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Carp mucus and its role in mucosal defense

Maria C. van der Marel

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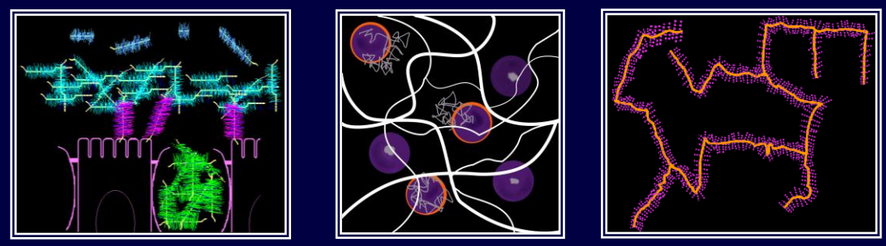

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Contents

The slimy is docile. Only at the very moment when I believe that I possess it, behold by a curious reversal, it possesses me. Here appears its essential character: its softness is leech-like…

(Sartre)

Chapter 1

General introduction

Body surfaces of multi-cellular organisms are defended by epithelia, which provide a physical barrier between the internal milieu and the pathogens-containing external world [1]. In fish, this barrier is formed by the integument and alimentary tract. Fish integument is a large organ that is continuous with the lining of all the body openings, and also covers the fins. In addition to its protective functions, fish skin may serve important roles in communication, sensory perception, locomotion, respiration, ion regulation, excretion, and thermal regulation [2]. The intestine of fish also performs many tasks [3]. The main function of the alimentary tract of any animal is the acquisition of food with subsequent assimilation of vital nutrients [4]. However, it also plays a role in osmoregulation and defense against pathogens [3].
The integumentary and intestinal barrier can be divided into the extrinsic, intrinsic, and immunological barriers. The extrinsic barrier is the first barrier pathogens and toxins encounter and is mainly formed by mucus which counters pathogens and toxins. The intrinsic barrier consists of epithelial cells and cell junctions. Immunological barriers are formed by the innate immune system and the adaptive immune system that are present in the intestinal wall. Active immune responses occurring in the skin are suggested by prevention of colonization at the skin by vaccination against bacteria (e.g. *Vibrio sp*.) and parasites (e.g. *Ichthyiophthyrius multifilliis*), but the mechanisms are still largely unknown. Immune cells, cytokines and antibodies were all described at the epithelial surface and were shown to respond to infections and vaccinations.
An infectious pathogen can enter the fish through the skin, gills, or the gut [5]. Infections occur only when pathogens can colonize or cross the above mentioned barriers [1]. Most aspects of barrier function are actively controlled. Increase in mucus production and infiltration of immune cells occur when the epithelium senses pathogens or tissue damage. Disturbances to the general homeostasis of the fish, as with other physiological systems, can affect the intestinal barrier. The barrier function is disturbed by factors, such as suboptimal water temperature, hypoxia, low water quality, suboptimal diet, acute and chronic stress, exposure to pathogens, and starvation [5].

Mucus

In mucus, the watery gel on the surface of the epithelium, immune functions usually prevent the entry of pathogens and toxins [1]. Mucus is a complex viscous adherent secretion that covers the surface of most epithelia and represents an interface between the environment and the interior milieu [6]. In vertebrates, mucus is a secreted viscous colloid produced by, and covering, mucous membranes, and is typically produced from mucous cells found in mucous glands. The range of roles for fish mucus is very large and includes respiration, ionic and osmotic regulation, reproduction, excretion, disease resistance, communication, feeding, nest building and protection [6]. Mucus is secreted in higher vertebrates, fish and amphibians, and all vertebrates, including fish, have more or less the same composition. Even the slime trail of snails and earthworms has a chemical composition similar to that of mammalian mucus [7]. Mucus lines all organs that are exposed to the external environment including the respiratory tract, the gastrointestinal tract, the reproductive tract, the oculo-rhino-otolaryngeal tracts and in fish and amphibians also the skin [8, 9]. In fish, mucus is considered to be predominantly found on the surface of skin and gills, but it also forms the gut lining being

merely produced by goblet cells [6]. Underneath the mucus layer, a dense forest of highly diverse glycoproteins and glycolipids are resent, the so-called glycocalyx [10]. Mucus is kept close to the surface of the intestine by the glycolayx, which consists of glycoprotein filaments extending from the interior of absorptive cells to the exterior forming a buffering network above the cell surface [11].
Mucus is primarily composed of water (approx. 95%) [8] and mucus glycoproteins (mucins and non-mucins) but also comprises many other substances. Substances that have been identified in mucus and include such chemical compounds as cytokines [12], peptides [13, 14], lysozyme [15], lipoprotein [16], complement [17], lectins [18-20], proteases [21] and antibodies [22-24]. Some components have a defensive purpose [9] whereas others may act to modulate the organization and properties of the gel [25].
In fish, the mucins contained in the intestinal goblet cells appear to be species-specific. Some species secrete predominantly negatively charged mucins [e.g. Shi drum Umbrina cirrosa; 26] and some secrete predominantly neutral mucins [e.g. common dentex Dentex dentex; 27]. There also appears to be heterogeneity in the glycoproteins within the goblet cells [26].

Mucus in health and disease

Mucus helps to protect epithelial surfaces in many different ways. It is a lubricant that protects against mechanical damage while enabling rapid removal of various types of aggression (such as pathogens or irritants) from the mucosal surfaces. It is a regulated nutrient medium in which whole ecosystems thrive, usually symbiotically [1]. Mucus allows exchange between the luminal content and the epithelial lining [9]. The major components of the mucosal barrier are expressed constitutively, as continuous mucus production and secretion are required to replace the mucus that is degraded by bacteria and, in the intestinal tract, which is lost with the movement of luminal contents [28]. Unlike the systemic immune system, the mucosal immune system continuously encounters, monitors and regulates a thriving array of microbes and toxins that are always present and continuously changing. The mucosal barrier itself is not a static barrier as its constituents and the released products are modulated by the microenvironment and by neural, endocrine and immune factors [28] (Fig. 1).
In the mucosal barrier, the recognition of pathogens leads to the production of host inflammatory factors that, in turn, induce the differentiation of secretory cells and alterations in mucus composition through altered transcription of mucins and glycosyltransferases that interact with them. The recognition of pathogen-associated molecular patterns (PAMPs) activates Toll-like receptor (TLR) and NOD-like receptor (NLR) signaling pathways, which leads to alterations in the expression of mucin and antimicrobial genes (Fig.1) [28].
The continuous regulation of mucous secretions in general helps to keep microbes and toxins out of the system. The majority of bacteria, yeasts, protozoa and worms that live in the mucous ecosystem of the human gut generally cause the host no trouble. However, many of them can switch from being commensals to pathogens if they become overgrown within this niche, especially if they penetrate the mucus blanket and adhere to the surfaces and/or penetrate the epithelial cells [1].

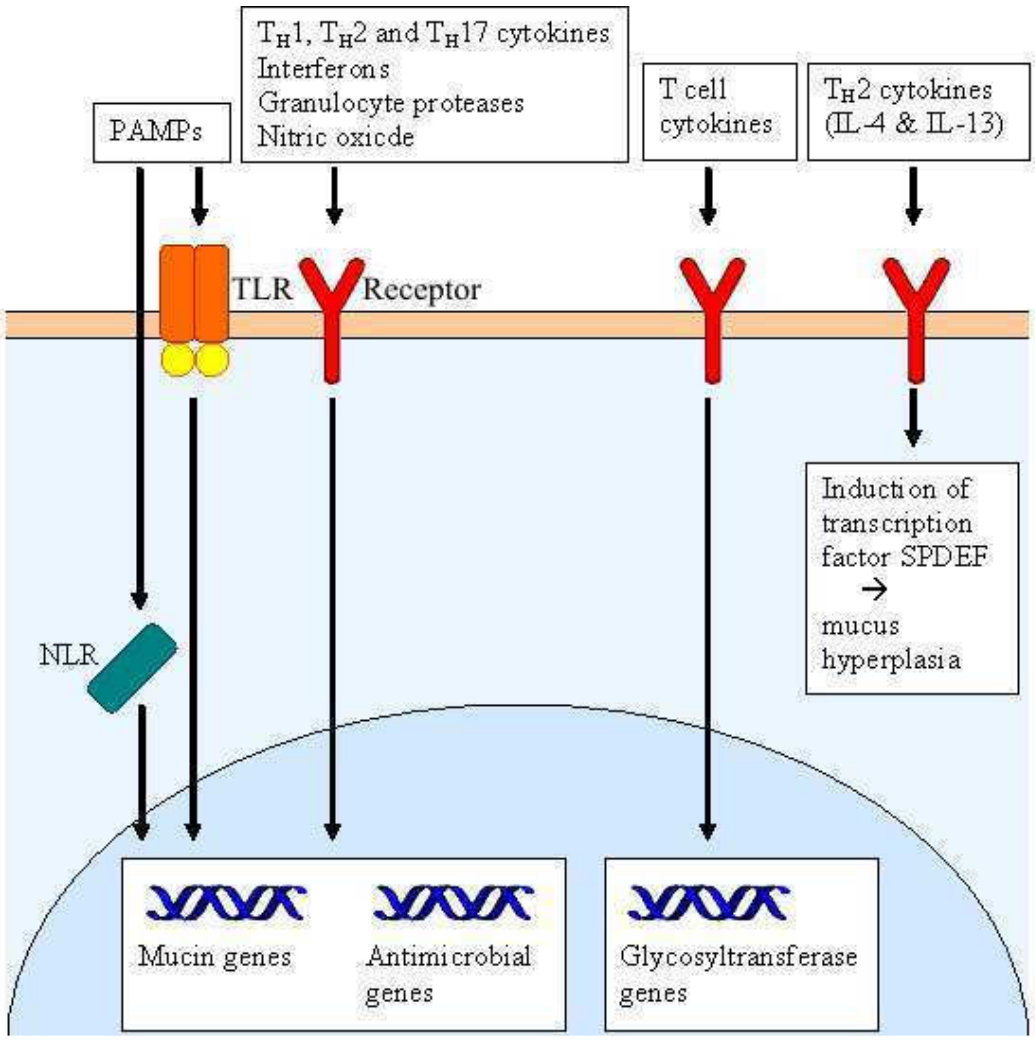

Fig. 1 Regulation of the mucus barrier adopted from McGuckin *et al.* [28]. The recognition of pathogens leads to the production of host inflammatory factors that, in turn, induce the differentiation of secretory cells and alterations in mucus composition through altered transcription of mucins and the glycosyltransferases that act on them. The recognition of pathogen-associated molecular patterns (PAMPs) activates Toll-like receptor (TLR) and NOD-like receptor (NLR) signaling pathways, which leads to alterations in the expression of mucin and antimicrobial genes. IL, interleukin; T_H, T helper cell.

In addition to its protective functions, mucus can also be involved in disease processes. Many commensal bacteria possess specific adhesins that bind specifically to mucus [29] and some parasitic organisms produce their own layers of mucus to evade the immune system [30]. Furthermore, a hyper- or hyposecretion of mucus is related to some diseases. In humans, hypersecretion of mucus is seen in cystic fibrosis [31], bronchitis, asthma [32] and in middle ear infections, with mucus gels serving as the matrix in which pathogens, allergens, and in the case of cholersterol gallstones trap nuclei and promote growth [33]. Hypersecretion can be induced as a response to proinflammatory cytokines, ATP, bacterial exoproducts (e.g. from *Pseudomonas aeruginosa*), and host proteases [34-38]. It is present in dry eye syndrome [39] and in some forms of ulcer disease. Additionally, mucus expression and composition is altered in cancers of epithelial origin [40].

Mucins

General

Mucus gels typically have mucin concentrations of 2 to 5%. In the gel, each mucin monomer is linked to other mucin monomers by non-covalent interactions and disulphide bonds [1]. Mucins are heavily glycosylated filamentous proteins which impart viscoelastic and rheological properties to mucosal layers. Mucins are built from a central core protein, the apo-mucin, to which carbohydrate side chains are attached (Fig. 2). Mucins have a high relative molecular mass (from several hundred to several thousand kDa) [9] and differ structurally and chemically from serum glycoproteins and proteoglycans of connective tissues [7]. Differences in glycosylation can be induced by altered assembly of carbohydrate side-chains or altered expression of core proteins [41].

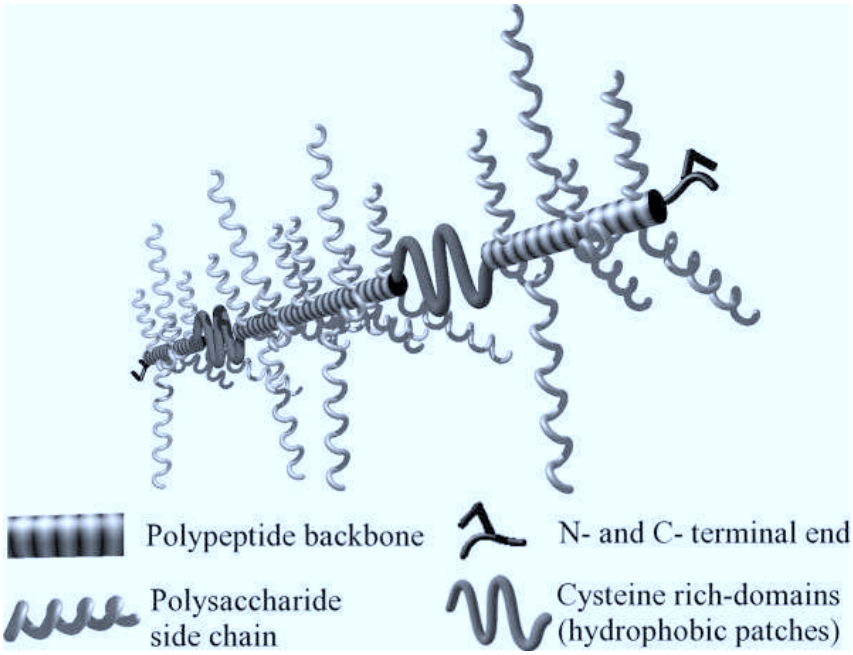

Fig. 2 Schematic view of a mucin molecule according to LaFitte *et al.* [42]

The primary functions of mucins are protection and lubrication of epithelial surfaces [9, 43]. Mucins have a negatively charged protruding structure and act as a selective barrier protecting the cell. Besides protection and lubrication, mucins appear to be also involved in more complex biological processes such as epithelial cell renewal and differentiation, cell signaling, and cell adhesion [44]. The secretion of mucins, which are stored in the vesicles of the goblet cells, is continuous (basal secretion) or can occur as a reaction on a specific stimulus (stimulated secretion) [45]. Mucins are known to be qualitatively and/or quantitatively abnormal in a number of diseases such as inflammatory bowel disease, respiratory disease and cancer [cf. 46].

Many mucins are specific to a particular mucosal surface forming an environmental niche that can support its own micro-ecosystem [1]. On the basis of their structural characteristics and physiological purposes, mucins have been categorized into two major types. One type is thought to be monomeric and primarily, but not exclusively, synthesized and located at the apial epithelial cell surface and is referred to as “membrane-bound” mucins. The secretory type consists of oligomeric mucins based on the cysteine-rich sequences located in the N- and C-terminal regions facilitating the formation of disulphide bridges and forming filamentous or networks of multimers that are apically secreted by goblet cells. These can be gel-forming or non-gel-forming, and are thought to be responsible for the rheological properties of mucus [25]. The main difference between members of these two types is the presence of a hydrophobic C-terminal transmembrane domain that serves to anchor membrane-bound mucins to the plasmalemma [44].
Cell-attached mucins range from 100 to 500 nm in length and, although much shorter than secreted mucins, are long enough to reach well beyond the glycocalyx where they help to make initial specific interactions between cells [1]. Membrane-anchored cell-surface mucin glycoproteins are a major constituent of the glycocalyx in all mucosal tissues [10]. Secreted mucins are extremely long molecules reaching up to several microns in length, longer than the diameter of many types of cells [1] and have a molecular mass between 0.5 and 40 MDa [47, 48]. These huge molecules are formed by end to end linkage of numerous mucin monomers, each about 1 micrometer in length [49, 50].
Cell surface mucins are cleaved into two subunits in the endoplasmic reticulum and inserted into the membrane and *N*-glycosylated. In the endoplasmic reticulum, secreted mucins are *N*-glycosylated and dimerised via their C-terminal domains. Both cell surface and secreted mucins are *O*-glycosylated in the Golgi apparatus. Following completion of *O*-glycosylation, the dimers of the secreted mucins undergo *N*-terminal oligomerization and are packed into granules for secretion (Fig. 3) [28].

Protein core

The apomucin is a linear polypeptide [51], which contains at least one protein domain [44]. Some mucin monomers are amongst the longest peptides known with a molecular mass exceeding 5 giga-Daltons and some are even polymorphic. Mucin monomers are encoded by different *mucin* genes (*MUC* for human and *Muc* for other species) [9]. Some are expressed in all or almost all epithelia studied indicating their involvement in general epithelial functioning. Expression of other mucins is confined to specific tissues. In a one single epithelium, two mucins can be highly expressed [46].
Centrally positioned within the apomucins, specific tandem repeated motifs (“tandem repeats”) are present [44]. Tandem repeats are particularly rich in proline, threonine and serine and are also designated as “PTS domains” [9]. Proline is presumed to be necessary to achieve a conformation that will permit the very close packing of the carbohydrate chains [7]. The oligosaccharide chains are bound to the hydroxyl side chains of serine and threonine and arranged in a “bottle brush” configuration around the protein core (Fig. 2). Many of the smaller membrane bound mucins are not considered true mucins since they only share the PTS repeats and glycosylation with other mucins [8]. In addition to the “bottle brush” regions of *O*-linked oligosaccharides (one to several hundred carbohydrate chains), there are *N*-linked sulphate-bearing oligosaccharides located near both ends of mucin monomers comprising 2

to 3 by weight % [1] (Fig. 2). Additional post-translational modifications, including sialylation or sulphation, are commonly observed on mature mucin glycoproteins [44].
The “bottle brush” regions are separated by “naked” protein regions which are cysteine-rich and are the most hydrophobic regions of mucins (Fig 2.). These naked proteins are sufficient hydrophobic that they may fold into globular beads which are stabilized by disulfide bonds [1] leading to enormous complexes [9]. About 90% of sulfhydryl groups in secreted mucins are involved in S-S bonds [1]. The “bottle brush” sections are resistant to proteolysis because they are protected by the carbohydrate sheath however, the naked regions can be hydrolyzed by a variety of proteolytic enzymes [7].

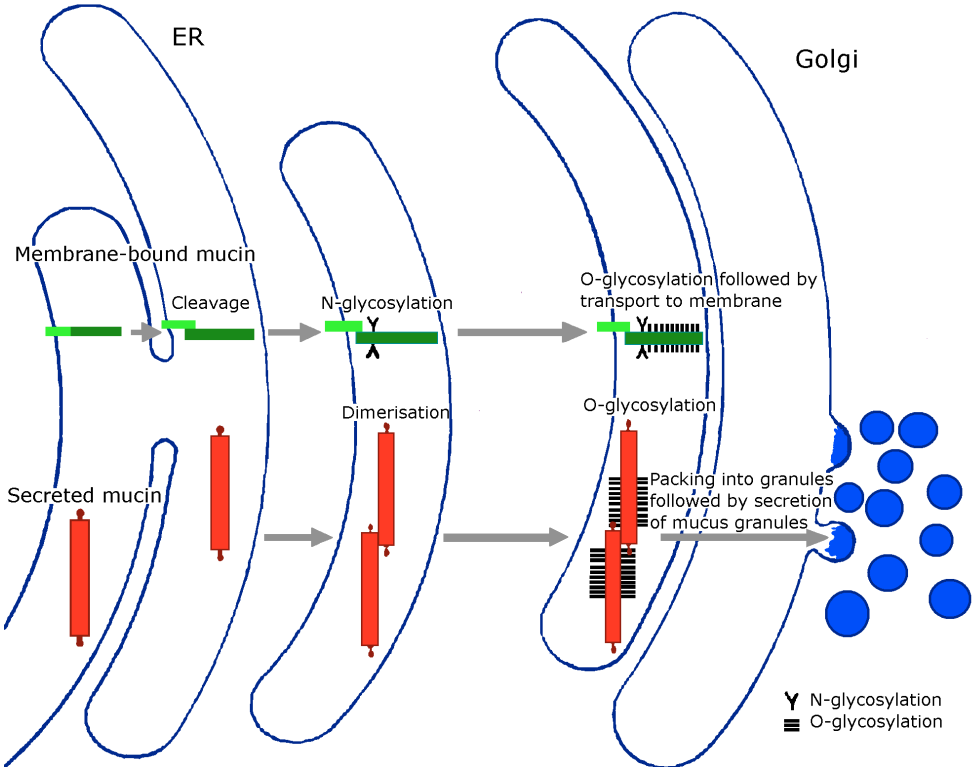

Fig 3 Mucin biosynthesis according to McGuckin *et al.* [28]. Cell surface mucins ( ) are cleaved into two subunits in the endoplasmic reticulum, inserted into the membrane and *N*-glycosylated ( ). In the endoplasmic reticulum, secreted mucins ( ) are *N*-glycosylated and dimerize via their C-terminal domains. Both cell surface and secreted mucins are *O*-glycosylated in the Golgi. Following completion of *O*-glycosylation, the dimers of the secreted mucins undergo N-terminal oligomerization ( ) and are packed into granules ( ) for secretion.

Carbohydrate side chains

The carbohydrate side chains constitute up to 80% of a mucins total mass [9, 25] and give an elongated and rigid structure to the molecule, while they also contribute to the rheological/viscoelastic properties of mucus secretions [44]. Due to their high glycosylation, many functions of the mucins depend on their carbohydrate chains, which offer wide possibilities of interactions with their environment in addition to participating to the mechanical properties of the mucus [9]. Furthermore, certain bacteria bind specific

oligosaccharide ligands. It is likely that the primary function of the oligosaccharide diversity is the enhancement of bacterial adherence and the facilitation of their removal by mucociliary transport. By providing competing receptors for cell-surface glycoconjugates, mucins may also trap bacteria and make them less successful in their attempts to colonize the epithelium. Thus, the array of oligosaccharides expressed on the mucins of an individual may play a key role in governing the susceptibility to infection [25]. Mucins contain negatively charged sugars [52] which interact to form the protective gel [53]. However, this also creates a negatively charged barrier that ions and water must cross to be absorbed [54].
The oligosaccharide chains consist of 5–15 monomers that exhibit moderate branching [8]. The chains are built up in a stepwise fashion through the transfer of one sugar at a time from sugar nucleotides by the action of specific glycosyltransferases. In this way the sequence of the sugars in the chains is exactly and genetically determined [7]. In the gastrointestinal tract, shorter, and perhaps sparser, carbohydrate side-chains are found in the goblet cell mucins of the lower crypt [41].
Typical mammalian mucins contain galactose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, sialic (neuraminic) acids, sulphate and mannose [8, 9, 25] but not uronic acid [7]. The *O*-glycosidic linkages are found between a hydroxylated amino acid (serine or threonine) and *N*-acetylgalactosamine [7-9, 25, 41].

ß-defensins

In addition to forming a relatively impervious gel, which acts as a lubricant, a physical barrier, and a trap for microbes, mucus also provides a matrix for a rich array of antimicrobial peptides (AMPs) [10]. AMPs are a part of the barrier function as they represent the first-line or host defense against pathogens [55]. As component of the innate immune system their role is to directly neutralize invading microbes [56] and act as part of the non-specific response of multi-cellular organisms but cannot recognize specific antigens [55]. Human neutrophils contain large amounts of three α-defensins (HNP-1-3), and smaller amounts of a fourth, HNP-4. AMPs are a group of small endogenous antibiotic peptides with a broad range of activity and a large diversity in structure and function. AMP consists of 18-45 amino acids including six to eight conserved cysteine residues. The molecules have a cationic or amphipathic charge which allow them to interact with bacterial cell membranes and develop antimicrobial activity by a pore forming mechanism [57]. Among the AMPs, the defensins are an important peptide family [55]. Defensins have been identified in many multicellular organisms including plants [58, 59], insects [60], invertebrates [61] and vertebrates [62]. Mammalian defensins can be classified into three subfamilies based on the arrangement of the canonical six cysteine motif and the disulfide bridges that stabilize the ß-sheet structure. The different subfamilies of defensins are thought to share a common ancestry, of which the ß-defensins (BDs) are probably the ancestral gene [63]. Defensins protect the skin and the mucous membranes of the respiratory, genitourinary and gastrointestinal tracts by acting as effectors of the innate immunity and as enhancers of antigen-specific humoral and cellular immune responses. Human BDs are endogenous antibiotics with broad bactericidal activity towards Gram-positive and Gram-negative bacteria and can affect a few pathogenic fungi, capsulate viruses and protozoa [55]. Neutrophils and almost all epithelial cells contain large

amounts of α-defensins and ß-defensins. Monocytes and macrophages generally lack defensins, but they release messengers that induce the synthesis of β-defensins in epithelial cells. In addition to their antimicrobial and immunomodulatory effects, defensins possess antiviral and toxin-neutralizing properties. Induction of β-defensins in epithelial cells is mediated by cell-surface Toll-like receptors or cytoplasmic peptidoglycan receptors that can recognize pathogen-associated molecules. Mutations in Nod2, a cytoplasmic peptidoglycan receptor, are associated with reduced levels of intestinal defensins and ileal Crohn's disease. Human defensin genes show marked copy-number polymorphism. High level constitutive expression of defensins may afford protection against HIV-1 and other defensin-sensitive pathogens.
Antimicrobial activity of defensins in fish has been documented as well [64]. Furthermore, human BDs are thought to be potent immunomodulaters indicating their important role in regulating both innate and adaptive immunity [65]. The expression of human BDs has been shown to vary between different tissues and have different induction patterns (constitutive expression vs. upregulation). This differential expression and induction suggest involvement of multiple signaling pathways in the regulation of these processes [65].

Aim and outline of this thesis

In fish, like higher vertebrates, the cells that form the intestinal epithelium are protected from pathogen and toxin offences by a layer of mucus [6]. Mucins are considered to be the major component of this protecting layer. Although all surfaces of fish are covered with mucus, knowledge about piscine mucins is scarce. Based on histochemical data, the number and content of intestinal goblet cells in fish and the composition of mucins is considered to be similar to that of mammals. However, nothing is known about the biochemical composition of piscine glycoproteins and their role in the first line of defense. This thesis investigates high molecular glycoproteins (HMG) in naive carp mucus of the intestine and skin. In **chapter two,** intestinal mucus HMGs and their glycosylation from adult carp at two ages was analyzed. As in mammals, mucus composition has been found to be age-related [66, 67].
As mentioned previously, mucus lines all of the organs that are exposed to the external environment. In contrast to most vertebrates, in fish this also includes the skin [8, 9]. As mucus HMG composition depends on the mucus origin, naive carp mucus was also monitored in skin and reported in **chapter three**.
Numerous interactions between microorganisms and the carbohydrate side chains of mammalian mucins as well as an influence on mammalian mucin gene expression have been demonstrated [references in 10, 68-70]. The mucus has a dual role in relation to microbiota as, on the one hand, it protects the underlying mucosa from undesired interactions with microbes such as pathogens, while on the other hand it provides an initial adhesion site, nutrient source, and matrix on or in which bacteria can proliferate and thrive. Interactions of bacteria with fish skin mucus have also been described [71]. In **chapter four**, carp were exposed to water with a high bacterial load. The influence of this challenge on carp skin HMGs is discussed.
The intestinal mucus HMGs that are described in **chapter two**, originated from the intestinal tract between intestinal bulb. In **chapter six** mucus HMGs from different intestinal segments

was isolated. Differences in the composition of mucus HMGs between these segments were examined. For mammals, a differential mucin expression was be found between various mucosal tissues [46]. In the human gastrointestinal tract site specific patterns of MUC gene expression have been observed [72]. Therefore, in **chapter five**, the gene expression of two mucin genes with homology to secreted mucin genes in other vertebrates is examined in different tissues, like skin, gills and intestine. The protective role of the mucus barrier does not solely rely on the mucins, but AMPs also play an important role. Human BDs are known to be expressed to a variable degree between different tissues [65]. In **chapter five** the gene expression of two carp β-defensins in different tissues are reported.

Continuous regulation of mucus secretions helps preventing inflx of microbes and toxins [1]. Dietary components are known to influence mucus quantity and quality as well as composition in mammals [73]. The influence of feeding the immunomodulatory β-glucan on mucin and ß-defensin gene expression in mucosal tissues of carp has also been monitored in **chapter five**. Alteration of mucin structure and/or function by β-glucans is possible as they interact with innate signaling pathways (including TLR signaling) in mucus producing cells [74]. Besides having a positive effect on health as found for β-glucan, food components may also have a negative effect on gut health.

Soybean meal (SBM) containing diets are known to induce an inflammatory response in the hindgut of certain fish species, like carp [75]. For inflammatory bowel diseases in humans genetic mutations in mucin genes, changes in sulphation, degree of glycosylation, mucin mRNA, protein levels and degradation of mucins have been described. In **Chapter six**, changes in mucus HMGs and staining for AMPs in different intestinal segments upon a SBM induced enteritis are monitored and discussed. Although continuous regulation of mucus secretions helps to keep microbes and toxins away most of the time, pathogens are s able to sometimeovercome the mucus barrier layer [1]. Some of the characteristics that promote bacterial colonization of the mucosal surface include chemotactic activity towards mucus [76] followed by bacterial adherence to mucins coating mucosal cells [77] and subsequent growth in mucus. These characteristics were examined for two bacteria strains using intestinal mucus in **chapter seven**. In **chapter eight** changes of intestinal mucus HMG samples upon different bacterial challenges are evaluated.

Finally, in **chapter nine**, the overall results obtained from this study, will be summarized and discussed and implications for future research are indicated.

Chapter 2

Intestinal mucus of common carp, *Cyprinus carpio* L.; biochemical and histochemical analysis

van der Marel, M.C.

This chapter is an updated version of the article “Biochemical and histochemical study on the intestinal mucosa of the common carp *Cyprinus carpio* L., with special consideration of mucin glycoproteins” [78, see appendix I] and contains additional information.

2

Abstract

Mucins are high molecular weight glycoproteins produced by specialized cells (goblet cells) and secreted on mucosal surfaces. Released glycoproteins form a biofilm protecting the underlying epithelium. Aim of the present study was to find and characterize biochemical and histochemical properties of intestinal mucin glycoproteins of virus and parasite-free common carps (*Cyprinus carpio* L.) and compare the results of secreted mucus from carp of two different ages.
The presence of carbohydrates in mucin glycoproteins could be demonstrated with histochemical methods, but generally, no obvious differences in specific staining for mucin glycoproteins were observed in contrast to biochemical techniques. Biochemical staining methods displayed differences in structure and composition of intestinal glycoproteins: Released intestinal glycoproteins contained two types of mucin glycoproteins: large mucins which were highly glycosylated and type 2 smaller mucins which were weakly glycosylated.
Thus, structure and composition of intestinal glycoproteins of carps were similar to those found in mammals. Differences in secreted mucus glycoproteins could be found for younger and older carp. The larger glycoproteins which are thought to form the mucus layer changed only slightly, where as the smaller glycoproteins showed more differences.

Introduction

Intestinal tissue is exposed to a permanent challenge of bacteria, parasites, viruses and toxins from the luminal content. The epithelial cells are protected against pathogen offences by a mucus layer, which covers the intestinal epithelium of all vertebrates including fish [6]. Mucin glycoproteins (“mucins”) are considered to be the major component of this protecting biofilm. Mucins are produced by goblet cells, which are scattered in many epithelial systems of aquatic vertebrates and particularly in the gut. The high molecular weight glycoproteins (HMG) form a water-insoluble layer of adherent mucus on epithelial cells, and have a high content of oligosaccharides. Oligosaccharides are believed to mediate the adhesion of microbial pathogens [79, 80] and to prevent glycoprotein degradation by proteases of microbial origin.
Although all surfaces of fish are covered with mucus, knowledge about piscine mucins is scarce. Based on histochemical data, the number and content of intestinal goblet cells in fish and the composition of secreted mucins in secretory tissues is considered to be similar to that of mammals; the production of secretory vesicles with neutral, acidic or sulphated glycoproteins has been described for fish [6, 81, 82]. Histochemical studies also indicate that fish with parasitic infection display goblet cell hyperplasia and hypertrophy, whereby their intracellular mucins shifted towards acidic and sulphated glycoprotein types [81, 83, 84].

However, nothing is known about the biochemical composition of piscine glycoproteins contributing to the intestinal first line of defence. Therefore, in the present study intestinal goblet cells of the common carp were characterized primarily by biochemical and histochemical methods to estimate their carbohydrate contents. The results provide a basis for further studies on intestinal glycoproteins of the carp.

Materials and Methods

Animals and sampling

Parasite and virus free sibling carp from a single crossing (E20xR8, Wageningen Agricultural University, the Netherlands) were used. Carp raised and kept in filtered recirculated tap water were used to examine the physiological mucus layer of carp. Hereto 12 to 18 month old carp (n=10) with a body weight of 88.3 ± 9.7 g and four year old carp (n=4) with a body weight of 92.8 ± 10.7 g were used.

Carp were starved three days before sampling to reduce faeces on intestinal mucus. For sampling, carp were killed by bath immersion with 500 mg l^{-1} tricaine (Sigma, Germany), weighed and subsequently dissected. The intestinal tract from directly behind the intestinal bulb until the anus was removed, weighed (younger carp: 1.33 ± 0.23 g, 4 year old carp 1.39 ± 0.38 g) and cooled on ice for further processing on ice. The intestines of all carp were used to isolate secreted mucus and of younger carp to isolate non-secreted mucus from the intestinal goblet cells. Mucus from all intestines was used to isolate high molecular weight glycoproteins (HMG). HMG from the younger carp was used for a lectin binding assay to determine the terminal glycosylation pattern. Furthermore 4 mm long gut sections were taken 4 cm behind the intestinal bulb, and fixed with Bouin´s solution and used for histochemistry.

Histochemistry

After fixation, samples were carefully dehydrated and embedded in paraffin wax. Sections of 5 µm thickness were stained for the presence of complex carbohydrates. Mucin carbohydrates were visualized with periodic-acid Schiff (PAS) Alcian Blue 8GX pH 1.0 (AB1.0) and pH 2.5 (AB2.5) as well as AB2.5/periodic-Acid-Schiff (AB-PAS) [85]. The AB1.0 method stains sulphated glycoconjugates; the AB2.5 method stains acidic glycoconjugates; the PAS reaction visualises neutral glycoconjugates and the AB-PAS visualises neutral and acidic glycoproteins. All sections were studied with a light microscope (Zeiss light microscope Axiphot, Zeiss, Germany).

In addition, terminal sugar residues were specifically stained with a variety of lectins (Table 1). Hereto, sections were incubated with several biotin labelled lectins in a concentration of 10 µg ml^{-1} in 0.1 M phosphate buffered salt solution (PBS), pH 7.2 for 30 min at room temperature. Lectin binding was visualized with peroxidase (PO) conjugated streptavidin and a diamino-benzidine-hydrogen peroxidase system (DAB, Biogenex, Germany). The sugar binding specificity was tested by the addition of 0.1 M inhibitory sugar solution [86]. To detect endogenous peroxidase activity, sections were also incubated with DAB alone.

The goblet cell staining, for pH-stains and for terminal sugars, was estimated on an arbitrary scale (no; very weak; weak; moderate; strong; very strong response).

Table 1 Lectins used in histochemistry and ELISA and the terminal sugars they bind to.

Acronym	Lectin	Binding specificity	Source
ConA*	*Canavalia ensiformis*	α–man and α–D-glc	Sigma, Germany
DBA*	*Dolchios biflorus*	α-galNAc	Sigma, Germany
MAL-1‡	*Maackia amurensis* I	αNeuNAc(2→3)gal	Vector, USA
PNA	*Arachis hypogaea*	β–gal(1→3)galNAc	Sigma, Germany
RCA-1*	*Ricinus communis* I	galNAc and β-gal	Vector, USA
SNA*	*Sambucus nigra*	αNeuNAc(2→6)gal	Vector, USA
UEA-I*	*Ulex europaeus* I	α-L-fuc	Sigma, Germany
WGA	*Tricium vulgare*	$(glcNAc)_2$ and NeuNAc	Sigma, Germany

* used in lectin ELISA.
‡ former known as MAA

Isolation of intestinal mucus HMG

Intestines of carp were opened longitudinally and cut into small pieces of 3-4 mm. Subsequently, glycoproteins were isolated as described previously [87]. In brief, to isolate secreted glycoproteins, tissue pieces were incubated for 20 minutes in 100 mL isolation buffer. The isolation buffer consisted of a phosphate buffer (PBS without calcium and magnesium (PAA, Germany), 1% sodiumpyruvate and 0.6% Hepes) with antibiotics (0.03% amphotericin B, 0.001% gentamycin, 100 IE/ml penicillin and 0.01% streptomycin) and a protease inhibitor (1% dithiothreitol). Intestinal tissue was removed from the isolation buffer and the isolation buffer containing the secreted mucus HMG was centrifuged for 30 min at 12 000 x g. The supernatant was collected and frozen at -20 °C until further processing.

Non-secreted mucus HMG were released from goblet cells by subsequent incubation of the tissue pieces for 30 min in a second buffer [87]. This buffer contained another protease inhibitor (0.02 % EDTA) and 0.005% dimercaptoethanol, but was otherwise equal to the first isolation buffer. Goblet cells were disrupted by means of an ultrasonic unit (Ultra Turrax T8, IKA-Werke, Germany). The suspension was centrifuged at 10,000 x g for 30 min, and the supernatant was collected and homogenised by gentle stirring. All samples were concentrated by ultrafiltration (Amicon, USA, exclusion limit 30,000 Da) to a final volume of 2 ml. Concentrated mucus samples were subjected to downward gel filtration on a 34 x 0.9 cm Sepharose CL-4B column (Sigma, Germany, flow rate 5.2 ml h^{-1}, fraction size 1.3 ml, 40 fractions). Aliquots of each fraction were used for carbohydrate content determination by periodic-acid-Schiff (PAS) reaction (absorbance at 550 nm) [88, 89] and for protein content determination by Bradford reaction (absorbance at 580 nm) [90].

From the optical density (OD) of the samples a biphasic profile (Fig. 1) was obtained after downward gel filtration and subsequent staining for carbohydrates and proteins. For further analysis of protein and for carbohydrate content, a distribution in three areas was made (peak 1: fraction 7-10, transition area: 11-15 and peak 2: 16-22, elution volume respectively 7.8-13, 13-19.5 and 19.5-28.6 ml). Mucin ratio between size areas (peak 1: TA: peak 2) was expressed as a percentage and was calculated with corrected carbohydrate ODs: (summated OD per size area) x (summated OD from fraction 7-22)$^{-1}$. Furthermore, the protein : carbohydrate ratio

(P:C) was calculated to determine the degree of glycosylation. A high glycosylation is reflected by a low P:C ratio as the amount of carbohydrates increases relatively to the protein core. Mucin amount was determined with the summated OD values of peak 1, TA and peak 2 by PAS reaction of the samples and of a standard of pig gastric mucin.

Determination of the terminal glycosylation pattern

The terminal glycosylation pattern was determined by lectin ELISA and the fractions were pooled per size area (peak 1, TA, peak 2). Fraction pools were incubated overnight in microtiter plates (Nunc Maxisorb, Germany) at room temperature. After blocking with 1% bovine serum albumin (BSA) solution in PBS, samples were incubated with biotin labelled lectins (10 µg ml^{-1} in 0.1 M PBS) for 30 min at room temperature. The following lectins were used: ConA, DBA, RCA-I, SNA, UEA-I (table 1). Lectin binding was visualized by streptavidin-horseradish- peroxidase (HRP) incubation for 30 min at room temperature and orthophenylenediamine (OPD)-peroxide (Dako Chemicals, Denmark). After 15 min, the reaction was stopped by the addition of 0.5 M sulphuric acid and the optical density was measured in a plate reader (BMG, Germany) at a wavelength of 485 nm.

To be able to compare the importance of the individual terminal sugars, the total OD of each lectin was divided by the total OD of all lectins and expressed as percentage. Furthermore, per lectin the distribution of the lectin binding over the size pools was calculated (OD size pool/ total OD).

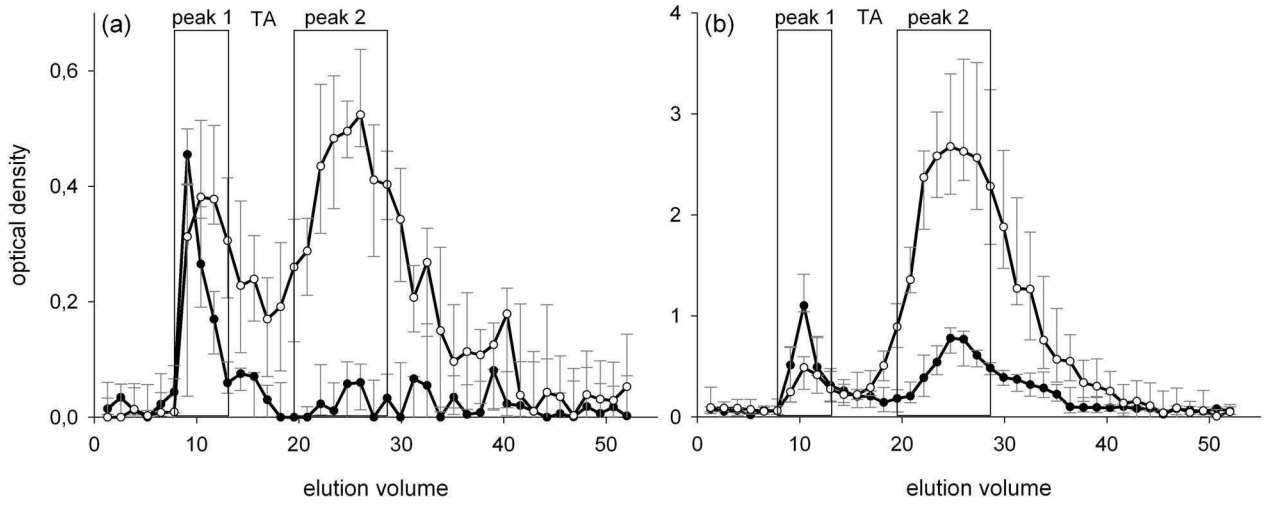

Fig. 1 Elution profiles of non-secreted (a) and secreted (b) intestinal mucus high molecular weight glycoproteins (HMG; per g gut weight) after staining for carbohydrates (□) and proteins (□). Values are median (25 -75% quartiles).

Statistics

If not indicated else, results are expressed as median value and 25% -75% quartiles. Data were statistically analysed with a t-test (for normally distributed data) or with a Rank Sum test (non-normally distributed data). Differences were considered significant at $p < 0.05$.

Results

Histochemistry

Goblet cells were regularly distributed in the intestinal epithelium between the enterocytes. Most goblet cells had a rounded, thick appearance and could be stained for carbohydrates. With the glycoprotein staining the presence of neutral, acidic and sulphated glycoconjugates could be monitored (Fig. 2).

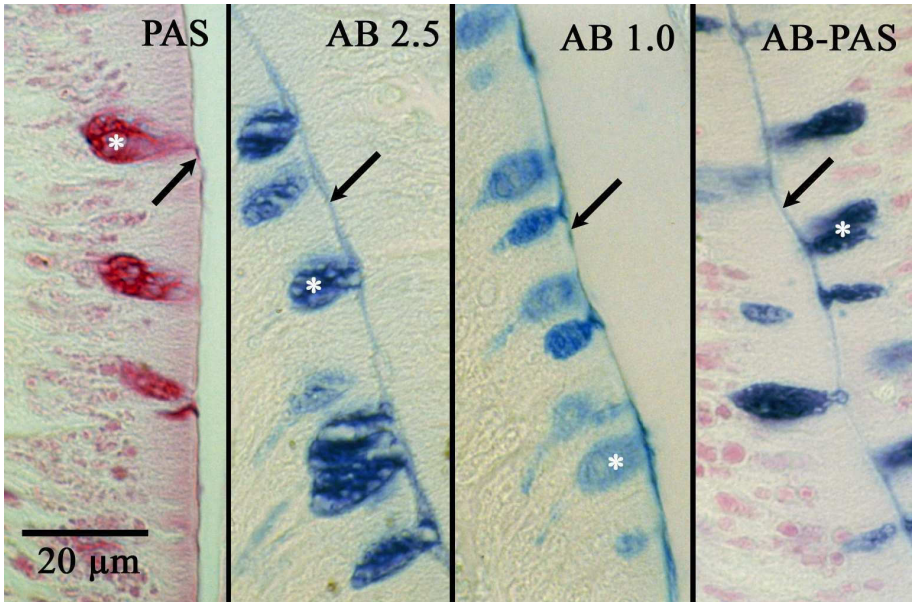

Fig. 2 General carbohydrate histochemical staining (a) PAS, (b) AB 2.5, (c) AB 1.0 and (d) AB-PAS of the intestinal mucosa of the common carp. Goblet cells (*) and mucous layer (→) covering the intestinal mucosa of common carp show a positive, intense staining.

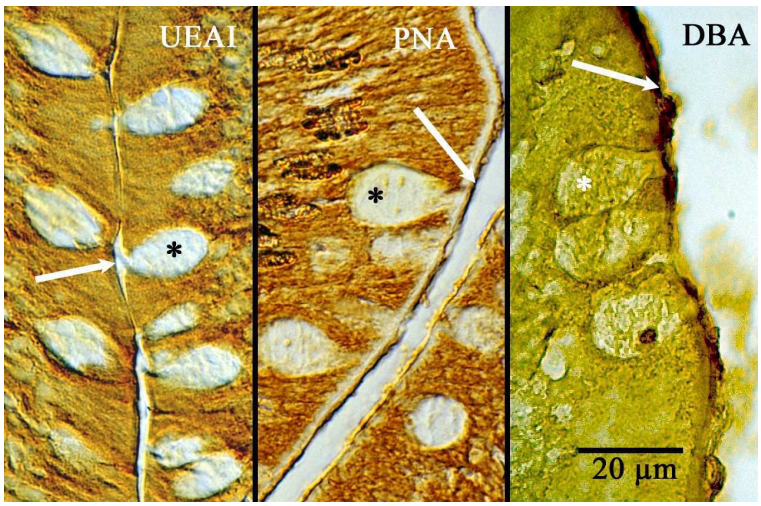

Fig. 3 Lectin histochemical staining (a) UEAI, (b) RCA and (c) DBA (see Table 1) on goblet cells (*), positive for the intestinal mucous layer (→).

Goblet cells responded in two different ways to the staining for sulphated glycoconjugates (AB 1.0). One population of cells was weakly stained, while a second population showed a stronger reaction. Overall the cells were stained very weak to weak. Acidic glycoconjugates (AB2.5) were weakly stained. Neutral glycoconjugates (PAS reaction) as well as neutral and acidic glycoproteins (AB-PAS) were stained weak to moderate.
Lectin staining of goblet cell content for specific carbohydrates (Fig. 3.) displayed a weak or negative reaction with UEA-I, WGA, as well as MAL-1. A faint to moderate reaction was observed with ConA, PNA, and SNA. A moderate reaction was observed with RCA and a moderate to strong reaction with DBA. Reactivity of mucus layer to all lectins used was generally stronger than reactivity of mucous cell content.

Isolation of intestinal mucus HMG

Non-secreted (epithelial) and secreted (luminal) glycoproteins could be isolated separately with the methods applied. From samples a biphasic profile (Fig. 1) was obtained after downward gel filtration and subsequent staining for carbohydrates and proteins. In between the first peak (peak 1), which contains the largest molecules and the second peak (peak 2) a transition area (TA) could be found.
The secreted mucin amount which could be isolated per mg of gut weight of the younger fish was approximately four times higher for secreted as for non-secreted mucus (Table 2). Total glycosylation was similar between non-secreted and secreted mucus. However, when observing the glycosylation of the three HMG size pools, a lower glycosylation (higher P:C) could be observed for the non-secreted glycoproteins than for the secreted glycoproteins.
The difference was significant for molecules in peak 1 and peak 2. Highest glycosylation could be observed in peak 1 and the lowest in peak 2 for both non-secreted as for secreted mucus. In peak 2, the highest percentage of protein could be found and for secreted mucus also the highest percentage of carbohydrates. When comparing non-secreted with secreted mucus in younger carp, significantly more protein and carbohydrates could be found in peak 1 and significantly less in peak 2.
When comparing older carp to younger carp, amount of secreted mucin which could be isolated per g per mg of gut weight was significantly higher in older carp. The secreted mucus of older carp had the lowest Protein:Carbohydrate ratio (P:C), indicating a higher glycosylation. Total glycosylation was significantly higher than that of younger carp. Glycosylation of molecules in peak 1 however, was hardly higher, but glycoproteins in TA and peak 2 had an approximately three times higher glycosylation. Secreted mucus of older carp had significantly more protein in peak 1 than in TA and also significantly more as the younger carp in peak 1 of their secreted mucus.

Terminal glycosylation pattern

The terminal glycosylation pattern was determined by lectin ELISA for the secreted and non-secreted mucus of younger carp. OD values of UEA-I (α-D-fucose) were around detection level and lectin binding of UEA-I was therefore not used for further analysis.
OD values of all other lectins had a high variation. Total lectin binding was higher for non-secreted mucus than for secreted mucus (Table 3). For both secreted- and non-secreted mucus the lectins which predominantly bound were DBA and ConA (Table 3). SNA bound relatively stronger to secreted mucus than to non-secreted mucus (Table 3).

Table 2 Total mucin weight of carp intestinal mucus, glycosylation expressed as Protein:Carbohydrate ration for the total mucus as well as for the individual size fractions. Also shown are protein and carbohydrate distribution over the three size areas (peak 1, TA, peak 2). Shown are median and 25% - 75% quartiles.

	Younger carp secreted mucus (ys)	Younger carp non-secreted mucus (yn)	Older carp secreted mucus (os)	Significant differences (P<0.05)
Mucin amount (g)	1.7 (1.3 - 1.9)	0.4 (0.4 - 0.6)	2.9 (2.4 - 3.3)	ys >yn; os > ys
Glycosylation (P:C)				
peak 1	0.5 (0.4 - 0.8)	1.1 (0.9 - 1.3)	0.3 (0.3 - 0.5)	ys < yn
TA	2.4 (1.3 - 2.9)	3.5 (2.7 - 4.5)	0.5 (0.4 - 0.7)	os < ys
peak 2	5.1 (3.9 - 5.8)	6.6 (6.1 - 8.9)	1.3 (1.1 - 1.5)	ys < yn; os < ys
Total	2.9 (2.5 - 3.5)	2.8 (2.3 - 3.1)	1.0 (0.9 - 1.0)	os < ys
Protein distribution (%)				
peak 1	8 (6 - 9)	27 (24 - 28)	13 (10 - 16)	ys < yn; os > ys
TA	11 (10 - 12)	19 (17 - 21)	8 (7 - 9)	ys < yn
peak 2	80 (80 - 85)	56 (54 - 57)	80 (76 - 82)	ys > yn
Carbohydrate distribution (%)				
peak 1	38 (32 - 40)	60 (54 - 70)	32 (30 - 33)	ys < yn
TA	14 (9 - 17)	17 (14 - 18)	14 (14 - 15)	
peak 2	50 (46 - 54)	24 (15 - 28)	54 (53 - 57)	ys > yn

Mucus glycoproteins from different size fractions for lectin binding were also analysed for their distribution over the size fractions. Generally, the distribution over the size fractions was different for non-secreted and secreted mucus. Non-secreted glycoproteins from peak 1, generally had lower amounts of oligosaccharides than those from TA, whereas secreted glycoproteins from TA had higher amounts of oligosaccharides than those from peak 2 (Table 3). For secreted mucus no or little lectin binding was found in peak 2, except for ConA.

Discussion

General mucus HMG characteristics

In mammals, the most abundant macromolecules in mucus are mucins, large polypeptides with tandemly repeated sequences and rich in threonine and/or serine with hydroxyl groups. These amino acids are O-linked to oligosaccharides [52]. In large intestinal mucins, carbohydrate content may account for up to 80% of its weight, but in gastric mucins approximately only 50% of its weight [91, 92]. For fishes, the presence of mucins was deducted from genomic sequences in the pufferfish, *Fugu rubripes* [93].

Table 3 Total OD, importance of the individual lectin (total OD lectin /total OD all lectins) and distribution over the size pools per lectin (OD size pool/ total OD)

	secreted mucus	non-secreted mucus	significant differences
Total OD			
DBA	3 (1 – 8)	25 (17 – 48)	non-secreted < secreted
ConA	5 (4 – 5)	19 (15 – 27)	non-secreted < secreted
RCA-I	2 (1 – 2)	5 (4 – 10)	non-secreted < secreted
SNA	3 (1 – 3)	4 (3 – 5)	
(total OD lectin /total OD all lectins) in percent			
DBA	22 (15 – 49)	55 (28 – 71)	
ConA	36 (27 – 51)	30 (21 – 44)	
RCA-I	12 (10 – 17)	10 (7 – 13)	
SNA	15 (11 – 22)	5 (4 – 9)	non-secreted < secreted
OD size pool/total OD) in percent			
DBA			
peak 1	55 (36 - 67)	17 (14 - 18) \	non-secreted < secreted
TA	44 (32 - 63)	66 (56 - 72)	
peak	0 (0 - 1)	15 (11 - 21	secreted < non-secreted
ConA			
peak 1	17 (14 – 22)	9 (5 – 11)	non-secreted < secreted
TA	68 (57 – 77)	24 (23 – 32)	non-secreted < secreted
peak 2	14 (8 – 22)	65 (55 – 73)	secreted < non-secreted
RCA-I			
peak 1	33 (29 – 34)	15 (14 – 20)	non-secreted < secreted
TA	67 (61 – 71)	64 (50 – 72)	non-secreted < secreted
peak 2	0 (0 – 0)	18 (8 – 24)	secreted < non-secreted
SNA			
peak 1	26 (11 – 30)	17 (15 – 20)	
TA	73 (68 – 83)	49 (45 – 64)	non-secreted < secreted
peak 2	2 (2 – 5)	32 (18 – 34)	secreted < non-secreted

In contrast to a variety of protective proteins and their functions described in skin and gut mucus of fish, knowledge about fish mucins is scarce. The present paper presents some specific features of these molecules, which -at least for mammals- are closely attributed to functional characteristics.

