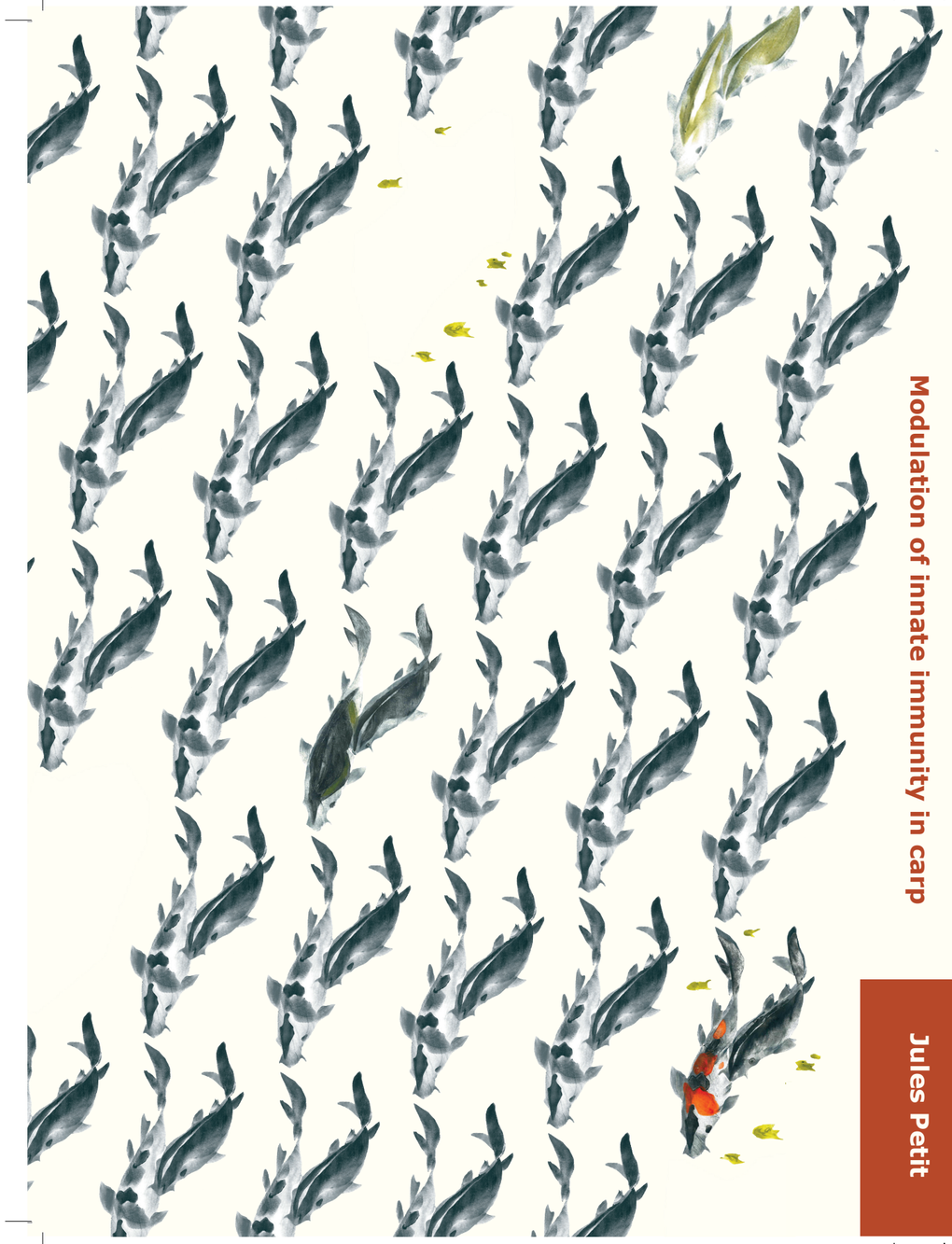


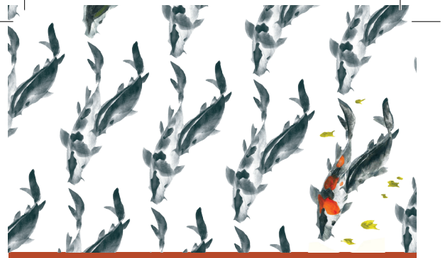
Modulation of innate immunity in carp

Jules Petit

Jules Petit

Modulation of innate immunity in carp

Diverse approaches to β-glucan-mediated responses



Invitation

To the public defence ceremony of my PhD thesis entitled:

Modulation of innate immunity in carp
Diverse approaches to β-glucan-mediated responses

On Friday, June 14th 2019 at 16:00 in the Aula of Wageningen University, Generaal Foulkesweg 1a, Wageningen

Jules Petit
jules.petit@wur.nl

The defence will be followed by a reception at the same location.

Paranymphs

Annelieke Wentzel
annelieke.wentzel@wur.nl
Mark Goldman
mark.goldman@wur.nl

Propositions

1. Trained immunity is a conserved feature of common carp macrophages (this thesis).

2. β-glucans activate a Dectin-1-like signalling pathway in carp macrophages (this thesis).

3. If an increase in ploidy can improve tastiness of strawberries (*inspired by Edger et al., 2019; Nature Genetics*), there is hope for the tetraploid common carp.

4. Social networking can never be about *connecting* if not interrupted by frequent and conscious *disconnecting*.

5. Although an inborn enthusiastic and positive attitude can be perceived as naive, it is of great advantage to one's scientific career.

6. Since time has become our most valuable asset, we should value more the cost of other people's willingness to spend time with us.

Propositions belonging to the thesis, entitled

Modulation of innate immunity in carp
Diverse approaches to β-glucan-mediated responses

Jules Petit
Wageningen, 14 June 2019

Modulation of innate immunity in carp

Diverse approaches to β-glucan-mediated responses

Jules Petit

Thesis committee

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Prof. Dr M. Kleerebezem, Wageningen University & Research
Prof. Dr H.A. Schols, Wageningen University & Research

This research was conducted under the auspices of the Graduate School Wageningen Institute of Animal Sciences.

Modulation of innate immunity in carp

Diverse approaches to β-glucan-mediated responses

Jules Petit

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 14 June 2019
at 4 p.m. in the Aula.

J. Petit
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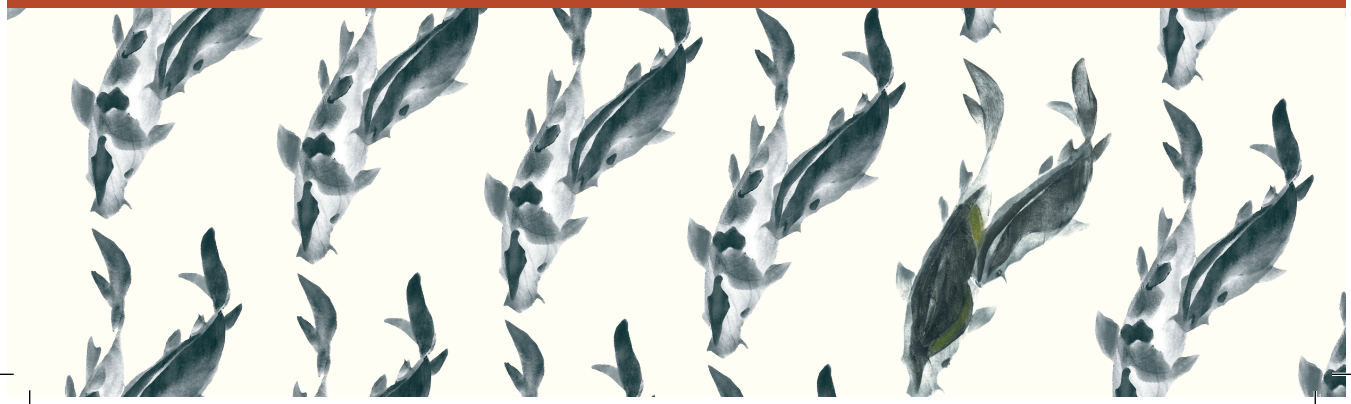


1

General introduction

NO Waste: working on a circular economy

The São Paulo Research Foundation (FAPESP) and The Netherlands Organisation for Scientific Research (NWO) agreed to finance a scientific collaboration aiming at the valorisation of β-glucan, a common 'waste' product from bioethanol production, as immuno-stimulant for use in aquaculture. This was aimed not only at strengthening the cooperation between the State of São Paulo in Brazil and the Netherlands, but is a subject well in frame with the Bio-based Economy research theme formulated by these two large research organisations.

In 1975, the Brazilian government launched a nationwide program to reduce the use of fossil fuels for automobiles, in favour of bio-ethanol. Currently in Brazil, approximately 40% of the traditional gasoline consumption is replaced by bio-ethanol produced from sugar cane (1, 2). The main industrial route of bio-ethanol production is through the fermentation of extracted sugar from sugar cane by *Saccharomyces cerevisiae* yeast (3). While the extracted sugar is used to produce ethanol, some of the left-over residues can be used as a source of renewable energy. A large portion of yeast cells used during the fermentation process, up to 90 – 95%, is recycled in the Brazilian ethanol industry, which results in yeast cells being reused at minimum twice a day for 200 – 250 days (4). A major constituent of yeast cell walls are β-glucans, comprising over 50% of the cell wall dry mass weight (5, 6). These β-glucans can be isolated and be used in feed for companion animals and for production animals.

The possibility to use waste products such as β-glucans from yeast cell walls as feed supplements can further improve waste reduction. This formed the rationale for a scientific collaboration between two university partners; the Cell Biology and Immunology (CBI) group at Wageningen University & Research and the Centro de Aquicultura da Unesp (Caunesp) at Universidade Estadual Paulista (UNESP), Campus de Jaboticabal, and the research unit of a commercial partner in Brazil (Biorigin). The scientific rationale for the project entitled "Use of branched 1,3/1,6 β-glucan, MacroGard®, a waste product of the production of sugar and ethanol from baker's yeast, to stimulate the innate immune system of farmed fish (short name NOWASTE)" took shape after earlier observations on the immuno-modulating ability of the β-glucan product, as published in two PhD theses of the Cell Biology and Immunology group (7, 8).

The aim of the NOWASTE project was to establish optimal protocols for the valorisation of β-glucan 'waste' from bioethanol production as immuno-stimulant for use in aquaculture, using β-glucans in the strategic improvement of fish health. Within the NOWASTE project, the CBI group was responsible for the characterization of molecular mechanisms underlying β-glucan immuno-modulatory effects in fish. Furthermore, the CBI group was responsible for provision of training to the Brazilian partners in relevant *in vitro* assays and for providing a theoretical framework that could support field studies in Brazil.

Growth and intensification of aquaculture

The aquaculture sector keeps growing rapidly and, as illustrated by the Food and Agriculture Organisation of the United Nations in their biyearly reports on 'State of World Fisheries and Aquaculture (SOFIA)', the consumption of fish has been growing with average rate of 3.2% per year since the 1960s (9). During this period the average growth of the world population was 1.6% per year, which means that fish consumption per capita has increased. In 2015, over 40% of the world population derived at least 20% of their animal protein intake from fish. With a growing consumption of fish and a growing world population, pressure on fisheries and aquaculture is increasing. In 2016, the total fisheries production accounted for 171 million tons, of which 47% could be ascribed to aquaculture (9). Almost 60% of this production could be accounted for by inland finfish production. The current expectation is that in 2020, total aquaculture production will surpass the total capture fisheries and the total aquaculture production for human consumption for 2030 is estimated to be 109 million tons alone.

In Brazil, the aquaculture sector is also growing fast, as illustrated by the tremendous increase from 30.000 tonnes in the 1990s to over 275.000 tonnes in 2003[1]. From 2005 until 2015, the Brazilian aquaculture yield has further grown with a staggering 123% to 574.000 tonnes per year[2] and in 2016 the FAO assessed the total aquaculture production of Brazil to be 581.000 tonnes, over 87% from inland finfish production, with an expected growth towards a total aquaculture production of 1.097.000 tonnes in 2030 (9).

The intensification of the aquaculture industry accommodated for the growth of the sector and the growing demand for fish, but it came with some drawbacks. Intensification of aquaculture led to increased stress due to environmental factors such as low water quality, high temperature and high fish density (10). Increased stress, lack of adaptation to local infectious agents, increased host contact rates due to high stocking density and intensive monoculture has resulted in a clear increase in disease incidence (11-14) and economic losses (14) over the past decades. The economic losses in the Brazilian aquaculture farms due to diseases were estimated to a total of 84 million US dollars per year (15).

[1] As reported by FAO (http://www.fao.org/fishery/countrysector/naso_brazil/en, accessed 20-02-2019)
[2] As reported by Embrapa on 13-12-2016 (Brazilian Agricultural Research Corporation commissioned by the Brazilian Ministry of Agriculture, Livestock, and Food Supply)

Immuno-modulation of fish through β-glucan supplementation

Using β-glucans to modulate the immune system of farmed fish can be a sustainable solution to reduce losses in the aquaculture sector, especially when derived as waste product. Further, the use of immuno-modulatory β-glucans to prevent disease and or increase resilience of fish in aquaculture in alternative to the use of antibiotics, might help reduce the detrimental effects of antibiotic spill over into the environment and food chain (16-18). Already in the 90's of the previous century it became evident that dietary supplementation or administration through injection of β-glucans could resulted in increased bacterial and viral resistance of fish [as reviewed by: (19-21)].

β-glucans are polysaccharides made by glucose linked by β-glycosidic bonds. β-glucans are classified as non-starch polysaccharides (NSPs), a complex group of molecules composed predominantly of linked monomers of hexoses and pentoses, e.g., galactose, glucose, arabinose, xylose and mannose, such as cellulose, hemi-cellulose (arabinoxylans and β-glucans) and pectin. Although nutritionists often refer to the adverse effects of NSPs in feed and to their little nutritional value due to their low digestibility, β-glucans in feed are often considered immuno-modulatory (22). β-glucans are naturally found in the cell wall of yeast, including *Candida albicans* and yeast of the *Saccharomyces* genus (including *S. cerevisiae*, or baker's and brewer's yeast) (Figure 1). Furthermore, these polysaccharides can also be found in the cell wall of plants, among others wheat, rye, barley and several *Echinacea* species, seaweeds, such as *Laminaria digitata*, fungi and even in the cell wall of several bacterial species (21, 23). Depending on the exact source of β-

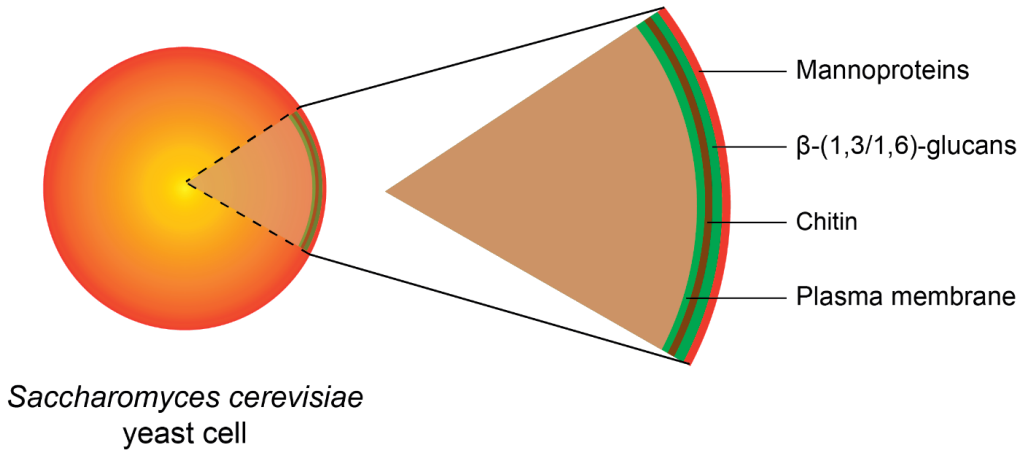


Figure 1. Schematic overview of a cross-section of the cell wall of *S. cerevisiae*. The outermost layer of the cell wall consists of mannoproteins, followed by a layer with β-(1,3/1,6)-glucans, a layer of chitin and the plasma membrane.

glucan, these polysaccharides exist as a wide variety of structurally diverse molecules with different β-glycosidic bonds and a linear or branched structure (Figure 2). Plant-derived β-glucans are often linear with a β-(1,3)-, β-(1,4)- and β-(1,3/1,4)-conformation, while yeast- and fungi-derived β-glucans are commonly branched with a β-(1,3/1,6)-conformation. β-glucan molecules can have a soluble or insoluble character and can have large differences in molecular weight (23). Probably the most widely used β-glucans in animal husbandry are the high molecular weight *S. cerevisiae* yeast-derived β-(1,3/1,6)-glucans. One of the best studied β-glucan preparations in aquaculture is MacroGard®, a mixture of β-(1,3/1,6)-glucans derived from *S. cerevisiae* (24). Although the effects of β-glucan supplementation in aquaculture have commonly been perceived as positive and beneficial for fish health [reviewed by: (19-21, 24)], no definitive underlying mechanisms explaining the immuno-modulatory effects of β-glucan in fish had been described at the start of this project.

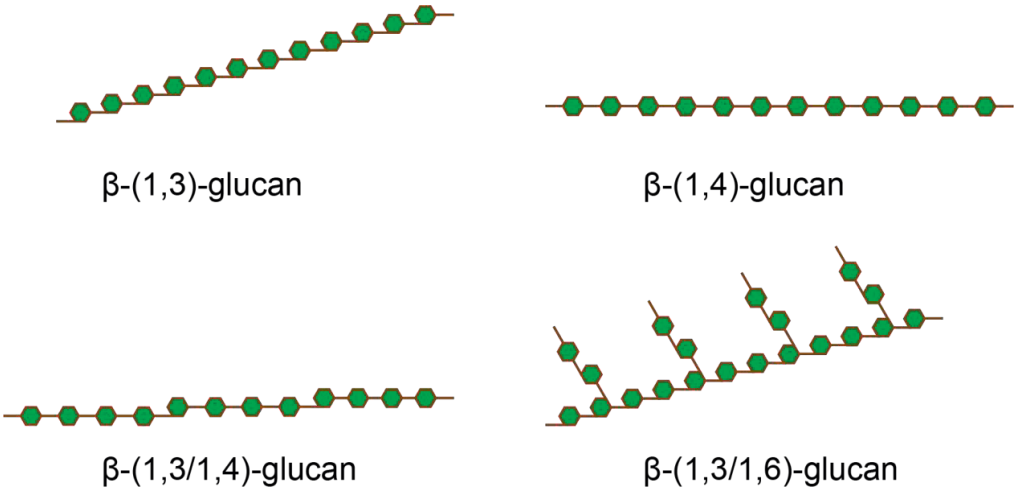


Figure 2. Schematic representation of different β-glucan structures. Depending on the position of the linkage, β-glucans exist in a wide range of different conformations. β-(1,3)-, β-(1,4)- and β-(1,3/1,4)-glucans exist as linear molecules and are often derived from plants or algae. β-(1,3/1,6)-glucans are branched and are often derived from fungi or yeasts.

Direct or indirect effects of β-glucan supplementation

There are several ways through which β-glucans might affect the immune system. Direct effects of β-glucan supplementation could be induced through sensing β-glucans as pathogen-associated molecular pattern (PAMP) via direct recognition by pattern recognition receptors (PRRs) on antigen sampling cells such as macrophages or dendritic cells. PRRs are germline-encoded innate immune

receptors capable of recognizing different PAMPs (25). There are many different classes of PRRs, of which the Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs) are well known. Different classes of PRRs recognize different classes of PAMPs, and within one class of PRRs many different ligands can be sensed. For example, for the TLR family, TLR4 may sense lipopolysaccharides (LPS) from Gram-negative bacteria whereas TLR3 may sense double stranded RNA. In general, upon binding of a PAMP to a PRR, a downstream signalling cascade will be activated which can finally result in different responses, such as production of inflammatory cytokines or pathogen-induced phagocytosis, the process here referred to as direct effects of β-glucans (Figure 3A).

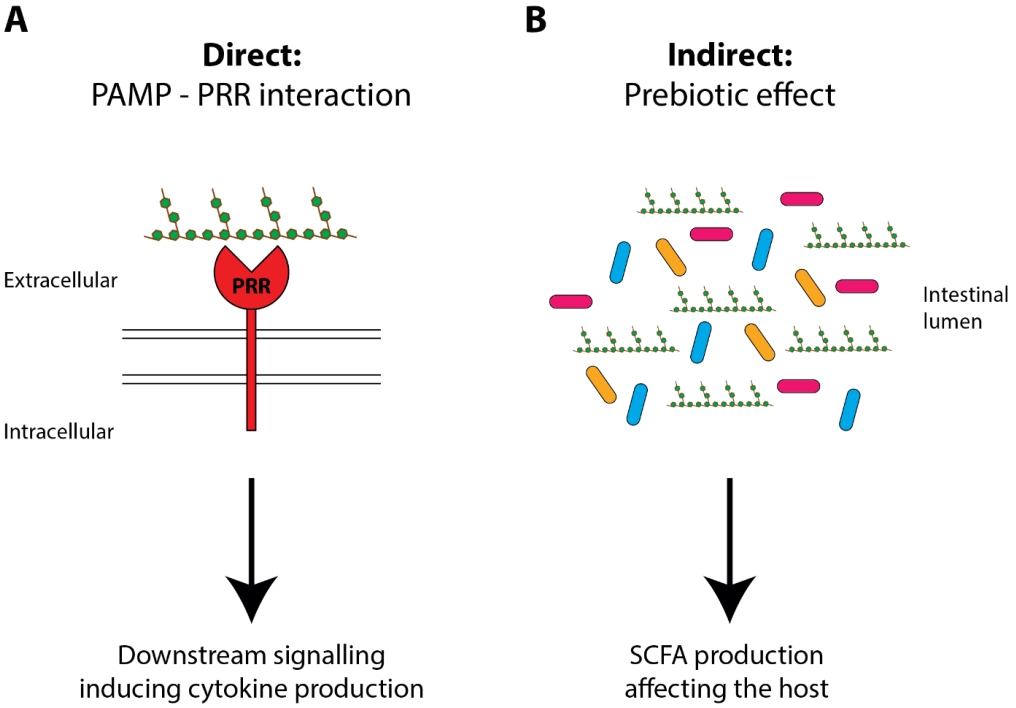


Figure 3. Schematic representation of a PAMP – PRR interaction and possible prebiotic β-glucan effect. A Branched β-glucan as pathogen associated molecular pattern (PAMP) binding to a C-type Lectin Receptor (CLR) as pattern recognition receptor (PRR). β-glucan ligates to the extracellular part of the CLR, which directly activates a signalling pathway leading to various downstream responses, such as the induction of a cytokine response. B branched β-glucans fermented by intestinal microbes. With respect to indirect prebiotic effects, part of the microbiota is capable of degrading and fermenting β-glucans present in the intestinal lumen. As a result, fermentation end-products such as short chain fatty acids (SCFAs) can be produced which in turn can affect the host immune system.

Supplementation with β-glucans can also have indirect effects, often referred to as prebiotic effects. Prebiotics can be defined as "selectively fermented ingredients that allow specific changes, both in the composition and/or activity of the gastrointestinal microflora that confer benefits upon host wellbeing and health"

(26). Supplementation with β-glucans could possibly provide a source of nutrient for specific intestinal microbes capable of fermenting these molecules. The fermentation end-products produced by the intestinal microbiota, such as short chain fatty acids (SCFAs), could then influence the host' immune responses, the process here referred to as indirect effects of β-glucans (Figure 3B).

Direct effects: Dectin-1, the 'main' β-glucan receptor in mammals

Direct effects of β-glucan supplementation could be induced by sensing β-glucans (PAMP) by a β-glucan receptor (PRR) on antigen sampling cells. To date, the best-described β-glucan receptor is Dectin-1, part of C-type Lectin Receptor (CLR) family and also known as C-type Lectin domain Family 7 member A (CLEC7A). This receptor belongs to the *C-type lectin super family V, NK cell receptors* and is a transmembrane receptor with a single carbohydrate recognition domain (CRD) and a cytoplasmic tail containing one immunoreceptor tyrosine-based activation motif (ITAM) (27-29). In mammals, Dectin-1 is predominantly expressed on cells from both the monocyte/macrophage and neutrophil lineages, where it acts as the major receptor for β-glucans (30). Research has shown that the ligation of β-(1,3)-glucan to Dectin-1 is dependent on a β-glucan binding cleft, formed by the spatial arrangement of three amino acid residues in the carbohydrate binding domain, tryptophan (W) and histidine (H) and tyrosine (Y). The 3-dimensional arrangement of these three amino acid residues in a triangular fashion forms a shallow hydrophobic surface groove, capable of accommodating and binding β-glucan chains through hydrophobic interactions (31-33). While the three residues are conserved in a WxHxxxxY motif in Dectin-1 in mammals, the same three residues are present in the binding domain of β-(1,3)-glucan recognition protein (GNBP3) of invertebrates, although not as a conserved motif (34). Dectin-1 signalling is activated following clustering in synapse-like structures, formed within minutes after activation by particulate β-glucans (35). Following the interaction with β-glucan, Dectin-1 induces intracellular signalling via the phosphorylation of its ITAM (6, 36). Dectin-1 homodimers with phosphorylated ITAM recruit and activate Syk kinase. Activated Syk kinase is responsible for the recruit and activate Syk kinase. Activated Syk kinase is responsible for the phosphorylation of protein-kinase C δ (PKCδ) which in turn phosphorylates the adapter protein Card9 (Caspase recruitment domain-containing protein 9) and the formation of a signalosome with the adapter proteins B cell chronic lymphocytic leukaemia and/or lymphoma 10 (Bcl10) and mucosa-associated lymphoid tissue lymphoma translocation gene 1 (Malt1), (37). The formation Card9-Bcl10-Malt1 signalosome results in activation of NF-κB, leading to an inflammatory profile typical of stimulation with β-glucans (38, 39). Activation of Syk is also required for the induction of reactive oxygen species (ROS) production in response to β-glucan stimulation (40). Alternatively, Dectin-1 can signal via the Raf-1 kinase pathway, independently of Syk kinase via Ras (Figure 4). Identification of Dectin-1 and/or other *C-type lectin super family V*

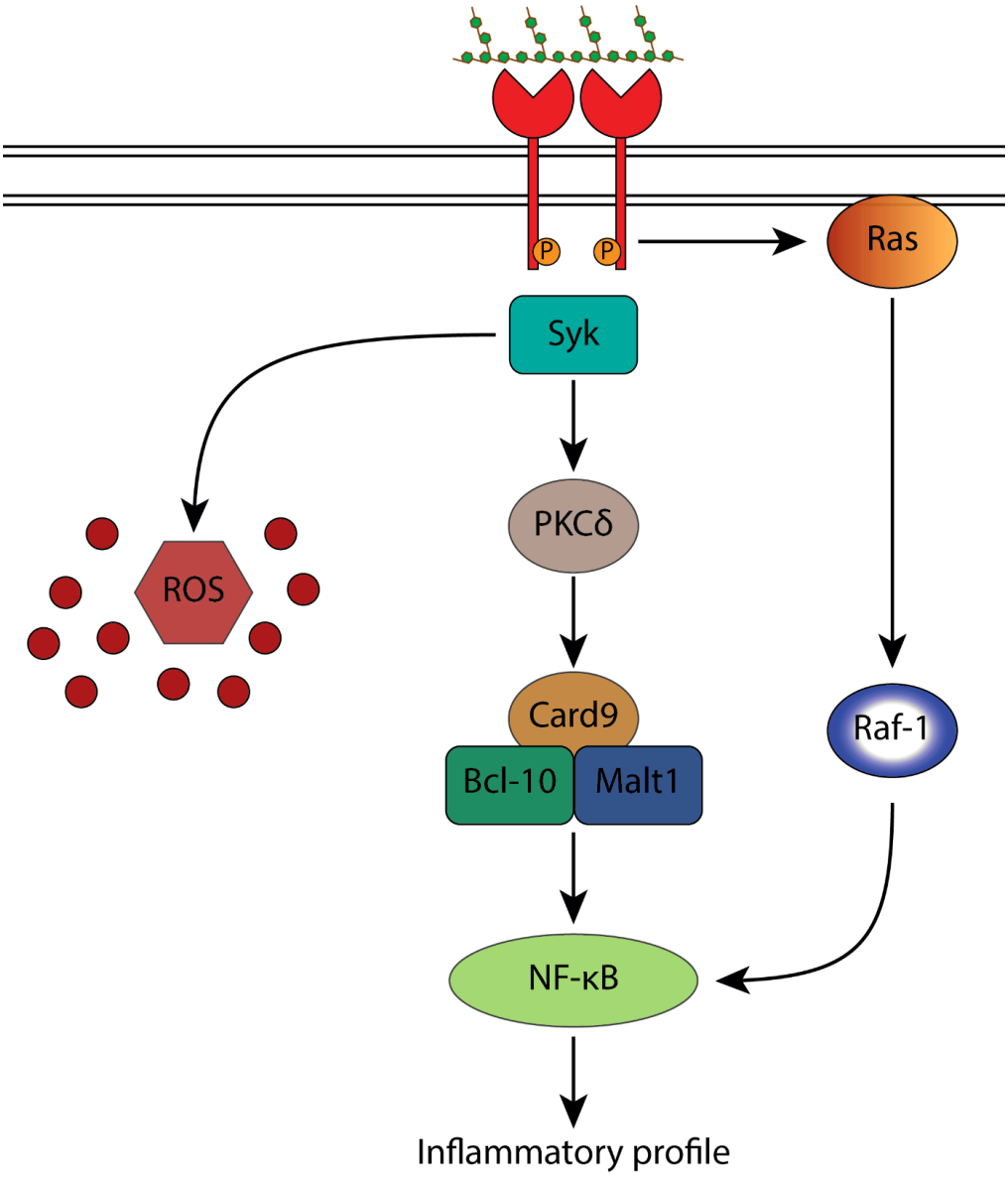


Figure 4. Schematic representation of Dectin-1 signalling. Schematic representation of the signalling of Dectin-1 after ligation with a ligand, e.g. β-glucans. Dectin-1 homodimers recruit Syk to phosphorylated ITAMs (Orange P), Syk in turn initiates downstream signalling. Syk induces the generation of reactive oxygen species (ROS) and phosphorylates protein-kinase C δ (PKCδ). PKCδ phosphorylates the adapter protein Card9, which facilitates complex formation with the adapter proteins Bcl-10 and Malt1. The formation Card9-Bcl10-Malt1 signalosome results in activation of NF-κB, which results in the inflammatory profile associated with β-glucan stimulation. Alternatively, Dectin-1 can activate a Syk independent pathway via Ras, which activates Raf-1 that results in the activation of NF- κB. [Adapted from (6, 36)].

NK cell receptors in fish genome assemblies has remained a challenge (28, 41, 42), leaving unanswered the question on the 'main' β-glucan receptor in fish.

Direct effects: Additional β-glucan receptors in mammals

There are several additional recognition mechanisms besides recognition by Dectin-1 that have been implicated in β-glucan sensing, i.e. the interplay of Dectin-1 with TLRs, the interaction of β-glucans with Complement Receptor 3 (CR3), the binding of lactosylceramide (LacCer) to β-glucans and the interaction of Scavenger Receptors (SRs) with β-glucans (reviewed by: (19, 21, 43)). A role for TLR mediated signalling was shown in mice; deficiency in the adaptor protein MyD88 or in TLR2 reduced zymosan-induced IL2 and IL12 production by macrophages, but not the production of ROS [(44, 45)]. Zymosan is a preparation of *S. cerevisiae* cell wall consisting of not only β-glucans, but also mannose and chitin (46). A role for CR3 in the response to β-glucans has also been noted. CR3 is composed of an α_m chain, CD11b, and a common β_2 chain, CD18. The α_m chain possesses a lectin domain capable of binding β-glucans (30, 47). It was shown that CR3 is the major receptor for β-glucan particles on human neutrophils and that ligation of the lectin-site primes cells towards iC3b opsonized molecules cytotoxicity (48, 49). Another role for sensing β-glucans can be played by LacCer, a glycosphingolipid with a specificity for the β-structure in β-glucans, leading to the induction of macrophage inflammatory protein-2 in mammalian leukocytes (50, 51). Finally, SRs have been shown to play a role and in uptake of β-glucans through competition binding studies (52-54). More recently, a study showed that two SRs, MARCO and CD204, can play a role in the opsonin independent uptake of zymosan in murine macrophages (55). It may be clear that also in fish several 'additional' β-glucan receptors may be present.

Direct effects: Trained immunity induced by β-glucans

Recently, β-glucans were connected to a new paradigm in innate immunity. Traditionally, innate immune responses have been viewed as rapid, relatively non-specific and lacking development of immunological memory. New insights have challenged this view, introducing a novel concept called trained immunity, defined as a heightened non-specific innate immune response to a secondary infection (56, 57), (Figure 5). The initial experiments that lead to the concept of trained immunity showed that *Rag*$^{-/-}$ mice exposed to a sub-lethal *Candida albicans* infection were better protected against a subsequent lethal dose of these yeasts, whereas monocyte-deficient mice did not show this increased protection, suggesting a form of memory present in the myeloid compartment. Interestingly, β-glucans present in the cell wall of *C. albicans* proved crucial for inducing trained immunity. Effects of trained immunity were measured *in vivo* up to 3 months post primary exposure.

In vitro re-exposure of human PBMC to *C. albicans* induced a significant IL6 and TNFα response, even after a resting period of six days in the absence of a stimulus following primary exposure to β-glucans (58). Subsequently, several studies have further unravelled the underlying mechanisms of trained immunity, showing both metabolic reprogramming (59-63) and epigenetic reprogramming (59, 60, 64-66) following β-glucan exposure. Before the observation and establishment of the phenomenon trained immunity in humans and mice (67) similar cross-specific protection was observed in invertebrates [reviewed by: (68)] which, typically without T- and B-lymphocytes, can build up a form of immunity, protecting the organism upon a secondary exposure. Given the evolutionary position of teleost fish, as early vertebrates with a fully developed immune system it is likely that trained immune responses should exist also in fish and include receptors for direct sensing of β-glucan products.

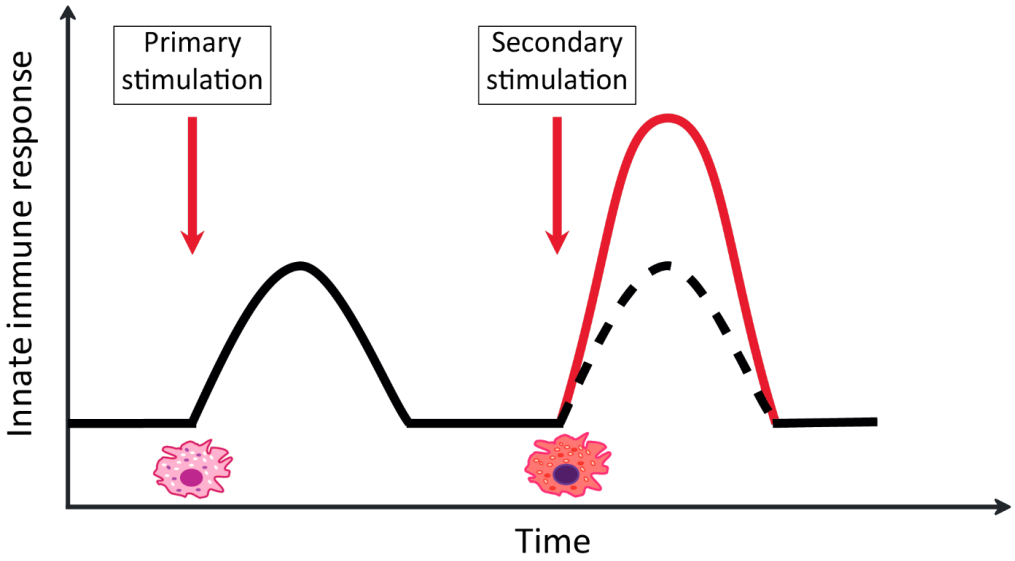


Figure 5. Schematic representation of trained immunity. Black line representing the innate immune response to stimulation, with the dotted black line representing the canonical perspective on the innate immune response to a second stimulation. The red line represents the observed innate immune response ascribed to trained immunity. During the first stimulation, infection or exposure, innate immune cells are reprogrammed, metabolically and epigenetically. Upon second stimulation, infection or exposure, these reprogrammed or 'trained' innate immune cells generate a heightened innate immune response.

Indirect effects: immuno-modulation through intestinal microbiota

Supplementation with β-glucans can also have prebiotic effects in the intestinal tract thereby influencing immune responses in an indirect manner. Upon supplementation, when administered orally, β-glucans may influence the

composition of the intestinal microbiota or act as a nutrient for particular groups of micro-organisms, thereby causing a shift in intestinal microbiota. Several genera of bacteria common to the gastro-intestinal tract (GIT) are able to degrade and ferment β-glucans. For example, members of the *Bacteroides* genus found in the GIT of humans can degrade barley-derived β-1,4-glucans (69) and fungal-derived β-1,6-glucans (70). Fermentation of β-glucans by bacteria will result in the production of short chain fatty acids (SCFAs), which in turn affect the host, as they can be sensed by SCFA receptors, G-protein coupled receptor (GPR) 41, GPR43 and GPR109A or directly enter host cells in a receptor-independent manner (71-73). Indeed, a study in mice showed that a diet enriched with β-glucans derived from oat bran led to higher SCFA levels in the caecum (74). Another study that compared the SCFA concentrations in the colon of mice fed five different dietary fibres, observed clear differences in SCFA accumulation and in microbiota composition, even though regulation of epithelial cell's gene expression was comparable between the different dietary fibres (75). In mice production of SCFAs by intestinal microbiota can affect the host and its immune responsiveness. In fact, shifts in the composition of the intestinal microbiota toward more lactic acid bacteria, led to an increase in the IFN-β response, induced upon recognition of double stranded RNA shed by these commensal bacteria (76). Of interest, β-glucans have been shown to stimulate growth of several commensal lactic acid bacteria, at least in humans (77), and preliminary data suggest effects of dietary β-glucan supplementation on the relative abundance of lactic acid bacteria in the intestinal microbiota of fish (78, 79). Thus, prebiotic effects of β-glucans on the immune system can be classified as indirect when based on shifts in intestinal microbiota, their metabolites, and when based on cellular mechanisms sensing these metabolites or other bacterial products (e.g. double-stranded RNA). No matter what, it may be clear that investigating the prebiotic effects of β-glucans on the immune system of fish is highly relevant.

Outline of this thesis

Reports on dietary supplementation with β-glucan and associated beneficial effects on fish health have been numerous and as a result β-glucan supplementation is widely applied in aquaculture. Although several studies have investigated the mechanisms of action and searched for potential candidate β-glucans receptors , no definitive mechanism has been reported that explains the immuno-modulatory effects of β-glucan in fish. The overall **aim of this thesis** was to explain the effects of β-glucans on carp immune responses, studying three different but not necessarily exclusive mechanisms: 1) long-lived effects on myeloid cells typical of trained immunity; 2) degradation and fermentation of β-glucans and associated shifts in composition of the intestinal microbiota and their metabolites; 3) regulation of downstream signalling upon β-glucan recognition in carp macrophages. In this thesis, the three mechanisms are discussed in this order.

First I discuss the possibility of upgrading waste products from bioethanol production to immuno-stimulants for intensive aquaculture (General introduction, **chapter 1**). Sensing of β-glucan by an unknown PRR could activate a downstream signalling cascade and pro-inflammatory response, either or not associated with trained immunity, whereas prebiotic effects of β-glucans could also be indirect when based on shifts in intestinal microbiota.

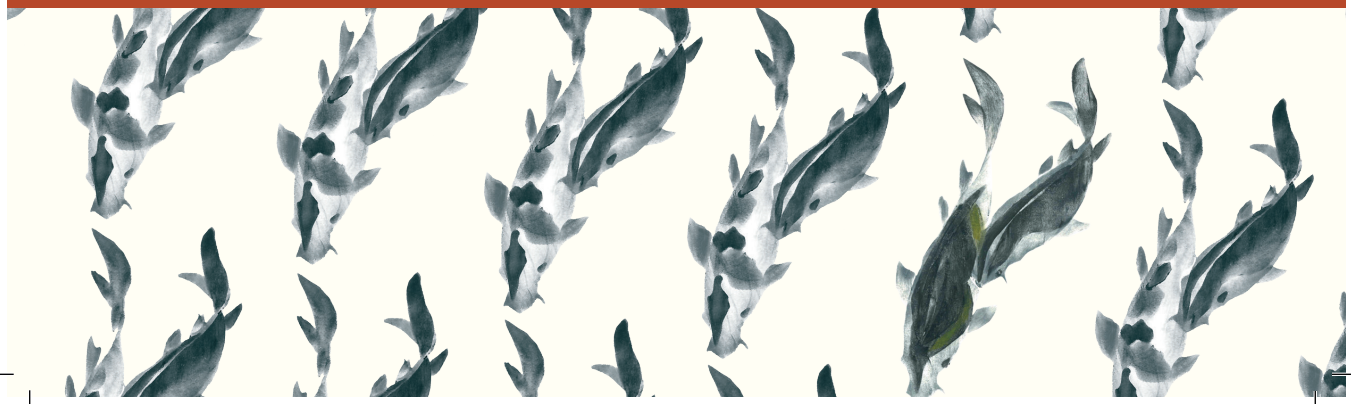
In **chapter 2** we review the current literature on β-glucan research in fish. We sought to update previous studies and reviews in the field and discuss possible mechanisms involved in the immuno-modulatory effects triggered by β-glucans, including changes in intestinal microbiota, receptor recognition and downstream signalling as well as the relatively new concept of trained immunity. We uncovered several studies with indications for long-lived effects of β-glucans in fish, but without further experimental evidence, were unable to ascribe these observations to trained immunity.

In **chapter 3** we sought to investigate conservation of trained immunity in carp, using an *in vitro* model based on a known NOD-ligand, peptidoglycan, previously shown to be crucial for the induction of trained immunity in mammals. Following establishment of the model, we investigated the ability of the soluble β-glucan laminarin to induce trained immunity in carp macrophages, and show that both ligands are suitable training stimuli in carp, effectively expanding the tools available to study trained immunity in teleost fish.

In **chapter 4** we set out to investigate the role of intestinal microbiota in the immuno-modulatory effects observed upon dietary β-glucan supplementation in fish. First, we characterised the normal intestinal microbiota of common carp by 16S rRNA sequencing. Next, in an *in vitro* batch culture experiment, we analysed the ability of carp intestinal microbiota to ferment β-glucans, and measured short chain fatty acid (SCFA) profiles. Finally, *in vivo* β-glucans supplementation was performed to analyse the effects of β-glucan on the intestinal microbiota composition and local gene expression.

In **chapters 5** and **6** we investigated the possibilities of using next generation sequencing (NGS) to address our questions related to the mechanisms underlining the immuno-modulatory effects of β-glucans. In **chapter 5** we first reviewed the current genome assemblies in teleost species and summarized current NGS-based studies investigating immune responses in carp and related species. We discuss the complexities and potential pitfalls of working with polyploid species such as common carp. In **chapter 6** we applied NGS-based transcriptome analysis to macrophages stimulated with β-glucans. We tested the hypothesis that immuno-modulatory effects of β-glucan in carp macrophages could be triggered by an unknown member of the C-type Lectin Receptor (CLR) family, different from Dectin-1. Regulation of the C-type lectin receptor signalling pathway suggested at least partial conservation of a β-glucan recognition cascade via a C-type lectin receptor. We conclude with the identification of several candidate receptors for β-glucans.

Finally, in **chapter 7**, the general discussion, I integrate the results of the previous chapters in a larger framework and I address both the limitations and opportunities for further research into β-glucan supplementation as immuno-stimulant for aquaculture. The findings of this project alongside the interpretation and perspectives offered, pave the way for future research into β-glucan immuno-modulation as well as tailored β-glucan supplementation strategies in aquaculture.



2

Long-lived effects of administering β-glucans: Indications for trained immunity in fish

Jules Petit, Geert F. Wiegertjes

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Abstract

Over the past decades, it has become evident that immune-modulation of fish with β-glucans, using injection, dietary or even immersion routes of administration, has stimulating but presumed short-lived effects on both intestinal and systemic immunity and can increase protection against a subsequent pathogenic challenge. Although the exact effects can be variable depending on, among others, fish species and administration route, the immune-stimulating effects of β-glucans on the immune system of fish appear to be universal. This review provides a condensed update of the most recent literature describing the effects of β-glucans on the teleost fish immune system. We shortly discuss possible mechanisms influencing immune-stimulation by β-glucans, including microbial composition of the gut, receptor recognition and downstream signalling. Of interest, in mammalian monocytes, β-glucans are potent inducers of trained immunity. First, we screened the literature for indications of this phenomenon in fish. Criteria that we applied include indications for at least one out of three features considered characteristic of trained immunity; (i) providing protection against a secondary infection in a T- and B-lymphocyte independent manner, (ii) conferring increased resistance upon re-infection and, (iii) relying on key roles for innate immune cell types such as natural killer cells and macrophages. We conclude that several indications exist that support the notion that the innate immune system of teleost fish can be trained. Second, we screened the literature for indications of long-lived effects on innate immunity of fish after administering β-glucans, a criterion which could help to identify key roles for macrophages on resistance to infection. We discuss whether β-glucans, as well-known immune-stimulants, are able to train the immune system of fish and argue in favour of further studies designed to specifically investigate this phenomenon in fish.

1. Introduction

Since the 1990's, when immune-stimulation of fish was still under early development, several investigations have suggested that the provision of β-glucans, either dietary or by supplementary injection, can potentiate the resilience of immune cells (80-82), reduce in vivo stress effects (81, 83) and protect teleost fish against subsequent challenges in vivo (84, 85). Indeed, provided they are applied as a prophylactic measure, it has become evident that β-glucans can be a potential immuno-stimulant, suitable for injection and dietary administration, with well-described but short-lived effects on intestinal immunity, systemic immunity and increased protection from a subsequent pathogenic challenge [reviewed by: (19)]. Yet, detailed knowledge of the receptors involved in recognition of β-glucans and of their downstream signalling is missing for teleosts, leaving obscure whether the observed potentiation should be attributed to direct effects on leukocytes or to indirect effects on, for example, the composition of microbial communities in the gut. Typically, studies investigating the effects of β-glucans have mostly focussed on relatively short-lived effects, in the order of days up to a few weeks, but recent insights in the field of innate immunity provide indications that β-glucans could also have effects for a longer period of time, possibly explained by the phenomenon 'trained immunity' (see paragraph 4.3).

β-glucans are found not only in the cell wall of yeast species, including *Candida albicans* and yeast of the *Saccharomyces* genus (*S. cerevisiae*, or baker's and brewer's yeast), but also in the cell wall of plants including wheat, rye and several *Echinacea* species, seaweeds, mushrooms and other fungi and even in the cell wall of several bacterial species [reviewed by: (21)]. In fact, β-glucans comprise a wide variety of structurally diverse molecules (Figure 1), which can be short or long, linear or branched and have a soluble or insoluble character but which all have in common that they are polymers comprised of repeating units of glucose, linked by β-glycosidic bonds (21, 86). Although β-glucans appear to have their immune-activating capacity in common, there can be clear differences in activity owing to the diversity in structure (21, 87). In particular, large molecular weight β-glucans have stimulatory effects on leukocytes which include the induction of phagocytic, cytotoxic and antimicrobial activities (21). Probably the best-studied and most-applied β-glucans are large molecular weight *S. cerevisiae* and *C. albicans* yeast-derived β-1,3/1,6-glucans.

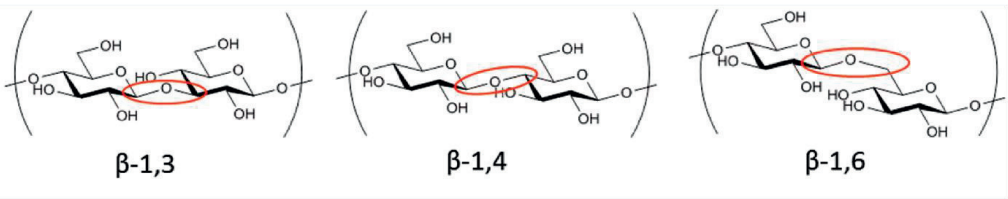

Figure 1. Structure of β-glucan molecules. Examples of different linkages between repeating glucose units, determining the biochemical structure of diverse β-glucans.

