The use of carbohydrates as an immunomodulator in carp (*Cyprinus carpio*)

by

Nawroz Omar Kareem

Thesis submitted partial fulfilment of the requirements for the degree of Doctor of Philosophy (Ph.D.)

March 2017

Keele University

To my parents

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Degree for which thesis being submitted: Doctor of Philosophy

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Date of submission 19/09/2016 Original registration date 02/04/2012

Name of candidate Nawroz O. Kareem

Research Institute Research Centre for Life Sciences, Faculty of Natural Sciences

Name of Lead Supervisor Professor David Hoole

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(c) The data and results presented are the genuine data and results actually obtained by me during the conduct of the research

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Abstract

Fish diseases are the most important challenge that is facing the development of the aquaculture industry. Recently, there has been substantial interest to control fish diseases through the use of immunomodulators, such as β-glucans that activate a range of immune parameters. These carbohydrates are extracted from different sources and thus vary in their effectiveness. To develop a new biomaterial, with improved therapeutic and biological activities, β-glucans were modified by sulphation, which is a final chemical modification process to obtain derivatives of sulphated polysaccharide.

In vitro experiments were used to establish the effect of different glucans and modified carbohydrates from animal and plant sources on the cytotoxicity and respiratory burst activity of carp pronephric cells and carp leukocytes cell lines (CLC). Dose response and administration time of glucans were examined using nitroblue tetrazolium (NBT) and MTT tetrazolium assays in CLCs. After screening 75 modified carbohydrates; only 9 were selected for their positive dose responses. The cellulose drive carbohydrate, sulphated tylose (CHO 1), induced greater respiratory burst activity in both cell types. The reproducibility of the sulphation process was established using a range of biological parameters, and analysing CHO 1 structure using FTIR and NMR analysis.

Carp immune responses were determined after injection with CHO 1 alone or as an adjuvant in vaccines against *Aeromonas hydrophila*. The CHO1 proved to be an effective immune adjuvant in fish, enhancing and modulating a range of innate and adaptive immune response including: serum lysozyme and complement activity, leukocytes numbers and the expression of immune-related genes in carp organs e.g. cytokines (IL1β, IFNγ), complement component3 and lysozyme, as well the antibody titre against *Aeromonas hydrophila* was significantly improved.

The potential of using CHO 1 and producing synthetic carbohydrates with identified structures maximise their role as adjuvants for vaccine or immunostimulant in anti-infective therapies has been established.

Acknowledgment

Today is the day that I would like to acknowledge the help and support of many people during my study. First of all, I would like to express my sincere appreciation to my supervisor Professor Dave Hoole for his dedication and guidance throughout my Ph.D. His inspiration and enthusiasm for the project made it a pleasure to conduct this thesis. Working with him had a great impact on my scientific knowledge and personal experience. I will be always grateful for his invaluable insights, and timely encouragement. I will remember the kindness and generosity of Dave and Jan Hoole, opening their home and spending wonderful time with me and my family.

Very special thanks are to Dr Mark Skidmore, my second supervisor for all his time and effort during my Ph.D. and for providing all the utilised carbohydrates and the row data of the structural analysis in this study.

I would also like to thank Professor Gwyn Williams for his guidance and support over the last years. I have greatly benefited from his feedbacks, expertise and discussion points on my presentations and reports.

I am truly grateful to my committee members Dr Kim Thompson, Dr Helen Price and Dr William Kirk for their willingness to examine my dissertation.

I would also like to gratefully thank Professor Alexandra Adams from the institute of Aquaculture in Stirling University, United Kingdom, for the useful discussions and her support to the study through providing the *Aeromonas hydrophila* vaccine. Also, I like to acknowledge Professor Dieter Steinhagen from Fish Disease Research Unit, University of Veterinary Medicine, Hannover, Germany, for sending the *Epithelioma papulosum cyprini* cell line (EPC) and his valuable advice during conference meeting.

Thanks to Lynsay Hadfield Ph.D. research student at Dr Mark Skidmore research group for analysing the sulphate determination of the modified carbohydrate.

I was fortunate working in a friendly environment at the school of Life Science at Keele University, many thanks for all the staffs and technicians for their support and help. I would like to thank Mrs Brenda Bould, Ms Lynsey Wheeldon, Ms Stefanie Jones, Miss Sarah Harris and Mr Alan Dudley for their support and assistance during the fish sampling processes, also for keeping our fish healthy.

I would like to thank a former Ph.D. students during my first few months in my Ph.D., Dr Joanna Miest, Dr Mohd Hamzah Mohd Nasir and Dr Nicolas Pionnier for their help and support. Also, I would like to thank my friends in UK Nana Efua Andoh, Nancy Dawam, Dr Mohd Hamzah Mohd Nasir, and Dr Imran Ullah and from my home country, Dr Rozhgar M. Ahmad and Banaz Jamal for their support and encouragement.

I have been blessed with a very loving and supportive family. I thank my husband and my best friend Dr Anwar Othman for standing with me through the many disappointments and celebrations over the last few years. I am forever grateful for all the love and support he has shown me. He has given up and sacrificed so much to make my career a priority in our lives. I am so thankful to have you as my life partner. I would like to thank my bright son Daryan, who have inspired me with his love and caring, and being the joy and the reason in my life.

I would like to thank those closest to me in my life. My brothers Huner, Aras and sisters Awaz, Jwan, Nigar and the rest of my family for being always there for me.

I dedicate this thesis to my parents, for made me the person that I am today.

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Abbreviations

ANOVA	analysis of variance
bp	base pair(s)
cDNA	complementary DNA
CLC	carp leucocyte cell line
CO_2	carbon dioxide
Ct	threshold cycle
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsRNA	double stranded rna
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EPC	epithelioma papulosum cyprini cell line
g	gram(s)
GALT	gut associated lymphoid tissue
GVB	gelatin veronal buffer
h	hour(s)
H_2O_2	hydrogen peroxidase
HBSS	hanks balanced salt solution
i.p.	intraperitoneally
IL	interleukin
iNOS	inducible no synthase
IU	international units
kg	kilogram(s)
KHV	koi herpes virus
LPS	lipopolysaccharide
M	molar(s)
mg	milligram
min	minute(s)

ml	millilitre(s)
mM	millimole(s)
mRNA	messenger RNA
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2h-tetrazolium, inner salt
MTT	3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NBT	nitroblue tetrazolium
ng	nanogram(s)
nm	nanometer(s)
NO	nitric oxide
O2 (-)	superoxide anion
OD	optical density or absorbance
p.i.	post infection/injection
PAMP	pathogen associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Poly(I:C)	polyinosinic:polycytidylic acid
qPCR	quantitative PCR
RBCs	red blood cells
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell park memorial institute 1640
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription PCR
sec	second(s)
SEM	standard error of means
SRBC	sheep red blood cells
SVCV	spring viremia of carp virus
TAE buffer	Tris-acetate-EDTA buffer

TLRs	toll like receptors
TNF-α	tumor necrosis factor α
U/mL	unit per millilitre
UV	ultraviolet
V	volt
v/v	volume per volume
w/v	weight per volume
WBCs	white blood cells
x g	units of gravity
α	Alpha
β	Beta
γ	gamma
µl	microliters(s)
µm	micrometre(s)
µg	microgram(s)
°C	degree Celsius

Chapter 1: General Introduction

1.1 Fresh water fish farming and production

The global increase in the human population has led to a greater demand in fish and shell fish production, for example an estimation of fish consumption per capita increased from an average of 9.9 kg in the 1960s to 19.2 kg in 2012 (highlighted in red arrow) (Figure 1-1). The annual fish consumption has also increased rapidly and reached 136 million tons obtained from captured fisheries and aquaculture in 2012 (FAO, 2014). To meet the demand for this protein source, aquaculture production has risen and become comparable to capture production. In 2012 aquaculture produced fish represented 42.2 % of total world fish production compared to 25.7 % in 2000 (FAO, 2014). Such an expansion has not been without issues in the production systems, which can bring about environmental stress and increase in disease.

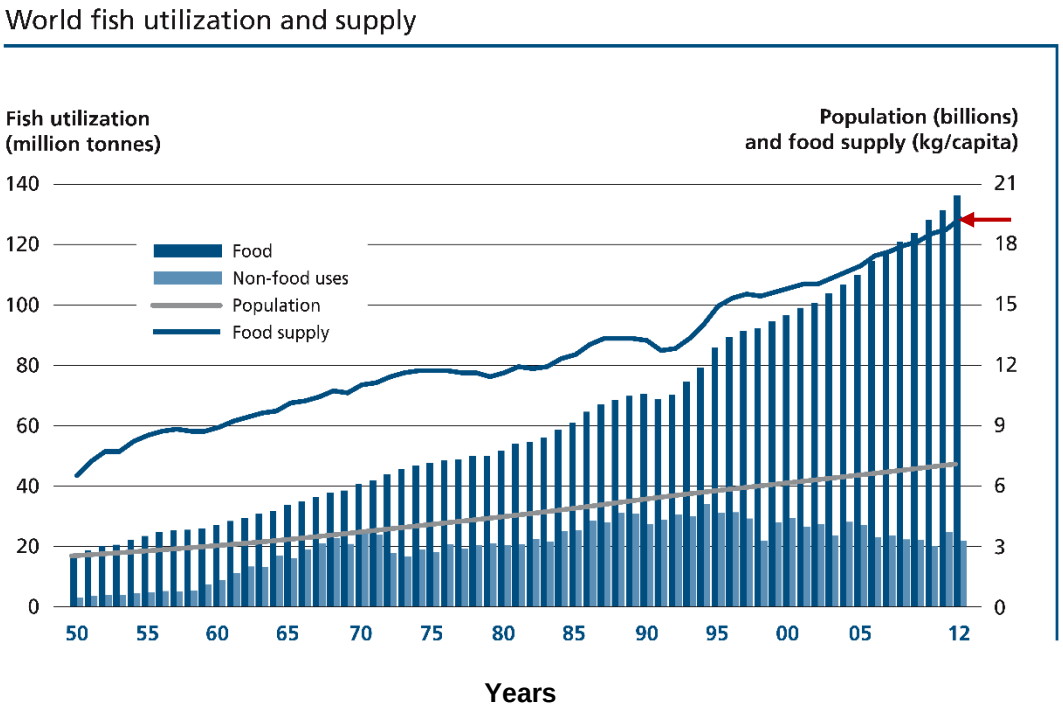


Figure 1-1: World fish utilization and supply between 1950-2012 (FAO, 2014).

The majority of aquaculture production is in Asia, where it comprises 54% more than captured fish production. Carp (*Cyprinus carpio*) is one of the main cultured fish species in the continent, most of which is consumed domestically (FAO, 2014). According to the FAO statistics in 2010 carp production reached 71.9% of the total global freshwater aquaculture production (FAO, 2012). (see Figure 1-2).

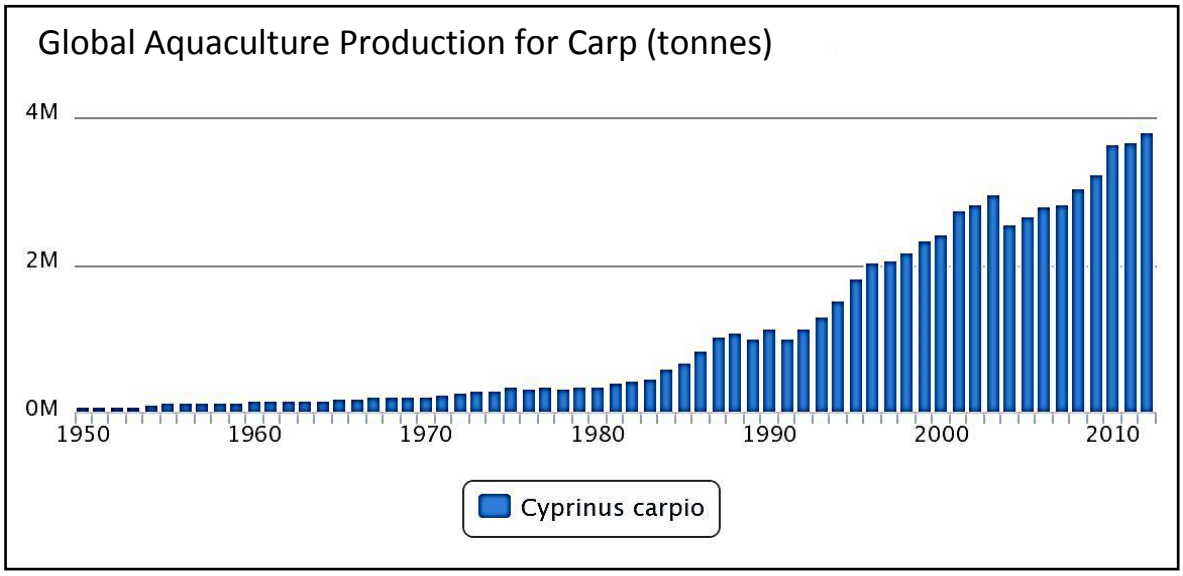


Figure 1-2: Common carp capture fisheries and aquaculture production in Europe (FAO, 2004-2014).

Common carp is one of the oldest cultured and domesticated fish in the world primarily due to it inhabiting fresh water and it being very adaptable in wild and cultured conditions. It is produced in a range of culture systems, for example, extensive production systems in stagnant water and cages, polyculture systems were it is bred synergistically with other species, and integrated with animal husbandry and/or plant production. Common carp has the capability to consume various feed sources, such as natural diets supplemented with farm made components and commercial feed (Billard, 1999). Currently, disease is considered as the main constraint to the fish culture, where it can spread rapidly between fish farms, impeding both economic and social development in many countries. Fish can

be infected due to the intensification of their rearing density that leads to stress and reduction in their immune-competence (Iguchi et al., 2003). There are several additional factors that facilitate disease outbreaks, such as fish handling, age, species, production system, pollution and seasonal temperature change (Hoole et al., 2001). The effect of stress was studied in carp, for example the effect of crowding conditions (25g l^{-1}) on the non-specific immune response of carp was studied and the results revealed a significant increase in stress indicators, such as plasma cortisol, glucose and chloride levels, as well as a significant reduction in the non-specific immune parameters such as phagocytic activity from the first day of the trial (Yin et al., 1995). Pathogens can be controlled in carp aquaculture through vaccination and antibiotic treatment. However, the development of antibiotic resistance and the financial expense of disease treatments as led to an increase search for different ways to strength the immune system and reduce infection occurrence. Recently, the vast majority of the researches and investigations have focused on specific immune system and vaccine development in fish. However, the use of immunostimulant development is one of the recent, promising fields of research that increase the activity of immune system and assist in disease resistance of fish (Tort et al., 2003). The development of such control strategies has meant an increased interest in the immune system of fish.

1.2 Fish immune system

Despite the presence of some important differences, the teleost fish (Osteichthyes) immune system does have some similarities to that of other vertebrates, as it comprise of both a nonspecific (innate) and specific (adaptive) immune response (Figure 1-3). Fish depend on the innate immunity from the early stages of embryogenesis, where it is a fundamental defence mechanism in fish and plays a key role in association with the adaptive immunity in disease protection (Uribe et al., 2011). Teleost are ectothermic animals, however the

innate immune parameters appear to be less affected by water temperature, while the adaptive immune mechanisms are temperature dependent (Magnadóttir, 2006). In addition, the adaptive immune system in fish is less developed than higher vertebrates and exhibits a slow lymphocyte proliferation and less antibody types in comparison to mammals (Plouffe et al., 2005). Figure 1-3 below and Table 1-1 show the major differences between innate and adaptive immunity.

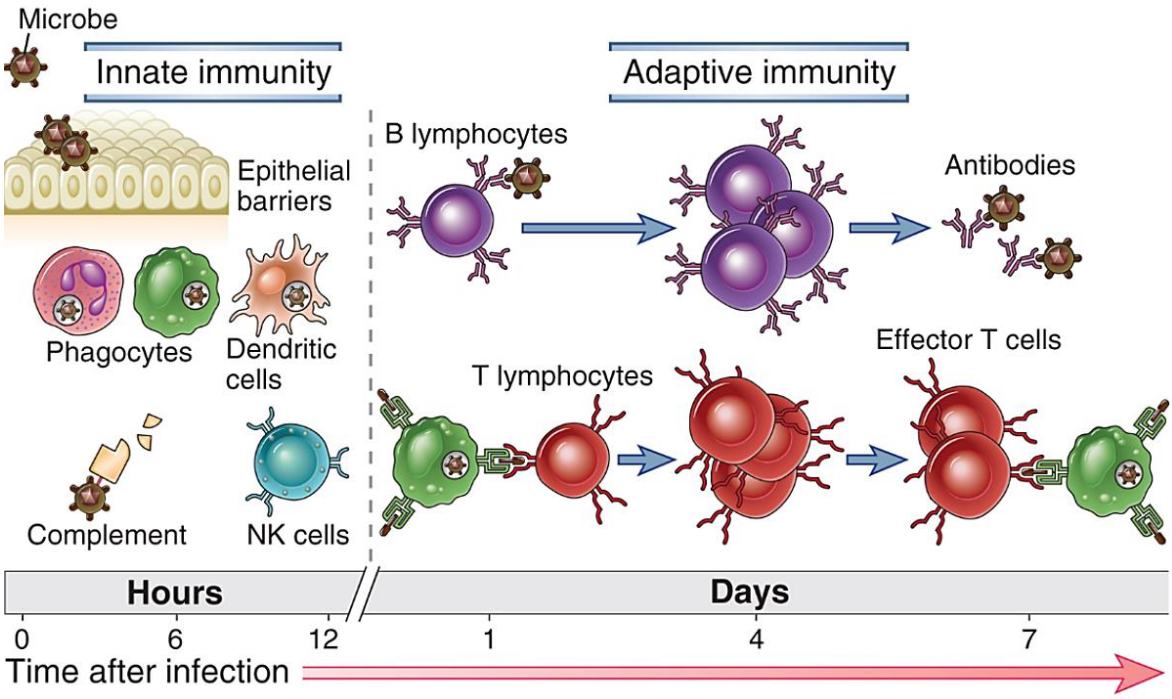


Figure 1-3: Innate and adaptive immunity in mammals. The mechanisms of innate immunity provide the initial defence against infections. Adaptive immune responses develop later and consist of activation of lymphocytes. The kinetics of the innate and adaptive immune responses are approximations and may vary in different infections (Abbas et al., 2014).

Table 1-1: Cells and effector molecules of the adaptive and innate immunity in vertebrates (Bayne and Gerwick, 2001).

	Adaptive	Innate
Cell	T-,B cells	NK cells, monocyte/macrophages, granulocytes (predominantly neutrophils)
Tissues	Thymus, pronephros	Pronephros, liver, spleen
Regulators	Cytokines	Cytokines
Humoral components	Igs	Complement system, clotting system, anti-proteases, metal binding proteins, lectins, lysozymes, antimicrobial peptides, opsonins
Kinetics	Slow	Fast

1.2.1 Immune organs

Teleosts lack hematopoietic bone marrow and lymph nodes component that are found in mammals. However, fish have an extensive series of lymphoid organs that include thymus, kidney, spleen, gut (GALT gut associated lymphoid tissue), which are involved in the hematopoiesis and immune response. In addition, liver tissue acts as a mediator of systemic and local immunity and an important site for immune regulation (Castro et al., 2014). Although, there are differences between fish species in the morphology of lymphoid organs, there are some generalization which can be made about the structure and function of the immune organs (Erik and Judith, 2008) (Figure 1-4).

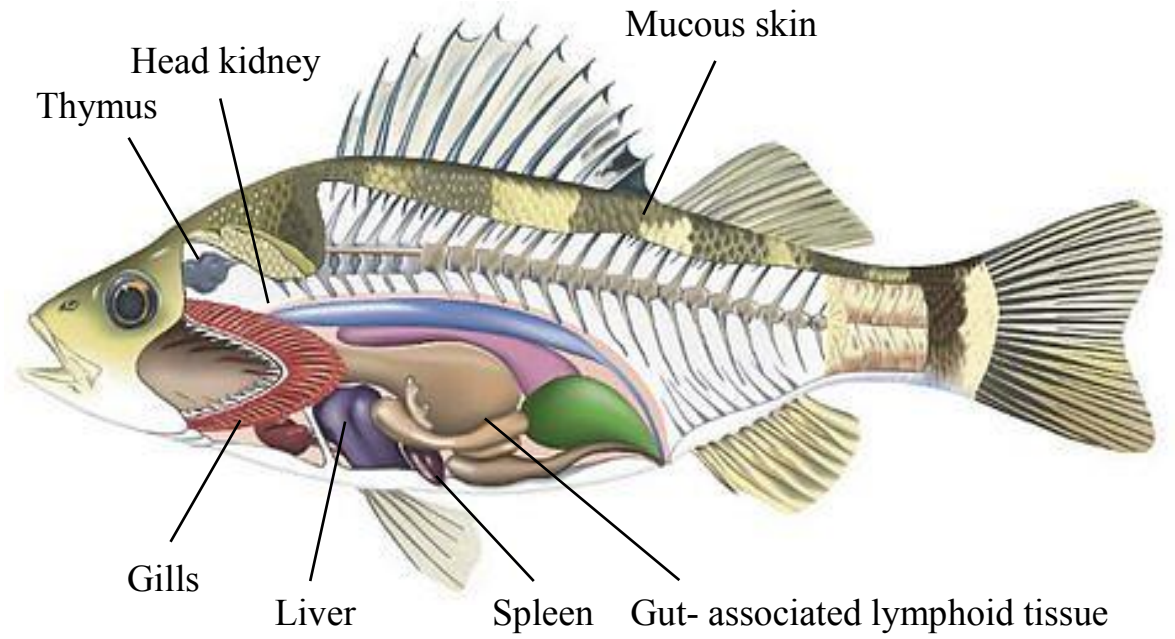


Figure 1-4: Shows the structure of immune organs in teleost fish (Tort et al., 2003).

The thymus is a primary lymphoid tissue that is located dorsolaterally above the opercular cavities. It has a heterogeneous cellular component, which comprises epithelial cells, macrophages and monocytes, myeloid cells, eosinophil granular cells, Hassell's body-like structures, nurse cells and neuroendocrine cells (Erik and Judith, 2008). The teleost thymus is involved in T-lymphocyte production, as it does in mammals and development of the cell mediated immune responses (Tort et al., 2003).

The pronephros (anterior kidney) is an important immune organ in teleost fish and is responsible for hematopoiesis, phagocytosis, antigen processing and formation of IgM and immune memory through melanomacrophagic centres (Tort et al., 2003). In the beginning of fish development, it is responsible for the production of immune cells and the early immune responses and possesses immunoglobulin positive (sIg+) B cells during ontogenesis (Hoole et al., 2001). In mature fish, the pronephros becomes the most important site of blood cell formation and immune functions, whilst the posterior kidney is concerned in blood filtration and/or urinary functions. As a result of slow blood flow in

kidney, there is a concentration of melanomacrophage aggregates or immune cells in pronephros. These melanomacrophage centres which contain reticular cells, macrophages, lymphocytes and plasma cells are involved in antigen trapping and play a role in immunologic memory (Secombes et al., 1982).

The spleen is a secondary immune organ involved in immune system activation and blood cell formation. It has a complex structure composed of a system of splenic ellipsoids, melanomacrophage centres and lymphoid tissue, which function in blood filtration and the trapping and processing of antigens. The smaller vessels or arterioles, commonly called ellipsoids, are surrounded by reticuloendothelial cells and melanomacrophage centres. (Hoole et al., 2001). The cells along the walls of the splenic arterioles are actively involved in the macrophage phagocytosis of antigens (Uribe et al., 2011). There is a suggestion that melanomacrophage centres are involved in antigenic stimulation, and this stimulation may persist for about a year, thus suggesting a role in immunologic memory (Yoffey, 1929, Shoemaker et al., 2001).

The physical and chemical barriers that protect the fish from infection comprise the scales, skin, gastrointestinal and gills. The skin plays an important role in preventing pathogen entry, the mucus contains soluble components comprising lectins, pentraxins, lysozymes, complement proteins, antibacterial peptides and immunoglobulin IgM and IgT (Uribe et al., 2011). The gut-associated lymphoid tissue consists of lymphocytes, macrophages, plasma cells and different type of granulocytes (Doggett and Harris, 1991). Within the gut, protection mechanisms include the secretion of hydrochloric acidity, which kills most of the invading microorganisms. In addition, the commensal bacteria in intestinal lumen inhabit every niche and reduce the pathogenic growth by competing for nutrition and microhabitat resources, and secretion of a bacterial substance such as peptide bacteriocins, hydrogen peroxide, and lactic acid. (Jutfelt, 2011). The liver is also an important organ,

involved in the immune system as sites of plasma protein production from parenchymal cells and are described as a phagocyte site due to the blood cell migration from other lymphohematopoietic tissues (Stolen and Fletcher, 1993, Zapata et al., 1996).

1.2.2 Innate (non-specific) immunity

The innate immune system, which is essential and earliest defence mechanism in teleost, responds to non- specific and danger signalling, and is not dependent on previous recognition of the invader, and is therefore also known as the non- specific immune system (Tort et al., 2003, Magnadóttir, 2006). Several factors make the innate immune system a fundamental defence mechanism in teleost. These factors include for example, the ectothermic nature and the evolutionary status of fish, which results in a limitation in adaptive immune system, the limitation in antibody repertoire, the slow lymphocytic proliferation and the affinity between maturation and memory of lymphocytes (Uribe et al., 2011). The innate immune system is characterised by being induced by external molecules, reacting rapidly to the pathogen (Tort et al., 2003). Recognition within the non-specific response is mediated by germline- encoded pattern recognition protein (PRP)/ receptor (PRR), which are specific molecular patterns such as polysaccharides, lipopolysaccharide (LPS), peptidoglycan, bacterial DNA, and viral RNA, that can be identified by innate immune receptors (Uribe et al., 2011).

The non- specific immune response comprises physical barriers and cellular and humoral (soluble) component. It has a range of defence parameters including growth inhibitors, lytic enzymes, the classic complement pathways, the alternative and lectin pathway, agglutinins and precipitins (opsonins and primary lectins), natural antibodies, cytokines, chemokines and antibacterial peptides (Uribe et al., 2011).

The innate immune system is affected by several factors, some of them are suppressive like water temperature, stress and fish density; and some are enhancing like the application of feeding additives and immunostimulators (Uribe et al., 2011).

In teleosts, the main cellular response relies on the recognition of the pathogen, phagocytosis and intracellular killing mechanism of the host to kill the pathogen. Different cell types are involved in this nonspecific cellular response. Cells comprise monocytes/ macrophages, granulocytes (neutrophilic, eosinophilic, and basophilic), and nonspecific cytotoxic cells (NCCs), all of which represent a primitive group of immune- effectors cells. Several generalisations have been made in morphology and function of immune cells between different specific species (Erik and Judith, 2008).

- Monocytes/ Macrophages

Macrophages are one of the major leukocytes in the innate cellular defence in fish. These cells are found in blood and secondary lymphoid tissues, and represent a mobile phagocytic cell population (Secombes, 1997). Carp macrophages appear in the head kidney at two days after fertilization, and after two weeks they are found in most lymphoid tissues (Erik and Judith, 2008).

Monocytes and macrophages are perhaps one of the most important leukocytes in the fish immune response because they also produce cytokines and are involved in the recognition of pathogens, engulfing invaders and destroying them intracellular through reactive oxygen/ nitrogen species and oxygen independent mechanism. Macrophages with neutrophils represent the first line of defence after the epithelial barrier. Macrophage defence mechanisms include surveillance, chemotaxis, phagocytosis and devastation of a target organism (Schepetkin and Quinn, 2006). These cells are also the primary antigen-presenting cells, and thus link the innate and acquired immune responses (Shoemaker et al., 2001). Pigmented macrophages are present in various fish species, and are primarily

found in both lymphoid (i.e. spleen, kidney, and thymus) and non-lymphoid (i.e. liver, gonads, and heart) tissues. Moreover, these pigments which appear from yellow to black in colour highlight that they contain hemosiderin, lipofuscin (ceroid), or melanin pigments (Erik and Judith, 2008).

- Granulocytes

Granulocytes are one of major players in the innate immune defence and there are several granulocytic (heterophilic) cell types in teleost. Granulocytes can be isolated from blood, lymphoid tissues and the peritoneal cavity. The functions of granulocytes during inflammation comprise phagocytosis, respiratory burst activity and chemotaxis (Secombes, 1997). They are highly mobile cells, are the first to immigrate to the site of inflammation and are involved in the initial stages of inflammation (12 to 24 h) in fish. They have the ability to produce cytokines, which recruit immune cells to the area of infection or damage (Shoemaker et al., 2001).

- Nonspecific Cytotoxic Cells (NCC)

Nonspecific cytotoxic cells are small lymphocytes, which lack cytoplasmic granules and have a pleomorphic nucleus. They are found in lymphoid tissues, gut and blood, similar to the natural killer cells in mammalian (Uribe et al., 2011, Secombes, 1997). Nonspecific cytotoxic cells have the ability in lyse target cells following receptor binding, and subsequent signalling to destroy the target. They are particularly important in parasitic and viral infections (Shoemaker et al., 2001).

Innate immune responses are initiated through the recognition of pathogens by the pattern recognition receptors (PRRs), expressed on the membranes or in the cytoplasm of most of the cells. Neutrophils and monocytes/macrophages, and dendritic cells express various and large amounts of PRRs that indicate their important role in recognition of pathogen and damaged cells, phagocytosis or induction of inflammation and the subsequent activation of

adaptive immunity (Abbas et al., 2014). Immune cells recognise molecular structure that are characteristic of pathogens such as bacterial lipopolysaccharides (LPS) and peptidoglycans, viral double stranded RNA and carbohydrates (like glucans). These substances are called pathogen associated molecular patterns (PAMPS) (Magnadóttir, 2006). In addition, during disease, inflammation or any cellular stresses that cause abnormal molecular complexes, the PRRs indirectly are able to detect of pathogens presence through danger associated molecular patterns (DAMPs) (van der Vaart et al., 2012). There is wide range of PRRs that are found on immune cells. Table 1-2 shows the location of these receptors on various cell types.

In teleost fish, the presence of PRRs such as TLRs, lectins, complement receptors, NLRs has been reported. For instance, 11 types of TLRs (TLR 1, 2, 3, 5, 7, 8, 9, 14, 21, 22 and 23) have been described as isotypes for TLR 1, 4, 8 and 21, suggesting they possess similar type of pathogen recognition mechanism similar to their mammalian counterparts (Aoki et al., 2008). TLRs, which trigger a rapid inflammatory response and prime adaptive immunity, also activate two pathways leading to: 1) the transcription factor nuclear factor-κB (NF-κB), and 2) activator protein-1 (AP-1). The result of both pathways activate transcription of pro-inflammatory cytokines such as interleukin-1, interleukin-6, or tumor necrosis factor alpha (Pietretti and Wiegertjes, 2014). The carbohydrate recognition receptors have also been studied in fish, for example the C-type lectin receptors (CLRs) are a large family of carbohydrate-binding proteins, comprising 17 groups which are present in vertebrates and occur as soluble serum proteins or transmembrane proteins (van der Vaart et al., 2012). The plasma membrane CLRs with specificity to different carbohydrates includes: mannose, glucose, N-acetylglucosamine and β-glucan (Abbas et al., 2014). Mannose receptors are the most studied CLRs in serum, involved in pathogen phagocytosis, binding to a variety of carbohydrate present on viruses, bacteria, fungi and

protozoa that activates the complement system (van der Vaart et al., 2012). Dendritic cell receptors that are associated with CLRs comprise each of dectin-1 and dectin-2 that recognise two life cycle stages of fungal organisms. Dectin-1 binds β-glucan, which is the major component of many types of yeast and dectin-2 recognizes high-mannose oligosaccharides on the fungus hyphae (Abbas et al., 2014). In response to the binding of their ligands on the cell surface of fungi, both dectins induce signalling events in dendritic cells that stimulate the production of cytokines and other proteins that promote inflammation and enhance adaptive immune responses (van der Vaart et al., 2012).

Table 1-2: Pattern recognition molecules of the innate immune system in vertebrates (Abbas et al., 2014).

Cells associated with PRRs	Location	Specific examples	PAMP/DAMP
Toll-like receptors (TLRs)	Dendritic cells, phagocytes, B cells, endothelial cells, and many other cells	TLRs 1-9	Various microbial molecules including bacterial LPS and peptidoglycans, viral nucleic acids
NOD-like receptors (NLRs)	Phagocytes, epithelial cells, and many other cells	NOD1/2 NALP family	Bacterial cell wall peptidoglycans, LPS, muramyl dipeptide and product of damaged cells
RIG-like receptors (RLRs)	Phagocytes, and many other cells	RIG-1, MDA-5	Viral RNA
C-type lectin like receptors	phagocytes	Mannose receptors	Microbial surface carbohydrate with terminal mannose and fructose
		Dectin	Glucan present in fungal cell wall
Scavenger receptors	phagocytes	CD36	Microbial diacylglycerides
N-Formyl met-leu-phe receptors	phagocytes	FPR and FPRL1	Peptide contain N-formylmethionyl residues

- Complement

Complement is an essential multicomponent defence mechanism of the innate immune system, and is a series of biological substances that helps the ability of antibodies and cells to attack pathogens. It includes the involvement of 35 soluble substances and membrane bound proteins (Holland and Lambris, 2002, Hoole et al., 2001). In mammals, the complement defence functions comprise killing the pathogen by creating pores in their surface membranes; the direct activation of complement mediated killing activity by antibody- antigen (Ag-Ig) complexes. The latter is an important effector mechanism for adaptive immune responses having a variety of roles in immune complex clearance; attracting phagocytic cells to an injury site. Complement proteins also mediate the opsonising pathogen to the surface of phagocytic cells via complement receptors, play an important link between adaptive and innate immune response, and modulate the adaptive immune response via specific receptors on lymphocyte surface and follicular dendritic cells (Holland and Lambris, 2002). Indeed, foreign substances and in some cases antibody is required to activate the complement cascade. Many researches have shown that each of the components within the complement cascade generate products, which clear antigenic molecules and immune complexes, and participate in the processes of inflammation and phagocytosis by macrophages and neutrophils (Shoemaker et al., 2001).

The complement system of many teleosts is thought to be similar to that occurring in mammals, which is activated via three routes: antibody-dependant (classical), alternative pathway and lectin pathways, the latter of which is activated by the binding between mannose binding lectin (MBL) to carbohydrate structure on microorganism (Figure 1-5). The alternative complement pathway is considered to be more important in fish immune defence as its actions are more pronounced in several fish species than in mammals (Magnadottir et al., 2005). Teleost C3 is the central complement molecule, being a part of all three pathways and has multiple isoforms i.e. at least five isoforms (Erik and Judith,

2008, Magnadottir et al., 2005). For example, in gilthead sea bream (*Sparus aurata*) five different forms of C3 (C3-1, C3-2, C3-3, C3-4, and C3-5) was characterized (Sunyer et al., 1997) and three functional C3 proteins (C3-1, C3-3, and C3-4) was identified and characterized in trout (Sunyer et al., 1996). This diversity in complement proteins is thought to expand the innate immune recognition range and provide evidence to understand the evolution of the C3 protein, and the formation and generation of a new C3-related gene family (Holland and Lambris, 2002).

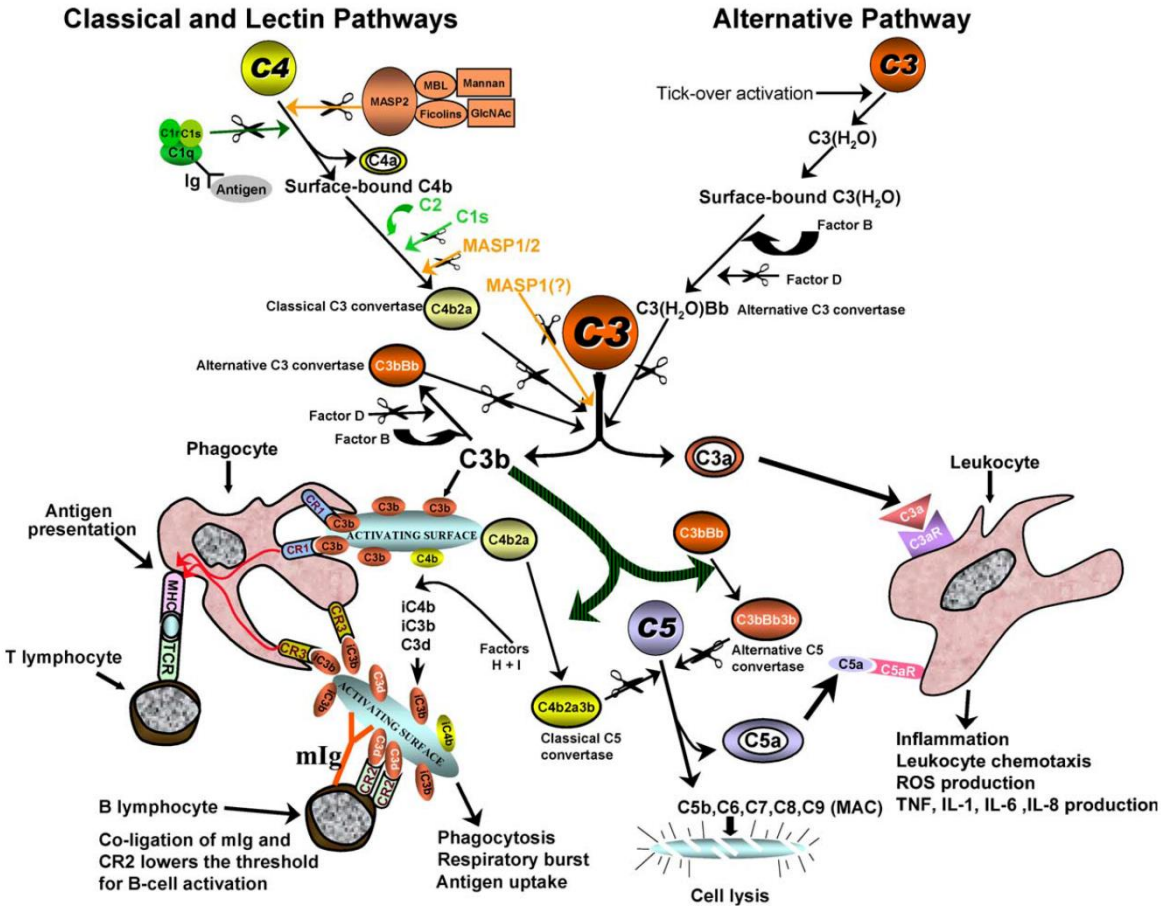


Figure 1-5: Complement activation pathways and functions in mammals. The classical, alternative or lectin complement pathways leads to the activation of C3 into C3b and C3a. The binding of C3b to complement activating surfaces (i.e., bacteria, fungi, and viruses) can be degraded into iC3b. Both C3b and iC3b bind to CR1, CR3 receptors and promote phagocytosis, respiratory burst, and antigen-uptake processes. The activated C4 through

the classical or lectin pathways can bind to an activating surface and promote its uptake. Antigen containing bound C3b or C4b molecules (or their degradation fragments) can be presented to T-lymphocytes. Antigen containing bound Ig and C3d lead to the colligation of the B cells receptor (BCR or mIg) and CR2/CD21 receptors on B cells, which in turn lowers the threshold for B cell activation. The C3b/C4b bound to a microorganism can lead to the formation of the membrane attack complex (MAC) which results in cell lysis. C5a and C3a anaphylatoxins generated during complement activation play a key role in inflammatory processes (Boshra et al., 2006).

- Interleukins (IL)

The interleukin (IL) subgroup of molecules are cytokines which are involved in the intracellular regulation of the immune system and are produced by a variety of immune cells mainly macrophages/ monocytes (i.e. IL 1), $CD4^+$ T helper cells (i.e. IL 2) and endothelial cells (Secombes et al., 2011). They act either as a pro-inflammatory or inhibitor role to particular a family member, and are able to promote cell growth, differentiation, and functional activation (Secombes et al., 2011, Shoemaker et al., 2001).

There are 35 interleukins described in mammals, although subdivisions for interleukins have been proposed, for example the IL-1 group where 11 members (IL-1F1–IL-1F11) are present, and the IL-17 group where 6 members (IL-17A–IL-17F) occur. As a result, the total number of interleukins may be greater than originally thought. Splice variants and allelic variation may increase this diversity further. Both IL-1β and IL-18 have clear homologues that have been discovered in fish (Secombes et al., 2011). In some fish species some interleukins have been extensively studied for example, interleukin IL-1β has been cloned and characterized in rainbow trout, where it activates phagocyte migration and phagocytosis, and IL-6 has recently been identified in carp (*Cyprinus carpio*) (Erik and Judith, 2008).

- Interferons (IFNs)

Interferons (IFNs) are heterogeneous family of proteins or glycoproteins, produced and released by host cells in response to the occurrence of pathogens. They have nonspecific antiviral function, able to inhibit virus replication and induce the expression of Mx and other antiviral proteins. There are three types of IFNs in mammals (α, β, and γ), which can be distinguished on the basis of biological and biochemical characteristics (Yano, 1997). Both IFN- α and IFN- β have similarities and are very often grouped together as they share 29% amino acid sequence homology and nucleotide homology, are acid stable (pH 2), act via a single receptor, and their genes are located on the same chromosome.

Interferons are important cytokines that regulate cell antiviral activity, cell growth, with immune modulatory and anti-tumor functions (Sen, 2000). Evidence shows that teleost fish cells can produce both interferons IFN- α and IFN- β in response to viral infections (Yano, 1997). In rainbow trout, a description of the induction of a protein with antiviral and macrophage activating factors were characterised by Graham and Secombes (1990). In addition, genes encoding IFN have been identified in zebra fish (*Danio rerio*) and catfish (*Ictalurus punctatus*). The antiviral Mx fish protein has also recently been shown to be induced by viral infection and poly I: C incubation (Erik and Judith, 2008).

- Tumor necrosis factor (TNF)

Tumor necrosis factor (TNF) is an important pro-inflammatory factor and mediator of host response to Gram-negative bacteria and also has a role in viral and parasitic infections (Secombes, 1997). It is produced by macrophages and is involved in several immune responses including cell proliferation, differentiation, up regulation, and stimulation of other cytokines. In addition, it is associate with cytotoxic reactions involving enhanced neutrophil migration and macrophage respiratory burst activity (Reyes-Cerpa et al., 2012). Necrosis, apoptosis and inhibition of intracellular replication has also been noted, and there

is evidence of the cooperation between TNF and IFNγ in the killing of target cells. There are some similarities in the TNF gene sequence between mammals and several fish species (Manning and Nakanishi, 1997, Reyes-Cerpa et al., 2012). Moreover, one form of TNF in fish has been noted to be similar to TNFα in mammals, and TNFα of mammals stimulates macrophage activity in fish and birds, suggesting the presence of bioactive native TNFα (Goetz et al., 2004). In catfish (*Ictalurus punctatus*), there is evidence that TNF is expressed in both lymphoid (spleen, head kidney, and thymus) and non-lymphoid (liver and gill) organs, as well as in peripheral blood leukocytes (Erik and Judith, 2008).

- Lysozyme

Lysozyme is an important component of the innate immune defence system and in fishes it mediates protection against microbial invasion and is able to lyse β-(1,4) linked glycoside bond of bacterial cell wall peptidoglycan. Lysozyme is involved in the defence against Gram- positive and negative bacteria, parasites and viruses, and has been found in mucus, lymphoid tissues, serum, and other body fluids. In several fish species, high levels of lysozyme have been found in yolk sac, fertilised eggs and larval stages, and is therefore thought to play an important role in protection of the embryo protection and in prevention of mother to progeny (vertical) transmission of some bacterial diseases like *Aeromonas salmonicida* (Magnadottir et al., 2005). Lysozyme, as an enzyme with antibiotic properties, also has broader activity in combating infections in fish and promotes phagocytosis by direct activation of neutrophils and macrophages, or indirectly by eliciting an opsonic effect (Saurabh and Sahoo, 2008). Lysozyme activity is affected by several parameters such as fish sex, age and size, season, water temperature, pH, toxicants, infections and degree of stressors (Saurabh and Sahoo, 2008).

1.2.3 Adaptive (specific) immunity

Adaptive immunity is a specific immune response involving a complex network of cells, proteins, genes and biochemical massages such as lymphocytes, antigens recognising immunoglobulins (antibodies and Ig family T cell receptors), MHC products, recombination-activating genes (RAGs) (Alvarez-Pellitero, 2008). The adaptive (acquired or specific) immune system is evoked after a few days of infection and may occur after the pathogen has overwhelmed or evaded the innate immune mechanism (Kum and Sekkin, 2011).