In accordance to Aristoteli and Willcox [94], histochemical methods were applied to identify structural components of intracellular glycoproteins, and lectin analyses to display the presence of their specific carbohydrates. As many fixatives change glycoprotein reactions by interactions between the fixatives themselves and glycoproteins [6]. In a second step, further biochemical investigations were done. Hereto, intestinal non-secreted and secreted mucin glycoproteins were isolated and analysed. The biochemical approach overcomes the difficulties of histochemical methods to fixate and quantify extracellular secreted glycoproteins.

With the histochemical methods, numerous scattered goblet cells containing neutral, acidic and sulphated glycoproteins could be detected in the intestinal epithelium. Acidic and sulphated glycoproteins are reported to inhibit bacterial adhesion and glycoprotein degradation by proteases, and goblet cells with such mucins usually are detected in parasite-infected fishes [81].

With biochemical methods, intestinal mucin glycoproteins were isolated separately from the luminal content and from the epithelium. By isolation with gelfiltration, a biphasic elution profile was received both for protein and carbohydrate contents. In rats, similar elution profiles were obtained, and the intestinal mucins displayed similar molecular sizes [87]. The first peak of the biphasic profile is thought to represent adherent mucins (AM) and the second peak contains the so-called luminal mucins (LM) [95]. Functionally, large molecules (AMs) are regarded to form the mucus layer, which adheres to the epithelium. Smaller molecules (LMs) are thought to represent the soluble, luminal mucus. Generally it can not be excluded that isolated small mucin molecules also contain different “non mucin” glycoproteins. Regarding to Enss *et al.*[95] isolated small glycoproteins are considered to represent mainly small mucin glycoproteins.

Molecules of peak 1 had biochemical characteristics of mucins with high molecular weight and high carbohydrate contents. This means that mucus HMG of high molecular weight were highly glycosylated, resembling mammalian HMG and this suggests that mucin glycoproteins in vertebrates are highly conserved.

Peak 2 HMG were less glycosylated than HMG from peak 1 or TA. In secreted mucus separation, these glycoprotein types are thought to represent soluble mucins, which originate from degradation of glycoproteins by proteases of microbial origin [94], or by digestive enzymes from the host. In non-secreted mucin glycoproteins, peak 2 molecules most likely represent small mucin glycoproteins, which are not completely synthesised yet [95]. Small molecules are generally less glycosylated and bind in lower intensities to carbohydrate specific lectins than glycoproteins with higher molecular weight. These aspects indicate that peak 2 HMG of epithelial separations contain glycoproteins in an early state of synthesis.

In mammals, the protein core of the glycoproteins is synthesised first and serves as a structural basis for subsequent glycosylation [45]. The dense molecular packing of the protein core with carbohydrate side chains in vesicles is fundamental for the general properties of mucosal glycoproteins, which includes protease resistance, swelling and gel formation due to the uptake of water and binding of ions [51]. In addition, carbohydrate side chains allow adhesion of bacteria [96], which also was recorded from the skin and the intestinal tract of fishes [97, 98].

In the present study, intestinal isolates of mucus HMG were investigated according to known mammalian oligosaccharides. Carbohydrate side chains in mucins from mammalian mucosal surfaces are composed of a limited array of oligosaccharides: N-acetyl-α-galactosamine (galNAc), N-acetyl-β-glucosamine ($(glcNAc)_2$), galactose, mannose, fucose and neuraminic-acid (NeuNAc) [45].

The presence of galNAc, $(glcNAc)_2$, galactose, mannose and NeuNAc in the content of piscine intestinal goblet cell was shown by lectin histochemistry and lectin ELISA for both non-secreted and secreted mucus HMG. Especially the terminal presence of mannose and galNAc was demonstrated. GalNAc is typically the first monosaccharide that connects serine or

threonine in particular forms of protein O-glycosylation. Mannose is a typical component in N-glycosylation [53], and is bound to the protein core early during synthesis [45].
Fucose (via UEA-I) however, in compliance with Fiertak & Kilarski [99], could not be detected by means of the lectin approach. In a gas chromatographic analysis of mucus isolated from the intestinal tract of the rainbow trout *Oncorhynchus mykiss*, however, fucose was demonstrated [100]. This lack of fucose, indicated in the present study, may have two reasons: In intestinal mucins of carp, fucose could be located in a position, which -by steric reasons- did not allow a lectin linkage. On the other hand, fucose is totally missing and does not play an important, physiological role in untreated carp.
In conclusion, goblet cells of the intestinal mucosa of carp secrete large glycoproteins, which in structure and composition show a high similarity to those found in mammals. Secreted molecules have a high molecular weight and are highly glycosylated with the similar order of monosaccharides at terminal positions as recorded for their mammalian counterparts. Such glycoproteins form the basis for the mucus layer, which in fishes covers the epithelia of the skin, the gills and the intestine, and shields piscine cells from environmental challenges.

Differences between intestinal mucus HMG

The significantly higher mucin amount per mg of gut weight in older carp (compared to younger carp) might be caused by an increased relief of the mucosal folds present in older fish. However, in older carp the submucosal tissues is also more pronounced. To obtain more information about differences in mucus amount between younger and older carp, the thickness of the mucosal layer had to be measured as well as the viscosity.
As glycosylation of peak 1 hardly differed between older and younger carp, the larger glycoproteins in the mucus, which are thought to play a major role in the formation of the structure of the mucus gel [87, 95], appeared not to be affected over time. Total glycosylation of older carp was significantly higher (lower (P:C) than that of younger carp. Glyosylation may differ with age [101] and in the present study an increased glycosylation (lower P:C ratio) in TA and peak 2 in older carp was found. This increased glycosylation might reflect the exposure to a larger array of micro-organisms. The smaller molecules are thought to be able to trap invading micro-organisms as micro-organisms can bind to certain carbohydrates [71, 102]. Viscosity may differ with age [101]. The older carp in this study had significantly more protein in peak 1 as in TA. The increased amount of the structure protein might indicate that the mucus of older fish is more firm. However, to be certain viscosity measurements must be made.
Mucus is produced in the goblet cells and their content is a mixture of mature and immature mucus glycoproteins, which are not yet fully glycosylated [95]. This is reflected in the lower glycosylation of the non-secreted glycoproteins compared to the secreted glycoproteins.
The mucus molecules that are important in the formation of the gel layer (peak 1) [87], are likely to have a lower turnover rate as the molecules that are thought to be able to trap invading micro-organisms (peak 2). Unchallenged mammals have a mucus layer which is continuously replaced [103]. A higher release rate of smaller mucus HMG would explain the significantly higher protein and carbohydrates percentages found in peak 1 and TA of non-secreted mucus compared to secreted mucus.
In conclusion, intestinal mucus HMG of younger and older carp differ, especially the HMG found in TA and peak 2 and less those of peak 1 which are thought to form the mucus layer.

Chapter 3

Characterization of skin mucus layer in common carp, *Cyprinus carpio* L.

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Abstract

Mucosal surfaces form the primary interface between the animal and its environment. The outermost constituent of this barrier is a mucus gel that mostly contains water and high molecular weight glycoproteins (HMGs). In fish, skin, gills and intestinal tract are the main mucosal surfaces. Goblet cells of the skin mucosa of fish secrete HMGs. HMGs of common carp skin mucus were analyzed using histological, histochemical and biochemical techniques.
HMGs were highly glycosylated and the presence of N-acetylgalactosamine, galactose, fucose, mannose and neuraminic acid could be demonstrated. A high glycosylation with fucose and neuraminic acid indicated a negative net charge of mucins, which reduces the ability of pathogens to attach. This study shows that HMGs from carp skin have a high similarity to those found in the intestinal mucus of carp and even in mammals. This confirms the idea of an evolutionary conserved mucosal barrier system.

Introduction

The fish mucosal surface of skin, gills and intestinal tract are the primary interface between the animal and its environment. The outermost constituent of this barrier is a mucus gel which forms a layer of adherent mucus covering the cells of the epithelium. This mucus blanket is secreted from goblet cells in the epithelium. Proposed roles of mucus in the fish system are numerous, and consist of respiration, ionic and osmotic regulation, reproduction, locomotion and defence against microbial infections (review Shephard *et al*. 1994). Mucus includes many components, two of which are predominantly important in gel formation. These two gel-forming components are water and high molecular weight glycoproteins (HMGs), called “mucins” [1, 51-53, 104].
Most studies on fish mucus have focused on the qualitative immunohistochemical staining of goblet cell contents [105-107], or on goblet cell numbers and distribution in response to a variety of stressors [107-116]. The few studies in which mucus was isolated focussed on viscosity [108, 117] or the characterisation of particular substances within the mucus [6, 112, 117, 118]. Studies directly dedicated to fish mucins are relatively rare. The available histological data indicates that fish skin mucins are quite similar in their composition to the mucins of mammalian epithelial tissues [115, 119, 120]. This study presents histological analysis and biochemical data on mucus HMGs in carp skin.

Materials and Methods

Fish

Parasite free, virus free and clinically healthy sibling common carp *Cyprinus carpio* L. from a single crossing (E20 x R8, Wageningen Agricultural University, Netherlands) were used throughout the study. Carp were raised and kept in filtered recirculated tap water at 20-23° C. For this study, 12-18 month old carp were removed from their aquarium in a small plastic container with water (to avoid damage to the mucosal layer) and euthanised by adding 500

mg tricaine methane sulfonate and 600 mg sodium bicarbonate (Sigma, Munich, Germany) per litre. All fish were weighed and measured.

Histology and histochemistry

Four carp with an average body weight of 92.5 g ± 14.2 were euthanised for histological and histochemical examination and then immersed in Bouin's fluid for four days at room temperature. From each fish, skin samples were taken from three different locations of the dorso-lateral body region: 5mm cranial, medial and caudal from the lateral line. Samples were de-calcified with ethylene diamino tetra acid (EDTA), washed, dehydrated in a series of graded ethanol and embedded in paraffin wax. From the embedded material, 4 µm sections were cut. Paraffin embedded material was stained with haematoxylin-eosin (HE) and for the presence of complex carbohydrates. Carbohydrate staining included AB 1.0 [121], AB 2.5, PAS and the combined staining AB2.5-PAS (Table 1).

Table 1 Carbohydrate staining of carp skin goblet cell HMGs

Acronym	Staining method	Binding specificity	Staining intensity‡
PAS	*Periodic-Acid Schiff*	periodate reactive vicinal diols (neutral glycoproteins)	moderate to strong
AB1.0	*AcianBlue-pH 1.0*	sulphated glyconjugates	moderate to strong
AB2.5	*AcianBlue-pH 2.5*	acidic glycoproteins	moderate to strong
AB2.5/PAS	*AcianBlue-pH 2.5/ Periodic-Acid Schiff*	acidic glycoproteins, periodate reactive vicinal diols and mixtures of the former two	moderate

‡ intensity was estimated on an arbitrary scale (none; faint; weak; moderate; strong; very strong response).

Since the stratum superficiale was only stained very weakly and the goblet cells stained intensively, the pH-stains were evaluated on the basis of the goblet cell reaction. Furthermore, the number of responding goblet cells was counted for the AB1.0 and AB 2.5 and HE stained sections in an area comprising all the cells above 100 epithelial stratum basal cells. The number of stained goblet cells was counted twice per fish and per location. As values differed largely between the three different locations and no correlation between location and number of stained goblet cells could be observed (data not presented), an average and standard deviation (SD) of all measurements per fish was calculated.

In addition, terminal sugar residues were specifically stained with different lectins (see Table 2). Paraffin embedded sections were incubated with several biotin labelled lectins in a concentration of 10 µg ml^{-1} in 0.1 M phosphate buffered salt solution (PBS), pH 7.2 for 30 min at room temperature. The following lectins were used: PNA, ConA, DBA, WGA, UEA-I (Sigma, Munich, Germany), MAL-1, RCA-1, and SNA (Vector, Burlingame, USA) (Table 2). Lectin binding was visualized with peroxidase (PO) conjugated streptavidin and a diamino-benzidine-hydrogen peroxidase system (DAB, Biogenex, Hamburg, Germany). The sugar binding specificity was tested by the addition of 0.1 M inhibitory sugar solution (Brooks et al. 1997). To detect possible endogenous peroxidase activity, sections were incubated with DAB alone. Goblet cell staining was estimated on an arbitrary scale (no; faint; weak; moderate; strong; very strong response).

Isolation of high molecular weight glycoproteins

Skin mucus was isolated as described previously [122]. Carp were transferred individually to a container filled with 100 ml of 100 mM ammonia bicarbonate, pH 7.8, agitated by hand for 10 min on ice and then removed from the container. The mucus containing solution was mixed with 100 ml of ice cold protease inhibitor phenyl methyl sulphonyl fluoride (PMSF, 1 mM), and ultracentrifuged at 13,000 x g for 30 min at 4°C (Beckman Coulter, Palo Alto CA). The supernatant was collected and stored at -80°C. Samples were concentrated by ultrafiltration (Amicon, Beverly, MA, exclusion limit 30 kDa) to a final volume of 2 ml.

Table 2 Lectin binding to carp skin goblet cell HMGs.

Acronym	Lectin	Target sugars	Lectin binding[≠]
PNA	*Arachis hypogaea*	β–gal(1→3)galNAc	none to faint
ConA	*Canavalia ensiformis*	α–man and α–D-glc	faint
DBA	*Dolchios biflorus*	α-galNAc	weak
MAL-1	*Maackia amurensis* I	αNeuNAc(2→3)gal	moderate
RCA-1	*Ricinus communis* I	galNAc and β-gal	none to faint
SNA	*Sambucus nigra*	αNeuNAc(2→6)gal	moderate
WGA	*Tricium vulgare*	$(glcNAc)_2$ and NeuNAc	faint to weak
UEA-I	*Ulex europaeus* I	α-L-fuc	none

≠ intensity was estimated on an arbitrary scale (no; faint; weak; moderate; strong; very strong response).

Size exclusion chromatography

From six euthanized carp (139.5 g ± 9.2) skin mucus was isolated and concentrated. Concentrated samples containing high molecular weight glycoproteins (HMGs) were subjected to downward gel chromatography on a 34 x 0.9 cm Sepharose CL-4B column (Sigma, Munich, Germany, flow rate 5.2 ml h^{-1}, fraction size 1.3 ml, separation range: >70 kDa). Fractions were analyzed for carbohydrate by the PAS reaction at 550 nm and for protein with the Bradford assay at 580 nm in a multiwell plate reader (BMG, Offenburg, Germany) [89, 90, 123].

After gel filtration, a biphasic elution profile was obtained for protein and for carbohydrate content which allowed a distribution in three areas (Peak I (fractions 7-10), transition area (TA; fractions 11-15) and Peak II (fractions: 16-22) with elution volumes of respectively 9.1-14.3, 14.3-20.8 and 20.8-29.9 ml). Protein ratio between size areas (Peak I: TA: Peak II) was expressed as a percentage and was calculated with corrected optical density (OD):
(summated OD size area) x $(\text{summated OD fraction 7-22})^{-1}$.

Carbohydrate ratio between size areas was calculated in the same way. The protein (P) : carbohydrate ratio (C) was calculated to determine the degree of glycosylation. Mucin amount was determined with the summated OD values of the samples by PAS reaction and the summated OD values of the pig gastric mucin standard.

As each fish has a different body surface, a body surface correction factor was used for the ODs of each fish. This was done to improve the comparison of the protein and carbohydrate data as well as mucin amount between different days. The body surface factor (1.82 (1.68 ± 1.91)) is a modified Fulton's condition factor [124] and includes the total fish length to account for the mucus on the fish tail.

This body surface factor was calculated as follows:
(body weight x 100) x (total length^3)$^{-1}$
All HMGs results were expressed as median and 25-75% percentiles.

Determination of the terminal glycosylation pattern

For mucus from the size exclusion chromatography, terminal glycosylation pattern was determined by lectin ELISA as described previously [78, 125]. Therefore fractions were pooled per size area (Peak I, TA, Peak II). Fraction pools were incubated in microtiter plates (Nunc Maxisorb, Wiesbaden, Germany) overnight at room temperature. After blocking with 1% bovine serum albumin (BSA) solution in PBS, samples were incubated with biotin labelled lectins (10 µg ml^{-1} in 0.1 M PBS) for 30 min at room temperature. The following lectins were used: ConA, DBA, RCA-I, SNA, UEA-I (Table 2). Lectin binding was visualized by streptavidin-horseradish- peroxidase (HRP) incubation for 30 min at room temperature and orthophenylenediamine (OPD)-peroxide (Dako Chemicals, Glostrup, Denmark). After 15 min, the reaction was stopped by the addition of 0.5 M sulphuric acid and the OD was measured in a plate reader (BMG, Offenburg, Germany) at a wavelength of 485 nm. Results were expressed as median and 25-75% percentiles. Furthermore, the binding of each lectin was expressed as the percentage of the total binding of all lectins tested and compared to the percentage binding to intestinal mucus HMGs. Also the binding of the lectins ConA, DBA, RCA and SNA to each fraction was expressed as the percentage of the total binding to all fractions and compared to the percentage binding to intestinal mucus HMGs.

High-performance liquid chromatography of mucus

Skin mucus samples were also analysed for the presences of monosaccharides by reverse-phase HPLC with the method described by Anumula [126]. In brief, concentrated mucus samples were hydrolysed with 20% trifluoroacetic acids and then derivatized with excess anthranilic acid in the presence of sodium cyanoborohydride. Antranilic acid derivatives of monosaccharides were separated on a HPLC column using a 1- butylamine-phosporic acid-tetrahydrofuran mobil phase. All the separations were carried out at room temperature using a flow rate of 1ml/min. Solvent A consisted of 0.25% 1-butylamine, 0.5 % phosphoric acid, 1% tetrahydrofuran (inhibited) in water. Solvent B consisted of equal parts of solvent A and acetonitrile. The elution program was 5% B isocratic for 25 min followed by a linear increase to 15% B at 50 min. The column was washed for 15 min with 100% B and equilibrated for 15 min with 5% B. Elution was monitored by measuring the fluorescence (excitation wavelength, 230 nm; emission wavelength, 425 nm). Sugars were identified by comparing retention times (RT) of peaks in the samples with RT of peaks of standard sugar solutions. Standard sugar solutions were galacotose (gal) RT 12.1, N-acetylneuraminic acid (NeuNAc) RT 12.6 min, glucose (glc) RT 12.8 min, mannose (man) RT 13.2, fucose (fuc) RT 18.2 min and N-Acetylgalactosamine (galNAc) RT 23.4. Of each identified peak in the samples, surface areas of eluted peaks were compared with the surface area of peaks of standard sugar solutions with a known amount of monosaccharide.

Results

Histological evaluation

The presence of neutral, acidic and sulphated glycoconjugates could be visualised in goblet cells of carp skin. In the area comprising all the cells above 100 epithelial stratum basal cells 47.1 (± 7.4) goblet cells could be observed (HE stain). In this area, 31.5 (± 6.7) cells containing carboxylated acid glycoconjugates (AB 2.5 positive; Fig. 1A) and 20.3 (± 5.2) cells containing sulphated glycoconjugates (AB 1.0 positive) could be found. In all reactions skin goblet cells were stained with a similar moderate intensity, only acidic glycoproteins stained somewhat stronger (Table 1).

Lectin histochemistry displayed the contents of skin goblet cells and showed no or a faint reaction with ConA, PNA, RCA-I and UEA-I (Table 2). With WGA, a faint to weak reaction was observed, while DBA, MAL-1 and SNA (Fig. 1B) produced a weak to moderate staining with goblet cells.

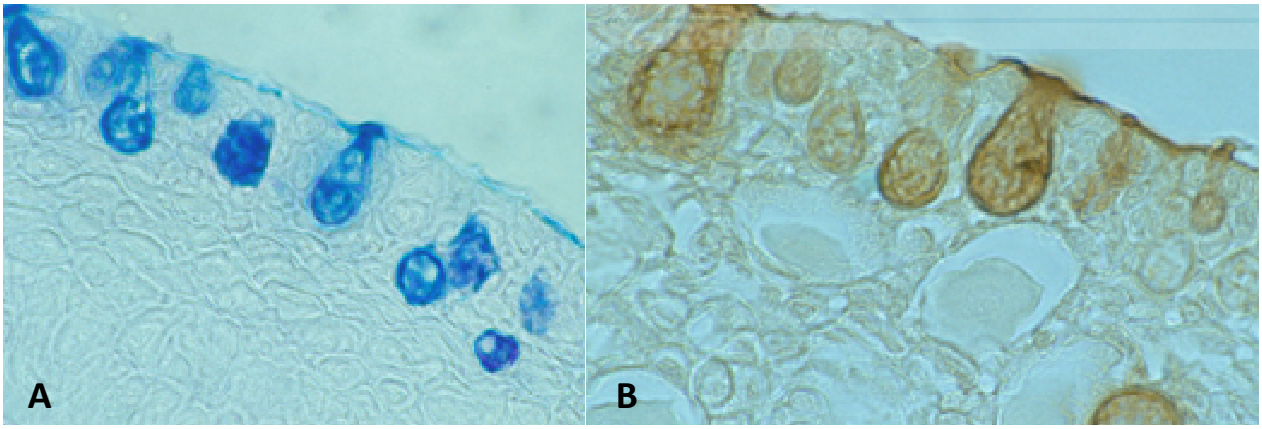

Fig. 1 Staining of acidic glycoproteins with AB2.5 (Fig. 1A) and lectin binding of SNA (Fig. 1B) in goblet cells and the mucus layer of carp skin.

Size exclusion chromatography

Released HMGs could be isolated from the skin of carp. With lyophilized pig gastric mucin as standard and corrected with body surface correction factor, the total glycoprotein content was calculated to be approximately 15.8 g (15.3 - 16.0) per fish. Glycosylation index (P : C ratio) of the HMGs was 1.2 (0.8- 1.7).

Table 3 Carbohydrate and protein ratios of carp skin HMGs. Values are median and 25 - 75 percentiles.

	Peak I:	TA:	Peak II
Protein ratio between size fractions (%)	13.9 (9.1 - 17.2)	30.1 (24.9 - 39.1)	52.1 (47.9 - 56.8)
Carbohydrate ratio between size fractions (%)	38.0 (37.0 - 45.8)	32.9 (24.8 – 38.7)	30.1 (29.4 – 36.6)
Protein/carbohydrate ratio per size fraction	0.6 (0.5 - 0.7)	1.3 (1.1 -1.4)	2.1 (0.9 - 3.0)

After downward gel filtration, a biphasic elution profile was obtained for both the protein and carbohydrate contents (Fig. 2). Between these peaks, a transition area with a lower glycoprotein content was found. In the peak I, the highest carbohydrate and the lowest protein amounts could be detected (Table 3). In peak II on the other hand, the highest protein and the lowest carbohydrate amounts could be detected. This low P : C ratio in the first peak and the somewhat higher PC ratio in the second peak (Table 3) indicates that HMGs from peak I have higher levels of glycosylation than HMGs of peak II. However, all three pooled skin mucus size fractions demonstrated a high level of glycosylation (low P:C ratio). The molecules in TA showed the highest glycosylation level.

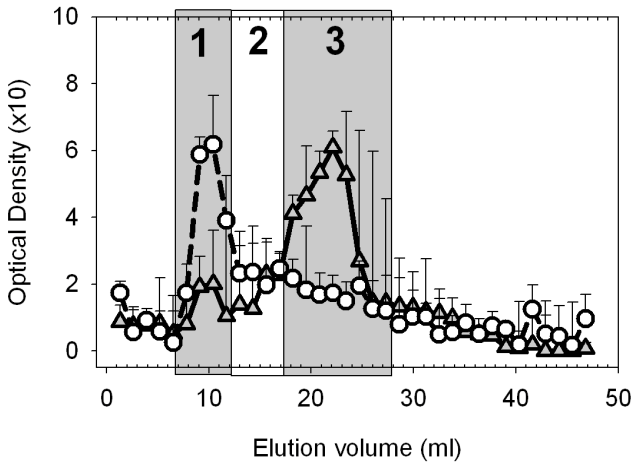

Fig. 2 Elution profile of protein (△) and carbohydrate (○) content of carp skin HMGs on a CL4B column. Values are median and 25 - 75 percentiles.

Terminal glycosylation pattern of skin HMG

The following lectins were bound to isolated glycoproteins: RCA-1, ConA, DBA and SNA (Table 4). The presence of fucose via a ligation of UEA-I could hardly be detected in the carp skin mucins. Skin mucins showed the highest lectin binding for DBA and ConA (Fig. 3), indicating the presence of high amounts of α-galNAc, α–man and α–D-glc. For ConA and SNA, lectin binding was almost equally distributed over the three size fractions (Fig. 4). DBA and RCA however showed a lower binding to peak II than to peak I and TA.

Table 4 Lectin binding to pooled size fractions of carp skin HMGs from the skin of the common carp. Values are median and 25 - 75 percentiles.

Lectin*	Peak I	TA	Peak II
UEA1	0.03 (0.01 ± 0.07)	0.04 (0.00 ± 0.08)	0.01 (0.00 ± 0.03)
SNA	0.30 (0.24 ± 0.35)	0.36 (0.31 ± 0.43)	0.25 (0.21 ± 0.30)
RCA	0.41 (0.25 ± 0.57)	0.32 (0.21 ± 0.45)	0.16 (0.10 ± 0.24)
DBA	1.70 (1.45 ± 1.81)	1.46 (1.14 ± 1.78)	0.40 (0.35± 0.75)
ConA	0.98 (0.78 ± 1.05)	1.18 (0.94 ± 1.33)	1.04 (0.85 ± 1.18)

* see Table 2I for specific binding to terminal sugars

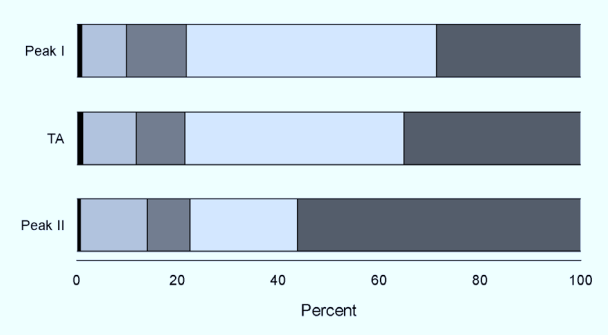

Fig. 3 Distribution of lectin binding (%) to skin mucus per HMG size fraction (Peak I, Transition Area (TA) and Peak II). Used lectins: UEA-I (■), SNA (▭), RCA-I (▭), DBA (▭), ConA (■)

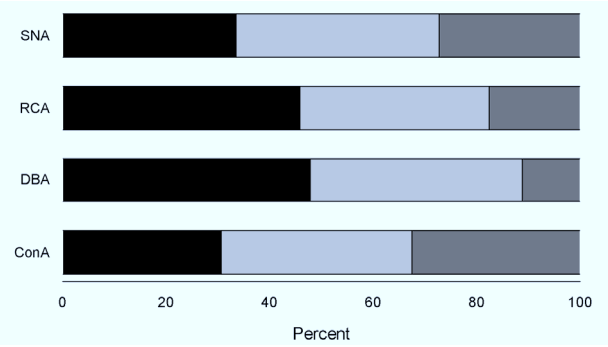

Fig. 4 Lectin binding: distribution over HMG size fractions (%) per lectin. Peak I (■), TA (▭), Peak II (▭).

Monosaccharide composition

Skin mucus samples were analysed for the composition of monosaccharides by reverse-phase HPLC. For all samples, a differentiation on basis of retention time could not be made between glc/man and NeuNAc/gal and hence they were analysed as a mix. Of the tested sugars fucose was the most predominant one and galNAc could not be detected (Table 5). Of the identified sugars (fuc, glc/man, NeuNAc/gal), a total of 3.0 ± 1.0 µg could be collected per fish. One peak at RT 16 min. could not be identified with the standard sugar solutions used. The peak had approximately the same area size as NeuNAc/gal.

Table 5 Monosaccharides that were quantified in hydrolysed carp skin mucus.

Tested monosaccharide	% of total
fuc	68. ± 16.5
galNAc	0.0 ± 0.0
glc/man	19.6 ± 8.8
NeuNAc/gal	12. ± 8.3

Discussion

In contrast to a variety of protective proteins and their functions described in the skin and gut mucus layer in fish, knowledge about its constituting molecules (so-called “mucins”) is scarce. However, fish gill and intestinal mucins appear to be similar to mammalian mucins [127]. The present paper presents some characteristics of mucin molecules which, at least for mammals, are closely attributed to functional characteristics.

In accordance to Aristoteli and Willcox (2003), histochemical methods and lectin analysis were applied to identify structural components of intracellular glycoproteins and to display the terminal glycosylation.

With histochemical methods, skin goblet cells were stained with a similar intensity (moderately), only acidic glycoproteins were stained somewhat more intensively. This indicates that skin goblet cells contain similar amounts of neutral, acidic and sulphated glycoproteins. Acidic and sulphated glycoproteins are reported to inhibit both the adhesion of some bacteria and the degradation by proteases of glycoproteins. Goblet cells with such mucins are more frequently found in parasite-infected fish [128]. When comparing skin and intestinal goblet cells comparable individuals [78], the skin goblet cells stained stronger for carbohydrates, indicating a higher or different amount of glycoproteins in skin. Furthermore, also lectin histochemistry suggests that glycoproteins in skin goblet cells differ from intestinal goblet cells as the lectins ConA, PNA, DBA, RCA-I, and UEA-I bound less and MAL-1 better to skin sections than to gut sections [78].

Parallel to the histochemical approach, mucin glycoproteins were isolated from the epithelium of carp skin for biochemical characterization to allow for a better quantification of extracellular secreted glycoproteins. After isolation and gel filtration, a biphasic elution profile was obtained for both protein and carbohydrate contents. Similar elution profiles were reported for intestinal mucus of rat and carp [78, 87].

The HMGs of peak I had similar biochemical characteristics to mammalian mucins with a high molecular weight, and as indicated by a low P:C ratio were highly glycosylated. These HMGs are considered to form a gel layer covering the underlying skin epithelium [51] and provide protection for epithelial cells from external challenges or pathogen penetration [80].

Furthermore, a second peak of smaller glycoproteins could be identified. These smaller HMGs co-elute with various “non mucin” glycoproteins, but smaller isolated HMGs are considered to represent mainly small mucin glycoproteins; they may be a result of bacterial mucin degradation [129], or may have been prematurely secreted as shown by a lower glycosylation (a higher P:C ratio). In the present study, peak II HMGs were less glycosylated than larger HMGs. However, the clearly higher glycosylation of skin HMGs of this size compared to similar sized intestinal HMGs [78], might indicate that other mucin-like glycoproteins are released next to the large mucin-like glycoproteins.

In general, the protein core of the glycoproteins is synthesized first and serves as a scaffold for subsequent glycosylation [45]. The dense molecular packing of the protein core with carbohydrate side chains is fundamental for the general properties of mucosal glycoproteins, which include protease resistance, swelling and gel formation due to the uptake of water and binding of ions [51]. In addition, glycans also interact with lectins of microorganisms or parasites and make the mucin domain resistant to proteolytic enzymes from pathogens [94]. As a consequence the dense glycosylation of smaller mucus molecules might play a role in the

defence against bacteria.
Carbohydrate side chains in mucins from mammalian mucosal surfaces are composed of a limited array of oligosaccharides: N-acetyl-α-galactosamin (galNAc), N-acetyl-β-glucosamin ($(glcNAc)_2$), galactose (gal), mannose (man), fucose and neuramic acid (NeuNAc) [45]. Oligosaccharides which are bound directly to the protein core of mucin HGMs are composed primarily of the O-linked galNAc, gal, the N-linked $(glcNAc)_2$, man, fucose and NeuNAc. The carbohydrate side chains O-linked to the protein core through galNAc are often terminated by fucose, NeuNAc or sulphated groups, which give the mucin a negative net charge at neutral pH [130].
For crude skin mucus samples of rainbow trout, *N*-acetylgalactosamine, *N*-acetylglucosamine, 2-deoxyribose, glucose, mannose, ribose, xylose, and inositol appeared as free carbohydrates. Hydrolysis of the mucus material liberated the monosaccharides fucose and galactose and increased amounts of *N*-acetylgalactosamine and *N*-acetylglucosamine [100] .
The unidentified sugar in this study is probably ribose [131]. Ribose and 2-deoxyribose were the predominant sugars in rainbow trout skin mucus [100]. Ribose is probably not a predominant sugar in carp skin mucus, as the peak area was not large.
Fucose is also a predominant sugar in hydrolysed rainbow trout skin mucus [100]. In the present study fucose was by far the most predominant sugar in carp skin mucus. In mammals, fucose content of intestinal mucus or anal gland mucus, may vary to a great extent but may be especially important for the viscoelasticity of the mucus [132]. Although fucose was the predominant monosaccharide detected by HPLC, no terminal bound fucose could be detected by lectin histochemistry and only in low levels in the lectin-ELISA. This result corresponds with previous findings on cyprinid intestinal mucins [78, 99]. The lack of fucose, is probably caused by steric hindrance, in which fucose could be located in a position that does not allow a lectin linkage.
Galactose could be detected in relatively high amounts in hydrolysed skin mucus samples of rainbow trout. Unfortunately in the present study, no differentiation could be made in the HPLC between galactose and NeuNAc. Terminal galactose could be detected by lectin histochemistry and lectin-ELISA, but only in small amounts. Slightly higher amounts of galactose are reported for carp intestinal mucins [78]. Especially immature mucins are thought to have a high amount of terminal galactose [133]. This may indicate that the smaller sized mucus molecules found in peak II are not immature as they are supposed to be for intestinal mucus.
The sugar galNAc could not be detected with the method used in this study for carp skin mucus. In skin mucus of rainbow trout galNAc was only present in a low concentration [100]. Although galNAc was not detectable by HPLC, galNAc does seem to be present as terminal galNAc was detected in low amounts by histochemistry with DBA. DBA stained stronger compared to PNA staining, indicating that a restricted amount of galNAc is present in the form of N-acetyl-β-1-3-galactosamine.
In mammals, $(glcNAc)_2$ forms a considerable part of the mucin carbohydrate side chains. The weak staining of the goblet cells, observed in this study, might be due to the demonstrated NeuNAc, since NeuNAc often binds as terminal sugars to glcNAc and hereby hides the glcNAc [134].

Also the binding of ConA was lower for skin mucins than for intestinal mucus of carp [78]. ConA, which detects primarily mannose in the core of N-glycosylated glycoproteins, could not be detected in skin goblet cells of rainbow trout [135].
NeuNAc is terminally bound to oligosaccharides under complete assembly [125] and gives a negative charge to glycoproteins [52]. NeuNAc is thought to protect core sugars against glycosidases, degradation and prevents adhesion of some pathogenic micro organisms, such as bacteria, fungi and virus particles on the skin surface [136]. The presence of NeuNAc in skin mucus of rainbow trout was not described [100]. In lectin histochemistry SNA and MAL-1 stained well, but in lectin ELISA SNA responded only weakly and MAL-1 was not tested. However, SNA was found in a relatively higher percentage in skin mucus compared to gut mucus [78].
In conclusion, goblet cells of the skin mucosa of carp secrete large glycoproteins which show a high similarity in structure and composition to those found in mammals and the intestinal mucus of carp. These findings confirm the idea of an evolutionary conserved barrier system. A biphasic profile was found for both carbohydrates and proteins, with peaks of similar molecule size ranges, which are comparable to intestinal mucus. As for intestinal mucus, secreted molecules from peak I were highly glycosylated. Such glycoproteins form the basis for the mucus layer, which in fishes covers the epithelia of skin, gills and intestinal tract, and shields piscine cells from environmental challenges. The presence of GalNAc, galactose, fucose, mannose and NeuNac acid could be demonstrated in skin mucus. GalNAc appears to be abundantly present and NeuNac might play a more important role in skin mucus than in gut mucus. HMGs from the second peak were also highly glycosylated indicating a different glycoprotein constitution or glycosylation between the skin and gut, which may be correlated to the different functions, although both organs can be considered as important mucosal barriers in fish.

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Chapter 4

Changes in skin mucus of common carp, *Cyprinus carpio* L., after exposure to water with a high bacterial load

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Abstract

Water in aquaculture systems may contain a high load of microorganisms. Reduction in overall bacterial tank water load improves fish health and growth parameters. In this study, the effect of an increase of overall bacterial load in tank water on carp skin mucus was assessed. Intracellular and released high molecular weight glycoproteins (HMGs) of carp skin mucus were analysed for changes using histological, histochemical and biochemical techniques. Increase of bacterial load did not induce obvious clinical responses in carp, but the skin of exposed carp responded quickly. The amount of skin mucus HMGs isolated increased as well as their total glycosylation. An increased goblet cell number was observed for all carbohydrate stainings, but most clearly for acidic glycoconjugates. A change in the terminal presence of some sugars was also seen. After the initial response of carp, an adaptation to the higher bacterial load in the water appeared to occur as mucins had a higher glycosylation. The changes observed suggest that these skin mucus adaptations are part of a primary defence mechanism of mucosal epithelia, even at a low pathogenic pressure.

Introduction

The mucosal surface of the skin, gills and intestinal tract is the primary interface between a fish and its environment. The outer constituent of this barrier is a mucous gel which forms a layer of adherent mucus covering the epithelial cells and is secreted from goblet cells in the epithelium. There are many proposed roles of mucus in fish, including respiration, ionic and osmotic regulation, reproduction, locomotion and defence against microbial infections ewed [reviewed by 6]. Mucus consists of many components, two of which are predominantly important in gel formation. These are water and glycoprotein conjugates with a large content of high molecular weight oligosaccharides, called 'mucins' [1, 51-53, 104]. In mammals, alterations in mucus composition are often associated with severe disease signs like diarrhoea. In fish, excess mucous production on skin and gills was reported after exposure to various noxes, such as adverse pH conditions, toxins or pathogenic micro organisms. Altered mucus secretion is considered to modulate exchange processes during respiration, ammonia excretion or ionic and osmotic regulation and thus may have a serious impact on fish physiology.

Altered mucus secretion is also considered an important factor in the protection against pathogen invasion. For infection, pathogens have to attach to and penetrate the mucous layer. The pathogenic effect of pathogens such as *Entamoeba histolytica*, [137] or *Helicobacter pylori* [138] to the epithelium of the mammalian intestinal tract was found to depend on the ability of these pathogens to colonize, degrade or alter the secretory profile of host mucins on infection. Peroral application of endotoxin [139] or motile aeromonads[140] has been found to alter the profile of secreted intestinal high molecular weight glycoproteins (HMGs) in carp. Lipopolysaccharides and one aeromonad induced mild alterations, whereas another strain of Aeromonas sp. induced bigger changes [140].

In recirculating aquaculture systems, the water may contain a high load of microorganisms. Empirical data during farm operations showed that a reduction of overall bacterial load of

tank water improved fish health and growth parameters. This observation lead to the study reported here in which the effect of water with a high bacterial load on the mucus cover of carp skin was investigated. Intracellular and released HMGs of carp skin mucus were analysed for changes using histological, histochemical and biochemical techniques.

Material and methods

Fish

Parasite free, virus free and clinically healthy sibling common carp (*Cyprinus carpio* L.) from a single crossing (E20 x R8, Wageningen Agricultural University, Netherlands) were used throughout the study. Carp were raised and kept in filtered recirculated tap water at 20-23° C. For this study, 12-18 months old carp were used. For mucus collection, the carp were killed by adding 500 mg tricaine methane sulphonate L−1 (Sigma). All fish were weighed and measured.

Water with a high bacterial load

To create tank water with a high bacterial load *Aeromonas hydrophila*, isolated from organically polluted water from a commercial duck farm, was added to the tank water. The *A. hydrophila* was biochemically characterized using the API 20 NE system (Bio Mérieux). The strain was tested negative for the type three secretion system (TTSS), which is required for pathogenesis of *A. hydrophila* [141], using the method of Yu *et al*. The bacteria were propagated on standard blood agar) and then suspended in 0.9 % saline.

In exposure experiments, *A. hydrophila* was added to tank water with a bacteria load of approximately 9 x 10^9 CFU ml^{-1}. Carp (n = 60) were exposed for 20 days. Before exposure and on day 1, 3, 6, 13 and 20 post-exposure, 10 carp were collected. On each sampling day, tank water was sampled for monitoring bacterial numbers which were counted in a cytometer under phase contrast optics. Before addition of *A. hydrophila*, the tank water had a bacterial load of 1 x 10^3 CFU. Concentrations of 9 x 10^4 CFU (day 1), 5 x 10^4 (day 3), 6 x 10^4 (day 6), 7 x 10^4 (day 13), and 6 x10^4 (day 20) CFU ml^{-1} were found after exposure. For sampling, carp were removed from their tank in a small plastic container with water (to avoid damage to the mucosal layer).). Of the ten fish sampled on each occasion, four carp (88.1 g ± 10.5) were processed for skin histology and six carp (132.9 g ± 19.3) for skin mucus collection.

Histology and histochemistry

Killed carp were immersed in Bouin's fluid for 4 days at room temperature. From each fish, skin samples were taken from three different locations of the dorsolateral body region: cranial, medial and caudal from the lateral line. Samples were de-calcified with ethylene diamino tetra acid (EDTA), washed, dehydrated in a series of graded ethanols, embedded in paraffin wax or glycol methacrylate (Technovit) and 4 µm sections cut. Glycol methacrylate embedded material was used to measure epidermis thickness, which was determined with the CUE 3 program (Olympus, Version 4.5; Galai Productions Ltd). Per fish and per location thickness was measured three times each from five different fields.

Paraffin-embedded material was stained with haematoxylin-eosin (H&E) and for the presence of complex carbohydrates. Mucin carbohydrates were visualized histochemically [85] with periodic-acid Schiff (PAS) alcian blue 8GX pH 1.0 (AB1.0) and pH 2.5 (AB2.5) as well as AB2.5/periodic-Acid-Schiff (AB-PAS). The AB1.0 method stains sulphated glycoconjugates; the AB2.5 method stains acidic glycoconjugates; the PAS reaction visualises neutral glycoconjugates. All sections were studied by light microscopy (Zeiss).
The pH-stains were evaluated on the basis of the goblet cell reaction. The number of responding goblet cells was counted for the AB1.0-, AB 2.5-, and H&E stained sections in an area comprising all the cells above 100 epithelial stratum basal cells. The number of stained goblet cells was counted twice per fish and per location and an average plus standard deviation of all measurements per fish was calculated. Besides the goblet cells, also eosiniphilic granular cells, rodlet cells and neutrophils were counted. Results are not shown as no differences between days post-exposure to water with a high bacterial load were observed.
Terminal sugar residues were specifically stained with different lectins (see Table 1). Paraffin-embedded sections were incubated with several biotin-labelled lectins at a concentration of 10 µg mL−1 in 0.1 m phosphate-buffered saline (PBS), pH 7.2 for 30 min at room temperature. The following lectins were used: PNA, ConA, DBA, WGA, UEA-I (Sigma), MAL-1 (formerly known as MAA), RCA-1 and SNA (Vector) (Table 1). Lectin binding was visualized with peroxidase (PO) conjugated streptavidin and a diamino-benzidine-hydrogen peroxidase system (DAB; Biogenex). The sugar-binding specificity was tested by the addition of 0.1 m inhibitory sugar solution [86]. To detect endogenous peroxidase activity, sections were incubated with DAB alone. Goblet cell staining was estimated on an arbitrary scale (no; faint; weak; moderate; strong; very strong response).

Table 1 Lectins used in histology and ELISA in this study and their target terminal sugars.

Acronym	Lectin	Target sugars
PNA	*Arachis hypogaea*	β–gal(1→3)galNAc
ConA	*Canavalia ensiformis*	α–man and α–D-glc
DBA	*Dolchios biflorus*	α-galNAc
MAL-1	*Maackia amurensis* I	αNeuNAc(2→3)gal
RCA-1	*Ricinus communis* I	galNAc and β-gal
SNA	*Sambucus nigra*	αNeuNAc(2→6)gal
WGA	*Tricium vulgare*	$(glcNAc)_2$ and NeuNAc

Isolation of HMGs from carp skin

Six euthanized carp were individually transferred to a container filled with 100 ml of 100 mM ammonia bicarbonate, pH 7.8 [122], agitated by hand for 10 min on ice and then removed from the container. The mucus-containing solution was mixed with 100 ml of ice cold protease inhibitor phenyl methyl sulphonyl fluoride (PMSF, 1 mM) and ultracentrifuged at 13 000 x g for 30 min at 4°C (Beckman CoulterA). The supernatant was collected and stored at -80°C. Samples were concentrated by ultrafiltration (exclusion limit 30 kDa; Amicon) to a final volume of 2 mL.

Concentrated samples containing HMGs were subjected to downward gel chromatography on a 34 x 0.9 cm Sepharose CL-4B column (flow rate 5.2 ml h^{-1}, fraction size 1.3 ml; Sigma). Fractions were analysed for carbohydrate by the PAS reaction at 550 nm and for protein by the Bradford at 580 nm in a multiwell plate reader (BMG) [89, 90, 123].

A biphasic profile (Fig. 1) was obtained from all samples after downward gelfiltration and subsequent staining for carbohydrates and proteins. The first peak of the biphasic profile is considered to represent mucins which adhere to the epithelium (EM), and the second peak mucin constituents mixed with luminal proteins (LM) [95]. For further analysis of protein and for carbohydrate content three size areas were used [Peak I (EM): fractions 7–10, transition area (TA): fractions 11–15 and Peak II (LM): fractions 16–22, elution volumes 9.1–14.3, 14.3–20.8 and 20.8–29.9 mL, respectively]. The mucin ratio between size areas (Peak I: TA: Peak II) was expressed as a percentage and was calculated with corrected carbohydrate ODs: (summated OD per size area) × (summated OD from fraction 7-22)$^{-1}$.

The protein:carbohydrate ratio (PC) was calculated to determine the degree of glycosylation. A high glycosylation is reflected by a low PC ratio as the amount of carbohydrates increases relative to the protein core.

Mucin amount was determined using the summed optical density (OD) values of the samples by PAS reaction and the summed OD values of a pig gastric mucin standard. As each fish has a different body surface, a size correction factor was used for the ODs of each fish to improve comparison of the skin mucin amount between sampling days. A size correction factor was used for protein and carbohydrate data from skin mucus from individual carp. This size correction factor [(weight × 100) × (total length3)−1; 1.74 (1.69 ± 1.95)] is a modified Fulton's condition factor [124] and includes the total fish length to account for the tail skin mucus.

Results were expressed as median and 25-75% quartiles and analysed using an ANOVA on ranks.

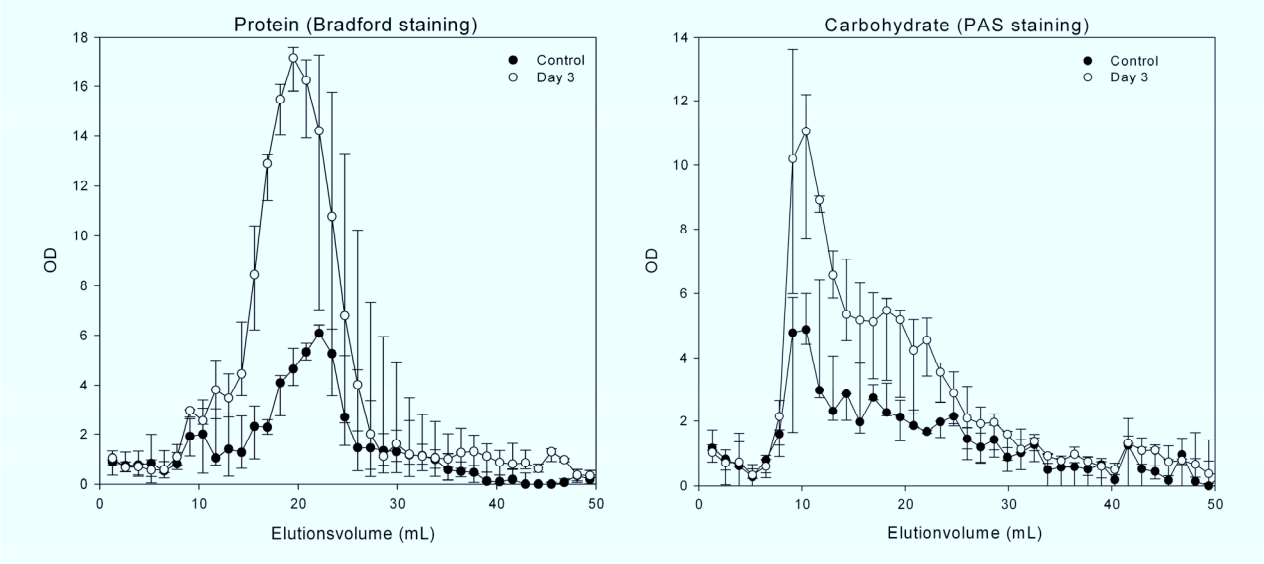

Fig. 1 Protein and carbohydrate content of glycoproteins (corrected with a modified Fulton's factor) in skin mucus eluted on a sepharose CL-4-B column on day 0 and day 3 post-exposure to water with a high bacterial load. Median and 25-75 quartiles of six carp.

Determination of the terminal glycosylation pattern

The terminal glycosylation pattern was determined by lectin ELISA as described before [78]. For this fractions were pooled per size area (Peak I, TA, Peak II). Fraction pools were incubated in microtiter plates (Nunc Maxisorb) overnight at room temperature. After blocking with 1 % bovine serum albumin solution in PBS, samples were incubated with biotin labelled lectins (10 µg ml^{-1} in 0.1 M PBS) for 30 min at room temperature. The following lectins were used: ConA, DBA, RCA-I, SNA, UEA-I (Table 1). Lectin binding was visualized by incubation with streptavidin-horseradish-peroxidase (HRP) and orthophenylenediamine (OPD)-peroxide (Dako Chemicals) for 30 min at room temperature. After 15 min, the reaction was stopped by the addition of 0.5 m sulphuric acid, and the optical density was measured in a plate reader (BMG) at a wavelength of 485 nm. Results were expressed as median and 25–75% quartiles.

Results

No clinical alterations were observed in fish during exposure to the water with a high bacterial load.

Histology and histochemistry

After exposure to water with a high bacterial load, epidermal thickness significantly decreased on days 6, 13 and 20 compared to the control. Differences between the number of goblet cells were not apparent with the naked eye because of a high variation in numbers. However, after counting, an increase in goblet cell number (with H&E stain) was observed (Table 2) after exposure. These changes were not significant.

With increasing duration of the exposure, the number of goblet cells containing acid glycoconjugates (AB 2.5 positive) was increased (significantly higher on days 6 and 20 compared to the control). The numbers of goblet cells with sulphated glycoconjugates (AB1.0 positive) remained unchanged. Staining intensity of the goblet cells was moderate and relatively uniform for all carbohydrate stains (Fig. 2). Staining intensity remained relatively stable after exposure to water with a high bacterial load (Fig. 2, Table 3).

Table 2 Epidermal thickness and number of stained goblet cells of carp (H&E, AB1.0, AB2.5) after *Aeromonas hydrophila* exposure.

	Days post *A. hydrophila* exposure					
	Control	1	3	6	13	20
Epidermis thickness (µm)	106.4 ± 18.8	97.6 ± 18.8	84.1 ± 14.4	81.1 ± 6.0[a]	79.5 ± 8.1[a]	78.2 ± 10.7[a]
Goblet cell number						
HE stain	42.0 ± 2.5	47.7 ± 4.9	44.2 ± 12.8	47.5 ± 3.1	45.6 ± 4.7	50.5 ± 9.4
AB1.0 stain	20.3 ± 5.5	20.5 ± 5.8	20.7 ± 6.8	24.4 ± 5.5	25.0 ± 6.8	21.6 ± 3.6
AB2.5 stain	32.8 ± 6.5	37.3 ± 6.0	32.6 ± 12.1	44.9 ± 7.0[b]	42.1 ± 8.0	48.1 ± 10.5[b]

[a]Epidermis thickness was significantly lower on day 6, 13 and 20 compared to control.
[b]The number of goblet cells which stained for acidic high molecular weight glycoproteins was significantly higher on days 6 and 20 compared to control.

