2014

Effect of dietary -glucan supplementation on growth, intestinal functionality and disease progression in selected cyprinids (Cyprinus carpio and Danio rerio)

Kuhlwein, Holger

Effect of dietary β-glucan supplementation on growth, intestinal functionality and disease progression in selected cyprinids (*Cyprinus carpio* and *Danio rerio*)

by

Holger Kühlwein

A thesis submitted to Plymouth University in partial fulfilment for the degree of

Doctor of Philosophy

(June 2013)

Department of Biomedical and Biological Sciences

Faculty of Science and Technology

This work was supported by the European Community's Seventh Framework Programme (FP7/2007-2013) under Grant agreement number PITN-GA-2008-214505

Copyright statement

Effect of dietary β-glucan supplementation on growth, intestinal functionality and disease progression in selected cyprinids (*Cyprinus carpio* and *Danio rerio*)

Abstract

Three experiments were conducted to investigate the effects of dietary supplementation of a ß-(1,3)(1,6)-D-glucan derived from the yeast *Saccharomyces cerevisiae*, on growth performance parameters and intestinal functionality in mirror carp (*Cyprinus carpio* L.) and on progression of a chronic, pre-existing mycobacteriosis in zebrafish (*Danio rerio*). The 1st experiment revealed that mirror carp fed diets containing 1% and 2% ß-glucan showed significant improvements in growth performance compared to fish fed both the control and the 0.1% ß-glucan containing diet. Equally, fish fed diets supplemented with 1% and 2% ß-glucan displayed significantly higher infiltration of leukocytes into the epithelial layer of in the anterior intestine. This effect was not observed in the posterior intestine. There were no significant differences in the intestinal absorptive surface area and number of goblet cells in either intestinal region. Compared to control fed fish, the haematocrit value was significantly elevated in fish fed the 2% ß-glucan diet. The blood monocyte fraction was significantly higher in fish fed the 1% and 2% ß-glucan diets. No significant changes were observed in the other blood parameters assessed.

In the 2nd experiment, culture-dependent microbiology unveiled that aerobic heterotrophic bacterial levels in mirror carp intestinal microbiota were unaffected by dietary β-glucan after two and four weeks. No effects were observed on the allochthonous lactic acid bacteria (LAB) populations at either time point, however, reduced autochthonous LAB populations were observed at week four. PCR-DGGE confirmed these findings through a reduction of the abundance of autochthonous LAB operational taxonomical units (OTUs) in β-glucan fed fish compared to the control fed

fish. DGGE analyses also revealed that dietary β-glucan reduced the number of OTUs and the species richness of the allochthonous microbiota after two weeks, but not after four weeks. In contrast, dietary β-glucan reduced the number of OTUs, the species richness and diversity of the autochthonous microbiota after two weeks, and those parameters remained reduced after four weeks. Intestinal microvilli length and density were significantly increased after four weeks in fish fed diets supplemented with 1% β-glucan.

The 3rd experiment comprised of two individual trials where dead or moribund fish displayed the classical clinical signs of mycobacteriosis. In experiment I (200 days), zebrafish were fed diets supplemented with 0% (control, C) or 0.1% ß-glucan (B). In experiment II (63 days), zebrafish were fed diet C or diet B continuously or both diets intermittently (CB) in weekly intervals. Dietary ß-glucan failed to improve survival rates in both experiments. In experiment II, histopathological analysis revealed absent (normal appearance), moderate and severe manifestations of granulomas. Ecological microbial community analysis of intestinal samples collected at day 63 showed significantly higher number of OTUs, species richness and diversity in treatments B and CB compared to the control treatment.

In conclusion, high dietary ß-glucan supplementation levels enhanced growth performance, leukocyte infiltration in the anterior intestine and the ultrastructure of the enterocytes in mirror carp without detrimental effects on intestinal morphology or the haemato-immunological parameters assessed. In addition, intestinal microbial communities were altered in healthy mirror carp and diseased zebrafish. It was also demonstrated that dietary ß-glucan failed to impact the progression of a pre-existing mycobacteriosis in zebrafish. Future research is required to investigate the underlying reasons for the observed effects by applying advanced molecular techniques, and whether these effects occur under commercial husbandry, or in different fish species.

All experimental work involving animals was carried out in accordance with the 1986 Animals Scientific Procedures Act under the Home Office project licence number 30/2644 and personal licence number 30/9104.

List of contents

List of Tables

List of Figures

List of Plates

Plate 5.3: (A) Normal zebrafish liver (a), spleen (b) with no signs of inflammation. (B) Overview of a severe infection. (C) Granuloma in kidney with central, caseous necrosis (asterisk). (D) Liver with scattered granulomas (arrows). Areas of the liver are brighter in colour than those in (A). (E) Granulomas in spleen with melanomacrophages (black arrows) and a granuloma in muscle tissue (blue arrow). (F) Multicentric necrotizing granulomas (arrows) in liver. (G) Granuloma in spleen with central necrosis (blue arrow), granuloma with caseous necrosis (black arrow), and a non-necrotizing granuloma in early stages of development (red arrow) just above a blood vessel (asterisk). (H) Multicentric granuloma (dotted line) in spleen containing 3 granulomas (arrows) with high amount of acid fast bacilli stained red. (I) Granuloma with caseous necrosis and rod shaped acid-fast bacilli (arrowheads). Scale bars: B = 500 µm; A, C, D, E, F, G and H = 200 µm; I = 20 µm. Staining: H&E (A-G) and modified Ziehl-Neelsen (H&I).

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

This study was supported in form of a Marie Curie Initial Training Network (project "NEMO: Training network on protective immune modulation in warm water fish by feeding glucans") by the European Community's Seventh Framework Programme (FP7/2007-2013) under Grant agreement number PITN-GA-2008-214505.

Word count: 45,882

Date _____

Signed _____

Conferences attended

Apr 2010	Fish Immunology Workshop (Wageningen, NL)
Oct 2010	EAS Aquaculture Europe (Porto, PT)
May 2011	Workshop "Common carp as a model organism for biological studies: propagation, husbandry and health control" – Polish Academy of Sciences, Institute of Ichthyobiology and Aquaculture (Golysz, PL)
Sep 2011	EAFP International Conference on diseases of fish & shellfish (Split, HR)
Mar 2012	WAS Aquaculture America (Las Vegas, USA)
Sep 2012	Conference Prebiotics and probiotics in medicine, veterinary sciences and aquaculture: the future (Keele University, UK)

Work presented

Sep 2009	3rd NEMO meeting Copenhagen, DK; PP-presentation: "NEMO – Plymouth: Growth and gut physiology"
Apr 2010	Fish Immunology Workshop – Wageningen, NL; Poster presentation: "β-glucan effects on gut function and growth of carp (*Cyprinus carpio* L.): plans for the 1st experiment"
Sep 2010	4th NEMO meeting Keele, UK; PP-presentation: "β-glucan effects on growth and gut function – preliminary results"
Sep 2010	NEMO official mid-term meeting Keele, UK; PP-presentation: "Report mid-term meeting"
April 2011	CRTB – Centre for Research in Translational Biomedicine, University of Plymouth, UK; Poster presentation: "β-glucan effects on growth and haematological indices of carp (*Cyprinus carpio* L.)"
May 2011	6th NEMO meeting Krakow, PL: Poster presentation: "Effects of β-glucan on growth, haematological indices and gut health of carp (*C. carpio* L.) – current progress"
Sep 2011	Holger Kühlwein, Daniel L Merrifield, Mark D Rawling & Simon J Davies. Poster presentation: "β-glucan effects on growth, haematological indices and intestinal integrity of carp (*Cyprinus carpio* L.)" EAFP - 15th International Conference on Diseases of Fish and Shellfish (Split, HR)
Mar 2012	Holger Kühlwein, Christyn J Bailey, Catherine Mead, Matthew J Emery, Daniel L Merrifield, Simon J Davies, Theodore Henry. Poster presentation: "Effects of dietary β-glucan on Zebrafish *Danio rerio* mycobacteriosis" WAS – Aquaculture America Conference (Las Vegas, USA)

April 2012 8th NEMO meeting London, UK; PP-presentation:
"Disease and beta-glucans: mycobacteriosis in Zebrafish (*Danio rerio*)"

April 2012 NEMO – CEFAS meeting Weymouth, UK; PP-presentation:
"Effects of dietary beta-glucans on healthy and diseased cyprinids"

Sep 2012 Holger Kühlwein, Matthew J Emery, Daniel L Merrifield, Simon J Davies. Poster presentation:
"Effects of β-glucans on intestinal microbiota in mirror carp (*Cyprinus carpio* L.)" Conference Prebiotics and probiotics in medicine, veterinary sciences and aquaculture: the future (Keele University, UK)

Oct 2012 1st Aquatic Animal Nutrition and Health Research Group Meeting, Plymouth University, UK: PP-presentation:
"Effects of β-glucans on intestinal microbiota in mirror carp (*Cyprinus carpio* L.)"

Oct 2012 Seminar Series of the School of Biomedical and Biological Sciences, Plymouth University, UK: PP-presentation:
"Effects of β-glucans in mirror carp (*Cyprinus carpio* L.)"

Jan 2013 2nd Aquatic Animal Nutrition and Health Research Group Meeting, Plymouth University, UK: PP-presentation:
"Effects of dietary ß-glucan on zebrafish (*Danio rerio*) mycobacteriosis"

Publications

May 2011 Mark D Rawling & Holger Kühlwein (2011).
"Preliminary effects of β-glucans on Nile tilapia health and growth performance" *International Aquafeed*, 14 (3), pp. 20-23
(non-peer-reviewed)

Feb 2012 Samad S Omar, Daniel L Merrifield, Holger Kühlwein, Peter E V Williams, Simon J Davies (2012).
"Biofuel derived yeast protein concentrate (YPC) as a novel feed ingredient in carp diets" *Aquaculture*, 330-333, pp. 54-62

May 2013 Holger Kühlwein, Daniel L Merrifield, Mark D Rawling, Andrew D Foey, Simon J Davies.
"Effects of dietary β-(1,3)(1,6)-D-glucan supplementation on growth performance, intestinal morphology and haemato-immunological profile of mirror carp (*Cyprinus carpio* L.)" *Journal of Animal Physiology and Animal Nutrition*. doi: 10.1111/jpn.12078

In preparation:

Holger Kühlwein, Matthew J Emery, Mark D Rawling, Glenn M Harper Daniel L Merrifield, Simon J Davies.
"Effects of a dietary β-(1,3)(1,6)-D-glucan supplementation on intestinal microbial communities and intestinal ultrastructure of mirror carp (*Cyprinus carpio* L.)"
Submitted to: *Journal of Applied Microbiology*

Holger Kühlwein, Christyn J Bailey, Catherine Mead, Matthew J Emery, Daniel L Merrifield, Simon J Davies, Theodore Henry.
"Effects of dietary β-glucan on survival, histopathology and intestinal microbiology on Zebrafish *Danio rerio* mycobacteriosis"

Acknowledgements

I needed a lot of help from a lot of people to finally complete this PhD project and I wish to express my deep gratitude to all of them.

First and foremost Prof. Simon Davies, my director of studies, for guiding, supporting and inspiring me, for believing in me and for always having an open door when there were problems. And for profound discussions about Dad's Army & Fawlty Towers, of course!

Dr. Daniel Merrifield not only for broad knowledge, help and ideas especially regarding microbiology and writing publications but also for being a great friend and sharing many a delicious dram of single malt whisky.

Dr. Andrew Foey for support and constructive comments from an immunological point of view, and Prof. Dieter Steinhagen for his help and advice. Dr. Ted Henry for his help with regards to the zebrafish experiment.

All the technicians, especially Matt Emery for his extensive help in the microlab, his patience, his infinite knowledge, lab supplies, great fishing trips and the Imperial March. Ben Eynon for his support in the aquarium and for taking care of my fish when I was absent, Mike Hockings for help with the histology, the electron microscopy team with Glenn Harper, Dr. Peter Bond and Dr. Roy Moate (they know why!), Liz Preston and many more. Also the MSc students Catherine Mead and Christyn Bailey for their major contributions to the zebrafish trial.

A huge "thank you!" must also go to my friends and colleagues, especially to my "partner in crime" and wingman Mark Rawling, Paul Waines, Ana Rodiles, Dan Leeming, Dave Peggs, Ben Standen, Arafat Ahmed, all the guys from project NEMO, the "Freedom Fields Park-8 o'clock-dog-walking-club", and all the friends we left behind in Germany, especially "the Meiers".

However, the greatest thanks must go to Tanja and Yogi who stood by my side and dared to go on this 4-year adventure with me, for all their love, support and understanding when they often had to spend time without me due to my work. I couldn't have done it without them. I'm also deeply grateful to both our families for their love and support reaching us from Bavaria!

List of Abbreviations

AIS	Acquired immune system
ANOSIM	Analysis of similarity
AU	Arbitrary units
BW	Body weight
CFU	Colony forming unit
DGGE	Denaturing gradient gel electrophoresis
FCR	Feed conversion ratio
GALT	Gut associated lymphoid tissue
IEL	Intraepithelial leukocyte
IIS	Innate immune system
IP	Intraperitoneal
LAB	Lactic acid bacteria
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MHC	Mean haemoglobin concentration
OTU	Operational taxonomical unit
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRR	Pathogen recognition receptor
RBC	Red blood cells
SGR	Specific growth rate
SIMPER	Similarity of percentages
WBC	White blood cells
WG	Weight gain

„Grau, teurer Freund, ist alle Theorie,
und grün des Lebens goldner Baum.“

Johann Wolfgang von Goethe: Faust I

“Grey, dear friend, is every theory
and green the golden tree of life.”

Chapter 1:

The importance of aquaculture and integration of β-glucans therein

1.1. The importance of aquaculture and the position of cyprinids

The world population is expected to increase from nearly 7 billion in 2011 to approximately 8 billion by 2025 and to over 9.5 billion by 2050 (UN, 2011). The dietary supply of food and specifically protein for this rising population is a major task for future generations and aquaculture plays an important role in this demand. The global annual food fish supply per capita increased from 9.9 kg in the 1960s to 18.1 kg in 2009; further estimates predict a supply of 18.8 kg in 2011 (FAO, 2012) and this requirement is likely to rise even more. This increase however is covered at a progressive rate by harvests from aquaculture production since the harvests from capture fisheries have been stagnating since the late 1980s; recent data from 2006 until 2011 (estimated) indicate annual capture yields fluctuating around 90 million tonnes (Figure 1.1). In contrast, aquaculture production faced a tremendous and persistent boom since the 1980s with mean annual growth rates of approximately 8.8%; the data for the recent years reflect this fact with the global production rising from 47.3 million tonnes in 2006 up to 63.6 million tonnes in 2011 (inland and marine, without aquatic plants), equivalent to an increment of over 34%.

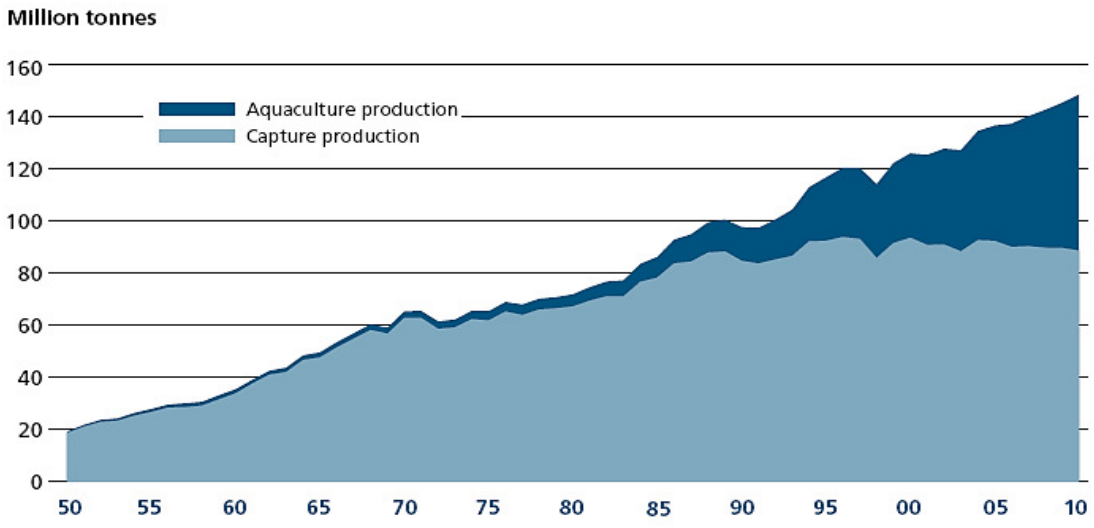

Figure 1.1: World capture fisheries and aquaculture production (from FAO, 2012)

Within aquaculture, the freshwater based production is prominent and accounted for nearly 62% of the total aquaculture production in 2010 (increasing from approximately 50% in 1980) whereas marine water based production supplies approximately 30% (declining from 40% in the same period). Within freshwater aquaculture the production of cyprinids plays a dominant role. In 2010 total worldwide cyprinid production accounted for 71.9% or 24.2 million tonnes of all freshwater aquaculture production. Cyprinid species produced (with descending share of production) include silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Hypophthalmichthys nobilis*), Indian major carps [catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus cirrhosus*)], grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*), goldfish (*Carassius auratus auratus*) and crucian carp (*Carassius carassius*) and other cyprinids (Figure 1.2).

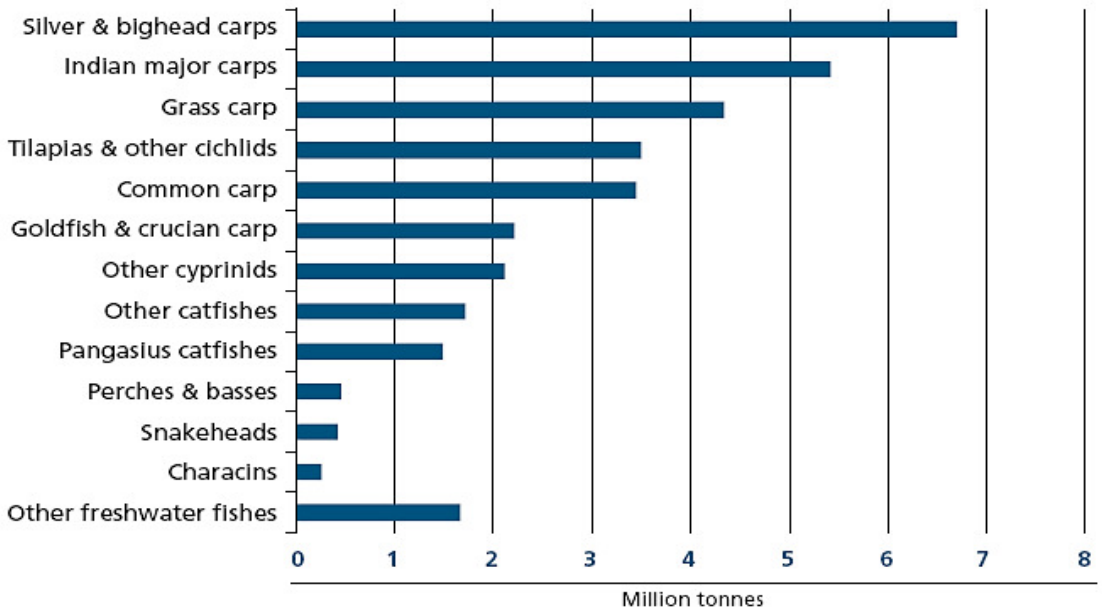

Figure 1.2: Aquaculture production of freshwater species/species group in 2010 (from FAO, 2012)

Another increasingly important, yet often overlooked sector of aquaculture is the ornamental fish industry, even though it does obviously not contribute to the food fish supply for human nutrition. According to FAO statistics (2012) however, the global total value of the industry has risen dramatically since the late 1980s. In 2009 the value (imports plus exports) accounted for nearly 700 million US$ compared to approximately 100 million US$ during the early 1980s (Figure 1.3). Many of the traded species belong to cyprinids as the recent study by Collins *et al*. (2012) indicates; the authors identified 172 ornamental cyprinid species with 91 species having been previously unrepresented in reference libraries. The most prominent representant of ornamental cyprinids is the Koi carp (*Cyprinus carpio koi*) reaching very high retail prices; unconfirmed reports mention a record price of 2.2 million US$ for one single koi carp.

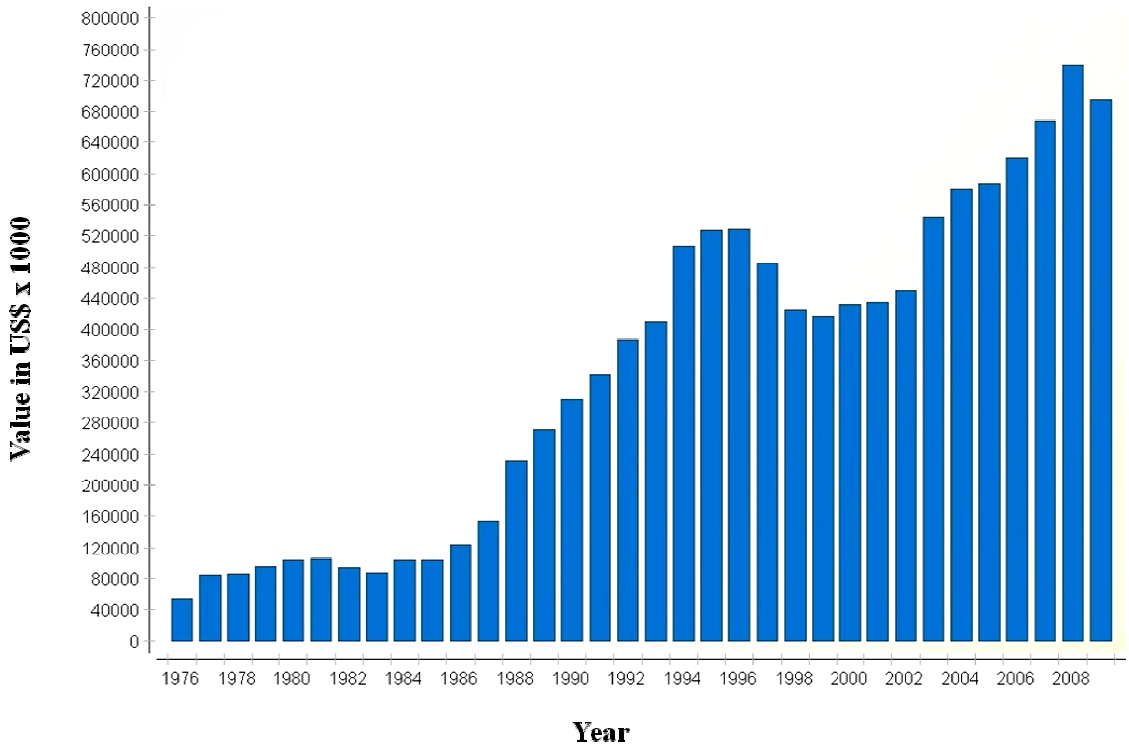

Figure 1.3: Annual total value (in US$) of the global ornamental fish industry (imports plus exports) from 1976 until 2009 (from FAO, 2012).

A third sector where a cyprinid species plays an integral part is the research sector. The zebrafish (*Danio rerio*) has gained considerably more attention over the past decades as an ideal research model organism in a variety of scientific fields, e.g. infection studies, cancer research, developmental biology, environmental toxicology, drug screening, evolution and aquaculture. The zebrafish information network (ZFIN, http://zfin.org/) reports global data from 1998 until 2012 about the annual number of publications released, the number of researchers within the field, the number of laboratories and the number of companies related to zebrafish research (Figure 1.4). It highlights the rapid boom of the field over the last decade. Recent ambitions have strived for standardization of nutritionally balanced zebrafish diets in order to minimize unwanted nutritional effects affecting the outcomes of experiments and in order to increase the confidence in the comparison of different experiments (Penglase *et al*., 2012).

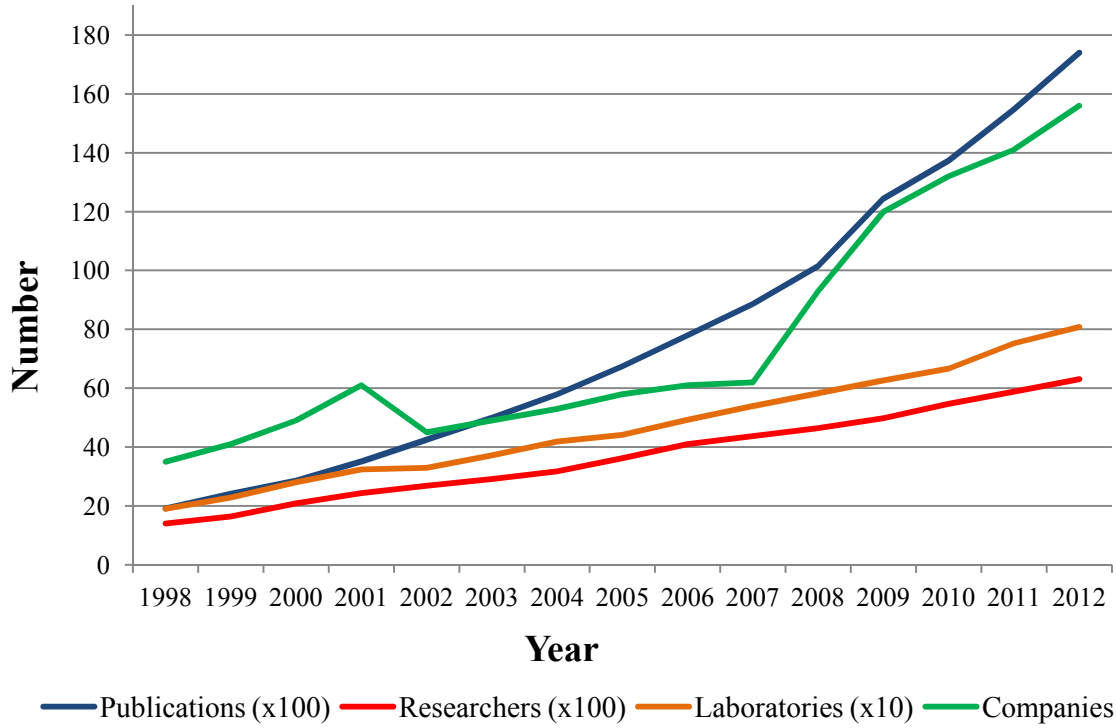

Figure 1.4: Registered number of publications, researchers, laboratories and companies with zebrafish information network (http://zfin.org/) in the years from 1998 until 2012

1.2. Aquaculture production, disease and prophylaxis

The continuously rising expansion and intensification of aquaculture production described in the previous paragraph also implicates a continuously rising burden of disease; this is elucidated by several major disease epidemics over the last two decades (Peeler and Feist, 2011). The most prominent large scale disease outbreak in all main salmon producing countries with severe economical and animal welfare consequences was the recent epidemic of infectious salmon anaemia (ISA). First reported in 1984 (Thorud and Djupvik, 1988) it caused massive production losses in the Faroe Islands (Lyngoy, 2003) and in the years 2007-2009 in Chile (Mardones *et al*., 2009). Delayed total losses of 2 billion US$ and 26,000 jobs have been predicted for the Chilean crisis for 2009-2011 (Asche *et al*., 2009). Another major disease with significant financial implications is the white spot syndrome virus (WSSV) in crustaceans; since its first emergence (presumably in China) in 1993 the disease spread rapidly and globally to all known production zones having caused total financial losses of at least 10 billion US$ to date (Stentiford, 2011). The most prominent severe disease concerning cyprinids in recent years was caused by the koi herpes virus (KHV). It was first described in Europe in 1996, the first major outbreak however was reported in Israel in 1998 in farms producing common carp and koi carp (Haenen *et al*., 2004); the disease spread to 90% of the carp farms in Israel until the end of 2000 with estimated financial losses of 3 million US$ per year. The disease further spread through international transport of carp and ornamental koi carp (Gilad *et al*., 2003) and is now present in Europe, US, Israel, South Africa, Japan and South-East Asia (Haenen *et al*., 2004). It is estimated that diseases in aquaculture overall account for global financial losses of several billion US$ per year (Defoirdt *et al*., 2011).

The most important objective in aquaculture production therefore is the maintenance of the fish's freedom of disease in order to sustain high growth rates and survival which in turn leads to a greater profitability (Gatlin III, 2002). The outbreak of a disease caused by a pathogen generally occurs when several factors coincide (Figure 1.5). A virulent pathogen is present in the first place in order to manifest its virulence in the host; pathogenic organisms that cause disease in fish include bacteria, viruses, fungi and parasites. Secondly, the host fish is susceptible to this pathogen; endogenous factors, e.g. age or the genetic constitution of a fish (Houghton *et al*., 1991; Wiegertjes *et al*., 1993; Arkush *et al*., 2002; Grimholt *et al*., 2003) can have an effect on susceptibility of the fish. Thirdly, the conditions in the fish's environment are adverse, i.e. stressors are present which can impair normal physiological processes and cause disease. Stressors include adverse water conditions (e.g. oxygen satiation, pH, ammonia, nitrite, carbon dioxide, temperature, salinity and turbidity), high stocking densities (overcrowding), interactions among fish, handling (harvesting, grading, transport, therapeutic treatments), frequent disturbances, inadequate nutrition (nutrient deficiencies, feeding regime, toxins in the diet) and a general lack of sanitation (Schaperclaus, 1991; Gatlin III, 2002).

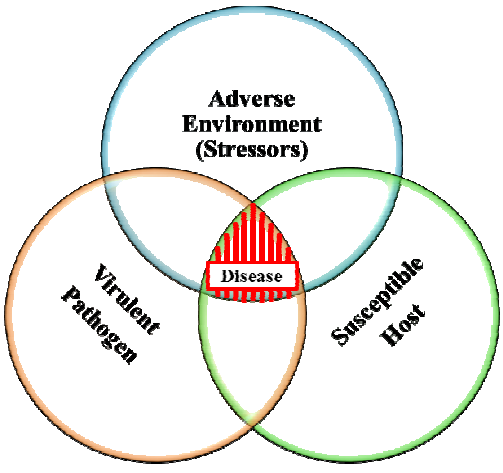

Figure 1.5: Only the simultaneous coincidence of a virulent pathogen, a susceptible host and adverse environmental conditions lead to the onset of a disease (adapted from Svobodova *et al*., 1993)

Depending on the persistence of the stressor exposure and the intensity, the physiological stress response by the organism can be acute or chronic. Acute stress has been shown to be beneficial by enhancing the immune reaction, e.g. in rats (Dhabhar and McEwen, 1997) and rainbow trout (Peters and Schwarzer, 1985; Möck and Peters, 1990; Demers and Bayne, 1997). Chronic stress response however is generally believed to suppress or dysregulate immune function and make the organism more susceptible to pathogens with the eventual risk of causing diseases (Gatlin III, 2002; Dhabhar, 2009).

The famous phrase "prevention is better than cure" was coined by Erasmus of Rotterdam (1466-1536) already half a millennium ago. This philosophy takes on much more significance in today's animal husbandry in such a way that research and industry constantly try to optimize the production conditions. Some of the stressors in aquaculture mentioned above can be avoided or, at least, minimized by good management and foresighted planning. Some stressors however will always be present to some degree in intensive aquaculture production, e.g. inadequate stocking densities, suboptimal or poor water quality, handling and transport. Therefore one popular means of trying to prevent disease outbreaks and mortalities is the dietary administration of immunostimulants in order to boost the fish's immune system. A lot of concerted research efforts have been put forward over the last two to three decades to find new and further characterize known immunostimulants. In recent years, research efforts have been additionally accelerated since the European Union has ratified a ban on the non-medical use of antibiotics in animal nutrition in the Regulation (EC) No 1831/2003 (EU, 2003), effective from 2006 onwards.

1.3. Defining the term immunostimulants

A possible definition of a substance, which acts as an immunostimulant, is the classification in groups of origin by Sakai (1999), even though that is independent on their mode of action. In this context, those studied in fish and shellfish trials can be generally divided into synthetic products (e.g. levamisole), bacterial/fungal substances (e.g. lipopolysaccharides, ß-glucans), animal and plant components (e.g. glycyrrhizin), diet components (e.g. vitamin C and E), hormones, cytokines and others. The use of immunostimulants in aquaculture strives for an enhanced disease resistance through increased immunological competence. A similar classification is mentioned by Galeotti (1998). This implicates that immunostimulants can be of either natural or synthetic origin. Raa (2000) defined immunostimulants as substances, which activate leukocytes and therefore may provide higher resistance against viral, bacterial, fungal and parasitic infections. In recent years it has been clarified, that foreign substances entering the body are recognized by pattern recognition receptors (PRRs) by means of the so-called pathogen-associated molecular patterns (PAMPs), highly conserved molecular motifs (Janeway, 1992, Medzhitov and Janeway, 2000, Didierlaurent *et al*., 2005). Against this background Bricknell and Dalmo (2005) redefined a former definition as different leukocytes may express different PRRs as follows: ’An immunostimulant is a naturally occurring compound that modulates the immune system by increasing the host’s resistance against diseases that in most circumstances are caused by pathogens‘. Thereby they limit them to naturally occurring substances.

A great range of immunostimulants have received attention in aquaculture over the last two to three decades. The immunostimulants however with probably the best scientific track record and most established on an industrial scale in the aquaculture industry are β-glucans.

1.4. What are β-glucans?

1.4.1. Sources of β-glucans

β-glucans are widely distributed in nature and can be found in the cell walls of yeasts, cereal grains, algae, bacteria and fungi (Zekovic *et al*., 2005). The most common sources for natural β-glucans with highly immunostimulating properties are yeasts and fungi. The fungus derived forms, lentinan (obtained from *Lentinus edodes*), scleroglucan (from genus *Sclerotium*) and schizophyllan (from *Schizophyllum commune*), are β-(1,3)(1,6)-D-glucans and have been extensively studied in human as well as in animal nutrition. One of the first studies, conducted in 1969 by Chihara *et al*. (1969), showed an inhibiting effect of lentinan on tumour growth in transplanted mice tumours after systemic infection. Lentinan and schizophyllan are nowadays used clinically in cancer therapy in Japan (Kaneko *et al*., 1989). An industrial schizophyllan product used in aquaculture is VitaStim. The most established yeast derived immunostimulating β-(1,3)(1,6)-D-glucans are extracted from the yeast *Saccharomyces cerevisiae*, which is utilized in commercial products such as Leiber® Beta-S or Macrogard®, EcoActiva™ (mixed with mannans) and Zymosan (mixed with mannans, proteins and nucleic acids). Krestin (also PSK) is a protein-bound β-glucan obtained from the fungus *Trametes versicolor*. But in contrast to the β-glucans mentioned previously, krestin has a β-(1,4)-linked backbone with β-(1,3) and β-(1,6) side chains, which bind to the protein moieties O- or N-glycosidicly (Tsukagoshi *et al.*, 1984). Laminaran provides a water soluble and an insoluble form of β-(1,3)(1,6)-D-glucan and is an extract from brown algae (*Laminariae* spp.) (Peat *et al*., 1958; Kim *et al*., 2000). An example of a microbial β-(1,3)-glucan is the linear curdlan, which is derived by fermentation from *Agrobacterium* sp. and *Alcaligenes* sp..

1.4.2. Chemical structure and solubility

Glucans are polysaccharides consisting of repeating D-glucose monomers. They can be branched or unbranched and are linked by O-glycosidic bonds, either α- or β-bonds. Examples for α-glucans are starch, dextran or glycogen, but there are no practical applications of these polysaccharides as immunostimulants. In this context, the β-glucans are highly important because of their properties as biological response modifiers. In yeasts they are located in the notably thick cell wall (100-200 nm), which consists of three layers. The cell wall is made up of 80-90% polysaccharides, mainly β-glucans and mannans. The β-glucans in the intermediate layer give strength and rigidity to the cell wall by forming a microfibrillar network. Humbel *et al*. (2001) analysed the cell wall of *Schizosaccharomyces pombe* and suggested, that this layer was comprised mainly of β-(1,6) branched β-(1,3)-glucan. Figure 1.6 shows the structure of a yeast cell wall (Sigma-Aldrich).

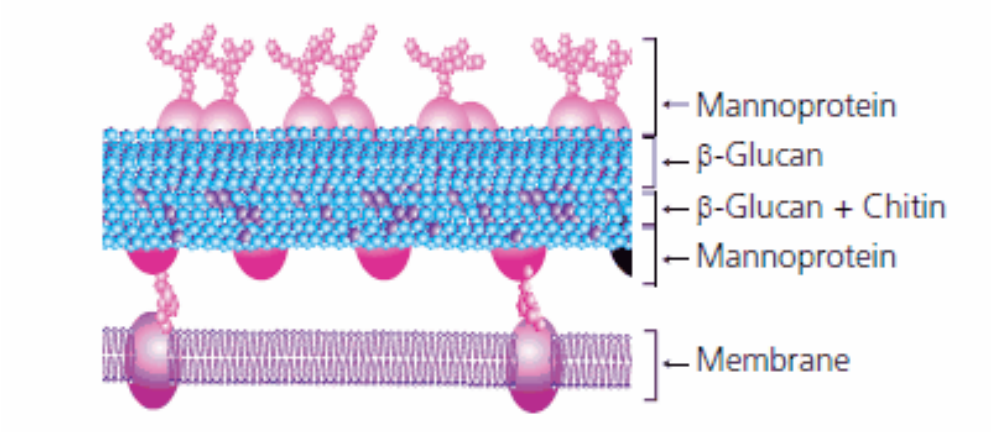

Figure 1.6: Typical structure of the yeast cell wall (source: www.sigmaaldrich.com)

Differences between various β-glucans occur regarding their molecular weight, primary structure (including linkage), degree of polymerization (DP) and branching (DB) and the conformation (triple/single helix or random coil) (Yadomae and Ohno, 1996; Kim *et al*., 2000; Yadomae, 2000). There are linear β-glucans with a mixed β-(1,3)(1,4)-linkage

backbone, e.g. from barley (Mantovani *et al*., 2008), which have shown positive effects on disease resistance in rainbow trout (Sealey *et al*., 2008). More eminent in this respect are the β-glucans only consisting of a backbone of β-(1,3)-linked-D-glucopyranosyl units. These glucose polymers can be linear or branched with randomly distributed single β-(1,6)-linked D-glucopyranosyl side chains, in which case it provides a comb-like structure (Bohn and BeMiller, 1995). Thereby the main and side chains can vary in length; the side chains support complex tertiary structures, stabilized by inter-chain hydrogen bonds (Mantovani *et al*., 2008), which also determines their properties as structural polysaccharides. The branched β-(1,3)(1,6)-D-glucans seem to be the most active among the immunostimulants (Bohn and BeMiller, 1995). Figure 1.7 shows the typical structure of a β-(1,3)(1,6)-D-glucan.

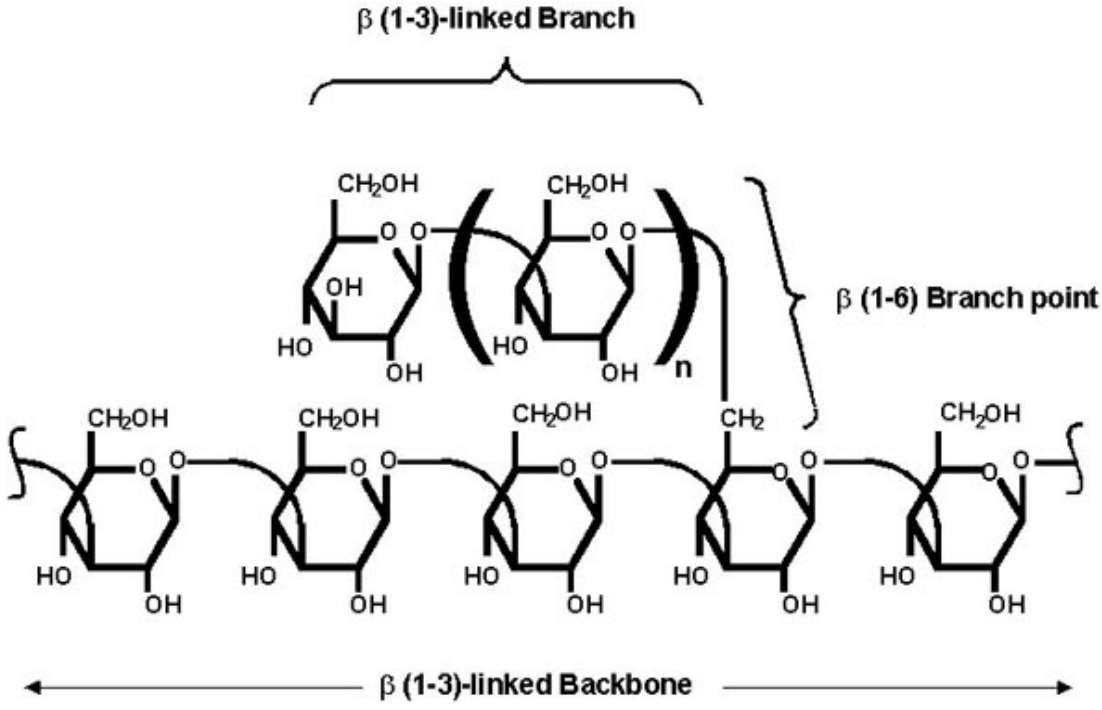

Figure 1.7: Typical primary structure of a yeast β-(1,3)(1,6)-D-glucan (source: www.beta13dglucan.org)

The critical properties for effective biological response modifiers are the molecular weight (MW) and the water solubility, which again depends on the degree of branching and the chemical modification (Suzuki *et al*., 1991; Williams *et al*., 1991; Williams *et al*., 1992). If the degree of polymerization (as a function of the DB) is more than 100, the β-glucans are insoluble in water and solubility increases as the DP decreases. The solubility in water ranges from insoluble (curdlan at room temperature) and gelling (scleroglucan) to completely soluble (laminaran) (Bohn and BeMiller, 1995). A classification in terms of solubility is: alkali-insoluble, acetic acid insoluble 1,3-β-glucans, alkali-soluble 1,3-β-glucans and highly branched 1,6-β-glucans (Zekovic *et al*., 2005). This passes an insolubility on most β-glucans and thus limits their application in *in vitro* experiments (Mantovani *et al*., 2008). Bohn & BeMiller (1995) mentioned in their review that in terms of clinical use the molecular weight of the most effective β-glucans is between 100 and 200 kDa with a degree of branching from 0.2 to 0.33 and a triple helical structure. Figure 1.8 shows as an example the triple helical structure of schizophyllan (Mizuno, 1999).

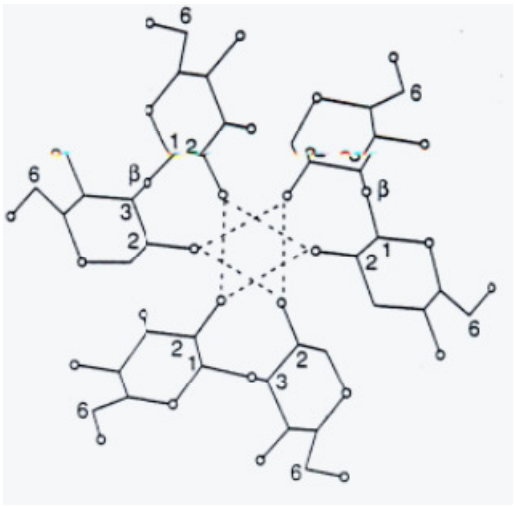

Figure 1.8: Model of the triple helical structure of schizophyllan (from Mizuno, 1999)

1.5. Teleost immunity

1.5.1. Lymphatic organs

The lymphatic organs of bony fish can be divided in primary and secondary organs. The head kidney (pronephros), trunk kidney (mesonephros) and thymus are the primary organs. The spleen and the different types of MALT (mucosa-associated lymphoid tissue) represent the secondary organs (Zapata *et al*., 1996); the MALT in fish is considered to be well developed and can be found in the intestine (GALT = gut-associated lymphoid tissue), gills and skin (Van Muiswinkel and Wal, 2006). The number of leukocytes in these organs is appreciable (Rombout *et al*., 1986; Rombout *et al*., 1989). The primary lymphatic organs are responsible for the generation and differentiation of lymphocytes (Hansen and Zapata, 1998), whereas the T-cells are processed in the thymus and the B-cells in the kidney. The secondary lymphatic organs could be regarded as the ’battlefield‘, the infiltrated antigens clash here with the maturated, immune competent lymphocytes. The main difference to the mammalian immune system is that fish don’t possess bone marrow and lymph nodes (Press and Evensen, 1999), and furthermore the fact that the kidney in fish is a lymphatic as well as an important haematopoietic organ (Faenge, 1986; Hansen and Zapata, 1998).

1.5.2. The immune system

The teleost immune system can be described on the basis of either compartments or functions. The classification via compartments takes place in a cellular and a humoral immune system. The functional categorization occurs in a nonspecific/innate immunity (IIS = innate immune system) and a specific/acquired/adapted immunity (AIS = adaptive immune system). The IIS comprises thereby physico-chemical barriers such as scales, skin and mucus layer, phagocytic and non-specific cytotoxic activity and non-

specific humoral activity like the complement system, lysozyme and C-reactive protein. The IIS is independent of any recognition mechanisms of pathogens (because of the phylogenetic highly conserved PAMPs), barely temperature dependent and the response is carried out rapidly (minutes to hours) (Ellis, 2001; Van Muiswinkel and Wal, 2006). An effective innate immune response is particularly important for embryonic and larval stages as the AIS is yet to fully develop (Ellis, 2001; Magnadottir, 2006). The AIS is a phylogenetic further development of the IIS. Fish are the first vertebrates with an AIS, which appears in the presence of lymphocytes, immunoglobulin, MHC (major histocompatibility complex) and T-cell receptor and therefore they can mount a specific response (Nakanishi, 2003). It works via recognition of specific antigenic structures and the development of so-called memory cells; building up a sufficient response against pathogens takes weeks to months and the AIS is temperature-dependent. But on balance, the teleost immune system is more simple and less differentiated compared to the mammalian immune system (Nakanishi, 2003). Figure 1.9 summarizes the different levels of the teleost immune system.

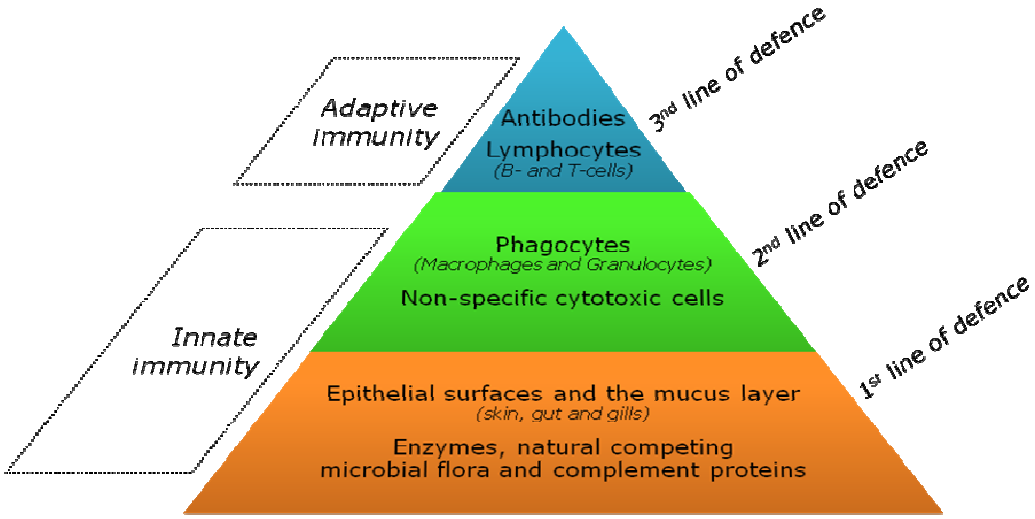

Figure 1.9: Structure of the teleost immune (adapted from: systemhttp://www.webmed.ch/Aktuell/Immunsystem.htm)

1.6. How are β-glucans recognized in vertebrates? – Receptors

As mentioned above, invading pathogens exhibit evolutionary conserved PAMPs, which are recognized by vertebrate immune cells via specific receptors, the PRRs, upon contact. Those receptors for β-glucan are present in all animals, in invertebrates as well as in vertebrates (Raa, 2000) and are important for the recognition of fungal pathogens. This is the reason why we can see effects both in shrimp and fish. The receptors, which are known to date for being involved in binding of β-glucan in vertebrates, are the complement receptor 3 (CR 3), Toll-like receptor (TLR) 2 and 6, lactosylceramide (LacCer) and dectin-1, which all prompt innate immune responses (Brown and Gordon, 2003; Chen and Seviour, 2007); but TLR 2 and 6 and dectin-1 take part in adaptive responses as well (Dalmo and Bøgwald, 2008). As known so far, these receptors are located on cell surfaces; recognizing plasma molecules are unknown to date, even though opsonisation takes part in the recognition of particulate glucans (Brown and Gordon, 2005). Dennehy and Brown (2007) suggest, that mainly Dectin-1 by itself, but also its collaboration with TLRs is most important in responding to infectious agents. The receptors can be found both on immune and non-immune cells including monocytes/macrophages, neutrophils, Langerhans cells, eosinophils, NK cells, endothelial cells, alveolar epithelial cells and fibroblasts (Brown and Gordon, 2003). A further potential receptor for β-glucans could be the scavenger receptor (SR); it is present on macrophages and dendritic cells as well as on certain endothelial cells (Peiser *et al*., 2002). Several studies implied soluble β-glucans as ligands, but no specific scavenger receptors have been identified to date (Rice *et al*., 2002). In contrast, the detection in invertebrates is carried out mainly in the hemolymph; the *Drosophila* scavenger receptor is the solely known cellular β-glucan receptor (Soltanian *et al*., 2009).

