mucin excretion and FOS ingestion, a double-blind placebo controlled crossover study design with 2 supplement periods of 2 weeks separated by 1 washout period of 2 weeks was set-up. Participants were stratified by age and then randomly divided in 2 groups. In total 34 healthy men consumed either lemonade with 20 g of FOS or 6 g of sucrose (placebo) per day. Faeces were collected the last day of each treatment. From the 34 individuals, 29 were included for faecal analysis [293].

DNA extraction from pure cultures and faecal samples. Bacterial DNA was extracted from *A. muciniphila* from 10 ml of culture and 0.2g of faeces using the QIAamp DNA stool mini kit (QIAgen, Westburg, Leusden, The Netherlands) [293]. In short, after addition of the lysis buffer ASL, cells were treated in a bead beater two times at maximum speed for 45 sec. After heating at 70°C for 5 min the protocol was continued according to the instructions of the manufacturer. Purified DNA was checked by eletrophoresis on agarose gel (1.2%) containing ethidium bromide

and quantity was assessed spectrophotometrically (ND-1000, NanoDrop Technologies, Wilmington, USA).

Primer design and PCR conditions. Two specific primers were designed from the variable regions of the 16S rRNA gene sequence of *A. muciniphila.* The GenBank program from NCBI (BLAST) was used to verify that both primers were only specific to the target organism. Both primers selected for detection of *A. muciniphila* were named based on the nomenclature from Alm *et al*. [4]. These were S-St-Muc-1129-a-a-20 (AM1) with the following sequence: 5' CAG CAC GTG AAG GTG GGG AC and S-St-Muc-1437-a-A-20 (AM2) with the sequence: 5' CCT TGC GGT TGG CTT CAG AT. Both primers were purchased from MWG (Ebersgerg, Germany). To check the specificity of the amplification, classic PCR was performed using DNA from 20 intestinal isolates and 96 cloned 16S rRNA genes from uncultured organisms as templates, which are representatives of the most dominant groups found in the GI tract. The optimal annealing temperature was optimised using gradient PCR from 52 to 70°C with a T1 thermocycler (Whatman Biometra, Göttingen, Germany). PCR mixtures of 20-µl contained 1X *Taq*DNA polymerase buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl], 3 mM MgCl2, 100 µM each deoxynucleoside triphosphate, 0.2 µM of each primer and 1.25 U of *Taq* polymerase (Life Technologies, Gaithersburg, MD, USA), and 1 µl of genomic or plasmid DNA corresponding approximately to 30-100 ng. The PCR conditions were the following: 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 52 to 70°C for 40 sec, 68°C for 30 s, and 72°C for 5 min (final extension). The specificity of the primers was confirmed using non-target organisms by analysing 5 µl PCR aliquots by electrophoresis on 1.2% (wt/vol) agarose gels to check the product size.

Quantitative PCR. Quantitative PCR was performed with a MyiQ Cycler apparatus (Bio-Rad, Veenendal, The Netherlands). All reactions were performed in duplicate in one run and in duplicate PCR runs. Samples were analyzed in a 25-µl reaction mix consisting of 12.5 µl Bio-Rad master mix SYBR Green (50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM of each dNTP, 0.625 U i*Taq* DNA polymerase, 3 mM $MgCl_2$, 10 nM fluorescein), 0.1 µM of each primer and 5 µl of template DNA, water or a pool of DNA extracted from intestinal non target bacteria. Standard curves of DNA from *A. muciniphila* were created using serial 10-fold dilution of the purified DNA corresponding to a range from 175 ng to 0.175 pg (corresponding to 10^8 to 10^2 cells, determined by FISH (Chapter 6)). For faeces 10 times diluted DNA, corresponding to approximately 30 ng of genomic DNA was used as template. As negative control a mix containing 10 ng DNA from each of the 4 intestinal strains (*Bifidobacterium bifidum, B. infantis, Bacteroides fragilis and Victivallis vadensis*) was used. The following conditions of_qPCR used

were: 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing temperature of 60°C for 20 sec, extension at 72°C for 30 sec and a final extension step at 72°C for 5 min. A melting curve was performed at the end of each run to verify the specificity of the PCR amplicons by slowly heating the final reaction mix to 95°C (0.5°C per cycle). Data analysis was performed using the Bio-Rad software. The percentage of *A. muciniphila* cells in faecal samples was determined by comparing the C_T values obtained to the standard curve.

Mucin determination. Mucins were extracted from freeze-dried faeces and quantified fluorimetrically, as described earlier [26]. Standard solutions of *N*-acetylgalactosamine (Sigma) were used to calculate the amount of oligosaccharide side-chains liberated from mucins. Faecal mucins are expressed as μmol oligosaccharide equivalents.

Statistical analysis. Correlation analysis and paired student's *t* -tests were performed for statistical analysis of the data. If $p<0.05$ was observed, the data sets were considered as significantly different.

Results

A previous double-blind, placebo-controlled, cross-over study of 2 x 2 weeks, with a washout period of 2 weeks was performed with 29 normal weight healthy men between 25 and 30 years [293].

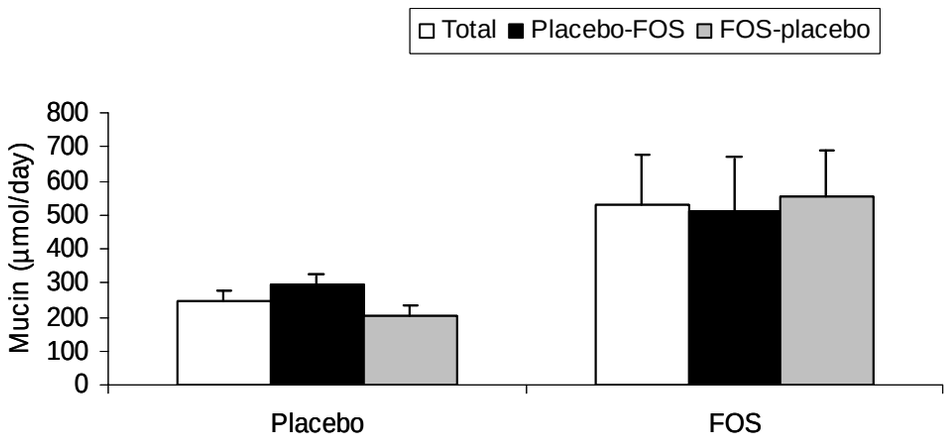
Mucin analysis

Faecal mucin excretion was determined during the FOS administration and placebo periods. During the FOS administration, the average of the mucin excretion within the 29 individuals was 248.6 (±150.5) µmol/day. After FOS ingestion, it reached 531.4 (±782.1) µmol/day [293]. Independent of the order of the FOS-placebo intake, there was in average a significant increase of the faecal mucin excretion ($p<0.05$). However, the mucin excretion varied between the different individuals as indicated by the relatively large SEM, notably in the FOS group (Fig. 7.1A).

Growth of *A. muciniphila* on FOS

In order to investigate whether *A. muciniphila* can use FOS as carbon and energy source, *A. muciniphila* was grown in a basal medium supplemented with FOS as sole carbon source alone or supplemented with a protein source. No growth was observed, indicating that *A. muciniphila* does not use FOS as sole carbon and energy source.

A



B

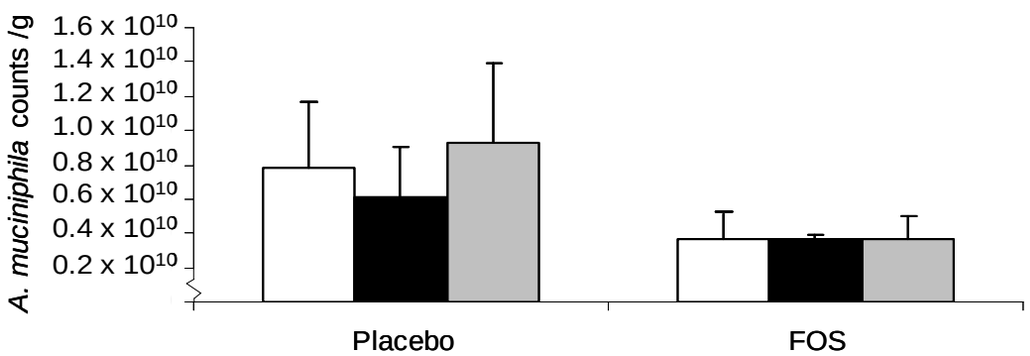


Figure 7.1. A. Impact of the order of the intake FOS and placebo on the excretion of mucin [293]. **B.** Numbers of *A. muciphila* in faeces. Results are expressed as mean ± SEM (n=29).

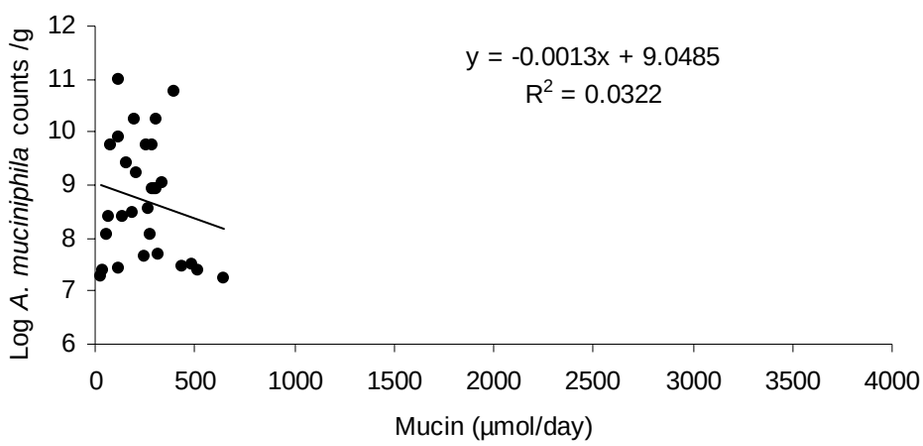
qPCR assays for quantification of *A. muciniphila*

In order to monitor the *A. muciniphila* population in faeces, two *A. muciniphila*-specific primers, AM1 and AM2, targeting part of the variable regions V7-V9 of its 16S rRNA gene were developed and validated. The PCR primers were specific for *A. muciniphila* at 60°C with amplification of a product of the expected size (327 bp). All other test strains and the cloned 16S rRNA genes from uncultured GI tract bacteria did not show any amplification during PCR. To determine the PCR detection limit a standard curve was made from serial dilutions of DNA isolated from pure cultures of *A. muciniphila*. A linear relationship was observed between the cell number and C_T values ($r^2 = 0.998$) when the cell numbers per reaction mixtures varied between 10^2 and 10^8 cells. Reliable quantification of cell numbers was not possible below 10^2 cells per reaction mix. This corresponds to a detection limit of 5 $x10^3$ cells / g faeces. Melting curve analysis of the amplicons obtained by qPCR generated a specific peak at 90°C 16S rRNA gene of *A. muciniphila*.

Quantification of *A. muciniphila* in human faeces

The number of *A. muciniphila* cells was estimated by qPCR with DNA extracted from the 58 samples. Based on qPCR analysis, *A. muciniphila* accounted between 1.76 x 10^7 and 5.85 x 10^{10} cells per gram (average of 7.76 ± 20.1 x 10^9) after the placebo period and between 1.25 x 10^7 and 2.70 x 10^{10} cells per gram (average of 3.67 ± 7.70 x 10^9) after FOS consumption (p=0.16) (Fig. 7.1B). Interestingly, the SEM was much higher in the placebo group than in the FOS group, indicating that the individual variability in faecal *A. muciniphila* numbers was less after FOS ingestion. Correlation analysis was performed to determine if there was a relation between these varying *A. muciniphila* numbers and the mucin excretion in the faeces. Since the mucin excretion was significantly different between placebo and FOS, they were treated separately for correlation analysis. During the placebo treatment, the *A. muciniphila* numbers tended to decrease with increasing mucin excretion, but this correlation was not significant (Fig. 7.2A).

A



B

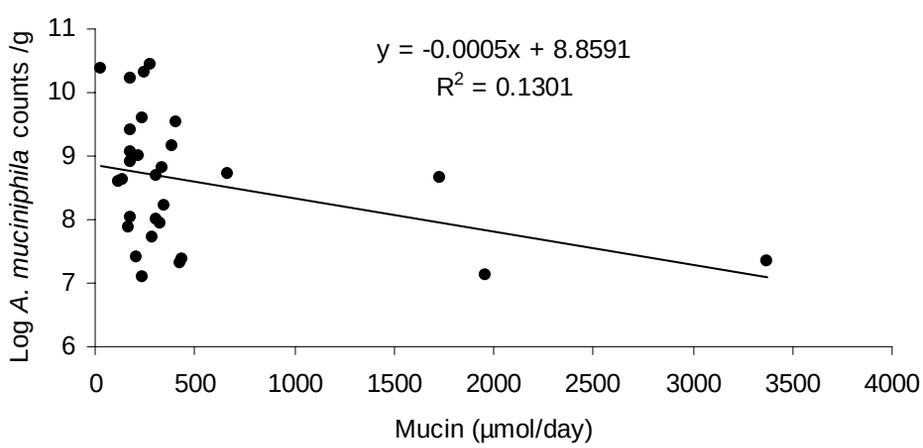


Figure 7.2. Diagrams showing the relation between the *A. muciniphila* number and the faecal mucin excretion after the placebo period (**A**) and after the FOS intake (**B**). The linear relation that best fitted the data is plotted. The equation and R^2 are presented.

However, a significant decrease of *A. muciniphila* ($p<0.05$) with increasing faecal mucin excretion was observed with FOS (Fig. 7.2B). The significance of this correlation could be affected by the three faecal samples which contained much higher faecal mucin content than the rest. Nevertheless, these results suggest a role for *A. muciniphila* in the degradation of mucin degradation in the human colon.

Impact of FOS on *A. muciniphila* population dynamics

Since a negative correlation was suggested for *A. muciniphila* numbers and faecal mucin excretion, the impact of FOS on the *A. muciniphila* population dynamics was examined. Independent of the FOS-placebo intake order, there was in average a lower number of *A. muciniphila* after intake of FOS (Fig. 7.1B), although this difference was not significant ($p=0.16$). However, this varied between the different individuals as indicated by the relatively large SEM. Remarkably, the fold-change in *A. muciniphila* numbers was dependent on the order of the FOS-placebo intake. The change in *A. muciniphila* numbers was lower when FOS was taken at the end of the trial (Fig. 7.3).

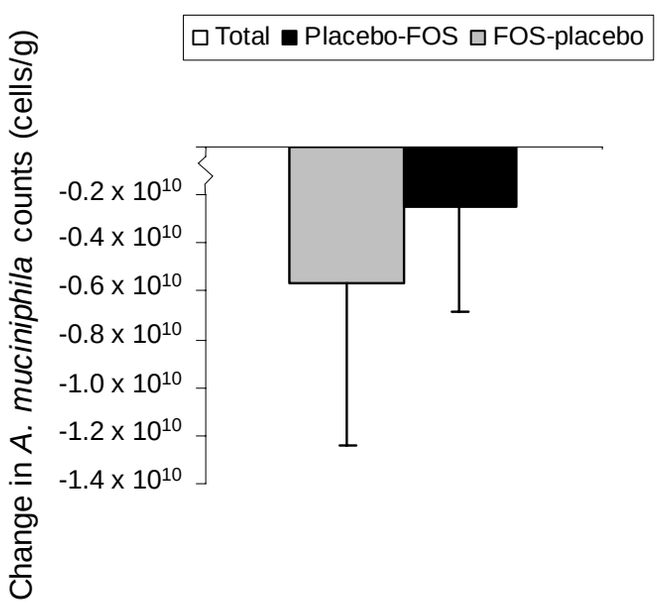


Figure 7.3. Diagram showing the impact of the order of the FOS-Placebo intake on the change in *A. muciniphila* numbers in faeces. The mean and SEM are indicated. The changes in cell numbers are determined by subtracting the *A. muciniphila* numbers after the FOS intake from the numbers found after placebo period.

This difference between the intake order indicates that there was less temporal variation per individual in *A. muciniphila* numbers when FOS was consumed after the placebo. Moreover, the impact of FOS on the *A. muciniphila* numbers varied between the different individuals. Depending on the individual FOS resulted in either an increase or a decrease in *A. muciniphila*

numbers. Since individual-specific effect of FOS on *A. muciniphila* population dynamics, but also on the mucin excretion were observed [293], we examined if these observations were confounding. From the 29 subjects, only 15 had a positive relationship between the change in *A. muciniphil*a numbers and the change in mucin excretion. This observation was not influenced by the order of FOS-placebo intake. This indicates that the type of impact of FOS on the changes in *A. muciniphila* numbers and faecal mucin excretion are independent of each other, and suggests the role of other GI tract bacteria in this interplay.

Discussion

A. muciniphila is a recently described bacterium that is specialised in mucin degradation and is a common dominant member of the GI tract microbiota in human. Some species belonging to the genera *Ruminococcus*, *Bacteroides* and *Bifidobacterium* have also been shown to degrade mucin [123]. Nevertheless, these organisms can grow in media containing a wide variety of carbon sources while *A. muciniphila* can be only maintained in a mucin-based medium. Despite its capacity to degrade mucin, little is known about the role of *A. muciniphila* in GI tract and how the mucin excretion and *A. muciniphila* numbers are correlated. Recently, a double-blind placebo-controlled trial was reported, involving 34 healthy men in which the impact of FOS consumption on the intestinal barrier was studied [293]. This report demonstrated that the number of bifidobacteria and lactobacilli, as well as mucin excretion, were significantly affected by FOS consumption in these individuals. In the present study, all accessible faecal samples (29) were analysed. Since FOS consumption had a significant effect on the faecal mucin excretion ($p<0.05$), we studied the impact of consumption of this prebiotic on the number of *A. muciniphila* and analysed whether FOS consumption, faecal mucin excretion and *A. muciniphila* numbers were correlated.

To determine the *A. muciniphila* numbers in faeces, a specific qPCR approach was developed and validated since it allows accurate and sensitive detection of fastidious microorganisms, such as *A. muciniphila* in a complex ecosystem and is relatively easy to use. After FOS consumption a significant correlation ($p<0.05$) was found between the decrease in *A. muciniphila* numbers and increasing mucin excretion. Although a similar correlation was observed after the placebo period, this was not significant. The difference in significance could for part be explained by three faecal samples with much higher mucin excretion values in the FOS group. Nevertheless, it was recently described that the carbon source utilisation by *A. muciniphila* is almost completely restricted to mucin and therefore, the most likely explanation

for the observed correlation is that *A. muciniphila* plays a role in the mucin degradation in the GI tract.

It was previously shown that FOS consumption induced an increase in bifidobacteria and lactobacilli, and that *Escherichia coli* numbers were not affected. These bacteria except *B. bifidum* are not known to degrade mucin. To assess if the observed mucin secretion is a harmful or beneficial event is difficult to state. While some studies argue that mucin excretion is an indication of mucosal irritation [293, 294], others claim that mucin excretion is a normal phenomenon after fibre intake, resulting from an increase of both short-chain fatty acids and mechanical stimulation induced by an increase of faecal material [94]. Moreover, an additional secretion of mucin in the colon would favour the transit time of faecal material and increase the protection of the epithelium due to the lubricant and protective properties respectively of mucin. Mucin degradation can be performed by both bacteria and transit of faecal material with a balance between mucin degradation and mucin synthesis [63]. The balance in the turnover or composition of mucins has been shown to be disturbed in case of intestinal diseases as reviewed by Corfield *et al*. [53]. In our study, the balance was apparently modified in the direction of an increase of faecal mucin excretion by the FOS consumption. This change can be interpreted in three ways: either there was an increase of mucin synthesis or a decrease of mucin degradation in the GI tract, or a combination of these. Mucin can be degraded by bacteria and passage of intestinal material through the GI tract. The role of bacteria in mucin degradation was shown using germ-free rats, in which faecal mucin was found intact indicating that the microbiota plays a major role in its degradation [133]. Moreover, after treatment of antibiotics, such as bacitracin, clindamycin or vancomycin, the faecal mucin profile tends to be similar to the one obtained in germ-free rats highlighting the role of bacteria in mucin degradation [41]. In a previous study, *A. muciniphila* was found to be a specialist in mucin utilisation [66]. In the current study, the increase of mucin excretion was correlated with a decrease of *A. muciniphila* after FOS consumption, which suggests that *A. muciniphila* plays a role in the mucin degradation in the GI tract. Based on qPCR counts, there was no significant difference in *A. muciniphila* numbers between placebo and FOS ($p=0.16$). Moreover, the changes were not consistent and varied from increases to decreases in number depending on the individual (Fig 7.2A). Despite all these different influences, in average the *A. muciniphila* numbers tend to be lower, from 7×10^9 to 3×10^9 after FOS digestion. Our growth studies indicated that *A. muciniphila* could not use FOS as sole carbon and energy source or supplemented with a protein source, and that FOS did not inhibit its growth on mucin. This suggests that the impact of FOS consumption was not a direct effect on the *A. muciniphila* population dynamics. Since other bacterial groups, such as

bifidobacteria and lactobacilli were stimulated by the use FOS, which resulted in a lower faecal pH and lactate concentration [293], the average reduction in *A. muciniphila* numbers might be a result of competition with microbial groups that are stimulated by FOS.

Numerous studies have shown that each individual has a specific microbial community in their GI tract [330, 335]. This also holds for the mucin-degrading community in faeces [65]. In the present study it was observed that the temporal variation in *A. muciniphila* numbers in faeces is largely individual-dependent. Remarkably, a similar effect was found for the faecal mucin excretion [293]. This means that despite the trends observed, the impact of FOS caused either an increase or decrease for both *A. muciniphila* numbers and mucin excretion, depending on the individual. As demonstrated in this study, these individual changes in mucin excretion and *A. muciniphila* numbers were not linked. What exactly determines this individual-specific impact of FOS on faecal mucin excretion and *A. muciniphila* numbers, and what the role of the other mucin-degrading community members is, has to be determined in future studies using diversity microarrays, such as the recently developed human intestinal tract chip (HITChip; [227]).

Overall, our study indicated that *A. muciniphila* has a role in the mucin degradation in the GI tract and therefore, *A. muciniphila* might be regarded as a biomarker for mucin degradation. FOS affected the population dynamics of *A. muciniphila* by stimulating other bacterial groups, such as lactobacilli and bifidobacteria. However, the degree of this impact varies between individuals, which is likely due to host-specific factors, such as differing GI tract community structures.

Acknowledgements

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CHAPTER 8

Gene Profiling of Mice Responses after Mono-Association with the Intestinal Mucin-Degrading Bacterium *Akkermansia muciniphila* and the Commensal *Lactobacillus plantarum*

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In this study, we analysed the global transcriptional responses of intestinal epithelia of germ-free mice after mono-association with two intestinal bacteria, *Akkermansia muciniphila*, a Gram-negative bacterium specialised in the degradation of mucin, and *Lactobacillus plantarum*, a Gram-positive lactic acid bacterium that can not degrade mucin. Two groups of six germ-free mice were mono-associated with each individual bacterium, and one group was kept germ-free. Intestinal biopsies from three anatomical sites (ileum, caecum and proximal colon) were sampled seven days after bacterial inoculation. Affymetrix mouse 430 v2.0 GeneChips were used to screen for genes which expression in the intestine had changed after colonisation. Microarray analysis of these intestinal samples using the whole genome arrays showed that both *A. muciniphila* and *L. plantarum* modulated a comparable number of genes, although slightly increased in response to *L. plantarum*. Moreover it was observed that depending on the anatomical location, the host response was found to be highly specific for each bacterium. For *A. muciniphila,* major responses included regulation of the immune response, cell proliferation, cell adhesion and apoptosis, while the major response to *L. plantarum* consisted of regulation of lipid metabolism.

Introduction

Interactions between microbes and their animal hosts usually take place at mucosal surfaces. These relationships are highly specific since particular microbes colonise definite areas creating microenvironments. The human gut microbiota includes more than 10^{15} bacterial cells with a common function and hence can be referred to as a virtual organ situated in an organ [208]. These enormous numbers of bacterial cells that interact with the intestinal epithelium render the relationships between bacteria and host complex to analyse. Moreover, the host has to face a paradox: tolerate commensals while respond to pathogens via elicitation of defence mechanisms [139].

Increasing availability of complete bacterial and host genomes and the application of powerful techniques such as DNA microarrays has allowed studying the details of the interactions between host and bacteria at the transcriptional level. Host-pathogens interactions have been most extensively studied due to their obvious consequences on host health. Nowadays, a number of studies related to interactions between host and commensal bacteria has emerged, especially those occurring in the GI tract. Microarrays have become an important tool to screen the global transcriptional responses, and have considerably improved our understanding of the host responses to bacterial colonisation and vice versa [56, 132]. *In vivo* models have shown their potency to elucidate host-microbes interactions. Moreover, the use of germ-free animals devoid of microorganisms has opened a new window to investigate the role of bacteria on intestinal functions [202].

The pioneers studying the impact of intestinal commensals were Gordon and co-workers, who showed in a series of studies based on microarray technology that the commensal bacterium *Bacteroides thetaiotaomicron*, colonised in germ-free mice, was able to trigger the mouse metabolism at its expense [278], activate the host immune system and angiogenesis [282], and increase fat storage [8]. These studies have demonstrated the mutualistic relationship between host and bacteria, the specificity of the host response to bacterial colonisation and in general the major role of commensals on host physiology. So far, only few studies based on germ-free animal models mono-associated with commensal bacteria have been performed, and most of the studies focused on mono-associated mice with *B. thetaiotaomicron* or bi-associated with *B. thetaiotaomicron* and several others commensals [276].

In the gut, interactions usually occur in or close to the mucus layer, which lubricates and protects the epithelium against different types of aggressors such as toxins and acid and bacterial invasion. The mucus layer is the interface where bacteria and epithelial cells are in close contact [157] and therefore, it is believed that primary contacts occur in this layer [277]. Mucus-

associated bacteria were found to be numerous in an intestinal *in vitro* system approaching 10^9cfu/ml [173]. In addition, 16S rRNA-based studies indicated that communities associated with the colonic mucosa are different from those in the faeces [154, 201, 336]. These studies indicate that mucosa-associated bacteria play an important role in this ecosystem by creating a microenvironment [277].

Some bacteria can use mucins as energy source, which confers them a growth advantage [175]. Recently, we have isolated a strict anaerobic and Gram-negative bacterium, *Akkermansia muciniphila*, that is specialised in mucin utilisation [66], and that accounts for approximately 0.1-3% of the total microbial community in humans (Chapter 6). Other studies based on PCR-amplified 16S rDNA clone libraries from human biopsies and faeces showed that this bacterium was frequently detected at significant levels in the GI tract [75, 112, 178, 313]. In addition, *A. muciniphila* has also been detected in mice and was estimated to represent approximately 4% of the bacterial community based on 16S rRNA analysis (Galic, personal communication). However, despite its regular detection in intestinal samples, the role of *A. muciniphila* in the gut and impact on the host are still unknown.

The objective of this study was to investigate the impact of *A. muciniphila* on host by comparing the global transcription of germ-free mice mono-associated with this mucin-utilising bacterium to that colonised by *Lactobacillus plantarum*, another commensal of mice and human that cannot utilise mucin, but has the capability to utilise a wide variety of carbon sources [143] and is known to display specific and differential responses at various sites in the murine GI tract [32, 179].

Material and methods

Animals. The study protocol was reviewed and approved by the Northern Stockholm Ethics Committee for Animal Experiments. Adult germ-free female NMRI-KI mice (45 – 65 days) were used (n = 18) for bacterial mono-association. The germ-free animals were inbred for >60 generations at the Laboratory of Medical Microbial Ecology at Karolinska Institute and they were housed in lightweight stainless-steel isolators [100]. All mice had free access to a steam-sterilised standard mouse chow (R36; Lactamin, Vadstena, Sweden) and to sterilised water. Artificial light was available between 6 a.m. and 6 p.m.; the temperature was 24 ± 2.2°C, and the humidity was 55% ± 10%. The germ-free status was checked weekly by inoculating faecal samples in different media incubated both aerobically and anaerobically at 20 and 37°C for up to 4 weeks.