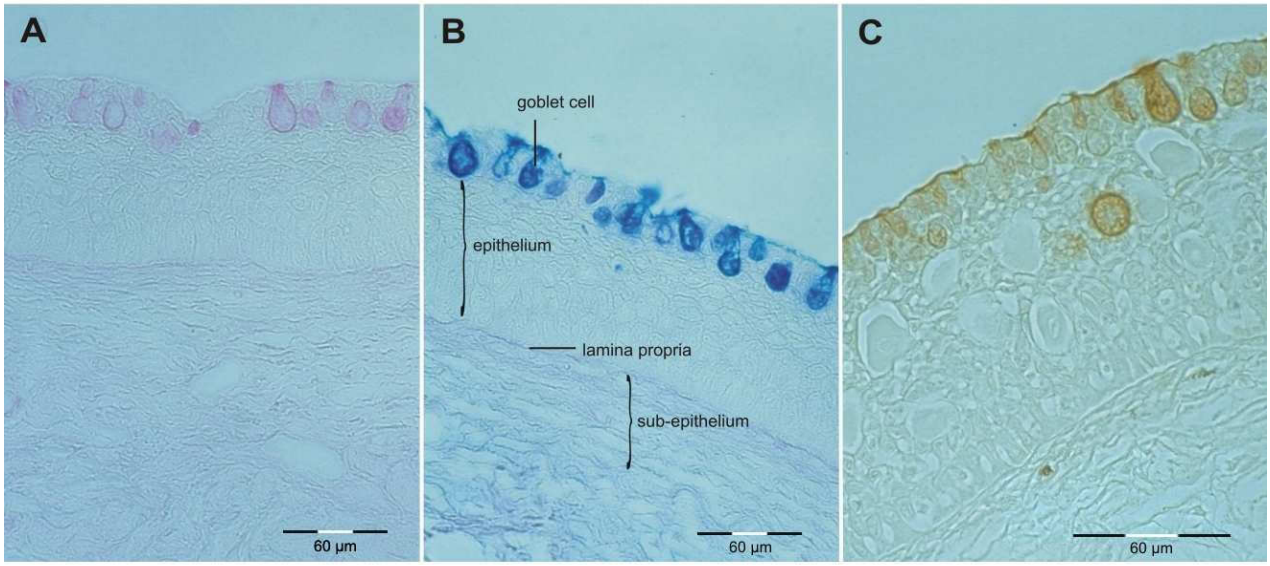

Fig. 2 Skin of carp stained for (a) neutral glycoconjugates (PAS), (b) sulphated glycoconjugates (AB1.0) and (c) αNeuNAc(2→6)gal (SNA). Sections are shown of untreated individuals. After bacterial exposure, changes in glycoprotein content were not obvious, but could be identified by statistical analysis

When lectin binding patterns were considered, the goblet cell content was not clearly changed in carp skin after exposure to water with a high bacterial load. However, an alteration in the terminal presence of αNeuNAc(2→3)gal was seen at day 1 post-exposure by a significantly reduced binding of MAL-1 (Table 3). The terminal presence of α-galNAc could be recognized at day 6 post-exposure by a significantly reduced binding of DBA (Table 3).

Release of HMGs from carp skin

After exposure to water with a high bacterial load, the amount of HMGs isolated increased and had doubled by day 3 (Table 4). Mucin quantity then decreased and by day 20 the quantity was significantly lower compared to day 3. By day 13, the relative amount of adherent mucins had increased while the relative amount of luminal mucins (LM) decreased (Table 4). However, this difference in the distribution of the mucin molecules over the different size fractions was not significant.

Directly after exposure, total glycosylation of mucus HMGs increased and then decreased as indicated by a higher PC ratio. On day 13, molecules of all size fractions had the highest PC ratio, which indicated reduced glycosylation throughout the range of HMG molecules. However, the main reduction in glycosylation was found for molecules from TA and LM (Table 5). The lowest PC ratio was found for molecules of all size fractions on day 20, which indicated an increased glycosylation throughout the range of HMG molecules.

Table 3 Intensity of carbohydrate staining and lectin binding to carp skin goblet cell high molecular weight glycoproteins after *Aeromonas hydrophila* exposure

	Days post *A. hydrophila* exposure					
	Control	1	3	6	13	20
Histology						
Periodic acid-Schiff (PAS)	Moderate to strong	Moderate	Moderate to strong	Moderate to strong	Moderate	Moderate to strong
AB/PAS	Moderate to strong	Moderate	Moderate	Moderate to strong	Moderate to strong	Moderate to strong
AB1.0	Moderate to strong	Moderate to strong	Moderate	Moderate to strong	Moderate to strong	Moderate to strong
AB2.5	Moderate to strong	Moderate	Moderate	Moderate to strong	Moderate to strong	Moderate to strong
Lectin binding						
ConA	No to faint	No to faint	No to faint	No to faint	No to faint	No to faint
DBA	Weak	Weak	Weak	No to faint[a]	Weak to moderate	Weak
MAL-1	Weak to moderate	Faint[a]	Weak to moderate	Weak to moderate	Weak	Weak to moderate
PNA	No to faint	No to faint	No to faint	No to faint	Faint	No to faint
RCA-1	No to faint	No to faint	No to faint	No to faint	No to faint	No to faint
SNA	Weak to moderate	Weak to moderate	Weak to moderate	Weak	Moderate	Weak to moderate
UEA-I	No to faint	No to faint	No to faint	No	No to faint	No
WGA	faint to weak	faint to weak	faint to weak	faint to weak	faint to weak	faint to weak

[a]Binding of MAL-1 was significantly lower on day 1 than on all other day, except for day 13. Binding of DBA was significantly lower on day 6 than on all other days.

Table 4 Percentage of mucin weight compared to control and distribution of carp skin mucin high molecular weight glycoproteins (%) over the three size fractions after *Aeromonas hydrophila* exposure (mean ± SD)

	Days post *A. hydrophila* exposure					
	Control	1	3	6	13	20
Mucin weight (%) compared to control	100 ± 14	143 ± 64	206 ± 34[a]	131 ± 60	133 ± 58	73 ± 39[a]
Distribution						
Adherent mucins	36 ± 17	42 ± 11	46 ± 14	50 ± 19	41 ± 16	45 ± 8
Transition area	35 ± 14	30 ± 3	31 ± 5	28 ± 6	32 ± 8	28 ± 11
Luminal mucins	29 ± 11	28 ± 8	23 ± 9	23 ± 13	27 ± 22	27 ± 13

[a]Mucin quantity on day 3 was significantly higher compared to control. On day 20, mucin quantity was significantly lower compared to day 3.

Table 5 Protein/carbohydrate ratio of skin high molecular weight glycoproteins (HMGs) from *Aeromonas hydrophila*-exposed carp of different size fractions (AM: Adherent mucins, TA: , LM: Luminal mucins). Protein/carbohydrate ratio is an index for glycosylation. Carbohydrate content was monitored by periodic acid-Schiff reaction, and the protein by Bradford assay

	Days post *A. hydrophila* exposure					
Size fractions	Control	1	3	6	13	20
AM	0.6 (0.5 - 0.7)	0.3 (0.2 – 0.3)	0.3 (0.3 - 0.6)	0.5 (0.4 – 0.6)	0.6 (0.4 – 0.6)	0.2 (0.2 – 0.4)
TA	1.3 (1.1 - 1.4)[a]	0.9 (0.7 – 1.2)[a]	2.4 (1.9 – 3.3)[a]	1.4 (1.1 – 2.7)	2.8 (1.6 – 3.3)[a]	0.8 (0.7 – 0.9)[a]
LM	2.1 (0.9 - 3.0)	2.2 (1.9 – 2.7)	3.2 (2.5 – 5.2)	2.8 (2.8 – 4.7)	5.3 (3.9 – 7.9)[b]	0.4 (0.4 – 0.6)[b]
Total	1.2 (0.8 - 1.7)	1.0 (0.9 - 1.2)	1.6 (1.6 - 1.8)[c]	1.6 (1.0 - 2.6)	2.3 (1.4 - 2.8)[c]	0.4 (0.4 - 0.5)[a,c]

[a]On day 3 and 13 the glycosylation of HMGs from TA was significantly lower compared to control and day 1. On day 20 the glycosylation of HMGs from TA was significantly higher compared to control and day 3 and day 13.
[b]On day 13 the glycosylation of luminal mucins was significantly lower compared to day 1. On day 20 the glycosylation of luminal mucins was significantly higher compared to control and days 1, 3 and 13.
[c]On day 3 and day 13 the total glycosylation of the HMGs was significantly lower compared to day 1. On day 20 the total glycosylation of the HMGs was significantly lower compared to control and days 3, 6 and 13.

Terminal glycosylation pattern of skin HMGs

Lectin binding to molecules from carp mucin size pools changed after exposure to water with a high bacterial load (Table 6). UEA-I binding was weak in all size fractions on all sampling days. Lectin binding to HMGs was lower on days 1, 3, 6 and 13 compared to the control. Binding of ConA, DBA, RCA and SNA was generally lowest on day 3. Lectin binding to HMGs was generally higher on day 20 compared to the control.

On day 20, binding to adherent mucin molecules was significantly higher than at day 3 for ConA, DBA and RCA-1. For DBA, binding to TA was also significantly higher on day 20 compared to day 3 and day 6; binding to LM was significantly higher on day 20 compared to day 1 and day 3. For RCA-1, binding to TA was also significantly higher on day 20 compared to day 3.

DISCUSSION

During the present study the bacterial load of the water was increased from 1×10^3 CFU ml^{-1} before exposure to approximately 5 till 9×10^4 CFU ml^{-1} after addition of *A. hydrophila*. Exposure to water with a high bacterial load did not induce obvious clinical responses in carp, but the skin of exposed carp responded quickly with increased mucous secretion. At 3 days post-exposure, significantly more (1.0 mg) HMGs were isolated from the skin of challenged carp, versus approximately 0.5 mg from control carp. This coincides with the reduced glycosylation of the HMG which suggests a hypersecretion of 'immature mucins' which would allow adherent bacteria to be washed off.

Table 6 Lectin binding to separated high molecular weight glycoproteins from the skin of the common carp exposed to *Aeromonas hydrophila*. Lectins were applied to glycoproteins from the pooled fractions of adherent mucins (AM), transition area (TA) and luminal mucins (LM). Shown are summated median OD values and 25 - 75 percentiles

		Days post *A. hydrophila* exposure					
		Control	1	3	6	13	20
ConA	AM	5.76 (4.75 - 7.90)	3.73 (3.35 - 5.27)	3.22 (1.61 - 4.30)[a]	3.90 (2.28 - 7.18)	2.43 (1.98 - 6.45)	11.18 (9.46 - 11.82)[a]
	TA	12.98 (9.58 - 22.77)	9.87 (4.98 - 16.21)	5.78 (2.81 - 7.08)	6.81 (3.65 - 11.17)	10.55 (9.90 - 16.25)	15.95 (7.38 - 24.56)
	LM	11.95 (7.70 - 15.23)	7.59 (4.96 - 9.91)	3.69 (1.24 - 5.38)	5.21 (4.17 - 8.74)	3.41 (1.34 - 9.72)	18.64 (13.14 - 23.64)
DBA	AM	9.24 (7.91 - 10.41)	6.31 (5.09 - 8.86)	4.21 (3.60 - 4.82)[a]	6.36 (5.30 - 9.10)	6.67 (4.07 - 15.07)	18.08 (9.88 - 25.93)[a]
	TA	5.72 (4.34 - 8.82)	1.36 (0.75 - 3.63)	2.00 (1.10 - 7.09)[a]	8.16 (3.79 - 9.69)[a]	4.56 (4.25 - 4.61)	30.01 (25.03 - 42.21)[a]
	LM	11.94 (11.22 - 14.15)	5.30 (2.92 - 9.11)[a]	2.36 (1.30 - 4.72)[a]	6.63 (6.08 - 9.94)	7.59 (3.09 - 19.26)	31.74 (13.89 - 51.58)[a]
RCA-I	AM	2.35 (1.65 - 2.85)	2.09 (1.71 - 2.97)	1.03 (0.36 - 1.49)*	2.72 (0.67 - 3.22)	2.40 (1.43 - 2.70)	7.49 (3.58 - 12.15)*
	TA	2.77 (0.60 - 3.66)	1.09 (0.39 - 1.90)	0.48 (0.14 - 1.55)*	3.28 (0.74 - 3.83)	1.56 (0.89 - 2.09)	8.23 (6.11 - 12.05)*
	LM	2.61 (2.33 - 2.80)	2.09 (1.51 - 2.90)	0.25 (0.15 - 0.91)	2.47 (0.68 - 3.28)	2.70 (1.75 - 4.44)	8.84 (4.25 - 14.60)
SNA	AM	2.12 (1.09 - 2.18)	1.83 (1.70 - 1.98)	0.63 (0.32 - 0.92)	1.38 (0.64 - 1.63)	1.23 (0.79 - 1.32)	3.50 (2.50 - 5.30)
	TA	3.20 (1.60 - 3.69)	2.32 (1.50 - 3.42)	0.94 (0.48 - 1.28)	1.82 (1.27 - 2.58)	1.86 (1.45 - 3.85)	6.02 (4.04 - 7.44)
	LM	3.52 (2.12 - 3.98	2.68 (2.43 - 2.88)	1.17 (0.56 - 1.29)	1.71 (1.21 - 1.98)	2.23 (0.39 - 2.47)	5.05 (3.48 - 7.55)
UEA-I	AM	0.22 (0.05 - 0.48)	0.28 (0.13 - 0.59)	0.10 (0.07 - 0.11)	0.21 (0.14 - 0.44)	0.26 (0.14 - 0.62)	0.11 (0.01 - 0.82)
	TA	0.19 (0.04 - 0;31)	0.14 (0.06 - 0.37	0.01 (0.00 - 0.62)	0.79 (0.19 - 1.24)	0.17 (0.10 - 0.46)	0.97 (0.39 - 1.88)
	LM	0.31 (0.08 - 0.49)	0.24 (0.13 - 0.59)	0.02 (0.00 - 0.20)	0.43 (0.11 - 0.79)	0.37 (0.01 - 0.51)	0.06 (0.02 - 0.27)

[a]On day 20, binding to adherent mucin molecules was significantly higher than at day 3 for ConA, DBA and RCA-1. For DBA binding to TA was also significantly higher on day 20 compared to day 3 and day 6, binding to LM was significantly higher on day 20 compared to day 1 and day 3. For RCA-1 binding to TA was also significantly higher on day 20 compared to day 3.

The mucous blanket is secreted from goblet cells in the epithelium, and mucous secretion in many studies is estimated from altered goblet cell numbers or staining properties in histological or histochemical observations [83, 112]. A rapid increase of skin mucus secretion after exposure of rainbow trout to acidified [142] or organically fertilised water [110] was suggested based on such histological investigations, as trout skin responded with an initial increase in the number of goblet cells, which was followed by a sharp decline because of mucus release from mature cells. In this study, an increase in goblet cell number was also observed for all carbohydrate stainings. The increase in the number of goblet cells was most clearly observed for the acidic glycoconjugates. A clear decrease in epidermal thickness was also observed. On days 6, 13 and 20 post-exposure, the thickness of the epidermis was significantly thinner compared to the control. A decrease in the epidermal thickness has been correlated with stress [143].

Only minor differences in the intensity of carbohydrate staining and lectin binding could be observed in the skin goblet cells after exposure to water with a high bacterial load. However, a significantly lower presence of terminal bound αNeuNAc(2→3)gal was found on day 1 compared to the other days. Neuraminic acid provides negative charge to the mucin molecules and reduces bacterial binding [144]. A decreased presence of terminal bound sugars on days 1, 3, 6 and 13 after exposure was also observed for released mucins, although the decrease was not significant. However, the decreased presence of the sugars was reflected on day 13 by a significantly increased P:C ratio (low glycosylation) for the TA and the LM.

On day 20 post-exposure, glycosylation had clearly increased compared to day 13 in the TA and the LM. This is in agreement with the higher binding of the lectins used, as found in the binding assay. Mannose (ConA) and galNAc (DBA, RCA-1) especially showed a higher binding.

In conclusion, this study indicates that carp skin mucosa responds rapidly to water with a high bacterial load, even if the bacteria involved are considered to be non-pathogenic, with an increased secretion of mucin molecules especially of low glycosylated ones. This corresponds to well-characterized reactions of mucosal tissue in mammals to bacterial challenges, which were associated with a goblet cell hyperplasia and an increased release of mucins, mainly of Muc2 and Muc5AC/Muc5B molecules [145 and references therein]. The release of these mucins from mucosal epithelia was found to be triggered by bacterial products or live bacteria ([146 and references therein]). Our data, together with previous observations on the application of lipopolysaccharide to the intestinal mucosa of carp [139] suggest that this is a primary defence mechanism of mucosal epithelia over a wide range of organisms.

After the initial response of carp to bacteria an adaptation to the higher bacterial load in the water seems to take place as mucins have a higher glycosylation. Similar responses were observed from the mucosa of germ-free rats [123] and zebrafish [147] after colonisation with the resident microbial flora.

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Chapter 5

Molecular cloning and expression of two β-defensin and two mucin genes in common carp (*Cyprinus carpio* L.) and their up-regulation after β-glucan feeding

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Supplemental files can be found on:
http://www.sciencedirect.com/science/article/pii/S1050464811004712

Abstract

In this study, we described the partial structure, mRNA tissue distribution and regulation of two carp *mucin* and two *β-defensin* genes. This is the first description of these genes in fish. The genes might provide relevant tools to monitor feed-related improvements of fish health under aquaculture conditions. Carp *mucin 2* and *mucin 5B* genes show a high similarity to their mammalian and avian counterparts. The carp *β-defensin 1* and *β-defensin 2* genes cluster together well with their piscine family members. The influence of a β-glucan immunomodulant on the expression of these genes in mucosal tissues could be confirmed for the first time. *Muc5B* expression was significantly increased in the skin. For *Muc2* no significant up- or down-regulation could be observed. Significantly higher expression levels of *β-defensin 2* in gills and both *β-defensin* genes in skin were found. Thus, the mucosal system can be influenced by the addition of β-glucans to the food.

Introduction

Body surfaces of multicellular organisms are defended by epithelia, which provide a physical barrier between the internal milieu and the external world. In fish, skin, gills and intestine are mucosal barriers, in which the epithelium is covered by a mucus overlay [6]. In this mucus layer, particles, bacteria or viruses are entrapped and removed from the mucosa by the water current or, in the intestinal tract, by peristaltic movements [148]. Infections occur only when a pathogenic organism can colonise and/or invade mucosal barriers [1]. Mucus is a complex fluid and its composition varies throughout the epithelial surface. The main components of the mucus layer are large filamentous, highly glycosylated glycoproteins called mucins. Mucins are strongly adherent and play a major role in the defence of the mucosae [9, 43]. Mucins give mucus its viscous properties and form a matrix in which a diverse range of antimicrobial molecules can be found [28].

Based on biochemical characterisation, 19 genes are currently assigned to the mucin family [see 149, 150] and are named "MUC-number" for humans or "Muc-number" for other species [9]. While in mammals the structure of mucin type genes and their critical role in the infection process in the gastrointestinal tract [145] or airways [151] are confirmed, to our knowledge mucin genes are yet to be identified in fish.

Besides mucins, antimicrobial peptides (AMPs) are a part of the barrier function as they are the first challenge for pathogens [28]. Fish mucus contains antimicrobial peptides such as piscidins [57] and defensins [9]. Defensins are most effective in killing microbes by compromising cell membrane integrity [152, 153]. Antimicrobial activity of defensins has been documented in mammals [review by 154] as well as in fish [64]. Homologues to β-defensin 1 (BD1) and β-defensin 2 (BD2) were recently identified from in silico studies in several fish species [155, 156].

Despite the physical barrier function of mucus and their bio-active substances, protection of fish against infectious diseases is a major challenge in aquaculture worldwide, and losses due to infectious diseases limit profitability. The use of antibiotics and vaccination has partially alleviated this problem. Probiotics and prebiotics, such as β-glucans, are gaining more and more interest for use in the therapy and prevention of human diseases as the antibiotic resistance development and antibiotic residues in fish have raised concern [157, 158]. For many fish species, the immune-modulatory activity of β-glucan has been reported [159-164]. Recent preliminary research data indicates that β-glucan promotes an antimicrobial response [165]. Furthermore, β-glucans can potentially affect mucin structure and/or function as they interact with innate signaling pathways in mucus producing cells.
In this study, we described the partial structure and mRNA tissue distribution of two carp mucin and two β-defensin genes. Furthermore, the influence of feeding the immunomodulant β-glucan on gene expression in mucosal tissues has been monitored in this study.

Materials and methods

Animals and sampling
For identification of genes and gene expression in naïve four year old, parasite and specified-virus free sibling carp (92.1 ± 4.6 g) from a single crossing (E20xR8, Wageningen University, the Netherlands) were used. Carp were fed with commercial carp feed (Pro Aqua, Skretting, Germany). Brain, liver, kidney, head kidney, spleen, skin, gills, first intestinal segment and second intestinal segment were used for mRNA tissue.
For the β-glucan experiment, ten month old (78.4 ± 9.0 g) UR (PAS-IIA, Poland) which were raised under pond conditions, were transferred to a recirculation system with 90 l aquaria. Carp were then fed with pellets (1% body weight per day) containing 0% β-glucans (supplied by TETRA, Germany). After two weeks the carp were divided into two treatment groups (n = 5 for each group). The first group continued on the 0% β-glucans diet, while the other group was fed with the same feed that was supplemented with β-glucans. Fish were sampled after 14 days of feeding, based on results of other groups [166, 167] and manufacturer's suggestions. Tissue samples for qPCR analysis were taken from skin, gills, first intestinal segment and second intestinal segment. See Supplementary File 1 for diet composition and information.

cDNA production

Total RNA was extracted using the Trizol reagent (Invitrogen, Germany) from 20 mg of collected tissue. Any remaining genomic DNA was digested with 2 U of DNase I (Fermentas, Germany). cDNA was synthesised from 900 ng total RNA. To find (partial) sequences of two mucin and two β-defensin genes, a mix of skin and intestine cDNA was synthesised with the SMART RACE cDNA amplification kit (Clonetech, USA). For gene expression, cDNA was synthesised using the 200 U Maxima RT and a mix of 25 pM random hexamer primers, 25 pM oligo dT(18) and 0.5 mM dNTP mix (Fermentas, Germany). cDNA samples were further diluted 1:20 prior to real-time quantitative PCR analysis.

Amplification and sequencing of carp mucin and β-defensin genes

For the secreted mucin genes Muc2 and Muc5 B as well as the β-defensin genes BD1 and BD2, primers were designed on the basis of known vertebrate sequences (Table 1). For carp Muc5B several primers were used (see Supplemental File 2 for primers and cloning strategy). The primers were used in an endpoint PCR, performed with the Advantage 2 PCR kit (Clonetech, USA) with a Mastercycler gradient (Eppendorf, Germany). Products amplified by PCR were ligated and cloned with the StrataClone PCR cloning kit (Stratagene, Germany). DNA was isolated from colonies with the NucleoSpin Plasmid kit (Macherey-Nagel, Germany) and sequenced (Eurofins MWG Operon, Germany).

Sequences were checked for homologues in the GenBank using the program BLAST (http://www.ncbi.nlm.nih.gov/blast). Structural analysis of the genes was conducted at the protein level (nucleotide translation using http://expasy.org/tools/dna.html). Sequences were aligned with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). Phylogenetic trees (Supplemental Files 3, 4 and 5) were depicted on the overall amino acid sequences by the neighbour-joining method implemented in the Mega5.05 [168]. Newly identified carp sequences were used to design qPCR primers (Table 1).

Table 1 Endpoint primers for partial sequences of *Mucin 2* (*Muc2*, 805 bp), *Mucin 2* (*Muc5B*, 3212 bp), *β-defensin 2* (*BD2*, 64 bp) and the full sequence of *β-defensin 1* (*BD1*, 204 bp) as well as qPCR primers for these genes and reference genes *40S ribosomal protein S11* (*40S*), *40S ribosomal protein S18*[b] (*18S)*.

Primer	Primer sequence (5'-3')	Gene	Usage
Muc2FW1	CAGCAYSTGGGGARACTTCCAC	*Muc2*	endpoint PCR
Muc2RV1	CATCGATGTTGTGTTCCTCAC		
FL1-fw	AATTATAAAAGGACAAGTGCTGACAGGTAG	*Muc5B*	endpoint PCR
5_rv3a	TCCGCAGGKYTKRTAGTGCC		
pDefB1_F1	tcatccgaagataccaac	*BD1*	endpoint PCR
pDefB1_R1	agggaacataattttcagtt		
Def2-128[a]	TGGACRTGTGGGTAYRGAGGACTCT	*BD2*	endpoint PCR
Muc2c_F	TGACTGCCAAAGCCTCATTC	*Muc2*	qPCR
Muc2c_R	CCATTGACTACGACCTGTTTCTC		
Muc5b_F	CAGCCCTCTTCCTCTTTCATC	*Muc5B*	qPCR
Muc5b_R	CCACTCATCTTTCCTTTCTCTTC		
Def1_rt1F	CTTGCTTGTCCTTGTCGT	*BD1*	qPCR
Def1_rt1R	CCCTTGCCACAGCCTAA		
Def2_rt2_F	GGGATTCGATTTGGACGTGTGG	*BD2*	qPCR
Def2_rt2_R	GTGGACAACCCTGGTGACTAACA		
q40S.FW1	CCGTGGGTGACATCGTTACA	*40S*	qPCR
q40S.RV1	TCAGGACATTGAACCTCACTGTCT		
Cyca_18S_qF1	AAACGGCTACCACATCCAA	*18S*	qPCR
Cyca_18S_qR1	TTACAGGGCCTCGAAAGAGA		

[a] Raceprimer.

[b] qPCR primers for the reference genes were designed by Gonzalez et al. [169].

Expression analysis

To perform plasmid based quantification recombinant plasmids were constructed. The PCR was performed using Advantage 2 PCR kit (Clontech, USA). The products were ligated into the pGEM–T Easy vector (Promega, USA) and propagated in JM109 competent E. coli bacteria (Promega, USA). The plasmids were isolated with the GeneJET Plasmid Miniprep Kit (Fermentas, Germany).

Quantitative real-time PCR (qPCR) was used for expression analysis. For each gene and each sample qPCR was performed in duplicate. The reactions were performed using the Maxima SYBR Green 2× mastermix (Fermentas, Germany), in Stratagene Mx3005P cycler (Agilent, USA). Briefly, mastermix was prepared as follows: 1× Maxima SYBR Green mastermix (with 10 nM of ROX), 200 nM of each primer, 5.0 µl of 20× diluted cDNA and nuclease free water to a final volume of 25 µl. The amplification program included an initial denaturation at 95 °C for 10 min, followed by 40 cycles with denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s. At the end of the run dissociation was performed.

For gene quantification of the samples, a standard curve from 10^2 to 10^7 of gene copies (Supplementary File 3) was prepared using the recombinant plasmids. Two reference genes were used (Table 1). For normalisation 40S ribosomal protein S11 was used as this one was the most stable reference gene between tissues [cf. 170]. The level of common carp Muc2, Muc5B, BD1 and BD2 gene expression was shown as copy number of the gene normalised against the reference gene (1 × 105 copies of 40S ribosomal protein S11).

Normalised copy number = mRNA copies per PCR for target gene/(mRNA copies per PCR for reference gene/10^5).

Differences in expression of the target genes in tissues from carp fed different β-glucan diets are shown as fold increase between the two feed groups: β-glucan enriched or control diet, according to the following formula:

Fold increase = normalised copy number of the target gene found in the tissue of β-glucan fed carp/average of normalised copy numbers of the target gene in tissue from non-β-glucan fed carp

Differences in gene expression upon β-glucan feeding were tested for significance ($p \leq 0.05$) by means of a Students t-test using SigmaPlot12 (Systat Software, USA).

RESULTS

Sequence analysis of mucin genes

The sequence found with the primers Muc2FW1 and Muc2RV1 showed a high similarity to Muc2 genes of zebrafish and other vertebrates and will be called Muc2 onwards. With the primers FL1-fw and 5_rv3a a 3212 bp sequence (excluding primers) was found. This sequence showed in a BLAST analysis a high similarity to the sequence of a mucin-like gene of zebrafish as well as to the sequence of Muc5B genes of zebrafish and of other vertebrates and will from this point on be referred to as Muc5B. The cDNA sequences for the mucin carp genes were deposited in GenBank (GenBank ID JF343440 and JF343438). As mucin genes have long sequences, only fragments that aligned with the carp genes are shown (Fig. 1 and Fig. 2).

A BLAST analysis revealed conserved domains in both mucin sequences: von Willebrand factor D domains, and C8 domain (Fig. 1 and Fig. 2). In addition, the Muc5B sequence contained Trypsin Inhibitor-Like (TIL) cysteine rich domains (Fig. 2). Phylogenetic analysis revealed that the translated amino acid sequences of carp Muc2 and Muc5B were closely clustered with sequences from zebrafish and other vertebrates, as seen in Supplementary File 4.

organism: latin name	organism: common name	GenBank ID	aa length
Cyprinuscarpio	common carp	JF343440	268
Danio rerio	zebrafish	XP_002667590	1597
Homo sapiens	human	Q02817	5179
Mus musculus	mouse	NP_076055	2319
Taeniopygia.guttata	zebra finch	XP_002198250	3329
Xenopus tropicalis	Western clawed frog	XP_002936080	3442

```
C.carpio      ------------------------------------------------------------
D.rerio       MEWR-----------------TSTVCMLLLALSGIQVD-----SKKVSPSNHVNSICSMW   38
H.sapiens     MGLP-----------------LARLAAVCLALSLAGGS---ELQTEGRTRYHGRNVCSTW   40
M.musculus    MGLP-----------------LARLVAACLVLALAKGS---ELQKEARSRNH---VCSTW   37
T.guttata     MGLR-----------------AASLLLLWLALSSAN------EIKKGRTRSHGHYVCSTW   37
X.tropicalis  MGIARRESRSSKGERRRVSTFMPAYGVKQIVLGIEKGIGTWEEEHHCRIRNHGHYVCSTW   60

C.carpio      -----------VYHPPGTCEYNLVSDCQSLIRQFSVHVKRTEH--NTGPKISRVSISIND   47
D.rerio       GNFHFKTFDGDVYQFPGMCEYNLVSDCQSLIRQFSIYVKRTER--STGPKISRVSITIND   96
H.sapiens     GNFHYKTFDGDVFRFPGLCDYNFASDCRGSYKEFAVHLKRGPGQAEAPAGVESILLTIKD  100
M.musculus    GDFHYKTFDGDVYRFPGLCDYNFASDCRDSYKEFAVHLKRGLGEAGGHSQIESILITIKD   76
T.guttata     GNHHFKTFDGDIYQFPGVCEYNFVSDCREAYKEFSVHIQRALN-SNGHPEIQYILVKIKD   96
X.tropicalis  GNLHYKTFDGDFYQYPGLCSYELASHCGEAYREFSVHVKHTNA--TGHPLVEKIVVTIKD  118
                        .:::** *.*::.*.*    ::*:::::::        . :. : :.*:*

C.carpio      IGIEFTEKQVVVNGEKVTLPVHVAGILVEENTIYTRLYSKMGITVMWNKEDAVMVELDSK  107
D.rerio       IAIELTENQVNVNEAKVTLPVHVSGILVEENTIYTRLYSKMGITVMWNKDDAVMVELDSK  156
H.sapiens     DTIYLTRHLAVLNGAVVSTPHYSPGLLIEKSDAYTKVYSRAGLTLMWNREDALMLELDTK  160
M.musculus    DTIYLTHKLAVVNGAMVSTPHYSSGLLIEKNDAYTKVYSRAGLSLMWNREDALMVELDSR  157
T.guttata     IMVYLKPNLVVVDGRIVKTPYYTSGVLIESNEIYSKIYAKLGLILMWNQQDALMVELDNK  156
X.tropicalis  VIVEIRSSLVVVNGQIAKTPYYSYGILLHKNDAYIKLYTKSGLTLMWNKEDAIMVELDPK  178
                : :    . ::   .. * :  *:*:...  * ::*:: *: :***::**:*:*** :

C.carpio      YSNRTCGLCGDFNGVPVYSEFIE-SGRRVGYTEFGNMHRVPNPTHVCEDPFENDDEQNVV  166
D.rerio       YSNRTCGLCGDFNGIPVYNEFIQ-SGRTVGYTEFGNMHRVPNPTHQCEDPFENVDEQNVV  215
H.sapiens     FRNHTCGLCGDYNGLQSYSEFLS-DGVLFSPLEFGNMQKINQPDVVCEDPEEEVAPAS--  217
M.musculus    FQNHTCGLCGDFNGMQTNYEFLSEEGIQFSAIEFGNMQKINKPEVQCEDPEAVQEPES--  215
T.guttata     FNNHTCGLCGDYNGIQIYNEFIK-GDASYNSITYGNMQKISKPNAKCEDPDETQALPS--  213
X.tropicalis  YNNQTCGLCGDYNGIPFYNEFIG-ESFHLTPVQFGNLQNIYDPAEHCTNPDETQIAETSF  237
              : *:*******:**:    **:   .       :**::.: .*   * :*        .

C.carpio      DKCEKFRADCADLLEDEKWSSCSWVLNPEPYIKACTNDICSSQPEDEDTSISALCATLSE  226
D.rerio       DQCEKYRADCADLLEDEKWSSCSWVLDPEAYIKACTNDLCNRQPEDEDT--TALCATLTE  273
H.sapiens     --CSEHRAECERLLTAEAFADCQDLVPLEPYLRACQQDRCRC---PGGD--TCVCSTVAE  270
M.musculus    --CSEHRAECERLLTSAAFEDCQTRVPVESYVRACMHDRCQC---PKGG--ACECSTLAE  268
T.guttata     --CNEHRDECQRLLTSPAFADCRLRLNLEMYIQACMQDKCAC---NGKDDTFCLCSTISE  268
X.tropicalis  --CSQYRSVCEEHLAHPAFTDCQSLLNTEAYIKACMLDMCSC---GQSQDAFCLCSTISE  292
                *.:.*  *   *    : .*   :  * *:**  * *           . *:*::*

C.carpio      YSRQCSHSGGTPPSWRTANFCAMKCPYNMVHSESGSPCMDTC------------------  268
D.rerio       YSRQCSHAGGNPPAWRTAKFCNVQCPYNMVHSESGSPCMDTCSHKDTNALCEEHNIDGCF  333
H.sapiens     FSRQCSHAGGRPGNWRTATLCPKTCPGNLVYLESGSPCMDTCSHLEVSSLCEEHRMDGCF  330
M.musculus    FSRQCSHAGGRPENWRTASLCPKKCPNNMVYLESSSPCVDTCSHLEVSSLCEEHYMDGCF  328
T.guttata     YSRQCSHAGGRPGEWRTQNFCPKTCPATMVYRESSSPCMDTCSHLQISSLCEEHYMDGCF  328
X.tropicalis  FSRQCSHAGGRPGTWRTENFCPKTCPGNMIYQESGSPCTSSCSRLEIHSLCEEHFMDGCF  352
              :******:** *  *** .:*   ** .::: **.*** .:*
```

Fig. 1 Multiple alignment of the amino acid (aa) sequences of carp *Muc2* with *Muc2*-(*like*) genes of zebrafish (predicted, partial), human, mouse, zebra finch (predicted) and Western clawed frog (predicted). Symbols indicate identities (*) and similarities (: and .). Conserved domains (von Willebrand factor D domain aa 2–121) and (C8 domain aa 179–248) are marked in the carp sequence.

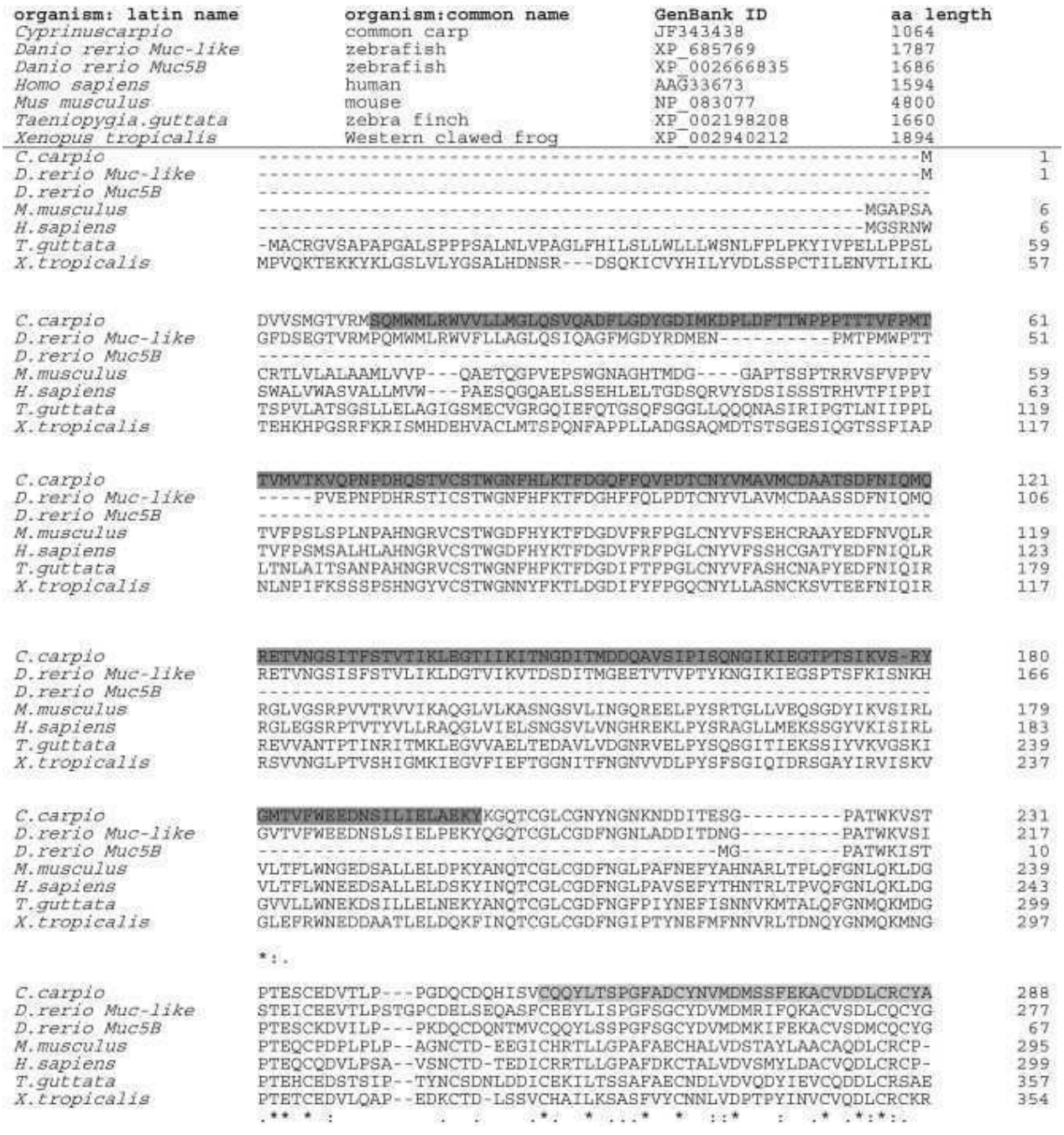

Fig. 2 Multiple alignment of the amino acid (aa) sequence of carp *Muc5B* with *Muc5B*-(*like*) genes of zebrafish (predicted), human, mouse, zebra finch (predicted) and Western clawed frog (predicted), as well as a *Muc-like* gene of zebrafish (predicted). Conserved domains (von Willebrand factor D domain aa 72–20, 409–571, 869–1027, C8 domain aa 254–321, 606–680 and TIL domain aa 324–380, 686–743) are marked in the carp sequence.

Sequence analysis of β-defensin genes

With the pDefB1_F1 and pDefB1_R1 the full coding sequence of a gene with high similarity to piscine *BD1-like* genes was found. With the race primer Def2-128 a partial sequence with high similarity to *BD2-like* genes of fish was found. The cDNA sequences for these β-defensin carp genes were deposited in GenBank (GenBank ID JF343439 and JF343441).

The translated amino acid sequence of the carp *BD1* and *BD2* genes were compared to known piscine *BD1* and *BD2* sequences by multiple alignment analysis (Fig. 3 and Fig. 4). The protein sequence in the regions containing the potential secondary structure of the β-defensins [as described by 155] were identical to the zebrafish sequence for *DB1*. Furthermore, the carp *BD1* protein sequence had a six-cysteine motif identical to piscine and higher vertebrate *BD1* (Fig. 3). As for carp BD2, alignment of one of the three possible secondary structure containing regions was made, as only partial protein sequences were obtained. In this region 7 out of 9 amino acids are identical for carp and *D. rerio*. The partial sequence is encoding two of six conserved cysteins (Fig. 4). Furthermore, the translated amino acid sequence of the carp genes was used in a phylogenetic analysis (Supplementary Files 5 and 6). The carp β-defensins (*BD1* and *BD2*) have highest similarity to those in *D. rerio*, but less to other fish species and cluster poorly with those from mammalian and avian species.

organism: latin name	organism: common name	GenBank ID	aa length
Cyprinus carpio	carp	JF343439	67
Danio rerio	zebrafish	NP_001075022	67
Tetraodon nigroviridis	tetraodon	CAJ57644	64
Takifugu rubripes	Japanese pufferfish	CAJ57646	66
Onchorhynchus mykiss	rainbow trout	NP_001117906	65
S.salar	Atlantic salmon	CK892029	66

```
C.carpio        MKPQSILVLLVLVVLALHCKENEAVSFPWSCASLSGVCRQGVCLPSELYFGPLGCGKGFLCCVSHFR
D.rerio         MKPQSIFILLVLVVLALHFKENEAASFPWSCASLSGVCRQGVCLPSELYFGPLGCGKGFLCCVSHFL
T.nigroviridis  MASYRAVFLALVVVLMLN--ENEAASFPWACPSLNGVCRK-VCLPTELFFGPLGCGKGFLCCVSHFL
T.rubripes      MASYRAVVLALLVVLVLNAVENEAASFPWTCPSLSGVCRK-VCLPTEMFFGPLGCGKGFQCCVSHFL
O.mykiss        MSCQRMVTLVLLVFLLLNVVEDEAASFPFSCPTLSGVCRK-LCLPTEMFFGPLGCGKGFLCCVSHF-
S.salar         MSCQRMVTLVLLVFLLLNIVENEAASFPFSCPTLSGVCRK-LCLPTEMFFGPLGCGKGFLCCVSHF-
                *     . * ::*.* *:  *:**.***::*.:*.****: :***:*::******** ** ******
                                                        β1          β2      β3
```

Fig. 3 Multiple alignment of the amino acid (aa) sequence of carp *β-defensin 1* with full *β-defensin 1*-(like) genes known from fish species. Symbols indicate identities (*) and similarities (: and .). Probable regions for *β*-strands based on human β-defensin 2 [155] are marked as well as conserved cysteins.

organism: latin name	organism: common name	GenBank ID	aa length
Cyprinus carpio	carp	JF343441	22
Danio rerio	zebrafish	CAJ57443	65
Tetraodon nigroviridis	tetraodon	BN000874	63
Epinephelus coioides	orange-spotted grouper	AY129305	63
Onchorhynchus mykiss	rainbow trout	CBB12547	62

```
C.carpio        ------------------------------------RRFCFDQEYIVSHQGCPRKY-------
D.rerio         MKKLGMIIFITLPALFAGNVHNAEVQIQNWTCGYGGLCRRFCFDQEYIVAHHGCPRRYRCCAVRF
T.nigroviridis  MKGLSLVLLVLLLMLAAG--EDSDSEMQYWTCGYRGLCRRFCYAQEYTVGHHGCPRRYRCCATRP
E.coioides      MKGLSLVLLVLLLMLAVG--EGNDPEMQYWTCGYRGLCRRFCHAQEYIVGHHGCPRRYRCCAVRS
O.mykiss        MGRLGLVMLVLLLLTAVQ---ADDTKVQGWTCGYRGACRKYCYAQEYMVGYHGCPRRLRCCALRF
                                          *:*. *** *.:****:
                                          β1      β2      β3
```

Fig. 4 Multiple alignment of the amino acid sequence of carp *β-defensin 2* with full *β-defensin 2*-(like) genes known from fish species. Symbols indicate identities (*) and similarities (: and .). Probable regions for carp *β*-strands based on human β-defensin 2 [155] are marked as well as conserved cysteins.

Tissue specific expression of carp mucins and β-defensins

Expression of carp *Muc* and *β-defensin* genes could not be detected in kidney, head kidney and spleen when cDNA of naïve carp was analysed by RT-qPCR. Low expression (30–100 normalised copy number) of *BD2* was detected in liver (Fig. 5). An intermediate expression, with 100–1000 normalised copy number, could be detected for *BD1* in skin. High expression with > 1000 normalised copy number was not found for *BD1* or *BD2* in any of the examined tissues. Low expression of *Muc5B* was detected in brain and liver (Fig. 5). High gene expression of *Muc2* in both the first and second intestinal segments and *Muc5B* in the skin and gills was seen.

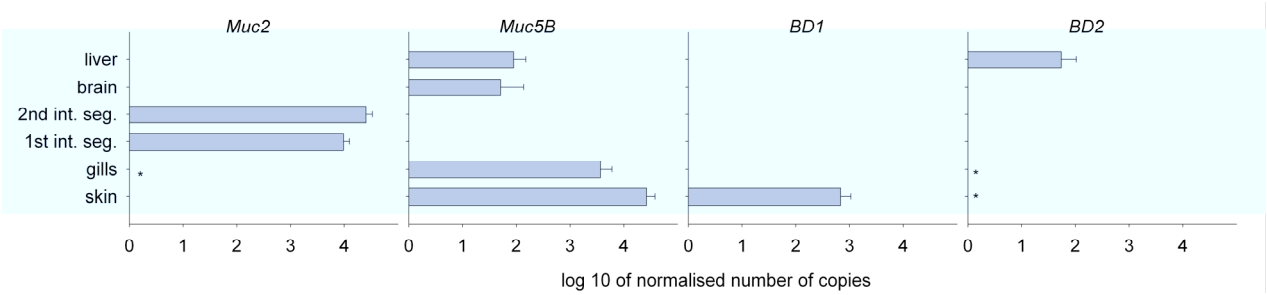

Fig. 5 Expression of carp *BD1*, *BD2*, *Muc5B* and *Muc2-like* in liver, brain, first intestinal segment, second intestinal segment, gills and skin from naïve fish. Data are presented as copy numbers of mRNA normalised against *40S* mRNA from the same sample. *copy numbers below 10 could be observed.

Influence of β-glucan feeding on expression of carp *mucins* and *β-defensins*

The expression of carp *Muc* and *β-defensin* genes was analysed in skin, gills and intestine from carp with a β-glucan feeding regimen (0.1% MacroGard) relative to the control fish (0% β-glucan). Differences in the expression could be observed in all tested tissues (Fig. 6) upon β-glucan feeding A significant increase of *BD1* and *BD2* mRNA could be detected in the skin (4.0 and 2.8 fold respectively) and of *BD2* mRNA in the gills (1.5 fold). Furthermore, a significant increase of *Muc5B* mRNA could be detected in the skin (2.7 fold). A non-significant decrease in the amount of *Muc2* and *Muc5B* mRNA could be observed in the gills (0.5 and 0.9 of the expression in control). *Muc2* mRNA was also non-significantly decreased in the first and second intestinal segment (0.7 and 0.9 of the expression in control) (Fig. 6).

Discussion

Homology of carp mucins with vertebrate mucins

In the present study two partial mucin-like sequences from common carp were cloned and sequenced. Accurate assembly of the mucin genes is difficult due to the large size of the central tandem repeats [39], which is probably why attempts to fully sequence the *mucin* genes in this study were unsuccessful. Carp mucin sequences had high homology to two mammalian and avian gel forming mucins:*Muc2* and *Muc5B*. For mammals it is known that gel forming mucins present strong structural similarities [9].

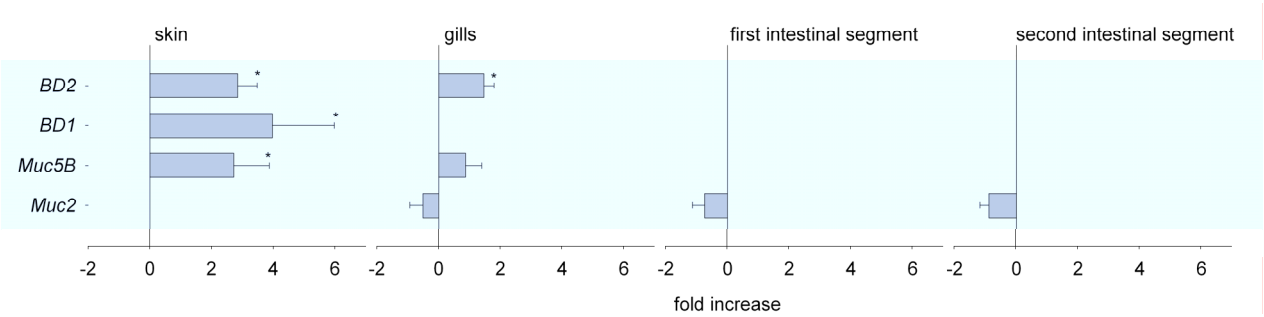

Fig. 6 Difference in the expression of carp *BD1*, *BD2*, *Muc5B* and *Muc2* in tissues from carp after MacroGard β-glucan feeding regimen relative to expression in these tissues from no β-glucan feeding control fish. Copy numbers of mRNA were normalised against *40S* mRNA from the same sample. The data are presented as a fold increase of normalised mRNA copies in tissues of carp fed with β-glucan feed to normalised mRNA copies in tissues of carp fed with non-β-glucan feed. * indicates significant ($p < 0.05$) increases as tested with a Students *t*-test.

However, for MUC-type mucins, unifying sequence homology is not seen, implying that they may have evolved through convergency [149]. Dekker et al. [149] therefore suggested an adaptation of mucin nomenclature to distinguish at least two separate families, one being the MUC-type mucins located within the human 11p15 locus. The most identifiable relationships are found for the mucins within this locus: *MUC2*, *MUC5AC*, *MUC5B* and *MUC6*. These mucins have von Willebrand factor domains containing several type D domains: D1 and D2 are present within the N-terminal propeptide, whereas the remaining D domains are required for multimerisation. Von Willebrand factor can also be found in secreted mucins of humans humans [52]. In both carp mucins, von Willebrand factor D domains were found, indicating a relationship to this MUC family, however these domains are also found in other non-mucin proteins [149]. However, the carp sequences found in the present study also share C8 domains with other vertebrate mucins. C8 domains contain 7 or 8 conserved cysteine residues that overlap with other domains. C8 domains can be found in disease-related proteins including von Willebrand factor and mucin humans [52]. The cystein-rich domains of *MUC2*, *MUC5AC* and *MUC5B* are so far unique to these mucins [149]. In the *Muc5B* sequence also TIL domains were found. TIL domains typically contain ten cysteine residues, that form five disulphide bonds. Gel forming mucins of the type observed in mammals have arisen by the combination of the VWD-VWE-TIL module with PTS, CysD, and cysteine-knot domains [171]. The presence of von Willebrand factor D, C8 and TIL domains strongly supports the conclusion that the carp mucins are real *Muc2* and *Muc5B* genes, instead of genes which only resemble those (*Muc-like* genes).

Analysis of tissues and organs for mucin expression in carp revealed a clear separation in the site of expression between the two mucins. *Muc5B* was expressed with low copy numbers in brain and liver and with high copy numbers in skin and gills. *Muc5B* appears to be homologous to mammalian *Muc5B*. *MUC5B* has been found to be mainly expressed in the mucous glands of the respiratory mucosa and salivatory glands, as well as in the gall bladder, pancreas and cervix [9]. Carp *Muc2* was only, but highly, expressed in the first and second intestinal segment. In human tissues this mucin is also expressed in the intestine, but can also

be found in bronchi [9]. In contrast to this, carp *Muc2* expression was not detected in the gills. Contrary to the respiratory tract of mammals, in the gills of carp *Muc5B* seems to be more important than *Muc2*.

Homology of carp β-defensins with piscine β-defensins

In addition to the mucin genes, two β-defensin encoding genes from carp were also partially sequenced in the present study. Defensins have a broad antimicrobial spectrum ranging from Gram-negative to Gram-positive bacteria, mycobacteria, fungi and enveloped viruses. Based on the organisation of three intra molecular disulphide bonds between cysteine residues, defensins are termed α- and β-defensins [172]. To our knowledge piscine β-defensins have so far only been identified through *in silico* studies [155, 156]. These defensin-homologues share the common features of vertebrate defensins, including small size, net cationic charge and six conserved cysteine amino acids in the mature region. Based on their cysteine arrangement, the identified fish defensin-*like* peptides resemble the β-defensin family members in birds and mammals [155]. In carp *BD1* and *BD2* these conserved cysteines were also present. The two sequences obtained from carp cluster well with, and are closely related to piscine *BD1* and *BD2* and cluster poorly with those from mammalian and avian species.

In human, *BD1* is constitutively expressed. Analysis of β-defensin expression in tissues from naïve carp revealed limited constitutive expression, as *BD1* was only expressed at intermediate levels (100–1000 normalised copy numbers) in skin and *BD2* only at low levels (10–100 normalised copy numbers) in liver. In contradiction to this, *Oncorhynchus mykiss* show a widespread constitutive expression at both mucosal and systemic sites, especially with high expression of *β-defensin 3* (*BD3*) and *β-defensin 4* (*BD4*). However, *BD1* and *BD2* were expressed at low levels [156]. For *D. rerio*, *BD2* was only expressed at low levels in the gut, while *BD1* and *BD3* were more highly expressed in all tissues examined [155]. Therefore, the expression profile of the β-defensin genes appears species-dependent.