Regarding the current knowledge in fish it has been shown, that in Atlantic halibut (*Hippoglossus hippoglossus*) larvae the intestinal epithelial layer is involved in the uptake of laminaran (Strand and Dalmo, 1997). Moreover, a receptor for β-glucan was located on salmon macrophages, granulocytes and non-specific cytotoxic cells (Engstad and Robertsen, 1993; Engstad, 1994; Engstad and Robertsen, 1994), and the receptor on macrophages appear capable of recognizing very short β-(1,3)-linkages (3 linked glucose molecules) and not the β-(1,6)-linkages (Engstad and Robertsen, 1994). Ainsworth (1994) detected on channel catfish neutrophils a receptor for the β-glucan component of zymosan, a cell wall preparation of *S. cerevisiae*. It has also been suggested, that CR3 is present in head kidney leukocytes of carp (Nakao *et al*., 2003).

1.7. β-glucans in aquaculture

1.7.1. Effects of β-glucans on growth

Reports on the influence of β-glucan on growth performance are conflictive. Although it is not the main objective of immunostimulant applications, several studies have demonstrated enhancing effects on growth performance (Table 1.1). Improved growth performance after 56 days dietary administration was reported in koi carp (*Cyprinus carpio koi*) (Lin *et al*., 2011) and large yellow croaker (*Pseudosciaena crocea*) (Ai *et al*., 2007) with *S. cerevisiae* β-glucan as well as rohu (*Labeo rohita*) fingerlings with a barley derived β-glucan (Misra *et al*., 2006). Also studies with *Penaeus* species have shown enhanced growth parameters 39 days after an initial 3 hour β-glucan bath immersion (Sung *et al*., 1994) or oral administration over 75-84 days (Felix *et al*., 2008; Van Hai and Fotedar, 2009; Zhao *et al*., 2012). Positive effects on growth rates were also seen in sea cucumber (*Apostichopus japonicus*) over 29 days (Gu *et al*., 2011; Zhao *et al*., 2011). Many other studies however did not observe such positive effects on

growth performance, e.g. in Nile tilapia (*Oreochromis niloticus*) (Whittington *et al*., 2005; Shelby *et al*., 2009), European sea bass (*Dicentrarchus labrax*) (Bagni *et al*., 2005), channel catfish (Welker *et al*., 2007) or hybrid striped bass (*Morone chrysops x Morone saxatilis*) (Li *et al*., 2009). Even negative effects on feed conversion were found after 3 (but not after 9) weeks with a medium and high β-glucan barley based diet or a wheat based basal diet containing 0.2% *S. cerevisiae* β-glucan compared to a wheat meal based control diet, when fed to rainbow trout (Sealey *et al*., 2008); also weight gain was significantly reduced after week 3 with the high β-glucan barley diet.

It is impossible up to date to elucidate logically, why growth enhancing effects can be seen under certain circumstances in some fish/shellfish species and not in others. Dalmo & Bøgwald (2008) suggest that the effects may depend on the concentration of β-glucan in the diet and its solubility, the fish species, temperature and length of the feeding period; they also mention that the intestine only absorbs soluble β-glucan and there are no endogenous enzymes which degrade particulate β-glucan making them available for absorption. They also hypothesize, when growth enhancing effects occur, that the β-glucans induce a localized intestinal immune response that in turn leads to resistance against pathogens which otherwise would cause reduced weight gain and possibly disease.

1.7.2. Immunostimulating effects of β-glucans in vitro

In vitro studies are a useful means to conduct very specific and isolated experiments under controlled conditions, it must be noted however that the results may not always fully translate into an *in vivo* system with its complex processes (Kiron, 2012). Several studies reported the *in vitro* effects of β-glucans on fish immune cells, nearly always macrophages isolated from the pronephros (Table 1.2). β-glucans enhanced the respiratory burst, one of the crucial innate immune responses on the cellular level, in

Table 1.1: Reported growth performance enhancing effects of β-glucans in aquaculture species

Species	β-glucan type	Feeding regime	Effects	Reference
Cyprinus carpio koi	*S. cerevisiae* β-glucan	0.09% inclusion fed to apparent satiation	↑ weight gain ↑SGR	Lin *et al*. (2012)
Labeo rohita	Barley derived β-glucan	0.025% and 0.5% inclusion fed at 3% of BW day^{-1}	↑SGR	Misra *et al*. (2006)
Pseudosciaena crocea	*S. cerevisiae* β-glucan	0.09% inclusion fed to apparent satiation	↑SGR	Ai *et al*. (2007)
Penaeus monodon	*S. cerevisiae* β-glucan	3 h bath immersion at 0.5, 1, and 2 mg/ml	↑body length ↑body weight	Sung *et al*. (2004)
Penaeus latisulcatus	β-(1,3)-glucan	0.2% inclusion fed at 3%-5% of BW day^{-1}	↑SGR ↑FCR	Van Hai and Fotedar (2009)
Penaeus monodon	*S. cerevisiae* β-glucan	0.1, 0.7 & 1.3% inclusion, fed at 10% of BW day^{-1} in weekly intervals	↑ weight gain ↑ SGR	Felix *et al*. (2008)
Litopenaeus vannamei	β-(1,3)-glucan	0.025% inclusion	↑ weight gain	Zhao *et al*. (2012)
Apostichopus japonicus	*S. cerevisiae* β-glucan	0.125% inclusion fed at 3% / 2% of BW day^{-1} for 3/5 weeks	↑body weight ↑SGR	Zhao *et al*. (2011)
Apostichopus japonicus	*S. cerevisiae* β-glucan	0.075 and 0.15% inclusion fed to apparent satiation	↑SGR	Gu *et al*. (2011)

isolated macrophages from Indian major carp catla (*Catla catla*) (Kamilya *et al*., 2006a) and Atlantic salmon macrophages (Sveinbjornsson and Seljelid, 1994; Dalmo and Seljelid, 1995). Three key studies however gave more detailed and contrary insights into this topic. Jorgensen and Robertsen (1995) showed that the commercial β-glucan MacroGard® raised the respiratory burst activity in Atlantic salmon macrophages at 0.1 and 1 μg/ml while a concentration of 10 μg/ml had no effects; but 50 μg/ml had even inhibitory effects. Castro *et al*. (1999) investigated the effects of 3 yeast β-glucans from *S. cerevisiae* and a fungus β-glucan from *S. commune* (0-500 μg/ml) on turbot

(*Psetta maxima*) and gilthead seabream (*Sparus aurata*) phagocytes. The cell responses in both species were similar. Overall, the authors hypothesize that low concentrations prepared the cells for a higher production of reactive oxygen species prior to a later following activation of the respiratory burst; but high concentration of β-glucans may exhaust the phagocytic cells due to a direct induction of the respiratory burst. Kudrenko *et al*. (2009) however reported in a recent study with barramundi (*Lates calcarifer*) macrophages that β-glucans of different sources (curdlan, paramylon, laminaran and purified yeast β-glucan) stimulate the respiratory burst at concentrations of 100 and 1000 µg/ml; but only two other stimulants in this experiment (LPS and Zymosan) were able to prime the macrophages at low concentrations (0.1 and 1 µg/ml). The authors suggest that the β-glucans activate macrophages in high concentrations, but do not prime them through the β-glucan receptor. They also investigated the effect of an acid environment on β-glucans after determining the stomach pH of the carnivorous barramundi, which was at pH 2 for up to 6h after feeding. The β-glucans, treated with HCl to simulate those conditions, lost their ability to activate macrophages. This circumstance might underline the relevance of the digestive system of the respective species in terms of the efficacy of orally delivered β-glucans. In contrast to stomachless herbivorous and omnivorous species, the β-glucans are exposed to a strongly acid environment in the stomach of carnivorous species. This might influence their physico-chemical properties and consequently their biological effects.

β-glucans also improved another important part of the cellular innate immune response. Phagocytic processes were increased in macrophages isolated from catla (Kamilya *et al*., 2006a) and Atlantic salmon (Dalmo and Seljelid, 1995) and enhanced pinocytosis was described in Atlantic salmon (Sveinbjornsson and Seljelid, 1994). Also acid

phosphatase activity was augmented in Atlantic salmon macrophages (Sveinbjornsson and Seljelid, 1994; Dalmo and Seljelid, 1995).

While the above described improved respiratory burst and phagocytic activity of catla macrophages translated into a higher bactericidal activity against *Escherichia coli* (Kamilya *et al*., 2006a), this effect was not observed in Atlantic salmon macrophages against a virulent and an avirulent strain of *Aeromonas salmonicida* (Jorgensen and Robertsen, 1995).

Another important part of innate immunity was activated, when Paulsen *et al*. (2001) administered a *S. cerevisiae* β-glucan to Atlantic salmon macrophages. The lysozyme production was increased in an almost linear dose-response relation from 1 to 250 µg/ml (up to 6-fold higher compared to controls); a concurrent enrichment of the lysozyme gene transcript occurred as well. Furthermore, macrophages of fish infected with the skin parasite *Ichthyobodo necator* showed increased lysozyme activity under ß-glucan stimulation.

1.7.3. Immunostimulating effects of β-glucans in vivo

Several methods have been used to administer β-glucans in *in vivo*-studies: injection intraperitoneal (ip), immersion bathing and oral delivery via feeding.

1.7.3.1. Injection

The ip injection – delivery into the body cavity – is a highly effective method with fast occurring effects and ensures that the desired dose is administered, but it's costly and labour-intensive, impractical for use in smaller fish and a stressful situation for treated fish (Galindo-Villegas and Hosokawa, 2004). In most of the studies the influence of the used β-glucans in association with disease challenges was examined (Table 1.3).

Table 1.2: Reported *in vitro* effects of β-glucans in aquaculture species

Species	β-glucan type	Effects	Reference
Catla catla	*P. florida* β-glucan	↑ respiratory burst ↑ phagocytic and bactericidal activity	Kamilya *et al*. (2006)
Salmo salar	β-(1,3)-polyglucose from curdlan	↑ respiratory burst ↑ pinocytic activity ↑ acid phosphatase	Sveinbjornsson and Seljelid (1994)
Salmo salar	Native laminaran	↑ phagocytotic index ↑ intracellular superoxide anion ↑ acid phosphatase activity	Dalmo and Seljelid (1995)
	sulphated laminaran	↑ intracellular superoxide anion ↑ acid phosphatase activity	
Salmo salar	*S. cerevisiae* β-glucan	↑ respiratory burst at low β-glucan levels →bactericidal activity against *A.salmonicida*	Jorgensen and Robertsen (1995)
Psetta maxima and Sparus aurata	*S. cerevisiae* and *S. commune* β-glucans	↑ respiratory burst with high β-glucan levels leading to exhaustion; priming effect with low β-glucan levels for ↑ respiratory burst	Castro *et al*. (1999)
Lates calcarifer	various β-glucans	↑ respiratory burst with high β-glucan levels no priming effect with low levels → respiratory burst after 6 h HCl treatment of β-glucans	Kudrenko *et al.* (2009)
Salmo salar	*S. cerevisiae* β-glucan	↑ lysozyme activity and gene transcript ↑ lysozyme production in *Ichthyobodo necator* infected fish	Paulsen *et al*. (2001)

Administration of β-glucans ip has been reported to significantly increase survival rates upon challenges with *E. tarda* (Yano *et al*., 1989; 1991) and *A. hydrophila* (Yano *et al*., 1991; Selvaraj *et al*., 2005; Rodriguez *et al*., 2009) in cyprinids (carp and zebrafish), upon challenges with *V. salmonicida*, *V. anguillarum* and *Y. ruckeri* (Robertsen *et al*., 1990) in Atlantic salmon and upon challenges with *E. ictaluri* (Chen and Ainsworth, 1992) in channel catfish. In these studies innate immune responses were elevated, likely having promoted the higher survival rates observed. For example, higher activities of

pronephros phagocytotic cells were detected in channel catfish (Chen and Ainsworth, 1992), and in carp where it likely lead to the rapid elimination of the challenge bacteria from the blood (Yano *et al.*, 1989); another non-challenge study by Brattgjerd *et al*. (1994) reported increased phagocytic activities of Atlantic salmon macrophages against *V. salmonicida*, but interestingly not against *R. salmoninarum*. Enhanced bactericidal activities of phagocytes against the respective pathogens were seen in carp (Selvaraj *et al*., 2005), zebrafish (Rodriguez *et al*., 2009) and channel catfish (Chen and Ainsworth, 1992). Furthermore, an augmented activation of the alternative complement pathway occurred in carp (Yano *et al*., 1989; 1991); in Selvaraj *et al*.'s (2005) study however the classical and alternative complement pathway remained unaffected. Higher levels of total blood leukocytes, the proportion of monocytes and neutrophils and a decrease of eosinophils and basophils as well as an enhancement of the respiratory burst of pronephros macrophages were detected in carp (Selvaraj *et al*., 2005). A higher respiratory burst in non-challenged Atlantic salmon macrophages was also observed by Brattgjerd *et al*. (1994). Another important part of the innate immune defence, the presence of lysozyme, was increased in the blood of non-challenged Atlantic salmon (Brattgjerd *et al*., 1994; Paulsen *et al*., 2003). The studies of Selvaraj (2005) and Chen and Ainsworth (1992) have also indicated β-glucan effects on the adaptive immune response when serum antibody titres against *A. hydrophila* and *E. ictaluri*, respectively, were increased following vaccination.

Interestingly, a recent study by Kim *et al*. (2009) also reported anti-viral effects of β-glucans when grass carp displayed increased survival rates upon a challenge with the grass carp haemorrhage virus. In addition, the authors showed augmented activities of antioxidant enzymes (superoxide dismutase and catalase) in erythrocytes and increased expression levels of the Mx gene, an anti-viral response gene.

Table 1.3: Reported effects of β-glucans after intraperitoneal injection in aquaculture species

Species	β-glucan type	Injection dose	Effects	Reference
Cyprinus carpio	lentinan schizophyllan scleroglucan	2, 5 and 10 mg kg^{-1}	↑ survival against *E. tarda* & *A. hydrophila* ↑ elimination of *E. tarda* from blood ↑ phagocytic index of pronephros cells ↑ alternative complement pathway	Yano *et al*. (1989) Yano *et al*. (1991)
Cyprinus carpio	*S. cerevisiae* β-glucan	100, 500 and 1000 µg	↑ survival against *A. hydrophila* ↑ total blood leukocytes, neutrophils and monocytes levels ↑ respiratory burst and bactericidal activity ↑ antibody titres against *A. hydrophila* → classical and alternative complement pathway	Selvaraj *et al*. (2005)
Danio rerio	*S. cerevisiae* β-glucan	0.5, 2 and 5 mg ml^{-1}	↑ survival against *A. hydrophila* ↑ bactericidal activity of myelomonocytic kidney cells	Rodriguez *et al*. (2009)
Ctenopharyngodon idella	*Poria cocos* β-glucan	10 mg kg^{-1}	↑ survival against grass carp haemorrhage virus ↑ SOD and catalase activity in erythrocytes ↑ Mx gene expression	Kim *et al*. (2009)
Salmo salar	*S. cerevisiae* β-glucan	0.1 – 2.5 mg $fish^{-1}$	↑ survival against *V. salmonicida*, *V. anguillarum* &*Y. ruckeri*	Robertsen *et al*. (1990)
Salmo salar	*S. cerevisiae* β-glucan	14.8 mg $fish^{-1}$	↑ serum lysozyme and macrophage respiratory burst ↑ phagocytic activity against *V. salmonicida* → phagocytic activity against *R. salmoninarum*	Brattgjerd *et al*. (1994)
Salmo salar	*S. cerevisiae* β-glucan	60 mg kg^{-1}	↑ plasma lysozyme activity and gene transcripts	Paulsen *et al*. (2003)
Ictalurus punctatus	*S. cerevisiae* β-glucan	50 and 750 µg $fish^{-1}$	↑ phagocytic index & bactericidal activity of phagocytes ↑ survival against *E. ictaluri* ↑ serum antibody titres against *E. ictaluri*	Chen & Ainsworth (1992)

1.7.3.2. Immersion

Very few studies have been conducted so far regarding the administration of β-glucans via bathing or immersion. Even though this could be a putatively efficient way of treating large quantities of fish, the majority of the studies have not shown any positive results. Immersion appears to be less successful as fish are probably not capable of absorbing particulate β-glucans, which are insoluble in water.

Selvaraj *et al*. (2005) conducted immersion studies with carp by bathing them at days 1, 3 and 5 with *S. cerevisiae* β-glucans. At day 7 the authors challenged the carp with *A. hydrophila*. No modifying effects occurred regarding the measured parameters, viz. survival, total and differential leukocyte counts, respiratory burst and bactericidal activity, antibody titres, classical and alternative complement pathway activity. Juvenile Chinook salmon (*Oncorhynchus tshawytscha*) received a vaccine of *A. salmonicida* either alone or in combinations with a commercial β-glucan (from *S. commune*) both in crude or pure form via a 15 min bathing (Nikl *et al*., 1993). The fish showed no enhanced protection to a challenge with virulent *A. salmonicida* 21 days after the vaccination. In contrast, Anderson *et al*. (1995) proved that glucan immersion led to higher but temporary resistance of rainbow trout against *A. salmonicida* after treatment for 3 days. This effect was non-existent after 14 days. A study with juvenile American white shrimp (*Litopenaeus vannamei*) reported a 2-fold increase of the superoxide anion generation and a 1.4-fold increase in superoxide dismutase activity in haemocytes and muscles after 6 hours immersion in a solution with a *S. cerevisiae* β-glucans (Campa-Cordova *et al*., 2002). The authors conclude that the β-glucans were competent to enhance the respiratory burst in haemocytes after a single bath treatment.

1.7.3.3. Oral administration

The oral administration of β-glucans incorporated in feeds would be the most preferable way as large quantities of fish could be treated more easily. As already mentioned under section *2.9.1 (Injection)*, administration of β-glucans has the potential to reduce mortalities due to pathogenic challenges by strengthening the host's innate immune defences. This phenomenon has also been observed when β-glucans had been delivered orally. Yet, the level of information about the mechanisms of action is low because most of the previous research has focused on the other ways of administration (Soltanian *et al*., 2009).

1.7.3.3.1. Other fish

Many studies have shown the protective effects of dietary β-glucans in fish upon challenges with various stressors relevant to aquaculture husbandry. β-glucans from *S. commune* and *S. cerevisiae* have been reported to enhance the survival upon challenges with *A. salmonicida* in chinook salmon (Nikl *et al*., 1993) and rainbow trout (Siwicki *et al*., 1994). Three commercial β-glucans were administered to gilthead seabream (Couso *et al*., 2003). All glucans and doses led to higher resistance against *Photobacterium damselae* while the effects were more distinct with high supplementation. A subsequent study was conducted with the *S. cerevisiae* β-glucan with the same feeding scheme twice in a row and dosages of 0.1, 0.5 and 1%; interestingly, the mortality caused by *P.damselae* was reduced with 0.1%, unaffected with 0.5% and even raised with 1% β-glucan compared to control groups. Higher survival rates after *S. cerevisiae* β-glucan administration have been reported in hybrid striped bass (Li *et al*., 2009), large yellow croaker (Ai *et al*., 2007) and channel catfish (Welker *et al*., 2011) upon challenges with *Streptococcus iniae*, *V. harveyi* and *E. ictaluri*, respectively.

Anti-viral properties of barley and *S. cerevisiae* β-glucans have been shown when rainbow trout challenged with the infectious haematopoietic necrosis virus (IHNV) displayed higher survival rates (Sealey *et al*., 2008).

More recently, positive effects of β-glucans were also revealed in parasite challenge studies. Rainbow trout infected with *Ichthyophthirius multifiliis* had lower infection rates when they were fed diets supplemented with high levels of paramylon (5%) from the algae *Euglena gracilis* (Jafaar *et al*., 2011) or yeast β-glucan (Lauridsen and Buchmann, 2010). Simultaneously, in both studies an increase in blood lysozyme activity occurred, possibly explaining the reduction in infection rates. Furthermore, dietary yeast β-glucans had distinctly reducing effects on the formation of *Loma salmonae* xenomas on the gills of rainbow trouts (Guselle *et al*., 2010) and *dactylogyrid monogeneans* infection rates in spotted rose snapper (*Lutjanus guttatus*) (Rio-Zaragoza *et al*., 2011).

β-glucans also revealed their positive effects when studies reproduced stressful situations common to aquaculture practise (Jeney *et al*., 1997; Volpatti *et al*., 1998). Rainbow trout having received β-glucans over four weeks prior to transportation stress displayed a minor reduction in innate immune parameters (respiratory burst, phagocytic activity, lysozyme) compared to control fed fish.

Where examined in those studies as well as in other non-challenge studies, enhanced activities of important parameters related to innate immunity were detected. β-glucans caused higher phagocytic activities of blood leukocytes in rainbow trout (Siwicki *et al*., 1994; Jeney *et al*., 1997; Volpatti *et al*., 1998) and spleen/pronephros macrophages in pike perch (Siwicki *et al*., 2009) and large yellow croaker (Ai *et al*., 2007), respectively. An increase of respiratory burst activity was observed in spleen phagocytes of gilthead seabream (Couso *et al*., 2003) and pikeperch (Siwicki *et al*., 2009) and pronephros macrophages of large yellow croaker (Ai *et al*., 2007) and blood leukocytes of rainbow

trout (Jeney *et al*., 1997; Volpatti *et al*., 1998). A higher neutrophil oxidative radical production in whole blood occurred in the studies of Siwicki *et al*. (1994) and Li *et al*. (2009), although no effects were seen on the respiratory burst activity of pronephros macrophages and blood serum lysozyme in Li *et al*.'s (2009) study. Furthermore, bactericidal and myeloperoxidase activity were enhanced in the study of Siwicki *et al*. (1994) in blood leukocytes without changes in haematocrit and total leukocyte levels. Also enhancing effects on blood serum lysozyme have been reported (Bagni *et al*., 2005; Ai *et al*., 2007; Siwicki *et al*., 2009; Welker *et al*., 2011). Alternative complement pathway activity was enhanced after short-term administration in sea bass (Bagni *et al*., 2005) but not after long-term administration in sea bass (Bagni *et al*., 2005) and large yellow croaker (Ai *et al*., 2007).

Other studies however led to contradictory results in challenge studies. Dalmo *et al*. (1998) reported that orally administered laminaran could not protect Atlantic salmon upon challenges with *A. salmonicida* and *V. salmonicida* when the diets were fed for 10 days prior to challenging. A *S. cerevisiae* β-glucan administered for 5 weeks had also no effect on survival rates of turbots challenged with *V. anguillarum* (deBaulny *et al*., 1996). A *S. cerevisiae* β-glucan supplemented to diets for Nile tilapia and fed for 14 weeks had no effect on survival rates when the fish were challenged with *S. iniae* after week 12 (Whittington *et al*., 2005). A series of three experiments included the administration of two *S. cerevisiae* β-glucans to Nile tilapia in varying concentrations, stocking densities and durations (2 or 4 weeks) (Shelby *et al*., 2009). Challenges with *S. iniae* and *E. tarda* did not reveal any protection. The same products fed for 4 weeks followed by 2 weeks of a control diet to channel catfish did not improve survival rates in a subsequent challenge with *E. ictaluri* (Welker *et al*., 2007).

Simultaneously, the measured innate immune parameters in those studies were in most instances not enhanced through dietary β-glucan supplementation. Total blood

leukocyte cell counts remained unaffected in channel catfish after four weeks of feeding (Welker *et al*., 2007) but were increased in turbot at days 8 and 21 after five weeks of feeding (deBaulney *et al*., 1996). No effects were seen on bactericidal and respiratory burst activity of blood leukocytes (Welker *et al*., 2007). Furthermore, lysozyme activity did not change in the studies of deBaulny *et al*. (1996), Shelby *et al*. (2009) and Welker *et al*. (2007); Whittington *et al*. (2005) reported the same and, beyond that, even a decline in the highest β-glucan group two weeks after the challenge. Complement activities were not affected by dietary β-glucans (deBaulny *et al*., 1996; Welker *et al*., 2007; Shelby *et al*., 2009).

1.7.3.3.2 Cyprinids

A few studies have investigated the effects of orally administered β-glucans on disease resistance and immune response in cyprinids. β-glucans were able to increase survival rates of carp (Kwak *et al*., 2003; Gopalakannan and Arul, 2010) and rohu (Misra *et al*., 2006) upon challenges with *A. hydrophila* and survival rates of rohu (Misra *et al*., 2006) upon a challenge with *E. tarda*. In these studies a simultaneous enrichment in total blood leukocyte counts (Misra *et al*., 2006; Gopalakannan and Arul, 2010) and macrophages/neutrophils (Kwak *et al*., 2003) occurred. Respiratory burst (Misra *et al*., 2006; Gopalakannan and Arul, 2010) and phagocytic activities (Kwak *et al*., 2003; Misra *et al*., 2006) as well as alternative complement pathway and bactericidal activity (Misra *et al*., 2006) were improved. Furthermore, serum lysozyme activities were augmented (Kwak *et al*., 2003; Misra *et al*., 2006; Gopalakannan and Arul, 2010). In one study however (Selvaraj *et al*., 2005), carp did not show increased survival upon an *A. hydrophila* challenge. Strikingly, the simultaneously observed unaffected cellular and humoral immune responses (total and differential blood leukocyte counts, respiratory burst, bactericidal, classical and alternative complement pathway activities) could explain the non-responsiveness in survival rates.

Table 1.4: Reported immunostimulating and disease resistance effects of β-glucans after oral delivery in cyprinid aquaculture species

Species	β-glucan type	Feeding regime	Effects	Reference
Cyprinus carpio	*S. cerevisiae* β-glucan	1, 2 and 4%	→ survival against *A. hydrophila* → total and differential blood leukocytes → bactericidal activity and respiratory burst → classical and alternative complement pathway ↑ slight increase of the antibody titre against *A. hydrophila*	Selvaraj *et al*. (2005)
Cyprinus carpio	*S. uvarum* β-glucan	1%	↑ survival against *A. hydrophila* ↑ total blood leukocytes and respiratory burst ↑ serum lysozyme activity	Gopalakannan and Arul (2010)
Labeo rohita	Barley β-glucan	0.01, 0.25 and 0.5%	↑ survival against *A. hydrophila* and *E. tarda* ↑ respiratory burst, phagocytic activity, lymphokine production index ↑ lysozyme, alternative complement & bactericidal activity ↑ total blood leukocytes, serum protein, globulin and albumin:globulin ratio ↓ serum glucose ↑ antibody titre against *E. tarda*; → antibody titre against *A. hydrophila*	Misra *et al*. (2006)
Cyprinus carpio	Schizophyllan	0.1%	↑ survival against *A. hydrophila* ↑ proportion of blood macrophages and neutrophils ↑ phagocytic activities and lysozyme	Kwak *et al*. (2003)
Tinca tinca	*S. cerevisiae* β-glucan	0.05, 0.1 and 0.2%	↑ survival against *A. hydrophila* ↑ respiratory burst and phagocytic activity ↑ proliferative response of lymphocytes ↑ serum lysozyme and Ig levels; → total serum protein, ceruloplasmin	Siwicki *et al*. (2010)
Cyprinus carpio koi	*S. cerevisiae* β-glucan	0.09%	↑ survival against *A. veronii* ↑ total blood leukocytes, respiratory burst, phagocytic & SOD activity ↑ lysozyme; → alternative complement pathway	Lin *et al*. (2011)

1.7.4. Effects of β-glucans on immunocompromised fish

An interesting question is how β-glucans influence the immune response in immuno-compromised organisms, that is, what effect does an immunostimulant have on an already challenged immune system? Only a few studies have been conducted with fish so far by "artificially" inducing a compromised immune system, using injections of cyclophosphamide (Kumari and Sahoo, 2006 a, b), mercuric chloride (El-Boshy *et al*., 2010) or aflatoxin B_1 (Sahoo and Mukherjee, 2001; 2002).

Two studies of Kumari and Sahoo with immunocompromised Asian catfish (*Clarias batrachus*) reported enhancing β-glucan effects on cellular and humoral innate immune responses (2006a) as well as on the adaptive immune response and the survival rates of vaccinated and non-vaccinated fish after an *A. hydrophila* challenge (2006b). Similarly, El-Boshy *et al*. (2010) observed significant increases of cellular and humoral innate immune responses and survival rates upon an *A. hydrophila* challenge in Nile tilapia fed β-glucans. Dietary β-glucans also increased innate immune responses and survival rates upon an *A. hydrophila* challenge in immunocompromised rohu, but no effect on adaptive immune responses was observed (Sahoo and Mukherjee, 2001). A similar, subsequent study however demonstrated augmented adaptive immune responses and survival rates of vaccinated and non-vaccinated immunocompromised rohu upon an *E. tarda* challenge (Sahoo and Mukherjee, 2002).

It therefore seems that dietary β-glucans are able to realize in immunocompromised fish the same positive effects regarding immune response and disease resistance as in healthy fish. But what are the effects of β-glucans on fish where naturally occurring diseases (being the reason for immunosuppression) are already manifest prior to the administration of the β-glucan? There is a lack of studies in this respect and there may be differences in immune responses and disease resistance compared to an artificially induced immunosuppression.

1.7.5 Use of β-glucans as adjuvants

The word adjuvant originates from the Latin word *adiuvare* which means *to aid*. By definition, "an adjuvant is a substance that helps and enhances the pharmacological effect of a drug or increases the ability of an antigen to stimulate the immune system". The aim is to increase efficacy and potency of the vaccine. They activate antigen-presenting cells (e.g. macrophages) and stimulate those to generate more of the signal molecules (e.g. cytokines) which activate other immune cells (Raa, 2000). β-glucans have the potential to act as such without causing the problems like oil emulsion based adjuvants, e.g. tissue damage or skeletal deformities (Dalmo and Bøgwald, 2008).

Early studies reported that injection of β-glucans together with *A. salmonicida* vaccines enhanced survival rates of coho salmon (*Oncorhynchus kisutch*) (Nikl *et al*., 1991) and Atlantic salmon (Rørstad *et al*., 1993) upon challenges with *A. salmonicida*. In contrast, the mortality of turbot upon a *V. anguillarum* challenge did not differ when an orally administered anti-vibriosis vaccine was given alone or adjuvanted with β-glucan (deBaulny *et al*., 1996).

In cyprinids, adjuvant effects of β-glucans on survival and immune responses have been reported in carp (Selvaraj *et al.*, 2006) and catla (Kamilya *et al*., 2006b) upon challenges with *A. hydrophila*. Moreover, Selvaraj *et al.* (2006) observed that the adjuvanticity depends on the administration route. Survival and immune responses were highest with ip injection even at low dosages and oral delivery required medium to high dosages. Bathing however did not enhance any of the analysed parameters and survival rates. Also, some immune parameters (IL-1β mRNA expression, classical and alternative complement pathway) were not affected independent of the delivery route.

Adjuvants are generally administered alongside the vaccine. Anderson *et al*. (1989) reported that the administration of levamisole prior to the vaccine suppressed the immune responses of rainbow trout against *Y. ruckeri*, mainly at high dosages. Time-

dependent adjuvanticity was studied when a *S. cerevisiae* β-glucan was given to turbots before, simultaneously and after the *V. damsela* vaccine (Figueras *et al*., 1998). Immune responses including antibody titres were higher when the glucan was given afterwards.

1.7.6. Administration strategies for use in aquaculture

As mentioned beforehand, the ip injection seems to be most effective in administering β-glucans; immersion appears to be less successful as fish are probably not capable of absorbing particulate β-glucans (Dalmo and Bøgwald, 2008) which are insoluble in water. But the oral delivery would be the easy and more cost-efficient way, if they are incorporated in the diets and given in the daily feeding process. There are two recognized strategies of oral delivery of any immunostimulant (Fig. 1.10), the continuous feeding or the pulse feeding (Bricknell and Dalmo, 2005). By continuous feeding the immune response can be enhanced and maintained until the immunostimulant delivery is stopped or after initial enhancement adverse effects occur and the immune response drops (possibly to even lower levels compared to fish fed no immunostimulant). This can occur through an acquired tolerance induced by a desensitisation of the immune system and even lead to an immune-suppression. A pulsative administration in 4-6 week intervals could potentially avoid this and can be applied for stressful periods with higher disease risks like seasonal temperature changes, transportation, prior to the breeding season, sea-water transfer of smolts, fishing dry of ponds, etc.

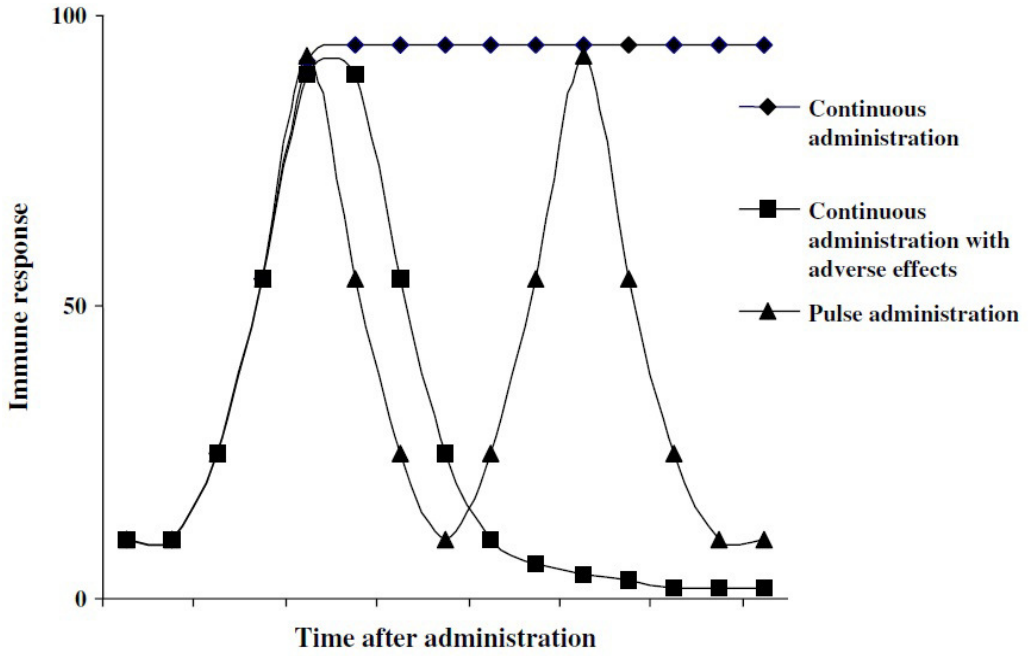

Figure 1.10: Possible outcomes after immunostimulant administration to fish (from Bricknell and Dalmo, 2005)

1.8 Recent advances in β-glucan research in cyprinids

Several recent studies gave insights into new aspects of β-glucan research in cyprinids, e.g. comprehensive gene expression studies relating to immune responses, first-line-of-defence-mechanisms and apoptosis, or the recently discovered neutrophil extracellular traps.

Namely, it has been shown that β-glucans protect neutrophil extracellular traps (NETs) against bacterial degradation by *A. hydrophila* nuclease in common carp (Brogden *et al*., 2012). This could possibly contribute to the frequently observed enhancing effect of β-glucans on survival rates in *A. hydrophila* challenge studies described in the previous paragraphs.

Miest *et al*. (2012) hypothesized that the known elevating effect of β-glucan on the production of reactive oxygen species may affect apoptosis. But the authors conclude from their studies that dietary β-glucan alone or combined with other pathogenic factors did not induce significant apoptosis in common carp immune organs.

Falco *et al*. (2012) proposed that the gene expression of pro-inflammatory cytokines may be down-regulated by dietary β-glucans in carp intestine. Similar results have been reported in rainbow trout spleen (Djordjevic *et al*., 2009). Furthermore, when carp were challenged with a non-lethal *A. salmonicida* infection, the gene expression of pro-inflammatory cytokines shortly after infection was overall down-regulated in the intestine but up-regulated in the pronephros (Falco *et al*., 2012). The authors suggest prevention from acute, possibly detrimental responses in the gut and increase of inflammatory responses in the pronephros.

β-glucans also influenced the mucosal systems of epithelial surfaces in carp (van der Marel *et al*., 2012). A significant increase of the gene expression of antimicrobial peptides, β-defensin 1 in skin and β-defensin 2 in skin and gills, occurred upon dietary β-glucan administration; yet, expression remained unaffected in the first and second intestinal segment. Furthermore, the β-glucans caused changes in the expression of mucins, the main components of the mucus covering the epithelial surfaces. The gene Muc5B was higher expressed in skin (significantly) and gills (non-significantly), respectively, whereas expression was unaffected in either intestinal region. Muc2 however was slightly reduced in both intestinal segments and in gills and unchanged in skin. Overall, this suggests a contribution to improved first-line-of-defence-mechanisms by dietary β-glucans.

Pionnier *et al*. (2013) observed enhancing effects of dietary β-glucans on C-reactive protein levels and alternative complement activity in serum of both healthy and *A. salmonicida* challenged carp. The expression pattern of the related genes displayed an organ and time dependent profile during infection stages: early on, an up-regulation of the gene expression was observed in pronephros followed by an up-regulation in the liver. Interestingly, in mid-gut the expression was up-regulated after 24 h but then down-regulated after 72 h.

1.9. Conclusions and aims of the study

As described in this literature review, numerous studies have shown that β-glucans are able to enhance growth performance, elicit immune reactions and improve disease resistance in aquatic species. It is also obvious, that beneficial results are not always observed and issues of reproducibility and consistency exist. Common to the studies investigating the effects of a dietary β-glucan administration so far is that they used semi-purified diets or even industrial diets. Thus, it is likely that substances with immunostimulating properties other than the supplemented β-glucans, e.g. nucleotides in fish meal or β-(1,3)(1,4)-D-glucans in cereal products, are present through the ingredients used. Consequently, this effect is likely to vary considerably between studies and, furthermore, the possibly resulting immunostimulating effects could potentially influence or disguise the actual immunostimulating effects of the supplemented β-glucan. The key scientific approach of this study was the use of highly purified basal diets in order to ascertain to the highest possible degree that the observed effects are deducible from the supplemented β-glucans. These diets, consistently used in all experiments, were supplemented with MacroGard®, a commercially available ß-(1,3)(1,6)-D-glucan derived from the yeast *Saccharomyces cerevisiae*.

The overarching aim of the study was to determine the effects of the orally administered MacroGard® on growth and intestinal functionality in mirror carp (*Cyprinus carpio* L.) (Chapters 3&4), and if an enhanced disease resistance can be achieved in zebrafish (*Danio rerio*), also a cyprinid, through direct effects or indirect effects (Chapter 5).

In summary of the above literature review, it is evident that there are still gaps in knowledge regarding β-glucan research in aquatic species. Even though there is information on effects of dietary β-glucan supplementation on growth performance, this information is, generally in teleosts, inconsistent and, specifically in carp, very scarce. The aim of the 1st experiment (Chapter 3) therefore was to evaluate the efficacy of

MacroGard® on growth performance and nutrient retention of mirror carp. In order to assess the fishes' health status and physiological response towards the supplemented ß-glucan, general haemato-immunological indices were determined at the end of the experiment. In addition, as little is known about localized intestinal immune responses in teleosts upon contact of orally administered ß-glucans and the mucus-layer/mucosa, another objective was to examine whether intestinal morphology and intestinal immune response were affected by dietary provision of ß-glucans.

Further important aspects increasingly having gained attention in aquaculture over the last decade are the intestinal microbiota and their interactions with the host, with dietary components and among each other. Surprisingly, to date almost no information however is available whether dietary β-glucans have an impact on these microbial communities (and/or vice versa) in aquatic species and, in consequence, possibly affect this network of interactions. The 2nd experiment (Chapter 4) therefore aimed at assessing the effects of dietary MacroGard® supplementation on the intestinal microbial communities in mirror carp. Moreover, little is known about β-glucan effects on the ultrastructure of the apical brush border of the enterocytes. Since possible alterations of the intestinal microbiota however may influence physiological processes in the mucosa, a further objective of the 2nd experiment was to also examine this aspect.

A plethora of dietary β-glucan studies have reported augmented disease resistance of aquatic species against various pathogens. Understandably, challenges are generally applied during or at the end of β-glucan administration. But an interesting question is if β-glucans influence the progression of a naturally developed disease existent in the fish prior to ß-glucan administration. Since literature is scarce in this regard and challenge tests could not be performed with mirror carp, the 3rd experiment (Chapter 5) dealt with the effects of dietary MacroGard® on survival rates, histopathology and intestinal microbiota of zebrafish suffering from a pre-existing, chronic mycobacteriosis.

Chapter 2:

General Methodology

2.1. Overview

The subsequently described general experimental conditions and analytical techniques were essential for the experiments conducted during this study. Further methods and techniques specific to individual experiments are described in their respective methodology sections. All experiments were carried out under the Home Office project licence number 30/2644 and personal licence number 30/9104 under the Animal Scientific Procedures Act of 1986.

2.2. Rearing facilities and water quality

All experiments with carp were undertaken in the freshwater recirculating system "B" within the aquarium facilities of the Aquatic Animal Health and Nutrition Research Group of Plymouth University. The total volume of this system accounts for approximately 3900 litres with each of the 18 polyethylene tanks having a capacity of 71 litres each (Plate 2.1). The system has been fed with municipal freshwater, and 99% recirculated aerated freshwater was provided at a rate of 25 litres per hour.

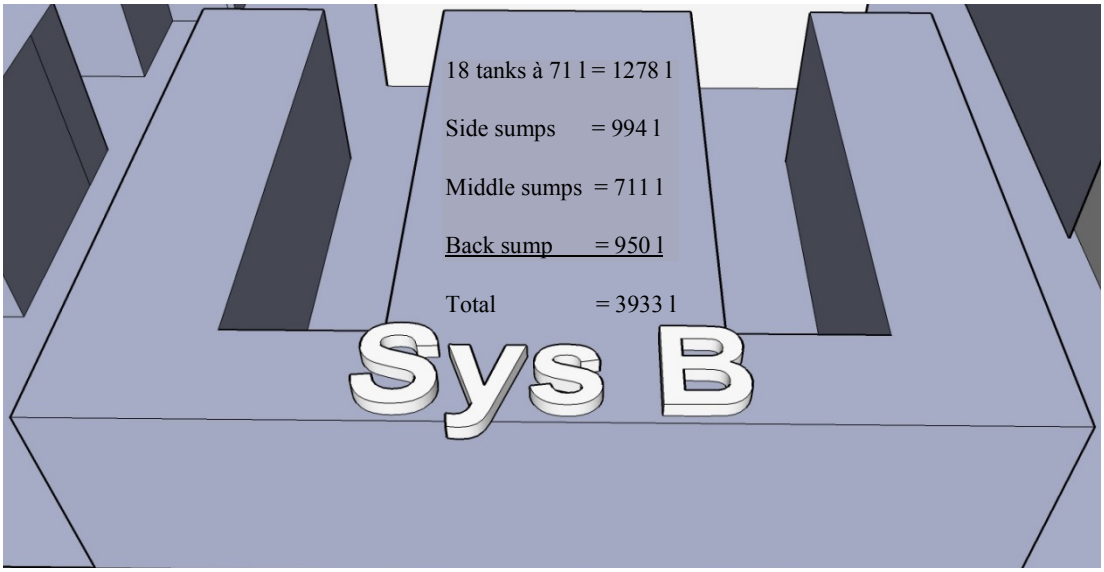

Plate 2.1: Outline of system "B" with technical data of the water holding capacity

Effluent water from the tanks was treated in three steps: a mechanical filtration using layers of different foam mattings and gauze, a biological filtration in which ammonia is oxidized over nitrite to nitrate, and a chemical filtration using UV-light. Mechanical filters were cleaned daily and a partial water exchange (about 10%) was done once a week in order to avoid accumulation of nitrogenous compounds. With the aid of a heating unit the average water temperature was maintained at 24 – 25°C. Water quality was monitored by daily measuring temperature, oxygen saturation and pH-value using an electronic meter (Hach HQ40d). Whenever necessary, pH-values were regulated by adding sodium bicarbonate ($NaHCO_3^-$) to the water circulation. Ammonium, nitrate and nitrite were recorded once a week with Hach Lange GmbH vials (Ammonia 0.015-2.0 mg/l, Nitrite 0.015-0.6 mg/l, and Nitrate 5-35mg/l) and a photo spectrometer (Hach Lange DR2800). Their content in the system was kept below the following limits: 0.1 mg/l for ammonia; 1 mg/l for nitrite and 50 mg/l for nitrate. An automatically controlled photoperiod was set to a 12/12 h light/dark cycle using fluorescent tubes installed above the tanks. Plate 2.2 shows Senior Technician Ben Eynon feeding carp in tank 5 of system “B”.

Plate 2.2: Feeding carp during the acclimatization period in tank 5 of system “B”.

2.3. Experimental fish and feeding

All mirror carp were delivered from Hampshire Carp Hatcheries, Hampshire, UK. The fish, weighing about 7 g on average, were carefully acclimatized over approximately six weeks feeding the control diet at a level of 2% of their body weight (BW) spread across three meals a day. A preliminary pre-grading was undertaken after three weeks and the main grading prior to the experiment start took place after six weeks. During the experimental period the carp were fed by hand at a fixed rate of 4% of their BW over four meals (8:30, 11:00, 14:00 & 17:00) for five days a week. On the morning of the sixth day 1% of BW was given to allow a sufficient starving period of 24 hours prior to weighing on the seventh day. After weighing, carp were fed 2% of the BW in the afternoon. The body weight was determined weekly as whole tank body mass. Afterwards the feed amounts for the following week were calculated based on those measurements.