Bacteria and growth conditions. Two bacterial strains were used in this study, the mucin-degrading *A. muciniphila* Muc^T (ATTC BAA-835) and the non mucin-degrading *L. plantarum* WCFS1 (NCIMB 8826). *A. muciniphila* was grown anaerobically in a basal mucin based medium as previously described [66] and *L. plantarum* was grown anaerobically at 37°C in Man-Rogosa-Sharpe broth (MRS; Le Pont de Claix, France).

Mono-association. The germ-free mice were mono-associated using established protocols [40]. In brief, 10 ml of cultures in late log phase of *A. muciniphila* Muc^T and *L. plantarum* WCFS1 were centrifuged (4500 rpm, 10 min). Pellets were resuspended in 1 ml of sterile anaerobic Phosphate Buffer Saline (PBS) and dispensed into sterile ampoules which were heat-sealed. The external surface of each ampoule was sterilised with chromsulfuric acid before transfer into respective isolators. Inside the isolators, the ampoules were broken, and 0.2 ml (10^9 cfu/ml) of *A. muciniphila* (n=6) was inoculated intragastrically since it is a strict anaerobe and *L. plantarum* (n=6) was inoculated orally. The germ-free control mice (n=6) were handled routinely. Each group of mice was housed in separate isolators.

Preparation of specimens. After 7 days of colonisation mice were killed by cervical dislocation and terminal ileum, caecum and ascending colon specimens were sampled. Intestinal contents were scrapped from the epithelium and were kept at -20 °C for DNA extraction and bacterial enumeration by quantitative PCR (qPCR). Tissues were flushed with PBS, and processed for different purposes. For RNA isolation, tissues were immediately preserved in 5 volumes of RNA later (Ambion, Austin, Texas, USA) and stored at 4 ºC until use. For histology, biopsies were fixed for 18 h at room temperature in 4% paraformaldehyde, pH 7.3 and subsequently processed for fluorescent *in situ* hybridisation (FISH).

Histology and fluorescent *in situ* hybridisation. After preservation in paraformaldehyde, tissue samples were washed in phosphate buffer, dehydrated in an ethanol gradient and embedded in paraffin. Five-µm thick sections were mounted on superfrost coated slides, dried and incubated at 37°C for 16 h. For fluorescent *in situ* hybridisation (FISH), slides were deparaffinised in xylene and dehydrated in an ethanol gradient. Sections were overlaid with 100 µl hybridisation buffer (0.9 M NaCl, 0.02M Tris-HCl [pH 8.0], 0.01% sodium dodecyl sulfate) containing an oligonucleotide mixture (5 ng/µl) consisting of the *A. muciniphila* (Cy3-labelled MUC-1437) and total bacterial (FITC-labelled EUB-338) probes (Table 8.1) (Biolegio BV, Nijmegen, The Netherlands). Hybridisation was carried out at 50°C for 16 h in a humid chamber. After

hybridisation, the tissue sections were washed with a washing buffer (0.02 M Tris-HCl pH 8, 0.9 M NaCl) for 10 min at 50°C. Counterstaining was carried out with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St Louis, MO, USA), and the slides were analysed with a Nikon E600 epifluorescence microscope equipped with an appropriate filter set.

Establishment of *A. muciniphila* in mice. To verify that mice were colonised with *A. muciniphila*, faecal samples (collected at day 3 and day 7) were diluted in mucin medium, incubated anaerobically at 37°C and inspected daily for growth for 6 days as previously described [66]. Exact enumeration of bacteria in intestinal samples was examined by a 16S rRNA quantitative PCR approach. In short, the genomic DNA from pure culture, ileal, caecal and colonic contents was isolated using the Fast DNA Spin kit (Qbiogene, Inc, Carlsbad, CA, USA). PCR amplification of bacterial 16S rRNA genes was performed on genomic DNA of *A. muciniphila* using specific primers set AM1 and AM2 (Table 8.1).

RNA extraction. To examine the intestinal transcriptome response in mice, total mouse RNA from the intestinal tissue segments (ileum, caecum and colon) was extracted with Trizol® following supplier's protocol (Trizol reagent, Invitrogen, Breda, The Nertherlands). RNA was purified, treated with DNase and concentrated using RNeasy mini kit (Qiagen, Hilden, Germany). RNA quantity and quality were assessed spectrophotometrically (ND-1000, NanoDrop Technologies, Wilmington, USA) and bionalyzer nano chips (Bioanalyzer 2100; Agilent, Amstelveen, The Netherlands).

Table 8.1. Primers and probes sequences used in this study (NA: Not applicable)

Gene name	Gene Symbol	Function	Accession number	Sequence (5'→3')
Mouse				
18S rRNA	18S	Ribosome biogenesis and assembly	X00686	F: CATTCGAACGTCTGCCCTATC R: CCTGCTGCCTTCCTTGGA
S100 calcium binding protein g	S100g	Cell adhesion	NM 009789	F: ATGTGTGCTGAGAAGTCTCCT R: CGCCATTCTTATCCAGCTCCTT
Glycosylation dependent adhesion molecule 1	Glycam1	Cell adhesion	NM 008134	F: GTCCTGCTATTTGTCAGTCTTG R: CCTGGTCTTGATTCTCTG
Lipoprotein lipase	Lpl	Lipid metabolism	NM 008509	F: GGGAGTTTGGCTCCAGAGTTT R: TGTGTCTTCAGGGGTCCTTAG
Carbonic anhydrase 1	Car1	Nitrogen metabolism	BC011223	F: GACTGGGGATATGGAAGCGAA R: TGCAGGATTATAGGAGATGCTGA
Regenerating islet-derived family, member 4	Reg4	Vascularisation	NM_026328	F: TGAGCTGGAGTGTCAGTCATA R: CAATCCACACAGGCAGGTTTC
Small proline-rich protein 2a	Sppr2a	Vascularisation	AV371678	F: CCTTGTCGTCCTGTCATGT R: GGCATTGCTCATAGCACACTAC
Bacteria				
16S rRNA *A. muciniphila*	AM1 and AM2	Ribosome biogenesis and assembly	NA	F: CAG CAC GTG AAG GTG GGG AC R: CCT TGC GGT TGG CTT CAG AT
16S rRNA *A. muciniphila*	MUC-1437-Cy3	Ribosome biogenesis and assembly	NA	CCTTGCGGTTGGCTTCAGAT
16S rRNA total bacteria	EUB-338 –FITC	Ribosome biogenesis and assembly	NA	GCTGCCTCCCGTAGGAGT

DNA microarray hybridisations and data analysis. Affymetrix GeneChip mouse genome 430 2.0 arrays (Affymetrix, Santa Clara, CA, USA) containing 45,000 probe sets for the analysis of around 39,000 transcripts and variants of the approximately 22,000 mouse genes, were used to assess the transcriptional response to *A. muciniphila* and *L. plantarum* in the ileum, caecum and colon. For each group and location, 2 µg of total RNA per mouse was subsequently pooled per group, and 10 µg were used for one cycle cDNA synthesis. Hybridisation, washing and scanning of GeneChip Mouse Genome 430 2.0 Array were done according to the manufacturer's protocol (http://www.affymetrix.com). Fluorometric data were processed by Affymetrix GeneChip Operating software (GCOS) to assess the transcription profiles. Chip to chip normalisation was performed using the Affymetrix Microarray Analysis Suite (MAS) 5.0 algorithm. High level analysis with D-Chip software was used to normalise the GeneChips, and with a model-based approach (PM-MM model), the differentially expressed genes were retrieved. All genes were considered differentially expressed when they gave a present call and gave a signal that met the requirements: $p<0.05$, fold change >2 or <-2. Further data analysis was performed using DNA-Chip Analyzer v1.3 (dChip http://biosun1.harvard.edu/complab/dchip/), David software v2.1 (http://david.abcc.ncifcrf.gov/) for Gene ontology (GO) term analysis, GenMapp

(http://www.genmapp.org/) for pathways analysis. Gene Ontologies describe gene function in relation to a large and growing context of biological knowledge at three levels: biological process, molecular function or cellular component, using a systematic classification [84]. Datasets were also analysed using the Ingenuity Pathways Analysis (IPA) software tool (http://www.ingenuity.com). IPA annotations follow the GO annotation principle, but are based on a proprietary knowledge base of over 1,000,000 protein-protein interactions. The IPA output includes metabolic and signaling pathways with statistical assessment of the significance of their representation being based on Fisher's Exact Test. This test calculates the probability that genes participate in a given pathway relative to their occurrence in all other pathway annotations.

Quantitative Reverse Transcription (qRT)-PCR. cDNA was synthesised using 500 ng of total RNA employed for microarray analyses using Superscript III reverse transcriptase; random primers according to supplier's protocol (Invitrogen). qPCR on 6 ng cDNA derived from non-pooled RNA was performed on a Bio-Rad iCycler (Bio-Rad Laboratories BV, Veenendaal, The Netherlands) using gene-specific primers (0.2 μM) for selected genes (Table 8.1) and 1× SYBR Green PCR Master Mix buffer (Bio-rad). Each pair was designed to generate a 100-200 bp amplicon were derived from Primerbank (http://pga.mgh.harvard.edu/primerbank/) and primers were purchased from Biolegio BV, (Nijmegen, The Netherlands). The specificity of the amplification was verified by melt curve analysis and evaluation of efficiency of PCR amplification. All data were normalised to an internal 18S mRNA control (ΔΔCT analysis).

Results

Colonisation of germ-free mice with *A. muciniphila* and its localisation

A single intragastric dose of 10^9 *A. muciniphila* cells was sufficient to obtain reproducible colonisation of germ-free mice. All *A. muciniphila*-associated mice were healthy throughout the study period. To verify that the *A. muciniphila* colonised the GI tract, DNA was extracted from intestinal contents (ileum, caecum and colon), and used for 16S rRNA qPCR enumeration. In all mice mono-associated with *A. muciniphila*, bacterial concentration present in the luminal contents reached at least 10^8 cells/g of content in the ileum, 10^{10}/g of content cells in the caecum and 10^9 cells/g of content in the colon. Subsequently, hybridisation with the specific oligonucleotide probe targeting one region of the 16S rRNA of *A. muciniphila*, MUC-1437 (Chapter 6) was performed on sections from the anatomical sites of mice mono-associated where *A. muciniphila* colonised. In most of the sections *A. muciniphila* cells were found in clumps and closely associated to the epithelium with a distance less than 50 μm (Fig. 8.1).

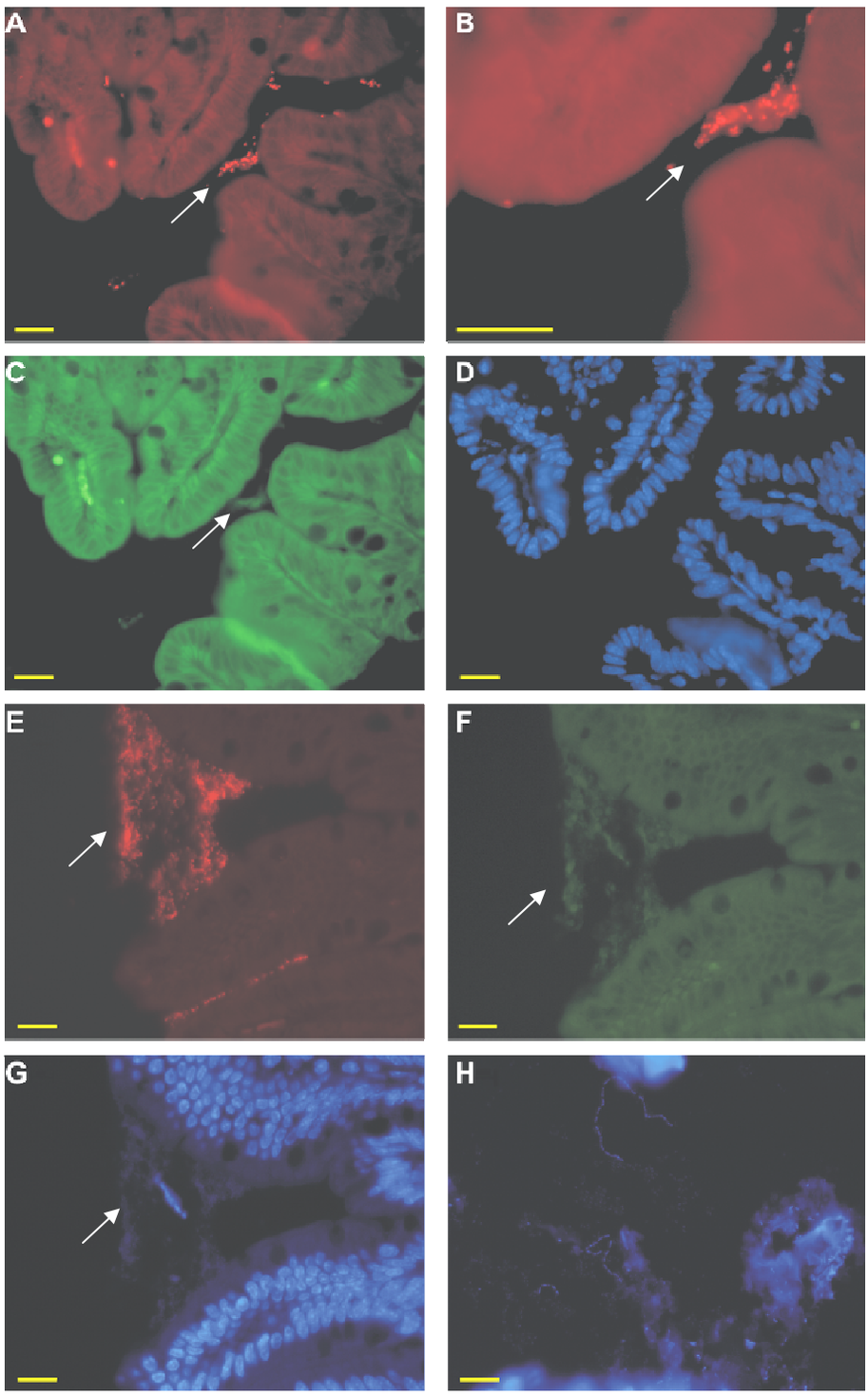


Figure 8.1. Micrograph of biopsy samples from ileum (A to D) and colon (E to G) of a mouse mono-associated with *A. muciniphila*. Cells were hybridised with the Cy3-labelled MUC-1437 (A, B, E), aggregates of *A. muciniphila* cells are zoomed (B), FITC-labelled EUB-338 (C, F) and a DAPI staining (D, G). Arrows indicate aggregates of *A. muciniphila*. Caecal biopsy from *L. plantarum* mono-associated mouse stained with DAPI (H). Bar represents 10 µm.

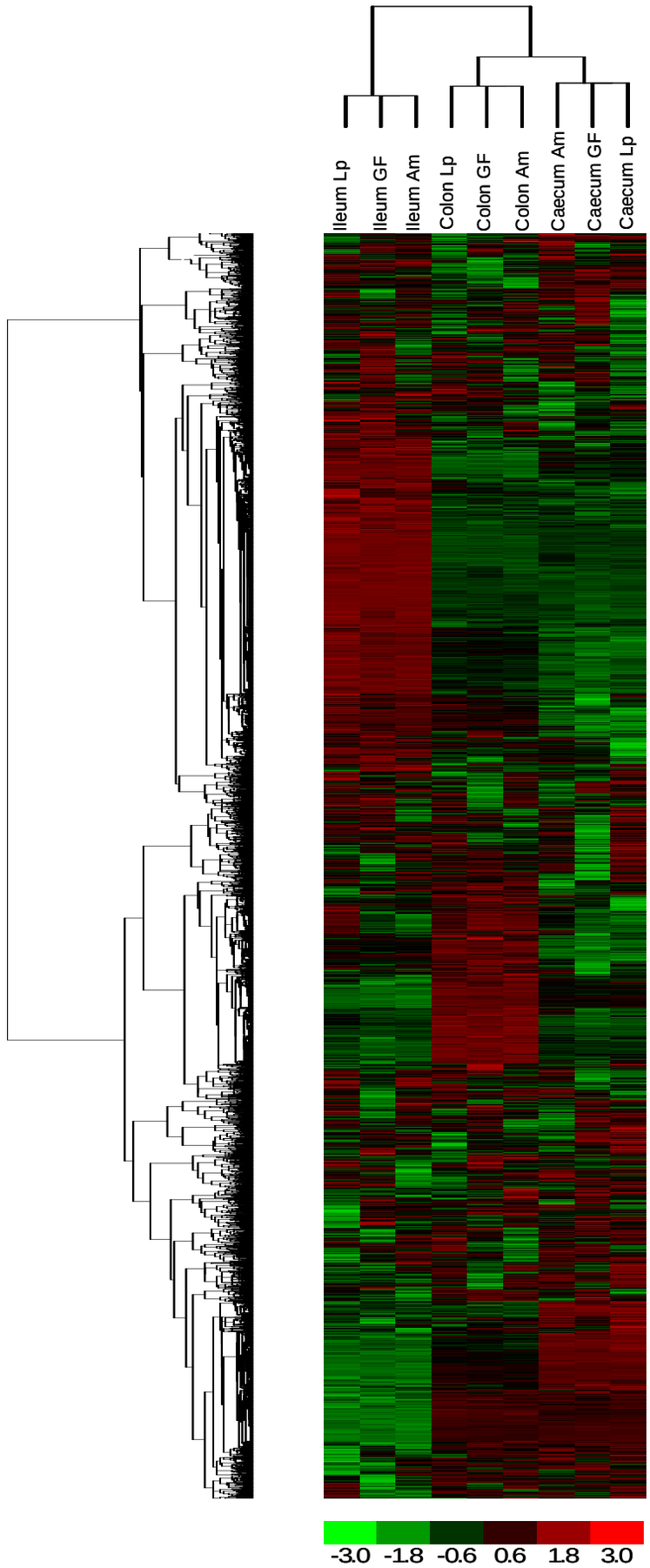


Figure 8.2. Hierarchical clustering of genes obtained using D-Chip software. Hierarchical clustering was performed on intensity values generated by Affymetrix MAS 5.0 software, showing differential responses in the three locations (ileum, caecum and colon) and with bacteria (*A. muciniphila* and *L. plantarum* compared to germ-free mice). Each column corresponds to one array (pool of six RNA) and each frame corresponds to one probe set. Red corresponds to an up-regulation of the gene expression and green to a down-regulation. Black corresponds to no change.

***A. muciniphila* induces host anatomical site specific responses**

To examine the impact of *A. muciniphila* colonisation on the host genes expression, the transcriptome of the host was analysed using microrrays. Modifications of the mice's transcriptome could be visualised by hierarchical clustering of the data sets (Fig. 8.2). Using D-chip software, genes were selected based on their intensity level, presence call, and fold change. The gene expression at each anatomical site of the mono-associated mice was compared to germ-free mice. Based on a cut-off value of ±2-fold change 22, 11 and 41 genes were significantly modulated in the ileum, caecum and colon respectively (Fig. 8.3).

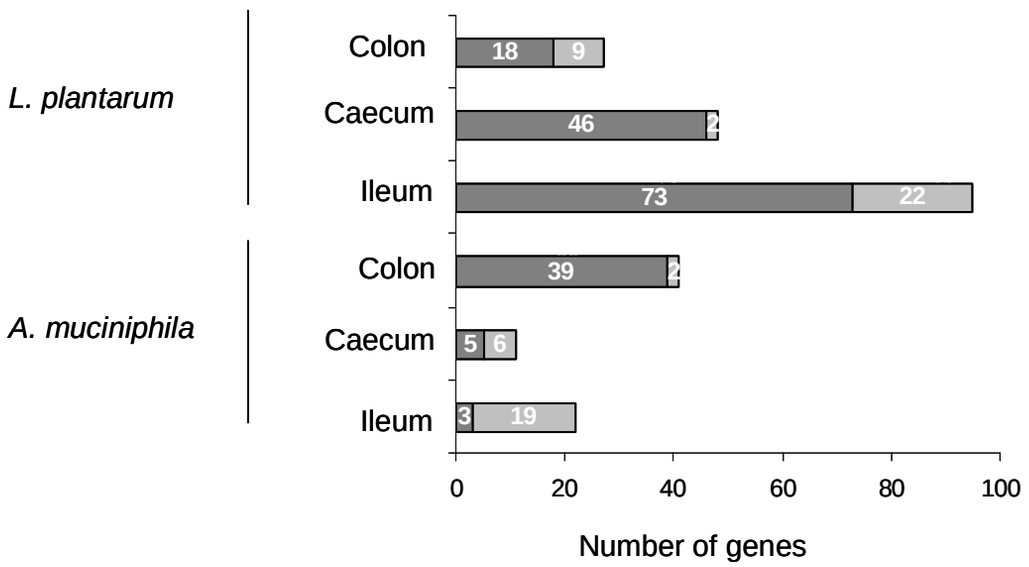


Figure 8.3. Number of genes regulated with a ±2-fold change in the ileum, caecum and colon of mice mono-associated with *A. muciniphila* and *L. plantarum*. Up-regulated genes are indicated in dark grey while down-regulated genes are indicated in light grey.

When comparing the specificity of the *A. muciniphila*-associated responses in ileum, caecum and colon, only very few genes showed similar expression patterns (Fig. 8.4A).

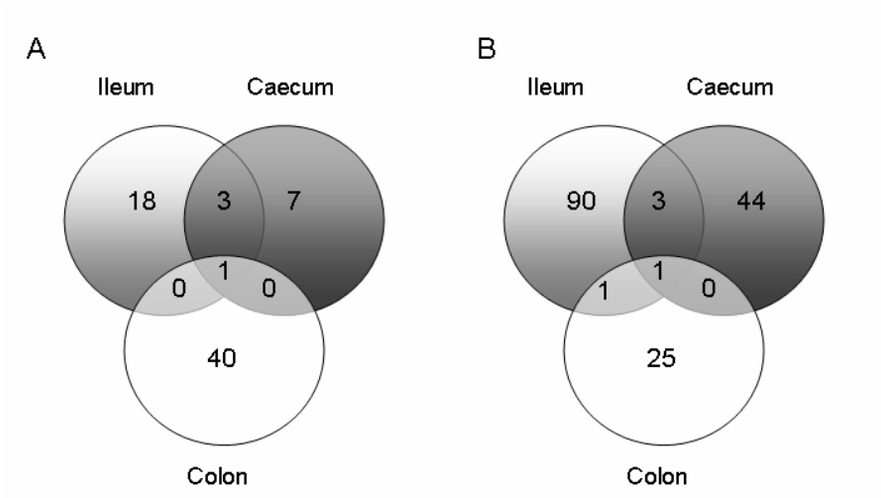


Figure 8.4. Venn diagrams representing the number of ±2-fold regulated genes in the ileum, caecum and colon of mice associated with *A. muciniphila* (A) and *L. plantarum* (B) interaction.

Only one out of 72 genes, S100 calcium binding protein g (S100g), was similarly regulated in the ileum, caecum and colon of mice associated with *A. muciniphila*. The greatest majority of genes were affected in a location-specific manner. When genes were classified based on their GO biological processes annotation, the immune response was found as the major response in all locations. In the ileum and caecum, it accounted for 23 and 27% respectively, while in the colon, 62 % of the significantly differentially expressed genes belonged to immune response. The majority of these genes were involved in formation and maturation of B- and T-cells and chemotaxis. According to the more specific Ingenuity Pathway Analysis output, *A. muciniphila* induced IL-2 and JAK/STAT signalling pathways in the caecum, leukocyte extravasation, T- and B-cell receptor signalling, and antigen processing and presentation pathways in the colon (Fig. 8.5), and genes involved in lipid metabolism and molecular transport in the ileum.

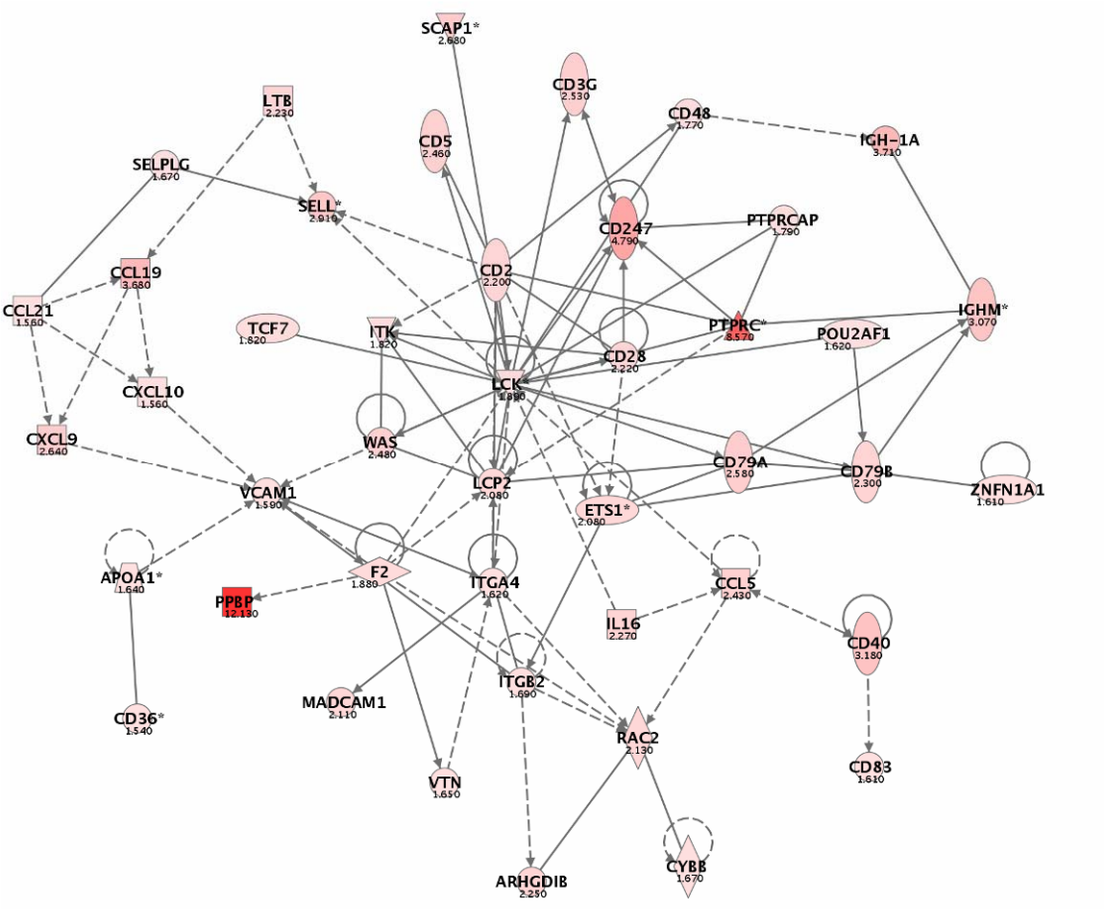
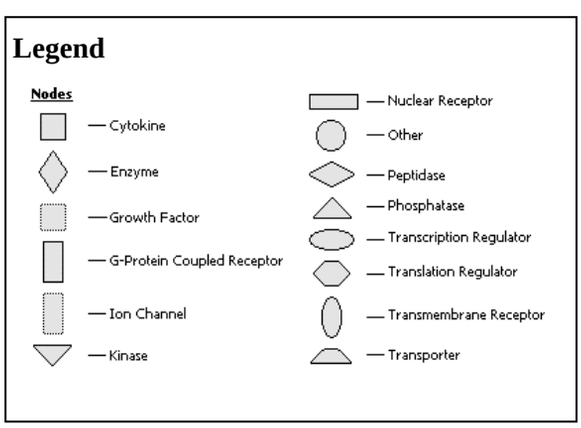


Figure 8.5. Gene networks of immune response generated using Ingenuity Pathway Analysis software of colonic biopsies from mouse mono-associated with *A. muciniphila*. Red/Pink representes up-regulated genes.



Other biological processes predicted to be regulated in the colon by *A. muciniphila* colonisation included cell and cytoskeleton organisation (5 %), transcription regulation (3%) and protein metabolism (3 %). A total of 19 % of the genes, including genes coding for hypothetical proteins and unannotated ORFs, could not be allocated to one of the classes and were therefore, assigned as unknown (Fig. 8.6A).