2. Oral administration of β-glucans stimulates immune responses in fish

As mentioned above, β-glucans can be a potential immuno-stimulant for fish with clear effects, in particular, on innate immunity (19, 21, 88, 89). These effects may not only depend on the branched structure but may also rely on the non-digestible nature of β-glucans (88). Non-digestible β-glucans may induce alterations in the composition of the gut microbiota and thereby indirectly influence the immune system (see paragraph 4.1) and/or the bacterial community in the gut may help to digest non-digestible oligosaccharides such as β-glucans into short-chain fatty acids with a physiological effect of their own (90). Alternatively, the linear β-1,3 backbone ends up undigested in the proximal part of the intestine, where a proportion is phagocytosed by neutrophilic granulocytes and/or macrophages and degraded by a reactive oxygen species-driven process (91). Of interest, in salmonids, the uptake of laminaran, a linear β-1,3-glucan, via the posterior intestine results in a systemic accumulation in, among others, heart and spleen (92) whereas anal intubation with FITC-labelled yeast particles reveals uptake by mononuclear cells in the intestinal lumen (93). The extent to which yeast and β-glucan particles are digested and/or taken up is still under debate, but it appears that the teleost intestine certainly is capable of uptake of β-glucans. The mechanisms behind antigen sampling and the cells involved in this process and present in the teleost gut are reviewed by Løkka and Koppang in this issue. Here, we build on previous reviews of the subject and add a discussion of the more recent literature (2008–2015 in particular) grouped by (super)order, differentiating salmonids, perciforms and cyprinids based on the assumption that the closer the phylogenetic relationship the more reliable the conclusions. While also briefly discussing other routes of administration such as injection and immersion, the more practical route of oral administration through the diet will receive most attention.

2.1 Salmoniformes (salmonids)

One of the first studies on the protective effects of β-glucans, describing that intraperitoneal injection with a β-1,3/1,6 'M' glucan from S. cerevisiae enhanced resistance against two different bacterial pathogens, was performed in Atlantic salmon (84). It may not come as a surprise that subsequent studies addressing immune-modulating effects of β-glucans were performed in salmonids in particular. Based on the initial observations that β-glucans do indeed have immune-stimulating capacities, at least when injected, subsequent studies often included more practical routes such as oral administration (Table 1). The common picture that emerges from the studies on oral administration in salmonids is a confirmation of the immune-stimulating capacity of β-glucans, although with variable outcomes when it comes to increasing resistance against pathogens. For example, β-glucan treatment appears to increase resistance of Atlantic salmon to sea lice of the species Lepeophtheirus salmonis but not Caligus elongates (94), for

which β-glucan treatment may even lead to a higher infestation (95). Of course, β-glucans should not be considered miracle compounds able to increase resistance to all pathogens at all levels of infection.

Table 1. Effects of oral administration of β-glucans in salmonid fish. Publications are grouped according to species, exact type of β-glucan used as immune stimulant and date of publication. The most pronounced outcomes of each study are summarized as "Results".

Species	Stimulant	Results	Reference
Rainbow trout (*Oncorhynchus mykiss*)	β-1,3/1,6-glucan (lentinan)	Decreased expression of pro-inflammatory genes in response to LPS (investigation by micro-array)	(96)
Rainbow trout	β-1,3/1,6-glucan	Increasing trend in lysozyme activity (not significant) observed in glucan fed trout Glucan fed trout show increased resistance against challenge with *Ichthyophthirius multifiliis* (white spot) Trend visible after short term feeding, significant after feeding for a longer time period	(97)
Atlantic salmon (*Salmo salar*)	β-1,3/1,6-glucan	No significant effect on diarrhoea-like conditions No implications on soybean meal induced enteritis No effect on number of sea lice (*Caligus elongatus*) infested fish or on number of sea lice per fish Significant lower salmon lice (*Lepeophteirus salmonis*) infestation (infested fish and lice per fish	(94)
Atlantic salmon	β-1,3/1,6-glucan	No effect on sea lice infestation, lice per fish even tends to be higher than control Histology showed no adverse effects of glucan enriched feed on intestines	(95)
Rainbow trout	β-1,3-glucan (*Euglena gracilis*) (≥98% purity)	No effect of β-glucan alone or as adjuvant on survival after *Yersinia ruckerii* challenge Down-regulation of expression of pro-inflammatory, acute phase and lysozyme related genes after challenge	(98)
Rainbow trout	β-1,3-glucan	Increased gene expression of cathelicidins 2 and IL-1β in gut epithelial cells Increased number of mucus secreting cells in the intestine	(99)

Studies investigating the effects of β-glucans on maintaining the integrity of the gut have found no adverse effects and provide evidence for an assumed favourable increase in frequency of mucus-secreting cells in the epithelial barrier (95, 99). Of interest, oral administration of rainbow trout with β-glucans appears to down-regulate the expression of immune-regulatory genes (e.g. IL-1β and lysozyme) in the presence of a microbial stimulus (96, 98), but up-regulate the expression of such genes (e.g. IL-1β and cathelicidins (host defense peptides)) in the absence of a microbial stimulus (98, 99). These apparent contrasting effects of β-glucans on the expression of immune-regulatory genes, in the presence or absence of a microbial stimulus, could possibly help explain the variable outcomes with respect to increased resistance against pathogens mentioned above.

To verify and help explain the initial field observations, the number of laboratory-based studies aiming to acquire more detailed knowledge of the immune-stimulating effects of β-glucans in salmonids, have increased considerably. It hasbecome clear that although the exact recognition receptors and downstream signalling routes still remain undefined, the immune-modulating effects of β-glucans on the immune system of salmonid fish should be considered stimulatory. Although the degree of disease protection offered by β-glucans clearly depends on, among others, the infectious agent, it should be noted that oral administration of β-glucans in salmonid species holds great potential as a prophylactic measure.

2.2 Perciformes (bass)

In Nile tilapia, β-glucans can rescue immune-compromised individuals treated with mercuric chloride, by feeding with a diet containing live *S. cerevisiae*, laminaran or purified β-glucans (from *S. cerevisiae*) (Table 2). The most pronounced effect was observed in fish fed with the purified β-glucans, where cellular and humoral immune parameters were restored to control levels and protection against subsequent challenge with *Aeromonas hydrophila* increased from 5% (control diet) to 60% survival (β-glucan enhanced diet) (100). In most of the recent studies performed on bass species, oral administration of β-glucans not only increases innate immune parameters, such as phagocytic capacity and oxidative burst, lysozyme and complement activity (100-103), but also increases protection against challenge with a number of bacterial pathogens including *A. hydrophila* and *Vibrio alginolyticus* (100, 103). In general, the observations on the immune-stimulating effects of β-glucans in perciforms (Table 2) support the findings in salmonids, with a comparable lack of mechanistic knowledge that could help explain recognition, signalling and immune-stimulation.

Table 2. Effects of oral administration of β-glucans in perciform fish. Publications are grouped according to species, exact type of β-glucan used as immune stimulant and date of publication. The most pronounced outcomes of each study are summarized as "Results".

Species	Stimulant	Results	Reference
Nile tilapia (*Oreochromis niloticus*)	β-1,3/1,6-glucan or laminaran	Increased phagocytic activity and index in immunocompromised (IMC) fish due to mercuric chloride exposure); Increased oxidative burst and neutrophil adhesion cells in IMC fish fed β-glucan or laminaran; Increased survival after challenge with Aeromonas hydrophila in normal and IMC fish fed β-glucan;	(100)
Orange spotted grouper (*Epinephelus coioides*)	Mixture of β-1,4; β-1,3 and β-1,6-glucans	Increased lysozyme activity, alternative complement activation, phagocytic activity and oxidative burst; Increased protection against *Vibrio alginolyticus* challenge;	(103)
Gilthead seabream (*Sparus aurata*)	β-1,3/1,6-glucan (99% pure)	Increased IL-1β and IFNγ expression; Increased phagocytosis and phagocytic index;	(101)
Red sea bream (*Pagrus major*)	Heat killed Lactobacillus plantarum (HKLP) in combination with β-1,3/1,6-glucan	β-glucans significant increase the effect of HKLP, with respect to the lysozyme activity, the bactericidal effect, the alternative complement pathway activation and the total serum protein concentrations	(102)

2.3 Cypriniformes (cyprinids)

Increasing attention has been given towards studying immune-modulating effects of β-glucans in cyprinids. As described above for salmonids and perciforms, also in cyprinids, oral administration of β-glucans stimulates a suite of innate immune parameters, again including stimulation of phagocytic capacity and oxidative burst, lysozyme and complement activity (104-107) (Table 3). Several studies in cyprinids have addressed the effect of β-glucan administration on (immune) gene expression (108-111). Among the investigated studies, continuous administration of β-glucans generally appears to result in an increased expression of pro-inflammatory genes, with a gradual decline over time depending on, among others, route of administration and immune organ under investigation (107, 108). Continuous oral administration (25 days) of β-glucans can result in the up-regulation of anti-apoptotic genes in gut and head kidney, and of both anti- and pro-apoptotic genes in the spleen of common carp (109). The effects of β-glucans on apoptosis were further investigated and show that, in vitro, β-glucans can have a significant effect on apoptosis, but only at very high concentrations (112). Taken together, these findings support the notion that oral administration of β-glucans may modulate the intestinal immune response and protect cyprinid fish from an acute (over)reaction (108, 111). Since stimulation requires very high doses of β-glucans (500 µg/mL) to significantly increase apoptosis in head kidney leukocytes (112), it does not appear that oral administration of β-glucans has major effects

on programmed cell death of leukocytes. Strikingly, continuous oral administration of β-glucans up-regulates the expression of TLR3, a pattern recognition receptor assumed important for the recognition and binding of viral double-stranded RNA, leading to the subsequent triggering of a type-I interferon (IFN) response (111). The link between oral administration of β-glucans and up-regulation of a receptor for viral pathogen-associated molecular patterns such as dsRNA does not appear an obvious one and requires further investigation, before it can help to explain the mechanism behind protective effects of β-glucans on resistance against viral pathogens. Overall, it is becoming clear that oral administration of β-glucans stimulates the innate immune system of cyprinids as it stimulates the innate immune system of salmonid and perciform fish species, suggesting that the capacity to stimulate the innate immune system of fish is a capacity intrinsic to (large molecular weight) β-glucans.

Table 3. Effects of oral administration of β-glucans in cyprinid fish. Publications are grouped according to species, exact type of β-glucan used as immune stimulant and date of publication. The most pronounced outcomes of each study are summarized as "Results".

Species	Stimulant	Results	Reference
Common carp (*Cyprinus carpio*)	β-1,3-glucan	Increased oxidative burst Increased lysozyme activity Increased protection against *Aeromonas hydrophila* challenge	(105)
Koi carp (*Cyprinus carpio koi*)	β-1,3-glucan, Chitosan or Raffinose	Increased white blood cell count (WBC) Increased oxidative burst, lysozyme activity, phagocytosis, bactericidal effect	(106)
Common carp	β-1,3/1,6-glucan	Down-regulation of pro-inflammatory genes in gut and head kidney Decrease in IgM titer after *Aeromonas salmonicida* challenge (i.p injection) Increased expression of pro-inflammatory genes in head kidney after challenge but down-regulation in gut	(108)
Common carp	β-1,3/1,6-glucan	No apoptosis in head kidney cells Up-regulation of several anti- and pro-apoptotic genes Differential responses between different organs Upon LPS injection increased expression of pro-apoptotic genes in head kidney, rest of tested organs no effect	(109)
Common carp	β-1,3/1,6-glucan	Increased expression of ß-Defensin 1 and 2 and Mucin5b in skin and ß-Defensin-2 in gills	(110)
Common carp	β-1,3/1,6-glucan	Increased basal CRP levels and alternative complement activation β-glucan augments the CRP and complement response to *Aeromonas salmonicida* challenge Differential effects observed between organs	(107)
Common carp	β-1,3/1,6-glucan	Reduced expression of immune-regulatory genes in the midgut (IL-1β, IL-10 and TNFα) Mx significantly increased upon poly(I:C) injection	(111)
Common carp	β-1,3/1,6-glucan	Increased serum complement activity Increased alternative complement activation	(104)

3. Injection and immersion routes of administration of β-glucans

Although oral administration of β-glucans clearly is among the most practical applications, other routes of administration have also been investigated (Table 4). Maybe not always cost-effective, but intraperitoneal (i.p.) injection certainly is an effective method to deliver β-glucans and stimulate the immune system. For example, a single dose of β-glucans injected i.p. in rainbow trout resulted in a level of protection against infection with the microsporidian, *Loma salmonae,* similar to the level of protection induced by a 3 weeks feeding trial using 10 times higher concentrations of β-glucans. Interestingly, the effects of the single i.p. injection could be measured for a prolonged period of up to 9 weeks in vivo (113) and up to 20 days ex vivo (no further time points measured) (114). Protective and stimulating effects on innate immunity after i.p. injection with β-glucans have also been observed in zebrafish. A single i.p. injection 6 days prior to challenge with *A. hydrophila* reduced the cumulative mortality, with similar although lower effects when injected only 2 days prior to challenge (115). Increasing the frequency of injections further increased the survival of the zebrafish. Although the effects of even a single i.p. injection appear to be rapid and universal, only relatively few studies have addressed this route of administration, while this route could be of wider interest. For example, verification of immune-stimulation after injection of β-glucans could be of interest for injection vaccination protocols.

A potentially interesting alternative application of immune-stimulation induced by β-glucans is provided by the immersion treatment of fertilized eggs, or gametes, of chum salmon (*Oncorhynchus keta*) against infection with *Saprolegnia spp.*: a short treatment of 3 minutes only was sufficient to provide a significant protection against spontaneous infection with this oomycete (116). This finding seems supported by the observation that both, pro- and anti-inflammatory genes were up-regulated after immersion of rainbow trout fry in a solution containing β-glucan (117). Comparable to these studies in salmonids, there has been an increasing interest in applying β-glucans as immersion treatment to cyprinid fish. Although it appears difficult to convincingly show that β-glucan administration by immersion can (also) have a systemic rather than only a local effect on the immune system (118), β-glucans can significantly improve wound healing of carp skin when applied to the water (119). Mucosal organs, including the skin, typically are covered by a layer of mucus which is continuously renewed to prevent pathogen attachment and serves as a vehicle for antimicrobial compounds, complement, and immunoglobulins (120). Administration of β-glucans by immersion, as modulators of mucosal surfaces of the skin or gills could be a promising new area of research, especially now that tools to reliably measure mucosal immunity are becoming available (121). Possible explanations for immune-stimulating effects of β-glucan immersion baths could be sought, for example, in effects on the composition of microbial communities in the skin mucus (see also paragraph 4.1) (122) or increased local populations of alternatively–activated macrophages expressing a

healing phenotype (123). Independent of the exact macrophage phenotype that would develop in the presence of β-glucans, given the most recent indications in humans that trained immunity can be stimulated via recognition of β-glucans by macrophages (see also paragraph 5), there is no doubt that modulation of macrophage function by β-glucans should remain an active area of research of fish immunology.

Table 4. Effects of routes of β-glucan administration other than oral. Publications are grouped according to species, route of administration and date of publication. The most pronounced outcomes of each study are summarized as "Results". The bold black border separates studies in *Salmoniformes* from studies in *Cypriniformes.*

Species	Stimulant	Route of administration	Results	Reference
Rainbow trout (*Oncorhynchus mykiss*)	β-1,3/1,6-glucan (*S. cerevisiae* or ProVale)	Intraperitoneal (i.p.) injection or oral administration	Hypertrophic lesion (xenoma) formation after *Loma salmonae* challenge reduced up to 92.5% (4 mg/mL) by i.p. injected research grade glucan Commercial glucan reduced xenoma formation by 64.5% (4 mg/mL) and 72.8% (10 mg/mL) Higher dose (20 mg/mL) abolished the reduction Oral administration of commercial glucan reduced xenoma formation dose dependently	(113)
Atlantic salmon (*Salmo salar*)	β-1,3/1,6-glucan (laminaran)	I.p. injection dissolved in PBS or encapsulated in nanoparticles	Two days post injection, significant up-regulation in TNFα, IL-1β and IL-10 expression Encapsulated β-glucan invoked a stronger increase in IL-1β expression than not encapsulated glucans	(124)
Atlantic salmon	β-1,3-glucan	I.p. injection	Macrophages show significantly increased oxidative burst Lysozyme and phagocytic activity at 10 and 20 days post injection (no further time point measured)	(114)
Chum salmon (*Oncorhynchus keta*)	β-1,3/1,6-glucans	Treatment of eggs or gametes with glucan solution	Increased embryo and juvenile survival Increased resistance against *Saprolegnia* spp. infection	(116)
Zebrafish (*Danio rerio*)	β-1,3/1,6-glucan	I.p. injection	Increased myelomonocytic cell counts Increased pro-inflammatory cytokine and chemokine expression Increased resistance against *Aeromonas hydrophila* challenge	(115)
Common carp (*Cyprinus carpio*)	β-1,3/1,6-glucan	Bath immersion	Significant increase in wound healing Effects on the cytokine expression profile but differential	(119)

4. Mechanisms influencing immune-stimulation by β-glucans

Immune-modulatory effects of β-glucan administration have been widely observed and are generally considered as stimulatory for the health status of fish (21, 88, 89, 122). One of the proposed modes of action indicates a prime role for the intestinal immune system, where induction of local intestinal inflammation after administration of β-glucans would result in a subsequent increased resistance against pathogens (19). A conclusive mode of action explaining the effects of β-glucans on the immune system of teleosts has yet to be uncovered.

4.1 Composition of microbial communities

The immune-stimulating effects of β-glucans not only depend on their branched structure but also rely on their non-digestible nature (88). Acid-treatment to mimic the effect of stomach-passage, completely abolishes the immune-stimulating effects of β-glucans on macrophages (125). So far, this disruptive effect of low pH values has been reported in a single study only, but it could be important to further investigate to which extent (treatments mimicking) digestive processes can abolish the immune-stimulating capacity of β-glucans when administered orally.

Although beyond the direct scope of this review, one of the modes of action of oral administration of β-glucans could be to induce alterations in the composition of the gut microbiota. Feeding common carp with β-glucan-supplemented diets can modulate the microbial communities in the gut (79). Two weeks of feeding appears to reduce diversity, species richness and number of taxonomic units in the autochthonous (mucosal associated, indigenous) microbiota, a reduction not observed after 4 weeks of feeding. However, in another study, two weeks of feeding resulted in a clear increase rather than reduction in microbial community diversity, possibly explained by differences in samples, size of fish and analysis techniques (126). In sea bass fed with β-glucans for 4 or 8 weeks, pyrosequencing of the intestinal microbiota revealed a transient alteration at the family taxonomic level in the composition of the autochthonous microbiota (127). It took a period of 4 weeks to completely shift the dominance within the microbial communities, which returned to the original composition after another 4 weeks of feeding. The data presented in these studies imply that effects of oral administration of β-glucans on the microbial composition in the gut are present, but could be transient and require further investigation. In line with these findings; the previously-mentioned effect of long-term feeding with β-glucans on TLR3 expression in the gut of carp could also be due to an indirect effect of β-glucans on the composition of the microbiota. In mice, a particular group of commensal bacteria present in the intestine have the ability to induce TLR3 expression, leading to the production of protective IFN-β (76). Given the immunological importance of the skin, especially in teleost fish (128), it should also be of interest to study changes in composition of the microbial community of the skin after bath treatment with β-glucans.

4.2 Receptor recognition and signalling

β-glucans are thought to be internalized by phagocytosis, permitting their destruction by reactive oxygen and nitrogen species and by lytic enzymes in the acidic environment of the phagolysosome, largely based on information in mammals [reviewed by:(86)]. Among the best characterized phagocytic receptors are the opsonising Fc-γ receptor (FcγR) and the complement receptor 3 (CR3). Teleost fish do express genes encoding for the alpha- (CD11(b)-like) and beta-unit (CD18) of CR3 (129, 130), but at least zebrafish appear to miss classical members of the FcRs, although the genome does contain ancestral FcR-like genes (131). A distinct class A member of the Scavenger Receptor (SR) family, MARCO (macrophage receptor with collagenous structure), is present in several fish species, and knockdown experiments in zebrafish confirmed a role for MARCO in rapid phagocytosis of *Mycobacterium marinum* (132). In contrast, a clear role for the class B SR family member, CD36, could not be demonstrated in zebrafish and common carp (133). Toll-like receptors such as TLR2 could possibly sense β-glucans and are expressed in several fish species (134), but the presence of the prototypical C-type lectin receptor (CLR) for β-glucan, Dectin-1, is limited to mammalian genomes (135).

In vitro modulation of fish leukocyte function by β-glucans is an active field of research that might help identify the exact receptor(s) involved and characterize (new) activation routes. For example, recent studies in common carp show that β-glucans can induce and increase robustness of Neutrophil Extracellular Traps (NETs) (136, 137). We studied the production of reactive oxygen and nitrogen species in response to β-glucans in common carp. Untreated zymosan and branched 1,3/1,6 β-glucans induce higher responses than zymosan depleted of TLR-stimulating activity and curdlan, both considered to be Dectin-1-specific ligands (138). The latter finding suggests recognition of β-glucans by multiple pattern recognition receptors on carp macrophages. Clearly, the relative importance of phagocytic receptors such as CR3, members of the SR and CLR superfamilies, and sensing receptors such as TLR2 with respect to β-glucan-stimulated immune responses has yet to be defined for fish. Identification of a C-type lectin receptor in fish with a role equivalent to the Dectin-1 receptor would be a crucial first step to subsequent studies of function based on, for example, the production of soluble receptors for β-glucan binding and carbohydrate competition assays.

4.3 Trained immunity

As mentioned earlier (paragraph 1), recent insights in the field of innate immunity provide indications that β-glucans could have effects over a longer timespan than initially anticipated, that might be explained by the phenomenon trained immunity. Traditionally, innate immune responses are characterized as rapid (hours-days), aspecific and without development of specific memory. In contrast, acquired immune responses are characterized as slow (days to weeks), highly specific

(variable receptors on T and B lymphocytes can specifically recognize almost any antigen) and expressing long-term memory (based upon clonal expansion of memory T and/or B cells). Already, the classification of innate immune responses as aspecific has been challenged and thus, based on the presence of numerous classes of pattern recognition receptors (25, 139) innate immunity no longer is classified as aspecific. At present, also the strict absence of a form of memory for innate immune responses is challenged by a new concept named trained immunity (Figure 2), which is characterized by three criteria: (i) it can be induced after a primary infection or immunization and subsequently provide protection against a secondary infection in a T- and B-lymphocyte independent manner, (ii) it may be less specific than the adaptive immune response but still confers increased resistance upon reinfection of the host and, (iii) innate cell types such as macrophages and natural killer cells are key players in the mechanism, which involves improved pathogen recognition and an increased inflammatory response (56).

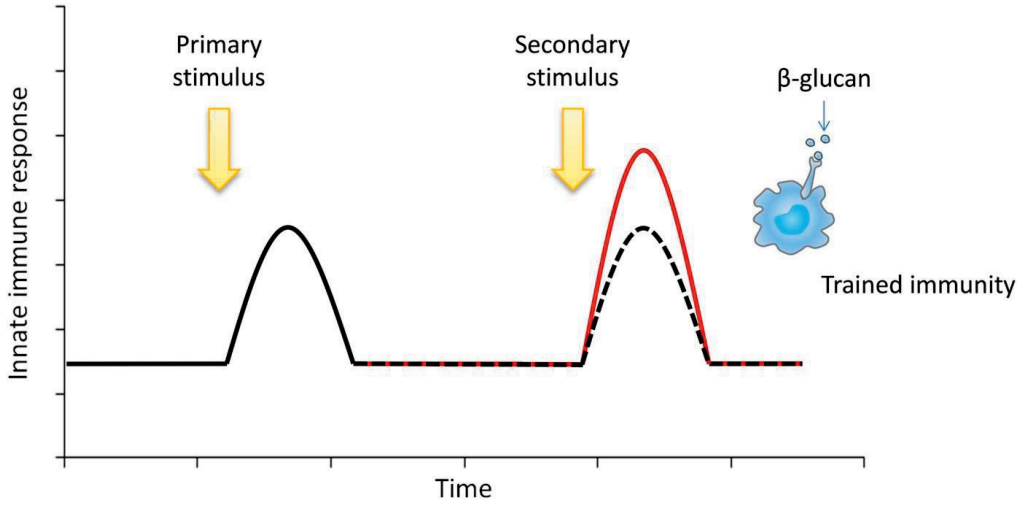

Figure 2. The concept of trained immunity. Classical view on repeated stimulation of innate immunity (dotted black line) and updated view including trained immune responses (solid red line), [adapted from: (57, 66)]. Arrows depict the moment of first and second stimulation with, for example, β-glucans. In the classical view, primary and secondary responses are of equal magnitude, whereas trained immunity results in a heightened secondary response facilitated by cells of the innate immune system.

Of particular interest with respect to the present review, are observations in humans and mice on long-lived immune-modulatory effects of purified β-glucans and/or *C. albicans* on the development of trained immunity. In humans, pre-incubation of peripheral blood monocytes (PBMCs) in vitro with a single dose of *C. albicans* or β-glucans thereof, results in an increased cytokine production of, among others, IL-6 and TNFα, upon secondary stimulation with the same glucans for a relatively long period of up to 2 weeks (58). Besides the increase in cytokine production, a metabolic shift is also observed; pre-incubated PBMCs have

significantly higher glucose consumption and lactate production than control cells (62). In mice, pre-treatment with highly purified β-glucans in vivo, protects animals from a subsequent infection with an unrelated pathogen, such as *Staphylococcus aureus* (140). The innate nature of this form of cross protection was investigated in recombination-activating gene (Rag) knock-out (KO) mice which lack functional B- and T-cells and thus lack functional adaptive immunity. The Rag-KO mice could be fully protected from a lethal infection with *C. albicans*, but only when pre-stimulated with a sub-lethal dose of *C. albicans* (58), a phenomenon proposed to be monocyte-dependent. Despite clear in vitro effects, oral administration of β-glucans appears not to induce pronounced long-lived effects in vivo, in humans (141). However, administration of Bacillus Calmette–Guérin (BCG) does clearly induce trained immunity also in vivo. Effects induced by vaccination with BCG (65, 142), prepared from attenuated live *Mycobacterium bovis*, support the proposed benchmarks of trained immunity that it can elicit cross-specific protection in a T- and B-cell independent manner with innate immune cell types such as macrophages acting as key players (56). Of evolutionary interest, long before the recent discussions on the presence of trained immunity in humans and mice (67), similar cross-specific protection was observed in plants (143-145) and invertebrates (68) which, typically without T and B lymphocytes, can build up a form of immunity able to protect the organism upon a secondary exposure. Owing to the basal position of teleost fish as early vertebrates, it makes evolutionary sense to expect that trained immunity could be an important mechanism determining immune-stimulation of fish by β-glucans.

5. Future perspectives

5.1 Evidence for the presence of trained immunity in fish

Although teleost fish are among the evolutionarily oldest vertebrates with both an innate and classical adaptive immune system (146), there are several examples where innate immune parameters are more active and more diverse in fish than in mammals (147). In line with these findings one would expect trained immunity should not only be present, but could have a pronounced role in the immune system of fish. There are at least a few studies providing preliminary evidence for the presence of a form of trained immunity in fish, primarily based on experiments with mycobacteria. Already in 1986, Olivier et al. observed a long-lived increase in phagocytic activity of peritoneal macrophages from Brook trout (*Salvelinus fontinalis*), for a period up to 33 days after i.p. injection with Modified Freund's Complete Adjuvant (MFCA) containing killed *Mycobacterium butyricum*. Only macrophages from trout injected with MFCA showed a significantly higher bactericidal activity (148). Almost three decades later, the efficacy of injection with (modified) *Mycobacteria* was further investigated using BCG. Vaccination of Japanese flounder (*Paralichthys olivaceus*) with BCG resulted in an up-regulation of pro-inflammatory cytokines and conferred protection against *Mycobacterium sp.* (149). Also vaccination of Amberjack (*Seriola dumerili)* with BCG led to protection

against challenge with *Mycobacterium sp.* (150). Importantly, these researchers could measure cross-specific protection, one of the proposed benchmarks of trained immunity. The cross-specific protection could be induced in Japanese flounder by BCG, shown by challenge with *Nocardia seriolae*, and was possibly mediated by bacteriolytic activity of the serum (151). The other benchmark of trained immunity; that cross-specific protection occurs in a T- and B-cell independent manner (56), was also studied in fish. Exposure of Rag-KO zebrafish to a sub-lethal infection with *Edwardsiella ictaluri* significantly protected the same animals from a subsequent lethal infection with the same bacteria. Crucially, protection could be transferred to naïve Rag-KO individuals by injection with kidney leukocytes from animals pre-exposed to the sub-lethal infection (152). Together, these studies provide first indications for our hypothesis that trained immunity should be present in teleost fish and provide arguments that the innate immune system of fish can be trained by pre-exposure to sub-lethal pathogens. It remains to be investigated if trained immunity indeed has the predicted, pronounced role in the immune defense of fish and is indeed mediated by innate immune cell types such as macrophages.

5.2 Trained immunity induced by β-glucans: revisiting the literature

In mammalian monocytes, β-1,3-glucans purified from *C. albicans* are potent inducers of trained immunity (58, 62, 140). Until this moment, no studies have (knowingly) investigated induction of trained immunity in teleost fish in after administering β-glucans. After a first screen of the literature for clear indications of β-glucan-induced trained immunity in fish, we realized it was difficult to unambiguously ascribe observed effects to at least one of the characteristic criteria defined for trained immunity [paragraph 5.3 and (56)]. Therefore, based on the assumption that innate immune responses generally are short-lived, we re-screened the literature for indications of long-lived effects on innate immunity after administering β-glucans (Table 5).

For example, a single i.p. injection of zebrafish (1–1.5 g body weight) with β-1,3/1,6-glucan provided partial protection against challenge with *A. hydrophila* at 6 days, but not at 2 or 4 days post-injection (115). Similarly, but spanning a much longer time period, i.p. injection of yellowtail (*Seriola quinqueradiata*) with β-1,3/1,6-glucan induced increased resistance upon challenge with *Pasteurella piscicida* for a prolonged period of up to 45 days post-injection (153). Also, i.p. injection of Blue Gourami (*Trichopodus trichopterus*) with β-1,3-D-glucan (laminarin), induced increased resistance upon infection with *A. hydrophila* for a prolonged period of 22 days post-injection, which was the last time point measured. Of interest, the latter study provided insights into possible protective mechanisms by measuring an increase in phagocytosis-induced oxidative burst, suggestive of a key role for innate immune cell types such as macrophages (154). Indeed, phagocytes could be responsible for long-lived effects induced by β-glucans since i.p. injection of Atlantic salmon with β-1,3-glucan leads to an increase in oxidative burst, phagocytosis and lysozyme activity of macrophages

(114). Most interesting in the present context; the latter study showed that increased macrophage activity was still measurable at 10-20 days post-injection, providing clear indications that single i.p. injections with β-glucans can induce long-lived effects in fish.

Table 5. Innate immune memory-related effects of β-glucan administration in fish. Publications are presented according to order of discussion in the text. The column "Experimental set up" gives a concise summary of the parameters such as timing and route of administration. The most pronounced outcomes of each study are summarized as "results".

Species	Stimulant	Experimental set up	Effects	Reference
Zebrafish (*Danio rerio*)	β-1,3/1,6-glucan	Intraperitoneal injection 6, 4 or 2 days prior to bacterial challenge.	Lowest cumulative mortality upon injection 6 days prior to the bacterial challenge	(115)
Yellowtail (*Seriola quinqueradiata*)	β-1,3/1,6-glucan	Intraperitoneal injection on day 0 followed by a bacterial challenge on 15, 25, 35 or 45 days post injection	Minor increase in RPS (relative percentage survival) upon challenge on day 15 and 45 post injection	(153)
Blue gourami (*Trichopodus trichopterus*)	β-1,3-D-glucan (Laminarin)	Intraperitoneal injection followed by bacterial challenge 14 or 22 days post injection, or by chemilumine-scence assay 8, 14 and 22 days post injection	Increased oxidative burst in phagocytes up to 22 days post injection Increased survival upon challenge 22 days post injection	(154)
Atlantic salmon (*Salmo salar*)	β-1,3-glucan	I.p. injection of β-glucan solution. Isolation of macrophages 10 and 20 days post injection.	Significant increased oxidative burst, lysozyme and phagocytic activity at 10 and 20 days post injection	(114)
Rainbow Trout (*Oncorhynchus mykiss*)	β-1,3/1,6-glucan	Oral administration of glucans. Subjects were fed control diet for 4 weeks, experimental diet for 2 weeks and finally 4 weeks of control diet.	Glucans increase ConA induced proliferation of PBLs Glucans increase Ab titer in units/uL Increase in complement activation 8 weeks post vaccination (vaccination on week 0)	(155)
Sea Bass *(Dicentrarchus labrax)*	β-1,3/1,6-glucan	Oral administration of glucans. Subjects were sampled 4 weeks after the last feeding cycle	Increased serum complement activity and lysozyme activity	(156)
Orange spotted grouper *(Epinephelus coioides)*	Mixture of β-1,4; β-1,3 and β-1,6-glucans	Oral administration for 12 days followed by bacterial challenge at 0, 3, 6, 9, 12 and 15 days after switching back to control diet.	Increased survival from bacterial challenge (up to 15 days after switching to control diet)	(103)

Continuous feeding with β-glucans for a number of subsequent days also appears to induce long-lived effects on the immune system of fish. For example, rainbow trout fed with β-1,3/1,6-glucans for a period of 2 weeks still showed higher antibody responses after vaccination against enteric redmouth disease and higher concanavalin A-induced proliferation of head kidney derived leukocytes 4 weeks after switching back to a control diet (155). Sea bass fed with β-1,3/1,6-glucan-enriched diets for 3 feeding cycles of 2 weeks followed by 10 weeks of control diet, showed higher serum complement and lysozyme activity than fish fed a control diet only (156). Grouper fed a diet containing a mixture of mushroom-derived β-1,4; β-1,3 and β-1,6-glucans for a continuous period of 12 days still showed higher protection against challenge with *V. alginolyticus* 15 days after switching back to a control diet (103).

6. Concluding remarks

The studies discussed here provide indications in existing literature for long-lived effects stimulated by β-glucans, possibly based on key roles for macrophages. There also are several studies that support the notion that the innate immune system of teleost fish can be trained. Whether β-glucan administration, either by injection, bath or oral route, indeed triggers trained immunity in a manner similar to what is observed for humans and mice requires more detailed studies specifically designed to investigate the phenomenon of trained immunity in fish. For example, it could be of interest to analyse, in detail, long-lived effects of stimulation with β-glucans in in vitro models based on purified populations of innate immune cell types, among which macrophages. Such studies would provide fundamental knowledge on mechanisms basal to trained immunity and conserved in cold- and warm-blooded vertebrates.

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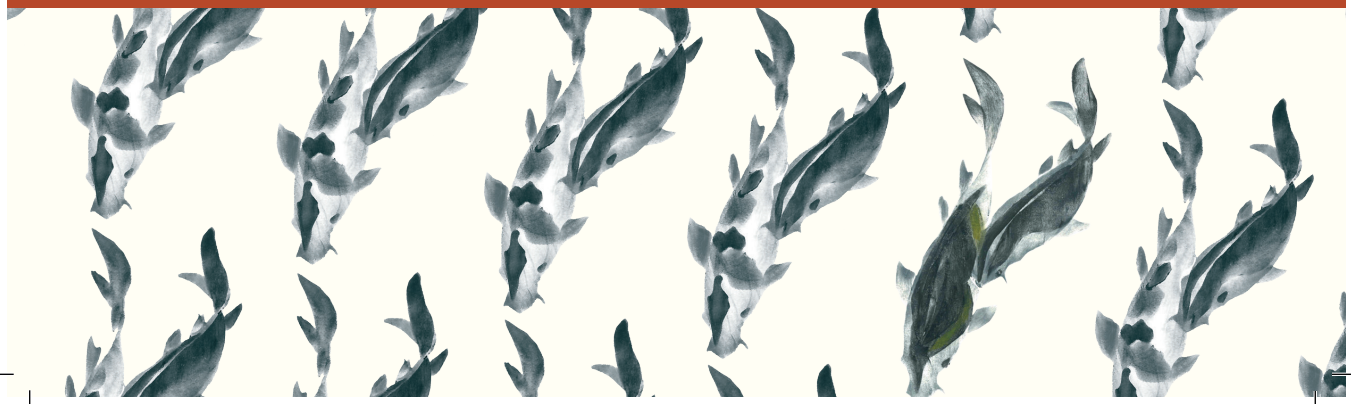

3

Evidence of trained immunity in teleost fish: conserved features in carp macrophages

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Abstract

Trained immunity is a form of innate immune memory best described in mice and humans. Clear evidence of the evolutionary conservation of trained immunity in teleost fish is lacking. Given the evolutionary position of teleosts as early vertebrates with a fully developed immune system, we hypothesize that teleost myeloid cells show features of trained immunity common to those observed in mammalian macrophages. These would at least include the ability of fish macrophages to mount heightened responses to a secondary stimulus in a non-specific manner. We established an *in vitro* model to study trained immunity in fish by adapting a well-described culture system of head kidney-derived macrophages of common carp. A soluble NOD-specific ligand and a soluble β-glucan were used to train carp macrophages, after which cells were rested for six days prior to exposure to a secondary stimulus. Unstimulated trained macrophages displayed evidence of metabolic reprogramming, as well as heightened phagocytosis and increased expression of the inflammatory cytokines *il6* and *tnfa.* Stimulated, trained macrophages showed heightened production of reactive oxygen and nitrogen species as compared to the corresponding stimulated but untrained cells. Measurement of the production of reactive oxygen species proved particularly informative to identify ligands able to train carp macrophages. We discuss the value of our findings for future studies on trained immunity in teleost fish.

Introduction

Traditionally, innate immune responses have been viewed as rapid, relatively non-specific and lacking immunological memory. New insights have challenged this view, introducing a novel concept referred to as trained immunity, which is defined as a heightened response to a secondary infection that can be exerted toward both homologous and heterologous microorganisms (56). Typical criteria of trained immunity include: 1) induction upon primary infections or immunizations and subsequent protection against a secondary infection, in a T- and B-lymphocyte independent manner, 2) a response that is less specific than an adaptive immune response but that still confers increased resistance upon reinfection of the host and, 3) the involvement of innate cell types such as NK cells and macrophages involved in improved pathogen recognition and an increased inflammatory response.

Initial experiments showed that *Rag*$^{-/-}$ mice exposed to a sub-lethal *Candida albicans* infection were better protected against a subsequent lethal dose of these yeasts, whereas monocyte-deficient mice did not show this increased protection, suggesting a form of memory present in the myeloid compartment. Furthermore, *in vitro* re-exposure of human PBMC to *C. albicans* induced a significant IL6 and TNFα response, even after a resting period of six days following primary exposure to *C. albicans*. Activation of the Dectin-1/RAF proto-oncogene serine/threonine-protein kinase (Raf-1) pathway in PBMCs by β-glucans present in the cell wall of *C. albicans* proved crucial for inducing trained immunity (58). Concurrently, non-specific protection induced by Bacillus Calmette-Guérin (BCG) vaccination also resulted in increased cytokine production in monocytes upon secondary exposure to unrelated pathogens in a T- and B-cell independent manner. This process, active for prolonged periods of up to three months after vaccination, proved dependent on recognition by nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and signalling via receptor-interacting serine/threonine-protein kinase 2 (Rip2) (65). These studies highlighted that the pathways linked to Dectin-1/Raf-1 (β-glucans) and/or NOD2/Rip2 (BCG) could be considered central to stimulation of trained immunity in monocytes.

Further studies that sought to unravel the underlying mechanisms of trained immunity revealed crucial roles for epigenetic modifications and metabolic reprogramming. Trained immunity, induced by exposure of monocytes to β-glucans or BCG, was associated with long-lived epigenetic modifications in the form of increased trimethylation of histone 3 (H3) lysine 4 (H3K4me3) and acetylation of H3 lysine 27 (H3K27ac), both activation markers (58, 62, 64), and with decreased trimethylation of H3 lysine 9 (H3K9me3), a repressor marker (60). These epigenetic modifications, positioned at promotor sites of immune genes, helped explain the heightened IL6 and TNFα response in PBMC after a resting phase of six days following primary exposure, and appear key to the inflammatory response associated with trained immunity. Epigenetic modifications were also noted at promotor sites of metabolic genes, among which the mechanistic target

of rapamycin kinase (mTOR) and hexokinase 2 (HK2), introducing metabolic reprogramming as a mechanism underlying trained immunity. Indeed, crucial to the onset of trained immunity appears to be a metabolic shift from oxidative phosphorylation towards glycolysis, orchestrated via the protein kinase B (Akt)/mTOR/hypoxia-inducible factor 1α (HIF1α) pathway (62, 64). Transcriptome analysis in mice, comparing constitutive expression of trained and untrained monocytes, revealed significantly higher expression of several genes associated with glycolysis, among others aldehyde dehydrogenase (*Aldh2*), ADP-dependent glucokinase (*Adpgk*) and bisphosphoglycerate mutase (*Bpgm*) (62). Further, activation of the cholesterol synthesis pathway was also noted as an important mechanism underlying trained immunity (61). The metabolic shift resulted in the accumulation of several metabolites such as fumarate, a substrate of the TCA cycle and mevalonate, an intermediate in the cholesterol synthesis pathway. Both metabolites could, by themselves, induce epigenetic modifications and induce cytokine profiles associated with trained immunity (59-61). In addition, blocking metabolic shifts towards glycolysis or glutaminolysis abolished these epigenetic modifications and cytokine profiles, illustrating the strong connections between epigenetic modifications, metabolic reprogramming and trained immunity.

Little or nothing is known about the conservation of aspects of trained immunity in fish, including the aspects described above. Two recent studies in zebrafish have touched upon such aspects, including observations on an increased antiviral state in *rag*$^{-/-}$ zebrafish associated with increased transcription of innate immune genes (157), and a study describing (the lack of) effects of pre-exposure to different pathogen associated molecular patterns on subsequent viral challenges (158). Given the evolutionary position of teleost fish as early vertebrates with a fully developed immune system (146), it is likely that innate immune cells of fish such as macrophages should possess the ability to express specific features of trained immunity. In fact, given that numerous examples exist of a relatively prominent role of innate immunity in fish (147, 159-162), trained immunity could be considered highly relevant to this group of cold-blooded vertebrates. Recently, a review summarized potential benefits and constraints of exploiting trained immunity to our benefit in larval aquaculture (163). We already summarized long-lived effects of β-glucans in fish (20) and hypothesized these might well be explained by features of trained immunity.

In this study, we sought to investigate the hypothesis that trained immunity is conserved in the teleost fish, common carp. To this end, a well-established *in vitro* culture of macrophages derived from the head kidney (164), the fish equivalent to mammalian bone marrow, was adapted to study the mechanisms of trained immunity in common carp. Given that the existence of a true Dectin-1/Raf-1-like pathway in fish is still elusive (42), but that a NOD2/Rip2-like pathway was proven to exist in zebrafish (165-167), we used a soluble NOD-specific ligand to train carp macrophages. Following a resting period of six days, alike the experimental set-up used to study trained immunity in human PBMC (168), unstimulated trained macrophages showed increased phagocytosis, elevated constitutive expression of

several immune- and glycolysis-related genes as well as a metabolic shift from oxidative phosphorylation towards glycolysis, the latter measured as increased production of lactate. Stimulated trained macrophages typically showed an increased inflammatory response measured as increased production of reactive oxygen species (ROS) and nitrogen radicals (NO). Altogether, our data provide evidence that innate immune cells of teleost fish, such as macrophages, possess the ability to express specific features of trained immunity.

Methods

Animals

European common carp (*Cyprinus carpio carpio* L.) of the R3 × R8 strain were used, which results from crossing the Hungarian R8 strain and the Polish R3 strain (169). Carp were bred and raised in the aquatic research facility (Carus-ARF) of Wageningen University, at 20 – 23°C in recirculating UV-treated water and fed pelleted dry food (Skretting, Nutreco) twice daily. All experiments were performed with the approval of the animal experiment committee of Wageningen University (DEC number: 2017.W-0034).

Macrophage culture and training stimulus

Carp were euthanized with 0.3 g/L Tricaine Methane Sulfonate (TMS) (Crescent Research Chemicals, Phoenix, USA) in aquarium water buffered with 0.6 g/L sodium bicarbonate and bled via the caudal vein. Head kidney was isolated and total head kidney leukocytes were separated on a Percoll™ (GE healthcare, Fisher Scientific) density gradient. Macrophages were obtained by culturing head kidney leukocytes in complete NMGFL15 medium at 17.5×10^6 cells/flask (75-cm^2, Corning (CORN430725U)) for 6 days at 27°C without CO_2 as previously described (164). From here onwards 6-days cultured, head kidney-derived macrophages will be referred to as 'macrophages'.

Culture flasks were placed on ice for 15 min, macrophages were harvested by gentle scraping, washed once with ice-cold PBS and centrifuged at 450 x *g* for 10 min. Subsequently, cells were resuspended in RPMI-1640 culture medium with 25 mM Hepes, supplemented with L-glutamine (2 mM), penicillin G (100 U/ml), streptomycin sulphate (50 mg/ml) ('cRPMI') and heat-inactivated pooled carp serum (2.5% v/v) ('cRPMI-2.5').

For selection of the optimal concentration of training stimulus, production of reactive oxygen species (ROS) and of nitric oxide (NO) was measured according to the protocol described below. For NO production, macrophages were seeded at a density of $5x10^5$ cells/well in 96-well culture plates (Corning, CORN3596) and stimulated for 24h with various concentrations of the NOD-ligand 'soluble sonicated peptidoglycan from *E. coli* K12' (PGN-ECndss, Invitrogen) resuspended in endotoxin-free LAL water, from now on referred to as 'PGN'. For ROS production,

macrophages were seeded at a density of $5x10^5$ cells/well in white 96-well plates (Corning, CLS3912) and stimulated for 2h with various concentrations of PGN.

Optimization of the training period

Macrophages were obtained as described above, and the training stimulus (1 µg/ml PGN) was added directly to the culture flask (20 µL of a 1 mg/mL solution in 20 mL medium) for 2h at 27°C without CO_2. Cells were harvested as described above and washed 3x in ice-cold PBS containing 0.02% (w/v) EDTA (PBS-EDTA) to remove any unbound stimulus. Cells were then seeded at a density of $5x10^5$ cells/well in 96-well culture plates and either incubated in cRPMI supplemented with heat-inactivated pooled carp serum (1.5% v/v) (cRPMI-1.5) or stimulated for additional 24h with 1 µg/ml PGN. NO production in the cell culture supernatants was measured as described below.

***In vitro* set-up for studying trained immunity**

Macrophages were trained by adding PGN directly to the flask at a final concentration of 1 µg/mL and incubated for 2h at 27°C in the absence of CO_2. Alternatively, laminarin (tlrl-lam, InvivoGen) at a concentration of 50 µg/mL was added (100 µL of a 10 mg/mL solution in LAL water, in 20 mL medium). As a control, cells were exposed to LAL water in a volume equal to the training stimulus. Cells were then harvested as described above and washed 3x in ice-cold PBS-EDTA to remove any unbound stimulus. The obtained cells will be referred to as 'trained macrophages' or 'untrained macrophages'. Sub-sets of trained and untrained macrophages were always analysed for cell viability with Trypan blue exclusion, and for reactivity with an NO assay, as described in section 2.6. The remainder of the cells was seeded at 2.5×10^6 macrophages in 20 mL of cRPMI-2.5 in 75-cm^2 culture flasks (T-75 TC treated, 0030711114, Eppendorf). Cells were incubated at 27°C for additional six days (resting period) before harvesting as described above. Subsequently, trained and untrained macrophages were washed once with ice-cold PBS, centrifuged at 450 x *g* for 10 min and resuspended in cRPMI. Cell growth, viability and morphology of trained macrophages after the resting period were comparable to that of untrained macrophages. A schematic overview of the *in vitro* set-up is depicted in Figure 1.

Reactive oxygen species production

Production of reactive oxygen species (ROS) was determined by a real time luminol-ECL assay, as previously described (170). Trained or untrained macrophages were seeded at a density of $5x10^5$ cells/well in white 96-well plates (Corning, CLS3912) and stimulated with: zymosan (tlrl-zyd, InvivoGen, 250 µg/mL; heterologous stimulus), PGN (10 µg/mL; homologous stimulus), cRPMI (unstimulated control), PMA (P8139, Sigma-Aldrich, 1 µg/mL; stimulated control). Chemiluminescence emission was measured in real time (every 2 min for 120 min)

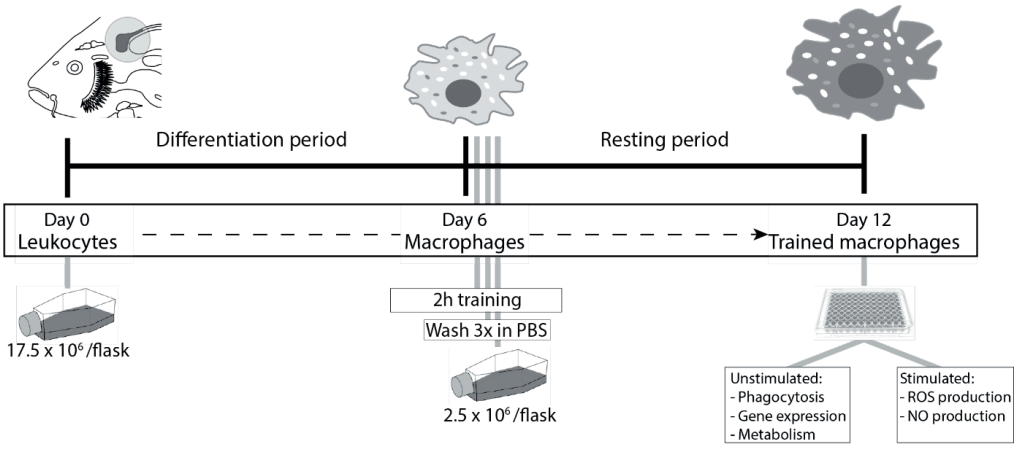


Figure 1. schematic representation of the *in vitro* experimental set up to obtain trained macrophages. On day 0, leukocytes are collected from common carp head kidney and cultured for 6 days to allow for differentiation into macrophages (differentiation period). On day 6, macrophages are or are not exposed to the training stimulus for 2h in the culture flask. Subsequently, cells are harvested and washed 3 times in ice-cold PBS-EDTA. Cells are transferred to new culture flasks at a fixed density per flask and cultured for another 6 days (resting period). On day 12, trained macrophages are harvested and used for subsequent analysis. Macrophages not exposed at day 6 were treated similarly and served as untrained controls.

with a FilterMax F5 Multi-Mode Microplate Reader at 27°C and expressed as area under the curve. Fold changes were calculated as the area under the curve of trained or untrained stimulated macrophages relative to untrained unstimulated control (cRPMI).