Influence of β-gluan feeding on mucin and β-defensin expression

β-Glucans derived from plants, bacteria or fungi are recognised by receptors from the innate immune system, like C-type lectins (Dectin-1 [173] and TLR2/6 [174]), and therefore have immune-modulatory properties when administered to mammals [174] and fish [175]. β-Glucans have been shown to be effective immune-modulators in a number of bacterial, viral and parasitic infections [176]. When prebiotics, such as β-glucans, promote health responses in fish, less chemotherapeutics may be required, holding the potential to increase efficiency and sustainability of aquaculture production [177]. The present data show an effect of β-glucan feeding on the expression of mucus-related genes in carp.

For the mucin encoding genes carp *Muc5B* and *Muc2*, differences in expression were observed in carp with different β-glucan feeding regimens. Consistent, but not significant, down-regulation of *Muc2* in the intestine and gills was seen in the glucan fed fish, while *Muc5B* was significantly increased in skin, with slight up-regulation in gills. An up-regulation of *Muc2* expression after β-glucan feeding was described in the intestine of chicken [178] and pigs [179], and an increased expression of *Muc2* and *Mub5B* has been observed in mammals after bacterial [174, 180-182] and nematode infections [183]. In addition to the mucin encoding genes, an effect of β-glucan feeding could also be observed on the expression of carp β-defensin genes. The expression levels for both carp β-defensins were significantly

higher in the skin of β-glucan fed carp, with *BD2* significantly higher in gills. A regulation of *BD1* and *BD*2 was not observed in the mucosal tissues of *O. mykiss* challenged with *Yersinia ruckeri*, but in these fish *BD3* was increased in gills [183]. The present study shows that carp β-defensins can be up-regulated, although their precise role in infections and immune defence remains to be elucidated.

Even though different mucin and defensin genes are expressed in skin and intestine, the regulation of both in the skin of carp after feeding β-glucans suggests that not only the mucosal system of the intestine, can be influenced. This underscores the interconnection of mucosal tissues in the body, potentially permitting the application of functional feed additives to improve fish skin health.

In summary, with the two β-defensins and two mucin genes (partially) sequenced in the present study, important molecules for monitoring the mucosal defence in carp, are now available for futher analysis. The mucin genes were highly conserved and showed a high similarity to *Muc2* and *Muc5B*. The β-defensins showed high similarity to piscine *BD*1 and *BD2*. The β-defensin expression in naïve carp was low (*BD1* intermediate expression in skin and *BD2* low expression in liver). Mucin expression on the other hand was high in certain mucosal tissues (*Muc5B*: skin and gills, *Muc2*: intestine). Expression levels of *BD1* (skin), *BD2* (skin, gills) and *Muc5B* (skin) could be significantly increased by the addition of β-glucans to the food. This indicates the relevance of these genes to monitor feed-related improvement of fish health under aquaculture conditions.

Acknowledgements

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Chapter 6

Differential soybean meal induced mucus composition in intestinal segments of common carp, *Cyprinus carpio* L.

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Abstract

The alimentary tract is a possible site where pathogens and toxins can enter. The alimentary tract is protected, amongst others, by mucus. In this study, tissue samples and crude mucus preparations from different parts of the intestinal tract of *Cyprinus. carpio* (from intestinal bulb onto the hindgut) were examined using histological, histochemical and biochemical techniques. Furthermore, the response of the intestinal mucosal layer and intestinal mucus of C. carpio to acute soybean meal (SBM)-induced enteritis was investigated.
In the present study, an indication for a different protein core of mucus high weight molecular glycoproteins (HMGs) for first and second segment could not be found. However, differences in mucus glycosylation could be found. Along the gut axis, the size of the major protein peaks were not similar, which can be caused by a different glycosylation. Also differences in staining for the antimicrobial peptide beta-defensin 2 were found.
Furthermore, changes in HMGs upon SBM-diet were found similar those found in inflammatory bowel disease IBD in humans. Initial changes include: changes in mucin composition, the presence of BD3 and of bacteria in internal organs. After the initial changes, all values measured returned back to the initial pre-SBM diet values.

Introduction

Barrier function of the alimentary tract

The main function of the alimentary tract of fish as well as any other vertebrates is the acquisition of food with subsequent assimilation of vital nutrients and defense against pathogens [4]. In fish the alimentary tract also plays a role in osmoregulation [3]. The intestinal barrier is formed by extrinsic, intrinsic, and immunological barriers. the first barrier pathogens and toxins encounter is the extrinsic barrier. This barrier is mainly formed by mucus that counters pathogens and toxins. The intrinsic barrier consists of epithelial cells and cell junctions. Molecules and cells of the innate and the adaptive immune system that are present in the intestinal wall form the immunological barriers.
The extrinsic barrier mucus consists mostly of two components which are both important in gel formation [1, 51-53, 104]. These components are water and high molecular weight glycoproteins (HMGs), called “mucins”. Mucins exhibit a high content of oligosaccharides and form a water-insoluble layer. Preventing pathogens from penetrating the mucus layer relies not only on mucus flow and mucin composition, but also on other mucus components such as antimicrobial peptides or lysozyme. Knowledge about fish mucus and especially about its HMGs is still scarce. In addition, the composition of the commensal microbiota and therefore also of potential pathogens in fish is still limited.
The gastrointestinal tract of fish can be subdivided into four topographical regions: the headgut, foregut, midgut and hindgut [184]. The headgut is composed of the mouth and pharynx and the foregut is formed by esophagus and stomach [185]. In carp as an agastric fish the foregut only comprises the esophagus. The midgut or intestine accounts for the greatest proportion of the gut length and here the chemical digestion of ingested food is continued and absorption of nutrients mainly occurs. In carp the anterior intestine bulges to form an

intestinal bulb, which functions in temporary food storage: however, gastric glands and a pylorus are lacking. The hindgut is the final section of the gut, which includes the rectum [186]

In the absorbing parts of the gut, there are differences in barrier function between regions. In teleosts, the anterior intestine including the pyloric ceca, which are present in some species, are the main nutrient-absorbing regions. The lumen in this region contains high nutrient concentrations, typically relative low numbers of bacteria, and is lined by an epithelium of high paracellular permeability. In the posterior intestine however, bacterial numbers are higher and free nutrient concentrations drop. As the nutrient content drops along the intestinal tract and bacteria and bacterial toxins rise in concentration, the need for a tighter barrier increases [5]. Therefore, in the absorbing parts of the gut, differences in the barrier function between regions are found.

Soybean meal and enteritis

Optimal feeding of fish has been subject of extensive study. As, in aquaculture, feed costs often comprise more than 50% of total production costs [187, 188] a cheap and reliable source of protein is needed for both economic and sustainability reasons. Soybean *Glycine max* L. has a great potential as protein and/or oil source for fish feed [189]. However, soybean meal (SBM) containing diets are known to have the potential to induce an inflammatory response in the hindgut of certain fish species. So far most studies have been conducted on *S. salar* and have mainly focused on the effect of SBM-containing diets on intestinal morphology, growth, enzyme activity and metabolism. A negative influence was observed for these parameters [190-194] and on disease resistance [195] as well as immune relevant genes [196]. In common carp *Cyprinus carpio* L. which were fed a diet where 20% of the protein was replaced by SBM, enteritis was induced in the hindgut [75]. Contrary to previous observations made with *S. salar*, *C. carpio* start to recover or adapt to the SBM feed from the fourth week after the SBM feeding.

In humans, increased gut permeability has been described following enteritis. If changes after enteritis persist a chronic inflammation can develop [197]. Chronic inflammatory bowel diseases (IBD) are well described for humans. In IBD, genetic mutations in mucin genes, changes in sulphation, degree of glycosylation, mucin mRNA, protein levels and degradation of mucins have been described [198]. In the research on SBM induced enteritis in fish no attention has been paid so far to the role of mucins.

The present study

In *C. carpio*, the presence of intestinal glycoproteins that are similar in structure and composition to those found in mammals has been documented [78], but until now data did not include a differentiation of functional intestinal segments based on mucus composition. In this study, tissue samples and crude mucus preparations from different parts of the intestinal tract of *C. carpio* (from intestinal bulb onto the hindgut) were examined using histological, histochemical and biochemical techniques. A distinction was made between non-secreted mucus HMGs present in the goblet cells and secreted mucus HMGs present in the lumen. Furthermore, in this study, the response of the intestinal mucosal layer and of intestinal mucus of *C. carpio* to acute SBM-induced enteritis was examined.

Materials and methods

Fish and rearing conditions

Clinically healthy, parasite and virus free *C. carpio* from a single crossing (Wageningen Agricultural University, Netherlands) were used throughout the study. From day 4 post fertilization until four weeks of age, larvae were fed *Artemia salina* nauplii.
To examine possible differences in mucin composition between intestinal segments, *C. carpio* (120 ± 21 g, strain E20 x R8) which had been fed with commercial trout pellets (Trouvit) were used.
To examine a possible effect of SBM on mucin composition *C. carpio* were transferred to another system and kept in recirculating, filtered, UV-sterilized water at 23 °C. *C. carpio* were then fed a fishmeal based diet without soybean protein (0SBM). For experimentation, fish were switched to an experimental diet (20SBM), in which fish meal, fish oil and wheat were exchanged for 20% SBM. Feed was produced as extruded sinking pellets (Skretting, Aquaculture Research Centre, Stavanger, Norway) and were formulated to be iso-nitrogenous and iso-energetic on a crude protein and a crude lipid basis. The fish (221.7 ± 47.2 g, stain R8 x R3) were manually fed 4% of their body weight per day, which was divided into two equal servings and given by hand. Both diets (0SBM and 20SBM) have been described earlier by Uran *et al.* [75].

Sampling

C. carpio were starved for two days before sampling to reduce fecal contamination of the intestinal mucus. *C. carpio* were euthanized with 0.03 % tricaine methane sulphonate (TMS; Crescent Research Chemicals, USA), buffered with 0.06% sodium bicarbonate to a pH of 7.2. The entire intestinal tract was removed, cooled on ice and divided into three parts: intestinal bulb, rest of the first segment and second segment. Small tissue pieces (3 - 4 mm) were taken from the intestinal segments for histology and the remaining tissue was used for mucus isolation.
Fish were sampled at the start of the experiment at week 0 (n=8) and weeks 1, 2 and 3. (at each of these time points n=5). Furthermore, one bacterial swab was taken from internal organs (liver, kidney and spleen) from these fish for microbiological examination. Swabs were cultivated on blood agar plates for two days at 25 °C. Bacteria were identified with the API system (BioMerieux, France). Differences to control were tested to One Way ANOVA on Ranks.

Histology and immunohistochemistry

By means of histology and immunohistochemistry, the composition and amount of complex carbohydrates within the mucus in the goblet cells as well as the presence of antimicrobial substances was examined. Hereto fresh intestinal tissue was immersed in Carnoy's fluid (staining for complex carbohydrates, segment analysis samples) [199] or Bouin's fluid. Samples were immersed in Bouin's fluid for at least four days at room temperature. Samples were immersed in Carnoy's for two hours at room temperature and then transferred to, and washed in isopropanol. All samples were then washed, dehydrated in a series of graded ethanol and embedded in paraffin wax. Subsequently, 5 µm paraffin sections were cut.

Mucin carbohydrates were visualized with periodic-acid Schiff (PAS), Alcian Blue 8GX pH 1.0 (AB1.0) as well as a AB2.5 (segment analysis samples) or AB2.5/Crossmann (OSBM and 20 SBM) [85]. The PAS reaction visualizes neutral glycoconjugates; the AB1.0 method stains sulphated glycoconjugates; the AB2.5 method stains acidic glycoconjugates. All sections were studied or photographed with a light microscope (Zeiss light microscope Axiphot, Zeiss, Germany).

The presence of antimicrobial substances (ß-defensin 2 (BD2), ß-defensin 3 (BD3) and lysozyme-muramidase (Lys)) was examined by immuno-histochemical indirect visualization. Hereto, sections were de-paraffinised in xylene and carefully hydrated through descending concentrations of ethanol. Sections for lysozyme staining were incubated for 30 min with 0.1% trypsin (pH 7.8, 37°C) to demask the proteins. Subsequently, all sections were pre-treated for 20 minutes with NGS (normal goat serum). Then sections were incubated with the first antibody which was diluted with 1% BSA (bovine serum albumin) in PBS (phosphate buffered saline). The polyclonal primary antibody BD2 was diluted 1:500, BD3 1:1000 and Lys 1:100, and all three were obtained from rabbit immunized with human peptides (Biologo, Germany). Following overnight incubation at 4°C, the reaction was detected by the EnVision system (Dako, Germany). Control sections were incubated without the first antibody to test for unspecific binding of the visualization substrate. Furthermore, sections were incubated without the primary, and without the secondary antibody, but with the visualization substrate to test for endogenous enzyme activity. Antibody specificity has been previously described [200].

The presence of antimicrobial substances in the goblet cells was estimated on an arbitrary scale (none, few, several, many) for both the number of folds with stained goblet cells and the number of stained goblet cells per fold. The carbohydrate-stains were evaluated on the basis of the goblet cell reaction. The number of responding goblet cells was counted in four intestinal folds each over a fixed length (390 µm) of epithelial lining per fold. The mean and standard deviation of all measurements per fish were calculated. Furthermore, staining intensity of the goblet cells (faint, weak, moderate, strong, very strong) was evaluated on an arbitrary scale. The number of goblet cells was statistically analyzed with a one way ANOVA. Goblet cell staining intensity was statistically analyzed with a Kruskall-Wallis one way ANOVA on ranks. Differences were considered as significant at a probability of error of $p \leq 0.05$.

Isolation of HMGs from intestinal mucus

For mucus isolation, the intestinal segments were opened longitudinally and cut into small pieces of 3-4 mm. Subsequently, secreted HMGs were isolated with isolation medium as described previously [140]. In brief, tissue pieces were incubated for 20 minutes in 100 ml isolation buffer containing antibiotics and protease inhibitors (Amphotericin B, dithiothreitol, sodium pyruvate, HEPES).

The isolation buffer was collected, centrifuged for 30 min at 12,000 x g, and the supernatant was collected and frozen at -20 °C until further processing. Non-secreted mucus HMGs were released from goblet cells by subsequent incubation of the tissue pieces for 30 min in a buffer containing EDTA, with the addition of the same antibiotics and protease inhibitors as in the isolation medium for secreted mucus. Goblet cells were disrupted by means of an ultrasonic unit (Ultra Turrax T8, IKA-Werke, Staufen, Germany).

The suspension was centrifuged at 10,000 g for 30 min, and the supernatant was collected and homogenized by gentle stirring. All samples were concentrated by ultrafiltration (Amicon, Beverly, MA/USA, exclusion limit 30 kDa) to a final volume of 2 ml and used for size exclusion chromatography.

High-performance liquid chromatography (HPLC)

Mucus samples for segment analysis were analyzed for the presences of monosaccharides by reverse-phase HPLC with the method described by Anumula [126]. In brief, the mucus samples were hydrolyzed with 20% trifluoroacetic acids and then derivatised with an excess of anthranilic acid in the presence of sodium cyanoborohydride. Antranilic acid derivatives of monosaccharides were separated on a HPLC column using a 1-butylamine-phosphoric acid-tetrahydrofuran mobile phase. All separations were carried out at ambient temperature using a flow rate of 1 ml min^{-1}. Solvent A consisted of 0.25% 1-butylamine, 0.5 % phosphoric acid, 1% tetrahydrofuran (inhibited) in water. Solvent B consisted of equal parts of solvent A and acetonitrile. The elution program was 5% of solvent B for 25 min followed by a linear increase to 15% of solvent B at 50 min. The column was washed for 15 min with 100% of solvent B and equilibrated for 15 min with 5% of solvent B. Elution was monitored by measuring the fluorescence (excitation wavelength, 230 nm; emission wavelength, 425 nm).

Sugars were identified by comparing retention times (RT) of peaks in the samples with RT of peaks of standard sugar solutions. Standard sugar solutions were galactose (gal) RT 12.1 min, N-acetylneuraminic acid (NeuNAc) RT 12.6 min, glucose (glc) RT 12.8 min, mannose (man) RT 13.2 min, fucose (fuc) RT 18.2 min and N-acetylgalactosamine (galNAc) RT 23.4 min. For each identified peak in the samples, surface areas of eluted peaks were compared with surface area of peaks of standard sugar solutions with a known amount of monosaccharide. Total sugar quantities and the monosaccharide percentage of the total sugar quantities were calculated. Data was analyzed with a t-test. Differences between intestinal segments and differences between secreted and non-secreted mucus of the same segment were considered as significant at a p value of ≤ 0.05.

Size exclusion chromatography of mucus samples

Size exclusion chromatography was performed on an ÄktaFPLC-System (GE Healthcare). Sepharose CL-4B (Sigma-Aldrich) was used in an XK 16/40 column (GE Healthcare). PBS at pH 7.2 was used as running buffer. Up to 2 ml of concentrated mucus samples were loaded on the column. In a steady buffer stream of 1 ml min^{-1} the proteins in the mucus concentrate were separated by size. Elution of the proteins was monitored online via UV-activity (280 nm) and conductivity.

To determine the molecular weight of the eluted proteins, solutions with known molecular weights were also monitored. Hereto apoprotein (8 kDa), FK506 binding protein (12 kDa), MBP2* protein (40 kDa) and MBP-β-galactosidase (158 kDa) were subjected to size exclusion chromatography (Fig. 1). Differences were statistically evaluated with an ANOVA on Ranks ($p < 0.05$).

Results

Histology

Goblet cells with sulphated, acidic and neutral glycoproteins were found in all gut regions. In all stainings and all intestinal segments the goblet cells had an oval to rounded form. For carp that were kept on normal trout feed, a significant difference in goblet cell number and staining intensity could be found for some stainings between the intestinal segments (Table 1). Goblet cells in the second segment showed a significantly weaker staining than in the first segment for the AB2.5 and PAS stain (acidic and neutral glycoconjugates, respectively). The number of goblet cells which were stained in the intestinal bulb was significantly lower than the number in the first and second segment for AB1.0 and PAS. The number of AB2.5 stained goblet cells was also lower in the intestinal bulb, but the differences were not significant.

Table 1 Number of stained goblet cells (mean ± SD) per 390 µm of epithelial lining per fold and median goblet cell staining intensity of three intestinal segments after staining for complex carbohydrates.

	intestinal bulb	1st segment	2nd segment
Goblet cell number			
AB1.0	14.5 ± 3.5[a]	20.4 ± 3.4[b]	18.6 ±3.0[b]
AB2.5	15.0 ± 3.8	19.2± 2.1	18.0 ± 3.5
PAS	14.5 ± 3.5[a]	20.4 ± 3.4[b]	18.6 ± 3.0[b]
Staining intensity			
AB1.0	strong	strong	strong
AB2.5	strong[ab]	strong[a]	moderate[b]
PAS	strong[ab]	very strong[a]	strong[b]

[ab] Different superscripts indicate significant differences between intestinal segments (One Way ANOVA, $p \leq 0.05$)

After switching from the 0SBM to the 20SBM diet, significant differences between the 0SBM and 20SBM fish could be found for some carbohydrate stainings in the goblet cell number (Table 2).

Table 2 Percent change in number of goblet cells compared to control after changing the diet from 0SBM to 20SBM. Values are averages ± standard deviation. Significant differences (One Way ANOVA, $p \leq 0.05$) to control are indicated with *.

Staining	Intestinal segment	Time point after diet change		
		Week 1	Week 2	Week 3
AB1.0	1st	106 % ± 12 %	68 %± 27%*	131% ± 19*
	2nd	88 % ± 3 %	93 % ± 26 %	85 % ± 9 %
AB2.5	1st	95 % ± 6 %	92 % ± 25 %	104 % ± 4 %
	2nd	59 % ± 20 %*	96 % ± 18 %	117 % ± 19 %*
PAS	1st	122 % ± 15 %*	97 % ± 66 %*	123 % ± 5 %*
	2nd	42 % ± 13 %*	64 % ± 4 %*	74 % ± 18 %*

For the sulphated glycoconjugates (AB1.0), the number of stained goblet cells was significantly lower on week 2 post diet change and significantly higher the week after. For the acidic glycoconjugates (AB2.5) significant differences were observed in the second intestinal segment. Here the goblet cell number was significantly lower at week 1 after switching to 20SBM and significantly higher at week 3 after switching to 20SBM. For the neutral glycoconjugates (PAS), significant differences could be found for both intestinal segments for all sampling points compared to the 0SBM. Here, a significantly higher goblet cell number was found for the first intestinal segment and a significantly lower number for the second segment. No clear effect of switching to 20SBM could be observed on the presences of supranuclear vacuoles.

Immunohistochemistry

For carp that were kept on a normal feeding regime, goblet cells could be stained with the antibodies against BD2, BD3 and lysozyme humoral substances (Table 3). However, goblet cells were mostly stained at the base of the intestinal fold. In the first segment only a few goblet cells were stained on the flank base. Although goblet cells were visible in flank and tips of the folds they showed low levels of antibody binding.

Table 3 Staining for the presence of antimicrobial peptides in the base and the flank base of intestinal folds of first and second segments of naïve carp. Number of intestinal folds in which goblet cells could be stained (folds stained) and the number of stained goblet cells per stained fold (goblet cells stained) are indicated. Shown are median (min – max).

humoral substances	presence of staining	1st segment	2nd segment
BD2	folds stained of:		
	base	many (few – many)	few (none – several)*
	flank base	few (none –few)	none (none – none)*
	goblet cells stained in:		
	base	several (few – several)	few (none – few)*
	flank base	few (none – few)	none (none – none)
BD3	folds stained of:		
	base	few (few – several)	few (none - several)
	flank base	none (few – none)	none (none – none
	goblet cells stained in:		
	base	few (few – several)	few (none – few)
	flank base	none (none – few)	none (none – none)
lys	folds stained of:		
	base	none (none – few)	none (none -few)
	flank base	none (none – none)	none (none – none
	goblet cells stained in:		
	base	none (none – few)	none (none – few)
	flank base	none (none – none)	none (none – none)

* indicates significant differences between first and second segment

For lysozyme and BD3, a few goblet cells per fold, and only a few folds with stained goblet cells could be observed at the base of the intestinal folds for both first and second segments. Only for BD2 could significant differences be observed between the first and second segment (Table 3, Fig. 1). The number of goblet cells per fold which could be stained was significantly less in the second segment when compared to the first segment, and they were present in both base and flank base. The number of folds (base) with stained goblet cells was significantly lower in the second segment compared to the first segment.

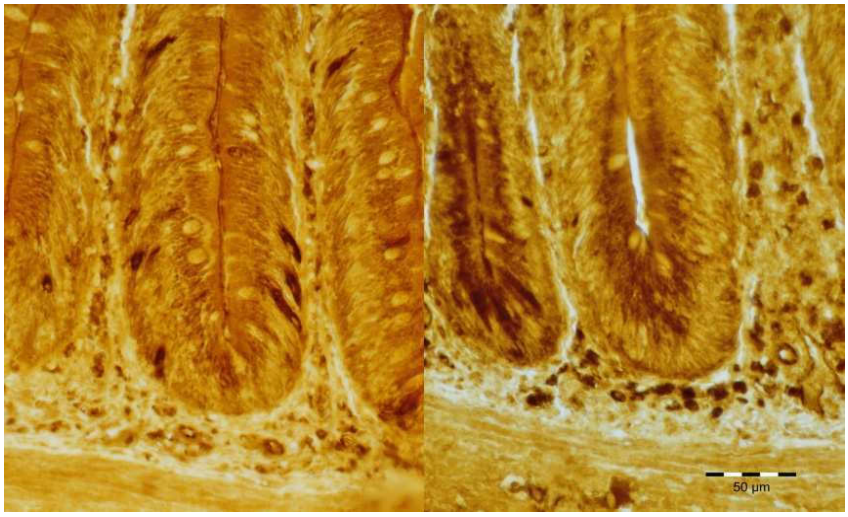

Fig. 1 Goblet cell staining at the fold base for BD2. Shown are sections of the first (left) and second (right) intestinal segment of carp fed with standard trout feed.

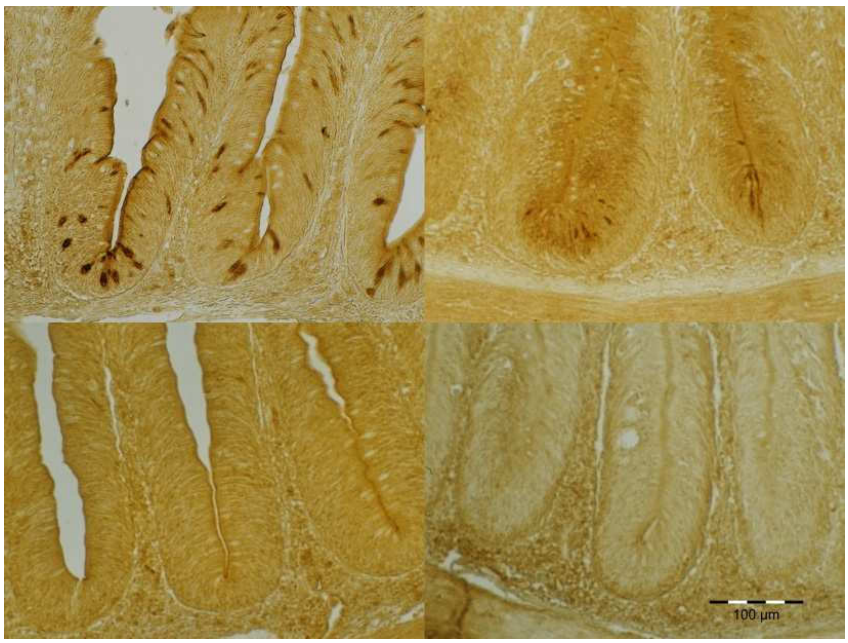

Fig. 2 Goblet cell staining for BD3. Shown are sections of the first (left) and second (right) intestinal segment of 0SBM (top) and day 7 after switching to 20SBM samples (bottom).

After switching from the 0SBM to the 20 SBM diet, significant differences to 0SBM could only be observed for BD3. These differences were observed only at the fold base between the control and week 1 fish post diet change (Fig 2.). The number of folds with stained goblet cells was significantly lower for the second intestinal segment at week 1 (none (none-few)) compared to the control (few (few - several)). The number of goblet cells per 390 µm of epithelial lining per fold was also significantly lower at week 1 compared to control. This was found for both first intestinal segment (none (none - none) compared to several (few - several)) and second intestinal segment (none (none - few) compared to few (few - several)).

HPLC

Mucus samples of naïve fish were analyzed for the presence of monosaccharides by reverse-phase HPLC (Table 4). In samples from the first segment NeuNAc/gal was abundantly present. For non-secreted mucus the percentage of NeuNAc/gal was significantly higher in the second segment when compared to first segment. For the second segment, glc/man and fuc could be frequently detected. GalNac could not be detected in the second segment. Therefore, the percentage of glc/man was significantly lower in the first than in the second segment for secreted mucus and for fuc in non-secreted mucus. One peak at RT 16 min could not be identified with the standard sugar solutions used. The peak area was large for both non-secreted and secreted mucus in the first segment (1.7 and 2.5 times the area of NeuNAc/gal). The area of the unidentified peak was smaller for the second segment. The size area was approximately that of the glc/man peak for the non-secreted mucus and for secreted mucus it was 1.2 times the size area of the glc/man peak.

Table 4 The presence of N-acetylneuraminic acid/galactose (NeuNAc/gal), glucose/mannose (glc/man), fucose (fuc) and N -acetylgalactosamine (galNAc) in non-secreted and secreted mucus from first segment (1-ns and 1-s respectively) and second segment (2-ns and 2-s respectively) of naïve carp.

	1-ns	1-s	2-ns	2-s	significant differences ($p<0.05$)
Total amount (µg)	16.0 ± 6.3	16.8 ± 6.1	2.4 ± 0.1	9.5 ± 0.2	2-ns < 2-s
% of total					
NeuNAc/gal	42.7 ± 5.7	39.0 ± 5.0	13.9 ± 2.6	27.7 ± 3.4	2-ns < 1-ns
glc/man	22.6 ± 6.8	29.3 ± 5.3	32.0 ± 4.6	47.0 ± 2.4	1-s < 2-s
fuc	15.4 ± 5.0	15.1 ± 4.7	54.2 ± 7.2	25.4 ± 1.0	1-ns < 2-ns
galNAc	19.3 ± 6.6	16.7 ± 4.7	0.0 ± 0.0	0.0 ±0.0	2-s < 1-s, 2-ns < 1-ns

Size exclusion chromatography

In all examined samples a biphasic profile could be observed. All samples showed a major protein residue between 120 and 180 kDa (Fig. 3) peaking at approximately 150 kDa. In all samples, a second major protein residue with molecules ranging between 12 and 70 kDa could be observed. The second major protein residue showed a high variation. In all residues a major peak at approximately 40 kDa with a second flanking peak at approximately 25 kDa was observed.

Although in all samples the peak at approximately 40 kDa and 25 kDa were visible, areas of the peaks were highly variable. Samples from the second intestinal segment showed a third peak in the second major residue at approximately 70 kDa. This peak was quite small with an average absorbence of 10 mAU but it was still very prominent (Fig. 3).

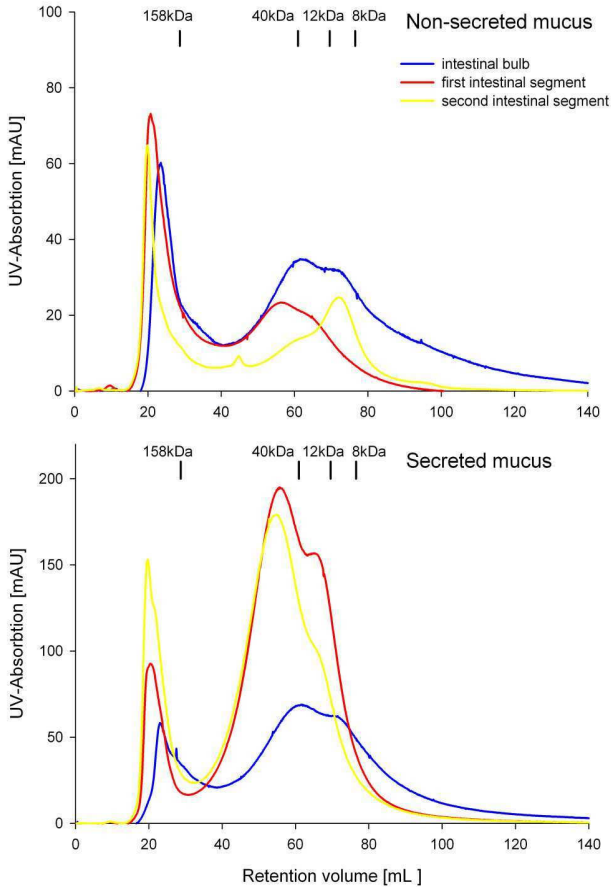

Fig. 3 Size exclusion chromatograms of non-secreted mucus (top) and secreted mucus (bottom) from the different intestinal segments of *C. carpio*. Indicated are standards for size exclusion chromatography with apoprotein (8 kDa), FK506 binding protein (12 kDa), MBP2* protein (40 kDa) and MBP-β-galactosidase (158 kDa).

Size areas of the major protein residue between 120 and 180 kD were also highly variable. However, this major protein residue is a single peak with little variation in retention time. Between non-secreted and secreted mucus samples from the same intestinal segment the retention time of the first peak was not significantly different. This was also true when samples from the first and second intestinal segment (peak at approximately 150 kDa) were compared. The protein peak from the mucus of the intestinal bulb had a slightly larger retention volume, indicating a slightly smaller protein size compared to the protein peaks from mucus of the first and second segments. Although size difference was small (approximately 10 kDa) the first major protein residue from the intestinal bulb had a significantly higher retention time compared to that of the first and second intestinal segment ($p < 0.001$).

All samples from the SBM experiment also showed a biphasic protein profile with a major protein peak at approximately 150 kDa. For non-secreted mucus in the first intestinal segment, switching to the 20SBM diet lead to a significant increase in peak height (8.0 fold increase on day 7 compared to day 0). The increase became less over time (7.2 fold (significant) on day 14 and 5.3 fold on day 20). No significant differences could be found for the peak height of the secreted mucus from the first intestinal segment and of both non-secreted and secreted mucus from the second intestinal segment.

Microbiological examination from the soybean experiment

None of the fish died during the soy bean feeding experiment. From the 23 fish that were sampled from only seven no bacteria could be cultured on blood agar plates. From all other carp facultative pathogenic bacteria (fluorescent and non-fluorescent *Pseudomonas* spp., *Aeromonas sobria*, *Aeromonas veronii* und *Cytophaga*-like bacteria) could be isolated

Bacterial content in the organs was for most fish (twelve out of sixteen) where bacteria could be isolated, low to moderate (Table 5). From none of the control fish or of the fish sampled three weeks after feed alteration, bacteria could be isolated in high numbers. On week 1 after feed alteration, significant higher bacteria intensity could be observed.

Table 5 Number of carp with a no, low, moderate and high bacterial content isolated from carp organs after switching to a 20SBM diet.

Bacterial content	Time point after diet change			
	Control (n=8)	Week 1*(n=5)	Week 2 (n=5)	Week 3 (n=5)
No	2	0	2	3
Low	3	0	1	1
Moderate	3	2	1	1
High	0	3	1	0

* Significant difference to control (p< 0 .05). Prevalence was compared to control and tested with One Way ANOVA.

Discussion

HMGs and antimirobial peptides in intestinal mucus

On fish mucin molecules relatively few studies have been conducted, however the data available indicate that fish mucus is similar in composition to the mucins secreted by mammalian goblet cells from epithelial tissues [78, 119, 120, 127, 201]. For *C. carpio*, mucus glycoproteins have so far been described under physiological circumstances and after bacterial challenge for *C. carpio* [78, 139, 140, 202]. For mammals, it is well known that different parts of the gastrointestinal tract have different mucus thicknesses [203, 204] and express different mucus genes [46, 205]. For carp so far two mucin genes have been found of which only one, *Muc2*, was expressed in the intestinal tract [206]. Since only one Muc gene has been identified in the intestinal tact it is not known wether different *Muc* gene profiles exist along the gut axis. However, morphological and functional differences have been previously found along the gut axis. Whethere these differens between different parts of

intestinal tract also include differences in mucus HMGs has to our knowledge not been extensively examined so far.

In this study, mucus glycoproteins within the goblet cells appeared to have a similar charge due to their glycosylation, as differences between the applied stainings could not be found. However, HPLC differences in the monosaccharide content of the mucus HMGs could be observed for both the first and second segments. Based on its molecular weight, the unidentified sugar in this study is probably ribose [131]. Ribose and 2-deoxyribose were the predominant sugars in rainbow trout skin mucus [100], but probably not in *C. carpio* skin mucus (unpublished data). For the first segment, the size area of the "ribose-peak" was the highest, indicating a different role for ribose in *C. carpio* skin and intestinal mucus. Fucose which is thought to be especially important for the viscoelasticity of the mucus, may vary strongly in intestinal mucus or anal gland mucus of mammals [132]. The fucose content of *C. carpio* intestinal mucus was dependent on the origin of the mucus (first or second segment). For non-secreted mucus in the second segment, fucose was the most predominant sugar, as was also observed for *C. carpio* skin mucus (unpublished data). Non-secreted mucus is thought to be present in a native form, whereas secreted mucus might be altered by environmental influences such as bacterial degradation. In the first segment NeuNAc/gal was predominant. The sugar galNAc could not be detected with the method used in the second segment, which was also the case for *C. carpio* skin mucus (unpublished data). In general, on the monosaccharide level, *C. carpio* skin mucus resembled the mucus of the second segment more than the mucus of the first segment.

Besides sugar analysis, protein content was examined by size exclusion chromatography. In all examined samples a biphasic profile could be observed. Non-secreted and secreted mucus samples from the different intestinal segments contained almost similar protein profiles: their residues are between 120 to 180 kDa and 12 to 70 kDa. The proteins found between 120 to 180 kDa most likely represent mucins which adhere to the epithelium, as they are similar to the adherent mucins (AMs) from previous reports [95]. The proteins found between 12 to 70 kDa probably consist of mucin constituents mixed with luminal proteins, the so-called luminal mucins (LM) [95]. Smaller molecules (LMs) probably represent the soluble luminal mucus. The AMs form a framework in which smaller molecules might be entrapped. This could explain why molecules smaller than the retention size of the ultrafiltration filter could be found.

LM might be essential for protection of the intestinal surface as higher bacterial adhesion to smaller mucus molecules has been observed [207]. Mucin degradation by pathogenic bacteria has been suggested earlier for *C. carpio* [140, 208] and might largely take place at the carbohydrate side chains of the mucins. In humans, it is known that bacteria with extracellular glycosidases may contribute to the damage of intestinal mucins [209]. In clinically healthy *C. carpio*, bacterial degradation of the protein core of mucus HMGs appears to be limited, since secreted and non-secreted mucus have comparable sized mucins indicated by their similar protein profiles.

However, between different intestinal segments differences in carbohydrate staining could be observed indicating differences in pH of the mucus. Differences between the first and second segment in the staining intensity of the glycoproteins in the goblet cells support the role of mucus in the defence against pathogens. This fits well in the immunological role as previously suggested for the second segment [210]. The mucus from the intestinal bulb might have also

a different function in defense against bacteria as differences in carbohydrate staining to that of first and second intestinal segment could be seen.
A possible different role for mucus in the defense against bacteria is underlined by the different staining for BD2. Staining for BD2 was more pronounced for tissue samples of the first than for the second segment. It is probable that under physiological circumstances, the release of BD2 in the first segment is high enough so that BD2 molecules can also be functional in the second segment. Staining for BD3 and lysozyme was low and similar between the first and second segment. Therefore under unchallenged conditions the humoral substances BD3 and Lys appear to be less important in *C. carpio*.
Besides the LMs also AMs were found. Functionally, large molecules (AMs) are regarded to form the mucus layer, which adheres to the epithelium. The main function of these proteins appears to be some kind of stabiliser or carrier for the mucus. In the present study, size areas of the major protein residue between 120 and 180 kD were variable in height, but were a single peak with little variation in retention time. Retention volume of the first peak were similar for samples from the first and second intestinal segment (peak at approximately 150 kDa), but was slightly but significantly larger (approximately 140 kDa) for mucus of the intestinal bulb. Size shifts of around 10 kDa are often caused by a difference in the glycosylation pattern of a protein. The smaller size of the molecules might play a role in food transportation, as smaller molecules are considered to be washed away easier. The intestinal bulb has a food storage function, with the mucus enveloping the food for easier transport. Furthermore a large part of the digestion has already taken place in the intestinal bulb.

Mucus alterations upon SBM

SBM is used in fish feed as it is a cheap source of protein. The use of soy bean in fish feed however, is sometimes causing problems as SBM containing diets are known to induce an inflammatory response in the hindgut of certain fish species. Contrary to previous observations made with *S. salar*, *C. carpio* start to recover or adapt to the SBM feed from the fourth week after the SBM feeding [75].
After SBM feeding, the second gut segment of *C. carpio* did not show external disease symptoms as found previously for carp by Uran *et al.* [75]. A clear loss of supranuclear vacualistion could not be found. A reduction in goblet cell number in the AB2.5 staining as found by Uran *et al.* [75] however could also be observed. This indicates that the carp in this study suffered from an acute but mild enteritis process. In carp, the enteritis process is accompanied by damages to the intestinal epithelium [75]. In the present study, a higher number of bacteria could be isolated from internal organs after SBM feeding. This might be explained by damage to the intestinal epithelium. The increased bacteria number in the internal organs indicates that the mucosal barrier as first line of defence was compromised, supporting the theory that the intestinal barrier was affected. For humans, increased gut permeability has been described following enteritis. If changes after enteritis persist a chronic inflammation can develop [197]. Between the control and week 3 after 20SBM feeding, no difference could be found in bacterial numbers in intestinal organs, indicating that the intestinal barrier function recovers over time.
All samples from the SBM experiment showed a biphasic protein profile with a major protein peak at approximately 150 kDa. The size of the large mucins did not change markedly. However, switching to the 20SBM diet led to a significant increase in peak height of 150 kDa

proteins in non-secreted mucus of the first segment, whereas the amount of 150 kDa in secreted mucus remained relatively stable. The obseved increase in peak heightbecame less over time. Mucus has a high turnover rate as mucus traps pathogens that can be removed from the body through a constant flow of mucus. The increase in the non-secreted mucus indicates that the SBM diet induces an increased mucus synthesis. The amount of newly synthesised mucus might increase the mucus flow, which would explain why the amount of secreted mucus remains stable. An increased mucus flow stimulates the removal of pathogens from the entire subsequent intestine, which might hereby prevent pathogens from entering the soy bean damaged intestinal epithelium. Changes in mucin levels during enteritis have been described for chronic inflammatory bowel diseases (IBD) in humans.

In IBD, genetic mutations in mucin genes, changes in sulphation, degree of glycosylation, mucin mRNA, protein levels and degradation of mucins have been described. Changes of immunological or bacterial factors during an initial or ongoing inflammation can influence mucin production, which could have further adverse effects on mucosal-bacterial interactions, hereby sustaining the chronic character of the inflammation [198]. In ulcerative colitis (a form of IBD) mucin protein levels [198], as well as the expression of MUC2 were reduced [211]. MUC2 is the structural component of the colonic mucus layer in humans [211]. Reduced Muc2 levels were also observed in interleukin (IL)-10 knockout mice that develop colitis [211]. The expression of mucin 2 proteins and the anti-inflammatory cztokine IL-10 by type 2 T helper cells and activated macrophages which induces its expression seems to be essential in the control of enteritis in mice. The increase in mucus glycoproteins as seen in the first intestinal segment in carp under SBM diet decreased over time, which indicates that mucus composition returns to the state before switching to the SBM-diet. An initial up-regulation of IL-10 at week 1, followed by a down-regulation to slightly below or to the level before the enteritis induction was observed previously for *C. carpio* [75].

In the present study, a 20SBM diet induced changes in the mucus, indicating that the mucosal layer showed altered mucus secretion upon 20SBM feeding. In the first segment the amount of sulphated glycoconjugates decreased, suggesting that similar to IBD reports [198] SBM feeding also leads to changes in mucus sulphation in *C. carpio*. In addition, mucus from SBM-fed *C. carpio* showed a different glycosylation pattern as was also reported for IBD [198]. The different glycosylation pattern is indicated by the significantly increased amount of neutral glycoconjugates in the first intestinal segment and the significantly decreased amount of acid and neutral glycoconjugates in the second intestinal segment.

Besides the changes in mucus HMs also a difference in staining for BD3 could be observed. One week after switching to the SBM-diet, the amount of goblet cells which were stained for the antimicrobial peptide BD3 was significantly reduced. This might indicate that BD3 was released to the intestinal lumen to fight pathogens, as BD3 in humans has a strong bactericidal activity [212]. No goblet cells could be stained at week 1 after the diet switch, this might indicate that BD3 was released more rapidly than it could be produced. After the initial depletion of the goblet cells, synthesis and secretion of BD3 seemed to return to their initial equilibrium, as on week 2 or 3, no differences compared to the control could be observed anymore.

Conclusion

In conclusion, no indication for a different protein core of mucus HMGS for first and second segment could be found. This is in contrast to what is found for humans and other mammalian species analyzed. In the present study, only differences in mucus glycosylation could be observed. Along the gut axis, between the intestinal segments and the intestinal bulb a difference in the size of the major protein peak could be observed. This difference however can also be caused by a different glycosylation. A difference between first and second segment could be observed for the antimicrobial peptide BD2.
Furthermore it was found that SBM-diet induces a change in mucins in *C. carpio* similar to that found in IBD in humans. Initial changes included; changes in mucin composition, the presence of BD3 and of bacteria in internal organs. After the initial changes, all values measured returned back to the initial pre-SBM diet values. This might indicate a recovery of the mucosal layer and thus a recovery of the primary barrier between the *C. carpio* and its surrounding. Changes in the mucosal layer form only a small part in the complex inflammation process of soybean mediated enteritis with its many symptoms such as the normal supranuclear vacuolisation of the absorptive cells in the intestinal epithelium [213]. The recovery of the extrinsic mucus layer may help *C. carpio* in recovering from SBM-enteritis and not developing a chronic inflammation, as through the restoration of the barrier the *C. carpio* is no longer constantly exposed to new pathogens.

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Chapter 7

Chemotaxis towards, adhesion to, and growth in carp gut mucus of two *Aeromonas hydrophila* strains with different pathogenicity for common carp, *Cyprinus carpio* L.

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Abstract

Characteristics that promote bacterial colonization of the intestinal mucosal surface were examined in two strains of the common fish pathogen *Aeromonas hydrophila*, with different pathogenicity. The characteristics examined were chemotactic activity towards mucus, bacterial adherence to mucus and growth in mucus. Intestinal gut mucus of healthy common carp was used. The results indicate that chemotaxis is not necessary for a bacterium to become pathogenic, but it may be a necessary parameter for a bacterium to be an obligate pathogen. Adhesion also seems to be a factor influencing pathogenicity. The results suggest that higher adhesion to mucus and subsequent growth is associated with differences in pathogenicity.

Introduction

Many infectious diseases are initiated by bacterial colonization of mucosal surfaces [77]. Aeromonas spp. are bacteria that are widespread throughout aquatic environments such as fresh surface water or marine water and are probably one of the leading causes of bacterial enteritis in humans [214]. *Aeromonas hydrophila* is a common pathogen and has been involved in heavy mortalities in farmed and feral fishes [215]. It is considered to be an opportunistic agent that only provokes clinical signs in stressed fish or fish affected by concurrent infections. In experimental infections, clinical signs could only be invoked when bacteria were injected intra-peritoneally [216]. Oral, anal or bath application is seldom used experimentally, as few or no pathological changes are observed when common disease indicators, such as blood parameters, are monitored [217, 218]. It appears that an intact mucosal surface is capable of forming a protective barrier against *A. hydrophila* [219].

The mucosal epithelial surface is protected against pathogens by the mucus layer and the mucins it contains. However, the ability of bacteria to bind to mucus components may actually facilitate the colonization of mucosal epithelial surfaces by immobilizing the bacteria in the mucus gel. For successful colonization, it is necessary that bacteria establish themselves in the mucus gel once they are bound to mucus. Normal erosion of the mucus gel is likely to wash away adherent bacteria. Therefore, to achieve successful colonization the replication rate of bacteria attached to mucus must be equal to or higher than the rate of erosion of the mucus layer itself. Thus, it would be advantageous for bacteria if they can use mucus as a nutrient source for their growth [71].

Some of the characteristics that promote bacterial colonization of the mucosal surface are chemotactic activity towards mucus (cell contact) [76] followed by bacterial adherence to mucins coating the mucosal cells[77] and subsequent growth in mucus. Several bacteria species are known to show chemotactic responses towards mucus [96, 97, 100, 220-222]. However different strains of the same species may show a different chemotactic behaviour. For instance, Hazen *et al.* [220] showed that different isolates of *A. hydrophila* have a different chemotactic index for largemouth bass skin mucus and that some isolates are non-chemotactic. Based on the chemotactic behaviour of the isolates examined, Hazen *et al.* [220] suggested that there could be a difference in pathogenicity between *A. hydrophila* strains.

This view is supported by the observation that different strains of bacteria may also show a different adhesion to mucus [223]. In a study of the intestinal bacterial microbiota of carp, Namba *et al.* [224] demonstrated that most isolates with high adhesive ability to intestinal mucus from carp belong to *Aeromonas* sp. In addition *A. hydrophila* can grow in mucus supplemented media [71, 225]. Whether different strains have a different growth in mucus is not known. Although Ascencio *et al.* [71] state that mucus can serve as a carbon and nitrogen source for *A. hydrophila*, it is not known whether carbon, nitrogen or phosphate are limiting factors for growth. In this study we examined chemotactic behaviour, adhesion and growth of two *A. hydrophila* strains isolated from fish.

Material and methods

Animals

Parasite and virus-free sibling common carp, *Cyprinus carpio* L., from a single crossing (R8S8 × R3S8, Wageningen University, The Netherlands) were used for mucus isolation. Carp were raised and kept in filtered re-circulated tap water. For all experiments, approximately 4-year-old carp with a mean body weight of 99.1 g (± 20.6 g) and a standard length of 14.1 cm (± 1.1 cm) were used. The carp were placed in a 400 L tank with filtered tap water 3 days before sampling, and were starved to reduce faecal contamination of intestinal mucus. Fish were killed by bath immersion with 500 mg L^{-1} tricaine (Sigma, Munich, Germany) and subsequently dissected. The entire intestinal tract from pseudogaster to anus was removed, weighed and cooled on ice.

Bacteria

Aeromonas hydrophila strain 38 (A38) was a gift from the Friedrich-Loeffler Institute, Federal Research Institute for Animal Health, Germany. *Aeromonas hydrophila* strain 60 (A60) was a gift from the Department of Veterinary Pathobiology, Royal Veterinary and Agricultural University Copenhagen, Denmark. Both *A. hydrophila* strains were isolated from carp with skin ulcers. *Edwardsiella tarda* was a gift from the Lower-Saxony State Office for Consumer Protection and Food Safety and was isolated from diseased catfish.

Bacteria were routinely grown in veal infusion medium and stored at 10^9 CFU mL^{-1} at −80 °C. To remove all possible nutrients, the bacterial suspension was centrifuged at 10 500 g for 10 min and the supernatant removed. For the infection and adhesion experiments, bacteria were resuspended in physiological salt solution. For the chemotaxis and growth experiments, bacteria were resuspended in a nine salt solution (NSS) (Marden, Tunlid, Malmcronafriberg, Odham & Kjelleberg 1985). NSS is a carbon, nitrogen and phosphorus free solution and was adjusted with 0.5% NaCl to 0.9% salinity.

Infection experiment

To determine whether the *A. hydrophila* strains used (A38 and A60) had different virulence, 100 carp were orally intubated either with physiological salt solution (control fish) or with 10^7 CFU of one of the *A. hydrophila* strains. All carp were intubated with a volume of 300 µL. Thirty-two carp were used per treatment. Carp were kept at 20 °C and eight fish per bacterial

strain were examined 1, 3, 6 and 10 days after oral intubation. Carp were killed, dissected for pathological–anatomical examination and swabs taken from liver, kidney and spleen for microbiological examination. Swabs were cultivated on blood agar plates for 2 days at 25 °C. Bacteria were identified with the API system (BioMerieux, France).

Isolation of mucus

Mucus was isolated from carp that were starved for 3 days to empty the gut. Intestines of untreated carp (mean gut weight 1.37 g ± 0.34 g) were opened longitudinally and cut into 3-4 mm small pieces. Subsequently, secreted luminal mucus was isolated with isolation medium as described previously [87, 139]. In brief, tissue pieces were incubated for 20 min in 100 mL isolation buffer containing protease inhibitors. The isolation buffer was collected and centrifuged for 30 min at 12 000 g. Subsequently, the supernatant was collected and frozen at -20 °C until further processing. All samples were concentrated by ultrafiltration (Amicon, Beverly, MA/USA, exclusion limit 30 000 Da) to a final volume of 2 mL. Mucus was sterilized by freezing at -80 °C for at least a week. Sterility was checked by plating out 100 µL of mucus and incubation for 2 days at 25 °C. For chemotaxis and growth experiments, the mucus was pooled (3-6 fish) and subsequently UV radiated for at least 1 h. For the adhesion experiments, mucus of eight fish was used. Protein content was determined by Bradford reaction (580 nm; BMG, Germany) [90]. Carbohydrate content was determined by the periodic acid-Schiff [226]reaction (540 nm) [88, 89].

Labelling of live bacteria

For adhesion experiments bacteria were stained with a green fluorescent nucleid acid stain (Syto 9, Invitrogen, Germany; 2 µL per 109 bacteria). Stained bacteria were spectrophotometrically analysed (470 nm excitation, 520 nm emission, BMG, Germany).

Chemotaxis

Chemotaxis assays were performed using a 10-well transmigration chamber (Neuroprobe, Gaithersburg, MD, USA). NSS with mucus (5 mg protein mL^{-1}) or without mucus (control) was placed in the lower wells. Bacterial suspension (10^9 CFU mL^{-1}) was placed into the upper wells of the transmigration chamber. Besides the two *A. hydrophila* strains, a strain of the obligate fish pathogenic bacterium *E. tarda* was used as a positive control. In all assays, NSS was used as a negative control in the upper and lower wells, to check for random migration. All treatments were done in duplicate. Upper and lower compartments of the transmigration chamber were separated by a polyvinylpyrrolidone (PVP)-treated polycarbonate filter with 2 µm pores (Neuroprobe). Filled transmigration chambers were incubated for approximately 16 h at 25 °C. Cells were harvested from upper and lower wells and spectrophotometrically analysed (470 nm excitation, 520 emission).