2.4. Measurement of growth related parameters

Important parameters for the measurement of growth of the fish are the mean body weight *BW* (g), the weight gain *WG* (g), the specific growth rate (%) and the feed conversion ratio *FCR* (g/g). The mean *BW* was determined by dividing the total body mass *BM* in each tank by the number of carp (*n*).

$$BW\ (g) = BM \div n$$

The *WG* per fish was calculated by subtracting the initial weight *Wi* from the final weight *Wf*:

$$WG\ (g) = Wf - Wi$$

The specific growth rate (SGR) describes the percentage weight gain per day within a certain period; it depends on the initial weight (*Wi)*, the weight (*Wt*) after a certain period and the amount of days (*t*) in this period. Positive values stand for weight gain, negative values for weight loss (*LN* = natural logarithm):

$$SGR\ (\%) = [LN(Wt) - LN(Wi)] \div t \times 100$$

The feed conversion ratio *FCR* (g/g) reflects to what extent the ingested feed (feed intake, *FI*) is converted into growth; it tells how much of this particular feed (in g) is needed to gain one g in weight:

$$FCR\ (g/g) = FI \div WG$$

The protein efficiency ratio (*PER* [g/g]) reflects the ability of the ingested dietary protein (= protein intake *PI*) to sustain growth, i.e. it measures the conversion of this protein into growth by means of the body weight:

$$PER\ (g/g) = WG \div PI$$

2.5. Composition of experimental diets

A nutritionally balanced basal diet fulfilling the requirements of carp was prepared by TETRA GmbH (Melle, Germany). Main ingredients were fish protein concentrate (Soprapeche CPSP 90) containing at least 83% crude protein and at most 10% lipids, and wheat starch (not less than 84% starch). The aim was the production of a highly purified diet with minimal immunologically active substances to avoid any unwanted effects originating from other sources than the β-glucan (MacroGard®). Among other possible reasons contamination could occur through the used ingredients. Therefore the relevant ingredients were subjected to immunological *in vitro* tests with carp phagocytes

such as respiratory burst (NBT) and nitric oxide assay as well as a cell viability test. The tests confirmed on the one hand, that MacroGard® seems to be a good immune stimulator at a concentration of 10 µg/ml or more, on the other hand the fish protein did not cause any effects. Ascorbic acid showed moderate effects at a concentration of 10 µg/ml. Four isonitrogenous and isocaloric extruded diets were produced with the basal diet by supplementing 0% (control diet), 0.1%, 1% and 2% w/w MacroGard®, a β-(1,3)(1,6)-D-glucan derived from the yeast *S. cerevisiae*. Table 2.1 shows the composition of the basal diet and the proximate analysis of the four experimental diets. Pellet size was 2.5 mm. Diets were stored in polythene bags and refrigerated at 4°C throughout the experiment.

2.6. Proximate analysis of diets and fish carcasses

Samples of the four diets as well as of fish were taken for a comprehensive chemical analysis in order to determine important parameters of nutrient metabolism at the end of the experiment. With regards to the fish 12 carp (pooled into four samples) from the stock were sampled for initial body composition at the experiment start. Similarly, at the end of the trial three carp per tank were sampled and pooled together for the final body composition. All samples were analysed according to AOAC protocols (2003).

2.6.1. Moisture

In order to examine the moisture content of the diets, ~ 6 g of the finely grounded material was placed into a crucible and put in a drying oven at 105°C until a constant weight was achieved (Gallenkamp Oven BS, OV-160; UK). The ratio of the sample weight after and prior to drying leads to the moisture content:

$$Moisture\ (\%) = wet\ weight\ (g) - dry\ weight\ (g) \div wet\ weight\ (g) \times 100$$

Table 2.2: Formulations (g per 1000g) and chemical composition (%) of the experimental diets; M = MacroGard®

Ingredients	Control	0.1% M	1% M	2% M
Fish protein concentrate	450.0	450.0	450.0	450.0
Wheat starch	410.0	409.0	400.0	390.0
Fish oil	45.0	45.0	45.0	45.0
Soybean oil	45.0	45.0	45.0	45.0
Cellulose	25.65	25.65	25.65	25.65
Min/trace element premix *	20.60	20.60	20.60	20.60
Vitamin premix †	2.50	2.50	2.50	2.50
Stabilized vitamin C ‡	1.10	1.10	1.10	1.10
Ethoxyquin	0.15	0.15	0.15	0.15
MacroGard®	0	1	10	20
Chemical composition (%DM)				
Dry matter §	92.9 ± 0.0	95.1 ± 0.0	94.8 ± 0.0	95.0 ± 0.1
Crude protein ¶	41.4 ± 0.1	42.4 ± 0.3	43.7 ± 0.3	42.8 ± 0.2
Crude lipid §	14.2 ± 0.0	14.5 ± 0.1	15.0 ± 0.4	15.1 ± 0.3
Ash §	4.6 ± 0.0	4.5 ± 0.0	4.6 ± 0.0	4.6 ± 0.0
Energy (MJ/kg) **	22.3 ± 0.1	22.4 ± 0.0	22.5 ± 0.2	22.2 ± 0.1
Calcium (g/kg)	3.98 ± 0.04	3.98 ± 0.07	3.98 ± 0.05	3.98 ± 0.16
Phosphorus (g/kg)	6.51 ± 0.05	6.49 ± 0.06	6.67 ± 0.09	6.87 ± 0.25

* Mineral/trace element premix per kg feed meal (prior to extrusion):
20.0 g Mono-calcium-phosphate, 10.7 g Manganese-(II)-sulfate, 5.7 g Zinc sulfate,
4.1 g Ferreous-(II)-sulfate, 0.1 g Cobalt-(II)-acetate

† Vitamin premix per kg feed meal (prior to extrusion):
64800 IU Vitamin A, 2700 IU Vitamin D3, 144 mg Vitamin E, 75.6 mg Vitamin B1,
57.6 mg Vitamin B2, 216 mg Calcium-D-pantothenate, 720 mg Niacin, 29 mg Vitamin B6,
126 µg Vitamin B12, 900 mg Inositol, 1134 mg Ascorbic acid;

‡ min. 35 % Vitamin C activity

§ $n = 3$

¶ $n = 5$

** $n = 2$

The moisture content of the pooled initial and final fish samples took place in a very similar way. The fish were placed in a tray with the body cavity opened and the viscera exposed. Then the three pooled fish in each tray were proceeded in the same drying process as the diet samples. The dried samples were subsequently grounded in a blender for further analysis.

2.6.2. Crude protein

In order to determine the protein content, the nitrogen content of 100 mg of the sample was measured by the Kjeldahl method. After a digestion step, nitrogen was quantified by titration with concentrated sulfuric acid (H2SO4) in a distillation unit (Vapodest 40; Gerhardt Laboratory Instruments; Germany). The value was then multiplied by the factor 6.25 and divided by the efficiency factor (EF) of the process to calculate the protein content:

$$Crude\ protein\ (\%) = [nitrogen\ (\%) \times 6.25] \div EF$$

2.6.3. Crude lipid

Lipid contents were determined using the Soxhlet method. Thus, lipids were extracted from ~3 g of sample with petroleum ether in the Soxtherm apparatus (model 41x; Gerhardt Laboratory Instruments; Germany).

$$Crude\ lipid\ (\%) = [lipid\ weight\ (g) \div sample\ weight\ (g)] \times 100$$

2.6.4. Crude ash

Ash contents were determined by incinerating ~ 500 mg of sample, placed in a ceramic crucible, at 550°C in a muffle furnace (Carbolite ESF3; UK) for 8 hours.

$$Crude\ ash\ (\%) = [ash\ weight\ (g) \div sampleweight\ (g)] \times 100$$

2.6.5. Gross energy

Gross energy was measured with a Parr Adiabatic Bomb Calorimeter 1356 (Parr Instrument Company, Illinois, US). Briefly, the sample (~1 g pressed into a pellet) was precisely weighed and placed into a nickel crucible inside the bomb with a fuse wire attached to the pellet. The bomb was then put in exactly 2 kg of water inside the instrument, which measured the amount of heat energy released from the bomb by the increase in water temperature after firing the bomb.

2.6.6. Mineral analysis and net mineral retention

Mineral contents of the diets and fish were analysed using an inductively coupled plasma optical emission spectrophotometer (ICP-OES) in triplicates. An acid digestion was conducted by placing approximately 150-200 mg of the dehydrated and homogenized samples into 10% nitric acid cleaned (24 hours) Kjeldahl boiling tubes. Subsequently, 10 ml of 70% nitric acid were added and the solution heated in a Gerhardt Kjeldatherm 40 tube digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) according to the following protocol: 60°C for one hour, 90°C for one hour, 110°C for 30 minutes, 135°C-140°C for 4 hours. A clear, pale yellow colour of the solution confirmed a successful digestion and the samples, once cooled down to room temperature, were diluted to 50 ml with deionised water. The mineral contents were then determined using a Varian 725-ES ICP-OES. In order to reduce dilution errors, all samples were standardised to indium which was included as internal standard (1 mg/l). Standard solutions containing 0, 0.5, 1, 5 and 10 mg/l of calcium, phosphorus, magnesium, potassium, sodium, sulphur and 0, 0.05, 0.1 0.5 and 1 mg/l of copper, zinc, iron and manganese were used to generate standard curves.

Net mineral retention (NMR) was then calculated with the known final (BM_f) and initial (BM_i) concentrations of the respective mineral in the body and the concentration of the mineral in the diet (DM) using the following formula:

$$NMR\ (\%) = \{(Wf \times [BM_f] - Wi \times [BM_i]) \div (FI \times [DM])\} \times 100$$

2.7. Light microscopy

For histological appraisal of the intestine starved fish (n = 6 per treatment) were sampled and analysis was conducted using light microscopy. Intestinal samples of ~1 cm length were taken from the anterior and posterior intestine.

2.7.1. Sample preparation and paraffin wax embedding

Intestinal samples were fixed in 10% neutral-buffered formalin immediately after dissection. After 48 hours the samples were transferred to 70% IMS (industrially methylated spirit). Subsequently, samples were dehydrated in a series of graded ethanol concentrations in an automated tissue processor (Leica TP1020) according to standard histological protocols and embedded in paraffin wax (Leica EG1150 H).

2.7.2. Sectioning and Staining

From each wax block multiple sets of 5 µm cross-sections were cut with a microtome (Leica) and mounted onto glass slides. Prior to staining the sections were cleared with histolene, re-hydrated in a series of graded ethanol concentrations (100% twice, 90%, 70% and 50%) and rinsed in distilled water for 2 minutes. In each specimen, multiple sets of sections were stained with a standard haematoxylin and eosin (H&E) stain. Finally, sections were cleared in histolene and mounted with a cover slip and DPX. Slides were examined under an Olympus light microscope, photographed with an Olympus digital camera and processed using Corel Photo-Paint X5.

2.8. Culture-independent microbiology

For evaluation of the microbial population allochthonous (digesta) and autochthonous (mucosal tissue) samples of the intestine were taken. Under aseptic conditions the body cavity was opened, the whole intestine excised and the digesta removed. The mucosal tissue samples were washed thoroughly three times with phosphate-buffered saline and homogenized in a macerator. All samples were stored at -20°C in sterile molecular grade 1.5 ml microcentrifuge tubes until further analysis.

2.8.1. DNA extraction

DNA was extracted from 200 mg of digesta and mucosal tissue samples using the QIAamp® Stool Mini Kit (Qiagen, Crawley, UK) with slight modifications of the manufacturer's instructions. A pre-treatment was included by incubating the samples with 500 µl of lysozyme (50 mg/ml in TE buffer) for 30 min at 37°C as described by Merrifield *et al*. (2009a) in order to enhance the lysis of the robust cell walls of gram positive bacteria. Four key steps characterize the extraction process: lysis, inhibitor removal, protein removal and purification.

Firstly, 750 µl of buffer ASL was added to the sample incubated with lysozyme, vortexed for 1 min and incubated for 10 min at 90°C. Then the sample was vortexed again and centrifuged for 1 min at 20.000 x *g*. 700 µl of supernatant were transferred to a new, sterile microcentrifuge tube and half an Inhibitex tablet was added (removal of inhibitors). The sample was vortexed immediately for 1 min and then left to stand for 1 min at room temperature. Subsequently, the sample was centrifuged at 20.000 x *g* for 6 min, the supernatant transferred into a new, sterile microcentrifuge tube and centrifuged again for 4 min. Then 300 µl of supernatant were transferred into a new, sterile microcentrifuge tube and 17 µl of Proteinase K as well as 300 µl of buffer AL were added (protein removal). The sample was vortexed for 15 sec and incubated for 15 min

at 70°C. Afterwards 300 µl of molecular grade 100% ethanol were added and the sample vortexed. For the purification step 600 µl of the sample was applied to a QIAamp spin column in a 2 ml collection tube and centrifuged for 1 min. The spin column was then transferred to a new collection tube, 500 µl of Buffer AW1 were added and the sample centrifuged for 2 min. The spin column was again transferred to a new collection tube, 500 µl of Buffer AW2 were added and the sample centrifuged for 5 min. Finally the spin column was again transferred to a new collection tube, 200 µl of Buffer AE were added and the sample was left to stand for 5 min. Then the sample was centrifuged for 3 min to elute the DNA into the collection tube. Subsequently, the sample was transferred to sterile microcentrifuge tubes and stored at 4°C until further analysis.

2.8.2. 16S rRNA amplification

The extracted DNA templates were subjected to a Polymerase Chain Reaction (PCR) for amplification of the V3 region of the 16S rRNA gene. The forward primer P3 (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGG AGGCAGCAG-3') which includes an additional 40 nucleotide-rich GC-sequence (GC-clamp) on its 5'-end, and the reverse primer P2 (5'-ATTACCGCGGCTGCTGG-3') from Muyzer *et al*. (1993) were used. Each single PCR reaction consisted of 25 µl ReadyMix^{TM} Taq PCR Reaction Mix with $MgCl_2$ (Sigma-Aldrich Company Ltd., Gillingham, England), 1 µl each of primer P2 and P3 (50 pmol/µl Eurofins MWG Operon, Ebersberg, Germany), 1 µl of DNA template and sterile, molecular grade water to adjust the final volume of the reaction to 50 µl. This yielded in a final concentration of 1.5 U Taq DNA polymerase, 10mM Tris-HCl, 50mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatine and 0.2mM dNTPs. The samples including a negative (sterile, molecular grade water as template) and a positive control (cultured *Arthrobacter globiformis* as

template, Microbiology Laboratory culture collection, Plymouth University, UK) then were run through a PCR reaction using a GeneAmp® PCR system 9700 (Perkin-Elmer, CA, USA) with the following parameters: touchdown thermal cycling at 94°C for 5 min and then 20 cycles of 94°C for 1 min, 65°C for 1 min (decreasing 1°C every 2nd cycle) and 72°C for 1 min; this was followed by 15 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. A final extension for 5 min at 72°C was performed. In order to ascertain a successful PCR amplification 8 µl of the PCR product mixed with 3 µl loading buffer was subjected to electrophoresis at 80 V for approximately 40 min on a 1.5% agarose gel containing 1 µl SYBR Safe DNA gel stain (Invitrogen, Carlsbad, US) per 10 ml of molten agarose. A Hyper ladder IV (Bioline, London, UK) and the negative/positive control (as described above) were run alongside the samples to identify the correct amplification of the V3 region (233 bp) or possible contaminations.

2.8.3. Denaturing gradient gel electrophoresis

A denaturing gradient gel electrophoresis (DGGE) was conducted with the PCR products (standardized to 40 ng/µl) using a DCode Universal Mutation Detection System (Bio-Rad laboratories, Italy). With the aid of a Bio-Rad mode 475 gradient delivery system (Bio-Rad laboratories, Italy) an 8% polyacrylamide gel (16 cm x 16 cm x 1 mm) was poured with a denaturing gradient of 40 – 60% (where 100% denaturant is 7 M urea and 40% formamide) and left to set for 2 hours. Plate 2.3 (left image) depicts the pouring of such a DGGE-gel. 20 µl of PCR product were mixed with 5 µl of loading buffer (4 ml glycerol, 6 ml 1x TE buffer, 25 mg bromophenol blue), and 20 µl of this mixture were run on the gel for 16 hours at 60°C and 65 V in 1x TAE buffer (66 mM Tris, 5 mM Na acetate and 1 mM EDTA). DGGE bands were visualized by staining the gel with 200 µl of 1 µl/10 ml SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Carlsbad, US) for 30 min. Finally, the gel was scanned in a Bio-Rad universal hood II (Bio-Rad

laboratories, Italy) and negative images were taken with the software Quantity One, version 4.6.3 (Bio-Rad laboratories, Italy) and optimized for analysis by enhancing contrast and greyscale. Bands of interest were selected for sequence analysis and samples were taken with molecular grade pipette tips comparable to a biopsy punch or excised with a sterile scalpel. Plate 2.3 depicts the excision of bands from a DGGE-gel under UV light. The DNA was eluted in 30 µl of molecular grade water at 4°C for at least 12 hours prior to re-PCR.

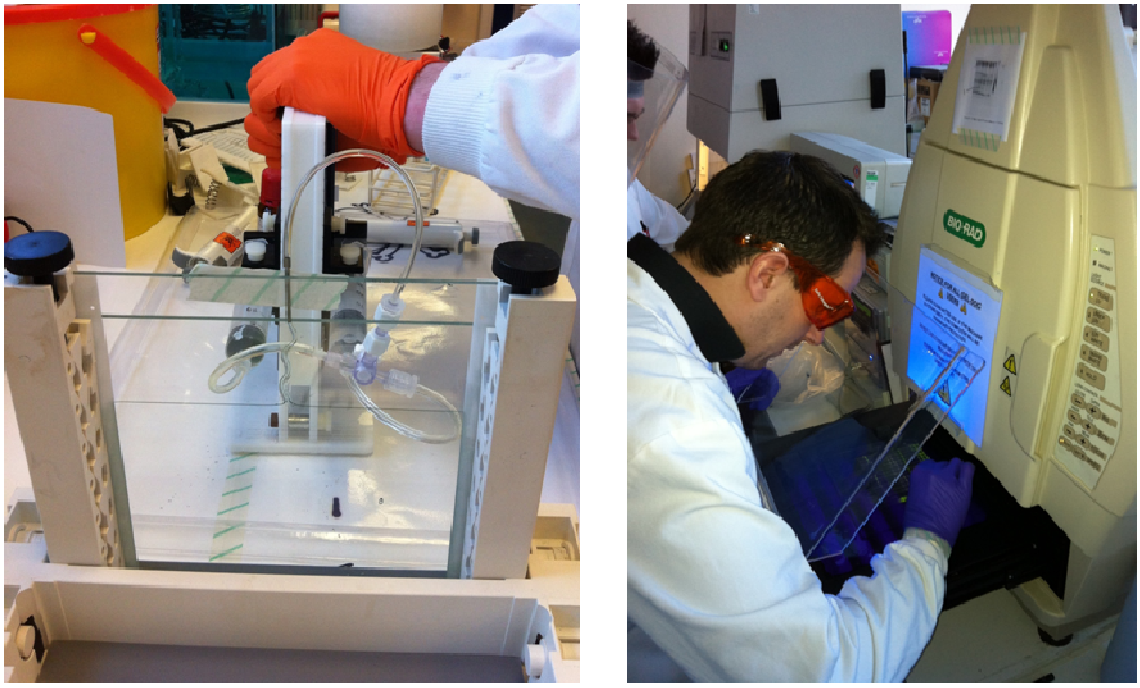

Plate 2.3: Pouring of a DGGE-gel with the Bio-Rad mode 475 gradient delivery system (left) and excising bands from a DGGE-gel under UV light (right).

2.8.4. 16S rRNA amplification of excised DGGE bands

The DNA template of the excised bands gained from the DGGE was subjected to a re-PCR using the same conditions as described under section 2.8.2 for further amplification. The only exception was that primer P1 was used instead of primer P3; primer P1 is the same primer as P3 but without the GC-clamp. Success of the PCR amplification was tested on a further agarose gel as described under section 2.8.2.

2.8.5. Purification of the PCR products and sequence analysis

Subsequently, 25 µl of the PCR product was purified using Diffinity Rapid Tips (West Henrietta, US) and then sequenced by GATC laboratories (GATC Biotech, Konstanz, Germany). The determined nucleotide sequences were submitted to a BLAST search in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to obtain the closest known alignment identities for the partial 16S rRNA sequences, hence retrieving taxonomic groups for which the sequences showed the highest similarities.

2.9. Statistical Analysis

Unless otherwise stated, all presented data are means ± standard deviation (SD) and the data were checked for homogeneity of variance and then subjected to a one way analysis of variance (ANOVA). Significant differences between control and treatment groups were determined by a post-hoc LSD (least significant difference) test and were accepted at the $P<0.05$ level. Statistical analysis was performed using SPSS statistics, version 18 (SPSS Inc., Chicago, US).

The images of the DGGE gels were subjected to a statistical analysis as described by Dimitroglou *et al*. (2009). The DGGE banding patterns were transformed into intensity matrices in order to evaluate similarities between treatments using the software Quantity one, version 4.6.3 (Bio-Rad Laboratories) after Schauer *et al*. (1992). Band intensities were measured and analysed using software Primer v6 (Clarke and Gorley, 2006) and similarity percentages (SIMPER) were performed. A 1-way analysis of similarity (ANOSIM) was used for pairwise comparison to determine differences between DGGE banding profiles (Abell and Bowman, 2005). Further tests were employed to investigate

changes in the intestinal microbiota and the results subjected to a one way analysis of variance (ANOVA) as described above:

- total number of OTUs (S)
- Margalef's species richness: $d = (S-1)/\log(N)$
- Pielou's evenness: $J' = H'/\log(S)$
- Shannon's diversity index: $H' = -\sum (p_i (\ln p_i)$

(N = total number of individuals (total intensity units), p_i = the proportion of the total number of individuals in the $_i$th species)

Chapter 3:

Effects of dietary β-(1,3)(1,6)-D-glucan supplementation on growth performance, intestinal morphology and haemato-immunological profile of mirror carp (*Cyprinus carpio* L.)

3.1. Abstract

In recent years aquaculture research has focused on probiotics, prebiotics, and β-glucans, in order to improve health status and growth performance. Information regarding the effects of β-glucan on growth performance and intestinal immunity of mirror carp (*Cyprinus carpio* L.) is scarce. An experiment was therefore conducted to investigate the effects of a yeast β-glucan preparation (MacroGard®) on growth performance, intestinal morphology and haemato-immunological indices of mirror carp. Carp (initial weight 11.1 ± 0.0g) were fed highly purified diets supplemented with 0% (control), 0.1%, 1% or 2% MacroGard® for 8 weeks.

Fish fed diets containing 1% and 2% MacroGard® exhibited significant improvements in weight gain, specific growth rate and feed conversion ratio compared to fish fed both the control and the 0.1% MacroGard® containing diet.

Histological appraisal of the intestine revealed a significantly higher infiltration of leukocytes into the epithelial layer of fish fed diets supplemented with 1% and 2% MacroGard® in the anterior intestine compared to fish fed the control and 0.1% MacroGard® diet. This effect was not observed in the posterior intestine. There were no significant differences in the intestinal absorptive surface area and number of goblet cells in either intestinal region.

At the end of the experiment the haematological status of the fish was examined. Compared to control fed fish, the haematocrit value was significantly elevated in fish fed the 2% MacroGard® diet. Furthermore, the blood monocyte fraction was significantly higher in fish fed the 1% and 2% MacroGard® diets. No significant changes were observed in the other blood parameters assessed.

The present study shows that high dietary β-glucan inclusion increases growth performance without detrimental effects on the health indicators assessed. Increased intraepithelial leukocytes in the anterior intestine may indicate a localized immune response; no detrimental effects on intestinal morphology were observed.

3.2. Introduction

Aquaculture remains the fastest growing agri-business sector with total global production (inland and marine, without aquatic plants) increasing to 63.6 million tonnes in 2011 from 47.3 million tonnes in 2006 (FAO, 2012). On a global scale cyprinid production is still the prominent sector of freshwater aquaculture (FAO, 2012), accounting for 71.9% (24.2 million tons) of freshwater production. The intensification of production can lead to increased incidences of stress and disease outbreaks. As a result a considerable effort has been made to find effective prophylactic measures to support and augment the immune response of fish reared under intensive conditions. Since the EU ratified a ban on non-medical use of antibiotics in animal nutrition in the Regulation (EC) No 1831/2003 (EU, 2003), effective from 2006 onwards, there has been a clear drive towards finding alternative dietary supplements to boost health and production.

Among the most well studied dietary additives are β-glucans. They are ubiquitous in nature and can be found in the cell walls of yeasts, cereal grains, algae, bacteria and fungi (Zekovic *et al*., 2005). The most abundant source of natural β-glucans with highly immuno-modulating properties are fungi and yeasts, where research effort has focused in particular on β-(1,3)(1,6)-D-glucans. A plethora of information is available regarding their positive effects on growth performance, immune responses and disease resistance upon oral delivery to a number of farmed animals such as swine (Dritz *et al*., 1995; Li *et*

al., 2006), poultry (Guo *et al*., 2003; Chen *et al*., 2008), salmonids (Nikl *et al*., 1993; Siwicki *et al*., 1994; Jeney *et al*., 1997; Lauridsen and Buchmann, 2010) and shellfish (Van Hai and Fotedar; 2009). For cyprinids it is also well documented that β-glucans can enhance the innate immune response and disease resistance (Kwak *et al*., 2003; Misra *et al*., 2006; Gopalakannan and Arul, 2010; Siwicki *et al*., 2010) but to the authors' knowledge the effects on growth performance are scarce.

The aim of the present investigation therefore was to evaluate the efficacy of a commercially available β-(1,3)(1,6)-D-glucan preparation from the yeast *Saccharomyces cerevisiae* (MacroGard®) on growth performance, nutrient retention and general haematological indices of mirror carp. As little is known about localized intestinal immune responses in teleosts upon contact of orally administered β-glucans and the mucus-layer/mucosa, another objective was to examine whether intestinal morphology and intestinal immune response was affected by dietary provision of β-glucans.

3.3. Material and Methods

3.3.1. Rearing facilities and water quality

The experiment was conducted as described under section 2.2 in system "B" within the rearing facilities of the University of Plymouth and water quality was monitored accordingly. Water temperature, oxygen saturation and pH were maintained at 25.0 ± 0.2°C, 89.1 ± 3.1% and 6.33 ± 0.48, respectively. Ammonium, nitrite and nitrate were determined once a week and the levels ranged between 0 - 0.281 mg/l, 0.004 - 0.084 mg/l and 8.15 - 52.92 mg/l, respectively.

3.3.2. Experimental fish and feeding

Four hundred and fifty mirror carp were obtained from Hampshire Carp Hatcheries, Hampshire, UK and carefully acclimatized as described under section 2.3. Thereafter, 25 carp were randomly allocated to each of the twelve 71L experimental tanks (n = 3 tanks per treatment). The initial average body weight at the start of the experiment was 11.14 ± 0.03g. Twelve of the remaining fish were sampled for analysis of the initial body composition. Feeding scheme and bulk weighing was conducted as described under section 2.3.

3.3.3. Measurement of growth related parameters

Growth related parameters were determined as described under section 2.4.

3.3.4. Proximate analysis of diets and fish carcasses

Proximate analysis of diets and fish carcasses was determined as described under section 2.6.

3.3.5. Net mineral retention

Net mineral retention in whole body was determined as described under section 2.6.6.

3.3.6. Blood sampling and general haemato-immunological parameters

At the end of the trial, blood was sampled from five fish per tank (n=15 per treatment). The fish were euthanized with tricaine methanesulfonate (MS-222; Pharmaq Ltd., Fordingbridge, UK), buffered with sodium bicarbonate ($NaHCO3^-$), followed by destruction of the brain (PIL № 30/9104 under PPL № 30/2644). Blood was withdrawn immediately from the caudal arch using a 1 ml syringe with a 25 gauge needle and transferred to 1.5 ml Eppendorf tubes.

3.3.6.1. Haematocrit

Blood samples were drawn into heparinised capillary tubes and sealed. The samples were centrifuged at 3600 *g* in a microhaematocrit centrifuge (Brown, 1988). After, the % packed cell volume (PCV) was read with a Hawksley reader and recorded as percentage of the total blood volume.

3.3.6.2. Haemoglobin

Drabkin's cyanide – ferricyanide solution was used to determine the haemoglobin levels in the blood (Drabkin, 1946). Four µl of blood sample was thoroughly mixed with 1 ml of Drabkin's solution (Sigma-Aldrich Ltd, Dorset, UK) and samples were stored at 4°C until further analysis. The haemoglobin contents were then determined by measuring the absorbance at 540 nm in a spectrophotometer and reading the values against a standard curve (derived from a dilution series from 0 - 180 mg/l of a cyanmethemoglobin standard).

3.3.6.3. Total blood cell counts

Twenty µl of blood sample were thoroughly mixed with 980 µl of Dacies solution (equal to a 1:50 dilution) and stored at 4°C until further analysis. Total erythrocyte and leukocyte cells counts were determined using a Neubauer haemocytometer.

3.3.6.4. Differential leukocyte counts

Blood smears were prepared by using 5 µl of blood according to Rowley *et al*. (1988). The slides were then left to air-dry and fixed in 100% methanol for 1 minute. Smears were then stained with the May-Grünwald-Giemsa (MGG) stain (Biostain Ready Reagents Ltd., Cheshire, UK) and rinsed with distilled water. Slides were air-dried at room temperature and mounted with DPX and a cover slip. Two hundred leukocytes per

slide were counted and classified according to their morphological appearance into lymphocytes, granulocytes and monocytes.

3.3.6.5. Serum glucose, total protein and albumin

In order to obtain serum, whole blood was incubated at 4°C for 12 hours in order to allow clotting. After, the samples were centrifuged at 2500 *g* for 5 minutes, the supernatant serum was carefully removed and the serum samples were stored at -80°C until further analysis. The levels of glucose in the serum samples were determined using the glucose oxidase – peroxidase method after Trinder (1969). Total protein levels in serum samples were determined according to the method of Bradford (1976). Albumin levels in serum were determined using the bromocresol green assay by Doumas *et al*. (1971). The albumin/globulin ratio was also determined for each respective group.

3.3.7. Intestinal histology

Histological appraisal of H&E stained sections of anterior and posterior intestine (consistently sampled from the middle of the respective region) was undertaken using light microscopy as described under section 2.7.1 in order to examine the intestinal perimeter ratio after Dimitroglou *et al*. (2009). Briefly, the internal perimeter (IP) of the intestinal lumen and the external perimeter (EP) of the intestine were analysed using Image J version 1.42 (National Institutes of Health) and the perimeter ratio was calculated (PR = IP/EP, arbitrary units, AU). The number of leukocytes (histologically stained cells) infiltrated into the epithelial layer (i.e. intraepithelial leukocytes, IELs) across a standardized distance of 100 enterocytes was determined as described by Ferguson *et al*. (2010). In addition, sections were also stained with Alcian blue-PAS (Periodic acid Schiff) in order to differentiate between acid and neutral glycoconjugates and to determine the number of goblet cells in five random mucosal folds per specimen which were then standardized to 100 μm of mucosal fold length. Briefly, sections were

stained with 1% Alcian blue 8GX (pH 2.5) for 60 minutes, rinsed with distilled water, stained with 0.8% periodic acid for 10 minutes, rinsed with distilled water three times for 3 minutes, stained with Schiff's reagent for 30 minutes, rinsed with distilled water and dehydrated in a series of graded ethanol concentrations (50%, 70%, 90% and twice 100%). For both the leukocyte and the goblet cell counts the average of the cell numbers from all specimens was calculated.

3.3.8. Statistical analysis

Statistical analysis was conducted as described under section 2.9.

3.4. Results

3.4.1. Growth performance

Carp displayed a high growth performance in all groups (Table 3.1) and no mortalities occurred during the experiment. After 8 weeks of feeding the final body weight increased by over 600%; the weight in groups fed with the 1% and 2% MacroGard® supplemented diet (69.0 ± 0.7g and 66.6 ± 1.9g, respectively) was significantly higher than the control (61.9 ± 2.4g; $P \leq 0.009$) and the 0.1% MacroGard® group (63.0 ± 1.2g; $P \leq 0.030$). Consequently, the weight gain of fish fed the 1% and 2% MacroGard® supplemented diets was significantly higher. The same was observed for the specific growth rates (SGR) where fish fed the 1% (3.26 ± 0.02%) and 2% (3.19 ± 0.05%) MacroGard® supplemented diet displayed an increased SGR compared to fish fed the control (3.06 ± 0.07%) and the 0.1% MacroGard® (3.09 ± 0.03%) supplemented diet. This is also reflected in a better feed conversion with the 1% group showing the lowest FCR of 0.86 ± 0.01, which was significantly superior to all other groups ($P < 0.029$). Fish from the 2% group displayed significantly better FCR than the 0.1% group

(P = 0.012) and a trend towards a lower FCR compared to the control group (P = 0.078). The protein utilisation was unaffected by dietary treatment.

Table 3.1: Growth performance of carp after 8 weeks feeding experimental diets (n=3).

	Control	**0.1% M**	**1% M**	**2% M**
Initial BW (g)	11.13 ± 0.02	11.16 ± 0.00	11.13 ± 0.05	11.15 ± 0.02
Final BW (g)	61.92 ± 2.44^{a}	62.99 ± 1.23^{a}	69.04 ± 0.70^{b}	66.64 ± 1.89^{b}
Weight gain (g)	50.79 ± 2.46^{a}	51.83 ± 1.23^{a}	57.91 ± 0.68^{b}	55.49 ± 1.87^{b}
SGR (%)	3.06 ± 0.07^{a}	3.09 ± 0.03^{a}	3.26 ± 0.02^{b}	3.19 ± 0.05^{b}
FCR (g/g)	0.90± 0.02^{ab}	0.92 ± 0.01^{a}	0.86± 0.01^{c}	0.88 ± 0.02^{b}
PER (g/g)	2.48 ± 0.05	2.45 ± 0.02	2.53 ± 0.02	2.51 ± 0.04

^{abc}different superscripts indicate a significant difference (P<0.05)

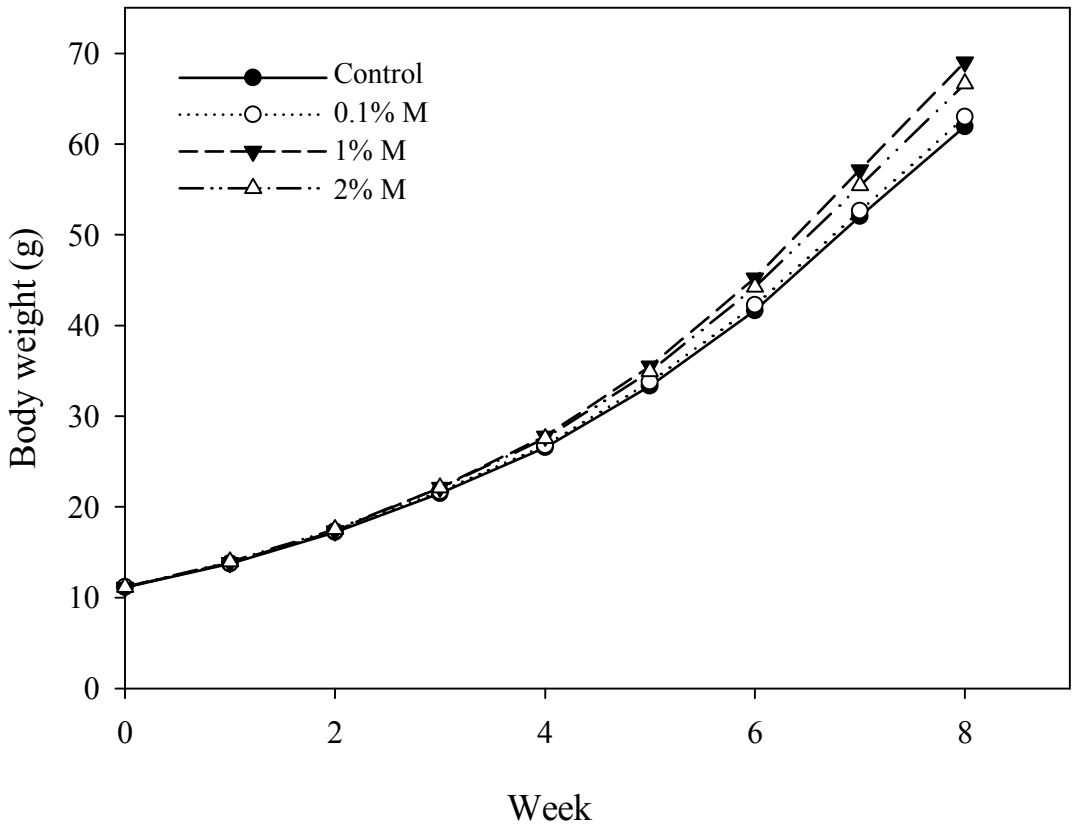

Figure 3.1: Mean body weight (g) of carp over the 8-week experimental period (n = 3).

3.4.2. Proximate analysis of diets and fish carcasses

Table 3.2 shows the body composition of the carp at the beginning and at the end of the experiment. No significant differences were seen in any of the parameters between control and treatment groups at the end of the trial. The nutrient retention is displayed in Table 3.3. Dietary protein was retained at levels of 47% - 49% and energy at 39% - 41%. There were no significant differences between treatments.

Table 3.2: Initial (*n*=4) and final (*n*=3) body composition of the experimental carp.

	Initial	**Control**	**0.1% M**	**1% M**	**2% M**
Moisture (%)	77.4 ± 0.4	72.7 ± 0.3	72.5 ± 0.4	73.0 ± 0.6	72.9 ± 0.3
Protein (%)	14.8 ± 0.1	15.1 ± 0.4	15.3 ± 0.3	15.0 ± 0.5	15.3 ± 0.4
Fat (%)	4.8 ± 0.5	9.3 ± 0.3	9.5 ± 0.3	8.9 ± 0.5	9.2 ± 0.3
Ash (%)	3.3 ± 0.1	2.4 ± 0.1	2.4 ± 0.2	2.4 ± 0.1	2.4 ± 0.1
Energy (MJ/kg)	5.2 ± 0.2	6.7 ± 0.1	6.6 ± 0.2	6.7 ± 0.2	6.7 ± 0.1

Table 3.3: Protein and energy retention efficiency (%) over the 8-week period (*n*=3).

	Control	**0.1% M**	**1% M**	**2% M**
Protein retention	49.3 ± 0.7	47.8 ± 0.8	47.4 ± 1.9	48.6 ± 0.9
Energy retention	40.4 ± 0.6	39.4 ± 0.9	41.1 ± 1.3	40.8 ± 0.3

3.4.3. Net mineral retention

The mean net mineral retention of carp over the course of the experiment is shown in table 3.4. Potassium was significantly higher retained (51.6 ± 0.9%) in fish fed the 1% MacroGard® supplemented diet compared to fish fed the control diet (48.6 ± 1.7%) (P = 0.007). All other elements assessed did not show any significant differences.

However, in several elements (except sulphur, iron and manganese) the mean retention showed a slight increase with 0.1% or 1% MacroGard® supplementation, whereas in fish fed the 2% MacroGard® supplemented diet the retention decreased to the values of the control group and in some cases even below that. Calcium showed retention of over 100% in all treatments indicating that the fish absorbed calcium from the water. Calcium retention increased from 116.6 ± 13.6% in the control to 126.6 ± 14.2% and 132.5 ± 11.3% in the 0.1% and 1% MacroGard® groups, respectively, whereas the retention in the 2% MacroGard® group was 122.3 ± 11.8%. Similarly, phosphorus retention improved from 55.3 ± 3.5% in the control to 58.8 ± 3.8% and 59.0 ± 3.6% in the 0.1% and 1% MacroGard® groups, respectively; fish fed the 2% MacroGard® supplemented diet only retained 54.7 ± 2.6% of the phosphorus. Also magnesium increased from 41.4 ± 2.7% (control) to 42.8 ± 1.8% and 42.0 ± 3.3% in the 0.1% and 1% MacroGard® groups, respectively. In the 2% MacroGard® group however, 38.8 ± 1.0% magnesium was retained. Also sodium retention increased from 21.4 ± 1.9% (control) to 22.5 ± 1.6% and 23.1 ± 0.7% (0.1% and 1% MacroGard®) and then decreased to 20.4 ± 0.5% (2% MacroGard®). Copper retention was 14.1 ± 2.4% in the control and was higher only in the 0.1% MacroGard® group (18.7 ± 4.3%); in the 1% MacroGard® group copper was retained at 14.7 ± 6.0%, whereas in the 2% MacroGard® group the level dropped to 11.9 ± 4.6%. Similarly, zinc retention increased from 70.2 ± 3.3% in the control group to 73.6 ± 3.5% and 74.0 ± 12.3% in the 0.1% and 1% MacroGard® groups, respectively, and decreased to 67.1 ± 2.0% in the 2% MacroGard® group.

Table 3.4: Mean net mineral retention (%) of carp over the 8-week experimental period (n = 3)

	Control	0.1% M	1% M	2% M
Calcium	116.6 ± 13.6	126.6 ± 14.2	132.5 ± 11.3	122.3 ± 11.8
Phosphorus	55.3 ± 3.5	58.8 ± 3.8	59.0 ± 3.6	54.7 ± 2.6
Magnesium	41.4 ± 2.7	42.8 ± 1.8	42.0 ± 3.3	38.8 ± 1.0
Potassium	48.6^{a} ± 1.7	50.1^{ab} ± 0.9	51.6^{b} ± 0.9	50.0^{ab} ± 0.2
Sodium	21.4 ± 1.9	22.5 ± 1.6	23.1 ± 0.7	20.4 ± 0.5
Sulphur	43.1 ± 1.6	44.6 ± 1.4	44.5 ± 0.8	43.2 ± 0.5
Copper	14.1 ± 2.4	18.7 ± 4.3	14.7 ± 6.0	11.9 ± 4.6
Zinc	70.2 ± 3.3	73.6 ± 3.5	74.0 ± 12.3	67.1 ± 2.0
Iron	10.2 ± 2.7	11.0 ± 2.5	11.0 ± 2.0	9.2 ± 1.2
Manganese	1.0 ± 0.2	1.1 ± 0.2	1.1 ± 0.1	1.0 ± 0.1

^{ab}different superscripts indicate a significant difference (P<0.05)

3.4.4. Haemato-immunological parameters

The haemato-immunological parameters are presented in Table 3.5. Significant differences in haematocrit levels (%PCV) were observed between the groups. Fish fed the 2% MacroGard® diet displayed higher %PCV (47.5 ± 3.3%) when compared to fish fed the control and 0.1% MacroGard® diets. Haemoglobin levels remained unaffected by MacroGard® supplementation and therefore the mean corpuscular haemoglobin concentration (MCHC) was significantly lower in the 2% MacroGard® fed group (18.8 ± 1.4%). Furthermore, fish fed the 1% and 2% MacroGard® supplemented diets showed significantly elevated levels of monocytes (3.7 ± 1.8% and 3.0 ± 1.4%, respectively) in the peripheral blood compared to the control group (1.6 ± 0.8%; Table 3.6). Plate 3.1 A-D illustrates micrographs of the different types of blood leukocytes (1000x

magnification). The other parameters were not significantly affected by dietary treatment.

Table 3.5: Haemato-immunological parameters after the 8 week period (*n*=15).

	Control	**0.1% M**	**1% M**	**2% M**
Haematocrit (%)	42.8 ± 4.0^{a}	42.2 ± 2.6^{a}	45.2 ± 5.9^{ab}	47.5 ± 3.3^{b}
MCHC (%)	22.2 ± 3.3^{a}	22.4 ± 2.0^{a}	20.2 ± 4.5^{ab}	18.8 ± 1.4^{b}
MCV (fL)	317.9 ± 60.9	309.8± 61.0	333.4 ± 53.1	332.7 ± 67.9
Haemoglobin(g/dl)	9.4 ± 0.8	9.6 ± 0.8	9.3 ± 1.3	9.0 ± 0.6
RBC (x 10^6 ml^{-1})	1.37 ± 0.19	1.44 ± 0.33	1.37 ± 0.17	1.47 ± 0.25
WBC (x 10^4 ml^{-1})	2.97 ± 0.71	3.24 ± 1.10	3.39 ± 0.76	3.06 ± 0.58
Glucose (mmol/l)	3.2 ± 0.7	2.7 ± 0.8	3.5 ± 1.1	3.2 ± 0.7
Serum protein (g/dl)	2.91 ± 0.2	3.37 ± 0.47	3.15 ± 0.47	3.25 ± 0.32
Serum albumin (g/dl)	1.08 ± 0.12	1.09 ± 0.07	1.12 ± 0.11	1.10 ± 0.09
Serum globulin (g/dl)	1.84 ± 0.25	2.21 ± 0.48	2.08 ± 0.44	2.15 ± 0.31
Albumin/Globulin	0.60 ± 0.14	0.52 ± 0.11	0.57 ± 0.14	0.52 ± 0.09

MCHC = mean corpuscular haemoglobin concentration; MCV = mean corpuscular volume; RBC = red blood cell count; WBC = white blood cell count.
[ab]different superscripts indicate a significant difference (P<0.05)

Table 3.6: Differential leukocyte cell counts (%) in the peripheral blood of carp after the 8-week feeding period (*n*=15)

	Control	**0.1% M**	**1% M**	**2% M**
Lymphocytes	89.9 ± 4.8	88.7 ± 5.8	87.5 ± 4.9	89.1 ± 3.8
Granulocytes	8.5 ± 4.6	8.8 ± 5.9	8.8 ± 4.7	7.9 ± 3.3
Monocytes	1.6 ± 0.8^{a}	2.5 ± 1.2^{ab}	3.7 ± 1.8^{c}	3.0 ± 1.4^{bc}

[abc]different superscripts indicate a significant difference (P<0.05)

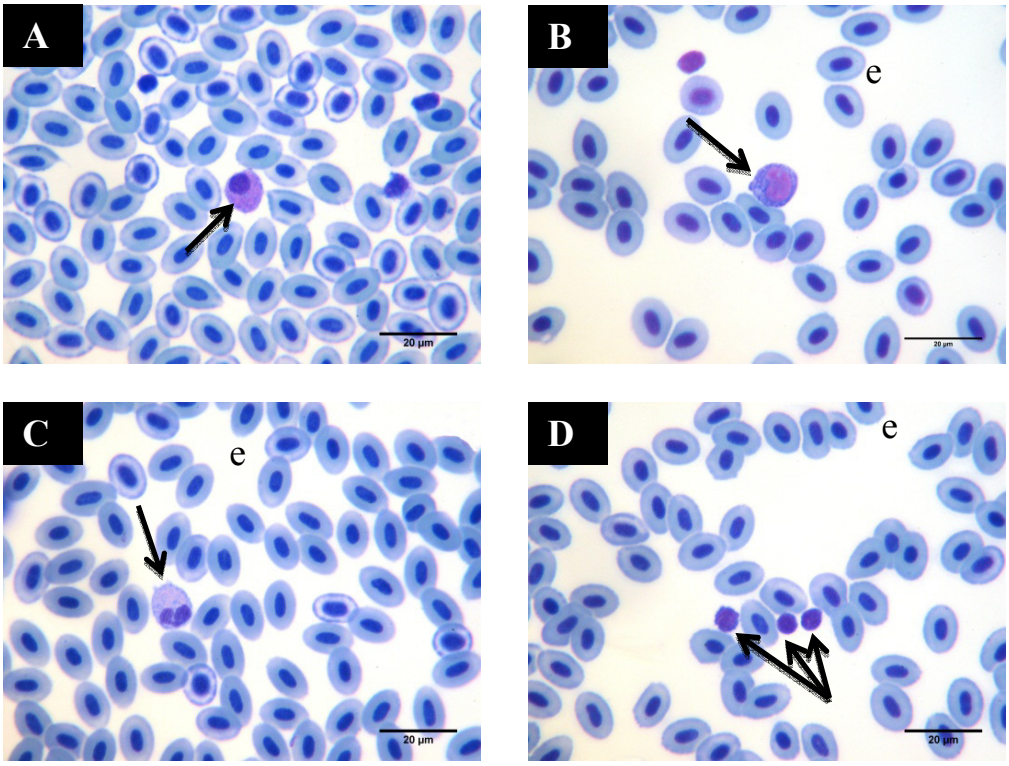

Plate 3.1: Micrographs of blood smears of carp after the 8-week period stained with May-Grünwald-Giemsa (MGG) depicting erythrocytes (e) and leukocytes (arrows): A = eosinophilic granulocyte, B = monocyte, C = neutrophilic granulocyte, D = lymphocytes; scale bar represents 20 µm.

3.4.5. Intestinal histology

Light microscopy revealed a normal arrangement of columnar enterocyte cells, with an apical microvilli brush border, forming the epithelium of the mucosal folds. No pathological lesions or necrotic cells were observed in any of the fish sampled from each treatment group. Acidic mucosubstances were almost exclusively predominant in the goblet cells and in the mucus covering the brush border. There were no significant differences between treatment groups with regards to the absorptive surface area (Figures 3.2, Plate 3.2) or the number of goblet cells in the epithelium in either anterior or posterior intestinal sections (Figure 3.3). However, a trend towards elevated goblet cell levels was evident in the anterior intestine with MacroGard® supplementation (P = 0.064). Plate 3.3 shows typical histology micrographs of transverse sections stained with Alcian blue – PAS (periodic acid Schiff) from each dietary treatment.