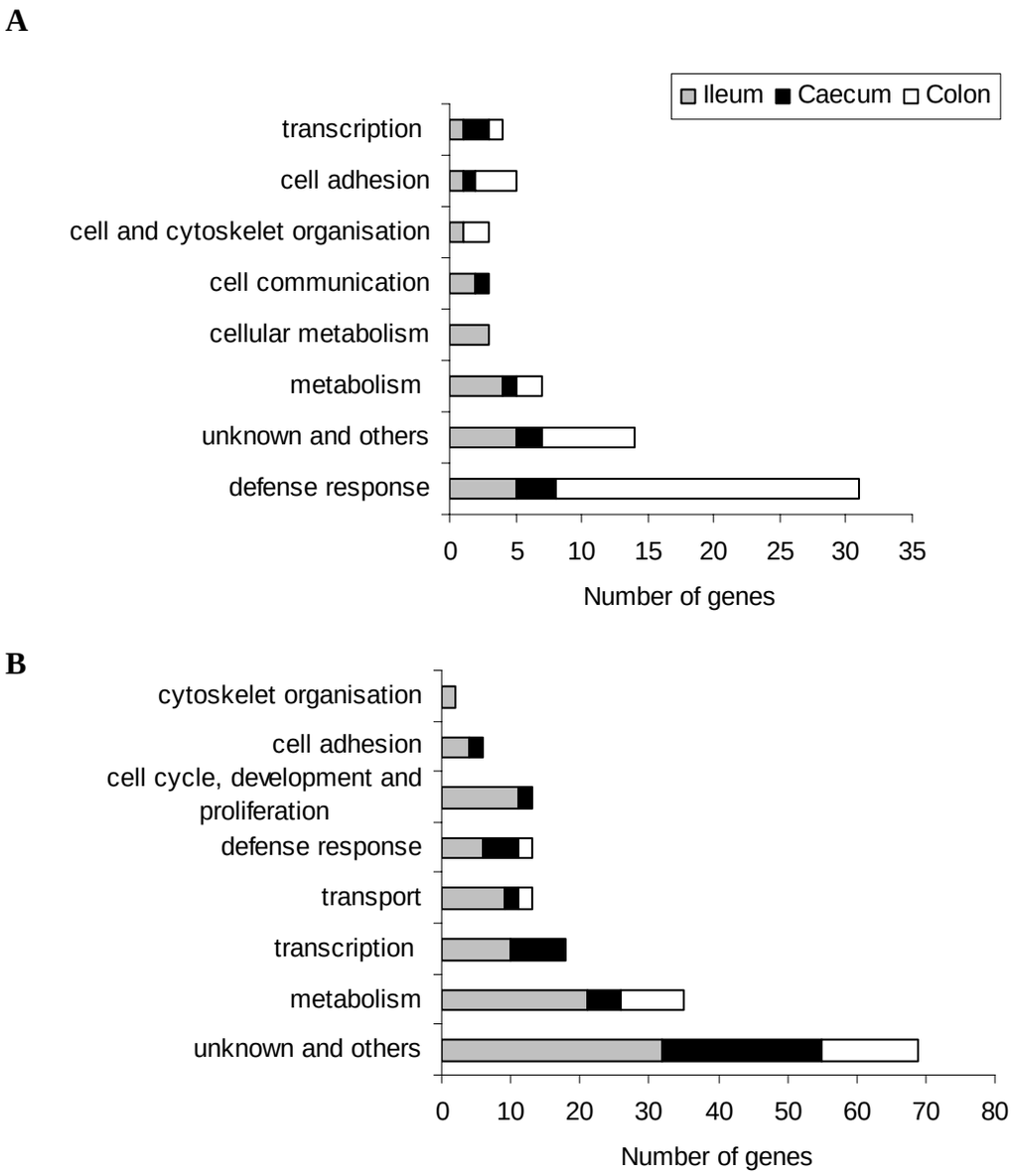


Figure 8.6. Bar diagram representing functional annotation of all differentially expressed genes in intestine of mice mono-associated with *A. muciniphila* (A) or *L. plantarum* (B).

Other genes affected by *A. muciniphila* were predicted to be involved in cell adhesion, cell-surface mediated signalling and capture and migration of leukocytes. They included selectin (+3.53 fold change), which is a cell surface adhesion protein located on lymphocytes and contains a lectin domain. The glycosylation-dependent adhesion molecule 1 (Glycam1), was up-regulated as well with a fold change of +4.35, and is a mucin-like endothelial glycoprotein. It is expressed specifically by the endothelial cells of peripheral and mesenteric lymph nodes, that acts as an adhesive ligand for selectin by presenting one or more O-linked carbohydrates to the lectin domain of this leukocyte cell surface selectin [13, 150]. Also the vascular adhesion molecule 1 (VCAM1) showed an up-regulation, which is important in cell-cell recognition, particularly in leukocyte-endothelial cell adhesion. This molecule interacts with the beta-1 integrin VLA4 on

leukocytes, and mediates both adhesion and signal transduction. The VCAM1-VLA4 interaction has been shown to play a role in leukocyte migration to sites of inflammation [161]. The last adhesion gene that was affected included the gene for sialophorin which was also up-regulated (+2.49). It has a high content of sialic acid and O-linked carbohydrate structures and is one of the major glycoproteins of thymocytes and T lymphocytes. It plays a role in the physicochemical properties of the T-cell surface and in lectin binding. Like Glycam1, sialophorin presents carbohydrate ligands to selectins.

Comparison of the response induced by *A. muciniphila* mono-associated mice and *L. plantarum* mono-associated mice compared to germ-free mice

Both, FISH and microarray examinations suggested that *A. muciniphila* is closely associated with the mucosa. Since this suggests that *A. muciniphila* is utilising mucin *in situ*, the impact of *A. muciniphila* on the host was compared to a non-mucin degrader, *L. plantarum*. Based on 16S rRNA-gene qPCR *L. plantarum* efficiently colonised the mice at levels similar to *A. muciniphila*, with exception of the small intestine where the colonisation was 10-fold higher (10^9 cells/g), data consistent with previous studies from *L. plantarum* in mice [179]. Biopsy sections from the three intestinal locations stained with DAPI showed that cells were visible, most likely corresponding to *L. plantarum* cells. These cells were not found in aggregates but randomly distributed (Fig. 8.1H).

The data generated from germ-free, *A. muciniphila* and *L. plantarum* mono-associated mice were compared for the three intestinal locations. When all arrays were compared, 30,159 out of the 45,000 probe sets had a present call in at least one of the nine arrays. In the ileum, 26,581 probe sets gave a signal (59%) in at least one of the three arrays corresponding to germ-free, *A. muciniphila* or *L. plantarum* treated mice. In the caecum and in the colon, 24,889 (55%), and 27,301 (60.67 %) probe sets gave a signal, respectively. This demonstrates the enormous potential of the GI tract to respond to the events happening in the mucosa and lumen.

Based on microarrays analysis, *L. plantarum* induced the expression of 95, 48 and 27 genes in the ileum, caecum and colon, respectively (Fig. 8.3). When comparing the global gene expression between *A. muciniphila* and *L. plantarum* in each of the three locations, the expression of only very few genes were regulated by both strains. In the ileum, 8.5 % of the differentially expressed genes (9 out of 95 genes), i.e. adipocyte C1Q, S100g, carbonic anhydrase 1 and 2, cytochrome P450, serum amyloid A1 and A2, and two unknown genes, showed similar expression patterns in mice mono-associated with *A. muciniphila* or *L. plantarum*. For the caecum, this only was the case for the nuclear receptor subfamily 2, group C, member 1 (1.75 %). Remarkably, no commonly regulated gene was found in the colon. Moreover, *L. plantarum*

showed more genes differentially expressed in the ileum and in the caecum, while *A. muciniphila* regulated more genes expressed in the colon.

The host genes response appeared as location-specific as was observed with *A. muciniphila*. No gene was commonly regulated between the locations by *L. plantarum* colonisation (Fig. 8.4B). Compared to *A. muciniphila*, only a small portion of genes regulated by *L. plantarum* was involved in immune response (6%). This was the case for Serum amyloid A (Saa) genes. *Saa1* and *Saa2* were both down-regulated in the ileum while *Saa3* was down-regulated in the colon. Saa proteins belong to the superfamily of acute phase proteins and their level in the blood increases dramatically in response to tissue injury and inflammation. They also act as cytokines, influencing cell adhesion, migration, proliferation and aggregation [300].

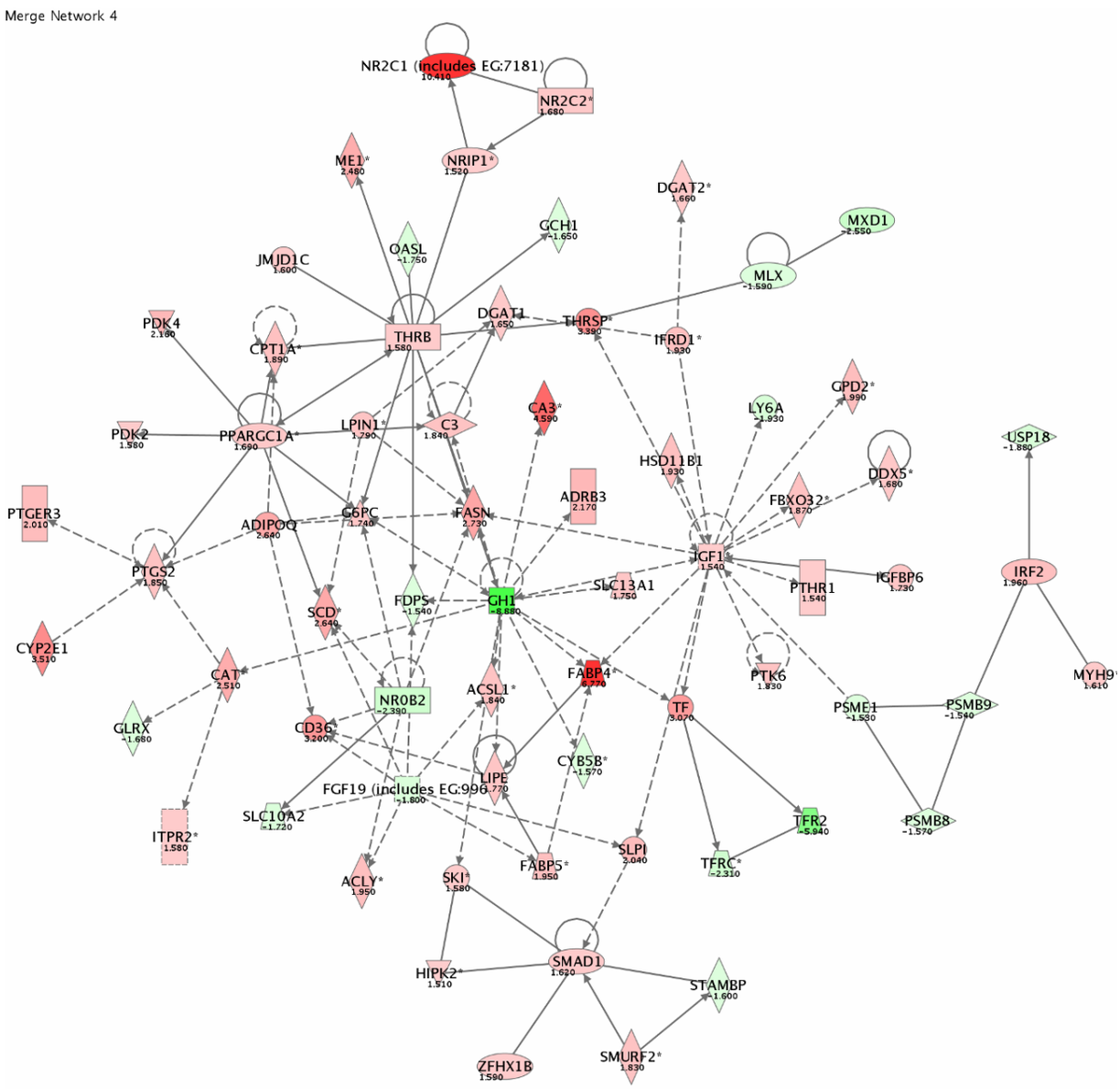


Figure 8.7. Gene networks of lipid metabolism and transport generated using IPA software of ileal biopsies from mouse mono-associated with *L. plantarum*. Green indicates down-regulated genes while pink/red indicates up-regulated genes. For the legend, see Fig. 8.5.

Looking in detail at the gene expression, *L. plantarum* induced mainly metabolic responses (22%) in the ileum and colon, immune response, cell and cycle development in the caecum (Fig 8.6B). Among the genes involved in metabolism, 52% of those genes belonged to the lipid metabolism, followed by the protein (24%), carbohydrates (19%) and amine (5%) metabolism in the ileum. In the lipid metabolism, most of the up-regulated genes were involved in β-oxidation. Other genes were included that for the lipoprotein lipase (+2.83), involved in the degradation of triglycerides into free fatty acids.

Ingenuity pathway analysis confirmed the major impact on lipid metabolism in the ileum (Fig. 8.7) and in the colon. Regulation of cell signalling (including integrin and ERK/MAP) was also an important feature of responses of ileal mucosa to *L. plantarum*. Interestingly, several genes encoding proteins involved in proteolytic degradation and receptors involved in antigen presentation pathway by MHC pathway I were down-regulated in the ileum upon exposure to *L. plantarum*. Up-regulation of cell signalling (Wnt/β-catenin, PI3k/Akt) was also a feature of responses of caecum mucosa to *L. plantarum*. The major cellular processes that were regulated involved genes regulating cell death and cellular proliferation. In the colon, in addition to regulation of (glycerophospho-)lipid and amino acid metabolism, genes involved in responses to antigen through the Fc-epsilon receptor I pathway were also found to be up-regulated.

Amongst other metabolic enzymes, the fucosyltransferase gene was up-regulated 1.75-fold in mice colonised by *L. plantarum*. These enzymes catalyse the transfer of fucose from GDP-fucose, with inversion of anomeric configuration, to various oligosaccharide acceptors [29, 164]. The most regulated gene found with *L. plantarum* was the carbonic anhydrase 1 (*Car1*) involved in cellular metabolism, with a fold change of -29.5. *Car1* belongs to the α-class of carbonic anhydrases and catalyses the reversible hydration of CO_2.

Nine genes encoding for solute transport molecules with varying functions were found to be affected by *L. plantarum* colonisation. What their exact role in regard to *L. plantarum* colonisation is, is not clear, but it is likely to assume that they are associated with the altered metabolism of the host.

Direct comparison between *A. muciniphila* mono-associated mice and *L. plantarum* mono-associated mice

The microarray data obtained from *A. muciniphila* mono-associated mice were directly compared to those obtained with *L. plantarum* mono-associated mice. This direct comparison revealed striking differences between the influences of both either of these bacteria on the host transcriptome. The highest regulated genes were similar to those obtained when compared to germ-free animals, and few genes were emerging after selection. In the ileum, caecum and colon,

84, 94 and 43 genes were significantly regulated (Table 8.3) with more genes up-regulated by *A. muciniphila* in the colon than in the ileum and caecum where *L. plantarum* induces more up- than down-regulation. No similar gene expression was observed in the three locations, while eight genes were commonly regulated between the ileum and caecum, i.e. the adipocyte, C1Q and collagen domain containing, carbonic anhydrase 3, CD36 antigen, cytochrome P450, fatty acid binding protein 4, orosomucoid 1, thyroid hormone responsive SPOT14 homolog and two unknown genes. Between the caecum and colon, two genes were common, *S100g* and one unknown gene, and one between the ileum and colon (*S100g*). When looking more in details, some genes involved in the reinforcement of the epithelial barrier such as *Sppr2a*, important in epithelial differentiation and permeability in the ileum and *Reg4* were up-regulated in the ileum and colon respectively of *A. muciniphila* mono-associated mice compared to *L. plantarum* mono-associated mice. One remarkable difference in antigen processing and presentation pathway responses in response to *A. muciniphila* and *L. plantarum* was observed, in different parts of the intestine. Whereas *A. muciniphila* induced an up-regulation of genes involved in MHC Pathway II antigen processing and presentation pathway in the colon, *L. plantarum* induced a down-regulation of genes involved in MHC Pathway I in the ileum.

Validation of the microarrays results

qRT-PCR analysis on selected genes was conducted on cDNA samples of individual mice to confirm the trends of change of transcriptional response of the mice genes as observed by microarrays analysis after colonisation with *A. muciniphila* and *L. plantarum*. Genes were selected based on their significant fold change on transcriptome and involved in different biological processes were selected (Table 8.1). Gene expression was normalised to the internal standard 18S rRNA, which expression was not differed between germ-free and mono-associated mice (data not shown). Although the absolute expression levels differed, the same trend was observed between microarray and qRT-PCR results (Fig. 8.8). The major difference in fold-changes was observed for *Car1*: while microarray data indicated a -29-fold-change, qRT-PCR results showed a -7.4 fold-change. This difference was explained by a differential expression between individual mice in the *A. muciniphila* mono-associated mice.

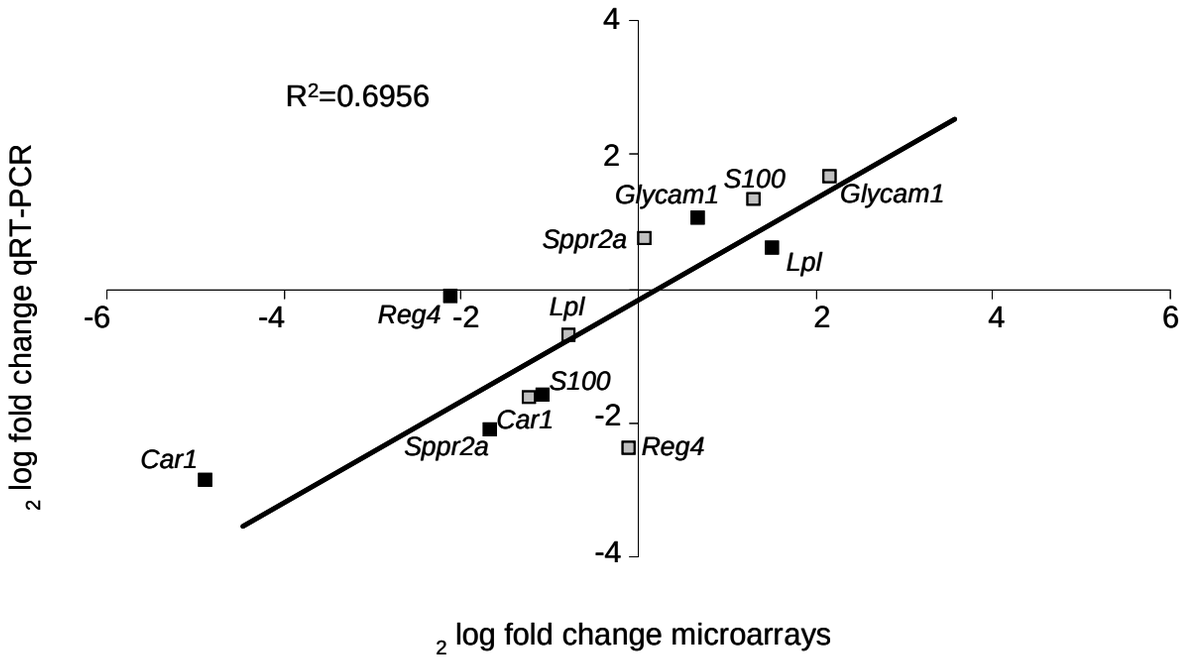


Figure 8.8. Comparison between qRT-PCR and microarray results. Expression levels of *S100g*, *Glycam1*, *Lpl*, *Car1*, *Reg4*, *Sppr2a* in ileal, caecal and colonic samples from *A. muciniphila* (■) and *L. plantarum* (■) mono-associated mice compared to the germ-free mice are indicated. Assays were performed in duplicate. Data were normalised to 18S ribosomal RNA and results are expressed as log fold change. The linear relation that best fitted the data is plotted.

Discussion

We determined the transcriptional profiles of the mouse intestinal mucosa to examine the impact of two commensal bacteria on changes in the epithelial transcriptome. Recently, microarrays have become a widely used tool for studying host-intestinal commensal bacteria interactions from the perspective of both host and bacteria in *in vivo* models [9, 68, 301]. In our study, we colonised two groups of germ-free mice with two commensal bacteria, i.e. the Gram- negative and strictly anaerobic *A. muciniphila* and the Gram-positive non mucin-degrading *L. plantarum*. Both bacteria colonised the GI tract seven days after a single inoculation.

The analysis of the host transcriptomic response on three intestinal anatomical regions, ileum, caecum and colon indicated that *A. muciniphila* and *L. plantarum* induced distinct intestinal responses. For each bacterium the regulated genes were specific for the anatomical location, even though in general the same biological functions were found. Amongst the genes with a fold change of 2, only few genes were commonly expressed in the *A. muciniphila* and *L. plantarum* mono-associated mice in the ileum, caecum and colon, suggesting that each bacterium showed a preferential site of host gene regulation. Interestingly, Marco *et al*. found that *L. plantarum* displays specific and differential responses at various sites along the mouse intestine [179], which could explain the differential host transcriptomic regulation on the various intestinal

sites. While a majority of the genes affected by *A. muciniphila* were found in the colon, *L. plantarum* affected gene expression mainly in the ileum. Preferential gene expression could be partly explained by the bacterial numbers found in the different intestinal locations. The 16S rRNA gene quantification showed that *A. muciniphila* and *L. plantarum* colonised at the same level the colon and caecum while *L. plantarum* outnumbered *A. muciniphila* by 10-fold in numbers in the ileum. While both bacteria colonised the caecum of the mice more efficiently than the other two sites, the host genes expression were lower for *A. muciniphila* and *L. plantarum* mono-associated mice. The caecum, which is a distinct anatomical site which connects the small intestine to the beginning of the colon, is a large pouch that receives luminal material from the small intestine. In mice, the caecum is relatively larger compared to humans. One of a remarkable feature of germ-free mice is a marked caecal enlargement compared to conventional mice, due to the water-retention of mucins [202].

Most of the genes affected by *A. muciniphila* were implicated in immune response, and with a higher proportion in the colon involving genes in the innate and adaptive immune response. While some genes were involved in chemotaxis and complement cascade, the majority of the genes were implicated in the maturation of B- and T- cell and cell adhesion. This might suggest that *A. muciniphila* is able to modulate the host immune system, possibly enhancing the intestinal barrier function. The differential host responses to *A. muciniphila* and *L. plantarum* could be due to the diverse components from the bacterial cell-wall. Components of Gram-negative bacteria, such as lipopolysaccharides (LPS), which are the major outer surface membrane components, have been reported to be strong stimulators of the innate immune system, while peptidoglycan including lipoteichoic acids are the major immunomodulators from the cell wall of Gram-positive bacteria. Grangette *et al.* reported recently that the composition of lipoteichoic acids from *L. plantarum* could modulate proinflammatory or antiinflammatory immune responses [93].

The different ecological niches of *A. muciniphila* and *L. plantarum* could be responsible for the differential host genes response. *A. muciniphila* cells were frequently found in aggregates close to the epithelial cells (less than 50 μm) as showed by FISH combined with microscopy. These microcolonies were also observed in growing cultures from *A. muciniphila* in a mucin-based medium and could be involved of the utilisation of mucin as energy source. In contrast, in biopsies from *L. plantarum* mono-associated mice, DAPI-stained cells were not observed in aggregates but were randomly distributed. Recently, a study from Van der Waaij *et al*., based on FISH on human intestinal biopsy samples concluded that there was no specific bacterial population close to the host epithelial cells [304]. However, this study did not focus on *A.*

muciniphila and since the bacterium is not detected efficiently with the EUB-338 probe, it may have escaped detection in the mucus layer.

Using *in vitro* models, Caco-2 and HT29-MTX cell lines, MUC2 and MUC3 were up-regulated after *A. muciniphila* co-incubation (Derrien *et al.*, unpublished data). However, in our mice study, no significant regulation of *MUC* genes was found. In germ-free mice, *MUC2*, *MUC3* and *MUC4* had a present call, suggesting that these genes were already expressed before bacterial colonisation. Interestingly, we could detect an up-regulation of the gene encoding for a mucin-like glycoprotein, Glycam1 in the colon of *A. muciniphila*-associated mice. Glycam1 acts as an adhesive ligand for L selectin by presenting O-linked carbohydrates to the lectin domain of this leukocyte cell surface selectin. One other form of GlyCam1 was found in breast milk suggesting that the protein might function in the gastrointestinal tract as a lubricant or defence line from colonisation by pathogens [71].

In contrast to *A. muciniphila*, *L. plantarum* colonisation changed the expression of a number of metabolic genes, especially those involved in the processing of the carbohydrates and in the breakdown and absorption of lipids. The increase of gene expression involved in lipid degradation indicates that *L. plantarum* could extract lipids from the host cells. These date are in agreement with previous observations with mice mono-associated with the single Gram-negative and strict anaerobe microorganism, *B. thetaiotaomicron* [8]. Previous studies performed on mono-association of this microbe demonstrated that it induced in general higher gene expression levels compared to our study. For instance, a 200-fold change was observed for a particular gene, Sppr2a in the ileum, while it was slightly up-regulated in *A. muciniphila* mono-associated mice and down-regulated in *L. plantarum* mono-associated mice. Sppr2a which appears to be involved in maintaining the barrier function of the villous epithelium [116]. In our study, neither *A. muciniphila* nor *L. plantarum* induced such a strong gene regulation. One other differential response to *B. thetaiotaomicron* was the regulation of *Reg* genes. Reg proteins contain a C-type lectin domain, and are involved in many physiological functions including proliferation and regeneration, cell survival, cell adhesion and resistance to apoptosis in liver, pancreas and GI tract [328]. Reg3 has been linked with inflammatory bowel diseases and colonisation of germ-free mice with *B. thetaiotaomicron* triggered epithelial expression of Reg3γ, suggesting intestinal strategies for maintaining symbiotic host-microbial relationships [44]. In our study *Reg4* was up-regulated in the colon of mice mono-associated with *A. muciniphila* compared to *L. plantarum*. *Reg4* expression has been correlated with colorectal cancer and is then contributing to cellular proliferation [328].

A potential explanation could be that *B. thetaiotaomicron* is more active than *A. muciniphila* and *L. plantarum* in the mouse intestine. Indeed genome sequencing revealed that *B. thetaiotaomicron* is a highly glycophile bacterium well adapted to the intestinal environment [322]. Other explanations could be that *B. thetaiotaomicron* produces particular metabolites from food fermentation, to which the mice are more responsive or that the level of colonisation. *B. thetaiotaomicron* was shown to colonise at a higher density the mice intestine approaching 10^{12} cells/g intestinal content in the caecum and distal colon [35] and $>10^{7}$ cells/g intestinal content in the ileum [119]. In contrast, *A. muciniphila* and *L. plantarum* did not exceed 10^{10} cells/g of mice intestinal content. In the human GI tract, lactobacilli belong to the minority species [288] with average counts of $\leq 10^{9}$ cfu/g faeces, while *Bacteroides* species belong to dominant species with average of 10^{12} cfu/g faeces [113, 195].

Our data confirm the specificity of the host responses towards different bacteria. Germ-free mice were used as a simplified host model, and therefore, the results have to be taken cautiously. In this model, mice were mono-associated, which is not obligatory representative of what occurs in the natural ecosystem. Consequently, in a germ-free animal model, interactions of a single microorganism and the host are expected to be more direct. Nevertheless germ-free animals are good experimental models to study direct interactions of controlled bacterial community on host genes expression.

In conclusion, the transcriptional profiling of the host response after colonisation with two commensal bacteria allowed insight into the regulation of important genes which are clearly distinct depending on the bacterium. *A. muciniphila* affected genes involved in early inflammatory response, likely due to the fact that *A. muciniphila* is affecting the mucus layer. In addition to the type of bacterium, there were also location-specific differences in host responses. Therefore, looking at a restricted area such as the caecum might be not sufficient for further extrapolation to predict human responses to commensal bacteria in other regions of the intestine. Further studies will combine validation of the biological functions and determination of the bacterial products between *A. muciniphila* (direct contact or soluble factors) with immune cells in the intestine.