$UV_{migrated\ bacteria} = UV_{lower\ well} / (UV_{upper\ well} + UV_{lower\ well})$

Chemotactic index (CI) was calculated with fluorescence values (UV) as:

$CI = UV_{migrated\ bacteria\ in\ assayed\ conditions} / UV_{migrated\ bacteria\ in\ negative\ control}$

Adhesion

For adhesion experiments, mucus of carp was separated by means of downward gel filtration on a 34 × 0.9 cm Sepharose CL-4B column (Sigma, flow rate 5.2 mL h^{-1}, fraction size 1.3 mL, 40 fractions). The fractions pooled into three fraction pools of glycoproteins with different molecular size (30-670 kDa, 670-2000 kDA, >2000 kDa). Twenty-five microlitres of each sample was added in triplicate to a 96-well black polystyrene plate (Nunc, Denmark). As positive controls, bovine serum albumin (BSA) (1 mg mL^{-1}) and pig gastric mucin (5 mg mL^{-1}, Invitrogen) were used. Then 75 µL of coating buffer (16.8 g sodium hydrogen carbonate, 21.2 g sodium carbonate per litre, pH 9.6) was added to each well and left overnight at room temperature. After washing with physilogical salt solution, 25 µL of 10^9 CFU mL^{-1} fluorescent labelled bacteria-solution (A38 or A60) was added. A strain of *E. tarda* served as positive control. Bacteria were forced to the plate bottom by centrifugation (163 g, 12 s) and then incubated for 30 min in the dark at room temperature. Plates were washed and liquid was removed by centrifugation. For spectrophotometrical measurements 50 µL of physiological salt solution was added and the fluorescence generated by adherent bacteria was recorded with a microplate reader (BMG, Germany). Data were corrected on the basis of carbohydrate content of the mucus fractions applied and gut weight was used as an indication of mucus quantity [78]. Binding of mucus to the plate was confirmed by a lectin binding assay using ConA as described by Neuhaus *et al.*[139].

Growth

Both *A. hydrophila* strains were incubated with and without mucus and compared for growth. 1×10^8 CFU were incubated in the following media:

(i) Complete energy and nutrient depletion (NSS)
(ii) NSS supplemented with mucus (0.8 mg protein mL^{-1})
(iii) veal infusion medium

A38 (5×10^7 CFU mL^{-1}) was also incubated in several experimental media. NSS was supplemented with 1.1 g L^{-1} ammonium sulphate, 0.27 g L^{-1} potassium hydrogen phosphate and 2.0 g L^{-1} glucose or mucus. The following media were used:

(i) Complete energy and nutrient depletion (NSS)
(ii) Carbon limitation: NSS supplemented with ammonium sulphate and potassium hydrogen phosphate
(iii) Nitrogen limitation: NSS supplemented with glucose and potassium hydrogen phosphate
(iv) Phosphorus limitation: NSS supplemented with glucose and with ammonium sulphate
(v) Enriched medium: NSS supplemented with ammonium sulphate, potassium hydrogen phosphate and glucose
(vi) NSS supplemented with mucus (1.7 mg/mL)

Growth was monitored after 24 h by measurement of the optical density (595 nm) and by serial dilution and plate counts. Fold increase after 24 hours was calculated as follows:

$CFU_{\text{after 24 h in total medium}} / CFU_{\text{inocculated}}$.

Statistics

The chi-square test was used to compare the impact of the two *Aeromonas* strains on fish mortality and bacteria counts in internal tissues in the infection study. For the adhesion study, results were analysed using a Rank sum test. For growth studies Student's *t*-test was used. $P < 0.05$ was considered to be significant.

Results

Infection experiment

Clinical changes

None of the fish given 10^7 CFU died during the infection experiment. However, after intubation some fish did show clinical signs, such as redness of one or more fins, fin erosions or congestion of blood vessels. Some control fish (4 of 32) showed external changes, but signs of infection were more often observed in the bacteria-treated fish, where external changes were seen more frequently in the A38-treated fish (14 of 32) than in the A60-treated fish (7 of 32). However, results were not significantly different ($P = 0.062$). Redness of the skin was only observed in one fish in the control and A60 groups. However, five fish from the A38 group had a reddened skin. In the A38 fish, congestion of blood vessels was also observed. Pathological changes of internal organs were not observed in any fish.

Microbiological examination

Bacteria were isolated from some fish treated with 1×10^7 CFU A38 and A60 and also from control fish. Bacteria were isolated from one or more of the examined organs on all sampling days and were identified as *Pseudomonas putida*, *Staphylococcus* spp., *Gaffkya tetragena*, *Acinetobacter lwoffii* and *Shewanella putrefaciens*. *Aeromonas hydrophila* was re-isolated from only one fish (A38, day 6). In general, bacteria were isolated more frequently from A38-treated fish (8 of 32) than from control or A60-treated fish (2 and 3 of 32, respectively).

Chemotaxis

Syto9 stained A38, A60 and *E. tarda* equally. Bacterial vitality (motility and percentage of mobile bacteria) was not negatively influenced by staining or the presence of mucus, as determined visually under a fluorescence microscope 0, 1, 2, 3 and 20 h after incubation at 25°C.

In the first migration experiment, *A. hydrophila* were incubated overnight with 5 mg mucus protein mL^{-1} as chemoattractant in the lower well of the chemotaxis chamber. In this assay, a CI less than one was recorded for the *A. hydrophila* strains examined. The A38 and A60 strains had a CI of 0.75 and 0.63, respectively. *E. tarda* on the other hand showed a high chemoattractive movement towards the mucus (CI = 3.52). In a second migration experiment, different mucus concentrations (0.3, 1 and 3 mg mucus protein mL^{-1}) were used in a 3 h chemotactic assay with the A38 strain. At low mucus concentrations (0.3 and 1 mg mL^{-1}), no differences in CI (CI = 0.95) were found. Only at a higher mucus concentration (3 mg mL^{-1} mucus protein), a slight difference in CI was found (CI = 1.28). The viscosity of mucus might slow bacterial movement. Therefore, the A38 strain was also incubated in a solution

supplemented with 3 mg mucus protein mL^{-1} and the movement towards NSS observed. More bacteria moved into NSS when incubated in mucus than when incubated with NSS (CI = 1.42). It appears that the viscosity of mucus at this specific concentration is not able to slow bacteria. The increased bacterial movement might indicate a chemokinetic effect of gut mucus on *A. hydrophila*.

Adhesion

Aeromonas hydrophila strain A38 adhered more strongly to mucus glycoproteins of all size fractions tested than the A60 strain (see Fig. 1). Bacteria from both strains adhered most strongly to molecules with a size of 670–2000 kDa and weakest to molecules with a size of 30–670 kDa. The fluorescence intensity of bacteria which adhered to mucus molecules of 30 to >2000 kDa was 13.3 times higher for the A38 than for the A60 strain. *Edwardsiella tarda* showed an even higher fluorescence intensity (59.5 times higher than the A60 bacteria adhering to mucus, see Fig. 1).

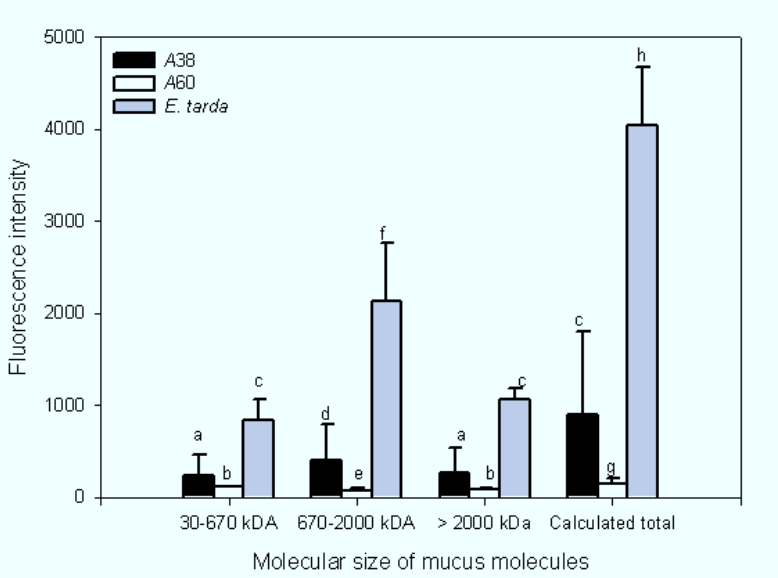

Fig. 1 Adhesion of the facultative pathogen *Aeromonas hydrophila* strains A38 and A60, and the obligate pathogen *Edwardsiella tarda* to intestinal carp mucus (n = 8) of different molecular size ranges. All values represent mean and standard deviation of absorbancy measurements of fluorescent labelled bacteria. Mean values with different superscripts are significantly different (Rank sum test, $P < 0.05$).

Growth

Bacterial growth was determined by measuring OD and by serial dilutions with plate counts for all cultures in the growth experiments. However, OD values were only used to estimate which dilution was necessary for the serial dilution assays. OD values were not used for data processing, since for mucus-supplemented NSS cultures no correlation was found between OD values and the CFU mL^{-1} cell culture.

The total yield of CFU after 24 h starvation in NSS was approximately the same as the amount of CFU with which the culture was inoculated (Figs 2 & 3). Both strains grew rapidly in the

normal culture medium (veal infusion medium, Fig. 2). In this medium, the A60 strain produced a 2.8 times higher yield than the A38 strain. Addition of mucus to NSS cultures clearly increased bacterial growth after 24 h. In this medium, the A38 strain produced a 2.3 times higher yield than the A60 strain. Veal infusion medium was a better medium for growth for the A60 strain than NSS supplemented with mucus (total yield four times higher). A38, on the other hand, grew better in mucus-supplemented NSS than in veal infusion medium (Fig. 2).

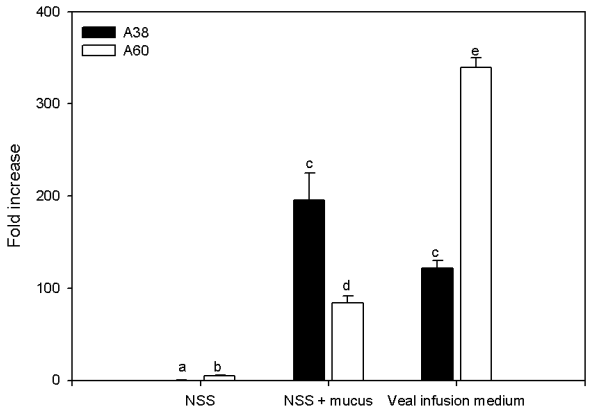

Fig. 2 Fold increase of cell-forming units of *Aeromonas hydrophila* strains A38 and A60 after 24 h cultivation in a nutrient depleted medium (NSS), a nutrient depleted medium supplemented with 0.8 mg mL^{-1} mucus protein and a normal culture medium (veal infusion medium). All values represent mean and standard deviation. Mean values with different superscripts are significantly different (Student's *t*-test, $P < 0.05$).

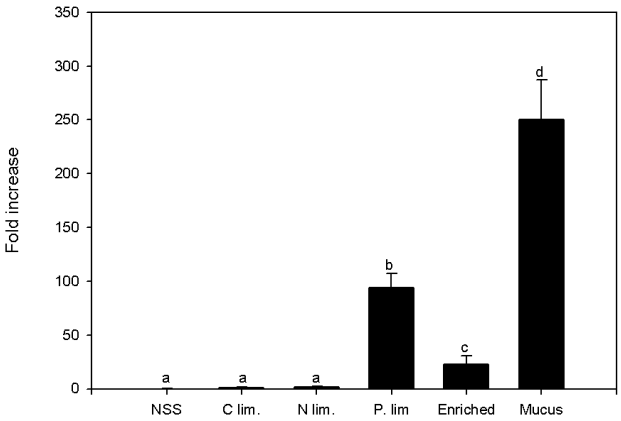

Fig. 3 Fold increase of cell-forming units of *Aeromonas hydrophila* strain A38 after 24 h cultivation in nutrient depleted media, complete nutrient depleted medium (NSS), carbon limited medium (C lim.), nitrogen limited medium (N lim.), phosphorus limited medium (P lim.), medium supplemented with carbon, nitrogen, phosphorus and glucose (Enriched) and medium supplemented with 2.0 mg mL^{-1} mucus protein (Mucus). All values represent mean and standard deviation. Mean values with different superscripts are significantly different (Student's *t*-test, $P < 0.05$).

A38 grew better in mucus-supplemented NSS than A60 and was therefore used to determine which nutrients are limiting for *A. hydrophila* and which may be obtained from mucus. Cultures with NSS supplemented with carbon, nitrogen and phosphorus from inorganic sources promoted bacterial growth differently: bacterial growth was only slightly increased in media with carbon or nitrogen depletion, in comparison with completely depleted medium (NSS). Medium with phosphorus depletion allowed up to 100-fold increase in bacteria compared with NSS. None of the inorganic supplements enhanced bacterial growth as much as supplementation with mucus (Fig. 3).

Discussion

Aeromonas *hydrophila* is a facultative pathogen of fish and can cause secondary infections [227]. In general, *A. hydrophila* can not cause disease in healthy, well-conditioned fish [228]. Carp used in the present infection experiments were healthy, parasite and virus free and kept in well-filtered tap water. It is therefore not surprising that no severe clinical signs were observed in this experiment after bacterial intubation [229]. Mild redness of fins and skin was rarely observed in control fish and fish treated with *Aeromonas hydrophila* strain A60. Facultative pathogens, commonly resident in fish, can cause these signs in stressed fish. The carp in this experiment were stressed due to transfer to their new environment, starvation and oral application.

Previous experiments with platys, *Xyphophorus* spp. [229] showed that *A. hydrophila* can induce mortality. Fish that died showed clinical symptoms, such as redness in gills, head and vent [229]. All survivors, however, did not show any pathological changes. These findings are in accordance with the results of the present infection experiment, where fish treated with 10^7 CFU A38 did not show severe clinical signs. *A. hydrophila* could be re-isolated more often from A38 h than from A60-treated fish. This is in agreement with Kawula *et al.* [229], who only re-isolated *A. hydrophila* from organs of fish which died from the infection.

Fish treated with A38 showed clinical signs more often than the A60-treated fish. In addition, bacteria were isolated more often from internal organs after A38 treatment. This suggests that *A. hydrophila* A38 is more capable of destabilizing the intestinal barrier. The higher occurrence of clinical signs and higher number of fish where bacteria could be isolated from internal organs indicate that A38 is more virulent than A60.

In order to act as a pathogen, bacteria need to have contact with the host. This can be coincidental or the result of directed chemotactic movement. In a chemotaxis, capillary assay movement towards mucus of fish was observed for some *A. hydrophila* strains [220]. The chemotaxis response for *A. hydrophila* strains towards mucus found in the present experiment and as reported by Hazen *et al.* (1982) is a low response (approximately 1). The present study showed that gut mucus of carp did not induce a chemotactic migration of the two *A. hydrophila* strains used, but it might induce an increased chemokinesis, which results in enhanced migration of the bacteria and thus allows it to more readily reach the fish epithelium.

Greater chemoattractive movement towards mucus from a different location is not to be expected as intestinal mucus is equally chemoattractant as skin mucus and more

chemoattractant than gill mucus [230]. O´Toole *et al.*(1999) found that a wild type *Vibrio anguillarum* had a much higher chemotactic response towards intestinal mucus (relative response approximately 140) than *A. hydrophila*. This large difference in chemotactic response might be a reason why *A. hydrophila* is an opportunistic pathogen and *V. anguillarum* is a more frequent causative agent of disease.

The mucus used in the chemotaxis experiment came from healthy virus and parasite-free fish. Hazen *et al.* (1982) found differences in chemotactic activity of *Aeromona*s bacteria towards surface mucus from healthy largemouth bass and surface mucus from red sore-infected largemouth bass. It is, however, debatable whether this difference is caused by excretion of antibodies as suggested by Hazen *et al.*, (1982), or by an alteration of the major constituent of mucus, glycoproteins. During infection, mucus glycoproteins (mucins) are known to have altered molecular size and glycosylation pattern [76, 139, 219, 231]. An up-regulation of the expression of an MUC-encoding gene that codes for the mucin protein core, is also recorded in the context of bacterial infection [232]. Altered diet also influences mucus composition [233]. Since diseased fish usually change or stop their food intake such an influence should be excluded. Therefore, when comparing mucus from diseased and non-diseased fish the mucus should always originate from artificially infected fish kept under controlled conditions. Mucus should also be corrected for protein content as it is known that total mucus production can be modulated in diseased fish [125, 139, 234, 235].

Bacteria must attach to host mucus in order to become pathogenic. However, adhesion to mucus is not necessarily a sign of pathogenicity or virulence. For instance, probiotics are chosen for their ability to bind to mucus [236]. Nevertheless, adhesion of pathogens to the mucosal surface is the first step in the pathogenesis of most infectious diseases [96, 237, 238]. In infection experiments, it is observed that more virulent strains of a bacterium show a higher adhesion than less virulent strains. This was reported for example in *Yersinia enterocolitica* [102, 239] and *Pseudomonas cepacia* [240]. The higher adherence of A38 therefore suggests that this strain has a higher virulence than A60.

When bacteria bind to mucus, they bind to the major constituent of mucus, i.e. glycoproteins [71]. Glycoproteins are highly glycosylated proteins [1, 51-53, 104], where the protein core is usually not accessible to pathogens due to the carbohydrate side-chains [241]. Therefore, bacteria usually bind predominantly to the carbohydrate side-chains of the glycoproteins [71, 77, 102, 239, 240, 242]. Mantle and Husar [102] found that some bacteria can bind better to mucus glycoproteins when some of the carbohydrate side-chains are cleaved off from the protein core. A possible explanation is that the bacteria can now bind to carbohydrates which lie closer to the protein core or even to the core itself [102, 240]. Glycoproteins of high molecular weight are considered to represent matured, fully glycosylated proteins. Smaller glycoproteins may either be newly synthesized glycoproteins, where not all the glycan side chains are already formed or they may be matured glycoproteins where glycan side chains have already been cleaved by bacteria or intestinal enzymes [96, 139, 243]. The higher adherence of *A. hydrophila* in this study to mucus molecules sized 670–2000 kDa compared with molecules of 30–670 kDa or >2000 kDa might therefore be explained by a difference in the carbohydrate side chains of the glycoproteins.

In order to become pathogenic, bacteria must be able to use mucus for growth once they have adhered to it. They need to multiply fast to prevent their being flushed out of the gut. Both *A. hydrophila* strains grew in mucus-supplemented media. A38 however had a higher

total yield than A60 when cultured under the same conditions. Both strains did not grow in nutrient depleted medium. This agrees with previous findings for *A. hydrophila* [71]. Culture of A38 in inorganic nutrient-rich (phosphorus limited or enriched) medium enhanced bacterial growth, but not as strongly as did mucus supplementation. This might indicate that the bacteria can use mucus as an energy source.

Optical density values were not used in the present experiments to process growth data, since for mucus-supplemented NSS cultures no correlation could be found between OD values and the bacterial content measured as CFU mL−1 cell culture. Aeromonas hydrophila utilizes mucus and therefore most likely degrades it. With increasing growth of bacteria, mucus might have a changed OD. Different bacteria might process mucus in a different way or to a different degree. Furthermore, different mucus composition (e.g. due to diet or disease) might also influence how bacteria process mucus. Therefore, OD measurements on mucus should be considered unreliable when monitoring bacterial growth. When only OD values are used to fit data to growth models [225], bacterial suspensions should be centrifuged and the bacteria diluted again in a known volume of a medium devoid of mucus. It is doubtful if growth models are fitted correctly when no reliable blank value is available.

The present study indicates that chemotaxis is not necessary for a bacterium to become pathogenic, as both of the *A. hydrophila* strains used did not move towards mucus. However, it might be a necessary parameter for an obligate pathogenic bacterium, as the pathogenic bacterium *E. tarda* cultured under the same conditions as *A. hydrophila* did show a strong chemotactic movement towards gut mucus.

Adhesion also seems to be a factor influencing pathogenicity as *E. tarda* showed a very high adhesion to mucus. A38 showed a 13 times higher adhesion to mucus than the A60 strain. This agrees with the findings of the infection experiment where A38 was apparently more pathogenic than the A60 strain.

The A38 strain also showed a higher growth in mucus than the A60 strain. A faster multiplication of bacteria in mucus increases its potential pathogenicity as the flushing out of bacteria due to mucus erosion is reduced. A higher adhesion and subsequent growth may lead to differences in pathogenicity.

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Chapter 8

Intestinal mucus of challenged carp

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This chapter is an updated version of the articles “Biochemical and histochemical effects of perorally applied endotoxin on intestinal mucin glycoproteins of the common carp *Cyprinus carpio*., with special consideration of mucin glycoproteins” [139, see appendix II] and “Changes of intestinal mucus glycoproteins after peroral application of *Aeromonas hydrophila* to common carp (*Cyprinus carpio*)” [140, see appendix III] and contains additional information.

Abstract

Mucins are high molecular weight glycoproteins produced by specialized cells (goblet cells) and secreted on mucosal surfaces. Released glycoproteins form a biofilm protecting the underlying epithelium. Aim of the present study was study the effect of different challenges on the intestinal mucus from common carp, Cyprinus carpio. Here to carp were exposed to a mechanical challenge, a challenge with a bacterial endotoxin or to a challenge with one of two strains of *A. hydrophila* strains (A38 and A60). Intestinal mucus was isolated and used to determine mucin weight, glycosylation, protein and carbohydrate distribution over different molecule size. Subsequently, terminal glycosylation was characterized by a lectin-binding assay.

With the lectin ELISA the five monosaccharides which primarily occur in the glycosylation of piscine mucins were tested and differences in terminal glycosylation were found. Changes occurred quickly after challenge.

With the isolation of the HMG and the determination of protein and carbohydrate content, differences between the control and treated fish could be observed for mucin content, glycosylation and distribution of carbohydrate and protein over the size fractions. After the bacterial challenges three trends could be observed. For each bacterial challenge each trend was observed at different times, indicating different mechanisms leading to the observed changes. The first trend indicates that a bacterial challenge leads to release of mucus from the goblet cells, but this release is not sufficient to keep the amount of mucus stable over the next days. The second trends indicates that a bacterial challenge leads to a degradation of secreted molecules followed by a release of newly synthesized high glycosylated molecules. The third trend indicates a shift in the equilibrium between release and degradation of mucus molecules.

Introduction

One of the major routes of infection in fish is through the gastrointestinal tract [244]. An overwhelming number of infectious diseases are initiated by bacterial colonisation of mucosal surfaces [77]. The epithelium is protected from chemical, enzymatic, and mechanical damage by overlaying mucus. This water-insoluble mucus layer on the epithelial cells also plays an important role in host defence mechanisms. Mucus consists mostly of water and high molecular weight glycoproteins (HMG), called “mucins”. Both are important in gel formation [1, 51, 53, 104]. Mucins are produced by goblet cells, which are scattered in many epithelial

systems of aquatic vertebrates and particularly in the gut. Mucus HMG exhibit a high content of oligosaccharides, which are believed to mediate adhesion of microbiata [79, 80, 92, 97] and to prevent the glycoproteins from degradation by proteases of microbial origin.
Based on histochemical data, the number and content of intestinal goblet cells in fish are considered to be similar to those of mammals. As in mammals, a production of secretory vesicles with neutral, acidic or sulphated glycoproteins has been described [6, 81]. Histological and histochemical studies also indicate that fishes with parasitic infections, like mammals, display goblet cell hyperplasia and hypertrophy, whereby as a chronic reaction their intracellular mucins shift towards acidic and sulphated glycoprotein types [81, 83, 84].
Although all fish surfaces are covered with a mucus layer, knowledge concerning piscine mucins and their responses to noxes is scarce. In the present study, the biochemical composition of carp glycoproteins and the changes upon a challenge is studied, using a mechanical, an endotoxin or a live bacteria challenge. Therefore intestinal goblet cells of the common carp were characterized with biochemical and histochemical methods.

Materials and Methods

Challenges

Parasite and virus free sibling carp from a single crossing (R3 x R8, Wageningen Agricultural University, The Netherlands) were used. Carp were raised and kept in filtered recirculated tap water. Carp were used to examine the effect of a mechanical challenge (induced by application of physiological salt solution) (body weight 88.3 ± 18.7, gut weight 1.10 ± 0.25 g), of an endotoxin challenge (LPS-experiment; body weight 76.4 ± 12.9, gut weight, 1.06 ± 0.2 g)), and of two live bacteria strains (*A. hydrophila*-experiment; body weight 81.6 ± 19.2, gut weight, 1.00 ± 0.28 g) on intestinal mucus (see Table 1).
Fish were treated by peroral application. Carp were starved three days before treatment to reduce faeces on intestinal mucus. Before intubation, the animals were anaesthetized by bath immersion with 150 mg l^{-1} tricaine (Sigma, Germany).
For LPS-experiment, 150 µg LPS of *Escherichia coli* O55:B5 (Sigma, Germany) per gram body weight dissolved in PBS was used. PBS was taken for the control fish. In the *A. hydrophila*-experiment, carp were perorally intubated with a physiological salt solution (control fish) or one of the two *A. hydrophila* strains (A38 or A60). These control fish were used in the mechanical challenge as treated fish. All fish were intubated with a total volume of 300 µl containing 10^7 CFU bacteria.

Bacteria

Two strains of motile Aeromonads were used, that were isolated from diseased commercially farmed carp. The two strains were examined by standard biochemical diagnostic methods and classified as *Aeromonas hydrophila*. *A. hydrophila* strain 38 (A38) was a gift from the Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Germany. *A. hydrophila* strain 60 (A60) was a gift from the Department of Veterinary Pathobiology, Royal Veterinary and Agricultural University Copenhagen, Denmark [245]. Bacteria were routinely grown on sheep blood agar at 25°C and stored in veal infusion medium at 10^9 bacteria mL^{-1} at

–80°C. To remove all possible nutrients, bacterial cells were harvested by centrifugation at 10,500 x g for 10 min and the supernatant was removed. Bacteria were re-suspended in physiological salt solution.

The used strains differed in adhesion ability to mucus, growth rate in mucus, *in vitro* cytotoxicity and presence of a type III secretion system (TTSS). A38 adhered more strongly to mucus and had a higher growth rate [208]. When monolayers of EPC-cells were incubated with A60 or with physiological salt solution, no changes in cell morphology or monolayer coherence were observed over a period of 24 h. However, when EPC-monolayers were incubated with A38 over this time period, the EPC-monolayer had detached completely from the bottom of the culture vessel, and only a few viable cells could be recognised [140]. On a genomic level the TTSS was found for A38, but not for A60 (unpublished data). For both *A. hydrophila*-strains, both live and dead bacteria were not detectable by periodic-acid-Schiff reaction.

Table 1 Experimental setup of the mechanical challenge, LPS-experiment and *A. hydrophila*-experiment

Age	Experiment (treatment)	Sampling time points after intubation (days)	Fish per time point (n=)
12-18 months	LPS-experiment (PBS: ctrl)	1, 2, 3, 5, 8	2
12-18 months	LPS-experiment (LPS)	1, 2, 3, 5, 8	8
ca 4 year	mechanical challenge (none: ctrl)	-	4
ca 4 year	mechanical challenge (NaCl), *A. hydrophila*-experiment (NaCl: ctrl)	1, 3, 6, 10	8
ca 4 year	*A. hydrophila*-experiment (A38)	1, 3, 6, 10	8
ca 4 year	*A. hydrophila*-experiment (A60)	1, 3, 6, 10	8

Sampling and analysis

For sampling, carp were killed by bath immersion with 500 mg l^{-1} tricaine (Sigma, Deisenhofen, Germany). They were weighed and subsequently dissected. The complete intestinal tract from behind the intestinal bulb to anus was removed, weighed and cooled on ice.

From the intestine of all fish secreted intestinal mucus was isolated. Furthermore, from the LPS-treated and control carp (Table 1) also non-secreted mucus (present in the intestinal goblet cells) was isolated. Isolated intestinal mucus was processed and the high molecular weight glycoproteins (HMG) were analysed by gelchromotography and subsequent protein and carbohydrate determination. Isolation, processing, analysis and calculations were done as described in Chapter 2.

From all untreated and control samples a biphasic profile was obtained after downward gel filtration and subsequent staining for carbohydrates and proteins. In between the first peak (peak 1), which contains the largest molecules and the second peak (peak 2) a transition area (TA) could be found. Gel filtration, carbohydrate and protein staining as well as subsequent calculations were done as described in Chapter 2.

Fractions from the gel chromatography were pooled into peak 1, peak 2 and a transition area (TA) between these peaks. These pools (except the mechanical challenge pool) were used for a lectin-ELISA (cf. Chapter 2) to determine terminal glycosylation.

Statistics

If not indicated else, results are expressed as median value and 25% -75% quartiles. Data were statistically analysed with an analysis of variance (ANOVA for normally distributed data, and ANOVA-on-Ranks for non-normally distributed data). Differences were considered significant at $p < 0.05$. If not indicated else, treatments were compared to the control group.

Results

Animals

All fish appeared healthy without clinical alterations throughout the study.

Isolation of intestinal mucus HMG

Mechanical challenged carp

Mucus remained relatively unchanged after a peroral application of PBS. No significant effect othe amount of mucus which could be isolated, was found (Table 2). However, total glycosylation was significantly higher on all days from carp exposed to a mechanical challenge compared to untreated carp and for TA on some days (Table 3).

Table 2 Total mucin weight (in g per mg gut weight) of carp intestinal mucus HMG of mechanically challenged, bacterial challenged as well as LPS challenged carp. Shown are median and 25% - 75% quartiles and significant differences to control ($p<0.05$).

Challenge	ctrl	day 1	day 2	day 3	day 6/5	day 10/8
Secreted mucus						
mechanic	2.9 (2.4 - 3.3)	2.2 (1.7 - 3.6)	No data	2.5 (2.1 - 3.0)	3.9 (3.7 - 4.1)	1.4 (1.4 - 1.5)
al	2.3 (1.6 - 3.5)	1.2 (0.8 - 1.6)	No data	1.2 (1.0 - 1.5)	1.5 (1.4 - 1.7)	1.0 (0.9 - 1.3)
A38	2.3 (1.6 - 3.5)	2.5 (1.6 - 3.3)	No data	3.6 (3.1 - 4.6)	2.9 (2.2 - 3.8)	1.8 (1.7 - 3.2)
A60	1.8 (1.6 - 1.9)	2.0 (1.5 - 2.1)	1.0 (0.7 - 1.0)	1.3 (1.1- 1.6	1.6 (1.5- 2.0)	1.8 (1.5 - 2.0)
LPS						
Non-secreted mucus						
LPS	0.6 (0.5 - 0.6)	0.4 (0.4 - 0.6)	0.6 (0.6 - 0.7)	0.8 (0.7 - 1.1)	0.9 (0.8 - 1.2)	0.7 (0.6 - 0.8)

7

A38/A60 challenged carp

The amount of mucus that could be isolated decreased significantly after oral intubation with *A. hydrophila* 38 whereas it did not significantly change after intubation with *A. hydrophila* 60 (Table 2). On day 3 and day 10 the amount of mucus which could be isolated was significantly lower for A38 compared to A60. On day 1 and 3 after A38 challenge, molecules of TA showed a significantly higher protein to carbohydrate ratio (P:C) (Table 3), indicating a lower glycosylation as well as a higher protein percentage. The significantly higher P:C ratio was also observed after the challenge with *A*60 on day 1 for TA, peak 2 as well as the total mucus. In contrast to the challenge with A38, the A60 challenge resulted in a significantly higher protein percentage for TA on day 6 and 10 (Table 4).

LPS challenged carp
Mucus amount which was secreted decreased significantly on day 2 after LPS challenge (Table 2). On day 2, molecules from TA and total mucus were less glycosylated. Also changes in protein and carbohydrate distribution were observed (Table 4, Table 5). The percentage of molecules found in TA increased significantly. On day 3 a significantly higher glycosylation of molecules in peak 2 could be observed. On day 5 a significantly higher amount of non-secreted mucus could be isolated compared to control.

Terminal glycosylation

As UEA-I results were below or around level of detection the results were not statistically analysed. After LPS challenge only few significant differences in terminal glycosylation could be observed (Table 6). A stronger binding of SNA (binding to αNeuNAc(2→6)gal) could be found on day2 for the secreted mucus after LPS-challenge. After this challenge ConA (binding to α–man and α–D-glc) showed a stronger binding compared to control in peak 1 on day2 for the non-secreted mucus and on all days for the secreted mucus.
No significant changes could be found in peak 2 after LPS or after A38/A60 challenge. However, after A38 and A60 significant changes in terminal glycosylation could be found in the total OD per tested sugar as well as for the percentage of the total OD in peak 1 and in TA. On day 10 the percentage of the OD found in TA was significantly lower for all lectins compared to control. Total OD on this day was higher for both A38 and A60 for RCA-1 (binding to galNAc and β-gal) as well as for SNA for A60. On day 1 only for A60 for conA a significantly lower total binding was observed and on day 3 a lower binding to RCA. On day 6 a higher binding could be observed for RCA for A38.

Discussion

Mechanical challenged carp

When carp were exposed to a mechanical challenge, total glycosylation and therefore the amount of carbohydrates relative to the amount of protein was significantly higher compared to untreated carp. The increased carbohydrate portion might be a microbial defence mechanism. In rats, changes in mucus composition were also observed after mechanical challenge and food uptake [123, 246, 247].

LPS

In the present study, carp were perorally intubated with LPS. Lipopolysaccharide (LPS)-protein-complexes are mainly synthesized by gram negative bacteria. LPS is an endotoxin, which is an integrated part of the bacterial membrane. Endotoxin has, at least in mammals, a local and systemic immune-inducing function in inflammation reactions [248, 249]. Toxic effects in fish are mediated by different factors as in mammals or have not been identified yet. In many in vitro studies on cells of lower vertebrates such as fish, extremely high concentrations of LPS (µg ml-1) have been used before immune responses could be induced [250].

Table 3 Glycosylation of intestinal mucus HMG, expressed as P:C ratio after a mechanically challenge, bacterial challenge as well as a LPS challenge. Shown are median and quartiles and significant (sign.) differences to control (p<0.05).

Challenge	ctrl	day 1	day 2	day 3	day 6/5	day 10/8	Sign,differences to ctrl
Peak 1							
Secreted mucus							
mechanical	0.3 (0.3 - 0.5)	0.2 (0.1 - 0.2)	No data	0.3 (0.2 - 0.3)	0.2 (0.2 - 0.3)	0.6 (0.3 - 0.7)	
A38	0.2 (0.1 - 0.3)	0.5 (0.2 - 0.8)	No data	0.4 (0.3 - 0.5)	0.3 (0.3 - 0.4)	0.6 (0.5 - 0.7)	
A60	0.2 (0.1 - 0.3)	0.3 (0.2 - 0.5)	No data	0.3 (0.2 - 0.4)	0.2 (0.1 - 0.2)	0.3 (0.1 - 0.4)	
LPS	0.4 (0.4 - 0.6)	0.6 (0.4 - 0.8)	0.5 (0.4 - 0.6)	0.7 (0.6 - 0.7)	0.6 (0.5 - 1.0)	0.7 (0.5 - 0.8)	
Non-secreted mucus							
LPS	1.2 (0.8 - 1.5)	2.7 (1.6 - 3.0)	1.3 (1.0 - 1.5)	1.1 (0.9 - 1.5)	1.5 (1.1 - 2.0)	0.7 (0.6 - 0.8)	ctrl < d1
TA							
Secreted mucus							
mechanical	0.5 (0.4 - 0.7)	0.2 (0.2 -0.3)	No data	0.2 (0.2 - 0.3)	0.3 (0.3 - 0.3)	0.6 (0.4 - 0.6)	d1, d3 < ctrl
A38	0.2 (0.2 - 0.3)	0.7 (0.4 - 1.1)	No data	0.8 (0.4 - 1.0)	0.4 (0.3 - 0.5)	0.5 (0.5 - 0.5)	ctrl < d1, d3
A60	0.2 (0.2 - 0.3)	0.5 (0.3 - 0.7)	No data	0.4 (0.3 - 0.4)	0.4 (0.3 - 0.5)	0.4 (0.3 - 0.4)	ctrl < d1
LPS	2.0 (1.6 - 2.4)	2.2 (1.9 - 2.6)	6.0 (5.4 - 7.3)	1.9 (1.5 - 2.3)	1.8 (1.7 - 2.4)	1.0 (1.0 - 1.5)	ctrl < d2
Non-secreted mucus							
LPS	1.7 (1.5 - 2.0)	3.9 (2.8 - 5.1)	1.8 (1.6 - 2.0)	1.7 (1.3 - 2.0)	2.4 (1.7 - 2.8)	1.7 (1.2 -2.2)	ctrl < d1
Peak 2							
Secreted mucus							
mechanical	1.3 (1.1 - 1.5)	0.8 (0.6 -0.9)	No data	0.7 (0.6 - 0.8)	0.6 (0.6 - 0.6)	0.5 (0.4 - 1.0)	
A38	0.7 (0.6 - 0.9)	1.1 (0.6 - 1.5)	No data	0.5 (0.4 - 0.8)	0.4 (0.4 - 0.6)	0.8 (0.6 - 0.8)	ctrl < d1
A60	0.7 (0.6 - 0.9)	1.0 (0.9 - 1.2)	No data	0.9 (0.8 - 0.9)	0.7 (0.6 - 0.8)	0.4 (0.4 - 0.8)	
LPS	5.0 (3.7 - 5.6)	4.0 (3.6 - 5.7)	5.8 (5.5 - 7.1)	3.4 (3.1 - 3.9)	3.5 (2.9 - 4.7)	4.3 (3.5 - 4.7)	d3 < ctrl
Non-secreted mucus							
LPS	4.5 (4.1 - 6.4)	4.6 (4.0 - 6.3)	2.0 (1.5 - 2.4)	0.9 (0.7 - 2.5)	2.5 (1.2 - 3.2)	3.6 (1.8 - 11.5)	
Total							
Secreted mucus							
mechanical	1.0 (0.9 - 1.0)	0.5 (0.4 - 0.6)	No data	0.4 (0.4 - 0.5)	0.4 (0.4 - 0.5)	0.7 (0.4 - 0.7)	d1, d3, d6, d10 < ctrl
A38	0.5 (0.4 - 0.5)	0.8 (0.6 - 1.0)	No data	0.5 (0.5 - 0.6)	0.4 (0.4 - 0.5)	0.6 (0.6 - 0.6)	
A60	0.5 (0.4 - 0.5)	0.8 (0.7 - 0.9)	No data	0.5 (0.5 - 0.5)	0.4 (0.4 - 0.5)	0.4 (0.3 - 0.4)	ctrl < d1
LPS	2.9 (2.2 - 3.2)	2.4 (2.2 - 3.2)	4.8 (4.4 - 5.0)	2.0 (1.9 - 2.4)	2.5 (1.8 - 2.6)	2.1 (2.0 - 2.4)	ctrl < d2
Non-secreted mucus							
LPS	2.3 (1.9 - 2.5)	4.1 (2.7 - 4.2)	1.7 (1.5 - 1.7)	1.2 (0.9 - 2.1)	2.0 (1.6 - 2.1)	1.4 (1.2 - 1.7)	ctrl < d1

Table 4 Protein distribution in percent of mucus HMG for peak 1 after a mechanically challenge, bacterial challenge as well as a LPS challenge. Shown are median and quartiles and significant differences to control (p<0.05).

	ctrl	day 1	day 2	day 3	day 6/5	day 10/8	Significant differences to ctrl
Peak 1							
Secreted mucus							
mechanical	13(10 - 16)	13(10 - 16)	No data	21(15 - 27)	16(13 - 16)	21(9 - 35)	
A38	16 (12 - 25)	23(13 - 24)	No data	25(12 - 33)	21(20 - 24)	30(25 - 35)	
A60	16(12 - 25)	12(8 -12)	No data	15(12 - 20)	19(14 - 22)	27(15 - 32)	
LPS	6(6 - 7)	7(5 - 8)	5(4 - 7)	7(6 - 9)	9(7 - 11)	9(6 - 10)	
Non-secreted mucus							
LPS	26(24 - 27)	31(24 -33)	29(26 - 38)	42(38 – 47)	35(31 - 43)	28(26 – 32)	ctrl < d3, d5
TA							
Secreted mucus							
mechanical	8(7 - 9)	7(5 -9)	No data	7(7 - 12)	7(6 - 8)	8(8 - 15)	
A38	8(7 - 12)	17(12 -18)	No data	21(15 - 23)	14(14 - 20)	12(10 - 16)	ctrl < d1, d3
A60	8(7 - 12)	11(7 -15)	No data	10(10 - 12)	16(11 - 17)	19(16 - 21)	ctrl < d6, d10
LPS	15(8 - 17)	31(25 -32)	35(23 - 38)	23(19 - 25)	23(20 - 28)	15(12 - 15)	ctrl < d1, d2, d3, d5
Non-secreted mucus							
LPS	20(18 - 23)	31(30 -32)	32(27 - 34)	32(29 - 35)	29(28 - 30)	30(29 - 39)	ctrl < d1, d2, d3, d5, d8
Peak 2							
Secreted mucus							
mechanical	80(76 - 82)	82(77 - 85)	No data	66(65 - 77)	78(72 - 81)	61(53 - 83)	
A38	77(63 - 83)	63(58 - 69)	No data	54(52 - 62)	64(61 - 65)	54(51 - 58)	
A60	77(63 - 83)	78(73 - 84)	No data	75(68 - 80)	69(56 - 70)	55(49 - 67)	
LPS	75(75 - 80)	63(60 - 68)	60(58 - 71)	70(66 - 72)	70(58 - 71)	76(74 - 82)	d1, d2, d5 < ctrl
Non-secreted mucus							
LPS	54(51 - 55)	38(36 - 44)	36(33 - 41)	22(21 - 27)	38(30 - 40)	40(28 - 45)	d1, d2, d3, d5, d8 < ctrl

Table 5 Carbohydrate distribution in percent of mucus HMG for peak 1 after a mechanically challenge, bacterial challenge as well as a LPS challenge. Shown are median and quartiles and significant differences to control (p<0.05).

Challenge	ctrl	day 1	day 2	day 3	day 6/5	day 10/8	Significant differences to ctrl
Peak 1							
Secreted mucus							
mechanical	32 (30 – 33)	29 (25 – 33)	No data	39 (33 – 43)	41 (32 – 50)	52 (41 – 58)	
A38	32 (27 – 43)	28 (25 – 35)	No data	29 (27 – 30)	29 (23 – 33)	31 (29 – 33)	
A60	32 (27 – 43)	32 (19 – 36)	No data	34 (29 – 45)	44 (37 – 47)	36 (26 – 48)	
LPS	40 (38 – 44)	30 (27 - 34)	27 (25 - 29)	31 (29 – 33)	33 (25 – 36)	33 (26 – 37)	d1, d2, d3, d5, d8 < ctrl
Non-secreted mucus							
LPS	54 (45 – 61)	43 (41 - 49)	40 (34 – 46)	46 (46 – 53)	42 (38 – 54)	58 (49 – 67)	
TA							
Secreted mucus							
mechanical	14 (14 – 15)	18 (16 – 20)	No data	19 (17 – 20)	15 (12 – 17)	9 (9 – 13)	
A38	18 (16 – 20)	16 (15 – 18)	No data	12 (11 – 17)	16 (15 – 24)	19 (17 – 20)	
A60	18 (16 – 20)	16 (15 – 19)	No data	18 (18 – 19)	13 (12 – 16)	21 (14 – 22)	
LPS	16 (16 – 18)	35 (31 – 36)	25 (24 - 30)	26 (23 – 27)	28 (24 – 29)	29 (24 – 34)	ctrl < d1, d2, d3, d5, d8
Non-secreted mucus							
LPS	23 (22 – 28)	29 (22 – 31)	29 (27 - 30)	26 (19 – 31)	22 (20 – 26)	28 (26 – 33)	
Peak 2							
Secreted mucus							
mechanical	54 (53 – 57)	53 (48 – 55)	No data	4537 – 49)	44 (38 – 51)	39 (34 – 46)	
A38	49 (41 – 53)	54 (48 – 56)	No data	58 (53 – 59)	53 (53 – 55)	51 (47 – 53)	
A60	49 (41 – 53)	53 (44 – 65)	No data	47 (38 – 53)	41 (40 – 47)	44 (37 – 52)	
LPS	45 (39 – 46)	37 (32 – 41)	47 (42 – 51)	43 (41 – 45)	41 (39 – 45)	37 (34 – 42)	
Non-secreted mucus							
LPS	21 (17 – 30)	28 (25 – 30)	30 (27 – 35)	25 (23 – 29)	30 (27 – 35	16 (10 – 18)	

Table 6 Significant differences in terminal glycosylation of mucus HMG between control (ctrl) and treated carp. Shown are total OD per tested sugar and per lectin the percentage of the total OD which could be found in a size pool.

	A. 38 secreted mucus	A. 60 secreted mucus	LPS challenge secreted mucus	LPS challenge non-secreted mucus
total OD per tested sugar	RCA, d6: ctrl < A38 RCA, d10: A38 < ctrl	ConA, d1: A60 < ctrl RCA, d3: A60 < ctrl RCA, d10: A60 < ctrl SNA, d1, d10: A60 < ctrl	SNA, d2: ctrl < LPS	
% of OD found in peak 1	DBA, d10: ctrl < A38 RCA, d10: ctrl < A38	DBA, d6: A60 < ctrl DBA, d10: ctrl < A60 SNA, d1, d10: ctrl < A60	ConA, d1, d2, d3, d5, d8: ctrl < LPS	ConA, d2: ctrl < LPS
% of OD found in TA	DBA, d10: A38 < ctrl ConA, d10: A38 < ctrl RCA, d10: A38 < ctrl SNA, d10: A38 < ctrl	RCA, d10:A60 < ctrl SNA, d10: A60 < ctrl		
% of OD found in peak 2				

In this study, the cell membrane products were capable to induce a significantly lower mucus amount on day 2 after challenge probably by the formation of pathogen-associated molecular patterns (PAMPs). It is likely that LPS has mimicked a bacterial challenge, as a large quantity of LPS was given. LPS is usually given a higher dose to fish as to mammals before inducing a reaction, because responses to LPS in fish are not as intense as they are in mammals [250].
The significant changes on day 2 in glycosylation, protein and carbohydrate distribution indicate a degradation of molecules from peak 1, as the amount of carbohydrates in peak 1 decreased although not significantly. This degradation of mucus might explain the significantly lower amount of mucus on this day caused by a washing out effect. The first peak (peak 1) of the biphasic profile is thought to represent mucins which adhere to the epithelium, while the second peak (peak 2) represents mucin constituents mixed with luminal proteins [95].
The changes on day 2 with the secreted mucus were preceded with similar non-significant changes in the non-secreted mucus on day 1, indicating that different mucus was quickly synthesized upon LPS administration and subsequently released. After day 1, the mucus molecules in peak 2 and TA, which was synthesized in the days after the LPS application, was higher glycosylated as well as the mucus which was secreted from day 3 onwards. Small mucus molecules that are higher glycosylated are thought to be able to trap bacteria more easily. On day 5 the amount of non-secreted mucus was significantly higher, probably to cope with ongoing secretion and maybe caused gene up-regulation.
Although changes in glycosylation were found, only a few significant differences in terminal glycosylation could be detected. This is in compliance with histological and lectin-histochemical data from these fish [139]. In response to endotoxin treatment no significant changes in goblet cell number and general carbohydrate histochemistry were observed and only slight alterations in the lectin binding pattern of goblet cell content were found. The

changes in terminal glycosylation as derived from the lectin-ELISA were predominantly found in the larger HMG on day 2. This indicates that the intestine responded quickly with a slight alteration of the terminal glycosylation of the HMG, which are thought to be the framework of the mucus layer.

A38/A60 challenged carp

In the present study two strains of *A. hydrophila* were used to mimic a bacterial challenge. *Aeromonas* spp. are Gram-negative bacteria, which are widespread in aquatic environments [251, 252]. They are commonly found in both fresh and salt water [252-256] and are also part of the normal intestinal microflora of healthy fish [257]. Bacterial virulence is determined by a complex array of bacterial traits. Some of these traits have been examined for different isolates of *A. hydrophila*, including chemotactic behaviour [208, 220], growth, adhesion [208]. Another bacterial trait which has been linked to virulence is the presence a type III secretion system (TTSS) which injects anti-host virulence determinants into host cells. TTSS has been found for different isolates of *A. hydrophila* [141, 258, 259].

The virulence factors, growth in mucus and adhesion [208] were more pronounced in A38 as in A60. Furthermore, in A38 a TTSS was found (unpublished data) and A38 has a clear *in vitro* cytotoxic effect [140], whereas A60 did not show a virulent effect for these factors.

The significant decrease of mucus amount after A38 challenge can be explained by the strong degradation of the mucus molecules and a possible washing out effect. Apparently the fish were not able to produce and secrete enough mucus to compensate for degradation and washing out. This might be caused by a possible down-regulation of the bacterial expression of MUC-genes. A60 appears to be less invasive as the mucus amount appears to be unaffected. Whether the mucus is hardly degraded or the mucus layer is restored through secreted mucus remains unclear.

On day 1 and 3 molecules of TA showed a significantly higher P:C ratio after A38 challenge, meaning that relative less carbohydrates were present. This observation is supported by the higher protein percentage on these days. This indicates that molecules are either degraded or that more molecules which are not ready synthesized are released, which may be supported by the medium sized HMG (in TA). On day 1 after the challenge with A60 a significantly higher P:C ratio was also observed on for TA, peak 2 as well as the total mucus. However, after A60 challenge a higher protein percentage for TA could only be observed on day 6 and day 10. The two *A. hydrophila* strains led to a similar response after challenge, however upon the A38 challenge a faster response could be observed. This difference is probably due to the faster growth rate of A38.

Differences in the P:C ratio were found in the first days after bacterial application which is accompanied by a changed terminal glycosylation (lectin-ELISA). However, most differences were found on the last day of sampling. All changes in terminal glycosylation pattern were found in the larger molecules. Whether the bacteria changed terminal glycosylation or if goblet cells produced HMG with a different terminal glycosylation is still unclear.

Trends after bacterial challenge

Terminal glycosylation

With the lectin ELISA the five monosaccharides (fucose, mannose, N-acetyl-α-galactosamine and N-acetyl-β-galactosamine, neuraminic acid) [6, 260], which primarily occur in the glycosylation of piscine mucins were tested and differences in terminal glycosylation were found. Oligosaccharides are believed to mediate the adhesion of microbial pathogens and to prevent glycoprotein degradation by proteases of microbial origin [71, 77, 102, 239, 240, 242]. OD-values of UEA-1 (α-L-fucose), were around or below detection level in naïve, control and treated samples. Therefore α-L-fucose does not seem to play an important role in the terminal glycosylation of intestinal mucus HMG of carp. In a gas chromatographic analysis of mucus isolated from the intestinal tract of the rainbow trout *Oncorhynchus mykiss*, however, fucose was demonstrated [100].

Changes in the other terminally bound sugars occurred quickly after challenge and for the bacterial challenge also on the last day of sampling. However, it is not unlikely that not all changes were monitored in the analysis, as lectin ELISA results showed a high variation.

General characteristics of intestinal mucus hmg

With the isolation of the HMG and the determination of protein and carbohydrate content, differences between the control and treated fish could be observed for mucin content, glycosylation and distribution of carbohydrate and protein over the size fractions. After the bacterial challenges three trends could be observed. Firstly, the increase and subsequent decrease of secreted mucus amount. This combined with the data from the non-secreted mucus indicates that a bacterial challenge leads to release of mucus from the goblet cells, but this release is not sufficient to keep the amount of mucus stable over the next days.

Secondly, upon bacterial challenge glycosylation of smaller molecules which are thought to bind to bacterial components, firstly decreased (day 1 for A38 and A60, day 2 for LPS) and then increased. This indicates a degradation of secreted molecules followed by a release of newly synthesized high glycosylated molecules able to trap bacteria. Thirdly after challenge relatively more protein could be found in TA, indicating a shift in the equilibrium between release and degradation of mucus molecules.

For each bacterial challenge each trend was observed at different times, indicating different mechanisms leading to the observed changes. That the mucosal system reacts differently after a LPS challenge compared to a bacteria challenge, might have following reasons. Firstly the LPS was from *E. coli* and not from *A. hydrophila*. Secondly, LPS is only the lipopolysaccharid from the cell membrane, but the bacteria consist of many more components. Thirdly, LPS will be washed out of the intestinal system, however, bacteria have the possibility to establish themselves and grow in the intestinal tract. The differences observed between A38 and A60 can be explained by the clear differences in several pathogenic factors.

Chapter 9

General discussion

Aim of this thesis

Body surfaces are defended by epithelia, which provide a physical barrier between the internal milieu and the pathogen-containing external world [9]. Protection of fish against pathogen entry from the surrounding water largely relies on the integrity of external surfaces in conjunction with the capacity to induce innate and adaptive immune responses. The importance of an intact fish skin for pathogen defense was clearly shown in live experiments on rag-1 deficient zebrafish, lacking adaptive immunity. They showed normal life expectancy in water containing large numbers of fish pathogens, however within three weeks of a fin injury, they died from bacterial infection [261].
External surfaces of fish are formed by a mucosal, non-keratinized epidermal tissue which is covered by a mucous layer [262]. Mucus is a complex viscous adherent secretion and represents an interface between the environment and the interior milieu [9]. The mucus gel is the first barrier pathogens encounter and protects the epithelial cells against chemical, enzymatic, microbial, and mechanical challenges [10]. Mucus is produced primarily by goblet cells and composed of gel forming macromolecules, mucins and other glycoproteins that can hold water [6]. The first general aim of this study was to gather information on the mucus layer of naïve carp.
The mucosal barrier is not a static barrier, as its constituents and their release are modulated by the microenvironment and by neural, endocrine and immune factors [28]. The immune functions in mucus usually prevent the entry of pathogens and toxins. For mammals, it is known that mucin glycosylation [72] and mucus secretion can be altered upon bacterial infection [77]. Infection of mucosal surfaces can result in a rapid release of stored mucin granules to bolster the barrier and exclude pathogens [1]. The second general aim of this study was to describe changes within the mucus upon different challenges.