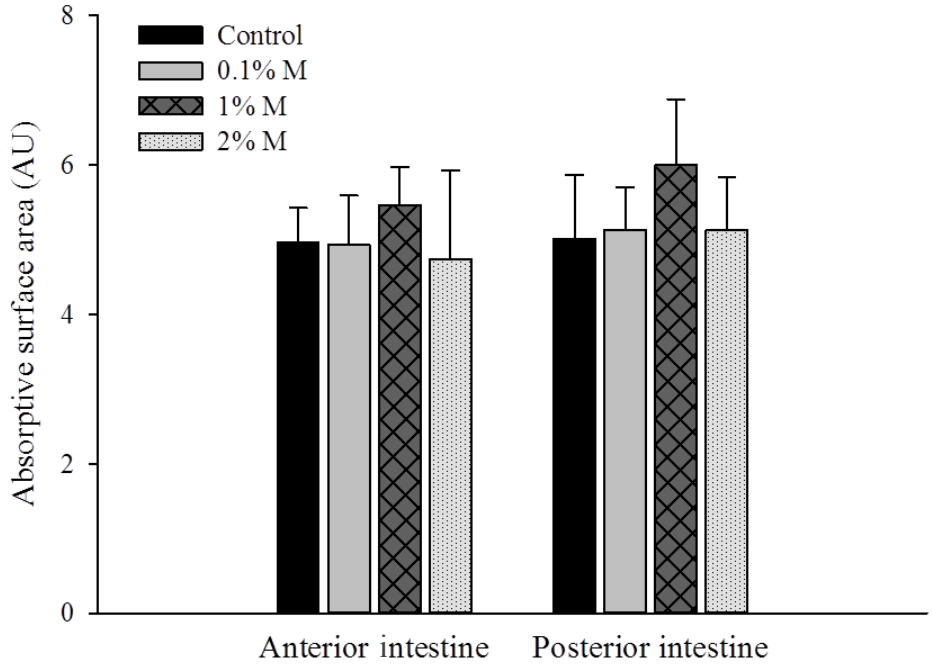

Fig. 3.2: Mean (±SD) absorptive surface area (arbitrary units) in anterior and posterior intestine after the 8-week period (n = 6).

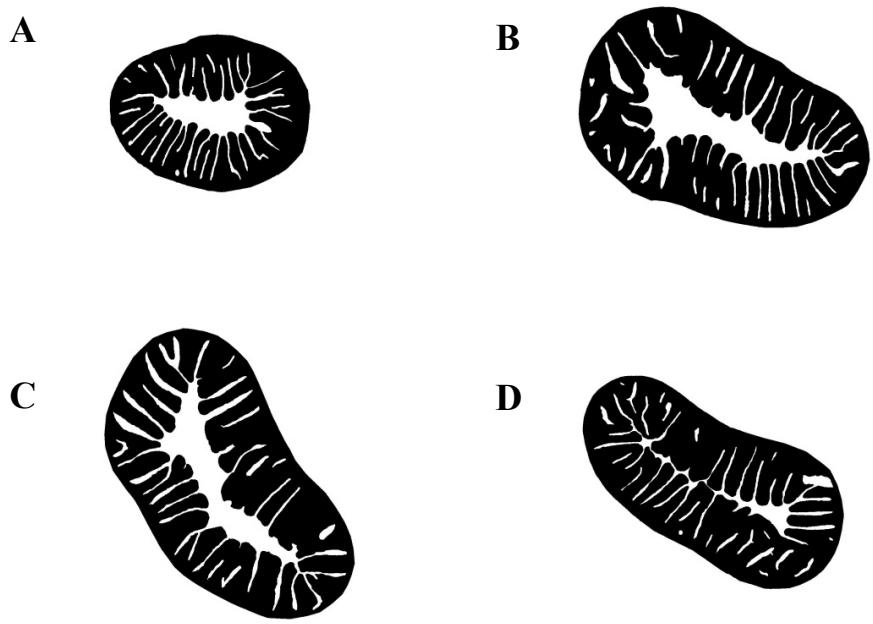

Plate 3.2: Black and white converted images of H&E stained transverse sections of posterior intestine of carp after the 8-week period showing the gross morphological architecture of the intestine; A = control diet, B = 0.1% M, C = 1% M and D = 2% M.

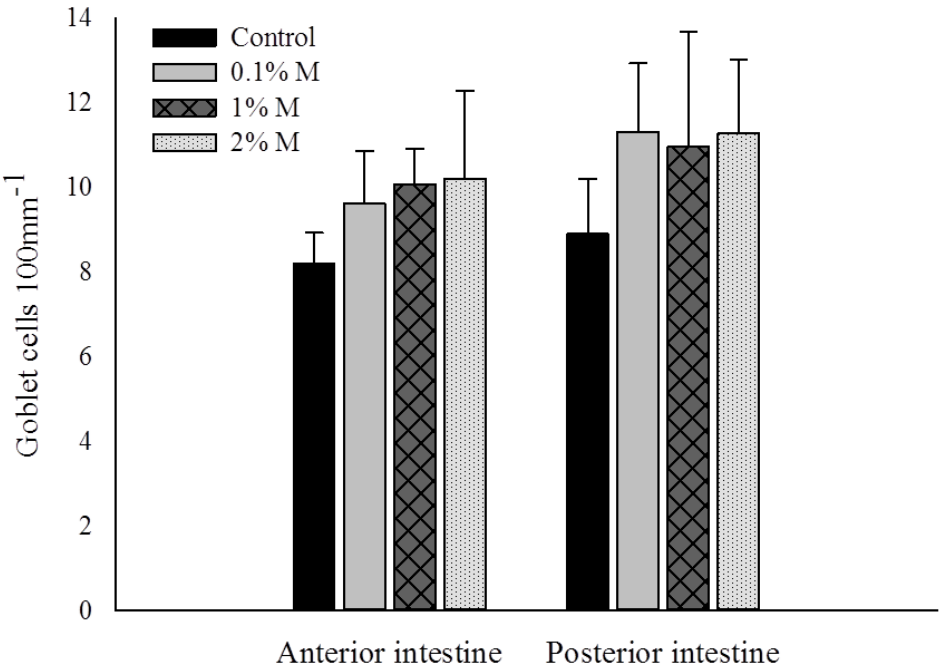

Fig. 3.3: Mean (± SD) number of goblet cells 100 μm^{-1} of mucosal fold in anterior and posterior intestine after the 8-week period (n = 6).

A significantly higher number of intraepithelial leukocytes (IELs) was observed in the anterior intestine of fish fed the 1% and 2% MacroGard® supplemented diets (Figure 3.4 and Plate 3.4); on average, 122 ± 7 and 116 ± 13 IELs, respectively, were found per 100 enterocytes compared to each 92 ± 9 and 92 ± 10, respectively IELs in fish fed the control and 0.1% MacroGard® containing diet (P < 0.05). Plate 3.4 shows typical histology pictures of transverse sections stained with H&E from each dietary treatment.

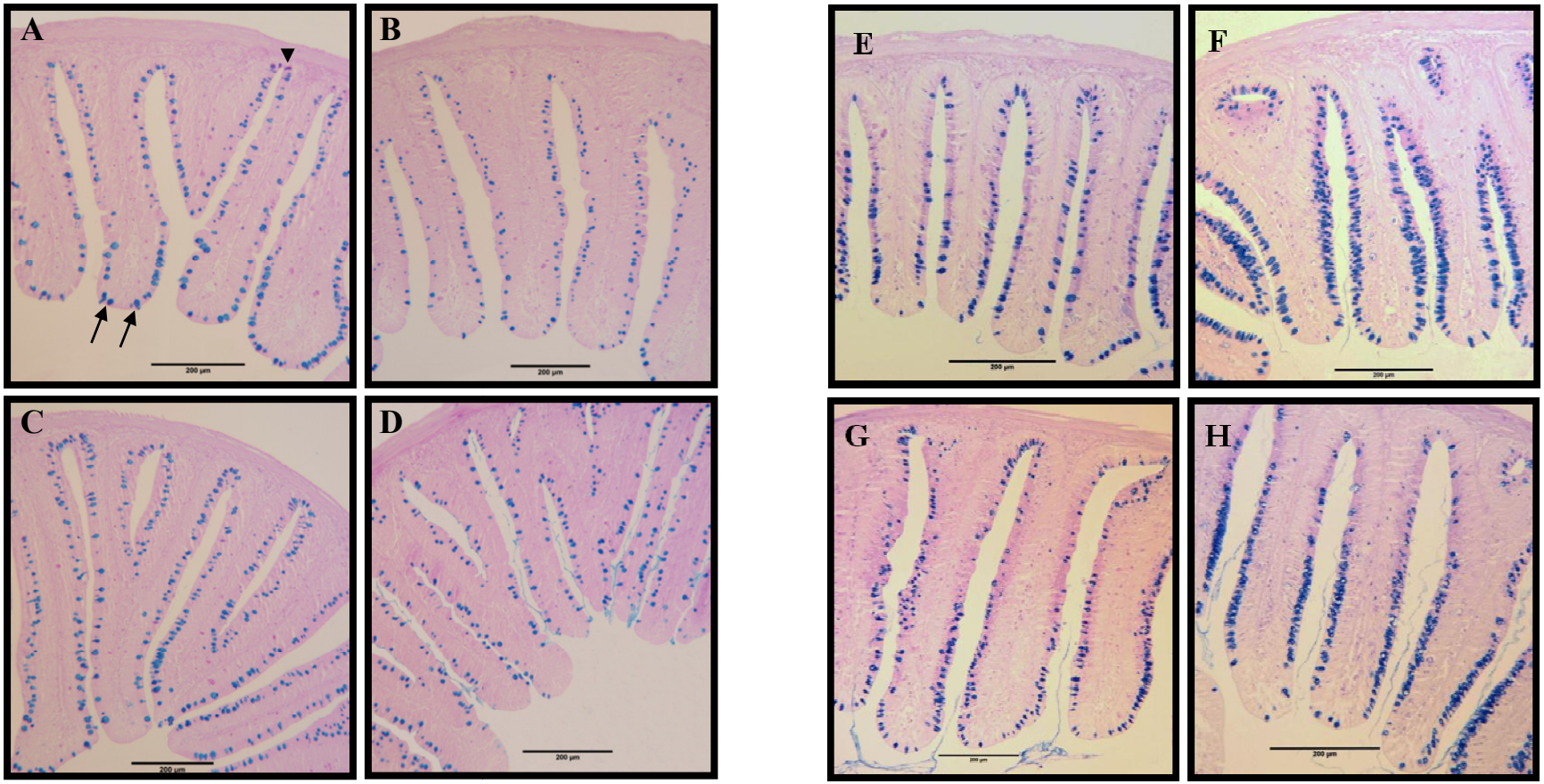

Plate 3.3: Histology micrographs of transverse sections of anterior (A-D) and posterior (E-H) intestine of carp after the 8-week period stained with Alcian blue-PAS visualizing the glycoconjugates within the goblet cells; arrows and arrowhead in A indicate the predominant acidic and the neutral glycoconjugates, respectively. A&E = control diet, B&F = 0.1% M, C&G = 1% M and D&H = 2% M. Scale bar represents 200 µm.

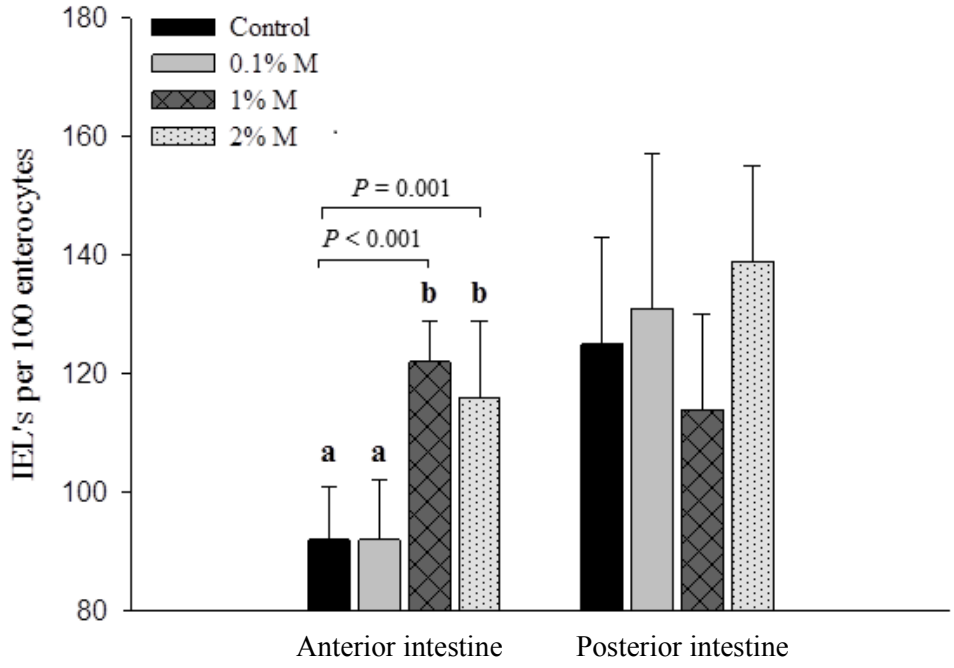

Fig. 3.4: Mean (± SD) infiltration of intraepithelial leukocytes (IELs) per 100 enterocytes in the anterior (AI) and posterior (PI) intestine after the 8-week period (n = 6); ^{ab} different letters indicate a significant difference between fish fed the 1% and 2% MacroGard® supplemented diet compared to fish fed the control and 0.1% MacroGard® supplemented diet in the anterior intestine (P<0.05).

3.5. Discussion

The findings of the present study indicate that high dietary levels of MacroGard® (1% and 2%) promote enhanced weight gain, specific growth rates and a better feed conversion of mirror carp whereas a low level of 0.1% MacroGard® did not show such an effect. Growth enhancing effects of β-glucans in mirror carp have not been reported previously to the authors' knowledge but superior weight gain and specific growth rates were reported recently in koi carp (*Cyprinus carpio koi*) fed β-glucan (from *S. cerevisiae*) at a level of 0.09%, to apparent satiation, for 56 days (Lin *et al*., 2011). Misra *et al*. (2006) found higher specific growth rates after 56 days in the cyprinid rohu (*Labeo rohita*) fed a diet containing comparatively low doses (0.025% and 0.05%) of a

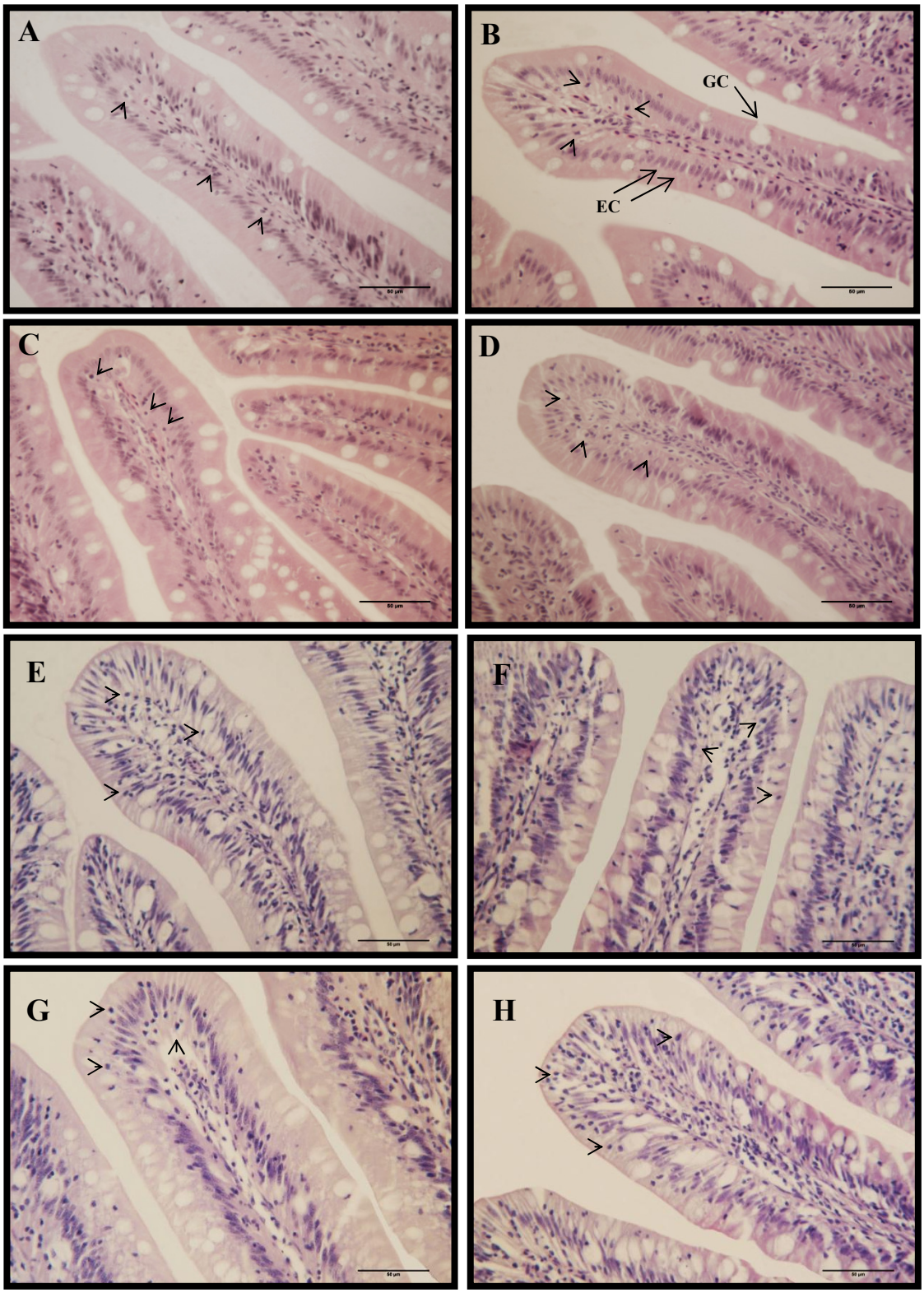

Plate 3.4: Transverse sections of the anterior (A-D) and posterior (E-H) intestine of carp after the 8-week period stained with H&E showing leukocytes (arrowheads) infiltrating from the lamina propria into the epithelial layer. (EC = enterocytes; GC = goblet cells; exemplary shown in B); A&E = control diet, B&F = 0.1% M, C&G = 1% M and D&H = 2% M. Scale bar represents 50 µm.

barley derived β-glucan at a rate of 3% of the body weight per day. Similarly, a *S. cerevisiae* derived β-(1,3)-glucan, fed to apparent satiation for 56 days to large yellow croaker (*Pseudosciaena crocea*) had positive effects on growth performance at 0.09% dietary inclusion but not at 0.18% inclusion (Ai *et al*., 2007). Other studies have not observed growth enhancing properties when feeding β-glucans to Nile tilapia (*Oreochromis niloticus*) (Shelby *et al*., 2009), channel catfish (*Ictalurus punctatus*) (Welker *et al*., 2007), European sea bass (*Dicentrarchus labrax*) (Bagni *et al*., 2005) or hybrid striped bass (*Morone chrysops x Morone saxatilis*) (Li *et al*., 2009). In the present study, whole body chemical composition, protein retention and energy retention remained unaffected by dietary β-glucan inclusion indicating that the enhanced growth performance with 1% and 2% MacroGard® supplementation was not caused by a higher retention of these nutrients.

At present, it is not clear what causes the improvements in growth observed with dietary β-glucans in previous studies and it is not evident why growth enhancing effects of β-glucans occur in some aquatic species and not in others. However, Dalmo and Bøgwald (2008) suggested that the effects may depend on the concentration of the β-glucan in the diet, its solubility, the fish species, the water temperature and length of the feeding period. They also hypothesize, when growth enhancing effects occur, that the β-glucans induce a localized intestinal immune response that in turn leads to resistance against pathogens which otherwise would cause reduced weight gain and possibly disease.

However, histology derived data on the effects of β-glucans on the intestine and its associated lymphoid tissue (GALT) are scarce but data derived from the present study may help to support this hypothesis. High dietary inclusion levels (1% and 2%) of MacroGard® in the present study caused an increased infiltration of leukocytes from the lamina propria into the epithelial layer (i.e. IELs) in the anterior intestine whereas the

low inclusion of 0.1% MacroGard® had no such effect. Similar findings have been reported in mice; Tsukada *et al*. (2003) reported that mice receiving 25 mg of a β-(1,3)(1,6)-D-glucan per day displayed elevated intraepithelial lymphocytes in the small intestine. The lower β-glucan dosage (10 mg) decreased the number whereas doubling the dosage (50 mg) yielded a similar amount of intraepithelial lymphocytes. Shen *et al*. (2007) reported similar findings in their mice studies. In the present study dietary supplementation of 1 and 2% MacroGard® significantly elevated the monocyte proportion of the peripheral blood leukocytes. Monocytes are key components of the innate immune response and respond quickly to sites of inflammation to replenish macrophage and dendritic populations (Gordon and Taylor, 2005; Shi and Pamer, 2011). The evaluation in the present study might be indicative of their transport to the anterior epithelium to bolster and replenish localized IEL levels. Further studies, such as the immunohistochemistry and the analysis of mucosal cytokines, are required to validate this hypothesis because it has recently been shown that orally administered β-glucans may down-regulate the gene expression of pro-inflammatory cytokines in carp intestine (Falco *et al*., 2012) and in rainbow trout spleen (Djordjevic *et al*., 2009). Even though absorption and pharmacokinetics of β-glucans in the teleost gastrointestinal tract are not known yet, it is possible that the different responses of the anterior and posterior intestinal regions might be due to differences in the β-glucan levels present in the intestinal regions. It is possible that β-glucans could be internalized by macrophages through phagocytic processes (Chan *et al*., 2009) as has been shown for soluble β-glucans in rats (Rice *et al*., 2005) and for particulate β-glucans in mice (Hong *et al*., 2004) or fermentative processes by the microbiota in the complex intestinal ecosystem (e.g. through β-glucanase activity (Chen and Seviour, 2007)) may reduce the level, or change the structure, of the β-glucans that reach the posterior

intestine. The experiment described in the subsequent chapter 4 investigated this hypothesis.

Histological analysis also revealed that the mucus composition in the goblet cells consisted predominantly of acid mucus which is consistent with the findings of Ngamkala *et al*. (2010) who administered 1% dietary β-glucan to Nile tilapia for two weeks prior to a challenge with *Aeromonas hydrophila*. The total number of goblet cells in the anterior intestine with high β-glucan supplementation in the present study was not significantly different from the control group but a trend towards elevation was evident. Ngamkala *et al*. (2010) did not observe corresponding changes in anterior or posterior intestine in their study. This could be related to species-specific differences, the shorter administration of β-glucan over 2 weeks and/or the type of β-glucan they used (sonicated curdlan, a linear bacterial β-glucan). Zhu *et al*. (2012) however reported an increase of intestinal goblet cells in their study of channel catfish fed diets supplemented with a *S. cerevisiae* yeast polysaccharide mixture (containing β-(1,3)(1,6)-D-glucan and mannan oligosaccharides (MOS)) for seven weeks. Unfortunately, the authors do not state exactly which intestinal region they sampled. The mucus produced and released by the goblet cells forms a layer covering the epidermal body surfaces (including the intestinal epithelium) and constitutes, among other functions, the first line of defence against external influences (Shephard, 1994). The mucus layer builds a trap for particles, bacteria and viruses, thereby hindering translocation to the internal environment, and subsequently the intestinal peristaltic processes expel those potentially pathogenic organisms (Mayer, 2003). It is likely therefore that an elevated number of goblet cells lead to a higher release of mucus in the intestine therefore improving first line of defence mechanisms.

The absorptive surface area, as determined by the internal/external surface area ratio, was not affected by the dietary treatments in the present study. However, using scanning

electron microscopy, Van Hai and Fotedar (2009) found increased surface structures (larger upper folds) of the intestines of western king prawns after six and 12 weeks of dietary supplementation of β-glucan. Studies with broiler chickens showed that supplemented yeast cell wall fractions from *S. cerevisiae*, as well as its main structural components β-glucan and mannoproteins, equally led to higher villus length in the jejunum (Moralez-Lopez *et al*., 2009). The same group found in a similar subsequent study that the same yeast cell wall fraction increased villus height, mucus thickness and number of goblet cells without changing the ileal content viscosity (Morales-Lopez *et al*., 2010). A number of studies focusing on MOS derived from *S. cerevisiae* showed enhancing effects on gut morphology (absorptive surface area) and ultrastructure (microvilli length and density) in rainbow trout (Dimitroglou *et al*., 2009) and sea bream (Dimitroglou *et al*., 2010), whereas effects on gut histology were absent in European sea bass (Torrecillas *et al*., 2007).

As haematological and biochemical indices are recognized as a useful means to assess health status and physiological response of an organism towards nutritional and environmental changes (Cnaani *et al*., 2004, Jawad *et al*., 2004, Schütt *et al*., 1997) the present study investigated a number of haemato-immunological parameters. Fish fed MacroGard® supplemented diets did not show significantly altered circulatory leukocyte cell counts, erythrocyte cell counts, serum glucose concentration, serum total protein, serum albumin or globulin levels or albumin/globulin ratio after 8 weeks. These findings are broadly in accordance with the study by Misra *et al*. (2006), who observed no effect on rohu serum total protein, serum albumin or globulin levels or albumin/globulin ratio after 8 weeks of feeding. However, some differences in these parameters were observed at earlier time points and glucose levels were reduced and peripheral leukocyte levels were elevated at 8 weeks. Peripheral leukocyte levels were also reported to be elevated in koi carp after 8 weeks feeding of 0.09% of a *S. cerevisiae*

β-glucan (Lin *et al*., 2011). Feeding tench (*Tinca tinca*) with MacroGard® (0.1% and 0.2%) led to higher serum globulin levels after 4 weeks but serum total protein was not affected (Siwicki *et al*., 2010). A previous study reported that MacroGard® and other immunostimulants enhanced both parameters in rainbow trout after 1 week of feeding (Siwicki *et al*., 1994).

In the present study haematocrit levels were significantly elevated in carp fed the 2% MacroGard® supplemented diet. The haemoglobin levels were unaffected by dietary β-glucan and thus, the MCHC levels were correspondingly lower in the same group. Increased haematocrit levels have been observed in channel catfish fed diets supplemented with 0.1% dietary MacroGard® for 4 weeks with no effect on haemoglobin concentration (Welker *et al*., 2007). Babicek *et al*. (2007) found increased haematocrit levels in rats fed a β-(1,3)(1,6)-D-glucan from *S. cerevisiae*. Higher haematocrit and haemoglobin levels were reported in Nile tilapia upon administration of high levels (0.1% to 0.5%) of baker's yeast from *S. cerevisiae* (Abdel-Tawwab *et al*., 2008). Other studies did not observe any changes in haematocrit levels, e.g. in spotted rose snapper (*Lutjanus guttatus*) (Del Rio-Zaragoza *et al*., 2011) or rainbow trout (Siwicki *et al*., 1994).

3.6. Conclusions

The current study demonstrates that high dietary inclusion levels of β-glucan can enhance growth performance and localized intestinal leukocyte infiltration in the anterior intestine of mirror carp without detrimental effects on carcass composition, intestinal morphology or the haemato-immunological parameters investigated. However, future research is needed in order to further investigate the underlying reasons for the growth promoting effects. Furthermore, detailed studies (e.g. using immunohistochemistry, gene expression and proteomics) of the localized immunity at

the mucosal level are required to explore the mechanisms and reasons for the increased IELs found in the present study. Future studies are also recommended to assess the gastrointestinal microbiota which could either be affected directly by selective utilisation of β-glucans by certain bacterial groups, or secondarily by changes in the host's localised immune status. Possible changes could alter the microbial balance which in turn might influence the host immunity or β-glucan availability.

Chapter 4:

Effects of a dietary β-(1,3)(1,6)-D-glucan supplementation on intestinal microbial communities and intestinal ultrastructure of mirror carp (*Cyprinus carpio* L.)

4.1 Abstract

The aim of this experiment was to assess the effects of a dietary *Saccharomyces cerevisiae* β-(1,3)(1,6)-D-glucan supplementation (MacroGard®) on mirror carp (*Cyprinus carpio* L.) intestinal microbiota and ultrastructure of the enterocyte apical brush border. The carp were fed either a control diet or diets supplemented with 0.1%, 1% or 2% w/w MacroGard®. Culture-dependent microbiology revealed that aerobic heterotrophic bacterial levels were unaffected by dietary MacroGard® after two and four weeks. No effects were observed on the allochthonous lactic acid bacteria (LAB) populations at either time point, however, reduced autochthonous LAB populations were observed at week four. PCR-DGGE confirmed these findings through a reduction of the abundance of autochthonous LAB observed taxonomical units (OTUs) in MacroGard® fed fish compared to the control fed fish. DGGE analyses also revealed that dietary MacroGard® reduced the number of OTUs and the species richness of the allochthonous microbiota after two weeks, but not after four weeks. In contrast, dietary MacroGard® reduced the number of OTUs, the species richness and diversity of the autochthonous microbiota after two weeks, and those parameters remained reduced after four weeks. Transmission electron microscopy revealed that intestinal microvilli length and density were significantly increased after four weeks in fish fed diets supplemented with 1% MacroGard®. This study indicates that dietary MacroGard® supplementation modulates intestinal microbial communities of mirror carp and influences the morphology of the apical brush border. To the author's knowledge this is the first study to investigate the effects of β-(1,3)(1,6)-D-glucans on fish gut microbial communities, using culture-independent methods, and the ultrastructure of the apical brush border of the enterocytes in fish.

4.2 Introduction

The intestinal microbial communities and their metabolites play an integral role in the ontogeny of teleosts. They influence the host's metabolic and nutritional balance, the immunological balance and physiological processes (Cerf-Bensussan and Gaboriau-Routhiau, 2010; Merrifield *et al*., 2010; Sekirov *et al*., 2010). Intestinal microbial communities consist of allochthonous (digesta-associated, transient) and autochthonous (mucosa-associated, indigenous) microbiota (Ringø and Birkbeck, 1999; Ringø *et al*., 2003). The piscine intestine itself is known to be one of the main portals for disease. Environmental changes, e.g. in the diet, can lead to imbalances in the intestinal microbial populations and disruption of the mucosal barrier, thus allowing for pathogenic invasion (Lødemel *et al*., 2001; Ringø *et al*., 2003; Vine *et al*., 2004; Birkbeck and Ringø, 2005; Ringø *et al*., 2007).

As a result efforts have been directed towards strengthening the intestinal health of fish reared under intensive aquaculture conditions, especially since the EU ratified the ban on non-medical use of antibiotics in animal nutrition from 2006 onwards (EU, 2003). Functional dietary supplements such as β-glucans are frequently employed as immunomodulators in animal husbandry. β-glucans have been shown to trigger innate immune responses, increase disease resistance and enhance growth performance of fish (Dalmo and Bøgwald, 2008; Soltanian *et al*., 2009). Although a number of studies have examined the effect of prebiotics on the teleost intestinal microbial communities, the effects of β-glucans on the intestinal microbial communities have received little attention. β-glucans, like prebiotics, are non-digestible β-glycosidicly linked monomeric units. Prebiotics selectively stimulate the growth and/or activity of intestinal bacteria associated with health and well-being (Gibson *et al*., 2004). Recent studies have shown that prebiotics can modulate the gut microbiota of fish; for example, inulin in Atlantic salmon (*Salmo salar* L.) (Bakke-McKellep *et al*., 2007) and Arctic charr (*Salvelinus*

alpinus L.) (Ringø *et al*., 2006a), and mannan oligosaccharides (MOS) in rainbow trout (*Oncorhynchus mykiss*) (Dimitroglou *et al*., 2009) and gilthead sea bream (*Sparus aurata*) (Dimitroglou *et al*., 2010). However, the effects of β-glucans, which are often utilized for their immunostimulatory properties, on the gut microbiota of fish have seldom been investigated. To the authors knowledge only one study has reported the effect of dietary β-(1,3)(1,6)-D-glucans (chrysolaminaran, found in phytoplankton, and MacroGard®) on gut microbial populations of fish (Skjermo *et al*., 2006). This study revealed some evidence of the potential for β-glucans to modulate or disrupt the gut microbiota of fish; however, due to the limited number of replicates and the culture – dependent methods used, no comprehensive conclusions can be drawn.

Recent investigations using culture – independent techniques have provided quantitative information on the vast diversity of bacterial species which inhabit the gastrointestinal (GI) tract of teleosts (Merrifield *et al*., 2010; Nayak, 2010). Using massively paralleled sequencing van Kessel *et al*. (2011) reported that the allochthonous microbiota of common carp (*Cyprinus carpio* L.) was highly diverse and composed of *Fusobacteria* (46%), *Bacteroidetes* (21%), *Planctomycetes* (12%), *Gammaproteobacteria* (7%), *Firmicutes* (ca. 4%) and *Verrucomicrobiae* (1%). Several studies have reported that members of the *Firmicutes* and *Bacteroides* produce β-glucanases [e.g. *Bacillus circulans* (Rombouts and Phaff, 1976a,b), *Arthrobacter* spp. (Kitamura *et al*., 1974; Vršanská *et al*., 1977; Doi and Doi, 1986), *Flavobacterium dormitator* (Mori *et al*., 1977)].

Therefore we hypothesize that the GI microbiota of fish maybe influenced by inclusion of dietary β-glucans. As β-glucans are becoming more commonly utilized in aquafeeds the potential impact on the gut microbiota warrants further investigation.

The aim of the present study was therefore to investigate the effects of a dietary administration of a *Saccharomyces cerevisiae* β-(1,3)(1,6)-D-glucan (MacroGard®) on the intestinal microbiota of mirror carp (*Cyprinus carpio* L.). Since possible changes in the composition of the intestinal microbiota may have implications for physiological processes in the mucosa, a further aim was to examine the ultrastructure of the apical brush border of the enterocytes.

4.3 Material and Methods

4.3.1 Rearing facilities and water quality

The experiment was conducted as described under section 2.2 in system "B" within the rearing facilities of the University of Plymouth and water quality was monitored accordingly. Water temperature, oxygen saturation and pH were maintained at 23.7 ± 0.4 °C, 94.1% ± 0.7 and 6.57 ± 0.50, respectively. Ammonium, nitrite and nitrate were determined once a week and the levels ranged between 0 - 0.039 mg/l, 0 - 0.004 mg/l and 16.11 - 31.02 mg/l, respectively.

4.3.2. Experimental fish and feeding

One hundred and fifty mirror carp were obtained from Hampshire Carp Hatcheries, Hampshire, UK and carefully acclimatized as described under section 2.3. Thereafter, 15 carp were randomly allocated to each of the eight 71L experimental tanks (n = 2 tanks per treatment). The initial average body weight at the start of the experiment was 11.3 ± 0.1g. The fish were fed the experimental diets as described under section 2.5 over a period of four weeks and bulk weighing and feeding regime was conducted as described under 2.3 with the exception that the body weight was determined after two weeks and the feeding rates were adjusted accordingly.

4.3.3. Microbiology sampling procedures

For evaluation of the intestinal microbial communities, mucosa and digesta samples from the posterior intestine were taken after week two (n = 4) and week four (n = 3). Fish were euthanized with an overdose of tricaine methanesulfonate (MS-222; Pharmaq Ltd., Fordingbridge, UK), buffered with sodium bicarbonate ($NaHCO3^-$), followed by destruction of the brain (PIL № 30/9104 under PPL № 30/2644). The body surface was cleaned with 70% ethanol, the body cavity was opened under aseptic conditions, the entire intestine excised and the digesta was removed by gentle squeezing (Merrifield *et al*., 2009a). The mucosal tissue samples were taken from the posterior end of the intestine and washed thoroughly three times with sterile phosphate-buffered saline (PBS) in order to remove adherent digesta. At this point, mucosa and digesta subsamples were used for culture-dependent analysis or stored at -20°C in sterile molecular grade 1.5 ml microcentrifuge tubes for later culture-independent analysis.

4.3.4. Culture – dependent analysis of the intestinal microbiota

One hundred mg of each sample (mucosa and digesta) was 10-fold diluted with sterile PBS and homogenized in a macerator (MSE, London, UK). Samples were serially diluted 10-fold with sterile PBS. One hundred µl were spread onto TSA (trypton soy agar, Oxoid, Basingstoke, UK) plates to determine aerobic heterotrophic bacterial levels and MRS (de Man, Rogosa, Sharpe agar, Oxoid, UK) plates to determine lactic acid bacteria (LAB) levels. Samples were plated in duplicate and incubated for 7 days at 25°C before enumeration of colony forming units (CFUs) from statistically viable plates (i.e. plates containing 30-300 colonies) (Merrifield *et al*., 2009b).

4.3.5. Culture – independent analysis of the intestinal microbiota

DNA extraction and PCR amplification of the 16S rRNA V3 region were performed as described under sections 2.8.1 and 2.8.2, respectively. Subsequently, the V3 PCR amplicons were subjected to a 40% - 60% Denaturing Gradient Gel Electrophoresis (DGGE) as described under section 2.8.3. Finally, the excised bands were re-amplified, then purified and sequenced as described under sections 2.8.4 and 2.8.5, respectively.

4.3.6. Transmission Electron Microscopy (TEM)

Samples from the posterior intestine of 5 fish per treatment (n = 5) were obtained from fish fed the control, the 0.1% and the 1% MacroGard® supplemented diets for TEM analysis. Rectangular samples of circa 2 mm^3 were taken both at week two and week four.

4.3.6.1. Sample preparation and resin embedding

Samples were fixed immediately after excision in 2.5% glutaraldehyde with 0.1 M cacodylate acid sodium salt solution (1:1 vol/vol), pH 7.2 and refrigerated at 4°C for later analysis (Merrifield *et al*., 2009b). Samples were then rinsed twice with 0.1 M sodium cacodylate buffer for 15 min each and post-fixed in buffered OsO_4 for 1 h. Afterwards, samples were rinsed again twice with 0.1 M sodium cacodylate buffer and dehydrated with graded alcohol solutions of 30%, 50%, 70%, 90% and 100 % (twice) for at least 15 min each. Alcohol was removed by gradual replacement with low viscosity resin of 30%, 50%, 70% and 100% for at least 12 h in each solution. A second incubation step in 100% resin for 24 h followed. Samples were placed in Beem® embedding capsules (Beem® Inc., West Chester, US) and the resin was polymerised at 60 °C (overnight).

4.3.6.2. Sectioning, staining and screening

Resin blocks were trimmed using a glass knife and then blocks were sectioned using a diamond knife (~80nm). Ultrathin sections from each sample were placed on copper grids and stained with saturated uranyl acetate for 15 min, rinsed with distilled water and post-stained with Reynolds lead citrate for 15 min (Reynolds, 1963). Sections were screened with a JSM 1200EX transmission electron microscope at 120 kV (Jeol; Tokyo, Japan).

TEM images were analysed using Image J version 1.42 (National Institutes of Health, USA) to measure the microvilli length as described by (Hu *et al*., 2007) with slight modifications. Briefly, the length of 10 well-orientated longitudinal microvilli was evaluated in 5 ultrathin sections per sample (50 measurements per sample, in total 250 measurements per treatment). Furthermore, the microvilli density was assessed after Daniels *et al*. (2010) with a slight modification. Briefly, the number of microvilli present on the enterocyte surface was evaluated and then standardized to 1 μm in 5 ultrathin sections per sample (5 measurements per sample, in total 25 measurements per treatment).

4.3.7. Statistical Analysis

Statistical analysis was performed as described under section 2.9.

4.4 Results

4.4.1. Culture – dependent analysis of the intestinal microbiota

The effects of a dietary MacroGard® supplementation on viable counts of aerobic heterotrophic bacteria and LAB in the carp intestine were determined by a culture-based approach using TSA and MRS agar plates, respectively. Figure 4.1 displays the allochthonous and autochthonous viable cell counts after week two and week four. After two weeks of feeding the levels of both allochthonous and autochthonous aerobic heterotrophic bacteria fluctuated at around log 6.5 CFU g^{-1} across treatments (Figure 4.1A). Figure 4.1B shows that the allochthonous LAB reached levels of approximately log 5 CFU g^{-1} (present in all replicates) whereas the respective autochthonous levels were lower at levels of log 3 CFU g^{-1} (detectable in three of the four replicates of the 0.1% MacroGard® group). After four weeks of feeding the allochthonous aerobic heterotrophic bacteria mirrored the results from week two with levels of around log 6.5 CFU g^{-1} (Figure 4.1C); the levels of the respective aerobic heterotrophic autochthonous bacteria however decreased from log 6 CFU g^{-1} in the control group to log 3 CFU g^{-1} in the 2% MacroGard® group (detectable in two of the replicates). A clear reduction in the abundance of autochthonous LAB was observed in fish fed β-glucans (Figure 4.1D). Indeed, levels were too low to be statistically viable (i.e. below 30 CFU on the lowest dilution plate) in two fish from the 1% MacroGard® group and one fish from the 2% MacroGard® group. The levels decreased from log 3.9 CFU g^{-1} in the control group to log 1 CFU g^{-1} in the 1% MacroGard® group and log 1.4 CFU g^{-1} in the 2% MacroGard® group.

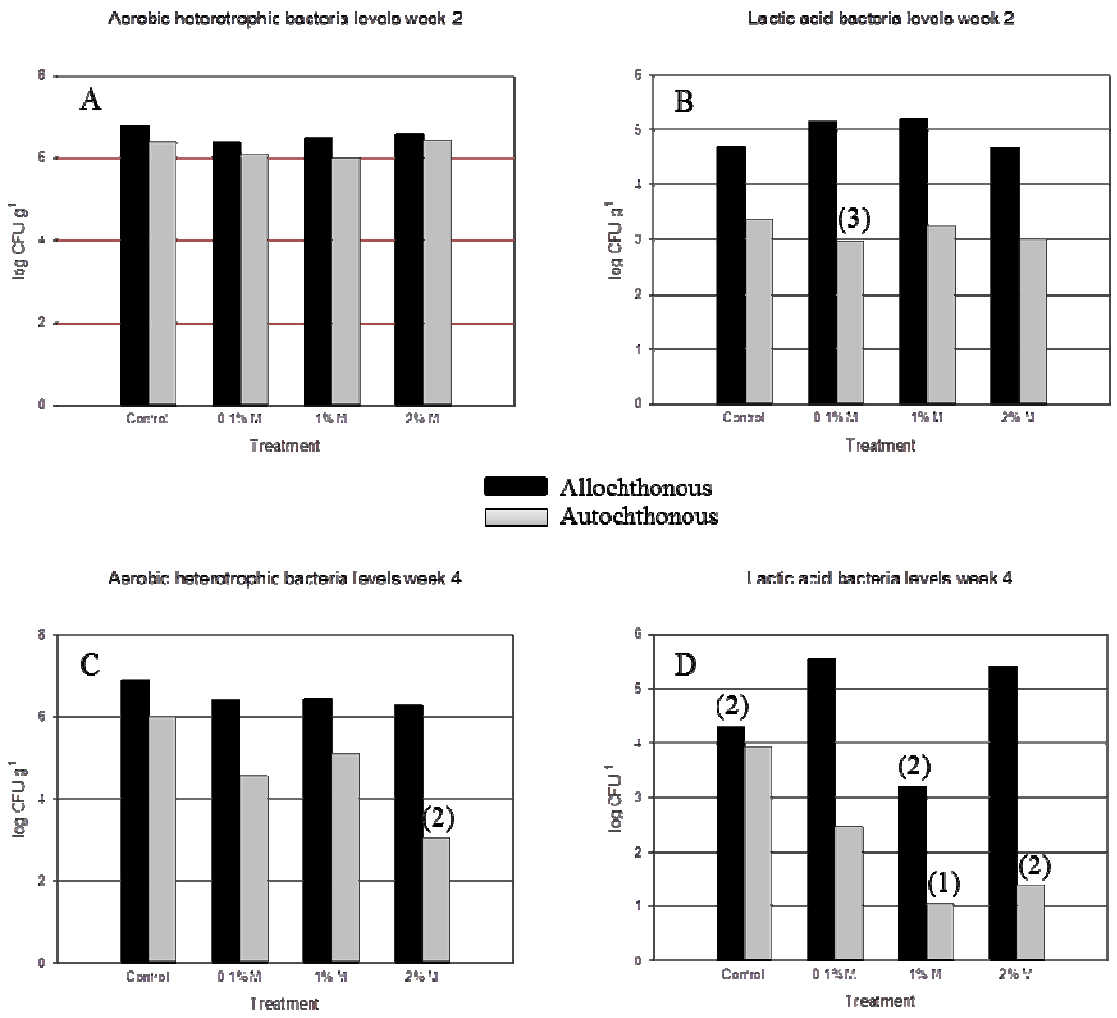

Figure 4.1A-D: Viable counts (CFU g^{-1}) of allochthonous and autochthonous aerobic heterotrophic bacteria and lactic acid bacteria in the carp intestine after two weeks (n = 4) and four weeks (n = 3) of feeding control (0%) or MacroGard® (0.1%, 1%, 2% M) supplemented diets. Statistically viable levels of bacteria were recovered from all replicates unless indicated by the number present in parenthesis.

4.4.2. Culture – independent analysis of the intestinal microbiota

The influences of dietary MacroGard® supplementation on the intestinal microbial diversity of carp were investigated using culture-independent techniques. Figures 4.2-4.5 display the 16S rRNA V3 PCR-DGGE fingerprints of the allochthonous and autochthonous microbiota after two and four weeks of feeding together with the respective dendrograms and the nonmetric multidimensional scaling analysis plots.

Tables 4.1 and 4.2 show the microbial ecological parameters derived from those PCR-DGGE fingerprints at week two and four, respectively.

Week two

Figure 4.2 illustrates low similarities between treatments (ca. 47% - 61%) of the allochthonous samples taken after two weeks. This is confirmed by the microbial ecological parameters displayed in Table 4.1 which show that dietary MacroGard® reduced the number of OTUs from 28.00 ± 3.27 in the control group to 23.00 ± 3.37 (P = 0.033) and 21.25 ± 2.22 (P = 0.007) in the 0.1% and 2% MacroGard® group, respectively; the reduced numbers of OTUs in the 1% MacroGard® group (24.00 ± 2.71) was approaching significance (P = 0.077). Similarly, the species richness was significantly reduced in all MacroGard® groups to 2.17 ± 0.28 (P = 0.020), 2.23 ± 0.24 (P = 0.038) and 1.99 ± 0.16 (P = 0.003) from 2.63 ± 0.28 in the control treatment. Species diversity, evenness and SIMPER remained unaffected.

Similarly, Figure 4.3 illustrates low similarities between treatments (ca. 54% - 78%) of the autochthonous microbiota after two weeks. This is confirmed by the microbial ecological parameters displayed in Table 4.1 which show that dietary MacroGard® reduced the number of OTUs from 34.00 ± 4.55 in the control group to 29.33 ± 2.08 (P = 0.088), 28.75 ± 2.06 (P = 0.044) and 25.00 ± 3.37 (P = 0.002) in fish fed the 0.1%, 1% and 2% MacroGard® supplemented diets, respectively. Additionally, the species richness decreased from 3.00 ± 0.36 in the control group to 2.63 ± 0.17 (P = 0.098), 2.60 ± 0.18 (P = 0.055) and 2.31 ± 0.27 (P = 0.003) and Shannon's diversity index decreased from 3.47 ± 0.13 in the control group to 3.31 ± 0.07 (P = 0.073), 3.30 ± 0.06 (P = 0.044) and 3.15 ± 0.17 (P = 0.001) in the 0.1%, 1% and 2% MacroGard® groups, respectively. Species evenness and SIMPER remained unaffected by dietary MacroGard®.