Ackowledgements

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Table 8.2. List of genes whose expression is significantly regulated by *A. muciniphila* compared to germ-free mice in the ileum, caecum and colon, excluding hypothetical and unknown gene product or unknown function.

Probe set	Gene	Function (GO)	Accession	Fold change
Ileum				
1417487_at	Fos-like antigen 1	Transcription	U34245	6.57
1448964_at	Calbindin 3, (vitamin D-dependent calcium binding protein)	Cell adhesion	NM_009789	2.72
1422651_at	Adipsin	Immune response	NM_013459	-2.19
1448752_at	Carbonic anhydrase 2	Cellular metabolism	NM_009801	-2.28
1416193_at	Carbonic anhydrase 1	Cellular metabolism	BC011223	-2.34
1422651_at	Adipocyte, C1Q and collagen domain containing	Lipid metabolism	NM_009605	-2.59
1422822_at	Immunoglobulin lambda chain, variable 1	Immune response	M94350	-2.65
1421613_at	H2A histone family, member Y3	Cell organisation and biogenesis	NM_026230	-2.66
1419075_s_at	Serum amyloid A 2	Immune response	NM_011314	-2.68
1450788_at	Serum amyloid A 1	Immune response	NM_009117	-2.73
1451962_at	Immunoglobulin kappa chain mRNA, complete cds.	Immune response	L41881	-2.74
1419582_at	Cytochrome P450, family 2, subfamily c, polypeptide 55	Transport	NM_028089	-2.75
1435535_at	DEP domain containing 5	Cell communication	AW550473	-2.95
	StAR-related lipid transfer (START) domain containing 5	Lipid metabolism	BI076697	-5.27
Caecum				
1422422_at	Defensin related cryptdin 4	Immune response	NM_010039	2.95
1448964_at	Calbindin 3, (vitamin D-dependent calcium binding protein)	Cell adhesion	NM_009789	2.59
1427856_a_at	Anti-DNA immunoglobulin light chain variable region, clone 4B2, partial cds.	Immune response	M18237	2.35
1450631_x_at	Defensin related cryptdin 13	Immune response	NM_007852	2.33
1439853_at	UDP-N-acetyl-alpha-D-galactosamine	Lipid metabolism	BF581125	2.24
1435535_at	DEP domain containing 5	Cell communication	AW550473	-2.68
1418605_at	Nuclear receptor subfamily 2, group C, member 1	Regulation of transcription	NM_011629	-3.06
1449316_at	Cytochrome P450, family 4, subfamily f, polypeptide 15	Transport	NM_134127	-3.76
1438364_x_at	Angiogenin, ribonuclease A family, member 4	Angiogenesis	AV060923	-4.68
1426929_at	Bruno-like 4, RNA binding	RNA metabolism	AV327653	-24.78

	protein (Drosophila)			
Colon				
1424825_a_at	Glycosylation dependent cell adhesion molecule 1	Cell adhesion	M93428	4.35
1449498_at	Macrophage receptor with collagenous structure	Transport	NM_010766	4.35
1448727_at	Transducin-like enhancer of split 6, homolog of Drosophila E(spl)	Regulation of transcription	NM_053254	4.09
1419480_at	Selectin, lymphocyte	Cell adhesion	M36005	3.53
1455530_at	Similar to Ig delta chain C region, membrane-bound form - mouse (LOC382646), mRNA	Immune response (B cell activation)	BE686052	3.25
1451513_x_at	Serine (or cysteine) proteinase inhibitor, clade A, member 1b	Immune response	BC012874	3.15
1426113_x_at	T-cell receptor alpha chain	Immune response (T cell activation)	U07662	3.14
1449277_at	Chemokine (C-C motif) ligand 19	Immune response (chemotaxis)	NM_011888	3.07
1417640_at	CD79B antigen	Immune response (B cell activation)	NM_008339	2.86
1422828_at	CD3 antigen, delta polypeptide	Immune response (T cell activation)	NM_013487	2.79
1425226_x_at	T-cell receptor beta, variable 13	Immune response (T cell activation)	M16120	2.72
1450912_at	Membrane-spanning 4-domains, subfamily A, member 1	Immune response (B cell activation)	BB236617	2.69
1425289_a_at	Complement receptor 2	Immune response (B cell activation)	M35684	2.68
1419178_at	CD3 antigen, gamma polypeptide	Immune response (T cell activation)	M58149	2.56
1418652_at	Chemokine (C-X-C motif) ligand 9	Immune response (chemotaxis)	NM_008599	2.55
1439034_at	sialophorin	Immune response (T cell activation)	BB160586	2.49
1418830_at	CD79A antigen (immunoglobulin-associated alpha)	Immune response (B cell activation)	NM_007655	2.48
1448964_at	Calbindin 3, (vitamin D-dependent calcium binding protein)	Cell adhesion	NM_009789	2.48
1427329_a_at	Immunoglobulin heavy chain 6 (heavy chain of IgM)	Immune response (B cell activation)	AI326478	2.46
1419206_at	CD37 antigen	Immune response (B cell activation)	BC019402	2.41
1450357_a_at	Chemokine (C-C motif) receptor 6	Immune response (chemotaxis)	NM_009835	2.38
1416295_a_at	Interleukin 2 receptor, gamma chain	Immune response	L20048	2.34

1419135_at	Lymphotoxin B	Immune response	NM_008518	2.28
1416514_a_at	Fascin homolog 1, actin bundling protein (Strongylocentrotus) purpuratus)	Cytoskelet	NM_007984	2.27
1455269_a_at	Coronin, actin binding protein 1A	Cytoskelet	BB740218	2.25
1436312_at	Zinc finger protein, subfamily 1A, 1 (Ikaros)	Regulation of transcription	AV317621	2.24
1417620_at	RAS-related C3 botulinum substrate 2	Immune response (chemotaxis)	NM_009008	2.13
1456494_a_at	Tripartite motif protein 30-like	Protein metabolism	BG068242	2.11
1422124_a_at	Protein tyrosine phosphatase, receptor type, C	Immune response (B cell activation)	NM_011210	2.07
1425738_at	Processed pseudo-IgK chain mRNA, VC-region, from hybridoma A23A41.	Immune response	M35669	-2.32

Table 8.3. List of genes retrieved from direct comparison between *A. muciniphila* and *L. plantarum* in the ileum, caecum and colon excluding hypothetical and unknown gene product or unknown function with *L. plantarum* as reference

Probe set	Gene	Function (GO)	Accession	Fold change
Ileum				
1416193_at	Carbonic anhydrase 1	Cellular metabolism	BC011223	12.67
1419582_at	Cytochrome P450, family 2, subfamily c, polypeptide 55	Transport	NM_028089	4.44
1416075_at	Salvador homolog 1 (Drosophila)	Transcription	NM_022028	3.61
1450618_a_at	Small proline-rich protein 2A	Cell differentiation	NM_011468	3.07
1450788_at	Serum amyloid A 1	Immune response	NM_009117	2.51
1449326_x_at	Serum amyloid A 2	Immune response	NM_011314	2.42
1439853_at	UDP-N-acetyl-alpha-D-galactosamine	Lipid metabolism	BF581125	2.15
1439260_a_at	Etonucleotide pyrophosphatase/phosphodieste rase 3	Cellular metabolism	BB039510	-2.18
1421145_at	Solute carrier family 26 (sulfate transporter), member 2	Transport	NM_007885	-2.22
1450391_a_at	monoglyceride lipase	Lipid metabolim	NM_011844	-2.25
1416021_a_at	Fatty acid binding protein 5, epidermal	Lipid metabolim	BC002008	-2.26
1437621_x_at	3-phosphoglycerate dehydrogenase	Amine metabolism	AV216768	-2.31
1438389_x_at	ATP citrate lyase	Lipid metabolim	BB424434	-2.46
1448842_at	Cysteine dioxygenase 1, cytosolic	Amine metabolism	NM_033037	-2.5
1417686_at	Lectin, galactose binding, soluble 12	Apoptosis	AF244979	-2.53
1416632_at	Malic enzyme, supernatant	Carbohydrate metabolism	BC011081	-2.55
1417184_s_at	Hemoglobin Y, beta-like embryonic chain	Transport	BC027434	-2.61
1450344_a_at	Prostaglandin E receptor 3 (subtype EP3)	Cell communication	NM_011196	-2.68
1426225_at	Retinol binding protein 4, plasma	Transport	U63146	-2.69
1417888_at	Tripartite motif protein 13	Cell cycle	NM_023233	-2.7
1449130_at	CD1d1 antigen	Immune response	NM_007639	-2.77
1418773_at	Fatty acid desaturase 3	Lipid metabolim	BE652876	-2.88
1436521_at	Solute carrier family 36 (proton/amino acid symporter), member 2	Transport	AI596194	-2.91
1417741_at	Liver glycogen phosphorylase	Carbohydrate metabolism	NM_133198	-2.95
1417714_x_at	Hemoglobin alpha, adult chain 1	Transport	NM_008218	-2.99
1420655_at	Adiponutrin	Lipid metabolism	NM_054088	-3.1
1424504_at	RAB22A, member RAS	Transport	BC006596	-3.16

	oncogene family			
1448700_at	G0/G1 switch gene 2	Cell cycle	NM_008059	-3.33
1435370_a_at	Carboxylesterase 3	Lipid metabolism	AI315015	-3.37
1423828_at	Fatty acid synthase	Lipid metabolim	AF127033	-3.45
1451263_a_at	Fatty acid binding protein 4, adipocyte	Lipid metabolim	BC002148	-3.56
1431056_a_at	Lipoprotein lipase	Lipid metabolim	AK017272	-4.92
1415965_at	Stearoyl-Coenzyme A desaturase 1	Lipid metabolism	NM_009127	-5.01
1450883_a_at	CD36 antigen	Immune response or transport	BB534670	-5.4
1417765_a_at	Amylase 1, salivary	Carbohydrate metabolism	NM_007446	-5.45
1460197_a_at	Tumor necrosis factor, alpha-induced protein 9	Cell differentiation	NM_054098	-5.5
1415994_at	Cytochrome P450, family 2, subfamily e, polypeptide 1	Transport	NM_021282	-6.47
1451054_at	Orosomucoid 1	Immune response	BE628912	-6.76
1422651_at	Adipocyte, C1Q and collagen domain containing	Lipid metabolim	NM_009605	-7.37
1424393_s_at	Alcohol dehydrogenase, iron containing, 1	Metabolism	BC026584	-7.75
1430584_s_at	Carbonic anhydrase 3	Cellular metabolism	BB213876	-15.99
Caecum				
1448964_at	Calbindin 3, (vitamin D-dependent calcium binding protein)	Cell communication	NM_009789	3.84
1426517_at	Guanine nucleotide binding protein, alpha z subunit	Cell communication	AI326356	3.3
1423436_at	Glutathione S-transferase, alpha 3	Metabolism	AI172943	2.75
1427912_at	Carbonyl reductase 3	Metabolism	AK003232	2.35
1415965_at	Stearoyl-Coenzyme A desaturase 1	Lipid metabolism	NM_009127	-2.13
1423693_at	Elastase 1, pancreatic	Protein metabolism	BC011218	-2.14
1456429_at	Mucosa associated lymphoid tissue lymphoma translocation gene 1	Apoptosis	BB296321	-2.23
1448290_at	Pancreatitis-associated protein	Immune response	NM_011036	-2.3
1417079_s_at	Lectin, galactose-binding, soluble 2	Apoptosis	NM_025622	-2.31
1458899_at	Ubiquitin specific protease 53	Protein metabolism	AV320152	-2.34
1438888_at	Germ cell-less homolog (Drosophila)	Cell differentiation	BM239632	-2.35
1421424_a_at	Alanyl (membrane) aminopeptidase	Angiogenesis	NM_008486	-2.36
1418777_at	Chemokine (C-C motif) ligand 25	Immune response	NM_009138	-2.37
1441200_at	Kruppel-like factor 3 (basic)	Regulation of transcription	BB327909	-2.37

1430529_at	Casein kinase 1, alpha 1	Protein metabolism	AK019176	-2.47
1457712_at	Chromodomain helicase DNA binding protein 8	Development	BG073323	-2.49
1440311_at	Sorbin and SH3 domain containing 1	Cytoskeleton organization	BB259710	-2.55
1426061_x_at	Cullin 4A	Cell cycle	BC007159	-2.61
1417023_a_at	Fatty acid binding protein 4, adipocyte	Lipid metabolism	NM_024406	-2.61
1437060_at	Similar to differentially expressed in hematopoietic lineages precursor (LOC239192), mRNA		AV290148	-2.63
1451054_at	Orosomucoid 1	Immune response	BE628912	-2.68
1432007_s_at	Adaptor protein complex AP-2, alpha 2 subunit	Protein metabolism	AK009735	-2.69
1450883_a_at	CD36 antigen	Immune response	BB534670	-2.69
1421578_at	Chemokine (C-C motif) ligand 4	Immune response	AF128218	-2.8
1440832_at	Angiogenin, ribonuclease A family, member 4	Immune response	BE199688	-2.84
1419233_x_at	Apolipoprotein A-I	Lipid metabolim	NM_009692	-2.98
1424737_at	Thyroid hormone responsive SPOT14 homolog (Rattus)		BC009165	-3.01
1417257_at	Carboxyl ester lipase	Lipid metabolim	BC006872	-3.03
1449434_at	Carbonic anhydrase 3		NM_007606	-3.05
1455442_at	Solute carrier family 6 (neurotransmitter transporter), member 19	Transport	BM119683	-3.09
1448872_at	Regenerating islet-derived 3 gamma	Immune response	NM_011260	-3.45
1453896_at	PDZ domain containing 3	Amine metabolism	AK015986	-3.57
1417867_at	Adipsin	Immune response	NM_013459	-3.58
1422651_at	Adipocyte, C1Q and collagen domain containing	Lipid metabolim	NM_009605	-3.69
1449316_at	Cytochrome P450, family 4, subfamily f, polypeptide 15	Transport	NM_134127	-3.74
1415994_at	Cytochrome P450, family 2, subfamily e, polypeptide 1	Transport	NM_021282	-3.94
1438211_s_at	D site albumin promoter binding protein	Regulation of transcription	BB550183	-5.56
1417074_at	CEA-related cell adhesion molecule 10		NM_007675	-13.92
Colon				
1448964_at	Calbindin 3, (vitamin D-dependent calcium binding protein)	Cell adhesion	NM_009789	5.18
1424797_a_at	Paired-like homeodomain transcription factor 2	Angiogenesis	U80011	4.97
1418931_at	Regenerating islet-derived family, member 4	Vascularisation	NM_026328	3.65
1448727_at	Transducin-like enhancer of	Regulation of	NM_053254	3.43

	split 6, homolog of Drosophila E(spl)	transcription		
1449498_at	Macrophage receptor with collagenous structure	Immune response	NM_010766	3.18
1425289_a_at	Complement receptor 2	Immune response	M35684	2.87
1419480_at	Selectin, lymphocyte	Cell adhesion	M36005	2.83
1424825_a_at	Glycosylation dependent cell adhesion molecule 1	Cell adhesion	M93428	2.74
1455530_at	Similar to Ig delta chain C region, membrane-bound form - mouse (LOC382646), mRNA	Immune response	BE686052	2.71
1423467_at	Membrane-spanning 4-domains, subfamily A, member 4B		BB199001	2.48
1436312_at	Zinc finger protein, subfamily 1A, 1 (Ikaros)	Transcription	AV317621	2.41
1419178_at	CD3 antigen, gamma polypeptide	Immune response	M58149	2.38
1450357_a_at	Chemokine (C-C motif) receptor 6	Immune response	NM_009835	2.37
1419135_at	Lymphotoxin B	Immune response	NM_008518	2.3
1427351_s_at	Immunoglobulin heavy chain 6 (heavy chain of IgM)	Immune response	BB226392	2.24
1455656_at	B and T lymphocyte associated	Immune response	BM240873	2.14
1426159_x_at	T-cell receptor beta, variable 13	Immune response	U46841	2.14
1419974_at	Sterol carrier protein 2, liver	Transport	C76618	-2.37
1420400_at	Seminal vesicle antigen-like 1		NM_027832	-2.44
1427782_a_at	Corticotropin releasing hormone receptor 1	Cell communication	AF369656	-2.53
1428352_at	Arrestin domain containing 2		AW542672	-2.89
1425738_at	Processed pseudo-IgK chain mRNA, VC-region, from hybridoma A23A41.	Immune response	M35669	-2.94
1419576_at	Homeo box B13	Cellular metabolism	BC013639	-3.38
1459366_at	RGM domain family, member B	Cell adhesion	AV235481	-4.4

CHAPTER 9

Summary, Concluding Remarks and Future Perspectives

Introduction

Intestinal mucus is an important interface between the luminal microbiota and the underlying epithelium. The major organic mucus components, mucins, can interact with bacteria in different ways and at this location, major interactions are believed to take place [144]. However, the specific mucus bacterial community has not been intensively studied. Of particular interest is the diversity and functional analysis of bacteria that grow on mucins, since mucins are also an energy source for intestinal bacteria, especially for those that reside in the colon, where nutrients are less present **(Chapters 1 and 2)**. To get more insight into the interactions taking place between mucin-degrading bacteria and the host, the work described here focussed on the bacterial community that is able to degrade mucin. This community was studied combining both cultivation-based and -independent approaches.

The diversity of mucin-degrading bacteria in the gut

Despite its importance, the ecology of human mucin-degrading bacteria has not been studied in detail. Previous cultivation studies showed that bacteria belonging to few genera including *Bifidobacterium*, *Ruminococcus*, *Bacteroides*, *Clostridium*, could be grown into pure culture using a basal media containing mucin as sole carbon source [121]. In order to analyse the diversity of mucolytic bacteria, we have used a strategy which combined the use of both molecular and cultivation-based approaches. In **Chapter 3,** mucolytic bacteria were enriched from six faecal samples inoculated on a mucin-limited medium and monitored by denaturing gel gradient electrophoresis (DGGE) of 16S rRNA gene amplicons. This molecular fingerprint approach showed that the profiles were highly diverse, with a similarity index of 57.4 ± 9.2 %, indicating that the mucin-degrading bacterial community differs between individuals. In addition, cloning and sequencing of 16S rRNA genes revealed that the majority (69%) of these genes derived from not-yet cultivated species. The 16S rRNA gene sequences that showed similarities higher than 98% to known database entries, were related to the Gram-positive genera *Clostridium* and *Ruminococcus* (*Clostridium* clusters IV, XIVa and XVIII) while the remaining ones were related to species belonging to *Verrucomicrobia* and *Proteobacteria.* This study provided evidence for the fact that faecal samples contain a diverse population of mucin-associated bacteria, the composition of which differs from one person to one other. However, this approach did not allow differentiating between micro-organisms responsible for mucin degradation and those that benefit from this degradation directly or indirectly.

A. muciniphila, a novel mucin-degrading bacterium isolated from human faeces

Chapter 4 reveals that the use of a targeted cultivation approach allowed the isolation of a novel microorganism, *Akkermansia muciniphila*, capable of growth on mucin as sole carbon and nitrogen source. This is the first intestinal member belonging to the recently discovered Verrucomicrobia phylum. Bacterial members of this phylum have been detected using molecular techniques in a variety of ecosystems, including soil, water and GI tract and few have been cultivated [125]. *A. muciniphila* has the remarkable characteristic that it is mainly using mucin as carbon and nitrogen source. Phylogenetic analysis revealed that *A. muciniphila* was distantly related to *Verrucomicrobium spinosum*. In addition, analysis of recent clone libraries of the 16S rRNA gene showed that *A. muciniphila* was detected as a dominant member of the colonic mucosa [75].

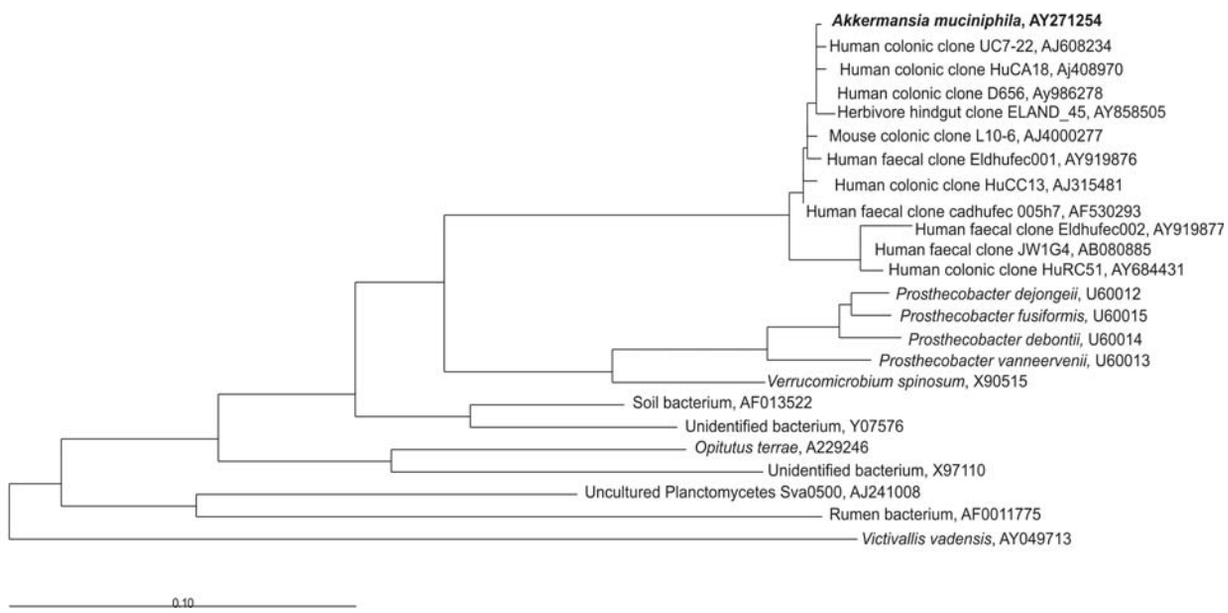


Figure 9.1. Phylogenetic tree of 16SrRNA sequences showing the position of *A. muciniphila* and the clones in the Verrucomicrobia division. The bar represents 10% divergence. Clones UC7-22 and cadhufec 005h7 were retrieved from clone libraries derived form ulcerative colitis and Crohn's disease patients, respectively.

Up to now, no less than 11 sequences corresponding to *A. muciniphila* and related species have been detected in 16S rRNA clone libraries, originating from human biopsies, faeces derived from healthy adults [75, 108, 112] (Saunier *et al*, unpublished) and inflammatory bowel diseases patients [178] (Hutson and Collins, unpublished), and in other animals such as mouse [250] and a herbivore [199] (Fig. 9.1).

This study showed that the use of a basal medium supplemented with a single carbon source could be an alternative to the use of complex and rich media for the isolation of new intestinal strains. Indeed, the cultivation of intestinal microorganisms has often been restricted to the use of rich media, which do not reflect the environment of the intestine. Another bacterium, *Victivallis vadensis* has been obtained in pure culture from human faeces using a similar basal medium supplemented with cellobiose as the sole carbon source [334]. Phylogenetic analysis based on 16S rRNA gene sequences indicated that *V. vadensis* is only distantly related to any other cultured bacterium and belongs to the newly proposed phylum Lentisphaerae [48]. Thus, the Verrucomicrobia and Lentisphaerae are two new phyla, containing both a single intestinal member isolated using a targeted-cultivation approach. Other studies targeting on butyrate-producing bacteria allowed the isolation of novel strains related to the genus *Roseburia* [73]. In the same research group, lactate or wheatbran-utilising bacteria were isolated from a medium containing a sole added energy source [74, 242]. In conclusion, it appears that the use of specific media containing a single carbon source is a targeted strategy in order for isolating novel microorganisms. While faeces can be used as inoculum, mucosal biopsies can serve as starting material to isolate bacteria and to investigate mucosal bacterial communities. Using an *in situ* viability assay based on flow cytometric analysis, Ben-Amor *et al.*, found that a large proportion of faecal bacteria is dead (32%), and thus are not able to be cultivated [16]. This indicates that sampling bacteria higher in the GI tract could improve the cultivation of microorganisms. However, due to the difficulty of the sampling of biopsy samples due to ethical and practical reasons, the isolation of mucosa-associated bacteria has been limited.

In order to get more quantitative data about the presence and diversity of *A. muciniphila* in the human gut, two different quantitative 16S rRNA-based approaches, were used to enumerate the species in a panel of faecal samples from healthy subjects, i.e. fluorescent in situ hybridisation (FISH) combined with flow cytometry (FCM) and quantitative PCR (qPCR), **(Chapters 6 and 7).** Compared to FISH, qPCR has the advantage of being more flexible, since DNA and not rRNA serves as the target molecule. Moreover, qPCR is highly sensitive and enables the detection of non-predominant species, in contrast to the FISH and culture-based methods. As a consequence, qPCR is increasingly being used for enumeration of bacteria in intestinal samples because of its specificity and sensitivity [69, 78, 96, 102, 103, 182, 216]. A specific oligonucleotide probe, named MUC-1437 **(Chapter 6)** and a pair of primers, AM1 and AM2 **(Chapter 7)**, both targeting the 16S rRNA sequence of *A. muciniphila*, were designed for FISH and qPCR, respectively based on alignments of 16S sequences from its closest relatives. FISH-FCM and qPCR were used to validate the newly designed probe and primers, respectively,

and to quantify *A. muciniphila* in faeces. Both techniques showed that *A. muciniphila* ranged from 10^6 to 10^{10} bacteria/g wet faeces with an average of $8.24 \pm 7.35 \times 10^8$ bacteria/g wet faeces for FISH-FCM and $7.76 \pm 20.1 \times 10^9$ cells/g wet faeces for qPCR. The numbers of *A. muciniphila* found in human faeces are equivalent to those from other important genera including *Ruminococcus*, *Eubacterium*, *Bifidobacterium* and outnumbers that of *Escherichia coli* and *Lactobacillus* spp. [335]. Moreover a high variability in the number of *A. muciniphila* between individuals was found. This is agreement with the variability found for the mucin-degrading bacteria based on the profiling of the 16S rDNA amplicons by DGGE **(Chapter 3)**.

The advantage of the FISH is that it allows the visualisation and localisation of bacterial cells in biopsy samples, when combined with microscopy. In **Chapter 8** the specific probe MUC-1437 was used to localise *A. muciniphila* cells in mouse intestinal biopsies. These cells were localised at a distance of less than 50 μm to the host cells.

Role of *A. muciniphila* in mucin degradation

A. muciniphila was found to produce a set of efficient enzymes that participate in mucin degradation **(Chapter 5)**, providing a potential competitive advantage compared to non-mucin-degrading bacteria [63]. Various glycosidases were detected in extracellular and intracellular fractions. However, the activity of these enzymes differed between the fractions. The secretion of diverse enzymes involved in mucin degradation makes *A. muciniphila* a fundamental player in mucin degradation. In contrast to other described microbes, *A. muciniphila* was found to degrade up to 85% of the mucin **(Chapter 5)**. *In vivo*, a significant correlation was observed between increasing faecal mucin excretion and decreasing *A. muciniphila* numbers as quantified by qPCR **(Chapter 7)**. This suggests a role for *A. muciniphila* plays in mucin degradation in the human GI tract.

Excessive mucin degradation can be linked with pathogenicity since enzymes that promote its erosion may contribute to weakening the mucus, the first defence layer of the host epithelium. However, some commensals and probiotic strains such as *Bifidobacterium bifidum* produce mucin-degrading enzymes [64] suggesting that mucin degradation is a general phenomenon. In healthy individuals, the turnover of mucin is balanced so that the layer remains relatively stable, while an excess or reduction of degradation has been observed in some intestinal disorders such as ulcerative colitis and Crohn's disease, respectively [53].

The regular detection of *A. muciniphila* in faeces from healthy individuals, and the healthy status of mono-associated mice **(Chapters 6, 7 and 8)** would favour to consider *A.*

muciniphila as a commensal. Moreover, increasing numbers of *A. muciniphila* have been detected in faecal samples from 6 and 12 months old babies (Collado M, personal communication).