Nitrogen radicals production

Production of nitrogen radicals (NO) was determined as nitrite accumulation using the Griess reaction, as previously described (171). Trained or untrained macrophages were seeded at a density of $5x10^5$ cells/well in 96-well culture plates (Corning, CORN3596) and stimulated with: zymosan (250 µg/mL; heterologous stimulus), PGN (10 µg/mL; homologous stimulus), cRPMI (unstimulated control), LPS (L2880, Sigma-Aldrich, 50µg/mL; stimulated control). After 15h at 27°C in the presence of 5% CO_2, nitrite production was measured at OD_{540}, using a FilterMax F5 Multi-Mode Microplate Reader and quantified using a sodium nitrite ($NaNO_2$) standard curve. Fold changes were calculated as production of nitrite by trained or untrained stimulated macrophages relative to untrained unstimulated control (cRPMI).

Phagocytosis analysis

Analysis of phagocytic capacity was performed by flow cytometry (FACS) as previously described (172), with minor modifications. Briefly, trained or untrained macrophages ($5x10^4$ cells/well in 96-well cell culture plates (Corning, CORN3596)) were incubated with fluorescent beads (PSF-001UM Red, MagSphere; cell:bead

ratio of 1:10) for 120 min at 27°C in the presence of 5% CO_2. Subsequently, macrophages were treated with 0.25% (v/v) trypsin-EDTA (Gibco, 11560626, Fisher Scientific) for 10 min, resuspended in ice-cold FACS buffer (0.5% (w/v) BSA (Roche), 0.01% (w/v) NaN_3 in PBS), washed twice with ice-cold FACS buffer and centrifuged at 450 x *g* for 5 min. Finally, macrophages were resuspended in FACS buffer and phagocytosis was quantified using a FACS Canto A (BD Biosciences); data were analysed using FlowJo V10 (BD Biosciences). Phagocytic activity was calculated as the relative proportion of cells that ingested at least 1 bead. Phagocytic capacity was calculated as the relative proportion of cells that ingested ≥3 beads. Fold changes were calculated as phagocytic activity or phagocytic capacity of trained unstimulated macrophages relative to untrained unstimulated controls.

Gene expression analysis

Gene expression was analysed by directly lysing 1.5 × 10^6 trained or untrained macrophages (n = 3 independent cultures) in RLT buffer, immediately after harvest on day 12. Total RNA was isolated using the RNeasy Kit (QIAGEN) including on-column DNase treatment, according to the manufacturer's instructions, and stored at −80°C. Prior to cDNA synthesis, 500 ng total RNA was treated with DNase I, Amplification Grade (Invitrogen), and cDNA was synthesized using random primers (300 ng) and Superscript III First-Strand Synthesis for RT-PCR (Invitrogen). cDNA samples were diluted in nuclease-free water prior to real-time PCR using the primers listed in Table I. Gene expression was measured by RT-qPCR analysis using ABsolute qPCR SYBR Green Mix (Thermo Scientific) in a Rotor-Gene 6000 (Corbett Research), and fluorescence data were analysed using Rotor-Gene Analysis software version 1.7. The relative gene expression of unstimulated trained versus untrained macrophages was measured immediately after the resting period (day 12). The relative expression ratio (R) was calculated according to the Pfaffl method (173) based on the take-off deviation of the unstimulated trained sample versus each of the unstimulated untrained controls at the same time point, and normalized relative to the *s11* protein of the 40s subunit as reference gene (Table I).

Table I. Overview of RT-qPCR primers used for in the current study. cypCar numbers identify ORFs in the draft carp genome (bioproject: PRJNA73579) that were also confirmed by RNA sequencing, LHQP number refers to the accession number of the associated scaffold.

Primer	Forward (5′ – 3′)	Reverse (5′ – 3′)	GenBank Accession No.
40s	CCGTGGGTGACATCGTTACA	TCAGGACATTGAACCTCACTGTCT	AB012087
il6a	CAGATAGCGGACGGAGGGGC	GCGGGTCTCTTCGTGTCTT	KC858890
il6b	GGCGTATGAAGGAGCGAAGA	ATCTGACCGATAGAGGAGCG	KC858889
tnfaa1	GAGCTTCACGAGGACTAATAGACAGT	CTGCGGTAAGGGCAGCAATC	AJ311800

tnfaa2	CGGCACGAGGAGAAACCGAGC	CATCGTTGTGTCTGTTAGTAAG TTC	AJ311801
tnfab1	GAAGACGATGAAGATGATACC AT	AAGTGGTTTTCTCATCCTCAA	cypCar_00029601, LHQP01065580
tnfab2	CTTGGACGAAGCCGATGAAGA C	ATCTTGTGACTGGCAAACA	cypCar_00023012, LHQP01037150
adpgk-1	GGCACCACTGAACTTCT	GCGTGACCTCTGAAAACAG	cypCar_00013411, LHQP01005743
adpgk-2	GCAAGCCGTGGATATTACA	GCGTGAGATGGAAGGA	cypCar_00024520, LHQP01021894
aldh2.1-1	TCCAGAACTTTCCCACAA	GCAGATAACCTCACCAGT	cypCar_00011521, LHQP01009285
aldh2.1-2	GATTCCTGCCCCGAGTC	TTCTCCACATCCGCCTTC	cypCar_00046381, LHQP01040595
bpgm-1	CGCCACCCCCCATTGAGGAGA	GCAGAGATGAGGACTGTTTG	cypCar_00001430, LHQP01009643
bpgm-2	CTAAACGAGCGGCACTAC	GGGCAGTTCCTCCTTT	cypCar_00018360, LHQP01029542

Lactic acid production

Production of lactic acid was measured using a lactate colorimetric assay (Kit II K627, Biovision), including an optional filtration step (Amicon 10K spin column Z677108-96EA, Sigma Aldrich), according to the manufacturer's instructions. Briefly, macrophages ($5x10^5$/well, 96-well culture plate (Corning, CORN3596)) were incubated in 150 µL cRPMI-1.5 for 24h at 27°C + 5% CO_2, after which supernatants from triplicate wells (from n = 5 independent cultures) were pooled and filtered. For each pooled sample, a volume of 10 µL was diluted 5x in lactate assay buffer and transferred to 96-well plates. Subsequently, 50 µL reaction mix composed of lactate substrate mix (2 µL), lactate enzyme mix (2 µL), and lactate assay buffer (46 µL), was added to each sample and incubated for 30 minutes at RT. Optical density was measured at 450nm and concentration of extracellular lactate was calculated based on a lactic acid calibration curve supplied by the manufacturer. Fold changes were calculated as lactic acid production of trained unstimulated macrophages relative to untrained unstimulated controls.

Intracellular fumarate accumulation

Accumulation of intracellular fumarate was measured using a fumarate colorimetric assay (K633, Biovision) according to the manufacturer's instructions, including an optional filtration step (Amicon 10K spin columns; as above). Briefly, macrophages ($5x10^5$/well, 96-well culture plate (Corning, CORN3596)) were incubated in 150 µL cRPMI-1.5 for 24h at 27°C, after which supernatants were removed and the macrophages were lysed in 50 µL fumarate assay buffer (n = 4 independent cultures). Cell lysates from duplicate wells were pooled and filtered as mentioned above. For each pooled sample, 25 µL were diluted 2x in fumarate assay buffer and transferred to a 96-wells plate. Subsequently, 100 µL reaction mix composed of fumarate developer mix (8 µL), fumarate enzyme mix (2 µL),

and fumarate assay buffer (90 µL), was added to each sample and incubated for 30 minutes at RT. Optical density was measured at 450 nm and concentration of intracellular fumarate was calculated based on a fumarate calibration curve supplied by the manufacturer. Fold changes were calculated as fumarate accumulation in trained unstimulated macrophages relative to untrained unstimulated controls.

Statistical analysis

Statistical analyses were performed using SPSS (v23.0) and differences were considered significant if $p \leq 0.05$. Data presented as 'fold change' were tested for significance after log transformation. Data used for phagocytosis activity and capacity were first logit-transformed. All data, after testing for Gaussian distribution using the Shapiro–Wilk test, were analysed as paired data to eliminate interference caused by high variability between individual cultures. Optimization of training period was tested with a Friedman test on untransformed $NaNO_2$ values (µM), followed by a Dunn's post-hoc test. Comparison between untrained and trained macrophages of ROS and NO production, and comparison of constitutive gene expression was performed with a linear mixed model, followed by an LSD post-hoc test. Comparison of phagocytic activity and phagocytic capacity was performed with a paired samples t-test. Comparison of lactate production and fumarate accumulation was performed with an independent samples T test. For multiple comparisons of the stimulatory effect of PGN versus laminarin, a multivariate analysis followed by LSD post-hoc test was used.

Results

Soluble peptidoglycan can be used as primary stimulus to induce trained immunity in carp macrophages

Given that induction of trained immunity in human and mouse monocytes could be achieved via stimulation with a NOD2 ligand, we used the NOD1/2-specific soluble peptidoglycan (PGN) as a ligand to stimulate carp macrophages. To determine the optimal concentration, on day 6 of culture, macrophages were stimulated with various concentrations of PGN. Induction of NO was measured in cell supernatants after 24h (Figure 2A) whereas ROS production was measured in real-time for 2h and expressed as relative light units (Figure 2B). A dose-dependent response was observed for both assays and based on this analysis, subsequent experiments were performed with a concentration of 1 µg/mL PGN as primary stimulus to induce trained immunity since this dose stimulated a robust but not maximal response.

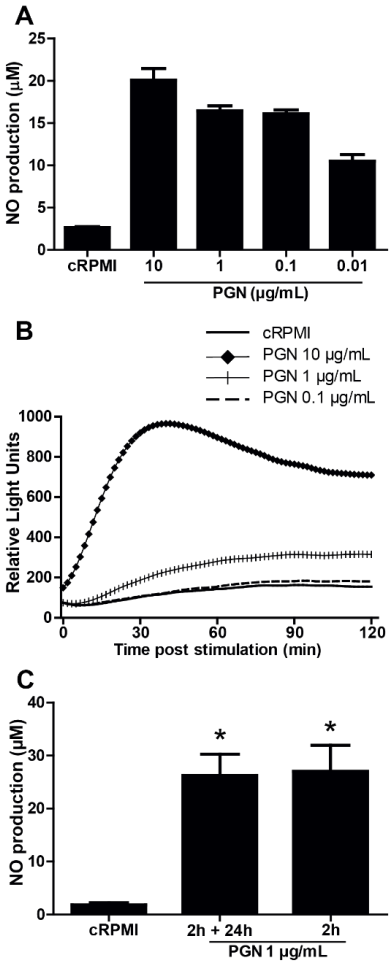


Figure 2. Peptidoglycan (PGN) induces a dose-dependent response in carp macrophages. After the differentiation period (day 6), carp macrophages were harvested, seeded in 96 well plates (5 x 10^5 cells/well) and stimulated with cRPMI or with the indicated concentration of PGN. A. Dose-dependent analysis of PGN on Nitric oxide (NO) production. NO production was measured as $NaNO_2$ in the supernatant 24h post-stimulation. Bars indicate mean + SD of triplicate measurements from one representative experiment out of n=3 performed independently. B. Dose-dependent analysis of PGN on ROS production. Real-time ROS production was measured immediately following stimulation. Lines indicate acquisition of light emission in one representative experiment out of n=3 performed independently. C. Time course analysis of PGN on NO production. Cells were stimulated for 2h by directly adding PGN or RPMI to the flask. Cells were then seeded and either incubated in cRPMI-1.5 or stimulated for additional 24h with PGN (2h + 24h). NO production was measured as $NaNO_2$ in the supernatant 24h post-seeding. Bars indicate mean + SEM of n=5 independently performed experiments. Asterisk (*) indicates significant difference relative to the cRPMI control as assessed with a Friedman test, followed by a Dunn's post-hoc test (Figure 2C).

After having determined the optimal concentration of the training stimulus, we next investigated the duration of the primary stimulation required to train macrophages. Previous studies reported that exposure for 24h to the training stimulus followed by a 6-day resting period, was required for optimal training of human or mouse macrophages (168). In our case we tested whether 24h or an even shorter period would be suitable to train carp macrophages. When carp macrophages were stimulated for a total of 26h (2h + 24h) or for only 2h with PGN, in all cases a significantly higher NO production was measured relative to the unstimulated untrained cells (RPMI) with no difference between the two durations of treatment (Figure 2C). This suggested that a stimulation period as short as 2h was sufficient to stimulate carp macrophages. Altogether, soluble PGN induced a dose-dependent production of NO and ROS in carp macrophages and a 2h primary stimulation with 1 µg/ml PGN followed by a 6-days resting period was selected to further investigate trained immunity in carp macrophages.

Trained macrophages show heightened innate immune responses

Next, we investigated whether the response of trained macrophages differed from that of untrained macrophages upon re-stimulation with either the training stimulus (PGN, homologous) or with a different stimulus (zymosan, heterologous), (for experimental set-up see Figure 1 and 3A). Stimulation with zymosan resulted in comparable kinetics of ROS production between trained and untrained macrophages, but the production of ROS was significantly higher in trained macrophages (Figure 3B). Constitutive ROS production was not different between untrained and trained macrophages (Figure 3C, cRPMI) whereas it was significantly higher in trained macrophages exposed to zymosan or PGN (Figure 3C). Similarly, exposure to the receptor-independent stimulus PMA, resulted in a significantly higher production of ROS in trained as compared to untrained macrophages (Figure 3D). With respect to production of NO, constitutive production was not different between untrained and trained macrophages (Figure 3E, cRPMI). Exposure to zymosan, but not to LPS or PGN, resulted in a significantly higher NO production in trained as compared to untrained macrophages (Figure 3E). Phagocytic activity and phagocytic capacity of macrophages was compared between unstimulated trained and unstimulated untrained macrophages. A significantly higher number of cells with internalized beads as well as higher number of beads per cell, was observed for trained as compared to untrained macrophages, indicative of a heightened phagocytic activity as well as phagocytic capacity of trained macrophages (Figure 3F). Altogether, measurement of ROS and NO production as well as measurement of phagocytosis showed heightened innate immune functions of trained carp macrophages.

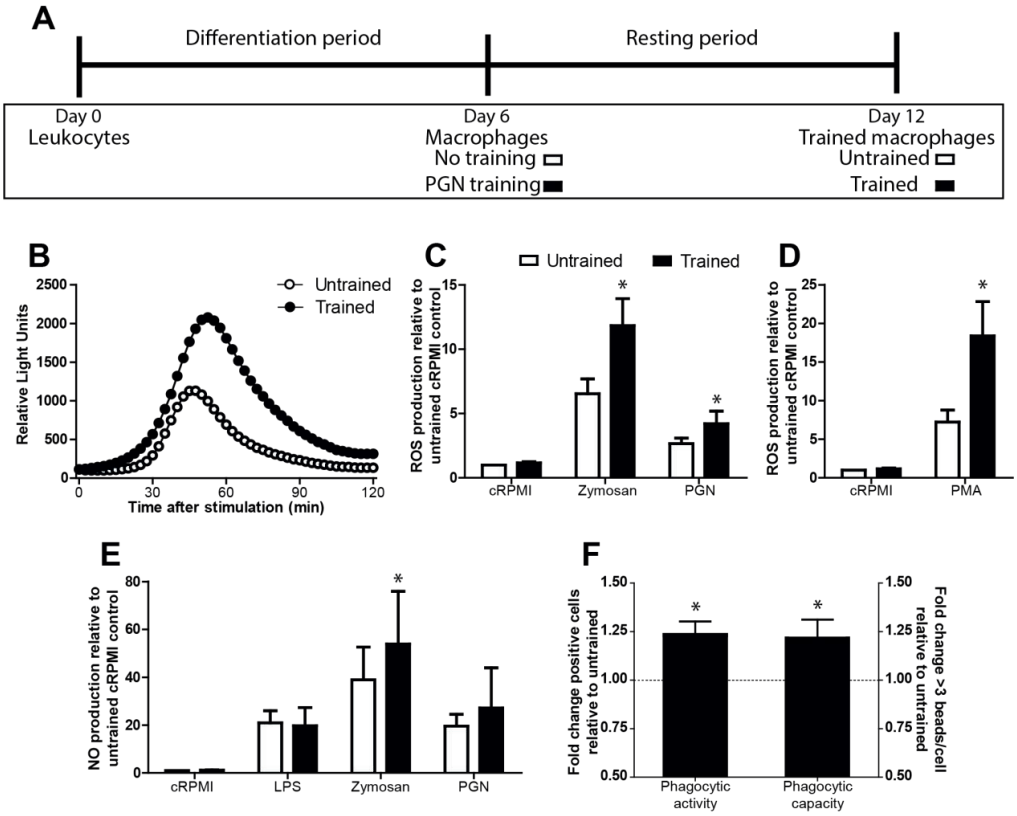


Figure 3. Trained carp macrophages show increased innate immune functions. A. Experimental *in vitro* set-up. Head kidney leukocytes were differentiated into macrophages for 6d. On day 6, macrophages were stimulated with 1 µg/ml PGN (trained) or vehicle control (untrained), followed by a resting period of 6 days. On day 12, trained or untrained macrophages were harvested and used for subsequent analyses. B-D. Trained or untrained macrophages were seeded in 96-well plates (5 x 10^5 cells/well) and stimulated with cRPMI, zymosan (250 µg/mL), PGN (10 µg/mL) or PMA (1 µg/mL). B. Real-time ROS production was measured immediately following stimulation. Lines indicate acquisition of light emission measured in one representative experiment out of n=11 independently performed experiments. C-D. Total ROS production relative to unstimulated, untrained macrophages (cRPMI); bars indicate mean + SEM of n=11 experiments performed independently. E. Trained or untrained macrophages were seeded in 96-well plates (5 x 10^5 cells/well) and stimulated with cRPMI, LPS (50 µg/mL), zymosan (250 µg/mL) or PGN (10 µg/mL). NO production was measured as $NaNO_2$ in the supernatant 15h post-stimulation. NO production is expressed relative to unstimulated untrained macrophages (cRPMI); bars indicate mean + SEM of n=7 experiments performed independently. F. Unstimulated trained or unstimulated untrained macrophages were analysed for their phagocytic activity (left y-axis) and phagocytic capacity (right y-axis) after 2h incubation with fluorescent beads (1 cell:10 beads). Fold change is expressed relative to the corresponding untrained cells; bars indicate mean + SEM of n= 6 experiments performed independently. Asterisk (*) indicates significant difference relative to the corresponding untrained sample as assessed by a linear mixed model one-way ANOVA, followed by a LSD post-hoc test (Figure 3C-E) or paired samples t-test (Figure 3F).

Trained macrophages show immune and metabolic reprogramming

Studies on trained human PBMC revealed a differential expression of IL6 and TNFα in response to microbial stimulation, which introduced metabolic reprogramming towards glycolysis as an important mechanism underlying trained immunity. Therefore, after having assessed that trained macrophages display heightened responses to stimulation (Figure 3), we also analysed in more details whether differences exist in the constitutive expression of inflammatory or metabolic genes between unstimulated trained and unstimulated untrained macrophages. Trained macrophages showed moderate but significantly increased expression of inflammatory genes, including *il6* and *tnfa*, each present in multiple copies (paralogues) in carp (Figure 4A). A trend towards increased *il1β* expression in trained macrophages was also observed (*p* value = 0.06), whereas *il10* and *inosb* expression remained unchanged (data not shown). With respect to the expression of metabolic genes, trained macrophages showed significantly increased expression of specific paralogs of *adpgk*, *aldh2.1* and *bpgm* (Figure 4B), suggestive of activation of the glycolysis pathway compared to untrained cells. Further evidence of metabolic reprogramming in unstimulated trained macrophages towards glycolysis was obtained upon measurement of extracellular lactate concentrations and intracellular accumulation of the metabolite fumarate. Trained macrophages showed significantly higher lactate values and a trend (*p* value = 0.077) towards higher accumulation of fumarate as compared to untrained cells (Figure 4C). Altogether, these data suggest that markers of immune and metabolic reprogramming were increased in trained macrophages.

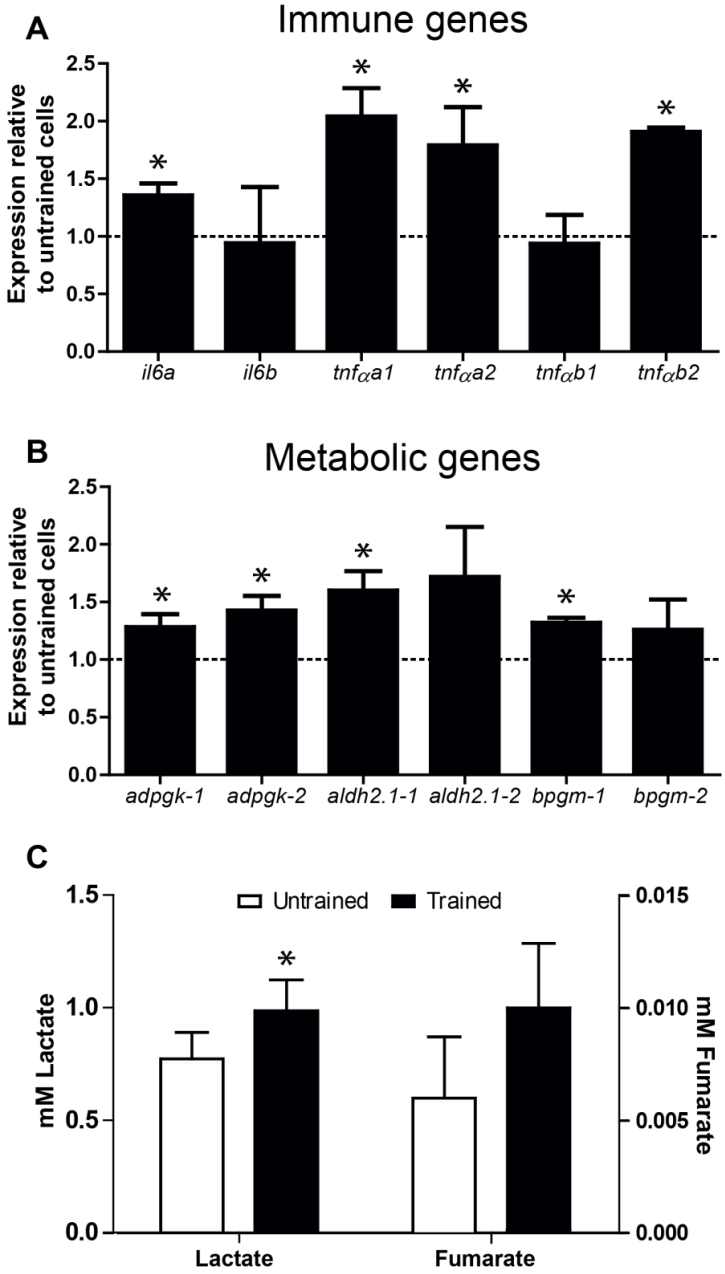


Figure 4. Trained carp macrophages show immune and metabolic reprogramming. Trained or untrained macrophages were obtained as described in figure 3. A-B. Gene expression of *il6* and *tnfa* paralogues (A) or selected metabolic genes and their paralogues (B) in unstimulated trained macrophages relative to unstimulated untrained controls; bars indicate mean + SEM of n=3 experiments performed independently. C. Lactate and Fumarate accumulation. Unstimulated trained or unstimulated untrained macrophages were seeded in 96-well plates (5 x 10^5 cells/well) and accumulation of extracellular lactate and intracellular fumarate was measured 24h later; bars indicate mean + SEM of n=5 (lactate) and n=4 (fumarate) experiments performed independently. Asterisk (*) indicates significant difference relative to the corresponding untrained sample as assessed by a linear mixed model one-way ANOVA, followed by a LSD post-hoc test (Figure 4A-B) or independent samples test (Figure 4C).

Soluble β-glucan laminarin is a potent training stimulus

In human PBMC, stimulation with BCG or β-glucans could induce trained immunity. We therefore investigated whether besides PGN, also the soluble β-glucan laminarin could induce trained immunity in carp macrophages (Figure 5). Training with laminarin, but not PGN, led to a marginal but significant increase in constitutive production of ROS as compared to untrained cells (cRPMI). Training with laminarin and subsequent exposure to zymosan or PMA, resulted in significantly heightened production of ROS, comparable to macrophages trained with PGN. Altogether, measurement of ROS production proved particularly informative to identify ligands able to train carp macrophages and revealed that not only NOD-ligands but also β-glucans can serve as suitable training stimuli of fish macrophages.

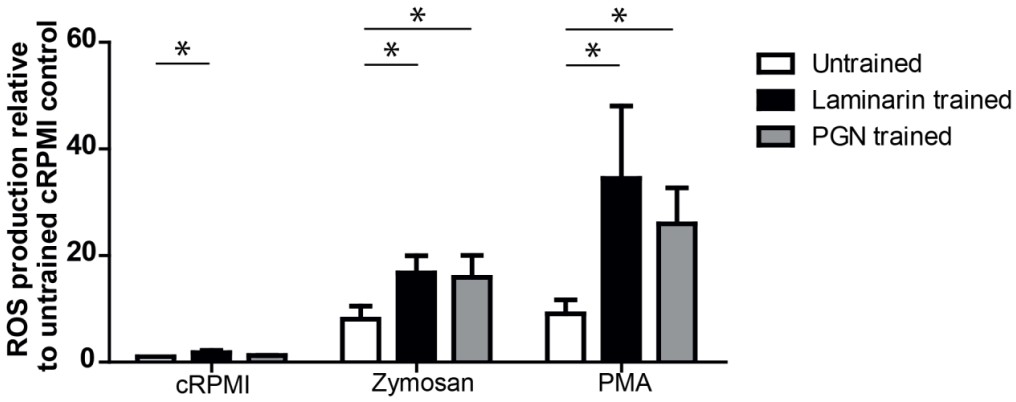


Figure 5. Exposure to laminarin leads to trained immunity in carp macrophages. On day 6, macrophages were trained with laminarin (20 µg/mL) or PGN (1 µg/mL) or left untrained (vehicle control), followed by a resting period of 6 days. On day 12, trained or untrained macrophages were harvested and seeded in 96-well plates (5 x 10^5 cells/well). ROS production was measured immediately following stimulation with either cRPMI, zymosan (250 µg/mL) or PMA (1 µg/mL) and expressed relative to the unstimulated untrained control (cRPMI); bars indicate mean + SEM of n=6 experiments performed independently. Asterisk (*) indicates significant differences relative to the corresponding untrained sample as assessed by a multivariate analysis, followed by LSD post-hoc test.

Discussion

In the present study, we adapted a well-described *in vitro* culture system of head kidney-derived macrophages to investigate conservation of trained immunity in teleost fish. A 2h *in vitro* exposure to a soluble NOD-specific ligand or to soluble β-(1,3/1,6)-glucan resulted in carp macrophages that displayed typical features of trained immunity for a period of at least six days. The use of soluble ligands allowed for thorough washing and removal of traces of ligands, a procedure we considered crucially important to exclude continuous re-stimulation of macrophages. Typical features of trained macrophages of carp included heightened phagocytosis and inflammatory responses following stimulation with (homologous or heterologous) microbial stimuli. The inflammatory profile displayed heightened production of reactive oxygen or nitrogen species, increased constitutive gene expression of selected immune and metabolic genes as well as an increased constitutive lactate production, illustrative of a metabolic shift. Measurement of the production of reactive oxygen species proved particularly informative to identify ligands able to train carp macrophages.

The present study provides *in vitro* evidence for conservation of several key features of trained immunity in macrophages of carp, a representative teleost fish, but provides no *in vivo* evidence for trained immunity. However, vaccination with BCG, a known stimulant of trained immunity in mice/human monocytes, has already been shown to provide cross-protection against *Mycobacterium* sp. infections in several fish species (149-151) including zebrafish (174), a well-known animal model closely related to carp. The non-specific protection provided by BCG injection suggests that also in fish, *in vivo* evidence for trained immunity already exists. In addition, zebrafish i.p.-injected with β-glucans and subsequently challenged with spring viremia of carp virus (SVCV), either showed a significant but minor increase in survival at 7 but not 35 days post-treatment (158), or a clear increase in survival at 14 days post treatment (175). Thus, relatively long-lived effects of immune stimulants not specifically associated with anti-viral immunity, provide at least circumstantial *in vivo* evidence for trained immunity in zebrafish. All of the above-described studies clearly indicate the complexity of *in vivo* experiments, not unique to experiments in fish, in which it is not easy to exclude the involvement of adaptive immunity and the possibility of continuous stimulation as opposed to a single training event, a clear advantage of our *in vitro* system based on soluble ligands and rigorous washing steps.

Zebrafish could prove especially informative for *in vivo* studies on trained immunity owing to the availability of *rag*$^{-/-}$ strains. Increased survival of *rag*$^{-/-}$ zebrafish upon lethal challenge with *Edwardsiella ictaluri* eight weeks post-exposure to an attenuated non-virulent strain of this bacterium (152) could be considered *in vivo* evidence of trained immunity, although persistence of attenuated bacteria and absence of non-specific cross-protection against another bacterium, *Yersinia ruckeri,* could not fully exclude the involvement of other protective mechanisms. Only recently, *rag*$^{-/-}$ zebrafish were shown to exhibit a constitutively heightened

innate immune activity, characterized by an increased antiviral state and associated resistance to a viral challenge with SVCV (157). In the latter study, also NK cell markers such as *cd8* and *nklysin* were constitutively higher expressed in *rag*$^{-/-}$ than in wild type zebrafish. This observation is of particular interest in view of the BCG-induced (176) and virus-induced (177, 178) memory-like NK cells, recently highlighted as cell types associated with trained immunity in mammals. It could thus be relevant to further study NK-like cells in relation to virus-induced trained immunity in the *rag*$^{-/-}$ zebrafish model, and to explore genes associated with an antiviral state as novel markers for trained immunity.

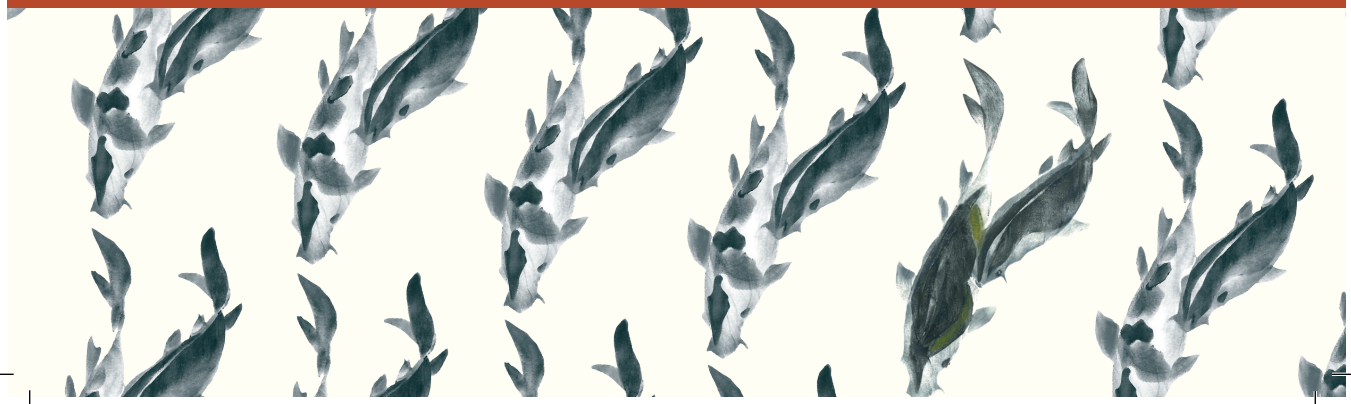
Teleost fish (e.g. carp, zebrafish) are poikilotherms, which allows for manipulation of temperature and thus allows for studies on temperature-associated effects on trained immunity *in vitro* and *in vivo*. Adaptive immunity, more than innate immunity, is considered sensitive to temperature change, reflected by reduced IgM serum concentrations and suppression of T cell responses at lower temperatures (179, 180). Thus, the use of poikilothermic animals opens the possibility to 'knock-down' adaptive immune responses in animals for which *rag*$^{-/-}$ strains are not available, and to study, *in vivo*, aspects of trained immunity, including the duration of cross-protection against a secondary infection in a T- and B-lymphocyte independent manner. Temperature–mediated 'knock-down' of adaptive immune responses in teleost fish might thus allow to unravel mechanisms underlying long-lived protection likely mediated by innate immune cells that remain active also at lower temperatures.

One of the determining and underlying mechanisms of trained immunity is based on long-lived epigenetic modifications that persist even after removal of the training stimulus (as reviewed by: (181)). For example, histone modifications such as trimethylation of H3K4 and mono acetylation of H3K27 have been associated with β-glucan- induced trained immunity of human PBMC. These epigenetic changes were strongly correlated with differential gene expression induced by β-glucan training in monocytes (58). In the same study, comparable epigenetic changes were observed in peritoneal macrophages isolated from *C. albicans*-infected mice, linking the observed *in vitro* histone modifications to an *in vivo* model of trained immunity. The onset of these epigenetic modifications is strongly connected to metabolic reprogramming, and dependent on specific metabolites such as fumarate and mevalonate (59, 60). In our *in vitro* model we investigated epigenetic modifications in trained macrophages by measuring (increased) constitutive expression of several immune- and glycolysis-related genes, and a metabolic shift from oxidative phosphorylation towards glycolysis. To study in more detail epigenetic modifications underlying trained immunity in fish, future studies could build on, for example, a recent chromatin immunoprecipitation (ChIP) sequencing study performed on SVCV-infected zebrafish (182). Another interesting aspect of trained immunity that could be studied *in vivo*, at least in oviparous fish, is transgenerational epigenetic reprogramming. Since primordial F2 germ cells are not present upon exposure of F0 individuals to potential training stimuli, one less generation is needed to prove transgenerational effects in

oviparous fish (183). In conclusion, the constitutive antiviral state of *rag*$^{-/-}$ zebrafish, the cold-blooded nature of teleost fish allowing "natural" knockdown of the adaptive immune system, and the suitability of oviparous fish for transgenerational experiments, all provide arguments in favour of studying conserved but also unique aspects of trained immunity in teleost fish.

Acknowledgements

Raphael Barbetta de Jesus and Fabiana Pilarski are greatly acknowledged for their discussions on the potential impact of trained immunity on aquaculture practise. Cassandra van Doorn is greatly acknowledged for her contribution to the initial cell culture optimization.



4

β-glucan immuno-modulation in common carp intestine: a role for microbiota and its metabolite

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Manuscript in preparation

Abstract

Dietary supplementation of fish with β-glucans has been widely associated with immunomodulation and commonly accepted as beneficial for fish health. However, to date the exact mechanisms of immunomodulation by β-glucan supplementation in fish are still largely unknown. In mammals a clear relation has been observed between high fibre diets and immunomodulation via intestinal microbiota and its metabolites. In this study, we first described the normal microbiota of common carp intestine by 16S rRNA sequencing. Based on the abundance of the genus *Bacteroides*, well known for their capacity to degrade and ferment carbohydrates, we hypothesized that common carp intestinal microbiota could ferment dietary β-glucans. Indeed, two different β-glucan preparations (curdlan and MacroGard®) were both fermented *in vitro* albeit with distinct fermentation dynamics and significant differences in production of short chain fatty acids (SCFA). MacroGard® more than curdlan lead to production of propionate, a SCFA with immunomodulatory properties. Single oral gavage of MacroGard® in nine-month-old carp significantly altered the intestinal microbiota. At seven days post-treatment, increased abundance of a member of the family *Rhodocyclaceae* was observed, which contains genera known to synthesize propionate using unusual transcarboxylase enzymes. Coinciding with the shift in microbial composition, an overall immunomodulation in the intestine could be observed as inhibition of expression of several pro-inflammatory genes (*il1β*, *il6, tnfa*). Based on our data, we discuss the possibility that fermentation of MacroGard® by specific bacteria, part of the normal microbiota of common carp intestine, can lead to a shift in microbial composition and associated production of the SFCA propionate, the increased presence of which could possibly explain (part of) β-glucan-induced immunomodulatory effects.

Introduction

Effects of immunomodulation by β-glucans have been widely studied in teleost fish. Regardless of administration route or fish species, β-glucans often are associated with immunomodulatory effects and increased resistance to both viral and bacterial infections (reviewed by: (19-21). Dietary supplementation of β-glucans may be frequently applied in aquaculture, but definitive mechanisms explaining the observed immunomodulatory effects of β-glucans in the intestine have yet to be described for fish.

In mammals, direct recognition of β-glucans is mediated by at least the C-type lectin receptor (CLR) Dectin-1 (27-29), whereas in invertebrates β-glucans can be recognized by β-glucan binding proteins (184). For both, mammals (43, 185) and invertebrates (89, 184), several non-exclusive pathways play a downstream role in the response to β-glucans, often mediating activation of the transcription factor NF-κB (38). Although in fish genomes no clear homologue of Dectin-1 could be identified so far (28, 42), in recent studies we could activate carp macrophages with curdlan (41, 138), considered a Dectin-1-specific (1,3)-β-glucan stimulus. We therefore proposed that immuno-modulatory effects of β-glucan in carp macrophages could include regulation of a downstream signalling pathway typical of CLR activation, and confirmed our hypothesis by pathway analysis of differentially expressed genes (DEGs), (41). Among the regulated genes were *card9* and *bcl10*, both members of the signalling complex shown to be downstream of Dectin-1. Not only did we observe regulation of the CLR signalling pathway, several candidate receptors could be identified based on regulation of expression upon stimulation with β-glucans, or based on the conservation of β-glucan binding motifs in their predicted translations to protein (41). As there is clear evidence for presence of macrophages and other antigen-sampling cells in the intestine (93, 186, 187), it is possible that recognition of β-glucans in the intestine occurs via a direct recognition mechanism of β-glucan receptors. Another mechanism could be indirect and rely on prebiotic effects inducing a shift in intestinal microbiota which would influence the microenvironment of the intestine. These mechanisms should not necessarily be exclusive.

The intestinal microbiota is of well-known importance to the host, for example via degradation and fermentation of non-starch polysaccharides and/or resultant production of metabolites (188-190). Several studies have shown the ability of fish microbiota to degrade carbohydrates, showing growth of specific intestinal bacterial monocultures on different carbohydrates among which β-glucan (191), and observing *in vitro* fermentation of several carbohydrates and production of short chain fatty acids (SCFAs) by intestinal microbes of common carp (*Cyprinus carpio*) or Nile tilapia (*Oreochromis niloticus*) (192, 193). In a comparison of the fermentability of different carbohydrates and produced SCFAs between Nile tilapia and European sea bass, both their microbiota were found capable of fermenting carbohydrates but with differing end-product profiles (194). Effects of β-glucan supplementation on the composition of the intestinal microbiota are not well

documented, although several studies did observe differences due to dietary β-glucan supplementation. In common carp, two weeks feeding with β-glucan-enriched diets induced shifts in the normal intestinal microbiota, manifested either as a reduction in species richness and absolute number of operational taxonomic units (OTUs), (79), or as an increase in microbial diversity (126). In seabass, four weeks feeding with β-glucan-enriched diets induced a shift towards more *Methylobacterium* (127), whereas in rainbow trout and feral common carp, 8 weeks feeding with yeast-derived probiotics rich in β-glucans induced a shift towards more lactate bacteria (78, 195). Overall, possibly owing to differences in diet formulation, sampling, microbial analysis and fish species, a consistent and distinct shift in intestinal microbial composition induced by β-glucans remains to be discerned.

We studied the role of β-glucan as prebiotic and hypothesized that common carp normal intestinal microbiota would be able to ferment β-glucans. We further hypothesized that, as a result of this fermentation process, *in vivo* treatment of carp with β-glucans would induce a shift in intestinal microbial composition and might result in a shift in production of short chain fatty acid (SCFA) metabolites. Finally, we hypothesized that this series of processes would contribute to the commonly observed immunomodulation of the host. To test our hypotheses, we first characterised the normal, active intestinal microbiota of common carp by 16S rRNA transcript sequencing. We also analysed the SCFA profile generated during *in vitro* fermentation and noticed β-glucan-dependent shifts in SCFA production. Last but not least, common carp were treated with a single oral gavage with β-glucans to analyse *in vivo* effects of β-glucans on intestinal microbiota and effects on regulation of local gene expression. Our data suggest that dietary β-glucan administration may have indirect immunomodulatory effects accompanied by a shift in intestinal microbiota and associated shift in SCFA profile, of which the interconnection may require further investigation.

Material and Methods

Animals

European common carp (*Cyprinus carpio carpio* L.) of the R3 × R8 strain were used, which originated from a cross between the Hungarian R8 strain and the Polish R3 strain (169). Carp were bred and raised in the aquatic research facility of Wageningen University at 20 – 23°C in recirculating UV-treated water and fed pelleted dry food (Skretting, Nutreco) twice daily. All experiments were performed with the approval of the animal experiment committee of Wageningen University (DEC number 2015098).

Collection of intestinal contents for 16s rRNA profiling

Nine-month-old fish were anaesthetized in 0.3 g/L Tricaine Methane Sulfonate (TMS) (Crescent Research Chemicals, Phoenix, USA) in aquarium water buffered

with 0.6 g/L sodium bicarbonate and bled via the caudal vein. The third segment of the intestine was isolated and intestinal content of the third segment was collected by scraping, snap-frozen in liquid nitrogen and stored at -80°C. A tissue sample of the third segment of the intestine was snap-frozen in liquid nitrogen and stored at -80°C.

Total bacterial RNA isolation

Prior to isolation of RNA from intestinal content, all samples were weighed. Total RNA from intestinal content was isolated using the RNeasy PowerMicrobiome Kit (Qiagen, 26000-50), following the manufacturer's instructions. Agarose gel electrophoresis and a ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) were used to control RNA yield and quality. RNA samples were stored at -80° C until further use.

16S rRNA profiling

Total community analyses were performed on initially five replicates per treatment similar as described for (196). Total community RNA was used for amplification and sequencing of the 16S rRNA, targeting the variable V3-V4 regions resulting in amplicons of approximately ~460 bp. Illumina 16S rRNA gene amplicon libraries were generated and sequenced at BaseClear (Leiden, the Netherlands). In short, barcoded amplicons from the V3-V4 region of 16S rRNA genes were generated using a 2-step approach. Ten ng genomic RNA was used as template for the OneStep RT-PCR kit (QIAGEN®) employed according to manufacturer's instructions with a total volume of 50 ul using the 341F (5'-CCTACGGGNGGCWGCAG-3') and the 785R (5'-GACTACHVGGGTATCTAATCC-3') primers appended with Illumina adaptor sequences. Control PCR reactions were performed alongside each separate amplification without addition of template. PCR products were purified using AMPure XP beads according to manufacturer's instructions and the size of the PCR products were checked on Fragment analyzer (Advanced Analytical) and quantified by fluorometric analysis. Purified PCR products were used for the 2nd PCR in combination with sample-specific barcoded primers (Nextera XT index kit, Illumina). Subsequently, PCR products were purified, checked on a Fragment analyzer (Advanced Analytical) and quantified, followed by multiplexing, clustering, and sequencing on an Illumina MiSeq with the paired-end (2x) 300 bp protocol and indexing. The sequencing run was analyzed with the Illumina CASAVA pipeline (v1.8.3) with demultiplexing based on sample-specific barcodes. The raw sequencing data produced was processed removing the sequence reads of too low quality (only "passing filter" reads were selected) and discarding reads containing adaptor sequences or PhiX control with an in-house filtering protocol. A quality assessment on the remaining reads was performed using the FASTQC quality control tool version 0.10.0.

Bacterial community analyses

The RDP extension to PANDASeq (197) named Assembler (198) was used to merge paired-end reads with a minimum overlap of 10 bp and at least a PHRED score of 25. Primer sequences were removed from the per sample FASTQ files using FLEXBAR version 2.5 (199). Sequences were converted to FASTA format and concatenated into a single file. All reads were clustered into OTUs using the UPARSE strategy by dereplication, sorting by abundance with at least two sequences and clustering using the UCLUST smallmem algorithm (200). These steps were performed with VSEARCH version 1.0.10 (201), which is an open-source and 64-bit multithreaded compatible alternative to USEARCH. Next, chimeric sequences were detected using the UCHIME algorithm (202) implemented in VSEARCH. All reads before the dereplication step were mapped to OTUs using the usearch_global method implemented in VSEARCH to create an OTU table and converted to BIOM-Format 1.3.1 (203). Finally, taxonomic information for each OTU was added to the BIOM file by using the RDP Classifier version 2.10 (204). All steps were implemented in a Snakemake workflow (205), which is available on Github https://github.com/nioo-knaw/hydra/tree/1.3.3.

For downstream analysis we took the obtained OTU table and prepared a "filtered table" using QIIME (1.9.1) custom scripts (206). First, we extracted from the OTU table the Bacteria domain using the command *split_otu_table_by_taxonomy.py*. Next, we discarded singletons, doubletons, Chloroplast and Mitochondria sequences using the command *filter_otus_from_otu_table.py*. With the "filtered_OTUtable", we first calculated the alpha diversity. Using the command *alpha_rarefaction.py*, the OTU table was rarefied to counts up to 3975 reads. The reason to use this value was because this was the lowest sequencing depth obtained from a sample. To calculate the diversity indexes, we used the *alpha_diversity.py* and *alpha_rarefaction* commands, obtaining Shannon, Observed OTUs and Chao1 metrics and the *compare_alpha_diversity.py* for calculating the statistical difference between the treatments. For the Beta diversity, the unrarefied "filtered_OTUtable" was first normalized using the R package metagenomeSeq (v.1.12) (207, 208). We used a cumulative-sum scaling (CSS) method to avoid the biases generated with current sequencing technologies due to uneven sequencing depth (209) accompanied by fitZIG contrasts. With the normalized OTU table Bray-Curtis dissimilarity matrices were calculated and used to build Principal Coordinate with Phyloseq package (v.1.10) (210). The bubble plots were drawn with TreeMap©. Firstly, using the command *alpha_rarefaction.py*, the OTU table was rarefied to counts up to 3975 reads. Subsequently, random forest analysis was performed, followed by the Boruta feature selection (211) on rarefied data using 1000 trees with R packages randomForest v4.6–7 and Boruta v3.0, respectively. Subsequently, heatmaps were constructed based on z-score transformed (count-average count/standard deviation of counts) functional annotations to improve normality and homogeneity of variances and using the pheatmap R package (212).

In vitro gas production technique

In vitro gas production technique is an *in vitro* batch culture method that can be used to assess the potential fermentability of monogastric feed ingredients (194, 213, 214). With this technique, an inoculum is prepared under strictly anaerobic conditions using freshly collected intestinal contents. The inoculum is incubated with a fermentable substrate of choice and with medium to support bacterial growth. Subsequently, accumulating gas during fermentation is measured so that a picture of the kinetics of fermentation is obtained. At the end of the fermentation period, samples are taken from the fermentation fluid for measurement of fermentation end-products and substrate utilization (215).

Collection of intestinal contents for in vitro fermentation

Naive carp were anaesthetized with 0.3 g/l Tricaine Methane Sulfonate (TMS) (Crescent Research Chemicals, Phoenix, USA) in aquarium water buffered with 0.6 g/L sodium bicarbonate and bled via the caudal vein. Subsequently, fish were put on ice and the intestine, without bulbus, was removed. The content of the intestine was collected in pre-weighed plastic tubes that were filled with CO_2 to ensure anaerobic conditions. Intestinal content of ten fish was pooled and five independent pools were used.

Substrates for *in vitro* fermentation

Four different substrates were used for *in vitro* gas production analyses, glucose, (D-glucose monohydrate; Merck, Darmstadt, Germany), PBS (Cell culture grade, Lonza), Curdlan (β-(1,3)-glucan extracted from *Alcaligenes faecalis*, Sigma-Aldrich) and MacroGard™ (a cell wall preparation of *S. cerevisiae* comprising at least 60% β-glucan (Zilor, São Paulo, Brazil)). Glucose was included as a readily fermentable substrate for comparative purposes (194).