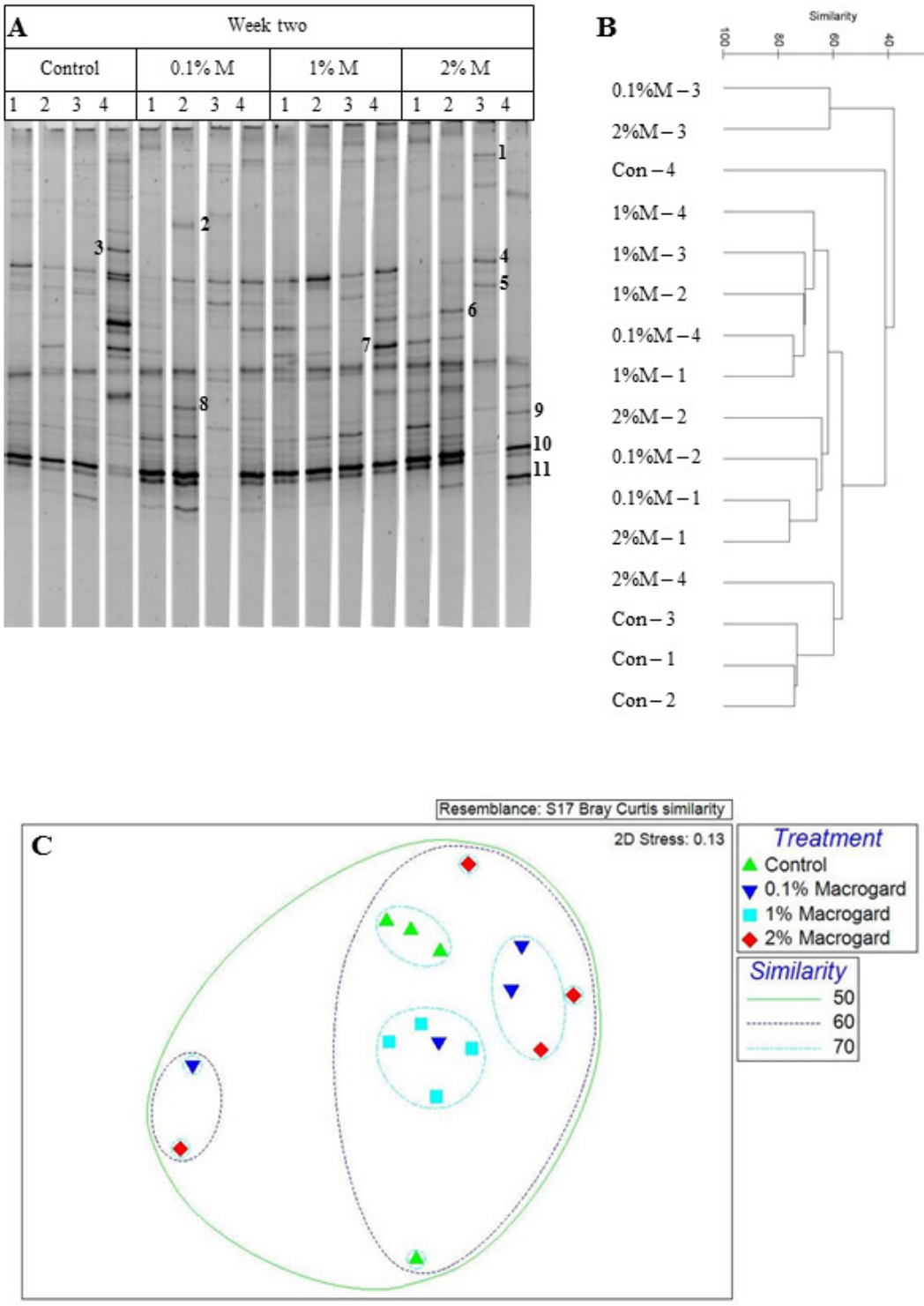

Figure 4.2: A: Denaturing gradient gel electrophoresis (DGGE) profiles of PCR - amplified products of the V3 region of the 16S rRNA gene from week 2 allochthonous samples (numbers indicate OTUs sequenced). B: Bray – Curtis dendrogram demonstrating the similarity. C: nonmetric multidimensional scaling analysis plots showing clusters at different similarity levels (%). Carp were fed control (0%) or MacroGard® (0.1%, 1%, 2% M) supplemented diets for 4 weeks.

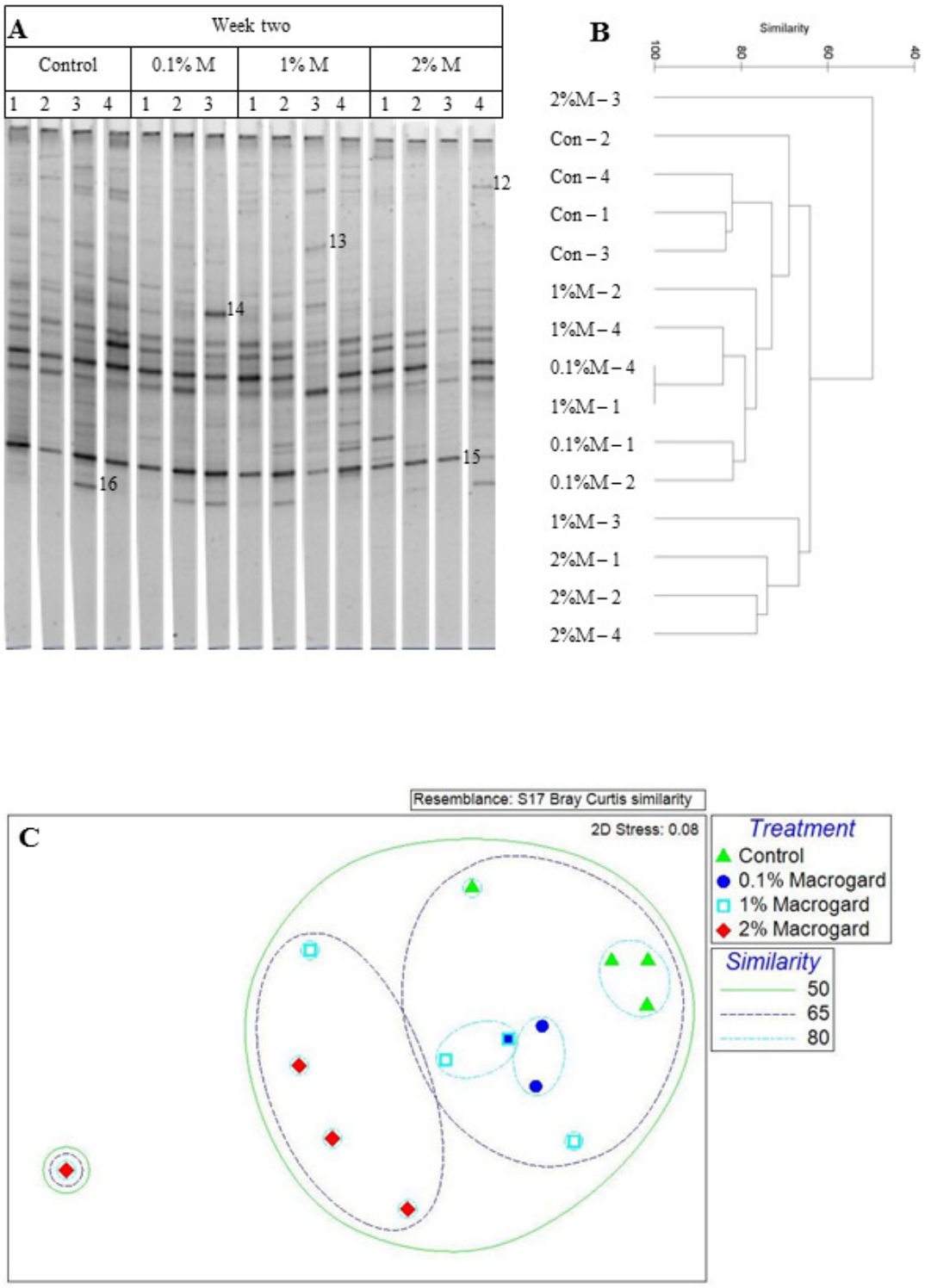

Figure 4.3: A: Denaturing gradient gel electrophoresis (DGGE) profiles of PCR – amplified products of the V3 region of the 16S rRNA gene from week 2 autochthonous samples (numbers indicate OTUs sequenced). B: Bray – Curtis dendrogram demonstrating the similarity. C: nonmetric multidimensional scaling analysis plots showing clusters at different similarity levels (%). Carp were fed control (0%) or MacroGard® (0.1%, 1%, 2% M) supplemented diets for 4 weeks.

Table 4.1: Microbial community analysis of the allochthonous and autochthonous microbial communities of carp from DGGE fingerprints after 2 weeks of feeding control (0%) or MacroGard® (0.1%, 1%, 2% M) supplemented diets.

Week 2 allochthonous	**Microbial ecological parameters**					**Similarity**			
	N	Richness	Evenness	Diversity	SIMPER (%)	Control	0.1% M	1% M	2% M
Control	28.00 ± 3.27^{a}	2.63 ± 0.28^{a}	0.95 ±0.01	3.16 ± 0.12	57.5 ± 17.9	100	50.16 ± 12.62	56.11 ± 6.25	46.99 ± 11.18
0.1% M	23.00 ± 3.37^{b}	2.17 ± 0.28^{b}	0.96 ± 0.02	3.00 ± 0.12	54.7 ± 18.3		100	60.66 ± 10.82	50.56 ±15.03
1% M	24.00 ± 2.71^{ab}	2.23 ± 0.24^{b}	0.95 ± 0.01	3.02 ± 0.11	67.5 ± 3.0			100	54.81 ± 8.06
2% M	21.25 ± 2.22^{b}	1.99 ± 0.16^{b}	0.96 ± 0.02	2.92 ± 0.11	42.0 ± 18.0				100

Week 2 autochthonous	**Microbial ecological parameters**					**Similarity**			
	N	Richness	Evenness	Diversity	SIMPER (%)	Control	0.1% M	1% M	2% M
Control	34.00 ± 4.55^{a}	3.00 ± 0.36^{a}	0.99 ± 0.00	3.47 ± 0.13^{a}	75.5 ± 8.1	100	73.10 ±4.75	67.48 ± 7.34	53.81 ± 8.78
0.1% M	29.33 ± 2.08^{ab}	2.63 ± 0.17^{ab}	0.98 ± 0.00	3.31 ± 0.07^{ab}	80.6 ± 1.5		100	77.72 ± 8.89	62.07 ± 9.64
1% M	28.75 ± 2.06^{b}	2.60 ± 0.18^{ab}	0.98 ± 0.00	3.30 ± 0.06^{b}	73.2 ± 9.2			100	64.62 ± 8.53
2% M	25.00 ± 3.37^{b}	2.31 ± 0.27^{b}	0.98 ± 0.00	3.15 ± 0.13^{b}	67.8 ± 7.9				100

N = number of operational taxonomical units, Richness = Margalef species richness, Diversity = Shannon's diversity index, SIMPER = similarity percentage within group replicates. Similarity = pairwise comparison of weighted Bray – Curtis similarity. ^{ab} Different superscripts indicate a significant difference (P<0.05)

Week four

At week four the allochthonous microbial profiles from each treatment group were highly similar (of ca. 82% - 90%) (Figure 4.4). The microbial ecological parameters revealed the total number of OTUs (28.67 - 32.00), the species richness (2.60 - 2.87), the species evenness (0.98), the species diversity (3.28 - 3.40) and the SIMPER similarity (85.81 - 95.17%) remained unaffected by dietary MacroGard® (Table 4.2).

However, Figure 4.5 illustrates that the similarity of the autochthonous microbiota remained low between treatments at week four (ca. 56% - 84%). Dietary MacroGard® supplementation reduced the number of OTUs from 28.00 ± 3.27 in the control group to 23.00 ± 3.37 ($P = 0.045$), 24.00 ± 2.71 ($P = 0.131$) and 21.25 ± 2.22 ($P = 0.004$) in fish fed the 0.1%, 1% and 2% MacroGard® supplemented diets (Table 4.2). Similarly, the species richness was reduced from 2.31 ± 0.32 in the control group to 1.83 ± 0.31 ($P = 0.052$), 1.99 ± 0.22 ($P = 0.163$) and 1.53 ± 0.09 ($P = 0.006$) and the Shannon diversity index from 3.14 ± 0.16 in the control group to 2.87 ± 0.19 ($P = 0.052$), 2.98 ± 0.14 ($P = 0.211$) and 2.69 ± 0.01 ($P = 0.005$) in the 0.1%, 1% and 2% MacroGard® groups, respectively. Species evenness was unaffected. A significant increase in SIMPER similarity in all MacroGard® groups was observed from 70.81 ± 1.85% in the control group to 82.48 ± 6.08% ($P = 0.006$), 87.36 ± 2.48% ($P = 0.001$) and 84.61 ± 3.78% ($P = 0.003$) in the 0.1%, 1% and 2% MacroGard® groups, respectively.

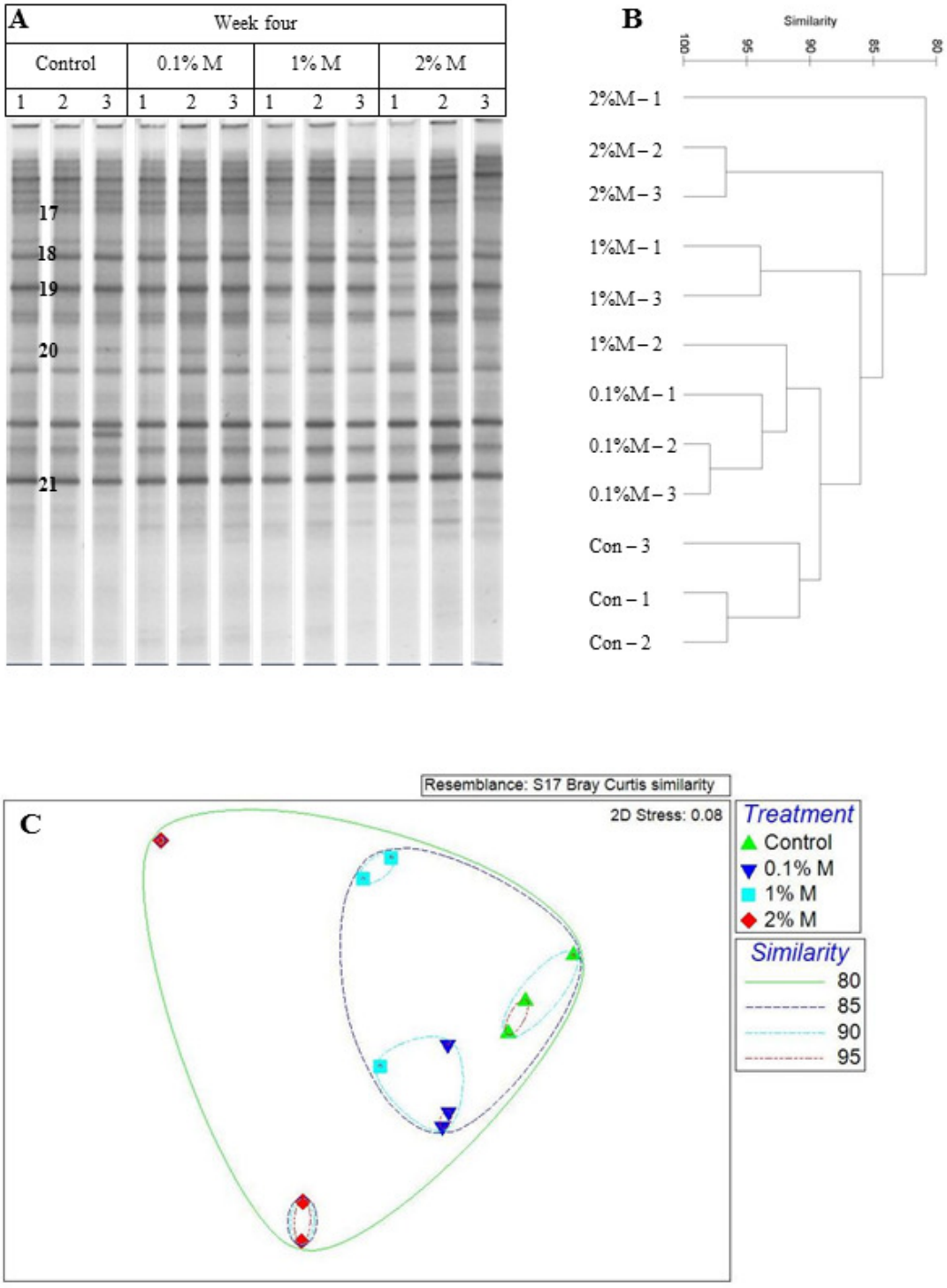

Figure 4.4: A: Denaturing gradient gel electrophoresis (DGGE) profiles of PCR – amplified products of the V3 region of the 16S rRNA gene from week 4 allochthonous samples. B: Bray – Curtis dendrogram demonstrating the similarity. C: nonmetric multidimensional scaling analysis plots showing clusters at different similarity levels (%). Carp were fed control (0%) or MacroGard® (0.1%, 1%, 2% M) supplemented diets for 4 weeks.

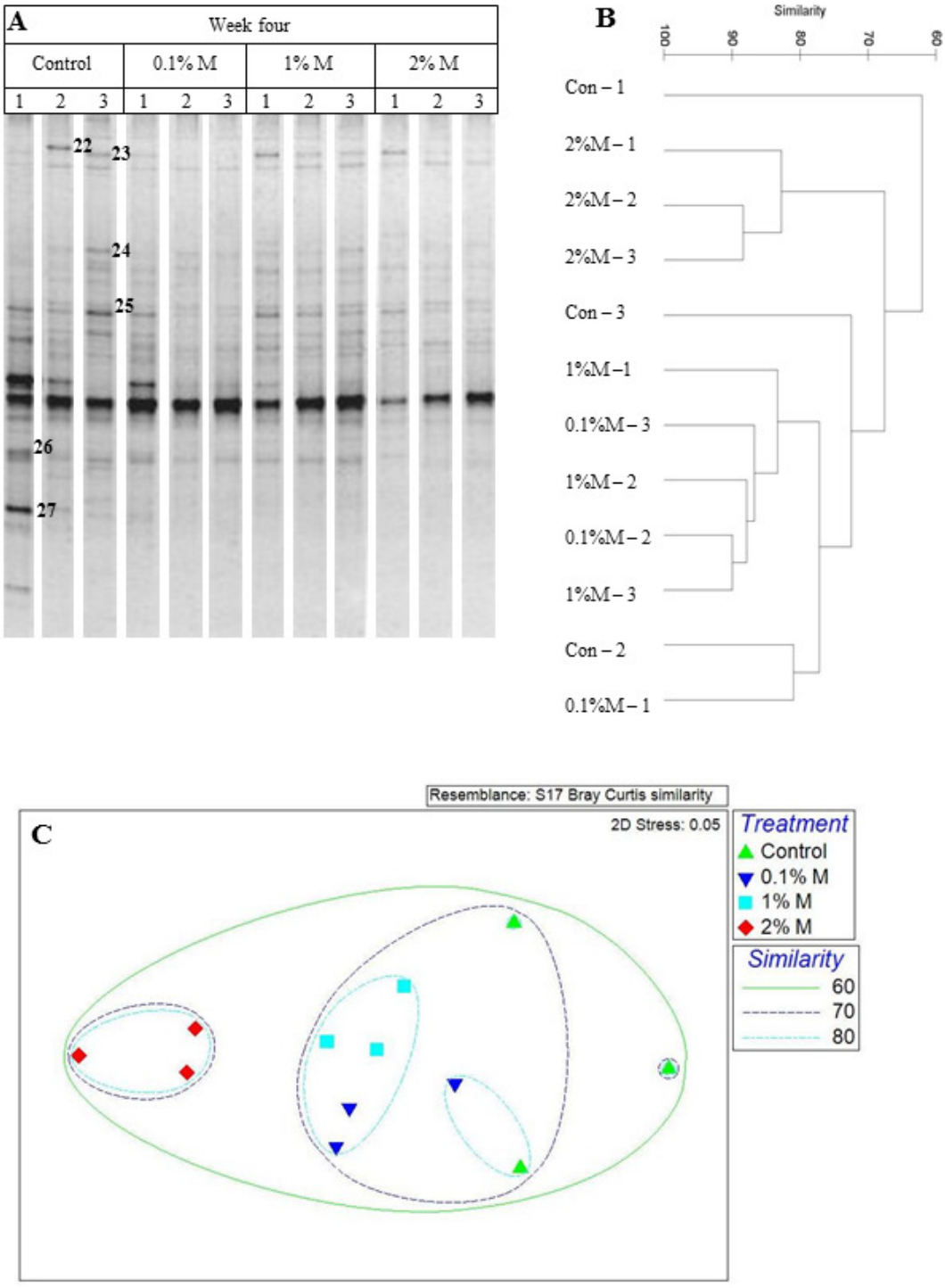

Figure 4.5: A: Denaturing gradient gel electrophoresis (DGGE) profiles of PCR – amplified products of the V3 region of the 16S rRNA gene from week 4 autochthonous samples. B: Bray – Curtis dendrogram demonstrating the similarity. C: nonmetric multidimensional scaling analysis plots showing clusters at different similarity levels (%). Carp were fed control (0%) or MacroGard® (0.1%, 1%, 2% M) supplemented diets for 4 weeks.

Table 4.2: Microbial community analysis of the allochthonous and autochthonous microbial communities of carp from DGGE fingerprints after 4 weeks of feeding control (0%) or MacroGard® (0.1%, 1%, 2% M) supplemented diets.

Week 4 allochthonous	**Microbial ecological parameters**					**Similarity**			
	N	Richness	Evenness	Diversity	SIMPER (%)	Control	0.1% M	1% M	2% M
Control	30.67 ± 1.15	2.76 ± 0.09	0.98 ± 0.00	3.36 ± 0.04	92.75 ± 3.34	100	89.62 ± 2.08	86.37 ± 1.87	81.91 ± 1.28
0.1% M	32.00 ± 0.00	2.87 ± 0.02	0.98 ± 0.00	3.40 ± 0.01	95.17 ± 2.47		100.00	87.67 ± 3.92	84.51 ± 4.96
1% M	28.67 ± 1.15	2.60 ± 0.08	0.98 ± 0.00	3.28 ± 0.04	90.53 ± 3.02			100.00	83.12 ± 3.50
2% M	31.67 ± 2.31	2.83 ± 0.17	0.98 ± 0.00	3.39 ± 0.08	85.81 ± 9.42				100

Week 4 autochthonous	**Microbial ecological parameters**					**Similarity**			
	N	Richness	Evenness	Diversity	SIMPER (%)	Control	0.1% M	1% M	2% M
Control	28.00 ± 3.27^{a}	2.31 ± 0.32^{a}	0.97 ± 0.01	3.14 ± 0.16^{a}	70.81 ± 1.85^{a}	100	70.00 ± 7.67	71.22 ± 5.82	55.77 ± 5.32
0.1% M	23.00 ± 3.37^{b}	1.83 ± 0.31^{ab}	0.97 ± 0.01	2.87 ± 0.19^{ab}	82.48 ± 6.08^{b}		100	83.67 ± 3.80	69.58 ± 7.06
1% M	24.00 ± 2.71^{ab}	1.99 ± 0.22^{ab}	0.98 ± 0.01	2.98 ± 0.14^{a}	87.36 ± 2.48^{b}			100	71.94 ± 6.46
2% M	21.25 ± 2.22^{b}	1.53 ± 0.09^{b}	0.97 ± 0.02	2.69 ± 0.01^{b}	84.61 ± 3.78^{b}				100

N = number of operational taxonomical units, Richness = Margalef species richness, Diversity = Shannon's diversity index, SIMPER = similarity percentage within group replicates. Similarity = pairwise comparison of weighted Bray – Curtis similarity. ^{ab} Different superscripts indicate a significant difference (P<0.05).

4.4.3. Sequence Analysis

A number of OTUs were excised for sequence analysis from the PCR-DGGE gels (Tables 4.3 and 4.4). These OTUs belonged to the phyla *Proteobacteria*, *Firmicutes*, *Fusobacteria* or were unidentified uncultured bacteria. Potentially pathogenic (e.g. *Aeromonas* spp., *Lactococcus garvieae*), probiotic (e.g. *Vagococcus* spp., *Weissella* spp., *Lactococcus* spp.) and beneficial (e.g. *Cetobacterium*) microbiota were found.

Week two

In the allochthonous microbiota after two weeks of feeding the relative abundance of *Lc. garvieae* (OTU 1) increased in fish fed the 1% and 2% MacroGard® supplemented diet (130.5% and 149.0% relative to the control abundance, respectively). Reductions in relative abundance were seen in an uncultured *Propionigenium* sp. (OTU 3) and an uncultured bacterium (OTU 6) with all MacroGard® concentrations. Minor changes in relative abundance were observed for *Aeromonas hydrophila* strains (OTU 8-10) and uncultured *Aeromonas* sp. (OTU 11) and *Aeromonas* sp. (OTU 2).

In the autochthonous microbiota after two weeks of feeding the abundance of LAB OTUs (*Weissella cibaria*, uncultured *Lactococcus* sp., *Vagococcus* sp.; OTUs 12-14) and Aeromonads (*Aeromonas* sp., *Aeromonas hydrophila*; OTUs 15 and 16) decreased with increasing dietary MacroGard® supplementation. The presence of *Vagococcus* sp. (OTU 14) was clearly reduced in all MacroGard® groups (0.1%M = 62.1%, 1%M = 45.0% and 2%M = 50.3%). The uncultured *Lactococcus* sp. (OTU 13) was less prominent only at the 1% and 2% MacroGard® concentrations (81.5% and 61.7%, respectively). The relative abundance of *Weissella cibaria* (OTU 12) was marginally decreased with all dietary MacroGard® concentrations (83.0%, 93.3% and 73.7%). *Aeromonas* sp. (OTU 15) decreased to 81.5% and 67.5% and *Aeromonas hydrophila* (OTU 16) decreased to 80.8% and 65.7% in the 1% and 2% MacroGard® groups.

Week four

In the allochthonous microbiota after four weeks of feeding the sequences retrieved from the excised bands were present in all samples and identified as *Lactobacillus sakei* (OTU 17), *Vagococcus* sp. (OTU 18), *Streptococcus* sp. (OTU 19), *Clostridium tetani* (OTU 20) and an uncultured bacterium (OTU 21). 0.1% MacroGard® supplementation had no effect on abundances but marginal reductions were observed at 1% and 2% MacroGard® supplementation.

In the autochthonous microbiota after four weeks of feeding OTUs identified as uncultured *Lactococcus* sp. (OTU 24) and *Vagococcus* sp. (OTU 25) were present in all samples, but the abundances were reduced in fish fed the MacroGard® supplemented diets. The uncultured *Lactococcus* sp. decreased to 83.0% and 85.2% with the 0.1% and 1% MacroGard® supplemented diets and further decreased to 69.9% with the 2% MacroGard® supplemented diet. Similarly, the *Vagococcus* sp. was reduced to 86.9% and 93.3% in fish fed the 0.1% and 1% MacroGard® supplemented diets and further reduced to 78.5% in fish fed the 2% MacroGard® supplemented diet. An uncultured *Cetobacterium* sp. (OTU 26) was marginally reduced in the 0.1% and 1% MacroGard® groups (79.9% and 80.0%, respectively) and clearly reduced in the 2% MacroGard® group (40.1%; absent in one replicate). *Lc. garvieae* (OTU 23) was absent in one control replicate and in 2 replicates of fish fed the 0.1% MacroGard® supplemented diet (38.9% abundance); the abundance was increased to 145.4% and 112.7% in the 1% and 2% MacroGard® groups, respectively. OTU 27, identified as *A. hydrophila*, was present in two of the three control replicates, but was not detectable in any of the replicates of the MacroGard® treatments.

Table 4.3: Closest relatives with similarity (%) to the respective relatives and mean relative abundance for the sequences obtained from the PCR-DGGE of the allochthonous and autochthonous microbial communities from carp after 2 weeks of feeding control (0%) or MacroGard® (0.1%, 1%, 2% M) supplemented diets.

Band №	Closest Relative	Identity (%)	Mean Relative Abundance (%)			
			Control	0.1% M	1% M	2% M
Week 2 allochthonous						
1	*Lactococcus garvieae*	100%	100%	89.8%	130.5%	149.0%
2	*Aeromonas* sp.	99%	-	+	-	-
3	Uncultured *Propionigenium* sp.	100%	100%	0.0%	75.9%	36.5%
4	*Vagococcus* sp.	100%	100%	79.9%	136.2%	60.4%
5	Uncultured bacterium	99%	100%	120.1%	120.3%	58.0%
6	Uncultured bacterium	100%	100%	57.9%	83.9%	57.4%
7	Uncultured *Propionigenium* sp.	100%	100%	43.3%	133.5%	65.4%
8	*Aeromonas hydrophila*	99%	-	+	+	+
9	*Aeromonas hydrophila*	98%	100%	116.8%	116.8%	111.3%
10	*Aeromonas hydrophila*	100%	100%	98.3%	123.7%	99.0%
11	Uncultured *Aeromonas* sp.	100%	-	-	+	+
Week 2 autochthonous						
12	*Weissella cibaria*	100%	100%	83.0%	93.3%	73.7%
13	Uncultured *Lactococcus* sp.	100%	100%	108.2%	81.5%	61.7%
14	*Vagococcus* sp.	100%	100%	62.1%	45.0%	50.3%
15	*Aeromonas* sp.	100%	100%	96.0%	81.5%	67.5%
16	*Aeromonas hydrophila*	100%	100%	89.0%	80.8%	65.7%

" – " = species absent; " + " = species present at least in one replicate.

Table 4.4: Closest relatives with similarity (%) to the respective relatives and mean relative abundance for the sequences obtained from the PCR-DGGE of the allochthonous and autochthonous microbial communities from carp after 4 weeks of feeding control (0%) or MacroGard® (0.1%, 1%, 2% M) supplemented diets.

Band №	Closest Relative	Identity (%)	Mean Relative Abundance (%)			
			Control	0.1% M	1% M	2% M
Week 4 allochthonous						
17	*Lactobacillus sakei*	94%	100%	105.3%	86.5%	87.8%
18	*Vagococcus* sp.	100%	100%	104.9%	93.1%	93.9%
19	Uncultured *Streptococcus* sp.	99%	100%	100.0%	92.5%	87.0%
20	*Chlostridium tetani*	93%	100%	104.9%	74.6%	95.8%
21	Uncultured *bacterium*	100%	100%	103.4%	93.2%	104.2%
Week 4 autochthonous						
22	Uncultured *bacterium*	97%	100%	0.0%	0.0%	0.0%
23	*Lactococcus garvieae*	100%	100%	38.9%	145.4%	112.7%
24	Uncultured *Lactococcus* sp.	100%	100%	83.0%	85.2%	69.9%
25	*Vagococcus* sp.	99%	100%	86.9%	93.3%	78.5%
26	Uncultured *Cetobacterium* sp.	99%	100%	79.9%	80.0%	40.1%
27	*Aeromonas hydrophila*	100%	100%	0.0%	0.0%	0.0%

4.4.4. Epithelial ultrastructure

The apical brush border of the enterocytes in the posterior intestinal region from fish fed either the control, the 0.1% or the 1% MacroGard® supplemented diets were examined using transmission electron microscopy. Overall, the epithelial architecture appeared healthy with integrated, columnar shaped enterocytes. Numerous, well-developed microvilli with the presence of associated terminal webs and no damage to tight junctional complexes, desmosomes or intercellular spaces was observed (Plate 4.1). The microvilli length and density of intestinal enterocytes was analysed (Table 4.5). Dietary MacroGard® supplementation did not affect microvilli length or density after two weeks of feeding. After four weeks of feeding however, the administration of 1% MacroGard® led to a significant increase of the microvilli length ($P < 0.025$) and density ($P < 0.038$) compared to fish fed the control and the 0.1% MacroGard® supplemented diet.

Table 4.5: Transmission electron microscopy analysis of the microvilli length (in µm) and the microvilli density (microvilli μm^{-1}) of posterior intestinal enterocytes of carp after 2 and 4 weeks of feeding control (0%) or MacroGard® (0.1%, 1% M) supplemented diets ($n = 5$).

		Control	**0.1% M**	**1% M**
Microvilli length	Week 2	1.38 ± 0.20	1.22 ± 0.14	1.42 ± 0.19
	Week 4	1.32 ± 0.15[a]	1.46 ± 0.15[a]	1.68 ± 0.12[b]
Microvilli density	Week 2	7.06 ± 0.25	7.28 ± 0.21	7.37 ± 0.40
	Week 4	7.21 ± 0.42[a]	7.36 ± 0.22[a]	7.85 ± 0.33[b]

[ab] different superscripts indicate a significant difference ($P<0.05$)

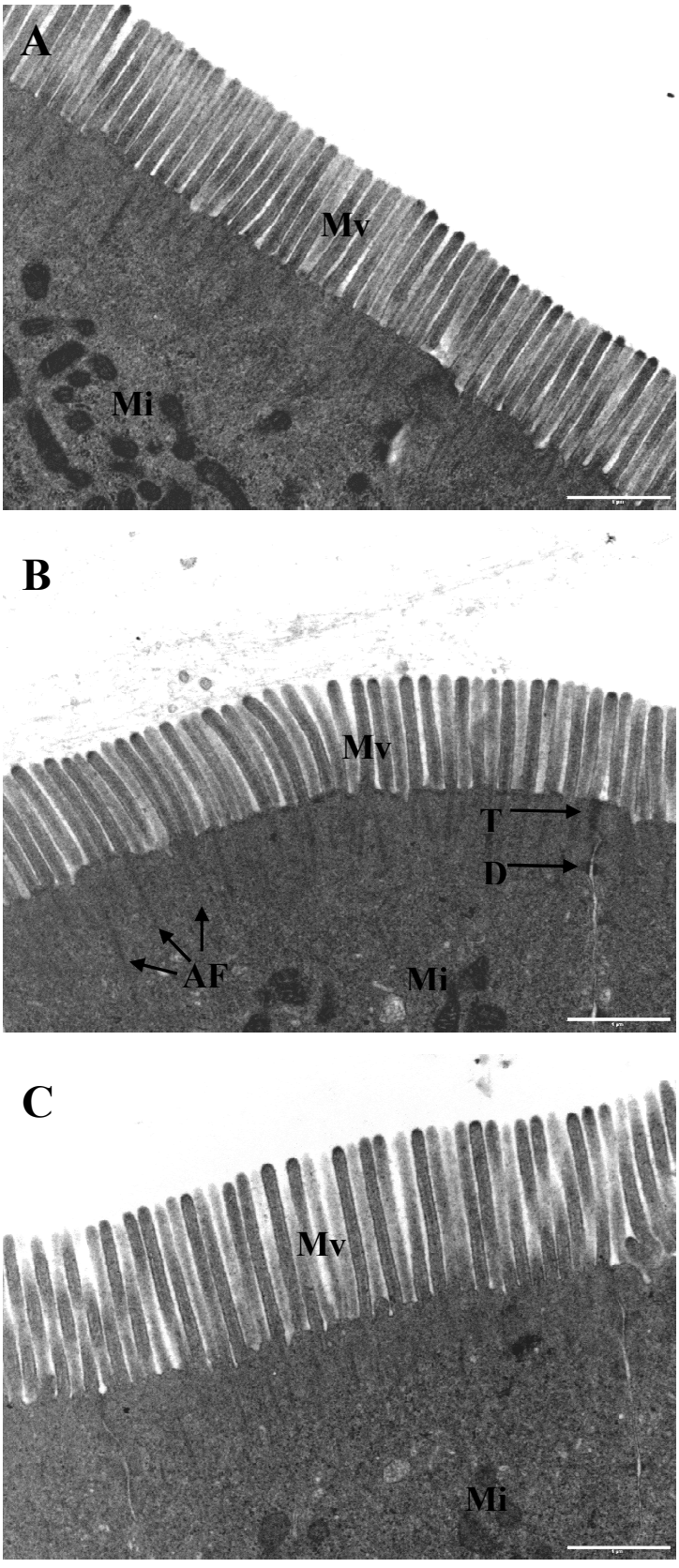

Plate 4.1: Comparative TEM micrographs displaying posterior intestinal microvilli of enterocytes of carp fed: (A) the control diet, (B) the 0.1% MacroGard® and (C) the 1% MacroGard® supplemented diet for 2 weeks. MV = microvilli, TJ = tight junction, D = desmosome, Mi = mitochondria, AF = actin filaments. Scale bar represents 1µm.

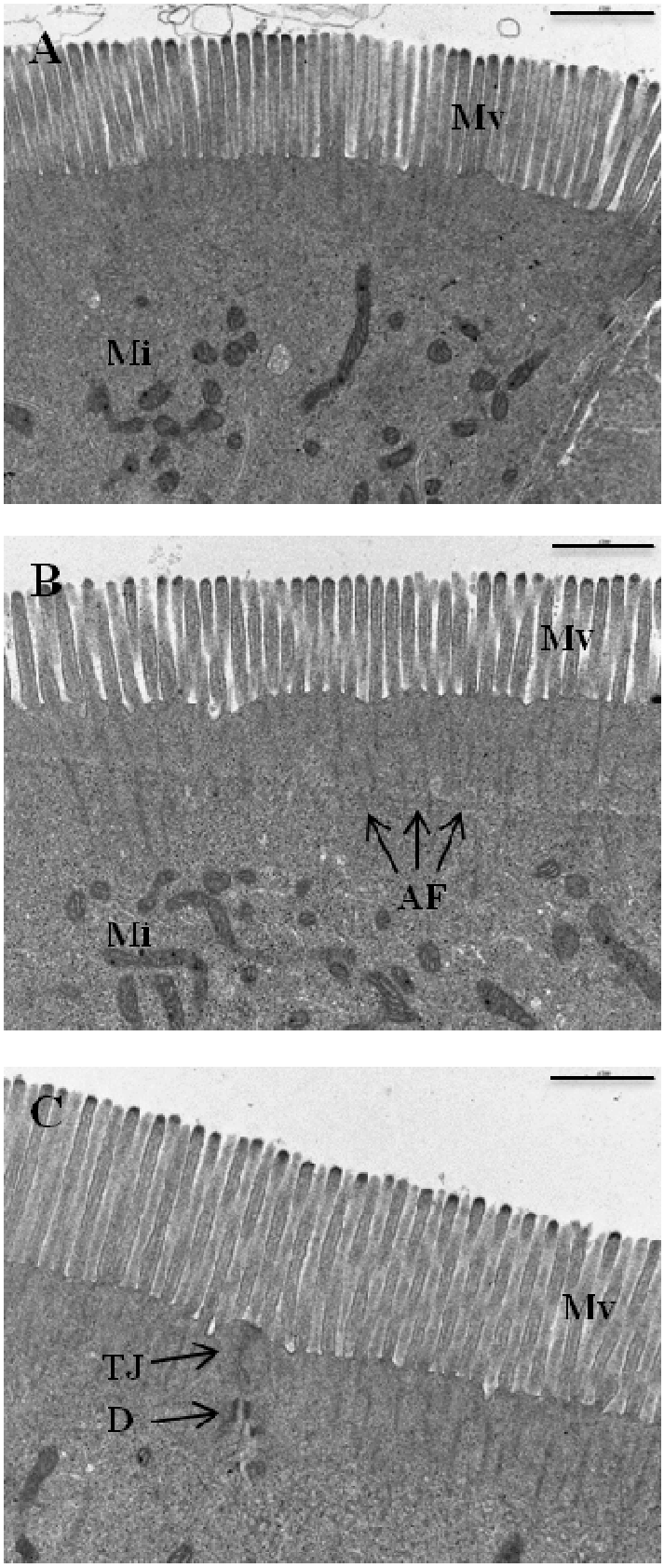

Plate 4.2: Comparative TEM micrographs displaying posterior intestinal microvilli of enterocytes of carp fed: (A) the control diet, (B) the 0.1% MacroGard® and (C) the 1% MacroGard® supplemented diet for 4 weeks. Note the increased microvilli length and density in (C). MV = microvilli, TJ = tight junction, D = desmosome, Mi = mitochondria, AF = actin filaments. Scale bar represents 1μm.

4.5 Discussion

The present study indicates for the first time that dietary β-(1,3)(1,6)-D-glucans may affect the intestinal microbial communities in teleosts. Carp intestinal microbial communities have been assessed using culture-dependent methods in the past and consisted mainly of aerobes and facultative anaerobes (Sugita *et al*., 1985) with *Aeromonas* species being highly abundant (Sugita *et al*., 1990; Namba *et al*., 2007). Enterobacteriaceae, *Pseudomonas*, *Moraxella*, *Acinetobacter* and *Micrococcus* (Sugita *et al*., 1990; Namba *et al*., 2007), *Bacteriodetes* (Sugita *et al*., 1990; Tsuchiya *et al*., 2008), *Plesiomonas*, *Flavobacterium*, *Staphylococcus*, *Streptococcus*, *Bacillus* and *Chlostridium* (Sugita *et al*., 1990), *Vibrio* (Namba *et al*., 2007) and *Cetobacterium* (Tsuchiya *et al*., 2008) have also been detected. It is however generally accepted that evaluation of intestinal microbial communities by using culture-based techniques alone is inaccurate and restricted to culturable bacteria (Cahill, 1990; Ringø *et al*., 2006b; He *et al*., 2011a). To the author's knowledge, there is no published information about the effects of β-glucans on teleost intestinal microbiota using culture-independent methods. The present study therefore applied culture-dependent and culture-independent PCR-DGGE methods to assess effects of a dietary β-(1,3)(1,6)-D-glucan (MacroGard®) supplementation on allochthonous and autochthonous microbial communities in mirror carp (*Cyprinus carpio* L.).

In the present study, cultivable allochthonous LAB levels were not affected by dietary MacroGard® at either sampling time point. Similarly, Smith *et al.* (2011) reported that laminarin, a low molecular weight (7.7 kDa), soluble β-1,3-1,6-D-glucan found in brown algae, had no effect on allochthonous lactobacilli and bifidobacteria levels in the pig colon. Contradictory results have been reported in rats, where elevated

allochthonous intestinal lactobacilli levels have been observed after dietary β-glucan supplementation (Dongowski *et al*., 2002; Snart *et al*., 2006).

In the present study, the autochthonous LAB levels were not affected after two weeks by dietary MacroGard® supplementation. After four weeks however, autochthonous LAB levels decreased with increasing MacroGard® supplementation. Sequence analyses from DGGE profiles identified a number of LAB and, confirming the culture based results, the abundance of the autochthonous LAB were reduced in the MacroGard® fed fish. After two weeks of feeding the abundance of *Weissella cibaria*, uncultured *Lactococcus* sp. and *Vagococcus* sp. (OTUs 12-14) decreased with increasing dietary MacroGard® supplementation. Similarly, after four weeks of feeding the abundance of uncultured *Lactococcus* sp. (OTU 24) and *Vagococcus* sp. (OTU 25) were reduced in fish fed the MacroGard® supplemented diets. To the authors' knowledge there is no information available on the effects of dietary β-glucan supplementation on autochthonous LAB in fish or mammals. In the present study, the total number of cultivable aerobic heterotrophic bacteria levels were not affected by MacroGard® supplementation which is in accordance with the preliminary results observed in Atlantic cod (*Gadus morhua*) Skjermo *et al*. (2006). Culture-independent analyses revealed that dietary MacroGard® reduced the number of phylotypes and the species richness of the allochthonous microbiota in the carp intestine after two weeks, but not after four weeks of feeding. For the autochthonous microbiota, dietary MacroGard® reduced the number of phylotypes, the species richness and diversity after two weeks, and those parameters remained reduced after four weeks of feeding. Sequence analysis identified that the abundance of autochthonous *A. hydrophila* was reduced with dietary MacroGard® whereas the effect on the abundance of allochthonous Aeromonad OTUs was more variable. These results demonstrate that the effect of MacroGard® on the bacterial communities in the intestine of carp is different, with a more pronounced effect

on the autochthonous microbiota which may be reflective of a different community composition on the mucosal surface, than in the lumen, or potentially the observed changes may be a result of structural or immunological changes in the host's epithelial brush border or mucus biochemistry.

After four weeks of feeding the posterior intestinal microvilli length and density were significantly increased in fish fed diets supplemented with 1% MacroGard® whereas after two weeks no differences were observed. To the authors' knowledge no report on the effects of β-glucans on the apical brush border morphology of teleost enterocytes has been published to date. However, a number of studies have reported similar effects with dietary mannanoligosaccharides (MOS) isolated from *Saccharomyces cerevisiae* yeast cell walls. Increased microvilli length was observed in red drum (*Sciaenops ocellatus*) when MOS and other prebiotic oligosaccharides were included in the diet at 1% (Zhou *et al*., 2010) and in Pacific white shrimp (*Litopenaeus vannamei*) with MOS supplementations from 0.2-0.8% but not with 0.1% (Zhang *et al*., 2012). In addition, increased microvilli length and density were reported in European lobsters (*Homarus gammarus* L.) (Daniels *et al*., 2010) and rainbow trout (*Oncorhynchus mykiss*) (Dimitroglou *et al*., 2009) fed MOS supplemented diets. The authors suggest that a well-developed, healthy mucosal epithelium may be advantageous in the defence of opportunistic indigenous bacterial infections in addition to a likely improved feed utilization.

4.6 Conclusions

The present study was the first to comprehensively investigate the time-dependent effects of a dietary immunostimulating β-(1,3)(1,6)-D-glucan (MacroGard®) on both allochthonous and autochthonous microbial communities in the teleost intestine. The

results indicated that dietary MacroGard® may affect the composition of the carp intestinal microbial communities. Furthermore, positive effects on intestinal microvilli length and density were also observed. Future research is required to validate whether the present observations occur under different environmental conditions, e.g. in commercial husbandry, or in fish species with different digestive systems, e.g. in carnivorous species in contrast to omnivorous, stomachless carp used in the present study. In addition, studies are required to elucidate the differences in the intestinal microbial activities and the impact of such changes on the host immune response and digestive activity.

Chapter 5:

Effects of a dietary β-(1,3)(1,6)-D-glucan supplementation on a chronic progressive *Mycobacterium haemophilum* mycobacteriosis in zebrafish (*Danio rerio*)

5.1 Abstract

Mycobacteriosis infections can cause economic losses in aquaculture and pose a threat to research facilities. Prophylactic dietary ß-glucan administration has been proven to increase resistance against bacterial pathogens in various fish species. Knowledge however about their possible therapeutic effects on chronic diseases such as mycobacteriosis is very limited. Two experiments were therefore conducted to examine the effects of a dietary *Saccharomyces cerevisiae* ß-glucan (MacroGard®) on chronic mycobacteriosis in adult zebrafish. Sequencing of PCR-amplified liver DNA extracts using *Mycobacterium* genus-specific primers identified *Mycobacterium haemophilum* as the causative pathogen. In the long-term study, experiment I (200 days), zebrafish were fed highly purified diets supplemented with 0% (control, C) or 0.1% MacroGard® (B), and survival rates were recorded. In the short-term study, experiment II (63 days), zebrafish were fed diet C or diet B continuously or both diets intermittently (CB) in weekly intervals. Survival rates, histopathological status and intestinal microbial communities were analysed. Dead and moribund fish displayed the typical clinical signs of mycobacteriosis. Dietary MacroGard® failed to improve survival rates (values ± SEM) in experiment I (C = 36.2 ± 4.2% *vs*. B = 20.0 ± 7.3%) and in experiment II (C = 72.5 ± 1.0% *vs*. B = 78.3 ± 0.3% *vs*. CB = 78.3 ± 1.6%). Histopathological analysis revealed absent (normal appearance), moderate and severe manifestations of granulomas, the hallmarks of mycobacteriosis. Ecological microbial community analysis of intestinal samples collected at day 63 showed significantly higher number of OTUs, species richness and diversity in treatments B and CB compared to the control treatment. In conclusion, MacroGard® failed to improve zebrafish resistance against mycobacteriosis irrespective of length or sequence of administration. Yet, since gaps in knowledge still exist, further research is required to eliminate the current ambiguities.

