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Short communication

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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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.

1. 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 [1]. 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 [2]. Infections occur only when a pathogenic organism can colonise and/or invade mucosal barriers [3]. 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 [4,5]. Mucins give mucus its viscous properties and form a matrix in which a diverse range of antimicrobial molecules can be found [6].

Based on biochemical characterisation, 19 genes are currently assigned to the mucin family [see 7,8] and are named "*MUC*-number" for humans or "*Muc*-number" for other species [5]. While in mammals the structure of mucin type genes and their critical role in the infection process in the gastrointestinal tract [9] or airways [10] 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 [6]. Fish mucus contains antimicrobial peptides such as piscidins [11] and defensins [5]. Defensins are most effective in killing microbes by compromising cell membrane integrity [12,13]. Antimicrobial activity of defensins has been documented in mammals (review by Selsted and Ouellette [14]) as well as in fish [15]. Homologues to *β-defensin 1* (*BD1*) and *β-defensin 2* (*BD2*) were recently identified from *in silico* studies in several fish species [16,17].

Despite the physical barrier function of mucus and their bioactive substances, protection of fish against infectious diseases is

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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 [18,19]. For many fish species, the immune-modulatory activity of β-glucan has been reported [20–25]. Recent preliminary research data indicates that β-glucan promotes an antimicrobial response [26]. Furthermore, β-glucans can potentially affect mucin structure and/or function as they interact with innate signalling 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.

2. Materials and methods

2.1. 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 [27,28] 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.

2.2. 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.

2.3. Amplification and sequencing of carp mucin and β-defensin genes

For the secreted mucin genes *Muc2* and *Muc5B* 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 [29]. Newly identified carp sequences were used to design qPCR primers (Table 1).

2.4. 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.

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	AATTATAAAAGGACAAGTGCTGAC AGGTAG	*Muc5B*	Endpoint PCR
5_rv3a	TCCGCAGGKYTKRTAGTGCC		
pDefB1_F1	TCATCCGAAGATACCAAC	*BD1*	Endpoint PCR
pDefB1_R1	AGGGAACATAATTTTCAGTT	*BD2*	Endpoint PCR
Def2-128[a]	TGGACRTGTGGGTAYRGAGGACTCT		
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] Race primer.
[b] qPCR primers for the reference genes were designed by Gonzalez et al. [1].

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. 30]. 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 (1×10^5 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).

3. Results

3.1. 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 (Figs. 1 and 2). A BLAST analysis revealed conserved domains in both mucin sequences: von Willebrand factor D domains, and C8 domain (Figs. 1 and 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.

3.2. 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 (Figs. 3 and 4). The protein sequence in the regions containing the potential secondary structure of the β-defensins [as described by 16] 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.

3.3. 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.

3.4. 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).

4. Discussion

4.1. Homology of carp mucin 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 [5]. However, for MUC-type mucins, unifying sequence homology is not seen, implying that they may have evolved through convergency [7]. Dekker et al. [7] 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

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

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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        -----------VYHFPGTCEYNLVSDCQSLIRQFSVHVKRTEH--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
                :******:** *  *** .:*   ** .::: **.*** .:*
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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.

[31]. 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 [7]. 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 [31]. The cystein-rich domains of *MUC2*, *MUC5AC* and *MUC5B* are so far unique to these mucins [7]. 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 [32]. 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 [5]. 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 [5]. 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*.

organism: latin name	organism:common name	GenBank ID	aa length
Cyprinuscarpio	common carp	JF343438	1064
Danio rerio Muc-like	zebrafish	XP_685769	1787
Danio rerio Muc5B	zebrafish	XP_002666835	1686
Homo sapiens	human	AAG33673	1594
Mus musculus	mouse	NP_083077	4800
Taeniopygia.guttata	zebra finch	XP_002198208	1660
Xenopus tropicalis	Western clawed frog	XP_002940212	1894

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C.carpio                ----------------------------------------------------------M          1
D.rerio Muc-like        ----------------------------------------------------------M          1
D.rerio Muc5B           ------------------------------------------------------------
M.musculus              ----------------------------------------------------MGAPSA          6
H.sapiens               ----------------------------------------------------MGSRNW          6
T.guttata               -MACRGVSAPAPGALSPPPSALNLVPAGLFHILSLLWLLLWSNLFPLPKYIVPELLPPSL         59
X.tropicalis            MPVQKTEKKYKLGSLVLYGSALHDNSR---DSQKICVYHILYVDLSSPCTILENVTLIKL         57

C.carpio                DVVSMGTVRMSQMWMLRWVVLLMGLQSVQADFLGDYGDIMKDPLDFTTWPPPTTTVFPMT         61
D.rerio Muc-like        GFDSEGTVRMPQMWMLRWVFLLAGLQSIQAGFMGDYRDMEN----------PMTPMWPTT         51
D.rerio Muc5B           ------------------------------------------------------------
M.musculus              CRTLVLALAAMLVVP---QAETQGPVEPSWGNAGHTMDG----GAPTSSPTRRVSFVPPV         59
H.sapiens               SWALVWASVALLMVW---PAESQGQAELSSEHLELTGDSQRVYSDSISSSTRHVTFIPPI         63
T.guttata               TSPVLATSGSLLELAGIGSMECVGRGQIEFQTGSQFSGGLLQQQNASIRIPGTLNIIPPL        119
X.tropicalis            TEHKHPGSRFKRISMHDEHVACLMTSPQNFAPPLLADGSAQMDTSTSGESIQGTSSFIAP        117