Impact of *A. muciniphila* on host physiology and transcriptome

A significant correlation was observed between increasing faecal mucin excretion and decreasing *A. muciniphila* numbers, indicating a role for this mucin-degrading bacterium plays a crucial role in the human GI tract **(Chapter 7)**. To gain more knowledge about the influence of mucin degradation on GI tract functioning in the host, the impact of *A. muciniphila* on the host was studied in a mice model by comparing the GI tract transcriptome between mono-associated and germ-free mice. Previous comparisons between conventional, mono-associated and germ-free mice have demonstrated that the intestinal microbiota has a crucial impact on the host. Such studies showed that many biochemical, physiological, and immunological characteristics of the animal host are strongly influenced by the presence of the conventional microbiota [289]. Therefore, to differentiate between the effect of bacterial colonisation and mucin degradation the impact of *A. muciniphila* on the mice GI tract was compared to that of *Lactobacillus plantarum*, a non mucin-degrading bacterium **(Chapter 8)**. In order to gain a better understanding of the global response in different GI tract regions of the host, whole genome profiling on high density oligonucleotide microarrays was applied to biopsies samples processed from the ileum, caecum and colon. The analysis of the microarray data indicated that both *A. muciniphila* and *L. plantarum* induced site and bacteria-specific responses. The major pathways affected by *A. muciniphila* were involved in immune response, cell adhesion and metabolism while metabolism was the strongest effect observed in the mice trancriptome after *L. plantarum* mono-association with immune response at a lesser extent (Fig 9.2). Hooper *et al*. observed that the commensal and strict anaerobe, *B. thetaiotaomicron*, a bacterium that can grown on a variety of substrates including mucin, was able to modulate host genes expression in a variety of physiological functions, including those for nutrient absorption, and immune response. Two other commensals, *Escherichia coli* and *Bifidobacterium infantis* did not induce the same response on the host, indicating that microbial interactions with the host's GI tract cells show microbe-specificity [116]. The high proportion of genes related to immune response led to the hypothesis that *A. muciniphila* is closely associated to the host cells. When fixed intestinal biopsies were hybridised with the specific probe MUC-1437, *A. muciniphila* cells were frequently localised in clumps close to the epithelial cells (distance estimated less than 50 µm), suggesting a potential impact on the cells.

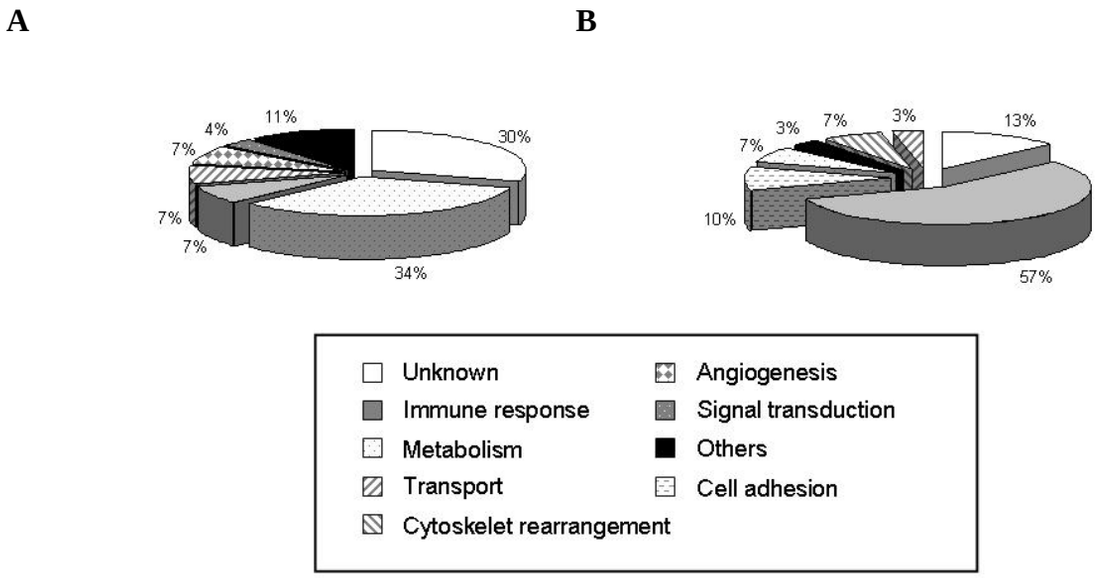


Figure 9.2. Proportion of genes regulated in mono-associated mice with *L. plantarum* (A) and *A. muciniphila* in the colon (B).

Perspectives and recommendations

This thesis has brought new insights into the mucin-degrading bacterial community, and especially the role of novel isolate, *A. muciniphila*. The approach that we used for its isolation, i.e. using mucin as a sole carbon and nitrogen source, has shown its efficiency to cultivate a not-yet cultivated microorganism. The further use of mucin-based media together with inocula from different locations in the intestine, will definitely allow the isolation of more of these bacteria, since we observed that mucin-degrading consortia are individual-specific. These and similar types of approaches will help in filling the phylogenetic gap observed between molecular and cultivation techniques.

A. muciniphila was found to be present at high numbers in stools from healthy individuals, and to produce mucin-degrading enzymes, which remain to be isolated and characterised. Frequently excessive mucin degradation is viewed as a pathogenicity factor since it disturbs the protective mucus layer. The role of mucin degradation of *A. muciniphila* in health and disease has not been investigated, and could be performed on intestinal samples derived from patients suffering from inflammatory bowel diseases, by combining quantification and localisation of the *A. muciniphila* in the mucosa.

When germ-free mice were mono-associated with *A. muciniphila*, the greatest majority of genes was involved in immune response **(Chapter 8),** which needs further biological validation. Future work could focus on association of germ-free mice with other selected microorganisms to verify change in host responses. Indeed, a recent study from Sonnneburg *et al*., indicated that the

host response to co-colonisation (*B. thetaiotaomicron* and *Bifidobacterium bifidum*) was distinct from the response to mono-association with either organism [276]. Moreover, other *in vivo* models could reflect better the conditions that take place in the GI tract. Indeed, a single micro-organism colonising the gut of germ-free mouse usually attains a much higher population level than it does in a conventional animal, where the microbe is faced with intense competition from the other members of the microbiota. Thus, an alternative would include a subtractive approach (conventional animal minus bacterial species). This could offer the ideal model to determine the impact of commensal bacteria on the regulation of expression of host genes [290]. However, human trials are the main but more challenging approaches to determine the direct impact of *A. muciniphila* on human cells.

Our study has brought information on molecular details of host responses, but little is known about details of the behaviour of *A. muciniphila* in the gut. In collaboration with Joint Genome Institute, the genome sequence of *A. muciniphila* is now being determined. This genome sequence will provide a wealth of information on its genetic potential and pave the way to comparative and functional genomics approaches to study its ecological role. Notably, studies on the mechanisms by which *A. muciniphila* associates to the host cells are a promising target for future work.

LITERATURE CITED

Literature cited

[1] **Allen, A.** 1983. Mucus - a protective secretion of complexity. *Trends Biochem Sci.* **8**, 169-173.

[2] **Allen, A. and G. Flemstrom**. 2005. Gastroduodenal mucus bicarbonate barrier: protection against acid and pepsin. *Am J Physiol Cell Physiol.* **288**, C1-19.

[3] **Allen, A., G. Flemstrom, A. Garner and E. Kivilaakso**. 1993. Gastroduodenal mucosal protection. *Physiol Rev.* **73**, 823-857.

[4] **Alm, E., D. Oerther, N. Larsen, D. Stahl and L. Raskin**. 1996. The oligonucleotide probe database. *Appl Environ Microbiol.* **62**, 3557-3559.

[5] **Amann, R., B. Binder, R. Olson, S. Chisholm, R. Devereux and D. Stahl**. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol.* **56**, 1919-1925.

[6] **Amann, R., L. Krumholz and D. Stahl**. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J Bacteriol.* **172**, 762-770.

[7] **Argueso, P., S. Spurr-Michaud, C. L. Russo, A. Tisdale and I. K. Gipson**. 2003. MUC16 mucin Is expressed by the human ocular surface epithelia and carries the H185 carbohydrate epitope. *Invest Ophthalmol Vis Sci.* **44**, 2487-2495.

[8] **Backhed, F., H. Ding, T. Wang, L. V. Hooper, G. Y. Koh, A. Nagy, C. F. Semenkovich and J. I. Gordon**. 2004. The gut microbiota as an environmental factor that regulates fat storage. *PNAS.* **101**, 15718-15723.

[9] **Baken, K., J. Ezendam, E. Gremmer, A. de Klerk, J. Pennings, B. Matthee, A. Peijnenburg and H. van Loveren**. 2006. Evaluation of immunomodulation by Lactobacillus casei Shirota: Immune function, autoimmunity and gene expression. *Int J Food Microbiol.* **112**, 8-18.

[10] **Baldwin, D., V. Vanchinathan, P. Brown and J. Theriot**. 2002. A gene-expression program reflecting the innate immune response of cultured intestinal epithelial cells to infection by *Listeria monocytogenes*. *Genome Biol.* **4**, R2.

[11] **Barcelo, A., J. Claustre, F. Moro, J. A. Chayvialle, J. C. Cuber and P. Plaisancie**. 2000. Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon. *Gut.* **46**, 218-224.

[12] **Bassett, D. E., M. B. Eisen and M. S. Boguski**. 1999. Gene expression informatics - it's all in your mine. *Nat Genet.* **21**, 51-55.

[13] **Baumheter, S., M. S. Singer, W. Henzel, S. Hemmerich, M. Renz, S. D. Rosen and L. A. Lasky**. 1993. Binding of L-selectin to the vascular sialomucin CD34. *Science.* **262**, 436-438.

[14] **Begley, M., C. G. M. Gahan and C. Hill**. 2005. The interaction between bacteria and bile. *FEMS Microbiol Rev.* **29**, 625-651.

[15] **Bell, A., L. Sellers, A. Allen, W. Cunliffe, E. Morris and S. Ross-Murphy**. 1985. Properties of gastric and duodenal mucus: effect of proteolysis, disulfide reduction, bile, acid, ethanol, and hypertonicity on mucus gel structure. *Gastroenterology.* **88**, 269-280.

[16] **Ben-Amor, K., H. Heilig, H. Smidt, E. E. Vaughan, T. Abee and W. M. de Vos**. 2005. Genetic diversity of viable, injured, and dead fecal bacteria assessed by fluorescence-activated cell sorting and 16S rRNA gene analysis. *Appl Environ Microbiol.* **71**, 4679-4689.

[17] **Ben Amor, K.** 2004. Microbial eco-physiology of the human intestinal tract: a flow cytometric approach. PhD thesis Wageningen University.

[18] **Ben Amor, K. and E. Vaughan.** 2006. Molecular ecology of the human intestinal microbiota.*In*: Ouwehand, A. and Vaughan, E. (Eds.), Gastrointestinal microbiology, Taylor & Francis New York.

[19] **Berg, J., L. Lindqvist, G. Andersson and C. Nord**. 1983. Neuraminidase in *Bacteroides fragilis*. *Appl Environ Microbiol.* **46**, 75-80.

[20] **Bergonzelli, G. E., D. Granato, R. D. Pridmore, L. F. Marvin-Guy, D. Donnicola and I. E. Corthesy-Theulaz**. 2006. GroEL of Lactobacillus johnsonii La1 (NCC 533) Is Cell Surface Associated: Potential Role in Interactions with the Host and the Gastric Pathogen Helicobacter pylori. *Infect Immun.* **74**, 425-434.

[21] **Bik, E. M., P. B. Eckburg, S. R. Gill, K. E. Nelson, E. A. Purdom, F. Francois, G. Perez-Perez, M. J. Blaser and D. A. Relman**. 2006. Molecular analysis of the bacterial microbiota in the human stomach. *PNAS.* **103**, 732-737.

[22] **Bingham, M.** 2003. Functional foods: dietary intervention strategies in autistic spectrum disorders. *Food Sci Technol Bull.* **1**, 1-15.

[23] **Bobek, L. A., H. Tsai, A. R. Biesbrock and M. J. Levine**. 1993. Molecular cloning, sequence, and specificity of expression of the gene encoding the low molecular weight human salivary mucin (MUC7). *J Biol Chem.* **268**, 20563-20569.

[24] **Boekhorst, J., Q. Helmer, M. Kleerebezem and R. J. Siezen**. 2006. Comparative analysis of proteins with a mucus-binding domain found exclusively in lactic acid bacteria. *Microbiology.* **152**, 273-280.

[25] **Boureau, H., D. Decre, J. P. Carlier, C. Guichet and P. Bourlioux**. 1993. Identification of a *Clostridium cocleatum* strain involved in an anti-*Clostridium difficile* barrier effect and determination of its mucin-degrading enzymes. *Res Microbiol.* **144**, 405.

[26] **Bovee-Oudenhoven, I. M., D. S. Termont, P. J. Heidt and R. Van der Meer**. 1997. Increasing the intestinal resistance of rats to the invasive pathogen Salmonella enteritidis: additive effects of dietary lactulose and calcium. *Gut.* **40**, 497-504.

[27] **Brayman, M., A. Thathiah and D. Carson**. 2004. MUC1: A multifunctional cell surface component of reproductive tissue epithelia. *Reprod Biol Endocrinol.* **2**, 4.

[28] **Breitbart, M., I. Hewson, B. Felts, J. M. Mahaffy, J. Nulton, P. Salamon and F. Rohwer**. 2003. Metagenomic Analyses of an Uncultured Viral Community from Human Feces. *J Bacteriol.* **185**, 6220-6223.

[29] **Breton, C., R. Oriol and A. Imberty**. 1998. Conserved structural features in eukaryotic and prokaryotic fucosyltransferases. *Glycobiology.* **8**, 87-94.

[30] **Brockhausen, I.** 2006. Mucin-type O-glycans in human colon and breast cancer: glycodynamics and functions. *EMBO Rep.* **7**, 599-604.

[31] **Brockhausen, I.** 2003. Sulphotransferases acting on mucin-type oligosaccharides. *Biochem Soc Trans.* **31**, 318-325.

[32] **Bron, P., C. Grangette, A. Mercenier, W. de Vos and M. Kleerebezem**. 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. *J Bact.* **186**, 5721-5729.

[33] **Brumel, J. and B. Finlay.** 2000. Bacterial adherence, colonization, and invasion of mucosal surfaces.*In*: Brogden, K., Roth, J., Stanton, T., Bolin, C., *et al.* (Eds.), Virulence mechanisms of bacterial pathogens, ASM Press, Washington D.C pp. 3-17.

[34] **Brunser, O., M. Gotteland, S. Cruchet, G. Figueroa, D. Garrido and P. Steenhout**. 2006. Effect of a milk formula with prebiotics on the intestinal microbiota of infants after an antibiotic treatment. *Pediatr Res.* **59**, 451-456.

[35] **Buck, B. L., E. Altermann, T. Svingerud and T. R. Klaenhammer**. 2005. Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. *Appl Environ Microbiol.* **71**, 8344-8351.

[36] **Bugaut, M. and M. Bentejac**. 1993. Biological effects of short-chain fatty acids in nonruminant mammals. *Annu Rev Nutr.* **13**.

[37] **Bunout, D., S. Hirsch, M. Pia de la Maza, C. Munoz, F. Haschke, P. Steenhout, P. Klassen, G. Barrera, V. Gattas and M. Petermann**. 2002. Effects of prebiotics on the immune response to vaccination in the elderly. *J Parenter Enteral Nutr.* **26**, 372-376.

[38] **Cabotaje, L., F. Shinnick, J. Lopez-Guisa and J. Marlett**. 1994. Mucin secretion in germfree rats fed fiber-free and psyllium diets and bacterial mass and carbohydrate fermentation after colonization. *Appl Environ Microbiol.* **60**, 1302-1307.

[39] **Campieri, M. and P. Gionchetti**. 2001. Bacteria as the cause of ulcerative colitis. *Gut.* **48**, 132-135.

[40] **Cardona, M., T. Midtvedt and E. Norin**. 2001. Probiotics in gnotobiotic mice: Short chain fatty acid production in vitro and in vivo. *Scand J Lan Anim Sci.* **28**, 75-84.

[41] **Carlstedt-Duke, B., T. Hoverstad, E. Lingaas, K. Norin, H. Saxerholt, M. Steinbakk and T. Midtvedt**. 1986. Influence of antibiotics on intestinal mucin in healthy subjects. *Eur J Clin Microbiol.* **5**, 634-638.

[42] **Carlstedt-Duke, B., T. Midtvedt, C. Nord and B. E. Gustafsson**. 1986. Isolation and characterization of a mucin-degrading strain of *Peptostreptococcus* from rat intestinal tract. *Acta Pathol Microbiol Immunol Scand* **94**, 293-300.

[43] **Carraway, K. L. and S. R. Hull**. 1991. Cell surface mucin-type glycoproteins and mucin-like domains. *Glycobiology.* **1**, 131-138.

[44] **Cash, H., C. Whitham, C. Behrendt and L. Hooper**. 2006. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. *Science.* **313**, 1126-1130.

[45] **Chavira, R. J., T. Burnett and J. Hageman**. 1984. Assaying proteinases with azocoll. *Anal Biochem.* **136**, 446-450.

[46] **Chen, Y., Y. H. Zhao, T. B. Kalaslavadi, E. Hamati, K. Nehrke, A. D. Le, D. K. Ann and R. Wu**. 2004. Genome-wide search and identification of a novel gel-forming mucin MUC19/Muc19 in glandular tissues. *Am J Respir Cell Mol Biol.* **30**, 155-165.

[47] **Chin, K. J., W. Liesack and P. H. Janssen**. 2001. Opitutus terrae gen. nov., sp. nov., to accommodate novel strains of the division 'Verrucomicrobia' isolated from rice paddy soil. *Int J Syst Evol Microbiol.* **51**, 1965-1968.

[48] **Cho, J.-C., K. L. Vergin, R. M. Morris and S. J. Giovannoni**. 2004. *Lentisphaera araneosa* gen. nov., sp. nov, a transparent exopolymer producing marine bacterium, and the description of a novel bacterial phylum, Lentisphaerae. *Environ Microbiol.* **6**, 611-621.

[49] **Christoph, M., A. Nikolaus, L. Thomas, A. Charalampos, K. Alexander, K.-M. Olga, S.-M. Anna, Z. Alexandra, S. Wolfgang and S. Gerd**. 2006. Aberrant intestinal expression and allelic variants of mucin genes associated with inflammatory bowel disease. *J Mol Med.* **84**, 1055-1066.

[50] **Cole, J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity and J. M. Tiedje**. 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucl Acids Res.* **33**, D294-296.

[51] **Conley, M. E. and D. L. Delacroix**. 1987. Intravascular and mucosal immunoglobulin A: two separate but related systems of immune defense? *Ann Intern Med.* **106**, 892-899.

[52] **Corfield, A. and A. Shukla**. 2003. Mucins: Vital components of the mucosal defensive barrier. *American Genomic Proteomic Technology*. 20-23.

[53] **Corfield, A. P., N. Myerscough, R. Longman, P. Sylvester, S. Arul and M. Pignatelli**. 2000. Mucins and mucosal protection in the gastrointestinal tract: new prospects for mucins in the pathology of gastrointestinal disease. *Gut.* **47**, 589-594.

[54] **Corfield, A. P., S. A. Wagner, J. R. Clamp, M. S. Kriaris and L. C. Hoskins**. 1992. Mucin degradation in the human colon: production of sialidase, sialate O-acetylesterase, N-acetylneuraminate lyase, arylesterase, and glycosulfatase activities by strains of fecal bacteria. *Infect Immun.* **60**, 3971-3978.

[55] **Crawley, S., J. Gum, J. Hicks, W. Pratt, J. Aubert, D. Swallow and Y. Kim**. 1999. Genomic organization and structure of the 3' region of human MUC3: alternative splicing predicts membrane-bound and soluble forms of the mucin. *Biochem Biophys Res Commun.* **263**, 728-736.

[56] **Cummings, C. A. and D. A. Relman**. 2000. Using DNA microarrays to study host-microbe interactions. *Emerg Infect Dis.* **6**, 513-525.

[57] **Cummings, J. and G. Macfarlane**. 1991. The control and consequences of bacterial fermentation in the human colon. *J Appl Bacteriol.* **70**, 443-459.

[58] **Daims, H., A. Bruhl, R. Amann, K. Schleifer and M. Wagner**. 1999. The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol.* **22**, 434-444.

[59] **de Vries, M.** 2006. Analyzing global gene expression of *Lactobacillus plantarum* in the human gastro-intestinal tract. PhD thesis Wageningen University.

[60] **Dekker, J., J. W. A. Rossen, H. A. Buller and A. W. C. Einerhand**. 2002. The MUC family: an obituary. *Trends Biochem Sci.* **27**, 126-131.

[61] **Delzenne, N. and N. Kok**. 1998. Effect of non-digestible fermentable carbohydrates on hepatic fatty acid metabolism. *Biochem Soc Trans.* **26**, 228-230.

[62] **Demigne, C., C. Morand, M. Levrat, C. Besson, C. Moundras and C. Remesy**. 1995. Effect of propionate on fatty acid and cholesterol synthesis and on acetate metabolism in isolated rat hepatocytes. *Br J Nutr.* **74**, 209-219.

[63] **Deplancke, B. and H. R. Gaskins**. 2001. Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. *Am J Clin Nutr.* **73**, 1131S-1141.

[64] **Derensy-Dron, D., F. Krzewinski, C. Brassart and S. Bouquelet**. 1999. Beta-1,3-galactosyl-N-acetylhexosamine phosphorylase from *Bifidobacterium bifidum* DSM 20082: characterization, partial purification and relation to mucin degradation. *Biotechnol Appl Biochem.* **29**, 3-10.

[65] **Derrien, M., D. Adawi, S. Ahrne, B. Jeppsson, G. Molin, N. Osman, J. Stsepetova, E. Vaughan, W. de Vos and A. Ouwehand**. 2004. The intestinal mucosa as a habitat of the gut microbiota and a rational target for probiotic functionality and safety. *Microb Ecol Health Dis.* **16**, 137-144.

[66] **Derrien, M., E. E. Vaughan, C. M. Plugge and W. M. de Vos**. 2004. *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *Int J Syst Evol Microbiol.* **54**, 1469-1476.

[67] **Desseyn, J.-L., V. Guyonnet-Duperat, N. Porchet, J.-P. Aubert and A. Laine**. 1997. Human mucin gene MUC5B, the 10.7-kb large central exon encodes various alternate subdomains resulting in a super-repeat. Structural evidence for a 11p15.5 gene family. *J Biol Chem.* **272**, 3168-3178.

[68] **Di Caro, S., S. Taob, A. Grillo, C. Elia, G. Gasbarrini, A. Sepulveda and A. Gasbarrini**. 2005. Effects of *Lactobacillus* GG on genes expression pattern in small bowel mucosa. *Digestive and Liver Disease.* **37**, 320-329.

[69] **Dick, L. K. and K. G. Field**. 2004. Rapid estimation of numbers of fecal Bacteroidetes by use of a quantitative PCR assay for 16S rRNA genes. *Appl Environ Microbiol.* **70**, 5695-5697.

[70] **Dinoto, A., T. M. Marques, K. Sakamoto, S. Fukiya, J. Watanabe, S. Ito and A. Yokota**. 2006. Population dynamics of *Bifidobacterium* species in human feces during raffinose administration monitored by fluorescence in situ hybridization-flow cytometry. *Appl Environ Microbiol.* **72**, 7739-7747.

[71] **Dowbenko, D., A. Kikuta, C. Fennie, N. Gillett and L. Lasky**. 1993. Glycosylation-dependent cell adhesion molecule 1 (GlyCAM 1) mucin is expressed by lactating mammary gland epithelial cells and is present in milk. *J Clin Invest.* **92**, 952-960.

[72] **Dubois, M., K. A. Gilles, J. Hamilton, P. Rebers and F. Smith**. 1956. Colorimetric method for determination of sugars and related substances. *Anal Chem.* **28**.

[73] **Duncan, S. H., G. L. Hold, A. Barcenilla, C. S. Stewart and H. J. Flint**. 2002. Roseburia intestinalis sp. nov., a novel saccharolytic, butyrate-producing bacterium from human faeces. *Int J Syst Evol Microbiol.* **52**, 1615-1620.

[74] **Duncan, S. H., P. Louis and H. J. Flint**. 2004. Lactate-Utilizing Bacteria, Isolated from Human Feces, That Produce Butyrate as a Major Fermentation Product. *Appl Environ Microbiol.* **70**, 5810-5817.

[75] **Eckburg, P. B., E. M. Bik, C. N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S. R. Gill, K. E. Nelson and D. A. Relman**. 2005. Diversity of the human intestinal microbial flora. *Science.* **308**, 1635-1638.

[76] **Egert, M., A. de Graaf, A. Maathuis, P. de Waard, C. Plugge, H. Smidt, N. Deutz, C. Dijkema, W. de Vos and V. K**. 2006. Identification of glucose-fermenting bacteria present in an in-vitro model of the human intestine by RNA-stable isotope probing. *FEMS Microbiol Ecol.* In press.

[77] **Fallani, M., L. Rigottier-Gois, M. Aguilera, C. Bridonneau, A. Collignon, C. A. Edwards, G. Corthier and J. Dore**. 2006. *Clostridium difficile* and *Clostridium perfringens* species detected in infant faecal microbiota using 16S rRNA targeted probes. *J Microbiol Methods.* **67**, 150.

[78] **Fite, A., G. T. Macfarlane, J. H. Cummings, M. J. Hopkins, S. C. Kong, E. Furrie and S. Macfarlane**. 2004. Identification and quantitation of mucosal and faecal desulfovibrios using real time polymerase chain reaction. *Gut.* **53**, 523-529.

[79] **Fleischmann, R., M. Adams, O. White, R. Clayton, E. Kirkness, A. Kerlavage, C. Bult, J. Tomb, B. Dougherty, J. Merrick*, et al.*** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science.* **269**, 496-512.

[80] Forstner, J. and G. Forstner. 1994. Gatrointestinal mucus *In*: Johnson, L. R. (Ed.), Physiology of the gastrointestinal tract, Raven Press, New York pp. 1255-1282.

[81] **Franks, A. H., H. J. M. Harmsen, G. C. Raangs, G. J. Jansen, F. Schut and G. W. Welling**. 1998. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol.* **64**, 3336-3345.

[82] **Freitas, M., C. Cayuela, J.-M. Antoine, F. Piller, C. Sapin and G. Trugnan**. 2001. A heat labile soluble factor from *Bacteroides thetaiotaomicron* VPI-5482 specifically increases the galactosylation pattern of HT29-MTX cells. *Cell Microbiol.* **3**, 289-300.

[83] **Gendler, S. J., C. A. Lancaster, J. Taylor-Papadimitriou, T. Duhig, N. Peat, J. Burchell, L. Pemberton, E. N. Lalani and D. Wilson**. 1990. Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin. *J Biol Chem.* **265**, 15286-15293.

[84] **Gene Ontology, C.** 2006. The Gene Ontology (GO) project in 2006. *Nucl Acids Res.* **34**, D322-326.

[85] **Gibbons, R. J., S. S. Socransky and B. Kapsimalis**. 1964. Establishment of human indigenous bacteria in germ-free mice. *J Bacteriol.* **88**, 1316-1323.

[86] **Gibson, G., E. Beatty, X. Wang and J. Cummings**. 1995. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. *Gastroenterology.* **108**, 975-982.

[87] **Gibson, G., H. Probert, J. Van Loo, R. Rastall and M. Roberfroid**. 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr Res Rev.* **17**, 259-275.

[88] **Gibson, G. and M. Roberfroid**. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr.* **125**, 1401-1412.

[89] **Gibson, G. R.** 1999. Dietary Modulation of the Human Gut Microflora Using the Prebiotics Oligofructose and Inulin. *J Nutr.* **129**, 1438-1441.

[90] **Gill, S. R., M. Pop, R. T. DeBoy, P. B. Eckburg, P. J. Turnbaugh, B. S. Samuel, J. I. Gordon, D. A. Relman, C. M. Fraser-Liggett and K. E. Nelson**. 2006. Metagenomic analysis of the human distal gut microbiome. *Science.* **312**, 1355-1359.

[91] **Gordon, H. and H. Pesti**. 1971. The gnotobiotic animal as a tool in the study of host microbial relationships. *Bacteriol Rev.* **35**, 390–429.