5.2 Introduction

The importance of zebrafish (*Danio rerio*) in research has risen over the last decade as many laboratories have utilised this species to develop protocols modelling cancer and infection studies for both teleosts and humans (van der Sar *et al*., 2004; Mione and Trede, 2010). For example, mycobacteriosis in zebrafish serves as a model for human tuberculosis due to the pathological similarities (Davis *et al*., 2002; Prouty *et al*., 2003; van der Sar *et al*., 2004). In addition, the zebrafish has also been established as a model for investigations of the intestinal microbiota, especially when gnotobiotic zebrafish models are used (Rawls *et al*., 2004; Bates *et al*., 2006). Mycobacterial infections frequently pose a threat to zebrafish facilities when mycobacteriosis is not the intended focus of research. Mycobacteria are widespread in aquatic environments making control of these pathogens by avoidance difficult; currently there are no effective treatments for mycobacteriosis in zebrafish available (Ramsay *et al.*, 2009).

Piscine mycobacteriosis is a systemic, predominantly chronic, progressive disease where clinical signs may not even occur (Gauthier and Rhodes, 2009). Seldom, acute forms however have been reported leading to substantial mortalities in aquaculture (Hedrick *et al*., 1987; Bruno *et al*., 1998). Studies with *M. marinum* and *Mycobacterium peregrinum* indicated that the natural route of infection in zebrafish is primarily through the gastrointestinal tract (Hariff *et al*., 2007). All tissues and organs may be affected and non-specific external clinical signs may include lethargy, loss of equilibrium, anorexia, skin, fin and scale alterations, exophthalmia, emaciation, ascites and skeletal deformities (Talaat *et al*., 1998; Decostere *et al*., 2004; Swaim *et al*., 2006; Watral and Kent, 2007; Whipps *et al.*, 2007; Gauthier and Rhodes, 2009). Granulomatous inflammation is the typical histopathological manifestation in teleosts suffering from mycobacteriosis (Gauthier and Rhodes, 2009). A granuloma is defined as “a compact (organized) collection of mature mononuclear phagocytes (macrophages and/or epithelioid cells)

which may or may not be accompanied by accessory features such as necrosis or the infiltration of other inflammatory leukocytes" (Adams, 1976). Size and structural organization of granulomas can vary considerably (Gauthier and Rhodes, 2009). The chronic proliferative form of the disease is characterized by granulomas; sub-acute and acute forms are associated with necrosis and acid-fast bacilli scattered diffusely among affected tissues including the kidney, liver and spleen, and often all visceral organs (Ferguson 2006).

As described in previous chapters, dietary ß-glucans have been reported to improve the immune response and disease resistance against bacterial pathogens in fish including cyprinids (Kwak *et al*., 2003; Misra *et al*., 2006; Gopalakannan and Arul, 2010). In zebrafish specifically, one study also reported increased survival rates upon an *Aeromonas hydrophila* challenge when ß-glucans were injected intraperitoneally (Rodriguez *et al*., 2009). Dietary ß-glucans also enhanced survival rates in artificially immune-suppressed fish (Sahoo and Mukherjee, 2001, 2002; Kumari and Sahoo, 2006b; El-Boshy *et al*., 2010) upon bacterial challenges. Literature however about the effects of dietary immunostimulants on mycobacteriosis is scarce. One study by Li and Gatlin (2005) indicated positive effects of partially autolyzed brewer's yeast (Brewtech®) and GroBiotic®-A, a mixture of partially autolyzed brewer's yeast, dairy ingredient components and dried fermentation products on survival rates of hybrid striped bass (*Morone chrysops* x *M. saxatilis*). Immunostimulants, such as ß-glucans, therefore could potentially have beneficial effects on the progression of chronic mycobacteriosis in teleosts.

Since Chapter 4 demonstrates comprehensively for the first time that dietary ß-(1,3)(1,6)-D-glucans modulate the intestinal microbiota in carp, such an effect could also occur in zebrafish. As already mentioned, the intestinal microbial communities and their metabolites are highly important by influencing, among other factors, the

immunological balance and physiological processes of teleosts (Cerf-Bensussan and Gaboriau-Routhiau, 2010; Merrifield *et al*., 2010; Sekirov *et al*., 2010).

Thus, two experiments were conducted to assess the efficacy of dietary β-glucan applications on zebrafish resistance to mycobacteriosis and to determine if the dietary provision of β-glucans influences the zebrafish gut microbiome. The aim of experiment I was to investigate the effects of a dietary β-(1,3)(1,6)-D-glucan (MacroGard®) on survival rates of zebrafish suffering from a chronic, pre-existing mycobacteriosis over a period of 200 days. The subsequent experiment II aimed at identifying the exact *Mycobacterium* spp. causing the mycobacteriosis in the facilities and examining the effects of MacroGard® on survival rates and severity of infection of zebrafish over 63 days. In order to avoid potential immunosuppression previous studies have suggested a pulse/intermittent feeding regime (Bricknell and Dalmo, 2005; Ringø *et al*., 2011) and therefore an intermittent diet was also included in experiment II.

5.3 Material and Methods

5.3.1 Rearing facilities and water quality

The experiments were conducted in the Zebrafish Research Facility at the University of Plymouth, UK and water quality was monitored as described under section 2.2. The water temperature and the pH were maintained at 28 ± 0.2 °C and between 6.8 and 7.2, respectively, with the water being partially re-circulated (30% water change per day). The photoperiod was maintained at a 12/12 h light/dark cycle.

5.3.2 Experimental fish and feeding

For both experiments, adult (approximately one year old) wild type zebrafish (*Danio rerio)* were obtained from the Zebrafish Research Facility at Plymouth University, UK.

For experiment I, 210 zebrafish (478 ± 91 mg) were randomly allocated in groups of 35 to each of the six 40L experimental tanks (n = 3 tanks per treatment). For experiment II, 360 zebrafish (503 ± 117 mg) were randomly allocated in groups of 40 to each of the nine 40L experimental tanks (n = 3 tanks per treatment). After allocation the fish were carefully acclimatized over three weeks prior to the experimental period whilst being manually fed the control diet. The basal diet consisted of the same ingredients as described under section 2.5 and was supplemented with 0% w/w MacroGard® (control diet) or 0.1% w/w MacroGard® and produced in flake form (Tetra GmbH, Melle, Germany). In experiment I fish were fed diet C or the experimental diet B. In experiment II fish were fed either 1] the control diet (C), 2] the experimental diet continuously (B) or 3] the control and experimental diet fed intermittently in weekly intervals (CB). At all times fish were manually fed twice per day to apparent satiation.

5.3.3 Cumulative mortality

Cumulative mortality was recorded over a period of 200 days and 63 days in experiment I and experiment II, respectively. Dead and/or moribund fish showing extensive signs of fish tuberculosis just prior to death (skin haemorrhages, abdominal distension, lethargy, emaciation, raised scales) were removed from the tanks and euthanized in 0.1 ppm buffered tricaine methanesulfonate (MS-222; Pharmaq Ltd., Fordingbridge, UK). Fish were stored at -20°C.

5.3.4 Sampling procedures

At the end of experiment II samples for histopathology, intestinal microbiology and confirmation and identification of mycobacteria were taken. Fish were euthanized in 0.1 ppm buffered tricaine methanesulfonate (MS-222).

5.3.4.1 Histopathology

On day 56 and day 63 six fish (2 per tank) were randomly sampled (n = 6 per time point) for histopathological examination and fixed in 10% neutral buffered formalin. Midsagittal sections (4-7 μm) were taken from the mid-trunk region to obtain a portion of multiple organ systems including the liver, kidney and spleen. Sections were decalcified for 30 min and processed for routine paraffin embedding and microtome sectioning. Sections were stained with haematoxylin and eosin (H&E). Sections were also stained for acid-fast bacilli with a modified Ziehl-Neelsen staining protocol (Luna, 1968). Briefly, sections were stained in carbol fuchsin for 60 minutes at room temperature, rinsed with tap water and then differentiated in 1% acid alcohol. After a further rinse with tap water, sections were counterstained with methylene blue (diluted 1:9 in distilled water) for 2 seconds, followed by a rinse with distilled water and then tap water. All preparing working steps (e.g. clearing, re- and de-hydration and mounting with a coverslip) were conducted as described under section *2.7.2*. Slides were examined under an Olympus light microscope, photographed with an Olympus digital camera and processed using Corel Photo-Paint X5. Kidney, liver, spleen, gastrointestinal tract, swim bladder, reproductive organs, and muscle tissue were both quantitatively and qualitatively examined for granulomas. To evaluate the extent of zebrafish organ and tissue inflammation, a severity of infection index, using an arbitrary scoring scale of the liver, spleen and kidney, was adapted and modified from Talaat *et al*. (1998). The index was defined as: score 1 = normal, 2 = moderate and 3 = severe. A score of normal indicates no granulomas. A score of moderate indicates that granulomas were focal or multifocal in an organ but not diffuse. A score of severe indicates where granulomas were multifocal or diffuse to an extent that normal tissue is almost unrecognizable.

5.3.4.2 Assessment of intestinal microbiota

For the evaluation of the intestinal microbial communities, intestinal samples from 5 fish per tank were isolated, pooled and homogenized after Merrifield *et al*. (2013) at day 56 and day 63, thus leading to 3 replicates per treatment, each representing 5 fish. Fish from the intermittent treatment CB were fed the control diet (day 56) and the MacroGard® supplemented diet (day 63) the week preceding the sampling, respectively. DNA extraction and PCR amplification of the 16S rRNA V3 region were performed as described under sections 2.8.1 and 2.8.2, respectively. Subsequently, the V3 PCR amplicons were subjected to a 40% - 60% Denaturing Gradient Gel Electrophoresis (DGGE) as described under section 2.8.3.

5.3.4.3 Confirmation and identification of mycobacteria

Liver tissue samples were taken from the initial pool of fish of each treatment in order to confirm mycobacteria presence and to verify that all fish had an equal probability of infection. Four random samples per treatment were collected from live fish which did not exhibit clinical signs of mycobacteriosis. Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN, Crawley, UK). A conserved region of the 16S rRNA gene of mycobacteria (924 bp product) was amplified by PCR using genus-specific primers after Talaat *et al*. (1997). Subsequently, 8 µl of the PCR product mixed with 2 µl loading buffer were subjected to electrophoresis on a 2% agarose gel containing 2.5 µl ethidium bromide (10mg/ml). Hyper ladder IV (Bioline, London, UK) as well as a negative and positive control (cultured *Mycobacterium phlei*, Microbiology Laboratory culture collection, Plymouth University, UK) were run alongside the samples to identify the correct amplification of the product. All samples displayed a band in the same position indicating that *Mycobacterium* DNA was present (Plate 5.1).

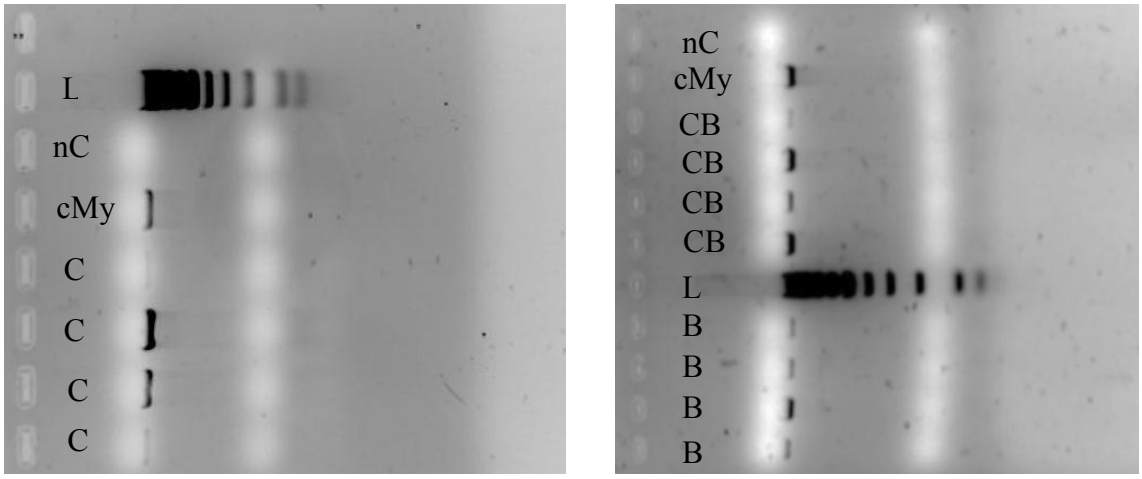

Plate 5.1: Presence/absence of *Mycobacterium* spp. within samples after DNA extraction from liver and subsequent PCR amplification of 16S rRNA using genus specific primers: L = ladder (1000 bp); nC = no template control; cMy = cultured *Mycobacterium phlei*; C = control treatment; CB = MacroGard® intermittent treatment; B = MacroGard® continuous treatment

Two PCR samples of each treatment were purified, sequenced and submitted to a BLAST search in GenBank as described under section 2.8.5. The closest relatives obtained were identical for all six samples which identified *Mycobacterium haemophilum* as the causative pathogen (Table 5.1).

Table 5.1: Closest relatives, identity (%) and accession numbers with the respective treatments and nucleotide length (Nt. length) obtained from the PCR amplification of the liver DNA extracts using *Mycobacterium*-specific primers

Treatment	Nt. length	Closest relative	Identity (%)	Accession №
C	887 nt	*Mycobacterium haemophilum*	99%	DQ851570
C	885 nt	*Mycobacterium haemophilum*	99%	DQ851570
B	884 nt	*Mycobacterium haemophilum*	99%	DQ851570
B	891 nt	*Mycobacterium haemophilum*	98%	DQ851570
CB	886 nt	*Mycobacterium haemophilum*	99%	DQ851570
CB	810 nt	*Mycobacterium haemophilum*	99%	DQ851570

5.3.5 Statistical Analysis

Kaplan-Meier analysis was used to generate survival curves, and a log-rank test was performed to compare the survival of fish in each experimental group. Histopathological data assuming sphericity (Mauchly's Sphericity test) or violating sphericity (Greenhouse-Geisser) was subjected to a repeated-measures ANOVA and significant differences revealed with a Bonferroni correction. The images of the DGGE-gels were statistically analysed as described under section 2.9. A significance level of $P < 0.05$ was used for all statistical tests. Statistical analysis was performed using SPSS statistics, version 18 (SPSS Inc., Chicago, US).

5.4 Results

5.4.1 *Clinical signs and survival rates Experiment I and II*

In the present study infected fish showed external clinical signs of mycobacteriosis as described elsewhere (Swaim *et al*., 2006; Whipps *et al*., 2007). Death was preceded by reduced feed intake, weakened mobility and loss of buoyancy for approximately one week. The fish either were often lethargic at the bottom of the tank or showed listless piping, remaining at the surface, constantly opening and closing their mouths in an attempt to increase gas exchange. Infected fish in both experiments presented clinical signs including severe haemorrhages on the trunk ventral to the lateral line and in close proximity to internal organs, localized granulomatous nodules, abdominal distension, raised scales, clamped fins, exophthalmia, ascites, emaciation and scoliosis (Plate 5.2).

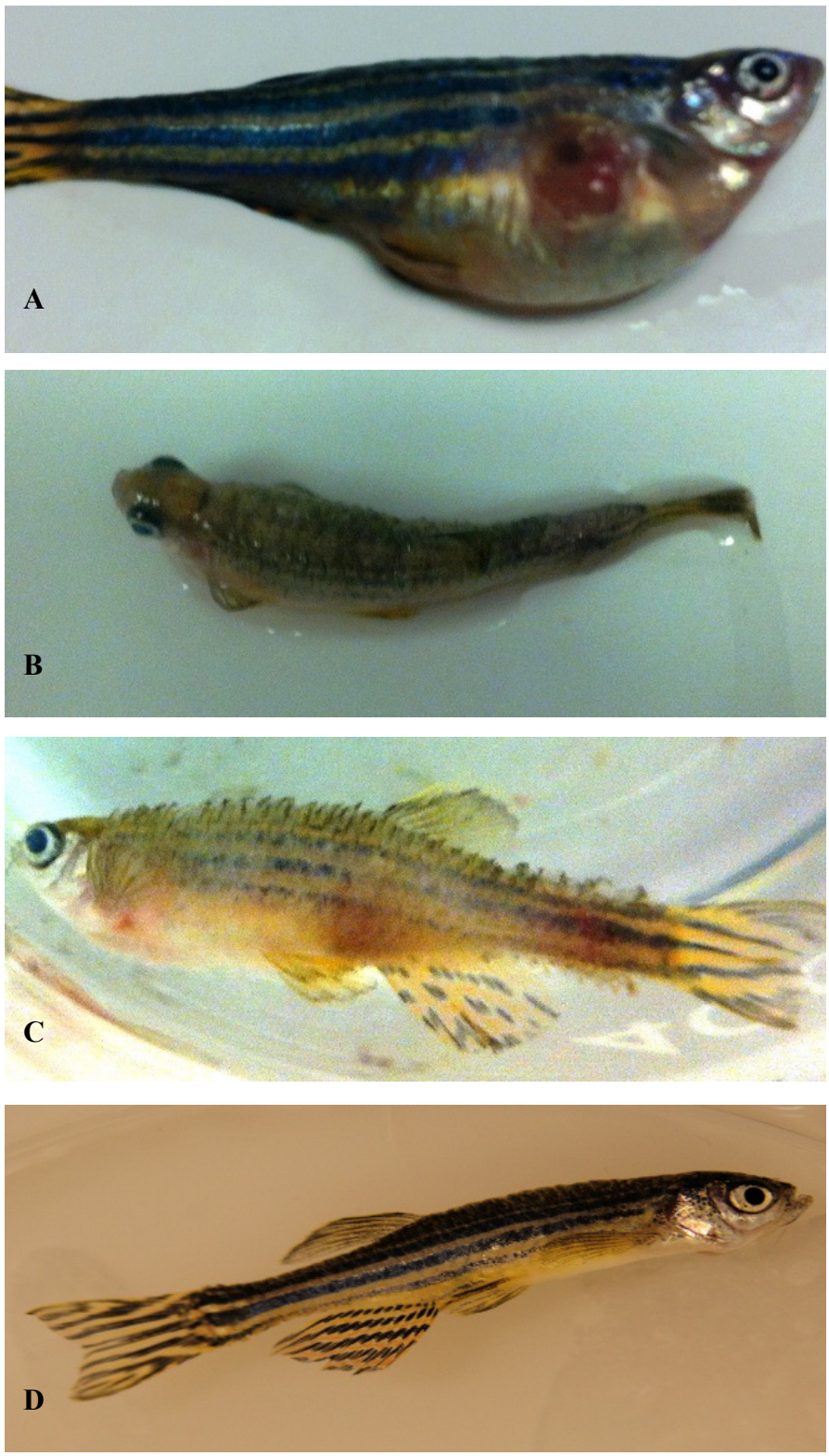

Plate 5.2: Examples of zebrafish from experiment II displaying clinical signs of mycobacteriosis. A = abdominal distension & granulomatous nodule; B = scoliosis & exophthalmia; C = raised scales and haemorrhages; D = emaciation.

After 200 days (experiment I) mean survival rates of fish fed the 0.1% MacroGard® supplemented diet were lower, at 20.0 ± 7.3%, but differences were not significantly (P = 0.21) different compared to fish fed the control diet, where 36.2 ± 4.2% of the individuals survived (Figure 5.1).

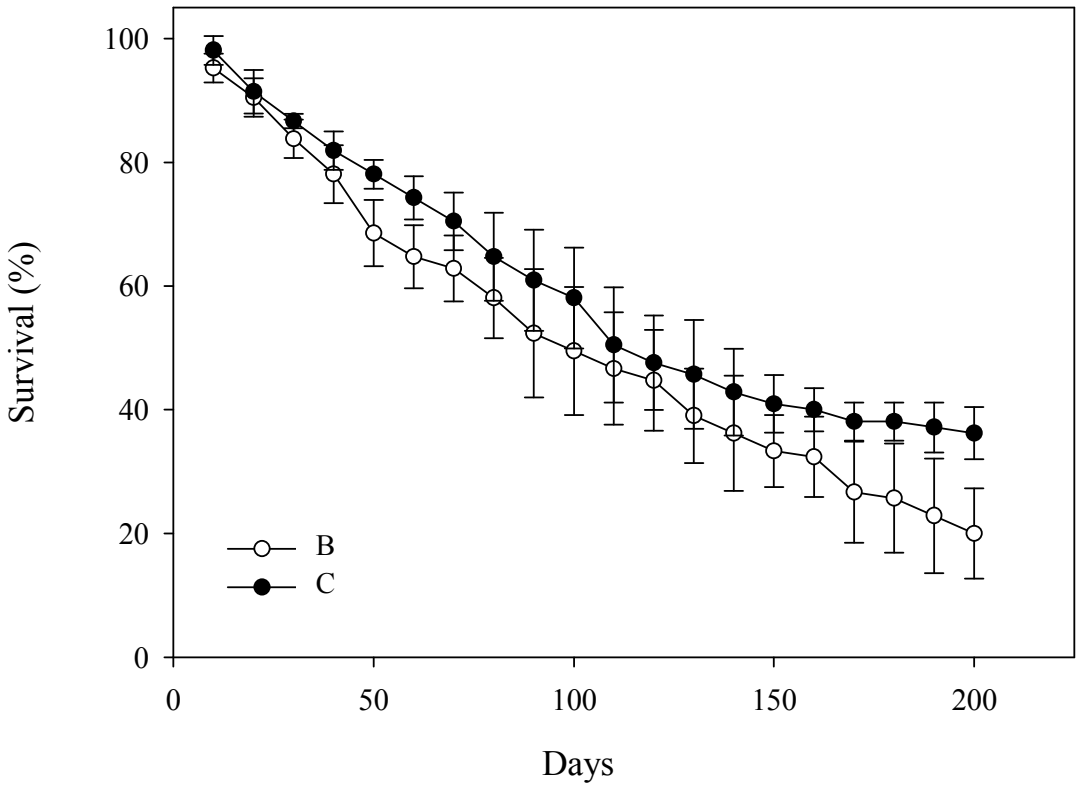

Figure 5.1: Mean survival (%) ± SEM of zebrafish suffering from *Mycobacterium haemophilum* mycobacteriosis fed either the diet C (●) or the diet B supplemented with 0.1% MacroGard® (○) over the 200 days experimental period.

As Figure 5.2 illustrates, after 63 days (experiment II) mean survival rates of fish fed the diet C (control) were marginally lower (72.5 ± 1.0%) compared to fish fed the 0.1% MacroGard® supplemented diets B (78.3 ± 0.3%) or CB (78.3 ± 1.6%) but differences were not statistically different ($P = 0.76$).

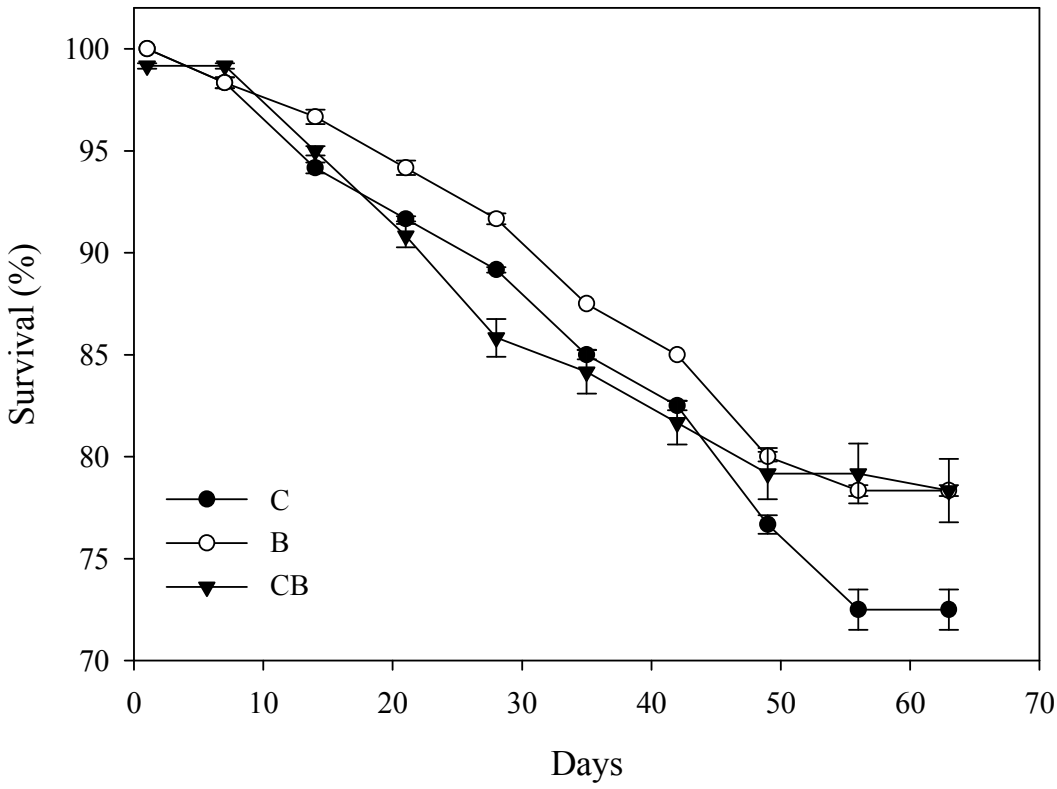

Figure 5.2: Mean survival (%) ± SEM of zebrafish suffering from *Mycobacterium haemophilum* mycobacteriosis fed either diet C (●) or the 0.1% MacroGard® supplemented diets B (○) or CB (▼) over the 63 days experimental period.

5.4.2 *Histopathology*

H & E staining revealed granuloma formation in the organs examined including kidney (Plate 5.3 C), liver (Plate 5.3 D, F) and spleen (Plate 5.3 E, G, H), gastrointestinal tract (Plate 5.3 B), swim bladder, ovaries and muscle tissue (Plate 5.3 E). Ziehl-Neelsen staining showed numerous rod-shaped acid-fast bacilli located in the granulomas (Plate 5.3 H and I). Foci of inflammation had a round pinkish amorphous area approximately 0.1 to 1.0 mm in diameter and included well-distinguished granulomas.

The majority of granulomas had necrotic centres and granular debris including numerous intracellular and extracellular acid fast-bacilli (Plate 5.3 H and I); but also necrotizing centres lacking and non-necrotizing centres displaying acid-fast bacilli under Ziehl-Neelsen stain were detected. Plate 5.3.G shows necrotizing and non-necrotizing centres of granulomas. Central necrosis often developed into caseous necrosis (e.g. Plate 5.3 C). Granulomas occasionally collected and grew into larger loosely organized groups, i.e. multicentric granulomas (Plate 5.3 F). These groups included granulomas with central necrosis with or without visible bacteria. Infrequently, accumulations were detected at the sites of inflammation which most likely comprised of melanomacrophages within the granulomas (Plate 5.3 E).

Normal, moderate and severe infections were found in each experimental group (Figure 5.6). Although, there was a trend for severe infection within the β-glucan supplemented diets, it was not statistically different to the control ($P = 0.07$).

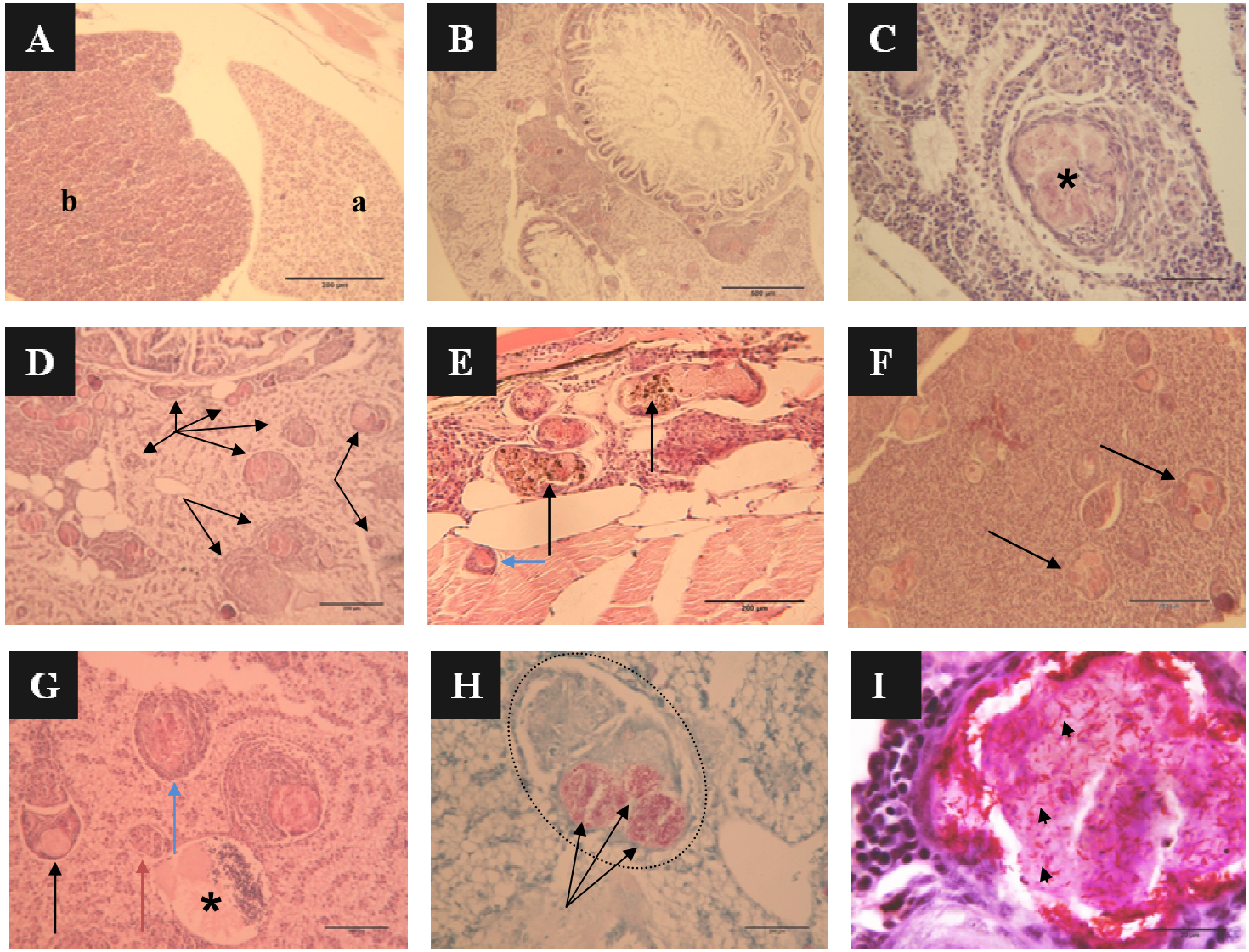

Plate 5.3:
(A) Normal zebrafish liver (a), spleen (b) with no signs of inflammation.

(B) Overview of a severe infection.

(C) Granuloma in kidney with central, caseous necrosis (asterisk).

(D) Liver with scattered granulomas (arrows). Areas of the liver are brighter in colour than those in (A).

(E) Granulomas in spleen with melanomacrophages (black arrows) and a granuloma in muscle tissue (blue arrow).

(F) Multicentric necrotizing granulomas (arrows) in liver.

(G) Granuloma in spleen with central necrosis (blue arrow), granuloma with caseous necrosis (black arrow), and a non-necrotizing granuloma in early stages of development (red arrow) just above a blood vessel (asterisk).

(H) Multicentric granuloma (dotted line) in spleen containing 3 granulomas (arrows) with high amount of acid fast bacilli stained red.

(I) Granuloma with caseous necrosis and rod shaped acid-fast bacilli (arrowheads).

Scale bars: B = 500 µm; A, C, D, E, F, G and H = 200 µm; I = 20 µm.
Staining: H&E (A-G) and modified Ziehl-Neelsen (H&I).

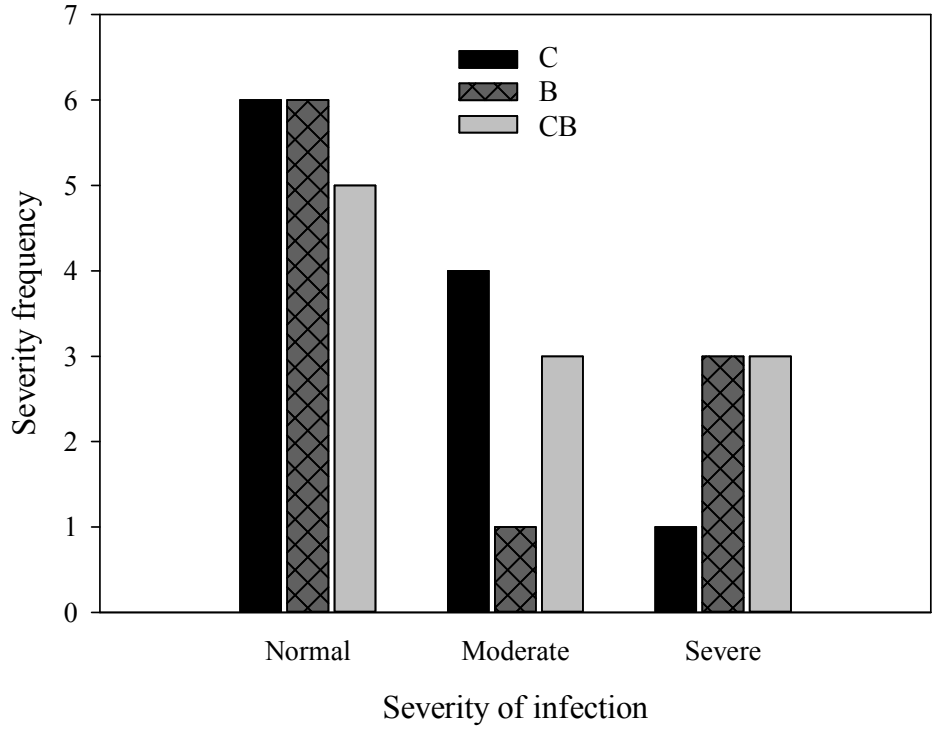

Figure 5.3: Frequency of severity of infection score after Talaat *et al*. (1998) for fish fed either the diet C (black bar), diet B (dark-grey, dashed bar) or CB (light-grey bar).

5.4.3 *Assessment of intestinal microbiota*

The influence of a dietary MacroGard® supplementation (0.1%) on the intestinal microbial diversity under a chronic mycobacteriosis condition in wild-type zebrafish (*Danio rerio*) was assessed using PCR-DGGE. Figures 5.4 and 5.5 display the 16S rRNA V3 PCR-DGGE fingerprints and the respective dendrograms of the intestinal microbiota after 8 weeks (day 56) and 9 weeks (day 63) of feeding, respectively. The respective microbial ecological parameters derived from PCR-DGGE fingerprints are presented in Table 5.2.

Figure 5.4 illustrates low similarities between treatments (ca. 68% - 72%) of the samples taken at day 56. Furthermore, the similarities within the replicates of each individual treatment (SIMPER) were low (ca. 62% - 79%), but CB (78.74 ± 7.43%) was

approaching significance towards a higher value than B (62.33 ± 2.88%, $P = 0.059$). There were no significant differences between treatments with regards to the number of OTUs, the species richness, evenness or diversity.

Figure 5.5 displays the equivalent after 63 days weeks of feeding when fish from the intermittent treatment CB were fed the 0.1% MacroGard® supplemented diet the week prior to sampling. It is evident that the three replicates from fish fed the control diet cluster together and display low similarity compared to fish fed both MacroGard® supplemented diets (C *vs*. B 73.83 ± 8.15% similarity, C *vs*. CB 74.72 ± 5.51% similarity). The similarity between treatment B and CB however was higher (89.92 ± 4.49%). Furthermore, the similarity within the group replicates was equally high for all treatments (approx. 88 - 90%). Irrespective of continuous or intermittent feeding regime, dietary MacroGard® also significantly ($P < 0.001$) increased the total number of OTUs from 21.33 ± 0.58% in the control group to 28.67 ± 1.53% in treatment B and 28.67 ± 0.58% in treatment CB. The species richness was also significantly ($P < 0.001$) higher with 2.70 ± 0.12% and 2.71 ± 0.06% in treatments B and CB, respectively, compared to the control (2.09 ± 0.03%). In addition, diversity was significantly higher ($P < 0.001$) in treatments B (3.31 ± 0.05%) and CB (3.31 ± 0.03%) compared to the control (3.05 ± 0.02%). The evenness remained unaffected.

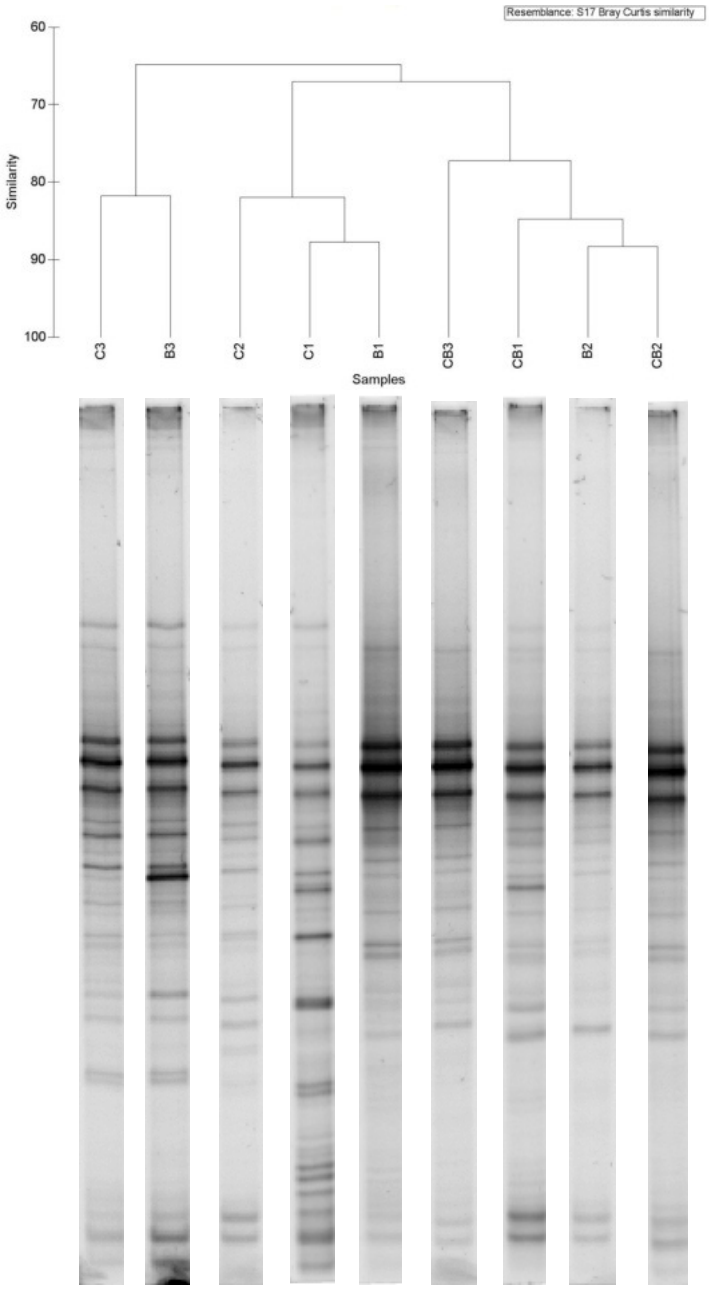

Figure 5.4: Denaturing gradient gel electrophoresis (DGGE) profiles of PCR – amplified products of the V3 region of the 16S rRNA gene from day 56 intestinal samples with the respective Bray – Curtis dendrogram demonstrating the similarity. Treatments: C 1-3 = control replicates, B 1-3 = replicates treatment B, and CB1-3 = replicates treatment B.

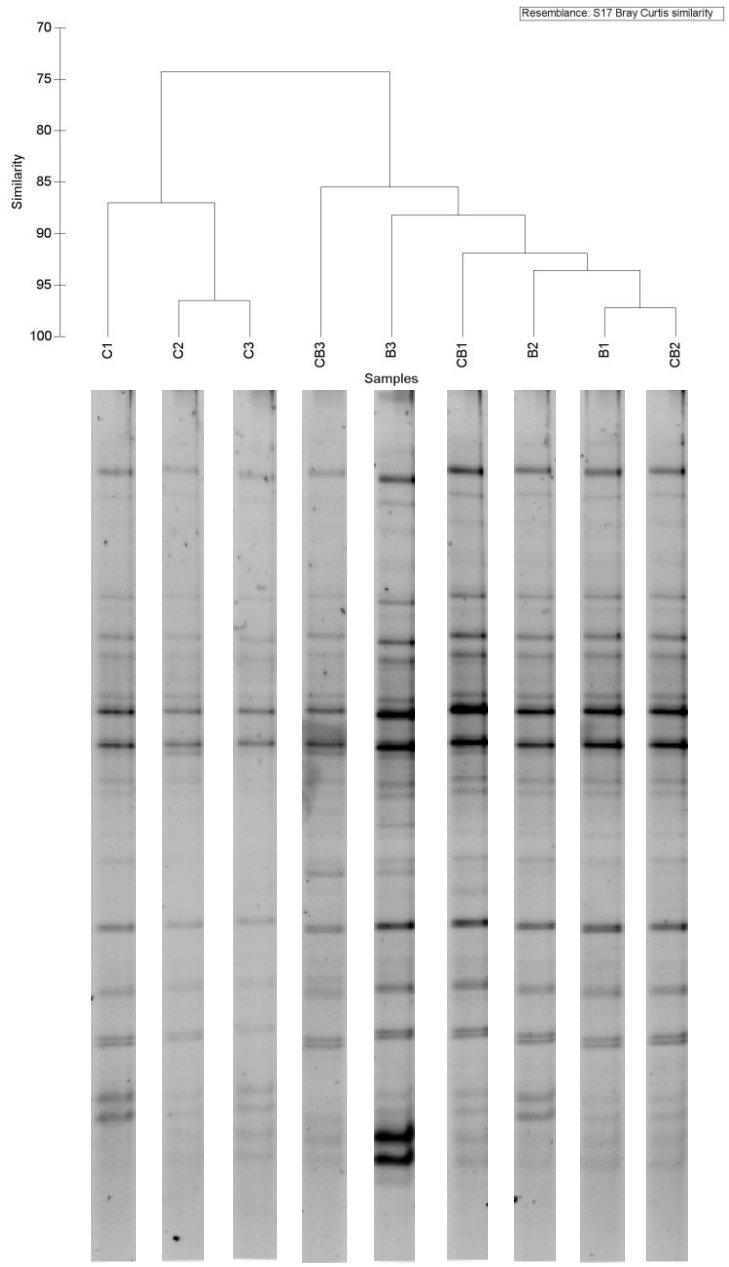

Figure 5.5: Denaturing gradient gel electrophoresis (DGGE) profiles of PCR – amplified products of the V3 region of the 16S rRNA gene from day 63 intestinal samples with the respective Bray – Curtis dendrogram demonstrating the similarity. Treatments: C 1-3 = control replicates, B 1-3 = replicates treatment B, and CB1-3 = replicates treatment B.

Table 5.2: Analysis of the intestinal microbial communities of zebrafish (n = 3) from PCR-DGGE fingerprints after 56 and 63 days of feeding control (C) or MacroGard® (0.1%) supplemented diets continuously (B) or intermittently (CB). On day 56 fish from treatment CB were fed the control diet, and on day 63 they were fed the MacroGard® (0.1%) supplemented diet the preceding week.

Day 56	**Microbial ecological parameters**					**Similarity**		
	N	Richness	Evenness	Diversity	SIMPER (%)	C	B	CB
C	23.33 ± 3.21	2.16 ± 0.29	0.98 ± 0.01	3.09 ± 0.13	69.69 ± 8.08	100	72.25 ± 11.28	67.68 ± 7.71
B	22.67 ± 2.52	2.10 ± 0.25	0.98 ± 0.00	3.06 ± 0.11	62.33 ± 2.88		100	71.47 ± 10.49
CB	21.67 ± 3.21	2.01 ± 0.30	0.98 ± 0.00	3.01 ± 0.16	78.74 ± 7.43			100

Day 63	**Microbial ecological parameters**					**Similarity**		
	N	Richness	Evenness	Diversity	SIMPER (%)	C	B	CB
C	21.33 ± 0.58^{a}	2.09 ± 0.03^{a}	1.00 ± 0.00	3.05 ± 0.02^{a}	90.15 ± 5.50	100	73.83 ± 8.15	74.72 ± 5.51
B	28.67 ± 1.53^{b}	2.70 ± 0.12^{b}	0.99 ± 0.01	3.31 ± 0.05^{b}	88.58 ± 3.93		100	89.92 ± 4.49
CB	28.67 ± 0.58^{b}	2.71 ± 0.06^{b}	0.99 ± 0.00	3.31 ± 0.03^{b}	88.36 ± 5.02			100

N = number of operational taxonomical units, richness = Margalef species richness, diversity = Shannon's diversity index, SIMPER = similarity percentage within group replicates. Similarity = pairwise comparison of weighted Bray – Curtis similarity. ^{ab} Different superscripts indicate a significant difference ($P < 0.05$).

5.5 Discussion

Piscine mycobacteriosis constitutes a threat to zebrafish research facilities, and pathogenic *Mycobacterium* spp. causing this disease in zebrafish include *M. marinum* (Prouty *et al*., 2003; Watral and Kent, 2007), *Mycobacterium fortuitum*, *M. chelonae*, *Mycobacterium abscessus* (Astrofsky *et al*., 2000) and *Mycobacterium peregrinum* (Kent *et al*., 2004). Also, the pathogenic *Mycobacterium haemophilum* (accession number DQ851570) identified as the causative agent for the severe mycobacteriosis in the present study has previously been described (Kent *et al*., 2004; Whipps *et al*., 2007) and considered as highly virulent species associated with severe disease (Whipps *et al*., 2012). Injecting 8 month old zebrafish with *M. haemophilum* at 1.2 x 10^5 CFU $fish^{-1}$ resulted in approx. 35% mortalities after 8 weeks (Whipps *et al*., 2007). Comparable mortality rates were observed in the present study: approx. 26% in the control group and 35% in the MacroGard® group in experiment I after 60 days (64% and 80%, respectively, after 200 days). In experiment II approx. 28% in the control group and approx. 22% in both the continuous and intermittent MacroGard® group deceased at day 63. It must be noted however that the fish in the present study were not injected with the pathogen but suffered from a mycobacteriosis caused by the ubiquitous presence of *M. haemophilum* within the facilities. The detection of *M. haemophilum* in biofilms from zebrafish research facilities has been reported elsewhere, indicating that biofilms constitute a reservoir and continuous source of infection in recirculating water systems (Whipps *et al*., 2007). This fact supports the observation that the zebrafish in the facilities used in the present study become infected in early life stages, suffering from a chronic form of mycobacteriosis which slowly develops into sub-acute and acute forms over time, possibly triggered by stressors, as described for other mycobacteriosis infections in fish (Ramsay *et al*., 2009).

As apparent from Figures 5.1 and 5.2, dietary β-glucan supplementation in the form 0.1% MacroGard® could not increase the survival rates of zebrafish suffering from severe *M. haemophilum* mycobacteriosis. Moreover, it was of no difference whether MacroGard® was administered over long- or short-term periods (experiment I, 200 days *vs*. experiment II, 63 days) or if administered in weekly intervals (experiment II). In contrast to the findings of the present study, Li and Gatlin (2005) observed a marginal, but significant, increase in survival rates of hybrid striped bass fed diets supplemented with partially autolyzed brewer's yeast (Brewtech®) and GroBiotic®-A, a mixture of partially autolyzed brewer's yeast, dairy ingredient components and dried fermentation products, under a progressing *in situ* infection with *M. marinum* which became well established at week 16. It is difficult however to compare these data to the data from the present study where a highly purified ß-glucan was used.