Adaptive immunity is responsible for: 1) recognising non-self invaders by antigen- specific lymphocytes; 2) rapid proliferation and differentiation of antigen- specific lymphocytes clones; 3) the memory and improved immune responses upon following exposure to antigen (Erik and Judith, 2008). Lymphocytes, the main cells of the adaptive immune response, are responsible for the diversity of antigen recognition, specify and memory, and are divided to B cell responsible for antibody production and T cell that mediate cell-mediate immunity. Moreover, the acquired response is slow to develop after a primary infection e.g. may take several weeks to develop in fish, but improves with time producing a lasting protection that relies on gene recombination to produce structures used for the recognition of antigens. The adaptive immune response is rapidly and highly specific to the pathogen antigens, which activate either T-lymphocytes i.e. cell- mediated immunity or B-lymphocytes (Mutoloki et al., 2014). In fish the adaptive immune response possesses genetic machinery, which is required to produce both humoral and cell mediated immune responses, that includes genes encoding recombination activating genes (RAGs), major histocompatibility complex (MHC) class I and II, T-cell receptors (TCRs), immunoglobulin heavy and light chains, cytokines, and various immune co-receptors (Erik and Judith, 2008). The immune mechanisms involved are however affected by different

factors such as endogen rhythms, environmental factors (the most important is the temperature) and fish species (Kum and Sekkin, 2011).

- Humoral immunity (Antibodies)

Humoral immunity includes antibodies (Ab), also known as immunoglobulins (Igs), which are Y-shape proteins produced by plasma cells, are used by the immune system to identify and neutralize foreign invaders. The antibody recognizes a unique part of the foreign target, the so-called antigen. They are highly specific to the antigen of the invading microbes (Kum and Sekkin, 2011). Specific antibody isotypes are produced by specific subset of B cells. The effector mechanisms of antibodies comprise neutralization, opsonisation, complement fixation, precipitation, and agglutination (Erik and Judith, 2008). Three classes of immunoglobulin isotypes have been identified in fish i.e. IgM, IgD, IgT (called IgZ in some teleost) (Mutoloki et al., 2014). IgM immunoglobulin is the major immunoglobulin in teleost, which contains eight antigenic combining sites, although some teleosts and in shark a monomer form of IgM occurs in the serum (Wilson and Warr, 1992). Similarities of binding affinities have, however, been noted between monomeric and tetrameric IgM in rainbow trout (Uribe et al., 2011). Antibody production occurs in plasma cells and plasma blasts located in head kidney, both cell types producing memory IgM responses (Sunyer, 2013). IgM is used as a marker for protection against several extracellular bacterial diseases such as furunculosis and vibriosis, and viral diseases such as pancreatic necrosis in Atlantic salmon (Munang'andu et al., 2013). Teleost vaccination increases IgM titer in the serum, but IgM affinity maturation is poor compared to IgG in mammals (Mutoloki et al., 2014). The antibodies in teleost are found in the skin, intestine, gill mucus, bile and systemically in the plasma. The immune response in the skin and gills are important because these organs are in direct contact with the environment. Also in the skin, intestine and gills, the specific antibodies can be generated without necessarily

generating a systemic response (Uribe et al., 2011). IgD is a second isotype, suggested to function as a pattern recognition molecule, and occurs in a variety of different molecular masses although all are present as a monomer in the serum (Mutoloki et al., 2014). In catfish an unique IgM^- IgD+ B cell subset and IgD armed granulocytes have been found, which is similar to B cell subsets in human, and thus suggests that IgD induces the release of antimicrobial, opsonizing, inflammatory and B cell–stimulating factors (Sunyer, 2013). IgT isotypes have recently have been discovered in some fish species and appear as a monomer in serum and specialise in gut mucosa immunity (Zhang et al., 2010). The IgT isotype is suspected to playing a key role in mucosal area such as skin and gills (Sunyer, 2013).

- Cell-Mediated Immunity

Cell-mediated immunity is an important defence mechanism in the control and elimination of intracellular pathogens, such as intracellular bacteria, parasites, and viruses. Fish cell-mediated immune responses comprise nonspecific cells (similar to natural killer cells), macrophages, granulocyte, and T cell related genes such as TCR, CD3, CD4 and CD8, in addition to major histocompatibility complex (MHC) class I and class II genes. The major histocompatibility complex (MHC)/T cell receptor system is primitive in Elasmobranchs and teleost groups although they may have a similarity to that present in mammals (Nakanishi et al., 1999). There are two classes of MHC (I and II) depending on the structural and functional different glycoproteins that offer antigenic peptides to T cells. The Class I molecules have an important role in cell-mediated immunity and involves peptides derived from cellular proteins to CD8+ve cytotoxic T cells. In many fish species, high sequence variability of MHC class I genes has been described (Nakanishi et al., 1999). Moreover, there are many cytokines which are enrolled in cellular immunity and simple polypeptides or glycoproteins that have been described as signalling molecules of

the immune system (Shoemaker et al., 2001). The cell mediated immunity is also representative in the phenomenon that is a graft- versus-host reaction (GVHR), which comprises an important role for CD4+ and CD8+ T- lymphocytes (Nakanishi et al., 2002). Finally, in many fish species, the delayed hypersensitivity responses (DHRs) response to bacterial and parasitic antigens occurs (Erik and Judith, 2008).

A number of key molecules need to be expressed on effector leukocytes and target cells as part of the adaptive immune response. For example, the specific cell-mediated cytotoxicity (CMC) CD8+ T lymphocytes, kill infected cells only, when their antigen receptor (TCR) corresponds to the MHC class I with a peptide bound of the target cell (Nakanishi et al., 2011). Furthermore, the possibility of vaccination young fish was suggested due to the expression of MHC class I molecules and CD8 at the larval stage, for example young trout up to 14 days old was able to destroy skin allografts in association with cytotoxic T lymphocytes (Uribe et al., 2011). In many fish species, T cell related genes such as TCR, CD3, CD4 and CD8, and MHC class I and II genes have been identified. In addition, reports exist on the utilisation of genetic information mRNA expression of T cell surface marker genes in alloantigen- or virus-specific effectors cells in some fish species (Nakanishi et al., 2011).

1.3 Fish health as a constraint to aquaculture

As highlighted previously, the increase in the World's human population is increasing the demand for protein from aquatic animals source, and has become a major important economic factor in several geographic areas. As a result, fish production has recently increased dramatically. Consequently, many disease problems have increased as a result of the intensification and expansion of aquaculture activities. Currently disease is considered as the main restriction to the fish culture for most aquatic species, impeding both economic

and social development in many countries. This situation may have arisen due to increase global trade of live aquatic animals, like translocation of brood stock, post larvae, fry and fingerlings, also the development and expansion of the ornamental fish trade. Recently many important diseases have emerged and spread, for example koi herpes virus (KHV) disease, which affects mainly common carp, *Cyprinus carpio* and causes up 90% dead in fish. This is leading to significant harmful effects on the aquaculture industry (Ardó et al., 2010, Oidtmann et al., 2011).

1.3.1 Prophylactic methods to control disease

There are a wide range of possible interventions that in theory can reduce the impact of disease and reduce the pathology induced. These include the application of specific diagnostic methods to detect disease at an early stage, sanitary prophylaxis and disinfection procedures, vaccination, chemotherapy, immunomodulation and the utilization of genetics of disease resistance (Jeney and Jeney, 1995). Whilst diseases caused by bacteria, fungi, and protozoan and metazoan pathogens have been controlled by chemicals and antibiotics, the application of vaccination used to strengthen the immune system in the fish to protect against infection has been used against a limited number of pathogens primarily of viral and bacterial origin. The application of these immunostimulators which act by enhancing the immune system by inducing activation or increasing activity of any of its components, is normally carried out by injection although immersion bath or internal treatment in fish feeds is the preferred method of choice (Herman, 1970).

- Chemicals

There are many general chemical treatments available for use on fish, such as formalin, malachite green, copper and salt. Unfortunately, they may have unwanted side effects and carcinogenic or toxic properties which can affect both the fish and the environment. Local

licensing laws restrict the application of controlled drugs; for example malachite green is forbidden for use on fish used for human consumption (Rintamaki-Kinnunen et al., 2005) although no restrictions, as yet, apply for usage in ornamental fish. Moreover, malachite green can be harmful for plant and fish species because of its ability to decrease the oxygen capacity of the water such as it toxicity to fish eggs. In addition, copper at medicinal concentrations is classified as unsafe for use for some plants or invertebrates. Formalin which forms paraformaldehyde is toxic, although can be used carefully with in correct dose rates and durations. There is also the possibility that after and during the application of chemical treatments that stress effects are induced in fish, which could weaken the immune response (Hoole et al., 2001).

- Environmental/physical treatments

There are several mechanism of disease control that include the alteration of physical and biological environment, such as: temperature; pH, filtration; using ultraviolet radiation; light and using ozone. These control procedures are very effective, for example, with white spot disease *Ichthyophthirius multifiliis* removing the top layer of gravel, which contains the reproductive tomont stage; reducing the hatched *Dactylogyrus* eggs by placing a mesh 50 cm from the bottom of the tank (Hoole et al., 2001).

1.3.2 Antibiotics

Antibiotics are natural or synthetic compounds that have the ability to kill or inhibit the growth of micro-organisms (Cañada-Cañada et al., 2009). Some antibiotics such as rifampin may have defined specificity and are thus are effective against a few types of bacteria. In contrast, others such as chloramphenicol and ampicillin, have a broader action and are effective against a wide range of organisms, the latter are known as broad-spectrum antibiotics (Hernández Serrano, 2005). Many antibiotics have been used, but two

in particular have been found to be particularly effective against pathogenic bacteria i.e. chlortetracycline and oxytetracycline. Giving an antibiotic to fish in food however may cause antibiotic resistance in intestinal bacteria (non-pathogenic), causing treatment-resistant illness (Hernández Serrano, 2005). Also antibiotic can result the accumulation of residues in tissues and immunosuppression (Huttenhuis et al., 2006). In addition high doses may affect the function of the liver and kidney, and may cause organ failure (Hernández Serrano, 2005).

1.3.3 Vaccination

Vaccination aims to prevent disease particularly viral and bacterial through the induction of long-term immunity by exposing the immune system to antigens from a specific pathogen or group of pathogens stimulating the immune system to develop a memory response and thus respond rapidly to future infection by the target pathogens. In fish, the nonspecific immune response plays an important role in disease protection, thus the use of adjuvants or immunopotentiators within the vaccine enhances the immune response, including long lasting effects and reducing the morbidity and mortality to an acceptable level (Anderson, 1992). Vaccines are given by injection, by immersion bath or as an oral vaccine. Injection vaccination has been reported to produce a greater immune response for example *Aeromonas salmonicida* vaccine in trout and β-glucan from *Saccharomyces cervisiae* injection in carp (Gudding and Goodrich, 2014, Selvaraj et al., 2005). The efficiency of different types of vaccines depends on their component and administration route. For example non-replicating vaccines include killed pathogens, which are either native or synthetic structure form or antigens such as *Aeromonas salmonicida* vaccine against furunculosis disease (Munang'andu et al., 2014). Replicating vaccines usually comprise live pathogens that have the infection properties for example the use of

Aeromonas hydrophila as a live attenuated vaccine against *Aeromonas salmonicida* infections in rainbow trout (Shoemaker and Klesius, 2014). DNA vaccines include a gene or genes encoding protective antigens such as Apex-IHN® vaccine for salmon against Infectious Hematopoietic Necrosis Virus IHNV (Biering and Salonius, 2014). Mucosal vaccine, for example the oral administration of an *Aeromonas hydrophila* biofilm vaccine in catla (*Catla catla*), rohu (*Labeo rohita*) and common carp (*Cyprinus carpio*).The high research and development costs are one of the major constraints in fish vaccine development and it impacts on the profits for fish production. In addition, because fish have a weak adaptive immunity, vaccines may not induce full protection or may do so for a limited time. Thus, the development of safe and effective vaccines is extremely desirable for improving cyprinid health (Hoole et al., 2001).

Commercially, vaccination plays an important role in fish farming and has been the main reason for the success of salmon cultivation. Vaccine development increased rapidly and there are commercial vaccines available for salmon, trout, channel catfish, European seabass and seabream, Japanese amberjack, yellowtail, tilapia and Atlantic cod (Sommerset et al., 2005). This efficient way of disease control lead to decline in the use of antibiotics in aquaculture. However, there are limitation in vaccine development in aquaculture, this includes the cost of vaccine production and delivery, vulnerability due to the handling and stress during the vaccination, the side effects post vaccination and fish size where fry and larval stages are more exposed to diseases and they are not large enough to be vaccinated and not developed a functional immune system (Sommerset et al., 2005). Tables 1-3 and 1-4 show major bacterial and viral fish diseases in relation to vaccine availability.

Table 1-3: Commercially available vaccines against bacterial diseases in farmed finfish (Sommerset et al., 2005).

Disease/pathogen	Affected fish species	Primary country(s)
Vibriosis (*Listonella anguillarum and V.* spp.)	Salmonids Cod/halibut Sea bass/ bream Amberjack/yellowtail	Globally
Coldwater vibriosis (*Vibrio salmonicida*)	Salmonids	Northern Europe Canada/USA
Wound disease (*Moritella viscosa*)	Salmonids	Northern Europe
Furunculosis (*Aeromonas salmonicida subsp.salmonicida*)	Salmonids	Northern Europe Canada/USA
Atypical *Aeromonas salmonicida*	Salmonids Various FW/SW species	Globally
ERM/Yersiniosis (*Yersinia ruckeri*)	Salmonids	FW Europe Chile Canada/USA
Piscirickettsiosis (*Piscirickettsia salmonis*)	Salmonids	Chile
Flavobacteriosis (*Flavobacterium psychrophilum*)	Salmonids, FW	Chile, Canada/USA
Columnaris (*Flavobacterium columnare*)	Channel catfish, Salmonids, FW	USA, Chile
Enteric septicaemia of catfish (*Edwardsiella ictaluri*)	Catfish species	USA
Bacterial kidney disease (*Renibacterium salmoninarum*)	Salmonids Rainbow trout	Chile, Canada/USA Italy, France, UK

Lactococciosis (*Lactococcus garvieae*)	Amberjack/yellowtail	Japan
Pasteurellosis (*Photobacterium damsela subspecies piscicida*)	Sea bream/sea bass Amberjack/yellowtail	Mediterranean Japan
Streptococciosis (*Streptococcus iniae*)	Tilapia	Asia

Table 1-4: Commercially available vaccines against viral diseases in farmed finfish (Dhar et al., 2014).

Virus	Vaccine type	Antigen	Delivery Route	Vaccine name	Licensed for use in country
DNA Viruses					
Koi herpes virus (KHV)	Attenuated viral vaccine	Attenuated virus	Immersion or injection	KV-3 (also known as Cavoy)	Israel, USA
Iridovirus	Inactivated viral vaccine	Inactivated virus	Intraperitoneal injection	AQUAVAC® IridoV	Singapore
Red sea bream iridiovirus	Inactivated viral vaccine	Inactivated virus	Intraperitoneal injection	Killed iridovirus vaccine, inactivated iridovirus-streptococcicosis-vibriosis combined vaccine	Japan
RNA viruses					
Salmon alphaviruses (SAV)	Inactivated viral vaccine	Inactivated virus	Intraperitoneal injection	PD Norvax® Compact PD	Norway, Chile, UK
Infectious hematopoi-etic necrosis virus (IHNV)	DNA Vaccine	Recombinant G protein	Intramuscular injection	APEX-IHN	Canada

Virus	Vaccine type	Antigen	Delivery Route	Vaccine name	Licensed for use in country
Spring viremia of carp virus (SVCV)[a]	Subunit vaccine	Recombinant G protein in baculovirus expression system	Intraperitoneal injection		Belgium
	Attenuated viral vaccine	Attenuated virus	Immersion		China
Infectious salmon anemia virus (ISAV)	Subunit vaccine	Recombinant hemagglutinin esterase protein	Oral	Centrovet, Chile	Chile
	Inactivated viral vaccine	Inactivated virus (Monovalent)	Intraperitoneal injection	Alpha Jects® Micro-1 ISA, Pharmaq AS, Norway	Chile, Finland, Ireland, Norway
	Inactivated viral vaccine	Inactivated virus (Multivalent)	Intraperitoneal injection	FORTE VI, Aqua Health Ltd., Novartis, Canada	Canada
	Inactivated viral vaccine	Inactivated virus (Multivalent)	Intraperitoneal injection	Microtek International Inc., British Columbia, Canada	Canada
Infectious pancreatic necrosis virus (IPNV)	Subunit vaccine	VP2 and VP3 capsid proteins	Oral	AquaVac® IPN Oral	Canada
	Subunit vaccine	VP2 protein (Trivalent SRS/IPNV/ *Vibrio*)	Intraperitoneal injection	SRS/IPNV/*Vibrio*	Canada, Chile
	Subunit vaccine	VP2 capsid protein	Intraperitoneal injection	Norvax® Minova -6	

Virus	Vaccine type	Antigen	Delivery Route	Vaccine name	Licensed for use in country
	Inactivated viral vaccine	Inactivated IPNV (Monovalent)	Intraperitoneal injection	Alpha Jects® 1000	Chile, Norway, UK
	Inactivated viral vaccine	Inactivated IPNV	Intraperitoneal injection	IPNV	Chile
	Inactivated viral vaccine	Inactivated IPNV	Intraperitoneal injection	Birnagen Forte	Canada

1.4 Immunomodulation

An immunomodulator is any substance that helps to regulate the immune system, which works as suppressors or/and stimulators of important components in the fish immune system, leading to an increase in protection against diseases (Zapata et al., 1997). Some immunomodulators are natural compounds and are present in organisms such as β-glucan, whilst others are available in pharmacologic preparations such as immunosuppressive treatments. Immunosuppressive therapies used in human studies include: antimetabolites, cytotoxic drugs, radiation, adrenocortical glucocorticosteroids, immunophilins, and therapeutic antibodies (Nelson and Ballow, 2003). Using the immunostimulation as a dietary supplement, helps animals to enhance the innate resistance to pathogens during high-stress periods, such as grading, reproduction, sea transfer and vaccination (Conceição et al., 2001). In addition, the use of immunomodulator improves larval survival by increasing the innate responses and larval growth rate enhancement, as reported in the use of β1-3, β1-6 glucans in common dentex (*Dentex dentex*) (Efthimiou, 1996) and Alginate in turbot (*Scophthalmus maximus*) (Conceição et al., 2001).

Pathogen control has been one of the most important limitations to the expansion of the fish production industry, and because of the side effects of fish disease treatments, the

expenses and a general desire of the public to employ more natural disease control strategies, have all led to the development of research aimed at strengthening the fish immune system through the application of immunomodulator compounds (Maudling, 2006).

1.4.1 The immunomodulatory effects of β-glucan

Glucan, a polysaccharide of D-glucose monomers, linked by glycosidic bonds, is an immunomodulatory compound obtained from the cell wall of many microorganisms and cereals, in certain mushrooms, molds, seaweed and algae. The most frequent sources are baker's yeast and brewer's yeast *Saccharomyces cerevisiae* (Novak and Vetvicka, 2008, Petravić-tominac et al., 2010), which has been investigated in clinical and laboratory studies. Initial studies were carried out by Luzio (1985) described the glucans as immunomodulators and having adjuvant properties with clinical potential in infectious diseases, neoplasia, radiation recovery, vaccine development, and control of hemopoeitic activity. Several studies e.g. Volman et al. (2008) have described the immunomodulatory effects of β-glucans to be produced either by parenteral (e.g. intravenous or subcutaneous) and enteral (dietary) administration. β-glucan has the ability to increase the resistance against infections, thought to occur primarily as a result of its effects on leukocytes activity in mammals, whilst in teleosts the pronephros (head kidney) is the main organ that produces leukocytes, thus this organ is important to the detection of β-glucan activity (Press and Evensen, 1999).

The immunomodulatory effects of β-glucan are on both the innate and adaptive immune system, however the stimulation of innate immunity to attack a pathogen via for example by the activation of complement and C-reactive protein (CRP) (Pionnier et al., 2013) may be important in the initial stages of the infection prior to the activation and action of the adaptive immunity. The direct effect of β-glucan on macrophages, neutrophils, natural

killer cells, B cells and T cells resulted in an enhanced immunity and the optimization of the resistance to pathogens (Gantner et al., 2003, Herre et al., 2004, Kim et al., 2011). The immune functions are improved by the engagement of β-glucan receptors, induction of phagocytosis; release certain cytokines (interleukin-1, interleukin-6, interferons) and the processing of antigens. β-glucan stimulates the production of cytokines from phagocytic cells and antibodies from B cells which enhance the vaccination efficiency (Raa, 2000). Also, cytokines induce the formation of new leukocytes in vertebrates including interleukin-1 (IL-1), interleukin 2 (IL-2), tumor necrosis factor-alpha (TNF-alpha), and interferon-gamma (IFN-gamma)(Meena et al., 2012). These immunostimulations affect defence mechanisms through several functions, such as encapsulation, coagulation, melanisation, and phagocytosis (Meena et al., 2012).

The immunostimulant activity leads to an increase in chemokinesis, chemotaxis, migration of macrophages to foreign particles, and enhance cytotoxicity and cytokine inflammatory responses (see Figure 1-6) (Kerékgyártó et al., 1996). As a result, IL-12 production increases and enhanced dendritic cells maturation is also noted (Chan et al., 2007). In this respect Murata et al. (2002) believed that there is an indirect effect on macrophage and dendritic cells activation and the production of IL12 and IFN-γ on T cells function, because in *in vivo* studies in mice, the β-glucan affected T cell function in tumour-bearing and malaria infected animals (Kim et al., 2011). Also, β-glucan has the ability to trigger a range of other cellular activities including the respiratory burst and the formation of reactive oxygen species (Novak and Vetvicka, 2008). In *in vivo* studies, the oral administered β-glucan activates leukocytes such as macrophages, granulocytes and monocytes, which increases the defence against infections, and supports the repair of damaged tissues in the body (Vetvicka et al., 2002). The latter modulatory effect of β-glucan may arise due to the enhanced collagen biosynthesises in skin and an increase in the

activity of fibroblasts that help to speed up wound healing. Also, it has been observed that the early arrival of macrophages to a wound area stimulates the release of growth factor and cytokines (Wei et al., 2002, Petravić-tominac et al., 2010).

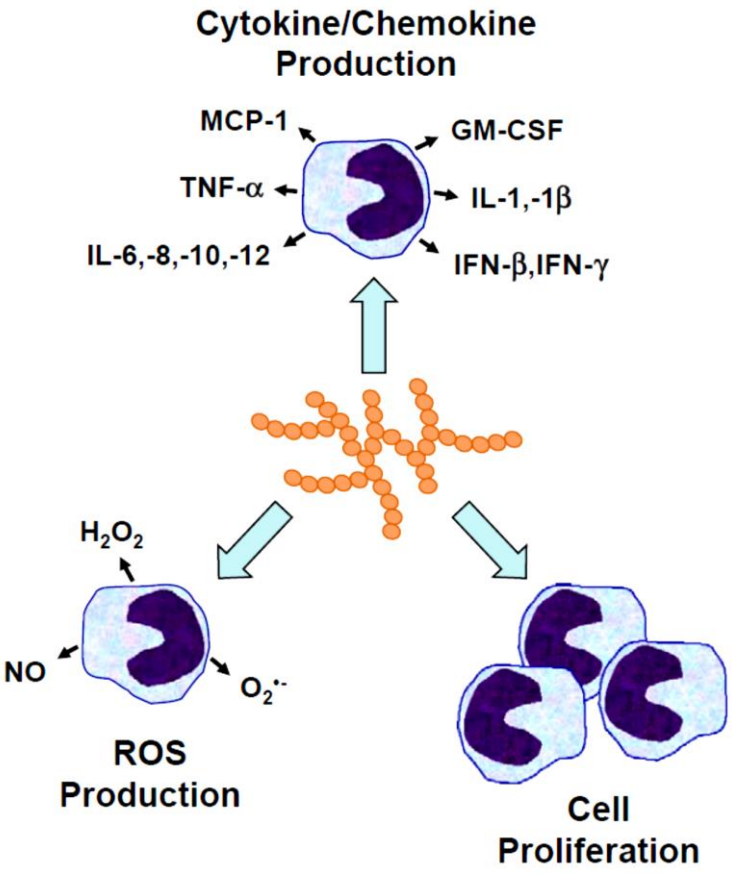


Figure 1-6: Plant polysaccharides activate a variety of macrophage responses. IL, interleukin; IFN, interferon; TNF-α, tumor necrosis factor α; GM-CSF, granulocyte/macrophage colony-stimulating factor; MCP-1, monocyte chemoattractant protein-1; NO, nitric oxide (Schepetkin and Quinn, 2006).

1.4.2 β-glucan recognition receptors

The innate immune system recognises β-glucan through pattern recognition receptors (PRRs) including Toll-like receptors TLRs, Dectin-1 (β-glucan receptor (βGR)), mannose receptor, complement receptors CR3, scavenger receptors and lactosylceramide (Gantner et al., 2003, Herre et al., 2004) as seen in Figure 1-7. The ligands of these receptors to glucan lead to activation of several pathways and triggers several protection mechanisms i.e. phagocytosis, induction of pathogen killing activity, production of inflammatory cytokines and chemokines, and initiates the development of adaptive immunity (Gantner et al., 2003). Different glucans associated with different or similar receptors on immune cells do not induce the immune response equally. For example, the scavenger receptors are a non-opsonic receptors, that have low affinity to attach to anionic β-glucans, which have been sulphated chemically or from natural source (algae) (Meena et al., 2012). Dectin-1 receptor has been identified as a major receptor for β-glucans on mammalian leukocytes (Herre et al., 2004).

The roles of Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are very important for the recognition of pathogen-derived molecules as β-glucan. Dectin-1 is one of C-type lectin receptors (CLRs) that is highly expressed receptors on monocytes/ macrophage and neutrophil surface. Also the highest expression of Dectin-1 receptors was reported in the liver, lung, thymus stomach and spleen in mouse (Herre et al., 2004). In

addition, several studies describe the C-type lectin receptors CLRs in different fish species, for example in Zebrafish (*Danio rerio*) (Chen et al., 2010); Atlantic salmon (*Salmo salar*) (Richards et al., 2003); Rainbow trout (*Oncorhynchus mykiss*) (Goetz et al., 2004) and in carp (*Cyprinus carpio*) (Pietretti et al., 2013).

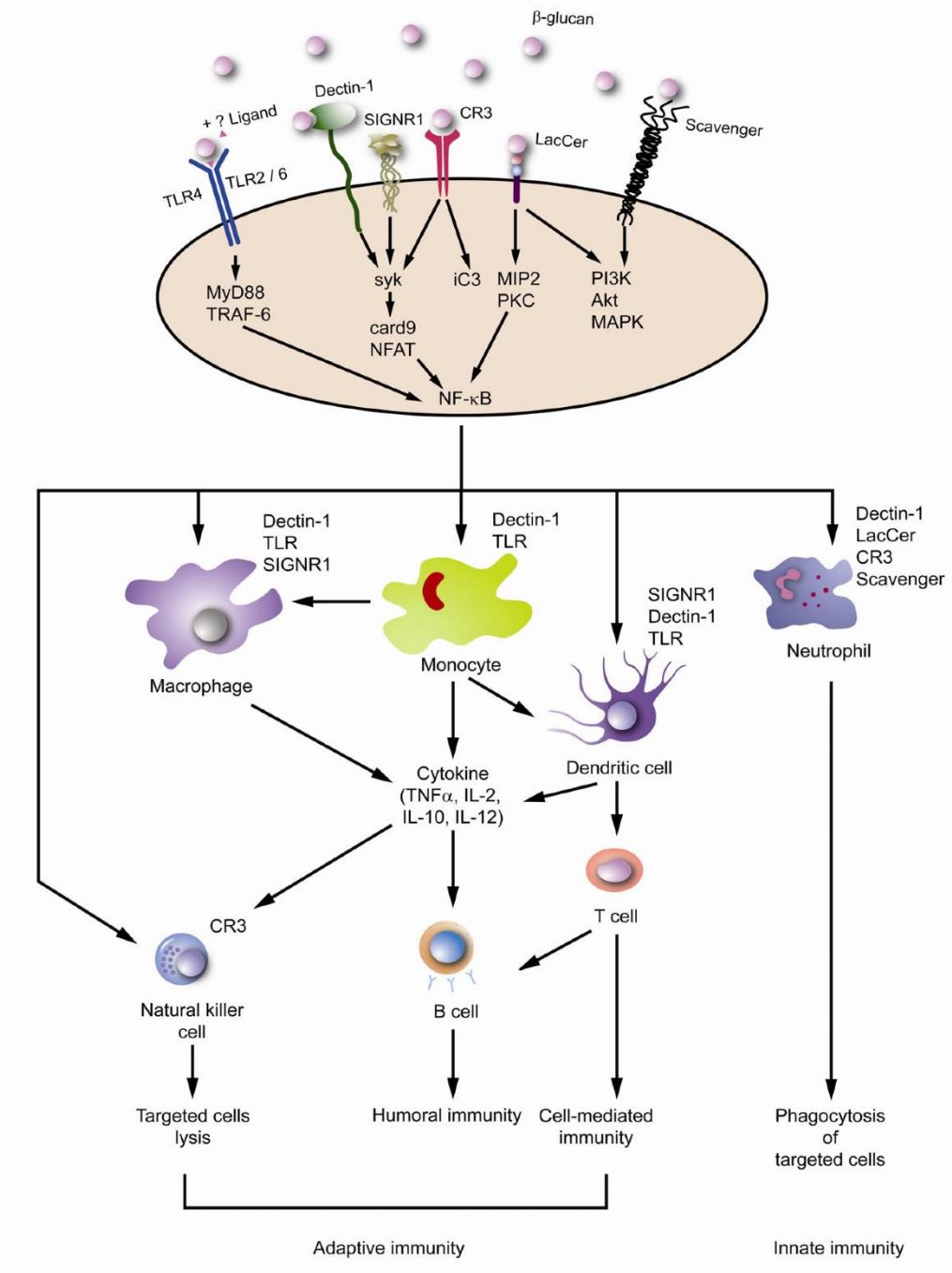


Figure 1-7: Immune activation induced by β-glucans. β-glucans can act on a variety of membrane receptors found on the immune cells, act singly or in combine with other ligands. Various signalling pathway are activated and their respective simplified downstream signalling molecules are shown. The reactors cells include monocytes, macrophages, dendritic cells, natural killer cells and neutrophils. Their corresponding surface receptors are listed. The immunomodulatory functions induced by β-glucans involve both innate and adaptive immune response (Chan et al., 2009).

The ability of Dectin-1 to induce the production anti-inflammatory cytokine IL10 from dendritic cells and respiratory burst activity in macrophage and neutrophil was reported in mice. In addition, the role of Dectin-1 alone or in association with TLR2 mediated the activation of pro-inflammatory responses of macrophage and induction the expression of IFNγ and IL 2 genes (Dalmo and Bogwald, 2008, Meena et al., 2012).

The identified TLR members in human genome includes: 1) TLR2 that recognises bacterial cell wall peptidoglycan and acylated lipopeptides; 2) TLR4 able to recognise G-negative bacterial LPS; 3) TLR5 responsible in bacterial flagellin recognition, while TLR3, 7, 8, and 9 recognise microbial nucleic acids (Boltaña et al., 2011). Similar members of the TLRs have been described in fish with conserved functions, however a noticeable differences were found for example in TLR4 and in some fish species extra TLRs was described (Boltaña et al., 2011). TLRs are capable of recognising pathogens through cell surface or the lysozyme/ endosome component, and induce the production of genes that regulate innate immunity and further develop an antigen-specific adaptive immunity (Arancibia et al., 2007). The presence of TLRs that are capable to recognising ligands is essential in the phagosome, whilst, the activation of nuclear factor (NF)-κB and production of inflammatory cytokines such as TNF-α, requires both TLR2 and TLR6 receptors in mouse macrophage cell line after treatment with zymosan particles (Underhill et al., 1999). The TLRs were described in many fish species, such as in zebrafish (*Danio rerio*),

japanese puffer (*Takifugu rubripres*) and green spotted puffer fish (*Tetraodon negroviridis*) (Roach et al., 2005); goldfish (*Carassius auratus*) (Stafford et al., 2003) and in common carp (*Cyprinus carpio*) where the presence of TLR2 and 3 have been reported (Ribeiro et al., 2010).

CR3 is another β-glucan membrane receptor that is highly expressed on neutrophils, monocytes and NK cells in comparison to macrophages, and plays an important role in phagocytosis and non-opsonic properties (Meena et al., 2012). β-glucan can activate macrophages through their ability to bind a specific receptor especially CR3 and Dectin-1. Figure 1-8 illustrate the engagement of macrophage receptors and β-glucan in mammals.

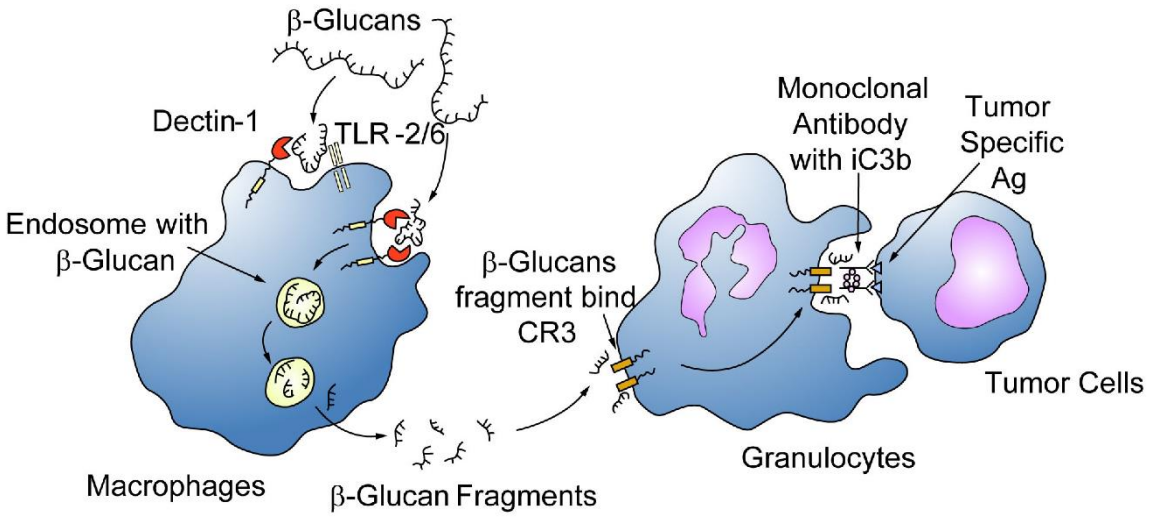


Figure 1-8: The uptake and subsequent actions of β-glucan on immune cells. β-glucans are captured by the macrophages via the Dectin-1 receptor with or without TLR-2/6. The large β-glucan molecules are then internalized and fragmented into smaller sized β-glucan fragments within the macrophages. They are carried to the marrow and endothelial reticular system and subsequently released. These small β-glucan fragments are eventually taken up by the circulating granulocytes, monocytes or macrophages via the complement

receptor (CR)-3. The immune response will then be turned on; one of the actions is the phagocytosis of the monoclonal antibody tagged tumor cells (Chan et al., 2009).

The complement receptor one (CR1) and three (CR3) are the major receptors in mammals that are involved in complement-mediated phagocytosis (Boshra et al., 2006). The CR1 require co-stimulatory signals such as IFN-γ or C5a to promote the attachment and phagocytosis of C3b/C4b-coated particles, while CR3 binding to the iC3b or the iC4b fragments is specific and does no need co-stimulatory signals and promotes natural killer (NK) cell activity for binding (Boshra et al., 2006). The CR3 has been identified in each of the following species: rainbow trout (*Oncorhynchus mykiss*) (Mikrou et al., 2009), Japanese flounder (*Paralichthys olivaceus*) (Aoki and Hirono, 2006) and in carp (*Cyprinus carpio*) (Nakao et al., 2011).

The interaction between β-glucan and lactosylceramide receptor also stimulated macrophage inflammatory protein (MIP)-2 and activate (NF)-κB which leads to the enhancement of neutrophil oxidative burst and antimicrobial functions (Meena et al., 2012).

The activity of previously described receptors leads to NF-κB activation and cytokine production (see Figure 1-7). The NF-κB signalling pathway has an important role in inflammation, immune response, apoptosis, cell growth, and differentiation (Correa et al., 2004), therefore this illustrates the association of β-glucans effects to the described physiological events.

1.4.3 Beta glucan structure, source and type

Several polysaccharides that originate from different sources have immunomodulatory effects. β-glucan is found in the cell wall of many microorganisms, cereals (wheat, rye, barley, and oat), certain mushrooms such as: Shiitake (*Lentinus edodes*), Maitake (*Grifola frondosa*), Reishi (*Ganoderma lucidum*), molds, seaweed (*Laminaria sp*.) and algae. The most frequent source of β-glucan is the fungus, baker's yeast (*Saccharomyces cerevisiae*) (Novak and Vetvicka, 2008, Petravić-tominac et al., 2010), whose cell wall consists of three layers: an inner layer of insoluble β-glucan (30–35%), middle layer – of soluble β-glucan (20–22%), external layer – of glycoprotein (30%) (Akramiene et al., 2007). These heterogeneous groups of glucose polymers are characterised by their backbone of β-(1, 3)-linked b-D-glucopyranosyl units with β-(1, 6)-linked side chains of varying distribution and length. The derived β-glucans, which originate from different sources, have therefore a range of molecular structures (Figure 1-9), that can cause difficulties in extraction and affects their activity (Meena et al., 2012). These authors also reported that β-glucan in oat and barley were linear with β (1, 4) and (1, 3) linkages; mushrooms have short β (1, 6)-linked branches from β (1, 3) backbone, whilst yeast has β (1, 6) branches further to the additional β (1, 3) regions.

There is evidence that the molecular weight of β-glucan may also affect the immunostimulatory ability. Accordingly, larger molecular weights activate leukocytes, stimulating their phagocytic, cytotoxic, and antimicrobial activities, and production of reactive oxygen species (ROS). Low molecular weight β-glucan has less cellular effects and very short glucans are believed to be inactive (Akramiene et al., 2007). Also the solubility is an additional factor that may affect the biological activity of β-glucan, as previous studies have shown that insoluble (1, 3/1, 6) β -glucans have greater biological activity than that of its soluble (1, 3/1, 4) counterparts (Ooi and Liu, 2000).

Furthermore, The present of the glycosidic bond in glucan molecules are of two types: 1) α-glucan, such as in dextran α-1,6 also in starch α-1,4- and α-1,6-glycosidic bonds 2) β-glucan, such as in cellulose with β-1,4; zymosan with β-1,3; laminarin with β-1,3- and β-1,6; lichenin with β-1,3 and β-1,4 glycosidic bond. The difference in glycosidic bond is added extra factor in the variability between glucans (Meena et al., 2012).

In addition to what has been described above on the variability of glucan effects, Table 1-5 shows the dependancy of β-glucan effects on dose, length of administration, environmental temperature and the species (Dalmo and Bogwald, 2008, Miest, 2013).

The complexity of β-glucan structure thus leads to the range of molecular forms that possibly have a greater or lesser ability to affect the immune system without over activating the immune response. They, thus are considered to act as biological response modifiers (BRM), which can have other physiological effects such as a decrease in levels of cholesterol and an ability to reduce sugar levels, which makes the β-glucan unique among immunostimulants (Meena et al., 2012).

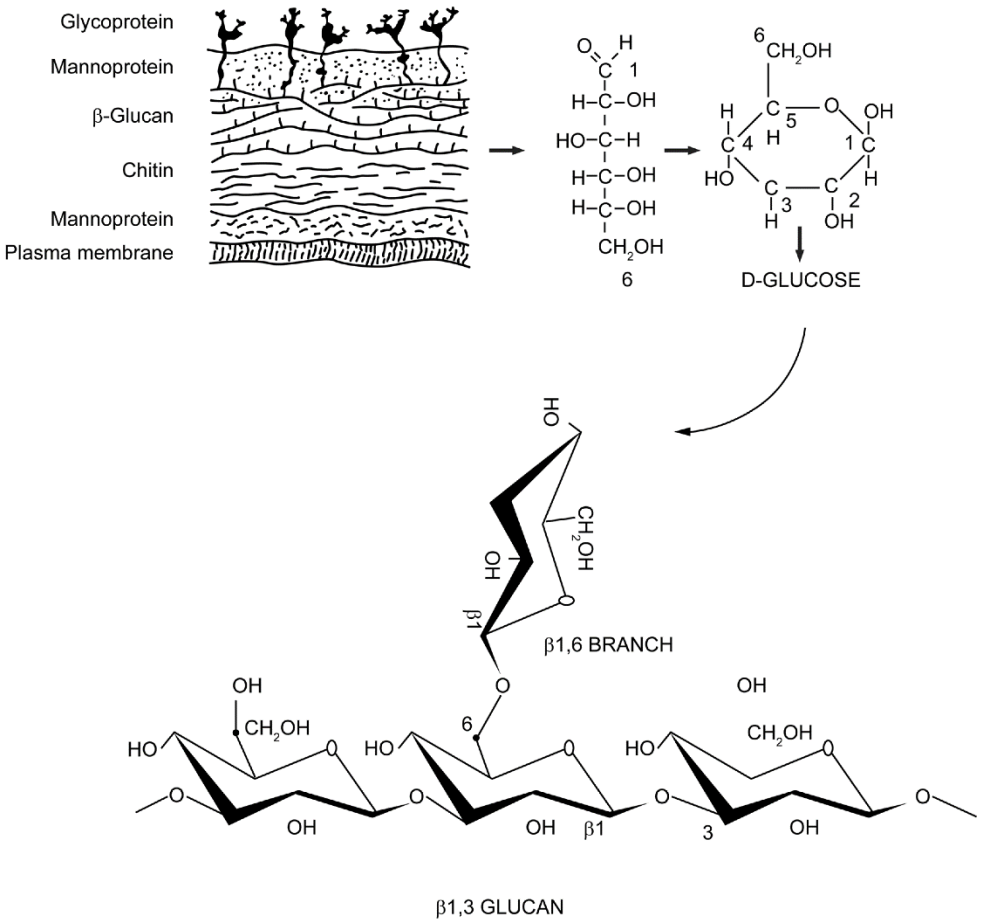


Figure 1-9: β-glucan is one of the key components of the fungal cell wall. The basic subunit of the fungal β-glucan is β-D-glucose linked to one another by 1→3 glycosidic chain with 1→6 glycosidic branches. The length and branches of the β-glucan from various fungi are widely different (Chan et al., 2009).