Mucus of naïve carp

Mucins

This thesis investigated carp mucus high molecular glycoproteins (HMG) and antimicrobial peptides of different mucosal tissues of naïve carp. With biochemical methods, carp mucus HMGs was isolated from the intestine (Chapter 2) and skin (Chapter 3). After isolation and gel filtration, a biphasic elution profile was received both for protein and carbohydrate contents of skin mucus HMGs and of secreted and non-secreted HMGs of intestinal mucus. Protein profiles of non-secreted and secreted mucus samples from the different intestinal segments had similar protein profiles. For intestinal mucus of rats, similar elution profiles were obtained [87]. Mucus is produced in the goblet cells and their content is a mixture of mature and immature mucus glycoproteins, which are not yet fully glycosylated [95]. This is reflected in the lower glycosylation of non-secreted mucus HMGs of all sizes compared to the secreted glycoproteins as found in this thesis.
The mucus HMGs of peak 1 from skin and intestine had similar biochemical characteristics to mammalian mucins with a high molecular weight, and as indicated by a low protein to carbohydrate (P:C) ratio were highly glycosylated. In this peak, for non-secreted and secreted

mucus samples from the different intestinal segments proteins between 120 to 180 kDa were found (Chapter 6). The proteins found in peak 1 probably represent mucins which adhere to the epithelium, the so-called adherent mucins (AMs). These HMGs which are thought to play a major role in the formation of the structure of the mucus gel [51, 87, 95] and provide protection for epithelial cells from external challenges or pathogen penetration [80]. Retention volume of the first peak was similar for samples from the first and second intestinal segment (peak at approximately 150 kDa), but was slightly but significantly larger compared to mucus of the intestinal bulb approximately (140 kDa). Size shifts of around 10 kDa are often caused by a difference in the glycosylation pattern of a protein, which might also have occurred here.

For all samples, a second peak of smaller glycoproteins could also be identified. In this peak, for non-secreted and secreted mucus samples from the different intestinal segments, proteins between 12 to 70 kDa were found. These smaller HMGs co-elute with various “non mucin” glycoproteins, but smaller isolated HMGs are considered to represent mainly small mucin glycoproteins. For skin mucus HMGs and intestinal HMGs of secreted and non-secreted mucus, peak 2 HMG were less glycosylated than HMG from peak 1 or the transition area (TA). These smaller HMGs may be a result of bacterial mucin degradation [129], or may have been prematurely secreted as shown by a lower glycosylation (a higher P:C ratio) or represent small mucin glycoproteins, which are not completely synthesized yet. Mucin can act as a carbon source for gut associated bacteria, certainly in the distal colon where the availability of carbohydrates is limited. Skin HMGs of peak 2 were clearly higher glycosylated compared to similar sized intestinal HMGs. This might indicate that other mucin-like glycoproteins are released next to the large mucin-like glycoproteins. This could be an indication that the glycogalyx of skin is different to that of the intestine.

For the general properties of mucosal glycoproteins, the carbohydrate side chains of mucus HMGs are fundamental [51]. Carbohydrate side chains in mucins from mammalian mucosal surfaces are composed of a limited array of oligosaccharides: N-acetyl-α-galactosamin (galNAc), N-acetyl-β-glucosamin ($(glcNAc)_2$), galactose (gal), mannose (man), fucose and neuramic acid (NeuNAc) [45]. O-linked carbohydrate chains are added to the apoprotein in the Golgi apparatus during biosynthesis via N-acetylgalactosamine (galNac) that is coupled to the hydroxyl group of either a serine or threonine residue via an O-glycosidic linkage. Then galactose and/or N-acetylglucosamine (glcNAc) residues are added to this initial galNAc to form the various core structures. The presence of terminally bound galNAc, galactose, mannose and neuramic NeuNAc in the content of piscine goblet cells was shown by lectin histochemistry for intestine (Chapter 3) and skin (Chapter 3). Staining for $(glcNAc)_2$ or fucose however was low.

Using HPLC, the monosaccharide content of mucus HMGs was analyzed. For both skin mucus and mucus from the second intestinal segment, the sugar galNAc could not be detected. Although galNAc was not detectable by HPLC, galNAc might to be present in low amounts as terminal galNAc, as this was detected in low amounts by histochemistry with DBA in skin. Also the fucose, which is thought to be especially important for the viscoelasticity [132], was dependent on the origin of the mucus. Fucose is found to vary strongly in intestinal mucus or anal gland mucus of mammals [132]. Fucose was the most dominant sugar for non-secreted mucus in the second segment as well as in skin mucus. In the first segment NeuNAc/gal was dominant. In general, on a monosaccharide level, *C. carpio* skin mucus (Chapter 3) resembled

the mucus of the second intestinal segment more than the mucus of the first segment (Chapter 2).
In this thesis, numerous goblet cells in the epithelium of intestine and skin could be stained with histochemical methods for neutral, acidic and sulphated glycoproteins. Acidic and sulphated glycoproteins are reported to inhibit bacterial adhesion and glycoprotein degradation by proteases, and goblet cells with such mucins usually are detected in parasite-infected fish [81]. Overall skin goblet cells (Chapter 3) stained stronger for carbohydrates compared to intestinal goblet cells (Chapter 2), indicating a higher or different amount of glycoproteins in skin. Goblet cells of different intestinal segments appeared to have similar charged HMGs, as differences between the applied carbohydrate stainings could not be found. Between different intestinal segments differences in carbohydrate staining could be observed indicating differences in pH of the mucus (Chapter 6). Differences between the first and second segment in the staining intensity of the glycoproteins in the goblet cells support the role of mucus in the defense against pathogens. This fits well in the immunological role suggested for the second intestinal segment [210]. The mucus from the intestinal bulb might have also a different function in defense against bacteria as differences in carbohydrate staining when comparing to carbohydrate staining of first and second intestinal segment.
When bacteria bind to mucus, they bind to the major constituent of mucus, i.e. glycoproteins [71]. The protein core is usually not accessible to pathogens due to the carbohydrate side-chains [241]. Therefore, bacteria usually bind predominantly to the carbohydrate side-chains of the glycoproteins [71, 77, 102, 239, 240, 242]. By offering binding sites similar to those of epithelial cells, mucin can prevent pathogen adhesion to the underlying epithelial cells, and further translocation into the mucosa. The array of oligosaccharides expressed on the mucins of an individual may play a key role in governing the susceptibility to infection [25]. In this thesis, the terminal presence of galNAc, galactose, mannose and neuramic NeuNAc could also be detected by lectin ELISA for skin mucus (Chapter 3) and non-secreted intestinal mucus (Chapter 2). Although terminal galactose could be detected by lectin histochemistry and lectin-ELISA, only small amounts were detected. Slightly higher amounts of galactose were found for carp intestinal mucins than for skin mucins. Especially immature mucins are thought to have a high amount of terminal galactose [133]. This may indicate that for skin, smaller sized mucus associated mucins are not immature, as they are thought to be for intestinal mucus.
Besides histological and biochemical analysis of naïve carp mucus, also partial sequences of two genes were found that show a high homology to mammalian and avian *Muc2* and *Muc5B* genes (Chapter 5). This is the first description of cloning and expression studies on fish mucin genes. The presence of von Willebrand factor D, C8 and TIL domains of the found genes strongly supports the conclusion that the carp mucins are real *Muc2* and *Muc5B* genes, instead of genes which only resemble those (*Muc-like* genes). Analysis of tissues and organs for mucin expression in carp revealed a clear separation in the site of expression between the two mucins. *Muc5B* was expressed with low copy numbers in brain and liver and with high copy numbers in skin and gills. Human *MUC5B* has been found to be mainly expressed in the mucous glands of the respiratory mucosa and salivary glands, as well as in the gall bladder, pancreas and cervix [9]. Carp *Muc2* was only, but highly, expressed in the first and second intestinal segment. In human tissues this mucin is also expressed in the intestine, but can also be found in bronchi [9]. In contrast to this, carp *Muc2* expression was not detected in the gills.

Contrary to the respiratory tract of mammals, in the gills of carp *Muc5B* seems to be more important than *Muc2*.

Antimicrobial peptides

In addition to the mucin genes, two ß-defensin encoding genes from carp were also partially sequenced in the present study (Chapter 5). Defensins have a broad antimicrobial spectrum ranging from Gram-negative to Gram-positive bacteria, mycobacteria, fungi and enveloped viruses [172]. To our knowledge piscine ß-defensins have so far only been identified through *in silico* studies. Fish defensin-homologs, as found in *in silico* studies, share the common features of vertebrate defensins, including small size, net cationic charge and six conserved cysteine amino acids in the mature region. Based on their cysteine arrangement, the identified fish defensin-*like* peptides resemble the ß-defensin family members in birds and mammals [155]. In carp *BD1* and *BD2* these conserved cysteins were also present.

Analysis of ß-defensin expression in tissues from naïve carp revealed limited constitutive expression, as *BD1* was only expressed at intermediate levels (100-1000 normalized copy numbers) in skin and *BD2* only at a low levels (10-100 normalized copy numbers) in liver. In contradiction to this, *O. mykiss* show a widespread constitutive expression at both mucosal and systemic sites, especially with high expression of *ß-defensin 3* (*BD3*) and *ß-defensin 4* (*BD4*). However, *BD1* and *BD2* were expressed at low levels [156]. For *D. rerio*, *BD2* was only expressed at low levels in the gut, while *BD1* and *BD3* were more highly expressed in all tissues examined [155]. Therefore, the expression profile of the ß-defensin genes appears species-dependent.

When staining the first and second segment for antimicrobial peptides (Chapter 6), no difference between segments was found for BD3 and lysozyme. Staining for these humoral substances was low and therefore BD3 and Lys appear to be less important in *C. carpio* under unchallenged conditions. Staining for BD2 was more pronounced for the first than for the second segment. It is probable that under physiological circumstances, the release of BD2 in the first segment is high enough so that BD2 molecules can also be functional in the second segment.

Factors influencing the mucus layer

The mucus layer is thought to be a dynamic fast responding system as evidenced by the reaction found after a short mechanical challenge (Chapter 8). When carp were exposed to a peroral application of a physiological salt solution, total glycosylation and therefore the amount of carbohydrates relative to the amount of protein was significantly higher compared to untreated carp. The increased carbohydrate portion might be a microbial defense mechanism. In rats, changes in mucus composition were also observed after mechanical challenge and food uptake [123, 246, 247].

The range of factors which can influence mucus production is large, for instance age-dependent glycosylation of mucins has been described for mammals [101]. In this thesis glycosylation of intestinal HGMs from peak 1 hardly differed between older and younger carp (Chapter 2). The HMGs responsible for the structure of the mucus gel therefore appear not to be affected over time. However, an increased glycosylation of HMGs from peak 2 was found for older carp compared to younger carp. This increased glycosylation might therefore reflect the exposure to a larger array of micro-organisms. The smaller molecules are thought to be

able to trap invading micro-organisms as micro-organisms can bind to certain carbohydrates [71, 102].
Starvation is one of the internal factors that can negatively affect the barrier function [5]. Fasting is a situation experienced by fish in the wild and seems to be well tolerated by many fish species [263-267]. Numerous species can starve for many months and then recover fully after refeeding [266]. Fasting experiments usually examine changes after several days, weeks or months. Extensive data on short term starvation, such as performed in this thesis to limit fecal contamination on mucus, is lacking for carp. In sea bass starved for 11 days, mucus lysozyme content doubled compared to the initial value [268]. Ketone concentration in mucus was significantly elevated in trout starved for 28 or 56 days [269]. The effect of starvation on fish mucus HMGs has not been examined. In the present thesis, all fish were starved for a short period to limit fecal contamination on mucus as this fecal contamination interferes with carbohydrate and protein analysis. How this starvation period influences structure and formation of mucin HMGs is unclear. However, for chicken, an effect of starvation on mucins has been found. Relative amounts of intestinal mucin mRNA and protein increased in the duodenum and jejunum of chicks starved for 72 hour, and mucus adherent layer thickness decreased throughout the small intestine. These changes may affect intestinal digestive function and defense [270].

Bacteria

Bacteria are capable of influencing the mucus layer. An overwhelming number of infectious diseases are initiated by bacterial colonisation of mucosal surfaces and this colonization is preceded by bacterial attachment to epithelial cells, or to mucins coating these mucosal cells [77]. In order to act as a pathogen, bacteria need to have contact with the host. This can be coincidental or the result of directed chemotactic movement. The first characteristic that promotes bacterial colonization of the mucosal surface is therefore chemotactic activity towards mucus (cell contact) [76]. The *A. hydrophila* strains A38 and A60 used in this thesis to test chemotactic activity towards mucus appeared to be not very pathogenic as none of the fish given 10^7 CFU died. This coincides with the low chemotactic response found for these strains in the present thesis (Chapter 7). A low chemotaxis response for *A. hydrophila* strains towards mucus was also reported by Hazen *et al.* [220]. Although chemotaxis appears not necessary for a bacterium to become pathogenic, it might be a necessary parameter for an obligate pathogenic bacterium, as the pathogenic bacterium *E. tarda* cultured under the same conditions as *A. hydrophila* did show a strong chemotactic movement towards gut mucus.
Once bacteria have cell contact they must adhere to mucins coating the mucosal cells in order to become pathogenic [77]. Although the mucus layer and the mucins it contains usually protect the mucosal epithelial surface against pathogens, the ability of bacteria to bind to mucus components may facilitate the colonization of mucosal epithelial surfaces by immobilizing the bacteria in the mucus gel. However, adhesion to mucus is not necessarily a sign of pathogenicity or virulence. For instance, probiotics are known for their ability to bind to mucus [236]. Nevertheless, adhesion of pathogens to the mucosal surface is the first step in the pathogenesis of most infectious diseases [96, 237, 238]. This coincides with the results from the present study. Here fish that were intubated with strain A38 showed more clinical symptoms than those intubated with A60 and A38 had a higher adherence to mucus than A60 (Chapter 7). In addition, bacteria were isolated more often from internal organs after A38

treatment. This suggests that *A. hydrophila* A38 is more capable of destabilizing the intestinal barrier. In other infection experiments, it was also observed that more virulent strains of a bacterium show a higher adhesion than less virulent strains [102, 239, 240]. This was also supported by the very high adhesion to mucus from *E. tarda*.

For successful colonization, it is necessary that after adherence bacteria establish themselves in the mucus gel. Normal erosion of the mucus gel is likely to wash away adherent bacteria. Therefore, to achieve successful colonization the replication rate of bacteria attached to mucus must be equal to or higher than the rate of erosion of the mucus layer itself. Thus, it would be advantageous for pathogenic bacteria if they can use mucus as a nutrient source for their growth [71]. Both *A. hydrophila* strains used in this thesis grew in mucus-supplemented media, but did not grow in nutrient depleted medium (Chapter 7). This agrees with previous findings for *A. hydrophila* [71]. Culture of the more pathogenic strain in inorganic nutrient-rich (phosphorus limited or enriched) medium enhanced bacterial growth, but not as strongly as did mucus supplementation. This might indicate that the bacteria can use mucus as an energy source (e.g. mucins act as a carbon source). The more pathogenic strain A38 did not only showed a higher adhesion but also had a higher total yield when cultured in mucus compared to the A60 strain. A faster multiplication of bacteria in mucus increases its potential pathogenicity as the flushing out of bacteria due to mucus erosion is reduced. A higher adhesion and subsequent growth may lead to differences in pathogenicity as found in this study.

In this thesis an effect of peroral application of a bacterial product (LPS), and two *A. hydrophila* strains on intestinal mucus (Chapter 8) and the effect of water with a high load of *A. hydrophila* on skin mucus (Chapter 4) was examined. Differences between the control and treated fish could be observed for mucin content, glycosylation of mucus HMG, differences in terminal glycosylation (intestine) and distribution of carbohydrate and protein over the size fractions.

After the bacterial challenges three response trends could be observed. Firstly, secreted mucus amount increased and subsequently decreased. This indicates that a bacterial challenge leads to release of mucus from the goblet cells, but this release is not sufficient to keep the amount of mucus stable over the next days.

Secondly, upon bacterial challenge glycosylation of smaller molecules which are thought to bind to bacterial components, firstly decreased (day 1 for A38 and A60, day 2 for LPS in intestinal mucus, day 13 for skin mucus) and then increased. This indicates a degradation of secreted molecules followed by a release of newly synthesized highly glycosylated molecules able to trap bacteria. Thirdly, after challenge relatively more protein could be found in TA, for both intestinal mucus as well as skin mucus. This indicates a shift in the equilibrium between release and degradation of mucus molecules.

For each bacterial challenge each trend was observed at different times, indicating different mechanisms leading to the observed changes. That the mucosal system reacts differently after a LPS challenge compared to a bacteria challenge, might have following reasons. Firstly the LPS was from *E. coli* and not from *A. hydrophila*. Secondly, LPS is only the lipopolysaccharide from the cell membrane, but the bacteria consist of many more components. Thirdly, LPS will be washed out of the intestinal system, however, bacteria have the possibility to establish themselves and grow in the intestinal tract. The differences observed between A38 and A60 can be explained by the clear differences in adhesion and

growth in mucus. For skin mucus changes could be observed at a later time point than for the intestinal mucus.Wether this late response is caused by the bacterial strain or by the origin of the mucus (skin instead of intestinal) could not be determined in this study. It remains to be clarified if different mechanisms take place upon the same bacterial challenge between different mucosal tissues.

Food

Besides bacteria, dietary factors (fibers, proteins, and anti-nutritional factors) have the potential to affect synthesis and secretion of mucin from the goblet cells [73]. For instance, supplementation with ovine serum immunoglobulin [271] or L-threonine [272] was found to alter mucin production. Probiotics modulate intestinal barrier function and modulate abnormal stimulation of MUC genes under inflammatory conditions [273, 274]. Furthermore, as mentioned before probiotics are known for their ability to bind to mucus [236].

Probiotics and prebiotics, such as β-glucans, are gaining more and more interest for use in the therapy and prevention of human diseases [157, 158]. For many fish species, the immunomodulatory activity of β-glucan has also been reported [159-164]. Recent preliminary research data indicate that β-glucan promotes an antimicrobial response [165]. In the present study β-glucans were fed to carp. β-Glucans can potentially affect mucin structure and/or function as they interact with innate signaling pathways in mucus producing cells [74]. The influence of feeding the immunomodulatory β-glucan on mucin and ß-defensin gene expression in mucosal tissues of carp has been monitored in this study.

ß-Glucans derived from plants, bacteria or fungi are recognized by receptors from the innate immune system, like C-type lectins (Dectin-1 [173] and TLR2/6 [174]), and therefore have immunomodulatory properties when administered to mammals [174] and fish [175]. ß-Glucans have been shown to be effective immunomodulators in a number of bacterial, viral and parasitic infections [176]. When prebiotics, such as ß-glucans, promote health responses in fish, less chemotherapeutics may be required, holding the potential to increase efficiency and sustainability of aquaculture production [177].

The present data show an effect of ß-glucan feeding on the expression of mucus-related genes in carp (Chapter 5). For the mucin encoding genes carp *Muc5B* and *Muc2,* differences in expression were observed in carp with different ß-glucan feeding regimens. Consistent, but not significant, down-regulation of *Muc2* in the intestine and gills was seen in the glucan fed fish, while *Muc5B* was significantly increased in skin, with slight up-regulation in gills. An up-regulation of *Muc2* expression after ß-glucan feeding was described in the intestine of chicken [178] and pigs [179], and an increased expression of *Muc2* and Mub5B has been observed in mammals after bacterial [174, 180-182] and nematode infections [183]. In addition to the mucin encoding genes, an effect of ß-glucan feeding could also be observed on the expression of carp β-defensin genes. The expression levels for both carp ß-defensins were significantly higher in the skin of ß-glucan fed carp, with *BD2* significantly higher in gills. A regulation of *BD1* and *BD*2 was not observed in the mucosal tissues of rainbow trout challenged with *Yersinia ruckeri*, but in these fish *BD3* was increased in gills [156]. The present study shows that carp ß-defensins can be up-regulated, although their precise role in infections and immune defense remains to be elucidated. In summary, expression levels of *BD1* (skin), *BD2* (skin, gills) and *Muc5B* (skin) could be significantly increased by the addition of ß-glucans to the food. This indicates the relevance of these genes to monitor feed-related improvement of

fish health under aquaculture conditions. Even though different mucin and defensin genes are expressed in skin and intestine, the regulation of both in the skin of carp after feeding ß-glucans suggests that not only the mucosal system of the intestine, can be influenced. This underscores the interconnection of mucosal tissues in the body, potentially permitting the application of functional feed additives to improve fish skin health.

Inflammation

Besides having a postive effect on mucus, dietary substances can also negatively influence mucus formation, structure and function. For instance, dextran sulfate sodium induces a colitis in mouse [275]. Muc gene expression was altered upon dextran sodium sulphate treatment and in mice fed soy *Glycine max* L. [276]. Soybean meal (SBM) containing diets are known to induce an inflammatory response in the hindgut of certain fish species. Soy bean meal is added to fish feed as it is a cheap and reliable source of protein, which is needed for both economic and sustainability reasons. Soybean has a great potential as protein and/or oil source for fish feed [189]. Between different fish species differences in the reaction upon soy bean have been observed. For instance, rainbow trout react less strongly to SBM-containing diets than Atlantic salmon [277]. Contrary to salmon, carp start to recover or adapt to the SBM feed from the fourth week after SBM feeding [75]

So far most studies have been conducted on *S. salar* and have mainly focused on the effect of SBM-containing diets on intestinal morphology, growth, enzyme activity and metabolism. A negative influence was observed for these parameters [190-194] and on disease resistance [195] as well as immune relevant genes [196]. The study on SBM-induced enteritis in carp conducted by Uran *et al.* [75] focused on the morphology of the hindgut by using histology and expression of pro- and anti-inflammatory cytokines in isolated intestinal epithelial leukocytes by real time quantitative PCR. In SBM-induced enteritis a significant increase in goblet cells can be observed for carp and salmon [75, 213]. However, so far no studies have been conducted on the effect of SBM-induced enteritis or a comparable intestinal inflammation on the composition of mucus. In this thesis, the response of the intestinal mucosal layer and of intestinal mucus of carp to acute SBM-induced enteritis was examined using bacterial, histological, histochemical and biochemical techniques (Chapter 6). An intact mucus layer is essential for fish health as a primary defect in mucins could breach the epithelial barrier or lead to altered mucosal-bacterial interactions [198].

After SBM feeding, the second gut segment of carp showed mild signs of an enteritis process comparable to that described for salmon and previously found for carp. The enteritis process was accompanied by an increase in goblet cells in the second segment which could be stained for acidic glycoconjugates as found previously for SBM-enteritis in carp by Uran *et al.* [75]. Damages to the intestinal epithelium as previously found however could not be observed.

Although no severe signs of an inflammation could be observed in the present study, a higher number of bacteria could be isolated from internal organs on week 1 and 2 after SBM feeding. This indicates that the mucosal barrier as first line of defense was compromised, supporting that the intestinal barrier was affected. In humans, increased gut permeability has been described following enteritis. If changes after enteritis persist a chronic inflammation can develop [197]. Chronic inflammatory bowel diseases (IBD) are well described for humans. In IBD, genetic mutations in mucin genes, changes in sulphation, degree of glycosylation, mucin mRNA and protein levels and degradation of mucins have been described. Changes of

immunological or bacterial factors during an initial or ongoing inflammation can influence mucin production, which could have further adverse effects on mucosal-bacterial interactions, hereby sustaining the chronic character of the inflammation [198]. Between the control and week 3 after 20SBM no difference could be found in bacterial numbers in intestinal organs, indicating that the intestinal barrier function had recovered.

In SBM-induced enteritis in fish, changes in mucus composition have previously not been studied. In the present study, 20SBM diet induced changes in the mucus, indicating that the mucosal layer showed altered mucus secretion upon 20SBM feeding. In addition, mucus from SBM-fed carp showed a different glycosylation pattern as was also reported for IBD [198]. The different glycosylation pattern is indicated by the significantly increased amount of neutral glycoconjugates in the first intestinal segment and the significantly decreased amount of acid and neutral glycoconjugates in the second intestinal segment.

The size of the mucins did not change markedly upon SBM-feeding. However, switching to 20SBM led to an increase in 150 kDa proteins in non-secreted mucus of the first segment, whereas the amount of 150 kDa in secreted mucus remained relatively stable. The increase became less over time. Mucus has a high turnover rate as mucus traps pathogens that can be removed from the body through a constant flow of mucus. The increase in the non-secreted mucus indicates that the SBM diet induced an increased mucus synthesis. An increased mucus flow stimulates the removal of pathogens from the entire subsequent intestine, which might hereby prevent pathogens from entering the soy bean damaged intestinal epithelium. Changes in mucin levels during enteritis have been described for chronic inflammatory bowel diseases (IBD) in humans.

Preventing pathogens from penetrating the mucus layer relies not only on mucus flow and mucin composition but also on other mucus components such as presence and bioactivity of antimicrobial peptides. Week 1 after switching to the SBM-diet the amount of goblet cells which were stained for the antimicrobial peptide BD3 was significantly reduced. This might indicate that BD3 was released to the intestinal lumen to fight pathogens, as BD3 in humans has a strong bactericidal activity [212]. No goblet cells could be stained at week 1 after the diet switch, indicating that BD3 was released more rapidly than it could be produced. After the initial depletion of the goblet cells, synthesis and secretion of BD3 seemed to return to their initial equilibrium, as on week 2 or 3 no differences to control could be observed anymore.

In conclusion, SBM-diet induces a change in mucins in carp similar to that found in IBD in humans. Initial changes included changes in mucin composition, and the presence of BD3 and of bacteria in internal organs. After the initial changes, all measured values returned back to the initial pre-SBM diet values. This might indicate a recovery of the mucosal layer and thus a recovery of the primary barrier between the carp and its surrounding. Changes in the mucosal layer form only a small part in the complex inflammation process of soybean mediated enteritis with its many symptoms such as the normal supranuclear vacuolization of the absorptive cells in the intestinal epithelium [213]. The recovery of the extrinsic mucus layer may help carp in recovering from SBM-enteritis and not developing a chronic inflammation, as through the restoration of the barrier the carp is no longer constantly exposed to new pathogens.

Final conclusion

In this thesis, I describe that carp mucus HMG from both skin and intestine are highly glycosylated, resembling mammalian HMG. With molecular techniques two genes, with similar domains as found in mammalian secreted mucin genes, were found and characterized. The genes have expression profiles similar to their counterparts in mammals. This suggests that mucin glycoproteins in vertebrates are highly conserved. It cannot, however, be excluded that in fish other mucin genes than in mammalians are also involved. No indication for a different protein core of mucus HMGS for first and second segment could be found. This is in contrast to what is found for humans. In the present study, only differences in glycosylation could be observed. Between these segments and intestinal bulb a difference in the size of the major protein peak could be observed. This difference, however, could also be caused by a different glycosylation. A difference between first and second segment could be observed for the antimicrobial peptide BD2. In this thesis, an alteration of mucus HMGs was found upon challenge with beta-glucan feeding, and soybeen feeding, bacterial endotoxin, and bacteria exposure. Furthermore, an effect of a mechanical challenge and age could be observed.
The studies presented here form only a very small part in the research which needs to be done to be able to understand mucin-bacteria interactions and the role of mucosal antimicrobial peptides and mucins in the defense against diseases in fish and how they might be influenced. Understanding these mechanics can help improving fish health in aquaculture. For instance, intestinal mucus HMG composition from salmonids, that are fed soybean and hereby suffering from an enteritis, is probably changed as they suffer from tissue damage. In salmonids this does not improve over time as it does in carp. Artificial upregulation of the mucin production may protect the intestine of salmon, hereby aiding in pathogen defense and possibly allowing tissue repair.

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Summaries and acknowledgements

Summary
Samenvatting
Zusammenfassung
Acknowledgements

Summary

The extrinsic barrier is the first barrier that protects fish from pathogens and toxins and is mainly formed by mucus. Although fish are completely both internally and externally covered with mucus, knowledge on mucus and especially on its major constituent mucins is limited.Mucins give mucus its viscous properties and form a matrix in which a diverse range of antimicrobial molecules can be found [28]. In this thesis, mucins from skin and intestine and antimicrobial peptides found in mucosal tissues are described. Also the impact of some external factors such as bacterial challenge is described.

With biochemical methods, carp mucus HMG was isolated from the intestine (Chapter 2) and skin (Chapter 3). After isolation and gel filtration, a biphasic elution profile was received both for protein and carbohydrate contents of skin mucus HMGs and of secreted and non-secreted HMGs of intestinal mucus. Proteins found in peak 1 are thought to be important for the structure of the mucus gel and had similar biochemical characteristics to mammalian mucins with a high molecular weight and high glycosylation. Non-secreted mucus HMGs of all sizes had a lower glycosylation compared to the secreted glycoproteins. Protein profiles of non-secreted and secreted mucus samples from the different intestinal segments (Chapter 6) had similar protein profiles

The protein core of mucins is transcribed by mucin genes. In this thesis, partial sequences of two genes were found for carp that show a high homology to mammalian and avian *Muc2* and *Muc5B* genes (Chapter 5). As for other vertebrates, in these genes von Willebrand factor D, C8 and TIL domains were present. *Muc5B* was highly expressed in skin and gills. Carp *Muc2* was highly expressed in the first and second intestinal segment.

Carbohydrate side chains of mucus HMGs are fundamental for the general properties of mucosal glycoproteins and provide an adhesion site for bacteria. In general, on a monosaccharide level, *Carp* skin mucus (Chapter 3) resembled the mucus of the second intestinal segment more than the mucus of the first segment (Chapter 6). By lectin histochemistry on skin (Chapter 3) and intestine (Chapter 2), the presence of terminally bound galNAc, galactose, mannose and neuramic NeuNAc in the content of goblet cell was shown. Between different intestinal segments (Chapter 6) differences in carbohydrate staining could be observed indicating differences in pH of the mucus.

In addition to the mucin genes, two ß-defensin encoding genes with cysteine arrangement resembling other vertebrate ß-defensins were partially sequenced (Chapter 5). Defensins have a broad antimicrobial spectrum ranging from Gram-negative to Gram-positive bacteria, mycobacteria, fungi and enveloped viruses. In naïve carp, *BD1* was only expressed at intermediate levels in skin and *BD2* only at low levels in liver. Staining for BD3 and lysozyme was low in both first and second intestinal segment (Chapter 6). Staining for BD2 was more pronounced for the first than for the second segment .

The barrier function of mucus can be disturbed by several factors and alterations in mucus HMG and antimicrobial peptides upon different stimuli were examined. The HMGs responsible for the structure of the mucus gel (peak 1) appeared not to be affected over time (Chapter 2). However, an increased glycosylation of HMGs from peak 2, which are thought to be able to trap invading micro-organisms, was found for older carp compared to younger carp.

An overwhelming number of infectious diseases are initiated by bacterial colonization of mucosal surfaces. Chemotactic activity towards mucus promotes bacterial adherence. Adhesion of pathogens to the mucosal surface is the first step in the pathogenesis of most infectious diseases. A faster multiplication of bacteria in mucus increases its potential pathogenicity as the flushing out of bacteria due to mucus erosion is reduced. A higher adhesion and subsequent growth may lead to differences in pathogenicity as found in this study (Chapter 7). The *A. hydrophila* strains appeared not pathogenic as none of the fish given 10^7 CFU died, had a. low chemotactic response and grew in mucus-supplemented media. The more pathogenic strain did not only show a higher adhesion, but also had a higher total yield when cultured in mucus compared to the less pathogenic strain.

In this thesis, the effect of peroral application of a bacterial product (LPS), and two *A. hydrophila* strains on intestinal mucus (Chapter 8) and the effect of water with a high load of *A. hydrophila* on skin mucus (Chapter 4) was examined. Differences between the control and treated fish could be observed for mucin content, glycosylation of mucus HMG, differences in terminal glycosylation (intestine) and distribution of carbohydrate and protein over the size fractions. After the challenges three response trends could be observed. each trend was observed at different times, indicating different mechanisms leading to the observed changes. Firstly, secreted mucus amount increased and subsequently decreased. Secondly, glycosylation of smaller molecules decreased and then increased again. Thirdly, relatively more protein could be found in for the middlesized mucus HMGs (transistion area).

Besides bacteria, dietary factors have the potential to affect synthesis and secretion of mucin from the goblet cells. In the present study, the influence of feeding the immunomodulatory β-glucan on mucin and ß-defensin gene expression in mucosal tissues of carp has been monitored (Chapter 5). Consistent, but not significant, down-regulation of *Muc2* in the intestine and gills was seen in the glucan fed carp, while *Muc5B* was significantly increased in skin, with slight up-regulation in gills. The expression levels for both ß-defensins were significantly higher in the skin of ß-glucan fed carp, with *BD2* significantly higher in gills.

Besides having a positive effect on mucus, dietary substances can also negatively influence mucus. Soybean meal (SBM) containing diets are known to induce an inflammatory response in the hindgut of certain fish. The response of the intestinal mucosal layer and of intestinal mucus of carp to acute SBM-induced enteritis was examined using bacterial, histological, histochemical and biochemical techniques (Chapter 6). After SBM feeding, the second gut segment of carp showed mild signs of an enteritis process comparable to that previously described for salmon and carp. SBM-diet induced a change in mucins in carp similar to that found in inflammatory bowel diseaes in humans. Initial changes included changes in mucin composition, the presence of BD3 and of bacteria in internal organs. After the initial changes, all measured values returned back to the initial pre-SBM diet values.

Samenvatting

De extrinsieke barrière is de eerste blokkade die vissen tegen pathogene organismen en toxines beschermt. Deze barrière wordt voornamelijk gevormd door slijm. Hoewel vissen volledig bedekt zijn met slijm (zowel intern als extern), is de kennis over dit slijm en vooral over het hoofdbestanddeel, de mucines, beperkt. Mucines geven slijm zijn viskeuze eigenschappen en vormen een matrix waarin een breed scala van anti-microbiële moleculen kan worden gevonden [28]. In dit proefschrift worden mucines van de huid en darm, alsmede anti-microbiële peptiden, die in deze mucosale weefsels worden aangetroffen, beschreven. Ook de invloed van een aantal externe factoren, zoals een bacteriële belasting, microbiële zijn onderzocht.

Met biochemische methoden werden karper slijm HMGs (hoog moleculaire glycoproteïnes) geïsoleerd uit de darm (Hoofdstuk 2) en de huid (Hoofdstuk 3). Na isolatie en gel filtratie werd een bifasische elutieprofiel verkregen. Dit elutieprofiel werd zowel gevonden voor proteïne en koolhydraat inhoud van huidslijm HMGs en ook van uitgescheiden en niet-uitgescheiden darmslijm HMGs. Proteïnes in piek 1 worden verondersteld belangrijk te zijn voor de structuur van de slijmgel. Deze proteïnes hadden biochemische eigenschappen (hoog molecuulgewicht en hoge glycosylering) vergelijkbaar met mucines van zoogdieren. Niet-uitgescheiden slijm HMGs hadden, ongeacht hun grootte, een geringere glycosylering dan de uitgescheiden glycoproteïnes. Slijmmonsters, zowel van niet-uitgescheiden als ook van uitgescheiden slijm, afkomstig van verschillende darmsegmenten (Hoofdstuk 6) hadden vergelijkbare proteïne profielen.

De proteïne kern van mucines wordt gekopieerd door mucine-genen. In dit proefschrift worden voor de karper partiële sequenties van twee genen beschreven, die een hoge homologie vertonen met *Muc2* en *Muc5B* genen van zoogdieren en vogels (Hoofdstuk 5). Net als bij andere gewervelden waren in deze genen ‘von Willebrand factor D’, C8 en TIL domeinen aanwezig. *Muc5B* had een hoge expressie in de huid en kieuwen en *Muc2* een hoge expressie in het eerste en tweede darmsegment van de karper.

Koolhydratenzijketens van slijm HMG zijn fundamenteel voor de algemene eigenschappen van mucosale glycoproteïnes en zijn belangrijke aanhechtingsplaatsen voor bacteriën. Karper huidslijm (Hoofdstuk 3) leek qua monosacharide niveau meer op het slijm van het tweede darmsegment dan het slijm van het eerste segment (Hoofdstuk 6). Via lectine histochemie op de huid (Hoofdstuk 3) en darm (Hoofdstuk 2) kon de aanwezigheid van eindstandig gebonden GalNAc, galactose, mannose en NeuNAc in slijmbekercellen worden getoond. Tussen darmsegmenten (Hoofdstuk 6) konden verschillen in koolhydraatkleuring worden waargenomen, een indicatie voor verschillen in de pH van het slijm.

Naast mucine-genen, konden twee ß-defensin coderende genen gedeeltelijk gesequenced worden. Deze twee ß-defensin genen hadden een cysteine rangschikking vergelijkbaar met die van gewervelde ß-defensines lijkt (Hoofdstuk 5). Defensines hebben een breed anti-microbieel spectrum variërend van Gram-negatieve, Gram-positieve bacteriën, mycobacteriën, schimmels en omhulde virussen. In de naïeve karper werd *BD1* alleen op intermediair niveaus in de huid geëxprimeerd en *BD2* alleen op een laag niveau in de lever.

Kleuring voor BD3 en lysozym was laag in zowel eerste als tweede darmsegment (Hoofdstuk 6). Kleuring voor BD2 was duidelijker voor het eerste dan voor het tweede segment.
De barrièrefunctie van slijm kan door diverse factoren verstoord worden. Veranderingen in mucus HMG en antimicrobiële peptiden na verschillende stimuli werden onderzocht. De HMG verantwoordelijk voor de structuur van de slijmgel (piek 1) bleek niet te worden beïnvloed door leeftijd (Hoofdstuk 2). Echter een toegenomen glycosylering van HMG uit piek 2, waarvan wordt aangenomen dat deze binnendringende micro-organismen kunnen vangen, werd gevonden.
Een overweldigend aantal infectieziektes begint met de bacteriële kolonisatie van mucosale weefsels. Chemotactische activiteit in de richting van slijm begunstigt bacteriële adhesie. Adhesie van ziekteverwekkers aan mucosale oppervlaktes is meestal de eerste stap in de pathogenese van infectieziektes. Een snellere vermenigvuldiging van bacteriën in slijm verhoogt de mogelijke pathogeniciteit, omdat het uitspoelen van bacteriën uit de darm als gevolg van slijmerosie wordt verminderd. Een hogere adhesie en daaropvolgende groei kan leiden tot verschillen in pathogeniciteit zoals in deze studie (Hoofdstuk 7) is beschreven. De *A. hydrophila* stammen leken niet pathogeen aangezien geen van de vissen, die met 10^7 kolonievormende eenheden (KVE) werden geïntubeerd, overleed Bovendien hadden deze stammen een geringe chemotactische respons. De meer pathogene stam had niet alleen hogere adhesie, maar ook een hogere totale opbrengst, als zij in een slijm-verijkt medium werd gekweekt, in vergelijking met de minder pathogene stam.
In dit proefschrift werd het effect van een perorale toediening van een bacterieel product (LPS) en twee *A. hydrophila* stammen op darmslijm (Hoofdstuk 8) en het effect van water met een hoge *A. hydrophila* belasting op huidslijm (Hoofdstuk 4) onderzocht. Verschillen werden tussen de controle vissen en de behandelde vissen waargenomen voor: 1) het mucinegehalte, 2) de glycosylering van slijm HMGs, 3) eindstandige glycosylering (darm) en 4) verspreiding van koolhydraten en proteïnes over de grootte fracties. Na de belastingen konden drie respons tendensen worden waargenomen waarbij elke tendens op een verschillende tijdstip werd waargenomen: 1) het gehalte aan uitgescheiden slijm nam toe en vervolgens af, 2) de glycosylering van kleinere moleculen nam af en vervolgens weer toe en 3) er waren relatief meer proteïnes te vinden in de middelgrote slijm HMGs Deze tendensen worden waarschijnlijk door verschillende mechanismen veroorzaakt.
Niet alleen bacteriën, maar ook voedingsfactoren bezitten het potentieel om de synthese en uitscheiding van mucines uit slijmbekercellen te beïnvloeden. In de huidige studie is de invloed van de toevoeging van immunomodulerende β-glucanen aan het karper voedsel op de genexpressie van mucines en ß-defensines in mucosale weefsels bestudeerd (Hoofdstuk 5). In de β-glucaan-gevoede karpers was een consequente, maar niet significante down-regulatie van *Muc2* in de darm en de kieuwen te zien, terwijl *Muc5B* expressie significant verhoogd was in de huid en een lichte verhoging in de kieuwen waarneembaar was. De expressie niveaus voor beide ß-defensines waren significant hoger in de huid van ß-glucaan-gevoede karpers en *BD2* expressie was significant hoger in de kieuwen.
Plantaardige stoffen niet alleen een positieve maar ook een negatieve invloed op slijm hebben. Van sojameel (SBM) bevattende voeding is bekend dat het in bepaalde vissoorten een ontstekingsreactie in de einddarm kan induceren. In *C. carpio* werd een acute SBM enteritis geïnduceerd en de reactie van het mucosale weefsel van de darm en het darmslijm werd onderzocht met behulp bacteriële, histologische, biochemische en histochemische

technieken (Hoofdstuk 6). Na SBM voeding, vertoonde het tweede darmsegment (einddarm) van de karper lichte enteritis, vergelijkbaar een vroegere beschrijvingen voor zalm en karper. De SBM-voeding leidde in de karper tot een verandering in mucines die vergelijkbaar is met “inflammatory bowel diseases” (inflammatoire darmziekten) bij mensen: veranderingen in mucine samenstelling, aanwezigheid van BD3 en bacteriën in inwendige organen. Echter, bij de karper, keerden, na deze eerste veranderingen, alle meetwaarden weer naar de oorspronkelijke waarden voor de SBM voeding terug.

Zusammenfassung

Die extrinsische Barriere ist die erste Barriere, die die Fische gegen Pathogene und Toxine schützt. Diese Barriere wird vor allem durch Schleim gebildet. Obwohl Fische komplett – sowohl intern als auch extern – mit Schleim bedeckt sind, ist der Wissensstand über Schleim und vor allem über seinen Hauptbestandteil, die Muzine, begrenzt. Muzine geben Schleim seine viskösen Eigenschaften und bilden eine Matrix, in der ein breites Spektrum von antimikrobiellen Molekülen gefunden werden kann. In dieser Arbeit werden Muzine der Haut und des Darms sowie antimikrobielle Peptide von mukosalen Geweben beschrieben. Auch die Auswirkungen von einigen externen Faktoren wie bakterielle Belastung werden diskutiert.

Mit biochemischen Methoden wurden Karpfenschleim-HMGs (high molecular weight glycoproteins: Glykoproteine mit einer hohen molekularen Masse) aus dem Darm (Kapitel 2) und der Haut (Kapitel 3) isoliert. Nach Isolierung und Gelfiltration wurde ein biphasisches Elutionsprofil gefunden. Dieses wurde sowohl für die Protein- und Kohlenhydratanteile der Hautschleim-HMGs als auch für sekretierte und nicht-sekretierte HMGs der Darmschleimhaut gefunden. Es wird angenommen, dass Proteine aus Peak 1 für die Gelstruktur des Schleims wichtig sind. Diese Proteine hatten ähnliche biochemische Eigenschaften wie Säugermuzine mit einem hohen Molekulargewicht und hoher Glykosylierung. Nicht-sezernierte Schleim-HMGs hatten, unabhänig von ihrer Größe, eine niedrigere Glykosylierung als sezernierte Glykoproteine. Protein-Profile von nicht-sezerniertem und sezerniertem Schleim aus den verschiedenen Darmabschnitten (Kapitel 6) waren ähnlich.

Der Protein-Kern der Muzine wird durch Muzingene transkribiert. In dieser Arbeit wurden Teilsequenzen von zwei Karpfen-Genen gefunden (Kapitel 5), die eine hohe Homologie zu *Muc2*- und *Muc5B*-Genen von Säugetieren und Vögeln haben. Wie bei anderen Wirbeltieren waren in diesen Genen “Von Willebrand-Faktor D”-, C8- und TIL-Abschnitte vorhanden. *Muc5B* war in Haut und Kiemen hoch exprimiert, Karpfen *Muc2* im ersten und zweiten Darmabschnitt.

Kohlenhydrat-Seitenketten von Schleim-HMGs sind von grundlegender Bedeutung für die allgemeinen Eigenschaften der Schleimhaut-Glykoproteine und bieten eine Adhäsionsstelle für Bakterien. Im Allgemeinen ähnelte der Hautschleim von *C. carpio* (Kapitel 3) dem Schleim des zweiten Darmabschnitts auf Monosaccharidebene mehr als dem Schleim des ersten Segments (Kapitel 6). Durch Lektin-Histochemie von Haut (Kapitel 3) und Darm (Kapitel 2) konnte das Vorhandensein von terminal gebundenem Acetylgalactosamin, Galaktose, Mannose und Neuraminsäure in den Becherzellen gezeigt werden. Zwischen verschiedenen Darmabschnitten (Kapitel 6) konnten Unterschiede in der Kohlenhydrat-Färbung beobachtet werden, was auf Unterschiede im pH-Wert des Schleims hindeutet.

Zusätzlich zu den Muzingenen wurden Teilsequenzen von zwei ß-Defensin kodierenden Genen gefunden. Diese Gene hatten eine Cystein-,Anordnung, die ähnlich zu ß-Defensinen anderer Wirbeltieren war (Kapitel 5). Defensine haben ein breites antimikrobielles Spektrum von Gram-negativen bis Gram-positiven Bakterien, Mykobakterien, Pilzen und behüllten Viren. In naiven Karpfen wurde *BD1* nur in mittleren Konzentrationen in der Haut und *BD2* nur in geringen Konzentrationen in der Leber exprimiert. Die Anfärbbarkeit für BD3 und Lysozyme

waren sowohl im ersten als auch im zweiten Darmsegment (Kapitel 6) gering. Die Anfärbbarkeit für BD2 war im ersten Darmsegment ausgeprägter als im zweiten Segment.
Die Barrierefunktion des Schleims kann durch mehrere Faktoren gestört werden. Veränderungen in Schleim-HMGs und antimikrobielle Peptide wurden nach verschiedenen Reizen untersucht. Die HMGs, die verantwortlich für die Gelstruktur des Schleims (Peak 1) sind, erschienen in verschiedenen Altersstufen unverändert (Kapitel 2). Für ältere Karpfen wurde im Vergleich zu jüngeren jedoch eine erhöhte Glykosylierung der HMGs aus Peak 2 gefunden. Es wird angenommen, dass diese HMGs in der Lage sind, eindringende Mikroorganismen einzufangen.
Der Großteil der Infektionskrankheiten wird durch bakterielle Besiedlung der Schleimhäute initiiert. Die chemotaktische Aktivität des Schleims fördert bakterielle Adhärenz. Adhärenz von Pathogenen an der Schleimhautoberfläche ist der erste Schritt in der Pathogenese vieler Infektionskrankheiten. Eine schnellere Vermehrung von Bakterien im Schleim erhöht die mögliche Pathogenität, da das Herausspülen der Bakterien durch Schleimerosion reduziert wird. Eine höhere Adhärenz mit darauffolgendem Wachstum kann, wie in dieser Studie gezeigt (Kapitel 7), zu Unterschieden in der Pathogenität führen. Die *A. hydrophila* Stämme erschienen nicht hochpathogen, da 1. keiner der Fische, die mit 10^7 KbE inkubiert wurden, starb, 2. die Stämme eine geringe chemotaktische Reaktion hatten und 3. sie in mit Schleim ergänzten Medien wuchsen. Der pathogenere Stamm zeigte nicht nur eine höhere Adhäsion an den Schleim, sondern im Vergleich mit einem weniger pathogenen Stamm auch einen höheren Keimgehalt, wenn das Medium mit Schleim angereichert wurde.
In dieser Arbeit wurde die Wirkung einer oralen Applikation eines bakteriellen Produktes (LPS) und von zwei *A. hydrophila* Stämmen auf die Darmschleimhaut (Kapitel 8) und die Wirkung von Wasser mit einer hohen *A. hydrophila* Belastung auf die Hautschleimhaut (Kapitel 4) untersucht. Unterschiede zwischen der Kontrollgruppe und behandelten Fischen konnten für Muzingehalt, Glykosylierung der Schleim-HMGs, in der terminalen Glykosylierung (Darm) und der Verteilung von Kohlenhydraten und Eiweißen in den Größenfraktionen beobachtet werden. Nach den Belastungen konnten drei Tendenzen beobachtet werden. Jede Tendenz wurde zu unterschiedlichen Zeiten bei allen Gruppen beobachtet, was auf verschiedene Mechanismen, die zu den beobachteten Veränderungen geführt haben, hindeutet. Erstens war die Menge des sekretierten Schleims erst erhöht und anschließend verringert. Zweitens war die Glykosylierung von kleineren Molekülen erst verringert und anschließend wieder erhöht. Drittens konnte relativ mehr Protein von den mittelgroßen Schleim-HMGs (Übergangsbereich) im Vergleich zur Kontrollgruppe gefunden werden.
Nicht nur Bakterien, aber auch diätetische Faktoren haben das Potenzial, die Synthese und Sekretion von Muzin aus den Becherzellen zu beeinflussen. In der vorliegenden Studie wurde der Einfluss der Fütterung mit immunmodulatorischem β-Glucan auf Muzin und ß-Defensin-Genexpression in Schleimhautgewebe von Karpfen überwacht (Kapitel 5). In Darm und Kiemen der mit β-Glucan gefütterten Karpfen wurde eine beständige, aber nicht signifikante Runterregulierung von *Muc2* gefunden. Im Gegensatz dazu war die *Muc5B*-Expression in der Haut deutlich erhöht und in den Kiemen leicht raufreguliert. Für die mit β-Glucan gefütterten Karpfen waren in der Haut die Expressionsniveaus beider ß-Defensine signifikant höher, ebenso *BD2* in den Kiemen.
Diätetische Substanzen können den Schleim nicht nur positiv, sondern auch negativ beeinflussen. Es ist bekannt, dass Sojabohnenmehl(SBM)-haltige Ernährung bei bestimmten Fischarten

eine entzündliche Reaktion im Enddarm induziert. Die Reaktion der Darmschleimhaut und des Darmschleims von Karpfen nach einer akuten SBM-induzierten Enteritis wurde mittels bakteriologischer, histologischer, histochemischer und biochemischer Techniken untersucht (Kapitel 6). Nach SBM-Fütterung zeigte der zweite Darmabschnitt von Karpfen milde Anzeichen einer Enteritis, die vergleichbar mit der zuvor für Lachse und Karpfen beschriebenen war. Bei Karpfen induziert eine SBM-Fütterung eine Änderung in den Muzinen, welche dem „inflammatory bowel disease“ (chronisch-entzündlichen Darmerkrankungen (CED)) beim Menschen ähnelte. Zu den anfänglichen Veränderungen zählen Veränderungen in der Muzin-Zusammensetzung, das Vorhandensein von BD3 und von Bakterien in inneren Organen. Nach den anfänglichen Veränderungen kehrten alle gemessenen Werte wieder auf die vor der SBM-Fütterung erhaltenen Ausgangswerte zurück.

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So far I have been able to avoid mentioning names. However, there is one person I want to acknowledge specially; my supervisor, promotor and boss Prof. Dr. Steinhagen. Lieber Dieter, ich bin Dir so dankbar, dass Du mir nach Hannover geholt hast und mir die Möglichkeit gegeben hast, im Institut anzufangen, und mich so viele Jahre begleitet, motiviert, betreut und „durchgefüttert“ hast. Mein Leben wäre ohne Dich ganz anders verlaufen.

About the author

Curriculum vitea
List of publications

Curriculum vitae

Maria Cornelia van der Marel was born in Leiderdorp, the Netherlands, on May 22 1978. In 1996 she started a four year full-time sandwich course, equivalent to a Bachelor of Science, in Animal husbandry and production with a specialization in Animal health care at Hogeschool Delft, the Netherlands. After graduating in 2000, she started an international Master of Science course in Aquaculture at Wageningen University, the Netherlands. A minor M.Sc. thesis, tittled 'Free amino acids and yolk proteins in eggs and developing larvae of alligator gar' was conducted at the University of Bergen, Norway. A major M.Sc. thesis, titled 'Improvement of spawning synchrony and artificial incubation of eggs in the Nile Tilapia, *Oreochromis niloticus*' was done at Wageningen University, the Netherlands. After her masters, she was enrolled in a European, Marie Curie Research Training Network entitled 'Integrated approach to the innate immune response to parasites in fish' (PARITY) at the Fish Disease Research Unit, University of Veternary Medicine Hannover, Germany. After the European Training Network she continued working at the University of Veterinary Medicine Hannover. She undertook research on the following subjects:

- Influence of coccidia on the intestinal tract of carp,
- carp mucus and its role in mucosal defense (this thesis),
- optimisation of a deniftrification unit in a filter system and its influence on fish health.