[92] **Goso, Y., K. Ishihara, S. Sugawara and K. Hotta**. 2001. Purification and characterization of alpha-L-fucosidases from *Streptomyces* sp. OH11242. *Comp Biochem Physiol B Biochem Mol Biol.* **130**, 375-383.

[93] **Grangette, C., S. Nutten, E. Palumbo, S. Morath, C. Hermann, J. Dewulf, B. Pot, T. Hartung, P. Hols and A. Mercenier**. 2005. From the Cover: Enhanced antiinflammatory capacity of a *Lactobacillus plantarum* mutant synthesizing modified teichoic acids. *PNAS.* **102**, 10321-10326.

[94] **Guarner, F.** 2006. Prebiotics and mucosal barrier function. *J Nutr.* **136**, 2269-.

[95] **Guarner, F. and G. Schaafsma**. 1998. Probiotics. *Int J Food Microbiol.* **39**, 237-238.

[96] **Gueimonde, M., S. Tolkko, T. Korpimaki and S. Salminen**. 2004. New real-time quantitative PCR procedure for quantification of *Bifidobacteria* in human fecal samples. *Appl Environ Microbiol.* **70**, 4165-4169.

[97] **Gum, J., S. Crawley, J. Hicks, D. Szymkowski and Y. Kim**. 2002. MUC17, a novel membrane-tethered mucin. *Biochem Biophys Res Commun.* **291**, 466-475.

[98] **Gum, J. R., Jr., J. W. Hicks, N. W. Toribara, E. M. Rothe, R. E. Lagace and Y. S. Kim**. 1992. The human MUC2 intestinal mucin has cysteine-rich subdomains located both upstream and downstream of its central repetitive region. *J Biol Chem.* **267**, 21375-21383.

[99] **Gum, J. R., Jr., J. W. Hicks, N. W. Toribara, B. Siddiki and Y. S. Kim**. 1994. Molecular cloning of human intestinal mucin (MUC2) cDNA. Identification of the amino terminus and overall sequence similarity to prepro-von Willebrand factor. *J Biol Chem.* **269**, 2440-2446.

[100] **Gustafsson, B. E.** 1959. Lightweight stainless steel systems for rearing germfree animals. *Ann N Y Acad Sci.* **78**, 17-28.

[101] **Guyonnet Duperat, V., J. Audie, V. Debailleul, A. Laine, M. Buisine, S. Galiegue-Zouitina, P. Pigny, P. Degand, J. Aubert and N. Porchet**. 1995. Characterization of the human mucin gene MUC5AC: a consensus cysteine-rich domain for 11p15 mucin genes? *Biochem J.* **305**, 211–219.

[102] **Haarman, M. and J. Knol**. 2006. Quantitative real-time PCR analysis of fecal *Lactobacillus* species in infants receiving a prebiotic infant formula. *Appl Environ Microbiol.* **72**, 2359-2365.

[103] **Haarman, M. and J. Knol**. 2005. Quantitative real-time PCR assays to identify and quantify fecal *Bifidobacterium* species in infants receiving a prebiotic infant formula. *Appl Environ Microbiol.* **71**, 2318-2324.

[104] **Hall, J.** 2006. Commensal bacteria in the GI tract: benefits of probiotic supplementation. *Glycosci Nutr.* **7**, 1-12.

[105] **Hane, B., K. Jager and H. Drexler**. 1993. The Pearson product-moment correlation coefficient is better suited for identification of DNA fingerprint profiles than band matching algorithms. *Electrophoresis.* **14**, 967-972.

[106] **Harmsen, H., H. Kengen, A. Akkermans, A. Stams and W. de Vos**. 1996. Detection and localization of syntrophic propionate-oxidizing bacteria in granular sludge by in situ hybridization using 16S rRNA-based oligonucleotide probes. *Appl Environ Microbiol.* **62**, 1656-1663.

[107] **Harmsen, H. J. M., G. C. Raangs, T. He, J. E. Degener and G. W. Welling**. 2002. Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. *Appl Environ Microbiol.* **68**, 2982-2990.

[108] **Hayashi, H., M. Sakamoto and Y. Benno**. 2002. Fecal microbial diversity in a strict vegetarian as determined by molecular analysis and cultivation. *Microbiol Immunol.* **46**, 819-831.

[109] **Hedlund, B. P., J. Gosink and J. Staley**. 1997. Verrucomicrobia div. nov., a new division of the Bacteria containing three new species of *Prosthecobacter*. *Antonie Leeuwenhoek.* **72**, 29-38.

[110] **Higuchi, T., T. Orita, S. Nakanishi, K. Katsuya, H. Watanabe, Y. Yamasaki, I. Waga, T. Nanayama, Y. Yamamoto, W. Munger, *et al.*** 2004. Molecular cloning, genomic structure, and expression analysis of MUC20, a novel mucin protein, up-regulated in injured kidney. *J Biol Chem.* **279**, 1968-1979.

[111] **Hobson, P. N.** 1969. Rumen bacteria. *Methods Microbiol.* **3B**, 133-139.

[112] **Hold, G. L., S. E. Pryde, V. J. Russell, E. Furrie and H. J. Flint**. 2002. Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis. *FEMS Microbiol Lett.* **39**, 33-39.

[113] **Holdeman, L., I. Good and W. Moore**. 1976. Human fecal flora: variation in bacterial composition within individuals and a possible effect of emotional stress. *Appl Environ Microbiol.* **31**, 359-375.

[114] **Hollingsworth, M. A. and B. J. Swanson**. 2004. Mucins in cancer: protection and control of the cell surface. *Nat Rev Cancer.* **4**, 45-60.

[115] **Hooper, L. V., P. G. Falk and J. I. Gordon**. 2000. Analyzing the molecular foundations of commensalism in the mouse intestine. *Curr Opin Microbiol.* **3**, 79-85.

[116] **Hooper, L. V. and J. I. Gordon**. 2001. Commensal host-bacterial relationships in the gut. *Science.* **292**, 1115-1118.

[117] **Hooper, L. V., T. Midtvedt and J. I. Gordon**. 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu Rev Nutr.* **22**, 283-307.

[118] **Hooper, L. V., T. S. Stappenbeck, C. V. Hong and J. I. Gordon**. 2003. Angiogenins: a new class of microbicidal proteins involved in innate immunity. *Nat Immunol.* **4**, 269-273.

[119] **Hooper, L. V., M. H. Wong, A. Thelin, L. Hansson, P. G. Falk and J. I. Gordon**. 2001. Molecular analysis of commensal host-microbial relationships in the intestine. *Science.* **291**, 881-884.

[120] **Hooper, L. V., J. Xu, P. G. Falk, T. Midtvedt and J. I. Gordon**. 1999. A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. *PNAS.* **96**, 9833-9838.

[121] **Hoskins, L., M. Agustines, W. McKee, E. Boulding, M. Kriaris and G. Niedermeyer**. 1985. Mucin degradation in human colon ecosystems. Isolation and properties of fecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins. *J Clin Invest.* **75**, 944–953.

[122] **Hoskins, L. and E. Boulding**. 1976. Degradation of blood group antigens in human colon ecosystems. I. In vitro production of ABH blood group-degrading enzymes by enteric bacteria. *J Clin Invest.* **57**, 63-73.

[123] **Hoskins, L. C. and E. T. Boulding**. 1981. Mucin degradation in human colon ecosystems. Evidence for the existence and role of bacterial subpopulations producing glycosidases as extracellular enzymes. *J Clin Invest.* **67**, 163-172.

[124] **Hoskins, L. C., E. T. Boulding and G. Larson**. 1997. Purification and characterization of blood group A-degrading isoforms of alpha -N-acetylgalactosaminidase from *Ruminococcus torques* strain IX-70. *J Biol Chem.* **272**, 7932-7939.

[125] **Hugenholtz, P., B. M. Goebel and N. R. Pace**. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J Bacteriol.* **180**, 4765-4774.

[126] **Hugenholtz, P., G. W. Tyson and L. L. Blackall**. 2001. Design and evaluation of 16S rRNA-targeted oligonucleotide probes for fluorescence *in situ* hybridisation.

[127] **Huijghebaert, S. M., J. A. Mertens and H. J. Eyssen**. 1982. Isolation of a bile salt sulfatase-producing *Clostridium* strain from rat intestinal microflora. *Appl Environ Microbiol.* **43**, 185-192.

[128] **Jacobs, L. and P. Huber**. 1985. Regional distribution and alterations of lectin binding to colorectal mucin in mucosal biopsies from controls and subjects with inflammatory bowel diseases. *J Clin Invest.* **75**, 112-118.

[129] **Janssen, P. H.** 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl Environ Microbiol.* **72**, 1719-1728.

[130] **Janssen, P. H., A. Schuhmann, E. Morschel and F. A. Rainey**. 1997. Novel anaerobic ultramicrobacteria belonging to the Verrucomicrobiales lineage of bacterial descent isolated by dilution culture from anoxic rice paddy soil. *Appl Environ Microbiol.* **63**, 1382-1388.

[131] **Jordan, N., J. Newton, J. Pearson and A. Allen**. 1998. A novel method for the visualization of the in situ mucus layer in rat and man. *Clin Sci.* **95**, 97-106.

[132] **Kagnoff, M. F. and L. Eckmann**. 2001. Analysis of host responses to microbial infection using gene expression profiling. *Current Opinion in Microbiology.* **4**, 246-250.

[133] **Kandori, H., K. Hirayama, M. Takeda and K. Doi**. 1996. Histochemical, lectin-histochemical and morphometrical characteristics of intestinal goblet cells of germfree and conventional mice. *Exp Anim.* **45**, 155-160.

[134] **Kapitany, R. and E. Zebrowski**. 1973. A high resolution PAS stain for polyacrylamide gel electrophoresis. *Anal Biochem.* **56**, 361-369.

[135] **Katayama, T., K. Fujita and K. Yamamoto**. 2005. Novel bifidobacterial glycosidases acting on sugar chains of mucin glycoproteins. *J Biosci Bioeng.* **99**, 457-465.

[136] **Kaur, N. and A. Gupta**. 2002. Applications of inulin and oligofructose in health and nutrition. *J Biosci.* **27**, 703-714.

[137] **Kelly, D., J. I. Campbell, T. P. King, G. Grant, E. A. Jansson, A. G. P. Coutts, S. Pettersson and S. Conway**. 2004. Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-[gamma] and RelA. *Nature Immunol.* **5**, 104-112.

[138] **Kelly, D. and S. Conway**. 2005. Bacterial modulation of mucosal innate immunity. *Mol Immunol.* **42**, 895.

[139] **Kelly, D., S. Conway and R. Aminov**. 2005. Commensal gut bacteria: mechanisms of immune modulation. *Trends in Immunology.* **26**, 326.

[140] **Kim, G.-B., S.-H. Yi and B. H. Lee**. 2004. Purification and characterization of three different types of bile salt hydrolases from *Bifidobacterium* strains. *J Dairy Sci.* **87**, 258-266.

[141] **Kimura, Y., Y. Nagata and R. Buddington**. 2004. Some dietary fibers increase elimination of orally administered polychlorinated biphenyls but not that of retinol in mice. *J Nutr.* **134**, 135-142.

[142] **Kirjavainen, P. V., A. C. Ouwehand, E. Isolauri and S. J. Salminen**. 1998. The ability of probiotic bacteria to bind to human intestinal mucus. *FEMS Microbiol Lett.* **167**, 185-189.

[143] **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. Kuipers, R. Leer, R. Tarchini, S. Peters, H. Sandbrink, M. Fiers*, et al.*** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *PNAS.* **100**, 1990-1995.

[144] **Kleessen, B. and M. Blaut**. 2005. Modulation of gut mucosal biofilms. *Br J Nutr.* **Suppl 1**, S35-40.

[145] **Kontiokari, T., K. Sundqvist, M. Nuutinen, T. Pokka, M. Koskela and M. Uhari**. 2001. Randomised trial of cranberry-lingonberry juice and Lactobacillus GG drink for the prevention of urinary tract infections in women. *Br Med J.* **322**, 1571-1575.

[146] **Lan, M. S., S. K. Batra, W. N. Qi, R. S. Metzgar and M. A. Hollingsworth**. 1990. Cloning and sequencing of a human pancreatic tumor mucin cDNA. *J. Biol. Chem.* **265**, 15294-15299.

[147] Lane, D. 1991. 16S/23S rDNA sequencing.*In*: Goodfellow, E. S. a. M. (Ed.), Nucleic acid techniques in bacterial systematics, J. Wiley & Sons, Chicester, England pp. 115-175.

[148] **Lapensee, L., Y. Paquette and G. Bleau**. 1997. Allelic polymorphism and chromosomal localization of the human oviductin gene (MUC9). *Fertil Steril.* **68**.

[149] **Larson, G., P. Falk and L. Hoskins**. 1988. Degradation of human intestinal glycosphingolipids by extracellular glycosidases from mucin-degrading bacteria of the human fecal flora. *J Biol Chem.* **263**, 10790-10798.

[150] **Lasky, L., M. Singer, D. Dowbenko, Y. Imai, W. Henzel, C. Grimley, C. Fennie, N. Gillett, S. Watson and S. Rosen**. 1992. An endothelial ligand for L-selectin is a novel mucin-like molecule. *Cell.* **69**, 927-938.

[151] **Lawson, A. J., S. L. W. On, J. M. J. Logan and J. Stanley**. 2001. Campylobacter hominis sp. nov., from the human gastrointestinal tract. *Int J Syst Evol Microbiol.* **51**, 651-660.

[152] **Lay, C., L. Rigottier-Gois, K. Holmstrom, M. Rajilic, E. E. Vaughan, W. M. de Vos, M. D. Collins, R. Thiel, P. Namsolleck, M. Blaut*, et al.*** 2005. Colonic microbiota signatures across five northern european countries. *Appl Environ Microbiol.* **71**, 4153-4155.

[153] **Lay, C., M. Sutren, V. Rochet, K. Saunier, J. Dore and L. Rigottier-Gois**. 2005. Design and validation of 16S rRNA probes to enumerate members of the *Clostridium leptum* subgroup in human faecal microbiota. *Environ Microbiol.* **7**, 933-946.

[154] **Lepage, P., P. Seksik, M. Sutren, M.-F. de la Cochetiere, R. Jian, P. Marteau and J. Dore**. 2005. Biodiversity of the mucosa-associated microbiota is stable along the distal digestive tract in healthy individuals and patients with IBD. *Inflamm Bowel Dis.* **1**, 473-480.

[155] **Lesourd, B. and S. Meaume**. 1994. Cell mediated immunity changes in ageing, relative importance of cell subpopulation switches and of nutritional factors. *Immunol Lett.* **40**, 235-242.

[156] **Lesuffleur, T., A. Barbat, E. Dussaulx and A. Zweibaum**. 1990. Growth adaptation to methotrexate of HT-29 human colon carcinoma cells is associated with their ability to differentiate into columnar absorptive and mucus-secreting cells. *Cancer Res.* **50**, 6334-6343.

[157] **Lievin-Le Moal, V. and A. L. Servin**. 2006. The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides, and microbiota. *Clin Microbiol Rev.* **19**, 315-337.

[158] **Ligtenberg, M. J., H. L. Vos, A. M. Gennissen and J. Hilkens**. 1990. Episialin, a carcinoma-associated mucin, is generated by a polymorphic gene encoding splice variants with alternative amino termini. *J Biol Chem.* **265**, 5573-5578.

[159] **Lindstedt, G., S. Lindstedt and B. E. Gustafsson**. 1965. Mucus in intestinal contents of germfree rats. *J Exp Med.* **121**, 201-213.

[160] **List, S., B. Findlay, G. Forstner and J. Forstner**. 1978. Enhancement of viscosity of mucin by serum albumin. *Biochem J.* **175**, 565-571.

[161] **Lobb, R. R., G. Chi-Rosso, D. R. Leone, M. D. Rosa, S. Bixler, B. M. Newman, S. Luhowskyj, C. D. Benjamin, I. G. Dougas and S. E. Goelz**. 1991. Expression and functional characterization of a soluble form of endothelial-leukocyte adhesion molecule 1. *J Immunol.* **147**, 124-129.

[162] **Lockhart, D. J. and E. A. Winzeler**. 2000. Genomics, gene expression and DNA arrays. *Nature.* **405**, 827.

[163] **Loesche, W.** 1969. Effect of bacterial contamination on cecal size and cecal contents of gnotobiotic rodents. *J Bacteriol.* **99**, 520-526.

[164] **Lopez-Ferrer, A., C. de Bolos, C. Barranco, M. Garrido, J. Isern, I. Carlstedt, C. A. Reis, J. Torrado and F. X. Real**. 2000. Role of fucosyltransferases in the association between apomucin and Lewis antigen expression in normal and malignant gastric epithelium. *Gut.* **47**, 349-356.

[165] **Loy, A., M. Horn and M. Wagner**. 2003. probeBase: an online resource for rRNA-targeted oligonucleotide probes. *Nucl Acids Res.* **31**, 514-516.

[166] **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb*, et al.*** 2004. ARB: a software environment for sequence data. *Nucl Acids Res.* **32**, 1363-1371.

[167] **Macfarlane, G. and G. Gibson**. 1991. Formation of glycoprotein degrading enzymes by *Bacteroides fragilis*. *FEMS Microbiol Lett.* **61**, 289-293.

[168] **Macfarlane, G., G. Gibson and J. Cummings**. 1992. Comparison of fermentation reactions in different regions of the human colon. *J Appl Bacteriol.* **72**, 57-64.

[169] **Macfarlane, G., S. Macfarlane and G. Gibson**. 1992. Synthesis and release of proteases by *Bacteroides fragilis*. *Curr Microbiol.* **24**, 55-59.

[170] **Macfarlane, G. T. and J. H. Cummings**. 1999. Probiotics and prebiotics: can regulating the activities of intestinal bacteria benefit health? *BMJ.* **318**, 999-1003.

[171] **Macfarlane, S., M. Hopkins and G. T. Macfarlane**. 2001. Toxin Synthesis and Mucin Breakdown Are Related to Swarming Phenomenon in *Clostridium septicum*. *Infect Immun.* **69**, 1120-1126.

[172] **Macfarlane, S., A. J. McBain and G. T. Macfarlane**. 1997. Consequences of biofilm and sessile growth in the large intestine. *Adv Dent Res.* **11**, 59-68.

[173] **Macfarlane, S., E. J. Woodmansey and G. T. Macfarlane**. 2005. Colonization of mucin by human intestinal bacteria and establishment of biofilm communities in a two-stage continuous culture system. *Appl Environ Microbiol.* **71**, 7483-7492.

[174] **Mack, D. R., S. Ahrne, L. Hyde, S. Wei and M. A. Hollingsworth**. 2003. Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells in vitro. *Gut.* **52**, 827-833.

[175] **Mackie, R., A. Sghir and H. Gaskins**. 1999. Developmental microbial ecology of the neonatal gastrointestinal tract. *Am J Clin Nutr.* **69**.

[176] **Mahida, Y. R. and S. Johal**. 2001. NF-KB may determine whether epithelial cell-microbial interactions in the intestine are hostile or friendly *Clin Exp Immunol.* **123**, 347-349.

[177] **Makarova, K., A. Slesarev, Y. Wolf, A. Sorokin, B. Mirkin, E. Koonin, A. Pavlov, N. Pavlova, V. Karamychev, N. Polouchine*, et al.*** 2006. Comparative genomics of the lactic acid bacteria. *PNAS.* **103**, 15611-15616.

[178] **Mangin, I., R. Bonnet, P. Seksik, L. Rigottier-Gois, M. Sutren, Y. Bouhnik, C. Neut, M. D. Collins, J.-F. Colombel, P. Marteau*, et al.*** 2004. Molecular inventory of faecal microflora in patients with Crohn's disease. *FEMS Microbiol Ecol.* **50**, 25-36.

[179] **Marco, M. L., R. S. Bongers, W. M. de Vos and M. Kleerebezem**. 2007. Spatial and temporal expression of *Lactobacillus plantarum* genes in the gastrointestinal tracts of mice. *Appl Environ Microbiol.* **73**, 124-132.

[180] **Marini, A., F. Negretti, G. Boehm, M. Li Destri, D. Clerici-Bagozzi, F. Mosca and M. Agosti**. 2003. Pro- and pre-biotics administration in preterm infants: colonization and influence on faecal flora. *Acta Paediatr Suppl.* **91**, 80-81.

[181] **Martin-Sosa, S., M.-J. Martin and P. Hueso**. 2002. The Sialylated Fraction of Milk Oligosaccharides Is Partially Responsible for Binding to Enterotoxigenic and Uropathogenic Escherichia coli Human Strains. *J Nutr.* **132**, 3067-3072.

[182] **Matsuki, T., K. Watanabe, J. Fujimoto, Y. Miyamoto, T. Takada, K. Matsumoto, H. Oyaizu and R. Tanaka**. 2002. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. *Appl Environ Microbiol.* **68**, 5445-5451.

[183] **Matsuo, K., H. Ota, T. Akamatsu, A. Sugiyama and T. Katsuyama**. 1997. Histochemistry of the surface mucous gel layer of the human colon. *Gut.* **40**, 782-789.

[184] **McCracken, V. J. and R. G. Lorenz**. 2001. The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity and microbiota. Microreview. *Cell Microbiol.* **3**, 1-11.

[185] **Mesbah, M. and U. W. Premachandran, WB**. 1989. Precise measurement of the G + C content of desoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol.* **39**, 159-167.

[186] **Midtvedt, A., B. Carlstedt-Duke and T. Midtvedt**. 1994. Establishment of a mucin-degrading intestinal microflora during the first two years of human life. *J Pediatr Gastroenterol Nutr.* **18**, 321-326.

[187] **Miller, R. and L. Hoskins**. 1981. Mucin degradation in human colon ecosystems. Fecal population densities of mucin-degrading bacteria estimated by a "most probable number" method. *Gastroenterology.* **81**, 759-765.

[188] **Mills, J. C., K. A. Roth, R. L. Cagan and J. I. Gordon**. 2001. DNA microarrays and beyond: completing the journey from tissue to cell. *Nat Cell Biol.* **3**, E175.

[189] **Minekus, M., P. Marteau, R. Havenaar and J. Huis in 't Veld**. 1995. A multicompartimental dynamic computer-controlled model simulating the stomach and small intestine. *Altern Lab Anim.* **23**, 197-209.

[190] **Minekus, M., M. Smeets-Peeters, A. Bernalier, S. Marol-Bonnin, R. Havenaar, P. Marteau, M. Alric, G. Fonty and J. Huis in't Veld**. 1999. A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. *Appl Microbiol Biotechnol.* **53**, 108-114.

[191] **Miniello, V., G. Moro and L. Armenio**. 2003. Prebiotics in infant milk formulas: new perspectives. *Acta Paediatr Scand.* **91**, 68-76.

[192] **Moniaux, N., W. M. Junker, A. P. Singh, A. M. Jones and S. K. Batra**. 2006. Characterization of human mucin MUC17: Complete coding sequence and organization. *J Biol Chem.*, M600302200.

[193] **Moniaux, N., S. Nollet, N. Porchet, P. Degand, A. Laine and J. Aubert**. 1999. Complete sequence of the human mucin MUC4: a putative cell membrane-associated mucin. *Biochem J.* **338**, 325-333.

[194] **Moore, E.** 1976. Physiology of intestinal and electrolyte absorption.

[195] **Moore, W. and L. Holdeman**. 1974. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. *Appl Microbiol.* **27**, 961-979.

[196] **Mueller, S., K. Saunier, C. Hanisch, E. Norin, L. Alm, T. Midtvedt, A. Cresci, S. Silvi, C. Orpianesi, M. Verdenelli, *et al.*** 2006. Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. *Appl Environ Microbiol.* **72**, 1027-1033.

[197] **Muyzer, G., E. C. de Waal and A. G. Uitterlinden**. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol.* **59**, 695-700.

[198] **Neef, A., A. Zaglauer, H. Meier, R. Amann, H. Lemmer and K. Schleifer**. 1996. Population analysis in a denitrifying sand filter: conventional and in situ identification of *Paracoccus* spp. in methanol-fed biofilms. *Appl Environ Microbiol.* **62**, 4329-4339.

[199] **Nelson, K. E., S. H. Zinder, I. Hance, P. Burr, D. Odongo, D. Wasawo, A. Odenyo and R. Bishop**. 2003. Phylogenetic analysis of the microbial populations in the wild herbivore gastrointestinal tract: insights into an unexplored niche. *Environ Microbiol.* **5**, 1212-1220.

[200] **Neurath, M. F., C. Becker and K. Barbulescu**. 1998. Role of NF-kappaB in immune and inflammatory responses in the gut. *Gut.* **43**, 856-860.

[201] **Nielsen, D. S., P. L. Moller, V. Rosenfeldt, A. Paerregaard, K. F. Michaelsen and M. Jakobsen**. 2003. Case study of the distribution of mucosa-associated *Bifidobacterium* species, *Lactobacillus* species, and other lactic acid bacteria in the human colon. *Appl Environ Microbiol.* **69**, 7545-7548.

[202] **Norin, E. and Midtvedt.** 2006. Born germ-free - Microbial dependent.*In*: Ouwehand, A. and Vaughan, E. (Eds.), Gastrointestinal microbiology, Taylor & Francis New York.

[203] **Norin, E. and T. Midtvedt**. 2000. Interactions of microflora associated characteristics of the host; non-immune functions. *Microb Ecol Health Dis.* **11**, 186-193.

[204] **Norin, K., B. E. Gustafsson, B. Lindblad and T. Midtvedt**. 1985. The establishment of some microflora associated biochemical characteristics in feces from children during the first years of life. *Acta Paediatr Scand.* **74**, 207-212.

[205] **Nubel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R. I. Amann, W. Ludwig and H. Backhaus**. 1996. Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. *J Bacteriol.* **178**, 5636-5643.

[206] **Nurmi, J., P. Puolakkainen and N. Rautonen**. 2005. *Bifidobacterium lactis* sp. 420 up-regulates cyclooxygenase (Cox)-1 and down-regulates Cox-2 gene expression in a Caco-2 cell culture model. *Nutr Cancer.* **51**, 83-92.

[207] **O'Connor, J. C., J. Julian, S. D. Lim and D. D. Carson**. 2004. MUC1 expression in human prostate cancer cell lines and primary tumors. *Prostate Cancer Prostatic Dis.* **8**, 36-44.

[208] **O'hara, A. and F. Shanahan**. 2006. The gut flora as a forgotten organ. *EMBO Rep.* **7**, 688-693.

[209] **Ohta, A., Y. Motohashi, K. Sakai, M. Hirayama, T. Adachi and K. Sakuma**. 1998. Dietary fructooligosaccharides increase calcium absorption and levels of mucosal calbindin-D9k in the large intestine of gastrectomized rats. *Scand J Gastroenterol.* **33**, 1062-1068.

[210] **Ott, S. J., M. Musfeldt, D. F. Wenderoth, J. Hampe, O. Brant, U. R. Folsch, K. N. Timmis and S. Schreiber**. 2004. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut.* **53**, 685-693.

[211] **Ouwehand, A., M. Derrien, W. de Vos, K. Tiihonen and N. Rautonen**. 2005. Prebiotics and other microbial substrates for gut functionality. *Curr Opin Biotechnol.* **16**, 212-217.

[212] **Pajari, A., J. Rajakangas, E. Paivarinta, V. Kosma, J. Rafter and M. Mutanen**. 2003. Promotion of intestinal tumor formation by inulin is associated with an accumulation of cytosolic beta-catenin in Min mice. *Int J Cancer.* **106**, 653-660.

[213] **Palframan, R., G. Gibson and R. Rastall**. 2003. Development of a quantitative tool for the comparison of the prebiotic effect of dietary oligosaccharides. *Lett Appl Microbiol.* **37**, 281-284.

[214] **Pallesen, L. T., L. Berglund, L. K. Rasmussen, T. E. Petersen and J. T. Rasmussen**. 2002. Isolation and characterization of MUC15, a novel cell membrane-associated mucin. *European Journal of Biochemistry.* **269**, 2755-2763.

[215] **Paulsen, F. P. and M. S. Berry**. 2006. Mucins and TFF peptides of the tear film and lacrimal apparatus. *Prog. Histochem. Cytochem.* **41**, 1.