Table 1-5: Effects of β-glucan on the fish's immune response. The arrows toward up means significant increases or up-regulation and toward down means significant reduction or down-regulation (Modified from (Dalmo and Bogwald, 2008, Miest, 2013))

Species	β-glucan specifications	Effects of β-glucan	Reference
Injections			
Carp (*Cyprinus carpio*)	ß-1,3-glucan (*Saccharomyces cerevisiae*)+LPS	Superoxide anion ↑ Adjuvant effect ↑ No effect on Classical/alternative complement Resistance *Aeromonas hydrophila* ↑	(Selvaraj et al., 2006)

Species	β-glucan specifications	Effects of β-glucan	Reference
Injections			
Rohu fingerlings (*Labeo rohita*)	Barley β-glucan Injections 0, 5, 10, 15 mg/kg body weight Injections at 0, 14, 28 and 42 d	Phagocytic activity ↑ Leukocytes count ↑ lysozyme activity ↑ 10 mg/kg bodyweight injections complement activity ↑ serum bactericidal activity ↑ *Aeromonas hydrophila* and *Edwardsiella tarda* Resistance ↑	(Misra et al., 2006b)
Channel catfish (*Ictalurus punctatus*)	yeast β-glucan 2 x i.p. injection of 5 and 75 µg/kg bodyweight of 3-14 days	No effect on bacterial killing No effect on H_2O_2 quantity (respiratory burst) Antibody concentration ↑ Resistance to *Edwardsiella ictaluri* challenge ↑ Phagocytosis ↑	(Chen and Ainsworth, 1992)
Hybrid Tilapia (*Oreochromis niloticus* X *O. mossambicus*) Japanese eel (*Anguilla japonica*)	Yeast β-glucan (MacroGard®) 2x injection of 10mg/kg analysis after 2d	Phagocytic activity ↑ Lysozyme activity ↑	(Wang et al., 2007)
Rainbow trout (*Oncorhynchus mykiss*)	yeast β-glucan 1 % (~29 µg/kg), 1-3 weeks	Bactericidal activity ↑at 2 weeks feeding O2- production ↑ at 2 and 3 weeks Lysozyme levels ↑ at 1-3 weeks	(Jørgensen et al., 1993)

Species	β-glucan specifications	Effects of β-glucan	Reference
Injections			
	ß-1,3-glucan (laminaran)	IL-1ß1 ↑, IL-1ß2 ↑, IL-6 ↑ C3-1 ↑, C3-2 ↑/↓, C3-3↓	(Lovoll et al., 2007)
Atlantic salmon (*Salmon salar*)	yeast glucan (~0.1 g/kg) 3-6 weeks	H_2O_2 ↑ Phagocytic activity ↑ at 3 weeks Lysozyme levels ↑ at 3 weeks	(Brattgjerd et al., 1994)
Atlantic salmon (*Salmon salar*)	β-1,3,1,6 yeast glucan 0.5 mg/fish + vaccines against furunculosis	Adjuvant ↑	(Rørstad et al., 1993)
Zebrafish (*Danio rerio*)	β-glucan 6, 4 and 2 days prior the challenge 0.5, 2, 5 mg/ml	Challenge with *A. hydrophila*: 5 mg/ml mortality ↓ 5 mg/ml bactericidal activity ↑ No influence on TNF-α, IL-1β gene expression	(Rodríguez et al., 2009)
Grass carp (*Ctenopharygodon idella*)	Fungal β-glucan, injection 15 days prior the challenge	Resistance against grass carp hemorrhage virus (GCHV) ↑ After viral infection Antiviral Mx gene expression ↑ than no β-glucan control	(Kim et al., 2009)
Catla (*Catla catla*)	Mushroom glucan	Adjuvant ↑ Antibody response ↑ No effect on resistance against *Aeromonas hydrophila*	(Kamilya et al., 2006)

Species	β-glucan specifications	Effects of β-glucan	Reference
Injections			
Carp (*Cyprinus carpio*)	Yeast β-glucan 100, 500, 1000 µg/fish Injections on day 1, 3, 5	Leukocytes count ↑ (max at 1000 µg/fish) Superoxide production ↑ IL-1β gene expression ↑ Challenge with *A. hydrophila* 500 and 1000 µg/fish: resistance ↑ No change in complement activity Bactericidal activity ↑ 500 and 1000 µg/ml: antibody titre against *A. hydrophila* ↑	(Selvaraj et al., 2005)

Species	β-glucan specifications	Effects of β-glucan	Reference
Dietary			
Pink snapper (*Pagrus auratus*)	EcoActiva	Macrophage respiratory burst ↑ Growth ↑	(Cook et al., 2003)
Carp (*Cyprinus carpio*)	Dietary yeast β-glucan 1, 2, 4 % 5 days	Antibody titre ↑ No change in complement pathway No change in Leukocytes count No change in superoxide anion production No change in resistance to *A. hydrophila*	(Selvaraj et al., 2005)
	Dietary yeast β-glucan	Lysozyme activity ↑ (max at day 30 of	(Gopalakannan and Arul, 2010)

Species	β-glucan specifications	Effects of β-glucan	Reference
Dietary			
	1.0 % 60 days	feeding) Resistance to *A. hydrophila* ↑	
Koi carp (*Cyprinus carpio koi*)	Dietary yeast β-glucan 0.5 % 56 days	Lysozyme ↑ Factors increase until 21d Phagocytic activity ↑ Respiratory burst ↑ Total leucocyte counts ↑ No effect on alternative complement pathway Growth ↑ Protection against *Aeromonas veronii* ↑	(Lin et al., 2011)
Rohu (*Labeo rohita* Ham.)	ß-1,3-glucan +levamisole	Antibody response ↑ Resistance against *Edwardsiella tarda* ↑	(Sahoo and Mukherjee, 2002)
Asian catfish (*Clarias batrachus*)	ß-1,3 yeast glucan	Antibody response ↑ Protection against *Aeromonas hydrophila* ↑ Superoxide anion ↑ Myeloperoxidase ↑ Phagocytic activity ↑	(Kumari and Sahoo, 2006)
Yellow croaker (*Pseudosciaena crocea*)	Dietary yeast β-glucan 0.09 %, 0.18 % 8 weeks	0.09 %: respiratory burst ↑, phagocytic activity ↑, lysozyme activity ↑ no effect on alternative complement activity 0.18 %: no effect on respiratory burst, phagocytic activity, lysozyme activity ↑, no effect on complement activity	(Ai et al., 2007)
Striped catfish *(Pangagianodon*	Fungal β-glucan 0.05, 0.1, or 0.2	0.1, 0.2 g/kg fungal β-glucan:	(Sirimanapong et al., 2015a)

Species	β-glucan specifications	Effects of β-glucan	Reference
Dietary			
hypophthalmus)	g/kg diet + 0.1% commercial yeast-derived beta-glucan 0, 1, 3, 7, 14, 21 and 28 days post-feeding (dpf), and also at 14 days post infection (dpi).	Respiratory burst activity ↑ 0.2% fungal β-glucan: Lysozyme activity on 7 dpf ↑ plasma anti-protease activity 21 dpf ↑ natural antibody titres by 3 dpf ↑ complement activity by 7 dpf ↑ 0.1% fungal β-glucan: complement activity ↑ after 14 dpi with *Edwardsiella ictaluri* No differences in mortalities, the control diet had the highest level of mortalities and the commercial yeast-derived beta-glucan and 0.2% fungal-derived beta-glucan the lowest.	
Rohu (*Labeo rohita*)	Dietary barley β-glucan dosages: 0.01 – 0.05 % 56 days	Superoxide anion production ↑ Phagocytic activity ↑ Lysozyme activity ↑ Haemolytic complement activity ↑ Serum bactericidal activity ↑ Growth rate ↑ Mortality ↓	(Misra et al., 2006a)
Sea bass (*Dicentrarchus labrax*)	Dietary yeast β-glucan (MacroGard®) 2 %	lysozyme activity ↑ complement activity ↑	(Bagni et al., 2000)

Species	β-glucan specifications	Effects of β-glucan	Reference
Dietary			
	Dietary yeast β-glucan (MacroGard®) 0.1 %	Day 15: Complement and lysozyme activity ↑ No change in lymphocyte count	(Bagni et al., 2005)
Rainbow trout (*Oncorhynchys mykiss*)	dietary yeast β-glucan 0.2 % 14 and 35 days feeding	Lysozyme activity ↑ Challenge with *Ichthyophthirius multifiliis*: Parasite count ↓ No difference between feeding periods	(Lauridsen and Buchmann 2010)

1.4.4 Zymosan

Zymosan, a model of microbial particle produced from the cell wall of *Saccharomyces cerevisiae* yeast, is used in immunological studies because of its ability to induce innate immune responses. Zymosan comprises repeating glucose units connected by β-1,3 glycosidic bonds, which includes β-1,3-glucans linked to chitin and β-1,6-glucan (Underhill, 2003). The roles of zymosan in recognition and internalisation depend on phagocytes receptors including β- glucan receptors. Zymosan induces inflammatory signals in macrophages and is associated with phagocytic receptors on macrophages which bind

zymosan and stimulate particle engulfment (Underhill, 2003). Several methods of preparing zymosan preparation and dissolvent have been described such as boiling, autoclaving and sonication in different solutions i.e. modified culture medium, saline solution, phosphate buffered saline solution (PBS) and sterile deionized water (Fuentes et al., 2011, Jorgensen and Robertsen, 1995, Sung et al., 1983, Underhill, 2003).

1.5 The aim of the thesis

The continued growth of the aquaculture industry is considered to be the fastest growing animal husbandry industry (Section 1.1), however fish diseases have become one of the most important challenges facing the expansion and development of fish production. Therefore, more attention must be paid toward disease prophylactic, diagnostic and therapeutic measures during farming. Nevertheless, disease control through antibiotics and chemotherapeutants has been widely criticized due to their negative impact, like accumulation of tissue residues, drug resistance development, immunosuppression and reduced consumer preference for food fish treated with antibiotics (Anderson, 1992).

In recent years there has been considerable interest in the use of immunostimulants in disease control, which enhance the non-specific defence mechanisms and promote early protection against infections (Misra et al., 2006a). Within the fisheries and aquaculture areas, there is now the realization that substances such as β-glucan have distinct advantages in maintaining health compared with the use of vaccines or the application of antibiotics. These carbohydrates can be sulphated to produce physical and chemical modified polymers in order to develop important therapeutic and biological activates such as anticoagulants, antitumor, and anti HIV therapies. However, the complexity of the β-glucan molecular creates problems both in predicting immune activity and in quality assurance issues within the industry. The Centre for Veterinary Medicine (CVM), and

Food and Drug Administration (FDA) conduct several examinations to approve aquaculture foods and drugs, which includes general and special examinations. The general examination includes: physical parameters (acidity (pH), homogeneity, presence of foreign particles and packaging test), examine the microbial contamination such as *Salmonella*, *Coliform* and *Vibrio*, and examination for heavy metals contamination such as lead Pb, cadmium Cd and mercury Hg. The special examination parameters include qualitative testing i.e identification/ composition of the product and quantitative testing (measure). Therefore, it is important that any product that is in the drug composition has identified structure and can be reproduced with every drug batch.

The main aim of this study is to investigate if β-glucan structures and forms affects its immunomodulator properties in carp. To achieve this aim it is important to establish an *in vitro* system used for screening and cytotoxicity testing of different β-glucans preparations (Chapter 3). This established *in vitro* system will be applied for screening the effect of different formulated carbohydrates that characterised with differences in their structure, form and degree of sulphation (Chapter 4). The most effective modified carbohydrate that will be defined as CHO 1 is a cellulose drive carbohydrate that will be analysed for its reproducibility, structure and degree of sulphation in Chapter 5. This modified carbohydrate CHO 1 can be used as immunomodulatory agents in carp after *in vitro* and *in vivo* examinations (Chapter 4-6). Finally, the role of CHO 1 as an adjuvant in *Aeromonas hydrophila* vaccine in carp is investigated (Chapter 6).

Chapter 2. Materials and Methods

2.1 Source and maintenance of fish

Carp, *Cyprinus carpio*, obtained from Fair Fisheries, Shropshire, UK, were maintained at Keele University in black plastic tanks (1 m×0.5 m) containing 225 litres dechlorinated, ozone depleted recirculating water, at 15 °C and pH 7. Approximately 25 fish were kept in each tank and were fed daily on commercial dry pelleted food (Tetra pond feed, see the Appendix for the composition) and kept on a 12h: 12h, light: dark cycle. The fishes were acclimated for at least 15 days prior to any experimentation. The condition factor K was calculated as (weight/ length^3) ×100.

Carp were acclimated to experimental conditions at least 14 days prior any experimentation and removed from the tanks by netting. For organ sampling, fish were sacrificed with a lethal dose (~ 0.2 %) of 2-Phenoxyethanol (Sigma, P1126) in aquarium water and dissected inside a laminar flow cabinet (Gelaire®, BSB 4A) under sterile conditions, organs were removed and processed as required for the study undertaken.

2.2 Cell biology techniques

2.2.1 Pronephric cell suspension preparation

Tissue sampling were carried out after fish were sacrificed as described in Section 2.1, blood was collected from the caudal vein before dissection. Fish were incised from the ventral median and head kidney (pronephros) was isolated and placed in modified RPMI medium (the composition described below) on ice in sterile conditions. A pronephric cell suspension was prepared using a modification of the procedure described by Kemenade et al.(1994).

Briefly, pronephros tissue was disrupted gently through a sterile cell strainer with 100 µm pore diameter (BD Falcon Cell strainer, Scientific Laboratory Supply, 352360) in 1 ml of modified RPMI medium that comprise RPMI with 0.3 g/L L-glutamine (Sigma, R7388), 0.5 % sterile water, 0.05 % pooled carp serum, penicillin (50 U/ml), and streptomycin (50 µg/ml) (Sigma, P4458), This modified medium will be referred to hereafter as RPMI+.

The pooled carp serum had been prepared previously by exsanguination of numerous *Cyprinus carpio* with sterile syringes and needles. Carp blood was stored in 4 °C overnight and serum was collected after centrifuging the blood for 5 min at 1400 x *g* (Boeco Germany U32-R), then fish serum was pooled and stored at -20 °C.

2.2.2 Cell viability and dilution

The cell density and viability was checked on every cell suspension by using the trypan blue exclusion test (Howard and Pesch, 1968, Hauton and Smith, 2004). The membrane of damage cells is permeable to trypan blue dye and hence stain blue, whilst viable cells exclude the trypan blue dye. The cell suspension was diluted 1:1 with trypan blue solution (0.4 %) (Sigma, T8154) and the total number of vital i.e. non-stained cells in five squares of a haemocytometer (Improved Neubauer, depth 0.1 mm, 1/400 mm^2) were determined under x 200 microscope magnifications (Olympus CH2) and percentage cell viability and concentration were determined thus:

Cell viability = (Number of cells excluding dye) / (Total number of cells) × 100

Viable cell count (cells/ml) = mean number of viable cells × 2 (dilution factor) × 10^4

The cell suspensions with at least 95% viability were used for experimentation and cells were diluted within RPMI+ to appropriate concentration depending on experimental requirement.

2.2.3 Cell separation using Percoll gradient

Silica colloid Percoll is a non-toxic density gradient medium, creating an isometric gradient due to particles size diversity and differences in sedimentation rate. Widely used to separate leukocytes population in teleost species through continuous and discontinuous gradient.

2.2.3.1 Percoll gradient preparation

Stock Isotonic Percoll (SIP) solution were prepared by diluted Percoll (Sigma, P1644) 9:1 with 10x RPMI medium (Sigma, R1145) and further dilution with RPMI+ to obtain different Percoll densities (1.020 and 1.083 g/ml), then 2ml of both densities were placed in a 15ml tube respectively starting from the highest density 1.083 g/ml (Vera-Jimenez et al., 2013, Kemenade et al., 1994).

2.2.3.2 Isolation of total leukocytes

The isolation of total leukocytes was carried out by placing the pronephric cell suspension, obtained as described in Section 2.2.1, on to the Percoll gradient and it was centrifuged at 800 x *g* for 25 min at 4℃ without braking of the centrifugation. Total leukocytes were collected at the interphase between densities 1.02 g/ml and 1.08 g/ml, followed by three times washing with RPMI+ and centrifugation at 800 x *g* for 10 min at 4℃. Cells were resuspended in 1 ml RPMI+ and counted as described in Section 2.2.2 before any assay was performed.

2.3 Cell line culture

2.3.1 Culture medium

The carp leukocytes cell line (CLC) and the epithelioma papulosum cyprini cell line (EPC) were cultured in L-glutamine free RPMI (Sigma, R0883) modified with 5 % Foetal bovine serum (FBS) (Sigma, F6178), 2.5 % pooled carp serum (heat inactivated, 30 min at 56 ºC in water bath), 50 U/ml penicillin-G, and 50 mg/ml streptomycin (Sigma, P4458). This modified medium will be referred to hereafter as CLC RPMI+ or EPC RPMI+.

2.3.2 Thawing

The CLC cell line, originating in Wageningen University, The Netherlands, was cryopreserved in 2010 by J. Miest (previous PhD student in D. Hoole research lab) and stored in liquid nitrogen. It was revived from stock in 2012 by thawing the cells immediately in a 37 ºC water bath (Clifton NE2-8D unstirred waterbath). The thawed CLC cell solution was transferred to 9 ml Hanks Balanced Salt Solution (HBSS) (Sigma, H9269) and centrifuged for 8 min at 750 x *g* at 19±1℃. The cell pellet was then resuspended in 1 ml fresh modified CLC RPMI+ and the cell viability determined with trypan blue (see Section 2.2.2). The cell solution was transferred to a T25 flask (Sarstedt, 83.3910.002) containing 4 ml of fresh CLC RPMI+ and the cells were left to adhere for at least 3 days before subculturing.

The mycoplasma free test was applied on CLC line using the EZ-PCR Mycoplasma Test Kit (Gene Flow, 20-700-20). This test depends on the detection of mycoplasma-specific 16S rRNA by PCR and gel electrophoresis. Briefly, 1 ml of supernatant from four flasks was centrifuged at 250 x *g* for 1 min to pellet any cellular debris. The supernatant was transferred to a sterile 15 ml tube (Sarstedt, 62.554.001) and centrifuged at 20000 x *g* for

10 min to sediment the mycoplasma. The supernatant was decanted carefully and the pellet resuspended in 50 µl Buffer solution (provided in the kit) and mixed by pipetting. The samples were heated at 95 ℃ for 3 min using a heat block (Techne, Dri-Block® DB 2H). The PCR reaction mix was prepared by adding 35 µl of DEPC-Treated Water (Invitrogen, AM9916) mixed with 10 µl Reaction mix (provided) and 5 µl sample. A positive control was prepared by mixing 1 µl of provided positive template control with 10 µl of Reaction mix and 39 µl of DEPC-Treated Water (Invitrogen, AM9916). Also to ascertain if the reaction products were free of contamination, a negative control comprising 40 µl of DEPC-Treated Water (Invitrogen, AM9916) and 10 µl Reaction mix (provided) was prepared. The PCR amplification carried out using the following program (Applied Biosystems, GeneAmp® PCR System 9700): 1 cycle: 94 °C – 30 sec; 35 cycles of: 94 °C – 30 sec; 60 °C – 120 sec and 72 °C – 60 sec; 1 cycle: 94 °C – 30 sec; 60 °C – 120 sec and 72 °C – 5 min. Gel electrophoresis was performed at 100 V with 20 µl of the PCR product on a 2 % agarose gel. Mycoplasma contamination is expected to give 270 bp PCR product.

The EPC cell line was obtained from Fish Disease Research Unit, Centre for Infection Medicine, University of Veterinary Medicine, Hannover, Germany. The EPC was revived as a cell monolayer in EPC RPMI+ culture medium in a flask and subcultured twice in fresh EPC RPMI+ medium before experimentation. Both cell lines were kept at constant temperature of 27 ℃ and 5% CO_2 incubator (L11738 Heto Cellhouse 170).

2.3.3 Subculturing of cells

The CLC and EPC cell line were subcultured every 4-5 days when it displayed 70 – 80 % confluence by diluting 1:3 with medium. Adherent cells were removed from the plastic culture flasks using three techniques:

2.3.3.1 Scraping

The spent medium was removed from the culture flask and stored in a sterile 15 ml tube (Sarstedt), 1 ml of the spent medium was then transferred to each new flask. The cells were detached from the culture flask by using a disposable sterile plastic scraper (Greiner Bio-one, 541070) and transferred to the tube with spent medium. The cells were centrifuged at 750 x *g* for 8 min at 19±1℃ (Heraeus Megafuge 1.0R) and the pellet re-suspended in 1 ml of fresh CLC RPMI+ or EPC RPMI+.

2.3.3.2 Trypsinisation

The spent medium was removed from the culture flask and placed in a sterile 15 ml tube (Greiner), 1 ml of this spent medium was then transferred to each new flask. The cell monolayer was washed gently with Hanks Balanced Salt Solution (HBSS) (Sigma, H9262), and removed from the flask. Approximately, 1-2 ml of 0.25x Trypsin-EDTA (Sigma, T4049) added to each flask for 1 min until more than 90% of cell had detached the flask. The collected spent medium was added to neutralize the trypsin. The cells were then centrifuged at 750 x *g* for 5 min at 19±1℃ (Heraeus Megafuge 1.0R) and the supernatant discarded. The cell pellet was re-suspended in 1 ml of either fresh CLC RPMI+ or EPC RPMI+.

2.3.3.3 Magnesium-free PBS

This is similar to the trypsinisation method (described above) except the washing step was carried out with magnesium-free PBS (Life technologies, 10010-015), which was then removed. The cells were then incubated with 3 ml of magnesium-free PBS and the cells

were checked for detachment till 70- 80 % of the cells had detached. Spent medium was added and the cells were processed using the same steps as described for the trypsinisation procedure (see above).

Both trypsinisation and PBS methods were used for cell detachment through the study.

2.3.4 Cryopreservation of cell lines

The cell lines were maintained by occasionally storing the cells in cryovials, which contained 0.5 ml of 2x freezing medium (CLC RPMI+ or EPC RPMI+) with 10 % DMSO (Fisher, BP 231). Cell lines were diluted to 1-2 x 10^6 cells/ml fresh CLC RPMI+ or EPC RPMI+ and 0.5 ml transferred to the cryovial and placed on ice. The cell solution was gradually frozen in a cell freezer (Nalgene, Cryo 1 °C freezing container) at -80 ºC overnight, and then transferred to liquid nitrogen.

2.4 Immunostimulant preparation

2.4.1 MacroGard®

The powder form of MacroGard® was provided by Biorigin (Brazil) and according to the certificate of analysis from the company; MacroGard® batch (batch 250813) consisted of 67.8 % carbohydrates, 5.1 % protein (dry matter), 14.2 % lipids, 6.5 % ash and 4.6 % moisture. MacroGard® was prepared as advised by Biorigin and briefly comprised dilution to required concentration in deionized autoclaved water (sH_2O) and sonication (2 x 30 sec at power 6, Sonics, vibra-cell). To ensure the sterility the stock solution was placed in a water bath (Grant) at 80 ºC for 20 min and left at 19±1 ℃ (approx. 20 ºC) to cool down.

The sonication step in the protocol helped to reduced particle size of MacroGard® and breaks up these long chains of β-glucan. According to the manufacturer's instruction,

MacroGard® size is up to 99 μm and consists of long chained β-glucan, which is insoluble and as result it is unavailable to the cells.

2.4.2 Sulphated MacroGard

The sulphated MacroGard has been provided by Dr Mark Skidmore, Keele University. The same source of powder form of MacroGard® provided by Biorigin (Brazil) was used to make the sulphated form. This sulphated MacroGard was prepared using chlorosulfonic acid (CSA method) as described elsewhere (Yoshida et al., 1995) with some modifications by Dr Mark Skidmore. Briefly, MacroGard® powder 500 mg was dissolved or suspended in the sulphating reagent which was consist of 5 ml dimethyltryptamine (Sigma, M2381) and (10 ml) Pyridine (Sigma-Aldrich, 270970) and (1-5 ml) Chlorosulfonic acid (Sigma-Aldrich, 571024), the mixture was cooled to 19±1℃ by an ice bath for 1 min, neutralized with 20% NaOH solution, and then the mixture was heated for 30 sec in a microwave and replaced in ice, this step repeated for 5 times. One ml of chlorosulfonic acid (Sigma-Aldrich, 571024) was added to the mixture and heated for 30 sec three times. As a result, a brown sediment was produced and to change the pH value, 4 ml of sodium hydroxide (Sigma-Aldrich, S8045) was added to the mixture, which was shaken well to remove the brown particles followed by adding 80 ml sodium acetate (Sigma-Aldrich, S2889) and left overnight after which the upper layer was removed. Some water were added enough to dissolve the sediment sugar and then dialyzed for at least 72h with distilled water.

2.4.3 Zymosan

Zymosan (Sigma, Z4250) was prepared using the same method as described for MacroGard® above and by Vera-Jimenez et al. (2013).

2.4.4 Soluble modified carbohydrates

All of the modified carbohydrates utilised in this study were prepared by dissolving the appropriate weight (dry carbohydrates) or volumes (liquid carbohydrates) in sterile water (Sigma, W3500) under sterile conditions and serial diluted to the required concentration for each experimental preformed.

2.5 Cell proliferation and viability assays

2.5.1 MTT assay

The tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) was utilised to determine cell proliferation in pronephric cell suspensions, and also in CLC and EPC cell lines. The viable cells are detected by the ability of dehydrogenase enzyme to convert a soluble yellow tetrazolium salt MTT into an insoluble dark purple formazan precipitate; this process indicates high level of mitochondrial activity in cells (see Figure 2-1).

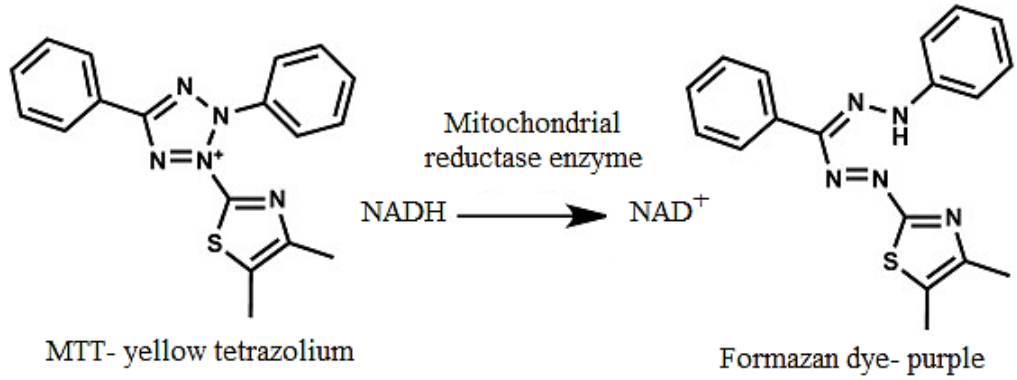


Figure 2-1: The reduction of MTT to formazan. The MTT assay is based on the constant activity of mitochondria in most of living cells, thereby the reflection of mitochondrial activity can be determine by the conversion of tertazolium salt (3-[4,5-dimethylthiazol-2-

yl]-2,5 diphenyl tetrazolium bromide) in MTT into formazan crystal only in viable cells (van Meerloo et al., 2011).

The MTT assay has been utilized to estimate cellular viability in a range of process including drug screening protocols (Twentyman and Luscombe, 1987, Vistica et al., 1991). MTT (Sigma, M2128) was prepared by dissolving 5 mg/ml in PBS (Life technologies, 10010-015) and the conversion were established in a flat bottom 96 well plate (Sarstedt, 83.1835.500) within a volume of 100 µl cell culture medium per well. Following the incubation of cells with immunostimulants, 10 µl of the MTT were add to each well. The plate was then incubated for 4 h at 27 C˚ with 5% CO_2 for MTT conversion to take place. Supernatant were then discarded and cells solubilised with 100 µl of Dimethyl sulfoxide (DMSO) (Fisher, BP 231) and measured spectrophotometrically on a microplate reader (BioTek EL800) at a 540 nm wavelength (Ferrari et al., 1990) (see Figure 2-2).

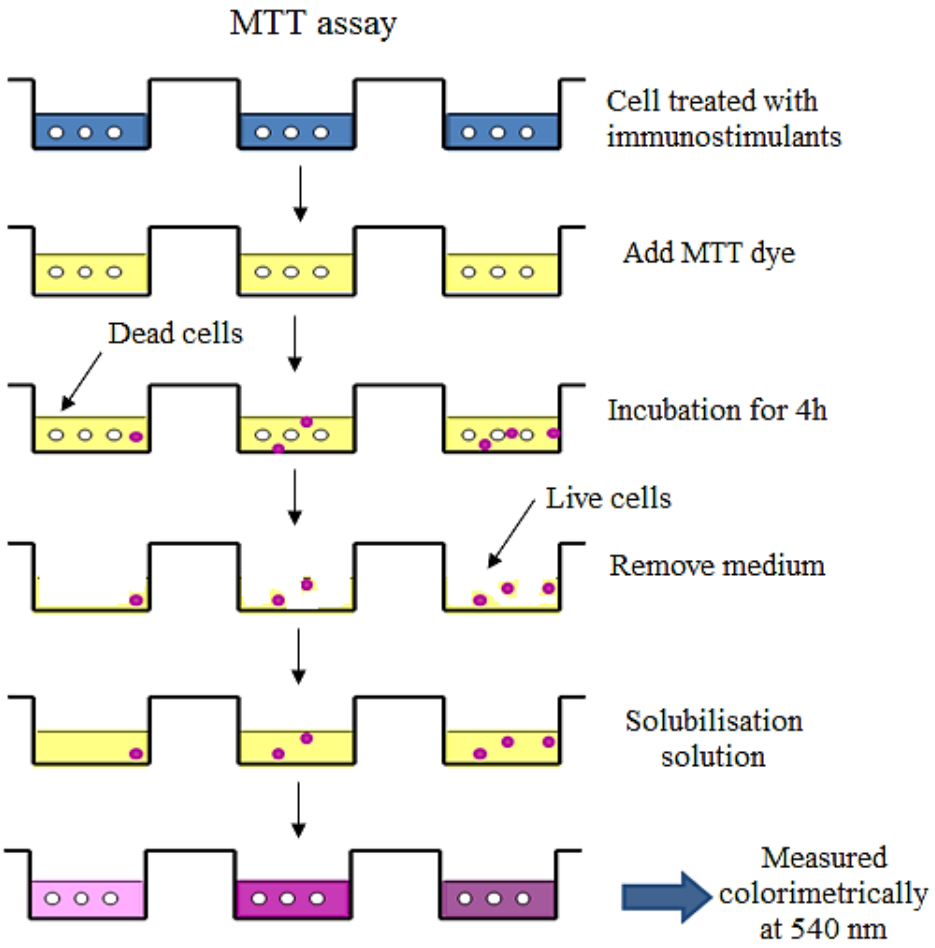


Figure 2-2: MTT assay protocol scheme. MTT produces a yellowish solution that is converted to dark purple, water-insoluble MTT formazan by mitochondrial dehydrogenases of living cells. The puple crystals are solubilized with acidified isopropanol and the intensity is measured colorimetrically at 540 nm.

2.6 Immune assay

2.6.1 Reactive oxygen species: Nitroblue tetrazolium (NBT) assay

Nitroblue tetrazolium (NBT), a yellow, water soluble powder, permeable to cells membrane, is used to detect the production of superoxide anion O_2 (-) in phagocytic cells in response to many immunostimulant by the detection of the reduction of NBT to blue formazan. The NBT assay was performed as described at Vera-Jimenez et al. (2013). Briefly, cells were placed in modified medium either RPMI+ or CLC RPMI+ as described

in Sections 2.2.1 or 2.3.1, except the RPMI medium used was phenol red free (Sigma, R7509). A cell monolayer was formed at the bottom of the flat 96 well plate (Sarstedt, 83.1835.500) by placing 100 µl of cell suspension in each well and incubated at 27 C° in 5% CO_2 for 2-3 h until the phagocytic cells attached to the well. The supernatant was then discarded, the cells washed with phenol red free Hank's balanced salt solution (HBSS) (Sigma, H8264) and 160 µl of modified RPMI+ or CLC RPMI+ containing NBT with concentration 1 mg/ml (Sigma,N6876) was added to each well. The respiratory burst activity was induced by adding 5 µl of different types and concentrations of the immunostimulants and a negative control comprising only 5 µl of sterile water (Sigma, W3500) was added in each trial. After the incubation at 27 C° in 5% CO_2, the supernatant was decant and cells were fixed with 100 µl ice cooled 100% methanol for 3 min and the plate was left to air dry. The membranes of the phagocytic cells were solubilised with 120 µl KOH (2 M) and 140 µl of DMSO added to dissolve blue formazan. The reduction of NBT was measured spectrophotometrically at 620nm using a microplate reader (Multiskan multisoft plate reader, Labsystems, Finland).

2.7 Molecular biology techniques

2.7.1 RNA extraction

Following appropriate *in vivo* treatments, organs were removed from the killed fish in each time period post-injection, placed in RNA later (using the recipe in the Appendix) and stored at -80℃. The RNA extraction was carried out at 19±1℃ using the RNeasy kit (Qiagen) and following the manufacturer's protocols. Briefly, a small amount of ≤ 30 mg

RNA later stabilized tissue was placed in 350 µl of RLT buffer (lysis buffer contain 1% β-mercaptoethanol; Sigma, M6250) in microcentrifuge tube and sonicated on iced water at high power (H, 320W) using a Bioruptor® Sonication System, diagenode for 5-10 min. The tube was centrifuged at 11600 x *g* (Micro-Centaur Centrifuge, SANYO) for 3 min to remove cell debris, the supernatant removed and mixed with 350 µl of 70% ethanol in new microcentrifuge. This mixture was transferred to an RNA spin column and centrifuge for 15 sec at ≥ 8000 x *g*, the column washed with 700 µl of RW1 and re-centrifuged span 15 s at ≥8000 x *g*. Two washing steps with 500 µl of RPE buffer followed, the first washing step was at ≥ 8000 x *g* for 15 sec, while the second step centrifugation was carried out for 2 min to ensure that ethanol was not carried over during RNA elution. At all washing steps the flow-through was discarded. The column was placed in a sterile centrifuge tube after removing the collection tube and the RNA was eluted by adding 30 µl DEPC treated water directly to the spin column membrane and centrifuged at ≥ 8000 x *g* for 1 min. The RNA concentration and purity was checked spectrophotometrically using nanodrop (Thermo Scientific, Nanodrop 1000) and samples were stored at -80℃.

2.7.2 Complementary DNA (cDNA) synthesis

2.7.2.1 DNase treatment of RNA samples prior to reverse transcriptase

A RQ1 RNase-Free DNase kit (Promega, M6101) comprising a DNase I (endonuclease), was used prior to RT-PCR to degrade any contamination with double-stranded and single-stranded DNA. The reaction was carried out as described in manufacture's protocol with slight modification, 1 µg of RNA samples were used within 7.75 µl volume (diluted with

DEPC treated water) and added to a mixture of 1x RQ1 RNase-free DNase buffer (1 µl), 1ng RQ1 RNase-free DNase (1 µl) (both supplied by Promega kit) and 10U of RNase inhibitor (0.25 µl) (Invitrogen, 10777-019). The reaction was incubated at 37℃ for 30 min, then 1 µl of stop solution (supplied by Promega kit) was added and the DNase was inactivated by incubating the samples at 65˚C for 10 min. Both incubations were carried out using a Thermocycler (Applied Biosystems, GeneAmp PCR System 9700).

2.7.2.2 Reverse transcription of RNA

Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen, 28025-013), an RNA-directed DNA polymerase, was used to synthesize a complementary DNA strand initiating from a single-stranded RNA in the presence of a primer. The manufacturer's protocol was followed to synthesise cDNA from RNA treated DNase samples. Briefly, a mixture of 500 ng of RNA sample, 1 µl of 50 µM random hexamers, 1 µl 10 mM dNTPs and 4.5 µl of DEPC water were heated at 65℃ for 5 min and quick chilled on ice. After a brief centrifugation, 4 µl 5X First-Strand Buffer, 2 µl 0.1 M DTT (both supplied with the (M-MLV RT) kit) and 1 µl RNaseOUT™ Recombinant Ribonuclease Inhibitor (40 units/µl) (Invitrogen, 10777-019) were added and mixed gently by pipetting up and down. The mixture was heated at 37℃ for 2 min and 1 µl (200 units) of M-MLV RT enzyme were add and mix thoroughly by pipetting. The reactions were carried out after the samples were incubated at 25℃ for 10 min followed by 50 min at 37℃, then inactivated by incubation at 70℃ for 15 min (Thermocycler: Applied Biosystems, GeneAmp PCR System 9700).

2.7.3 Gel electrophoresis

Gel electrophoresis is a laboratory technique for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on size and electrical charge. In gel electrophoresis, the molecules to be separated are moved by an electrical field through a gel that contains small pores. The migration speed of these molecules through the pores in the gel depend on the macromolecules lengths. Also the concentration of agarose gel varied between 0.5- 2 % dependent upon the molecule size of the products to be analysed; lower density gel are required for the analysis of large and heavy products, while high density gels are useful for the analysis of small and light molecules. This means that a small DNA molecule will travel a greater distance through the gel than will a larger DNA molecule. The appropriate amount of agarose gel at concentration 1% (Hi pure low EEO Bio Gene, 300-300) was added to 50 ml of TAE 1X buffer (see Appendix) and heated for 1 min in the microwave to dissolve. The dissolved agarose was allowed to cool, before 5 µl of ethidium bromide (Sigma, E1385) was added. The mixture was poured into gel chamber with comb and left to set.

The chamber with the gel was located into a gel tank (Mini Sub cell GT, BioRad) filled with TAE 1x buffer and the comb removed. The product was mixed with 6x orange blue loading buffer (5 µl PCR product to 1 µl loading buffer, Promega G190A) and loaded into the well in the gel. A DNA ladder 100 bp DNA ladder (Promega, G210A) was also mixed with loading buffer and placed into one of the wells to give an indication of the product size. The electrophoresis was run at 100 V (BioRad, Power Pack 300) and analysed with a UV camera (Syngene, Gene genius) and corresponding software (Syngene, Gene Snap 7.12). Finally, after the DNA, RNA, or protein molecules have been separated using gel electrophoresis, bands representing molecules of different sizes can be detected.

2.7.4 QPCR primer design and verification

A primer is a strand of nucleic acid that is used as a starting point to amplify target region from a DNA template in process of DNA synthesis. This study targeted a range of immune related proteins that have been identified in public database i.e Gene Bank for *Cyprinus carpio* and their primer sequences were obtained from previous published papers (see Table 2-1). To avoid the similarity of the studied primers with other genes, the forward and reverse primers were inserted into NCBI BLAST program and checked in a *Cyprinus carpio* and nucleotide collection. Primers supplied by Eurofins MWG Operon (United Kingdom) were aliquoted with DEPC water into final concentration of 10 pmol/µl and incorporated in a quantitative real time PCR as described in Section 2.7.5. The ribosomal gene 40S was confirmed to be the most stable reference gene in common carp, i.e. it displayed least up- or down-regulation under the experimental treatments (Miest et al., 2012). The housekeeping gene 40S was used to normalise the expression of target genes and the x-fold gene expression was calculated in relation to the control group of each experimental duration.

Table 2-1: List of used qPCR primers

Function	Gene name	Primers sequences	Gene bank accession numbers	References
House keeping	40S	FW: 5′ CCGTGGGTGACATCGTTACA 3′ RV: 5′ TCAGGACATTGAACCTCACTGTCT 3′	AB012087	(Huttenhuis et al., 2006)
Nitric oxide production	iNOS	FW: 5′ AACAGGTCTGAAAGGGAATCCA 3′ RV: 5′ CATTATCTCTCATGTCCAGAGTCTCTTCT 3′	AJ242906	(Huttenhuis et al., 2006)
Pro-inflammatory cytokines	IL1β	FW: 5′ AAGGAGGCCAGTGGCTCTGT 3′ RV: 5′ CCTGAAGAAGAGGAGGCTGTCA 3′	AJ245635	(Falco et al., 2012)
	IL6 Family	FW: 5′ GCAGCGCATCTTGAGTGTTTAC 3′ RV: 5′ CTGCTGCTCCATCACTGTCTTC 3′	AY102632	(Falco et al., 2012)
Anti-inflammatory cytokines	IL-10	FW: 5′ GCTGTCACGTCATGAACGAGAT 3′ RV: 5′ CCCGCTTGAGATCCTGAAATAT 3′	AB110780	(Huttenhuis et al., 2006) (Falco et al., 2012)

Continues of table

Function	Gene name	Primers sequences	Gene bank accession numbers	References
Pro-inflammatory chemokines	qCXCα	FW: 5′ CTGGGATTCCTGACCATTGGT 3′	AJ421443	(Forlenza et al., 2009)
		RV: 5′ GTTGGCTCTCTGTTTCAATGCA 3′		
Anti-viral cytokines	IFN-γ2b	FW: 5′ GCACATCCTGTCTTCCTACGGTTC 3′	AM168523	(Wang et al., 2011)
		RV: 5′ GCTTCATCCTGACTATCCTTCTCC 3′		
Viral resistance protein	Carp Mx	FW: 5′ CTGAGAATATTGGAGATCAGATCAAGAG 3′	FJ263281	(Kitao et al., 2009)
		RV: 5′ GTCAACATTACATGGCACCACAA 3′		
Antibacterial activity	Lysozyme-C	FW: 5′ GTGTCTGATGTGGCTGTGCT 3′	AB027305	(Yuan et al., 2008)
		RV: 5′ TTCCCCAGGTATCCCATGAT 3′		
Complement system activator	C3	FW: 5′ CCCTGGACAGCATTATCACTC 3′	AB016210-5	(Huttenhuis et al., 2006)
		RV: 5′ GATGGTCGCCTGTGTGGT 3′		

2.7.5 Polymerase Chain Reaction (PCR)

Principle

PCR is a sensitive technique which enables rapid amplifications of specific short segment of longer DNA molecule. The PCR helps to detect and identify a sequenced target gene using visual techniques depending on size and electrical charge and analyses their relative expression and is thus called qualitative real-time PCR. Furthermore, the quantitative real-time PCR (qPCR) is used to quantify the expressed gene that is present in the sample through the detection and quantification of the fluorescent dyes that non-specifically intercalate with double-stranded DNA and sequence-specific DNA (Garibyan and Avashia, 2013). The amplification reactions include basic components: DNA or cDNA template, forward and reveres primers, DNA polymerase, nucleotides, reaction buffer and magnesium. The reactions are carried out in thermal cycler, which has a thermal block allowing temperature to be raised and lowed precisely over time, thus defining the PCR cycles of amplification. Each cycle of PCR includes three steps: denaturation where the double-stranded DNA has to be heated above the melting point allows the strands to separate; annealing in which the temperature is then lowered to allow the specific primers to bind to the target DNA segments; extension where the temperature is raised again allowing the DNA polymerase to extend the primers by adding nucleotides to the developing DNA strand (Figure 2-4). With each repetition of these three steps, the amount of target sequence will be doubled.

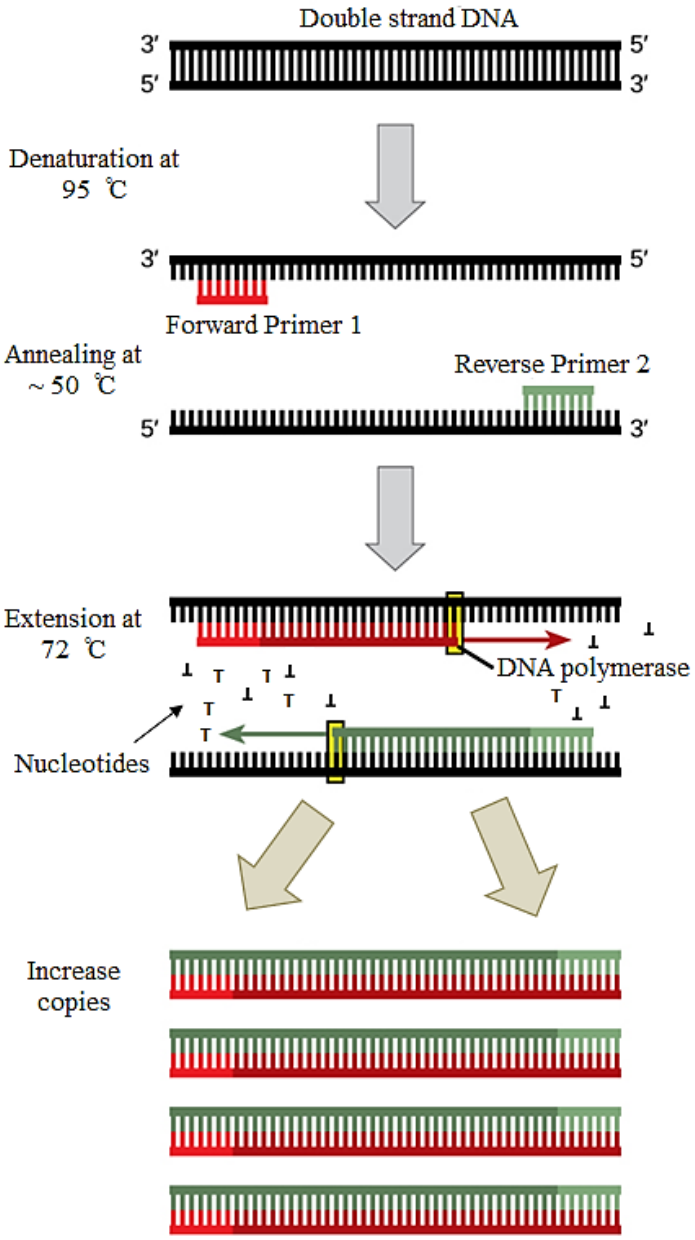


Figure 2-4: The principle of DNA amplification by the polymerase chain reaction (PCR). The diagram shows the three general steps to the process that are repeated for a number of cycles to exponentially increase the number of copies of a specific target region.

Method

The PCR reaction is detected when the fluorescent signal is accumulated, the number of required cycles for the fluorescent signal to exceed background level is called C_t value or "cycles to threshold". This value is inversely proportional to the amount of target sequence in the sample and that means high C_t values represent a low amount of target sequence while a low C_t equivalents to high amount of target sequence.

To quantify the real-time quantitative PCR result of the target sequence, the $\Delta\Delta C_t$ method of relative quantification with SYBR Green I detection (see Figure 2-5) was utilised to determine the expression level of target genes through the fold changes compared to the calibrator (control) samples. SYBR Green I dye, a highly sensitive fluorescent stain, has an important property of being a sequence-independent in DNA binding and an undetectable fluorescent in its free form. It is efficient, when it is working with a large panel of gene due to its ability to bind to all dsDNA molecules. However SYBR Green I detection lacks specificity due to the possible amplification of nonspecific PCR products (Ferreira et al., 2006).

Standard curve preparation

In each experiment where the gene expression was determined, a standard curve was calculated for each studied primer. For this purpose, a cDNA template was prepared by taking an equal volume from all the cDNA samples in one microcentrifuge tube with the last one being serially diluted in increments of 3 fold, forming range of cDNA concentrations between 25 and 0.03 ng/ml. The amplification of each dilution was performed in duplicate. The average Ct values from each dilution are then plotted versus the log cDNA dilution concentration to generate a standard curve. The standard curve was determined for each of the target genes and housekeeping gene in each studied tissue. The efficiency was calculated from the slope of the linear regression of the standard curve and

the efficiency was equal to $E = 10^{(-1/slope)}$ (Pfaffl, 2001). The real-time PCR efficiency vary with high linearity ($r^2 > 0.98$) and the acceptable efficiency value for PCR reaction is between 1.9- 2.1 which corresponds to 90-110 % respectively (Pfaffl, 2004).