C.carpio                TVMVTKVQPNPDHQSTVCSTWGNFHLKTFDGQFFQVPDTCNYVMAVMCDAATSDFNIQMQ        121
D.rerio Muc-like        -----PVEPNPDHRSTICSTWGNFHFKTFDGHFFQLPDTCNYVLAVMCDAASSDFNIQMQ        106
D.rerio Muc5B           ------------------------------------------------------------
M.musculus              TVFPSLSPLNPAHNGRVCSTWGDFHYKTFDGDVFRFPGLCNYVFSEHCRAAYEDFNVQLR        119
H.sapiens               TVFPSMSALHLAHNGRVCSTWGDFHYKTFDGDVFRFPGLCNYVFSSHCGATYEDFNIQLR        123
T.guttata               LTNLAITSANPAHNGRVCSTWGNFHFKTFDGDIFTFPGLCNYVFASHCNAPYEDFNIQIR        179
X.tropicalis            NLNPIFKSSSPSHNGYVCSTWGNNYFKTLDGDIFYFPGQCNYLLASNCKSVTEEFNIQIR        117

C.carpio                RETVNGSITFSTVTIKLEGTIIKITNGDITMDDQAVSIPISQNGIKIEGTPTSIKVS-RY        180
D.rerio Muc-like        RETVNGSISFSTVLIKLDGTVIKVTDSDITMGEETVTVPTYKNGIKIEGSPTSFKISNKH        166
D.rerio Muc5B           ------------------------------------------------------------
M.musculus              RGLVGSRPVVTRVVIKAQGLVLKASNGSVLINGQREELPYSRTGLLVEQSGDYIKVSIRL        179
H.sapiens               RGLEGSRPTVTYVLLRAQGLVIELSNGSVLVNGHREKLPYSRAGLLMEKSSGYVKISIRL        183
T.guttata               REVVANTPTINRITMKLEGVVAELTEDAVLVDGNRVELPYSQSGITIEKSSIYVKVGSKI        239
X.tropicalis            RSVVNGLPTVSHIGMKIEGVFIEFTGGNITFNGNVVDLPYSFSGIQIDRSGAYIRVISKV        237

C.carpio                GMTVFWEEDNSILIELAEKYKGQTCGLCGNYNGNKNDDITESG---------PATWKVST        231
D.rerio Muc-like        GVTVFWEEDNSLSIELPEKYQGQTCGLCGDFNGNLADDITDNG---------PATWKVSI        217
D.rerio Muc5B           ----------------------------------------MG---------PATWKIST         10
M.musculus              VLTFLWNGEDSALLELDPKYANQTCGLCGDFNGLPAFNEFYAHNARLTPLQFGNLQKLDG        239
H.sapiens               VLTFLWNEEDSALLELDSKYINQTCGLCGDFNGLPAVSEFYTHNTRLTPVQFGNLQKLDG        243
T.guttata               GVVLLWNEKDSILLELNEKYANQTCGLCGDFNGFPIYNEFISNNVKMTALQFGNMQKMDG        299
X.tropicalis            GLEFRWNEDDAATLELDQKFINQTCGLCGDFNGIPTYNEFMFNNVRLTDNQYGNMQKMNG        297

                        *:.

C.carpio                PTESCEDVTLP---PGDQCDQHISVCQQYLTSPGFADCYNVMDMSSFEKACVDDLCRCYA        288
D.rerio Muc-like        STEICEEVTLPSTGPCDELSEQASFCEEYLISPGFSGCYDVMDMRIFQKACVSDLCQCYG        277
D.rerio Muc5B           PTESCKDVILP---PKDQCDQNTMVCQQYLSSPGFSGCYDVMDMKIFEKACVSDMCQCYG         67
M.musculus              PTEQCPDPLPLP--AGNCTD-EEGICHRTLLGPAFAECHALVDSTAYLAACAQDLCRCP-        295
H.sapiens               PTEQCQDVLPSA--VSNCTD-TEDICRRTLLGPAFDKCTALVDVSMYLDACVQDLCRCP-        299
T.guttata               PTEHCEDSTSIP--TYNCSDNLDDICEKILTSSAFAECNDLVDVQDYIEVCQDDLCRSAE        357
X.tropicalis            PTETCEDVLQAP--EDKCTD-LSSVCHAILKSASFVYCNNLVDPTPYINVCVQDLCRCKR        354
                        .** * :          .  .    .*.  * ...*  *  ::*   :  .* .*:*:.
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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.

4.2. 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 [33]. To our knowledge piscine β-defensins have so far only been identified through *in silico* studies [16,17]. 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 [16]. 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 [17]. 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 [16]. Therefore, the expression profile of the β-defensin genes appears species-dependent.