Inoculum preparation and measurement of cumulative gas production

Pooled intestinal content was immediately transported to the laboratory after collection where it was weighed and transferred to a beaker. The contents of the beaker were stirred and flushed with a constant gentle stream of CO_2. Pre-warmed (25 °C) anaerobic, sterile saline (9 g/L NaCl) was added in a ratio of 1:5 (W/V) to ensure sufficient amount of inoculate. The diluted material was homogenized using a vortex mixer and strained through a double layer of cheese cloth with 16 threads per cm in both directions. From the resulting inoculate, 5 mL was then dispensed into a pre-warmed 300 mL fermentation bottle, containing 0.5 g substrate and 82 mL of medium. Three replicate bottles for each substrate per inoculate were used. The medium consisted of a basal solution containing micro-nutrients required by the microbial population for growth, a bicarbonate solution, a vitamin/phosphate solution and a reducing agent. The composition of the medium is described in detail by (216). Subsequently, bottles were immediately attached to an automated gas production system (217). Within this system, pressure sensors detect a fixed pressure, after which a computer software program allows opening of a valve to

release gas, the time at which this occurred was recorded. Bottles were incubated for 168 hours at 25 °C, equal to the body temperature of carp.

Curve fitting and statistics of cumulative gas production

A monophasic model as described by (218) was fitted to the profile of the cumulative gas production of each fermentation bottle according to the equation G = A/(1 + (C/t)B), where G is the total millilitre gas produced per gram organic matter (OM) at time t; A is the asymptotic gas production (mL/g OM); B is the switching characteristic of the curve; C is the half time (time at which half of the asymptote is reached) and t is the time (h).

The maximum rate of gas production (Rmax) and the time at which it occurs (Tmax), were calculated according to (219) as

$$R_{\max} = (A(C^B)B(T_{\max}{}^{(-B-1)}))/(1 + (C^B)(T_{\max}{}^{(-B)}))^2 \text{ and}$$
$$T_{\max} = C(((B-1)/(B+1))^{(1/B)})$$

For each bottle, curve fitting was done using the nonlinear least-squares regression procedure NLIN (SAS Inst. Inc., Cary, NC, USA). One-way analysis of variance using the GLM procedure of SAS (SAS Inst. Inc.) was used to test the effect of substrate on gas production parameters and fermentation end-products. The average of replicate bottles per substrate per inoculum was considered as the experimental unit. The effect of replicate bottles was tested separately and was not significant for any of the parameters. It was therefore excluded from the model and thus became part of the error term.

Sampling and analyses of fermentation liquid

At the end of the incubation period, pH of the fermentation fluids was recorded and samples were collected for analysis of ammonia (NH_3), SCFAs and lactate. Analysis of NH_3 and SCFA were determined as described previously (220). For NH_3 determination, 750 µL reaction fluid was deproteinised by 750 µL trichloroacetate (100 g per litre of trichloroacetate neutralised by NaOH). Samples were centrifuged at 14 000 × g at 20 °C for 10 min. Concentration of NH_3 in the supernatant was determined using a Skalar Autoanalyzer SA1050 (Skalar Analytical BV, Breda, the Netherlands). For SCFA analysis, 750 µL reaction fluid was acidified with 750 µL internal standard (isocaproic acid) in a 40 g L^{-1} *o*-phosphoric acid solution. SCFAs were analysed using a gas chromatograph (Fisons HRGCMega 2, CE Instruments, Milan, Italy) with a split/splitless injector operated in split mode (split ratio 1: 9) and fitted to a flame ionisation detector, using a capillary column (30m, internal diameter 0.53 mm, film thickness 1.0 µm; Agilent J&W HP-FFAP, Santa Clara, CA, USA) with hydrogen as the carrier gas (25 kPa pressure) and isocaproic acid used as an internal standard. The starting temperature of the column was set at 80 °C for 1 min, followed by a 20 °C min^{-1} increase to 120 °C, followed by a 6.1 °C min^{-1} increase to 205 °C, where temperature was maintained for 2 min. Lactate was measured using the Lactate Colorimetric Assay Kit II (K627, Biovision) according to the manufacturer's instructions, including the optional filtration step with

Amicon 10K spin columns (Z677108-96EA, Sigma Aldrich, centrifugation for 20 minutes at 21.100x *g*). Briefly, samples were tested in four different dilutions; undiluted, 3⅓ times, 10 times and 50 times diluted in "Lactate Assay Buffer" and 50 µL sample or dilution was transferred to a 96-wells plate. Subsequently, 50 µL reaction mix composed of Lactate Substrate Mix (2 µL), Lactate Enzyme Mix (2 µL), and Lactate Assay Buffer (46 µL), was added to each sample and incubated for 30 minutes at room temperature. Optical density was measured at 450 nm and concentrations of extracellular lactate were calculated based on a lactate calibration curve supplied in the kit.

Oral gavage and tissue sampling

Carp of 9 months (20–40 g, n = 20 per group) were starved overnight and anaesthetized in 0.15 g/L Tricaine Methane Sulfonate (TMS) (Crescent Research Chemicals, Phoenix, USA) in aquarium water buffered with 0.3 g/L sodium bicarbonate. Anaesthetized fish received an oral gavage with 100µL of PBS (Lonza, LO BE17-516F) or MacroGard® dissolved in PBS (10 mg/mL, 1 mg/mL per fish) using a 200 µL pipet. At 7 and 14 days post-gavage, fish were anaesthetized in 0.3 g/L Tricaine Methane Sulfonate (TMS) (Crescent Research Chemicals, Phoenix, USA) in aquarium water buffered with 0.6 g/L sodium bicarbonate and bled via the caudal vein. The third segment of the intestine was isolated and intestinal content of the third segment was collected by scraping, snap-frozen in liquid nitrogen and stored at -80 °C. A tissue sample of the third segment of the intestine was snap-frozen in liquid nitrogen and stored at -80 °C.

Total RNA isolation from intestinal samples

Total RNA from isolated tissue samples was isolated using the RNeasy mini Kit (Qiagen, 74106), including on-column DNase treatment, according to the manufacturer's instructions, and stored at −80°C. Prior to cDNA synthesis, 1000 ng total RNA was treated with DNase I, Amplification Grade (Invitrogen), and cDNA was synthesized using random primers (300 ng) and Superscript III First-Strand Synthesis for RT-PCR (Invitrogen). cDNA samples were diluted in nuclease-free water prior to real-time quantitative PCR (RT-qPCR) analysis.

Gene expression analysis

Gene expression was measured with RT-qPCR using ABsolute qPCR SYBR Green Mix (Thermo Scientific) in a Rotor-Gene 6000 (Corbett Research), and fluorescence data were analysed using Rotor-Gene Analysis software version 1.7. The relative expression ratio (R) of each sample was calculated according to the Pfaffl method (173) based on the take-off deviation of sample versus each of the PBS controls and normalized relative to the s11 protein of the 40s subunit as reference gene (see Table 1).

Table 1. Overview of RT-qPCR primers used for in the current study. cypCar numbers identify ORFs in the draft carp genome (bioproject: PRJNA73579) that were also confirmed by RNA sequencing, LHQP number refers to the accession number of the associated scaffold.

Primer	Forward (5′ – 3′)	Reverse (5′ – 3′)	GenBank Accession No.
40s	CCGTGGGTGACATCGTTACA	TCAGGACATTGAACCTCACTGTCT	AB012087
cxca	GGGTGTAGATCCACGCTGTC	CTTTACAGTGTGGGCTTGGAG	AJ550164
cxcb	GCTGCCTGCTTGTTGTAGAG	ATCTGTTTTGGAGGAACCA	AB082985
il1b	AAGGAGGCCAGTGGCTCTGT	CCTGAAGAAGAGGAGGAGGCTGTCA	AJ245635
il6a	CAGATAGCGGACGGAGGGGC	GCGGGTCTCTTCGTGTCTT	KC858890
il6b	GGCGTATGAAGGAGCGAAGA	ATCTGACCGATAGAGGAGCG	KC858889
tnfaa1	GAGCTTCACGAGGACTAATAGACAGT	CTGCGGTAAGGGCAGCAATC	AJ311800
tnfaa2	CGGCACGAGGAGAAACCGAGC	CATCGTTGTGTCTGTTAGTAAGTTC	AJ311801
tnfab1	GAAGACGATGAAGATGATACCAT	AAGTGGTTTTCTCATCCTCAA	cypCar_00029601, LHQP01065580
tnfab2	CTTGGACGAAGCCGATGAAGAC	ATCTTGTGACTGGCAAACA	cypCar_00023012, LHQP01037150
il10a	CGCCAGCATAAAGAACTCA	TGCCAAATACTGCTCAATGT	cypCar_00007086, LHQP01030085
il10b	CGCCAGCATAAAGAACTCGT	TGCCAAATACTGCTCGATGT	cypCar_00012555, LHQP01021640
tlr3.1	GTTATCCCTGGCGCATAATA	TCTTCAATAATTGGTAAGGATGATG	KF387571
tlr3.2	GTTTATCCCTGGAGCATAACT	CTTCAATAACTGGTAAAGACGAAC	KF387572
mx1	ACAATTTGCGGTCTTTGAGA	CCCTGCCATTTCTCTTCG	cypCar_00015892, LHQP01004675
mx2	GCTTACGGTCTCTGGGG	TGGTTTCATCTTTAGTTCTTATCATC	cypCar_00029512, LHQP01026950
mx3	ACAAAGGACAATAACTGGCG	GAGGTCAGGAACATCACTG	cypCar_00017679, LHQP01012215
mx4	CTAGAGTTGCCACTGCC	TCCAGTTGAATCCACTTCG	cypCar_00025664, LHQP01010684
mx5	ACTGAAGTGTGTGTTTTTGG	CAGACCTGGTAGATAAAGGAG	cypCar_00012158, LHQP01006771
stat1	GAGACGGAGGAATCACC	GGATGTCTGGGTAAAGGTAG	cypCar_00011644, LHQP01012446 and cypCar_00037712, LHQP01035256.1

Results

Characterization of intestinal microbial composition implies β-glucan fermentation capacity

Analysis of 16S ribosomal RNA rather than DNA provided insight into the active intestinal microbial communities (221). Furthermore, identification of bacterial microbiota up to family level allowed us to investigate the presence of bacteria with theoretical fermenting capacities. Intestinal content of the third segment of the intestine from n=5 individual fish was collected for 16S rRNA sequencing, revealing a total of n=55 active operational taxonomic units (OTUs) in the intestinal content of unhandled carp.

By far, the most represented Phyla in the carp intestinal microbiota were *Fusobacteriaceae* (71% of total reads), whereas *Bacteroidaceae* (21%), *Proteobacteria* (5%) and *Firmicutes* (2%) were considerably less frequent (Figure 1A). The majority of *Proteobacteria* belonged to the order of *Gammaproteobacteria* (95%), while the rest belonged to the *Betaproteobacteria* (4%). Looking at the distribution per Family in the active microbiota, the most abundant family were *Fusobacteriaceae* (71%), followed by *Bacteroidaceae* (16%), *Porphyromonadaceae* (5%), *Vibrionaceae* (3%), *Aeromonadaceae* (1.5%) and *Erysipelotrichaceae* (1.4%) (Figure 1B). Finally, looking at putative genus level it becomes clear that the majority of *Fusobacteriaceae* are member of the *Cetobacterium* genus, as 71% of the total reads belong to this genus, whereas all

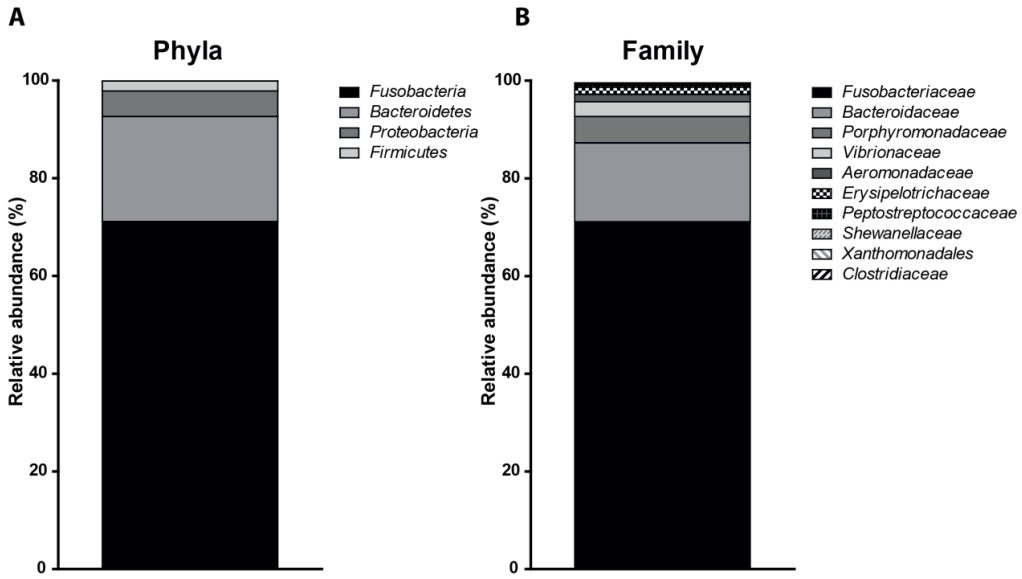

Figure 1. Relative abundance of active bacteria in the intestinal microbiota of common carp. Characterization with 16S rRNA sequencing of the intestinal microbiota of unhandled common carp. A. Relative abundance of phyla. B. Relative abundance of family. *Xanthomonadales* refers to family *Xanthomonadales Incertae sedis*.

further reads belong to a single OTU. The most active genera are *Bacteroides* (16%, 2 OTUs), *Vibrio* (3%), *Aeromonas* (1.5%) and two different uncultured genera of the families *Porphyromonadaceae* (4.5%, 3 OTUs) and *Erysipelotrichaceae* (1.1%, 1 OTU). Although the sequencing effort could not identify bacteria at species level, abundance of the *Bacteroides* genus well known to express β-glucan degrading genes (70), could imply that carp microbiota is able to ferment β-glucans.

In vitro batch analysis confirms fermentation of β-glucans

In order to investigate the β-glucan fermentation ability of the normal intestinal microbiota of carp, *in vitro* fermentation was performed (Figure 2A). Through an *in vitro* batch culture system, fermentation of different substrates could be analysed. As a measure for fermentation, cumulative gas production was measured over time. As a negative control, minor cumulative gas production was

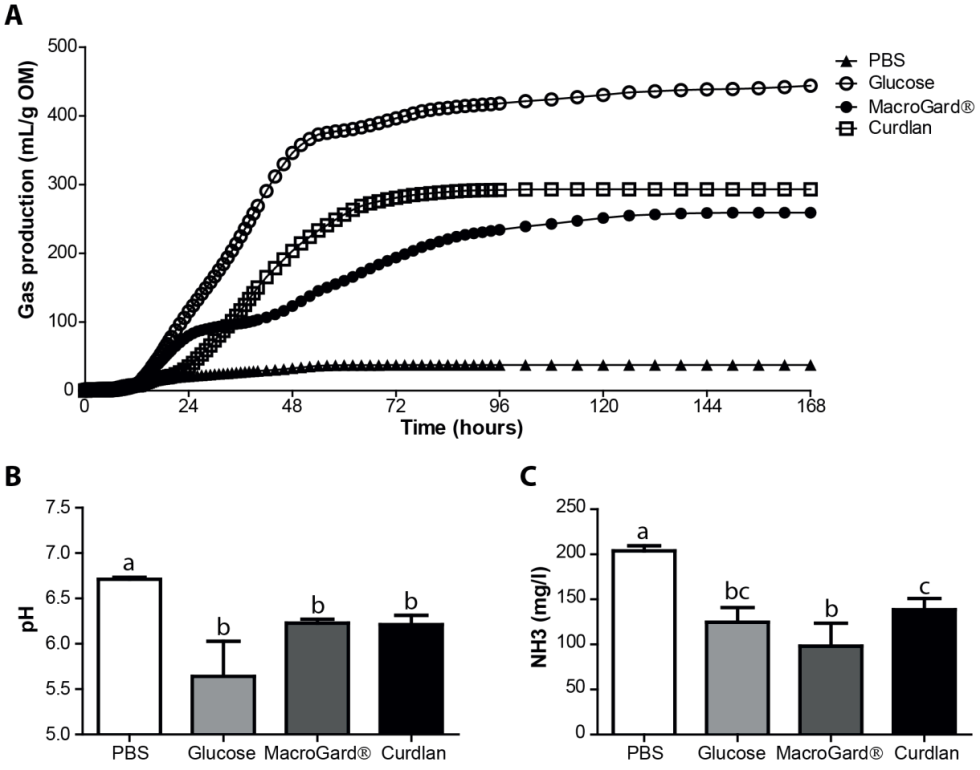

Figure 2. Carp normal microbiota is capable of *in vitro* fermentation of different β-glucans. A. Cumulative gas production over 168h as a result of *in vitro* fermentation of PBS, glucose, MacroGard® and curdlan by carp normal intestinal microbiota. Data shown as mean of n=5 independent intestinal pools. B. pH of fermentation fluids after 168h of *in vitro* fermentation (mean + SD, n = 5). Significant differences between groups were assessed by Welch's ANOVA followed by Games-Howell test. C. Ammonia (NH_3) accumulation in fermentation fluids after 168h of *in vitro* fermentation (mean + SD, n = 5). Significant differences between groups were assessed by one-way ANOVA followed by Tukey test. Groups with different letters are statistically different from one another.

observed in the PBS group, similar to gas production in the negative control without substrate (data not shown). As a positive control, fermentation of the readily digestible mono-saccharide glucose was observed, resulting in the highest cumulative gas production. Two different β-glucan preparations were studied; curdlan, a high molecular weight linear polymer consisting of β-1-3-linked glucose residues from *Alcaligenes faecalis* and MacroGard®, a branched polymer β-(1,3/1,6)-glucose feed additive. In comparison, fermentation of curdlan resulted in the most continuous and highest cumulative gas production, with fermentation of MacroGard® showing an intermediate plateau around 24 – 36 hours. No matter the different dynamics of gas production between curdlan and MacroGard®, carp normal intestinal microbes were able to ferment β-glucans.

Analysis of fermentation liquid following *in vitro* fermentation revealed differences in pH and NH_3 between substrates. At initialization of *in vitro* fermentation, pH per substrate and per inoculate did not differ (data not shown). In comparison, a (non-significant) trend towards lower pH was observed in the glucose group, compared to the two β-glucan treated groups. No difference in pH was observed between MacroGard® and curdlan treatment (Figure 2B). Ammonia production was significantly higher in the PBS group compared to all other groups (Figure 2C). Interestingly, NH_3 production was different between curdlan and MacroGard®, providing further evidence for differences in fermentation dynamics between the two β-glucans.

In vitro fermentation of β-glucans results in significantly increased SCFA levels

From the *in vitro* batch culture analysis it became evident that the intestinal microbiota of carp can ferment β-glucans up to a certain degree. Metabolites produced by the microbiota during fermentation such as SCFA are necessary components for immune homeostasis (222). Accumulation of SCFA metabolites produced during β-glucan fermentation was analysed in the *in vitro* fermentation liquid. Significant differences in SCFA profiles between the readily-fermentable substrate glucose and the two independent β-glucan preparations could be observed (Figure 3). Overall, a clear production of acetate, butyrate and propionate was observed after fermenting either curdlan or MacroGard® *in vitro*. Comparing the two different β-glucan preparations showed no differences in acetate production but revealed an interesting inverted trend for butyrate and propionate levels. After fermentation of curdlan, higher butyrate concentrations were measured, while after fermentation of MacroGard® higher propionate concentrations were measured (Figure 3B-C). In both β-glucan groups total SCFA levels were higher than in the glucose group, however, no differences were observed between both β-glucan groups (Figure 3E). Overall, differential production of SCFAs could be observed in both β-glucan groups, with fermentation of MacroGard® specifically resulting in an increase in propionate.

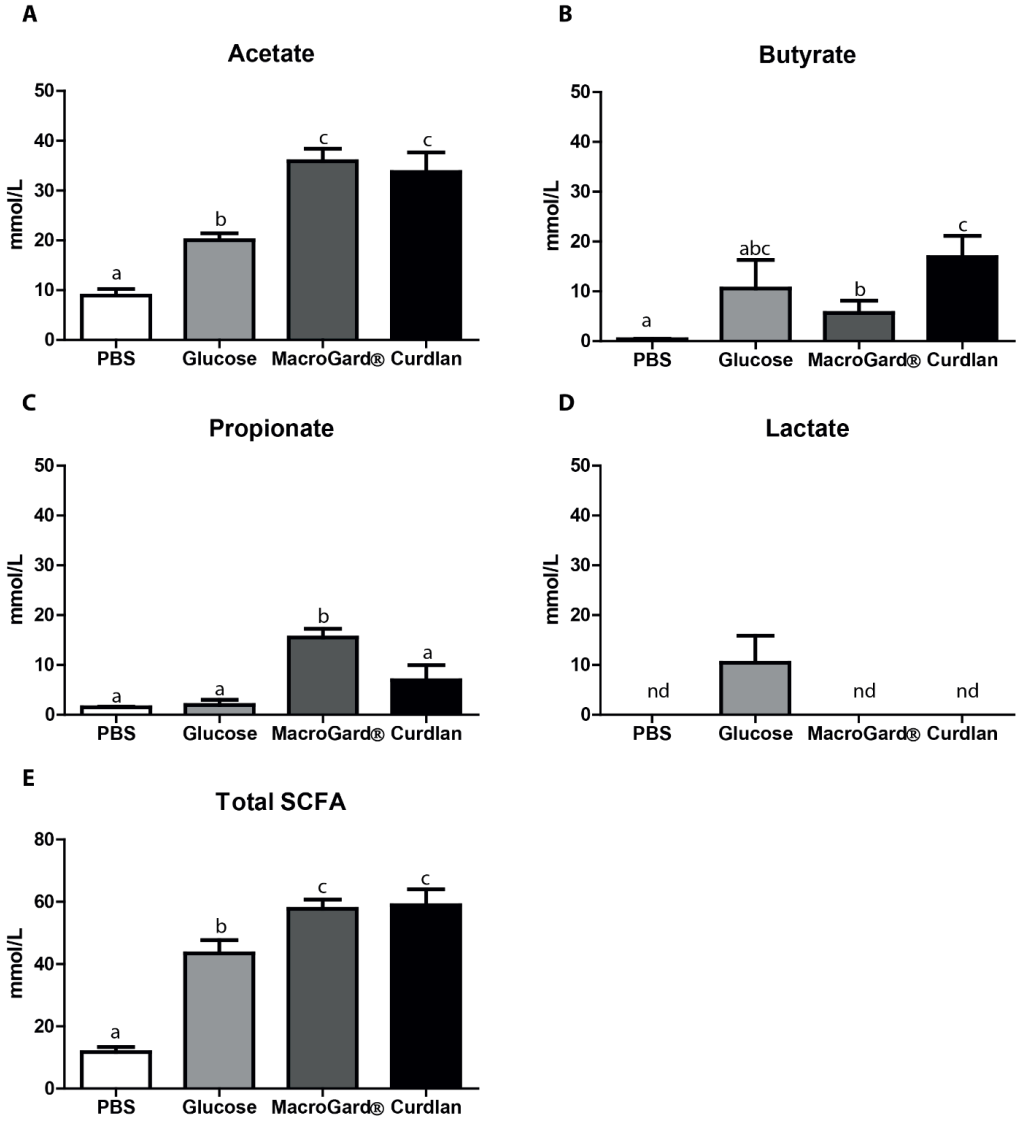

Figure 3. Differential SCFA profiles after *in vitro* fermentation of β-glucan. SCFA levels in the fermentation liquid following 168h *in vitro* fermentation of PBS, glucose, MacroGard® or curdlan. A-E. Acetate, butyrate, propionate and lactate, or total sum. Total SCFA levels were calculated by adding up all analysed SCFA levels including branched chain short chain fatty acids. Bars indicate mean + SD of n=5 independent intestinal pools. Significant differences between groups were assessed by one-way ANOVA followed by Tukey test (4A, D and E) or by Welch's ANOVA followed by Games-Howell test (4B and C). Groups with different letters are statistically different from one another.

Oral gavage with β-glucans can alter microbiota composition of carp

In vitro fermentation of β-glucans by intestinal microbes of carp revealed both the capacity to ferment β-glucans as well as an accumulation of propionate after fermentation of MacroGard®. We progressed to investigate the effects of *in vivo* β-glucan supplementation on the intestinal microbiota, 7 and 14 days after a single oral gavage treatment with PBS or MacroGard®. Overall, analysis of diversity indicated a trend towards a higher number of OTUs upon either PBS or β-glucan treatment compared to the unhandled group (Fig. 4A), however, only PBS-treated control fish on day 7 showed a significantly higher number of OTUs compared to those in the unhandled group ($p<0.05$) (Fig 4A, Supplementary table 1). The alpha diversity matrices, a measure for the mean species diversity in a single group, showed no significant differences between unhandled, control and treatment groups (Supplementary table 1).

Taking into account the OTU abundance, both control (PBS) and MacroGard®-treated groups were separated based on a principle component analysis (Figure 4B). To further investigate the differences in abundance between these two treatment groups two different statistical analyses were used, cumulative sum scaling (CSS) (Figure 4C-D; Supplementary table 2) and random forest analysis followed by the Boruta feature selection (Supplementary figure 1, Supplementary table 3). Specifically looking at increased OTUs in the treatment groups, identified by both CSS and the random forest analysis, revealed increased abundance of members belonging to the families *Rhodocyclaceae* and *Streptococcaceae* on day 7 post-treatment with MacroGard®. On day 14 post-treatment, there was only a single OTU increased by the treatment, belonged to the *Rhodocyclaceae*. Although the single oral gavage as a treatment was subtle, the abundance of several OTUs were shifted due to the treatment with β-glucans.

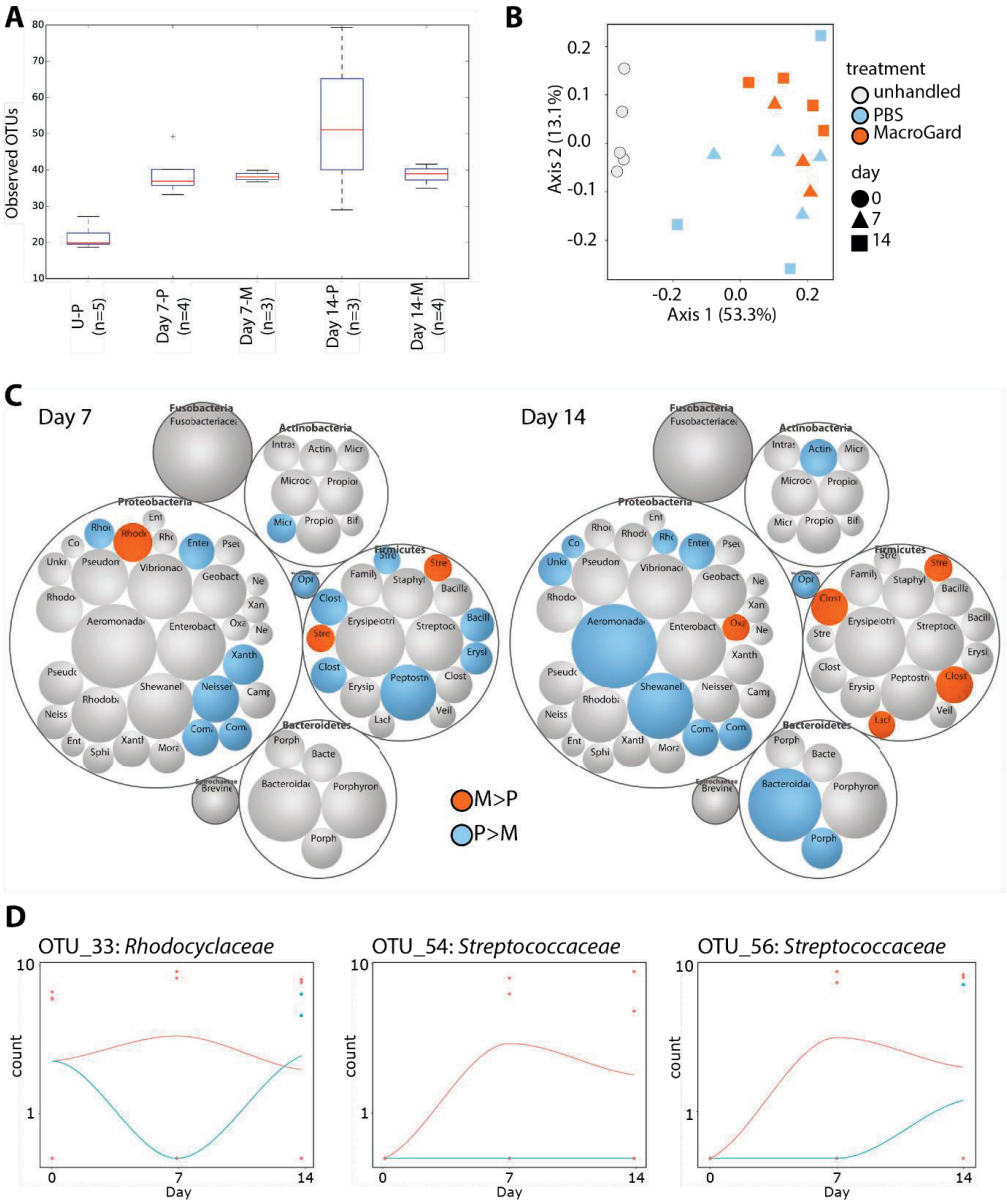

Figure 4. Oral gavage with β-glucans induces a shift in intestinal microbial community of carp. A. Number of observed operational taxonomic units (OTUs; alpha diversity) of the intestinal microbiota showing a higher diversity after treatment with PBS (P) and MacroGard® (M) compared to unhandled (U) fish. B. Principal component analysis of microbiota showing a shift in community upon β-glucan treatment. C. Differences based on cumulative sum scaling and fitZIG contrasts showing significantly more abundant OTUs between PBS- and MacroGard®-treated carp at day 7 and day 14 after gavage. The size of each circle indicates the abundance of the respective family overall in all samples. D. Three OTUs were significantly more abundant in MacroGard®-treated carp at day 7 showing different dynamics in time.

In vivo treatment with β-glucans modulates expression of immune genes

To follow up on the above-observed shift in carp intestinal microbiota *in vivo*, simultaneous modulation of local immune gene expression was analysed. Therefore, we analysed the gene expression from intestinal tissue samples collected at the exact same location at which the intestinal content was collected for 16S rRNA sequencing. Based on an earlier observation following feeding with MacroGard® (111), expression of several cytokines was examined. We found significant down-regulation of *cxca*, *il1b, il6a* and *tnfαa2* at 7 days (Figure 6A) and sometimes 14 days (Figure 6B) post-treatment with MacroGard®. In addition, based on an original hypothesis of sensing dsRNA from lactate bacteria (76), an increase of which could not readily be confirmed by the β-glucan treatment-

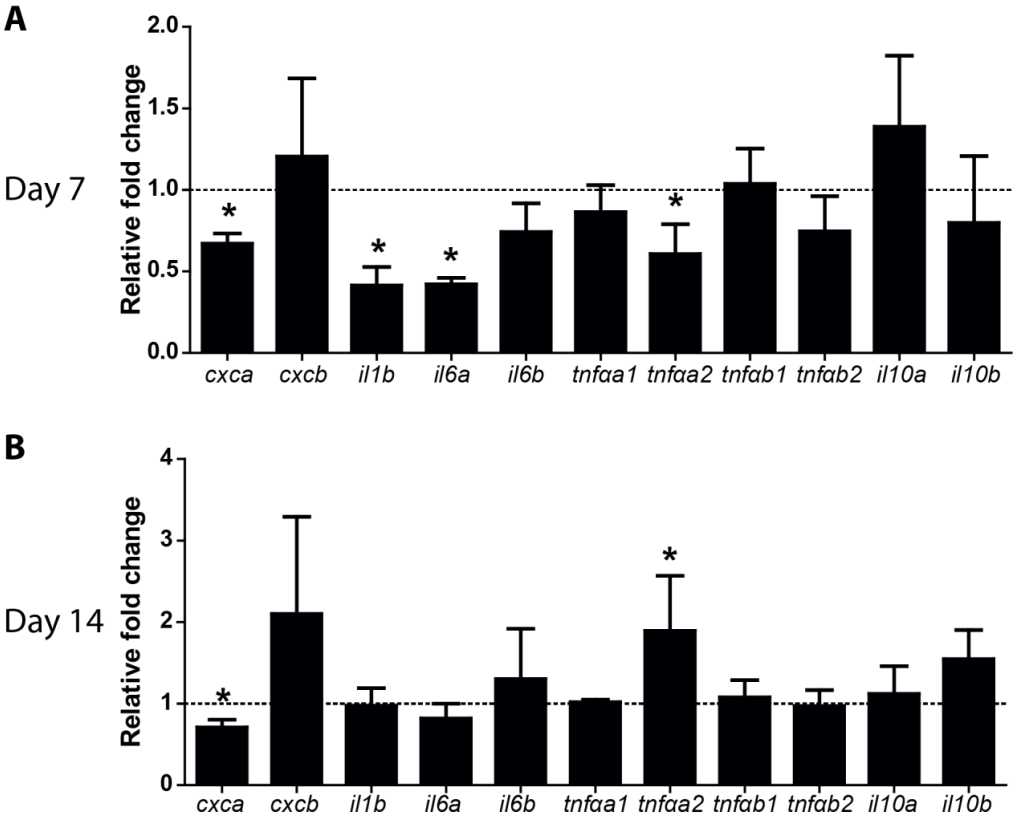

Figure 5. Oral gavage with β-glucans alters cytokine gene expression in the third segment of carp intestine. Gene expression at day 7 (A) and day 14 (B) in the third gut segment of the treated group compared to control group (dotted line), as measured by RT-qPCR. A. Significantly lower expression of *cxca, il1b, il6a and tnfαa2* can be observed. A trend towards lower expression of *il6b* (p = 0.062) and *tnfαb2* (p = 0.090) could be observed in the treated group. B. Significantly lower expression of *cxca* and a significant increase in *tnfαa2* expression could be observed in the treated group. Asterisk (*) indicates significant difference between expression between the group orally gavaged with PBS and the group orally gavaged with MacroGard® as assessed by one-way ANOVA.

induced shift, measurement of relevant gene expression was also included. We found significant down-regulation of *tlr3.1* and *mx3* at 7 days (Supplementary figure 2A) and up-regulation of *mx4* at 14 days (supplementary Figure 2B). Taken together, we observed several immunomodulatory effects on gene expression following *in vivo* treatment with MacroGard®, coinciding with a shift in intestinal microbial composition.

Discussion

To investigate whether β-glucans can alter composition or activity of the intestinal microbiota we first characterized the active intestinal microbiota of unhandled carp. We observed, among others, a prominent presence of the genus *Bacteroides*, which are well-known for their ability to degrade carbohydrates. The mere presence of the *Bacteroides* genus is interesting because some species of the *Bacteroides* genus have been shown able to specifically degrade β-glucans (70). Subsequently, we confirmed a theoretical β-glucan fermenting ability of carp microbiota by *in vitro* batch culture system, in which we used intestinal microbiota of carp to test fermentation of two different β-glucan preparations. As a result of this *in vitro* fermentation, we observed a production of several SCFAs, of which propionate could be specifically linked to the fermentation of MacroGard®. To follow up, we investigated the effects of a single oral gavage with MacroGard® on the intestinal microbiota and on local immune gene expression. We observed an increase in abundance at seven days post treatment with β-glucans of three OTUs, one belonging to the family *Rhodocyclaceae* and the other two belonging to *Streptococcaceae*. Coinciding with this increase, we also observed a clear reduction in IFN related and cytokine gene expression.

We identified four abundant phyla in our carp normal intestinal microbiota (*Fusobacteria*, *Bacteroidetes*, *Gammaproteobacteria*, *Firmicutes*) using 16S rRNA analysis. A comparable division of phyla in carp normal intestinal microbiota was also found previously using 16S rRNA pyrosequencing (223), suggesting that indeed this could be considered the core intestinal microbiota of common carp. In fact, despite possible fish species-specific differences with respect to relative abundance (223-226), the same four phyla appear common to intestines of fishes. Of the phylum *Fusobacteria,* the genus *Cetobacterium* clearly dominated abundance and appears to do so in omnivorous and carnivorous more than in herbivorous fishes (225). The genus *Bacteroides* appeared as the second most abundant microbial species and is particularly interesting with respect to fermentation of carbohydrates.

The genus *Bacteroides* has been linked to carbohydrate degradation and is capable of degrading even highly complex carbohydrates (69, 227, 228). Degradation of carbohydrates is achieved with so-called polysaccharide utilization loci (PUL) of which there can be many different ones, each related to the degradation of a specific glycan or group of related glycans (229). Members of the *Bacteroides* found in the gastro-intestinal tract of humans have been shown able to degrade

several different β-glucans, among which barley-derived β-1,4-glucans (69) and fungus- derived β-1,6-glucans (70). The presence of the *Bacteroides* genus in the normal intestinal microbiota might suggest that carp should be capable of degrading β-glucans. We thus performed an *in vitro* batch culture experiment with two different β-glucans as substrates; MacroGard®, a branched β-1,3/1,6-glucan isolated from the yeast *Saccharomyces cerevisiae* and commonly used in aquaculture practices, and curdlan, a linear β-1,3-glucan isolated from the bacteria *Alcaligenes faecalis*. Each showed distinct but slightly different fermentation curves, possibly in part related to 'contamination' of MacroGard® with products such as chitin and mannose (46), of which the latter could inhibit carbohydrate uptake of bacteria under specific conditions (230). Fermentation also led to higher butyrate production associated with curdlan and higher propionate production associated with MacroGard®. Again, the difference might be related in part to the presence of products such as chitin because, at least in tilapia, intestinal microbiota were able to ferment chitin resulting in acetate and propionate but no butyrate (193). Regardless of the above, *in vitro* fermentation of β-glucans by the intestinal microbiota of carp resulted in increased gas and SCFA production.

Several studies in mice have connected oral intake of fibres to increased SCFA levels, both local and systemic. Moreover, these studies showed connections between increased SCFA levels and reductions in pathogenesis. For example, mice fed with oat bran derived β-glucan enriched diets had higher SCFA levels in the caecum, especially after having been fed diets with practically insoluble β-glucan of the heavy molecular weight (74). Mice fed with high fibre diets showed systemic increases in propionate levels, while oral supplementation with propionate reduced allergy induced inflammation in the lungs (231). Earlier studies in fish have hinted at the ability of fish microbiota to degrade carbohydrates, observing fermentation of several carbohydrates and production of SCFAs by intestinal microbes of tilapia, European sea bass or common carp (192-194) or showing growth of monocultures of fish intestinal bacteria on different carbohydrate sources, including β-glucan (191). While the connections between differential SCFA levels due to diet and reduced pathogenesis have not been made in fish, here we show that the intestinal microbiota of fish can play a role in the fermentation of carbohydrates.

Studies in mammals have suggested connections between intake of high fibre diets and reduced systemic inflammation could be mediated by the increased expression of receptors for SCFAs such as the G-protein coupled receptors GPR41 and GPR43 (also known as free fatty acid receptor 2 or FFAR2) (232). SCFAs activities can be both anti- and pro-inflammatory and would be mediated by GPR41, GPR43, to lesser extent GPR109A, or via their effect on histone deacetylases (HDACs) (as reviewed by (72)). At present, these free fatty acid receptors have not been described for fish, but it would be of great interest to study expression of free fatty acid receptors in the context of our findings.

We observed changes in the SCFA profile after *in vitro* fermentation and observed an increase in *Rhodocyclaceae* and *Streptococcaceae* genera *in vivo*. Although the

change in SCFA profile associated with fermentation of β-glucans might explain the observed modulation of immune gene expression in the intestine, there might be other mechanisms at play that could modulate the immune response in the intestine. In mice, commensal lactate bacteria can induce an antiviral IFN response via shedding of double stranded RNA which, recognized by intestinal dendritic cells (DCs) via a *Tlr3* mediated mechanism, can lead to the production of IFNβ, which in turn dampens the expression of IL6 and TNFα (76). In fish, several studies observed effects of β-glucan supplementation on the presence of lactate bacteria (78, 79), although this shift did not stand out in our present study, at seven days post treatment there was a significant increase in a OTU belonging to the *Streptococcus* genus, belonging to the order of *Lactobacillales* (lactate bacteria). In a previous study we noticed a concurrent increase in *mx* and *tlr3* expression in the intestine of carp fed with β-glucans (111). Another study also observed prolonged *mx* expression following β-glucan supplementation (233). In our present study we again noticed in carp intestine modulation of expression of anti-viral genes such as *mx*, suggesting immunomodulation by the increased presence of dsRNA should not be excluded.

No matter the presence in the fish intestine of free fatty acids or dsRNA and their receptors, or not, immunomodulation was evident. In a previous study we reported on the inhibition of expression of several pro-inflammatory genes in the intestine of carp following 25 days of dietary supplementation with β-glucans. Expression of *il1β*, *tnfα* and *il10* in the intestine was strongly inhibited in this *in vivo* feeding experiment (111), an observation that was partly confirmed by our *in vivo* single gavage study. In mammals, propionate and butyrate are known to regulate inflammatory responses in epithelial cells and leukocytes (232, 234-237). It remains to be determined whether the inhibition of expression of pro-inflammatory genes in carp intestine was modulated by SCFAs or by bacterial substances.

In the end, the observed effects of β-glucans on the microbiota and on the intestinal gene expression cannot be definitively mechanisms, either direct effects of the β-glucans on the intestine or indirect effects through prebiotic effects on the intestinal microbiota. The coinciding change in microbiota and immuno-regulation on day 7 post-treatment at the same location in the intestine, could argue for prebiotic effects from β-glucans on the intestinal microbiota resulting in immunomodulation in the host via SCFAs. At the same time, a regulation of *tlr3* and *mx* paralogs was also observed following β-glucan supplementation, coinciding with an increase in a *Lactobacillales* genus on day 7 post-treatment. Therefore, we cannot rule out that the intestinal immuno-modulation is a result of the production of other molecules by the microbiota, such as double stranded RNA. It can be of interest to analyse the effects of β-glucans on sterile intestinal samples, for example with *ex vivo* stimulation of gut tissue, to investigate whether β-glucans directly affect the gene expression in the intestine resulting in regulation of several immune genes or whether the microbiota plays a crucial part in this effect. Such an experimental setup would allow for selectively testing the effects of SCFAs and other microbiota produced molecules. Although not conclusive, the present study

has provided insights into possible indirect mechanisms of β-glucan immuno-modulation and paves the road for future studies to unravel a definitive mechanism of action through which β-glucan supplementation can modulate immune responses.

Acknowledgements

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Supplementary material

Supplementary table 1. Comparison of alpha diversity matrices of operational taxonomic units (OTUs) detected in guts of unhandled carp (U0) and after 7 or 14 days of gavage with PBS (P) and MacroGard (M) treated carp. Significant differences were determined on rarefied data using two-sample t-test will be nonparametric using 999 Monte Carlo permutations[1].

Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
Observed_OTUs							
B_P7	A_U0	39.05	6.06	21.54	3.08	4.96	0.03
C_M7	A_U0	38.23	1.31	21.54	3.08	7.73	0.07
D_P14	A_U0	53.13	20.59	21.54	3.08	2.92	0.15
E_M14	A_U0	38.63	2.45	21.54	3.08	7.98	0.09
B_P7	C_M7	39.05	6.06	38.23	1.31	0.19	1.00
D_P14	C_M7	53.13	20.59	38.23	1.31	1.02	1.00
E_M14	C_M7	38.63	2.45	38.23	1.31	0.21	1.00
B_P7	D_P14	39.05	6.06	53.13	20.59	-1.09	1.00
B_P7	E_M14	39.05	6.06	38.63	2.45	0.11	1.00
D_P14	E_M14	53.13	20.59	38.63	2.45	1.18	1.00
Chao1							
B_P7	A_U0	43.57	7.82	29.50	5.05	2.88	0.21
C_M7	A_U0	38.69	1.56	29.50	5.05	2.65	0.74
D_P14	A_U0	59.35	21.67	29.50	5.05	2.55	0.69
E_M14	A_U0	39.21	2.64	29.50	5.05	3.07	0.23
B_P7	C_M7	43.57	7.82	38.69	1.56	0.90	1.00
D_P14	C_M7	59.35	21.67	38.69	1.56	1.34	1.00
E_M14	C_M7	39.21	2.64	38.69	1.56	0.26	1.00
B_P7	D_P14	43.57	7.82	59.35	21.67	-1.14	1.00
B_P7	E_M14	43.57	7.82	39.21	2.64	0.92	1.00
D_P14	E_M14	59.35	21.67	39.21	2.64	1.56	1.00
Shannon							
B_P7	A_U0	1.48	0.45	1.43	0.39	0.18	1.00
C_M7	A_U0	2.41	0.48	1.43	0.39	2.75	0.42
D_P14	A_U0	1.83	0.69	1.43	0.39	0.91	1.00
E_M14	A_U0	2.50	0.87	1.43	0.39	2.18	0.89
B_P7	C_M7	1.48	0.45	2.41	0.48	-2.22	1.00
D_P14	C_M7	1.83	0.69	2.41	0.48	-0.98	1.00
E_M14	C_M7	2.50	0.87	2.41	0.48	0.14	1.00
B_P7	D_P14	1.48	0.45	1.83	0.69	-0.67	1.00
B_P7	E_M14	1.48	0.45	2.50	0.87	-1.80	1.00
D_P14	E_M14	1.83	0.69	2.50	0.87	-0.93	1.00

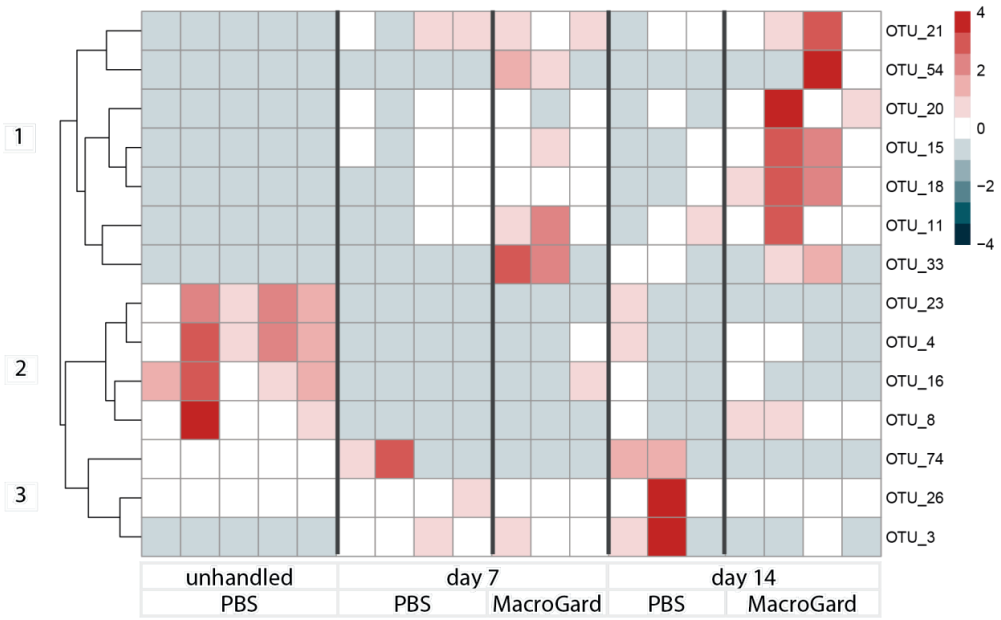
[1] Note: std indicates the standard deviation, t stat indicates t-statistics

Supplementary table 2. Cumulative sum scaling analysis of significantly different operational taxonomic units (OTUS) that were identified by 16S rRNA amplicon sequencing of RNA between MacroGard (M) and PBS (P) treatment on day 7 and 14 days post treatment. Significance is based on cumulative sum scaling and fitZIG contrasts.