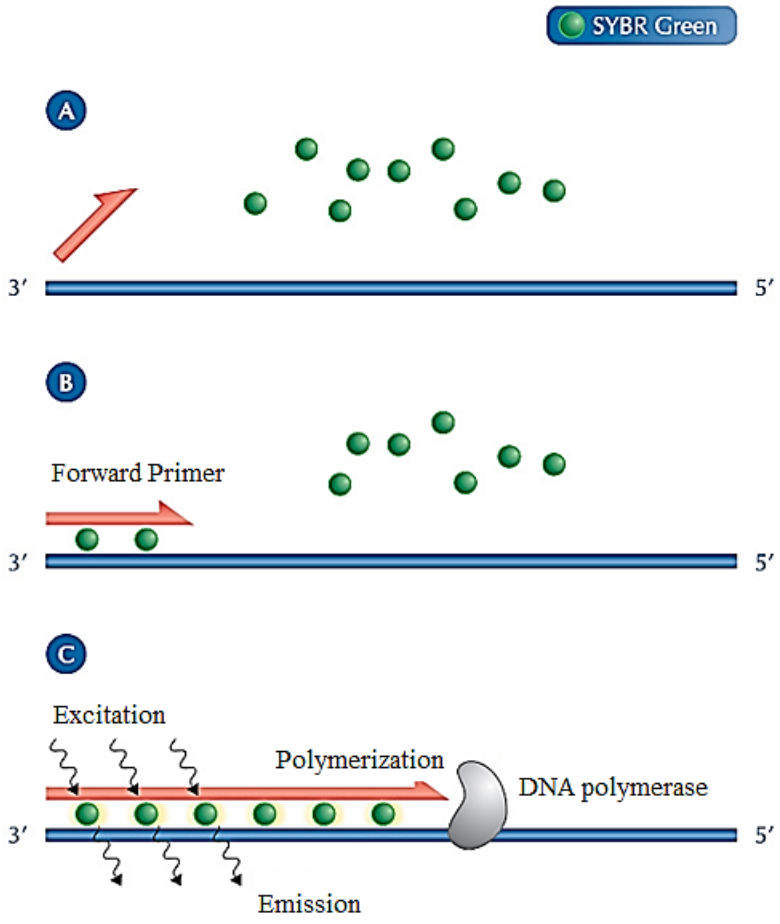


Figure 2-5: SYBR Green Chemistry. A) DNA is denatured and SYBR Green molecules are free in the reaction mix. B) Primers anneal and SYBR Green molecules bind to the dsDNA. C) DNA polymerase elongates the template and more SYBR Green molecules bind to the product formed resulting in exponential increase in the fluorescence level (http://www.thermoscientificbio.com/applications/pcr-and-qpcr/introduction-to-qpcr/).

Quantitative real time PCR

The real-time PCR reaction was performed using the SensiFAST™ SYBR® Hi-ROX Kit (Bioline, BIO-92020). A range of immune related gene primers in carp were used as listed in Table 2-1 and the ribosomal 40S acted as reference gene for the analysis. The PCR reaction carried out in 96 well PCR plate (Applied Biosystems, MicroAmp®), briefly, 2 µl of cDNA were added to 10 µl SensiFAST, 0.8 µl of 10 µM forward and reverse primers each (final concentration at the reaction mix is equal to 400 nM) and completed to 20 µl with DEPC treated water (Invitrogen). The PCR plate was sealed with film cover and centrifuged 660 x *g* for 4 min (Boeco, U-32R). The reaction was carried out in an ABI 7000 real-time cycler (Applied Biosystems) with two step cycling: first step was 1 cycle at 95°C for 2 min to activate the polymerase, the second step was 40 cycles at 95°C for 5 sec were the denaturation took a place and then 30 sec at 62°C were the annealing/extension occurred. Melting curve of PCR products were determined after each run between 60 and 95°C.

Data analysis

The $\Delta\Delta C_t$ method was carried out as explained in Livak and Schmittgen (2001), arithmetic formula were used to calculate relative expression level in comparison to control group (calibrator). The C_t values of target gene were normalised against housekeeping gene to reduce the variation between the amounts of cDNA samples. The fold difference is equal to the difference of corrected C_t values between the control and treatment, therefore the relative gene expression is equal to $2^{-\Delta\Delta Ct}$, the $\Delta\Delta C_t$ was calculated as described below:

$$\Delta\Delta C_t = \Delta C_{t\ samples} - \Delta C_{t\ control}$$

$$\Delta C_{t\ samples} = C_{t\ target\ gene\ for\ samples} - C_{t\ housekeeping\ gene\ for\ samples}$$

$$\Delta C_{t\ control} = C_{t\ target\ gene\ for\ control} - C_{t\ housekeeping\ gene\ for\ control}$$

The relative expression method required amplification efficiency to validate the $\Delta\Delta C_t$ calculation (Livak and Schmittgen, 2001). The validity of ΔΔCt method is dependent on the efficiencies of all amplifications, and is supposed to be approximately equal to 2 and equal between reactions (Livak and Schmittgen, 2001). The results from both methods ΔΔCt and Pfaff's mathematical ratio was established to be comparable, and relies on the primer efficiency to determine the standards (Pfaffl, 2001).

2.8 Statistics

The statistical analysis was carried out using GraphPad Prism 5 and SPSS 21, all data was presented as mean ± standard error. The statistics specific for each experiment will be described in the corresponding chapters and the significant was defined as $p \leq 0.05$. The data were tested for normality and equal distribution of variance. The unequally distributed data were normalised with log10 transformation or in case of percentage data, arc-sin transformation was utilised.

Chapter 3. Establishment of an *in vitro* system to determine the effects of different sources of beta glucan on cell viability and respiratory burst activity.

3.1 Introduction

Immunostimulants mainly induce several cellular and immunological activities that enhance the protection against infections in fish. They have been noted for their ability to stimulate phagocytic cells and increase bactericidal activities, mediated by the induction of phagocytosis, leucocyte migration and the production of cytokines (e.g. IL-1, TNFα), nitric oxide (NO) and reactive oxygen species. They also promote the activity of natural killer cells, complement pathways, lysozyme and antibody responses in fish (Sakai, 1999).

These immunological activities have been monitored using a range of assays and techniques *in vivo* and *in vitro*. For example:

- Cell viability and cytotoxicity monitored by either Trypan blue; ^{3}H thymidine (determine DNA polymerase activity); flow cytometry; formazan-based assays MTT or MTS, WST and XTT (Wittstock et al., 2001, Ferrari et al., 1990, Gerlier and Thomasset, 1986, Maghni et al., 1999).
- Phagocytic activity recorded by either counting stained cells that ingested yeast or pathogen-associated molecular patterns PAMPs on bacteria under the microscope; flow cytometry (antigen specific antibody) RT-luminol assay and NBT assay (colorimetric or microscopy) (Vera-Jimenez et al., 2013, Hyung Sim et al., 2006).
- Lysozyme activity assessed using a turbidimetric assay, lysoplate assay and staining procedure (Stolen et al., 1990).

- Complement levels measured through the classical complement pathway titre (CH50) and alternative complement pathway assay (ACH50) in serum of fish (Zhang et al., 2003, Stolen et al., 1990).

In addition, molecular biological techniques have been widely utilised to monitor immunological activities by measuring the gene expression associated with a range of immune proteins and immunological cytokines.

One of the early defence mechanisms of the innate immune system is the respiratory burst activity associated with phagocytosis. Respiratory burst involve in pathogen recognition through PAMPs (pathogen associated molecular pattern) that are preserved in microbial structure, and also the recognition of damaged associated molecular pattern (DAMPs) that are associated with damaged tissues and cell death (Volman et al., 2008). There is also evidence that immunostimulants enhance the mitogen activity through concanavalin A or lipopolysaccharides production, which induces proliferation in lymphocyte cells and increased antibody responses (Sakai, 1999, Meena et al., 2012). Therefore in this study, the respiratory burst activity using a NBT assay, and cell viability assay based on MTT were used to examine the ability of beta glucans from different sources to stimulate cells.

Phagocytosis in granulocytes and monocytes (macrophages) is associated with a burst of oxygen consumption and as a result, production of superoxide anions O_2 (-) and hydrogen peroxidase H_2O_2 (see Figure 2-3). Both are reactive oxygen species products which play an important role in defence mechanism by killing pathogens and induce several antioxidant enzymes.

The NBT assay can be used to detect intracellular superoxide anion O_2 (-) produced by phagocytic cells and has been used to detect superoxide anion O_2 (-) produced in monocytes and macrophages that are not detectable by the conventional microscopic NBT assay (Hyung Sim et al., 2006).

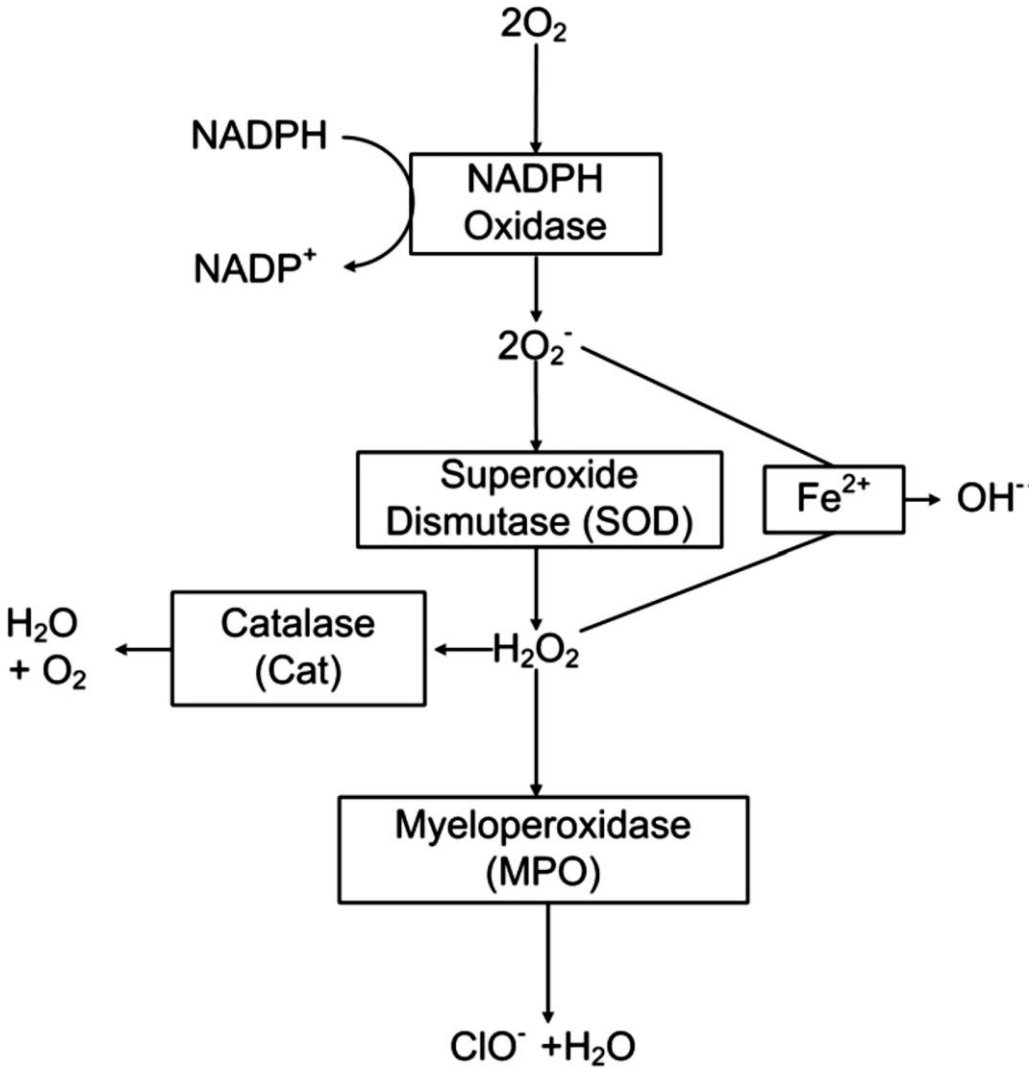


Figure 3-1: A representation diagram of the respiratory burst main products. The membrane-associated enzyme nicotinamide adenine dinucleotide phosphate (NADPH) catalyses the reduction of molecular oxygen (O_2) to superoxide anion (O_2^-), using NADPH as the electron donor. Further reduction of oxygen produces hydrogen peroxide (H_2O_2), which occurs either as a spontaneous dismutation, or as a catalysed reaction by a family of enzymes called Superoxide dismutase (SOD). Additional reactions of O_2^- and H_2O_2 may lead to the formation of hydroxyl radicals (OH^-), especially in the presence of iron through the Fenton or HabereWeiss reactions. The interaction of H_2O_2 with the enzyme myeloperoxidase (MPO) can produce hypochlorous acid and other toxic metabolites, or H_2O_2 is dismutated to water and molecular oxygen by the enzyme catalase (Vera-Jimenez et al., 2013).

The effects of beta glucan are known to be dose dependent and related to administration time. For example, in common carp the administration of beta glucan (*Saccharomyces cervisiae*) by injection induced an increase in phagocytic activity, total blood leucocyte cells count and bacterial killing activity at seven days post challenge with *Aeromonas hydrophilia*. The effects were increased depending on beta glucan doses i.e. 100, 500 and 1000 µg/ fish (Selvaraj et al., 2005). In another trial the effect of beta glucan was investigated on European sea bass (*Dicentrarchus labrax*) at different concentrations i.e. 250, 500 and 1000 ppm and feeding period 4, 7, 10, 14, 21 and 25 days. The result showed a significant increase of respiratory burst activity at 250 ppm beta glucan feed after 21 days of feeding compared with fish fed no immunostimulant (Bonaldo et al., 2009). An investigation determined the effect of different doses of beta glucan studied in *Anabas testudineus* spawns against a fungal infection *Saprolegnia parasitica*. Beta glucan exposure at all doses for 3 h enhanced lysozyme activity, bactericidal activity and NBT activity and reduced mortality in fish exposed to 10 mg/L and 15 mg/L (Das et al., 2013). The dose dependency of beta glucan was also observed on common carp where it induced a significant increase in apopotosis in pronephros cells at concentrations of 500 µg/ml and higher after 6 h incubation (Miest and Hoole, 2015).

Therefore, the aim of this chapter was to establish an *in vitro* system for screening and cytotoxicity testing of different beta glucans. This *in vitro* system will be applied for screening the effect of different formulated carbohydrates. This chapter evaluated different aspects which include: 1)different beta glucan sources that have differences in solubility i.e. MacroGard® is insoluble and sulphated MacroGard is soluble; 2) different beta glucans concentrations tested at different exposure time; 3) the use of different cell types i.e. primary cells from pronephros and cell lines, because primary cells derived from different fish however is the most represented to the *in vivo* condition but it can behave differently

in culture conditions depending on the genetics; and 4) determining MTT and MTS assays sensitivity, because the MTS is a one-step kit that does not need medium removal step as in the MTT assay.

3.2 Materials and Methods

3.2.1 MTT assay optimization

The MTT assay is a colorimetric assay, which determines the activity of cellular enzymes that are involved in the conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide) to formazan crystal by cellular metabolic activity of viable cells. Cell density and background colour are correlated directly with the absorbance.

Initial experiments were carried out to determine pronephric cell density and MTT solvent solution type. Two fish were sacrificed as described in Section 2.1, pronephric cells suspension were prepared as mentioned briefly at Section 2.2.1 and the density and viability of harvested cells were determined by using the trypan blue exclusion test (see Section 2.2.2). Pronephric cells suspension were serially diluted 1:2 dilution with RPMI+ medium, started with the highest cell density 5×10^6 cells/ml.

The MTT assay was performed in 96 well plate (Sarstedt, 83.1835.500), 100 µl of the different density of pronephric cells suspensions between (5- 0.0195) $\times10^6$ cells/ml were plated into each well and incubated overnight at 27℃ and 5% CO_2 incubator (L11738 Heto Cellhouse 170), followed by adding 10 µl of MTT solution (5 mg/ml) to each well and incubation for 4 h at 27℃ and 5% CO_2 (L11738 Heto Cellhouse 170). After incubation the microplate was centrifuged at 200 x *g* for 5 min, supernatant were decanted carefully and formazan crystal solubilised within 100 µl of either 10% SDS solution in 0.01N HCl or DMSO (Fisher, BP 231) was added and incubated overnight at 19±1℃ (approx. 20℃) in humid chamber or 100 µl of DMSO (Fisher, BP 231) and incubated for ten min at 27℃ at 5% CO_2 incubator (L11738 Heto Cellhouse 170). Samples were mixed and measured spectrophotometrically on a microplate reader (Multiskan multisoft plate reader, Labsystems, Finland) at 540 nm wavelength.

3.2.2 Viability in pronephric cells

3.2.2.1 MTT assay

To determine the dose effect of MacroGard® on cell viability, six fishes were killed as described in Section 2.1 and pronephric cells suspensions were isolated (see Section 2.2.1) and density and viability checked (see Section 2.2.2). One hundred µl of pronephric cells (5×10^5 cells/well) were seeded into flat bottom 96 well plate. MacroGard® solution was prepared as explained in Section 2.4.1 and cells were exposed to 5 µl of MacroGard® at concentrations 2.5, 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150 µg/ml. In addition a negative control i.e. 5 µl sterile water treated cells was utilised and both treated and control cells were then incubated for 24h at 27℃ and 5% CO_2 (L11738 Heto Cellhouse 170). After the incubation period, the MTT assay carried out as described in Section 2.5.1 and data was obtained after samples were read at 540 nm wavelength (Multiskan multisoft plate reader, Labsystems, Finland). The data were analysed for the dose effect of MacroGard® on cell viability with one- way ANOVA and for the individual dose effect with a subsequent Tukey's post- hoc test.

The effect of the solubility of MacroGard® on cell proliferation was then determined by sulphating the MacroGard® as described in Chapter 2.4.2. Sulphation increased the solubility of the β glucan which together with the original MacroGard® form were tested on pronephric cells. Four fishes were used to isolate pronephric tissue as described in Section 2.1., and pronephric cell density and viability was determined as described in Sections 2.2.1 and 2.2.2. Both of MacroGard® and sulphated MacroGard were prepared as detailed in Section 2.4.1 and 2.4.2 respectively. Each immunostimulant was utilised at concentrations of 2.5, 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150 µg/ml and a negative control (5 µl sterile water treated cells) was also included. Five µl of either MacroGard® or sulphated MacroGard were added to each wells containing pronephric cells at 5×10^5

cells/well then incubated for 24h at 27℃ with 5% CO_2. After the incubation, the MTT assay carried out as explained in Section 2.5.1 and the reduction of MTT assay in each well was then measured at 540 nm using a microplate reader (Multiskan multisoft plate reader, Labsystems, Finland).

The data of the dose effect of both immunostimulants were analysed with a one- way ANOVA and the Tukey's post- hoc test was used to determine the individual dose effect.

3.2.3 Viability in cell lines

3.2.3.1 MTT assay

Whilst the use of pronephric cells is appropriate as this organ represents one of the major immune organs in fish, the cell population obtained is heterogeneous and will vary in individual cell type per fish. To reduce this variability the sulphated MacroGard was tested on cell lines. Carp leucocyte cell line (CLC) are adherent cells, originated from peripheral blood of carp female (Faisal and Ahne, 1990), widely used in carp research i.e. apoptotic studies (Vidal et al., 2009) and macrophage function (Weyts et al., 1997) . CLC line can be stimulated with lipopolysaccharide (LPS), interferon gamma and tumour necrosis factor (TNF) and possess phagocytic activity, it is therefore similar in characteristics to monocytes / macrophages (Weyts et al., 1997). *Epithelioma papulosum cyprini* (EPC) are adherent cells, reported to be from Carp epidermal herpes virus-induced hyperplastic lesions (Fijan et al., 1983), but recently disputed that EPC cell line to be derived from Fathead Minnow (*Pimephales promelas*) (Winton et al., 2010). Both cell lines were maintained and cultured as described in Section 2.3 and prior to use the cell culture conditions and facilities were confirmed to be free from Mycoplasma contamination as described in Section 2.3.2 and Figure 3-2 illustrates the absence of the Mycoplasma contamination.

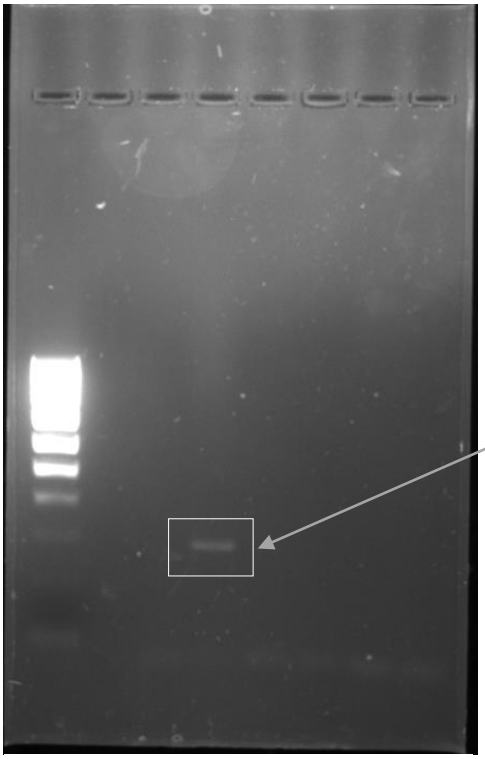


Figure 3-2: Mycoplasma contamination test in CLC line. The cDNA from four CLC line culture flasks were examined for Mycoplasma using Mycoplasma Test Kit following manufacturer's instructions. M = DNA ladder 100 lanes (Bioline, 33053), - C = negative control (DNA free), + C = positive control (Kit provided), S 1- 4 = samples (from 4 culture flasks).

The MTT assay was used to examine the effect of MacroGard® on CLC line viability. Briefly, cells were added at a density of 1×10^4 cells in 100 µl of CLC RPMI+ and treated with range of MacroGard® concentration i.e. (2.5, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 125, 150 µg/ml) or with a negative control (sterile water cells) in a volume of 5 µl per well. Following an incubation period of either 24 or 48h at 27℃ with 5% CO_2, the MTT assay was utilised as described in Section 2.5.1 and plates were read at 540nm wavelength. Data were presented as a percentage of cell viability and the statistical analysis was carried out using a two way ANOVA with significance defined as $p \leq 0.05$.

Furthermore, a similar experiment was carried out to determine the concentrations and incubation time effect of sulphated MacroGard on both cell lines (CLC and EPC) at different doses and exposure times. The MTT assay performed in 96 well plate, cells were seeded at concentration 1×10^4 cells in 100 µl culture medium either CLC RPMI+ or EPC RPMI+. Sulphated MacroGard was prepared as mentioned in Section 2.4.2 and cells exposed to concentrations of 1, 1.5, 2.5, 5, 10, 15, 20, 25, 150 µg/ml in a volume of 5 µl per well. In addition, a control comprising sterile water was also included. The plate samples were kept at 27℃ with 5% CO_2 for either 24 or 48 h. The cell viability test (MTT) was carried out after the incubation period as detailed in Section 2.5.1. Data were analysed with two- way ANOVA and the Tukey's post-hoc test was used to determine the individual dose effect. Cell viability was expressed as percentage of absorbance increases at cells treated with sulphated MacroGard in comparable to control.

3.2.3.2 MTS assay

MTS assay is a quantitative colorimetric method for the determination of the number of viable cells in proliferation or chemosensitivity assays. The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, G3580) used contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] regent and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. The MTS tetrazolium compound (Owen's reagent) is bioreduced by cells into a coloured formazan product that is soluble in tissue culture medium. This conversion is accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells (Berridge and Tan, 1993).

This experiment was performed on CLC and EPC cell lines at 1×10^4 cells per well in a flat bottom 96 well plate (Sarstedt, 83.1835.500) using 100 µl/well. The cells were exposed to sulphated MacroGard (Biorigin) prepared as described in Section 2.4.2 at a range of concentrations (1, 1.5, 2.5, 5, 10, 15, 20, 150 µg/ml) together with a negative control (5 µl of sterile water treated cells). Incubation was carried out at 27 C˚ in 5% CO_2 for 24 and 48 h. Following the incubation period, 20 µl of the One Solution (Promega, G3580) was added to each well, and then the plates were incubated for 4 h at 27 C˚ in 5% CO_2, then read at 490 nm absorbent (Multiskan multisoft plate reader, Labsystems, Finland) (Huang et al., 2004). Data was presented as a percentage of cell viability, was analysed using a two way ANOVA and the differences between the treatments was determined with Tukey's post- hoc test.

3.2.4 Reactive oxygen production

3.2.4.1 Pronephric cells

The modified NBT assay can be used as a diagnostic and tracing technique to determine the effectiveness of immunostimulants (Anderson, 2004). As described in Section 2.6.1 the NBT assay is used to detect the production of superoxide anion O_2 (-) in phagocytic cells and therefore, was used to detect the induction of respiratory burst activity by MacroGard® and sulphated MacroGard in pronephric cells. Both MacroGard® and sulphated MacroGard were prepared as explained in Section 2.4.1 and 2.4.2 respectively, and diluted in sterile water at concentration 2.5, 5, 10, 15, 25, 50, 100, 150 µg/ml. The pronephric cells suspension was prepared from four fishes (see Section 2.1) and the viability checked as mentioned in Section 2.2.2. A 96 well plate was used for the assay and 5×10^5 cells were added to each well in 100µl RPMI+ medium and processed as described in Section 2.6.1. Cells were treated with 5 µl of either MacroGard® or sulphated MacroGard at the concentrations indicated above, and a negative control (5 µl sterile water) was included in each plate. Following a 24h incubation period at 27℃ and 5% CO_2 incubator (L11738 Heto Cellhouse 170) the NBT assay was completed as explained in Section 2.6.1 and then the plates were read at 620 nm absorbent (Multiskan multisoft plate reader, Labsystems, Finland).

The result was analysed using a two- way ANOVA followed by Tukey's post-hoc test. Tested data were compared with that of the corresponding negative controls. The level of significance for all analysis was chosen as $p \leq 0.05$.

3.2.4.2 CLC Cell line

To investigate the effect of different immunostimulants on a homogenous cell population the CLC cell line was utilised. The NBT assay was utilised to determine reactive oxygen production, and the dose and incubation time effects of MacroGard®, sulphated MacroGard and zymosan (Sigma, Z4250) at three incubation periods 1, 6 and 24h. MacroGard®, sulphated MacroGard and zymosan were prepared as described as in Sections 2.4.1, 2.4.2 and 2.4.3 respectively, and utilised at concentrations 10, 25, 50 µg/ml prepared in sterile water. One hundred µl of CLC RPMI+ was added to the wells of a 96 well plate at a cell density of 1×10^4 cells, and after the cells were allowed to attach to the well for 2 h, the supernatant was discarded and cells washed with phenol red free Hank's balanced salt solution (HBSS) (Sigma, H8264) and 160 µl of CLC RPMI+ containing NBT (Sigma, N6876) (1 mg/ml) were add to each well. Cells were exposed with 5 µl of either MacroGard® or sulphated MacroGard or zymosan (Sigma, Z4250) at concentrations indicated above and a negative control (5 µl sterile water) was presented in each plate. The plates were incubated for either 1, 6 or 24h with the NBT at 27℃ with 5% CO_2. The NBT assay was completed as explained in Section 2.6.1 and then the plates were read at 620 nm (Multiskan multisoft plate reader, Labsystems, Finland). The respiratory burst activity of stimulated CLC line was analysed using a two-way ANOVA and the dose effects of MacroGard®, sulphated MacroGard and zymosan concentrations in comparison to the negative controls were analysed using Tukey's post-hoc test. The level of significance for all analysis was chosen as $p \leq 0.05$.

3.3 Results

3.3.1 MTT assay optimization

It is essential that, prior to analysing the effects of the immunostimulants on a cell population, the optimal conditions of MTT are determined for a particular cell density used. Figure 3-3 illustrates the relationship between cell number and absorbance. It can be seen that pronephric cells at 0.1563×10^5 cells/ml gave an optical density equal to the negative control (no cells to eliminate background colour) at all dissolvent types. Thus using a low cell number may give a false result. Furthermore, prolonged incubation with DMSO for 24 h gave the highest background optical density and thus reduced the effectiveness of the assay. Therefore, when a correlation analysis was carried out to evaluate the effectiveness of the solvent, a strong correlation ($p < 0.0001$) was established between the MTT assays solvent used, SDS exposed for 24h and DMSO exposed for 10 min (see Figure 3-4). This result shows the ability of DMSO to dissolve the formazan crystal in less exposure time (in 10 min) in comparison to SDS.

It was shown that when a pronephric cell suspension was used at a density of 5×10^5 cells/100µl, an optical density of 0.5715 was recorded after 24 h incubation. This value is less than the plate reader linear measurement range which can measure up to 2.5 absorbance units.

Hence the standard experimental protocol that was established involved the use of 5×10^5 pronephric cells in 100µl, DMSO as a formazan dissolvent and reading the plate 10 min after the addition of the solvent.

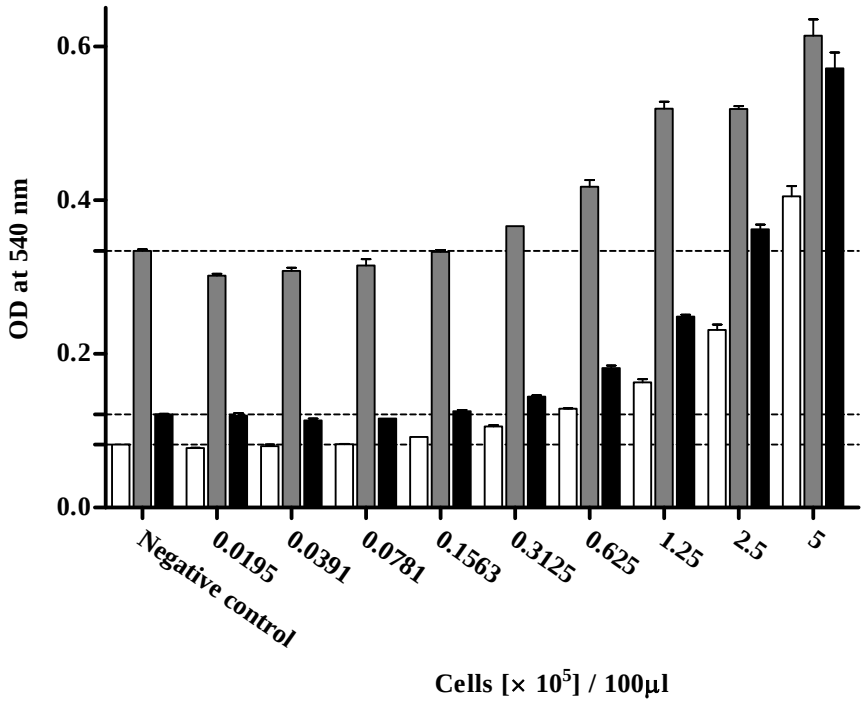


Figure 3-3: Standardisation of the MTT assay for measuring cell viability. Carp pronephric cells were used at concentration of (5- 0.0195) $\times 10^5$ cells/well and different solvent at different exposure time were used in the development of a standardised MTT assay. Bars □= 10% SDS solvent exposed for 24h, ■= DMSO solvent exposed for 24h, ■ = DMSO solvent exposed for 10 min and negative control= culture medium. Mean ± SEM, n= 2 wells replicates.

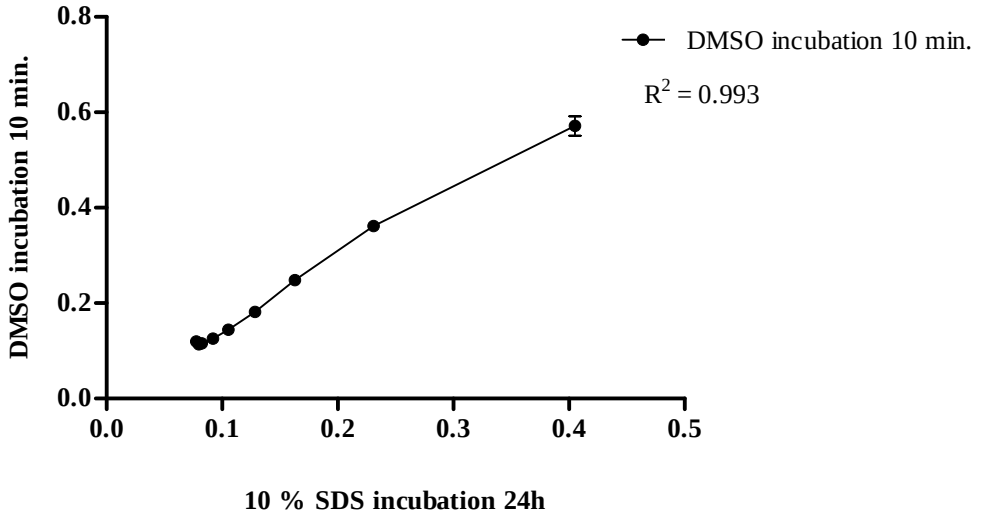


Figure 3-4: The correlation between SDS and DMSO solvent used in the MTT assay. Strong relation observed between the optical densities of viable cells utilised 10 % SDS exposed for overnight and DMSO exposed for 10 min. The correlation value was 0.9965, $P < 0.0001$ and the stat software GraphPad Prism 5 used to analyse the data.

3.3.2 Viability in pronephric cells

3.3.2.1 MTT assay

The MTT assay was used as an initial screening method to assess the effect of immunomodulatory carbohydrates on cell viability. An initial *in vitro* experiment was performed to determine the dose effect of MacroGard® on carp pronephric cells obtained from 6 fishes. The MTT assay performed at 24h after the addition of the MacroGard®. The mean absorbance for 3 replicate wells for each fish was calculated and values were subtract from the corresponding control (absorbance of test cells - absorbance of control wells). Although, the dose effect of MacroGard® on cell viability was found to be not significantly different between treatments ($P \leq 0.05$) (Figure 3-5), a noticeable decrease in cell viability after 24h was observed at concentrations of MacroGard® higher than 50 µg/ml.

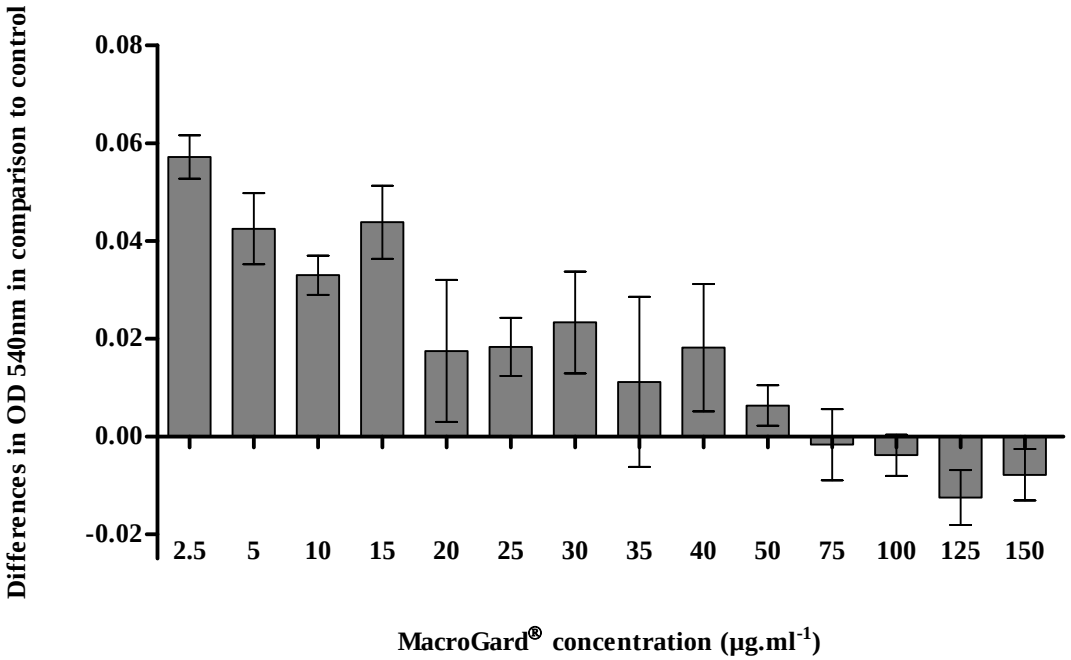


Figure 3-5: Dose effect of MacroGard® on the viability of pronephric cells (MTT assay). The cell viability were determined after cells (5×10^5 cells/well) were treated for 24 h with a range of MacroGard® concentrations i.e. (2.5- 150 µg/ml). A one-way ANOVA was performed, and the bars represent the mean differences from control of 3 wells of cells from 6 fish ± SEM ($p \leq 0.05$).

Following on from the previous experiment, the dose effect of MacroGard® and the sulphated form of MacroGard® on pronephric cell viability was compared using cells isolated from 4 carp (see Figure 3-6). These cells were exposed to either MacroGard® or sulphated MacroGard at concentrations of 2.5- 150 µg/ml for 24 h. Both MacroGard® and sulphated MacroGard did not significantly affect cell viability when used at any tested concentrations ($P \leq 0.05$). A similar trend for both MacroGard® and sulphated MacroGard can be observed and caused a non-significant decrease in viability when used at concentrations between 50 – 150 µg/ml. This preliminary results show that both MacroGard® and sulphated MacroGard are non-toxicity to the pronephric cells suspension when used at concentrations less than 50 µg/ml.

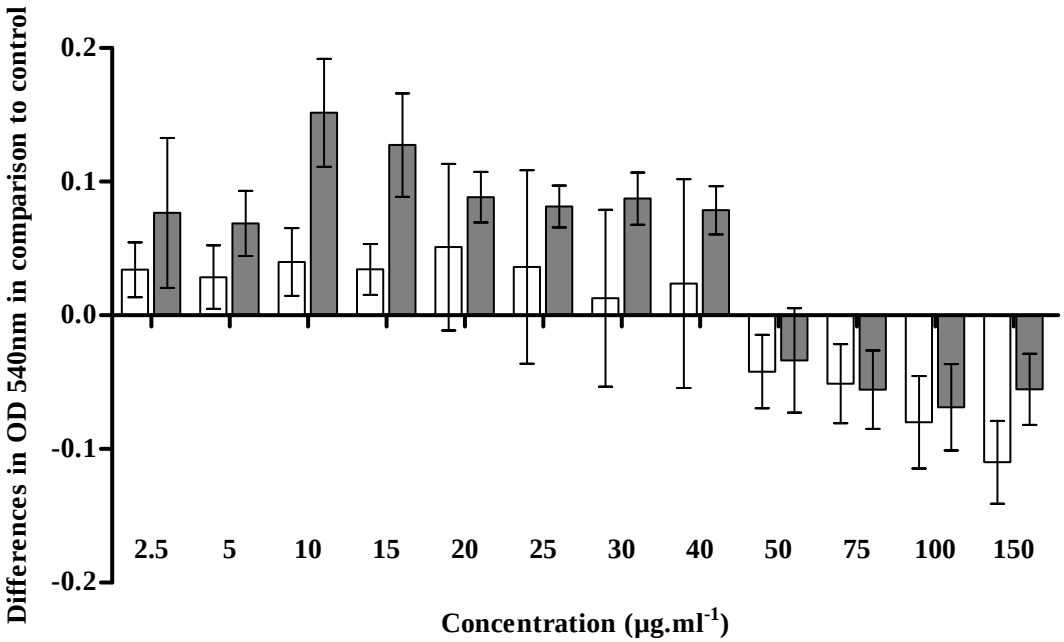


Figure 3-6: The effect of MacroGard® and sulphated MacroGard on pronephric cells viability. The MTT assay were determined on pronephric cell (5×10^5 cells/well) after cells were treated for 24h with □ MacroGard® (2.5-150 µg/ml) or ■ sulphated MacroGard (2.5-150 µg/ml). Statistical analysis was performed using a one-way ANOVA. Bars represent the mean differences from control of 3 wells of cells from 4 fish ± SEM ($p \leq 0.05$).

3.3.3 The effects of MacroGard® and sulphated MacroGard on cell lines viability.

3.3.3.1 MTT assay

The heterogeneous nature of the cell types obtained from carp pronephros whilst represents the situation forms *in vivo*, but introduces variability into the standardisation process. Hence a homogeneous cell line, CLC, grows relatively quickly and need minimal care was utilised. The viability of the CLC line was evaluated in the presence of MacroGard® at concentrations between 2.5 - 150 µg/ml and incubated for either 24 or 48h (Figure 3-7). The percentage difference in cell viability comparable to the control wells was calculated ([absorbance of test cells/absorbance of control wells] × 100) and plotted against the concentrations of the MacroGard®. MacroGard® did not induce a significant differences compared to control cells after 24 h incubation. However, after 48h incubation a

significant effect was observed ($P < 0.0001$) and significant increases in cell viability comparable to control was observed at 2.5 µg/ml ($P \leq 0.006$), 10 µg/ml ($P \leq 0.044$) and 15 µg/ml ($P \leq 0.015$) MacroGard® concentrations. MacroGard®, therefore had no toxic effects on CLCs at doses less than 50 µg/ml after 24 h and at all tested concentrations after 48 h incubation.

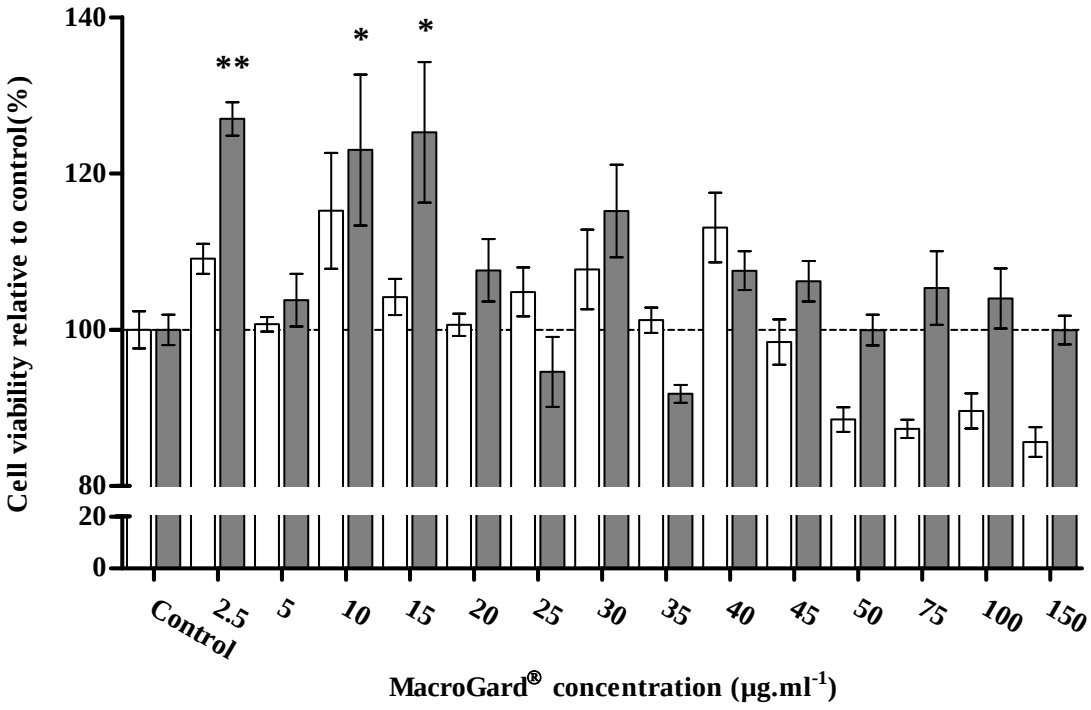


Figure 3-7: Percentage of differences in CLC line viability after 24h and 48h of MacroGard® exposure. The viability was determined with MTT assay, the range of MacroGard® concentrations used was 2.5- 150 µg/ml. Cell were seeded at 1×10^4 cells/well and optical density was read at 540 nm after 24 or 48h. Statistical comparison was performed by one-way ANOVA ($F = 9.154$, $P < 0.0001$ for 24h; $F = 5.47$, $P < 0.0001$ for 48h), the significant differences between the MacroGard® concentrations in comparison to control (5 µl sterile water). *= $p \leq 0.05$ and **= $p \leq 0.01$. The grid line shows the control level and the data represent as a mean ± SEM of six wells replicates. Incubation time labelled with □ = 24h and ■ = 48h.

The dose and incubation period dependency of the possible effects of sulphated MacroGard on cell viability was also measured and compared on two different fish cell lines i.e. CLC and EPC (Figure 3-8). The cell lines were incubated with sulphated MacroGard at concentrations 1- 150 µg/ml for 24 and 48 h. The result was calculated and expressed as percentage of differences in viable cells ([absorbance of test cells/absorbance of control wells] × 100) and each mean was calculated from 6 replicate wells.