[216] **Penders, J., C. Vink, C. Driessen, N. London, C. Thijs and E. Stobberingh**. 2005. Quantification of *Bifidobacterium* spp., *Escherichia coli* and *Clostridium difficile* in faecal samples of breast-fed and formula-fed infants by real-time PCR. *FEMS Microbiol Lett.* **243**, 141-147.

[217] **Peuranen, S., K. Tiihonen, J. Apajalahti, A. Kettunen, M. Saarinen and N. Rautonen**. 2004. Combination of polydextrose and lactitol affects microbial ecosystem and immune responses in rat gastrointestinal tract. *Br J Nutr.* **91**, 905-914.

[218] **Plugge, C. M.** 2005. Anoxic media design, preparation, and considerations. Methods in Enzymology, Academic Press p. 3.

[219] **Plugge, C. M., E. G. Zoetendal and A. J. M. Stams**. 2000. Caloramator coolhaasii sp. nov., a glutamate-degrading, moderately thermophilic anaerobe. *Int J Syst Evol Microbiol.* **50**, 1155-1162.

[220] **Porter, J. and R. W. Pickup**. 2000. Nucleic acid-based fluorescent probes in microbial ecology: application of flow cytometry. *J Microbiol Methods.* **42**, 75-79.

[221] **Potier, M., L. Mameli, M. Belisle, L. Dallaire and S. Melancon**. 1979. Fluorometric assay of neuramidase with a sodium (4-methylumbelliferyl-alpha-D-N-acetylneuraminate) substrate. *Anal Biochem.* **94**, 287-296.

[222] **Pratt, W., S. Crawley, J. Hicks, J. Ho, M. Nash, Y. Kim, J. Gum and D. Swallow**. 2000. Multiple transcripts of MUC3: evidence for two genes, MUC3A and MUC3B. *Biochem Biophys Res Commun.* **275**, 916-923.

[223] **Probert, H., J. Apajalahti, N. Rautonen, J. Stowell and G. Gibson**. 2004. Polydextrose, lactitol, and fructo-oligosaccharide fermentation by colonic bacteria in a three-stage continuous culture system. *Appl Environ Microbiol.* **70**, 4505-4511.

[224] **Probert, H. and G. Gibson**. 2002. Bacterial biofilms in the human gastrointestinal tract. *Curr. Issues Intest. Microbiol.* **3**, 23-27.

[225] **Pullan, R. D., G. A. Thomas, M. Rhodes, R. G. Newcombe, G. T. Williams, A. Allen and J. Rhodes**. 1994. Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. *Gut.* **35**, 353-359.

[226] **Rabiu, B. A. and G. R. Gibson**. 2002. Carbohydrates: a limit on bacterial diversity within the colon. *Biol Rev.* **77**, 443-453.

[227] **Rajilić-Stojanović, M.** 2007. Diversity of the human gastrointestinal microbiota: novel perspectives from high throughput analyses. PhD thesis Wageningen University.

[228] **Rappe, M. S. and S. J. Giovannoni**. 2003. The uncultured microbial majority. *Annu Rev Microbiol.* **57**, 369-394.

[229] **Rappuoli, R.** 2000. Pushing the limits of cellular microbiology: Microarrays to study bacteria-host cell intimate contacts. *PNAS.* **97**, 13467-13469.

[230] **Rawls, J. F., B. S. Samuel and J. I. Gordon**. 2004. From The Cover: Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *PNAS.* **101**, 4596-4601.
[231] **Rhodes, J. M.** 1997. Colonic mucus and ulcerative colitis. *Gut.* **40**, 807-808.
[232] **Rigottier-Gois, L., A.-G. Le Bourhis, G. Gramet, V. Rochet and J. Dore**. 2003. Fluorescent hybridisation combined with flow cytometry and hybridisation of total RNA to analyse the composition of microbial communities in human faeces using 16S rRNA probes. *FEMS Microbiol Ecol.* **43**, 237-245.
[233] **Rigottier-Gois, L., V. Rochet, N. Garrec, A. Suau and J. Doré**. 2003. Enumeration of Bacteroides species in human faeces by fluorescent in situ hybridisation combined with flow cytometry using 16S rRNA probes. *Syst Appl Microbiol.* **26**, 110-118.
[234] **Robbe, C., C. Calliope, E. Maes, M. Rousset, A. Zweibaum, J.-P. Zanetta and J.-C. Michalski**. 2003. Evidence of regio-specific glycosylation in human intestinal mucins. *J Biol Chem.* **278**, 46337-46348.
[235] **Robbe, C., C. Capon, B. Coddeville and J. Michalski**. 2004. Structural diversity and specific distribution of O-glycans in normal human mucins along the intestinal tract. *Biochem J.* **384**, 307-316.
[236] **Roberton, A. and R. Stanley**. 1982. In vitro utilization of mucin by *Bacteroides fragilis*. *Appl Environ Microbiol.* **43**, 325-330.
[237] **Roberton, A. M., C. G. McKenzie, N. Sharfe and L. B. Stubbs**. 1993. A glycosulphatase that removes sulphate from mucus glycoprotein. *Biochem J.* **293**, 683-689.
[238] **Robertson, A. and D. Wright**. 1997. Bacterial glycosulphatases and sulphomucin degradation. *Can J Gastroenterol.* **11**, 361-366.
[239] **Rogers, A., K. Pilowsky, P. Zilm and N. Gully**. 1991. Effects of pulsing with xylitol on mixed continuous cultures of oral streptococci. *Aust Dent J.* **36**, 231-235.
[240] **Roos, S. and H. Jonsson**. 2002. A high-molecular-mass cell-surface protein from Lactobacillus reuteri 1063 adheres to mucus components. *Microbiology.* **148**, 433-442.
[241] **Ruiz-Palacios, G. M., L. E. Cervantes, P. Ramos, B. Chavez-Munguia and D. S. Newburg**. 2003. *Campylobacter jejuni* binds intestinal H(O) antigen (Fucalpha 1, 2Galbeta 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. *J Biol Chem.* **278**, 14112-14120.
[242] **Rumney, C., S. Duncan, C. Henderson and C. Stewart**. 1995. Isolation and characteristics of a wheatbran-degrading *Butyrivibrio* from human faeces. *Lett Appl Microbiol.* **20**, 232-236.
[243] **Ruseler-van Embden, J., L. van Lieshout, M. Gosselink and P. Marteau**. 1995. Inability of *Lactobacillus casei* strain GG, *L. acidophilus*, and *Bifidobacterium bifidum* to degrade intestinal mucus glycoproteins. *Scand J Gastroenterol.* **30**, 675-680.
[244] **Ruseler-van Embden, J. G. H., R. van der Helm and L. M. C. van Lieshout**. 1989. Degradation of intestinal glycoproteins by *Bacteroides vulgatus*. *FEMS Microbiology Letters.* **58**, 37-42.
[245] **Rycroft, C., M. Jones, G. Gibson and R. Rastall**. 2001. A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. *J Appl Microbiol.* **91**, 878-887.
[246] **Salminen, S., C. Bouley, M. Boutron-Ruault, J. Cummings, A. Franck, G. Gibson, E. Isolauri, M. Moreau, M. Roberfroid and I. Rowland**. 1998. Functional food science and gastrointestinal physiology and function. *Br J Nutr.* **80**, S147-171.
[247] **Salminen, S., M. Laine, A. von Wright, J. Vuopio-Varkila, T. Korhonen and T. Mattila-Sandholm**. 1996. Development of selection criteria for probiotic strains to access their potential in functional food. A nordic and European approach. *Biosci Microflora.* **2**, 23-28.
[248] **Salyers, A. A.** 1979. Energy sources of major intestinal fermentative anaerobes. *Am. J. Clin. Nutr.* **32**, 158-163.
[249] **Salyers, A. A.** 1977. Fermentation of mucins and plant polysaccharides by anarerobic bacteria from the human colon. *Appl Environ Microbiol.* **34**, 529-533.
[250] **Salzman, N. H., H. de Jong, Y. Paterson, H. J. M. Harmsen, G. W. Welling and N. A. Bos**. 2002. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. *Microbiology.* **148**, 3651-3660.
[251] **Samuel, B. S. and J. I. Gordon**. 2006. A humanized gnotobiotic mouse model of host-archaeal-bacterial mutualism. *PNAS.* **103**, 10011-10016.
[252] **Sanguinetti, C. J., E. Dias Neto and A. J. Simpson**. 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *Biotechniques.* **17**, 914-921.
[253] **Sansonetti, P.** 2002. Host-pathogen interactions: the seduction of molecular cross talk. *Gut.* **50**, 2iii-8.
[254] **Satchithanandam, S., M. Vargofcak-Apker, R. Calvert, A. Leeds and M. Cassidy**. 1990. Alteration of gastrointestinal mucin by fiber feeding in rats. *J Nutr.* **120**, 1179-1184.
[255] **Savage, D.** 1977. Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol.* **31**, 107-133.
[256] **Savage, D. C.** 1972. Associations and physiological interactions of indigenous microorganisms and gastrointestinal epithelia. *Am J Clin Nutr.* **25**, 1372-1379.
[257] **Scheppach, W. and F. Weiler**. 2004. The butyrate story: old wine in new bottles? *Curr Opin Clin Nutr Metab Care.* **7**, 563-567.
[258] **Schlesner, H.** 1987. *Verrucomicrobium spinosum* gen. nov., sp. nov.: a fimbriated prosthecate bacterium. *Syst Appl Microbiol.* **10**, 54-56.

[259] **Schley, P. and C. Field**. 2002. The immune-enhancing effects of dietary fibres and prebiotics. *Br J Nutr.* **87**, S221-230.

[260] **Schmidt-Wittig, U., M. Enss, M. Coenen, K. Gartner and H. Hedrich**. 1996. Response of rat colonic mucosa to a high fiber diet. *Ann Nutr Metab.* **40**, 343-350.

[261] **Scholz-Ahrens, K. E., G. Schaafsma, E. G. H. M. van den Heuvel and J. Schrezenmeir**. 2001. Effects of prebiotics on mineral metabolism. *Am J Clin Nutr.* **73**, 459S-464.

[262] **Schultsz, C., F. M. Van Den Berg, F. W. Ten Kate, G. N. Tytgat and J. Dankert**. 1999. The intestinal mucus layer from patients with inflammatory bowel disease harbors high numbers of bacteria compared with controls. *Gastroenterology.* **117**, 1089-1097.

[263] **Schwiertz, A., G. Hold, S. Duncan, B. Gruhl, M. Collins, P. Lawson, H. Flint and M. Blaut**. 2002. *Anaerostipes caccae* gen. nov., sp. nov., a new saccharolytic, acetate-utilising, butyrate-producing bacterium from human faeces. *Syst Appl Microbiol.* **25**, 46-51.

[264] **Seregni, E., C. Botti, S. Massaron, C. Lombardo, A. Capobianco, A. Bogni and E. Bombardieri**. 1997. Structure, function and gene expression of epithelial mucins. *Tumori.* **83**, 625-632.

[265] **Shankar, V., M. Gilmore, R. Elkins and G. Sachdev**. 1994. A novel human airway mucin cDNA encodes a protein with unique tandem-repeat organization. *Biochem J.* **300**, 295-298.

[266] **Shankar, V., P. Pichan, R. Eddy, V. Tonk, N. Nowak, S. Sait, T. Shows, R. Schultz, G. Gotway, R. Elkins*, et al.*** 1997. Chromosomal localization of a human mucin gene (MUC8) and cloning of the cDNA corresponding to the carboxy terminus. *Am J Respir Cell Mol Biol.* **16**, 232-241.

[267] **Shimizu, M. and K. Yamauchi**. 1982. Isolation and characterization of mucin-like glycoprotein in human milk fat globule membrane. *J Biochem.* **91**, 515-524.

[268] **Shirazi, T., R. J. Longman, A. P. Corfield and C. S. J. Probert**. 2000. Mucins and inflammatory bowel disease. *Postgrad Med J.* **76**, 473-478.

[269] **Silverman, H. S., M. Sutton-Smith, K. McDermott, P. Heal, S.-H. Leir, H. R. Morris, M. A. Hollingsworth, A. Dell and A. Harris**. 2003. The contribution of tandem repeat number to the O-glycosylation of mucins. *Glycobiology.* **13**, 265-277.

[270] **Silvestri, L., R. Hurst, L. Simpson and J. Settine**. 1982. Analysis of sulfate in complex carbohydrates. *Anal Biochem.* **123**, 303-309.

[271] **Simmering, R., D. Taras, A. Schwiertz, G. Le Blay, B. Gruhl, P. Lawson, M. Collins and M. Blaut**. 2002. *Ruminococcus luti* sp. nov., isolated from a human faecal sample. *Syst Appl Microbiol.* **25**, 189-193.

[272] **Skelly, B., P. Trexler and J. Tanami**. 1962. Effect of a *Clostridium* species upon cecal size of gnotobiotic mice. *Proc Soc Exp Biol Med.* **10**, 455-458.

[273] **Smirnov, A., D. Sklan and Z. Uni**. 2004. Mucin dynamics in the chick small intestine are altered by starvation. *J. Nutr.* **134**, 736-742.

[274] **Smith, E. and G. Macfarlane**. 1997. Dissimilatory amino Acid metabolism in human colonic bacteria. *Anaerobe.* **3**, 327-337.

[275] **Sokol, H., P. Seksik, L. Rigottier-Gois, C. Lay, P. Lepage, I. Podglajen, P. Marteau and J. Dore**. 2006. Specificities of the fecal microbiota in inflammatory bowel disease. *Inflamm. Bowel Dis.* **12**, 106-111.

[276] **Sonnenburg, J., C. Chen and J. Gordon**. 2006. Genomic and metabolic studies of the impact of probiotics on a model gut symbiont and host. *PLoS Biol.* **4**.

[277] **Sonnenburg, J. L., L. T. Angenent and J. I. Gordon**. 2004. Getting a grip on things: how do communities of bacterial symbionts become established in our intestine? *Nat Immunol.* **5**, 569-573.

[278] **Sonnenburg, J. L., J. Xu, D. D. Leip, C.-H. Chen, B. P. Westover, J. Weatherford, J. D. Buhler and J. I. Gordon**. 2005. Glycan foraging in vivo by an intestine-adapted bacterial symbiont. *Science.* **307**, 1955-1959.

[279] **Specian, R. D. and M. G. Oliver**. 1991. Functional biology of intestinal goblet cells. *Am J Physiol Cell Physiol.* **260**, C183-193.

[280] **Staley, J., J. Bont and K. Jonge**. 1976. *Prosthecobacter fusiformis* nov. gen. et sp., the fusiform caulobacter. *Antonie Van Leeuwenhoek.* **42**, 333-342.

[281] **Stams, A., J. Van Dijk, C. Dijkema and C. Plugge**. 1993. Growth of Syntrophic Propionate-Oxidizing Bacteria with Fumarate in the Absence of Methanogenic Bacteria. *Appl Environ Microbiol.* **59**, 1114-1119.

[282] **Stappenbeck, T. S., L. V. Hooper and J. I. Gordon**. 2002. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *PNAS.* **99**, 15451-15455.

[283] **Stewart-Tull, D., R. Ollar and T. Scobie**. 1986. Studies on the *Vibrio cholerae* mucinase complex. I. Enzymic activities associated with the complex. *J Med Microbiol.* . **22**, 325-333.

[284] Strunk, O. and W. Ludwig, in: http://www.mikro.biologie.tu-Muenchen.de/pub/ARB/documentation/arb.ps (Ed.), Department of Microbiology,Technical University of Munich, Munich, Germany 1995.

[285] **Suau, A., R. Bonnet, M. Sutren, J.-J. Godon, G. R. Gibson, M. D. Collins and J. Dore**. 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl Environ Microbiol.* **65**, 4799-4807.

[286] **Swidsinski, A., A. Ladhoff, A. Pernthaler, S. Swidsinski, V. Loening-Baucke, M. Ortner, J. Weber, U. Hoffmann, S. Schreiber, M. Dietel*, et al.*** 2002. Mucosal flora in inflammatory bowel disease. *Gastroenterology.* **122**, 44-54.

[287] **Szentkuti, L., H. Riedesel, M. Enss, K. Gaertner and W. von Engelhardt**. 1990. Pre-epithelial mucus layer in the colon of conventional and germ-free rats. *J Mol Histol.* **22**, 491-497.

[288] **Tannock, G.** 1990. The microecology of lactobacilli inhabiting the gastrointestinal tract. *Advances in Microbial Ecology.* **11**.

[289] **Tannock, G.** 2001. Molecular assessment of intestinal microflora. *Am J Clin Nutr.* **73**, 410S-414S.

[290] **Tannock, G., C. Crichton, G. Welling, J. Koopman and T. Midtvedt**. 1988. Reconstitution of the gastrointestinal microflora of lactobacillus-free mice. *Appl Environ Microbiol.* **54**, 2971-2975.

[291] **Taras, D., R. Simmering, M. D. Collins, P. A. Lawson and M. Blaut**. 2002. Reclassification of Eubacterium formicigenerans Holdeman and Moore 1974 as Dorea formicigenerans gen. nov., comb. nov., and description of Dorea longicatena sp. nov., isolated from human faeces. *Int J Syst Evol Microbiol.* **52**, 423-428.

[292] **Ten Bruggencate, S. J. M., I. M. J. Bovee-Oudenhoven, M. L. G. Lettink-Wissink, M. B. Katan and R. Van der Meer**. 2004. Dietary fructo-oligosaccharides and inulin decrease resistance of rats to salmonella: protective role of calcium. *Gut.* **53**, 530-535.

[293] **Ten Bruggencate, S. J. M., I. M. J. Bovee-Oudenhoven, M. L. G. Lettink-Wissink, M. B. Katan and R. van der Meer**. 2006. Dietary fructooligosaccharides affect intestinal barrier function in healthy men. *J Nutr.* **136**, 70-74.

[294] **Ten Bruggencate, S. J. M., I. M. J. Bovee-Oudenhoven, M. L. G. Lettink-Wissink and R. Van der Meer**. 2005. Dietary fructooligosaccharides increase intestinal permeability in rats. *J Nutr.* **135**, 837-842.

[295] **Thim, L., F. Madsen and S. S. Poulsen**. 2002. Effect of trefoil factors on the viscoelastic properties of mucus gels. *Eur J Clin Invest.* **32**, 519-527.

[296] **Topping, D. L. and P. M. Clifton**. 2001. Short-Chain Fatty Acids and Human Colonic Function: Roles of Resistant Starch and Nonstarch Polysaccharides. *Physiol Rev.* **81**, 1031-1064.

[297] **Toribara, N. W., A. M. Roberton, S. B. Ho, W. L. Kuo, E. Gum, J. W. Hicks, J. R. Gum, Jr., J. C. Byrd, B. Siddiki and Y. S. Kim**. 1993. Human gastric mucin. Identification of a unique species by expression cloning. *J Biol Chem.* **268**, 5879-5885.

[298] **Troost, F., M. Kleerebezem, W. M. de Vos and R.-J. Brummer.** GutImpact Tallinn 2006.

[299] **Tsai, H., D. Sunderland, G. Gibson, C. Hart and J. Rhodes**. 1992. A novel mucin sulphatase from human faeces: its identification, purification and characterization. *Clin Sci.* **82**, 447-454.

[300] **Uhlar, C. M. and A. S. Whitehead**. 1999. Serum amyloid A, the major vertebrate acute-phase reactant. *Eur J Biochem.* **265**, 501-523.

[301] **Ukena, S., A. Westendorf, W. Hansen, M. Rohde, R. Geffers, S. Coldewey, S. Suerbaum, J. Buer and F. Gunzer**. 2005. The host response to the probiotic *Escherichia coli* strain Nissle 1917: Specific up-regulation of the proinflammatory chemokine MCP-1. *BMC Medical Genetics.* **6**, 43.

[302] **Vahouny, G., T. Le, I. Ifrim, Satchithanandam, S and M. Cassidy**. 1985. Stimulation of intestinal cytokinetics and mucin turnover in rats fed wheat bran or cellulose. *Am J Clin Nutr.* **41**, 895-900.

[303] **Van de Bovenkamp, J. H. B., J. Mahdavi, A. M. Korteland-Van Male, H. A. Buller, A. W. C. Einerhand, T. Boren and J. Dekker**. 2003. The MUC5AC Glycoprotein is the Primary Receptor for Helicobacter pylori in the Human Stomach. *Helicobacter.* **8**, 521-532.

[304] **van der Waaij, L., H. Harmsen, M. Madjipour, F. Kroese, M. Zwiers, H. van Dullemen, N. de Boer, G. Welling and P. Jansen**. 2005. Bacterial population analysis of human colon and terminal ileum biopsies with 16S rRNA-based fluorescent probes: commensal bacteria live in suspension and have no direct contact with epithelial cells. *Inflamm Bowel Dis.* **11**, 865-871.

[305] **van der Waaij, L. A., F. G. M. Kroese, A. Visser, G. F. Nelis, B. D. Westerveld, P. L. M. Jansen and J. O. Hunter**. 2004. Immunoglobulin coating of faecal bacteria in inflammatory bowel disease. *Eur J Gastroenterol Hepatol.* **16**, 669-674.

[306] **van der Waaij, L. A., P. C. Limburg, G. Mesander and D. van der Waaij**. 1996. In vivo IgA coating of anaerobic bacteria in human faeces. *Gut.* **38**, 348-354.

[307] **Vandekerckhove, T. T. M., A. Coomans, K. Cornelis, P. Baert and M. Gillis**. 2002. Use of the Verrucomicrobia-specific probe EUB338-III and fluorescent in situ hybridization for detection of "Candidatus Xiphinematobacter" cells in nematode hosts. *Appl Environ Microbiol.* **68**, 3121-3125.

[308] **Vandenbroucke, K., W. Hans, J. Van Huysse, S. Neirynck, P. Demetter, E. Remaut, P. Rottiers and L. Steidler**. 2004. Active delivery of trefoil factors by genetically modified *Lactococcus lactis* prevents and heals acute colitis in mice. *Gastroenterology.* **127**, 502-513.

[309] **Vaughan, E., F. Schut, H. Heilig, E. Zoetendal, W. de Vos and A. Akkermans**. 2000. A molecular view of the intestinal ecosystem. *Curr Issues Intest Microbiol.* **1**, 1-12.

[310] **Veal, D. A., D. Deere, B. Ferrari, J. Piper and P. V. Attfield**. 2000. Fluorescence staining and flow cytometry for monitoring microbial cells. *J Immunol Methods.* **243**, 191-210.

[311] **Venter, J. C., M. D. Adams, E. W. Myers, P. W. Li, R. J. Mural, G. G. Sutton, H. O. Smith, M. Yandell, C. A. Evans, R. A. Holt*, et al.*** 2001. The Sequence of the Human Genome. *Science.* **291**, 1304-1351.

[312] **Wallner, G., R. Amann and W. Beisker**. 1993. Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry.* **14**, 136-143.

[313] **Wang, M., S. Ahrné, B. Jeppsson and G. Molin**. 2005. Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes. *FEMS Microbiol Ecol.* **54**, 219.

[314] **Williams, S. J., M. A. McGuckin, D. C. Gotley, H. J. Eyre, G. R. Sutherland and T. M. Antalis**. 1999. Two novel mucin genes down-regulated in colorectal cancer identified by differential display. *Cancer Res.* **59**, 4083-4089.

[315] **Williams, S. J., D. J. Munster, R. J. Quin, D. C. Gotley and M. A. McGuckin**. 1999. The MUC3 gene encodes a transmembrane mucin and is alternatively spliced. *Biochem Biophys Res Com.* **261**, 83.

[316] **Williams, S. J., D. H. Wreschner, M. Tran, H. J. Eyre, G. R. Sutherland and M. A. McGuckin**. 2001. MUC13, a novel human cell surface mucin expressed by epithelial and hemopoietic cells. *J Biol Chem.* **276**, 18327-18336.

[317] **Willis, C., J. H. Cummings, G. Neale and G. R. Gibson**. 1996. In vitro effects of mucin fermentation on the growth of human colonic sulphate-reducing bacteria. *Anaerobe.* **2**, 117-122.

[318] **Willis, C. L., J. H. Cummings, G. Neale and G. R. Gibson**. 1996. *In vitro* effects of mucin fermentation on the growth of human colonic sulphate-reducing bacteria. *Anaerobe.* **2**.

[319] **Wilson, K. and R. Blitchington**. 1996. Human colonic biota studied by ribosomal DNA sequence analysis. *Appl. Environ. Microbiol.* **62**, 2273-2278.

[320] **Wong, J., R. de Souza, C. Kendall, A. Emam and D. Jenkins**. 2006. Colonic health: fermentation and short chain fatty acids. *J Clin Gastroenterol.* **40**, 235-243.

[321] **Wright, D., D. Rosendale and A. Robertson**. 2000. *Prevotella* enzymes involved in mucin oligosaccharide degradation and evidence for a small operon of genes expressed during growth on mucin. *FEMS Microbiol Lett.* **190**, 73-79.

[322] **Xu, J., M. K. Bjursell, J. Himrod, S. Deng, L. K. Carmichael, H. C. Chiang, L. V. Hooper and J. I. Gordon**. 2003. A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. *Science.* **299**, 2074-2076.

[323] **Yao, K., T. Ubuka, N. Masuoka, M. Kinuta and T. Ikeda**. 1989. Direct determination of bound sialic acids in sialoglycoproteins by acidic ninhydrin reaction. *Anal Biochem.* **179**, 332-335.

[324] **Yin, B., A. Dnistrian and K. Lloyd**. 2002. Ovarian cancer antigen CA125 is encoded by the MUC16 mucin gene. *International Journal of Cancer*. **98**, 737-740.

[325] **Yin, B. W. T. and K. O. Lloyd**. 2001. Molecular cloning of the CA125 ovarian cancer antigen. Identification as a new mucin, MUC16. *J Biol Chem.* **276**, 27371-27375.

[326] **Yolken, R., J. Peterson, S. Vonderfecht, E. Fouts, K. Midthun and D. Newburg**. 1992. Human milk mucin inhibits rotavirus replication and prevents experimental gastroenteritis. *J Clin Invest.* **90**, 1984-1991.

[327] **Younes, H., C. Demigne and C. Remesy**. 1996. Acidic fermentation in the caecum increases absorption of calcium and magnesium in the large intestine of the rat. *Br J Nutr*. **75**, 301-314.

[328] **Zhang, Y., L. Ding and M. Lai**. 2003. Reg gene family and human diseases. *World J Gastroenterol.* **9**, 2635-2641.

[329] **Zhou, J. S., P. K. Gopal and H. S. Gill**. 2001. Potential probiotic lactic acid bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) do not degrade gastric mucin in vitro. *Int J Food Microbiol.* **63**, 81-90.

[330] **Zoetendal, E. G., A. D. L. Akkermans and W. M. De Vos**. 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl Environ Microbiol.* **64**, 3854-3859.

[331] **Zoetendal, E. G., K. Ben-Amor, A. D. Akkermans, T. Abee and W. M. de Vos**. 2001. DNA isolation protocols affect the detection limit of PCR approaches of bacteria in samples from the human gastrointestinal tract. *Syst Appl Microbiol.* **24**, 405-410.

[332] **Zoetendal, E. G., K. Ben-Amor, H. J. M. Harmsen, F. Schut, A. D. L. Akkermans and W. M. de Vos**. 2002. Quantification of uncultured *Ruminococcus obeum*-like bacteria in human fecal samples by fluorescent in situ hybridization and flow cytometry using 16S rRNA-targeted probes. *Appl Environ Microbiol.* **68**, 4225-4232.

[333] **Zoetendal, E. G., C. T. Collier, S. Koike, R. I. Mackie and H. R. Gaskins**. 2004. Molecular ecological analysis of the gastrointestinal microbiota: A review. *J Nutr.* **134**, 465-472.

[334] **Zoetendal, E. G., C. M. Plugge, A. D. L. Akkermans and W. M. de Vos**. 2003. *Victivallis vadensis* gen. nov., sp. nov., a sugar-fermenting anaerobe from human faeces. *Int J Syst Evol Microbiol.* **53**, 211-215.

[335] **Zoetendal, E. G., E. E. Vaughan and W. M. de Vos**. 2006. A microbial world within us. *Mol Microbiol.* **59**, 1639-1650.