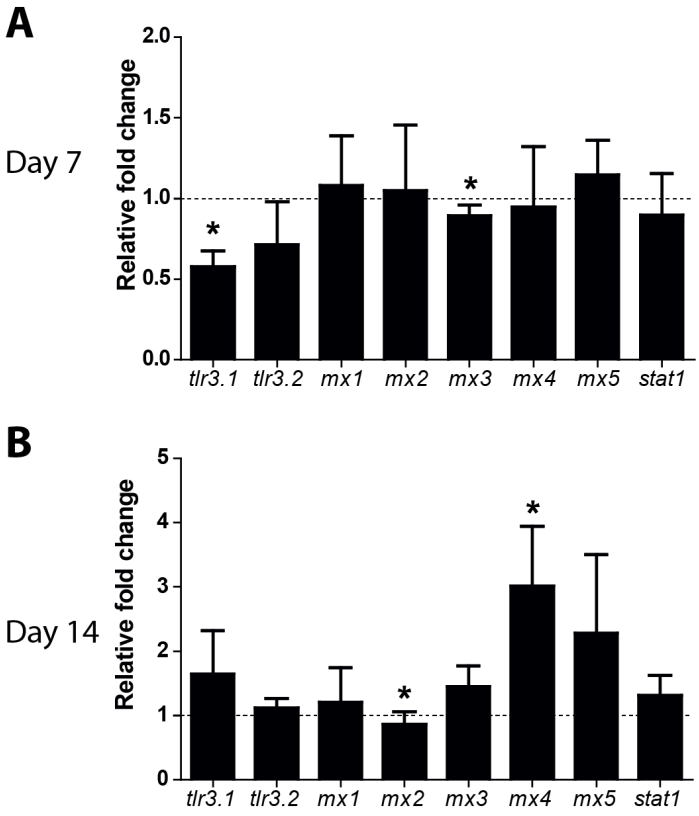
OTUid	Phylum	Family	logFC	adj.P.Val
Day 7 M>P				
OTU_33	Proteobacteria	Rhodocyclaceae	-6.16	0.00
OTU_54	Firmicutes	Streptococcaceae	-4.74	0.02
OTU_56	Firmicutes	Streptococcaceae	-3.20	0.04
Day 7 M<P				
OTU_74	Proteobacteria	Enterobacteriaceae	4.99	0.00
OTU_27	Proteobacteria	Comamonadaceae	5.83	0.01
OTU_41	Firmicutes	Erysipelotrichaceae	4.38	0.01
OTU_39	Proteobacteria	Xanthomonadales_IS	4.35	0.01
OTU_55	Firmicutes	Streptococcaceae	4.38	0.02
OTU_70	Proteobacteria	Rhodocyclaceae	3.41	0.02
OTU_51	Firmicutes	Bacillaceae	4.77	0.02
OTU_26	Proteobacteria	Comamonadaceae	2.88	0.03
OTU_57	Verrucomicrobia	Opitutae_vadinHA64	3.78	0.04
OTU_12	Firmicutes	Peptostreptococcaceae	3.21	0.03
OTU_46	Actinobacteria	Micrococcaceae	4.00	0.04
OTU_53	Firmicutes	Clostridiaceae_1	3.18	0.04
OTU_49	Firmicutes	Clostridiaceae_1	2.38	0.10
OTU_31	Proteobacteria	Neisseriaceae	2.11	0.10
Day 14 M>P				
OTU_69	Proteobacteria	Oxalobacteraceae	-5.02	0.00
OTU_54	Firmicutes	Streptococcaceae	-4.09	0.02
OTU_53	Firmicutes	Clostridiaceae_1	-4.19	0.03
OTU_79	Firmicutes	Lachnospiraceae	-3.60	0.03
OTU_29	Firmicutes	Clostridiaceae_1	-2.80	0.04
Day 14 M<P				
OTU_9	Proteobacteria	Unknown_Family	9.71	0.00
OTU_26	Proteobacteria	Comamonadaceae	6.45	0.00
OTU_57	Verrucomicrobia	Opitutae_vadinHA64	5.94	0.00
OTU_74	Proteobacteria	Enterobacteriaceae	5.16	0.00
OTU_27	Proteobacteria	Comamonadaceae	5.75	0.00
OTU_6	Proteobacteria	Shewanellaceae	3.73	0.00
OTU_3	Proteobacteria	Aeromonadaceae	3.82	0.00
OTU_153	Proteobacteria	Comamonadaceae	3.83	0.01
OTU_114	Proteobacteria	Rhodocyclaceae	3.93	0.02
OTU_36	Actinobacteria	Actinomycetaceae	3.41	0.03
OTU_23	Bacteroidetes	Porphyromonadaceae	5.40	0.03
OTU_4	Bacteroidetes	Bacteroidaceae	3.02	0.07

Supplementary table 3. Random forest analysis of significantly different operational taxonomic units (OTUS) that were identified by 16S rRNA amplicon sequencing of RNA between MacroGard (M) and PBS (P) treatment on day 7 and 14 days post treatment. Significance is based on using random forest analysis followed by the Boruta feature selection.

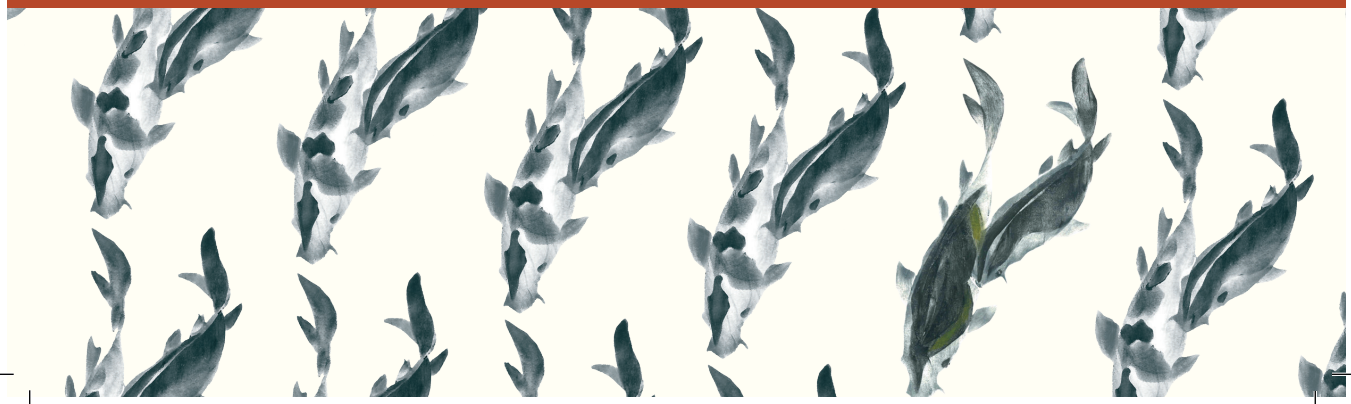
OTUid	Phylum	Family	Layer importance
M>P in both day 7 and day 14			
OTU_21	*Proteobacteria*	*Geobacteraceae*	9.77566216
OTU_54	*Firmicutes*	*Streptococcaceae*	3.772245573
OTU_20	*Proteobacteria*	*Rhodobacteraceae*	10.69023545
OTU_15	*Firmicutes*	*Streptococcaceae*	9.265338095
OTU_18	*Firmicutes*	*Staphylococcaceae*	7.802709367
OTU_11	*Proteobacteria*	*Pseudomonadaceae*	9.719296364
OTU_33	*Proteobacteria*	*Rhodocyclaceae*	4.012672004
Unhandeled > M,P			
OTU_23	*Bacteroidetes*	*Porphyromonadaceae*	7.161350787
OTU_4	*Bacteroidetes*	*Bacteroidaceae*	8.489835637
OTU_16	*Proteobacteria*	*Vibrionaceae*	7.138475572
OTU_8	*Bacteroidetes*	*Porphyromonadaceae*	10.76910099
P>M in both day 7 and day 14			
OTU_74	*Proteobacteria*	*Enterobacteriaceae*	6.200066198
OTU_26	*Proteobacteria*	*Comamonadaceae*	6.506197169
OTU_3	*Proteobacteria*	*Aeromonadaceae*	4.325133066



Supplementary figure 1. Treatment with a single oral β-glucan gavage alters intestinal microbial community of carp. Significant differences were identified by random forest analysis with Boruta feature selection (average z-scores of 1000 runs).



Supplementary figure 2. A. Interferon (IFN) pathway related gene expression seven days post-treatment. Significantly lower expression of *tlr3.1* and *mx3* can be observed. A trend towards lower *tlr3.2* (p = 0.084) expression can be observed in the treated group. B. IFN related gene expression 14 days post-treatment. Significantly lower expression of mx2 and increased expression of mx4 can be observed. A trend towards increased tlr3.2 (p = 0.096) and mx3 (p = 0.054) expression can be observed in the treated group. Asterisk (*) indicates significant difference in gene expression between the group treated with an oral gavage with PBS and the group treated with an oral gavage with MacroGard® as assessed by one-way ANOVA.



5

Genomic and transcriptomic approaches to study immunology in cyprinids: What is next?

Jules Petit, Lior David, Ron Dirks, Geert F. Wiegertjes

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Abstract

Accelerated by the introduction of Next-Generation Sequencing (NGS), a number of genomes of cyprinid fish species have been drafted, leading to a highly valuable collective resource of comparative genome information on cyprinids (*Cyprinidae*). In addition, NGS-based transcriptome analyses of different developmental stages, organs, or cell types, increasingly contribute to the understanding of complex physiological processes, including immune responses. Cyprinids are a highly interesting family because they comprise one of the most-diversified families of teleosts and because of their variation in ploidy level, with diploid, triploid, tetraploid, hexaploid and sometimes even octoploid species. The wealth of data obtained from NGS technologies provides both challenges and opportunities for immunological research, which will be discussed here. Correct interpretation of ploidy effects on immune responses requires knowledge of the degree of functional divergence between duplicated genes, which can differ even between closely-related cyprinid fish species. We summarize NGS-based progress in analysing immune responses and discuss the importance of respecting the presence of (multiple) duplicated gene sequences when performing transcriptome analyses for detailed understanding of complex physiological processes. Progressively, advances in NGS technology are providing workable methods to further elucidate the implications of gene duplication events and functional divergence of duplicates genes and proteins involved in immune responses in cyprinids. We conclude with discussing how future applications of NGS technologies and analysis methods could enhance immunological research and understanding.

1. Cyprinids are a biologically and economically important family

The cyprinid family is, economically, one of the most important and biologically, one of the most diverse fish families worldwide. Cyprinid species live in a wide range of different habitats including fresh, brackish and salt water, variable temperatures, water depths and oxygen concentrations. The entire cyprinid family includes approximately 3,000 species, whereas the combined aquaculture production of the 3 most-cultured cyprinid species alone, including grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*) and common carp (*Cyprinus carpio*), accounts for almost 20% of the aquaculture production worldwide (FAO – fisheries and aquaculture[1]). Further prominent members of the cyprinid family are species from the *Carassius* genus including goldfish, the major Indian carps and zebrafish (*Danio rerio*) (Figure 1). Of the most-cultured species; grass carp provides both an important protein source in China and is used in many countries as a biological control for aquatic weeds, whereas silver carp, also a herbivorous species, has been long cultured in polyculture in China. Aquaculture of common carp is found in more than 100 countries and serves several purposes, such as food consumption, but also angling and production of ornamental koi.

Carassius species are of economic importance to aquaculture mostly in Asia, and are a genus commonly known as Crucian carps, although this term is most often specifically used to refer to *C. carassius*. Maybe the best-known member of this genus is the ornamental goldfish (*C. auratus*), which was bred from the Prussian carp (*C. gibelio*). The 'major Indian carps' is a common name for three species which together form the backbone of Indian aquaculture: Catla (*Catla catla*), the most produced in India, Rohu (*Labeo rohita*) and Mrigal (*Cirrhinus mrigala*). Although their names indicate each belong to a different genus; their exact phylogenetic relationship is still under debate, particularly true for the position of Catla [see also Figure 1, (238)].

As a prominent and probably the most-studied member of the cyprinid family, the zebrafish has become a widely-accepted animal model species for, among others, human diseases and disorders, drug discovery and screening, and toxicological assessments (239-242). Since the publication of the early teleost genomes, including early builds of the zebrafish genome at the Wellcome Trust Sanger Institute since 2001, but also genomes of the fugu (*Takifugu rubripes*) (243), green spotted pufferfish (*Tetraodon nigroviridis*) (244), and medaka (*Oryzias latipes*) (245), the number of sequenced fish genomes has increased steadily for years, and steeply with the advent of Next Generation Sequencing (NGS, see also

[1] http://www.fao.org/fishery/statistics/en (accessed on: 2017-02-12)

info Box 1), (246). To date, at least, 108 genome assemblies (among which 30 are mitochondrial and 16 obsolete versions) from 73 different teleost fishes, have been submitted to the National Center for Biotechnology Information (NCBI)[2]. Surprisingly, the number of published genomes of cyprinids is relatively few, and only includes grass carp, common carp, zebrafish, cavefish (*Sinocyclocheilus complex*), Amur ide (*Leuciscus waleckii*), and fathead minnow (*Pimephales promales*). It stands out that, despite their economic relevance, there are no genomes available of silver carp, *Carassius* genus and the major Indian carps. The relative scarcity of cyprinid genomes is a good example of the difficulties still faced when trying to obtain high quality reference genomes for non-model species, despite the use of NGS technologies. Maybe the gap will be filled as a result of large-scale efforts aiming to sequence and assemble many more teleost genomes such as the 10K genome project, which includes several cyprinid species (247), helped by the latest developments in NGS technologies.

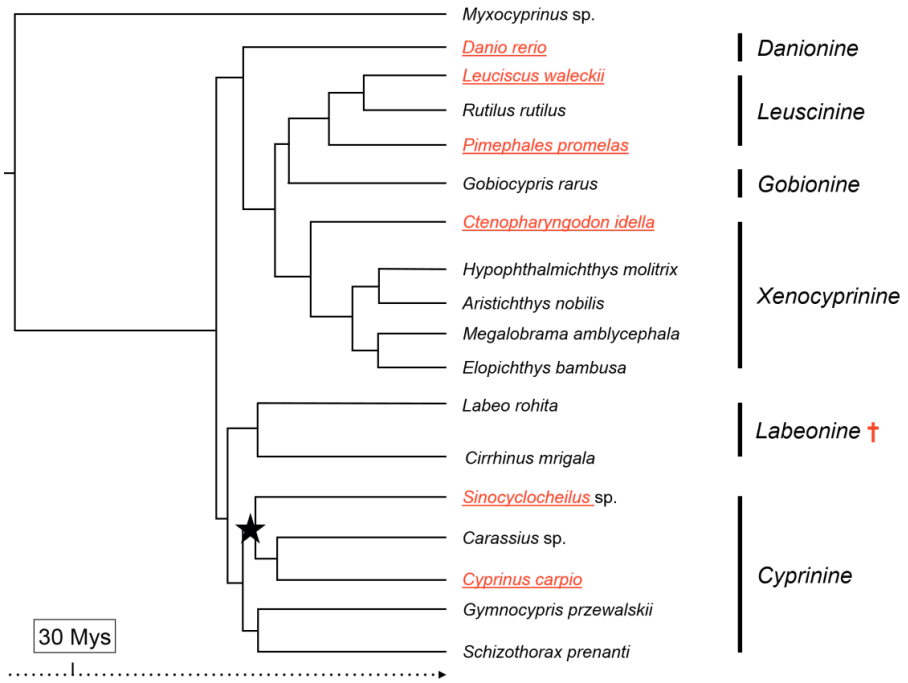

Figure 1. Phylogenetic relationships among key cyprinids with estimates of divergence time based on (238). Myxocyprinus asiaticus was chosen as outgroup. Mys: million years. Species with a have a submitted or published genome assembly are depicted in red and underlined. †: the phylogenetic position of the third major species of Indian carp (Catla catla) is still under debate. The black star represents a likely position for the fourth round of WGD relevant to common carp (Cyprinus carpio) and the Carassius spp., whereas the exact position of the WGD relevant to Sinocyclocheilus spp. is unknown.

[2] https://www.ncbi.nlm.nih.gov/genome/browse/ (accessed on: 2017-01-16)

BOX 1: The generations of sequencing technologies

First generation sequencing started in 1977 with the introduction of Sanger's "chain termination" technique (248). Sanger sequencing generates individual reads of up to one kilobase in length. The best-known example of a genome sequence assembled from Sanger reads is the human genome (249, 250). **Second generation sequencing,** also originally referred to as next generation sequencing, started around 30 years later, when mass parallelization and miniaturization became possible via pyrosequencing technology (251). Pyrosequencing was incorporated into the Roche 454 sequencer platform and was quickly followed by Solexa/Illumina and SOLiD (Applied Biosystems) sequencing, three competing platforms that use different technologies for parallelization and miniaturization. All three platforms can generate millions of reads simultaneously, ranging in size from less than a hundred (SOLiD) to few hundreds of base pairs (Illumina, Roche 454). Due to the massive throughput, second generation sequencing resulted in a greatly reduced cost price per sequenced base**.** Draft genome sequences assembled from Illumina reads are often fragmented and the scaffolds contain many sequence gaps, mostly caused by repeat regions that could not be resolved by the short reads. **Third generation sequencing** refers to very recent techniques based on single molecule sequencing (SMS), which combine generation of long reads with large amounts of sequence information. Examples of platforms are PacBio sequencing and the sequencing device from Oxford Nanopore Technologies. In this review, second and third generation sequencing will be clustered under the term **Next Generation Sequencing (NGS)**. No distinction will be made between second and third generation sequencing, unless explicitly mentioned.

2. Genome assemblies provide a basis for studying immune responses across cyprinid species

Sequencing of the zebrafish genome has greatly contributed to the present broad basis of genomic and functional studies not only in the zebrafish itself but in many other fish species, and turned zebrafish into a common reference for many, if not all, immunological studies in teleost fish. In its current assembly version, about 25,000 genes have been mapped on the genome (Table 1). In this review, we will mention the zebrafish as reference but will not further discuss genome or transcriptome studies in zebrafish. For a comprehensive overview of the advances in genomic and transcriptomic research in zebrafish we refer to, among others, a recent special issue of Methods in Cell Biology (Volume 135, 2016). It is, however, important to remember here that, being the most-complete and best-annotated cyprinid genome publicly available, the zebrafish provides a reference for new genetic data on any cyprinid fish species.

Common carp most likely is the one cyprinid species of economic relevance that has the most-advanced NGS-based version of the genome. Already in 2011, a preliminary draft combining a *de novo* assembly based on RNAseq with existing EST and mRNA sequences from GenBank was used to mine for a specific set of immune genes (252). Subsequently, using genomic DNA from a single individual of European genetic background with double-haploid homozygous status, an updated and more complete genome assembly was published (253). The choice for a double-haploid individual greatly facilitated the genome assembly of this

difficult species of tetraploid origin (254). The assembly published by Henkel and colleagues in 2012 was improved with long read sequencing (Pacific Bioscience) and combined with verification of gene expression, which resulted in a new genome assembly and a total number of 50,527 genes submitted to NCBI (255).

In parallel, a second major genome assembly of common carp was drafted based on a different (Songpu) strain of East-Asian genetic background (256) with 52,610 genes submitted to NCBI. For the latter assembly, contigs were built on single-end sequencing, whereas the scaffold assembly combined BAC-end sequences with paired-end and mate-pair sequences from different sequencing platforms (i.e. Roche 454, Illumina HiSeq and SOLiD). Using 3,470 single nucleotide polymorphisms (SNPs) and 773 microsatellites, a first attempt was made to anchor these scaffolds into 50 linkages groups (256). A comparison of size and number of scaffolds between the different genome assemblies (Table 1) shows differences which might be related to differences in sequencing strategies and platforms, or to the number of individuals sequenced and/or degree of homozygosity of the sequenced individuals. It is also conceivable that genetic differences between common carp of European and East-Asian background (257) might account for some of the differences between common carp genome assemblies. Aligning the two major genome assemblies of common carp revealed preservation of syntenic relationships in 1.13 Gbp (255).

Grass carp genome resources have been relatively few given its economic relevance, and included a genetic linkage map based on 279 markers, identified by a combination of microsatellite and EST-cloning (258). In 2015, a draft genome (Table 1) was published and opened the door to more in-depth genomic investigations. Independent draft assemblies of male and female grass carp resulted in different sizes of the two assemblies. The female genome was annotated, resulting in >27,000 genes and was anchored based on the previously-published genetic linkage map. The male genome assembly was mainly generated to analyse genetic variation between the female and the male genome sequences. Of the 279 markers, a total of 64% could be used for anchoring the scaffolds and predicted genes. Despite the genomic progress, the above-discussed assemblies for the common carp and grass carp genomes were primarily based on short-read sequencing and thus, novel long-read technology platforms are expected to further improve the current genome assemblies in the near future.

There are (at least) two genome sequencing projects that have selected particular members of the cyprinid family to study environmental effects, sometimes including effects on immune responses. The *Sinocyclocheilus* is a genus of cyprinid cavefish which, like other cavefish including the Mexican tetra (not discussed here), have adapted to their unique habitat and thus show differential grades of regression in several features including eyesight, presence of scales and skin pigmentation; all complex phenotypes of evolutionary interest. Three representatives of the *Sinocyclocheilus* genus, residing in three different habitats, were sequenced and three genome assemblies were generated (Table 1), (259).

Of particular interest here, based on computational annotation, the study found a correlation between expansion of the immune system and specific habitats of the three species. The cave-restricted species (*S. anshuiensis*) had fewer copies of particular immune-related genes, including a clear reduction in the copy number of some MHC class II genes (DPA1, DQB1), but more copies of other genes, including particular toll-like receptors (TLR8, TLR18) (259). This study potentially provides a first glimpse of a genetic basis for cave adaptation and the effects of speciation on immune-related gene evolution.

Another example provides the Amur ide (*Leuciscus waleckii*), of interest because of its evolutionary adaptation to an extreme alkaline habitat (260). The recent annotation of this genome assembly resulted in a total of 23,560 genes. Besides revealing gene family expansion and gene family contraction upon comparison with zebrafish and grass carp, the Amur ide genome assembly also revealed differences between individuals living under alkaline stress and individuals residing in relatively normal conditions (e.g. analysis of genetic diversity revealed copy number expansion in 10 genes between individuals living under alkaline stress and living under normal conditions). As discussed above, genome assemblies for cyprinid species of particular biological interest may allow for a first investigation of processes such as regression, or adaptation to extreme environments. Most certainly, the unravelling of such complex processes will be further facilitated by comparative analyses of genomes of several cyprinid species from diverse habitats.

Fathead minnow (*Pimephales promelas*) is a cyprinid probably best known for the almost universal use of the *epithelioma papulosum cyprini* (EPC) cell line for *in vitro* replication of several viruses (261). This cell line was originally reported to be from common carp epidermal herpes virus-induced hyperplastic lesions, but later recognized as derived from fathead minnow (262), a temperate species of the cyprinid family (Figure 1). Although the genome of this cell line may have diverged from its original host species through accumulation of mutations after many *in vitro* passages, the availability of a whole animal-based fathead minnow genome assembly (263), (Table 1) will improve the mapping and facilitate future transcriptome analyses of (innate) immune responses in this popular cell line. The application of NGS to controlled *in vitro* environments makes transcriptome analysis of cell lines a powerful tool to investigate complex processes such as innate immune responses to virus infection.

Table 1: Summary of currently available genomes from the cyprinid family. Information derived from Ensembl and NCBI[3]. In column Size, (A) refers to total size of assembled scaffolds; (P) refers to predicted size of the genome. Coverage is based on the statistics derived from NCBI, if they were available.

Species	Chromosome number (2*n*)	Ploidy level	Genome size (Gbp)	Contig number	Contig size N50 (Kbp)[4]	Scaffold number	Scaffold size N50 (Kbp)[4]	Sequencing coverage	Genetic linkage groups	Predicted genes	Accession or BioProject number	Reference
Zebrafish (*Danio rerio*)[5]	50	Diploid	1.48 (A)	66,213	411	14,463	1,274	NA	25	24,147	GCA_000002035.1	Ensembl
			1.41 (A)	28,064	1,073	4,560	1,551	NA	25	26,206	GCA_000002035.2	(264)
			1.37 (A)	22,851	1,258	3,398	2,181	NA	25	25,832	GCA_000002035.3	Ensembl
Common Carp (*Cyprinus carpio*)	100	Tetraploid	1.23 (A)	1,086,163	2	NA	NA	NA	NA	NA	PRJNA73579	(252)
			1.4 – 1.5 (P), 1.4 (A)	754,106	5	511,891	17.2	33x	NA	NA	PRJNA73579	(253)
			1.83 (P), 1.69 (A)	53,088	68.4	9,378	1,000	130x	50	52,610	GCA_000951615.2	(256)
			1.38 (A)	427,338	7	80,028	66.7	12x	NA	50,527	GCA_001270105.1	(255)
Grass carp (*Ctenopharyngodon idella*)	48	Diploid	0.9 – 1.07 (A), 0.89 (P)	16,682 *(N80)*	41	164,368	6,457	95x	24	27,263	PRJEB5920	(265)
Cavefish (*Sinocyclocheilus grahami*)	96	Tetraploid	1.75 (A) – 1.79 (P)	168,074	29	31,277	1,156	88x	NA	42,109	GCA_001515645.1	(259)
Cavefish (*S. rhinocerous*)	96	Tetraploid	1.65 (A) – 1.89 (P)	314,963	19	164,173	946	48x	NA	40,333	GCA_001515625.1	(259)
Cavefish (*S. anshuiensis*)	96	Tetraploid	1.63 (A) – 1.81 (P)	254,423	17	85,682	1,284	60x	NA	40,470	GCA_001515605.1	(259)
Amur ide (*Leuciscus waleckii*)	50	Diploid	0.75 (A) – 0.9 (P)	38,277	37	4,888	447	237x	24	23,560	GCA_900092035.1	(260)
Fathead minnow (*Pimephales promelas*)	50	Diploid	1.22 (A)	215,176	7	73,057	60	120x	NA	NA	GCA_000700825.1	(263)

[3] Ensembl (http://www.ensembl.org/Danio_rerio/Info/Annotation, visited 2017-01-05) and National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/assembly/, visited 2017-01-05).

[4] Contig N50 is calculated by sorting all contigs by length. Starting from the longest contig, the lengths of each contig are summed, until the sum of the largest sequences equals 50% of the total length of all contigs in the assembly. The contig N50 is the length of the shortest contig in this list. The scaffold N50 is calculated in the same fashion as the contig N50 but uses scaffolds rather than contigs.

[5] The zebrafish genome details consider Zv8, Zv9 and GRCz10.

Most of all, it is the combination of several cyprinid genome assemblies that provides a highly-valuable resource for comparative genome analysis. The added value of NGS resources for such comparative analyses is tremendous and will help to study similarities and differences in genome evolution, immune genes and many other processes or mechanisms (266). This is supported by studies observing a high conservation of syntenic relationships after comparative analyses of genome sequences or genetic maps. For example, comparison of grass carp, and Rohu, with zebrafish revealed 88% of the grass carp genes are located in syntenic blocks (265) and revealed 87% sequence similarity, on average, between Rohu and zebrafish transcripts (267). Comparative analysis also revealed two cross-chromosome rearrangements for two grass carp linkage groups, whereas two zebrafish chromosomes aligned to a single linkage group of the grass carp, suggesting a chromosome fusion. Another example is provided by the recent genetic map of the goldfish, which revealed a clear degree of conservation of synteny, not only between goldfish and zebrafish but also between goldfish and common carp (268). Phylogenetically, however, the *Carassius* genus is positioned closer to the *Cyprinus* genus than to zebrafish (Figure 1), (238, 269).

A compelling case for comparative genomics based on NGS was made by a recent study of the gene 'TLR4 interactor with leucine-rich repeats' (*tril*), first detected in an RNAseq data set of common carp (270). Surprisingly, despite a high degree of synteny surrounding the *tril* gene in the genome of several fish species, assembly Zv9 suggested the absence of *tril* in the relevant area of the zebrafish genome. Subsequently, detection of three overlapping ESTs for zebrafish *tril* allowed for the retrieval of the full nucleotide sequence of zebrafish *tril*. Based on this information the Genome Reference Consortium (GRC) undertook efforts to re-sequence the relevant area absent in the zv9 assembly. This case provides an excellent example of the added value of accessing multiple genomes, and transcriptomes, from related cyprinid fish species, because it is the collective set that provides the most valuable resource of genetic information for the cyprinid fishes. This would not be possible without the advent of NGS.

Realizing the efforts placed in drafting a genome assembly for, for instance the common carp, it is important to note few points relevant also to other cyprinids. In spite of the great improvements, the genome state is still largely fragmentary (Table 1), hence its annotation is still partial and heavily relies on the annotation of the better assembled and better annotated genome of the zebrafish. These imperfections are even more pronounced in genome assemblies of other cyprinids, with potential implications on the interpretation of NGS results that rely on the genome assembly as a reference. Therefore, despite the already invested efforts and the surge in cyprinid genome assemblies, there is still room for further improvement of the genome sequence in order to enhance interpretation of NGS based results. While cyprinid genome assemblies are being improved, considerable insights have already been gained in complex biological systems including immunology, mostly by utilizing the existing genomes and NGS platforms for transcriptome analyses.

3. Transcriptome analyses of immune responses – from basal to challenge conditions

3.1 Gene expression under basal conditions

Studying transcription levels and regulation of genes is pivotal for evaluating immune responses and traditionally this was carried out using RT-qPCR on small numbers of genes. As large-scale transcriptome analyses became more feasible and affordable, initially as DNA microarrays, later as NGS-based RNAseq, expression analysis of many genes in parallel has become widely-accepted and commonly-applied. In common carp, a transcriptomic approach was quickly adopted and a *de novo* assembly based on RNAseq was combined with existing EST and mRNA sequences from GenBank. The combined dataset was mined for a specific set of TIR domain containing immune genes and, using these genes as a reference, 162 contigs from a very fragmented genome could be stitched together into 39 scaffolds (252). *De novo* transcriptome assemblies have also been employed for data mining and gene identification in crucian carp, identifying 120,000 unigenes of which 6,000 could be assigned to known 'Kyoto Encyclopedia of Genes and Genomes' (KEGG) pathways, including almost 600 genes categorized as 'immune genes' (271). Unigenes can be output of *de novo* assembly using Trinity or alike; combining reads into contigs, extended with sequence clustering (272). Unigenes should not be confused with genes because upon annotation several unigenes can still result in only a single unique gene. Hence, conclusions based on expression analysis of unigenes should be interpreted carefully as they might not always accurately represent actual gene expression.

In 2014 another *de novo* transcriptome of the crucian carp was published with the main purpose to identify immune genes (273). Of the total of >78,000 transcripts, 7,500 transcripts could be aligned with fish-specific genes from the NCBI database, of which only 77 were deemed immune-relevant. Similar to these studies, a study in common carp employed transcriptome analysis and generated a *de novo* transcriptome, comprising 36,811 contigs of which 28,000 had a significant hit with the NCBI nr database corresponding to >19,000 unique genes, of which 441 were classified as immune system-related by KEGG analysis (274). The relatively low number of immune-related genes identified in the two above-mentioned studies may reflect some of the difficulties faced by *de novo* assembly of transcriptomes and suggest that the present Gene Ontology (GO) and KEGG analyses are not yet optimized for the investigation of immune responses in fish.

Other NGS-based transcriptome studies also addressed basal (immune) gene expression in cyprinid species such as silver carp (275), blunt snout bream (*Megalobrama amblycephala*) (276), *Schizothorax prenanti* (277) and the Tibetan naked carp (*Gymnocypris przewalskii*), which is of interest due to its ability to cope with hypoxic environments (278-280), but none have so far identified clear immune response profiles. Maybe more powerful examples of NGS-based transcriptome analysis should be sought in studying gene expression under basal

conditions, such as the transcriptome atlas of common carp (255). Immune response profiles of leukocytes, under basal conditions, can be addressed by NGS-based transcriptomic approaches studying, for example, primary cell cultures of common carp macrophages for validation of purity and confirmation of cell type-specific gene expression as well as polarization states (Wentzel and Wiegertjes, unpublished data), or in unbiased transcriptome analyses of common carp leukocyte cell populations sorted with cross-reactive monoclonal antibodies raised against related, Ginbuna crucian carp (*C. auratus langsdorfii*) (281), confirming the purity of sorted sub-populations (Embregts and Forlenza, unpublished data).

Given its increased acceptance and application, some constraints of transcriptomic analysis should also be noted. Gene identification, and hence gene expression studies, are still limited by imperfect genome assemblies and often rely on comparison to databases of known genes, significantly limiting the discovery of unique and species-specific genes and transcripts. Furthermore, the technical aspects of NGS sample preparation and analysis could still benefit from further improvement to increase standardization and reproducibility of results. Standardization and quality controls are extremely important prerequisites to meaningful comparative analyses, especially when based on different data sets. Furthermore, the statistical challenge of comparing expression levels of many genes in parallel, creates trade-offs between discovery of subtle changes and false positives. Therefore, at this stage, as NGS-based transcriptome analyses of non-model species gains momentum but costs are still considerable, it is important to stress the need for biological replicates, proper controls and rigorous data analyses to obtain reliable and reproducible knowledge. Limiting transcriptome analyses to enrichment of differentially-expressed genes in gene ontology categories and biochemical pathways or gene classifications with KEGG often reduces the outcome to generalized conclusions. Nevertheless, already the currently-available cyprinid transcriptome datasets provide powerful screening tools to guide further detailed studies to understand the functions and roles of genes and immune pathways and are added value for cyprinid immunology.

3.2 Responses to bacterial challenges

Besides transcriptome analysis of basal conditions, many studies use a form of exogenous challenge to identify genes of interest with respect to pathogenic conditions. *Aeromonas hydrophila* is a Gram-negative bacterium of high economic relevance in Asia and has been examined in several cyprinids, which makes it interesting to try and identify putative common transcriptomic responses. In common carp, the kinetics of the antibacterial immune response in spleen at 4, 12 and 24 hours after *A. hydrophila* challenge showed a total of 2,900 differentially-expressed genes at least one time point (282). The differentially-expressed genes were further analysed with GO analysis, however no distinct interpretation regarding the kinetics of the challenge and the kinetics of the differentially-expressed genes was performed. At a first glance, it appears that a similar number of differentially-expressed genes is upregulated at 12 and 24 hours post challenge,

however the number of genes significantly down-regulated at 24 hours is higher than at any other time point, potentially indicating the induction of control mechanisms.

In grass carp, *A. hydrophila* challenge revealed 700 differentially-expressed genes in spleen and kidney between early and late moribund fish including an enrichment of several immune-related pathways identified by KEGG and GO analysis. This study also included a small RNA transcriptome to investigate differences in micro RNA (miRNA) between these fish (283). A computational prediction of miRNA targets revealed several inversely-expressed target genes, of which more than half were related to 'immune and disease' pathways. A follow-up study identified 61 conserved and 124 candidate novel miRNAs and uncovered 21 differentially-expressed miRNA between the susceptible and resistant grass carp (284). As above, classification as resistant or susceptible was based on time-of-death, without direct evidence for a genetic component involved in the disease resistance, which means the differences in miRNA expression are not necessarily directly related to *A. hydrophila* resistance*,* although the data do provide information on the onset of different immune pathways elicited by this bacterial infection.

Independent of putative genetic differences, others identified at least 105 differentially-expressed genes involved in the immune response of grass carp to *A. hydrophila* (285). Also, transcriptome analysis of intestine samples identified 549 differentially expressed genes in the intestine of grass carp 24 hours after challenge with *A. hydrophila* (286). Challenging grass carp with *A. hydrophila* at a temperature 6 degree higher than normal resulted in more than 3,000 differentially-expressed genes, of which 90 genes were immune-related (287). Comparable numbers of immune-related differentially-expressed unigenes (88 unigenes) were identified in a transcriptome study addressing the kinetics of the immune response to *A. hydrophila* challenge, up to 72 hours after challenge, again in grass carp (288). The analysis of kinetics of gene expression profiles of this challenge showed a gradual increase over time of the total number of differentially expressed genes, however further analysis of these differentially-expressed genes would be required to make clear conclusions on the kinetics (288). In conclusion, although there are several transcriptome studies on infection of grass carp with *A. hydrophila*, it remains hard to identify common immune responses. A quick comparison of immune responses in the spleen of grass carp and common carp may point at a common regulation of the complement system after challenge with *A. hydrophila*, an outcome which would require confirmation by further and more detailed analysis. In general, extensive analyses of kinetics of gene expression after pathogenic challenges appear to be missing, in particular regarding time points later than a few days post-infection. Analysis of later time points, in particular, would be expected to provide the presently-missing transcriptomic information on adaptive immune responses.

In Rohu (Indian carp), fish were examined for differential gene expression after selective breeding for resistance to *A. hydrophila* infection (289). Indeed, several

contigs with differential expression between resistant and susceptible fish could be identified, although the results were confounded by the pooling of RNA samples and the environmental impact (289). Nevertheless, several contigs carrying genes of the major histocompatibility complex, heat shock proteins, serum lectin and glycoprotein genes appeared associated with resistance (290), providing a start for future immunological analyses of immune responses to *A. hydrophila* in Rohu and a start for the selection of strains with improved immunity. Challenging blunt snout bream with *A. hydrophila* resulted in the identification of 150,000 unigenes, of which only a small part was differentially regulated by the bacterial infection (291). Analysis of small RNA libraries generated from liver tissue after *A. hydrophila* infection predicted 61 and 44 differentially-expressed miRNAs at 4 and 24 hours after infection, respectively (292). GO and KEGG analysis of predicted target genes of these differentially-expressed miRNAs suggested an enrichment in the target genes of several immune related pathways, such as TGF-β signalling and TLR signalling.

Future transcriptome analyses should integrate multiple studies to increase reliability and generalizability, and identify robust gene expression signatures that otherwise would remain unidentifiable in the individual studies (293). Possibilities for such analyses are growing rapidly with the increasing number of available datasets in public databases, including Gene Expression Omnibus (GEO) (294) and ArrayExpress (295). Meta-analyses, not only within the same species, but also comparative transcriptome analyses among cyprinids might reveal common immune responses. Of course, confounding factors such as age, for example, will need to be taken into account, as illustrated by a transcriptome analysis of 1,700 differentially-expressed genes in the spleen of one-year-old compared with three-year-old grass carp (296). Luckily, efforts on transcriptome data across teleost species are starting, using identical experimental procedures, to reduce confounding factors and further facilitate comparative analyses (e.g. the Phylofish project (297)). Further, at present the majority of the transcriptome studies in cyprinids that address bacterial challenge have focused on *A. hydrophila*: despite the significant economic implications of these infections, additional transcriptome analysis of bacterial challenges would certainly widen insights into common antibacterial mechanisms.

3.3 Responses to viral challenges

Although viral pathogens can have devastating consequences for aquaculture of cyprinids, relatively few studies addressed transcriptome changes following viral challenges. In common carp, the effects of cyprinid herpes virus 3 (CyHV-3, or koi herpes virus) was investigated in spleen at 24 hours after infection (298). Of 70,000 unigenes, a total of 22,000 were differentially regulated in virus-infected tissue, whereas subsequent KEGG analysis resulted in 12,000 differentially-expressed unigenes classified into 256 pathways, a quarter of which were mapped into pathways belonging to the immune system. In grass carp, several transcriptome studies addressed immune responses following infection with grass

carp reovirus (GCRV). Expressed sequence tag sequencing of head kidney of GCRV-infected grass carp and uninfected grass carp resulted in >22,000 differentially-expressed tags between the two groups, which mapped to 3,000 unigenes that were further analysed with GO annotation (299). A follow-up study compared multiple tissues and time points of moribund and surviving fish, and noted a global distribution of differentially-expressed genes appeared among all tissues, which might imply GCRV causes a multi-organ disease (300). In addition, transcriptome analysis after GCRV challenge could also identify alternative splicing; 21% of the total number of genes that were differentially expressed between moribund and surviving fish aligned with 2 or more unique transcripts, suggestive of a considerable amount of alternative splicing in head-kidney and spleen (301).

Of interest, injection with poly(I:C) can also be used to identify anti-viral transcriptomes. Intramuscular injection of *Schizothorax prenanti* with this synthetic mimic of viral infection induced several differentially-expressed genes in the spleen at 12 hours after injection (302). Last but not least, viral challenge of cell lines, *in vitro*, eliminates many of the environmental impacts and may thus allow for controlled anti-viral responses. To investigate the kinetics of infection with spring viraemia of carp virus (SVCV), transcriptomes of EPC cells were investigated 3, 6 and 24 hours after infection (303). Differential expression analysis followed by KEGG analysis showed an increase in infectious disease-associated genes with an early stress response, but the majority of differentially-expressed genes were found at a relatively late (24 hours) time point post-infection. Clearly, only few studies addressed transcriptome responses induced by viruses that are potentially threatening cyprinid aquaculture. As with anti-bacterial (immune) responses, it could be of great interest to compare in meta-approach anti-viral immune responses to different viruses in the same species, or against the same virus in different but related species, potentially revealing common antiviral mechanisms.

3.4 Responses to non-infectious agents

NGS-based transcriptome analyses have also been used to investigate differential gene expression after non-infectious challenges. Both, intraperitoneal injection with growth hormone (304), or insulin (305), revealed modulating effects on immune responses in the liver which were subtle and would probably have been overlooked with other methods of expression analysis, such as a RT-qPCR, often based on pre-determined sets of genes. Transition from a carnivorous to a herbivorous diet is a challenge to the metabolic flexibility of grass carp (265, 306-309), as are the effects of starvation (310), and it could be of great interest to examine effects on the immune system in these datasets. Studies in roach (*Rutilus rutilus*) analysed the toxicity of several chemicals and downstream effects, revealing disruptions of the regulation of several physiological process, including the immune system (311). Similarly, a study analysing the transcriptome of blunt snout bream gills exposed to ammonia observed, besides differential gene

regulation, differential regulation of miRNAs (312). Interestingly, an opposite correlation was observed with 250 predicted target genes, showing either significant upregulation if the miRNA was significantly down regulated, or vice versa.

Clearly, the above-discussed complex physiological and immunological relationships have only been touched upon and remain topics of interest that require more detailed investigation by RNAseq, as well as more rigorous and detailed interpretation of RNAseq data in the future. Of course, the new and massive data output characteristic of NGS requires that novel analysis and visualization methods support the full appreciation of future NGS-based studies. As such, it will be more important than ever to focus on true biological insights rather than on the novelty of the approach. Besides these important points, the correct analysis of immune responses in cyprinids is unique because complicated by the effects of a recent WGD event in several family members, affecting degree of conservation and retention of duplicated genes, assembling genome sequences and interpreting transcriptome analyses.

4. Polyploidy and retention of duplicated genes complicate analysis of immune responses in cyprinids

The variation in ploidy levels between cyprinid species (238, 313) makes this family highly interesting biologically, with at least, diploid, triploid, tetraploid and hexaploid family members. All teleost fishes are generally believed to have undergone three whole genome duplication (WGD) events, two at the root of the vertebrate lineage (314, 315) and one teleost-specific WGD (316-318). Several cyprinid species have undergone an additional WGD (thus fourth round, see Figure 1), which is estimated to have occurred very recently in evolutionary terms, with estimates suggesting only 12 million years ago (MYA) for common carp (319-321). In comparison, the additional WGD event that occurred in the salmonid ancestor has been dated back to an earlier time of 80 - 95 MYA, approximately (322-324).

Genome duplication can occur by either auto- or allopolyploidization, terms already coined in 1926 (325). Autopolyploidization is the duplication of the species' own genome whereas allopolyploidization is the doubling of chromosomes following an interspecific hybridization. The two types of genome duplications are different not only by the process but also by the outcome with respect to the similarity in sequence of the duplicated genes following the duplication, which is higher in autopolyploid individuals. Furthermore, while in allotetraploid species the inheritance follows a disomic pattern like in diploids, in autotetraploid species the inheritance often follows a full or partial tetrasomic pattern, which further increases gene conversion and non-allelic recombination between duplicated loci, as was exemplified for the MHC class I and II genes in the polyploid frog *Xenopus ruwenzoriensis* (326). Salmonid species provide a clear example for

autopolyploidization, whereas common carp and *Carassius* spp. provide examples for allopolyploidization. Most likely, the ancestor of common carp and the *Carassius* genus experienced a WGD, after which their speciation occurred. This hypothesis is supported by an overall 2:2 conservation of genome-wide synteny between common carp and goldfish (268, 327, 328). In either case, auto- or allopolyploidization through a WGD event instantly doubles the gene copy number, creating both sequence and functional redundancy, which alleviates the evolutionary constraints on gene sequences and allows their faster evolution (314) (duplicated genes, see also box 2). Based on studies in yeasts, plants and last but not least the polyploid *Xenopus* family of clawed frogs, it is hypothesized that a WGD is generally followed by re-diploidization; the polyploid individual will retain the duplicated set of chromosomes but 'rapidly' lose one copy or the other (329-332).

BOX 2: Duplicated genes evolutionary terminology

Genes can have multiple copies that share sequence similarity and therefore, possibly also common functionalities. The terms used to describe gene copies come from their evolutionary history. The terms homologue, orthologue and paralogue are often misused or confused. To circumvent further confusion this review will use the definitions as proposed by (333). The term **homologous genes** refers to genes that show sequence similarity because they share a common evolutionary ancestor. Paralogues and orthologues are subdivisions of homologues based on how these copies have evolved. **Orthologous genes** are genes that originate from a single ancestral gene in the most recent common ancestor, but have diverged due to speciation and diversification events. **Paralogous genes** are genes that originate from gene duplication, usually within one species (ancestral or extant). **Co-orthologues** refers to two or more genes that are collectively orthologues to one or more genes in another species, thus co-orthologues originate from a single ancestral gene in the most recent common ancestor. For example, zebrafish *NOS2a* and *NOS2b* are paralogues of one another, and *NOS2ba* and *NOS2bb* in common carp are paralogues in carp and co-orthologues of *NOS2b* in zebrafish. **Ohnologues** refer to paralogous genes that have arisen due to whole genome duplication, named such in honour of the scientist (Ohno) who conceived the theory on the evolutionary roles of duplication and fate of duplicated genes (314).

Commonly, there are three evolutionary fates described for duplicated genes that shape their status in the genome: non-functionalization (334), sub-functionalization (335) and neo-functionalization (314), (see also info Box 3). Functional divergence of duplicated genes (sub- and neo-functionalization), in particular, can be a strong driver underlying the expansion of particular gene families, and is thus very relevant for studies unravelling immune responses in cyprinid fishes. An extensive investigation of re-diploidization was recently published along with the generation of the Atlantic salmon (*Salmo salar*) genome assembly (324). Although a study in salmonids, it can help understand the process of re-diploidization and aid future studies in cyprinids. The authors show that 20% of the genes duplicated as a result of the ancient teleost-specific WGD, and 55% of the genes duplicated as a result of the salmonid-specific WGD, are still retained as two functional copies, without bias for any of the two WGD events. This estimate

is in line with a previous estimate in rainbow trout (*Oncorhynchus mykiss*) (323). Further investigation of the fate of duplicated genes in salmon revealed a high degree of neo- instead of sub-functionalization with over 60% of the investigated duplicated gene pairs showing signatures of tissue-dependent divergent regulation, signifying the expressional divergence of ohnologues.

BOX 3: Fate of duplicated genes

Following gene duplication, most notably due to whole genome duplication, evolutionary constraints on sequence evolution are reduced and therefore, in general, duplicates evolve faster than singletons. Furthermore, polyploidy is a transient state and duplicated genomes go through a re-diploidization evolutionary process involving loss of duplicated gene copies. The loss of different copies of duplicated genes in different species is referred to as **divergent resolution** (318). Currently, three different divergence paths for duplicated genes are widely accepted (336). **Non-functionalization** refers to the situation where one copy becomes a pseudogene due to mutations, eventually leading to gene loss. **Neo-functionalization** refers to the situation where one copy acquires a mutation that confers a new function, which was not part of its ancestral gene function, and the other copy retains its original function. **Sub-functionalization** refers to the situation where subsets of the ancestral functions are divided between the copies. While non-functionalization explains the loss of gene copies, neo- and sub-functionalization are explanations for why many gene copies are retained. This evolutionary rational stresses the importance of studying the functions of (immune) gene families and gene copies in a copy specific manner.

In common carp, retention of duplicated genes in the most conserved genomic regions appears high (>92%), although examined in only 736 pairs and in double-conserved syntenic (DCS) regions (337). DCS regions are genomic blocks containing genes positioned on one chromosome in a diploid reference species (here, diploid zebrafish), but on two different chromosomes in the duplicated (here, tetraploid common carp) genome. Retention rate can be over-estimated by focusing the analysis on such stretches of highly similar regions, the DCS regions. Indeed, a previous analysis based on microsatellite loci estimated the retention rate in common carp to be lower with 60 – 70% (319). Regardless, sequence similarity of the analysed duplicated genes was invariably high (>90%), both at nucleotide and amino acid level (337). Also, the regulation of these highly conserved duplicated genes was relatively conserved, although less conserved than their coding sequence similarity, showing co-expression of duplicated genes in most cases. However, about half of the co-expressed genes investigated showed significant expression divergence in one or more tissues. Besides divergent expression, a proportion of the analysed duplicated genes showed functional divergence, implied by functional domain annotation compared to zebrafish, highlighting once more the importance of these duplicated genes and asking for a critical analysis of immune responses in polyploid species.