Both concentration and incubation time of the sulphated MacroGard significantly affected the CLC line viability (24h incubation $F = 1.361$, $P \leq 0.231$, $F = 31.345$, $P < 0.0001$; 48h incubation $F = 2.268$, $P \leq 0.032$, $F = 39.637$, $P < 0.0001$). Sulphated MacroGard induced a significant increase in CLC line growth after 48h incubation at concentrations 1.5 µg/ml (0.857 ± 0.039) and 2.5 µg/ml (0.753 ± 0.025) at *p* values equal to 0.015, 0.04 respectively. In addition, the results indicated a highly significant decrease in CLC line viability after exposing the cells to sulphated MacroGard at a concentration of 150 µg/ml for 24 or 48 h ($P < 0.0001$). Sulphated MacroGard at doses of 25 µg/ml and less did not induce a cytotoxic effect on the CLCs after 24 and 48 h and a high dose (150 µg/ml) reduced viable CLCs at incubation times.

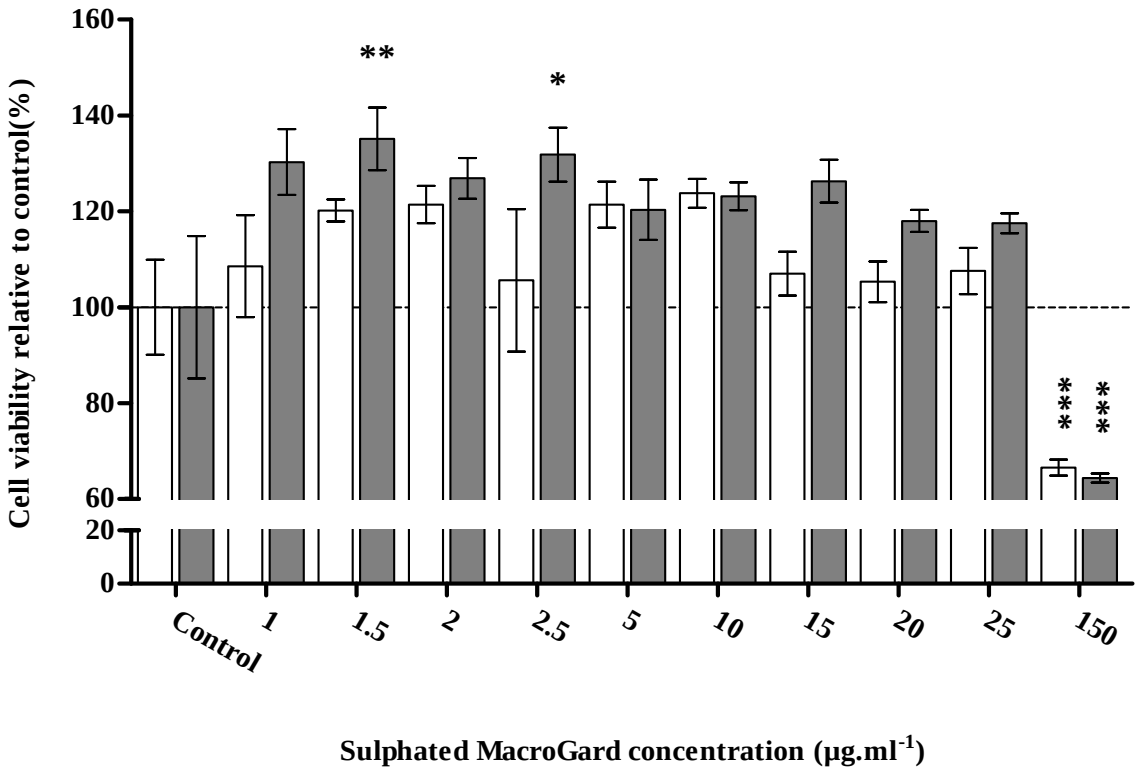


Figure 3-8: Effect of incubation time and sulphated MacroGard concentrations on CLC line viability. The percentage of cell viability was determined using MTT assay. Cells were exposed to different sulphated MacroGard doses (1- 150 µg/ml) over 24 and 48 h. Statistical comparison was performed using a one-way ANOVA and Tukey's post hoc test. The significant differences between the sulphated MacroGard concentrations in comparison to control (5 µl sterile water) was indicated by *= $p \leq 0.05$, **= $p \leq 0.01$ and ***= $p \leq 0.0001$. The dotted line shows the value for control wells, which was used as a reference of the tested data. The data represents the mean ± SEM of six wells replicates. Incubation time labelled as □ = 24h and ■ =48h.

The MTT assay was performed on the EPC cell line (Figure 3-9), to determine if different concentrations of sulphated MacroGard had a significant effect in cell viability ($F = 5.969$, $P < 0.0001$). After 24h incubation, a significant decrease in cell viability occurred at concentration 20, 25 µg/ml ($P \leq 0.001$) and 30 -150 µg/ml ($P \leq 0.0001$) in comparable to control.

The prolong incubation with sulphated MacroGard significantly affected the viability of the EPC cell line (F = 20.795, 19.33, $P < 0.0001$) at all doses tested. This revealed that the sulphated MacroGard was highly toxic to the EPC cells at 20 µg/ml and higher concentrations after 24 h incubation and at all experimented doses after 48 h.

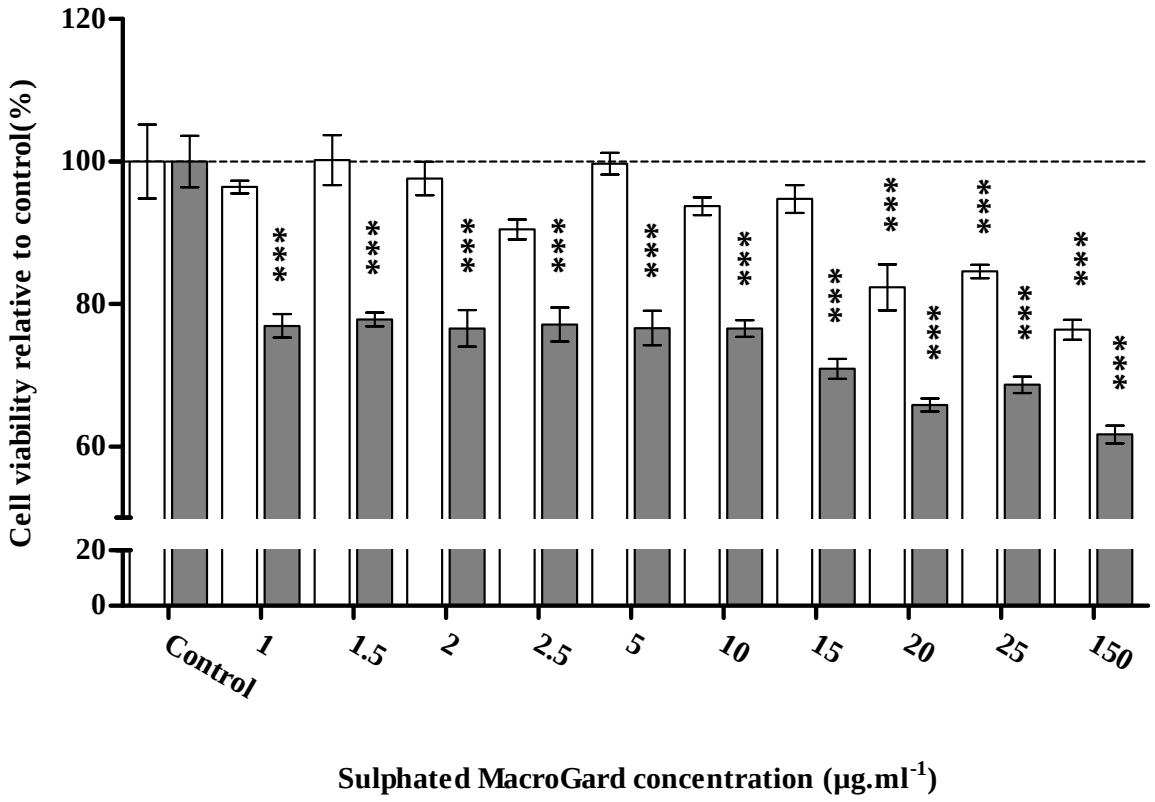


Figure 3-9: The effect of sulphated MacroGard dose and exposure time on the viability of EPC cell lines using MTT assay. Statistical analysis was performed using a one-way ANOVA and Tukey's post hoc test. The significant differences between cells exposed to different concentrations of sulphated MacroGard in comparison to control (5 µl sterile water) are indicated as ***= $p \leq 0.0001$ and the dotted line shows the value of the control level. The data are displayed as mean ± SEM of six wells replicates. Incubation time □ = 24h and ■ = 48h.

3.3.3.2 MTS assay

The first question was to find whether dose and incubation time of the immunostimulants tested affected the viability of both the primary cells and cell lines used in the current chapter.

Having ascertained the optimisation and sensitivity of the MTT assay, this was tested using a compatible assay to MTT assay, the MTS assay to assess the effect of sulphated MacroGard on the viability of the CLC and EPC cell lines after 24 and 48h incubation. The optical density was calculated and presented as the percentage of differences in cell viability comparable to control ([absorbance of test cells/absorbance of control wells] × 100) for 6 wells replicates of a 96 well plate. As shown in figure 3-10, it appeared that sulphated MacroGard caused a significant decrease in the viability of the CLC line at a concentration of 150 µg/ml after 24 ($P \leq 0.00003$) and 48h ($P \leq 0.017$), compared to the comparable control values. However, the viability of cells at all other concentrations used were not different to these of the control wells for the same two time periods examined.

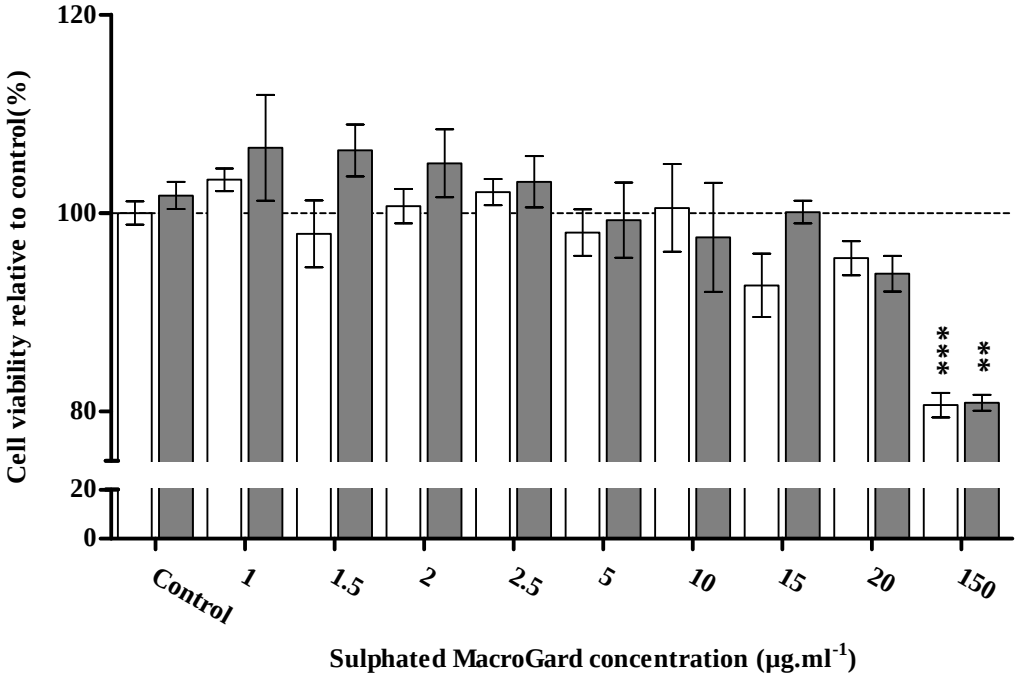


Figure 3-10: The effect of sulphated MacroGard on CLC line viability (MTS assay). CLC lines were stimulated with range of sulphated MacroGard doses 1- 150 µg/ml at two different incubation periods. Cell were seeded at 1×10^4 cells/well and optical density was read at 490 nm after 24 or 48 h. Statistic comparison was performed using a one-way ANOVA (24 h $P < 0.0001$; 48 h $P \leq 0.0003$) and the differences between concentrations of the corresponding control wells performed **= $p \leq 0.01$, ***= $p \leq 0.001$ and the dotted line shows the values obtained from the control wells. Data represent mean ± SEM of six replicate wells. Incubation time labelled with □ = 24 h and ■ = 48 h.

Interestingly, significant decrease comparable to time matched control in EPC line viability was observed (Figure 3-11) at concentrations 10 µg/ml ($P \leq 0.021$), 15 µg/ml ($P \leq 0.001$), 20 µg/ml ($P \leq 0.011$) and 150 µg/ml ($P \leq 0.001$) of sulphated MacroGard after 24h incubation and at concentrations of sulphated MacroGard 1.5 µg/ml ($P \leq 0.033$), 2 µg/ml ($P \leq 0.0004$) and between 2.5 - 150 µg/ml ($P < 0.0001$) after 48h incubation.

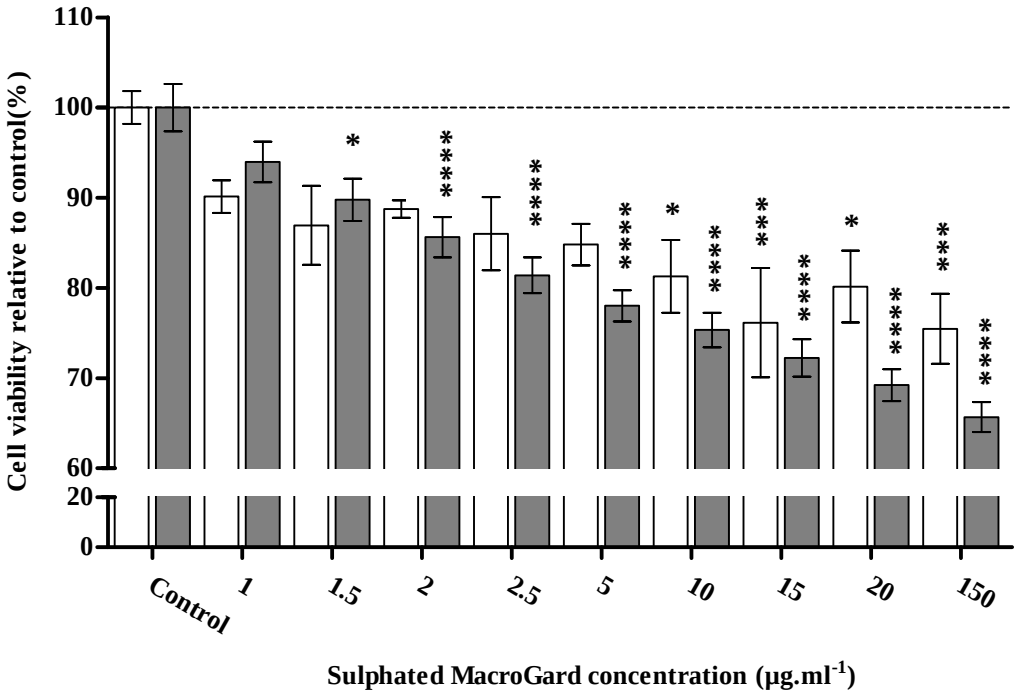


Figure 3-11: The effect of sulphated MacroGard on EPC cell line viability (MTS assay). EPC cell lines were exposed to range of sulphated MacroGard concentrations 1-150 µg/ml at 24 or 48h. Cell were seeded at 1×10^4 cells/well and optical density read at 490 nm after 24 or 48h. Statistic comparisons were performed using a one-way ANOVA (24h $p \leq 0.001$; 48h $p < 0.0001$), the significant differences between treatments in comparison to control performed with * = $p \leq 0.05$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$ and the grid line shows the control level. Data represent mean ± SEM of six wells replicates. Incubation time labelled with □ = 24h and ■ = 48h.

To ascertain the sensitivity between the cell viability utilised by MTT and MTS assay, the previous data were rearranged and presented as percentage of differences in viable cells in comparison to control (5 µl sterile water) after exposure to different concentrations of sulphated MacroGard at 24 or 48h. The performance of both assays can be observed more clearly in Figure (3-12). MTT assay was more sensitive and illustrate cell proliferation/ viability in CLCs in both incubation period (24 and 48 h) and also in EPC cells lines after 24 h. It can be conclude that the MTT assay and CLCs are more suitable to check the effect of sulphated MacroGard on cell viability.

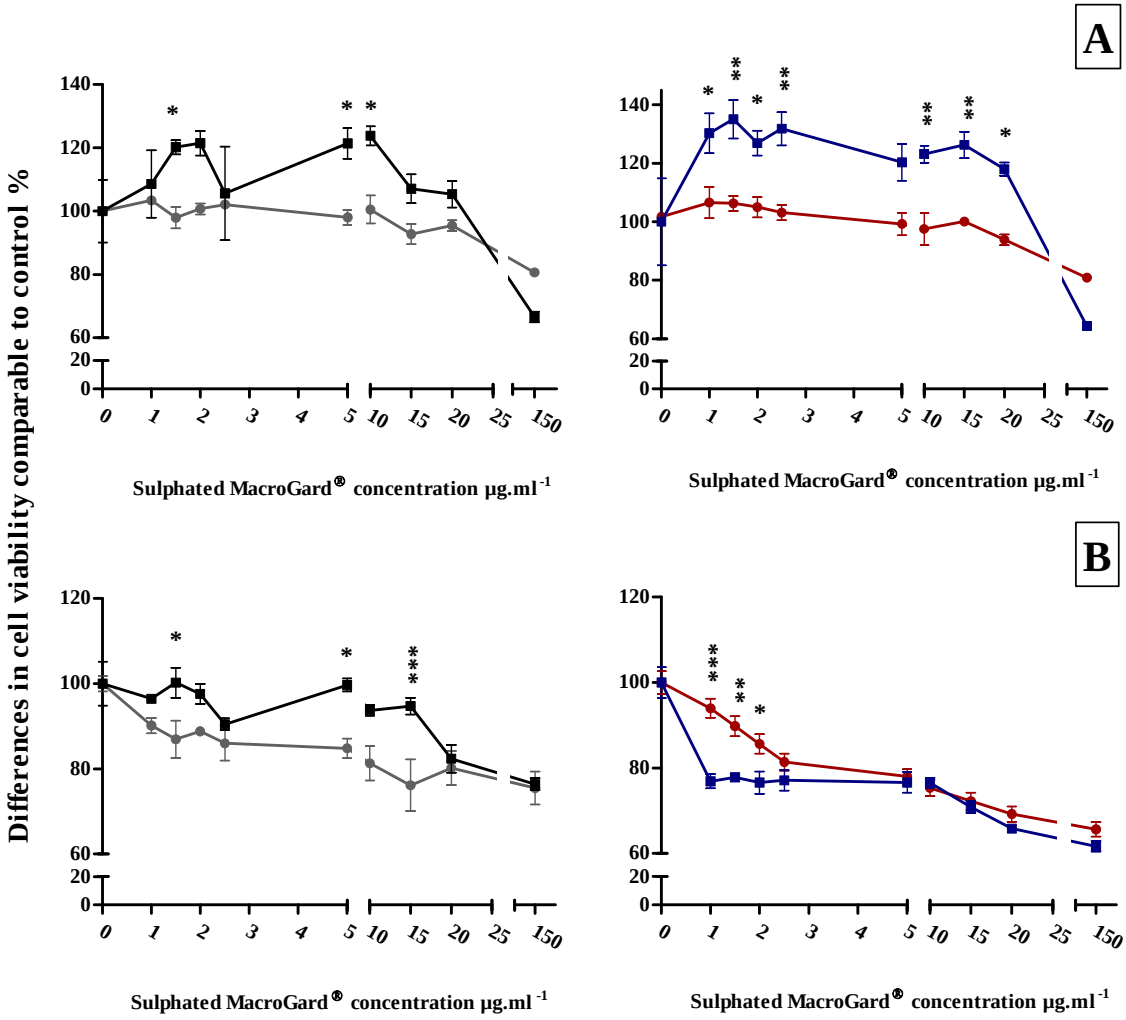


Figure 3-12: Comparison of the percentage of difference in cell viability monitored utilising the MTT and MTS assay. The effect of sulphated MacroGard on CLC line [A] and EPC cell line [B] viability, both cell lines were exposed to range of sulphated MacroGard concentrations 1-150 µg/ml. Cells were seeded at (1×10^4 cells/well) and optical density was read at 490 nm after 24 h (■ = MTT assay, ● = MTS assay) or 48 h (■ = MTT assay, ● = MTS assay). Statistical comparison was performed by two-way ANOVA and Bonferroni post tests used to analyse the significant differences between assays at each sulphated MacroGard concentration. Statistical significant defined * = $p \leq 0.05$, ** = $p \leq 0.01$ and *** = $p \leq 0.001$.

3.3.4 The effect of beta glucans on cells reactive oxygen production

3.3.4.1 Pronephric cells

Both of MacroGard® and sulphated MacroGard were tested at nine different concentrations between 2.5–150 µg/ml for their reactive oxygen production effect after 24h incubation. The dose dependency for pronephric cells isolated from 4 fish are shown in figure 3-13, with the optical density being recorded from three wells of cells from four fishes ± SE ($p \leq 0.05$). The statistical analysis with a one way ANOVA revealed the significant effects of both MacroGard® ($F = 2.597$, $P \leq 0.011$) and sulphated MacroGard ($F = 6.31$, $P \leq 0.000001$) on superoxide production in pronephric cells. Superoxide production decreased significantly correspond to the control wells after cells were treated with sulphated MacroGard at concentrations of 25 µg/ml ($P \leq 0.00006$), 100 µg/ml ($P \leq 0.048$) and 150 µg/ml ($P \leq 0.035$).

This analysis illustrated the inability of sulphated MacroGard to induce the respiratory burst activity in pronephric cells suspension.

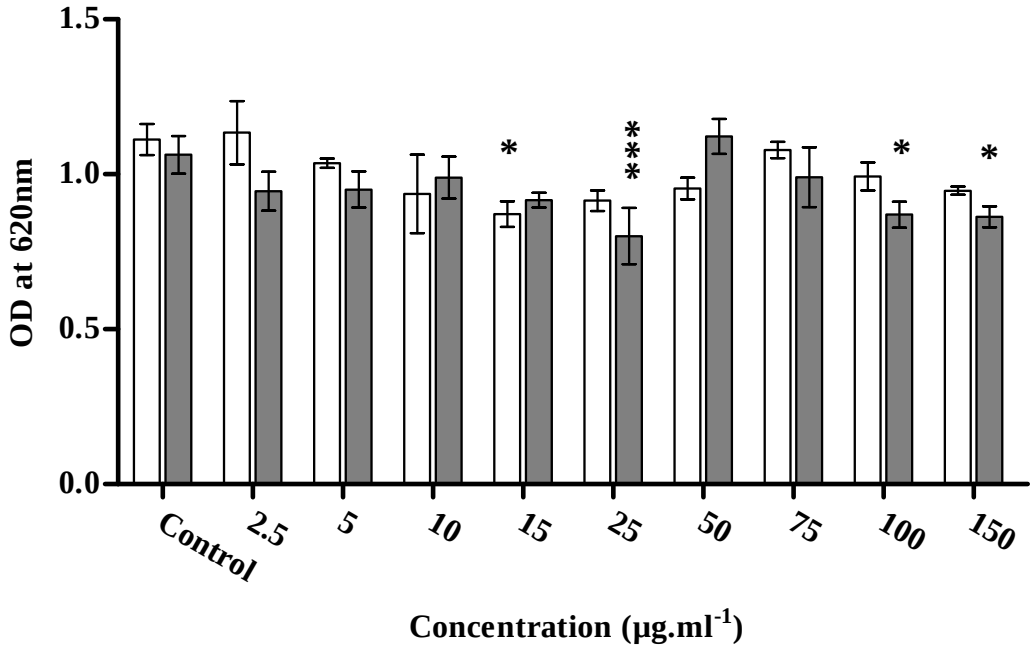


Figure 3-13: Measurement of the respiratory burst activity of carp pronephric cells using NBT assay. Pronephric cells at a density of 5×10^5 cells/well stimulated either with MacroGard® = □ or sulphated MacroGard = ■ at concentration of 2.5 -150 µg/ml for 24h. NBT assay was used to determine superoxide anion production and the optical density read at 620 nm after 24h. Statistical comparison was performed using a one-way ANOVA ($p \leq 0.05$) and the significant differences between treatments in comparison to control performed with *= $p \leq 0.05$ and *** = $p \leq 0.001$. Data represent mean ± SEM of 3 replicate wells from 4 fishes.

3.3.4.2 Cell lines

A parallel experiment was performed to assess the effect of MacroGard®, sulphated MacroGard and zymosan reactive oxygen species production in CLCs line. The aim was to find a suitable concentration and incubation period for these immunostimulants to be used as a positive control in subsequent chapters to examine the effect of different carbohydrates as immunostimulants. In order to assess the effect of MacroGard®, sulphated MacroGard and zymosan on the respiratory burst activity of CLC lines, dose (10, 25, 50 µg/ml) and exposure time (1, 6, 24 h) were used. The mean of optical density of four well replicates was plotted against treatments concentrations (see Figure 3-14). The statistical difference between treatment concentrations was determined in relation to the control (sterile water treated cells) at each time points.

MacroGard® exposure time (F = 59.615, $P \leq 0.0001$) and the interaction between dose and exposure time (F =3.384, $P \leq 0.009$) had a significant effects on reactive oxygen species production. Elevated significant increase of ROS production was observed at 10 ($P \leq 0.008$), 25 ($P \leq 0.0004$) and 50 ($P \leq 0.001$) µg/ml of MacroGard® after 24h incubation.

However, at all MacroGard® concentrations there was no significant detection in ROS production after 1 and 6h incubation.

Further statistical analysis revealed a significant effect of sulphated MacroGard doses (F = 4.106, $P \leq 0.013$), exposure times (F = 92.033, $P \leq 0.0001$) and the interaction between the two factor (F =4.354, $P \leq 0.002$). Sulphated MacroGard induced an almost similar effect to MacroGard® after 24h incubation, and there was a significant increase in ROS production at all concentrations used (i.e. 10 ($P \leq 0.013$), 25 ($P \leq 0.006$) and 50 ($P \leq 0.001$) µg/ml). When cells were exposed to sulphated MacroGard for one hour, a significant decrease in ROS production was observed at 10 ($P \leq 0.0004$) and 25 ($P \leq 0.019$) µg/ml concentration.

The two way ANOVA of zymosan treatments revealed a significant effect of exposure time (F = 321.275, $P \leq 0.0001$) and the interaction between dose and exposure time (F =22.098, $P \leq 0.0001$). At all-time points there was a significant difference detected between treatments, zymosan exposure for 6 h induced a significant increase in ROS production at 10 ($P \leq 0.001$), 25 ($P \leq 0.041$) and 50 ($P \leq 0.002$) µg/ml. Furthermore, incubation for 24h induced a significant increase in ROS production at 10, 25 ($P < 0.0001$) and 50 ($P \leq 0.003$) µg/ml concentration. A reduction ($P < 0.0001$) in ROS production occurred however at all zymosan concentrations (i.e. 10, 25 and 50 µg/ml) after one hour incubation. The time dependency was highly significant for the immunostimulants used and 24 h incubation increased ROS production in all treatments.

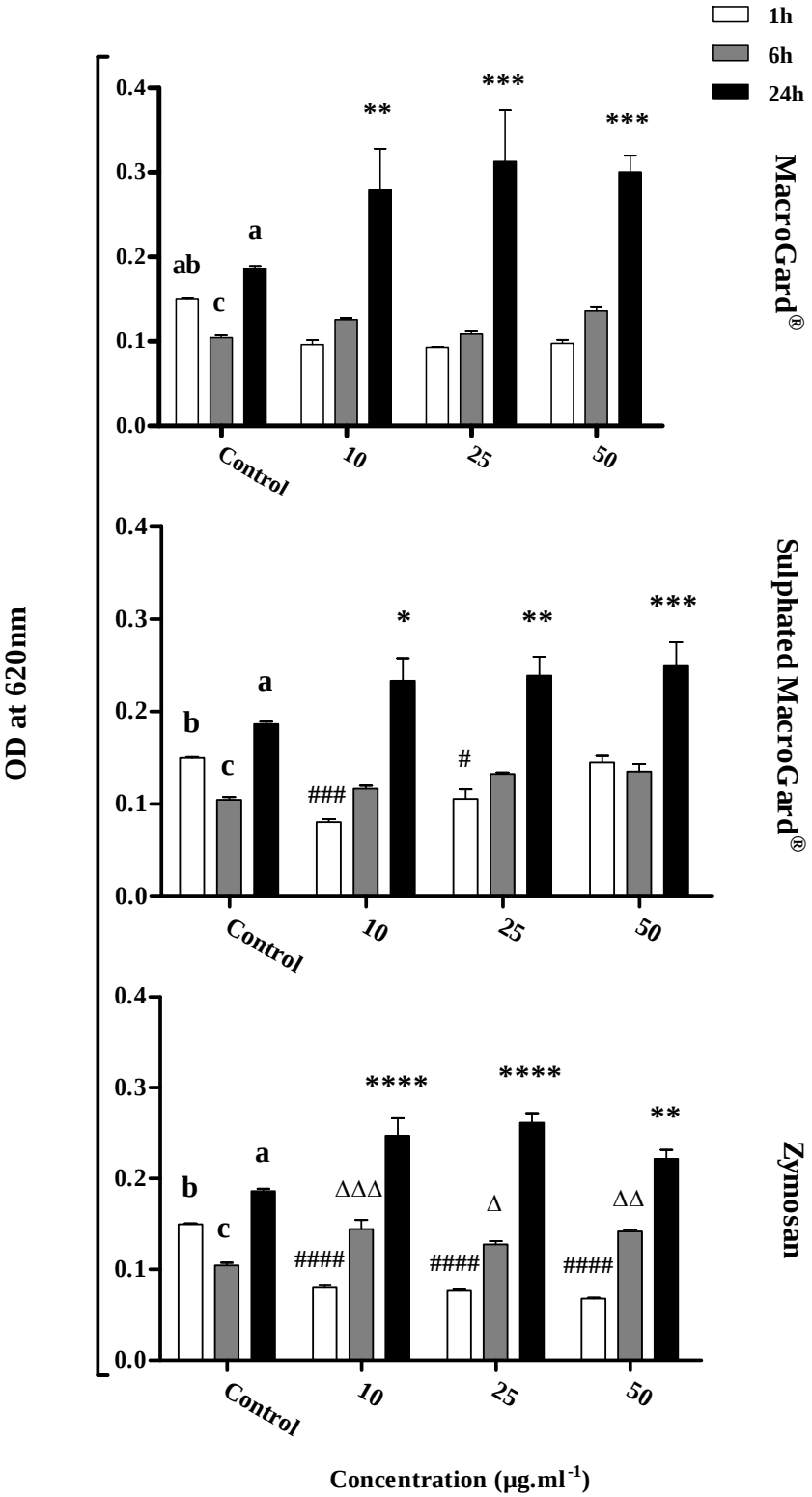


Figure 3-14: Comparison of superoxide anion production by CLCs line stimulated with various beta glucan sources. The cells were seeded at 1×10^4 cells/well and NBT assay was used to determine the reactive oxygen species after cells were stimulated with either

MacroGard®, sulphated MacroGard or zymosan at concentrations 10, 25, 50 µg/ml. The optical density was read at 620 nm after 1, 6 and 24h. Statistical comparison was performed using a two-way ANOVA and data is displayed as the mean ± SEM of four wells replicates. The significant differences between treatments in comparison to time matched control performed with # = $p \leq 0.05$, ### = $p \leq 0.001$ and #### = $p \leq 0.001$ for the 1h incubation; Δ = $p \leq 0.05$, ΔΔ = $p \leq 0.01$ and ΔΔΔ = $p \leq 0.001$ for the 6h incubation; * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ and ****= $p \leq 0.001$ for the 24h incubations. The different letters at control group in each time point means they are significantly different at $p \leq 0.05$. Incubation time dependency were labelled as □ = 1h, ▒ = 6h, ■ = 24h.

3.4 Discussion

3.4.1 MTT viability assay optimization

In this investigation, the cytotoxicity of different forms of beta glucan was determined using MTT assay, it is a colorimetric assay measure live and proliferative cells (Mosmann, 1983). The MTT assay has been used widely *in vitro* studies performed in fish to determine for example, the metabolic activity of blood phagocytes cells in European eel (*Anguilla anguilla*) (Siwicki and Robak, 2011); the dose effect of kynurenic on the viability and mitogenic response of blood and spleen lymphocytes cells isolated from of rainbow trout (*Oncorhynchus mykiss*) (Malaczewska et al., 2014); peripheral blood and kidney lymphocytes of brook trout (*Salvelinus fontinalis*) proliferation in responses to mitogens (Daly et al., 1995) and the effect of nanocolloids on rainbow trout (*Oncorhynchus mykiss*) leukocytes proliferation (Malaczewska and Siwicki, 2013).

As described in Section 2.5.1, the MTT assay depends on the ability of viable cells to convert the water soluble tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT), into water insoluble formazan crystals that dissolved in a variety of organic solvents (Twentyman and Luscombe, 1987). The amount of produced formazan crystal is directly in proportional to cell number and type (Mosmann, 1983) and there are different factors affect the assay, such as the culture medium (Vistica et al., 1991), the organic dissolvent and the duration to dissolve the formazan (Twentyman and Luscombe, 1987). Therefore, optimization of the MTT assay conditions was established, which included cell number, the dissolvent type and exposure time. A linear increase in optical density was found with the increases in cell number, which is comparable to observations made by Mosmann (1983). Twentyman and Luscombe (1987) suggested that DMSO was the best solvent to dissolve the formazan crystal, similar to the conclusions made in the present study comparing 10% SDS and DMSO (for 10 min) solvent, hence DMSO was

used as solvent for dissolving the formazan crystal (with a 10 min incubation) in subsequent experiments. The DMSO solvent gave less background colour when the plate test was read after 10 min compared with overnight incubation. This is probably due to the fact that DMSO, when added to a small volume of medium containing unconverted MTT (even in the absence of cells), leads to the production of formazan, which takes place over several hours i.e. the intensity of the colour is related to time (Twentyman and Luscombe, 1987) thus, it is important to read the plates as soon as possible after the addition of the DMSO.

3.4.2 Beta glucan time and dose dependency effects on cell viability and respiratory burst activity

In aquaculture, the exposure time and dose dependency effects of different beta glucans have received some attention, particularly in relationship to their effects on different fish species and the different routes of administration. The dose effects of beta glucan have been ascertained in a range of studies utilising different concentrations, for example *in vitro* (Miest and Hoole, 2015, Kepka et al., 2014, Hsiao et al., 2015, Hauton and Smith, 2004, Pietretti et al., 2013, Jorgensen and Robertsen, 1995, Castro et al., 1999, Vera-Jimenez and Nielsen, 2013) and *in viv*o by feeding (Ai et al., 2007, Bonaldo et al., 2009, Kühlwein et al., 2013) or by injection (Selvaraj et al., 2005). These studies established the immunomodulatory effect of beta glucan and the importance that this substance may play in increasing resistance against pathogens. However, few studies have been carried out to determine the beta glucans effect on cell cytotoxicity/ viability and proliferation on individual cells or populations of cells.

The most extensively area of research relates to the effects of beta glucan on mammalian cells. For example, the 24 h incubation of beta glucans at doses less than 50 µg/ml did not

affect mouse microglia cell line viability (Jung et al., 2007), whilst a decrease in lymphocyte proliferation was observed with beta glucan (MacroGard®) concentrations higher than 50 µg/ml in porcine peripheral blood (Sonck et al., 2010).

In comparison limited studies have been carried out *in vitro* for aquaculture species to ascertain the effect of beta glucan on cell viability and proliferation. For example studies on tilapia hepatocyte cells were examined for their proliferation ability after 1, 2, 3 and 4 days incubation with mushroom beta glucan at concentrations 5 and 10 mg/ml, the results indicated an enhancement in cell proliferation and cellular viability (Hsiao et al., 2015). A dose effect of different beta glucans was noted to induce cytotoxicity in granulocytes from the lobster *Homarus gammarus* (Hauton and Smith, 2004), and MacroGard® dose dependency on common carp pronephric cell suspension was noted to induce a significant increase in apoptosis levels at 500 µg/ml or higher after 6 h of incubation (Miest and Hoole, 2015).

In this present study the MacroGard® and sulphated MacroGard (Figures 3-5 and 3-6) concentrations between 2.5- 150 µg/ml did not induce a significant increase or decrease in pronephric cell viability after 24 h incubation, but a non-significant decrease in viability level was observed at concentrations higher than 50 µg/ml; this result is similar to the finding Jung et al. (2007) and Sonck et al. (2010) for mammalian cells. In drugs trials, the concentration of drugs that is just sufficient to produce a specific and minimal detectable effect is called the threshold dose. Therefore, the decrease in cell viability at higher concentrations of beta glucan might be because of the threshold concentration of these substance and possible toxicity at certain concentrations (Kepka et al., 2014, Miest and Hoole, 2015). However, since beta glucan activates phagocytic cells and induce the respiratory burst activity and reactive oxygen species production (Castro et al., 1999, Vera-Jimenez et al., 2013) it is possible that the high doses of beta glucan reduce the viable cells

due to the toxic level of reactive oxygen species production (Kepka et al., 2014, Miest and Hoole, 2015). Therefore it was important to determine the dose effect of beta glucan on respiratory burst activity in carp leucocyte cells and for that reason a rapid and precise colorimetric assay nitroblue tetrazolium (NBT) was used for this purpose. Previous research indicated that the respiratory burst is dependent on beta glucan dose administration. For example *in vitro* studies carried out on the effect of different beta glucans at concentrations (0-500 µg/ml) on turbot and gill head pronephric cells, revealed an increase in respiratory burst activity at high concentration (100- 500 µg/ml) (Castro et al., 1999). A similar response was observed in carp pronephric cells after exposure to MacroGard® and zymosan at 50 and 100 µg/ml concentrations (Vera-Jimenez et al., 2013, Pietretti et al., 2013). However, Atlantic salmon (*Salmo salar L*.) macrophages treated for 24 h with yeast beta glucan induced respiratory burst activity at doses 0.1 and 1 µg/ml, while 10 µg/ml had no effect and 50 µg/ml was inhibitory (Jorgensen and Robertsen, 1995). My results indicated no significant increases in respiratory burst activity in responses dose dependency of MacroGard® at concentrations between 2.5–150 µg/ml and sulphated MacroGard at 2.5-75 µg/ml, except for concentrations 15 µg/ml for MacroGard® and 25, 100, 150 µg/ml for sulphated MacroGard that induced inhibitory effects in carp pronephric cell. This may have happened because of the heterogeneity of the pronephric cell population, where the respiratory burst activity is related to the stage of cell differentiation and activation (Jorgensen and Robertsen, 1995) or may be the decrease of NBT reduction in response to beta glucan was less evident and did not exhausted the cells (Castro et al., 1999).

Consequently, carp leukocytes cell line (CLC) was used to overcome the heterogeneity in cell types present in pronephric cells and obtain an uniform cell population to perform immune response studies *in vitro* for fish (Vidal et al., 2009, Weyts et al., 1997). The dose

and exposure time dependency of different glucan were determined using the CLC line at 1, 6 and 24 h incubation, the results revealed the time response for all of beta glucans tested. Both of MacroGard® and sulphated MacroGard promoted a significant respiratory burst production after 24 h incubation, while no differences was observed after 1 and 6 h incubation in MacroGard® at all used doses. Interestingly, sulphated MacroGard at low doses of 10 and 25 µg/ml induced a decrease in respiratory burst activity after one hour incubation. The same effect was also observed in zymosan treatment; 1 h incubation promoted a significant decrease in respiratory burst activity at 10, 25 and 50 µg/ml, the reduction of formazan increased significantly within the incubation period for all zymosan concentrations tested, and promoted a higher stimulation effect at 10, 25 µg/ml and less at 50 µg/ml after 24 h. Similar outcomes have been found by Kudrenko et al. (2009), where low doses of zymosan (0.1, 1 µg/ml) needed 24 h incubation to prime the respiratory burst activity in barramundi (*Lates calcarifer*) macrophages while pure beta glucans had no effects, suggesting that because of zymosan derived from yeast cell wall it might react through different receptors that are not specific to beta glucan like mannose receptors.

3.4.3 Cytotoxicity assay factors and sensitivity

Once the dose and exposure time response of beta glucan was ascertained, the next step was to establish the feasibility of using homogenous fish cell lines as a tool to screen the activity of different forms of beta glucan. The cell type is an important factor in cytotoxic assays, as they provide the inherent toxicity information for screening compounds due to the relative metabolic incompetence of these cells compared to normal cells (Hamid et al., 2004). Although the primary cells are difficult to handle and depend on the genetic variation between individuals, they are a superior model and provide valuable information about the situation *in vivo* (Hamid et al., 2004). Therefore, both of CLC and EPC cell line

were used to determine the effect of beta glucan on cell viability. The effect of MacroGard® on both pronephric and CLC line was similar after 24 h incubation and there was a non-significant decrease in cell viability at concentrations 50 µg/ml and higher and after prolonged incubation with MacroGard® induced CLC line proliferation at 2.5, 10 and 15 µg/ml. This result confirmed the suitability of CLC line for this study and agreed with the conclusion of Weyts et al. (1997) investigation about the suitability of CLC line to monitor macrophage phagocytosis, respiratory burst and cytokine production.

The EPC cell line (*Epithelioma papulosum cyprinid)*, originating from carp epidermal herpes virus-induced hyperplastic lesions (Fijan et al., 1983), and has been used in several cytotoxicity studies (Kammann et al., 2001, Park and Choi, 2014, Tan et al., 2008). In my investigation the dose and time response of beta glucan induced a significant decrease in EPC cell line viability at concentrations ≤ 20 µg/ml after 24 h incubation and at ≤ 1 µg/ml after 48 h incubation. This reduction in viability is possibly due to the derive of the EPC cell line which is from a skin tumour of carp, since *in vitro* investigations reported the cytotoxic effect of beta glucan on prostatic cancer cells apparently through oxidative stress, leading to apoptosis (Fullerton et al., 2000).

The sensitivity of the MTT assay was compare to the MTS assay, using both CLC and EPC cell lines after exposure to different beta glucan doses over 24 and 48 h. Although both tests demonstrated similar trends of cell viability, there was significant differences between the assays. The MTT assay gave a higher percentage of CLC viability at 24 and 48 h incubation. The MTS is an alternative assay to MTT, the color intensity of the formazan dye is correlated to the number of viable cells. However, some chemicals or phytochemicals may change the activity of succinate dehydrogenase or interact with MTT directly and washing the cells before adding MTT to reduce the interference (Wang et al., 2010).

In summary an *in vitro* system was established for screening different modified carbohydrates. This *in vitro* system comprised evaluation of the effect of different modified carbohydrates at one concentration (if it is possible 50µg/ml) on CLCs respiratory burst activity (NBT assay) and viability (MTT assay) after 24 h incubation. Although, the sulphation process produced a soluble form of MacroGard® but its effect on cell viability and respiratory burst activity were similar to MacroGard®, therefore the sulphated MacroGard was not taken forward in work described in the next chapters.

Chapter 4. Determination of the immunomodulatory effect of modified carbohydrates utilising the *in vitro* cell system.

4.1 Introduction

The innate immune system in fish is the first line of defence against disease infections, the protection being dependent on the mobilisation of cellular and humoral nonspecific components. The main and earliest mediator of fish innate immune responses to pathogen invaders is phagocytosis, the phagocytic cells being supported by humoral factors such as the complement system and lysozyme activity (Dügenci et al., 2003). There are many compounds that modulate the biological responses of immune cells and enhance disease resistance, and are thus consider to be the most promising alternative to antibiotic treatments (Dügenci et al., 2003, Schepetkin et al., 2005).

Many immunostimulants have been identified in fish, such as synthetic chemicals: Levamisole (Siwicki, 1987, Siwicki, 1989, Siwicki et al., 1990, Jeney and Anderson, 1993b); bacterial derivatives substance such as beta glucan (Jorgensen and Robertsen, 1995, Kühlwein et al., 2013, Pionnier et al., 2013, Rodríguez et al., 2009, Samuel et al., 1996, Selvaraj et al., 2005, Selvaraj et al., 2006, Skov et al., 2012) and lipopolysaccharide (LPS) (Kadowaki et al., 2013, Morrison et al., 2004, Nayak et al., 2011); polysaccharides such as Chitin and Chitosan (Gopalakannan and Arul, 2006); vitamins such as vitamin C (Verlhac et al., 1996); cytokines such as interferon (Tamai et al., 1993) and growth hormone (Sakai et al., 1995). Of these compounds, polysaccharides including those derived from bacteria are non-pathogenic and most potent immunomodulating agents. They are able to modulate both of cellular and humoral immune responses, also affecting macrophage function and complement activation (Tzianabos, 2000). Beta glucans, one of the natural polysaccharides that are found in cell wall of many plants, fungus and bacteria,

They are glucose homopolymers, and the most important forms being beta 1, 3 and 1, 6 glucans (Meena et al., 2012). Beta glucans are able to protect and enhance the immune system through their ability to bind directly to leukocytes and activate them providing optimum defence against any possible infections (Meena et al., 2012). The source of beta glucan leads to differences in structure, side chain branching and length, which can cause difficulties in extractions resulting in changes in their biological activities. For example, in mice, intraperitoneal injection with low, medium or the high molecular weight glucans, all with low (<5%) β-(1,6)-linkages, or high molecular weight glucan with high 1,6-linkages (>20%), induced peritoneal cell activation and increased the proportion of neutrophils and eosinophils. Macrophages showed an altered morphology, increased intracellular acid phosphatase, increased LPS-stimulated NO production and increased PMA-stimulated superoxide production (Cleary et al., 1999). Therefore several investigations have been directed towards identifying the structure and biological characteristic of the effective part of the molecule and the soluble form of these carbohydrates in order to evaluate the potential for clinical use (Tzianabos, 2000).