List of publications

van der Marel M., Caspari N., Neuhaus H., Meyer W., Enss M.-L., Steinhagen D. Characterization of skin mucus layer in common carp, *Cyprinus carpio* L.. *In preparation*

van der Marel M., Pröpsting, M. J., Battermann, F., Jung-Schroers, V., Hübner, A., Rombout, J. H. W. M., Steinhagen, D.) Differences between intestinal segments and soybean meal induced changes in intestinal mucus composition of common carp *Cyprinus carpio* L.. S*ubmitted*

Meyer, K., Bergmann, S. M., **van der Marel, M.**, Steinhagen, D. (2012) Detection of Koi Herpesvirus (KHV): Impact of extraction method, primer set and polymerase on the sensitivity of PCR *Aquaculture Research,* **43**, 835-842. DOI: 10.1111/j.1365-2109.2011.02897.x

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Neuhaus, H., **van der Marel, M.**, Caspari, N., Meyer, W., Enss, M. L., Steinhagen, D. (2007) Biochemical and histochemical effects of perorally applied endotoxin on intestinal mucin glycoproteins of the common carp *Cyprinus carpio*. *Diseases of Aquatic Organisms*, **77**, 17-27. DOI: 10.3354/dao01836

Neuhaus, H., **van der Marel, M.**, Caspari, N., Meyer, W., Enss, M. L., Steinhagen, D. (2007) Biochemical and histochemical study on the intestinal mucosa of the common carp *Cyprinus carpio* L., with special consideration of mucin glycoproteins *Journal of Fish Biology*, **70**, 1523-1534. DOI:10.1111/j.1095-8649.2007.01438.x

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Appendix I

Biochemical and histochemical study on the intestinal mucosa of the common carp *Cyprinus carpio* L., with special consideration of mucin glycoproteins

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Biochemical and histochemical study on the intestinal mucosa of the common carp *Cyprinus carpio* L., with special consideration of mucin glycoproteins

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The biochemical and histochemical properties of intestinal mucin glycoproteins of virus and parasite-free common carp *Cyprinus carpio* were investigated. The presence of carbohydrates in mucin glycoproteins could be demonstrated by histochemical methods, but generally, no obvious differences in specific staining for mucin glycoproteins were observed in contrast to biochemical techniques. Biochemical staining methods displayed differences in structure and composition of intestinal glycoproteins. Released intestinal glycoproteins contained two types of mucin glycoproteins: type 1 mucins displayed a size of >2000 kDa, and were highly glycosylated, while type 2 mucins ranged between 700 and 70 kDa, and were weakly glycosylated. In epithelial (intracellular) glycoproteins, mainly N-acetyl-α-galactosamine and mannose were found, while in luminal (extracellular) glycoproteins in addition sialic acid was evident. Fucose was not detected. Thus, structure and composition of intestinal glycoproteins of common carp were similar to those found in mammals.

Key words: carp; fishes; glycoproteins; intestinum; mucus.

INTRODUCTION

Intestinal tissue is exposed to a permanent challenge of bacteria, parasites, viruses and toxins from the luminal content. The epithelial cells are protected from pathogen attack by a mucous layer, which covers the intestinal epithelium in vertebrates (Shephard, 1994). Mucin glycoproteins ('mucins') are considered to be the major component of this protecting biofilm, produced by goblet cells, which are scattered in many epithelial systems of aquatic vertebrates and

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particularly in the gut. The high molecular mass glycoproteins form a water-insoluble layer of adherent mucus on epithelial cells, and have a high content of oligosaccharides. Oligosaccharides are believed to mediate the adhesion of microbial pathogens (Karlsson *et al.*, 1991; Moncada *et al.*, 2003) and to prevent glycoprotein degradation by proteases of microbial origin.

Although all surfaces of fishes are covered with mucus, knowledge about piscine mucins is scarce. Based on histochemical data, the number and content of intestinal goblet cells in fishes and the composition of secreted mucins in secretory tissues is considered to be similar to that of mammals. As in mammals, a production of secretory vesicles with neutral, acidic or sulphated glycoproteins was described (Shephard, 1994; Meyer *et al.*, 2001; Bosi *et al.*, 2005). Histological and histochemical studies also indicate that fishes with parasitic infection display, like mammals, goblet cell hyperplasia and hypertrophy, whereby their intracellular mucins shifted towards acidic and sulphated glycoprotein types (George & Nadakal, 1981; Dezfuli *et al.*, 2002; Bosi *et al.*, 2005).

Nothing is known, however, about the biochemical composition of piscine glycoproteins contributing to the intestinal first line of defence. Therefore, in the present study intestinal goblet cells of the common carp *Cyprinus carpio* L. were characterized primarily by biochemical analyses; histochemical methods were applied on goblet cell carbohydrate contents.

MATERIALS AND METHODS

ANIMALS

Parasite and virus free sibling common carp ($n = 10$) from a single crossing (E20xR8, Wageningen Agricultural University, Wageningen, The Netherlands) were used (Wiegertjes *et al.*, 1995). Common carp were raised and kept in filtered re-circulated tap water. For all experiments 12–18 months old fish with a mean ± S.E. body mass of 88·3 ± 9·7 g and a mean ± S.E. standard length (L_S) of 14·4 ± 0·7 cm were used. The fish were placed in a 400 l tank with filtered tap water 5 days before sampling, and were starved to empty the guts.

The common carp were killed by bath immersion with 500 mg l^{-1} Ms-222 tricaine methane sulphonate (Sigma, Munich, Germany) and subsequently dissected. The entire intestinal tract from pseudogaster to anus was removed, weighed and cooled on ice.

HISTOLOGY AND HISTOCHEMISTRY

For histological and histochemical examination, 10 gut samples with a length of 4 mm were taken, 4 cm behind the pseudogaster and fixed with Bouin's solution. Samples were carefully dehydrated and embedded in paraffin wax. Sections of 5 μm thickness were stained histologically with haematoxylin-eosin (H.E.), and mucin carbohydrates were visualized histochemically with alcian blue 8GX pH 1 (AB 1.0) and pH 2·5 (AB 2.5) as well as AB 2.5/periodic-acid-Schiff (AB-PAS). All sections were studied with a light microscope (Zeiss light microscope axiphot; Zeiss, Jena, Germany). The AB 1.0 method stains sulphated glycoconjugates; the AB-PAS reaction visualizes either acidic or neutral glycoconjugates (Pearse, 1972; Table I). In addition, terminal sugar residues were characterized with different lectins. Lectins were indicated with biotin [10 μg ml^{-1} in 0·1 M phosphate buffered saline (PBS), pH 7·2, 30 min at room temperature]. The lectins and their sugar specificity are listed in Table I. Lectin binding was visualized with a very sensitive streptavidin-diamino-benzidine-hydrogen

TABLE I. Characterization of intestinal, intracellular goblet cell mucin carbohydrates concerning charge (PAS, AB-PAS, AB 1.0, AB 2.5) and terminal sugars (ConA, DBA, MAA, PNA, RCA, SNA, UEA I, WGA)

Acronym	Staining	Binding specificity	Histochemical goblet cell staining intensity
Conventional histochemistry			
PAS	Periodic-acid-Schiff	Neutral glycoproteins	2–3
AB-PAS	Alcianblue-PAS	Neutral and acidic glycoproteins	2–3
AB 1.0	Alcianblue-pH 1.0	Sulphated glycoproteins	1–2
AB 2.5	Alcianblue-pH 2.5	Acidic glycoproteins	2
Lectin histochemistry			
ConA*	*Canavalia ensiformis*	α-D-mannose	2–3
DBA*	*Dolichos biflorus*	N-acetyl-α-D-galactosamine	4
MAA	*Maackia amurensis*	Neuraminic-acid-α-2-3-galactose	1–2
PNA	*Arachis hypogyea*	β-D-galactose	2–3
RCA*	*Ricinus communis*	N-acetyl β-D-galactosamine	3
SNA*	*Sambucus nigra*	Neuraminic-acid-α-2-6-galactose	2–3
UEA I*	*Ulex europaeus*	Fucose-α1-2-galactose	1–2
WGA	*Triticum vulgaris*	N-acetyl β-D-glucosamine	1–2

1, weak; 2, weak to moderate; 3, moderate; 4, moderate to strong; 5, strong reaction.
*Used in lectin-binding assay.

peroxidase-system (DAB, Biogenex, Super Sensitive System, Hamburg, Germany) (Brooks *et al.*, 1997).

ISOLATION OF MUCIN GLYCOPROTEINS

Intestines of common carp with a mean ± S.E. mass of 1·33 ± 0·23 g were opened longitudinally and cut into small pieces of 3–4 mm. Subsequently, secreted luminal glycoproteins were isolated with isolation medium as described previously (Enss *et al.*, 1996). In brief, tissue pieces were incubated for 20 min in 100 ml isolation buffer containing antibiotics and protease inhibitors. The isolation buffer was collected, centrifuged for 30 min at 12 000 g, and the supernatant was collected and frozen at −20° C until further processing. Epithelial glycoproteins were released from goblet cells by subsequent incubation of the tissue pieces for 30 min in a buffer containing antibiotics, protease inhibitors and ethylene diamine tetra acetic acid (EDTA) (Enss *et al.*, 1996). Goblet cells were disrupted by means of an ultrasonic unit (Ultra Turrax T8; IKA-Werke, Staufen, Germany). The suspension was centrifuged at 10 000 g for 30 min, and the supernatant was collected and homogenized by gentle stirring. All samples were concentrated by ultrafiltration (Amicon, Beverly, MA, U.S.A.; exclusion limit 30 000 Da) to a final volume of 2 ml.

Concentrated mucous samples were subjected to downward gel filtration on a 34 × 0·9 cm Sepharose CL-4B column (Sigma, Munich, Germany; flow rate 5·2 ml h^{-1}, fraction size 1·3 ml, 40 fractions). For calibration pig gastric mucin (PGM, molecular mass >2000 kDa), thyroglobulin (molecular mass 670 kDa), ferritin (molecular mass 450 kDa) and bovine serum albumin (BSA, molecular mass 69 kDa) were used (Sigma). Aliquots of each fraction were determined for carbohydrate contents by periodic-acid-Schiff (PAS) reaction (550 nm) and for protein contents by Bradford reaction (580 nm; BMG, Offenburg, Germany) (Dubois *et al.*, 1956; Bradford, 1976; Enss *et al.*, 1992). Glycoprotein contents were calculated (CGC) in mg glycoprotein g^{-1} gut with lyophilized pig gastric mucin as a standard *via* PAS reaction.

DETERMINATION OF THE TERMINAL GLYCOSILATION PATTERN *VIA* LECTIN-BINDING ASSAY

Aliquots of each fraction (200 µl) were incubated over night at room temperature in 96-well-microtiter plates (Nunc Maxisorb, Wiesbaden, Germany). Subsequently, they were blocked with 1% BSA in phosphate buffered saline (PBS), and then incubated with biotin labelled lectins (10 µg ml^{-1} in 0·1 M PBS) for 30 min at room temperature. The following lectins were used: *Canavalia ensiformis* (ConA), *Dolichos biflorus* (DBA), *Ricinus communis* (RCA), *Sambucus nigra* (SNA) and *Ulex europaeus I* (UEAI) (Table I). Lectin binding was visualized by subsequent incubation with streptavidin-horseradish-peroxidase for 30 min at room temperature and orthophenyl-diamine (OPD) (DAKO Chemicals, Glostrup, Denmark). After 15 min, the reaction was stopped by addition of 0·5 M sulphuric acid, and the optical density was read in a microplate reader (BMG, Offenburg, Germany) at 485 nm (Enss *et al.*, 1995, 1996).

If not indicated results are expressed as median values with 25 and 75% quartiles.

RESULTS

ANIMALS

All fish appeared healthy throughout the study, no clinical alterations were observed during maintenance.

HISTOLOGY AND HISTOCHEMISTRY

Goblet cells were regularly located in the intestinal epithelium between the enterocytes. Most goblet cells had a rounded, thick appearance, and were filled with visible carbohydrate content. Glycoprotein staining intensity varied from very weak to moderate, whereby the presence of neutral, acidic and sulphated glycoconjugates could be demonstrated (Fig. 1 and Table I). Goblet cells responded in two different ways to AB 1.0 (staining of acid glycoconjugates): one population of cells was stained rather weakly, a second population showed

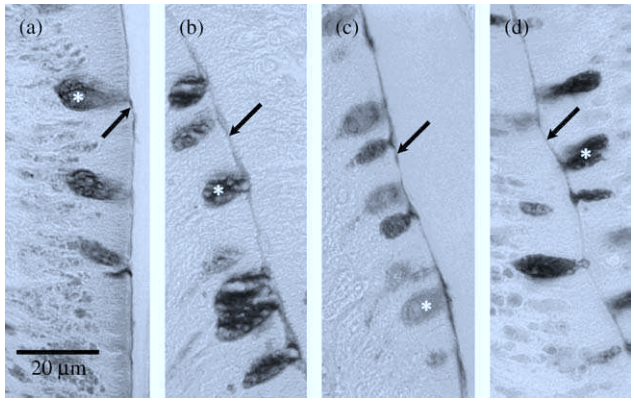

FIG. 1. General carbohydrate histochemical staining (a) PAS, (b) AB 2.5, (c) AB 1.0 and (d) AB-PAS (see Table I) of the intestinal mucosa of the common carp. Goblet cells (*) and mucous layer (→) covering the intestinal mucosa of common carp show a positive, intense staining.

a stronger reaction. Lectin staining of goblet cell content for specific carbohydrates displayed a weak or negative reaction with UEA I (indicating fucose), WGA (indicating N-acetyl-β-glucosamine) and MAA (indicating neuraminic-acid-α-2-3-galactose), a weak to moderate reaction with ConA (indicating mannose), PNA (indicating N-acetyl-β-1-3-galactosamine) and SNA (indicating neuraminic-acid-α-2-6-galactose), a moderate reaction with RCA (indicating N-acetyl-β-galactosamine) and a moderate to strong reaction with DBA (indicating N-acetyl-α-galactosamine) (Fig. 2 and Table I). Reactivity of mucous layer to used lectins was generally stronger than reactivity of mucous cell content (Fig. 2).

ISOLATION OF MUCIN GLYCOPROTEINS

Epithelial and luminal glycoproteins could be isolated separately with the methods applied. Epithelial glycoproteins were found with a total amount of 0·52 (0·23–0·91) mg, and luminal glycoproteins with a total amount of 2·22 (1·58–2·83) mg (Table II). When monitoring fractions from downward gel filtration, a biphasic elution profile was obtained for both, protein and carbohydrate contents (Fig. 3). For epithelial and luminal glycoproteins, large molecules with a molecular mass of >2000 kDa were eluted in the first peak (PI), and glycoproteins in the range of 70–700 kDa in the second peak (PII). Between these peaks, a transition area (TA) with fractions of fewer glycoproteins was found. Profiles for carbohydrate and protein contents were comparable. For epithelial glycoproteins, the amount of CGC in peak I was five times higher (0·26 mg CGC) than the amount in TA and PII (0·05 mg). For luminal glycoproteins, 0·54 mg CGC were found in peak I, 0·23 mg CGC in peak II and 0·79 mg CGC in TA (see Table II).

In order to determine the degree of glycosilation, the protein:carbohydrate ratio (PC) was calculated. Molecules of PI were highly glycosylated with a PC of 1·1 for epithelial and 0·6 for lumin glycoproteins. Molecules of PII were less glycosylated with a PC of 8·9 for epithelial glycoproteins and of

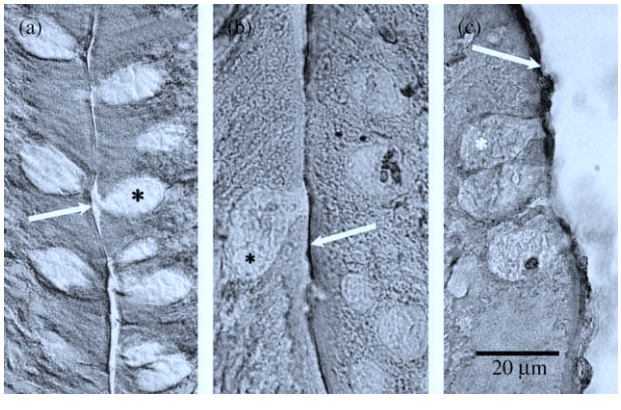

FIG. 2. Lectin histochemical staining (a) UEAI, (b) RCA and (c) DBA (see Table I) on goblet cells, positive for the intestinal mucous layer (→). Glycoprotein contents of goblet cells (*) showed weak (UEAI), moderate (RCA) and moderate to strong (DBA) positive staining.

TABLE II. Isolation of intestinal epithelial and luminal glycoproteins from the common carp on Sepharose CL-4B column. Carbohydrate content was monitored by periodic-acid-Schiff (PAS) and protein by Bradford. Values are medians and 25–75 percentiles of the glycoprotein content per g gut mass from 10 fish

	Epithelial glycoproteins		Luminal glycoproteins	
Glycoprotein fraction	Optical density	CGC (mg g^{-1} gut)	Optical density	CGC (mg g^{-1} gut)
PI (fraction 7–10)				
Carbohydrate	1·24 (0·94 and 1·47)	0·26	2·54 (1·88 and 3·26)	0·54
Protein	1·38 (1·02 and 1·68)		1·42 (0·94 and 1·47)	
TA (fraction 11–15)				
Carbohydrate	0·23 (0·07 and 0·55)	0·05	1·11 (0·58 and 1·51)	0·23
Protein	1·09 (0·69 and 1·39)		2·13 (1·67 and 2·84)	
PII (fraction 16–22)				
Carbohydrate	0·34 (0·09 and 0·62)	0·07	3·75 (2·91 and 4·42)	0·79
Protein	3·04 (2·64 and 3·53)		16·64 (13·95 and 20·1)	
Total (fraction 1–40)				
Carbohydrate	2·46 (1·10 and 4·27)	0·52	10·54 (7·44 and 13·32)	2·22
Protein	7·36 (4·60 and 10·03)		28·32 (21·9 and 38·31)	

CGC, calculated glycoprotein content based on the PAS-reaction, lyophilized pig gastric mucin served as standard; PI, fractions with glycoproteins >2000 kDa; PII, fractions with glycoproteins between 70 and 700 kDa; TA, fractions with molecules between 700 and 2000 kDa.

4·4 for lumin glycoproteins. TA had a PC of 4·7 for epithelial and of 2·0 for lumin glycoproteins. Carbohydrate and protein content of glycoproteins are shown in Table II.

TERMINAL GLYCOSYLATION PATTERN

The following lectins bound to isolated glycoproteins: RCA (N-acetyl-α-D-galactosamine), ConA (α-D-mannose), DBA (N-acetyl-β-D-galactosamine) and SNA (NeuNAc-β-2-6-galactose). UEA I (α-D-fucose) did not bind. The binding pattern for epithelial and luminal glycoproteins is shown in Fig. 4. Generally, the total amount of mucin glycoproteins in PI, TA and PII was higher for luminal mucus than for epithelial mucus (with the exception of DBA) indicating N-acetyl-α-galactosamine. When analysing mucin glycoproteins from different fractions for lectin binding, the following glycosilation pattern became obvious: epithelial glycoproteins from PI generally had higher amounts of oligosaccharides than those from TA, whereas luminal glycoproteins from TA generally had higher amounts of oligosaccharides than those from PII (with exception of DBA) (Fig. 5).

DISCUSSION

In contrast to a variety of protective proteins and their functions described in the skin and gut mucous layer in fishes, knowledge about its constituting molecules, the so-called ‘mucins’ are scarce. The present paper presents some

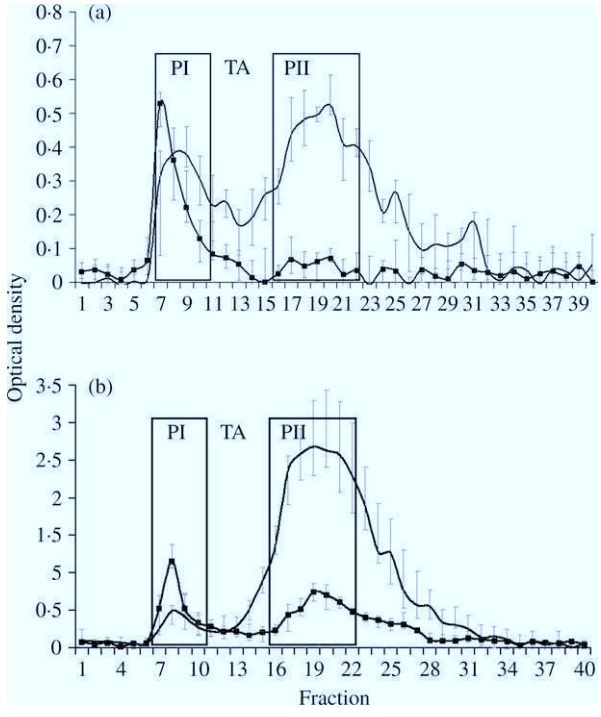

FIG. 3. Elution profiles of (a) epithelial and (b) luminal intestinal mucin glycoproteins from the common carp per g gut isolated (Sepharose CL-4B column, fraction volume: 1·3 ml). The carbohydrate content (■) was monitored by periodic acid-Schiff reaction (PAS), the protein content (▬) by Bradford-assay. Values are mean ± 25 − 75 percentiles of samples ($n = 10$). PI, glycoproteins >2000 kDa; PII, elution fractions with glycoproteins between 70 and 700 kDa; TA, elution fractions with molecules between 700 and 2000 kDa.

specific features of these molecules, which, at least for mammals, are closely attributed to functional characteristics.

In accordance to Aristoteli & Willcox (2003), histochemical methods were applied to identify structural components of intracellular glycoproteins, and lectin analyses to display the presence of their specific carbohydrates. In a second step, intestinal cellular and luminal mucin glycoproteins could be isolated for further biochemical investigations. This technique overcomes the difficulties of histochemical methods to fixate and quantify extracellular secreted glycoproteins: many fixatives change glycoprotein reactions by interactions between the fixatives themselves and glycoproteins (Shephard, 1994).

With the histochemical methods numerous scattered goblet cells could be detected in the intestinal epithelium. They showed different staining intensities, indicating different amounts of neutral, acidic and sulphated glycoproteins. Acidic and sulphated glycoproteins are reported to inhibit bacterial adhesion and glycoprotein degradation by proteases, and goblet cells with such mucins are usually detected in parasite-infected fishes (Bosi *et al.*, 2005).

With biochemical methods intestinal mucin glycoproteins were isolated separately from the luminal content and from the epithelium, consisting of molecules with a molecular mass >2000 kDa and glycoproteins with a molecular

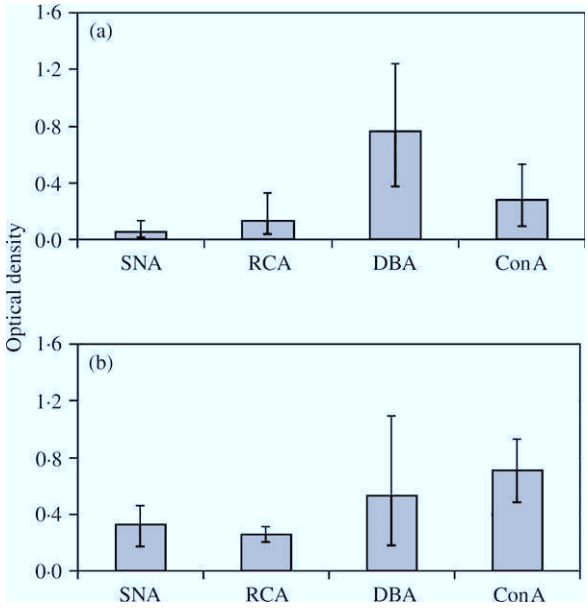

FIG. 4. Semi-quantitative anaylsis of lectin binding to separated (a) epithelial and (b) luminal glycoproteins from the intestine of the common carp. Binding specificity of used lectins: SNA, neuraminic acid; RCA, β-D-galactosamine; DBA, α-D-galactosamine; ConA, α-D-mannose. The lectins were applied to glycoproteins from the pooled fractions of PI, TA and PII (Fig. 3). Values are medians and 25–75 percentiles of measurements from 10 fish.

mass between 70 and 700 kDa. Glycoproteins of high molecular mass were highly glycosylated, the smaller glycoproteins carried less carbohydrates. By isolation with gel filtration, a biphasic elution profile was noted both for protein and carbohydrate content. In rats, similar elution profiles were obtained, and the intestinal mucins displayed similar molecular sizes (Enss *et al.*, 1996). Functionally, large molecules are regarded to form the mucous layer which adheres to the epithelium. Smaller molecules are thought to represent the soluble, luminal mucus. Generally it can be considered that isolated small mucin molecules also contain different 'non mucin' glycoproteins. Enss *et al.* (1996) isolated small glycoproteins and considered then to represent mainly small mucin glycoproteins.

For fishes, the presence of mucins was deducted from genomic sequences in the pufferfish *Takifugu rubripes* (Temminck & Schlegel) (Lang *et al.*, 2004). In the present study, molecules of peak I had biochemical characteristics of mucins with high molecular mass and high carbohydrate contents. These intestinal isolates of high molecular glycoproteins were investigated according to known mammalian oligosaccharides, supposing highly conservated mucin glycoproteins in vertebrates. In mammals, the most abundant macromolecules in mucus are mucins. These are large polypeptides with tandemly repeated sequences, which are rich in threonine and serine with hydroxyl groups. These sequences (amino acids) are *O*-glycosidically linked to oligosaccharides (Perez-Vilar & Hill, 1999). In the large intestinal mucins, carbohydrate content may account for up to 80% of its mass, in gastric mucin *c.* 50% (Carlstedt *et al.*, 1985; Bell *et al.*, 2003).

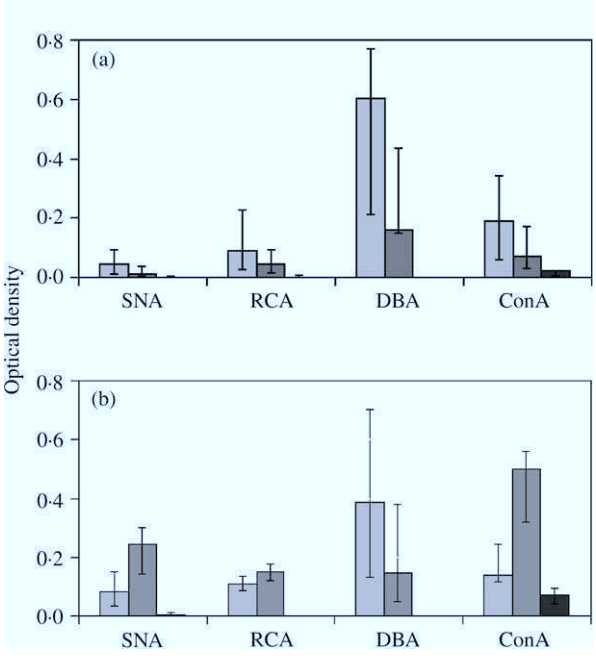

FIG. 5. Semi-quantitative anaylsis of lectin binding to separated (a) epithelial and (b) luminal glycoproteins from the intestine of the common carp. Binding specificity of used lectins: SNA, neuraminic acid; RCA, β-D-galactosamine; DBA, α-D-galactosamine; ConA, α-D-mannose. The lectins were applied to glycoproteins isolated by gel filtration and collected in PI (□), TA (▩) and PII (■) (see Fig. 3). Values are medians and 25–75 percentiles of measurements from 10 fish.

Carbohydrate side chains in mucins from mammalian mucosal surfaces are composed of a limited array of oligosaccharides: N-acetyl-α-galactosamine, N-acetyl-β-glucosamine, galactose, mannose, fucose and neuraminic acid (Forstner, 1995). The presence of the monosaccharides mannose and sialic acid in the content of piscine intestinal goblet cell was shown by lectin histochemistry and in mucin glycoprotein preparations isolated from the intestinal mucosa. Luminal glycoproteins of the common carp contained sialic acid, mannose and N-acetyl-β-galactosamine, as determined by lectin-binding assays, but the epithelial mucins had a much lower degree of glycosylation with sialic acids. Mainly a binding of DBA, which interacts with N-acetyl-β-galactosamine, was observed. Glycosylation with sialic acid and sulphated oligosaccharides gives glycoproteins a negative charge (Perez-Vilar & Hill, 1999). In human gastric mucins sulphated oligosaccharides are involved in the binding of bacteria, such as *Helicobacter pylori*, or in fishes sialic acid and sulphated oligosaccharides are involved in binding of *Aeromonas veronii* (Guzman-Murillo & Ascencio, 2000; Guzman-Murillo *et al.*, 2000). In mammalian cells during oligosaccharide assembly, the addition of sialic acid is the last event of synthesis (Bell *et al.*, 1998). The low binding of lectins with a specificity for sialic acid- to glycoproteins indicates the presence of high amounts of not completely glycosylated, 'non-mature' molecules in epithelial mucins. Fucose (*via* UEAI), however, in compliance with Fiertak & Kilarski (2002), could not be detected

by means of the lectin approach. In a gas chromatographic analysis of mucus isolated from the intestinal tract of the rainbow trout *Oncorhynchus mykiss* (Walbaum), however, fucose was demonstrated (O'Toole *et al.*, 1999). This lack of fucose, indicated in the present study, may be due to two reasons: in intestinal mucins of common carp, fucose could be located in a position, which, by steric reasons, did not allow a lectin linkage, or fucose is totally missing and does not play an important, physiological role in untreated common carp.

Peak II glycoproteins were less glycosylated than peak I or TA glycoproteins with a molecular mass between 70 and 700 kDa. In luminal mucin separation, these glycoprotein types were thought to represent soluble mucins, which originate from degradation of glycoproteins by proteases of microbial origin (Aristoteli & Willcox, 2003), or by digestive enzymes from the host. In intracellular mucin glycoproteins, PII molecules probably represent small mucin glycoproteins, which are not completely synthesized yet (Enss *et al.*, 1996). Small molecules are generally less glycosylated and bind in lower intensities to carbohydrate specific lectins than glycoproteins with higher molecular mass. These aspects indicate that PII glycoproteins of epithelial separations contain glycoproteins in an early state of synthesis. In mammals, the protein core of the glycoproteins is synthesized first and serves as a structural basis for subsequent glycosylation (Forstner, 1995). The dense molecular packing of the protein core with carbohydrate side chains in vesicles is fundamental for the general properties of mucosal glycoproteins, which includes protease resistance, swelling and gel formation due to the uptake of water and binding of ions (Bansil *et al.*, 1995). In addition, carbohydrate side chains allow adhesion of bacteria (Tse & Chadee, 1991), which also was recorded from the skin and the intestinal tract of fishes (Jöborn *et al.*, 1997; Bordas *et al.*, 1998).

Goblet cells of the intestinal mucosa of common carp secrete large glycoproteins, which in structure and composition show a high similarity to those found in mammals. Secreted molecules from the surface of the intestinal epithelium have a high molecular mass of >2000 kDa, and are highly glycosylated with the similar array of monosaccharides at terminal positions as recorded for their mammalian counterparts.

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Appendix II

Biochemical and histochemical effects of perorally applied endotoxin on intestinal mucin glycoproteins of the common carp *Cyprinus carpio*

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Biochemical and histochemical effects of perorally applied endotoxin on intestinal mucin glycoproteins of the common carp *Cyprinus carpio*

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ABSTRACT: Mucins are high molecular weight glycoproteins produced by goblet cells and secreted on mucosal surfaces. We investigated biochemical and histochemical properties of intestinal mucins of virus- and parasite-free common carp *Cyprinus carpio* in response to a single peroral application of endotoxin (lipopolysaccharide = LPS). Intracellular mucins were quantified histochemically by their carbohydrate content and characterized by specific, lectin-based methods. In addition, secreted epithelial (intracellular) and luminal (extracellular) mucins were isolated and separated by downward gel filtration. Carbohydrate and protein content were determined photometrically. Subsequently, terminal glycosylation was characterized by a lectin-binding assay. A peroral endotoxin application altered intestinal secretion and composition of intestinal mucin glycoproteins in common carp. A statistically significant decrease in mature luminal mucins was demonstrated, linked to a new biosynthesis of intracellular mucin glycoproteins. Simultaneous changes in the glycosylation pattern of isolated mucins were found. The intestinal mucosal system is purported to provide a removal mechanism for bacterial noxes by increasing secretion of mucins inducing a flushing-out effect, in combination with altered glycosylation patterns that change adhesion properties. Consequently, pseudofaeces of fish, which are a common sign of intestinal parasitical infections, may also be interpreted as an elimination mechanism for strong bacterial noxes.

KEY WORDS: Carp · Mucus · Intestinum · Endotoxin · Lipopolysaccharide

INTRODUCTION

Intestinal tissue of vertebrates is exposed continually to challenges from bacteria, parasites, viruses and toxins from the luminal contents. Intestinal epithelial cells are protected from pathogens by a mucus layer, which covers the intestinal epithelium in vertebrates such as mammals and fish (Shephard 1994). Mucin glycoproteins ('mucins') are considered to be the major component of this protective biofilm, which is produced by goblet cells that are scattered in many epithelial systems of aquatic vertebrates, particularly in the gut. High molecular weight glycoproteins (HMGs) form a water-insoluble layer of adherent mucus on epithelial cells. HMGs have a high content of oligosaccharides that are believed to mediate adhesion of microbial pathogens (Carlstedt et al. 1985, Karlsson et al. 1991, Bordas et al. 1998, Perez-Vilar & Hill 1999, Moncada et al. 2003) and protect the glycoproteins from degradation by proteases of microbial origin.

Although all fish surfaces are covered with this mucus layer, knowledge of piscine mucins and their responses to noxes is scarce. Based on histochemical data, the number and content of intestinal goblet cells

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in fish are considered to be similar to those of mammals. As in mammals, there is production of secretory vesicles with neutral, acidic or sulphated glycoproteins (Shephard 1994, Bosi et al. 2005). Histological and histochemical studies also indicate that fishes with parasitic infections (like infected mammals) exhibit goblet cell hyperplasia and hypertrophy, whereby as a chronic reaction their intracellular mucins shift towards acidic and sulphated glycoprotein types (George & Nadakal 1981, Dezfuli et al. 2002, Bosi et al. 2005).

However, to our knowledge little information is available on the biochemical composition of piscine glycoproteins and their response to enteric bacterial infections. We mimicked bacterial infection by an oral application of endotoxin. Endotoxins are lipopolysaccharide (LPS)-protein complexes synthesized mainly by gram-negative bacteria. LPS is an integrated part of the bacterial membrane. Endotoxin has, at least in mammals, a local and systemic immune-inducing function in inflammation reactions (Raetz 1990, Raetz & Whitfield 2002). Toxic effects are mainly mediated by cell receptors (TLR-4) and leucocyte mediators; however, there are numerous key differences between fish and mammals. In many *in vitro* studies on cells of lower vertebrates, such as fish, extremely high concentrations of LPS (µg ml^{-1}) have been used to induce immune responses. In fish (in contrast to mammals), some cell integrins and molecules for signal transduction are totally missing, or have yet to be identified (Iliev et al. 2005).

Here, intestinal goblet cells of the common carp were characterized primarily by biochemical analyses of responses to single oral endotoxin applications. Additionally, histochemical methods were applied to the analysis of goblet cell carbohydrate contents.

MATERIALS AND METHODS

Carp specimens. Parasite- and virus-free sibling carp (n = 50) from a single cross (E20 × R8, Wageningen Agricultural University, The Netherlands) were used (Wiegertjes et al. 1995). The carp were raised and kept in filtered recirculated tap water. For all experiments, 12 to 18 mo old carp with a mean (±SE) body weight of 76.36 g (±12.94 g) and a mean (±SE) length of 14.09 cm (±0.92 cm) were used. The carp were placed in a 400 l tank with filtered tap water 5 d before applying endotoxin; the fish were starved to reduce faeces on intestinal mucus. For the oral application of endotoxin (150 µg g^{-1} bodyweight dissolved in phosphate-buffered saline, PBS; LPS of *Escherichia coli* O55:B5; Sigma) via intubation, the carp were anaesthetized by adding 150 mg l^{-1} tricaine (Sigma) and weighed. For sampling, carp were killed by bath immersion with 500 mg l^{-1} tricaine and subsequently dissected. The complete intestinal tract from pseudogaster to anus was removed, weighed and kept on ice. Samples were collected from 8 endotoxin-treated carp and from 2 carp treated with PBS on Days 1, 2, 3, 5 and 8 after intubation.

Histology and histochemistry. For histological examination, 4 mm long gut samples were taken (n = 4 d^{-1}) 4 cm behind the pseudogaster and fixed in Bouin's solution. The samples were dehydrated and embedded in paraffin wax. Sections 5 µm thick were stained with haematoxylin-eosin (H&E), and with alcian blue 8GX pH 1 (AB1.0) at pH 2.5 (AB2.5), as well as with AB2.5/periodic acid-Schiff (AB-PAS) for the detection of mucin carbohydrates. The AB1.0 method stains sulphated glycoconjugates; the AB-PAS reaction visualises neutral and acidic glycoconjugates (Pearse 1972, Brooks et al. 1997, Table 1). In addition, terminal mucin sugar residues were characterized with various biotinylated lectins (10 µg ml^{-1} in 0.1 M PBS, pH 7.2, 30 min at room temperature; lectins and their sugar specificities are listed in Table 1). Lectin binding was visualised with peroxidase (PO), conjugated streptavidin and a diamino-benzidine-hydrogen peroxidase system (DAB, Biogenex, Super Sensitive System) according to the instructions of the manufacturer.

Isolation of HMGs. Intestines of carp (mean ± SE intestinal weight of 1.06 ± 0.2 g) were opened longitudinally and cut into small pieces of 3 to 4 mm. Subsequently, secreted luminal glycoproteins were isolated with isolation medium as described by Enss et al. (1996a,b). In brief, tissue pieces were incubated for 20 min in 100 ml isolation buffer containing antibiotics and protease inhibitors. The isolation buffer was collected, centrifuged for 30 min at 12 000 × *g*, and the supernatant was collected and frozen at –20°C until further processing. Epithelial glycoproteins were released from goblet cells by subsequent incubation of the tissue pieces in a buffer containing antibiotics, protease inhibitors and EDTA for 30 min (Enss et al. 1996a,b). Goblet cells were disrupted by means of an ultrasonic unit (Ultra Turrax T8, IKA-Werke). The suspension was centrifuged at 10 000 × *g* for 30 min, and the supernatant was collected and homogenised by gentle stirring. All samples were concentrated by ultrafiltration (Amicon; exclusion limit 30 000 Da) to a final volume of 2 ml. Concentrated mucus samples were subjected to downward gel filtration on a 34 × 0.9 cm Sepharose CL-4B column (Sigma; flow rate 5.2 ml h^{-1}, fraction size 1.3 ml, 40 fractions). For calibration, pig gastric mucin (PGM; molecular weight >2000 kDa), thyroglobulin (molecular weight = 670 kDa), ferritin (molecular weight = 450 kDa) and bovine serum albumin (BSA, molecular weight = 69 kDa) were used (Sigma). Aliquots of each fraction were

Table 1. *Cyprinus carpio*. Histochemical and lectin histochemical characterization of intestinal, intracellular goblet cell mucin carbohydrates by charge (PAS, AB-PAS, AB1.0, AB2.5) and sugars via lectin linkage (ConA, DBA, MAA, PNA, RCA, SNA, UEA I, WGA) after application of endotoxin (staining reaction: 1 = weak; 2 = weak to moderate; 3 = moderate; 4 = moderate to strong, 5 = strong reaction); ctr: control (application of phosphate buffered solution)

Acronym	Staining method	Binding specificity	Goblet cell histochemical staining intensity after endotoxin application					
			ctr	Day 1	Day 2	Day 3	Day 5	Day 8
Conventional histochemistry								
PAS	Periodic-acid Schiff	Neutral glycoproteins	3	2–3	1–2	1–2	2–3	2–3
AB-PAS	Alcian blue PAS	Neutral and acidic glycoproteins	3	2–3	3	2–3	3	3
AB1.0	Alcian blue pH 1.0	Sulphated glycoproteins	1–2	2	2	2	1–2	1–2
AB2.5	Alcian blue pH 2.5	Acidic glycoproteins	1–2	1–2	1–2	2	1–2	2
Lectin histochemistry								
ConA	*Canavalia ensiformis*	α-D-mannose	3	2–3	2–3	1–2	3	2–3
DBA	*Dolichos biflorus*	N-acetyl-α-D-galactosamine	2–3	3–4	3–4	3–4	4–5	3–4
MAA	*Maackia amurensis*	Neuraminic-acid-α-2-3-galactose	1–2	1–2	1–2	1	1–2	1–2
PNA	*Arachis hypogyea*	β-D-galactose	2–3	3	4	4–5	3	3
RCA	*Ricinus communis*	N-acetyl β-D-galactosamine	2–3	4–5	4–5	2–3	2–3	2–3
SNA	*Sambucus nigra*	Neuraminic-acid-α-2-6-galactose	2	2	2–3	2	2–3	2
UEA I	*Ulex europaeus*	Fucose-α1-2-galactose	1–2	1–2	1	1–2	1	1–2
WGA	*Triticum vulgaris*	N-acetyl β-D-glucosamine	1–2	1–2	1–2	2–3	2–3	1–2

determined for carbohydrate content by the PAS reaction (absorbance at 550 nm) and for protein content by the Bradford reaction (absorbance at 580 nm; BMG) (Dubois et al. 1956, Bradford 1976, Enss et al. 1992). Glycoprotein content was calculated (CGC) in mg HMG g^{-1} gut, with lyophilised pig gastric mucin as a standard via PAS reaction. Endotoxin contents of pooled mucin glycoprotein fractions were determinated with the *Limulus* test (Morita et al. 1978).

Determination of the terminal glycosylation pattern in mucin glycoproteins via lectin-binding assay. Aliquots of mucin glycoprotein fractions (200 µl) from 4 carp from each sampling day were incubated overnight at room temperature in 96-well microtiter plates (Nunc Maxisorb). Subsequently, nonspecific binding sites were blocked with 1% BSA in PBS, and then incubated with biotin labelled lectins (10 µg ml^{-1} in 0.1 M PBS) for 30 min at room temperature. The following lectins were used: *Concanavalia ensiformes* (Con A), *Dolichos biflorus* (DBA), *Ricinus communis* (RCA), *Sambucus nigra* (SNA) and *Ulex europaeus I* (UEA I) (see Table 1). Lectin binding was visualised by subsequent incubation with streptavidin-horseradish-peroxidase for 30 min at room temperature and orthophenyl-diamine (OPD) (DAKO Chemicals). After 15 min, the reaction was stopped by addition of 0.5 M sulphuric acid, and the optical density (OD) was read in a microplate reader (BMG) at 485 nm (Enss et al. 1995, 1996a,b). Carp from sampling Days 2 and 3 treated with PBS served as controls.

Statistics. Unless otherwise indicated, OD is expressed as median value and 25 to 75% quartiles. Analysis of variance (ANOVA) and Dunn's multiple comparison tests (treatments versus control group) were performed. Differences were considered significant at $p < 0.05$. For some sampling points, the data were not statistically significant because of individual variations in the carp used. Urlaub et al. (1998) as well as Enss et al. (1996a,b) also found high individual variations in rats and mice. In concurrence with these studies, if data were not statistically significant, they were used as indicators of trends in mucin composition (Table 2).

RESULTS

Carp specimens

All fish appeared healthy throughout the study, no clinical alterations were observed during maintenance.

Histology and histochemistry

The intestinal mucosa of control carp was formed by a continuous lining of columnar enterocytes. Goblet cells were located regularly in this epithelium between the enterocytes. Most goblet cells of controls had a rounded, thick appearance, and were filled with visible carbohydrate contents. Glycoprotein staining intensity varied from very weak to moderate, indicating the presence of neutral, acidic and sulphated glycoconjugates (Fig. 1, Table 1). Goblet cells responded in 2 different ways to AB 1.0 (staining of acid glycoconjugates). One population of cells was stained rather

Table 2. *Cyprinus carpio*. (a) Epithelial and (b) luminal glycoproteins isolated from intestinal goblet cells after administration of endotoxin. OD: optical density; CGC: calculated glycoprotein content; PI: Peak I, fractions with glycoproteins >2000 kDa; PII: Peak II, fractions with glycoproteins between 70 and 700 kDa; TA: transition area, fractions with molecules between 700 and 2000 kDa (see Figs. 1 & 2). ctr: control; *$p < 0.05$

	Glycoproteins of ctr carp (OD)		CGC (mg g^{-1} gut)	Content after application of endotoxin (% of control)					
	Median	Quartiles		ctr	Day 1	Day 2	Day 3	Day 5	Day 8
(a) Epithelial									
PI (fraction 7–10)									
Carbohydrate	1.19	0.82/1.56	0.25	100	80	97	150*	165*	161*
Protein	1.35	1.07/1.81		100	154*	103	167*	200*	93
TA (fraction 11–15)									
Carbohydrate	0.54	0.30/0.76	0.11	100	94	154*	163*	174*	167*
Protein	1.09	0.80/1.52		100	214*	126	177*	189*	139*
PII (fraction 16–22)									
Carbohydrate	0.43	0.08/ 0.61	0.09	100	128	207*	181*	293*	84
Protein	2.81	2.34/3.67		100	102	57*	50*	105	65*
Total (fraction 1–40)									
Carbohydrate	2.81	1.05/4.36	0.59	100	106*	163*	226*	223*	155*
Protein	7.52	5.23/10.53		100	138*	92*	108	146*	82
(b) Luminal									
PI (fraction 7–10)									
Carbohydrate	3.11	2.57/3.86	0.66	100	75	35*	60*	80*	84
Protein	1.48	0.98/2.03		100	105	77	95	117	98
TA (fraction 11–15)									
Carbohydrate	1.26	1.02/1.50	0.27	100	228*	79	118	168*	189*
Protein	3.84	2.62/5.02		100	159*	198*	117	106	66
PII (fraction 16–22)									
Carbohydrate	3.33	2.73/3.77	0.71	100	92	57*	68*	93	96
Protein	16.94	13.97/19.39		100	74*	73*	88*	69*	77*
Total (fraction 1–40)									
Carbohydrate	11.26	7.62/14.78	2.39	100	94	45*	69*	88*	117
Protein	33.01	24.81/41.38		100	91	88	91	74*	92

weakly and a second population showed a stronger staining reaction. Lectin staining of goblet cell content for specific carbohydrates in control fish produced weak reactions with UEA I indicating fucose, WGA indicating N-acetyl-β-glucosamine, and MAA indicating neuraminic-acid-α-2-3-galactose; there were generally weak to moderate reactions with DBA indicating N-acetyl-α-galactosamine, PNA indicating N-acetyl-β-1-3-galactosamine, SNA indicating neuraminic-acid-α-2-6-galactose, and RCA indicating N-acetyl-β-galactosamine, and a moderate reaction with Con A indicating mannose (Fig. 2, Table 1).

No significant changes in goblet cell number and general carbohydrate histochemistry were observed in response to endotoxin treatment. Goblet cell shape generally changed slightly to a smaller and narrower appearance than in the controls by Day 1 post application (p. appl.) of endotoxin. There were, additionally, slight alterations in the lectin-binding pattern of goblet cell content. Mucin glycoproteins had a stronger binding reaction with RCA (β-D-galactosamine) by Days 1 and 2, with PNA (β-D-galactose) by Days 2 and 3, with WGA (β-D-glucosamine) by Day 3, and with DBA (α-D-galactosamine) by Day 5 p. appl. Con A (mannose) had an increasing reaction by Days 2 and 5 p. appl., with a weaker binding by Day 3 p. appl. There was no cell infiltration into the tela submucosa throughout the study (Figs. 1 & 2).