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

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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 [167] are marked as well as conserved cysteins.

4.3. Influence of β-glucan 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 [34] and TLR2/6 [35]), and therefore have immune-modulatory properties when administered to mammals [35] and fish [36]. β-Glucans have been shown to be effective immune-modulators in a number of bacterial, viral and parasitic infections [37]. 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 [38]. 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 [39] and pigs [40], and an increased expression of *Muc2* and *Mub5B* has been observed in mammals after bacterial [35,41–43] and nematode infections [44]. 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 *BD2* was not observed in the mucosal tissues of *O. mykiss* challenged with *Yersinia ruckeri*, but in these fish *BD3* was increased in gills [17]. 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

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

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C.carpio          ------------------------------------RRFCFDQEYIVSHQGCPRKY-------
D.rerio           MKKLGMIIFITLPALFAGNVHNAEVQIQNWTCGYGGLCRRFCFDQEYIVAHHGCPRRYRCCAVRF
T.nigroviridis    MKGLSLVLLVLLLMLAAG--EDSDSEMQYWTCGYRGLCRRFCYAQEYTVGHHGCPRRYRCCATRP
E.coioides        MKGLSLVLLVLLLMLAVG--EGNDPEMQYWTCGYRGLCRRFCHAQEYIVGHHGCPRRYRCCAVRS
O.mykiss          MGRLGLVMLVLLLLTAVQ---ADDTKVQGWTCGYRGACRKYCYAQEYMVGYHGCPRRLRCCALRF
                                                  *:*:. *** *.::****:
                                                  β1        β2       β3
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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 [18] are marked as well as conserved cysteins.

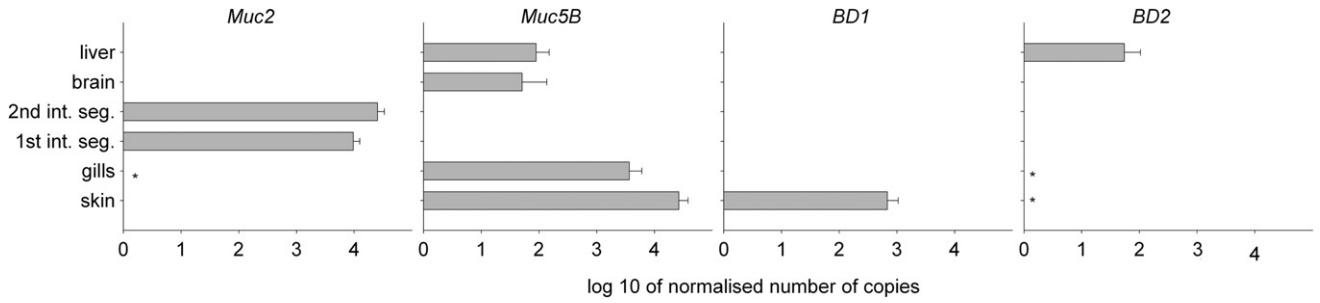

Fig. 5. Expression of carp *BD1*, *BD2*, *Muc5B* and *Muc2-like* in liver, brain, first intestinal segment, second intestinal segment, gills and skin from naïve fish. Data are presented as copy numbers of mRNA normalised against *40S* mRNA from the same sample. *copy numbers below 10 could be observed.

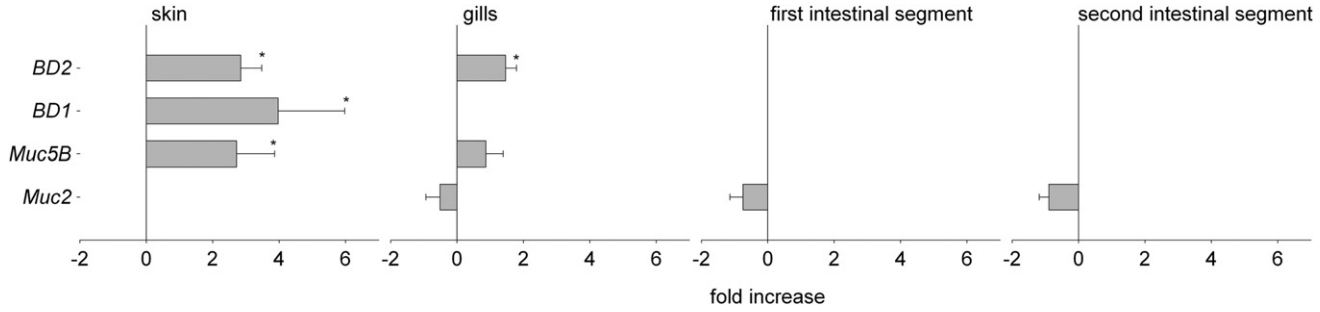

Fig. 6. Difference in the expression of carp *BD1*, *BD2*, *Muc5B* and *Muc2* in tissues from carp after MacroGard β-glucan feeding regimen relative to expression in these tissues from no β-glucan feeding control fish. Copy numbers of mRNA were normalised against *40S* mRNA from the same sample. The 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.

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.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsi.2011.12.008.

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