To obtain polysaccharides from crude material, boiling and enzyme treatment can be used to obtain the insoluble and soluble fractions. Furthermore, several biochemical reactions are employed to solubilised the insoluble beta glucan to produce a linear triple helix structure, such as phosphorylation, sulphation and amination (Tzianabos, 2000). Sulphatation, a final chemical modification process to obtain derivatives of sulphated polysaccharide, is a modification carried out either under non homogeneous or homogeneous conditions. Homogeneous media are more applicable as they increase the efficiency of the sulphation method (Chopin et al., 2015). Many polysaccharides have been sulphated and examined for their biological activity from different sources such as oat beta glucan (Chang et al., 2006), red microalgae (Geresh et al., 2002), and rice bran (Ghosh et

al., 2010), Konjac glucomannan (Bo et al., 2013). Various investigations have reported that the biological activity of sulphated polysaccharides and their diverse physiological functions, comprise antioxidant, antitumor, immunomodulatory, inflammation, anticoagulant, antiviral, antiprotozoan, antibacterial, antilipemic effects (Patel, 2012) (see Figure 4-1).

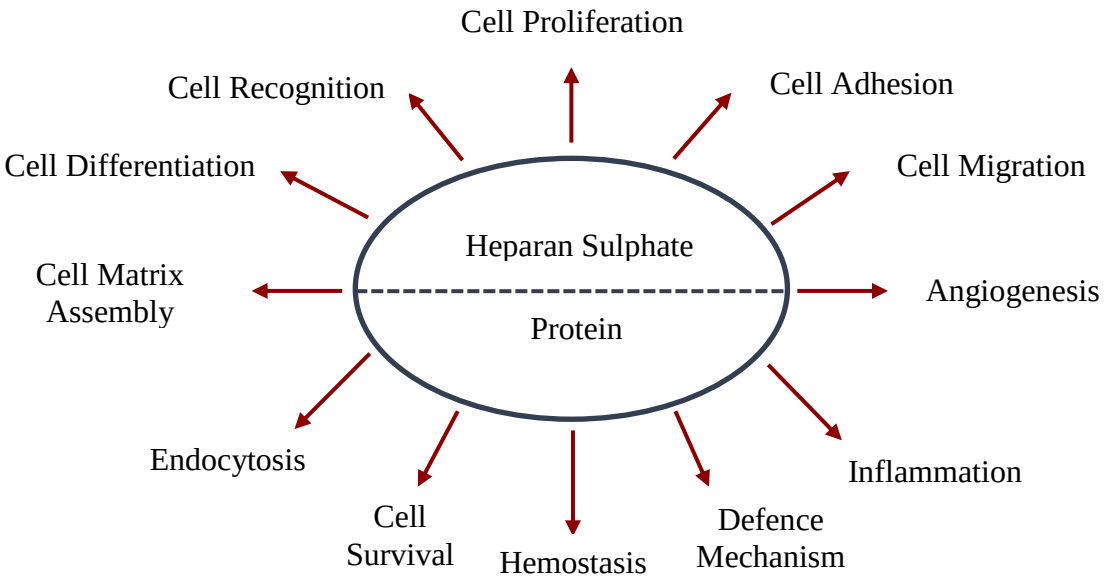


Figure 4-1: Biological activities modulated by the interaction of proteins with heparan sulphate (Dreyfuss et al., 2009).

The biological activity of sulphated polysaccharides can be affected by the degree of sulphation, molecular weight, constituent sugars, conformation and dynamic of the sulphated polysaccharides, also the distribution of sulphate groups on the constituent polysaccharides (Wijesekara et al., 2011).

The aim of this present chapter was to examine if modified carbohydrates (the sulphated drivers of a range of polysaccharides) would influence both CLCs line and carp pronephric cells viability and respiratory burst activity. As a result of initial screening of various modified carbohydrates, several were chosen based on their effectiveness as

immunostimulants for their dose responses on CLCs viability and respiratory burst activity. Finally the most effective one was examined for its dose response and exposure time in both cell types and studied in detail to identify its structure and biological effect in the following chapter.

4.2 Materials and Methods

4.2.1 Modified carbohydrate screening

The established *in vitro* system described in Chapter 3 was used to test the immunomodulatory effects of different soluble modified carbohydrates. Different modified carbohydrates (75 in total) were supplied kindly by Dr Mark Skidmore (Keele University) as listed in table 4-1, to determine their effects on fish immune cells. The whole screening process for the 75 carbohydrates was carried out in two experiments.

The first initial screening was carried out on 48 modified carbohydrates to determine their effects on CLCs respiratory burst activity. Tests were carried out blind i.e. not knowing the structural alterations that had been made to the polysaccharides utilising the CLC line (see Section 2.3.1) whose density and viability was checked as described in Section 2.2.2 and seeded at 2 $\times 10^4$ cells/well in 96 flat bottom well plates (Sarstedt, 83.1835.500). The respiratory burst activity was monitored utilising the NBT assay (as in Section 2.6.1). Briefly the CLCs were incubated with a range of modified carbohydrates at concentration either 5 µg/ml (based on the concentration of the modified carbohydrates) or 50 µg/ml (based on the *in vitro* system that established earlier in Chapter 3) for 24 h. For each experimental microplate used, different beta glucan sources were determined as positive references i.e. zymosan (Sigma, Z4250) at concentration 5 and/ or 50 µg/ml and MacroGard® at concentration 50 µg/ml, also a negative control that comprise 5 µl of sterile water (Sigma, W3500). After the incubation period, the formazan blue were solubilised and read at 620 nm. Data was analysed statistically using one way ANOVA and Tukey's post hoc test as described earlier (Section 2.8) with significance defined as $p \leq 0.05$. The modified carbohydrates that induced a highly significant increase in respiratory burst

activity in comparable to control and which had least complicated chemical structure were chosen to assess their concentration response as outlined in the following Section 4.2.2.

The second experiment examined the concentration response of 27 modified carbohydrates for their effects on CLCs respiratory burst activity utilising the NBT assay as described above using a more extensive range of concentrations 2.5- 150 µg/ml over a period of 24 h exposure to the cell line. Five µl of sterile water (Sigma, W3500) and MacroGard® at concentration 50 µg/ml were also included on each plate as a negative and positive control respectively. The optical density of three wells replicates were measured at 620 nm and the statistical analysis one way ANOVA and Tukey's post hoc test was carried out as described earlier (Chapter 2.8) with significant defined as $p \leq 0.05$.

Table 4-1: List of used modified carbohydrates

No.	Sample ID	The original source of the modified carbohydrates
1.	CHO 1	Sulphated Tylose
2.	CHO 2	Sulphated Gum Ghatti
3.	CHO 3	Sulphated Gumm Accroides
4.	CHO 4	Sulphated Ethyl Cellulose
5.	CHO 5	Sulphated Gellan Gum
6.	CHO 6	Sulphated Alginic Acid
7.	CHO 7	Sulphated Xanthan Gum
8.	CHO 8	Sulphated Locust Bean Gum
9.	CHO 9	Sulphated Gum Arabic
10.	CHO 10	Sulphated Styrax
11.	CHO 11	Sulphated Starch
12.	CHO 12	Sulphated Inulin
13.	CHO 13	Sulphated Carboxymethyl Cellulose
14.	CHO 14	Sulphated I-Carrageenan
15.	CHO 15	Sulphated Amylopectin
16.	CHO 16	Sulphated Karaya Gum

No.	Sample ID	The original source of the modified carbohydrates
17.	CHO 17	Sulphated Guar
18.	CHO 18	Sulphated Agarose
19.	CHO 19	Sulphated Arabic Acid
20.	CHO 20	Sulphated Amylose
21.	CHO 21	Sulphated Polygalacturonic Acid
22.	CHO 22	Sulphated Pullulan
23.	CHO 23	Sulphated Levan
24.	CHO 24	Sulphated Paramylon (WAKO)
25.	CHO 25	Sulphated Fucogalactan (DEXTRA)
26.	CHO 26	Sulphated K-Carrageenan (DEXTRA)
27.	CHO 27	Sulphated Lambda-Carrageennan (DEXTRA)
28.	CHO 28	Sulphated Konjac Glucomannan (DEXTRA)
29.	CHO 29	Sulphated Chitosan (DEXTRA)
30.	CHO 30	Sulphated Psyllium Seed Gum (DEXTRA)
31.	CHO 31	Sulphated Welan (DEXTRA)
32.	CHO 32	Sulphated Sodium Alginate (DEXTRA)
33.	CHO 33	Sulphated Propylene Glycol Alginate (DEXTRA)
34.	CHO 34	Sulphated Taramind Gum
35.	CHO 35	Sulphated Tara Gum
36.	CHO 36	Gum Ghatti
37.	CHO 37	Gumm Accroides
38.	CHO 38	Ethyl Cellulose
39.	CHO 39	Alginic Acid
40.	CHO 40	Xanthan Gum
41.	CHO 41	Locust Bean Gum
42.	CHO 42	Gum Arabic
43.	CHO 43	Arabic Acid
44.	CHO 44	Amylose
45.	CHO 45	Laminarin
46.	CHO 46	Maltohexaose
47.	CHO 47	Maltopentose

No.	Sample ID	The original source of the modified carbohydrates
48.	CHO 48	K-Carrageenan (DEXTRA)
49.	CHO 49	Porcine mucosal heparin
50.	CHO 50	NAc heparin
51.	CHO 51	2-de-O-S heparin
52.	CHO 52	6-de-O-S heparin
53.	CHO 53	2-de-O-sulfated and N-acetylated heparin
54.	CHO 54	6-de-O-sulfated and N-acetylated heparin
55.	CHO 55	2 and 6 de-O-sulfated and N-sulfated heparin
56.	CHO 56	2 and 6 de-O-sulfated and N-acetylated heparin
57.	CHO 57	Arixtra
58.	CHO 58	Orgaran
59.	CHO 59	BLH (Bovine Lung Heparin)
60.	CHO 60	Tinzaparin
61.	CHO 61	EU Heparin
62.	CHO 62	Sulodexide
63.	CHO 63	DeNS Enoxaparin
64.	CHO 64	Heparan Sulphate (Porcine intestinal mucosa)
65.	CHO 65	Dalteparin
66.	CHO 66	Chondroitin Sulphate (4S)
67.	CHO 67	Enoxaparin
68.	CHO 68	Dextran sulfate
69.	CHO 69	Reviparin
70.	CHO 70	N-Propyl Heparin
71.	CHO 71	N-Butyl Heparin
72.	CHO 72	Chondroitin Sulphate (6S)
73.	CHO 73	Dermatan Sulphate De-N-sulphated (NH2)
74.	CHO 74	Protamine sulfate
75.	CHO 75	Hyaluronic Acid

4.2.2 The dose dependency effect of selected modified carbohydrates

From the first initial screening experiment performed in Section 4.2.1, only nine carbohydrates were chosen as a result of their significant effectiveness compared to the control and their original structure, for further analysis. Therefore, the concentration dependency effects of those selected carbohydrates were evaluated on CLCs line for respiratory burst activity and viability.

4.2.2.1 Respiratory burst activity

In this experiment different concentrations of each modified carbohydrate selected (i.e. CHO: 1, 2, 3, 5, 9, 22, 24, 26, 28) were examined for their effect on CLCs respiratory burst activity. Briefly, cells were placed at density 2 $\times 10^4$ cells/well and treated with serially diluted carbohydrates at concentrations of 1- 150 µg/ml for CHO 1, CHO 2, CHO 5 and CHO 9, and at concentrations between 0.5- 25 µg/ml for CHO 3, CHO 22, CHO 24, CHO 26 and CHO 28. The difference in concentration utilised depended on the availability of the carbohydrate for the test. Each experimental 96 well plate also comprised two positive controls i.e. different beta glucan sources, zymosan (Sigma, Z4250) (5 or 50 µg/ml), MacroGard® (5 or 50 µg/ml) and a negative control (5 µl of sterile water Sigma, W3500) in order to test the normality of the cells used.

The oxidative burst activity was measured using the NBT assay as described in Section 2.6.1 and plates were read at an optical density 620 nm after 24 h incubation. Data was analysed statistically using a one way ANOVA and Tukey's post-hoc analysis used to compare the dose response to the negative control with $p \leq 0.05$.

4.2.2.2 Cell viability

The concentration dependency of previous selected modified carbohydrates were examined for their effects on CLCs viability. For this purpose, a serial dilution of carbohydrates CHO 1, CHO 2, CHO 5 and CHO 9 at concentrations 1- 150 µg/ml were prepared and exposed to CLCs at a density 2×10^4 cells in 100 µl in 96 well plate. A negative control comprising 5 µl sterile water and positive controls, 50 µg/ml of MacroGard® and zymosan (Sigma, Z4250) were also included. After 24 h the MTT assay was performed as detailed in Section 2.5.1 and optical density read at 540 nm. One way ANOVA and Tukey's post-hoc test were used to analyse the data at p value ≤ 0.05.

4.2.2.3 Viable cells count using a haemocytometer

The previous protocols had based on activity in the appropriate tests utilised highlighted that one of the selected carbohydrate, CHO 1 was both active and non-toxic in CLC line. In order to further ascertain the lack of toxicity of this selected carbohydrate, a haemocytometer cell count was used to determine cell viability in CLCs utilising the trypan blue exclusion test as described in Section 2.2.2. Briefly, 500 µl of CLC lines 2×10^5 cells/ml in RPMI CLC+ medium were distributed in 24 well plate (Greiner bio-one, 662160) and three replicate wells were prepared for each treatments. Different concentrations of CHO 1 (1, 2.5, 50, 150 µg/ml) were prepared and 15 µl/well were added, a negative control comprising 15 µl/well of sterile water (Sigma, W3500) was also included. An additional control included adding 15 µl/well of MacroGard® at a concentration of 50 µg/ml. Plates were incubated for 24 h at 27℃ and 5% CO_2 incubator (L11738 Heto Cellhouse 170). After the incubation period, culture medium was aspirated

from all wells, and the cells were washed once with pre-warmed RPMI CLC+ medium. 250 µl of 0.25x Trypsin-EDTA (Sigma, T4049) was added to each well for 1 min until more than 90% of cells had detached. The wells were washed three times with 500 µl of RPMI CLC+ medium and cells collected in 15 ml tubes (Sarstedt, 62.554.001). The cells were then centrifuged at 750 x *g* for 5 min at 19±1℃ (Heraeus Megafuge 1.0R) and the supernatant discarded. The cell pellet was re-suspended in 500 µl of fresh CLC RPMI+. Cells count was carried out as described in Section 2.2.2. The data was analysed using a one way ANOVA and CHO 1 concentrations were compared to the negative control by Tukey's post-hoc test at p value ≤ 0.05.

4.2.3 The time and dose dependency effects of CHO 1 on CLC line

It was important to evaluate the exposure time of CHO 1 to induce the respiratory burst activity of CLCs, thus a dose and time response of CHO 1 were examined in the CLC line. The NBT assay utilised in 96 well plates, cells were distributed at 2×10^4 cells/well and treated with various concentration of CHO 1 between 1- 150 µg/ml at different incubation periods for 1, 3, 6 and 9 h. A negative control (5 µl sterile water) and MacroGard® at concentration 50 µg/ml were measured on each experimental microplate. The rest of NBT assay was performed as described in Section 2.6.1 and the soluble formazan blue were measured at an optical density of 620 nm. At each time point data were analysed using a one way ANOVA and Tukey's post-hoc analysis. A two way ANOVA and Bonferroni post-tests were used to compare the time response of each doses with significant defined as $p \leq 0.05$.

4.2.4 The dose effect of CHO 1 on pronephric cells suspension

This experiment was designed to establish if the effect of CHO 1 is reproducible in primary cells as in cell line. For this purpose, four carps at average weight 55.62 ± 11.9 g and average length 13.5 ± 1.1 cm were killed as described in Chapter 2 (Section 2.1) and the pronephros tissues isolated and prepared as mentioned in Section 2.2.1. Pronephros cell suspensions were counted, checked for their viability as described in Section 2.2.2, distributed in to 96 well plates at concentration 2×10^5 cells/well and exposed to CHO 1 at concentrations between 1- 150 µg/ml to determine its effects on pronephros cell viability and respiratory burst activity. Each experimental microplate included a negative control (5 µl/well of sterile water) and MacroGard® at 50 µg/ml. Both NBT and MTT assay were carried out after 24 h incubation as described in Sections 2.6.1 and 2.5.1 respectively. The data was statistically analysed using a one way ANOVA and Tukey's post-hoc test with p value ≤ 0.05.

4.3 Results

4.3.1 Modified carbohydrate screening

The first initial screening experiments carried out utilise the *in vitro* system to determine the effect of a range of modified carbohydrate on the respiratory burst activity of CLCs line. The NBT assay was performed for 24 h after cells were exposed to either 5 or 50 µg/ml of forty eight different modified carbohydrates. The results indicated only seventeen modified carbohydrates stimulated a significant increases in CLCs respiratory burst activity (Figures 4-2, 4-3 and 4-4).

Exposing CLCs to zymosan at 50 µg/ml (used as positive control) and CHO 1, CHO 2, CHO 3, CHO 5 (Figure 4-2 A) and CHO 14 (Figure 4-2 B) led to significantly elevated oxidative burst level after 24 h, and the both of CHO 1 (0.169 ± 0.031) and CHO 14 (0.218 ± 0.056) had oxidative burst level 2X greater compared to the control.

In addition, there were significant influences of CHO 18, CHO 19, CHO 22, CHO 24, CHO 25, CHO 26 (Figure 4-3 C); CHO 27, CHO 28, CHO 29, CHO 31, CHO 34 (Figure 4-3 D) and carbohydrate CHO 48 (Figure 4-4 F) on CLC cells respiratory burst in comparison to control. Nine modified carbohydrates that induced a highly significant increase in CLCs respiratory burst activity compared to that of the control and which had less complicated original structure (Table 4-2). Further concentration response were

examined to the selected modified carbohydrates in relevant to cell viability and respiratory burst activity, presented later in Section 4.3.2.

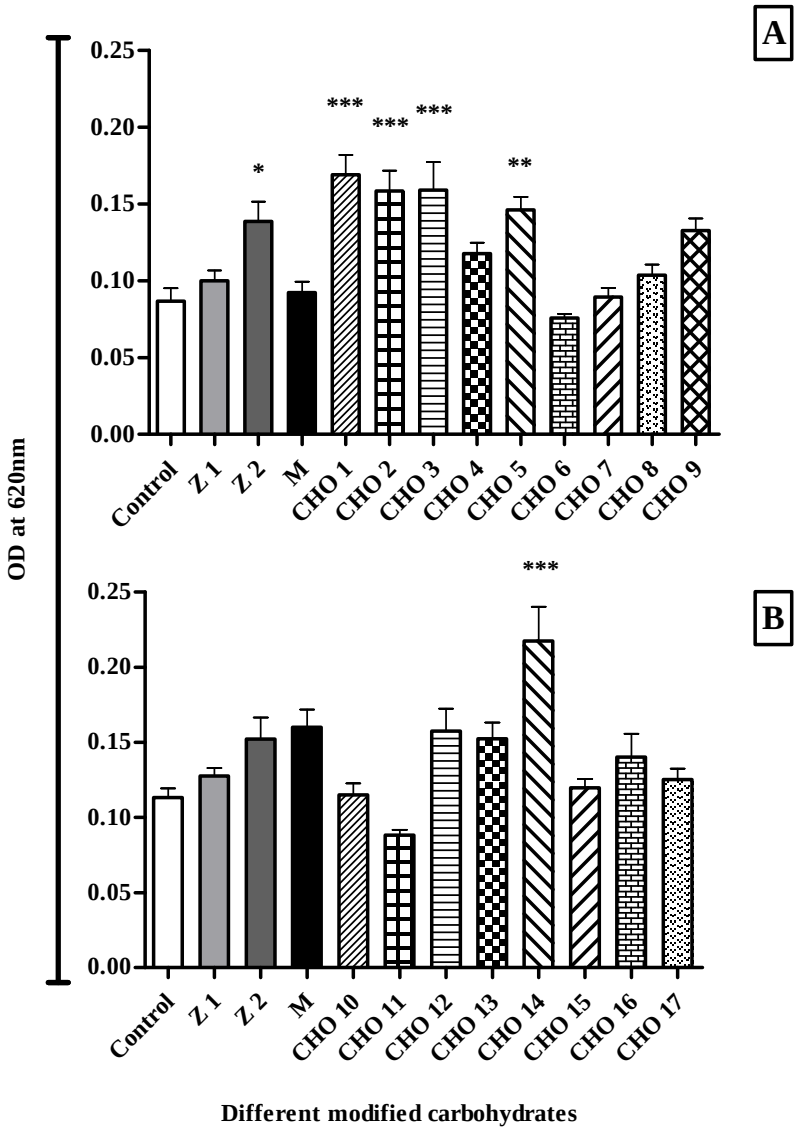


Figure 4-2: Modified carbohydrate screening effects utilised by NBT assay. CLC lines at density (2×10^4 cells/well) were stimulated with: zymosan at 5 µg/ml = **Z 1** and 50 µg/ml = **Z 2**; MacroGard® at 50 µg/ml = **M** and a range of modified carbohydrates at concentration 5 µg/ml (CHO: 3, 6, 7, 12, 13, 14, 15, 16, 17) or at 50 µg/ml (CHO: 1, 2, 4, 5, 8, 9, 10, 11) for 24 h incubation. Optical density at 620 nm was used to read the experimental plates. Statistical comparison was performed by one-way ANOVA and Tukey's post-hoc, the treatment differences in comparable to control performed with * = $p \leq 0.05$ ** = $p \leq 0.01$ and *** = $p \leq 0.001$. Bars represent mean ± SEM of six wells replicates. The letter **A** and **B** refer to different experiments.

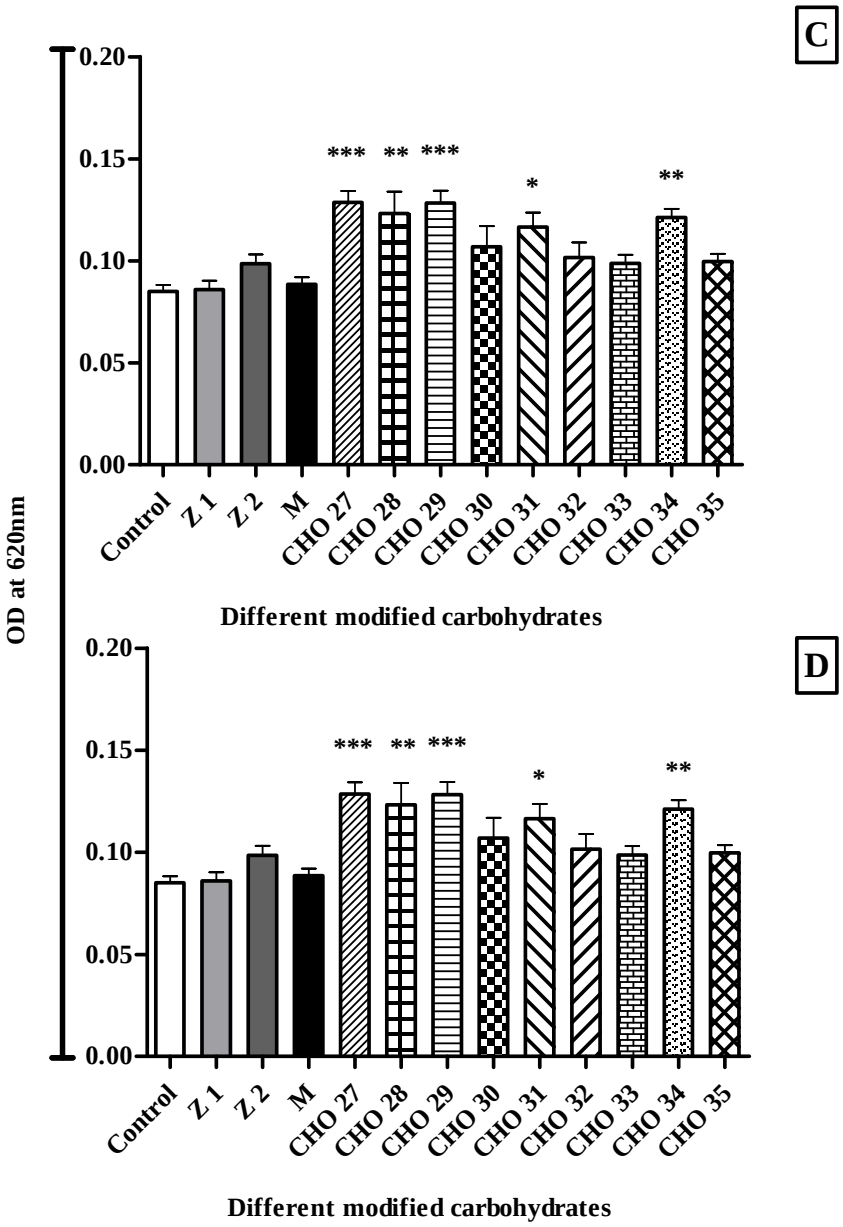


Figure 4-3: Screening different modified carbohydrate utilised by NBT assay. CLC lines were seeded at (2×10^4 cells/well) and stimulated with each of: zymosan at 5 µg/ml = **Z 1** and 50 µg/ml = **Z 2**; MacroGard® 50 µg/ml = **M** and range of modified carbohydrates at concentration 5 µg/ml for 24 h incubation. The cells respiratory burst level were determined at optical density 620 nm. The statistical significant differences in comparable to control performed with * = $p \leq 0.05$ ** = $p \leq 0.01$ and *** = $p \leq 0.001$. Data represent mean ± SEM of six wells replicates. The letter **C** and **D** refer to different experiments.

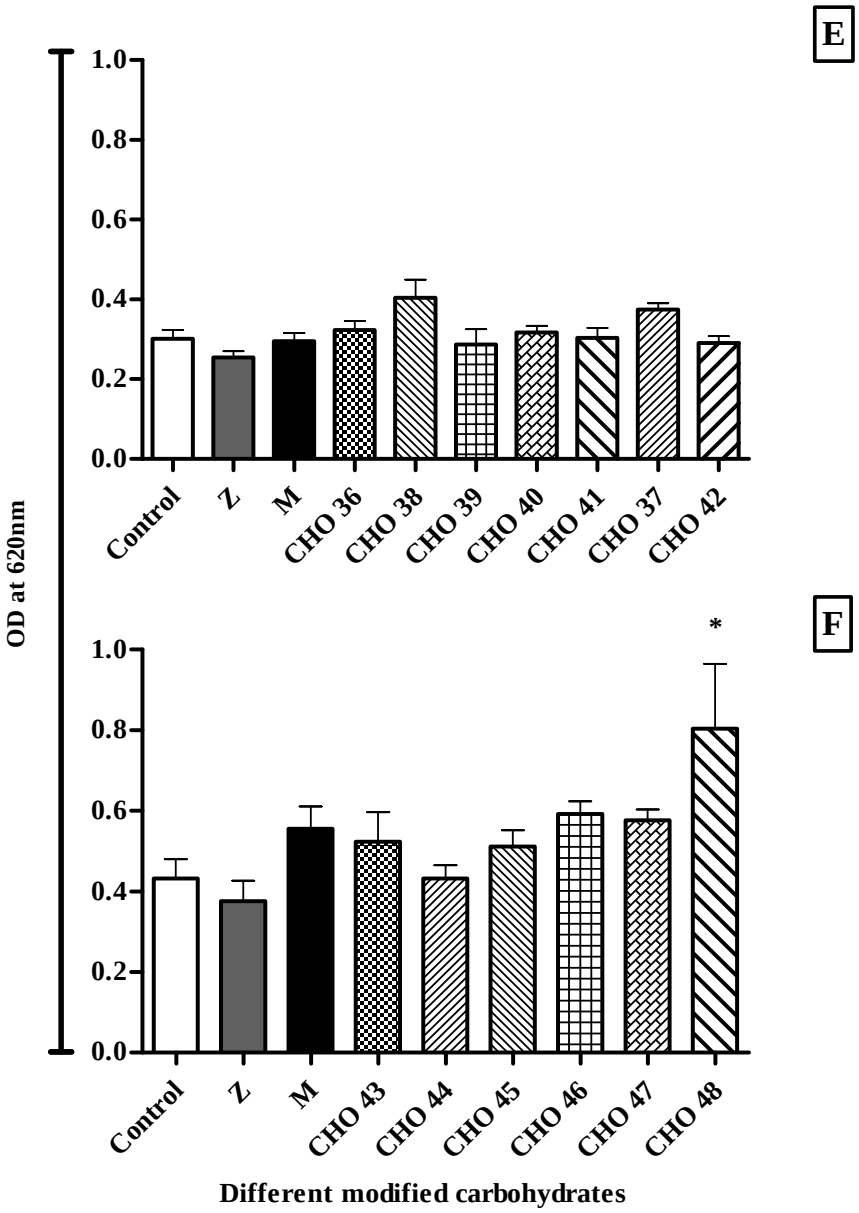
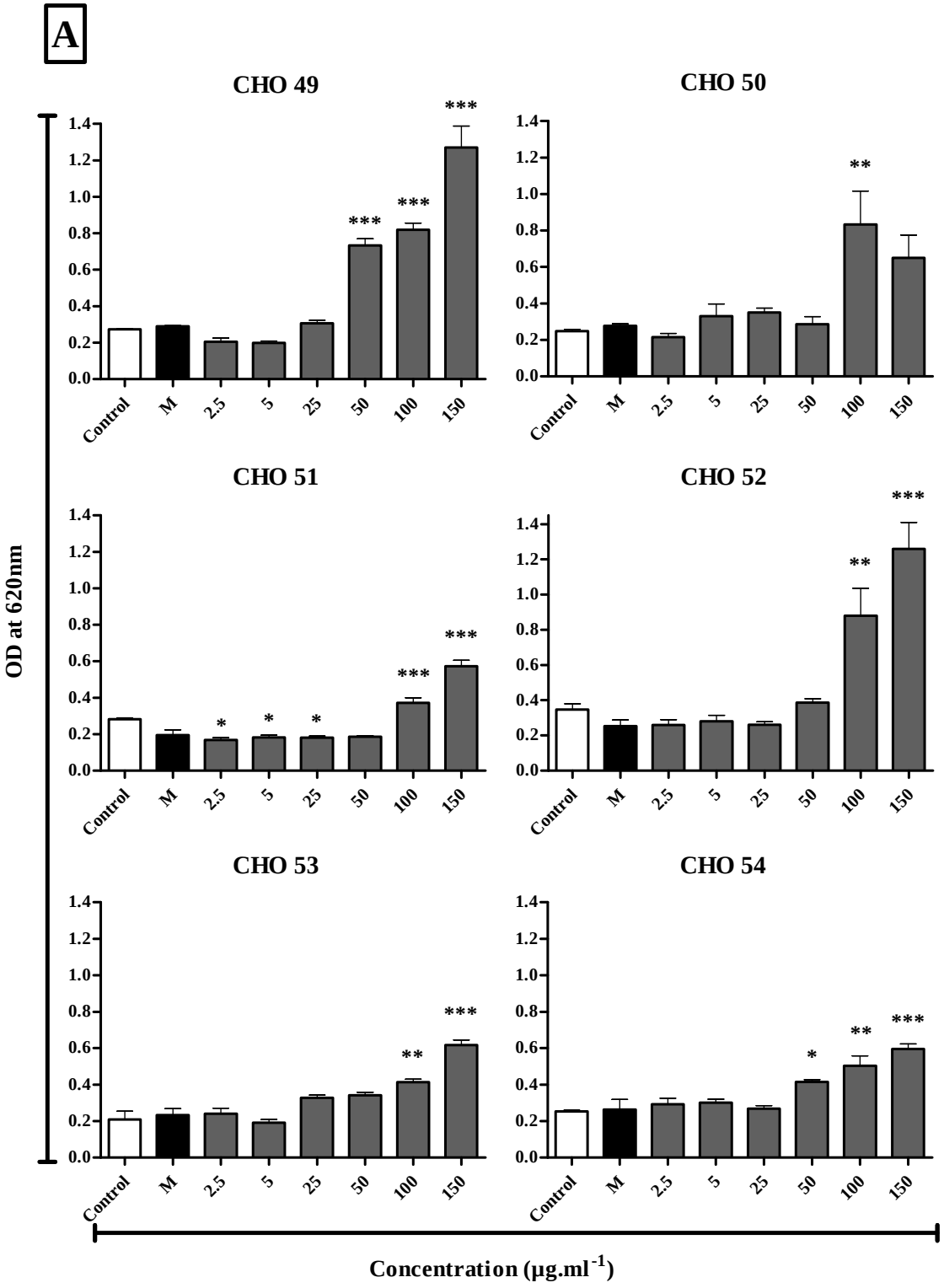


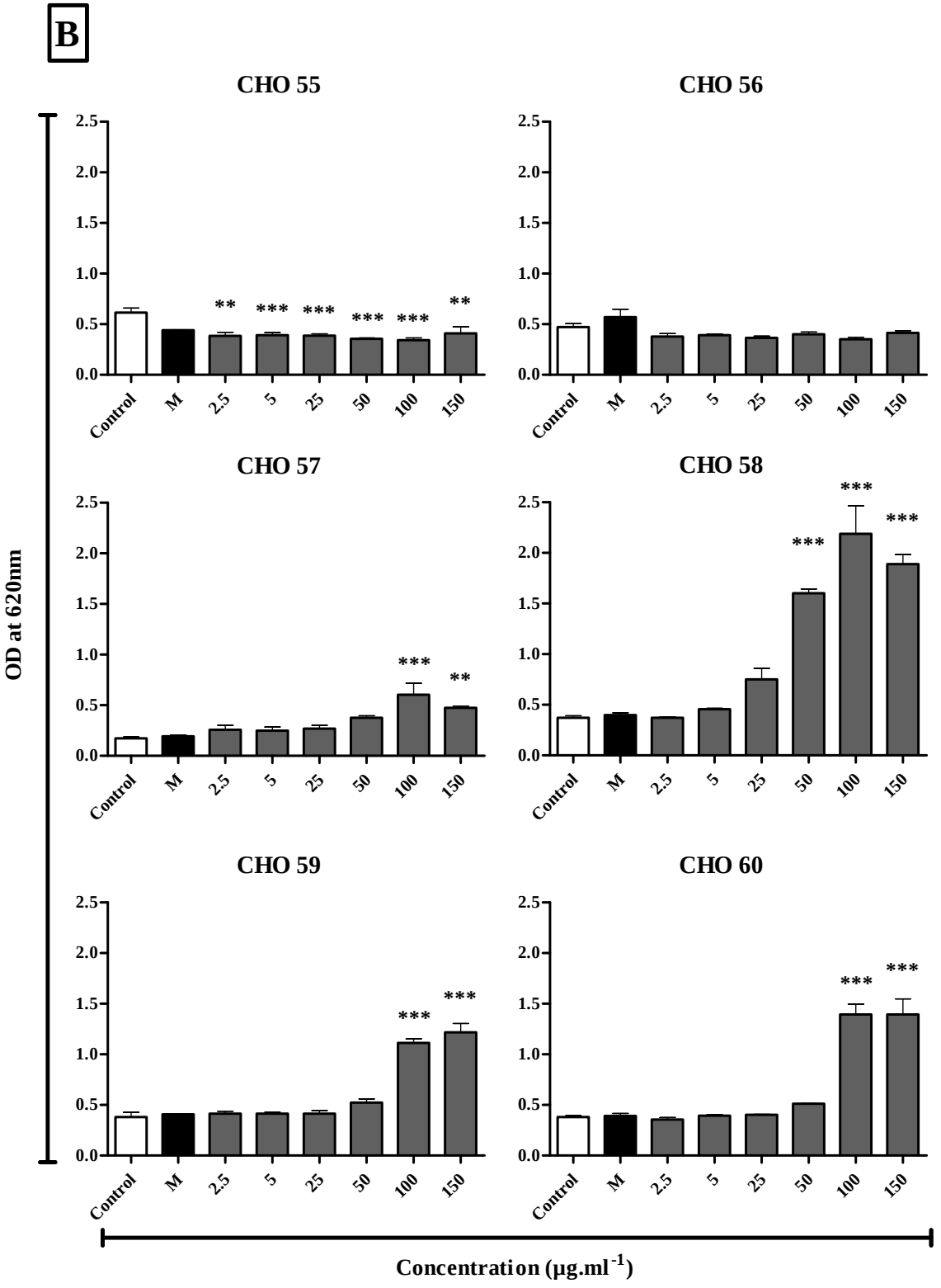
Figure 4-4: The effect of modified carbohydrate on CLCs oxidative burst activity. The CLC lines (2×10^4 cells/well) were stimulated with: zymosan at 50 µg/ml= **Z**; MacroGard® at 50 µg/ml= **M**; various modified carbohydrates at concentration 50 µg/ml and the NBT assay performed for 24 h incubation. One way ANOVA used to analyse the data, CHO 052 was significantly different in comparable to control with $p \leq 0.05$ (*). Data represent mean ± SEM of six wells replicates. The letter **E** and **F** refer to different experiments.

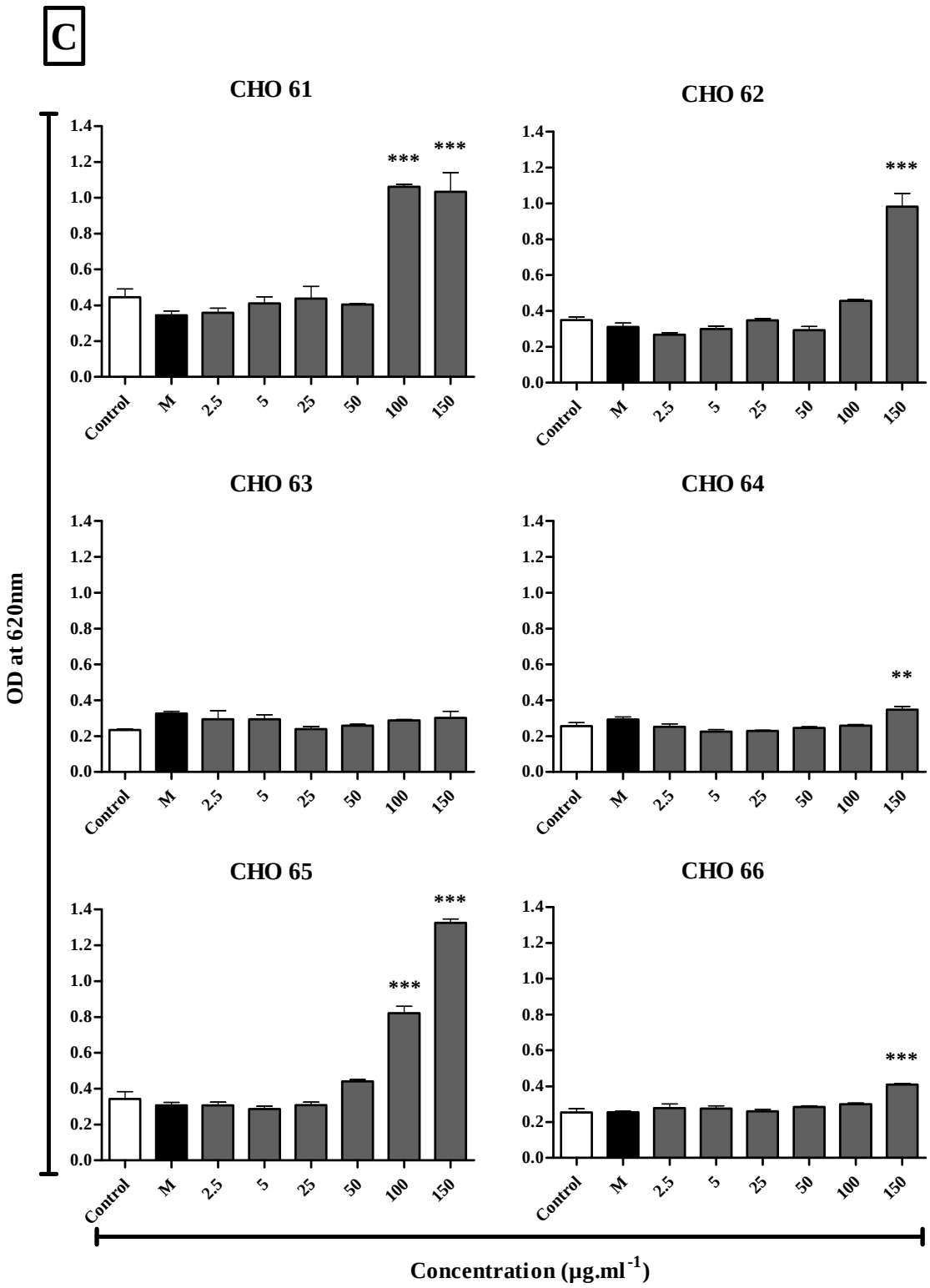
Table 4-2: Summary of the modified carbohydrates that had a significant effect in respiratory burst activity from figures 4-2, 4-3 and 4-4.

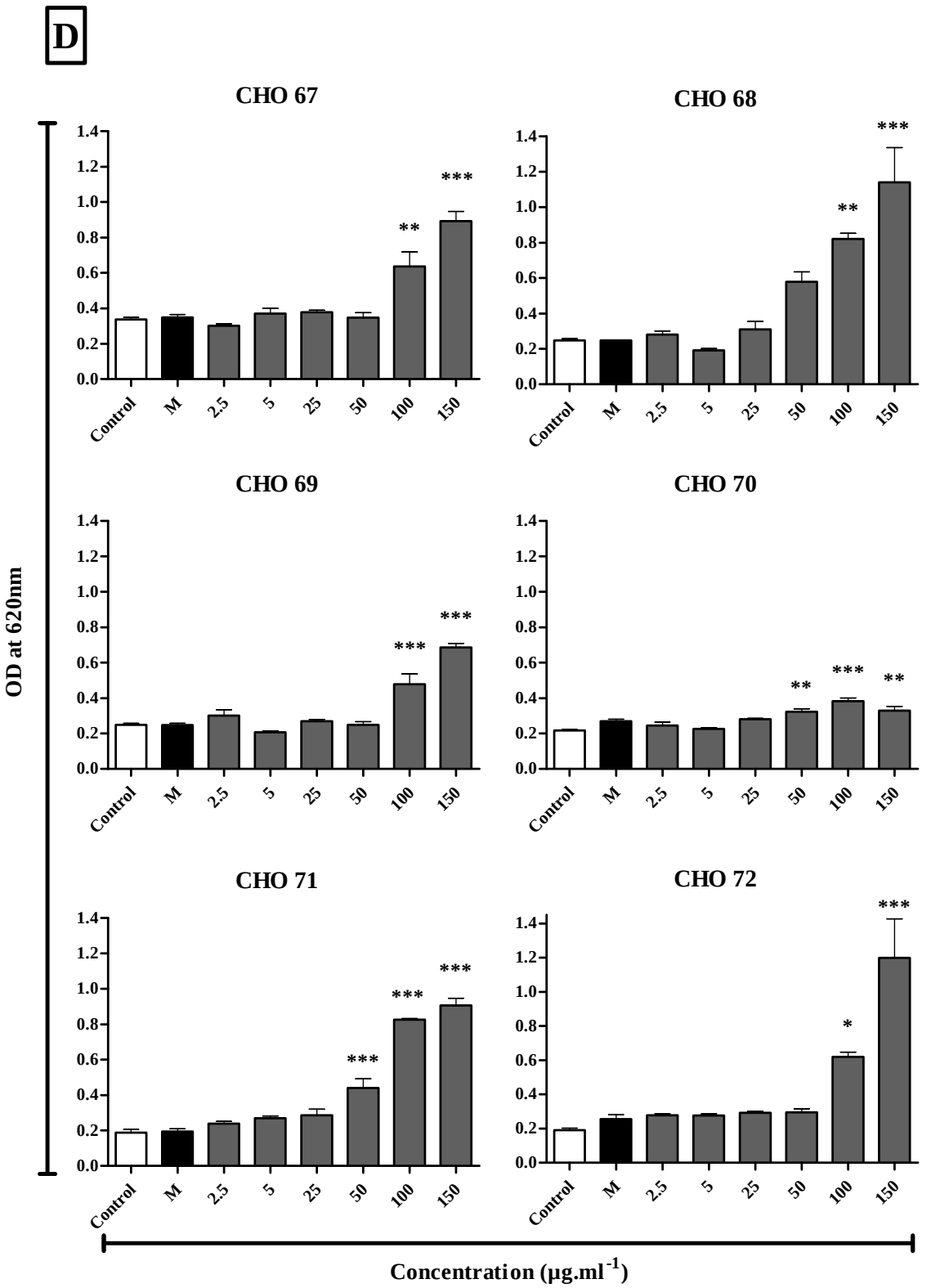
Modified carbohydrates used concentration	Modified carbohydrates
5 µg/ml	CHO 3, CHO 14, CHO 18, CHO 19, CHO 22, CHO 24, CHO 25, CHO 26, CHO 27 CHO 28, CHO 29, CHO 31, CHO 34
50 µg/ml	CHO 1, CHO 2, CHO 5, CHO 48

The sulphation degree of different carbohydrates was utilised to determine their dose response in comparable to the negative control (Figure 4-5). The result of analysing the 27 selected carbohydrates at greater range of concentrations revealed that the majority of tested carbohydrates induced an increase in NBT reduction at high concentrations, for example carbohydrate (CHO) 49, 54, 58, 70 and 71 were stimulated cells between concentrations 50- 150 µg/ml. Others i.e. CHO 50, 52, 53, 57, 59, 60, 61, 65, 67, 68, 69, 72, and 74 stimulated CLCs at doses 100 and/ or 150 µg/ml. In addition, carbohydrates CHO 62, 64, 66 and 75 stimulate increases in NBT reduction only at 150 µg/ml. In contrast, some of these carbohydrates CHO 51 and 55 caused a decrease in respiratory burst activity, whilst others had no effects i.e. CHO 56, 63 and 73 (see A- E Figures at 4-5).