The consequences of WGD events and associated effects of polyploidization can have their impact on proper analysis of immune responses. Divergent resolution is a process that operates independently in different species and will result in

different retention of duplicated (immune) genes even between related species, as signified by species-specific genes found in grass carp (265), Rohu (290) and common carp (253). These species-specific genes are easily filtered out by automated annotations, but might be highly interesting from a comparative point of view. Also, tissue-specific expression of duplicated (immune) genes requires careful examination to prevent misinterpretation of immune responses or, when based on examination of only one of the two paralogs, even wrong conclusions. Whereas NGS techniques can rapidly improve reference and other currently available genomes, it can be prone to errors as a result of polyploidy effects: high similarity between duplicated regions can obscure proper genome assembly. Indeed, indications for so-called assembly collapses in homologous regions with the highest sequence similarity (>95%) were observed in the salmon genome (324). These regions were highlighted by an increased read depth alignment and reduced scaffold length and accounted for almost 10% of the chromosome positioned sequences, signifying the difficulties in assembling genomes of polyploidy species.

There is some relief to be found in the knowledge that recent advancements in NGS will help to further resolve difficulties in transcriptome analyses and genome assemblies, including mapping of duplicated genes which, currently, is still largely based on short reads prone to error, signifying the need for high-throughput, long read sequencing platforms. Further, genome assemblies can also be improved by employing existing genetic variance within species to specifically study each gene copy, generate genetic maps, and anchor genomic scaffolds.

5. Employing genetic variation to improve genome assemblies and study specific gene copies

Ideally, we wish *de novo* assembly of NGS sequencing data to result in a perfect genome assembly. However, with the current technologies, *de novo* assembly typically yields many contigs and a fair number of assembly errors, reflected by the fragmented nature of the current cyprinid genome assemblies (Table 1). In principle, following *de novo* assembly, scaffolding (i.e. the process of putting together contigs in the order of the chromosome) could be improved by comparative mapping to other fish genomes, but this becomes more complicated depending on species diversity in both sequence and genome organisation. In addition, correct assembly is even more complicated for polyploid species with duplicated genomes: the higher the sequence similarity between paralogous regions is, the more difficult it becomes to assemble short sequence reads into their correct contig/scaffold. Furthermore, to avoid sequence mismatches and allow for a more stringent assembly of contiguous reads, genome sequencing is often performed on DNA of a highly inbred homozygous individual. However, this particular approach is methodology-driven and does not always fully respect the biology of the species. For duplicated genomes such as found in several cyprinids,

residual allelic variation can confound genetic variation between paralogs and complicate the genome assembly. One solution to assist scaffolding is to use genetic linkage maps which build on identifying large numbers of polymorphic markers and ordering them one relative to the other based on their segregation pattern in families. While paralogous polymorphisms in parents show independent assortment in progeny, genetically-linked markers are co-inherited and thus, co-segregate in the mapping population, allowing correct assignment of the DNA marker to linkage groups, even in duplicated genomes (Figure 2), (319). This is why using genetic linkage maps to improve scaffolding and genome sequence assembly is even more valuable for polyploid species.

The possibility to obtain large amounts of sequence information by NGS not only allows for sequencing genomes of single reference individuals but also permits comparison of different individuals and identification of genetic polymorphisms within and between populations (246, 338-340). In principal, to detect and genotype polymorphisms, NGS is usually applied to libraries with reduced genomic representation such that multiple individuals can be reliably analysed with sufficient coverage (341, 342). Depending on the specific protocols, these methods typically yield anywhere between thousands to several ten-thousands DNA markers per studied population in a cost-effective manner (Figure 2). Based on this unprecedented resolution, both existing and new research questions in multiple fields such as ecology, population genetics, quantitative genetics, breeding and immunology could be addressed with higher accuracy (339, 343-346). Thereby, NGS platforms have revolutionized not only genome and transcriptome analyses but also identification of genetic variation and the use of DNA markers. In the future, newly-developed sequencing technologies are expected to succeed in producing reliable contiguous genome sequence assemblies, which will relegate the need for genetic linkage maps. At this moment, however, genetic linkage maps can already greatly improve scaffolding and genome assembly, especially of non-model species. Since in genetic linkage maps, marker position and marker order are determined based on recombination data in segregating families, the same markers can also be mapped to existing contigs and scaffolds allowing ordering of the latter (324, 347-349). Because the large numbers of DNA markers typically show a genome-wide distribution, this generally results in the constructing of dense genetic linkage maps with a total number of linkage groups corresponding to the number of chromosomes, which is the final goal for the genome assembly.

The utility of DNA markers and genetic linkage maps go beyond improving scaffolding of the genome sequence. Having numerous DNA markers organized into a dense genetic map help in studying the function of duplicated genes, regardless whether a gene had been individually duplicated or duplicated as part of a segmental or whole-genome duplications. Often, the presence of polymorphic gene sequences in the genome assembly suggests a gene has multiple copies. Distinguishing multiple copies based on their sequence variation allows for studying more accurately their genomic organization, positioning, expression

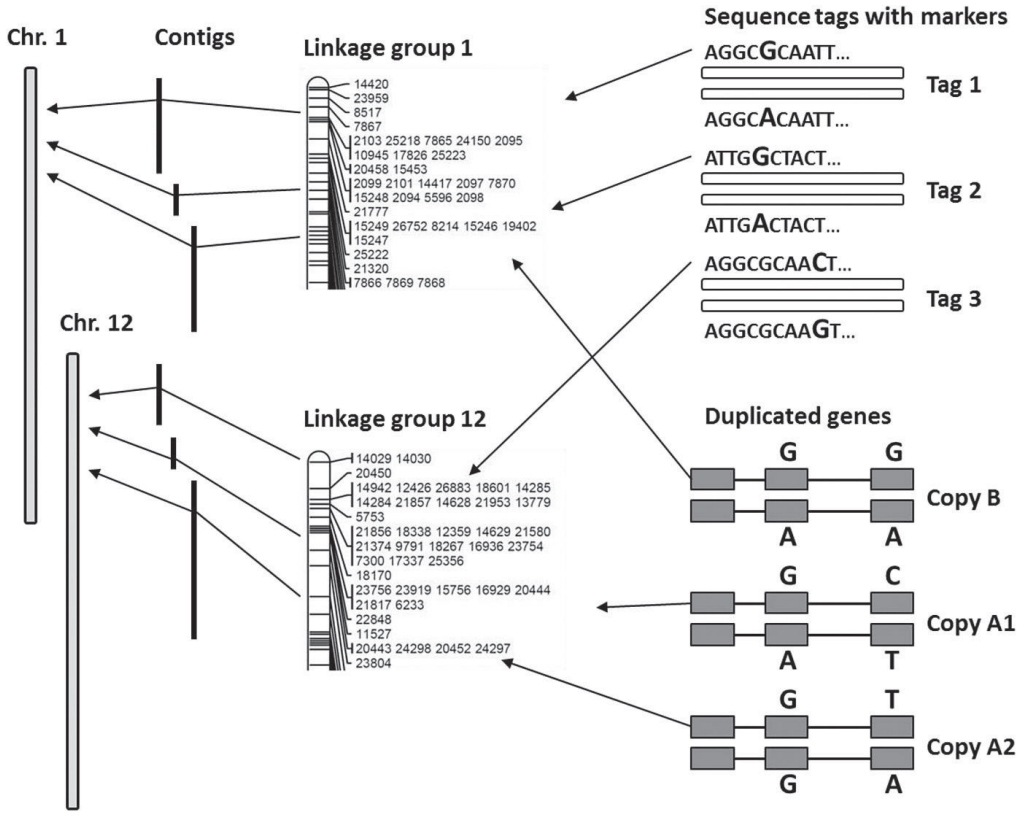

Figure 2. Improving scaffolding and resolving genomic organization of duplicated genes using DNA markers. NGS can generate information on many polymorphic markers by sequencing reduced representation libraries of multiple individuals. Information on the segregation of these markers in the mapping population allows for the construction of dense genetic linkage maps that group markers into linkage groups, and order them within each group. Depending on the number of recombination events, several markers might be assigned to the same position. The marker segregation pattern in the mapping population allows for correctly assigning tags with similar sequence, such as tags 1 and 3, each to a separate linkage group whereas often, in *de novo* sequence assemblies, such short reads might be collapsed to the same position. Similarly, any set of duplicated genes that has polymorphisms between the alleles of the same copy and between the different copies, can be assigned to linkage groups if copy-specific markers have been developed and genotyped. DNA markers are part of short sequence tags and these are used to identify the contigs that contain each of the markers. Based on the linkage groups, contigs with markers in them are grouped and the order of the markers is used to scaffold the contigs into chromosomes. Note that for both, sequence tags and duplicated gene copies, the polymorphisms in them in combination with genetic linkage mapping allow for determining their genomic organization and correct evolutionary history. In this example, copies A and B are part of a duplication of all or part of the chromosome, while copies A1 and A2 are local tandem duplicates.

regulation and function. For example, interleukin 10 (IL10) was found to have two copies in the genome of the common carp (350). Taking advantage of the polymorphism between these copies, their functional divergence was shown by association with disease resistance and at the level of gene expression (350, 351). Although common carp IL10 was previously studied as a single gene (352), to date we appreciate that each copy might function differently and thus, interrogating duplicated genes in a copy-specific manner is essential for a complete understanding of immune responses. Furthermore, using IL10a- and IL10b-specific polymorphisms, the two copies were shown to segregate independently, and are thus not the result of a tandem duplication but locate to different chromosomes (253, 255, 256). Often, genes that locate in tandem in the same genomic region are subject to regional regulation (353) and thus, might diverge more slowly in their regulation. Both copies of IL10 share good similarity in their upstream regulatory sequences but do not share genomic location. Resolving the genomic organization of IL10 in the polyploid common carp and studying its copies in a copy-specific manner is just one example of using polymorphism information from genetic linkage maps with genome assembly to improve understanding the function of the immune system.

6. What is next?

Further technological developments will continue to have major impacts on the improvement of current and new (cyprinid) genome assemblies. By now, first and second generation sequencing, requiring clonal amplification of the template molecules prior to the actual sequencing, may be considered major developments of the past four decades. The introduction of single molecule sequencing (SMS) marked essentially the start of third generation sequencing and will continue to have a major impact during the next decade. Pioneered by Helicos Biosciences (354), the actual breakthrough came in 2011 when Pacific Biosciences launched their single-molecule real-time (SMRT) sequencing platform onto the market. The PacBio technology uses single DNA polymerase molecules to produce long reads from single molecule templates. Initially, maximum read lengths were in the range of a few kilobases and PacBio data were mainly used to support second-generation-sequencing-based de novo assembly of bacterial genomes (e.g. (355)). The average error rate of PacBio reads is relatively high (11-15%), but individual reads can be corrected using, for example, more accurate Illumina reads. Alternatively, a reliable consensus sequence can be deduced from high coverage (>50-fold) PacBio reads via bioinformatics tools. The latest PacBio systems show much improved average read lengths of more than ten kilobases, which allows resolving long genomic repeat regions that cannot be unravelled using short Illumina reads. For example, *de novo* assembly of the human genome sequence using PacBio reads only has already contributed to a much-improved version of this reference genome (356). A complementary platform for scaffolding contigs is provided by the Irys® system of BioNanoGenomics. This system uses micro-fluidics to run extremely long (up to 1 Mb) individual DNA fragments in their linear

form through long and narrow glass channels to produce an optical map based on the relative positions of fluorescently-labelled restriction enzyme sites. The pattern of restriction sites in the optical map can subsequently be compared to in-silico predictions of restriction sites based on the available genome assembly, together facilitating scaffolding of the contigs ideally resulting in more contiguous genome sequences (e.g. (357)).

Currently, maybe the most promising development is linked with the commercial launch in 2014 of a small sequencing device by Oxford Nanopore Technologies, named MinION. This technology is completely different from all previous sequencers and is based on individual DNA molecules that are guided through protein nanopores; the typical distortion of the electric current as different DNA nucleotides pass through the nanopores is immediately translated into sequence reads. The MinION device is not only small, cheap and mobile but also allows for the production of hundreds of thousands of very long sequence reads in a single run. With this technology, limitation of read length appears to be restricted by the integrity of the DNA after purification and the error rates, but single reads of about 400 kb have already been reported using the current chemistry. This technology and its future development will greatly facilitate *de novo* assembly of high quality genome sequences for any organism. The first bacterial genome assembled from nanopore sequencing reads alone was published in 2015 (358) and complete vertebrate genomes assembled from nanopore reads alone are now emerging (359).

The PromethION, a larger version of the MinION with a predicted output of more than five terabases per day, will soon enter the market and further impact NGS applications. It is expected that assembly of intact haploid genome sequences for any organism will soon become possible and, especially relevant for cyprinids, identification of orthologous genes based on homology and synteny will become a challenge of the past. Also, these technologies hold a promise for identification of many sequence variants by sequencing genomes of multiple individuals from the same species. Once this will be made possible, continuous reference to genomes of model species such as the zebrafish, required to guide scaffolding and annotation of genomes of new species, will no longer be essential. These independent genome assemblies thus will also identify species- and even individual-specific features such as genomic rearrangements, gene duplications and repetitive elements variation, allowing even more accurate studies on the functions of gene families. Of course, some challenges will still remain even with very long reads, such as stitching correctly the chromosomes of heterozygous individuals in populations containing allelic variation affecting gene function. No matter the exact technology, the present rapid increase in high-quality genome assemblies will further facilitate investigation of evolutionary effects of WGD events on fish genomes, and thus be of great importance to the understanding of the fish' immune system.

Advances in NGS are rapidly leading to a flood of RNA sequencing data, highlighting the crucial importance for new analysis and visualization methods to be employed by bio-informaticians and biologists to critically interpret NGS-based studies and properly address biological questions. In spite of the relatively unbiased approach of full transcriptome analyses, the current standard of analysis is often biased, because it is restricted to the listing of regulated pathways identified based on sub-optimal allocation of genes to pathways identified in mammalian species or, at best, in zebrafish. Reflections on the biological significance of differentially expressed pathways for immune responses often remain elusive. Furthermore, such pathway analyses are commonly based on diploid species, obscuring information on duplicated genes, potentially leading to misinterpretation. Finally, most of the transcriptome studies in cyprinid species, as discussed here, addressed immune responses over periods of a few days, leaving opportunies for future research on the dynamics of immune responses over longer periods of time. A systemic analysis of gene expression profiles over periods of days to weeks should highlight the transition from innate towards adaptive immune responses and could reveal intriguing patterns of gene expression during the adaptive phase.

Maybe one of the most exciting immunological applications of NGS will come from sequencing single cells, which application already revealed cell-to-cell variation in seemingly homogenous cell populations of human leukocytes, revealing both functional and regulatory diversity within a cell fraction (360, 361). Single-cell sequencing of mRNA has recently been applied to zebrafish, revealing a highly-coordinated transcriptional framework through which hematopoietic cells progress upon their commitment and differentiation towards thrombocytes (362). Furthermore, single-cell mRNA sequencing in zebrafish revealed new subsets within specific epicardial cells and thereby facilitated identification of new potential markers (363). Single-cell sequencing would be a highly-suitable approach for further investigation of the theory on micro-heterogeneity in apparently clonal immune cells. Very likely, NGS-based analyses will also shed light on the mechanisms proposed for the cell alacrity (364), including genome-wide epigenetic modifications, which are increasingly recognized as important regulators of immune responses.

We also foresee great potential to apply the latest NGS approaches for detailed analyses of tissue-specific sub-regions such as lymphocyte aggregates in spleen after infection (365), lymphoid aggregates in nasal immune tissue (366), regions in the hindgut important for antigen uptake (367, 368), interbranchial immune tissue (369), and so forth. Investigation of gene expression in these specific regions and immune tissues, either under basal conditions or after bacterial or viral challenge, might reveal details of immune responses to a depth not possible before. These analyses will provide indications towards the organisation and regulation of lymphoid aggregates and tissues during homeostasis and pathogenic challenge in species, such as the cyprinids, lacking highly-organized lymphoid tissues. Another highly interesting application of NGS is dual transcriptome

analysis, where transcriptional changes of both pathogen and host are analysed simultaneously (370) and may improve our current understanding of host – pathogen interactions. Last but not least, NGS will facilitate studies into biological effects of (immune) gene duplications. Variation in ploidy, in combination with a relative conservation of genome organisation and synteny, make the cyprinids a family particularly suitable to this purpose. To this end, novel and high quality genomic and transcriptomic data should prevent misinterpretations of transcriptomic data by a proper distinguishing of paralogous regions. Thereby, NGS will prove essential in studies on genome duplication effects on immune responses in cyprinids.

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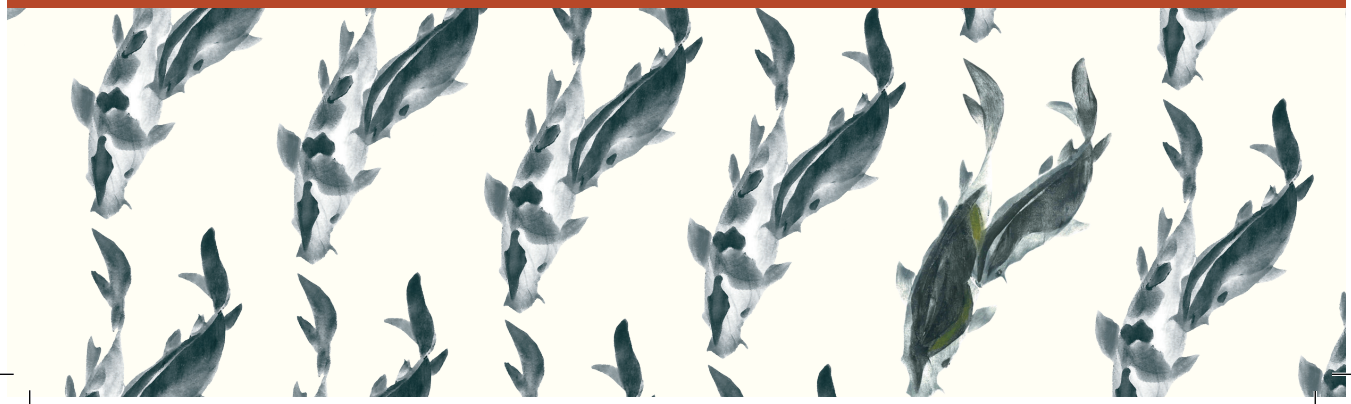

6

Studies Into β-Glucan Recognition in Fish Suggests a Key Role for the C-Type Lectin Pathway

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Abstract

Immune-modulatory effects of β-glucans are generally considered beneficial to fish health. Despite the frequent application of β-glucans in aquaculture practice, the exact receptors and downstream signalling remains to be described for fish. In mammals, Dectin-1 is a member of the C-type lectin receptor (CLR) family and the best-described receptor for β-glucans. In fish genomes, no clear homologue of Dectin-1 could be identified so far. Yet, in previous studies we could activate carp macrophages with curdlan, considered a Dectin-1-specific β-(1,3)-glucan ligand in mammals. It was therefore proposed that immune-modulatory effects of β-glucan in carp macrophages could be triggered by a member of the CLR family activating the classical CLR signalling pathway, different from Dectin-1. In the current study, we used primary macrophages of common carp to examine immune modulation by β-glucans using transcriptome analysis of RNA isolated six hours after stimulation with two different β-glucan preparations. Pathway analysis of differentially expressed genes (DEGs) showed that both β-glucans regulate a comparable signalling pathway typical of CLR activation. Carp genome analysis identified 239 genes encoding for proteins with at least one C-type Lectin Domains (CTLD). Narrowing the search for candidate β-glucan receptors, based on the presence of a conserved glucan-binding motif, identified 13 genes encoding a WxH sugar-binding motif in their CTLD. These genes, however, were not expressed in macrophages. Instead, among the β-glucan-stimulated DEGs, a total of six CTLD-encoding genes were significantly regulated, all of which were down-regulated in carp macrophages. Several candidates had a protein architecture similar to Dectin-1, therefore potential conservation of synteny of the mammalian *Dectin-1* region was investigated by mining the zebrafish genome. Partial conservation of synteny with a region on the zebrafish chromosome 16 highlighted two genes as candidate β-glucan receptor. Altogether, the regulation of a gene expression profile typical of a signalling pathway associated with CLR activation and, the identification of several candidate β-glucan receptors, suggest that immune-modulatory effects of β-glucan in carp macrophages could be a result of signalling mediated by a member of the CLR family.

Introduction

Immunomodulation by β-glucans has been widely studied in teleost fish. Regardless of the administration route or fish species, β-glucan administration often has an immune stimulatory effect and can result in increased resistance to both viral and bacterial infections (reviewed by: (19-21). For both, mammalian vertebrates (43, 185) and invertebrates (89, 184), specific mechanisms responsible for β-glucan recognition and/or downstream signalling have been described. Yet for teleost fish, despite the frequent application of β-glucans in aquaculture practice, the exact mechanisms underlying the induced effects are ill described.

In mammals, several non-exclusive pathways play a role in the recognition and down-stream signalling after β-glucan stimulation, however the best-described β-glucan receptor is Dectin-1, also known as C-type Lectin domain Family 7 member A (CLEC7A). Dectin-1 is a *C-type lectin super family V, NK cell receptors* transmembrane receptor with a single carbohydrate recognition domain (CRD) and a cytoplasmic tail containing one ITAM motif (27-29). Dectin-1 is predominantly expressed on cells from both the monocyte/macrophage and neutrophil lineages, where it acts as the major β-glucan receptor (30). Ligation of β-(1,3)-glucan to Dectin-1 is dependent on two specific amino acid residues in the CRD; tryptophan (W) and histidine (H), separated by a third residue (WxH motif) (31). Additionally, a tyrosine (Y) residue separated from histidine in the CRD by 4 residues (WxHxxxxY motif) is crucial for shaping the β-glucan binding cleft (32). This β-glucan binding cleft is formed by spatial arrangement of tryptophan, histidine and tyrosine in a triangular fashion resulting in a shallow hydrophobic surface groove, capable of accommodating and binding β-glucan chains through hydrophobic interactions (31-33). Also true for invertebrates, the same three residues are present in the binding domain of β-(1,3)-glucan recognition protein (GNBP3), although not as a conserved motif (34). Dectin-1 signalling is activated following clustering in synapse-like structures formed within minutes after activation by particulate β-glucans (35). Following the interaction with β-glucan, Dectin-1 signals via Syk kinase and the adaptor protein Card9 to send a downstream signal through Bcl10 and Malt1 to the transcription factor NF-κB (38). Activation of NF-κB leads to an inflammatory profile typical of stimulation with β-glucans.

The presence or absence in fish genomes of Dectin-1, along with the entire superfamily V of CLRs (NK cell receptors), is debated (42). Although initially a cKLR from *Paralabidochromis chilotes* was described as a member of the superfamily V (371), and 28 distinct KLR loci identified a particular chromosomal region in Nile tilapia (*Oreochromis niloticus*) (372), a subsequent and thorough phylogenetic analysis suggested these receptors to be members of superfamily II rather than superfamily V (42). At the same time, it is important to realize that this phylogenetic analysis was primarily based on an early genome assembly of a single fish species, *Fugu rubripes* (42), indicating that the presence of true superfamily

V CLR members in fish genomes has not been systematically investigated in other teleosts.

Functional and therefore indirect evidence of the presence of β-glucan receptor(s) in fish exists for at least Atlantic salmon (*Salmo salar*) macrophages, channel catfish (*Ictalurus punctatus*) neutrophils and seabream (*Sparus aurata*) leukocytes, based on observations that pre-treatment of these cell types with β-glucans reduces the uptake of yeast (*Saccharomyces cerevisiae*) glucan particles, zymosan or whole yeast cells (373-375). In carp, injection of β-glucans induced a complement receptor 3 (CR3)-dependent rosette formation of leukocytes and deposition of iC3b and C3d fragments on zymosan (130, 376). β-glucan was also shown to regulate the expression of several pattern recognition receptors including *tlr2* in primary macrophages of European eel (*Anguilla anguilla*) (377), regulation of *tlr3* and *cxc* receptors in common carp (111, 378), the purinergic receptor *p2x4* in Japanese flounder (*Paralichthys olivaceus*) (379) and *nod2* and *tlr2* in zebrafish (*Danio rerio*), (175). Although these genes were regulated by β-glucan stimulation, their involvement in the recognition of β-glucans remains to be confirmed. Oral intubation of Atlantic salmon (*S. salar*) with β-glucan resulted in the up-regulation of *syk* kinase and three salmon CLRs with one or two ITAMs and a single WxH motif (380, 381), alike the Dectin-1 architecture described above. We reasoned that macrophages could provide an informative starting point for investigating signalling pathways induced upon β-glucan stimulation. Indeed, primary macrophages of common carp have been shown to respond to curdlan and to zymosan depleted of Toll Like Receptor (TLR) stimulating properties; both considered Dectin-1-specific ligands in mammals (138). We therefore hypothesized that immune-modulatory effects of β-glucan in carp macrophages could be triggered by an unknown member of the CLR family, different from Dectin-1.

Here, we used primary macrophages of common carp for a whole transcriptome analysis of differentially expressed genes (DEG) induced by two different β-glucans. Analyses of gene ontology revealed comparable profiles and a clear regulation of the CLR pathway by both β-glucans. Subsequent investigation of the common carp genome for candidate β-glucan receptors identified a number of genes based on their architecture or expression profile, all encoding proteins with at least one C-type Lectin Domain (CTLD). Preliminary phylogenetic analysis of the CTLD sequences of candidate proteins showed no clustering with CTLD sequences of known group V members. Synteny analysis of the genome of zebrafish, a close relative of common carp, identified two CTLD-encoding genes with apparent conservation with mammalian CLR group V members, namely *clec4c* and *sclra*. Overall, our study identifies several teleost CLRs of interest for future functional studies aimed at further specifying the modulatory effects of β-glucans on the fish immune system.

Methods

Animals

European common carp (*Cyprinus carpio carpio* L.) of the R3 × R8 strain were used, which originate from a cross between the Hungarian R8 strain and the Polish R3 strain (169). Carp were bred and raised in the aquatic research facility of Wageningen University, Carus, at 20 – 23°C in recirculating UV-treated water and fed pelleted dry feed (Skretting, Nutreco) twice daily. All experiments were performed with the approval of the animal experiment committee of Wageningen University.

In vitro culture of head kidney-derived macrophages

Fish were anaesthetized with 0.3 g/l Tricaine Methane Sulfonate (TMS) (Crescent Research Chemicals, Phoenix, USA) in aquarium water buffered with 0.6 g/l sodium bicarbonate and bled via the caudal vein. Carp head kidney-derived macrophages were cultured for 6 days, as described previously (164), and will be referred to as macrophages.

Macrophage stimulation

Macrophages were harvested by placing culture flasks on ice for 15 minutes and by gentle scraping. Cell suspensions were centrifuged at 450x *g* for 10 minutes at 4°C. Macrophages were resuspended in complete NMGFL (incomplete-NMGFL-15 medium supplemented with 2.5% heat-inactivated pooled carp serum and 5% bovine calf serum (Invitrogen Life Technologies) with 100 U/mL of penicillin and 50 µg/mL streptomycin) (164). Subsequently, macrophages were seeded in 24-well flat-bottom culture plates (Corning™ 3526, FischerScientific) at 1.5 x 10^6 macrophages/300µL per well. For stimulation of the cells, curdlan (C7821, Sigma Aldrich) (a high molecular weight linear polymer consisting of β-1-3-linked glucose residues from *Alcaligenes faecalis*) and MacroGard® (a cell wall preparation of *S. cerevisiae* comprising 91% β-glucan (Zilor, São Paulo, Brazil)) were used (46). β-glucans were prepared as previously reported (138). Cells were stimulated with β-glucan preparations at 25 µg/mL, a concentration at which both β-glucan preparations were previously shown to induce considerable nitric oxide production in carp macrophages (138). For each stimulus at least three independent cultures were used and each stimulation was performed in technical triplicate.

After 6 hours of stimulation, three replicate wells were pooled and 4.5 x 10^6 macrophages were lysed in 350 µL RLT buffer (QIAgen, Netherlands) and stored at -80°C until RNA extraction. Total RNA was extracted using the RNeasy Mini kit according to the manufacturer's protocol (QIAgen) including on-column DNase treatment with the RNase-free DNase set (QIAgen). RNA was stored at -80°C until use.

Illumina sequencing and data analysis

RNA quality and concentration was checked on a Bioanalyzer (Agilent 2100 total RNA Nano series II chip, Agilent). RNAseq libraries were prepared from 0.5 µg total RNA using the TruSeq® Stranded mRNA Library Prep kit according to the manufacturer's instructions (Illumina Inc. San Diego, CA, USA). Similar to the previous carp study (255), all RNAseq libraries were sequenced on an Illumina HiSeq2500 sequencer as 1 × 50 nucleotides single-end reads. Image analysis and base calling were performed using the Illumina pipeline. Using TopHat (version 2.0.5), (382), reads were aligned to the latest published genome assembly of common carp (BioProject: PRJNA73579), (255). For each independent sample at least 10 million raw reads were sequenced, on average 65% of the raw reads could be mapped to annotated genes of this carp genome assembly. Secondary alignments of reads were excluded by filtering the files using SAMtools (version 0.1.18), (383). Aligned fragments per predicted gene were counted from SAM alignment files using the Python package HTSeq (version 0.5.3p9) (384).

Differential gene expression was analysed using the bioinformatics package DESeq 2.0 (v1.22.2), (385) or edgeR (v3.24.3), (386, 387) from Bioconductor (v3.8), (388) in R statistical software (3.1.2), (208). Statistical analysis was performed using a paired design with unstimulated cells as control and performed for curdlan and MacroGard® independently (n=3 independent cultures for curdlan and for MacroGard®). The paired design allowed for a better comparison between independent cultures, reducing noise generated by culture to culture differences (385). For DESeq 2.0, *p*-values were adjusted using Benjamini & Hochberg corrections for controlling false discovery rate and results were considered statistically significant when *p*-adjusted ≤ 0.05. For edgeR, genes were considered significantly regulated if both p-value ≤ 0.05 and FDR ≤ 0.05. Only genes identified as significantly regulated by both, DESeq 2.0 and edgeR, were used for subsequent analyses (Supplementary tables 1 and 2 for curdlan- and MacroGard®-DEGs, respectively). Venn diagrams were generated with the webtool from the University of Ghent Bioinformatics and Evolutionary Genomics group[1].

Gene Ontology annotation and enrichment analysis

The common carp genome has been annotated against Ensembl zebrafish GRCz10 (255). Due to its tetraploid nature because of an additional genome duplication event (253, 319, 389), the common carp generally has two copies of each zebrafish gene. However, for gene ontology (GO) and KEGG analysis only single IDs were used, resulting in a dataset with unique Ensembl zebrafish IDs (curdlan DEGs n=421 unique genes and MacroGard® DEGs n=638 unique genes). Gene Ontology (GO) analysis was performed with GOrilla (390). Using differentially

[1] http://bioinformatics.psb.ugent.be/webtools/Venn/

expressed genes as a target list and the entire list of annotated common carp genes as a background list, GO term enrichment was analysed (Supplementary table 3 and 4 for curdlan and MacroGard®, respectively). FDR *q*-values were calculated by adjusting *p*-values using Benjamini & Hochberg method for controlling false discovery rate, GO terms were considered statistically enriched when FDR ≤ 0.05.

Independent KEGG analysis (391) was performed using the stable Ensembl zebrafish ID's of each differentially expressed gene in KOBAS v3.0 (392), using the well-annotated zebrafish genome as a reference list. KOBAS was run with Chi-square test and for FDR correction the Benjamini and Yekutieli method was used. Pathways were considered significantly over-represented if the corrected *p*-value was $p \leq 0.05$. Recently, a zebrafish-specific KEGG pathway map for the CLR pathway was released (dre04625). As this map was not yet incorporated in the KOBAS analysis, we performed manual mapping using the "userdata mapping" feature on the zebrafish-specific KEGG pathway for C-type lectin receptor signalling.

Genome search for CTLD-encoding common carp sequences

The conceptual translation of all annotated carp gene sequences as submitted to NCBI (from here on referred to as carp proteins) (BioProject: PRJNA73579, (255)), was used to perform a Protein family (Pfam) domain search using CLC Main Workbench v8.0[2] with the Pfam Database v31 (393). Proteins without a C-type lectin domain (CTLD) (PF00059) were filtered out. Subsequently, using the PatmatDB feature from EMBOSS in the public Galaxy server (v5.0.0) at Wageningen University and Research centre (WUR, The Netherlands[3]), protein sequences containing an immunoreceptor tyrosine-based activation motif (ITAM) were identified using the signature YxxL/I sequence. Presence of a WxH motif within the CTLD was investigated using the PatmatDB feature from EMBOSS in Galaxy (v5.0.0) using the signature WxH. Alternatively, presence of a WxHxxxxY motif within the CTLD was investigated using the signature WxHx(1,4)Y sequence, allowing for 1 – 4 random residues between histidine and tyrosine. Transmembrane regions of all proteins with at least one CTLD, were predicted using TMHMM Server v. 2.0[4]. These analyses highlighted a restricted number of proteins with one or more CTLDs and characterized the number of ITAM motifs, WxH motifs and transmembrane regions present in the conceptual translations. Subsets of candidate receptors were selected from the restricted number of proteins based on the following three criteria: 1) presence of a conserved WxH motif in the CTLD; 2) corresponding expression of ≥ 50 reads per kilobase million

[2] https://www.qiagenbioinformatics.com
[3] http://galaxy.wur.nl
[4] http://www.cbs.dtu.dk/services/TMHMM/

(RPKM) in unstimulated macrophages (n=5); 3) differential regulation of expression in carp macrophages stimulated with β-glucans. Automatic annotation of candidate receptors was manually verified using BLASTx against the nr database from NCBI.

The CTLD sequences of identified candidate receptors were aligned with CTLD sequences from selected fish CLRs, selected mouse CLRs, several chicken CLRs (394) and with CTLDs from human CTLD-encoding genes (PF00059) present in Ensembl (GRCh38.p12) using MUSCLE v3.8 (395). In case of more than one CTLD sequence per protein, the first CTLD was designated 1, the next 2 and so on. Subsequently, Model Selection feature of MEGA-X (396) was used to calculate the most appropriate amino acid substitution model using all sites of the alignment as input data. The evolutionary history was inferred by using the Maximum Likelihood (ML) method based on the Whelan and Goldman model (397) allowing for Gamma distribution (+G) with 4 Gamma categories and using the rate variation model allowed for some sites to be evolutionarily invariable ((+I), 0.19% sites). The bootstrap consensus tree inferred from n=500 replicates was taken to represent the evolutionary history of the taxa analysed (398). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (+G, parameter = 1.6778). The final alignment involved a total of n=223 CTLD sequences.

As a fourth criterion for the identification of candidate receptors, conservation of synteny with the mammalian NK cell receptor cluster was added. Synteny analysis relied on genomic location data from NCBI and Ensembl Gene Summary databases. The GRCh38.p12, GRCm38.p6 and the Zv10 primary genome assemblies were used for human, mouse, and zebrafish genomic location data, respectively. For genomic location of carp genes the latest common carp assembly (255) was used.

Results

β-glucan stimulation of macrophages leads to regulation of the CLR signalling pathway

Transcriptome analysis of macrophages stimulated with curdlan retrieved a total of n=528 differentially expressed genes (DEGs) (Supplementary table 1) of which almost 85% were up-regulated. Transcriptome analysis of macrophages stimulated with MacroGard® retrieved a total of n=781 DEGs (Supplementary table 2), of which almost 80% were up-regulated. Subsequent comparison of the expression profile of both DEG datasets revealed a comparable profile, with n=291 DEGs that followed concordant expression patterns, only in one case discordant

regulation was observed as up-regulation by curdlan and down-regulated by MacroGard® (Figure 1).

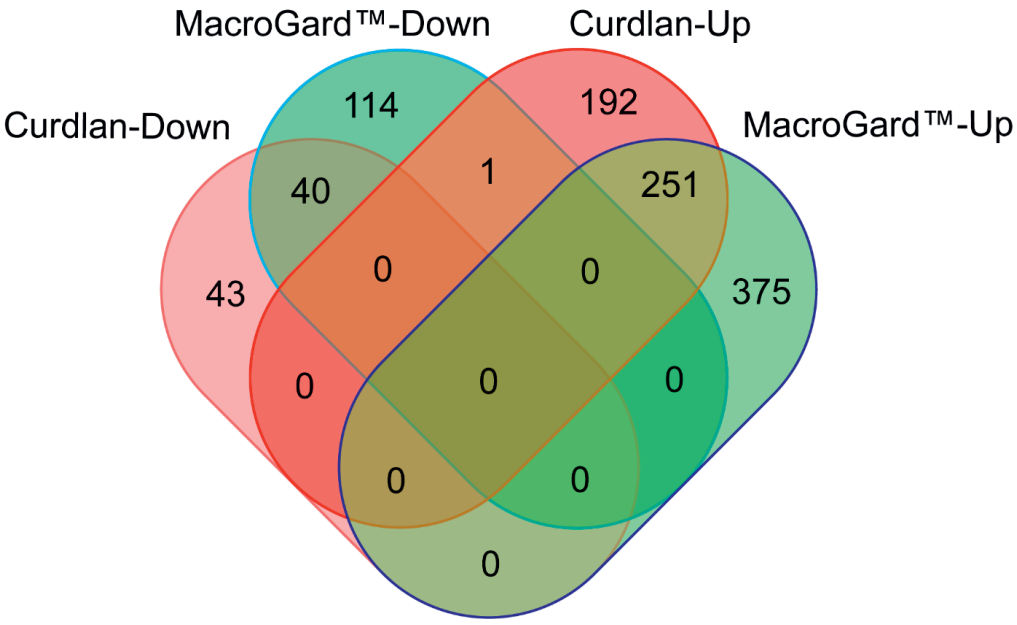

Figure 1. Venn diagram comparing differentially expressed genes (DEGs) regulated upon stimulation of common carp macrophages with β-glucans. Venn diagram of the DEGs regulated by curdlan (red) or MacroGard® (green). Macrophages were stimulated for 6 hours with 25 µg/mL of either curdlan or MacroGard® and collected for RNAseq analysis.

Automated gene ontology (GO) analysis of the two DEG datasets using GOrilla could map approximately 65% of the DEGs to GO terms. GO term enrichment analysis revealed 9 and 37 GO terms significantly enriched (FDR q-value ≤ 0.05) among curdlan-DEGs and MacroGard®-DEGs, respectively. For both datasets, the GO term with smallest FDR q-value was "Immune system process (GO:0002376)". Within the domain Biological Process, all significantly enriched GO terms in the curdlan-DEG dataset (n=7), were also significantly enriched in the MacroGard®-DEG dataset (n=25) (Supplementary tables 3 and 4), suggesting that the gene expression profile regulated by curdlan is also regulated by MacroGard®.

Automated annotation of the two DEG datasets to zebrafish KEGG pathways resulted in the mapping of the curdlan-DEGs to n=92 different pathways and of the MacroGard®-DEGs to n=112 different pathways. Four pathways were significantly over-represented in both DEG datasets, and a further nine unique pathways were significantly over-represented only in the MacroGard®-DEGs (Table 1). The overlap in the over-represented KEGG pathways in the two datasets supports the notion that in carp macrophages, the profile induced by curdlan is also induced by MacroGard®.

Table 1: Over-represented KEGG pathways following automated KEGG analysis of differentially expressed genes (DEGs) in macrophages stimulated with curdlan or MacroGard®. Curdlan-DEGs (n=356 genes) could be mapped to n=92 pathways, of which n=4 were significantly over-represented. MacroGard®-DEGS (n=541 genes) could be mapped to n=112 pathways of which n=13 were significantly over-represented. KEGG analysis was performed with KOBAS v3.0 with Chi-square test and the Benjamini and Yekutieli method for FDR correction. Pathways were considered significantly over-represented at corrected p-value ≤ 0.05.

Pathway	Curdlan	MacroGard®
Phagosome		**X**
Cytokine-cytokine receptor interaction	**X**	**X**
Lysosome		**X**
Apoptosis	**X**	**X**
Herpes simplex infection		**X**
Toll-like receptor signalling pathway		**X**
NOD-like receptor signalling pathway	**X**	**X**
VEGF signalling pathway		**X**
Arachidonic acid metabolism		**X**
Metabolic pathways		**X**
Phosphatidylinositol signalling system		**X**
ECM-receptor interaction	**X**	**X**
Adipocytokine signalling pathway		**X**

Automated annotation did not include the zebrafish-specific KEGG map for the "C-type lectin receptor signalling pathway" (dre04625) which has become available only recently. Manual mapping of curdlan- and MacroGard®-DEGs showed regulation of a large number of genes associated with the C-type lectin receptor pathway (Figure 2). Further, not only end-products of the CLR signalling pathway, such as cytokines which are often shared among pathways, but also several upstream molecules such as *card9*, *bcl10*, *malt1* and *calm* were regulated (see Figure 2). The manual mapping suggests that in carp macrophages, both β-glucans induced expression of genes typically associated with C-type lectin receptor signalling.

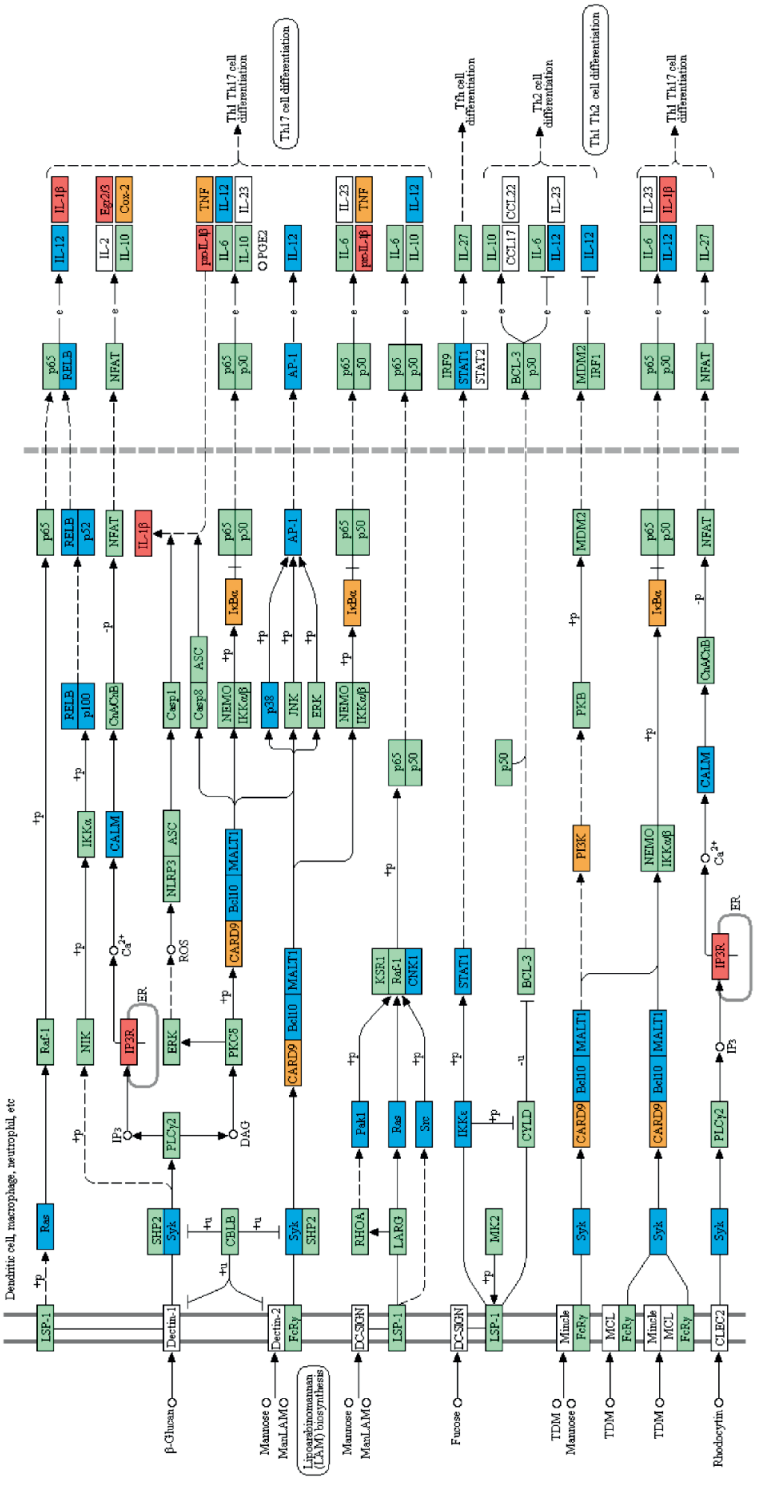

Figure 2: Regulation of the C-type lectin receptor signalling pathway by curdlan and MacroGard®. DEG datasets were manually mapped on the pathway-map dre04625 (https://www.genome.jp/kegg-bin/show_pathway?dre04625). The original reference pathway is indicated by white boxes, the organism-specific pathway is indicated by green boxes, confirming the presence of these genes in the zebrafish genome. Genes regulated after stimulation of carp macrophages with curdlan are indicated by red boxes, with MacroGard® by blue boxes, and by both curdlan and MacroGard® by orange boxes. The symbol O represents chemical compounds, DNA or other molecules. Black solid lines represent known molecular interaction or relation (►: activating, ⊥: inhibiting), dotted lines represent indirect links or unknown reactions, where +p, –p, +u and –u represent phosphorylation, dephosphorylation, ubiquitination and deubiquitination, respectively. Lines interrupted by e refer to gene expression interactions specifically.

Search for candidate receptors

Following the above-described mapping of DEGs to the CLR pathway, we continued with screening all 50,527 conceptually translated carp proteins for the presence of at least one CTL domain (CTLD), narrowing down the search for candidate receptors to a total of n=239 proteins. These CTLD-containing proteins were further characterized for the presence of ITAM sequences and transmembrane helices (data not shown). Studies have shown that the presence of a WxH motif or a WxHxxxxY motif in the carbohydrate binding region of Dectin-1 determines the β-glucan binding capacities of Dectin-1 (31-33). This criterion was used to further narrow down the search for candidate receptors, identifying a subset of n=13 carp proteins with a WxH motif (Table 2). We could not identify proteins with a WxHxxxxY motif present specifically in their CTLD. Interestingly, in four WxH-containing proteins, we could also identify a transmembrane region and one or two ITAMs and thus a 'complete' Dectin-1-like architecture. However, based on the RNAseq analysis, none of these candidates was significantly regulated by β-glucan stimulation or were constitutively expressed (higher than 10 reads per kilobase million) in unstimulated macrophages (Table 2), suggesting that these 13 candidates could not likely explain the functional responses to β-glucans in our current experimental set up.

Table 2: Candidate receptors with a WxH signature sequence in their CTL domain. Description refers to the closest BLASTx hit upon re-blasting the identified genes of interest and accession numbers refer to protein sequences in NCBI. Number of conserved CTL domains (CTLDs), ITAM sequences and transmembrane domains (TM) in the protein sequence are included. RPKM refers to the average (n=5) reads per kilobase million in unstimulated macrophages and is a measure for gene expression. cypCar codes identify common carp genes (*Cyprinus carpio*). Names or numbers in brackets refer to genes with identical BLASTx hits (likely owing to the additional genome duplication event in common carp). Proteins with architecture similar to Dectin-1 are highlighted in grey. Candidate receptors are ordered numerically by cypCar code.

Description	Accession number	CTLD	ITAM	TM	RPKM	cypCar
CD248 molecule, endosialin A precursor	NP_001092698.3	1	2	1	0.00	00001997
C type lectin receptor A	NP_001117051.1	1	3 (4)	1	0.45 (0.00)	00016746, (00029396)
Aggrecan	BAJ61837.1	1	3	0	0.00	00023554
Novel protein with Lectin C-type domains precursor	NP_001093528.1	3	2	0	0.01	00031652
Novel protein similar to lectins	CAI21223.1	1 (2)	1	0	0.00	00037242, (00037243)
Collectin 12	BAU33575.1	1	2	1	0.12	00040460
C-type mannose receptor 2-like	XP_026052210.1	3 (1)	1 (4)	0	0.02 (0.00)	00041550, (00047187)
Secretory phospholipase A2 receptor-like	XP_026074237.1	2	1	0	0.00	00045110
C-type lectin 1	AEH76769.1	1	1	1	6.67	00046286
Macrophage mannose receptor 1-like	XP_026074235.1	2	1	1	0.00	00048442

As the above-mentioned screening for WxH motif did not identify candidate receptors expressed or regulated in carp macrophages, we widened again our search and used constitutive expression or differential regulation as new criteria to narrow down the search for candidate receptors. Screening of all n=239 CTLD-containing proteins for constitutive expression of their corresponding gene identified a subset of n=12 genes that were expressed at an arbitrary threshold set at >50 Reads Per Kilobase Million (RPKM), corresponding to on average 1.5% of β-actin expression. Screening for regulation identified a subset of n=6 candidate receptors as differentially expressed after stimulation with β-glucans, all of which overlapped with the expressed subset (Table 3). Of interest, all six were down-regulated by β-glucan stimulation and two out of the six candidate receptors (both Asialoglycoprotein receptor orthologues) were regulated by both β-glucans. The finding that these candidate receptors were all regulated could suggest involvement in the response to β-glucan simulation of carp macrophages.