In general, pathogenic mycobacteria mainly infect macrophages (or other macrophage-like phagocytic cells) and are able to escape killing by the cell-intrinsic immune responses of macrophages and subsequently proliferate within the macrophages (Hagedorn and Soldati, 2009). In doing so, mycobacteria ironically exploit the host's defence mechanism of phagocytosis which acts to protect the host from infection. Literature on *M. haemophilum* mycobacteriosis in teleosts and literature on the effects of ß-glucans on mycobacteriosis is limited. A recent *in vitro* study however using human macrophages was able to illustrate the interaction between mycobacteria and ß-glucans (Betz *et al*., 2011). Treating the macrophages with a particulate *S. cerevisiae* ß-glucan prior to, or after infection, was able to decrease cell association and intracellular survival of the *M. tuberculosis* vaccine Bacillus Culmette Guerin (BCG; an attenuated strain of *Mycobacterium bovis*). In contrast, no effect was observed when a virulent *Mycobacterium tuberculosis* (Erdman strain, ATCC #25618) was used despite the fact that the ß-glucan increased oxidative burst and pro-inflammatory cytokines,

mediated through Dectin-1. The authors hypothesize that *M. tuberculosis* is equipped with a unique mechanism to inhibit downstream-signalling of Dectin-1 (potentially by preventing induction of NF-κB and pro-inflammatory responses), thus evading the macrophage's killing machinery. Indeed, *M. tuberculosis* has been shown to prevent phagosome maturation in macrophages and survive inside these vesicles; *M. marinum* can survive inside phagosomes but furthermore escape into the cytosol and spread to neighbour cells (van der Vaart *et al*., 2012). It is conceivable that the virulent *M. haemophilum* is equipped with similar mechanisms which could be one reason why the ß-glucan administration did not impact survival rates in the present study.

In contradiction to the findings of Betz *et al*. (2011), a study by Hetland and Sandven (2002) reported that particulate MacroGard®, as used in the present study, successfully reduced the number of intracellular *M. tuberculosis* cells in mouse macrophages when incubated together with the bacteria; however, the authors used a different strain *M. tuberculosis* (strain $H_{37}R_v$). The interesting finding however in this study was that this effect was not observed when the MacroGard was added after *M. tuberculosis*. This could also be indicative for the findings of the present study where the mycobacteriosis was well established and already existing prior to the start of the ß-glucan administration. One could hypothesize that the zebrafish were in an immuno-compromised state to such an extent that the immunostimulating effects of β-glucans described frequently in healthy fish (Dalmo and Bøgwald, 2008; Soltanian *et al*., 2009) do not occur and provide protection against mycobacteriosis post infection. Immuno-suppressive effects of mycobacteriosis have indeed been demonstrated in human tuberculosis caused by *M. tuberculosis* (Fenton and Vermeulen, 1996) and by *Mycobacterium leprae* cell wall lipids in mice (Moura and Mariano, 1997).

A further possible explanation could be that the recognition of the pathogen associated molecular patterns (PAMPs) of both the β-glucan and the mycobacteria by the immune system occurs partially via the same pattern recognition receptors (PRRs). β-glucan recognition by the mammalian innate immune system occurs via several receptors including scavenger receptors, complement receptor 3 and lactosylceramide (Dalmo and Bøgwald, 2008), but Dectin-1 either alone, or in interaction with Toll-like receptor (TLR) 2, is considered the main receptor (Brown *et al*., 2003; Gantner *et al*., 2003; Dennehy and Brown, 2007; Dostert and Tschopp, 2007). In contrast, β-glucan receptor identification in teleosts is limited so far, but the assumption that teleost and mammalian cells share many similar receptors may lead to a prediction of the effects of immunostimulants in fish (Ringø *et al*., 2011). The presence of zTLR2 however, the homologue of human TLR2, in zebrafish has been reported by Meijer *et al.* (2004); furthermore, gene expression of zTLR 2 (and other TLRs) was up-regulated when adult zebrafish were infected with *M. marinum* and showed clinical signs of mycobacteriosis. A pro-inflammatory immune response of mouse macrophages against various *Mycobacterium* spp. was mediated by TLR2 *in vivo* (Underhill *et al*., 1999) and by Dectin-1 along with TLR2 *in vitro* (Yadav and Schorey, 2006). If the zebrafish, and indeed the macrophages, are already infected by the mycobacteria, then the macrophages are stimulated to a high degree (and possibly already compromised). In consequence, it might be questionable whether the macrophages can be stimulated any further beyond its current level by ß-glucan if the signalling pathways are mediated through the same receptors. And even if the macrophages can yet be stimulated, it may be too late if the mycobacteria have already compromised too many of the macrophages. In two cases in Yadav and Schorey's study (2006) however the immune response was independent of dectin-1 indicating that the mediation of the immune response could also depend on the *Mycobacterium* strain.

Determining the effects of dietary ß-glucan supplementation on the intestinal microbiota under the chronic progressive zebrafish mycobacteriosis was an important aspect of this experiment. To the author's knowledge no information is available in this respect in teleosts. In the present study the microbial ecological parameters were altered. The numbers of OTUs and the species richness and diversity were elevated at day 63 (but not at day 56) in fish fed MacroGard® supplemented diets compared to the control group. As with survival rates and histopathology, the outcome regarding the microbial ecological parameters was irrespective of the method of ß-glucan administration (continuous or intermittent). Surprisingly, no change was seen in the continuously fed ß-glucan group at day 56; one would expect a similar increase as that observed at day 63. Similarly, in the intermittent group the increase at day 63 occurred when the fish were fed the control diet in the preceding week; a time-delayed effect could explain this phenomenon. In contrast to these findings, the 2nd experiment (Chapter 4) demonstrated reductions of the numbers of OTUs and the species richness and diversity in apparently healthy mirror carp fed ß-glucan supplemented diets, and sequence analysis revealed that mainly the abundance of LABs, but also of some Aeromonads decreased, while the abundance of a few species increased. Hence, it would be interesting to identify which intestinal bacteria species are present under mycobacterial infection to detect in consequence of which species the abundance is increased. Of utmost interest would be if the causative pathogen *M. haemophilum* is present and if its abundance changes with dietary ß-glucan administration. Furthermore, identifying the bacteria species could help in elucidating why changes of the intestinal microbial communities occurred at day 63, but not at day 56. Therefore further studies are required to identify the bacteria species by sequencing.

5.6 Conclusions

The present study clearly demonstrates that both continuous and intermittent dietary administration of MacroGard fails to improve histopathological findings or reduce the severe mortalities of zebrafish suffering from chronic, pre-existing mycobacteriosis caused by *M. haemophilum*. Further research is required to characterize the immunological processes under such circumstances in fish. Whether an improvement of resistance can be attained by ß-glucan supplementation prior to *Mycobacterium* infection remains to be seen and future studies should address this topic. Furthermore, the determination of the microbiota present in the intestine under this chronic disease, both with and without ß-glucan influence, by quantitative analyses and sequencing should be conducted.

Chapter 6: General Discussion

Each of the three experiments conducted during this research programme investigated aspects which have not, or only marginally, been in the focus of research related to the dietary administration of ß-glucans in humans and/or animals including fish. Emphasis shall be put on the overarching scientific approach of using highly purified basal diets of the same composition in each experiment. The aim was to minimize the presence of immunostimulating substances other than the supplemented MacroGard® originating from the various ingredients of the diets. From literature it is evident that all studies related to dietary ß-glucan supplementation in teleosts have been conducted using commercial or, at best, semi-purified diets. Important ingredients of fish feed however are known to contain immunostimulating substances. For example, standard fish meal contains significant amounts of nucleotides (Mateo *et al*., 2004) and cereals like wheat, barley, oat and rye contain ß-(1,3)(1,4)-D-glucans (Sealey *et al*., 2008). Dietary nucleotides (Li and Gatlin, 2006; Ringø *et al*., 2011) are potent immunostimulants in teleosts; the knowledge about cereal ß-glucans in this respect is still restricted, but Ringø *et al*. (2010) state that barley ß-glucans are highly likely to be able to induce immune responses. Such substances, undesired in the present studies, would most likely induce "background" immune responses themselves as a side-effect thereby possibly masking the true immunostimulating effect of the supplemented MacroGard®.

It was particularly striking to note that in the experiments with mirror carp (*Cyprinus carpio* L.) (Chapters 3 and 4) the diets supplemented with 0.1% MacroGard®, the actual dosage recommended by the supplier for industrial applications, had no effects on the parameters assessed (except on the intestinal microbial communities). Only high dietary MacroGard® levels of 1% and 2% exerted enhancing effects on parameters like growth performance, some haematological indices (e.g. blood monocyte fraction), intraepithelial leukocytes in the anterior gut mucosa and the enterocytes' microvilli length and density (not assessed for 2% MacroGard® supplementation). This

phenomenon may underpin the importance of the just described lack of background immunostimulation through the use of highly purified diets. Consequently, a most interesting question would be to conduct *ceteris paribus* experiments with a typical industrial basal diet and to compare the results with the results gained in Chapters 3 and 4 and, in addition, whether such results could be achieved under commercial husbandry conditions.

The first experiment described in Chapter 3 was concerned with the effects of MacroGard® on growth performance, haemato-immunological profile and intestinal morphology in mirror carp. Some information has been available about growth and haematology from literature (as described in Chapter 1), information about ß-glucan effects on intestinal morphology however has been very limited. High dietary MacroGard® supplementation significantly increased growth performance. As discussed in Chapter 3, the reasons for growth performance enhancing effects observed with dietary ß-glucans still remain unclear. Future research is required to elucidate these mysteries and novel technology should be able to achieve this. Tacchi *et al*. (2011) analysed the transcriptomic responses in liver and muscle tissue of Atlantic salmon (*Salmo salar* L.) fed diets supplemented with a "functional feed" premix containing nucleotides, mannan oligosaccharides, fructooligosaccharides, vitamin C and vitamin E. The fish displayed a decreased expression of protein turnover genes, reduced circulating plasma proteins and a down-regulation of immune response genes suggesting a decrease in whole body metabolic demands. The changes appear to result in reduced energy expenditure in fish and enhanced growth and performance; the feed conversion of fish fed the "functional feed" diet was improved by 4.1%. Unfortunately, the authors do not state whether the difference was significant compared to the control group, and no

detailed information is given about other growth performance parameters like weight gain or specific growth rate. However, such an approach could be applied to a ß-glucan study like the present one. Dalmo and Bøgwald (2008) suggest that ß-glucans induce a localized intestinal immune response that in turn leads to resistance against pathogens which otherwise would cause reduced weight gain and possibly disease. The present study indicated that a localized intestinal immune response may occur through an enhanced infiltration of intraepithelial leukocytes (IELs). Using transcriptomic technology, the expression patterns of a plethora of genes could be analysed simultaneously in intestinal tissue and the influence of dietary ß-glucan supplementation on immune modulation and intestinal health could be assessed. This could possibly help linking the enhanced growth performance to the enhanced infiltration of intraepithelial leukocytes observed in the present study.

Future studies should also aim at exactly identifying the types of IELs in order to assess whether or not the increased infiltration of IELs in the anterior intestine in the present study is caused by an increased infiltration of one certain type of leukocyte. Animal studies indicate that dietary ß-glucans entering the proximal small intestine are captured by macrophages (Chan *et al*., 2009). It is therefore conceivable that a higher number of macrophages migrates to the epithelium to respond to a high delivery of ß-glucans (such as the 1% and 2% MacroGard® in the present study) during intestinal passage. Immunohistochemistry would be a useful means for the identification of leukocytes. Inami *et al*. (2008) used myeloperoxidase and acid phosphatase staining to identify granulocytes and macrophage-like cells, respectively, in cod (*Gadus morhua* L.) intestine and rectum. Monoclonal antibodies used in carp studies in order to identify leukocytes are WCI-12 for B-cells, WCL-15 for monocytes and macrophages in tissue sections (Forlenza *et al*., 2008) and WCL38 for mucosal T-cells (Rombout *et al*., 2011). TCL-BE8 binds to carp neutrophilic granulocytes (strong affinity), monocytes (low

affinity), and basophilic granulocytes (intermediate affinity) (Forlenza *et al*., 2008). Using these available tools future studies should be able to shed light on the increased intestinal IEL presence observed in the present study.

No research has been conducted previously on the effects of ß-glucans on intestinal microbial communities using advanced methods and ultrastructure of the apical brush border of enterocytes in teleosts which were investigated using mirror carp in the second experiment in Chapter 4. Dietary MacroGard® clearly altered intestinal microbial communities in a time- and tissue dependent manner as assessed by culture-based and culture-independent methods. The limitations of culture-based methods however are known (Cahill, 1990; Ringø *et al*., 2006b; He *et al*., 2011a). It should furthermore be noted that PCR-DGGE, the culture-independent method used here, is a semi-quantitative method (Merrifield *et al*., 2013), and future studies should ideally apply next generation sequencing methods to investigate the effects of dietary ß-glucans on intestinal microbial communities, e.g. full-length sequencing or 454 pyrosequencing, allowing for a much higher taxonomic resolution (Sekirov *et al*., 2010). Several recent studies employed such sequencing methods in teleosts and demonstrated, how vastly diverse in general the intestinal microbial communities are in salmonids, e.g. rainbow trout (*Oncorhynchus mykiss*) (Desai *et al*., 2012) and cyprinids like common carp (van Kessel *et al*., 2011), zebrafish (*Danio rerio*) (Roeseler *et al*., 2011) and grass carp (*Ctenopharyngodon idellus*) (Wu *et al*., 2012). Even though next generation sequencing methods will advance current knowledge about the entirety of microbiomes in different teleosts and under different conditions (like dietary pre- and probiotic administration), they cannot give insights into the microbiome's contribution towards functionality and metabolic processes in health and disease (Sekirov *et al*., 2010). Looking even further

ahead, future studies should therefore try to use meta'omic approaches (metagenomics, metaproteomics, metabolomics and metatranscriptomics) which will enable researchers to study complex ecosystems and their interactions (Figure 6.1; Segata *et al*., 2013). DNA-based metagenomics will not only allow to likely detect all members of the microbial communities present in the sample but also to predict their biological functions, e.g. by environmental shotgun sequencing (Venter *et al*., 2004) which can be also applied for a parallel, comparative analysis of different samples (Eisen, 2007). Metagenomics however only allow insights into the functional and metabolic diversity of microbial communities but cannot give information about their actual metabolic activity (Simon and Daniel, 2011) or microbial abundance. This can be achieved by RNA-based metatranscriptomic studies giving insights into gene expression of microbial communities, but this method is currently still subjected to limitations. Such methods however would significantly increase current knowledge about possible influences of orally administered ß-glucans on the complex network of the intestinal microbiome and the host, and the interactions herein.

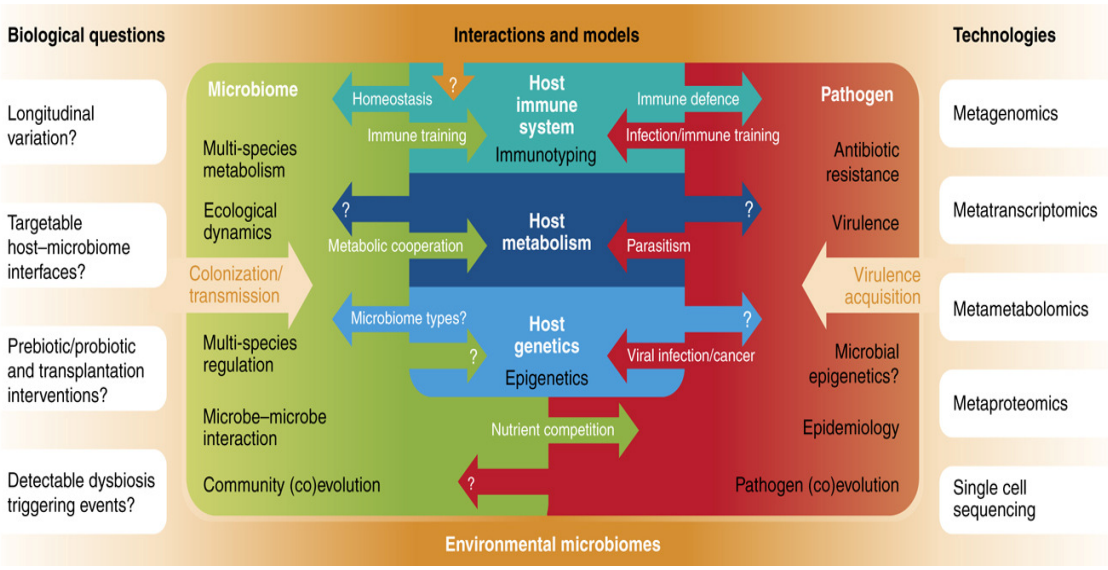

Figure 6.1: "Open biological questions in microbial community biology, and emerging technologies and models for their exploration. Microbial communities are complex biological entities interacting with the environment, host organisms, and transient microbes. Predictive models for most of the interactions within these ecosystems are currently rare, but several studies have begun to provide key insights." (from Segata *et al*., 2013)

The importance of the relationship between the intestinal microbiome and the host has been highlighted by two recent, pioneering studies in zebrafish (Rawls *et al*., 2004; Bates *et al*., 2006). Using germ-free zebrafish models, Rawls *et al*. (2004) observed that the microbiota stimulated intestinal epithelial proliferation and influenced enterocyte morphology, and Bates *et al*. (2006) demonstrated that the intestinal gut mucosa failed to differentiate fully in germ-free larvae. Reintroduction of the microbiota however caused a reversion of this deficiency in the study of Bates *et al*. (2006). These studies demonstrate the significance of the microbiota regarding gut development, possibly connecting the observed shifts in both autochthonous and allochthonous microbial populations with the observed increased microvilli length and density in the present study under the influence of the dietary supplementation of 1% MacroGard®. It can however not be determined whether the enhancing effects on microvilli length and density in the present study have been resulting from direct effects of the ß-glucan on the enterocytes or from secondary effects through the observed shifts in the microbial communities possibly caused by ß-glucans. Therefore, most interesting for future research would be to study the direct effects of dietary ß-glucans and other prebiotics in germ-free fish models to exclude influences of the microbiota.

The third experiment described in Chapter 5 examined the effects of dietary MacroGard® on disease resistance and intestinal microbial communities of zebrafish (*Danio rerio*) during a chronic progressive, pre-existing mycobacteriosis. This mycobacteriosis was caused by *Mycobacterium haemophilum* about which information is eminently limited in teleosts. As described, MacroGard® failed to improve mortality rates and histopathological findings caused by the mycobacteriosis. Even though much is known about the pathology of mycobacterial infections in general, the fundamental

immunological processes are not well defined (Li and Gatlin, 2005). Understanding the host-pathogen interactions is highly important in order to be able to develop effective prophylactic or therapeutic measures. Recent studies however have advanced knowledge using zebrafish *Mycobacterium marinum* models. Transcriptomic studies gained new knowledge into immune gene expression with *M. marinum* infected zebrafish larvae (van der Vaart *et al*., 2012) and adult zebrafish (van der Sar *et al*., 2009) in early stages of infection, in adult zebrafish and larvae during acute and chronic disease (Rotman *et al*., 2011) and in reactivation of dormant mycobacteria in adult zebrafish (Parikka *et al*., 2012). In future however, researchers need to examine the degree of translation of these transcribed genes into proteins using proteomic technology as already suggested above in the context of microbiota (metaproteomics), and in succession, what the effects of these proteins on the disease are. For example, it seems evident so far that tumor necrosis factor α (TNFα) plays a significant role in controlling *M. marinum* infections since TNFα receptor knockdown zebrafish exhibited enhanced intracellular mycobacterial growth, granuloma formation, necrotic death of macrophages and granuloma breakdown eventually leading to higher mortality (Clay *et al*., 2008). Furthermore, the balance between the pro-inflammatory TNFα and anti-inflammatory lipoxins is important in the control of the disease (Tobin *et al*., 2010). And finally, referring back to the present study, all of these aspects are required to be equally researched for *M. haemophilum* infections in zebrafish, of course.

Further research also needs to identify the pathogen responses and the associated mechanisms towards the host immune response. For example, it should be clarified whether *M. haemophilum* is equipped with similar mechanisms to evade macrophage immune response as *Mycobacterium tuberculosis* (Betz *et al*., 2011) and *M. marinum* (van der Vaart *et al*., 2012). The present findings may be indicative for that. But future studies should also address whether or not dietary ß-glucan administration prior to

M. haemophilum mycobacteriosis disease can counteract the disease or, at least, alleviate the symptoms, following the philosophy of a prophylactic approach. Growth inhibiting effects on *M. tuberculosis* have indeed been successfully demonstrated *in vitro* when MacroGard® was simultaneously administered to mouse macrophages (Hetland and Sandven, 2002). Whether this effect can be observed *in vivo* and against *M. haemophilum* remains to be illustrated.

The importance of the microbiome has been already mentioned and in recent years a lot of research effort has been put into investigating reasons and effects of dysbiosis (meaning imbalances) of the microbiome. In humans, dysbiosis has been connected to inflammatory bowel disease, diabetes mellitus, obesity, cardiovascular disease and cancer (Pflughoeft and Versalovic, 2012; Brown *et al*., 2012). The only link so far between mycobacteria and intestinal microbiota with regards to chronic disease is present in inflammatory bowel disease in humans and the Johne's disease in cattle. Among others factors, *Mycobacterium avium* ssp. *paratuberculosis* (MAP) has been suggested to be involved in the pathogenesis of Crohn's Disease and Ulcerative colitis (Sekirov *et al*., 2010; Friswell *et al*., 2010). A general dysbiosis of the microbiota has been described in patients, it is still not clarified however whether the dysbiosis is cause or consequence of the disease. Several studies reported decreased numbers of members of *Firmicutes* and the *Clostridium* cluster IV with a simultaneous increase in the number of *Bacteroides*. Remarkable is that several studies detected a lower abundance of the commensal, anti-inflammatory bacterium *Faecalibacterium prausnitzii* (*Firmicutes*) in both digesta and mucosa samples (Sokol *et al*., 2008; Joossens *et al*., 2011). Alterations of the intestinal microbial communities were observed in the present zebrafish study induced by dietary MacroGard®. Future studies however need to identify the microbiota by sequencing to discover which species are present in zebrafish infected with *M. haemophilum* with or without dietary ß-glucan influence. Furthermore, studies need

to include a negative control (fish uninfected with *M. haemophilum*) which was a drawback of this study due to the unavailability of healthy zebrafish. Ideally, those studies should employ the already discussed meta'omic approaches to further illustrate the influence of the microbiome on the host under this chronic progressive disease. Another interesting aspect is to assess possible changes of the microbial communities under mycobacterial infection when the ß-glucan was administered prior to infection since the gastrointestinal tract has been reported as the primary route of infection for *M. marinum* and *Mycobacterium peregrinum* (Harriff *et al*., 2007) likely indicating the same route for *M. haemophilum*.

Reflecting from the two carp experiments, future research is required to narrow down the supplementation range between 0.1% and 1% MacroGard® in order to detect at which minimum dosage level similar alterations of the parameters assessed occur as they did with 1% and 2% dietary MacroGard®. Namely, it is important to be aware that such high supplementation levels of 1% or even 2% of MacroGard® or comparable products are critical from an economic point of view. The prices for products like MacroGard® are currently considerable with approximately 35 €/kg due to the complex and cost-intensive processing. Increasing the dosage from 0.1% to 1% MacroGard® by ten-fold means that the portion of costs per ton of feed mixture would amount to approximately 350 €. This is economically not feasible considering current main commodities prices of approximately 1400 €/t for standard fish meal, 1800 €/t for fish oil, 830 €/t soya oil or 233 €/t for wheat (Source: The World Bank; as of April 2013). In contrast however, reflecting from the zebrafish experiment, future research should investigate if supplementation levels higher than the 0.1% MacroGard® used could possibly have a positive impact on the progression of a chronic, pre-existing mycobacteriosis.

As a final note, the results of the current study with its overarching approach of using highly purified diets also provide manifold incentives for further investigations. In the here presented carp experiments the ß-glucan effects were only examined in juvenile specimen over comparatively short periods of four and eight weeks, respectively. A topic of high interest is whether the here observed enhancing effects on growth, haematological and intestinal morphological parameters as well as the changes in the intestinal microbial communities can be observed in common carp of different life stages, e.g. in fry from first feeding onwards or in adult fish. In addition, the potential of dietary ß-glucans with regards to spawning should be given attention to determine, if positive effects on brood stock and the problems related to spawning occur, and if in consequence improvements in egg quality and quantity, and beyond that, in larval survival could also be achieved. Furthermore, long-term experiments should be conducted to examine whether these effects can persist over several months or possibly over the whole growth phase up to marketable size or whether suppressing effects occur since immunosuppression through long-term application of immunostimulants has been proposed (Bricknell and Dalmo, 2005; Ringø *et al*., 2011). Consequently, trials with various pulsative administration strategies of dietary ß-glucans should be included. Since traditional carp production is subject to changing weather conditions during the course of the year, the aspect of temperature is of high importance and should be given serious consideration. The present study examined the ß-glucan effects on a chronic, pre-existing zebrafish mycobacteriosis; tests on other cyprinid diseases of chronic and non-chronic nature are required. The use of ß-glucans from different commercial suppliers might results in differing outcomes of studies since the product properties will likely vary due to distinctions in the product's origin (e.g. baker's yeast *vs*. brewer's yeast *vs*. yeast from bioethanol production) and extraction technique. For example, the degree of purification is higher for some products, and for brewer's yeasts it is known

that they contain humolones (α-acids) and lupolones (ß-acids), bitter principles from hops exhibiting bacteriostatic effects. Therefore, comparison experiments will be of interest to determine the product efficacies under the plethora of approaches described. Innovative combinations of various yeast cell wall components/yeast autolysates among each other or combinations with other pre- and probiotics might further advance product development in this important sector. In conclusion, all these mentioned aspects shall also be investigated in other aquatic species relevant to aquaculture production, like in different fish species and in the various crustaceans to overall warrant efficient and sustainable production in the future. In 2050, it is highly likely that at least 9 billion people on planet earth will need food, and aquaculture plays an increasingly important role in the system!

References

ABDEL-TAWWAB, M., ABDEL-RAHMAN, A. M. & ISMAEL, N. E. M. 2008. Evaluation of commercial live bakers' yeast, *Saccharomyces cerevisiae* as a growth and immunity promoter for Fry Nile tilapia, *Oreochromis niloticus* (L.) challenged in situ with *Aeromonas hydrophila*. *Aquaculture, 280, 185-189.*

ABELL, G. C. J. & BOWMAN, J. P. 2005. Ecological and biogeographic relationships of class *Flavobacteria* in the Southern Ocean. *FEMS Microbiology Ecology*, 51, 265-277.

ADAMS, D. O. 1976. The granulomatous inflammatory response. *American Journal of Pathology*, 84, 163-192.

AI, Q., MAI, K., ZHANG, L., TAN, B., ZHANG, W., XU, W. & LI, H. 2007. Effects of dietary beta-1,3 glucan on innate immune response of large yellow croaker, *Pseudosciaena crocea*. *Fish & Shellfish Immunology*, 22, 394-402.

AINSWORTH, A. J. 1994. A beta-glucan inhibitable zymosan receptor on channel catfish neutrophils. *Veterinary Immunology and Immunopathology*, 41, 141-152.

ANDERSON, D. P., SIWICKI, A. K., DIXON, O.W. & LIZZIO, E.F. 1989. Immunostimulation by levamisole in Rainbow trout (*Salmo gairdneri*) *in vivo*. In: AHNE, W. & KURSTAK, E. (eds.) *Viruses of Lower Vertebrates*. Berlin, Heidelberg, Germany. Springer.

ANDERSON, D. P., SIWICKI, A. K. & RUMSEY, G. L. 1995. Injection or immersion delivery of selected immunostimulants to trout demonstrate enhancement of nonspecific defense mechanisms and protective immunity. In: SHARIFF, M., SUBASIGHE, R. P. & ARTHUR, J. R. (eds.) *Diseases in Asian Aquaculture Fish Health Section*. Manila, Philippines: Asian Fisheries Society.

AOAC 2003: Official methods of analysis. In: H. Horwitz (ed.), *Official methods of analysis of the association of Official analytical chemists*. Association of Official Analytical Chemists Inc, Arlington, VA.

ARKUSH, K. D., GIESE, A. R., MENDONCA, H. L., MCBRIDE, A. M., MARTY, G. D. & HEDRICK, P. W. 2002. Resistance to three pathogens in the endangered winter-run chinook salmon (*Oncorhynchus tshawytscha*): effects of inbreeding and major histocompatibility complex genotypes. *Canadian Journal of Fisheries and Aquatic Sciences*, 59, 966-975.

ASCHE, F., HANSEN, H., TVETERAS, R. & TVETERAS, S. 2009. The Salmon Disease Crisis in Chile. *Marine Resource Economics*, 24, 405-411.

ASTROFSKY, K. M., SCHRENZEL, M. D., BULLIS, R. A., SMOLOWITZ, R. M. & FOX, J. G. 2000. Diagnosis and management of atypical *Mycobacterium* spp. infections in established laboratory zebrafish (*Brachydanio rerio*) facilities. *Comparative Medicine*, 50, 666-672.

BABÍČEK, K., ČECHOVÁ, I., SIMON, R. R., HARWOOD, M. & COX, D. J. 2007. Toxicological assessment of a particulate yeast (1,3/1,6)-β-d-glucan in rats. *Food and Chemical Toxicology*, 45, 1719-1730.

BAGNI, M., ROMANO, N., FINOIA, M. G., ABELLI, L., SCAPIGLIATI, G., TISCAR, P. G., SARTI, M. & MARINO, G. 2005. Short- and long-term effects of a dietary yeast beta-glucan (Macrogard) and alginic acid (Ergosan) preparation on immune response in sea bass (*Dicentrarchus labrax*). *Fish & Shellfish Immunology*, 18, 311-325.

BAKKE-MCKELLEP, A. M., PENN, M. H., MORA SALAS, P., REFSTIE, S., SPERSTAD, S., LANDSVERK, T., RINGØ, E. & KROGDAHL, Å. 2007. Effects of dietary soyabean meal, inulin and oxytetracycline on intestinal microbiota and epithelial cell stress, apoptosis and proliferation in the teleost Atlantic salmon (*Salmo salar* L.). *British Journal of Nutrition*, 97, 699-713.

BATES, J. M., MITTGE, E., KUHLMAN, J., BADEN, K. N., CHEESMAN, S. E. & GUILLEMIN, K. 2006. Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. *Developmental Biology*, 297, 374-386.

BETZ, B. E., AZAD, A. K., MORRIS, J. D., RAJARAM, V. S. & SCHLESINGER, L. S. 2011. ß-Glucans inhibit intracellular growth of *Mycobacterium bovis* BCG but not virulent *Mycobacterium tuberculosis* in human macrophages. *Microbial Pathogenesis*, 51, 233-242.

BIRKBECK, T. H. & RINGØ, E. 2005. Chapter 10 Pathogenesis and the gastrointestinal tract of growing fish. In: W.H. HOLZAPFEL, P. J. N. S. G. P. R. Z. & SALEK, E. (eds.) *Biology of Growing Animals*. Elsevier.

BOHN, J. A. & BEMILLER, J. N. 1995. (1->3)-beta-D-glucans as biological response modifiers: A review of structure-functional activity relationships. *Carbohydrate Polymers*, 28**,** 3-14.

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72**,** 248-254.

BRATTGJERD, S., EVENSEN, O. & LAUVE, A. 1994. Effect of injected yeast glucan on the activity of macrophages in Atlantic salmon, *Salmo salar* L, as evaluated by in vitro hydrogen-peroxide production and phagocytic capacity. *Immunology*, 83**,** 288-294.

BRICKNELL, I. & DALMO, R. A. 2005. The use of immunostimulants in fish larval aquaculture. *Fish & Shellfish Immunology*, 19**,** 457-472.

BROGDEN, G., VON KÖCKRITZ-BLICKWEDE, M., ADAMEK, M., REUNER, F., JUNG-SCHROERS, V., NAIM, H. Y. & STEINHAGEN, D. 2012. ß-Glucan protects neutrophil extracellular traps against degradation by *Aeromonas hydrophila* in carp (*Cyprinus carpio*). *Fish & Shellfish Immunology*, 33, 1060-1064.

BROWN, B. A. 1988. *Routine haematology procedures*., Philadelphia, Leo and Febiger.

BROWN, G. D. & GORDON, S. 2003. Fungal beta-glucans and mammalian immunity. *Immunity*, 19**,** 311-315.

BROWN, G. D. & GORDON, S. 2005. Immune recognition of fungal beta-glucans. *Cellular Microbiology*, 7**,** 471-479.

BROWN, G. D., HERRE, J., WILLIAMS, D. L., WILLMENT, J. A., MARSHALL, A. S. J. & GORDON, S. 2003. Dectin-1 mediates the biological effects of beta-glucans. *Journal of Experimental Medicine*, 197**,** 1119-1124.

BRUNO, D.W., GRIFFITHS, J., MITCHELL, C.G., WOOD, B.P., FLETCHER, Z.J., DROBNIEWSKI, F.A. & HASTINGS, T.S., 1998. Pathology attributed to *Mycobacterium chelonae* infection among farmed and laboratory-infected Atlantic salmon *Salmo salar*. *Diseases of Aquatic Organisms*, 33, 101–109.

CAHILL, M. M. 1990. Bacterial flora of fishes: A review. *Microbial Ecology*, 19**,** 21-41.

CAMPA-CORDOVA, A. I., HERNANDEZ-SAAVEDRA, N. Y., DE PHILIPPIS, R. & ASCENCIO, F. 2002. Generation of superoxide anion and SOD activity in haemocytes and muscle of American white shrimp (*Litopenaeus vannamei*) as a response to beta-glucan and sulphated polysaccharide. *Fish & Shellfish Immunology*, 12**,** 353-366.

CASTRO, R., COUSO, N., OBACH, A. & LAMAS, J. 1999. Effect of different beta-glucans on the respiratory burst of turbot (*Psetta maxima*) and gilthead seabream (*Sparus aurata*) phagocytes. *Fish & Shellfish Immunology*, 9**,** 529-541.

CERF-BENSUSSAN, N. & GABORIAU-ROUTHIAU, V. 2010. The immune system and the gut microbiota: friends or foes? *Nature Reviews Immunoly*, 10**,** 735-744.

CHAN, G., CHAN, W. & SZE, D. 2009. The effects of beta-glucan on human immune and cancer cells. *Journal of Hematology & Oncology*, 2**,** 25.

CHEN, D. & AINSWORTH, A. J. 1992. Glucan administration potentiates immune defense mechanisms of channel catfish, *Ictalurus punctatus* Rafinesque. *Journal of Fish Diseases*, 15**,** 295-304.

CHEN, J. Z. & SEVIOUR, R. 2007. Medicinal importance of fungal beta-(1 -> 3), (1 -> 6)-glucans. *Mycological Research*, 111**,** 635-652.

CHEN, K.-L., WENG, B.-C., CHANG, M.-T., LIAO, Y.-H., CHEN, T.-T. & CHU, C. 2008. Direct Enhancement of the Phagocytic and Bactericidal Capability of Abdominal Macrophage of Chicks by β-1,3–1,6-Glucan. *Poultry Science*, 87**,** 2242-2249.

CHIHARA, G., MAEDA, Y., HAMURO, J., SASAKI, T. & FUKUOKA, F. 1969. Inhibition of Mouse Sarcoma 180 by Polysaccharides from *Lentinus edodes* (Berk.) Sing. *Nature*, 222**,** 687-688.

CLARKE, K. R. & GORLEY, R. N. 2006. *PRIMER v6: User Manual/Tutorial*. Plymouth, UK, Primer-E.

CNAANI, A., TINMAN, S., AVIDAR, Y., RON, M. & HULATA, G. 2004. Comparative study of biochemical parameters in response to stress in *Oreochromis aureus*, *O. mossambicus* and two strains of *O. niloticus*. *Aquaculture Research*, 35**,** 1434-1440.

COLLINS, R. A., ARMSTRONG, K. F., MEIER, R., YI, Y., BROWN, S. D. J., CRUICKSHANK, R. H., KEELING, S. & JOHNSTON, C. 2012. Barcoding and Border Biosecurity: Identifying Cyprinid Fishes in the Aquarium Trade. *PLoS ONE*, 7**,** e28381.

COUSO, N., CASTRO, R., MAGARINOS, B., OBACH, A. & LAMAS, J. 2003. Effect of oral administration of glucans on the resistance of gilthead seabream to pasteurellosis. *Aquaculture*, 219**,** 99-109.

DALMO, R. A. & SELJELID, R. 1995. The immunomodulatory effect of LPS, laminaran and sulfated laminaran [beta (1,3)-D-glucan] on Atlantic salmon, *Salmo salar* L., macrophages *in vitro*. *Journal of Fish Diseases*, 18**,** 175-185.

DALMO, R. A., MARTINSEN, B., HORSBERG, T. E., RAMSTAD, A., SYVERTSEN, C., SELJELID, R. & INGEBRIGTSEN, K. 1998. Prophylactic effect of ß(1,3)-D-glucan (laminaran) against experimental *Aeromonas salmonicida* and *Vibrio salmonicida* infections. *Journal of Fish Diseases*, 21, 459-462.

DALMO, R. A. & BØGWALD, J. 2008. ß-glucans as conductors of immune symphonies. *Fish & Shellfish Immunology*, 25**,** 384-396.

DANIELS, C. L., MERRIFIELD, D. L., BOOTHROYD, D. P., DAVIES, S. J., FACTOR, J. R. & ARNOLD, K. E. 2010. Effect of dietary *Bacillus* spp. and mannan oligosaccharides (MOS) on European lobster (*Homarus gammarus* L.) larvae growth performance, gut morphology and gut microbiota. *Aquaculture*, 304**,** 49-57.

DAVIS, J. M., CLAY, H., LEWIS, J. L., GHORI, N., HERBOMEL, P. & RAMAKRISHNAN, L. 2002. Real-Time Visualization of *Mycobacterium*-Macrophage Interactions Leading to Initiation of Granuloma Formation in Zebrafish Embryos. *Immunity*, 17**,** 693-702.

DEBAULNY, M. O., QUENTEL, C., FOURNIER, V., LAMOUR, F. & LEGOUVELLO, R. 1996. Effect of long-term oral administration of beta-glucan as an immunostimulant or an adjuvant on some non-specific parameters of the immune response of turbot *Scophthalmus maximus*. *Diseases of Aquatic Organisms*, 26**,** 139-147.

DECOSTERE, A., HERMANS, K. & HAESEBROUCK, F. 2004. Piscine mycobacteriosis: a literature review covering the agent and the disease it causes in fish and humans. *Veterinary Microbiology*, 99, 159-166.

DEFOIRDT, T., SORGELOOS, P. & BOSSIER, P. 2011. Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Current Opinion in Microbiology*, 14**,** 251-258.

DEL RIO-ZARAGOZA, O. B., FAJER-ÁVILA, E. J. & ALMAZÁN-RUEDA, P. 2011. Influence of β-glucan on innate immunity and resistance of *Lutjanus guttatus* to an experimental infection of *dactylogyrid monogeneans*. *Parasite Immunology*, 33**,** 483-494.

DEMERS, N. E. & BAYNE, C. J. 1997. The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. *Developmental & Comparative Immunology*, 21**,** 363-373.

DENNEHY, K. M. & BROWN, G. D. 2007. The role of the ß-glucan receptor Dectin-1 in control of fungal infection. *Journal of Leukocyte Biology*, 82**,** 253-258.

DESAI, A. R., LINKS, M. G., COLLINS, S. A., MANSFIELD, G.S., DREW, M. D., VAN KESSEL, A. G. & HILL, J. E. 2012. Effects of plant-based diets on the distal gut microbiome of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 350-353, 134-142.

DHABHAR, F. S. 2009. Enhancing versus Suppressive Effects of Stress on Immune Function: Implications for Immunoprotection and Immunopathology. *Neuroimmunomodulation*, 16**,** 300-317.

DHABHAR, F. S. & MCEWEN, B. S. 1997. Acute Stress Enhances while Chronic Stress Suppresses Cell-Mediated Immunity *in Vivo*:A Potential Role for Leukocyte Trafficking. *Brain, Behavior, and Immunity*, 11**,** 286-306.

DIDIERLAURENT, A., SIMONET, M. & SIRARD, J. C. 2005. Innate and acquired plasticity of the intestinal immune system. *Cellular and Molecular Life Sciences*, 62**,** 1285-1287.

DIMITROGLOU, A., MERRIFIELD, D. L., MOATE, R., DAVIES, S. J., SPRING, P., SWEETMAN, J. & BRADLEY, G. 2009. Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Animal Science*, 87**,** 3226-3234.

DIMITROGLOU, A., MERRIFIELD, D. L., SPRING, P., SWEETMAN, J., MOATE, R. & DAVIES, S. J. 2010. Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed utilisation, intestinal histology and gut microbiota of gilthead sea bream (*Sparus aurata*). *Aquaculture*, 300**,** 182-188.

DJORDJEVIC, B., SKUGOR, S., JORGENSEN, S. M., OVERLAND, M., MYDLAND, L. T. & KRASNOV, A. 2009. Modulation of splenic immune responses to bacterial lipopolysaccharide in rainbow trout (*Oncorhynchus mykiss*) fed lentinan, a beta-glucan from mushroom *Lentinula edodes*. *Fish & Shellfish Immunology*, 26**,** 201-209.

DOI, K. & DOI, A. 1986. Cloning and expression in *Escherichia coli* of the gene for an *Arthrobacter* beta-(1-3)-glucanase. *Journal of Bacteriology*, 168**,** 1272-1276.

DONGOWSKI, G., HUTH, M., GEBHARDT, E. & FLAMME, W. 2002. Dietary Fiber-Rich Barley Products Beneficially Affect the Intestinal Tract of Rats. *The Journal of Nutrition*, 132**,** 3704-3714.

DOSTERT, C. & TSCHOPP, J. 2007. DEteCTINg fungal pathogens. *Nature Immunology*, 8**,** 17-18.

DOUMAS, B. T., ARD WATSON, W. & BIGGS, H. G. 1971. Albumin standards and the measurement of serum albumin with bromcresol green. *Clinica Chimica Acta*, 31**,** 87-96.

DRABKIN, D. L. 1946. Spectrophotometric studies XIV. The crystallographic and optical properties of the hemoglobin of man in comparison with those of other species. *Journal of Biological Chemistry*, 164**,** 703-723.

DRITZ, S. S., SHI, J., KIELIAN, T. L., GOODBAND, R. D., NELSSEN, J. L., TOKACH, M. D., CHENGAPPA, M. M., SMITH, J. E. & BLECHA, F. 1995. Influence of dietary beta-glucan on growth performance, nonspecific immunity, and resistance to *Streptococcus suis* infection in weanling pigs. *Journal of Animal Science*, 73**,** 3341-3350.

EISEN, J. A. 2007. Environmental shotgun sequencing: Its potential and challenges for studying the hidden world of microbes. *PLoS Biology*, 5(3): e82.

EL-BOSHY, M. E., El-ASHRAM, A. M., ABDELHAMID, F., M. & GADALLA, H., A. 2010. Immunomodulatory effect of dietary *Saccharomyces cerevisiae*, ß-glucan and laminaran in mercuric chloride treated Nile tilapia (*Oreochromis niloticus*) and experimentally infected with *Aeromonas hydrophila*. *Fish & Shellfish Immunology*, 28, 802-808.

ELLIS, A. E. 2001. Innate host defense mechanisms of fish against viruses and bacteria. *Developmental & Comparative Immunology*, 25**,** 827-839.

ENGSTAD, R. E. 1994. *Yeast ß-glucan as an immunostimulant in Atlantic salmon (Salmo salar L.): Biological effects, recognition and structural aspects*. Tromso, University of Tromso.

ENGSTAD, R. E. & ROBERTSEN, B. 1993. Recognition of yeast cell wall glucan by Atlantic salmon (*Salmo salar* L) macrophages. *Developmental and Comparative Immunology*, 17**,** 319-330.

ENGSTAD, R. E. & ROBERTSEN, B. 1994. Specifity of a beta-glucan receptor on macrophages from Atlantic salmon (*Salmo salar* L). *Developmental and Comparative Immunology*, 18**,** 397-408.

EU 2003. Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. *The European Parliament and the Council of the European Union.*

FAENGE, R. 1986. Lymphoid organs in sturgeons (*Acipenseridae*). *Veterinary Immunology and Immunopathology*, 12**,** 153-161.

FALCO, A., FROST, P., MIEST, J., PIONNIER, N., IRNAZAROW, I. & HOOLE, D. 2012. Reduced inflammatory response to *Aeromonas salmonicida* infection in common carp (*Cyprinus carpio* L.) fed with β-glucan supplements. *Fish and Shellfish Immunology*, 32**,** 1051-1057.

FAO 2012. *The State of World Fisheries and Aquaculture 2010*.

FELIX, N., JEYASEELAN, P. M. J. & KIRUBAKARAN, C. J. 2008. Growth improvement and enhanced disease resistance against *Vibrio alginolyticus* using ß-glucan as a dietary supplement for *Penaeus monodon* (Fabricius). *Indian Journal of Fisheries*, 55**,** 247-250.

FENTON, M. J. & VERMEULEN, M. W. 1996. Immunopathology of Tuberculosis: Roles of Macrophages and Monocytes. *Infection and Immunity*, 64, 683-690.

FERGUSON, H. W. 2006. *Systemic Pathology of Fish. A Text and Atlas of Normal Tissue Responses in Teleosts, and Their Responses in Disease*. London, Scotian Press.

FERGUSON, R. M. W., MERRIFIELD, D. L., HARPER, G. M., RAWLING, M. D., MUSTAFA, S., PICCHIETTI, S., BALCÁZAR, J. L. & DAVIES, S. J. 2010. The effect of *Pediococcus acidilactici* on the gut microbiota and immune status of on-growing red tilapia (*Oreochromis niloticus*). *Journal of Applied Microbiology*, 109**,** 851-862.

FIGUERAS, A., M. SANTARÉM, M. & NOVOA, B. 1998. Influence of the sequence of administration of beta-glucans and a *Vibrio damsela* vaccine on the immune response of turbot (*Scophthalmus maximus* L.). *Veterinary Immunology and Immunopathology*, 64**,** 59-68.

FORLENZA, M., SCHARSACK, J. P., KACHAMAKOVA, N. M., TAVERNE-THIELE, A. J., ROMBOUT, J. H. W. M. & WIEGERTJES, G. F. 2008. Differential contribution of neutrophilic granulocytes and macrophages to nitrosative stress in a host–parasite animal model. *Molecular Immunology*, 45, 3178-3189.

GALEOTTI, M. 1998. Some aspects of the application of immunostimulants and a critical review of methods for their evaluation. *Journal of Applied Ichthyology*, 14**,** 189-199.

GALINDO-VILLEGAS, J. & HOSOKAWA, H. 2004. Immunostimulants: towards temporary prevention of diseases in marine fish. In: CRUZ-SUAREZ, L. E., RICQUE-MARIE, D., NIETO LOPEZ, M. G., VILLARREAL, D., SCHOLZ, U. & GONZALES, M., eds. *Avances en nutricion acuicola VII*, Hermosillo, Sonora, Mexico. Memorias del VII Simposium Internacional de Nutricion Acuicola.

GANTNER, B. N., SIMMONS, R. M., CANAVERA, S. J., AKIRA, S. & UNDERHILL, D. M. 2003. Collaborative Induction of Inflammatory Responses by Dectin-1 and Toll-like Receptor 2. *The Journal of Experimental Medicine*, 197**,** 1107-1117.

GATLIN III, D. M. 2002. Nutrition and Fish Health. In: HALVER, J. E. & HARDY, R. W. (eds.) *Fish Nutrition*. 3 ed. San Diego, California, USA: Academic Press.

GAUTHIER, D. T. & RHODES, M., W. 2009. Mycobacteriosis in Fishes: A review. *The Veterinary Journal*, 180, 33-47.

GIBSON, G. R., PROBERT, H. M., VAN LOO, J., RASTALL, R. A. & ROBERFROID, M. B. 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition Research Reviews,* 17**,** 259-275.

GILAD, O., YUN, S., ADKISON, M. A., WAY, K., WILLITS, N. H., BERCOVIER, H. & HEDRICK, R. P. 2003. Molecular comparison of isolates of an emerging fish pathogen, koi herpesvirus, and the effect of water temperature on mortality of experimentally infected koi. *Journal of General Virology,* 84**,** 2661-2667.

GOPALAKANNAN, A. & ARUL, V. 2010. Enhancement of the innate immune system and disease-resistant activity in *Cyprinus carpio* by oral administration of ß-glucan and whole cell yeast. *Aquaculture Research,* 41, 884-892.

GORDON, S. & TAYLOR, P. R. 2005. Monocyte and macrophage heterogeneity. *Nature Reviews Immunology,* 5**,** 953-964.