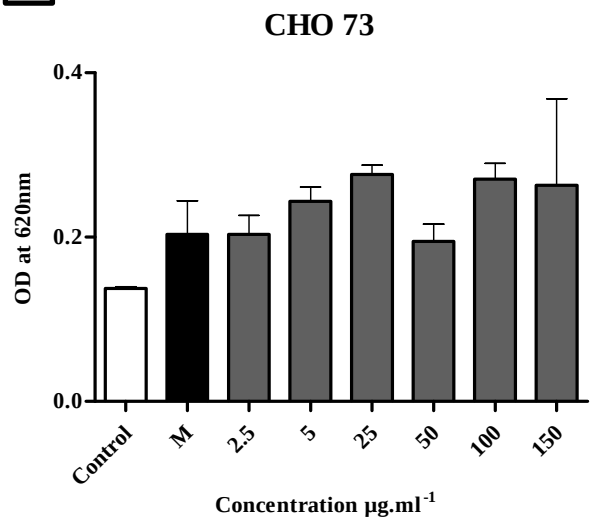

A
CHO 49
CHO 50
CHO 51
CHO 52
CHO 53
CHO 54
OD at 620nm
Concentration (µg.ml-1)
Control
M
2.5
5
25
50
100
150

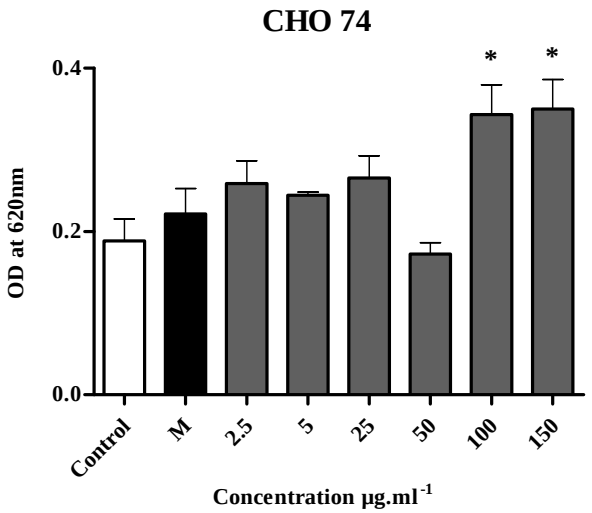

B
CHO 55
CHO 56
CHO 57
CHO 58
CHO 59
CHO 60
OD at 620nm
Concentration (µg.ml-1)
Control
M
2.5
5
25
50
100
150

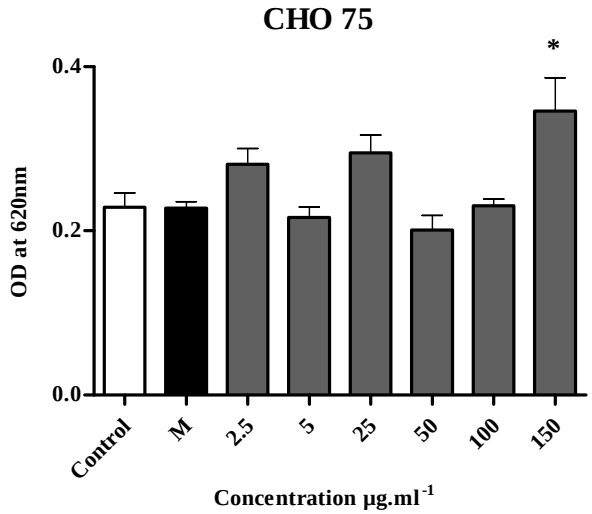

C
CHO 61
CHO 62
CHO 63
CHO 64
CHO 65
CHO 66
OD at 620nm
Concentration (µg.ml-1)
Control
M
2.5
5
25
50
100
150
***
**


D
CHO 67
CHO 68
CHO 69
CHO 70
CHO 71
CHO 72
OD at 620nm
Concentration (µg.ml-1)
Control
M
2.5
5
25
50
100
150

E


CHO 73
OD at 620nm
0.4
0.2
0.0
Control
M
2.5
5
25
50
100
150
Concentration µg.ml-1


CHO 74
OD at 620nm
0.4
0.2
0.0
*
*
Control
M
2.5
5
25
50
100
150
Concentration µg.ml-1


CHO 75
OD at 620nm
0.4
0.2
0.0
*
Control
M
2.5
5
25
50
100
150
Concentration µg.ml-1

Figures 4-5 A– E: Illustrate the dose response of various modified carbohydrates. CLC lines respiratory burst activity measured by NBT assay after cells were stimulated with MacroGard® at 50 µg/ml= **M** and range of modified carbohydrates at concentrations between 2.5-150 µg/ml over 24 h incubation. Cell were seeded at (2×10^4 cells/well) and optical density was read at 620 nm. Statistic comparison was performed using a one-way ANOVA and the differences between concentrations in comparable to control performed with *= $p \leq 0.05$ **= $p \leq 0.01$ and ***= $p \leq 0.001$. Bars represent mean ± SEM of three wells replicates. The letters **A** throw **E** is a different experimental plates test.

4.3.2 The response of CLC line to different concentrations of modified carbohydrates:

4.3.2.1 Respiratory burst activity

The pervious screening experiments presented in Figures 4-2, 4-3, 4-4 and 4-5 identified potential immunostimulant carbohydrates, therefore the dose dependency effects of selected modified carbohydrates were carried out to ascertain the immunostimulant effect and the response concentration. For that purpose, the CLCs were exposed to a serial dilution of selected modified carbohydrates (CHO 1, CHO 2, CHO 5 and CHO 9) at concentrations between 1- 150 µg/ml (Figure 4-6) and CHO 3, CHO 22, CHO 24 and CHO 26 at concentrations between 0.5- 25 µg/ml (Figure 4-7). The statistical analysis revealed a clear trend of significant increasing in CLCs respiratory burst activity after exposure to CHO 1 ($F = 68.668$), CHO 2 ($F = 13.051$) and CHO 5 ($F = 13.131$) all at p values less than (0.0001).

The concentration dependency effects of CHO 1 raised significantly at concentrations i.e. 25 µg/ml ($p \leq 0.022$), 50, 75 and 150 µg/ml ($p < 0.0001$), and the concentration 150 µg/ml induced an increase in respiratory burst activity four and half times higher than the control (Figure 4-6).

Moreover, a significant increase in oxidative burst level appeared after the exposure of both modified carbohydrates CHO 2 at concentrations 75 and 150 µg/ml ($p < 0.0001$) and CHO 5 at concentrations 50 µg/ml ($p \leq 0.009$), 75 µg/ml ($p < 0.0001$) and 150 µg/ml ($p < 0.011$). In contrast, there was no significant increase in respiratory burst activity associated with the concentration dependency of modified carbohydrate CHO 9.

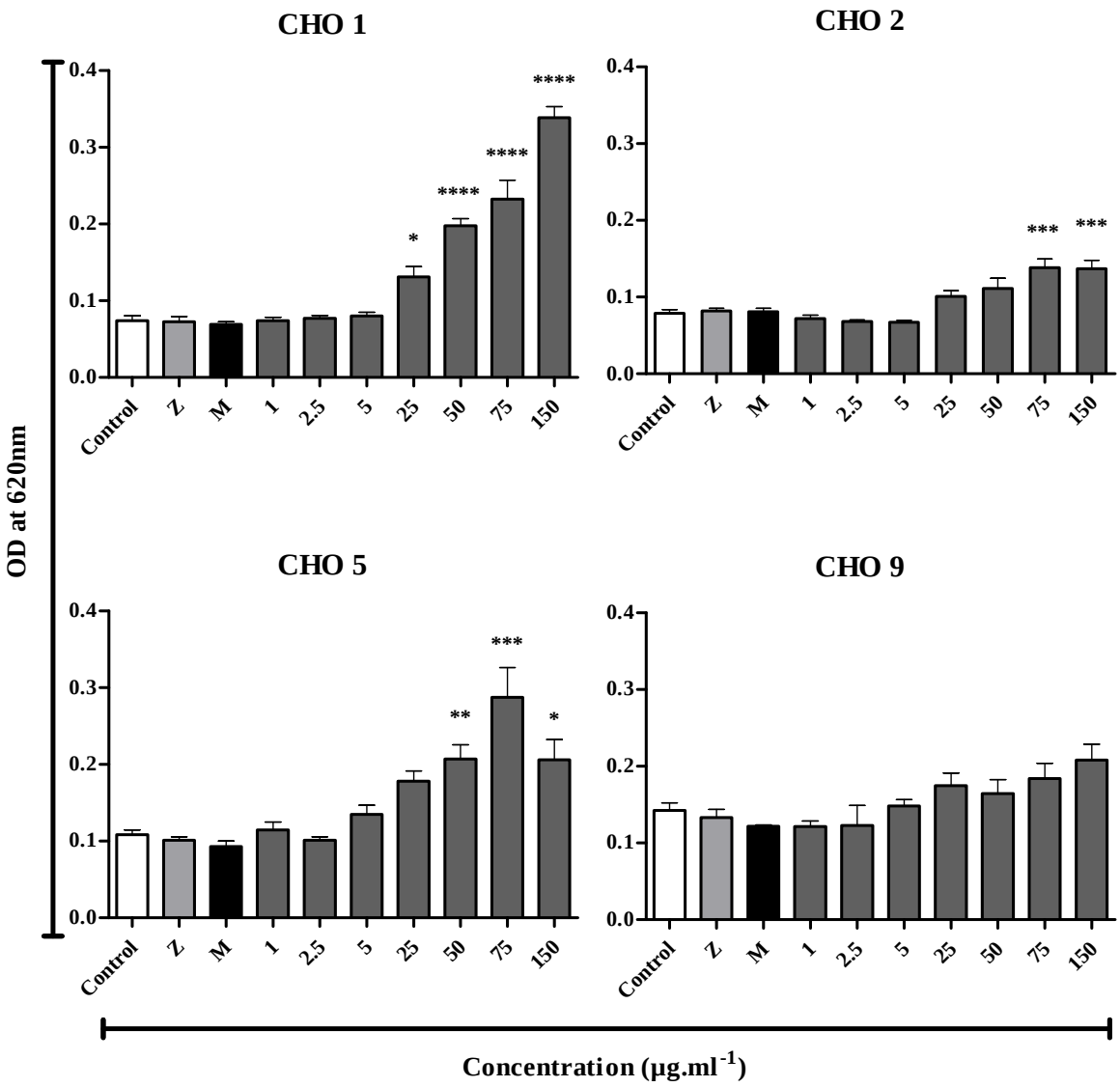


Figure 4-6: The dose response effect of modified carbohydrates on CLCs respiratory burst level. Cell were seeded at (2×10^4 cells/well) and stimulated with zymosan at 50 µg/ml= **Z**, MacroGard® at 50 µg/ml= **M** and range of modified carbohydrates at concentration between 1- 150 µg/ml for 24 h incubation. Statistical analysis one-way ANOVA and Tukey's post-hoc analysis were used and the differences between concentrations in comparable to control (non- treated cells) performed with *= $p \leq 0.05$, **= $p \leq 0.01$ and ***= $p \leq 0.0001$. Data represent mean ± SEM of six wells replicates.

Further statistical tests revealed the significant effect of serial diluted modified carbohydrates on CLCs respiratory burst activity (Figure 4-7). There was no clear concentration response trend of the effects of CHO 3, CHO 22, CHO 26 and CHO 28 on CLCs respiratory burst activity (Figure 4-7). Although, the concentration dependency of CHO 3 induced an increase in cells oxidative burst activity ($F = 17.364$, $p < 0.0001$), which started at 5 µg/ml and continued at 15 and 25 µg/ml, but the limitation in CHO 3 stock concentration made using higher concentration impossible.

Furthermore, only one concentration of CHO 22 (10 µg/ml) and CHO 28 (25 µg/ml) recorded a significant increase in CLCs respiratory burst activity compared to the control. Low doses of CHO 26 (1-10 µg/ml) stimulated significant respiratory burst activity ($F = 9.818$, $p < 0.0001$) although higher concentrations had no effects.

In contrast, CHO 24 affected CLCs respiratory burst activity ($F = 37.507$, $p < 0.0001$) reaching the optimum level at 10 µg/ml (0.209 ± 0.028) compared to control (Figure 4-7).

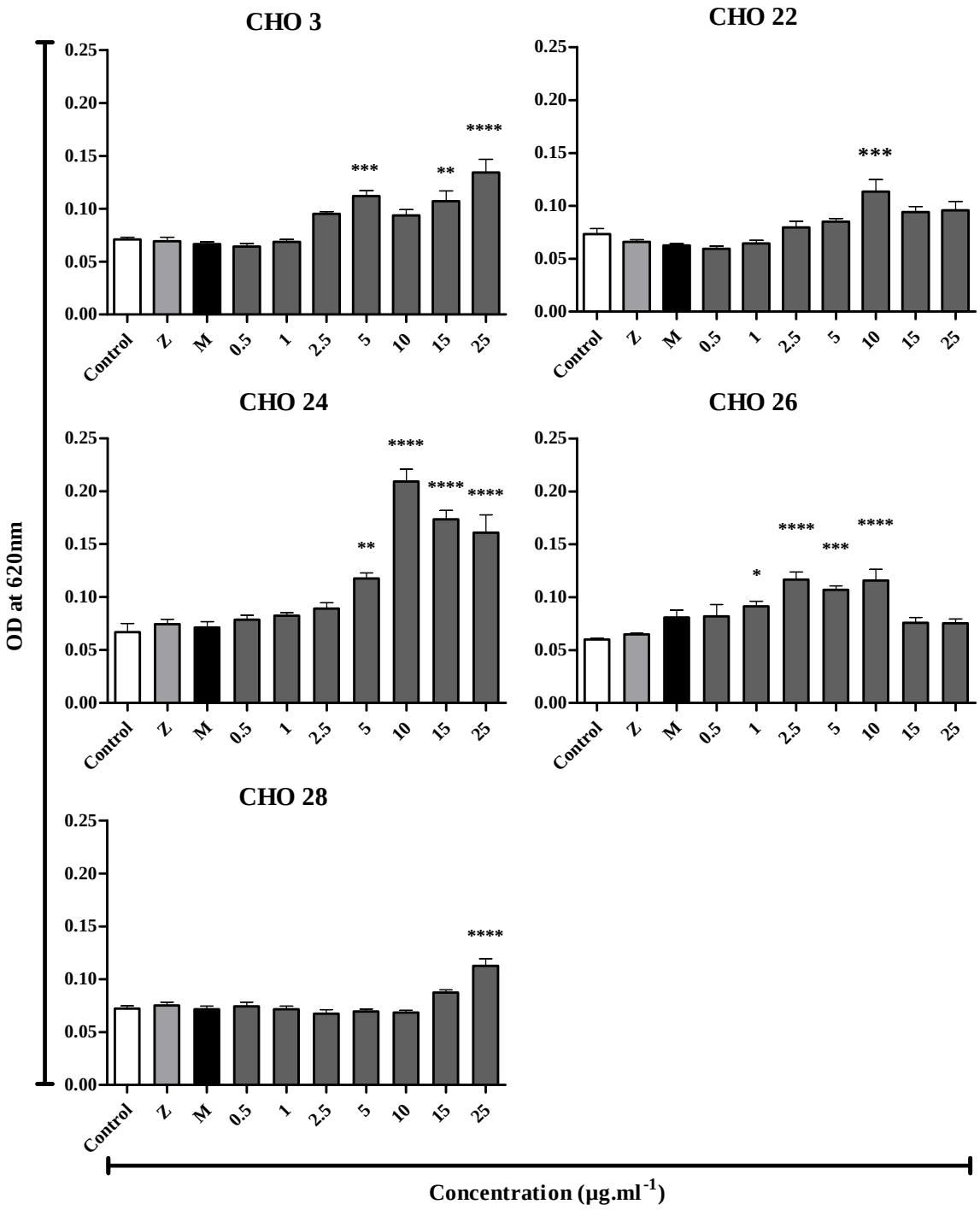


Figure 4-7: The reduction in formazan blue after dose dependency effect of modified carbohydrates on CLC lines. Cells were stimulated with zymosan at 50 µg/ml= **Z**, MacroGard® at 50 µg/ml= **M** and range of modified carbohydrates at concentration between 0.5-25 µg/ml for 24 h incubation. Statistical analysis one-way ANOVA and Tukey's post-hoc analysis were used ($p \leq 0.05$) and the differences between concentrations in comparable to control performed with * = $p \leq 0.05$, **= $p \leq 0.01$, ***= $p \leq 0.001$ and ****= $p \leq 0.0001$. Data represent mean ± SEM of six wells replicates.

4.3.2.2 Cell viability

- **MTT assay**

To ascertain that the dose dependency of modified carbohydrate were not cytotoxic to the CLC line, the viability test MTT assay was performed on CLC line after exposure to different concentrations (1- 150 µg/ml) of CHO 1, CHO 2, CHO 5 and CHO 9 for 24 h. Both of CHO 1 and CHO 9 did not reduce cell viability in CLC lines (Figure 4-8) and particularly, CHO 1 at concentrations 1 and 2.5 µg/ml ($P \leq 0.0011, 0.002$ respectively) actually promoted an increase in cell viability.

In contrast, a significant reduction in CLC lines viability occurred after exposure to CHO 2 at concentration 150 µg/ml and CHO 5 at concentrations between (1-150 µg/ml) see Figure 4-8. From the previous results, the modified carbohydrate CHO 1 was chosen for further analysis to ascertain the immunomodulatory effect of this carbohydrates.

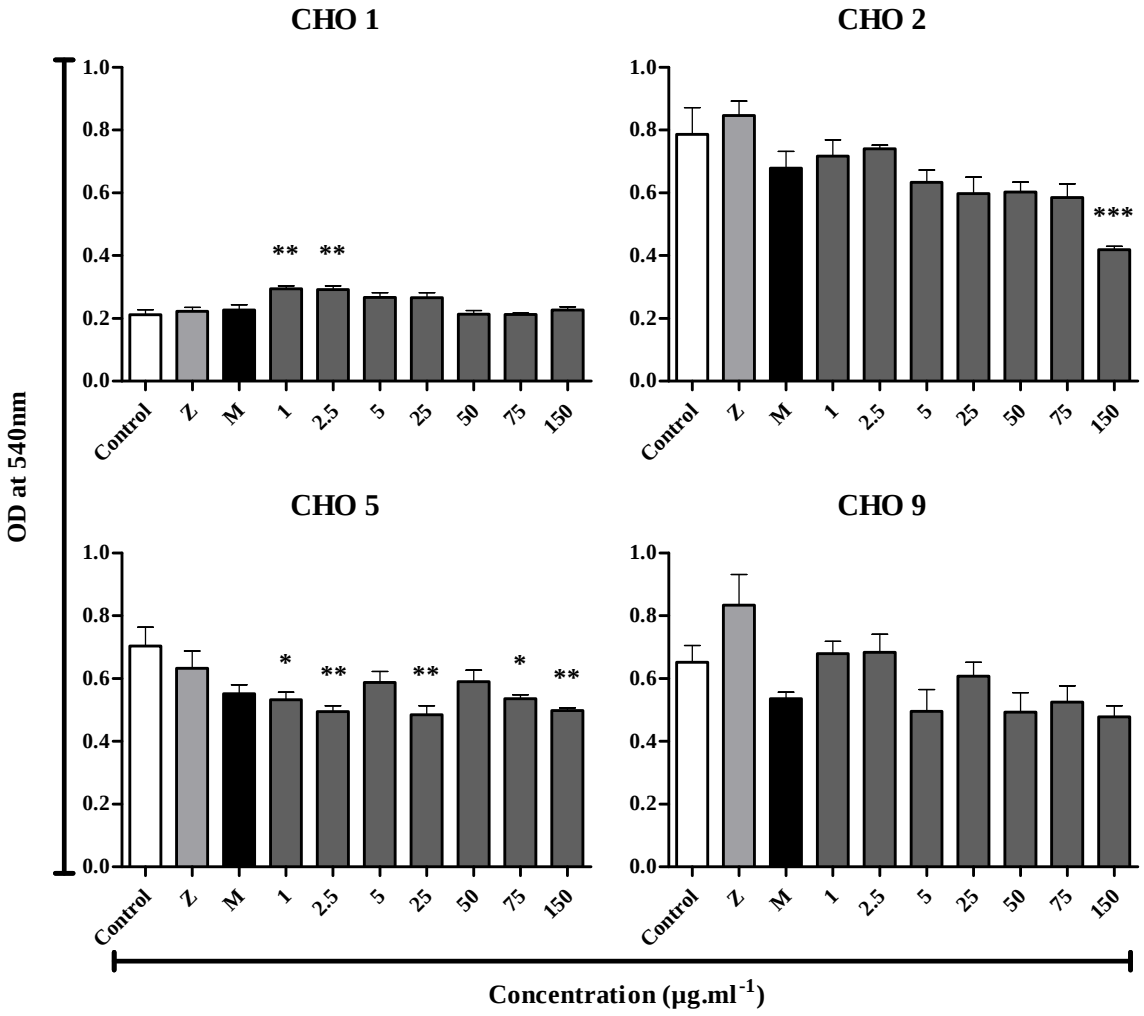


Figure 4-8: Viability of CLC line exposed to serial dilutions of modified carbohydrates. Cells at density (2×10^4 cells/well) stimulated with zymosan = **Z**, MacroGard® = **M** at 50 µg/ml and range of modified carbohydrates concentrations 1-150 µg/ml for 24 h. Statistic comparison was performed using a one-way ANOVA ($p \leq 0.05$) and the significant differences between treatments in comparison to control performed with *= $p \leq 0.05$, **= $p \leq 0.01$ and ***= $p \leq 0.001$. Data represent mean ± SEM of 6 wells replicates.

- **Vital dye staining (trypan blue)**

In order to confirm the previous result which revealed a the non-toxicity of modified carbohydrate CHO 1 on CLCs line, the cells (1×10^5 cells/well) were treated with MacroGard® at 50 µg/ml and CHO 1 at various concentrations (1, 2.5, 50 and 150 µg/ml) for 24 h incubation. Cells were harvested and counted after staining using trypan blue dye, the viable cells (non-stained) were counted and the analysed result revealed a significant increase in viable cell number after exposure to 2.5 µg/ml CHO 1 ($p \leq 0.026$) (Figure 4-9). Moreover, there was no effect compared to the control at the other concentrations of MacroGard® and CHO 1 utilised.

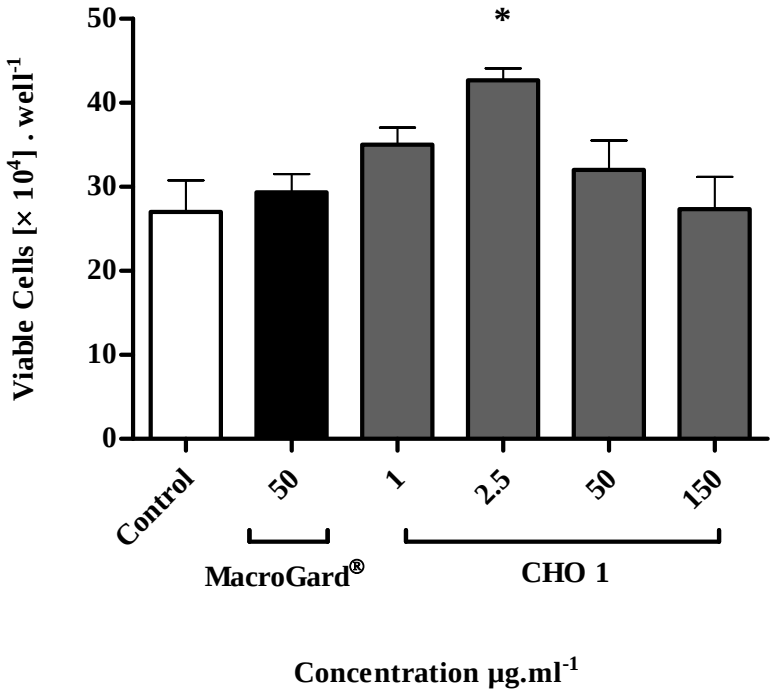


Figure 4-9: The CLC line count utilised by trypan blue viability assay. Cells were exposed to serial dilution of modified carbohydrates CHO 1 (1-150 µg/ml) and MacroGard® at 50 µg/ml concentration for 24h. Statistic comparison was performed using a one-way ANOVA ($F= 4.019$, $p \leq 0.022$) and the significant differences between treatments in comparison to control performed with *= $p \leq 0.05$. Data represent mean ± SEM of 3 wells replicates of 24 well plates.

4.3.3 The time and dose dependency effects of CHO 1 on CLC line

To further clarify the exposure period that the CLC line needs to be exposed to CHO 1 to induce NBT reduction, cells were subjected to different CHO 1 concentrations (1- 150 µg/ml) and incubated for four periods 1, 3, 6 and 9 h. The statistical analysis revealed that CHO 1 affect respiratory burst activity in CLC line after 1 ($F = 14.84$, $p < 0.001$), 3 ($F = 15.51$, $p < 0.001$) and 9 ($F = 18.63$, $p < 0.001$) hours incubation. In addition, CHO 1 concentrations 50, 100 and 150 µg/ml stimulated CLC line after 1, 3 and 9 h incubation. There were no significant dose effects of CHO 1 at 6 h incubation (see Figure 4-10).

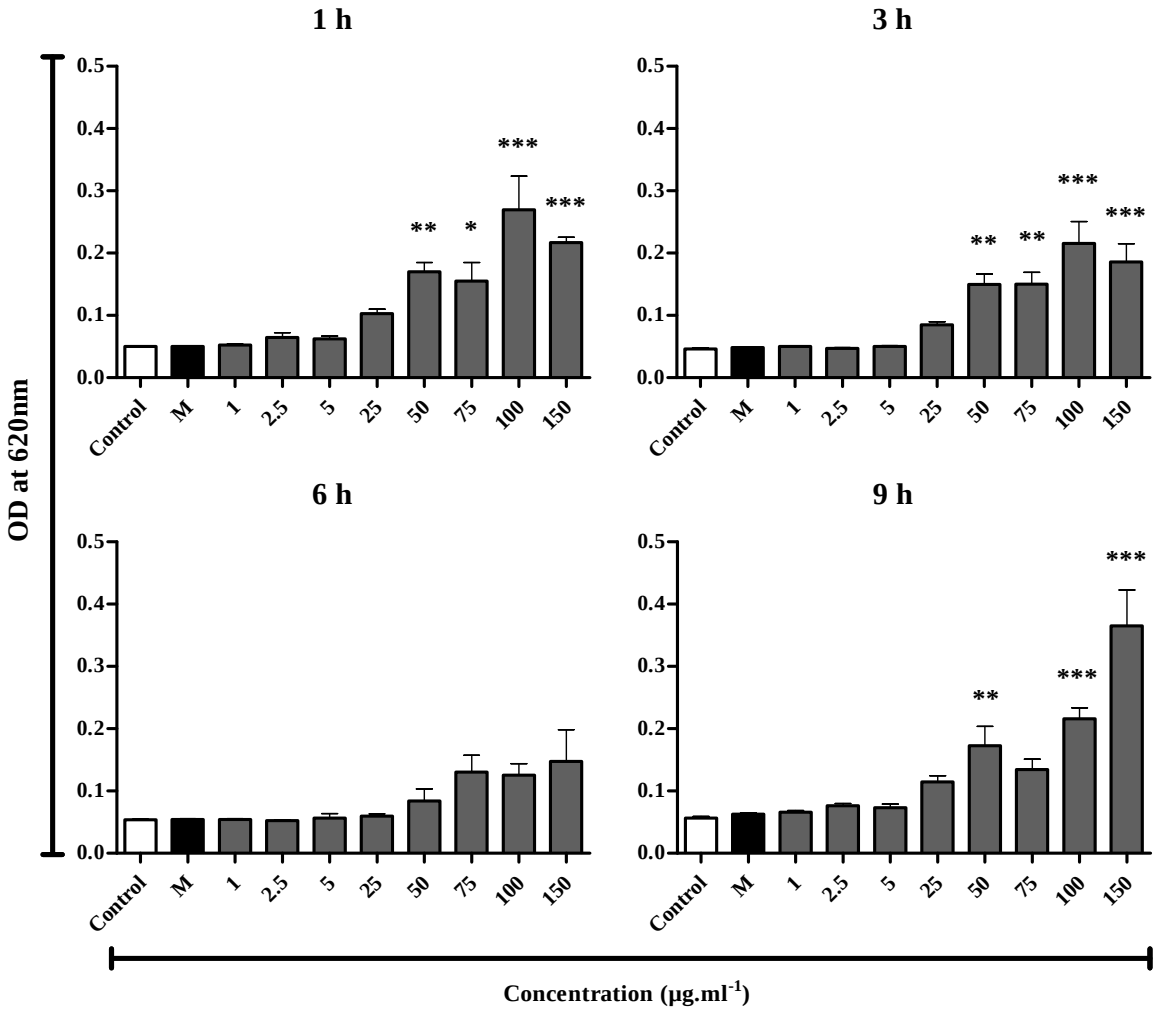


Figure 4-10: The time and dose response effect of CHO 1 on the respiratory burst activity of CLCs line. CLC line at density (2×10^4 cells/well) were exposed to MacroGard® at 50 µg/ml concentration and different CHO 1 concentration (1-150 µg/ml) for either 1, 3, 6 or 9 h incubation periods. The significant differences from the control performed as *= $p \leq 0.05$, **= $p \leq 0.01$ and ***= $p \leq 0.001$. The data are displayed as mean ± SEM of six wells replicates.

4.3.4 The concentrations effects of CHO 1 on pronephric cell suspension

This investigation expected if the effect noted for CHO 1 on a cell line could be replicated in a primary cell suspension, pronephric cells isolated from carp were utilised. CHO 1 influenced the NBT reduction in pronephros cells ($F= 55.83$, $P < 0.0001$) and significantly increased activity compared to the control at concentration of 25 µg/ml with stimulants obtained 2x greater than the control value at 150 µg/ml (3.094 ± 0.236) (Figure 4-11). Furthermore, the viability of pronephric cells, measured with the MTT assay (Figure 4-11), was not significantly affected by the concentrations were used. These results illustrated the reproducible effects of CHO 1 in pronephric cells to those seen in the CLC line, therefore highlighting the possibility of having the same effects in live fish.

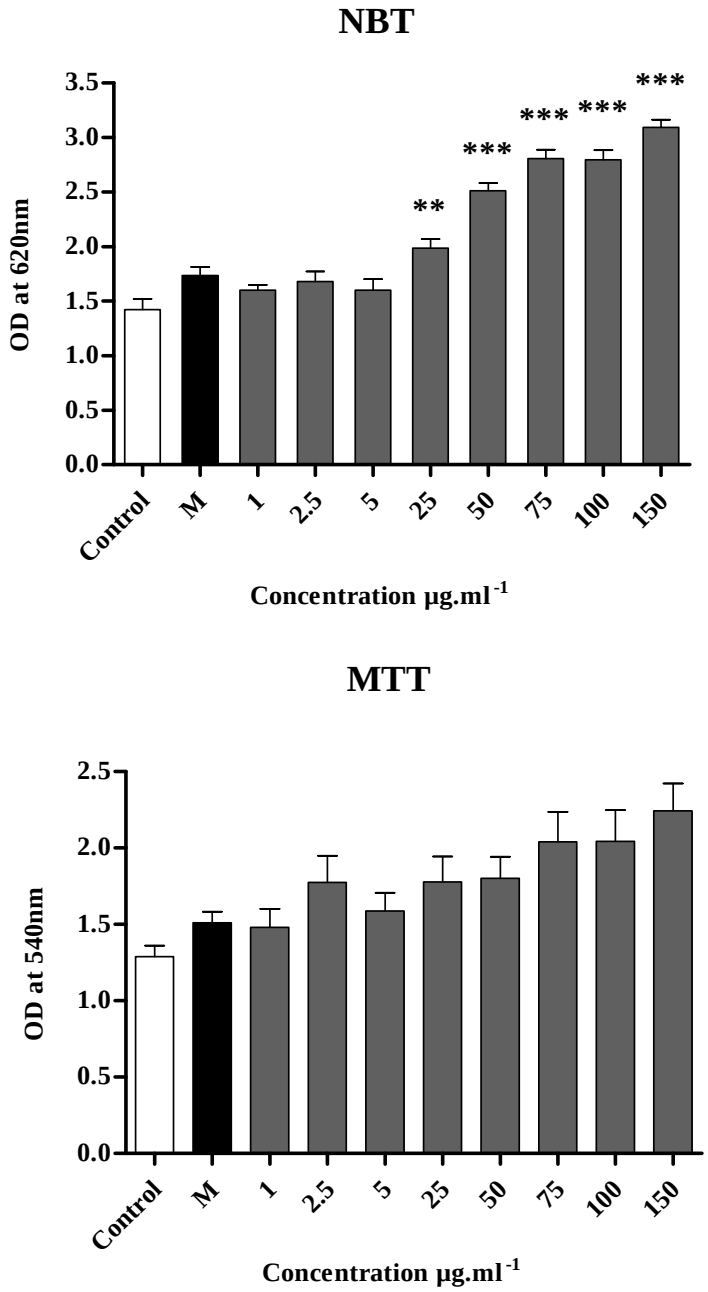


Figure 4-11: The dose response of pronephric cells to varying concentrations of CHO 1. The dose effect of CHO 1 on pronephric cells ascertained using NBT and MTT assays after 24 h incubation. Pronephric cells at density of 2×10^5 cells/well were exposed to MacroGard® at 50 µg/ml concentration and different CHO 1 doses (1-150 µg/ml). The significant differences from the control performed as **= $p \leq 0.01$ and ***= $p \leq 0.001$. The data are displayed as mean ± SEM of 3 replicate wells for 4 fish.

4.4 Discussion

4.4.1 Identification for the effective modified carbohydrates

The first part of this chapter aimed to identify the most immunostimulatory modified carbohydrate using the *in vitro* system developed earlier in Chapter 3. Thus systems complies with the 3R's philosophy for experimental design in the use of animals for experimental research (i.e. Replacement, Reduction and Refinement). Evaluation of immunostimulants was first performed using an *in vitro* system to measure the cellular and humoral immune mechanism. For example, two lympho-haematopoietic stromal cell lines derived from the spleen (trout splenic stroma, TSS) and the pronephros (trout pronephric stroma-2, TPS-2) of rainbow trout (*Oncorhynchus mykiss*), as well as in primary cultures of rainbow trout head kidney macrophages were used as *in vitro* system to determine the inflammatory responses of the previous cells after their exposure to the immunostimulants LPS, levamisole and poly I:C (Fierro-Castro et al., 2013). The most promising carbohydrates could be subjected to *in vivo* evaluation based on immune parameters such as phagocytosis, antibody production, free radical production, lysozyme activity, natural cytotoxic activity, complement activity, macrophage activating factor (MAF), nitroblue tetrazolium reaction (NBT), etc. (Kum and Sekkin, 2011).

The respiratory burst activity was determined in CLCs after exposure to different sulphated polysaccharides. These modified carbohydrates were labelled with codes and the sources were unknown until the results were obtained. Each experimental microplate comprised wells incorporating zymosan and/or MacroGard®, which acted as a control for the capability of each individual cell preparation to respond to an immunostimulant. The first screening was established using one concentration of those modified carbohydrates either at 5 or 50 µg/ml and compared to the control cells that the source of the carbohydrate affected the results obtained. Significant differences were observed for the sulphated

polysaccharides produced from gum (CHO: 2, 3, 5, 19, 31); seaweeds (CHO: 14, 18, 25, 26, 27) and starches (CHO: 22, 24), also each of tylose (CHO 1), konjac (CHO 28) and chitosan (CHO 29) induced significant respiratory burst activity in CLCs line. Some of these polysaccharides in a sulphated and/or non-sulphated form are ingredients or used as an additive in food manufacture, or used in pharmaceutical and drugs production because of their physical properties such as emulsification (Deshmukh et al., 2012). In addition, they have been previously characterised with potent immunomodulatory properties, for example different molecular weight polysaccharides isolated from *Juniperus scopulorum* induced a potent immunomodulatory activity in murine macrophages for the high molecular weight 200 and 680 kDa, polysaccharides through enhancing the respiratory burst, nitric oxide production and induce macrophages to secrete inflammatory and anti-inflammatory cytokines (Schepetkin et al., 2005). In a study on white shrimp, the oral administration of polysaccharides extracted from *Panax ginseng* (GSP group) at levels of 0.4 g/kg induced an increase in immune enzyme activity and modified immune gene expression after 84 days of feeding (Liu et al., 2011). Gopalakannan and Arul (2006) reported feeding common carp for 90 days with feed containing chitin (1%), chitosan (1%), levamisole (250 mg/kg of feed) stimulated the lysozyme activity, NBT reduction and enhanced the relative percentage survival (RPS) after the challenge with *Aeromonas hydrophila*. In another investigation, the effect of sulphated Konjac glucomannan was determined on human T cells line, and revealed that sulphated konjac had a potent anti HIV activity at concentrations of 1.2- 1.3 µg/ml which was almost as high as that of an acquired immune deficiency syndrome (AIDS) drug (Bo et al., 2013).

Although the remaining modified carbohydrates from the first screening did not show significant differences in respiratory burst activity compared to the control this may be because of the concentration used, the choice of incubation periods or the substance did not

have an immunomodulation property. Therefore the next screening carried out determined the dose responses of CLCs line exposed to more modified carbohydrates. Interestingly, most of the tested modified carbohydrates in Figure 4-5 induced a significant respiratory burst activity in CLCs line at high concentration between 50- 150 µg/ml except for CHO 55 which induced significant decrease in respiratory burst activity compared to control, also each of CHO 56, 63, 73 did not affect CLCs respiratory burst activity. Using the correct doses of immunostimulants is very important to achieve the desire result as mentioned in previous discussion Section 3.4.2. Several investigations have revealed that the high concentration of immunostimulant enhanced the immune responses and fish survival. For instance, *in vitro* trials revealed that the high doses (100- 500 µg/ml) of beta glucan promoted an increase phagocytic activity in turbot and gill head pronephros cells (Castro et al., 1999). Likewise *in vivo* investigation showed that the intraperitoneally injection of 500 µg of beta glucan enhanced the Relative Percent Survival (RPS) in common carp, also injected fish with glucan at (100, 500, 1000 µg/ml) concentrations caused an increase in total blood leukocytes counts, neutrophils, monocytes, and elevated the expression of interleukin-1beta on the 7th day post injection (Selvaraj et al., 2005). In contrast, Sakai (1999) reported that the effect of immunostimulants are not directly dose dependant and at high concentration may inhibit the immune responses. A similar conclusion was found when macrophages from Atlantic salmon (*Salmo salar* L.) were treated for 24 h with yeast beta glucan, the low doses 0.1 and 1 µg/ml induced respiratory burst activity while 10 µg/ml had no effect and 50 µg/ml was inhibitory (Jorgensen and Robertsen, 1995).

4.4.2 The dose responses of CLCs to selected modified carbohydrates

The initial screening examination helped to conclude the selection of nine modified carbohydrates to determine their dose effect on cell viability and respiratory burst activity. The dose effect of CHO 1, 2, 3, 5, 9, 22, 24, 26 and 28 on CLCs respiratory burst activity was measured after 24 h incubation, and a significant dose response were observed with all of selected modified carbohydrates except for the CHO 9 which did not induce the respiratory burst activity in CLCs line. Interestingly, CHO 1 promoted a rapid increase in respiratory burst activity which started at 25 µg/ml concentration and reached more than four and half times higher than the control. This linear dose/ effect relationship is unusual for immunostimulant, for most often the effect occurs at certain intermediate concentrations and disappears or even become toxic at high concentrations (Kum and Sekkin, 2011). This result led to investigate whether this steep increase was not caused by an over-stimulation resulting in exhaustion to the immune cells. The dose effect of CHO 1 were tested on CLCs viability, and no cytotoxic effects was observed after 24 h incubation, in contrast to a significant increase in cells detected at a concentration of 1 and 2.5 µg/ml utilised by MTT assay and at 2.5 µg/ml determined from cell count after 24 h incubation. This was a promising result, encouraging the debate whether the modification (sulphation) was the reason behind this biological effect. In a previous study it was shown that the soluble form of beta 1-3 glucan had some protective properties against infection in mice. The results of that study showed an increase in neutrophils in blood stream, enhancement in bone marrow proliferation and *in vitro* phagocytic activity to E. coli bacteria (Tzianabos, 2000).

The following step was to evaluate the incubation period of CHO 1, thus different concentrations of CHO 1 were exposed to CLCs line for 1, 3, 6 and 9 h. The dose response

of CLCs respiratory burst activity to CHO 1 was reproducible and a concentration of 150 µg/ml induced respiratory burst activity 6x higher than the control after 9 h incubation. The duration of the CHO 1 treatment for 9 and 24 h was not significantly different at 150 µg/ml thus 24 h incubation was concluded to be the effective incubation period for CHO 1.

4.4.3 The responses of pronephric cells to CHO 1 modified carbohydrate

It was important to examine the effect of CHO 1 on carp pronephric cells and to see whether the effect is reproducible in primary fish cells. Pronephric cells from carp were exposed to a variety of concentrations of CHO 1 and after 24 h incubation cell viability and respiratory burst activity measured. Interestingly, the concentration effect of CHO 1 on pronephric cells was similar to that obtained in the CLCs line, in that there was no cytotoxic effect and, at concentration of 150 µg/ml, CHO 1 induced respiratory burst activity twice as high than the control. The modified carbohydrate CHO is a sulphated form of Tylose MH 1000 (Sigma 93802), it is a cellulose drive carbohydrate that comprise methyl-hydroxyethyl cellulose ethers (MHEC). Tylose is used in materials to provide water retention, binding, thickening, film forming and colloid properties. The biological properties have been reported on modified cellulose. For example, hydroxyethyl methylcellulose (HEMC) affects fat metabolism in mice and possess hypolipidemic properties (Ban et al., 2012a). The increasing of HEMC viscosity may be useful in the management of high fat diet-induced hyperglycemia and oxidative stress in mice (Ban et al., 2012b). Although there appears to an absence of published reports on the effect of this cellulose drive on the immune system, these results led to a more detailed investigation on the biological effects of this modified cellulose.

In conclusion it has been demonstrated in this chapter that the *in vitro* system established in Chapter 3 can successfully be applied to screen a range of modified carbohydrates on

CLCs line. Amongst the tested modified carbohydrate, a cellulose drive carbohydrate CHO 1, has been identified and appeared to be a promising novel immunostimulant in both the CLC line and primary pronephric cells, however more research is required to elucidate the exact immunostimulation mechanism and the structural form of this carbohydrate and this will be taken forward in the next chapter.