Table 3: Candidate receptors expressed in carp macrophages. Candidates were selected based on a minimal expression of ≥ 50 RPKM in unstimulated carp macrophages or differential expression following stimulation of macrophages with β-glucans. Description refers to the closest BLASTx hit, with associated NCBI protein accession code. Number of conserved CTL domains (CTLDs), ITAM sequences and transmembrane domains (TM) in the protein sequence are included. RPKM refers to the average (n=5) reads per kilobase million in unstimulated macrophages and is a measure for gene expression. CRD and MG refer to differentially expressed genes in macrophages after stimulation with curdlan (CRD) or MacroGard® (MG) based on RNAseq analysis (highlighted in grey). cypCar codes identify common carp genes (*Cyprinus carpio*). Names or numbers in brackets refer to genes with identical BLASTx hits (duplicated genes). Candidate proteins are ordered based on their expression (RPKM).

Description	Accession number	CTLD	ITAM	TM	RPKM	CRD	MG	cypCar
zgc:174904	NP_001170922.1	1	0	1	836		X	00031274
Asialoglycoprotein receptor 1-like isoform 1	XP_026096377.1	2	1	1	654	X	X	00032252
Mannose receptor c type 1	ALS87701.1	2	2 (6)	0	467 (354)			00046879, (00034006)
Asialoglycoprotein receptor 1-like isoform X2	XP_026098956.1	1	0	1	239	X	X	00032253
Ladderlectin-like	XP_026129733.1	1	0	0	215			00046054
C-type lectin domain family 4 member E-like	XP_026087308.1	1	0	1	200			00044344
C-type lectin domain family 4 member C	NP_001313501.1	1	1	1	165		X	00024225
Macrophage mannose receptor 1-like	XP_026142830.1	2 (5)	1 (7)	1	92 (60)		X	00047225, (00023855)
CD209 antigen	XP_003197805.3	1	0	0	83			00049168
C-type lectin domain family 4 member E	XP_002660626.3	1	0	0	60			00014657

Figure 3. Molecular phylogenetic analysis of CTLD sequences from C-type lectin receptors by Maximum Likelihood. The large red or green-coloured clusters identify typical CLR superfamily group V members (red) and most group II members (green). Species are indicated as h_ for human, m_ for mouse, g_ for chicken, d_ for zebrafish, s_ for salmon f_ for fugu and cypCar_ for carp. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are indicated, whereas values of partitions with bootstrap values lower than 10% are not shown. Carp CTLD sequences from candidate receptors (in italics) containing a conserved WxH motif are highlighted in blue, expressed in macrophages at RPKM ≥ 50 are highlighted in red and regulated by β-glucan stimulation of macrophages are highlighted in green. Asterisks (*) denote candidates identified independently with synteny analysis (see Figure 4). Underlined cypCar codes refer to candidates specifically addressed in the discussion section.

Combining the subsets of candidate β-glucan receptors identified based on criteria 1 (WxH motif), 2 (RPKM ≥ 50) and 3 (differential regulation) resulted in a total of n=25 proteins of specific interest, containing n=39 CTLD sequences. To investigate the phylogenetic relation between these candidates of interest and known CLR family members, CTLD sequences were aligned. The phylogenetic tree revealed a clear subdivision of the cypCars related to the carp CTLD proteins. None of the CTLD sequences from the identified candidates clustered with family V members, the group containing Dectin-1 (Figure 3). Instead, seven CTLD sequences from WxH motif-containing candidates (criterion 1) clustered with human CLEC20A, among human CLR group II members, and seven other WxH-candidates clustered together with human Attractin. Maybe not surprisingly, four of the constitutively expressed CTLD sequences (criterion 2) clustered with CTLD sequences from macrophage mannose receptors, group VI family. Six CTLD sequences from the differentially regulated subset (criterion 3) also clustered with macrophage mannose receptors, the other six CTLD sequences in this subset followed no specific clustering pattern. Overall, preliminary phylogenetic analysis suggests the absence of group V members in our set of candidate receptors.

Synteny analysis of zebrafish and carp CTLD-encoding genes

Investigation of the carp CTLD containing proteins identified several candidates with architecture similar to mammalian Dectin-1. In mammalian genomes *Dectin-1* is located in the NK cell receptor cluster, which contains several CLRs. This NK cell receptor cluster shows conserved synteny between human chromosome 12 (hCHR12) and mouse chromosome 6 (mCHR6) (399). Conservation of synteny describes preservation of co-localization of genes on chromosomes of different species and might therefore serve as an additional criterion (criterion 4) for the identification for candidate receptors involved in the recognition of β-glucans. Synteny analysis can be best performed in a well-assembled genome. The genome assembly of zebrafish, a close relative of common carp, is among the best assembled genomes in teleost fish, including large chromosome scaffolds that can be used for synteny analysis, in contrast to the carp genome which is still largely fragmented into small scaffolds (253, 255). The NK cell receptor cluster in human includes, among others, *DECTIN-1, DECTIN-2,* and *MINCLE*. Regions surrounding the NK cell receptor cluster on hCHR12 and mCHR6 showed conserved synteny with regions on zebrafish chromosome 16 (zCHR16), based on co-localization of *pex5, clstn3, lpcat3, gnb3, cops7a,* and *znf384* (Figure 4). Intriguingly, this region of zCHR16 also includes two CTLD-encoding genes, *clec4c* (NCBI Gene ID: 563797) and *sclra* (NCBI Gene ID: 564061), highlighting them both as genes of interest for our study.

Further investigation of synteny between the zebrafish region of interest on zCHR16 and carp scaffolds revealed partial conservation of synteny with four scaffolds. Two of these scaffolds contained *cypCar_00024225* and *cypCar_00032523*, putative paralogues of zebrafish *clec4c*; of which *cypCar_00024225* is a CTLD-encoding gene, already identified in this study as

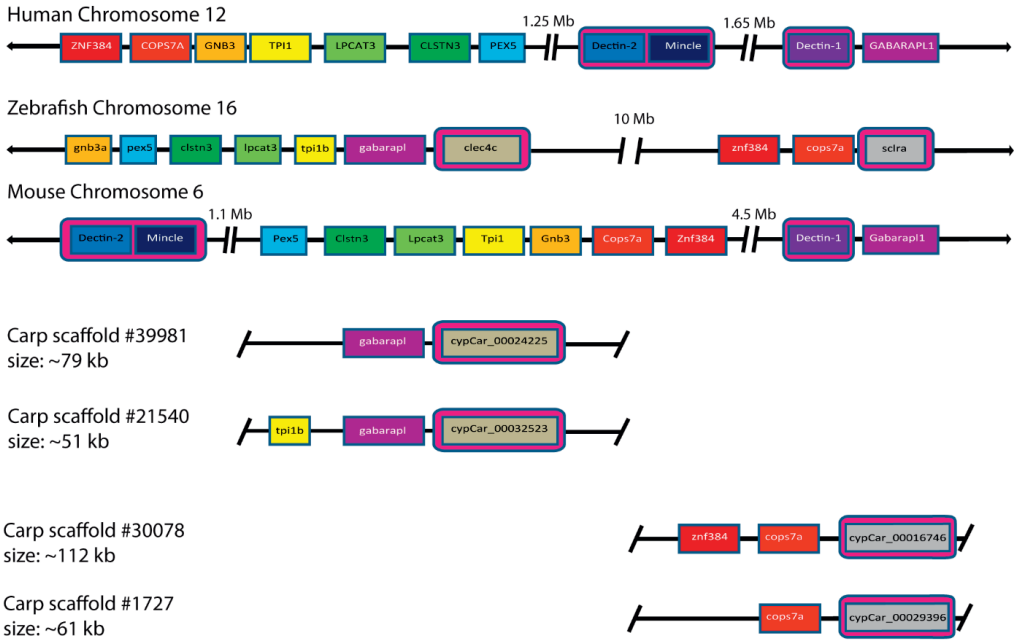

Figure 4. Synteny analysis of mammalian CLR group V cluster on human chromosome 12 and mouse chromosome 6 shows partial conservation with zebrafish chromosome 16 and four carp scaffolds. Partial conservation of synteny between zebrafish chromosome 16 and the mammalian CLR group V cluster can be observed. Among the syntenic genes, two zebrafish CLTD-encoding genes are located, *clec4c* (NCBI Gene ID: 563797) and *sclra* (NCBI Gene ID: 564061). Synteny of zebrafish *clec4c* and *sclra* with *Dectin-1*, *Dectin-2*, and *Mincle* is highlighted by pink boxes. Synteny of carp *cypCar*s is based on conservation of genes surrounding a CTLD-encoding gene for both *clec4c* and *scrla*. Large gaps between loci are indicated with breaks and chromosomal representations are not drawn to scale. Owing to the tetraploid nature of carp, two corresponding scaffolds for each zebrafish gene are shown.

candidate receptor based on its regulation in macrophages after stimulation with MacroGard® (criterion 3). The two other scaffolds contained *cypCar_00016746* and *cypCar_00029396*, putative paralogues of zebrafish *sclra*; both of which are CTLD-encoding carp genes, already identified in this study as candidate receptors based on their conserved WxH-motif (criterion 1). The synteny analysis provided a fourth criterion, additional to the previously formulated criteria (WxH motif, constitutive expression and differential regulation), to identify candidate receptors for β-glucan recognition. A Venn diagram, graphically representing the different subsets of candidate β-glucan receptors identified by the four criteria can be found Supplementary figure 1.

Discussion

Primary macrophages of common carp had previously been shown to respond to prototypical Dectin-1 ligands, which led to the hypothesis that the CLR pathway must play an important role in the recognition of β-glucans in carp macrophages. In our approach, we used head kidney-derived carp macrophages as a starting cell population to test our hypothesis and investigate activation of the CLR pathway upon stimulation with β-glucans. Indeed, pathway analysis of differentially expressed genes confirmed our hypothesis that β-glucans regulate a downstream signalling pathway typical of CLR activation. Further, we could identify in the transcriptome of β-glucan-stimulated carp macrophages, several differentially expressed genes with a C-type lectin domain. These data are of high interest for further functional studies on the mechanisms underlying β-glucan-induced immunomodulation in teleost fish.

We used two different β-glucans: curdlan, a linear polymer of β-(1,3)-linked glucose and considered a Dectin-1-specific ligand, and MacroGard®, a branched polymer of β-(1,3/1,6)-glucose widely-applied as feed additive in aquaculture. Overall, MacroGard® regulated a higher number of differentially expressed genes than curdlan, possibly owing to differences in purity, source, degree of polymerization, and nature of the glycosidic bonds in the β-glucans (23). Regardless of the extent of gene regulation, manual mapping of the DEGs revealed a clear regulation of the CLR signalling pathway (KEGG) for both β-glucans. Indeed, up-regulation of homologues of all three players of the *card9-Bcl10-Malt1* complex, previously shown to play a crucial role in β-glucan signalling through the CLR pathway (38), strongly supports regulation by the CLR pathway. We continued our study by identifying candidate genes encoding for proteins with one or more C-type Lectin Domains (CTLD), which could be of potential interest with respect to recognition of β-glucans, using a recently published database of RNAseq-validated gene predictions for carp (255). We used four criteria to identify candidate receptors in carp macrophages: 1) conservation of the glucan binding WxH-motif in the CTLD; 2) constitutive expression higher than 50 RPKM; 3) differential regulation upon stimulation with β-glucans; 4) conservation of synteny with mammalian NK cell receptor cluster.

Based on criterion 1 (conserved WxH motif) we identified two candidates (*cypCar_00016746* and *cypCar_00029396*) of which the CTLD clustered together with known CTLD sequences from zebrafish and from Atlantic salmon, known as *salmon C-type lectins sclra* and *sclrb* (381). Although the carp *sclr* paralogues were not constitutively expressed in macrophages (criterion 2), both salmon *sclr*s have been associated with the response to β-glucans (380). Interestingly, the mammalian WxHx[4]Y motif was not conserved, however a motif with five rather than four residues (WxHx[5]Y) separating histidine from tyrosine was conserved between all sequences. All three residues are considered crucial to form the β-glucan binding cleft of mammalian Dectin-1 (32), and also present in invertebrate β-glucan binding proteins (GNBP3), but not as a WxHx[5]Y motif (32, 34).

Although not constitutively expressed in macrophages, carp *sclr* could well play a role in β-glucan binding in other cell types.

Based on criterion 2 (constitutive expression ≥ 50 RKPM), we identified a further 13 candidate receptors, of which six were differentially regulated (criterion 3). Without exception, all six genes were down-regulated upon stimulation with β-glucans, which could possibly be explained by a need to restrict *de novo* protein synthesis and duration of signalling to prevent over-stimulation (400-403). Possibly, analysis of protein and/or gene expression at different time points could show up-regulation. In Atlantic salmon, three *sclr*s were up-regulated seven days after oral administration of MacroGard®, concomitantly with *syk* kinase (380). We similarly noticed a concomitant regulation of *syk*, suggesting the expression of CTLD-encoding genes and *syk* is co-regulated. No matter what, the observed modulation of gene expression strongly suggests involvement of CLR family members upon recognition of β-glucans by carp macrophages.

Based on criterion 3 (regulation of gene expression), we identified several additional candidate receptors. Among these, the CTLD-encoding gene with the highest expression and regulation in carp macrophages (*cypCar_00031274*), clustered together with three other genes of interest: i) a CTLD-encoding gene already identified in this study based on criterion 2 (*cypCar_00044344)*, ii) an unknown zebrafish full length cDNA (*zgc174904*) encoding a protein with a CTLD, a transmembrane helix and a WxHx[5]Y motif just outside the borders of the CTLD and iii) an Atlantic salmon C-type lectin receptor-c (*sclrc*), different from the ones previously mentioned in criterion 1. The salmon *sclr* genes were first identified in (suppression subtractive) EST libraries (381), while a follow up study revealed up-regulation of all three *sclr* genes after oral intubation of Atlantic salmon with MacroGard® (380). The salmon *sclrc* gene contains a WxH motif within the CTLD while the carp candidate genes (both, *cypCar_00031274* and *cypCar_00044344*) contain a WxHx[5]Y. Interestingly, the latter motif was found just outside the boundaries predicted for a CTLD, which suggests that further manual scrutiny of predicted domains in fish sequences could expand the list of currently identified domains of interest. Overall, the fact that closely-related *sclr* genes were identified as candidate receptors in both, salmon and carp, suggests a potential role of this CTL receptor in the response to β-glucans in fish and supports a need for its further characterization of function.

Based on criterion 4 (synteny in the zebrafish genome), we identified two CTLD-encoding genes of interest; putatively named *clec4c* and *sclra*. Three out of four corresponding duplicates at syntenic regions of the carp genome were already identified as genes of interest based on the selection criteria discussed above. This means that next to the *sclr* genes, we could identify *clec4c* as another gene of interest deserving further attention as candidate receptor for β-glucan. Taken together, our broad NGS approach helped us describe a clear regulation of the CLR pathway and identify a number of CTLD-containing candidate receptors for β-glucan binding. As proteins, these receptors would form a good starting point for

future sugar binding assays and for further functional characterization with e.g. glycome microarrays, a high-throughput method devised to analyse β-glucan-binding proteins through an oligosaccharide microarray, followed by mass-spectrometric sequencing (404, 405). Altogether, the candidates discussed in this study should help pave the way to future functional studies that could ultimately lead to the identification of β-glucan receptor(s) in fish.

Author Contributions

JP, MF and GW contributed to the design of the experiments, acquisition of samples, and analysis of data. GW acquired funding. RW and EC contributed with the phylogenetic and synteny analysis. CO contributed with reagents, materials and analysis tools. JP, RW, MF and GW wrote the manuscript.

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Acknowledgements

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Supplementary material

Supplementary tables 1 and 2 are excluded due to the size of the tables. The tables can be accessed at
https://www.frontiersin.org/articles/10.3389/fimmu.2019.00280/full.

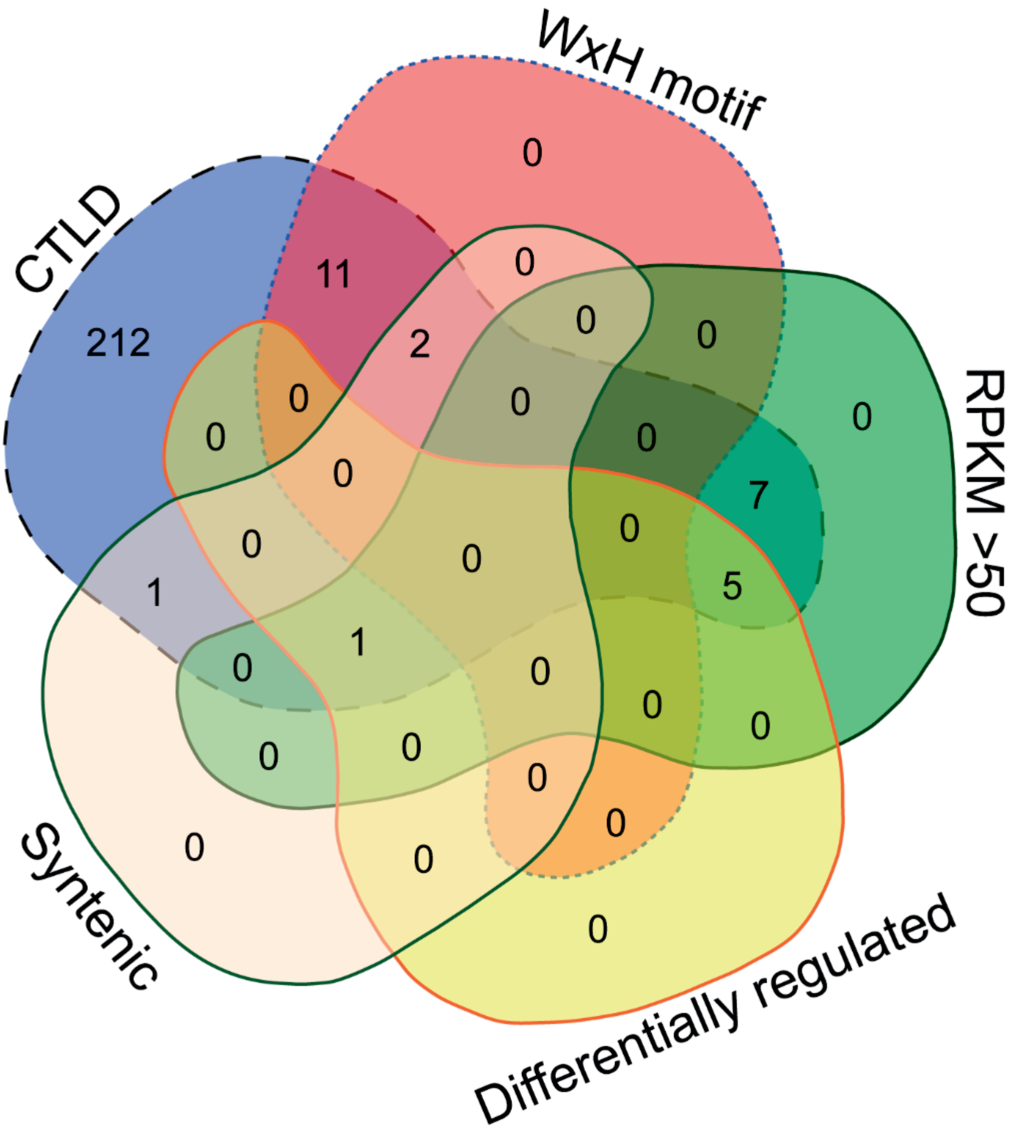
Supplementary table 3. GOrilla GO enrichment analysis of curdlan differentially expressed genes (DEGs). False discovery rate (FDR) correction is performed using the Benjamini and Hochberg (1995) method.

Domain	GO Term	Description	FDR q-value	# DEG
GO molecular function	GO:0032813	tumor necrosis factor receptor superfamily binding	1.99E-02	6
GO molecular function	GO:0005164	tumor necrosis factor receptor binding	9.94E-03	6
GO Biological Process	GO:0002376	immune system process	2.02E-03	22
GO Biological Process	GO:0043207	response to external biotic stimulus	4.39E-03	15
GO Biological Process	GO:0009607	response to biotic stimulus	3.22E-03	15
GO Biological Process	GO:0006955	immune response	7.22E-03	15
GO Biological Process	GO:0006952	defense response	1.19E-02	14
GO Biological Process	GO:0070309	lens fiber cell morphogenesis	2.07E-02	3
GO Biological Process	GO:0006954	inflammatory response	4.28E-02	9

Supplementary table 4. GOrilla GO enrichment analysis of MacroGard® differentially expressed genes (DEGs). False discovery rate (FDR) correction is performed using the Benjamini and Hochberg (1995) method.

Domain	GO Term	Description	FDR q-value	# DEG
GO Molecular Function	GO:0005126	cytokine receptor binding	4.12E-03	15
GO Molecular Function	GO:0004896	cytokine receptor activity	3.48E-03	11
GO Molecular Function	GO:0005164	tumor necrosis factor receptor binding	4.97E-02	6
GO Molecular Function	GO:0046961	proton-transporting ATPase activity, rotational mechanism	3.98E-02	6
GO Molecular Function	GO:0060589	nucleoside-triphosphatase regulator activity	4.79E-02	16
GO Molecular Function	GO:0042625	ATPase coupled ion transmembrane transporter activity	4.67E-02	9
GO Molecular Function	GO:0019829	cation-transporting ATPase activity	4.21E-02	9
GO Molecular Function	GO:0098772	molecular function regulator	4.41E-02	48
GO Molecular Function	GO:0005096	GTPase activator activity	4.88E-02	14
GO Biological Process	GO:0002376	immune system process	8.35E-08	35
GO Biological Process	GO:0006952	defense response	1.31E-07	25
GO Biological Process	GO:0006955	immune response	4.38E-07	25
GO Biological Process	GO:0043207	response to external biotic stimulus	1.45E-06	23
GO Biological Process	GO:0009607	response to biotic stimulus	1.35E-06	23
GO Biological Process	GO:0051707	response to other organism	2.95E-05	19
GO Biological Process	GO:0050896	response to stimulus	1.16E-04	78
GO Biological Process	GO:0051704	multi-organism process	1.10E-04	19
GO Biological Process	GO:0007165	signal transduction	2.69E-04	101
GO Biological Process	GO:0006954	inflammatory response	7.08E-04	13
GO Biological Process	GO:0009605	response to external stimulus	5.60E-03	26
GO Biological Process	GO:0098542	defense response to other organism	6.04E-03	11
GO Biological Process	GO:0009617	response to bacterium	6.62E-03	11

GO Biological Process	GO:0006935	chemotaxis	7.28E-03	13
GO Biological Process	GO:0060326	cell chemotaxis	1.09E-02	10
GO Biological Process	GO:0019221	cytokine-mediated signaling pathway	1.90E-02	9
GO Biological Process	GO:0042742	defense response to bacterium	2.18E-02	8
GO Biological Process	GO:0070309	lens fiber cell morphogenesis	2.35E-02	3
GO Biological Process	GO:0042330	taxis	2.62E-02	13
GO Biological Process	GO:0016477	cell migration	2.49E-02	27
GO Biological Process	GO:0042221	response to chemical	3.17E-02	31
GO Biological Process	GO:0048870	cell motility	4.05E-02	27
GO Biological Process	GO:0034097	response to cytokine	4.32E-02	6
GO Biological Process	GO:0040011	locomotion	4.35E-02	28
GO Biological Process	GO:0051336	regulation of hydrolase activity	4.28E-02	17
GO Cellular Component	GO:0005773	vacuole	3.01E-02	13
GO Cellular Component	GO:0005768	endosome	4.41E-02	14
GO Cellular Component	GO:0009897	external side of plasma membrane	4.49E-02	9



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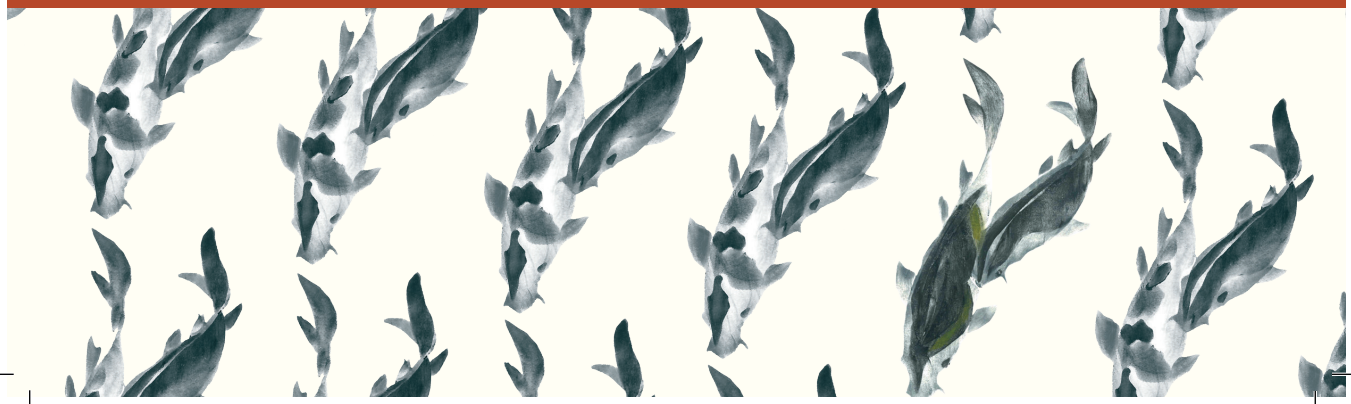
Supplementary figure 1. Venn diagram visualizing all C-type lectin domain (CTLD) encoding genes and the separate filtering criteria for potential β-glucan receptor candidates. All CTLD-encoding genes are identified with a protein family (Pfam) search of the protein sequence (blue shape). Four independent criteria were used to highlight candidate β-glucan receptors genes: 1) conservation of the glucan binding WxH-motif in the CTLD (red shape); 2) expression higher than 50 RPKM in carp macrophages (green shape); 3) differential regulation by stimulation of carp macrophages with β-glucans (yellow shape); 4) CTLD-encoding genes located in a region with conserved synteny to the mammalian CLR group V cluster are highlighted (orange shape).



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General discussion

The tremendous growth of the aquaculture sector seen over the past decennia is associated with an intensification of the production process. This has led to an increase incidence of infectious diseases. To prevent these undesired effects, it has become relatively common to supplement fish feed with immuno-stimulants such as yeast-derived β-glucans. Several studies have reported generally positive effects, including increased resilience to stress and bacterial infections. Yet, to explain current effects of β-glucan supplementation, there is a need for a better understanding of the underlying mechanisms influencing the immune system. In the general introduction, we proposed to investigate three different mechanisms that could play a part in the observed effects of β-glucan supplementation in fish:

1. *Long-lived effects on myeloid cells, typical of trained immunity;*
2. *Degradation and fermentation of β-glucans and associated shifts in composition of the intestinal microbiota and their metabolites;*
3. *Regulation of downstream signalling upon β-glucan recognition in carp macrophages*

Looking back, we started with reviewing the literature for potentially overlooked, long-lived effects of β-glucan supplementation that could possibly be ascribed to trained immunity [**Chapter 2,** (20)]. Next, we established an *in vitro* model of trained immunity that led us to conclude that not only the NOD-like receptor ligand PGN, but also the soluble β-glucan laminarin, can induce trained immunity in common carp macrophages (**Chapter 3**). The second mechanism addressed was the ability of common carp intestinal microbiota to ferment β-glucans, resulting in the production of potentially immunomodulating short chain fatty acids (**Chapter 4**). The third mechanism dealt with the direct recognition of β-glucans and subsequent signalling, which was studied using next generation sequencing (NGS) [**Chapter 5,** (389)]. The subsequent study confirmed the regulation of the C-type lectin receptor (CLR) signalling pathway and led to the identification of several CLRs as potential candidates for recognition of β-glucans [**Chapter 6,** (41)].

Looking forward, here I discuss the implications of these findings and will position them in a theoretical framework against relevant literature. I will not only discuss the three proposed mechanisms, but also discuss the possible relations between these three as well as new mechanisms. Finally, I will elaborate on the relevance of my thesis by addressing the following question: *"Will the description of mechanisms underlying immuno-modulatory properties of β-glucan have an impact on current practise in aquaculture?"*

Trained immunity: a critical reflection and outlook

Although the basic mechanisms leading to the induction of trained immunity in human monocytes and carp macrophages may be comparable, there are also differences in approaches and read-outs. For example, mammalian monocytes

trained with particulate *Candida* derived β-glucans produce reduced amounts of ROS upon non-specific stimulation (168), whereas carp macrophages trained with soluble *Laminaria* β-glucans produced increased amounts of ROS. Possibly, this outcome could be ascribed to differences between these two training stimuli with regard to solubility and/or origin, certainly when realizing that different preparations of laminarin can already result in different immune responses in mammalian macrophage cell lines (406). Alternatively, this outcome could be ascribed to differences between monocytes and macrophages. The majority of *in vitro* studies on trained immunity in mammals have been performed with isolated monocytes, while we studied a population of mature macrophages not devoid of progenitors (164, 407, 408). Noteworthy, the common carp myeloid cell culture is derived from head-kidney, the haematopoietic organ in fish equivalent to mammalian bone marrow (409). Of interest, a recent study in mice showed that β-glucan-induced trained immunity can alter haematopoiesis, illustrated by an increased frequency of myeloid biased multipotent progenitor cells in the bone marrow and a more pronounced response to LPS of myeloid cells pre-exposed to β-glucans (410). The effect of β-glucans on myeloid cell progenitors, is an area of interest that deserves more attention, especially when it comes to long-lived effects associated with trained immunity.

From a fundamental perspective, the induction of trained immunity in carp macrophages is of high interest because it provides insight in the evolutionary conservation of innate immune responses to β-glucans. From an applied perspective, it can be equally interesting to enhance innate immune responses given the constantly high pathogen pressure in intensive aquaculture. Trained immunity in fact, is defined as a form of innate immune memory that results in enhanced inflammatory and antimicrobial properties of innate immune cells, providing an increased nonspecific response to subsequent infections and improved survival of the host (411). The non-specific nature of trained immunity especially, strongly supports observations on increased resistance to bacteria (103, 105), viruses (412, 413) and parasites (94, 97) upon dietary β-glucan supplementation. Evidence for antigen sampling in teleost intestine (93, 186) provides a cellular basis to these observations in fish, but in humans oral supplementation of β-glucans did not induce trained immunity (141). Yet, in our review (20) we could identify seven field studies where indications for long-lived effects of β-glucans were observed, three of which used oral supplementation of β-glucans (103, 155, 156). These studies would suggest that induction of trained immunity via oral administration of β-glucans might be possible, at least in fish.

To date, several studies have shown that the ability to induce trained immunity is not unique to β-glucans, nor to Bacillus Calmette–Guérin (BCG), another common stimulus associated to trained immunity in mammals [reviewed by (411)]. Pre-exposure of human monocytes to endogenous oxidized low-density lipoprotein (oxLDL) (414), fungal chitin (415), raw milk or specific milk proteins (416), parasite infected red blood cells (417), all appear able to induce trained immunity. In addition, several cellular metabolites have been shown to induce trained

immunity-like profiles in myeloid cells, such as fumarate, a substrate of the tricarboxylic acid cycle and mevalonate, an intermediate in the cholesterol synthesis pathway (59-61), highlighting the links between metabolism and innate immunity and providing new avenues for the (fish) feed industry to develop immuno-modulatory feeds.

Future studies addressing the potential for reprogramming of the innate immune system, should also address physiological costs of trained immunity. In mammals, maladaptive (trained) immunity can result in tissue damage or chronic inflammation [reviewed by: (418, 419)]. For example, while exposure to oxidized LDL (oxLDL) can induce trained immunity-like characteristics (414), the associated long-term epigenetic reprogramming and the role of oxLDL in the development of artherosclerosis, might explain the persistent inflammatory profile of macrophages in atherosclerotic plaques. Recently, a study in mice connected Western diet and systemic inflammation with trained immunity. The authors observed long-lived transcriptional and epigenetic reprogramming, development of potentially harmful monocytes and an immunological over-reaction which might result in a maladaptive trained immunity response or in auto-immune disorders (420). It may be evident that there can be down sides to trained immunity related to a risk of persistent inflammation and associated tissue damage, but also increased metabolic costs of trained immunity should be considered. Sustained inflammatory responses can change the allocation of nutritional and energy resources with negative effects on growth efficiency (421, 422). It will thus be important to address the dynamics of trained immunity induced by dietary supplementation with β-glucans and study the physiological costs of feed supplementation strategies in aquaculture.

From a fundamental perspective, it is of interest to take the *in vitro* observations on trained immunity further and study innate immune responses *in vivo*, for example in *rag*$^{-/-}$ zebrafish. These mutant lines do not have functional B- and T-cells and thus no functional adaptive immune response (160). A tentative design could be as follows. First, trained immunity could be induced by a single intraperitoneal (i.p.) injection of β-glucans. Subsequently, fish would be exposed to different pathogenic challenges at different time intervals, for example at 7, 14, 21, and 28 days post injection. The different challenges are of interest because of differences in innate memory in *rag*$^{-/-}$ zebrafish observed upon challenge with intracellular (152) or extracellular bacteria (160). The different time intervals are of interest because of earlier observations in zebrafish challenged with spring viremia carp virus showing effects at 7 but not 35 days post-injection with β-glucan (158). Preferably, the aforementioned experiments could be performed in crosses between *rag*$^{-/-}$ zebrafish and transgenic zebrafish with fluorescent neutrophils (423) or macrophages (424) and for example combined with flow cytometry-based ROS production in "trained fish". This combination of approaches could confirm conservation of trained immunity in fish *in vivo*, validate the use of a single β-glucan i.p. injection to induce trained immunity and provide insight in the kinetics of trained immunity in fish.

From an applied perspective, field studies on trained immunity should probably combine several approaches to validate assumptions made in individual studies; the whole will be greater than the sum of the parts, and a tentative design could be as follows. Using i.p. injection with β-glucan as a positive control, fish could be fed diets supplemented with different concentrations of β-glucans administered at different frequency and duration of exposure. As a crucial part, all groups should be switched to a control diet for a certain period (resting period, as in **Chapter 3**) to allow the organism to clear the presence of β-glucans, followed by challenge with different pathogens at different time intervals, as suggested above. This would provide insight in the duration of trained immunity "memory" *in vivo*. In larger-sized fish, innate immune cells could be sampled for *ex vivo* measurement of reactivity to stimulation with different PAMPs, as in previous studies using head-kidney derived carp macrophages (164). Yet, field studies can never fully exclude a role of non-innate immune cells in the induction of trained immunity, no matter how carefully designed the experiment is. Given that a recent study showed that T cell-derived IFNγ could be crucial for induction of trained immunity, at least in alveolar macrophages (425), "contamination" with other cell types (e.g. lymphocytes) during field studies on trained immunity may be considered irrelevant to those interested in practical applications and rightfully be considered of academic interest.

Intestinal function: from microbiology to immunity

Prebiotics are defined as ingredients selectively fermented by the intestinal microbiota affecting the composition and activity of the microbiota itself (26). They are considered ingredients that could be important for "feeding the microbiota". Probiotics are defined as live microorganisms that can provide health benefits when consumed, generally by improving or restoring intestinal microbiota (426). While there is appreciation of the beneficial effects of prebiotics and probiotics by the aquaculture sector [reviewed by: (427-430)], a knowledge gap remains on the underlying mechanisms of such health benefits. In mice, a connection was made between a high-fibre diet and reduced allergy-induced inflammation in the lungs acting via short-chain fatty acids (SCFAs) produced by the microbiota (231).

SCFAs are fatty acids with less than six carbon atoms and produced as end products of fermentation of dietary fibres by the intestinal microbiota. SCFAs can have modulatory effects on inflammation via two major but different routes: 1) through receptor independent inhibition of histone de-acetylases (HDACs) of the host; 2) through activation of host SCFA receptors such as G-protein coupled receptor (GPR) 41, GPR43 and GPR109A [also known as free fatty acid receptor (FFAR) 3, FFAR2 and hydroxycarboxylic acid receptor 2 (HCAR2)]. Neither of the two routes have been described in detail in fish nor has modulation of the immune system by SCFAs produced by intestinal microbiota been widely studied in fish.

Histone de-acetylases (HDACs) are an evolutionary-conserved family of proteins present from bacteria to humans. HDACs mediate hypo-acetylation which is generally associated with a closed chromatin structure, thereby blocking or repressing gene transcription due to the inaccessibility of promotor regions for transcription factors [reviewed by: (72, 431, 432)]. The presence of SCFAs can inhibit HDACs and thus result in hyper-acetylation, which is associated with an open chromatin structure, facilitating the binding of transcription factors to promotor regions. The effects of these epigenetic changes can be diverse, dependent on the region of acetylation. Within an immunological context, butyrate and propionate are currently thought to be the two most important SCFAs. Butyrate and propionate have both been shown to inhibit LPS-induced TNF-α production via inhibition of NF-κB and increased IL10 production (73, 433). Moreover, butyrate was shown to reduce phagocytosis and ROS production by neutrophils (434). Given that epigenetic reprogramming has been claimed an important factor driving trained immunity [as reviewed by: (181)] and given that ROS and TNFα are inflammatory factors frequently associated with trained immunity (this thesis), it is of interest to study the potential influence of SCFAs on trained immunity in fish.

SCFA receptors have not yet been described in fish. In zebrafish, four predicted genes on chromosome 16 and three predicted genes on chromosome 19 are currently associated with FFAR2 and 3, however no validation nor characterization of these genes has been performed. In the latest common carp assembly, n=4 genes are annotated as mammalian FFAR2 and n=8 genes are annotated as mammalian FFAR3. In human and mouse monocytes, both FFAR2 and FFAR3 are constitutively expressed but down-regulated upon differentiation into macrophages (435). Our NGS data set of carp macrophages showed constitutive expression of two putative *ffar* genes with reasonable values of 27-47 reads per kilobase million (RPKM), corresponding to 0.5-1% of the constitutive β-actin expression. One of these two genes was even up regulated in macrophages stimulated with MacroGard®. This suggests that innate immune cells of carp such as macrophages could possibly express SCFA receptors and might be responsive to modulation by SCFAs such as butyrate and propionate. In a prelimary experiment we investigated the modulatory effect of different SCFAs, *in vitro*, on LPS-stimulated head kidney leukocytes (HKL) of carp (Figure 1). Butyrate in particular had a clear immunomodulatory effect.

In light of the presence of high number of macrophages in the carp intestine (187), it appears reasonable to hypothesize that SCFAs produced in response to shifts in the composition of intestinal microbiota, can be recognized by receptors present on these macrophages, thereby modulating local immune responses. Of course, since the current annotation of the carp genome is still largely based on prediction and previous *de novo* transcriptome data (253, 255), the next step should be validation of the putative *ffar* genes in carp. Only following their validation as receptors for SCFAs, subsequent studies can address organ expression and receptor dependent effects of different SCFAs. Clearly, receptor independent

effects of SCFAs, for example on HDACs, should not be neglected and both routes of action should be addressed in future studies.

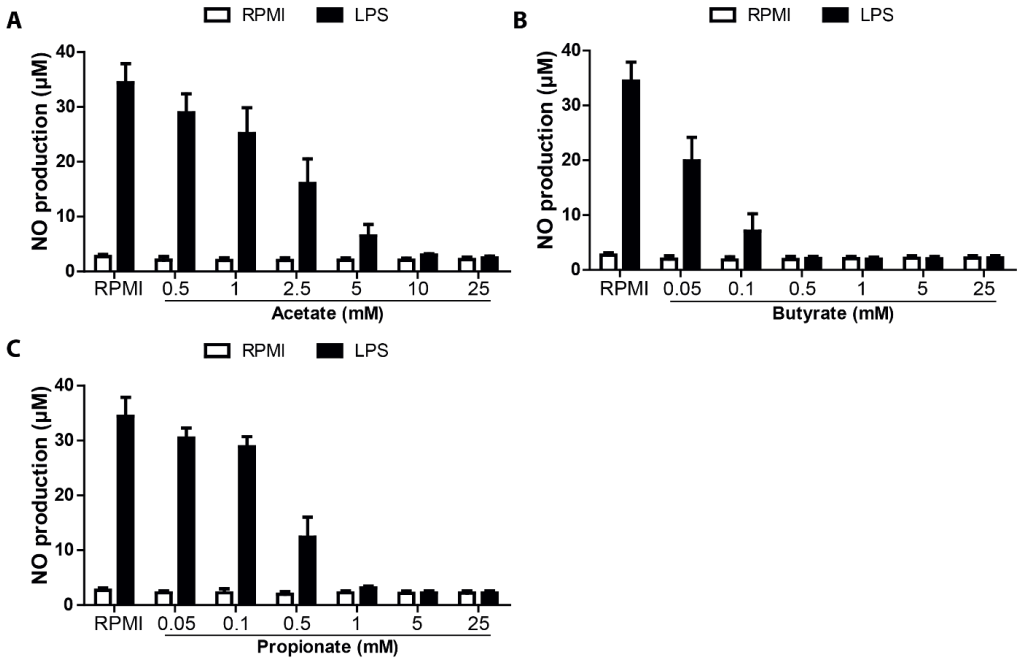


Figure 1. SCFA reduce LPS-induced production of nitric oxide (NO) in head kidney leukocytes (HKL). Cells were stimulated with LPS (30 µg/mL) for 96 hrs in the presence or absence of the SCFAs, acetate, butyrate and propionate. **A.** NO production of LPS-stimulated HKL in the presence of acetate. **B.** NO production of LPS-stimulated HKL in the presence of butyrate. **C.** NO production of LPS-stimulated HKL in the presence of propionate. Bars indicate mean + SD of n=3 independent experiments.

Regulation of the CLR pathway: zooming in

A genome-wide study could not identify true *C-type lectin super family V* members in the genome of Fugu (*Takifugu rubripes*) (42), and neither did we observe clustering of carp C-type lectin domain (CTLD) sequences with CTLD sequences from mammalian family V members (41). The receptor for β-glucans therefore may not be conserved between mammals and fish, but we did observe regulation of several elements of the C-type lectin receptor signalling pathway upon β-glucan stimulation in carp macrophages (Figure 2). Interestingly, we observed regulation of all three components of the Card9-Bcl10-Malt1 signalling complex, which strongly hints towards involvement of this complex in the response to β-glucan. This signalling complex is activated by PKCδ that is recruited by activated *syk*, downstream of Dectin-1. In contrast, we observed a down-regulation of *syk* following stimulation of macrophages with β-glucans (40, 44), which might suggest signalling via a syk- independent mechanism. In this study two different β-glucan preparations were used: curdlan, a linear β-(1,3)-glucan considered a Dectin-1-specific ligand in mammals, and MacroGard®, a branched β-(1,3/1,6)-glucose

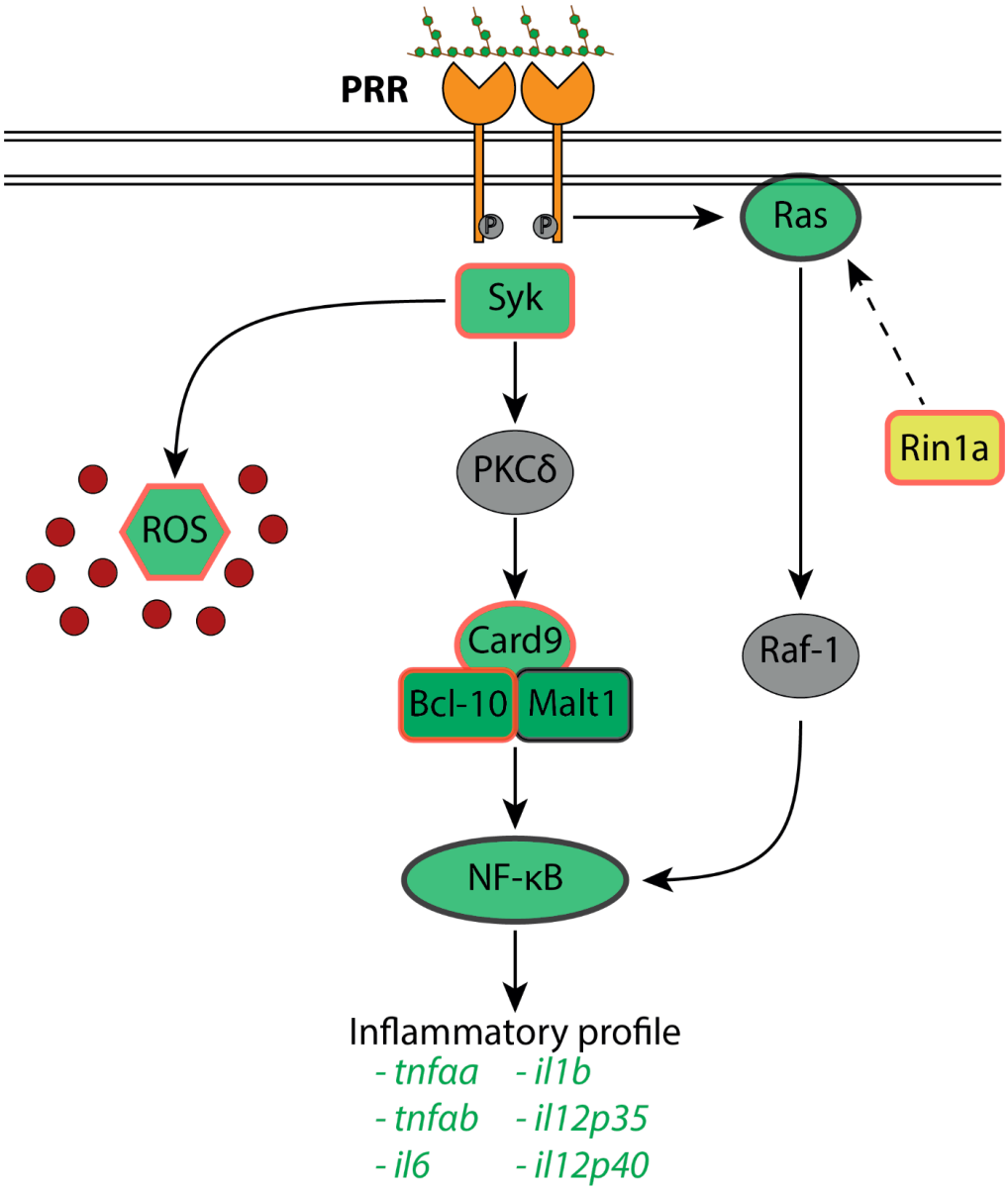


Figure 2. β-glucan regulated carp genes superimposed on the mammalian Dectin-1 signalling pathway. Orange PRRs represent proposed candidates based on the investigation of CLR family members in the carp genome. Signalling molecules depicted in green were identified as genes regulated in macrophages stimulated with β-glucans (**Chapter 6**). Signalling molecules depicted in grey were not regulated. Molecules marked by a red border were also observed regulated in separate experiments, either functional (ROS production) or by qPCR (*syk, card9* and *bcl-10*). Gene regulation of the list under inflammatory profile was validated by qPCR. The yellow Rin1a was specifically regulated at transcription level. Rin1a can inhibit Ras-regulated Raf-1 activation.

widely-applied as feed additive in aquaculture. We later included a third β-glucan preparation zymosan, a commonly used yeast preparation and compared the differentially expressed genes (DEGs) regulated by all three different β-glucan preparations. By comparing the overlap in regulation between the three datasets we aimed to identify a common β-glucan-regulated gene set (Figure 3A). Although the absolute number of genes regulated by zymosan (n = 224) was lower than for MacroGard® (n = 789) or curdlan (n = 528), there was a major overlap in DEGs, indicative of a common β-glucan-regulated gene sets.

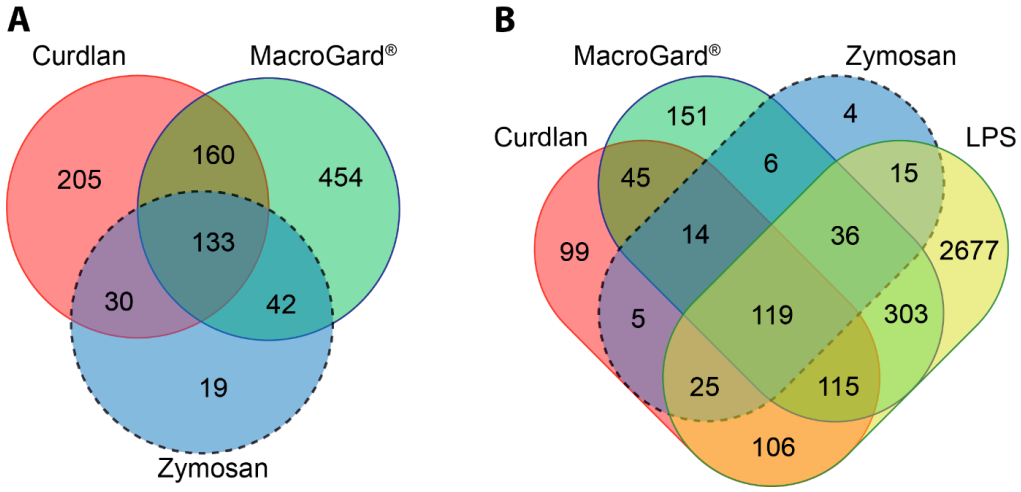


Figure 3. Venn diagram comparing differentially expressed genes (DEGs) regulated upon stimulation of common carp macrophages with different PAMPs. **A.** Venn diagram of the DEGs regulated by stimulation of macrophages with three different β-glucan preparations. Macrophages were stimulated for 6 hours with 25 µg/mL of either curdlan, MacroGard® or Zymosan and collected for RNAseq analysis. **B.** Venn diagram comparing the DEGs regulated by the three different β-glucan preparations shown in A. with DEGs regulated by stimulation of macrophages with LPS (30 µg/mL) for 6h.