Isolation of mucin glycoproteins/HMGs

We were able to isolate epithelial and luminal glycoproteins separately. When we monitored fractions from downward gel filtration for protein and carbohydrate contents, a biphasic elution profile was obtained (Figs. 3 & 4). Among epithelial and luminal mucin glycoproteins, large molecules with a molecular weight >2000 kDa were eluted in a first peak (PI), and molecules in the range of 70 to 700 kDa were eluted in a second peak (PII). Between these peaks, a transition area (TA) of fractions with a lower amount of glycoproteins was found. From the intestines of controls, epithelial glycoproteins were isolated at a concentration of 0.59 mg g^{-1} gut weight, and luminal glycoproteins at a concentration of 2.93 mg g^{-1} gut weight

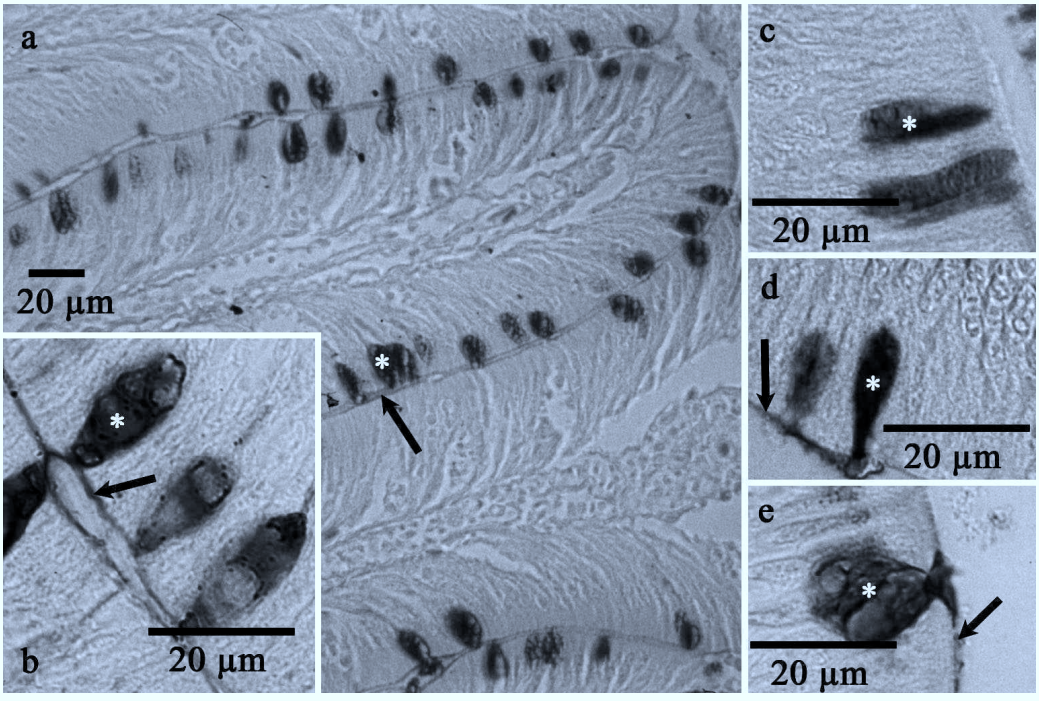

Fig. 1. *Cyprinus carpio*. General carbohydrate histochemical staining of the intestinal mucosa: (a) AB2.5 control, (b) AB-PAS control, (c) AB2.5 Day 1 post application, (d) AB1.0 Day 2 post application, (e) PAS Day 5 post application. Goblet cells (*) and mucus layer (arrows) covering the intestinal mucosa have positive, intense staining. See Table 1 for definition of stain acronyms

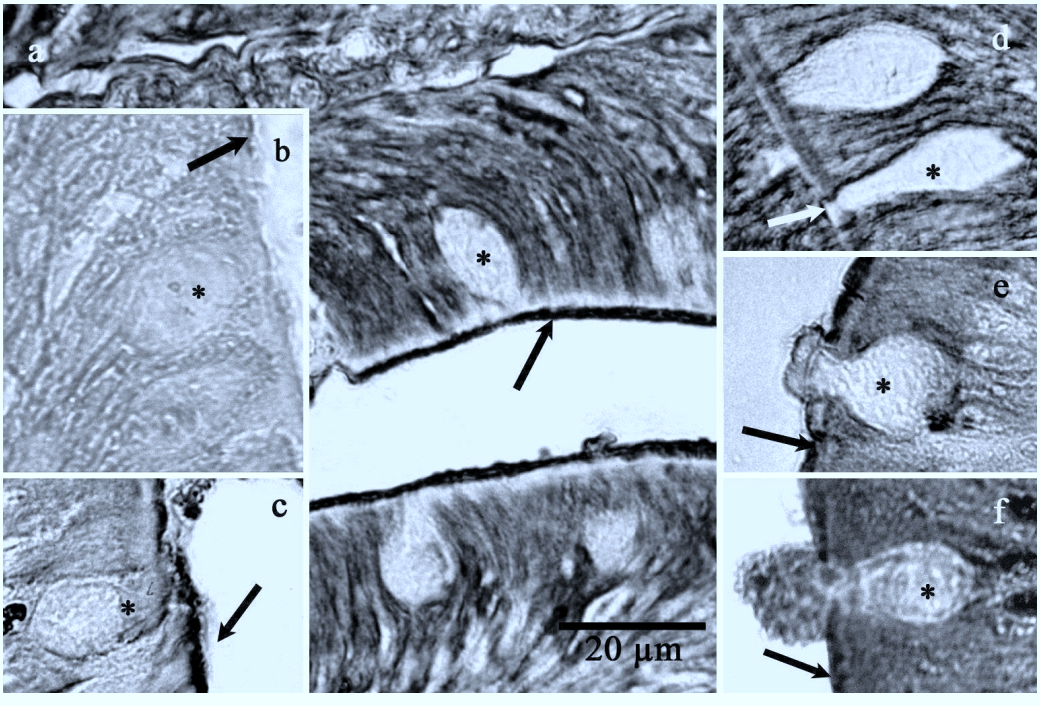

Fig. 2. *Cyprinus carpio*. Lectin histochemical staining of goblet cells, positive for the intestinal mucus layer (arrows). (a) DBA Day 3 post application, (b) PNA Day 2 post application, (c) RCA Day 2 post application, (d) UEA I Day 2 post applictation, (e) WGA Day 2 post application, (f) ConA Day 2 post application. Glycoprotein contents of goblet cells (*) showed a very weak (d) to moderate/strong (b,c) positive staining (see Table 1 for data and definitions of stain acronyms). All panels to the same scale as (a)

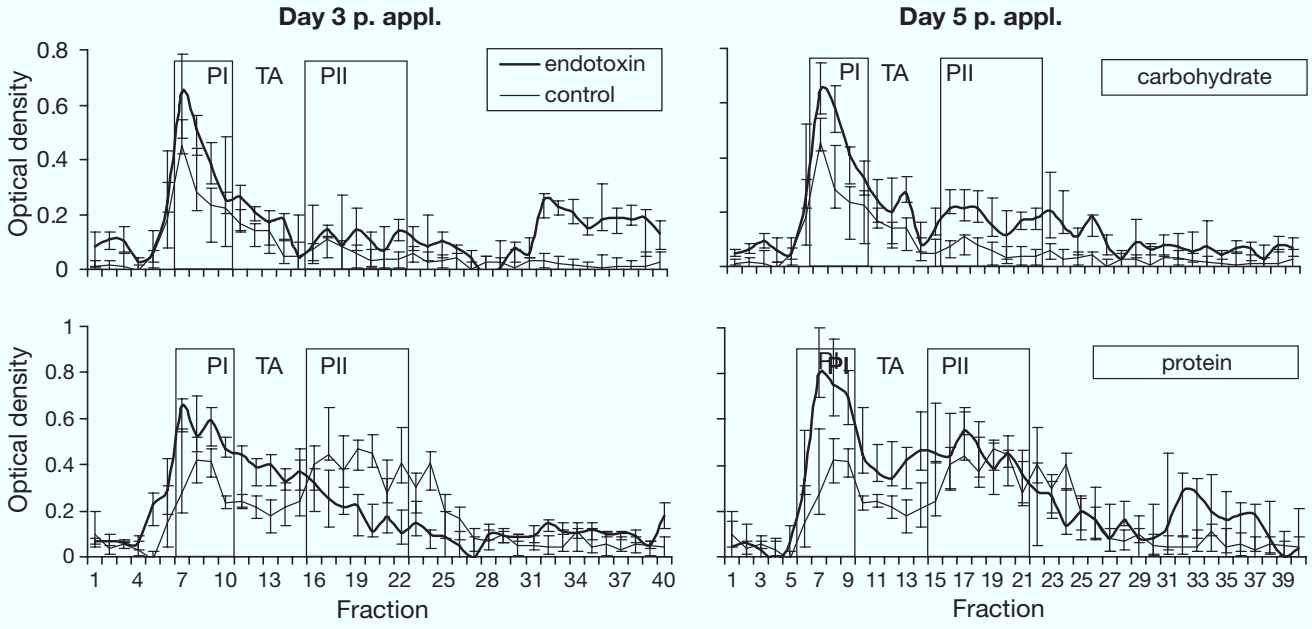

Fig. 3. *Cyprinus carpio*. Elution profiles of epithelial glycoproteins from the gut (g^{-1} gut weight) isolated on a Sepharose CL4B column (fraction volume: 1.3 ml) by Days 3 and 5 after a single, peroral application of endotoxin, as described in 'Materials and methods'. p.appl: post application. Carbohydrate content was monitored by periodic acid-Schiff reaction (PAS), and protein content by the Bradford-assay. Medians and 25th–75th percentiles of samples from 8 individuals are shown. PI: Peak I, glycoproteins exceeding 2000 kDa; PII: Peak II, elution fractions with glycoproteins between 70 and 700 kDa; TA: transition area, elution fractions with molecules between 700 and 2000 kDa (control = oral application of phosphate buffered solution, pool from all sample days)

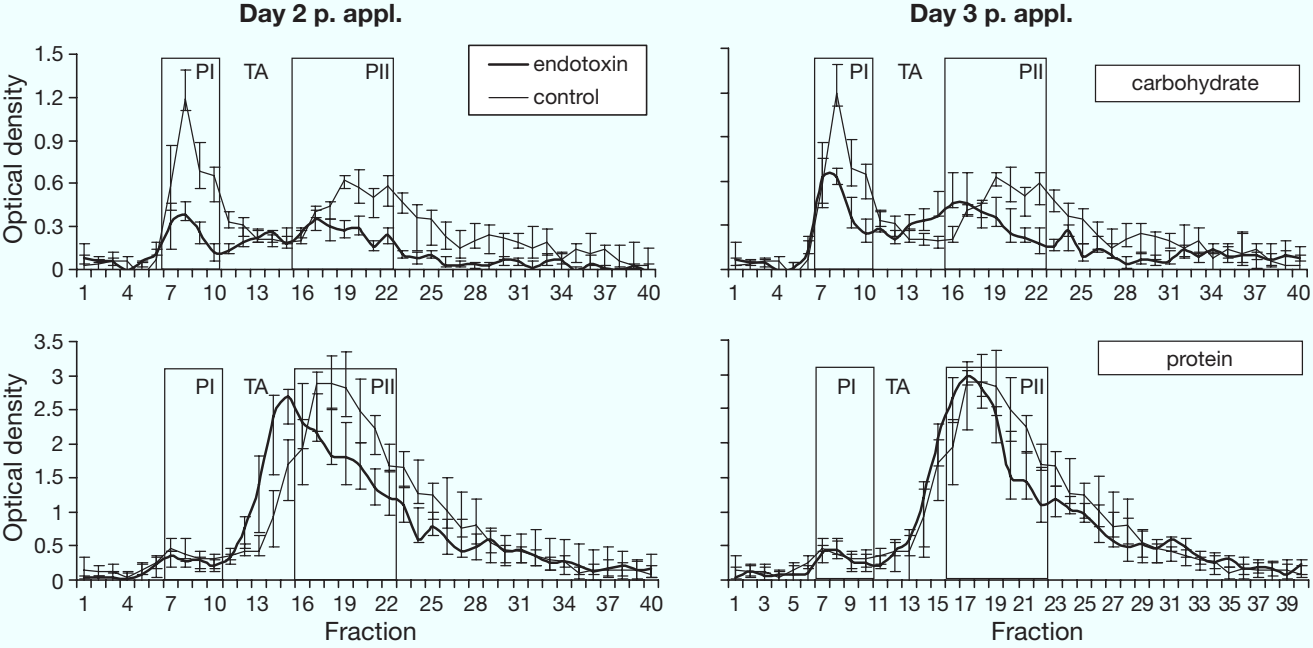

Fig. 4. *Cyprinus carpio*. Elution profiles of luminal glycoproteins from the gut (g^{-1} gut weight) isolated on a Sepharose CL4B column (fraction volume: 1.3 ml) by Days 2 and 3 after a single, peroral application of endotoxin as described in 'Materials and methods'. p.appl.: post application. Carbohydrate content was monitored by periodic acid-Schiff reaction (PAS), and protein content by the Bradford-assay. Medians and 25th–75th percentiles of samples from 8 individuals are shown. PI: Peak I, glycoproteins exceeding 2000 kDa; PII: Peak II, elution fractions with glycoproteins between 70 and 700 kDa; TA: transition area, elution fractions with molecules between 700 and 2000 kDa (control = oral application of phosphate buffered solution, pool from all sample days)

(Table 2). For epithelial mucin glycoproteins, the calculated concentration (mg g^{-1} gut weight: CGC) was 2.5 times higher in PI (0.25 mg g^{-1}) than in TA and PII (0.11 mg g^{-1} and 0.09 mg g^{-1}, respectively). For luminal mucin glycoproteins, 0.66 mg g^{-1} CGC was found in PI, 0.27 mg g^{-1} in PII and 0.71 mg g^{-1} in TA (Table 2b).

In response to endotoxin, the amounts of isolated epithelial mucin glycoproteins changed as follows. Calculated by carbohydrate content, epithelial glycoproteins increased by Day 3 and Day 5 in PI, TA and in PII ($p < 0.05$) (Table 2a). The relative OD of protein content also increased in PI and TA by Days 3 and 5 ($p < 0.05$). In contrast, the relative OD of PII molecules decreased by Day 3 ($p < 0.05$, Table 2a).

In luminal glycoproteins, carbohydrate content decreased in PI, PII ($p < 0.05$) and TA by Day 2. The protein content of luminal glycoproteins was reduced by Day 2 in PII ($p < 0.05$) and PI.

Using the *Limulus* test, we measured endotoxin content of pooled mucin glycoprotein fractions from PI. Endotoxin content increased from a mean concentration of 4.53 µg g^{-1} mucin in controls to 5.33 µg g^{-1} mucin (Day 1). Subsequently, endotoxin content decreased to 3.59 µg g^{-1} mucin (Day 2) and to 3.56 µg g^{-1} mucin (Day 3).

Terminal glycosylation pattern of isolated mucin glycoproteins

The following lectins bound to the isolated glycoprotein: ConA (specificity for α-D-mannose), DBA (specificity for N-acetyl-β-D-galactosamine), RCA (specificity for N-acetyl-α-D-galactosamine) and SNA (specificity for Neuraminic-β-2-6-galactose). UEA I (specificity for α-D-fucose) did not bind or had a weak binding reaction. The binding pattern of lectins applied to epithelial and luminal mucin glycoproteins is shown in Figs. 5 & 6.

As indicated by lectin binding, the glycosylation of separated glycoproteins changed by Days 1, 2 and 3 in response to endotoxin. For epithelial mucin glycoproteins, these changes were generally manifested in mucin glycoproteins from PI. By Day 1, the amount of β-galactosamine had increased, while sialic acid decreased in mucin glycoproteins from TA. By Day 2, mannose and sialic acid had increased in mucin glycoproteins from PI, and β-galactosamine increased by Day 3 (Figs. 5 & 6). It was only by Days 2 and 3 after endotoxin application that traces of fucose could be demonstrated in mucin glycoproteins of PI (data not shown).

For luminal glycoproteins, changes in glycosylation patterns generally appeared in molecules of PI and TA. By Day 1 after endotoxin application, the overall presence of mannose and sialic acid had decreased. In glycoproteins isolated on Day 2, mannose, sialic acid and β-galactosamine had increased, while by Day 3 the presence of all oligosaccharides analyzed had increased (Fig. 6). Glycoproteins containing fucose were not detected in PI or TA until Day 3 (in low quantities: 0.01 OD [0.01 – 0.02]; data not shown) after LPS application.

DISCUSSION

The intestinal tract of fishes is colonized by large numbers of bacteria (Trust & Sparrow 1974), and due to the ingestion of food particles covered in biofilm, there is a constant supply of additional microorganisms (Cahill 1990). Cells from the intestinal tissue are continually exposed to bacterial colonization. The mucus gel that covers the luminal surface of the intestine is thought to shield cells from noxes in gut contents (Neutra & Forstner 1987). Little is known about how the mucus layer of fishes responds to bacterial influx and prevents bacterial invasion of intestinal tissues.

In mammalian systems, infection of the gut by pathogenic bacteria has been mimicked by oral application of endotoxin from the cell wall of gram-negative bacteria. In fish, many pathogenic and enteric bacteria are gram-negative (Trust & Sparrow 1974, Jöborn et al. 1997, Bordas et al. 1998, O'Toole et al. 1999), which prompted us to apply endotoxin as a model substance. Because of poor knowledge on specific modifications of intestinal fish mucus, the aim of the present study was to describe reactions of intestinal mucosa and mucins of common carp to a single peroral application of endotoxin simulating strong bacterial colonisation.

Generally, intestinal and skin mucins of carp are similar to mucins of mammals. From the piscine intestinal mucosa, we isolated 2 different kinds of glycoproteins, viz. secreted glycoproteins from the surface of the intestinal epithelium (luminal glycoproteins) and epithelial glycoproteins stored in goblet cells. By gel filtration, a biphasic elution profile was obtained. Both preparations contained molecules >2000 kDa and between 70 and 700 kDa. Glycoproteins of high molecular weight were highly glycosylated, and the smaller glycoproteins carried less carbohydrate. A similar elution profile has been obtained in rats (Enss et al. 1996a,b).

Subsequent to secretion, mucin monomers form a polymer, which in the gut is a mixture comprising water, peptides, lipids and various serum and cellular macromolecules as well as indigenous bacteria (Neutra & Forstner 1987, Bansil et al. 1995). Mucin glycoproteins are believed to mimic cellular carbohydrate structures and thus entrap microbial pathogens (Karlsson et al. 1991). In rats and mice, an increased intestinal mucin secretion occurs upon a single application of endotoxin (Enss et al. 1996a,b). This enhanced mucus production was thought to expel pathogenic bacteria and contribute to a protection of the epithelium (Enss

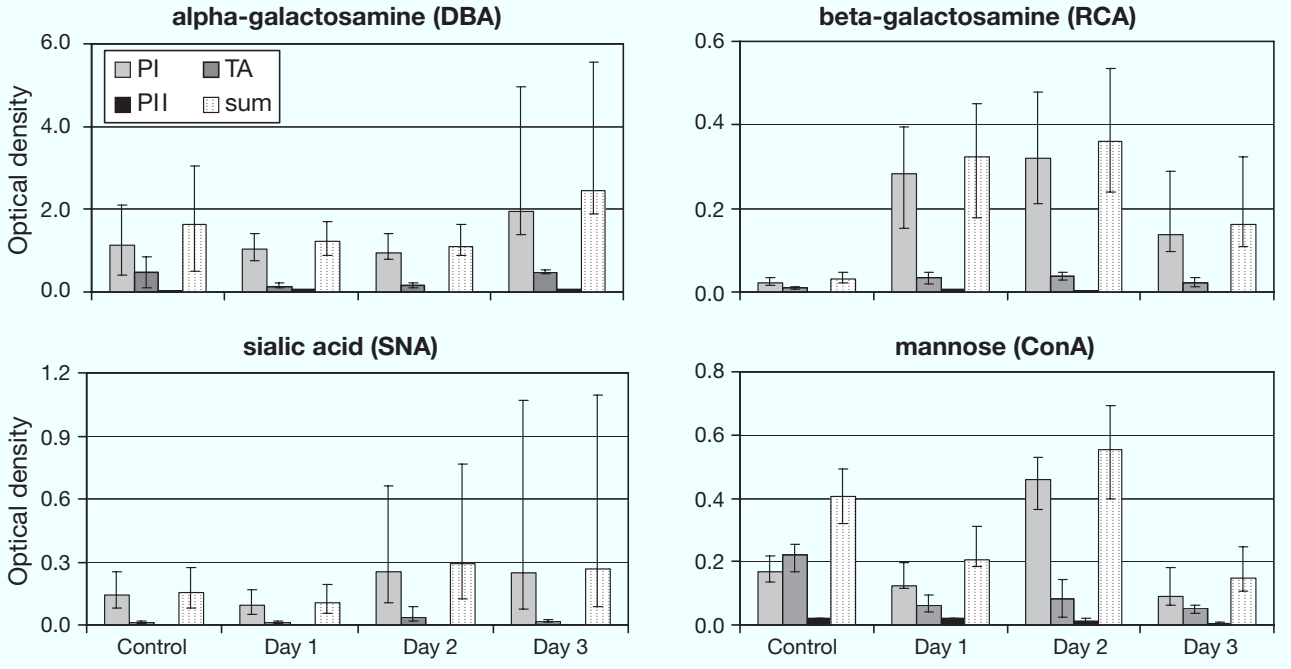

Fig. 5. *Cyprinus carpio*. Epithelial glycoproteins from the intestine. Semi-quantitative anaylsis of lectin binding to glycoproteins of control (pool from 2 fish by Days 2 and 3) and endotoxin-treated fish by Days 1, 2 and 3 after application of endotoxin. Binding specificity of lectins used: SNA, neuraminic acid; RCA, β-D-galactosamine; DBA, α-D-galactosamine; ConA, α-D-mannose. Lectins were applied to glycoproteins from the pooled fractions of Peak I (PI), TA (transition area), Peak II (PII) (displayed in this order and sum) from Figs. 1 & 2. Medians and 25th–75th percentiles of measurements of material from 4 carp are shown (control: oral application of phosphate buffered solution). See Table 1 for definitions of stain acronyms

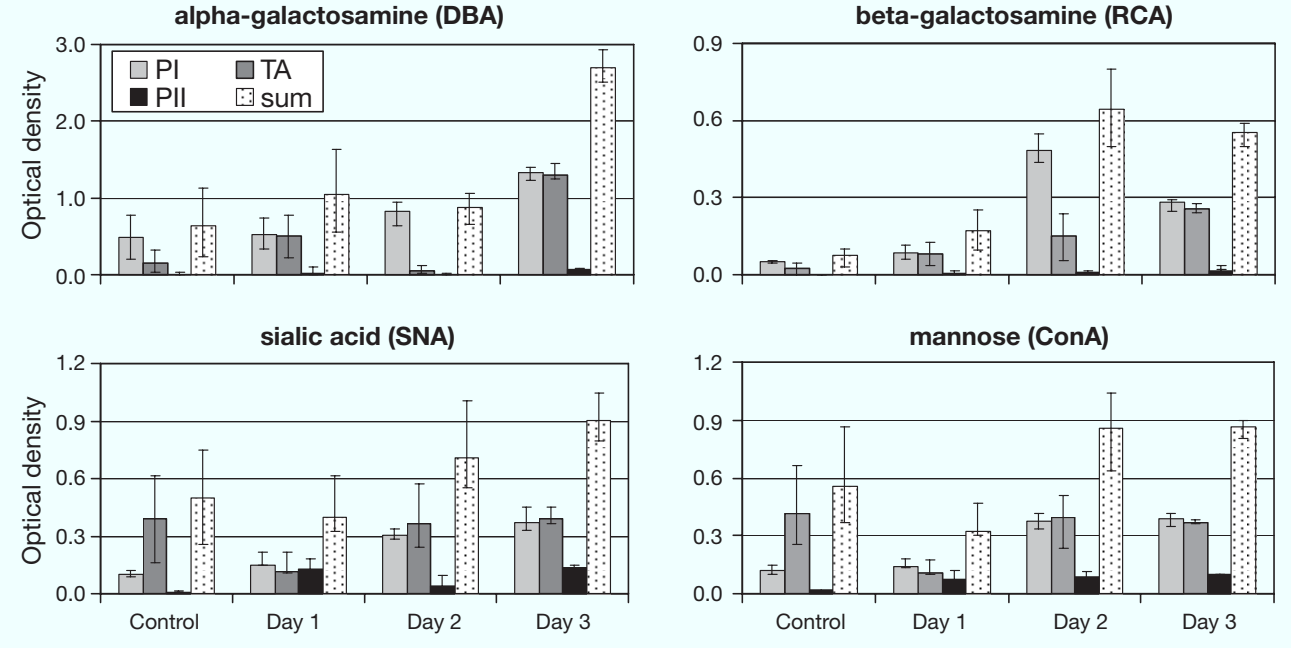

Fig. 6. *Cyprinus carpio*. Luminal glycoproteins from the intestine. Semi-quantitative anaylsis of lectin binding to glycoproteins of control (pool from 2 fish by Days 2 and 3) and endotoxin-treated fish by Days 1, 2 and 3 after application of endotoxin. Binding specificity of lectins used: SNA, neuraminic acid; RCA, β-D-galactosamine; DBA, α-D-galactosamine; ConA, α-D-mannose. Lectins were applied to glycoproteins from the pooled fractions of Peak I (PI), TA (transiition area), Peak II (PII) (displayed in this order and sum) from Figs. 1 & 2. Medians and 25th–75th percentile of measurements of material from 4 carp are shown (control: oral application of phosphate buffered solution). See Table 1 for definitions of stain acronyms

et al. 1995, 1996a,b). In mammalian species, endotoxin (as an integrated part of bacterial membranes) induces inflammatory reactions up to endotoxic shock level. Fish, however, are resistant to endotoxic shock as a result of differences in the cellular mechanisms of endotoxin recognition (Iliev et al. 2005). Thus, similar to many *in vitro* studies on leucocytes of different fish species, e.g. carp, extremely high concentrations of LPS (in comparison to mammals) are used to demonstrate probable effects on intestinal mucosa (Pelegrin et al. 2002, MacKenzie et al. 2003, Stafford et al. 2003, Zou et al. 2003, Hirono et al. 2004).

In the present study, the oral application of endotoxin to carp induced an enhanced secretion of mainly fully developed mucins (PI) by Day 1, which led to a substantial loss of luminal, adherent mucus by Day 2 (Fig. 4, Table 2b). Increased amounts of glycoproteins in fractions from the TA indicates a stimulated secretion of smaller, immature mucins. Tse & Chadee (1991) described an initial secretion of incompletely synthesized mucins, and postulated an incomplete glycosylation with a reduced amount of sialic-acid related to enteric infections. This view was supported by the results of the present study, which showed that by Day 1, epithelial and luminal mucin glycoproteins had a reduced content of sialic-acids (Figs. 5 & 6). During stepwise assembly of mucin oligosaccharides, terminally linked sialic-acids shield mucin glycoproteins from degradation by bacterial enzymes (Forstner & Forstner 1994, Forstner 1995, Aristoteli & Wilcox 2003, Schauer 2004).

In controls, a positive *Limulus* test seemed to indicate endotoxin integrated into the cell walls of the autochthonal flora. An increasing endotoxin content of mucins by Day 1 subsequent to oral application may be interpretated as an adhesion of endotoxin to fully developed mucin glycoproteins. Induced loss of mucin glycoproteins also led to a decreased amount of endotoxin (Days 2 and 3).

Endotoxin-stimulated secretion of intestinal glycoproteins in carp might also act as a first intestinal clearance mechanism for bacterial noxes. Subsequent to the luminal loss of mucin glycoproteins, epithelial mucin glycoprotein content increased by Days 3 and 5, indicating that an endotoxin stimulus may also induce a new biosynthesis of mucins.

Many intestinal parasite infections are associated with pseudofaeces, which in clinical studies are often regarded as a common sign for these intestinal infections (Wildgoose 2001). The loss of luminal mucin glycoproteins in response to endotoxin stimulus confirms the view that pseudofaeces may also be interpreted as a support/signal for the elimination of intestinal bacterial noxes. However, the massive expulsion of mucus glycoproteins results in a thinner mucus cover of the intestinal epithelium (Fig. 5, Day 2). This might facilitate an increased risk of bacterial invasion after bacterial dissemination in the gut. However, this thinner mucus layer might promote the uptake of oral vaccines. Thus, further studies should determine whether an endotoxin application prior to a delivery of an oral vaccine may reduce muco-adherence of vaccines and, in consequence, increase their absorption.

The glycosylation of piscine mucins takes place principally in 5 monosaccharides, fucose, sialic acid, mannose, N-acetyl-α-galactosamine and N-acetyl-β-galactosamine (Fletcher et al. 1976, Alexander & Ingram 1992, Shephard 1994). During the present study, most changes in glycosylation pattern were seen by Days 1 through 3 after application of endotoxin. In histochemical stainings of the gut sections, these changes were not very obvious, but they were evident in mucus samples examined by biochemical techniques. Therefore, biochemical methods seem to be more suitable for the characterization of intestinal glycoproteins than histochemical techniques. In agreement with the findings of Fiertak & Kilarski (2002), our study of carp mucus demonstrated (in general) no fucose, but large amounts of N-acetyl-α-galactosamine and N-acetyl-β-galactosamine (via DBA and RCA) as main terminal residues. However, by Day 3 after the stimulus, a faint positive lectin reaction for fucose was measured. General changes in the glycosylation pattern and the occurrence of fucose in intestinal mucins in response to endotoxin provided evidence for a second microbial clearance mechanism, viz. altered glycosylation can enhance bacterial adherence. This mechanism may be seen as an adaptive clearance and protection system. In contrast to mammalian systems, this microbial clearance system seems to be mediated mainly by local intestinal reactions and not by systemic responses of leucocytes. The endotoxin stimulus did not induce an obvious cell infiltration into gut tissue.

In summary, a peroral endotoxin stimulus altered the intestinal secretion and composition of mucin glycoproteins in carp, obviously linked to a simultaneous increase in their biosynthesis. The mucosal system seems to provide an adaptive removal mechanism for bacterial noxes by increased secretion of mucins with a modified glycosylation pattern. This may lead to altered bacterial adherence with a combined cleansing effect (Carlstedt et al. 1985, Karlsson et al. 1991, Bordas et al. 1998, Abraham et al. 1999, Moncada et al. 2003). Changes in mucin synthesis and secretion seem to be mediated primarily by local reactions in the gut and not by systemic leucocyte mechanisms. Consequently, the formation of pseudofaeces in fish (that in clinical diagnoses are a common indicator for intestinal parasitical infections) may also be interpreted as an indication of the presence of strong bacterial noxes.

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Appendix III

Changes of intestinal mucus glycoproteins after peroral application of *Aeromonas hydrophila* to common carp (*Cyprinus carpio*)

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Changes of intestinal mucus glycoproteins after peroral application of *Aeromonas hydrophila* to common carp (*Cyprinus carpio*)

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ABSTRACT

Mucus forms a biofilm that protects the underlying epithelium. Interactions between this mucus layer and bacteria are important in most infectious diseases. *Aeromonas hydrophila*, a widespread bacterium in the aquatic environment, is often isolated during disease outbreaks in fish. This study investigates biochemical properties of intestinal mucus glycoproteins of common carp, *Cyprinus carpio* L., in response to peroral application of two strains of *A. hydrophila* (strains 38 and 60).

Application of *A. hydrophila* altered intestinal secretion and composition of intestinal mucus glycoproteins in carp. Application of *A. hydrophila* 60 resulted in a significant increase in mucus glycoproteins. In contrast a striking reduction in the amount and molecular size of mucus glycoproteins was observed upon administration of *A. hydrophila* 38. Simultaneous changes in the glycosylation pattern of isolated glycoproteins were found.

The intestinal mucosal system is supposed to provide a removal mechanism for bacterial noxes by increased secretion of mucins inducing a washing out effect. Data from this study indicate that modulation of secretion and glycosylation depends on the applied *A. hydrophila* strain. Application of *A. hydrophila* 60 leads to an increased secretion of small mucus glycoproteins, which may lead to a flushing out of the bacteria and therefore to an elimination of bacterial noxes. Manifestation of the bacteria seemed to be prevented. In contrast the application of *A. hydrophila* 38 apparently destroys the mucus layer, leaving the intestinal mucosa insufficiently protected against further pathogens, which then have the capability to invade the underlying tissue and induce disease.

1. Introduction

One of the major routes of infection in fish is through the gastrointestinal tract (Ringo et al., 2007). An overwhelming number of infectious diseases are initiated by bacterial colonisation of mucosal surfaces (Abraham et al., 1999). The epithelium is protected from chemical, enzymatic, and mechanical damage by overlaying mucus. This mucus layer also plays an important role in host defence mechanisms. Mucus consists mostly of water and glycoproteins with a high molecular weight, called "mucins". Both are important in gel formation (Bansil et al., 1995; Cone, 1999; Perez-Vilar and Hill, 1999; Strous and Dekker, 1992; Verdugo, 1990). Mucus glycoproteins exhibit a high content of oligosaccharides and form a water-insoluble layer of adherent mucus on epithelial cells. The glycosylation of piscine mucins primarily occurs with five monosaccharides (fucose, mannose, N-acetyl-α-galactosamine, N-acetyl-β-galactosamine, and neuraminic acid) (Alexander and Ingram, 1992; Shephard, 1994). Oligosaccharides are believed to mediate the adhesion of microbial pathogens and to prevent glycoprotein degradation by proteases of microbial origin (Abraham et al., 1999; Ascencio et al., 1998; Mantle and Husar, 1993, 1994; Sajjan et al., 1992; Sanford et al., 1989).

Aeromonas spp. are Gram-negative bacteria, which are widespread in aquatic environments (Khushiramani et al., 2008; Rodriguez et al., 2008) and is commonly found in both fresh and salt water (Austin and Austin, 2007; Cipriano, 2001; Doukas et al., 1998; Rodriguez et al., 2008; Sahoo et al., 2008) and is also part of the normal intestinal microflora of healthy fish (Trust and Sparrow, 1974). The bacterium is considered to be an opportunistic agent, which does not cause problems in conditionally healthy fish (Geiger, 2001). It is known that differences in pathogenicity between *Aeromonas hydrophila* strains exist. Bacterial virulence is determined by a complex array of bacterial traits that allow pathogenic bacteria to cause a disease. These traits have been examined for different isolates of *A. hydrophila* including chemotactic behaviour (Hazen et al., 1982; Van der Marel et al., 2008), growth, adhesion (Van der Marel et al., 2008) and injection of anti-host virulence determinants into the host via a type III secretion system (TTSS) (Froquet et al., 2007; Tan et al., 2008; Yu et al., 2004).

Little is known about the response of piscine intestinal glycoproteins to enteral bacterial infections. Oral application of endotoxin (LPS, lipopolysaccharide from *E. coli* O55:B5) leads to a change in intestinal mucus glycoprotein composition (Neuhaus et al., 2007). In the present study, an enteral bacterial infection was mimicked by oral application of two strains of live *A. hydrophila* to common carp. Responses of intestinal mucus glycoproteins were examined by biochemical analysis.

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2. Materials and methods

2.1. Animals

Parasite and virus free sibling common carp (*Cyprinus carpio* L.) (n=96) from a single crossing (R8S8×R3S8, Wageningen Agricultural University, Wageningen, The Netherlands) were used. Carp were raised and kept in filtered recirculated tap water. Approximately 4 year old carp with a mean body weight±S.E. of 81.8 g±19.9 g and a mean standard length±S.E. of 14.1 cm±1.1 cm were used. Carp were placed in 100-l tanks with filtered tap water three days before bacterial application, and were starved to reduce faeces on intestinal mucus. For sampling, fish were killed by bath immersion with 500 mg l^{-1} tricaine methane sulfonate (Sigma, Munich, Germany). Subsequently, fish were dissected and the entire intestinal tract from pseudogaster to anus was removed, weighed and cooled on ice for mucus isolation.

2.2. Bacteria

Two strains of motile Aeromonads were used, that were isolated from diseased commercially farmed carp, examined by standard biochemical diagnostic methods and classified as *A. hydrophila*. *A. hydrophila* strain 38 was a gift from the Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Germany. *A. hydrophila* strain 60 was a gift from the Department of Veterinary Pathobiology, Royal Veterinary and Agricultural University Copenhagen, Denmark (Nielsen et al., 2001). Fish which were incubated with 10^8 CFU of *A. hydrophila* 38 died, whereas fish incubated with 10^8 CFU of *A. hydrophila* 60 did not (unpublished results).

Bacteria were routinely grown on sheep blood agar at 25 °C and stored in veal infusion medium at 10^9 bacteria ml^{-1} at −80 °C. To remove all possible nutrients, bacterial cells were harvested by centrifugation at 10,500 ×g for 10 min and the supernatant was removed. Bacteria were resuspended in physiological salt solution.

2.3. Infection experiment

To infect fish physiological salt solution (control fish) or one of the two *A. hydrophila* strains (*A. hydrophila* 38 and 60) was perorally administered. All fish were intubated with a total volume of 300 µl containing 10^7 CFU bacteria. Fish were kept at 20 °C. Samples were taken on days one, three and six after oral intubation. Per day and treatment eight fish were examined. Intestines between pseudogaster and anus with a mean weight±S.E. of 0.98 g±0.26 g were dissected out and used for mucus isolation. Furthermore, swabs were taken of liver kidney and spleen for microbiological examination. Method and results of this examination have been described previously (Van der Marel et al., 2008).

2.4. Mucus isolation

Intestines were opened longitudinally and cut into small pieces of 3–4 mm. Subsequently, secreted luminal mucus was isolated with isolation medium. Tissue pieces were incubated for 20 min in 100 ml isolation buffer containing protease inhibitors (PBS with 1% dithiothreitol, 0.03% Amphotericin B, 1% sodiumpyruvate and 0.6% Hepes). Gut tissue was removed from the isolation buffer and the isolation buffer was centrifuged for 30 min at 12,000 ×g. Subsequently, the supernatant was collected and frozen at −20 °C until further processing. All samples were concentrated by ultrafiltration (Amicon, Beverly, MA/USA, exclusion limit 30 kDa) to a final volume of 2 ml. Concentrated mucus samples were subjected to downward gel filtration on a Sepharose CL-4B-column (Sigma, Munich, Germany, flow rate 5.2 ml h^{-1}, fraction volume 1.3 ml, 40 fractions). For calibration, pig gastric mucin (molecular weight more than 2000 kDa), thyroglobulin (molecular weight 670 kDa) and bovine serum albumin (molecular weight 69 kDa) were used (Sigma, Munich, Germany). Aliquots of each fraction were used for carbohydrate content determination by periodic-acid–Schiff (Peppas and Huang, 2004) reaction (absorbance at 550 nm) (Dubois et al., 1956; Mantle and Allen, 1978) and for protein content determination by Bradford reaction (absorbance at 580 nm) (Bradford, 1976). Results were expressed per mg gut weight. Carbohydrate content of 2.5 mg ml^{-1} lyophilised pig gastric mucin (PGM) was measured via PAS reaction and used as a standard to calculate glycoprotein content (CGC) (OD fraction/OD PGM). Results are expressed as mean value and standard deviation. Live and dead bacteria were also centrifuged in isolation medium and concentrated to examine any possible influences on the measurement of carbohydrate content. Both live and dead bacteria were not detectable by periodic-acid–Schiff reaction.

2.5. Terminal glycosylation

Terminal glycosylation pattern of mucus glycoproteins was determined via a lectin-binding assay. Aliquots of mucus glycoprotein fractions (200 µl) were incubated overnight at room temperature in 96-well-microtiter plates (Nunc Maxisorb, Wiesbaden, Germany). Subsequently, they were blocked with 1% BSA in phosphate buffered saline (PBS), and then incubated with biotin labelled lectins (10 µg ml^{-1} in 0.1 M PBS) for 30 min at room temperature. The following lectins were used: *Canavalia ensiformes* (ConA, binds to α-man and α-D-glc), *Dolchios biflorus* (DBA, binds to α-galNAc), *Ricinus communis* I (RCA-1, binds to galNAc and β-gal), *Sambucus nigra* (SNA, binds to α-NeuNAc(2→6) gal) and *Ulex europaeus* I (UEA I, binds to α-L-fuc). Lectin binding was visualised by subsequent incubation with streptavidin–horseradish-peroxidase and orthophenyl-diamine (DAKO chemicals, Glostrup, Denmark) for 30 min at room temperature. After 15 min, the reaction was stopped by addition of 0.5 M sulfuric acid, and the optical density was read in a microplate reader (BMG, Offenburg, Germany) at 485 nm (Enss et al., 1995; Enss et al., 1996a; Enss et al., 1996b). Results are expressed as median value and quartiles.

2.6. Cytotoxicity in vitro

A cytotoxicity assay was performed to recognize possible differences in the toxicity of the two *A. hydrophila* strains used. Therefore an established monolayer-cultured fish cell line from carp epithelium, *Epithelioma papulosum cyprini*-cells (EPC-cells), was used. The cells were maintained in Eagle's minimal essential medium supplemented with 10% foetal calf serum, 200 IU ml^{-1} penicillin, 0.2 mg ml^{-1} streptomycin and 7.96 mg ml^{-1} non-essential amino acids. Cultures were maintained in a refrigerated incubator at 20 °C under normoxic atmosphere. Cells were detached for subcultivation using a trypsin solution (0.2% EDTA, 0.5% trypsin) in Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate buffered saline. EPC-cells were incubated with 10^7 CFU *A. hydrophila* in 24-well microtiter plates for 2, 6 and 24 h. After incubation the cell layer was examined microscopically.

2.7. Statistics

Data of mucus isolation and of lectin-binding assay were statistically analysed with a Kruskal Wallis Test. Differences between treatment groups were considered as significantly at a probability of error at $P<0.05$.

3. Results

3.1. Mucus glycoproteins

When monitoring fractions from downward gel filtration, a biphasic elution profile was obtained for both, protein and carbohydrate contents

(Fig. 1). Large molecules with a molecular weight >2000 kDa were eluted in a first peak (PI, fraction 5–10) and molecules in the range of 30–670 kDa in a second peak (PII, fraction 14–25). Between these peaks a transition area (TA) of fractions with a lower amount of glycoproteins and molecules in the range of 670–2000 kDa was found.

Mucus glycoproteins were isolated at a concentration of 1.92± 0.43 mg g^{-1} gut weight (day three after application) to 2.65± 0.51 mg g^{-1} gut weight (day six after application) from the gut of controls (Table 1). The amount of mucus glycoproteins, which could be isolated, changed in response to *A. hydrophila* 38 and 60 when compared to the control (Table 1). The calculated glycoprotein content decreased significantly on days one, three and six after application of *A. hydrophila* 38 in TA and PII ($P<0.05$). The total calculated glycoprotein content had decreased significantly ($P<0.05$) on days one and six after application. On day one after application the second peak in the elution profile was completely missing (Fig. 1). On day three the elution profile showed neither a first nor a second peak and on day six the normal biphasic elution profile could be seen (Fig. 1).

After application of *A. hydrophila* 60 calculated glycoprotein content had significantly increased on days one, three and six after application in PII ($P<0.05$) and on day three after application also in TA (Table 1). The total calculated glycoprotein content had increased significantly ($P<0.05$) on day three after application. On day six after application of *A. hydrophila* 60 the calculated glycoprotein content in total had decreased and reached the level of controls.

3.2. Lectin-binding assay

The following lectins bound strongly to the isolated glycoproteins: ConA (specificity for α-D-mannose), DBA (specificity for N-acetyl-β-D-galactosamine) and RCA (specificity for N-acetyl-α-D-galactosamine). SNA (specificity for Neu-β-2-6-galactose) and UEA (specificity for α-D-fucose) showed a weak binding reaction. Overall, the binding characteristics of lectins to unseparated glycoproteins as well as to mucus glycoproteins separated by downward gel electrophoresis varied widely. Mannose was predominantly found in molecules with a molecular size in a range of 30–670 kDa, while the terminal presence of other glucans, as indicated by lectin binding, was highly variable. Upon administration of the bacteria, increased glycosylation of intestinal glycoproteins with fucose and neuraminic acid could be observed. As indicated by UEA binding the content of fucose in glycoproteins had already increased one day after bacterial administration. This increase was statistically significant ($P<0.05$) in carp treated with *A. hydrophila* 60. After application of *A. hydrophila* 38 a reinforced glycosylation of intestinal mucus with fucose and neuraminic acid was evident three days post application ($P<0.05$, see Fig. 2).

3.3. In vitro cytotoxicity of bacteria strains

When monolayers of EPC-cells were incubated with *A. hydrophila* 60 or with physiological salt solution, no changes in cell morphology or monolayer coherence could be observed over a period of 24 h. EPC-monolayers incubated with *A. hydrophila* 38 were still coherent after

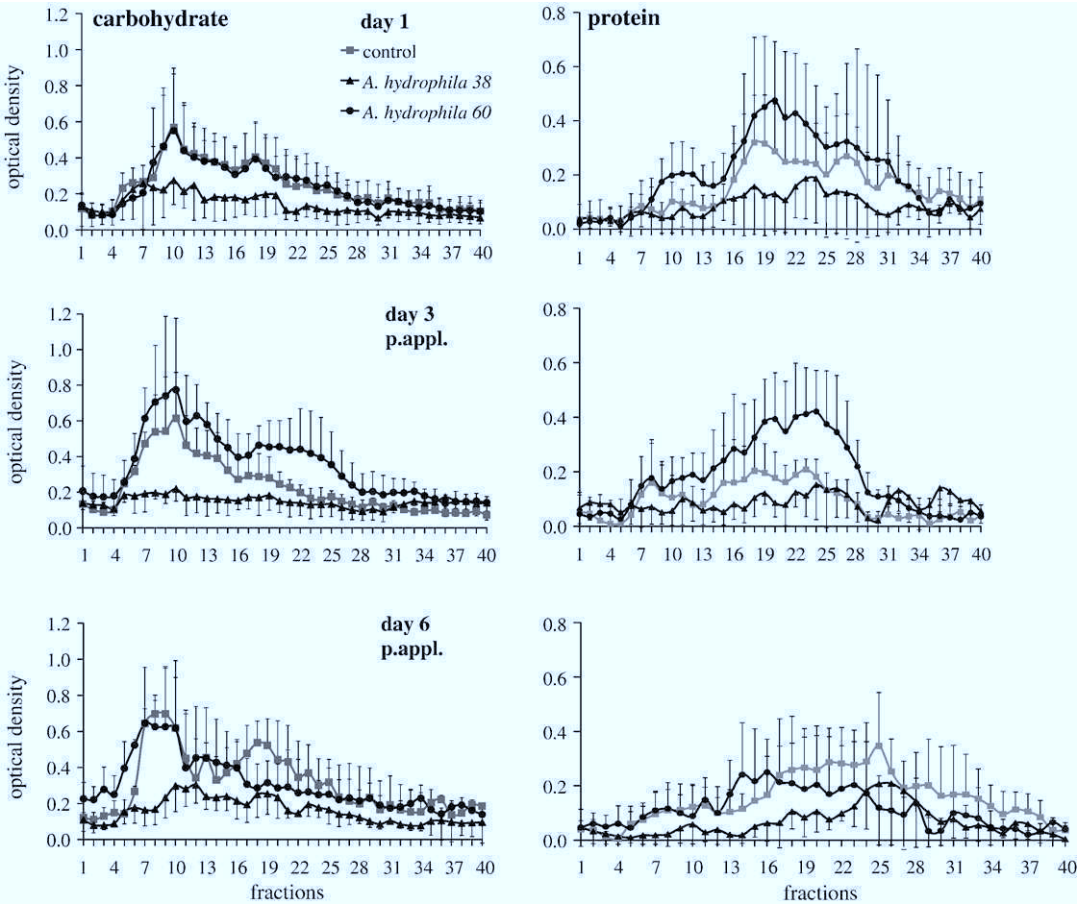

Fig. 1. Carbohydrate and protein contents of luminal gut mucus glycoproteins. Profiles of the carbohydrate and protein content of luminal gut mucus glycoproteins (g^{-1} gut weight) eluted on a sepharose CL-4-B column on days 1, 3 and 6 post application (p.appl.) of physiological salt solution (0.9%), *A. hydrophila* 38 and *A. hydrophila* 60. Carbohydrate content was monitored by periodic acid–Schiff reaction (PAS), and protein content by Bradford-assay. Mean values and standard deviations of samples from 8 individuals are shown.

Table 1
Mucus glycoproteins from intestines of carp after administration of *A. hydrophila* 38 and 60 and physiological salt solution (control)

Molecular weight (kDa)		Glycoproteins of control carp (OD) mean value (standard deviation)	CGC (mg g^{-1} gut)	Content (% of controls) after application of	
				A. hydrophila 38	*A. hydrophila* 60
Day 1					
>2000	Carbohydrate	2.08 (0.94)	0.44	66	92
	Protein	0.41 (0.32)		73	146
670–2000	Carbohydrate	1.27 (0.44)	0.27	50*	96
	Protein	0.26 (0.15)		65	219
30–670	Carbohydrate	3.74 (1.68)	0.79	49*	100
	Protein	2.73 (1.20)		58	152*
Total	Carbohydrate	9.68 (3.95)	2.05	58*	97
	Protein	5.97 (1.39)		58*	135
Day 3					
>2000	Carbohydrate	2.73 (0.94)	0.58	42	127
	Protein	0.55 (0.41)		67	133
670–2000	Carbohydrate	1.29 (0.39)	0.27	40*	140*
	Protein	0.28 (0.17)		86	193
30–670	Carbohydrate	3.01 (0.85)	0.64	60*	172*
	Protein	2.05 (0.68)		58	199*
Total	Carbohydrate	9.04 (2.01)	1.92	66	155*
	Protein	3.71 (1.34)		93	192*
Day 6					
>2000	Carbohydrate	3.07 (2.75)	0.65	39	112
	Protein	0.58 (0.27)		29	95
670–2000	Carbohydrate	1.25 (0.61)	0.27	66*	104
	Protein	0.33 (0.18)		27*	127
30–670	Carbohydrate	4.87 (2.75)	1.03	51*	77*
	Protein	2.93 (0.92)		40*	81
Total	Carbohydrate	12.48 (2.41)	2.65	51*	99
	Protein	6.04 (1.07)		45*	75

OD: optical density; CGC: calculated glycoprotein content.
* $P<0.05$.

2 h, but some EPC-cells were detached from the monolayer after 6 h of incubation. After 24 h the EPC-monolayer had detached completely from the bottom of the culture vessel.

4. Discussion

A. hydrophila is considered to be a facultative pathogen, since it does not lead to disease symptoms in conditionally fit fish (Geiger, 2001), but can lead to clinical symptoms in stressed fish. This is in compliance with the present study where no severe, but only mild clinical symptoms occurred in healthy, parasite and virus free carp which were kept in well filtered tap water.

In the present study cytotoxicity of two *A. hydrophila* strains was examined. *A. hydrophila* 38 but not *A. hydrophila* 60 induced a lysis of EPC-cells. In former studies with *A. hydrophila* and EPC-cells only virulent, but no avirulent strains of the bacterium lead to cytotoxic effects (Leung et al., 1996). Recent experiments have shown that TTSS is required for *A. hydrophila* pathogenesis (Yu et al., 2004). The ability to lyse EPC-cells might therefore indicate the presence of TTSS in *A. hydrophila* 38. In order to become pathogenic, *A. hydrophila* must overcome the mucosal layer of the skin or the intestine.

In several studies it has been found that the composition and the amount of secreted mucus is changed by bacterial noxes and by oral application of LPS from the cell membrane of gram negative bacteria in rats (Enss et al., 1996b), mice (Urlaub, 1998) and carp (Neuhaus et al., 2007). In rats also the colonic wall permeability can be modified by commensal bacteria (Garcia-Lafuente et al., 2001). In the present study oral application of *A. hydrophila* caused a modulation of the amount and composition of the intestinal mucus layer. The two different *A. hydrophila* strains induced different changes in the mucus cover of the intestinal epithelium.

After the application of *A. hydrophila* 60 an increase in the total amount of mucus glycoproteins and in particular a higher amount of smaller molecules could be observed. This suggests that mature as well as immature mucus molecules were discharged from the goblet cells. These observations correspond to the increased amount of secreted mucus found in humans (Leiper et al., 2001). Also peroral application of lipopolysaccharides (LPS) of bacteria can lead to an increased gut mucus secretion (Neuhaus et al., 2007). An increased mucus secretion is considered to cause a flushing out of invading bacteria along with the mucus of the gut lumen and can therefore be understood as an effective defence mechanism of the host (Smirnova et al., 2003). This mechanism most likely prevents clinical infections in many cases. This is in accordance with the present microbiological examination, where only low numbers of bacteria and not *A. hydrophila* could be isolated from organs from fish treated with *A. hydrophila* 60. It is most likely that mucus and applicated bacteria were flushed out. Overall, the intestinal mucosa reacted in a similar way after application of *A. hydrophila* 60 or LPS. In fish, responses to LPS are not as intense as they are in mammals (Iliev et al., 2005).

After application of *A. hydrophila* 38 there was a considerable decrease in the total amount of mucus. Three days after application there

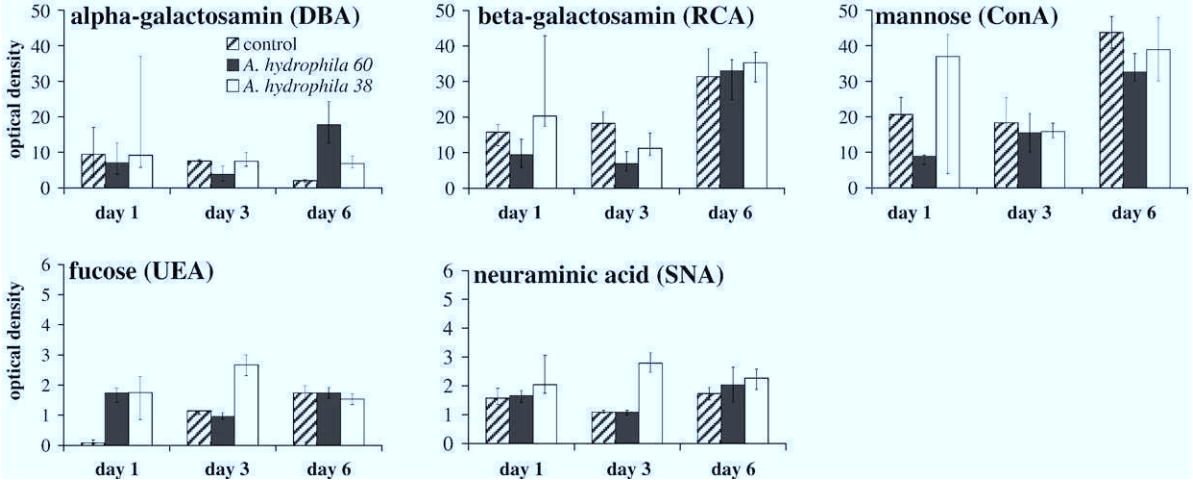

Fig. 2. Intestinal mucus glycoproteins. Semi-quantitative analysis of lectin binding to mucus glycoproteins of fish treated with *A. hydrophila* 38 or *A. hydrophila* 60 and control fish (treated with physiological salt solution) on days 1, 3 and 6 after application. Binding specificity of lectins used: DBA: α-D-galactosamine; RCA: β-D-galactosamine; ConA: α-D-mannose; UEA: fucose; SNA: neuraminic acid. Lectins were applied to glycoproteins with a molecular size between 70 kDa and >2000 kDa. Medians and 25th–75th percentiles of measurements of material from 4 carp are shown.

was almost no mucus left in the gut lumen. In particular there was a considerable reduction of large glycoproteins with a molecular size over 2000 kDa and of molecules with a molecular size between 670 and 2000 kDa. Since only high molecular size molecules are able to perpetuate the viscosity of mucus and to protect the epithelium against noxes (Bansil et al., 1995; Perez-Vilar and Hill, 1999), it can be assumed that the gut epithelium was not adequately protected against pathogens. This assumption is supported by the microbiological examination of internal organs. Bacteria were re-isolated more often from fish treated with *A. hydrophila* 38 than from fish treated with *A. hydrophila* 60. The re-isolated bacteria were either part of the normal gut microflora or ubiquitous in water (Van der Marel et al., 2008).

It can be assumed that the intestinal barrier was damaged in carp where bacteria were re-isolated and that *A. hydrophila* 38 is more capable of destabilizing the intestinal barrier than *A. hydrophila* 60. The reduced amount of mucus in the gut lumen after application of *A. hydrophila* 38 can be caused by either a massive loss of intestinal mucus or by mucus degradation. It is however unclear, if goblet cells massively released mucus during the first 24 h after application of *A. hydrophila* 38 or if the bacteria inhibited the secretion of mucus from the goblet cells. The examination of different strains of *Yersinia enterocolitica* showed that virulent strains are able to degrade intestinal glycoproteins whereas non virulent strains are not (Mantle and Rombough, 1993). Both *A. hydrophila* strains, especially strain 38, are able to grow in media enriched with intestinal glycoproteins and can use these glycoproteins as carbon and nitrogen source (Van der Marel et al., 2008). It is therefore probable that *A. hydrophila* 38 is able to degrade mucus glycoproteins thereby destroying the mucus layer.

Besides a modulation of mucin molecular size a modulation of the mucin glycosylation has been found in carp after application of LPS (Neuhaus et al., 2007). In the present study, a modulated terminal mucin glycosylation could also be observed. Most pronounced changes were seen by day six after application of *A. hydrophila*. Especially the amount of mannose and N-acetyl-β-galactosamine had increased on day six. The amount of mannose in mammalian mucus is low in high molecular weight glycoproteins, but higher in low molecular weight glycoproteins (Szentkuti and Enss, 1998). This is in compliance with the low amount of mannose found for molecules with a molecular weight of >2000 kDa and the high amount found for molecules with a molecular weight of 30–670 kDa. An increased amount of mannose and N-acetyl-β-galactosamine indicates an increased amount of not fully matured glycoproteins.

In addition, mucus glycoproteins from bacteria treated carp had an increased neuraminic acid terminal glycosylation. An increased amount of neuraminic acid in secreted mucus was reported in previous studies after an irritation of the mucosa (Enss et al., 1992) and in patients which suffered from inflammatory bowel disease (Rhodes, 1997). In the present study only low amounts of neuraminic acid could be measured in the mucus from untreated carp. Three days after application of *A. hydrophila* the amount of neuraminic acid in mucus had significantly increased in carp treated with *A. hydrophila* 60 compared to carp treated with *A. hydrophila* 38. Therefore, *A. hydrophila* 38 seems to irritate the mucosa to a greater extent than *A. hydrophila* 60. In mammals, fucose content of intestinal mucus or anal gland mucus, may vary to a great extent but may be especially important for the viscoelasticity of the mucus (Tsukise et al., 2000).

Fucose could only be measured in very low amounts in control carp. One day after bacterial application fucose could be detected in mucus from treated carp, especially those treated with *A. hydrophila* 38, but still in low amounts in control carp. After application of LPS, similar changes could be observed (Neuhaus et al., 2007). Fucose-glycosylated glycoproteins may act as a trap for bacteria (Lu and Walker, 2001). Bacteria can adhere to these fucose-glycosylated glycoproteins and can be washed off the gut lumen. Thus, the increased amount of fucose can be interpreted as a defence mechanism against the bacteria.

In summary, this study shows that *A. hydrophila* can modulate intestinal secretion and composition of mucus glycoproteins in carp. Mucus of these carp consisted of a higher amount of small molecules, which suggested an increased secretion of newly synthesised, immature glycoproteins. The mucosal system seems to provide an adaptive removal mechanism for bacterial noxes by secretion of mucins of different molecular size with a modified glycosylation pattern. There are indications that the diverse responses of the mucosa depend on the applied *A. hydrophila* strain. *A. hydrophila* 60 apparently could be eliminated very quickly from the intestinal tract due to an increased mucus secretion. *A. hydrophila* 38 seemed to destroy or lead to a destruction of the mucus layer, leaving the intestinal mucosa insufficiently protected for further pathogens.

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