[336] **Zoetendal, E. G., A. von Wright, T. Vilpponen-Salmela, K. Ben-Amor, A. D. L. Akkermans and W. M. de Vos**. 2002. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. *Appl Environ Microbiol.* **68**, 3401-3407.

Appendix

Samenvatting

Het menselijke maagdarmkanaal is verantwoordelijk voor het omzetten en opnemen van componenten uit ons voedsel, waarbij ze hulp krijgt van een enorme massa aan bacteriën die “de microbiota van de darm” of kortweg “de microbiota” wordt genoemd, welke voor het overgrote deel uit bacteriën bestaat. Naast het omzetten en opnemen van voedselcomponenten is de darm ook zeer belangrijk voor ons immuunsysteem. Er bestaat een nauw verband tussen de microbiota, voeding en gezondheid, en zonder deze microbiota is een gezond leven vrijwel onmogelijk. De darmwand bestaat uit een enkele laag epitheelcellen die bedekt is met een mucuslaag die 50-500 µm dik is, en ervoor zorgt dat micro-organismen in de darm het lichaam niet kunnen binnendringen. De mucuslaag bestaat voor het overgrote deel uit water met mucines als belangrijkste organische component. Bacteriën die toegang hebben tot de mucines hebben een groot voordeel in de darm aangezien ze hieruit continu hun nutriënten kunnen halen. Deze mucine afbrekende bacteriën spelen dus een belangrijke rol in de darm. Desondanks is er weinig bekend van deze bacteriën mede omdat mucine een complex hoog-moleculair substraat is. Het werk, beschreven in dit proefschrift, heeft zich gericht op de identificatie van de mucine afbrekende bacteriën in de darm van de mens, hoe deze mucine kunnen afbreken en wat voor interactie ze hebben met de gastheer.

Hoofdstuk 1 geeft een overzicht van het maagdarmkanaal van de mens en de daarbij behorende microbiota, waarbij extra nadruk wordt gelegd op de bacteriële populaties waarvan bekend is dat ze mucine kunnen gebruiken. Mucine is een van de belangrijkste componenten die de gastheer wordt geproduceerd en die door de microbiota gebruikt kan worden als substraat (in **Hoofdstuk 2** worden naast mucus ook andere van de gastheer afkomstige subtraten behandeld). Hierbij is gekeken naar de complexiteit van het mucine en welke enzymen door de mucine-afbrekende bacteriën geproduceerd worden om dit molecuul af te breken. Aangezien het overgrote deel van de darmbacteriën nog niet gekweekt kan worden, is naast het toepassen van kweektechnieken ook aandacht besteed aan de verschillende kweekonafhankelijke technieken welke gebaseerd zijn op de diversiteit van 16S ribosomaal RNA (rRNA) genen om de microbiota-samenstelling te beschrijven. Tot slot wordt er ingegaan op de verschillende manieren waarop de interacties tussen geselecteerde bacteriestammen en model gastheren op transcriptie niveau bestudeerd kunnen worden.

Om inzicht te krijgen in de populaties die betrokken zijn bij mucine afbraak in de darm, zijn zowel kweekafhankelijke als -onafhankelijke methoden toegepast (**Hoofdstuk 3**). Mucine afbrekende bacteriën zijn opgehoopt, uit ontlasting van 6 verschillende volwassen individuen, in

een mucine-gelimiteerd medium die vervolgens geanalyseerd werd met een 16S rRNA gen-gebaseerde vingerprint techniek genaamd “Denaturing Gradient Gel Electrophoresis”. Uit deze analyse is gebleken dat de mucine-geassocieerde bacteriële communities divers van samenstelling zijn en per individu verschillen. Identificatie van de dominante bacteriën in deze ophopingculturen, met behulp van kloneren en sequentie analyse van de verschillende 16S rRNA genen, gaf aan dat deze mucine-geassocieerde bacteriën tot de phyla firmicutes, delta- en gamma proteobacteria en Verrucomicrobia behoren, en dat 69% hiervan bestaat uit nieuwe nog niet eerder gecultiveerde soorten. Op basis van deze bevindingen is geconcludeerd dat er in de menselijke darm een diverse mucine-geassocieerde gemeenschap van tot nu toe onbekende bacteriën aanwezig is, waarvan uit toekomstig onderzoek moet blijken welke rol zij spelen bij de afbraak van mucine.

Uit de hoogste verdunning van een ophoping uit ontlasting van een persoon is een nieuw bacteriële isolaat verkregen dat een nieuw genus belichaamd (**Hoofdstuk 4**). Uit fysiologische karkaterisatie van dit nieuwe isolaat, dat *Akkermansia muciniphila* is genoemd, bleek dat dit organisme zeer efficiënt groeide op mucine, maar vrijwel niet op andere geteste substraten. Fylogenetische analyse op basis van 16S rRNA gen-sequenties gaf aan dat *A. muciniphila* tot de subdivisie 1 van het phylum “*Verrucomicrobium*” behoort. Dit phylum is nog maar 10 jaar geleden beschreven en tot dusver zijn er geen andere darmisolaten beschreven die hiertoe behoren.

Gezien de zeer geringe substraatspecificiteit kan *A. muciniphila* beschouwd worden als een specialist wat betreft mucine afbraak. Om in de darm te kunnen concurreren met andere bacteriën, moet *A. muciniphila* een scala aan enzymen bezitten om mucine efficiënt te kunnen afbreken. Gezien de complexe structuur van mucine, is de samenwerking van dit scala aan enzymen die specifiek de verschillende groepen (oligosacchariden, sulfaten, siaalzuren, aminozuren) of verbindingen daartussen afbreken nodig, om mucine volledig te kunnen afbreken. De identificatie van een aantal enzymen, die betrokken zijn bij de *in vitro* afbraak van mucine, zijn in **Hoofdstuk 5** beschreven. Deze enzymen, waarvan het merendeel glycosidasen, zijn zowel intra- als extracellulair gevonden. Een opmerkelijke waarneming was dat *A. muciniphila* 85% van de mucine *in vitro* kon afbreken. Een dergelijke efficiënte afbraak van mucine is nooit eerder beschreven voor een bacterie.

Naast de isolatie van *A. muciniphila* uit ontlasting van een persoon zijn haar 16S rRNA genen gedetecteerd in kloonbanken afkomstig uit andere ontlastingmonsters en darmbiopten van de mens en muis. Om meer inzicht te krijgen in de diversiteit en populatiegrootte van *A. muciniphila* in de menselijke darm is zowel een specifieke 16S rRNA oligonucleotide probe voor

kwantitatieve fluorescente *in situ* hybridisatie (FISH: **Hoofdstuk 6**) als een primerpaar voor kwantitatieve PCR (**Hoofdstuk 7**) ontwikkeld en gevalideerd. Zowel FISH als kwantitatieve PCR tellingen gaven aan dat *A. muciniphila* in vrijwel alle geteste personen voorkwam met een gemiddelde van 1.3% van de totale microbiota. Deze waarnemingen geven aan dat *A. muciniphila* tot de algemeen voorkomende soorten van de menselijke darm-microbiota behoort, en zelfs bekende bacteriën als *Escherichia coli* en lactobacillen in aantallen overschrijdt.

Omdat *A. muciniphila* een specialist is in mucine degradatie en een algemeen voorkomende darmbacterie is, is bestudeerd of er een relatie bestaat tussen de *A. muciniphila* populatiegrootte en mucine productie in mensen. Aangezien in een placebo gecontroleerde cross-over studie is aangetoond dat mucine productie significant hoger is na consumptie van fructooligosacchariden (FOS), hebben we de relatie tussen mucine uitscheiding, FOS consumptie en *A. muciniphila* aantallen in ontlasting bestudeerd (**Hoofdstuk 7**). Zowel na consumptie van placebo als FOS was een negatieve correlatie tussen mucine-uitscheiding en *A. muciniphila* aantallen te zien, echter deze was alleen significant na FOS consumptie. Aan de andere kant werd geen correlatie gevonden tussen FOS consumptie en *A. mucinphila* aantallen en dat de relatie tussen deze twee verschilt per individu. Op basis hiervan is geconcludeerd dat *A. muciniphila* zeer waarschijnlijk een rol speelt in de mucine afbraak *in vivo* in de darm, en dat FOS-consumptie een indirect effect heeft op de *A. muciniphila* aantallen doordat het de groei van andere bacteriële populaties stimuleert.

Aangezien *A. muciniphila* zowel in mens alsook in de muis een algemeen voorkomende darmbacterie is, is haar effect op bacterie-vrije muizen bestudeerd en vergeleken met het effect van *Lactobacillus plantarum*, een bacterie die geen mucine kan afbreken (**Hoofdstuk 8**). Transcriptoom analyse met behulp van een DNA chip (GeneChip arrays 430 Mouse genome, Affymetrix) met RNA geïsoleerd uit biopten van de dunne darm, caecum, en dikke darm gaf aan dat de reactie van de gastheer bacterie- en locatiespecifiek is. Hoewel beide bacteriën een vergelijkbaar aantal genen reguleerden, waren de regulatie van de immuun reactie, celdeling, cel aanhechting en apoptose specifiek voor *A. muciniphila*, terwijl regulatie van vetmetabolisme door *L. plantarum* geïnduceerd werd. De resultaten verkregen met de DNA chip zijn bevestigd door middel van kwantitatieve PCR analyse van een aantal geselecteerde genen.

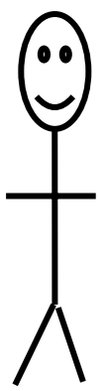
De resultaten beschreven in dit proefschrift hebben veel nieuwe inzichten gegeven in de ecologie van mucine-afbrekende bacteriële gemeenschappen in de darm en in het bijzonder die van *A. muciniphila*, het eerste isolaat behorend tot het *Verrucomicrobium* phylum, welke een veel voorkomende en dominante bewoner is van de menselijke darm.

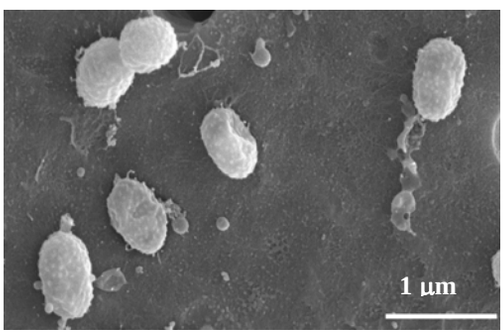
Résumé en français

Ce chapitre s'adresse plus particulièrement aux personnes non initiées à la microbiologie et pour lesquelles le résumé de thèse est expliqué de manière plus simplifiée.

Le monde des bactéries

Sur Terre, 13 à 14 millions d'espèces sont recensées. La très grande majorité des espèces non décrites sont des procaryotes, ou bactéries (Fig.1) et dont l'importance a longtemps été sous-estimée.





Etre humain
Taille moyenne (France): 1.70 m
Durée de vie : 80 ans
Population humaine sur Terre: 6.5 milliards

Bactérie
Taille moyenne: 1-10 μm
Temps de génération: 20-40 min
Population bactérienne sur Terre : $5x\ 10^{30}$

Figure 1. Différentes caractéristiques homme/bactérie.

La majorité des bactéries sont perçues à tort comme néfastes car certaines sont responsables de graves maladies. Pourtant la plupart des bactéries restent inoffensives ou sont même bénéfiques à l'homme. Ces dernières sont généralement connues sous le terme de probiotiques et sont fréquemment ajoutées dans certaines préparations dont les produits laitiers.

Le tube digestif de l'homme : un énorme réservoir de bactéries

Le système digestif de l'homme est un lieu privilégié pour la croissance des bactéries (Fig. 2). Il a été estimé qu'il contient jusqu'à 1 000 milliards de bactéries (1 000 000 000 000) par gramme de contenu intestinal. De plus, ces bactéries sont 10 fois plus nombreuses que nos cellules. En

d’autres termes, nous serions composés de 90% de cellules bactériennes et de seulement 10% de cellules humaines! Ceci implique leur rôle probable dans le développement de l’être humain. Par ailleurs, cette pléthore de la flore intestinale explique l’intérêt porté à cet écosystème. La diversité microbienne du tractus digestif a été estimée à plus de 1000 espèces. Plusieurs études ont mis en exergue le fait qu’une faible proportion de bactéries intestinales est actuellement connue. En effet, il a été estimé que 50% à 60% des bactéries intestinales restent non cultivées à ce jour, car ces microorganismes ont des besoins spécifiques tels que l'absence d’oxygène ou la présence de nutriments particuliers.

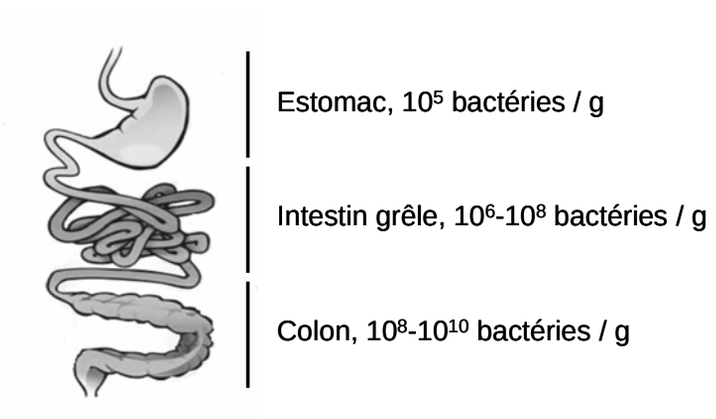


Figure 2. Représentation simplifiée du tractus digestif, et nombre de bactéries présentes par gramme de contenu intestinal.

La majorité des bactéries restent inoffensives (commensales) et nous sont même bénéfiques voire primordiales puisqu’elles nous aident, entre autres, à assimiler l’énergie alimentaire et certains éléments nutritifs. Par exemple la vitamine B12 est synthétisée par les bactéries de la flore intestinale**.**

Les techniques d’analyse des bactéries

Les bactéries ont longtemps été seulement étudiées par des techniques dites classiques, c'est-à-dire basées sur leur isolement en milieu de culture, étape obligatoire à toute étude ultérieure. Cependant les bactéries isolées ne reflètent pas la composition de l’écosystème. Récemment l’avènement de techniques moléculaires basées sur l'analyse de l’ADN ribosomal (ADNr) 16S a permis d’évaluer plus rapidement la diversité microbienne au sein d’un écosystème complexe. L’ADNr 16S est une molécule ubiquitaire, qui joue un rôle essentiel dans la synthèse protéique et demeure donc conservée au fil du temps. Cette molécule peut être vue comme "carte d’identité" des bactéries (Fig. 3). Récemment l’avènement de techniques moléculaires basées sur l’ADN ribosomal (ADNr) 16S a permis d’analyser plus rapidement la diversité microbienne au sein d’un

écosystème complexe. En effet cette molécule, est présente dans toutes les bactéries et joue un rôle essentiel dans la synthèse protéique. Sa séquence nucléotidique présente des régions hautement conservées et communes à toutes les bactéries, tandis que d'autres régions sont spécifiques d'espèce. L'avantage majeur des techniques moléculaires est qu'elles ne nécessitent pas l'isolement et la culture des bactéries et offrent une alternative aux méthodes de culture classiques. Elles comprennent tout d'abord une étape d'amplification des molécules d'ADNr présentes dans un échantillon, puis leur séquençage. On établit alors une "empreinte moléculaire" des bactéries présentes dans un écosystème donné sans passer par l'étape de culture.

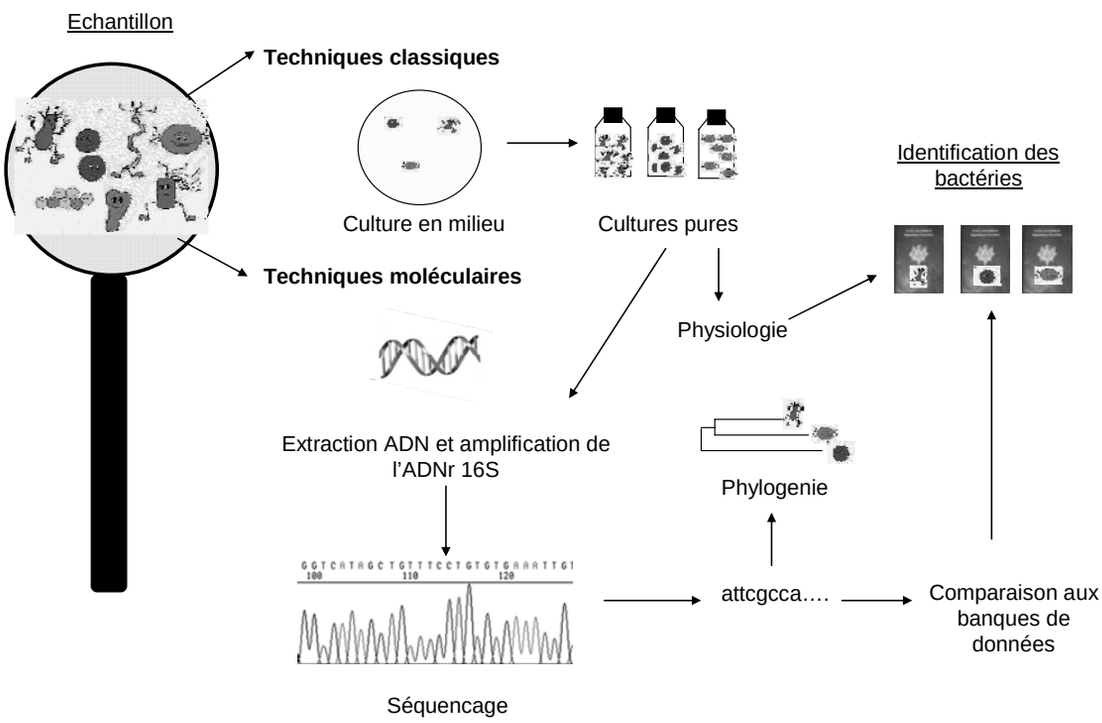


Figure 3. Représentation schématique des méthodes classiques et moléculaires d'identification des bactéries présentes dans un écosystème.

Interactions hôte-bactérie

Etant donné le nombre important de bactéries côtoyant les cellules intestinales, on peut penser que des interactions spécifiques ont lieu dans l'intestin. Les cellules intestinales sont recouvertes d'une fine couche protectrice de mucus qui empêche en conditions normales les bactéries, virus, toxines présentes dans la lumière intestinale d'agresser l'hôte. Chez une personne saine, la dégradation de la couche de mucus intestinal est due à l'action d'enzymes et au frottement des matières en cours de digestion. Cette dégradation est compensée par des sécrétions de mucus fraîchement synthétisé garantissant l'intégrité de la barrière protectrice. Le principal composant du mucus, responsable de ses propriétés gélifiantes est la mucine, composée de protéines fortement glycosylées. Les mucines constituent une fraction non négligeable des nutriments disponibles pour les bactéries intestinales et plusieurs travaux ont montré que le mucus est colonisé par une grande variété de micro-organismes (Fig. 4).

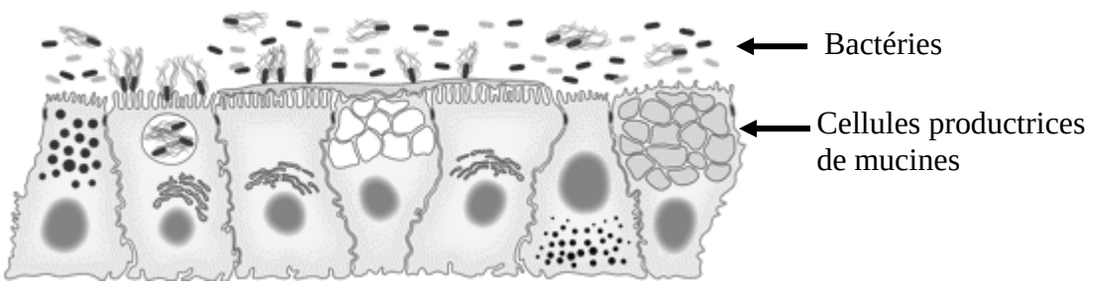


Figure 4. Représentation schématique de l'épithélium intestinal colonisé par des bactéries. Reproduit avec autorisation de l'auteur (Lievin-Le Moal V. and A.L. Servin. 2006 Clin Microbiol 19:315-37)

But et résumé du travail de thèse

Le but de ce projet était d'étudier les bactéries utilisant les mucines comme source d'énergie, et d'évaluer leurs interactions avec l'hôte.

Dans un premier temps, la diversité des bactéries intestinales dégradant les mucines a été évaluée par l'utilisation de techniques moléculaires basées sur l'analyse de l'ADNr 16S**.** Pour cela, des échantillons fécaux ont été inoculés dans un milieu contenant de la mucine comme source d'énergie et l'ADNr 16S amplifie et séquencé. L'étude a mis en évidence que les bactéries participant au consortium dégradant les mucines sont variées selon les individus. Afin de sélectionner des bactéries dégradant strictement les mucines, l'isolement a été poursuivi sur ce même milieu contenant de la mucine comme seule source d'énergie. Cette approche a permis d'aboutir à l'isolement d'une bactérie, *Akkermansia muciniphila*, qui représente un nouveau

genre et qui se situe phylogénétiquement au sein d'une nouvelle division. Sa propriété de dégrader la mucine a été approfondie par l'analyse des enzymes impliquées et, notamment plusieurs glycosidases participant à la dégradation des chaines oligosaccharidiques périphériques. *A. muciniphila* étant une bactérie nouvellement découverte, sa présence dans des échantillons fécaux a été mesurée/évaluée a l'aide d'une sonde spécifique de son ADNr16S. L'analyse a montré qu'*A. muciniphila* représente un pourcentage conséquent de la population microbienne fécale (1%). De plus, une relation négative entre la concentration en mucine et le nombre d'*A. muciniphila* a été établie suggérant un rôle vraisemblable d'*A. muciniphila* dans la dégradation des mucines *in vivo*. Enfin, afin d'appréhender le rôle d'*A. muciniphila* sur les cellules hôtes, une expérience de mono-association de souris axéniques (dépourvues de bactéries) avec *A. muciniphila* a été effectuée. Des puces à ADN comprenant les gènes représentant le génome de la souris ont été utilisées afin de cribler les gènes dont l'expression était modifiée après inoculation d'*A. muciniphila.*

Cette étude a mis en évidence qu'*A. muciniphila* induit une faible réponse immunitaire suggérant une faible proximité avec les cellules intestinales.

Acknowldegements

This work has been the result of 5 years spent at the laboratory of Microbiology. For me, these years have been full of events. Many people contributed to these and I would like to acknowledge them in this part.

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Petia, I have met you not so long time ago but we had first talks around our opposite bench and then outside and then you passed the difficult test to be able to keep Enora for some evenings and finally I am delighted you will have a small family soon. I am very happy that you accepted to support me as a paranimph.

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My last words are for Antoon, who has been a great mentor of moleco. I have been honoured to be able to make your work in the gut microbiology more recognised. We will remember you forever, and I hope that this small book will contribute to this.

Muriel

About the author

Muriel Derrien was born on the 25th December 1977 in Landerneau (France). After achieving her high school in Landerneau in 1995, she started to study science at the "Université de Bretagne Occidentale" in Brest from September 1995 to June 2000. After finishing her "maîtrise (equivalent to four years studies), she performed a stage during three months in the laboratory of "Toxines et pathogénie bactériennes" at the Institut Pasteur in Paris where she studied the germination of *Bacillus anthracis*. Then she moved to Rennes to follow the master of "Microbiologie fondamentale et Appliquée". During this, she performed a nine months study at the "Laboratoire de Recherches de Technologie Laitieres" at the INRA of Rennes where she studied the autolysins of *Lactobacillus heleveticus*, used as starter in the cheese emmental. This work was carried out under the supervision of Sylvie Lortal and was succeeded in July 2001. She worked then 3 months in the laboratory of "Amélioration des plantes" at the INRA of Rennes. Soon after that, she started her PhD thesis in the laboratory of microbiology in Wageningen University which was a workpackage within the EU project, under the supervision of Willem de Vos until august 2006. From October 2006 till April 2007, she was appointed in the WCFS project B-017 to study the oral bacteria and the mucus layer.

List of publications

Ouwehand A.C., **M. Derrien,** W.M. de Vos, K. Tiihonen and N. Rautonen. 2005. Prebiotics and other microbial substrates for gut functionality. *Curr Opin Biotechnol*. **16,** 212-217.

Derrien, M., E.E. Vaughan, C.M. Plugge and W.M. de Vos**.** 2004. *Akkermansia muciniphila*, a novel human intestinal mucin-degrading bacterium*. Int J Syst Environ Microbiol*. **54,** 1469-76.

Derrien M., D. Adawi, S. Ahrné, B. Jeppsson, G. Molin, N. Osman, J. Štšepetova, E.E. Vaughan, W.M. de Vos and A.C. Ouwehand. 2004. The intestinal mucosa as a habitat of the gut microbiota and a rational target for probiotic functionality and safety. *Microb Ecol Health and Disease*. **16,**137-144.

Te Biesebeke R., R. Boesten, E.S Klaassens, C.C.G.M. Booijink, M.C. de Vries, **M. Derrien,** D.P.A. Cohen, F. Schuren., E.E. Vaughan, M. Kleerebezem and W.M. de Vos. 2004. Microbial functionality in the human gastrointestinal tract. *Microbes and Environments*. **19**, 276-280.

Derrien M., C.M. Plugge, W.M. de Vos and E.G. Zoetendal. Genus *Akkermansia*. In :Bergey's manual of systematic bacteriology. In press

Derrien M., C.M. Plugge, W.M. de Vos and E.G. Zoetendal. Genus *Victivallis*. In :Bergey's manual of systematic bacteriology. In press

Derrien M., S. Ten Bruggencate, W.M. de Vos and E.G. Zoetendal. Mucin secretion and fructooligosaccharides in the intestine: the role of the mucin-degrading bacterium *Akkermansia muciniphila.* To be submitted.

Derrien M., K.B.A. Ben-Amor and W.M. de Vos. The mucin-degrader *Akkermansia muciniphila* is an important member of the human intestinal tract. To be submitted.

Derrien, M., E. Norin, P. Van Baarlen, E. Peters, E.G. Zoetendal, M. Muller and W.M. de Vos. Gene profiling of mice responses after mono-association with the intestinal mucin-degrading bacterium *Akkermansia muciniphila* and the commensal *Lactobacillus plantarum.* To be submitted.

Education

- ***Courses***

Ecophysiology of gastrointestinal tract, Wageningen, 2003	1.4
Bioinformatics Technology 1, Wageningen, 2003	2.0
Food Fermentation, Wageningen, 2004	1.0
Genetics and Physiology of Food Associated Microorganisms, Wageningen, 2004	1.0
Safe handling with radioactive materials and sources, Larenstein, 2002	1.4
ARB course, Wageningen, 2003	0.3
European training workshop, Paris, 2003	2.8

- ***Meetings***

Darmendag, Groningen, The Netherlands, 2006	0.3
Gut Impact meting Tallin, Estonia, 2006	0.6
RRI INRA Meeting, Aberdeen, 2006	1.0
PROEUHEALTH workshop, Sitges, Spain, 2004	1.0
EU and Microfunction project meetings London 2002, Tartu 2003, London 2004	1.0
PROEUHEALTH workshop, Saariselka, Finland, 2002	1.0
PROEUHEALTH workshop, Taormina, Italy, 2003	1.0
GI tract meeting, Rikilt, Wageningen, 2002	0.3
NL array platform, Utrecht, 2002	0.3
ID array symposium, Wageningen, 2002, 2003	0.6
Darmendag, Lelystad 2002	0.3
FEMS 1st Congress of European Microbiologists, Ljubljana, Slovenia, 2003	1.4
IUMS The world of microbes, Paris, 2002	1.7

- ***General courses***

PhD/Postdoc meetings (Laboratory of Microbiology) 2002-2006	3.0
Journal club, WUR 2004, 2005	0.7

- ***Optional***

Preparing PhD research proposal	6.0
VLAG PhD week 2002	1.1
PhD trip to Japan	2.5
Rowett Research Institute, Aberdeen, UK 2003 (3 weeks)	2.0
Karolinska Institute, Stochkolm, Sweden, 2005 (1 week)	0.7
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