GRIMHOLT, U., LARSEN, S., NORDMO, R., MIDTLYNG, P., KJOEGLUM, S., STORSET, A., SAEBØ, S. & STET, R. M. 2003. MHC polymorphism and disease resistance in Atlantic salmon *(Salmo salar)*; facing pathogens with single expressed major histocompatibility class I and class II loci. *Immunogenetics*, 55**,** 210-219.

GU, M., MA, H., MAI, K., ZHANG, W., BAI, N. & WANG, X. 2011. Effects of dietary β-glucan, mannan oligosaccharide and their combinations on growth performance, immunity and resistance against *Vibrio splendidus* of sea cucumber, *Apostichopus japonicus. Fish & Shellfish Immunology,* 31**,** 303-309.

GUO, Y., ALI, R. A. & QURESHI, M. A. 2003. The influence of β-glucan on immune responses in broiler chicks. *Immunopharmacology and Immunotoxicology,* 25**,** 461-472.

GUSELLE, N. J., SPEARE, D. J., FRED MARKHAM, R. J. & PATELAKIS, S. 2012. Efficacy of intraperitoneally and orally administered ProVale, a yeast β-(1,3)/(1,6)-D-glucan product, in inhibiting xenoma formation by the microsporidian *Loma salmonae* on Rainbow trout gills. *North American Journal of Aquaculture,* 72, 65-72.

HAENEN, O. L. M., WAY, K., BERGMANN, S. M. & ARIEL, E. 2004. The emergence of koi herpesvirus and its significance to European aquaculture. *Bulletin of the European Association of Fish Pathologists,* 24**,** 293-307.

HAGEDORN, M. & SOLDATI, T. 2009. *Mycobacterium marinum.* In: SCHAIBLE, U. E. & HAAS, A. (eds.) *Intracellular Niches of Microbes.* Weinheim, Germany.Wiley-VCH Verlag GmbH & Co. KGaA.

HANSEN, J. D. & ZAPATA, A. G. 1998. Lymphocyte development in fish and amphibians. *Immunological Reviews,* 166**,** 199-220.

HARRIFF, M. J., BERMUDEZ, L. E. & KENT, M. L. 2007. Experimental exposure of zebrafish, *Danio rerio* (Hamilton), to *Mycobacterium marinum* and *Mycobacterium peregrinum* reveals the gastrointestinal tract as the primary route of infection: a potential model for environmental mycobacterial infection. *Journal of Fish Diseases,* 30, 587-600.

HE, S., ZHOU, Z., MENG, K., ZHAO, H., YAO, B., RINGØ, E. & YOON, I. 2011. Effects of dietary antibiotic growth promoter and *Saccharomyces cerevisiae* fermentation product on production, intestinal bacterial community, and nonspecific immunity of hybrid tilapia (*Oreochromis niloticus* female × *Oreochromis aureus* male). *Journal of Animal Science,* 89**,** 84-92.

HEDRICK, R. P., McDOWELL, T., GROFF, J., 1987. Mycobacteriosis in cultured striped bass from California. *Journal of Wildlife Diseases*, 23, 391-395.

HETLAND, G. & SANDVEN, P. 2002. ß-1,3-Glucan reduces growth of *Mycobacterium tuberculosis* in macrophage cultures. *FEMS Immunology and Medical Microbiology,* 33, 41-45.

HONG, F., YAN, J., BARAN, J. T., ALLENDORF, D. J., HANSEN, R. D., OSTROFF, G. R., XING, P. X., CHEUNG, N.-K. V. & ROSS, G. D. 2004. Mechanism by Which Orally Administered β-1,3-Glucans Enhance the Tumoricidal Activity of Antitumor Monoclonal Antibodies in Murine Tumor Models. *The Journal of Immunology,* 173**,** 797-806.

HOUGHTON, G., WIERGERTJES, G. F., GROENEVELD, A. & VAN MUISWINKEL, W. B. 1991. Differences in resistance of carp, *Cyprinus carpio* L., to atypical *Aeromonas salmonicida. Journal of Fish Diseases,* 14**,** 333-341.

HU, C. H., XU, Y., XIA, M. S., XIONG, L. & XU, Z. R. 2007. Effects of Cu2+-exchanged montmorillonite on growth performance, microbial ecology and intestinal morphology of Nile tilapia (*Oreochromis niloticus*). *Aquaculture,* 270**,** 200-206.

HUMBEL, B. M., KONOMI, M., TAKAGI, T., KAMASAWA, N., ISHIJIMA, S. A. & OSUMI, M. 2001. *In situ* localization of ß-glucans in the cell wall of *Schizosaccharomyces pombe. Yeast,* 18**,** 433-444.

INAMI, M., TAVERNE-THIELE,A. J., SCHRØDER, M. B.,KIRON, V.& ROMBOUT, J. H. W. M. 2009. Immunological differences in intestine and rectum of Atlantic cod (*Gadus morhua* L.)*. Fish & Shellfish Immunology,* 26, 751-759.

JAFAAR, R. M., SKOV, J., KANIA, P. W. & BUCHMANN, K. 2011. Dose Dependent Effects of Dietary Immunostimulants on Rainbow Trout Immune Parameters and Susceptibility to the Parasite *Ichthyophthirius Multifiliis. Journal of Aquaculture Research and Development,* S3:001. doi:10.4172/2155-9546.S3-001

JANEWAY, C. A. 1992. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunology Today,* 13**,** 11-16.

JAWAD, L. A., AL-MUKHTAR, M. A. & AHMED, H. K. 2004. The relationship between haematocrit and some biological parameters of the Indian shad, *Tenualosa ilisha* (Family *Clupeidae*). *Animal Biodiversity and Conservation,* 27**,** 47-52.

JENEY, G., GALEOTTI, M., VOLPATTI, D., JENEY, Z. & ANDERSON, D. P. 1997. Prevention of stress in rainbow trout (Oncorhynchus mykiss) fed diets containing different doses of glucan. *Aquaculture,* 154**,** 1-15.

JORGENSEN, J. B. & ROBERTSEN, B. 1995. Yeast ß-glucan stimulates respiratory burst activity of Atlantic salmon (*Salmo salar* L) macrophages. *Developmental and Comparative Immunology,* 19**,** 43-57.

KAMILYA, D., GHOSH, D., BANDYOPADHYAY, S., MAL, B. C. & MAITI, T. K. 2006a. In vitro effects of bovine lactoferrin, mushroom glucan and *Abrus* agglutinin on Indian major carp, catla (*Catla catla*) head kidney leukocytes. *Aquaculture,* 253**,** 130-139.

KAMILYA, D., MAITI, T. K., JOARDAR, S. N. & MAL, B. C. 2006b. Adjuvant effect of mushroom glucan and bovine lactoferrin upon *Aeromonas hydrophila* vaccination in catla, *Catla catla* (Hamilton). *Journal of Fish Diseases,* 29**,** 331-337.

KANEKO, Y., CHIHARA, G. & TAGUCHI, T. 1989. Activity of lentinan against cancer and AIDS. *International Journal of Immunotherapy,* 5**,** 203-213.

KENT, M. L., WHIPPS, C. M., MATTHEWS, J. L., FLORIO, D., WATRAL, V., BISHOP-STEWART, J. K., POORT, M. & BERMUDEZ, L. 2004. Mycobacteriosis in zebrafish (*Danio rerio*) research facilities. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 138**,** 383-390.

KIM, Y. S., KE, F. & ZHANG, Q. Y. 2009. Effect of ß-glucan on activity of antioxidant enzymes and Mx gene expression in virus infected grass carp. *Fish & Shellfish Immunology,* 27**,** 336-340.

KIM, Y. T., KIM, E. H., CHEONG, C., WILLIAMS, D. L., KIM, C. W. & LIM, S. T. 2000. Structural characterization of beta-D-(1 -> 3,1 -> 6)-linked glucans using NMR spectroscopy. *Carbohydrate Research,* 328**,** 331-341.

KIRON, V. 2012. Fish immune system and its nutritional modulation for preventive health care. *Animal Feed Science and Technology,* 173**,** 111-133.

KITAMURA, K., KANEKO, T. & YAMAMOTO, Y. 1974. Lysis of vaible yeast cells by enzymes of *Arthrobacter luteus.* II. Purification and properties of an enzyme, zymolase, which lyses viable yeast cells. *Journal of General and Applied Microbiology,* 20**,** 323-344.

KUDRENKO, B., SNAPE, N. & BARNES, A. C. 2009. Linear and branched ß(1-3) D-glucans activate but do not prime teleost macrophages *in vitro* and are inactivated by dilute acid: Implications for dietary immunostimulation. *Fish & Shellfish Immunology,* 26**,** 443-450.

KUMARI, J. & SAHOO, P. K. 2006a. Non-specific immune response of healthy and immunocompromised Asian catfish (*Clarias batrachus*) to several immunostimulants. *Aquaculture,* 255, 133-141.

KUMARI, J. & SAHOO, P. K. 2006b. Dietary immunostimulants influence specific immune response and resistance of healthy and immunocompromised Asian catfish *Clarias batrachus* to *Aeromonas hydrophila* infection. *Diseases of Aquatic Organisms,* 70, 63-70.

KWAK, J. K., PARK, S. W., KOO, J. G., CHO, M. G., BUCHHOLZ, R. & GOETZ, P. 2003. Enhancement of the non-specific defence activities in carp (*Cyprinus carpio*) and flounder (*Paralichthys olivcaces*) by oral administration of schizophyllan. *Acta Biotechnologica,* 23**,** 359-371.

LAURIDSEN, J. H. & BUCHMANN, K. 2010. Effects of short- and long-term glucan feeding of rainbow trout (*Salmonidae*) on the susceptibility to *Ichthyophthirius multifiliis* infections. *Acta Ichthyologica Et Piscatoria,* 40**,** 61-66.

LI, J., LI, D. F., XING, J. J., CHENG, Z. B. & LAI, C. H. 2006. Effects of β-glucan extracted from *Saccharomyces cerevisiae* on growth performance, and immunological and somatotropic responses of pigs challenged with *Escherichia coli* lipopolysaccharide. *Journal of Animal Science,* 84**,** 2374-2381.

LI, P. & GATLIN, D. M. 2005. Evaluation of the prebiotic GroBiotic®-A and brewers yeast as dietary supplements for sub-adult hybrid striped bass (*Morone chrysops* x *M. saxatilis*) challenged *in situ* with *Mycobacterium marinum. Aquaculture,* 248, 197-205.

LI, P. & GATLIN, D. M. 2006. Nucleotide nutrition in fish: Current knowledge and future applications. *Aquaculture,* 251, 141-152.

LI, P., WEN, Q. & GATLIN, D. M. 2009. Dose-dependent influences of dietary ß-1,3-glucan on innate immunity and disease resistance of hybrid striped bass *Morone chrysops* x *Morone saxatilis. Aquaculture Research,* 40**,** 1578-1584.

LIN, S., PAN, Y., LUO, L. & LUO, L. 2011. Effects of dietary β-1,3-glucan, chitosan or raffinose on the growth, innate immunity and resistance of koi (*Cyprinus carpio koi*). *Fish and Shellfish Immunology,* 31**,** 788-794.

LØDEMEL, J. B., MAYHEW, T. M., MYKLEBUST, R., OLSEN, R. E., ESPELID, S. & RINGØ, E. 2001. Effect of three dietary oils on disease susceptibility in Arctic charr (*Salvelinus alpinus L.*) during cohabitant challenge with *Aeromonas salmonicida ssp. salmonicida. Aquaculture Research,* 32**,** 935-945.

LUNA, L. G. 1968. *Manual of histologic staining methods of the Armed Forces Institute of Pathology,* New York, McGraw-Hill.

LYNGOY, C. 2003. Infectious Salmon Anemia in Norway and the Faroe Islands: An Industrial Approach. In: MILLER, O., CIPRIANO, R. C. (tech. coords.). *International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium.* 3–4 September 2002; New Orleans, LA. Tech. Bull. 1902. Washington, DC: U.S. Department of Agriculture, Animal and Plant Health Inspection Service; U.S. Department of the Interior, U.S. Geological Survey; U.S. Department of Commerce, National Marine Fisheries Service.

MAGNADOTTIR, B. 2006. Innate immunity of fish (overview). *Fish & Shellfish Immunology,* 20**,** 137-151.

MANTOVANI, M. S., BELLINI, M. F., ANGELI, J. P. F., OLIVEIRA, R. J., SILVA, A. F. & Ribeiro, L. R. 2008. ß-Glucans in promoting health: Prevention against mutation and cancer. *Mutation Research/Reviews in Mutation Research,* 658**,** 154-161.

MARDONES, F. O., PEREZ, A. M. & CARPENTER, T. E. 2009. Epidemiologic investigation of the re-emergence of infectious salmon anemia virus in Chile. *Diseases of Aquatic Organisms,* 84**,** 105-114.

MATEO, C. D. & STEIN, H. H. 2004. Nucelotides and young animal health: Can we enhance intestinal tract development and immune function? In: *Nutritional Biotechnology in the Feed and Food Industries.* Proceedings of Alltech's 20th Annual Symposium. UK, Nottingham University Press.

MAYER, L. 2003. Mucosal Immunity. *Pediatrics,* 111**,** 1595-1600.

MEDZHITOV, R. & JANEWAY, C., JR. 2000. Innate immune recognition: mechanisms and pathways. *Immunological Reviews,* 173**,** 89-97.

MEIJER, A. H., GABBY KRENS, S. F., MEDINA RODRIGUEZ, I. A., HE, S., BITTER, W., EWA SNAAR-JAGALSKA, B. & SPAINK, H. P. 2004. Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish. *Molecular Immunology,* 40**,** 773-783.

MERRIFIELD, D. L., BURNARD, D., BRADLEY, G., DAVIES, S. J. & BAKER, R. T. M. 2009a. Microbial community diversity associated with the intestinal mucosa of farmed rainbow trout (*Oncoryhnchus mykiss Walbaum*). *Aquaculture Research,* 40**,** 1064-1072.

MERRIFIELD, D. L., DIMITROGLOU, A., BRADLEY, G., BAKER, R. T. M. & DAVIES, S. J. 2009b. Soybean meal alters autochthonous microbial populations, microvilli morphology and compromises intestinal enterocyte integrity of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases,* 32**,** 755-766.

MERRIFIELD, D. L., DIMITROGLOU, A., FOEY, A., DAVIES, S. J., BAKER, R. T. M., BØGWALD, J., CASTEX, M. & RINGØ, E. 2010. The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture,* 302**,** 1-18.

MERRIFIELD, D. L., SHAW, B. J., HARPER, G. M., SAOUD, I. P., DAVIES, S. J., HANDY, R. D. & HENRY, T. B. 2013. Ingestion of metal-nanoparticle contaminated food disrupts endogenous microbiota in zebrafish (*Danio rerio*). *Environmental Pollution,* 174**,** 157-163.

MIEST, J. J., FALCO, A., PIONNIER, N. P. M., FROST, P., IRNAZAROW, I., WILLIAMS, G. T. & HOOLE, D. 2012. The influence of dietary ß-glucan, PAMP exposure and *Aeromonas salmonicida* on apoptosis modulation in common carp (*Cyprinus carpio*). *Fish & Shellfish Immunology,* 33, 846-856.

MIONE, M. C. & TREDE, N. S. 2010. The zebrafish as a model for cancer. *Disease Models & Mechanisms,* 3**,** 517-523.

MISRA, C. K., DAS, B. K., MUKHERJEE, S. C. & PATTNAIK, P. 2006. Effect of long term administration of dietary beta-glucan on immunity, growth and survival of *Labeo rohita* fingerlings. *Aquaculture,* 255**,** 82-94.

MIZUNO, T. 1999. The extraction and development of antitumour-active polysaccharides from medicinal mushrooms in Japan. *International Journal of Medicinal Mushrooms,* 1**,** 9-29.

MÖCK, A. & PETERS, G. 1990. Lysozyme activity in rainbow trout, *Oncorhynchus mykiss* (Walbaum), stressed by handling, transport and water pollution. *Journal of Fish Biology,* 37**,** 873-885.

MORALES-LÓPEZ, R., AUCLAIR, E., GARCÍA, F., ESTEVE-GARCIA, E. & BRUFAU, J. 2009. Use of yeast cell walls, β-1, 3/1, 6-glucans and mannoproteins in broiler chicken diets. *Poultry Science,* 88**,** 601-607.

MORALES-LÓPEZ, R., AUCLAIR, E., VAN IMMERSEEL, F., DUCATELLE, R., GARCÍA, F. & BRUFAU, J. 2010. Effects of different yeast cell wall supplements added to maize- or wheat-based diets for broiler chickens. *British Poultry Science,* 51**,** 399-408.

MORI, H., YAMAMOTO, S. & NAGASAKI, S. 1977. Multiple forms of the lytic glucanase of *Flavobacterium dormitator* var. *glucanolyticae* and the properties of the main component enzyme. *Agricultural and biological chemistry* 41**,** 611-613.

MOURA, A. C. N. & MARIANO, M. 1997. Lipids from *Mycobacterium leprae* cell wall suppress T-cell activation *in vivo* and *in vitro. Immunology,* 92, 429-436.

MUYZER, G., DE WAAL, E. C. & UITTERLINDEN, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology,* 59**,** 695-700.

NAKANISHI, T. 2003. Immunological control of fish diseases [Online]. Available: http://www.soi.wide.ad.jp/class/20030032/slides/05/1.html.

NAKAO, M., FUJIKI, K., KONDO, M. & YANO, T. 2003. Detection of complement receptors on head kidney phagocytes of the common carp *Cyprinus carpio. Fisheries Science,* 69**,** 929-935.

NAMBA, A., MANO, N. & HIROSE, H. 2007. Phylogenetic analysis of intestinal bacteria and their adhesive capability in relation to the intestinal mucus of carp. *Journal of Applied Microbiology,* 102**,** 1307-1317.

NAYAK, S.K.2010. Probiotics and immunity: A fish perspective. *Fish & Shellfish Immunology,* 29, 2-14.

NGAMKALA, S., FUTAMI, K., ENDO, M., MAITA, M. & KATAGIRI, T. 2010. Immunological effects of glucan and *Lactobacillus rhamnosus* GG, a probiotic bacterium, on Nile tilapia *Oreochromis niloticus* intestine with oral *Aeromonas* challenges. *Fisheries Science,* 76**,** 833-840.

NIKL, L., ALBRIGHT, L. J. & EVELYN, T. P. T. 1991. Influence of 7 immunostimulants on the immune-response of coho salmon to *Aeromonas salmonicida. Diseases of Aquatic Organisms,* 12**,** 7-12.

NIKL, L., EVELYN, T. P. T. & ALBRIGHT, L. J. 1993. Trials with an orally and immersion-administered ß-1,3 glucan as an immunoprophylactic against *Aeromonas salmonicida* in juvenile chinook salmon *Oncorhynchus tshawytscha. Diseases of Aquatic Organisms,* 17**,** 191-196.

PAULSEN, S. M., ENGSTAD, R. E. & ROBERTSEN, B. 2001. Enhanced lysozyme production in Atlantic salmon (*Salmo salar* L.) macrophages treated with yeast ß-glucan and bacterial lipopolysaccharide. *Fish & Shellfish Immunology,* 11**,** 23-37.

PAULSEN, S. M., LUNDE, H., ENGSTAD, R. E. & ROBERTSEN, B. 2003. *In vivo* effects of ß-glucan and LPS on regulation of lysozyme activity and mRNA expression in Atlantic salmon (*Salmo salar* L.). *Fish & Shellfish Immunology,* 14**,** 39-54.

PEAT, S., WHELAN, W. J. & LAWLEY, H. G. 1958. 141. The structure of laminarin. Part I. The main polymeric linkage. *Journal of the Chemical Society (Resumed),* 724-728.

PEELER, E. J. & FEIST, S. W. 2011. Human intervention in freshwater ecosystems drives disease emergence. *Freshwater Biology,* 56**,** 705-716.

PEISER, L., MUKHOPADHYAY, S. & GORDON, S. 2002. Scavenger receptors in innate immunity. *Current Opinion in Immunology,* 14**,** 123-128.

PENGLASE, S., MOREN, M. & HAMRE, K. 2012. Lab animals: Standardize the diet for zebrafish model. *Nature,* 491**,** 333-333.

PETERS, G. & SCHWARZER, R. 1985. Changes in hemopoietic tissue of rainbow trout under influence of stress. *Diseases of Aquatic Organisms,* 1**,** 1-10.

PIONNIER, N. P. M., FALCO, A., MIEST, J. J., FROST, P., IRNAZAROW, I., SHRIVE, A. & HOOLE, D. 2013. Dietary ß-glucan stimulate complement and C-reactive protein acute phase responses in common carp (*Cyprinus carpio*) during an *Aeromonas salmonicida* infection. *Fish & Shellfish Immunology,* 34, 819-831.

PRESS, C. M. & EVENSEN, O. 1999. The morphology of the immune system in teleost fishes. *Fish & Shellfish Immunology,* 9**,** 309-318.

PROUTY, M. G., CORREA, N. E., BARKER, L. P., JAGADEESWARAN, P. & KLOSE, K. E. 2003. Zebrafish-*Mycobacterium marinum* model for mycobacterial pathogenesis. *FEMS Microbiology Letters,* 225**,** 177-182.

RAA, J. The use of immune-stimulants in fish and shellfish feeds. In: CRUZ-SUAREZ, L. E., RICQUE-MARIE, D., TAPIA-SALAZAR, M., OLVERA-NOVOA, M. A. & CIVERA-CERECEDO, R. (eds.). *Avances en Nutricion Acuicola V.* 19.-22. Noviembre 2000; Merida, Yucatan, Mexico. Memorias del V Simposium Internacionale Nutricion Acuicola.

RAMSAY, J. M., WATRAL, V., SCHRECK, C. B. & KENT, M. L. 2009. Husbandry stress exacerbates mycobacterial infections in adult zebrafish, *Danio rerio* (Hamilton). *Journal of Fish Diseases,* 32, 931-941.

RAWLS, J. F., SAMUEL, B. S. & GORDON, J. I. 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences*, 101, 4596-4601.

REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology,* 17**,** 208-213.

RICE, P. J., ADAMS, E. L., OZMENT-SKELTON, T., GONZALEZ, A. J., GOLDMAN, M. P., LOCKHART, B. E., BARKER, L. A., BREUEL, K. F., DEPONTI, W. K., KALBFLEISCH, J. H., ENSLEY, H. E., BROWN, G. D., GORDON, S. & WILLIAMS, D. L. 2005. Oral delivery and gastrointestinal absorption of soluble glucans stimulate increased resistance to infectious challenge. *Journal of Pharmacology and Experimental Therapeutics,* 314**,** 1079-1086.

RICE, P. J., KELLEY, J. L., KOGAN, G., ENSLEY, H. E., KALBFLEISCH, J. H., BROWDER, I. W. & WILLIAMS, D. L. 2002. Human monocyte scavenger receptors are pattern recognition receptors for (1 -> 3)-beta-D-glucans. *Journal of Leukocyte Biology,* 72**,** 140-146.

RINGØ, E. & BIRKBECK, T. H. 1999. Intestinal microflora of fish larvae and fry. *Aquaculture Research,* 30, 73-93.

RINGØ, E., MYKLEBUST, R., MAYHEW, T. M. & OLSEN, R. E. 2007. Bacterial translocation and pathogenesis in the digestive tract of larvae and fry. *Aquaculture,* 268**,** 251-264.

RINGØ, E., OLSEN, R. E., MAYHEW, T. M. & MYKLEBUST, R. 2003. Electron microscopy of the intestinal microflora of fish. *Aquaculture,* 227**,** 395-415.

RINGØ, E., SPERSTAD, S., MYKLEBUST, R., MAYHEW, T. M. & OLSEN, R. E. 2006a. The effect of dietary inulin on aerobic bacteria associated with hindgut of Arctic charr (*Salvelinus alpinus* L.). *Aquaculture Research,* 37**,** 891-897.

RINGØ, E., SPERSTAD, S., MYKLEBUST, R., REFSTIE, S. & KROGDAHL, Å. 2006b. Characterisation of the microbiota associated with intestine of Atlantic cod (*Gadus morhua* L.): The effect of fish meal, standard soybean meal and a bioprocessed soybean meal. *Aquaculture,* 261**,** 829-841.

RINGØ, E., OLSEN, R. E., GIFSTAD, T. Ø., DALMO, R. A., AMLUND, H., HEMRE, G.-I. & BAKKE, A. M. 2010. Prebiotics in aquaculture: a review. *Aquaculture Nutrition,* 16, 117-136.

RINGØ, E., OLSEN, R. E., VECINO, J. L. G., WADSWORTH, S. & SONG, S. K. 2011. Use of immunostimulants and nucleotides in aquaculture: a review. *Journal of Marine Science Research and Development,* 1:104. doi:10.4172/2155-9910. 1000104

ROBERTSEN, B., RORSTAD, G., ENGSTAD, R. & RAA, J. 1990. Enhancement of nonspecific disease resistance in Atlantic salmon, *Salmo salar* L, by a glucan from *Saccharomyces cerevisiae* cell walls. *Journal of Fish Diseases,* 13**,** 391-400.

RODRIGUEZ, I., CHAMORRO, R., NOVOA, B. & FIGUERAS, A. 2009. ß-Glucan administration enhances disease resistance and some innate immune responses in zebrafish (*Danio rerio*). *Fish & Shellfish Immunology,* 27**,** 369-373.

ROESELERS, G., MITTGE, E. K., STEPHENS, W. Z., PARICHY, D. M., CAVANAUGH, C. M., GUILLEMIN, K. & RAWLS, J. F. 2011. Evidence for a core gut microbiota in the zebrafish. *The ISME Journal,* 5, 1595-1608.

ROMBOUT, J. H. W. M., BOT, H. E. & TAVERNE-THIELE, J. J. 1989. Immunological importance of the second gut segment of carp. *Journal of Fish Biology,* 35**,** 167-178.

ROMBOUT, J. W. H. M., BLOK, L. J., LAMERS, C. H. J. & EGBERTS, E. 1986. Immunization of carp (*Cyprinus carpio*) with a *Vibrio anguillarum* bacterin: Indications for a common mucosal immune system. *Developmental & Comparative Immunology,* 10**,** 341-351.

ROMBOUT, J. W. H. M., ABELLI, L., PICCHIETTI, S., SCAPIGLIATI, G. & KIRON, V. 2011. Teleost intestinal immunology. *Fish & Shellfish Immunology,* 31, 616-626.

ROMBOUTS, F. M. & PHAFF, H. J. 1976a. Lysis of Yeast Cell Walls. Lytic ß-(1→3)-Glucanases from *Bacillus circulans* WL-12. *European Journal of Biochemistry,* 63**,** 121-130.

ROMBOUTS, F. M. & PHAFF, H. J. 1976b. Lysis of Yeast Cell Walls. Lytic ß-(1→6)-Glucanase from *Bacillus circulans* WL-12. *European Journal of Biochemistry,* 63**,** 109-120.

RØRSTAD, G., AASJORD, P. M. & ROBERTSEN, B. 1993. Adjuvant effect of a yeast glucan in vaccines against furunculosis in Atlantic salmon (*Salmo salar* L.). *Fish & Shellfish Immunology,* 3**,** 179-190.

ROWLEY, A. F., HUNT, T. C., PAGE, M. & MAINWARING, G. 1988. Fish. In: ROWLEY, A. F. & RATCLIFFE, N. A. (eds.) *Vertebrate Blood Cells.* Cambridge: Cambridge University Press.

SAHOO, P. K. & MUKHERJEE, S. C. 2001. Effect of dietary ß-1,3 glucan on immune responses and disease resistance of healthy and aflatoxin B1-induced immunocompromised rohu (*Labeo rohita* Hamilton). *Fish & Shellfish Immunology,* 11, 683-695.

SAHOO, P. K. & MUKHERJEE, S. C. 2002. The effect of dietary immunomodulation upon *Edwardsiella tarda* vaccination in healthy and immunocompromised Indian major carp (*Labeo rohita*). *Fish & Shellfish Immunology,* 12, 1-16.

SAKAI, M. 1999. Current research status of fish immunostimulants. *Aquaculture,* 172**,** 63-92.

SCHAPERCLAUS, W. 1991. Causes, development and prevention of fish diseases. In: SCHAPERCLAUS, W., KULOW, H. & SCHRECKENBACH, K. (eds.) *Fish Diseases.* Rotterdam: A.A. Balkema.

SCHAUER, M., MASSANA, R. & PEDROS-ALIO, C. 2000. Spatial differences in bacterioplankton composition along the Catalan coast (NW Mediterranean) assessed by molecular fingerprinting. *FEMS Microbiology Ecology,* 33, 51-59.

SCHÜTT, D. A., LEHMANN, J., GOERLICH, R. & HAMERS, R. 1997. Haematology of swordtail, *Xiphophorus helleri*. I: blood parameters and light microscopy of blood cells. *Journal of Applied Ichthyology,* 13**,** 83-89.

SEALEY, W. M., BARROWS, F. T., HANG, A., JOHANSEN, K. A., OVERTURF, K., LAPATRA, S. E. & HARDY, R. W. 2008. Evaluation of the ability of barley genotypes containing different amounts of beta-glucan to alter growth and disease resistance of rainbow trout *Oncorhynchus mykiss. Animal Feed Science and Technology,* 141**,** 115-128.

SEGATA, N., BOEMIGEN, D., TICKLE, T. L., MORGAN, X. C., GARRETT, W. S. & HUTTENHOWER, C. 2013. Computational meta'omics for microbial community studies. *Molecular Systems Biology,* 9, 666.

SEKIROV, I., RUSSELL, S. L., ANTUNES, L. C. M. & FINLAY, B. B. 2010. Gut Microbiota in Health and Disease. *Physiological Reviews,* 90**,** 859-904.

SELVARAJ, V., SAMPATH, K. & SEKAR, V. 2005. Administration of yeast glucan enhances survival and some non-specific and specific immune parameters in carp (*Cyprinus carpio*) infected with *Aeromonas hydrophila. Fish & Shellfish Immunology,* 19**,** 293-306.

SELVARAJ, V., SAMPATH, K. & SEKAR, V. 2006. Adjuvant and immunostimulatory effects of ß-glucan administration in combination with lipopolysaccharide enhances survival and some immune parameters in carp challenged with *Aeromonas hydrophila. Veterinary Immunology and Immunopathology,* 114**,** 15-24.

SHELBY, R. A., LIM, C., YILDIRIM-AKSOY, M., WELKER, T. L. & KLESIUS, P. H. 2009. Effects of Yeast Oligosaccharide Diet Supplements on Growth and Disease Resistance in Juvenile Nile Tilapia, *Oreochromis niloticus. Journal of Applied Aquaculture,* 21**,** 61 - 71.

SHEN, J., REN, H., TOMIYAMA-MIYAJI, C., SUGA, Y., SUGA, T., KUWANO, Y., IIAI, T., HATAKEYAMA, K. & ABO, T. 2007. Potentiation of intestinal immunity by micellary mushroom extracts. *Biomedical Research,* 28**,** 71-77.

SHEPHARD, K. L. 1994. Functions for fish mucus. *Reviews in Fish Biology and Fisheries,* 4**,** 401-429.

SHI, C. & PAMER, E. G. 2011. Monocyte recruitment during infection and inflammation. *Nature Reviews Immunology,* 11**,** 762-774.

SIMON, C. & DANIEL, R. 2011. Metagenomic Analyses: Past and Future Trends. *Applied and Environmental Microbiology,* 77, 1153-1161.

SIWICKI, A. K., ANDERSON, D. P. & RUMSEY, G. L. 1994. Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis. *Veterinary Immunology and Immunopathology,* 41**,** 125-139.

SIWICKI, A. K., ZAKES, Z., TERECH-MAJEWSKA, E., KAZUN, K., LEPA, A. & GLABSKI, E. 2010. Dietary Macrogard reduces *Aeromonas hydrophila* mortality in tench (*Tinca tinca*) through the activation of cellular and humoral defence mechanisms. *Reviews in Fish Biology and Fisheries,* 20**,** 435-439.

SIWICKI, A. K., ZAKES, Z., TERECH-MAJEWSKA, E., KOWALSKA, A. & MALACZEWSKA, J. 2009. Supplementing the feed of pikeperch [*Sander lucioperca* (L.)] juveniles with MacroGard and its influence on nonspecific cellular and humoral defense mechanisms. *Aquaculture Research,* 40**,** 405-411.

SKJERMO, J., STØRSETH, T. R., HANSEN, K., HANDÅ, A. & ØIE, G. 2006. Evaluation of ß-(1→3, 1→6)-glucans and High-M alginate used as immunostimulatory dietary supplement during first feeding and weaning of Atlantic cod (*Gadus morhua* L.). *Aquaculture,* 261**,** 1088-1101.

SMITH, A. G., O'DOHERTY, J. V., REILLY, P., RYAN, M. T., BAHAR, B. & SWEENEY, T. 2011. The effects of laminarin derived from *Laminaria digitata* on measurements of gut health: selected bacterial populations, intestinal fermentation, mucin gene expression and cytokine gene expression in the pig. *British Journal of Nutrition,* 105**,** 669-677.

SNART, J., BIBILONI, R., GRAYSON, T., LAY, C., ZHANG, H., ALLISON, G. E., LAVERDIERE, J. K., TEMELLI, F., VASANTHAN, T., BELL, R. & TANNOCK, G. W. 2006. Supplementation of the Diet with High-Viscosity Beta-Glucan Results in Enrichment for *Lactobacilli* in the Rat Cecum. *Applied and Environmental Microbiology,* 72**,** 1925-1931.

SOLTANIAN, S., STUYVEN, E., COX, E., SORGELOOS, P. & BOSSIER, P. 2009. Beta-glucans as immunostimulant in vertebrates and invertebrates. *Critical Reviews in Microbiology,* 35**,** 109-138.

STENTIFORD, G. D. 2011. Diseases of commercially exploited crustaceans: Cross-cutting issues for global fisheries and aquaculture. *Journal of Invertebrate Pathology,* 106**,** 3-5.

STRAND, H. K. & DALMO, R. A. 1997. Absorption of immunomodulating ß(1,3)-glucan in yolk sac larvae of Atlantic halibut, *Hippoglossus hippoglossus* (L). *Journal of Fish Diseases,* 20**,** 41-49.

SUGITA, H., MIYAJIMA, C., KOBAYASHI, H. & DEGUCHI, Y. 1990. Distribution of Microflora in the Intestinal Tract of Carp *Cyprinus carpio. Nippon Suisan Gakkaishi,* 56**,** 1133-1138.

SUGITA, H., TOKUYAMA, K. & DEGUCHI, Y. 1985. The Intestinal Microflora of Carp *Cyprinus carpio,* Grass Carp *Ctenopharyngodon idella* and Tilapia *Sarotherodon niloticus. Bulletin of the Japanese Society of Scientific Fisheries,* 51**,** 1325-1329.

SUNG, H. H., KOU, G. H. & SONG, Y. L. 1994. Vibriosis resistance induced by glucan treatment in tiger shrimp (*Penaeus monodon*). *Fish Pathology,* 29**,** 11-17.

SUZUKI, T., OHNO, N., ADACHI, Y., CIRELLI, A. F., COVIAN, J. A. & YADOMAE, T. 1991. Preparation and biological activities of sulfated derivatives of (1-3)-beta-D-glucans. *Journal of Pharmacobio-Dynamics,* 14**,** 256-266.

SVEINBJORNSSON, B. & SELJELID, R. 1994. Aminated ß-1,3-D-polyglucose activates salmon pronephros macrophages in vitro. *Veterinary Immunology and Immunopathology,* 41**,** 113-123.

SVOBODOVA, Z.,LLOYD, R., MACHOVA, J. & VYKUSOVA, B. 1993. Water quality and fish health. *EIFAC Technical paper,* No. 54. Rome, FAO.

SWAIM, L. E., CONNOLLY, L. E., VOLKMAN, H. E., HUMBERT, O., BORN, D. E. & RAMAKRISHNAN, L. 2006. *Mycobacterium marinum* Infection of Adult Zebrafish Causes Caseating Granulomatous Tuberculosis and Is Moderated by Adaptive Immunity. *Infection and Immunity,* 74**,** 6108-6117.

TACCHI, L., BICKERDIKE, R., DOUGLAS, A., SECOMBES, C. J. & MARTIN, S. A. M. 2011. Transcriptomic responses to functional feeds in Atlantic salmon (*Salmo salar*). *Fish & Shellfish Immunology,* 31, 704-715.

TALAAT, A. M., REIMSCHUESSEL, R. & TRUCKSIS, M. 1997. Identification of mycobacteria infecting fish to the species level using polymerase chain reaction and restriction enzyme analysis. *Veterinary Microbiology,* 58**,** 229-237.

TALAAT, A. M., REIMSCHUESSEL, R., WASSERMAN, S. S. & TRUCKSIS, M. 1998. Goldfish, *Carassius auratus*, a Novel Animal Model for the Study of *Mycobacterium marinum* Pathogenesis. *Infection and Immunity,* 66**,** 2938-2942.

THORUD, K. & DJUPVIK, H. 1988. Infectious salmon anaemia in Atlantic salmon (*Salmo salar* L.). *Bulletin of the European Association of Fish Pathologists,* 8**,** 109-111.

TORRECILLAS, S., MAKOL, A., CABALLERO, M. J., MONTERO, D., ROBAINA, L., REAL, F., SWEETMAN, J., TORT, L. & IZQUIERDO, M. S. 2007. Immune stimulation and improved infection resistance in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides. *Fish & Shellfish Immunology,* 23**,** 969-981.

TRINDER, P. 1969. Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. *Journal of Clinical Pathology* 22**,** 158-161.

TSUCHIYA, C., SAKATA, T. & SUGITA, H. 2008. Novel ecological niche of *Cetobacterium somerae*, an anaerobic bacterium in the intestinal tracts of freshwater fish. *Letters in Applied Microbiology,* 46**,** 43-48.

TSUKADA, C., YOKOYAMA, H., MIYAJI, C., ISHIMOTO, Y., KAWAMURA, H. & ABO, T. 2003. Immunopotentiation of intraepithelial lymphocytes in the intestine by oral administrations of β-glucan. *Cellular Immunology,* 221**,** 1-5.

TSUKAGOSHI, S., HASHIMOTO, Y., FUJII, G., KOBAYASHI, H., NOMOTO, K. & ORITA, K. 1984. Krestin (PSK). *Cancer Treatment Reviews,* 11**,** 131-155.

UN 2011. 2011 World Population Data Sheet. Washington D.C.

UNDERHILL, D. M., OZINSKY, A., SMITH, K. D. & ADEREM, A. 1999. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. *Proceedings of the National Academy of Sciences,* 96**,** 14459-14463.

VAN DER MAREL, M., ADAMEK, M., GONZALEZ, S. F., FROST, P., ROMBOUT, J. H. W. M., WIEGERTJES, G. F., SAVELKOUL, H. F. J. & STEINHAGEN, D. 2012. Molecular cloning and expression of two ß-defensin and two mucin genes in common carp (*Cyprinus carpio* L.) and their up-regulation after ß-glucan feeding. *Fish & Shellfish Immunology,* 32, 494-501.

VAN DER SAR, A. M., APPELMELK, B. J., VANDENBROUCKE-GRAULS, C. M. J. E. & BITTER, W. 2004. A star with stripes: zebrafish as an infection model. *Trends in Microbiology,* 12**,** 451-457.

VAN DER VAART, M., SPAINK, H. P. & MEIJER, A. H. 2012. Pathogen recognition and activation of the innate immune response in zebrafish. *Advances in Hematology,* 2012, Article ID 159807.

VAN HAI, N. & FOTEDAR, R. 2009. Comparison of the effects of the prebiotics (Bio-Mos® and β-1,3-D-glucan) and the customised probiotics (*Pseudomonas synxantha* and *P. aeruginosa*) on the culture of juvenile western king prawns *(Penaeus latisulcatus* Kishinouye, 1896). *Aquaculture,* 289**,** 310-316.

VAN KESSEL, M., DUTILH, B., NEVELING, K., KWINT, M., VELTMAN, J., FLIK, G., JETTEN, M., KLAREN, P. & OP DEN CAMP, H. 2011. Pyrosequencing of 16S rRNA gene amplicons to study the microbiota in the gastrointestinal tract of carp (*Cyprinus carpio* L.). *AMB Express,* 1**,** 41.

VAN MUISWINKEL, W. B. & WAL, V.-V. D. 2006. The Immune System of Fish. In: WOO, P. T. K. (ed.) *Fish Diseases and Disorders.* 2 ed. Oxfordshire, UK: CAB International.

VENTER, J. C., REMINGTON, K., HEIDELBERG, J. F., HALPERN, A. L. , RUSCH, D., EISEN, J. A., WU, D., PAULSEN, I., NELSON, K. E., NELSON, W., FOUTS, D. E., LEVY, S., KNAP, A. H., LOMAS, M. W., NEALSON, K., WHITE, O., PETERSON, J., HOFFMAN, J., PARSONS, R., BADEN-TILLSON, H., PFANNKOCH, C., ROGERS, Y.-H. & SMITH, H. O. 2004. Environmental Genome Shotgun Sequencing of the Sargasso Sea. *Science,* 304, 66-74.

VINE, N. G., LEUKES, W. D., KAISER, H., DAYA, S., BAXTER, J. & HECHT, T. 2004. Competition for attachment of aquaculture candidate probiotic and pathogenic bacteria on fish intestinal mucus. *Journal of Fish Diseases,* 27**,** 319-326.

VOLPATTI, D., D'ANGELO, L., JENEY, G., JENEY, Z., ANDERSON, D. P. & GALEOTTI, M. 1998. Nonspecific immune response in fish fed glucan diets prior to induced transportation stress. *Journal of Applied Ichthyology-Zeitschrift Fur Angewandte Ichthyologie,* 14**,** 201-206.

VRŠANSKÁ, M., KRÁTKÝ, Z. & BIELY, P. 1977. Lysis of intact yeast cells and isolated cell walls by an inducible enzyme system of *Arthrobacter* GJM-1. *Zeitschrift für allgemeine Mikrobiologie,* 17**,** 391-402.

WATRAL, V. & KENT, M. L. 2007. Pathogenesis of *Mycobacterium* spp. in zebrafish (*Danio rerio*) from research facilities. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 145**,** 55-60.

WELKER, T. L., LIM, C., YILDIRIM-AKSOY, M., SHELBY, R. & KLESIUS, P. H. 2007. Immune response and resistance to stress and *Edwardsiella ictaluri* challenge in channel catfish, *Ictalurus punctatus*, fed diets containing commercial whole-cell yeast or yeast subcomponents. *Journal of the World Aquaculture Society,* 38**,** 24-35.

WELKER, T. L., LIM, C., YILDIRIM-AKSOY, M. & KLESIUS, P. H. 2011. Effect of short-term feeding duration of diets containing commercial whole-cell yeast or yeast subcomponents on immune function and disease resistance in channel catfish, *Ictalurus punctatus*. *Journal of Animal Physiology and Animal Nutrition,* 96, 159-171.

WHIPPS, C. M., DOUGAN, S. T. & KENT, M. L. 2007. *Mycobacterium haemophilum* infections of zebrafish (*Danio rerio*) in research facilities. *FEMS Microbiology Letters,* 270**,** 21-26.

WHIPPS, C. M., LIEGGI, C. & WAGNER, R. 2012. Mycobacteriosis in Zebrafish Colonies. *ILAR Journal,* 53, 89-94.

WHITTINGTON, R., LIM, C. & KLESIUS, P. H. 2005. Effect of dietary ß-glucan levels on the growth response and efficacy of *Streptococcus iniae* vaccine in Nile tilapia, *Oreochromis niloticus*. *Aquaculture,* 248**,** 217-225.

WIEGERTJES, G. F., DALY, J. G. & VAN MUISWINKEL, W. B. 1993. Disease resistance of carp, *Cyprinus carpio* L.: identification of individual genetic differences by bath challenge with atypical *Aeromonas salmonicida*. *Journal of Fish Diseases,* 16**,** 569-576.

WILLIAMS, D. L., MCNAMEE, R. B., JONES, E. L., PRETUS, H. A., ENSLEY, H. E., BROWDER, I. W. & DILUZIO, N. R. 1991. A method for the solubilization of a (1-3)-beta-D-glucan isolated from *Saccharomyces cerevisiae. Carbohydrate Research,* 219**,** 203-213.

WILLIAMS, D. L., PRETUS, H. A., MCNAMEE, R. B., JONES, E. L., ENSLEY, H. E. & BROWDER, I. W. 1992. Development of a water-soluble, sulfated(1-3)-beta-D-glucan biological response modifier derived from *Saccharomyces cerevisiae. Carbohydrate Research,* 235**,** 247-257.

WU, S., WANG, G., ANGERT, E. R., WANG, W., LI, W. & ZOU, H. 2012. Composition, Diversity, and Origin of the Bacterial Community in Grass Carp Intestine. *PLoS ONE,* 7(2): e30440.

YADAV, M. & SCHOREY, J. S. 2006. The β-glucan receptor dectin-1 functions together with TLR2 to mediate macrophage activation by mycobacteria. *Blood,* 108**,** 3168-3175.

YADOMAE, T. 2000. Structure and biological activities of fungal beta-1,3-glucans. *Yakugaku Zasshi-Journal of the Pharmaceutical Society of Japan,* 120**,** 413-431.

YADOMAE, T. & OHNO, N. 1996. Structure-activity relationship of immunomodulating (1,3)-β-D-glucans. *Recent Research Developments in Chemical and Pharmacological Sciences,* 1**,** 23-33.

YANO, T., MANGINDAAN, R. E. P. & MATSUYAMA, H. 1989. Enhancement of the resistance of carp *Cyprinus carpio* to experimental *Edwarsiella tarda* infection by some B-1,3-glucans. *Nippon Suisan Gakkaishi,* 55**,** 1815-1819.

YANO, T., MATSUYAMA, H. & MANGINDAAN, R. E. P. 1991. Polysaccharide-induced protection of carp, *Cyprinus carpio* L, against bacterial infection. *Journal of Fish Diseases,* 14**,** 577-582.

ZAPATA, A. G., CHIBA, A. & VARAS, A. 1996. Cells and tissues of the immune System of fish. In: IWAMA, G. & NAKANISHI, T. (eds.) *The fish immune system.* San Diego, London, Boston, New York, Sydney, Tokyo, Toronto: Academic Press.

ZEKOVIC, D. B., KWIATKOWSKI, S., VRVIC, M. M., JAKOVLJEVIC, D. & MORAN, C. A. 2005. Natural and modified (1-3)-beta-D-glucans in health promotion and disease alleviation. *Critical Reviews in Biotechnology,* 25**,** 205-230.

ZFIN. [Online] Available: http://zfin.org/.

ZHANG, J., LIU, Y., TIAN, L., YANG, H., LIANG, G. & XU, D. 2012. Effects of dietary mannan oligosaccharide on growth performance, gut morphology and stress tolerance of juvenile Pacific white shrimp, *Litopenaeus vannamei. Fish & Shellfish Immunology,* 33**,** 1027-1032.

ZHAO, H.-X., CAO, J.-M., WANG, A.-L., DU, Z.-Y., YE, C.-X., HUANG, Y.-H., LAN, H.-B., ZHOU, T.-T. & LI, G.-L. 2012. Effect of long-term administration of dietary β-1,3-glucan on growth, physiological, and immune responses in *Litopenaeus vannamei* (Boone, 1931). *Aquaculture International,* 20**,** 145-158.

ZHAO, Y., MA, H., ZHANG, W., AI, Q., MAI, K., XU, W., WANG, X. & LIUFU, Z. 2011. Effects of dietary β-glucan on the growth, immune responses and resistance of sea cucumber, *Apostichopus japonicus* against *Vibrio splendidus* infection. *Aquaculture,* 315**,** 269-274.

ZHOU, Q.-C., BUENTELLO, J. A. & GATLIN III, D. M. 2010. Effects of dietary prebiotics on growth performance, immune response and intestinal morphology of red drum (*Sciaenops ocellatus*). *Aquaculture,* 309**,** 253-257.

ZHU, H., LIU, H., YAN, J., WANG, R. & LIU, L. 2012. Effect of yeast polysaccharide on some hematologic parameter and gut morphology in channel catfish (*Ictalurus punctatus*). *Fish Physiology and Biochemistry,* 38**,** 1441-1447.