Comparison of the DEGs regulated by all three β-glucan preparations with an unpublished DEG dataset of macrophages stimulated with LPS (Figure 3B), identified 14 unique β-glucan-regulated genes not present in the LPS dataset (Table 2). Of special interest is *rin1a*; orthologous to mammalian Rin1 (Rab and Ras interactor protein). In mammals, Rin1 can directly interact with Ras and a membrane trafficking GTPase Rab5, regulating receptor-dependent endocytosis (436). Furthermore, Rin1 and Raf-1 actually compete for binding of Ras, and possibly the signal transduction of Ras is determined by the competition between Rin1 and Raf-1 (437). Although the conservation of the interactions between Rin1a, Raf-1 and Ras has not been confirmed in fish, the up-regulation of *rin1a* again hints at a role for the syk-independent pathway (Figure 2).

Table 1. Tentative role of genes uniquely regulated in carp macrophages stimulated with β-glucans. List of DEGs all regulated in carp macrophages by three different β-glucan preparations but not in macrophages stimulated with LPS for 6h, identifying 14 unique β-glucan-regulated genes not present in the LPS dataset. Order of genes based on cypCar code (not shown).

Gene code	Description	Putative relevance
sh2d3cb	SH2 domain containing 3Cb (Chat)	Involved in monocyte adherence and regulation of lymphocyte adherence (438, 439).
ripk2	Receptor-interacting serine/threonine-protein kinase 2	Regulator of inflammatory responses and involved in multiple different pathways in innate and adaptive immune response, among others TLR, NLR and IL1R signalling (440).
fthl31	ferritin, heavy polypeptide-like 31	Macrophages are a major source of serum ferritin in mammals and increased serum ferritin levels can be associated to inflammation (441).
il10ra	interleukin 10 receptor, alpha	Involved in the regulation of inflammation (442). Upregulated upon murine lungs exposed to curdlan (443).
ctsla	Cathepsin La	Differentially expressed in antigen presenting cells and involved in regulating MHC class II presentation (444, 445).
Bcl2lp14	Apoptosis facilitator Bcl-2-like protein 14	Regulator of apoptosis facilitation (446).
cdkn1cb	Cyclin-dependent kinase inhibitor 1Cb	Negative regulator of proliferation and highly downregulated in M2 macrophages (447).
f3b	Coagulation factor IIIb	F3 enables the initiation of blood coagulation cascades, furthermore, F3 can induce M2-like profile in macrophages (448).
zgc92242	Uncharacterized protein	This gene is identified based on cDNAs isolated as part of the Zebrafish Gene Collection (ZGC) but not further characterized.
anxa2a	Annexin A2a	Annexin A2 tetramers can act as recognition protein for bacterial and viral pathogens and induce TNFα, IL1β and IL6 (449).
rsad2	radical S-adenosyl methionine domain containing 2 (viperin)	Important interferon stimulated gene with antiviral properties [reviewed by: (450)] and potentially involved in macrophage polarization (451).
siglec12	sialic acid-binding Ig-like lectin 12	CD33 related siglec, but its natural ligand remains to be determined (452).
tpcn2	Two pore segment channel 2	Sodium ion channel on intracellular endosomes and lysosomes (453).

Binding proteins, an underappreciated group of receptors

Several pattern recognition proteins (PRPs) have been described in invertebrate that are capable of recognizing pathogen epitopes and are able to bind β-glucans, such as β1,3-Glucan recognition protein (βGRP)/Gram-negative bacteria-binding protein 3 (GNBP3) and Lipopolysaccharide- and β-1,3-Glucan-binding Protein (LGBP) [reviewed by: (21, 89, 454)]. However, a motif search in the annotated carp genome [BioProject: PRJNA73579, (255)], using CLC Main workbench[5] and a conserved βGRM[6] motif based on a comparison of the putative β-glucan recognition motif of six different invertebrate LGBPs, provided no significant hits. Yet, considering the evolutionary position of teleost fish as early vertebrates and the apparent absence in fish genomes of genes orthologous to mammalian *Dectin-1*, it is plausible that fish can (also) express receptors similar to those described in invertebrates.

The family of PGRPs is present in genomes from insects to mammals with diverse functions in antimicrobial defence (455). In the genome of *Drosophila melanogaster*, where PGRPs are extensively studied, 13 different *pglyrp* encoding genes are present. These can be spliced into 19 different proteins, classified into secreted PGRP-short (S) or PGRP-long (L) that are either secreted, transmembrane, or intracellular (456). A study in beetle, *Holotrichia diomphalia*, reported binding of β-(1,3)-glucans by two secreted PGRPs with high sequence similarity to human PGLYRP-1, and activation of subsequent innate immune responses (457). In mammals, PGRPs were first classified as short, long and intermediate in analogy to insect PGRPs, but have been renamed into PGLYRP-1, -2, -3 and -4. In fish, currently, three genes have been identified, *pglyrp-2, -5 and* -6, with *pglyrp-2 and -6* showing the closest phylogenetic relation to PGLYRP-2 and *pglyrp-5* showing the closest relation to PGLYRP-1 (458). One study in grass carp studied the binding of Pglyrp proteins to β-glucans (459). In this study, four splice variants of grass carp Pglyrp-6 were shown to interact with β-glucans and with *S. cerevisiae* yeast cells, potentially suggesting a role for Pglyrp proteins in recognition of or binding to β-glucans. More recently, a study reported the identification of common carp *pglyrp-5* and *-6* (460), but no functional characterization was performed of the identified genes. We could identify four different carp *pglyrp* genes; *pglyrp-2* and *-6,* and two paralogs of *pglyrp-5* in the most recent common carp genome assembly. Based on RNAseq analysis[7], we found that none of these genes were constitutively expressed in carp macrophages. However, in two out of three RNAseq data sets from macrophages

[5] https://www.qiagenbioinformatics.com
[6] Conserved sequence used as search query: FHxWRxDWTxxxMxxYVDx
[7] The RNAseq dataset published in Chapter 6 was used for this analysis.

stimulated with MacroGard®, both *pglyrp-5* paralogs were up-regulated. This prompted us to perform further experimental work.

Stimulation of carp macrophages with zymosan, curdlan or MacroGard® for 6h resulted in increased gene expression of both paralogs of *pglyrp-5* (Figure 3A). Stimulating carp macrophages with zymosan pre-treated with pglyrp5-1 and -2 induced increased ROS production (Figure 3B). These preliminary experiments show that β-glucans not only regulate *pglyrp5* expression but also that pre-treatment of β-glucans with pglyrp5 can alter the response of macrophages to β-glucans. Although further research would be required, these preliminary data hint at a possibly conserved role of pglyrp5 proteins in the response to β-glucans in fish.

Are β-glucans unique?

This thesis focussed on the use of β-glucans to induce immuno-modulation. A possible alternative to the use of β-glucans can be the use of marine sulphated polysaccharides (MSPs). MSPs are biologically active components of marine algae (seaweeds), which are among the fastest growing plant organisms in nature. These

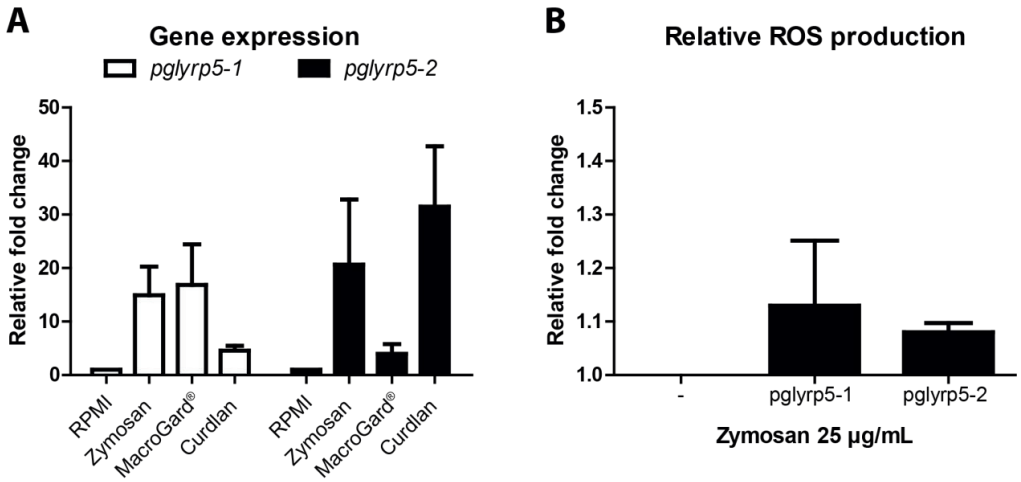


Figure 4. Preliminary results on a relation between stimulation with β-glucans and Pglyrp5 activity in carp macrophages. Bars indicate mean + SEM of n = 3 experiments performed independently. **A.** Expression of both paralogs of *pglyrp5* was analysed with qPCR using paralog-specific primers. RNA was isolated from carp macrophages after 6h stimulation with the indicated β-glucans (25 µg/mL). Fold change is calculated relative to macrophages stimulated with culture medium (RPMI). **B.** ROS production after stimulation of carp macrophages with Pgrp5-pre-treated zymosan (25 µg/mL). Zymosan was pre-treated for 1h with transfection supernatant from VERO cells transfected with pcDNA3-empty (-), pcDNA3-pglyrp5-1 or pcDNA3-pglyrp5-2. Following incubation for 1h, zymosan was centrifuged, washed with fresh culture medium and used to stimulate macrophages. Fold change ROS production is expressed as area under the curve of ROS production for 2h as measured with a luminol assay. Fold change is calculated relative to macrophages stimulated with zymosan pre-treated with supernatant from pcDNA3-empty-transfected VERO cells.

macroalgae are widely investigated for their sugar and carbohydrate content (461) that can be fermented to produce biogas or ethanol-based bio-fuels, much alike the process of sugar cane fermentation used in Brazil (see introduction to this thesis). However, the presence of MSPs can severely inhibit the fermentation process, reducing the efficiency of ethanol or biogas production, providing a good reason to extract MSPs. Of interest here, a broad range of biological properties have been ascribed to MSPs, including anticoagulant, antiviral and broader immuno-modulatory activities.

MSPs are common to the cell wall of all marine algae, but their concentrations differ among the three major groups of marine algae, *Rhodophyceae* (red algae), *Chlorophyceae* (green algae), and *Phaeophyceae* (brown algae). Well-known MSPs from green algae are ulvans, highly sulphated water-soluble polysaccharides with a backbone composed of sulphate, rhamnose, xylose, iduronic and glucuronic acids. Ulvans have been thoroughly investigated for their immuno-modulatory effects in mammals and other warm-blooded vertebrates (462, 463), but their potential for the aquaculture sector has yet to be appreciated, illustrated by only few published studies in fish. In turbot (*Scophthalmus maximus*), ulvan stimulated the respiratory burst of leukocytes *in vitro* (464) and increased *il1β* expression (465), a pro-inflammatory activity that could be abolished by de-sulphation. In Nile tilapia (*Oreochromis niloticus*), dietary supplementation of ulvan could increase the white blood cell count and phagocytic activity of isolated leukocytes (466). It may be obvious that in fish the exact immuno-modulatory mechanisms induced by MSPs such as ulvan, are still largely unknown.

Similar to what was discussed in this thesis for β-glucans, effector and or recognition mechanisms important for immuno-modulation by ulvan might be (partially) conserved between warm-blooded vertebrates and fish. In chicken, a recent *in vitro* study showed that ulvan can activate monocytes after recognition by TLR2 and TLR4, leading to an increase in NO production and transcription of several cytokines, among which *il1β*, *ifna*, *ifnγ*, *il8* and *inos* (467). Furthermore, *in vivo* dietary supplementation of ulvans was immuno-modulatory. At present, conserved roles may be suspected but remain to be shown for most Tlrs in fish, and recognition of specific ligands has not been unambiguously proven for Tlr2 nor for Tlr4 [reviewed by: (134)]. Nevertheless, there is no reason to believe that MSPs should not have an immuno-modulatory effect on fish cells. Further studies into the exact immuno-modulatory effects of MSPs in fish should focus on innate immune cell types such as macrophages, their receptors and their functional responses.

Are my data relevant to aquaculture practice?

At the beginning of this discussion chapter, I promised to not only look forward by discussing the inter-relations and implications of my findings but also discuss the relevance of my thesis by focussing on the following question: *"Will the description*

of mechanisms underlying immuno-modulatory properties of β-glucan have an impact on current practise in aquaculture?"

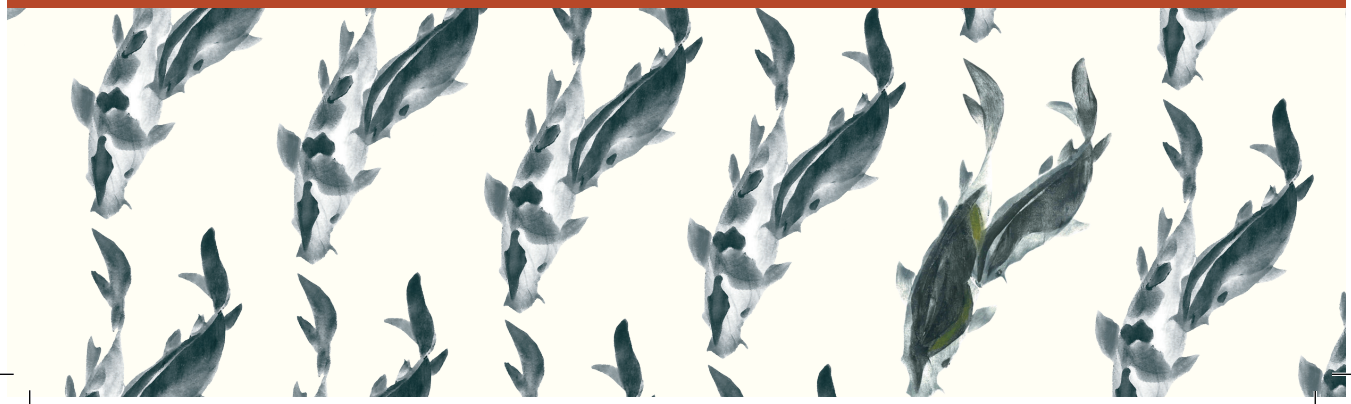
Throughout this chapter I already addressed this question in different sections and discussed: i) trained immunity as a form of innate immune memory that provides an increased nonspecific response to subsequent infections and improved survival of the host, the latter of obvious interest to aquaculture practise. I mentioned this could come at a metabolic cost that could have an impact on growth performance; the optimal energetic balance between metabolic cost for prevention of infections and fighting pathogens remaining unknown for now. Further I discussed that in field studies on trained immunity "contaminations" with cell types other than those of the innate immune system may be rightfully considered of academic interest only. ii) Prebiotics and their assumed beneficial effects on the immune system, possibly mediated by SCFAs produced by the intestinal microbiota, leading to nutritional concepts such as "feeding the microbiota", despite the current knowledge gap. iii) The use of other prebiotic polysaccharides such as MSPs and the striking parallels between ulvans from seaweed and β-glucans from yeast when it comes to bio-ethanol production and use of "waste" materials as feed additives for animals, including fish.

At present, products such as β-glucans and ulvans are mostly restricted to fish feed for species of high economic value in intensive aquaculture systems such as Atlantic salmon, mostly for economic reasons. Yet, for these species prevention of disease by vaccination is also common, and an alternative is not always available and/or economically viable for fish species kept in less intensive (pond) systems, such as tilapia. This would argue in favour of a new focus and associated marketing of "healthy feeds" at lower costs for the farmers interested in less intensive aquaculture systems. This might require use of immune-modulatory products of reduced purity, at lower production costs, the exact working mechanisms of which should be verified against the present "pure" products.

Over the last few years, access to fish genomes and thus the possibilities for NGS-based approaches to analyse fish immune responses have increased considerably. With the emergence of affordable bench-top equipment such as based on nanopore technology, large-scale RNA sequencing might rapidly replace specific detection by PCR and might even come to the farm. Yet, gene duplications common to fish genomes continue to complicate the correct interpretation of these large data sets and will require continued involvement of both, bioinformaticians and biologists. This is where my review of the current understanding of NGS approaches to the immune system of fish (389) may be relevant to practise.

Last but not least, looking ahead, improved understanding of the effects of dietary supplementation with immuno-modulatory compounds such as β-glucans or other could help achieve concepts such as "personalized feeding" based on the concept of "personalised nutrition". The ultimate goal of personalised nutrition is to enable individual guidance by predictive knowledge of personal health to diets that prevent disease and maximize health [reviewed by: (468)]. Improving fish health

by personalised diets would reduce the need to employ other measures to improve health, such as antibiotics or expensive vaccination strategies. Yet, this goal is not easily applied to individual fish in aquaculture systems but could be applicable to individual species in aquaculture. This would imply optimisation of fish feed to requirements of species with different metabolic, physiological and/or genetic status, and more. In this thesis, I have used multiple approaches to provide a scientific base to achieve such goal on the long term, inspired by new accurate and predictive measures of health, based on molecular signatures of metabolites, proteins, transcripts, genes, and microbiota.



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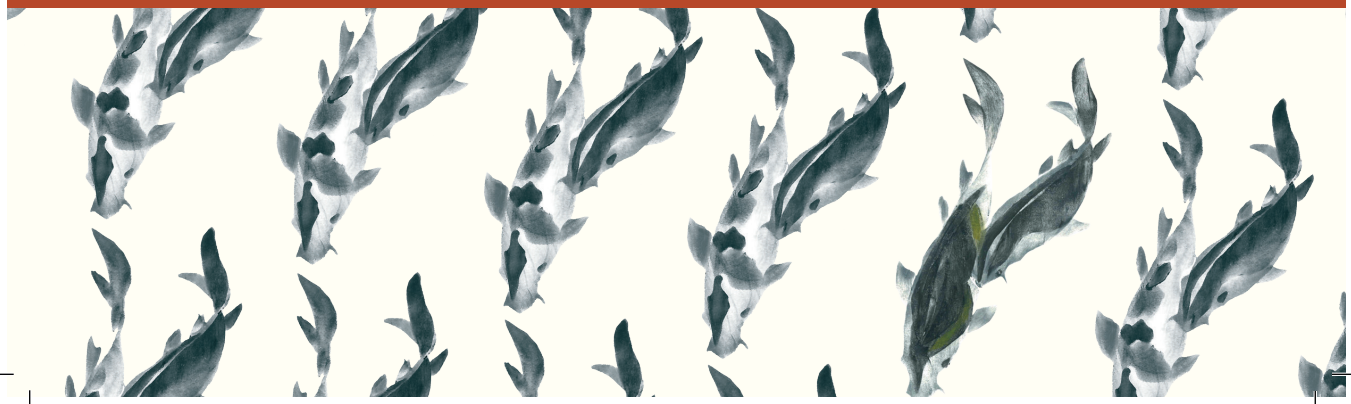

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Summary (English)

Samenvatting (Nederlands)

Summary

The past decades the aquaculture sector has grown tremendously to meet the growing demand for animal protein sources. The intensification of the aquaculture industry accommodated for the growth of the sector and the growing demand for fish, however increased stress, lack of adaptation to local infectious agents, increased host contact rates due to high stocking density and intensive monoculture have resulted in a clear increase in disease incidence. Overall, the immuno-modulation induced by β-glucan supplementation in fish have been well studied and are regarded as positive and beneficial for fish health. Dietary β-glucan supplementation is widely applied in aquaculture but no definitive mechanism of action has been described to date in fish. Improved understanding of the underlying mechanisms explaining the immuno-modulatory effects of β-glucans would allow for optimization of feeding and supplementation strategies.

In light of this, the main aim of this thesis was to characterize mechanisms of modulation of innate immune responses of carp by β-glucans. Using present mammalian literature as a starting point, we investigated three potential mechanisms that could be at play in the immuno-modulatory effects of β-glucan supplementation in fish, 1) long-lived effects on the myeloid cell compartment due to the induction of trained immunity by β-glucans; 2) degradation and fermentation of β-glucans by the intestinal microbiota, resulting in immuno-modulation via metabolites produced by the intestinal microbiota; 3) direct receptor recognition and downstream signalling, inducing innate immune modulation.

In **chapter 1**, I start with delineating the rationale behind the research project and scientific collaboration between and The Netherlands Organisation for Scientific Research (NWO). Subsequently, I provide the theoretical framework for this thesis by first introducing the increasing pressure on the aquaculture industry. This increasing pressure results in intensification of the sector and increased disease incidence. Subsequently, I discuss the use of β-glucans to modulate the immune system of farmed fish. Finally I treat different mechanisms potentially explaining the effects observed in fish after supplementation with β-glucans, differentiating between direct and indirect effects.

First in **chapter 2** we review the current literature on β-glucan research in fish. We sought to update previous reviews in the field, and grouped recent literature by (super)order, differentiating salmonids, perciforms and cyprinids based on the assumption that the closer the phylogenetic relationship the more reliable the conclusions. Subsequently, we review evidence in fish studies for proposed effector mechanisms involved in the immune-stimulatory effects induced by β-glucans, including intestinal microbiota, receptor recognition and downstream signalling, and a relatively new immunological concept of trained immunity. Subsequently, we revisited the screened literature for overlooked indications of long-lived effects of β-glucans in fish. We uncovered several studies with indications for long-lived

effects in fish, however due to suboptimal experimental set-ups it was not yet possible to ascribe these observations to trained immunity.

As we observed indications for long-lived effects of β-glucans in fish in the current literature, but were made under suboptimal experimental conditions to ascribe the long-lived effects to trained immunity, we established in **chapter 3** an *in vitro* model to investigate the conservation of trained immunity in fish. In mammals, the two main induction routes for trained immunity are via Dectin-1 or via NOD2. To date a definitive receptor and signalling pathway for β-glucans remains elusive in fish, however evidence for the conservation of NOD receptors and their downstream signalling cascade in fish is available, therefore experimental model was established with a NOD-ligand. Unstimulated trained macrophages displayed evidence of metabolic reprogramming, as well as heightened phagocytosis and increased expression of the inflammatory cytokines *il6* and *tnfa.* Stimulated, trained macrophages showed heightened production of reactive oxygen and nitrogen species as compared to the corresponding stimulated but untrained cells. After establishment of the *in vitro* model, we tested laminarin, a soluble β-glucan, for its capacity to induce trained immunity in carp macrophages and observed a clear increase in ROS production in stimulated, laminarin trained macrophages compared to stimulated untrained macrophages.

After we observed conservation of trained immunity in fish and could induce trained immunity-like profiles in macrophages with β-glucans, we set out to investigate other mechanisms at play in the immuno-modulation by β-glucans. In **chapter 4** we investigate whether or not the intestinal microbiota could be of importance in the effects of dietary β-glucan supplementation. First we characterised the active microbiota of naive common carp by 16S rRNA sequencing. We hypothesized based on the abundance of several genera, that the intestinal microbiota should theoretically be able to ferment β-glucans. Subsequently, an *in vitro* batch culture experiment was performed, confirming not only the capacity of carp intestinal microbiota to ferment β-glucans but also revealed a specific propionate production for one of the analysed β-glucans. Subsequently, a single oral gavage with this β-glucan was performed to analyse *in vivo* effects on the intestinal microbiota and the local gene expression. At day 7 post treatment, the β-glucan treated grouped showed a shift in active microbiota and regulation of the pro-inflammatory genes, *il1β*, *il6* and *tnfa* in the intestine. Taking the present literature on immune-modulatory effects of metabolites produced by the intestinal microbiota upon degradation and fermentation of dietary fibres into account, we conclude by hypothesizing that the regulation observed in the intestine after β-glucan supplementation can be a consequence of the microbiota fermenting β-glucans.

Finally, to investigate the last mechanisms proposed at the beginning of this project, we investigated possibilities of employing next generation sequencing (NGS) techniques to address this mechanisms. In **chapter 5** we reviewed the current genome assemblies in teleost species and summarized current NGS-based

studies investigating fish immune responses. We discuss the complexities and potential pitfalls of using NGS approaches with polyploid species, like common carp. Finally, we provided an outlook for coming technological advantages, which could be employed in addressing more complex issues such as genome assembly in a tetraploid species. After assessing the state of art of NGS-based studies in fish immunology, in **chapter 6** we used a NGS based transcriptome analysis of macrophages stimulated with β-glucans. Using a primary culture of common carp macrophages ascertained a reduction in background due to other leukocytes. Based on differential gene expression, we could discern regulation of several signalling pathways. Furthermore, manual pathway analysis revealed the regulation of the C-type lectin receptor signalling pathway. As the regulation of this pathway suggests at least partial conservation of a β-glucan recognition cascade via a C-type lectin receptor, we investigated the current common carp genome assembly for candidate receptors. To this end, we investigated the CLR family members identified in the latest carp genome assembly and based on expression and structural architecture, we proposed several candidate receptors.

Finally, I integrate the results of the previous chapters in a larger framework and discuss the implications of these findings for future research and applications of β-glucans in aquaculture in **chapter 7**. I start with reflecting on the observed induction of trained immunity in carp and discuss the parallels and differences between our observations and the studies on trained immunity in mammals. Furthermore, I discuss both the potential and the possible dark-side of trained immunity for the aquaculture sector. Finally I propose two future studies which might provide useful insights from a fundamental and an applied point of view. I continue by discussing our observations on the effects of β-glucans on carp intestinal microbiota. Besides placing our observations in a broader context, I discuss the potential implications of the presence of fermentation products and β-glucans in light of trained immunity. Subsequently, I take our observations on the regulation of the C-type lectin receptor signalling pathway a step further by comparing the dataset to new datasets. By comparison to macrophages stimulated with a different β-glucan, zymosan, or with LPS, as a common pro-inflammatory agent, we can identify a specific β-glucan-regulated set of genes. I continue with the investigation of a β-glucan recognition mechanism, peptidoglycan recognition proteins (PGRPs) and the conservation of its role from invertebrates to fish. We observe indications for a role for the PGRPs in the response to β-glucans. Finally, I recognize that there are other fish in the sea of immuno-modulatory substances and discuss the use of marine sulphated polysaccharides as an alternative to β-glucans. Although during the discussion I already reflected on the relevance of our observations to the aquaculture practice, I consciously reflect on relevance of my data by addressing both its short- and long-term implications on the aquaculture sector.

The diverse angles of approach taken in the studies in this thesis have allowed a better understanding of the underlying mechanisms possibly explaining effects of β-glucan supplementation in common carp. This improved understanding of β-

glucan induced immuno-modulation will provide a strong base for future studies. In the long-term, the findings in this thesis might aid in achieving concepts such as “personalized feeding”, similar to “personalized nutrition”. Fish health could be improved through these optimized feeding strategies and reduce the need to employ other measures to improve health, such as antibiotics or vaccination strategies.

Samenvatting

Om aan de toenemende vraag naar dierlijke eiwitbronnen te kunnen voldoen is de aquacultuur sector de laatste decennia gigantisch gegroeid. De aquacultuur sector heeft deze groei kunnen bewerkstelligen dankzij sterke intensivering. Deze intensivering heeft echter een keerzijde, namelijk een duidelijke toename aan infecties. Deze toename is onder andere het gevolg van zowel een toename aan stress en vis tot vis contact, inherent aan een hoge visdichtheid, alsmede een gebrek aan aanpassing aan de lokale ziekteverwekkers. De immuun-modulatie als gevolg van β-glucaan suppletie van visvoeders, is over het algemeen goed bestudeerd en wordt beschouwd als positief voor de gezondheid van de vissen. Ondanks de brede toepassing van β-glucaan suppletie in de aquacultuur sector zijn de definitieve mechanismen nog niet beschreven. Betere kennis van de mechanismen die aan de immuun-modulatie door β-glucaan suppletie ten grondslag liggen, zou verdere optimalisatie en verbetering van de voer- en suppletiestrategieën mogelijk kunnen maken.

Met dat in het achterhoofd was het hoofddoel van dit proefschrift het karakteriseren van de onderliggende mechanismen van de immuun-modulatie door β-glucanen in karpers. Op basis van de huidige literatuur op het gebied van immuun-modulatie door β-glucanen in zoogdieren, zijn er drie potentiele mechanismen onderzocht die een rol kunnen spelen bij de immuun-modulatie veroorzaakt door β-glucanen in vissen. De onderzochte mechanismen zijn de volgende: 1) langdurige effecten op het myeloïde cel compartiment door de inductie van 'trained immunity' door β-glucanen; 2) de afbraak en fermentatie van de β-glucanen door de microbiota in de darm, waarbij metabolieten geproduceerd door de microbiota indirect leiden tot immuun-modulatie; 3) directe activatie van een receptor door β-glucanen die via signaalcascades uiteindelijk het aangeboren immuun systeem moduleert.

In **hoofdstuk 1**, begin ik met het uiteenzetten van de rationaal achter het onderzoeksproject en achter de wetenschappelijke samenwerking tussen de São Paulo Research Foundation (FAPESP) en de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO). Vervolgens zet ik het theoretische kaderwerk voor deze thesis uiteen door de toenemende druk op de aquacultuur industrie te introduceren. Deze toenemende druk heeft ertoe geleid dat de sector sterk heeft moeten intensiveren, wat leidde tot een toename in ziekte incidentie. Vervolgens bediscussieer ik het gebruik van β-glucanen om het immuunsysteem van kweekvissen te moduleren. Tenslotte, bespreek ik verschillende mechanismen die mogelijk de geobserveerde effecten van β-glucanen in vissen zouden kunnen verklaren, hierbij differentieer ik tussen directe en indirecte effecten.

In **hoofdstuk 2**, behandelen we de huidige literatuur over onderzoek naar β-glucanen in vissen. We wilden eerdere reviews over dit onderwerp updaten om op die manier een concreet overzicht van de huidige literatuur op het gebied van immuun-modulatie door β-glucanen in vissen te krijgen. We hebben daartoe

recente literatuur gegroepeerd per (super)orde, waarbij we onderscheid maken tussen zalmachtigen, baarsachtigen en karperachtigen. Dit onderscheid maken we omdat we aannemen dat nauwere fylogenetische verwantschappen tot betrouwbaardere conclusies kunnen leiden. Vervolgens analyseren we de huidige literatuur voor indicaties dat de voorgestelde effector mechanismen een rol zouden kunnen spelen in de immuun-modulatie door β-glucanen in vissen. Deze eerder genoemde mechanismen zijn de effecten via de microbiota in de darm, directe receptor herkenning leidend tot activatie van de signaalcascade en het relatief nieuwe concept trained immunity. Vervolgens, zijn we opnieuw door de literatuur gegaan op zoek naar mogelijke langdurige effecten van β-glucanen in vissen die over het hoofd gezien zijn. We vinden een aantal studies waar dergelijke langdurige effecten in vissen beschreven worden, echter doordat de experimenten niet opgezet zijn om deze langdurige effecten te onderzoeken kunnen we deze observaties niet toeschrijven aan trained immunity.

Omdat we indicaties voor langdurige effecten van β-glucanen in vis vonden in de literatuur, maar we vanwege suboptimale opzet van experimenten deze observaties niet konden toeschrijven aan trained immunity, hebben we in **hoofdstuk 3** een *in vitro* model opgezet om de evolutionaire conservatie van trained immunity in vissen te onderzoeken. In zoogdieren zijn de twee best beschreven routes om trained immunity te induceren via Dectin-1 of via NOD2. Tot de dag van vandaag is er nog geen definitieve receptor-route of signaalcascade voor β-glucanen in vis beschreven, er is echter wel bewijs voor evolutionaire conservatie van de NOD receptoren en hun signaalcascade in vissen. Derhalve hebben we het experimentele model opgezet met een NOD-ligand. Ongestimuleerde getrainde karper macrofagen lieten naast kenmerken van metabole herprogrammering ook een toename in fagocytose activiteiten en een toename in expressie van de *il6* en *tnfa* genen zien. Gestimuleerde, getrainde macrofagen lieten verhoogde productie van zuurstof radicalen en stikstof radicalen zien, in vergelijking met gestimuleerde ongetrainde macrofagen. Na de opzet van het model hebben we getoetst of laminarin, een oplosbare β-glucaan, ook in staat was om trained immunity te induceren in karper macrofagen. Ook na training met laminarin zagen we een duidelijke toename in de productie van zuurstof radicalen in gestimuleerde getrainde macrofagen ten opzichte van gestimuleerde ongetrainde macrofagen.

Nadat we de evolutionaire conservatie van trained immunity in vissen hadden geobserveerd en we profielen gelijkend op trained immunity konden induceren in karper macrofagen met behulp van β-glucanen, zijn we andere potentiele onderliggende mechanismen van de β-glucaan geïnduceerde immuun-modulatie gaan onderzoeken. In **hoofdstuk 4** hebben we onderzocht of de darm microbiota een rol speelde in de immuun-modulatie na suppletie van het dieet met β-glucanen. Daartoe hebben we eerst de actieve microbiota van naïeve karpers geanalyseerd met 16S rRNA sequencing. We hebben toen op basis van de aanwezigheid van verschillende genera, de hypothese gesteld dat de microbiota van karpers in staat zou moeten zijn om β-glucanen te fermenteren. Vervolgens

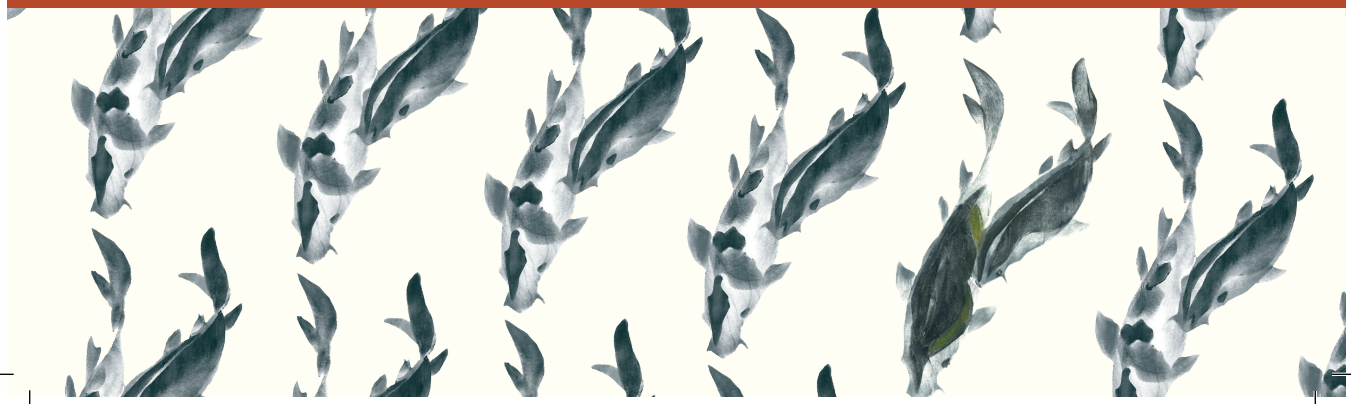
hebben we een *in vitro* fermentatie experiment uitgevoerd waarmee we aantoonden de darm microbiota van karpers in staat is om β-glucanen te fermenteren en observeerden we specifieke propionaat productie na fermentatie van een van de geanalyseerde β-glucanen. Daarna hebben we, om de *in vivo* effecten van β-glucanen op de darm microbiota en de lokale genexpressie te onderzoeken, karpers een enkele behandeling gegeven met orale β-glucaan spoeling. Op dag 7 na de behandeling, zagen we een verandering in de actieve microbiota en zagen we een duidelijke regulatie van pro-inflammatoire genen, *il1β*, *il6* en *tnfa* in de darm. In het licht van de huidige literatuur op het gebied van de immuun-modulatoire effecten van de metabolieten, geproduceerd door de microbiota na degradatie en fermentatie van voedingsvezels, formuleren we tenslotte de hypothese dat de geobserveerde regulatie in de darm na een β-glucaan supplement, het gevolg kan zijn van de fermentatie van die β-glucanen door de microbiota.

Om het laatste mechanisme te onderzoeken wat aan het begin van het project werd geopperd, hebben we eerst de mogelijkheid om gebruik te maken van next generation sequencing (NGS) in relatie tot dit mechanisme onderzocht. In **hoofdstuk 5** hebben we de huidige genomen van beenvissen bekeken en hebben we op NGS gebaseerde studies naar vissen immunologie samengevat. Daarnaast hebben we de complexiteiten en mogelijke valkuilen van het gebruik van NGS in polyploïde soorten, zoals de karper, bediscussieerd. Tot slot, bieden we een vooruitblik naar toekomstige technologische ontwikkelingen die een uitkomst kunnen bieden bij genoom analyses van complexe genomen. Vervolgens hebben we in **hoofdstuk 6** zelf, met behulp van NGS, een transcriptoom analyse uitgevoerd van macrofagen gestimuleerd met β-glucanen. Om achtergrond ruis vanuit andere cel typen te minimaliseren hebben we een primaire macrofagen celkweek gebruikt als uitgangspunt. Op basis van differentiële genexpressie profielen, konden we met geautomatiseerde analyses, de regulatie van verschillende signaalcascades identificeren. Daarnaast hebben we met handmatige analyses vast kunnen stellen dat de C-type lectin receptor signaalcascade gereguleerd werd. De regulatie van deze cascade suggereert dat een deel van de respons op β-glucanen evolutionair geconserveerd is tussen zoogdieren en karpers. Daartoe hebben we de C-type lectin receptoren in het karper genoom geanalyseerd. Op basis van expressie van de genen en de structurele architectuur van hun bijbehorende eiwitten, konden we tot slot een aantal kandidaat receptoren voorstellen.

Tot slot, integreer ik alle voorgaande hoofdstukken in een groter kader en bediscussieer ik de implicaties van mijn bevindingen voor toekomstig onderzoek en voor de toepassing van β-glucanen in de aquacultuur in **hoofdstuk 7**. Ik begin met een reflectie op de geobserveerde inductie van trained immunity in karper en bediscussieer overeenkomsten en verschillen tussen onze observaties en de studies in zoogdieren naar trained immunity. Daarnaast bediscussieer ik de potentie maar ook de mogelijke keerzijde van trained immunity voor de aquacultuur sector. Tenslotte stel ik twee mogelijke studies voor die bruikbare

inzichten kunnen bieden voor zowel de fundamentele en de toegepaste kant van het verhaal. Ik vervolg met een discussie over onze geobserveerde effecten van β-glucanen op de karper darm microbiota. Naast dat ik onze observaties in een bredere context plaats, bediscussieer ik mogelijke implicaties van de aanwezigheid van fermentatieproducten en β-glucanen in de darm, in de context van trained immunity. Vervolgens neem ik onze observaties van de gereguleerde C-type lectin receptor signaalcascade een stap verder door onze dataset met nieuwe datasets te vergelijken. Door onze gepubliceerde datasets te vergelijken met transcriptoom datasets van macrofagen gestimuleerd met een andere β-glucaan, zymosan, of met LPS, als een algemeen inflammatie inducerend ligand, ben ik in staat om een specifieke β-glucaan gereguleerde genen set te identificeren. Ik vervolg de discussie met het analyseren van een, tot dan toe, nog niet genoemd herkenningsmechanisme van β-glucanen, de peptidoglycaan herkennings eiwitten (PGRPs), en de mogelijke evolutionaire conservatie van die rol van invertebraten naar vissen. Tenslotte, bediscussieer ik dat er meer vissen in de zee van immuun-modulatoire stoffen zwemmen en bediscussieer ik het gebruik van zogenaamde mariene sulfaat polysachariden als een alternatief op β-glucanen. Ondanks dat ik gedurende de discussie al reflecteer op de relevantie van onze bevindingen voor de aquacultuur sector, sluit ik af door bewust de korte termijn en mogelijke lange termijn implicaties van mijn observaties voor de aquacultuur sector te behandelen.

De diverse invalshoeken die we hebben genomen voor de studies in dit proefschrift hebben geleid tot een beter begrip van de onderliggende mechanismen die mogelijk de immuun-modulatoire effecten van β-glucanen op karpers verklaren. Deze verbeterde kennis van de β-glucaan geïnduceerde immuun-modulatie kan als een sterke basis dienen voor toekomstige studies. Op de langere termijn, kunnen de bevindingen in dit proefschrift mogelijk bijdragen aan het bereiken van concepten als "gepersonaliseerd voeren", vergelijkbaar met "gepersonaliseerde voeding". De gezondheid van vissen zou verbeterd kunnen worden door de optimalisatie van voerstrategieën, wat vervolgens de noodzaak van inzet om andere maatregelen om de gezondheid te verbeteren, zoals antibiotica en vaccinatie, kan verminderen.



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About the author

List of Publications

Overview of completed training activities

Acknowledgements

About the author

Jules Petit was born on the 18th of November, 1989 in Nijmegen. He enrolled in a three-year program for gifted children during the first three years of his high school (Mondriaan College, Oss). After three years, the program ended and Jules enrolled in regular Athenaeum education. From his early days Jules was fascinated by oceans, seas, water and their inhabitants. In 2007, Jules started his BSc Biology at the Wageningen University, with the aim to become a marine ecologist. During his BSc period Jules spent a full year, performing extracurricular activities, as a board member of a student association. During this he was, together with seven other board members, responsible for the daily organization of an association with 600 active members.

After Jules finished his BSc, he came to the conclusion that another great interest was in immunology, and therefore continued his MSc studies in Biology with the specialization "Health and Disease". During his major MSc thesis he was able to combine these two passions in the form of independent research on the characterization of carp interleukin-12 and its possible use as an adjuvant during vaccination. He performed this research under the supervision of Dr Maria Forlenza at the Cell Biology and Immunology group of the Wageningen University. He continued his studies during an internship abroad at the Virologie et Immunologie Moléculaires department of the Institut national de la recherche agronomique (INRA) at Jouy-en-Josas, France. Under the supervision of Christelle Langevin and Pierre Boudinot he investigated finTRIMs and their potential role in antiviral responses. Jules obtained his BSc degree in 2012 and his MSc degree in 2014.

In January 2015, Jules started his PhD project at the Cell Biology and Immunology group of the Wageningen University under the supervision of Geert Wiegertjes and Maria Forlenza. His aim was to explain the effects of β-glucans on carp immune responses. The most important findings of his work are presented in this thesis. During this project there was a strong collaboration with the Brazilian partners reflected by several visits to Brazil and an exchange between labs of four weeks.

While finishing his PhD thesis, Jules already started as a Post-doctoral researcher at the Aquaculture and Fisheries group under the supervision of Geert Wiegertjes and Johan Schrama. In his current position he is investigating the effects of marine sulphated polysaccharides on the immune system of several important aquaculture fish species.

List of publications

Petit, J.; de Bruijn, I.; Brugman, S.; Pellikaan, W.F.; Forlenza, M.; Wiegertjes, G.F. β-glucan immuno-modulation in common carp intestine: a role for microbiota and its metabolites. *Manuscript in preparation*

Petit, J.; Embregts, C.W.E.; Forlenza, M.; Wiegertjes, G.F. Evidence of trained immunity in teleost fish: conserved features in carp macrophages. The Journal of Immunology, *Accepted for publication* (**2019**)
doi: 10.4049/jimmunol.1900137

de Jesus, R.B.; **Petit, J.;** Pilarski, F.; Wiegertjes, G.F.; Koch, J.F.A.; de Oliveira, C.A.F.; Zanuzzo, F.S. An early β-glucan bath during embryo development increases larval size of Nile tilapia. Aquaculture Research (**2019**)
doi: 10.1111/are.14047

Petit, J.; Bailey, E.C.; Wheeler, R.T.; de Oliveira, C.A.F.; Forlenza, M.; Wiegertjes, G.F. Studies Into β-Glucan Recognition in Fish Suggests a Key Role for the C-Type Lectin Pathway. Frontiers in immunology 10, article 280 (**2019**)
doi: 10.3389/fimmu.2019.00280

Embregts, C.W.E.; Rigaudeau, D.; Veselý, T.; Pokorová, D.; Lorenzen, N.; **Petit, J.;** Houel, A.; Dauber, M.; Schütze, H.; Boudinot, P.; Wiegertjes, G.F.; Forlenza, M. intramuscular Dna Vaccination of Juvenile carp against spring Viremia of carp Virus induces Full Protection and establishes a Virus-specific B and T cell response. Frontiers in immunology 8, article 1340 (**2017**)
doi: 10.3389/fimmu.2017.01340

Petit, J.; David, L.; Dirks, R.; Wiegertjes, G.F. Genomic and transcriptomic approaches to study immunology in cyprinids: What is next? Developmental & Comparative Immunology 75, 48-62 (**2017**)
doi: 10.1016/j.dci.2017.02.022

Petit, J.; Wiegertjes, G.F. Long-lived effects of administering β-glucans: indications for trained immunity in fish. Developmental & Comparative Immunology 64, 93-102 (**2016**)
doi: 10.1016/j.dci.2016.03.003

Overview of completed training activities

The Basic Package	**2 ECTS**
WIAS Introduction Day	2015
Research Integrity & Ethics and Animal Science (RI-EPAS)	2017

Disciplinary Competences	**19 ECTS**
Writing a literature review: "Long-lived effects of administering β-glucans: Indications for trained immunity in fish	2015
Advanced course immunology UMC	2015
Participant 16th International Fish Immunology Workshop,	2015
Statistics and R for the Life Sciences (EDx, Harvard)	2015
Bioinformatic Methods I (Coursera, University of Toronto)	2015
Bioinformatic Methods II (Coursera, University of Toronto)	2016
Summer Frontiers 2016 – Systems Biology of Innate Immunity	2016
Epigenesis and Epigenetics (VLAG)	2017
Genomics and Bioinformatics Workshop (IMPRESS, Leiden)	2017
Advanced statistics of Life Science (WIAS)	2017

Professional Competences	**2 ECTS**
Competence Assessment (WIAS)	2015
Participant WPC symposium (WPC)	2015
Employability outside Academia (PCDI)	2018

Presentation Skills	**4 ECTS**
Wellcome Trust Innate Immune memory, Cambridge (poster)	2015
The 13th ISDCI conference, Murcia, Spain (poster)	2015
European Macrophage and Dendritic Cell Society (poster)	2016
Epigenesis and Epigenetics course, Wageningen (Oral)	2017
19th International Fish Immunology Workshop (Oral)	2018
WIAS Science Day 2018 - best oral presentation prize (oral)	2018
The 14th ISDCI conference, Santa Fe, USA (Oral)	2018
The 14th ISDCI conference, Santa Fe, USA (Poster)	2018

Teaching Competences	**6 ECTS**
MSc cours: "Human and Veterinary Immunology" (4x)	2015-2018
Practical supervision "International Fish Immunology Workshop" (3x)	2016-2018
Supervising major Master thesis (3x)	2015-2018
Supervising minor Master thesis	2017
Review of Research Master Cluster proposals	2017
Presentation on Supervisor-PhD - WIAS Introduction day (3x)	2017-2018

Education and Training total:	**32.5 ECTS**

Completion of the training activities is in fulfilment of the requirements for the education certificate of the Graduate School of the Wageningen Institute of Animal Sciences (WIAS). One ECTS equals a study load of 28 hours.

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The acknowledgements. Commonly, the last written chapter, but also the first and most well-read chapter. Four years of research are not accomplished on your own and I would like to thank everybody who feels that he or she has contributed in any form to my research or to my physical and mental health. There are some people I would like to thank in particular.

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Secondly, off course my other promotor **Maria**. Already during my Master thesis, I got infected with your enthusiasm for science. You were the one that planted a seed in my mind about doing a PhD and look where we are now! Thank you for all your help and advice on experiments. Thank you for every time I had a "short question", which resulted in us spending an hour discussing results, experiments or future opportunities.

Ik wil graag ook alle technicians en studenten bedanken die hebben meegeholpen aan mijn onderzoeksproject. **Trudi**, **Marleen**, **Ben** en **Erik**, dank jullie wel voor alle hulp en zorgen dat ik in zo'n geweldig lab heb kunnen werken als waar ik in heb kunnen werken. Naast het geweldige lab, ben ik ook dankbaar dat we zo'n goede proeffaciliteit hebben waar ik altijd terecht kon voor nieuwe donoren. **Menno**, **Wian**, **Sander**, **Truus** en **Emily**, bedankt voor jullie werk. **Cassandra**, **Mark**, **Vignesh** (volgens mij ben je goed genoeg in Nederlands om dit te snappen) en **Koyan**, jullie waren allemaal hartstikke goede studenten en ik wil jullie dan ook bedanken voor al jullie inzet en het verrichtte werk tijdens jullie MSc thesis.

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