Chapter 5. Structural and biological characterisation of formulated carbohydrate (CHO 1)

5.1 Introduction

Carbohydrates are involved in many important biological processes such as carriers of cellular information, and involvement in infection and inflammation, and also their crucial role in metabolic pathways i.e. glycolysis, pyruvate, Krebs cycle, the pentose phosphate, PDH reaction, glycogenesis, glycogenolysis, gluconeogenesis (Albers, 2008). Carbohydrates polymers that comprise more than 20 units of monosaccharides are classified as polysaccharides. Sulphated polysaccharides occur both in animals e.g. glycosaminoglycans and in plants such as carrageenans. They are present in a variety of locations such as on the external cell membrane and in the external matrix space between cell membrane and the mucosa. Such carbohydrates have several biological roles like cell to cell communication, acting as a barriers between tissues, cell adhesion, reservoirs for growth factors and barriers to protect against pathogens (Toida et al., 2003). Natural polysaccharides can be sulphated to produce physical and chemical modified polymers in order to develop new biomaterials, bestow important therapeutic and biological activities such as anticoagulant: sulphated oat beta glucan (Chang et al., 2006); antitumor: sulphated α-(1-3)-D-glucan from fruiting bodies of *Ganoderma lucidum* (Zhang et al., 2000); anti HIV infection: sulphated curdlan (Yoshida et al., 1995) and sulphated konjac glucomannan (Bo et al., 2013). These biological functions are dependent upon the binding of highly specific carbohydrate structure (negative sulphated group) to protein (positive peptide sequence). This binding is affected by the degree of sulphation with a suitable ionic groups, water solubility, molecular weight and chain confirmation of the polysaccharides (Toida et al., 2003, Bo et al.,2013).

Cellulose ethers are one of important modified and widely abundant polysaccharides, characterised with a linear, regular polymer of 1-4 link β-D-glucopyranosyl units, which gives a rigid structure with intramolecular hydrogen bonds (Nasatto et al., 2015). Tylose is one of the commercially available cellulose ethers. It is a methyl hydroxyethyl cellulose (MHEC) where methyl groups (-CH3) substitute the hydroxyls (OH^-) at C-2, C-3 and/or C-6 positions of anhydro-D-glucose units (Figure 5-1). The sulphation comprises replacement of the hydroxyl, ethyl and methyl groups with a sulphite group (SO_3^{-2}) of polysaccharides at the substitution position. Cellulose has a three hydroxyl group on each glucose molecule, the average number of replaced hydroxyl groups per glucose molecule is called degree of substitution (DS) and is usually between 0- 3. The distribution and the degree of substitution is the main factor that affects the solubility, for example MHEC is soluble in water at a degree of substitution between 1.3- 2.5 (Nasatto et al., 2015).

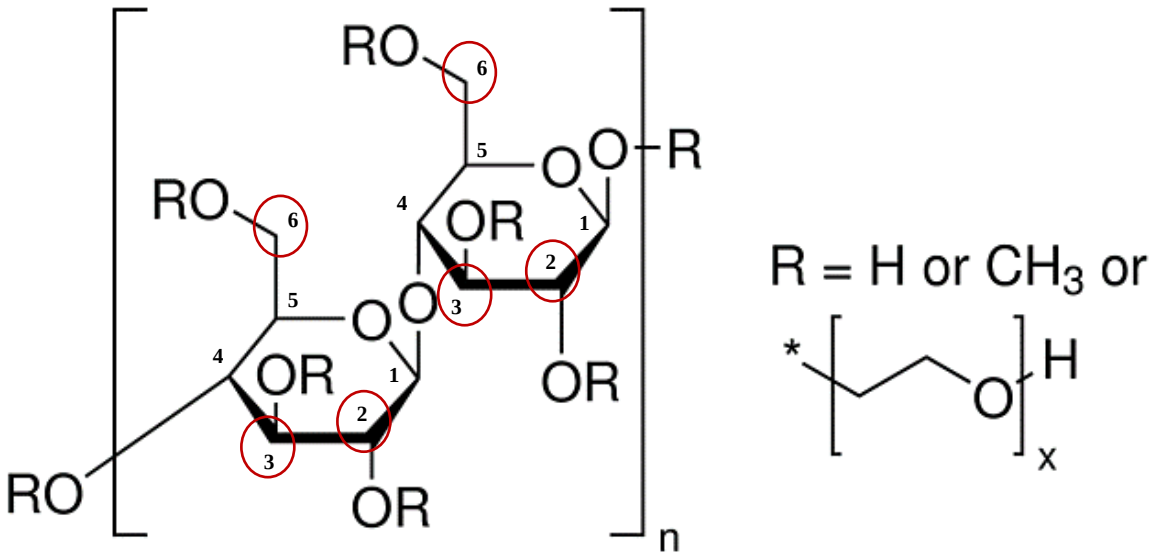


Figure 5-1: Structure of cellulose where n equals the number of anhydroglucose units. Substitute at positions C-2, C-3 and/or C-6 highlighted with red rings [Tylose MH 1000 (Sigma 93802)].

The common sulphating agent comprises chlorosulphonic acid, sulphuric acid, and sulphur trioxide, then completed with reagents such as pyridine, trimethyl amine and triethyl amine. The reactions carried out under anhydrous conditions for maximum yield. The most common solvent used are pyridine, dimethylformamide DMF, concentrated sulphuric acid and liquid sulphur trioxide (Geresh et al., 2002).

The sulphated carbohydrate structure and degree of substitution can be characterised through NMR spectra, IR spectroscopy, elemental analysis and high performance liquid chromatography HPLC. In a study on beef lung heparin, both of ^{1}H and ^{13}C NMR spectral assignments were used to determine the chemical shift position after the sulphation process (Yates et al., 1996). While the chemical shift position were determined in sulphated rice bran glucan using FTIR spectroscopy and both of ^{1}H, ^{13}C NMR spectral assignments (Ghosh et al., 2010).

The importance of carbohydrate sulphation for regulation and control of biological functions has recently been realised. The addition of a sulphation group leads the carbohydrates being recognised by a specific receptor such as in heparin the presence of sulphates in the ATIII binding region. Therefore the essential components for the biological activity of heparin are referred to 2-O-sulphate, 3-O-sulphate, 6-O-sulphate, and N-sulphate groups (Albers, 2008). Furthermore, the ability of heparan sulphates to interact with target proteins leads to a range of biological activities as seen in Figure 4-1. The variation in heparans' structure means it is able to interact and bind with various proteins like growth factors, chemokines, morphogens, extracellular matrix components, and enzymes (Dreyfuss et al., 2009).

Several *in vitro* studies revealed that the sulphation process alters the biological activity of the carbohydrate, as highlighted in a study on leukaemia cells, sulphated lentinan exhibited highly antioxidant activity and anti-proliferation, in contrast to native lentinan (Feng et al.,

2010). Another investigation showed the immunomodulation of sulphated derivative of carrageenan from red algae *Kappaphycus striatum* at concentration 200µg/g/day in mice, the resulted in increases in natural killer cells, and enhancement of antitumor effect and immunity (Yuan et al., 2010).

Therefore, the aim of this chapter was to produce the modified and sulphated carbohydrate CHO1 from the native carbohydrate (Tylose) and determine its effect on pronephric cells respiratory burst activity, and to confirm the sulphation process has taken place by analysing the chemical shifts in the carbohydrate. Also, its biological effects on both established cell types in the *in vitro* system Chapter 3 i.e. carp leukocytes cells line (CLCs) and carp pronephric cells was determined and compared to Tylose and different beta glucans.

5.2 Materials and Methods

5.2.1 Modified carbohydrate sulphation process

In the previous chapter CHO 1 was selected for further analysis and it was therefore necessary to define, as far as possible, the structure of this substance for further production. Therefore, the sulphated Tylose was prepared using chlorosulfonic acid (CSA) method as described elsewhere (Yoshida et al., 1995) with some modifications made by Dr Mark Skidmore (Keele University). Briefly, Tylose powder MH 1000 (Sigma 93802) (0.5 g) was dissolved in sulphating reagent, which consisted of 5 ml dimethylformamide (Sigma, M2381), 10 ml Pyridine (VWR, A12005) and 1-5 ml chlorosulfonic acid (Sigma, 571024). The mixture was cooled to 19±1℃ on ice for 1 min, and then the mixture was heated at 90℃ for 4 h. The mixture was again placed on ice and slowly neutralized with 7 ml of sodium hydroxide extra pure 50% (w/v) solution (VWR, 191543M). The mixture was sealed and incubated overnight in -20℃ cold room. Ethanol precipitations was carried out by adding 80 ml of saturated sodium acetate (Sigma, S2889) to the mixture, which was then incubated overnight at -20℃ cold room. After the incubation, the upper liquid layer was removed and the precipitated sugar was dissolved in deionized water and then dialysed in dialysis membrane with a cut off > 7kDa (VWR, 734-0667) for at least 72 h against distilled water. The sulphated Tylose (CHO 1) was lyophilised and tested for effects on fish immune cells.

To ensure the newly sulphated Tylose (CHO 1) has the same biological effects as that described in Chapter 4, two carps with an average weight of 99.98 ± 4.5 g and length of 16.75 ± 0.25 cm were sacrificed 2.1 as described in Chapter 2, cells were prepared, counted and checked their viability as described in Section 2.2.1 and 2.2.2 respectively.

The *in vitro* experimental design for the *in vitro* study described in chapter 4 was used, cells were distributed in 96 well plate (Corning, 3596) at (2×10^5 cell/100 µl) and exposed to a range of CHO 1 concentrations between 2.5- 250 µg/ml at volume 5 µl/well for 24 h. A negative control comprised 5 µl of sterile water (Sigma, W3500). After the incubation period, the NBT assay was performed as explained in Chapter 2 (Section 2.6.1) and the formazan blue were solubilised and read at 620 nm (Multiskan multisoft plate reader, Labsystems, Finland). Data was analysed statistically using one way ANOVA and Tukey's post-hoc test was carried out as described earlier (chapter 2.8) with significance defined as $p \leq 0.05$.

5.2.2 Structural characteristics

5.2.2.1 Fourier transform infrared (FTIR) spectrometer

To investigate the structure of CHO 1 carbohydrate and to confirm that the sulphation of Tylose had occurred, a sample of both Tylose and CHO 1 were used to obtain an infrared spectrum and recorded with Attenuated Total Reflection Fourier transform infrared (ATR-FTIR) spectrometer (Thermo Nicolet™ iS™10 FT-IR Spectrometer). The analysis was kindly performed by Dr Mark Skidmore at Keele University in collaboration with the Department of Structural and Chemical Biology at University of Liverpool.

The FT-IR is a widely used technique in organic synthesis, polymer science, petrochemical engineering, pharmaceutical industry and food analysis and measures the samples capability to absorb light at different wavelengths. When the infrared radiation is passed through a sample, some is absorbed by the sample and some of it is passed through (transmitted). The spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. Each molecular structures produce a unique infrared

spectrum (Derrick et al., 1999). This makes infrared spectroscopy useful for several types of analysis such as:

• Identify unknown materials

• Determination of the quality or consistency of a sample

• Determination of the amount of components in a mixture

Essential FTIR software was used to analyse the data, the spectra being interpreted with the correlation between the absorbance bands with bonds, and therefore functional groups in the molecule. It was important to determine the X-axis that provides the absorption number labelled with Wavenumber at ranges 400- 4000 and the Y-axis of the spectrum labelled as Percent Transmittance at ranges 0- 100. The next step was to characterise the peaks in the IR spectrum, which can be segregated into four regions depending on the functional groups absorbed in the first region as seen in Figure 5-2. The identification of the compound can be achieved by determining the wavenumber of the large peaks that provides the necessary data to read the spectrum and analysing peaks in the Figure print region (The fourth region of the IR spectrum) (Coates, 2000).

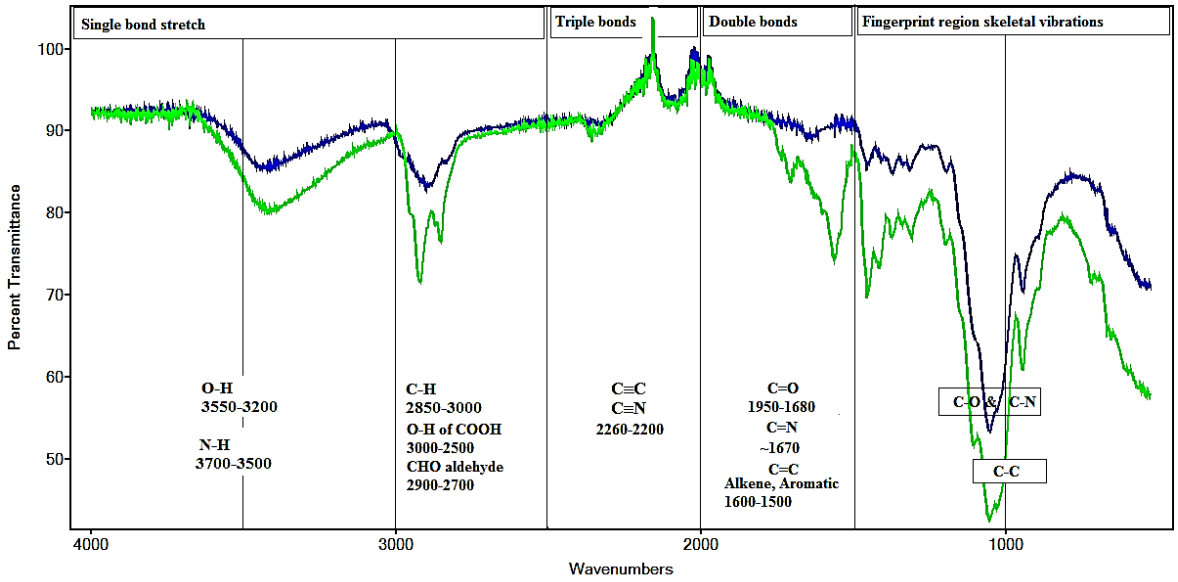


Figure 5-2: The major regions in FTIR spectrum for Tylose (in blue colour) and CHO 1 (in green colour). The infrared spectrum separated into four regions. The first is single bond region ranges from 4,000 to 2,500. The second is the triple bond region ranges from 2,500 to 2,000. The third is double bond region ranges from 2,000 to 1,500. The fourth is finger print region ranges from 1,500 to 400.

5.2.2.2 Nuclear magnetic resonance (NMR) spectroscopy

NMR or nuclear magnetic resonance spectroscopy is a technique used to determine the unique structure of an organic compound, through the carbon-hydrogen framework of the compound in which nuclei in a magnetic field absorb and re-emit electromagnetic radiation. This power is dependent on the strength of the magnetic field and the magnetic properties of the isotope of the atoms. The combined information from NMR and other instrumental methods including infrared and mass spectrometry, may lead to the determination of the entire structure of a molecule (Koch, 2003).

The most commonly studied nuclei are ^{1}H and ^{13}C, although nuclei from isotopes of many other elements (e.g. ^{2}H, ^{6}Li, ^{10}B, ^{11}B, ^{14}N, ^{15}N, ^{17}O, ^{19}F, ^{23}Na, ^{29}Si, ^{31}P, ^{35}Cl, ^{113}Cd, ^{129}Xe, ^{195}Pt) have been studied by high-field NMR spectroscopy.

The ^{1}H NMR spectrum was recorded kindly by Dr Mark Skidmore for both carbohydrates Tylose and CHO 1 using Bruker Tensor 27 spectrometer at 300 MHz at Keele University Chemistry Department. The data was interpreted using Spinworks 4 software: the number, position and intensity of the spectra signals determine the compound structure. ^{1}H NMR chemical shift plays a major role in functional groups identification. Figure 5-3 indicates important examples to the determination of the functional groups.

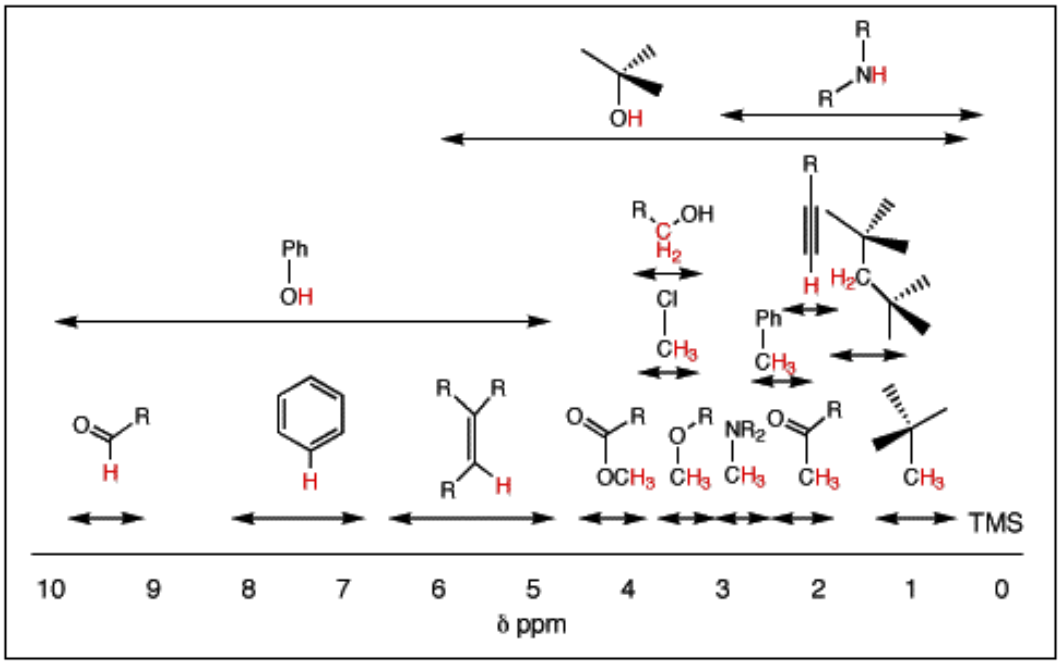


Figure 5-3: ^{1}H NMR chemical shift ranges for organic compound (Koch, 2003). Tetramethylsilane TMS is the accepted internal standard for calibrating chemical shift for 1H NMR spectroscopy in organic solvents.

5.2.2.3 Sulphate determination assay

It is a colorimetric quantitative method that determines small amounts of sulphate in polysaccharides. The reaction of sodium rhodizonate with a known concentration of barium chloride solution produce a red precipitate. The solubility of this red precipitate is significantly greater than that of the barium sulphate. The addition of sulphate solution causes to barium sulphate precipitation and the red colour disappearance (Terho and Hartiala, 1971). This analysis was carried out kindly by Lynsay Hadfield a Ph.D. research student at Dr Mark Skidmore research group, the method was based on Terho and Hartiala (1971). Briefly, a serial of 1:2 dilution was prepared for both of CHO1 and dextran separately starting from a concentration 25 µg/ml. 30 µl of either of samples (CHO 1), standards (Dextran), or double distilled water was pipetted in triplicate to each well of a 96

well plate. Followed by adding 30 µl of absolute ethanol and 60 µl of $BaCl_2$ buffer (see Appendix). Then 180 µl of sodium rhodizonate (see Appendix) was added and the plate was shaken well to develop the colour, the plate was allowed to stand for 10 min in the dark at 19±1℃. The intensity of the colour was measured spectrophotometrically (Beckman DU) at 520 nm. The colour remains stable for 30 min. The titration curve of dextran was used to determine the sulphate content of CHO 1. Dextran sulphate contains approximately 17% sulphur which is equivalent to approximately 2.3 sulphate groups per glucosyl residue. A titration curve was constructed by plotting delta absorbance that calculated by subtracting the absorbance value of Dextran from the absorbance value of distilled water, against the amount of sulphate in Dextran. The curve equation was used to calculate the amount of sulphate in CHO 1 as described in (Terho and Hartiala, 1971).

5.2.3 The effect of Tylose and CHO 1 on cell respiratory burst activity

The biological activities of tylose were determined on CLCs line to evaluate whether the sulphation changed the structure and effects of complex carbohydrates. For this purpose, the CLCs line maintained as described in Section 2.3.1 at density of 2×10^4 cell/100 µl were stimulated with range of concentrations of either Tylose or CHO 1 between 2.5-250 µg/ml. The concentrations 200 and 250 µg/ml were used to determine the maximal dose efficacy of Tylose and CHO 1. After 24 h incubation, the cells respiratory burst activity was determined with NBT assay (2.6.1). The data was analysed with a two way ANOVA and the differences between groups ascertained with a Bonferroni post-hoc tests and Tukey's post-hoc analysis was used to determine the differences with in one group. Significance was defined at $p \leq 0.05$.

5.2.4 Comparison of the effects of beta glucans and CHO 1 in CLCs line

To establish if the modified carbohydrate (CHO 1) had the similar immunogenic properties as the other carbohydrate immunostimulants, therefore its proliferation and respiratory burst activity was compared to MacroGard® and zymosan.

5.2.4.1 MacroGard® and CHO 1

Both of respiratory burst activity and cell viability were measured in CLCs line after exposure to different concentrations (2.5-250 µg/ml) of either MacroGard® or CHO 1 carbohydrates. Thus, CLCs were seeded at density 2×10^4 cells/well in two pairs of 96 well microplates and treated with 5 µl of either MacroGard® or CHO 1, using sterile water (Sigma, W3500) as a negative control. After 24 h incubation, the cells in each pair of experimental plates was utilised to determine cell viability i.e. MTT assay Section 2.5.1 or respiratory burst activity i.e. NBT assay Section 2.6.1. The statistical analysis was carried out utilising two way ANOVA with "Treatment" and "Concentration" as the variables with both of Tukey's and Bonferroni post-hoc tests. Significant was defined as $p \leq 0.05$.

5.2.4.2 Zymosan, MacroGard® and CHO 1

A similar experimental design as described above was repeated to measure the effect of different concentrations at 1- 150 µg/ml of either zymosan, MacroGard® or CHO 1 on CLCs respiratory burst activity. Briefly, cells at a density of 2×10^4 cells/well was distributed in three 96 well plates and treated for 24 h with 5 µl/well of different concentrations of the carbohydrates or with sterile water (Sigma, W3500). After the incubation period, the NBT assay performed as described in Section 2.6.1, and the statistical analysis of data was performed using a two way ANOVA and both of Tukey's and Bonferroni post-hoc tests, with significance defined as $p \leq 0.05$.

5.3 Results

5.3.1 Biological effects of the modified carbohydrate CHO 1 after the sulphation process

To ensure the newly synthesised tylose (CHO 1) has the same biological activity in carp leukocytes, as previous produced forms the dose dependency effects of CHO 1 was carried out to ascertain the immunostimulant effect and the reproducibility of CHO 1 production. Briefly, the pronephric cells were exposed to a serial dilution of the newly sulphated tylose (CHO 1) at concentrations between 2.5- 250 µg/ml as seen in Figures 5-4 and cells respiratory burst activity assessed using a NBT assay. The statistical analysis revealed a significant increase in cells respiratory burst activity after exposure to CHO 1 ($F = 83.86$, $P < 0.0001$). The concentration dependency effect of CHO 1 significantly increased at concentrations i.e. 50 µg/ml ($p \leq 0.001$), 100, 150, 200 and 250 µg/ml ($p < 0.0001$). At a concentration of 200 µg/ml the respiratory burst activity of cells increased nearly eight fold higher than the control (Figure 5-4). This result revealed the production of CHO 1 carbohydrate was reproducible and had the same immunological activity in carp leukocytes cells as that described in Chapter 4.

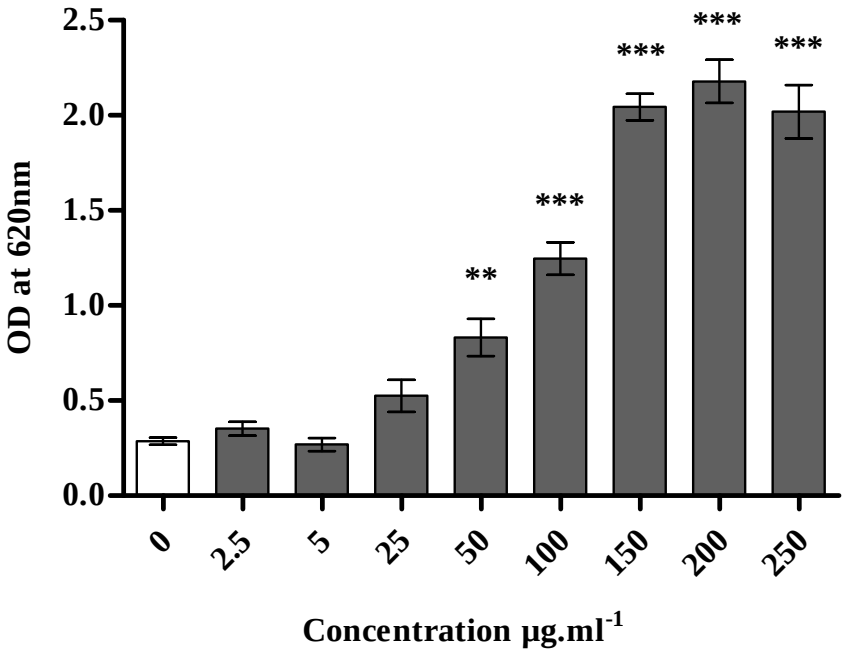


Figure 5-4: The dose response of CHO 1 on pronephric cells respiratory burst activity. The respiratory burst activity determined by NBT assay for this purpose cells were distributed at (2×10^5 cells/well) simulated with rang of sulphated CHO 1 concentrations between 2.5-250 µg/ml for 24h. Statistic comparison was performed by one-way ANOVA and Tukey's post-hoc test ($p \leq 0.05$) and the significant differences between concentrations in comparison to control performed with **= $p \leq 0.001$ and ***= $p < 0.001$. Data represented mean ± SEM of 3 wells replicates of two fishes.

5.3.2 Structural characteristic

5.3.2.1 Fourier transform infrared (FTIR) spectrometer

The sulphation of CHO 1 confirmed by FTIR and ^{1}H NMR analysis. The data at Figure 5-5 presents the FTIR spectra for Tylose and CHO 1in the 400-4000 cm^{-1} region. CHO 1 exhibited three strong absorption bands at 3431, 2900 and 1054, which is associated with O-H, C-H and C-O bonding respectively. Interestingly, several new bands and chemical shifts values were observed in the CHO 1 carbohydrates at wavenumber regions 2800-3000, 2300- 2400, 1340- 1770 and 700-1270 cm^{-1}, those bands are clearly highlighted in Figure 5-5. The appearance of absorptions bands at several areas as detailed in table 5-1 could be due to the vibrations of sulphate groups such as band at ~1051 cm^{-1}. Vibration of S=O groups and the C=S bands would give rise to FTIR bands at 1502 and 1238 cm^{-1}, indicating the sulphation had taken place successfully.

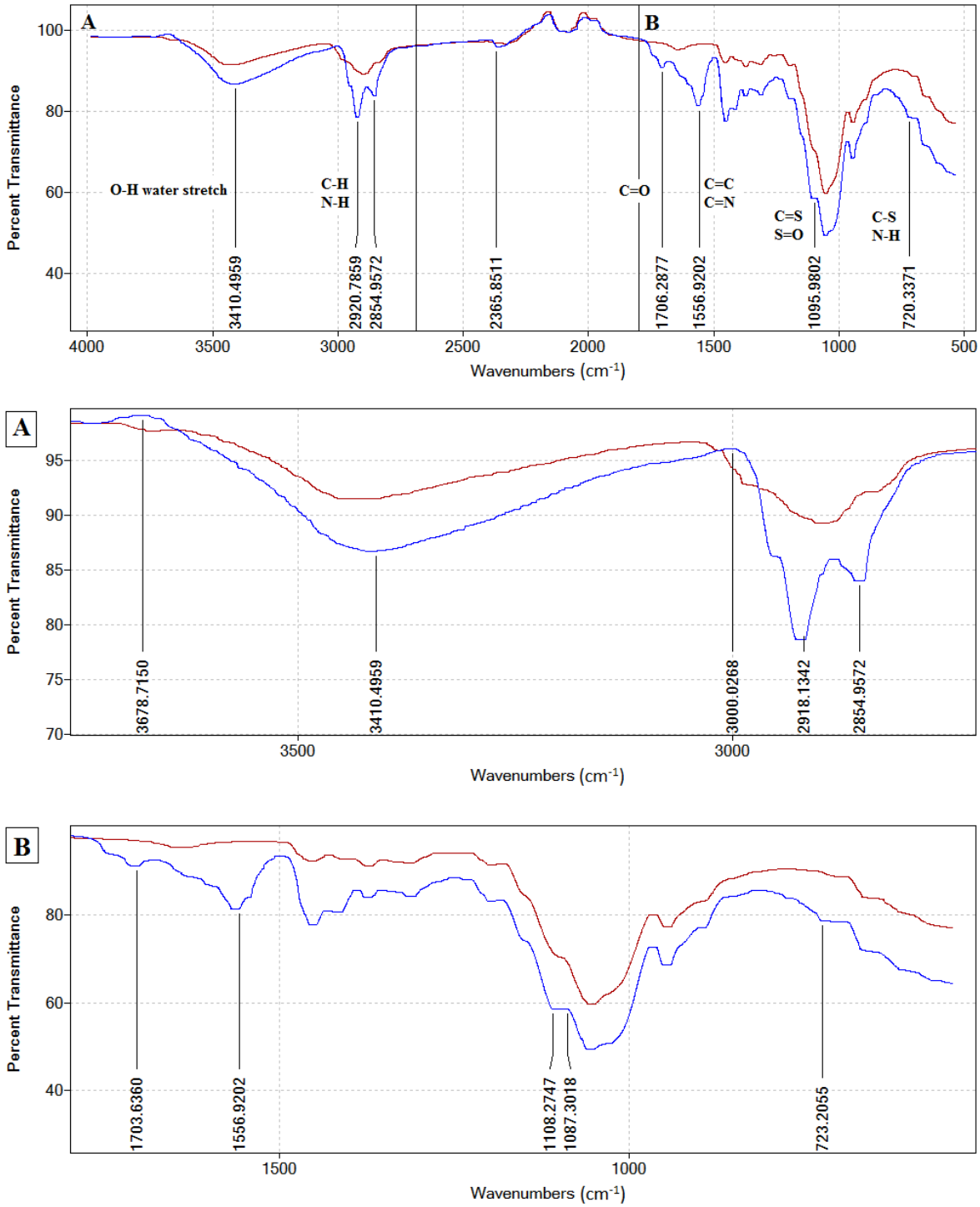


Figure 5-5: FTIR spectroscopy analysis for both of sulphated and non-sulphated Tylose. The infrared analysis for Tylose (Sigma, 93800) presented with red line and CHO 1 with blue line at 400-4000 cm^{-1} region. Different regions were highlighted and measured its wavenumber to illustrate the chemical shits that caused by the sulphation process. A and B Figures are the regions that have clearly a chemical shifts from tylose due to the sulphation process, therefore enlarged in separated graphs.

Table 5-1: The different FTIR peaks of CHO 1 from tylose that has remarked sulphate groups.

Peaks no.	CHO 1 peaks at wavenumbers (cm^{-1})	Peaks height	Wavenumbers range (cm^{-1}) (Tipson, 1968)	Remarks (Tipson, 1968)
1.	1051.9523	49.6275	1050-1020	S=O >S=O sulfoxide
2.	1238.1254	87.9255	1235-1212 1230-1150	C=S $(RO)_2C=S$ thioketone $(RO)_2SO_2$ sulfuric ester
3.	1312.7081	84.117	1340-1280	S=O R_2SO_2, sulfone
4.	1377.1684	83.3550	1440-1350 1420-1330	S=O $(RO)_2SO_2$ sulfuric ester S=O $ROSO_2R'$ sulfuric ester
5.	1423.1686	80.6564	1420-1330	S=O $ROSO_2R'$ sulfuric ester
6.	1502.1782	93.2925	1500-1470	C=S -N-C=S
7.	1673.8993	92.4397	~1675	C=S thioester
8.	2359.8463	95.6000	~ 2400	S-H thiol, H bonded
9.	2367.3876	95.6602		
10.	3417.9057	86.4224	3600-3100 3550-3195	H-O water crystallisation C=O Carbonyl

5.3.3 Nuclear magnetic resonance (NMR) spectroscopy

The ^{1}H NMR spectra of both of Tylose and CHO 1 recorded in dimethyl sulfoxide-d6 (D6MSO) and deuterium oxide (D_2O) are shown in Figure 5-6 and 5-8 respectively. The ^{1}H NMR analysis for Tylose displayed signals at three regions: first at 1.232 ppm (**a** Figure 5-6), possibly due to the H atoms bound to sp3 carbons (alkanes); and the intense signals found at 2.503 ppm might be due to the D6MSO. At 3.361 ppm the profile might be due to the H atoms bound to sp3 carbons bonded to at least one heteroatom (**b** and **c** Figure 5-6). There are difference in the chemical shifts between tylose and CHO 1 ^{1}H NMR, the appearance of strong signal at 4.7 ppm in CHO 1 spectrum could be due to the H atoms bound to non-aromatic sp2 carbons of alkenes. However, both carbohydrates recorded signals at an area between 3.24- 3.45 ppm (see Figure 5-6 c and 5-7).

The same ^{1}H NMR data for tylose and CHO 1 were interpreted to obtain two dimensional images as displayed in Figure 5-8. The intense signal area was different between tylose and CHO 1 due to the sulphation process. It can be seen for tylose spectra the intense signalling area is between 3.4- 3.2 ppm and for CHO 1 is between 4.8- 4.6 ppm.

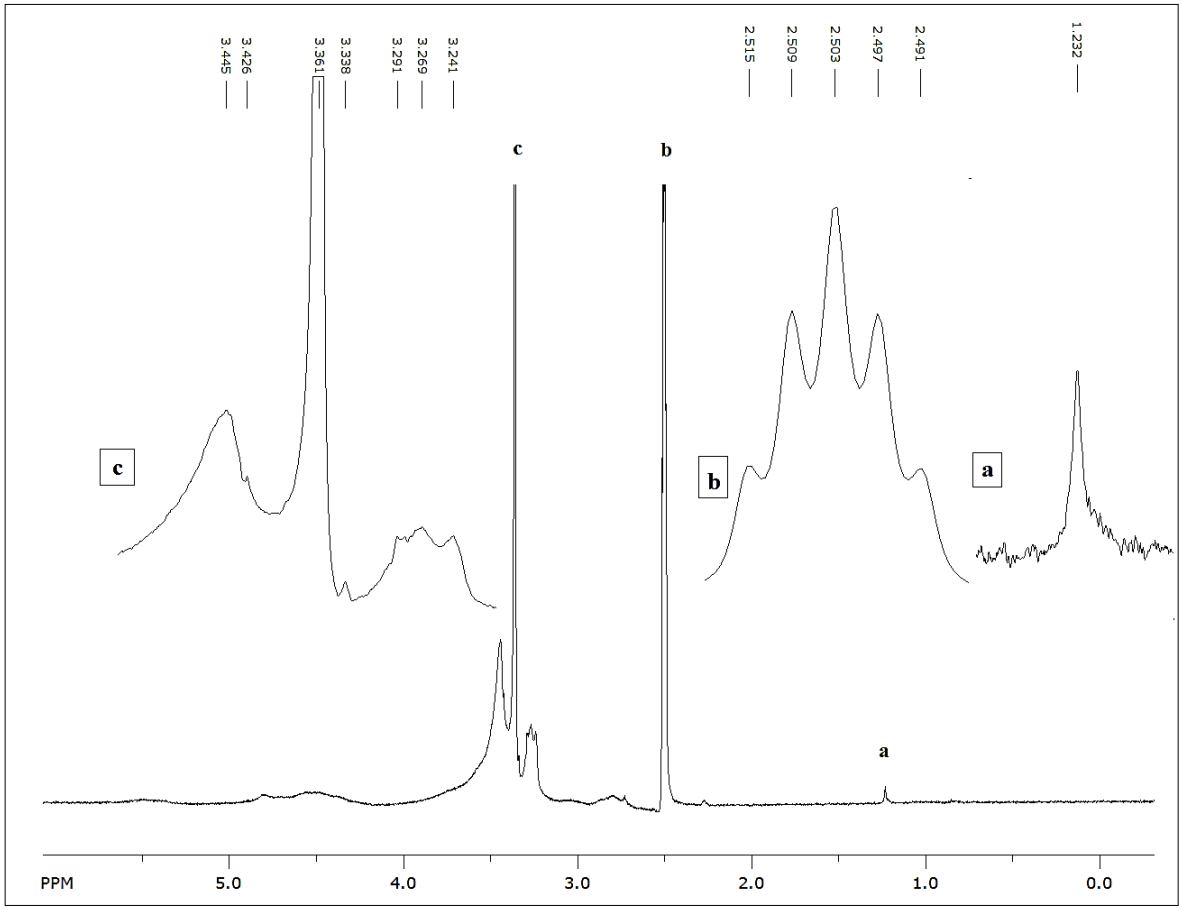


Figure 5-6: ^{1}H NMR spectra derivative at 300MHz of Tylose dissolved in D6MSO at 37℃. The chemical shifts were occurred at three regions **a** (1.232 ppm), **b** (between 2.49- 2.52 ppm) and **c** (3.36 ppm).

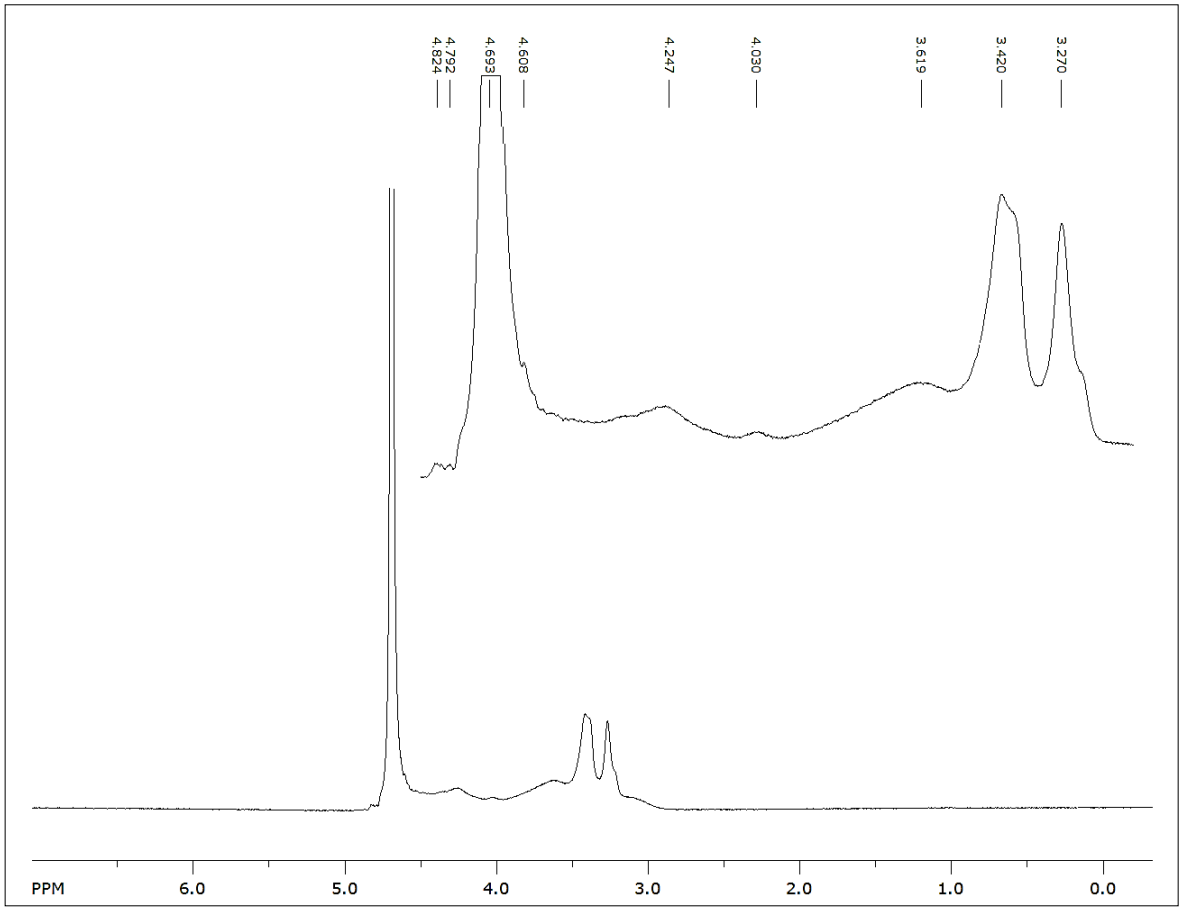


Figure 5-7: 1H NMR spectra derivative at 300MHz of CHO 1 dissolved in D2O at 37℃. The chemical shifts were occurred at three main regions, at 3.27, 3.42 and 4.69 ppm.

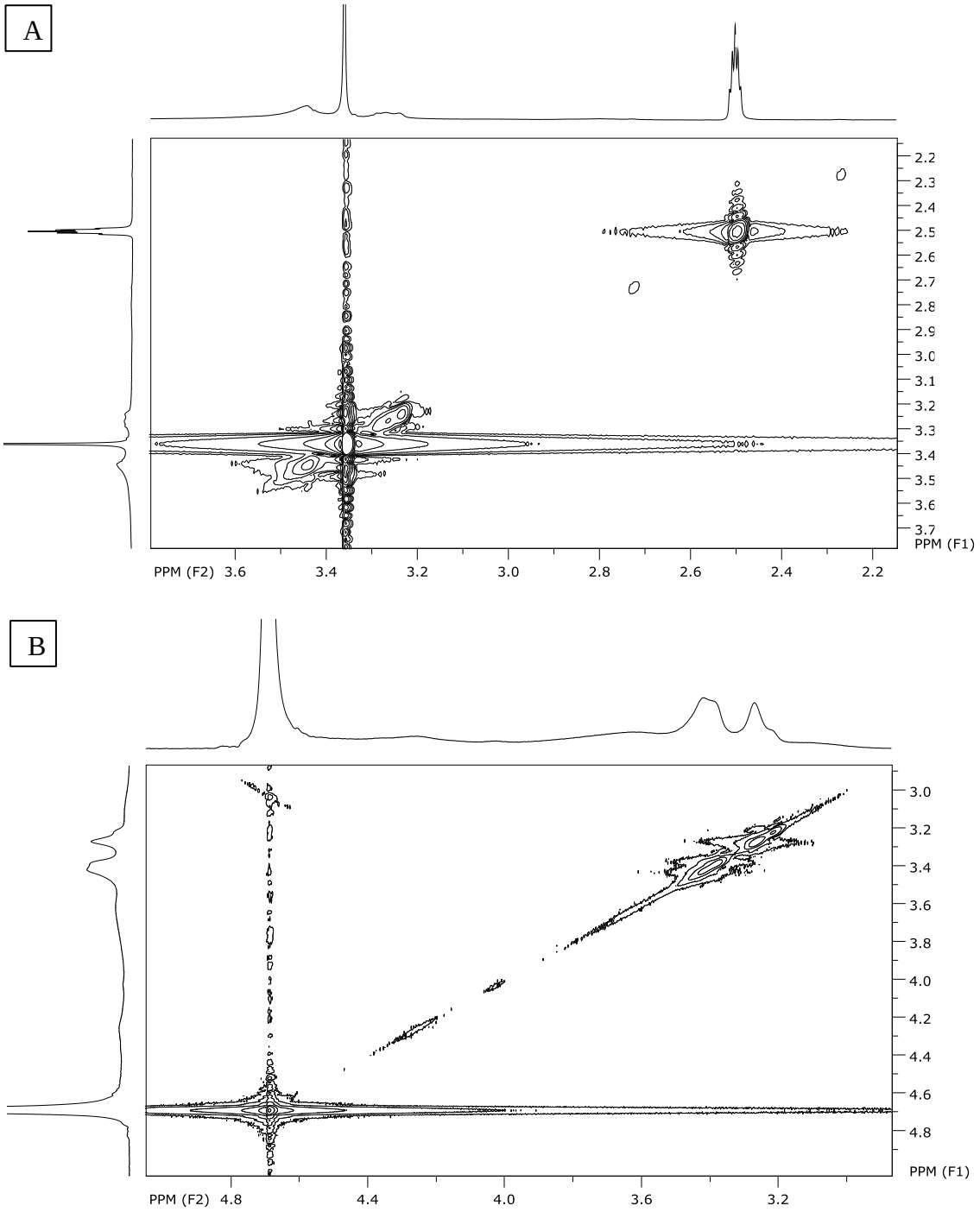


Figure 5-8: The two dimension proton to proton double-quantum filtered (^{1}H-^{1}H DQF) COSY spectrum for Tylose and CHO 1. The COSY spectra for tylose (A) and CHO 1 (B) are illustreate the two dimentional chmical shifts differences in both carbohydrate, also it is a very useful for structure clarification and provide correlations between coupled spins. Both of PPM F1 and F2 are the proton spectum for tylose and CHO 1 saperatly. The analysis carried out at Keele university chemistry department using Bruker high-resolution NMR spectrometers operating in the range of 300-600 MHz.

5.3.4 Sulphate determination assay with sodium rhodizonate

The analysis revealed that the dextran sulphate content used was 5.25 nmoles/µg of disaccharieds, and from the titration curve (Figure 5-9) the amount of sulphate in CHO 1 was determined. CHO 1 sulphate conent was between 0.98 and 2.49 nmoles/µg of disaccharieds of CHO 1. This shows that the modified carbohydrate CHO 1 was less sulphated in comparison to dextran and each 2 monosaccharied of its structure has been substituted with an average of 1.74 nmoles of SO^{-3} in substitution position.

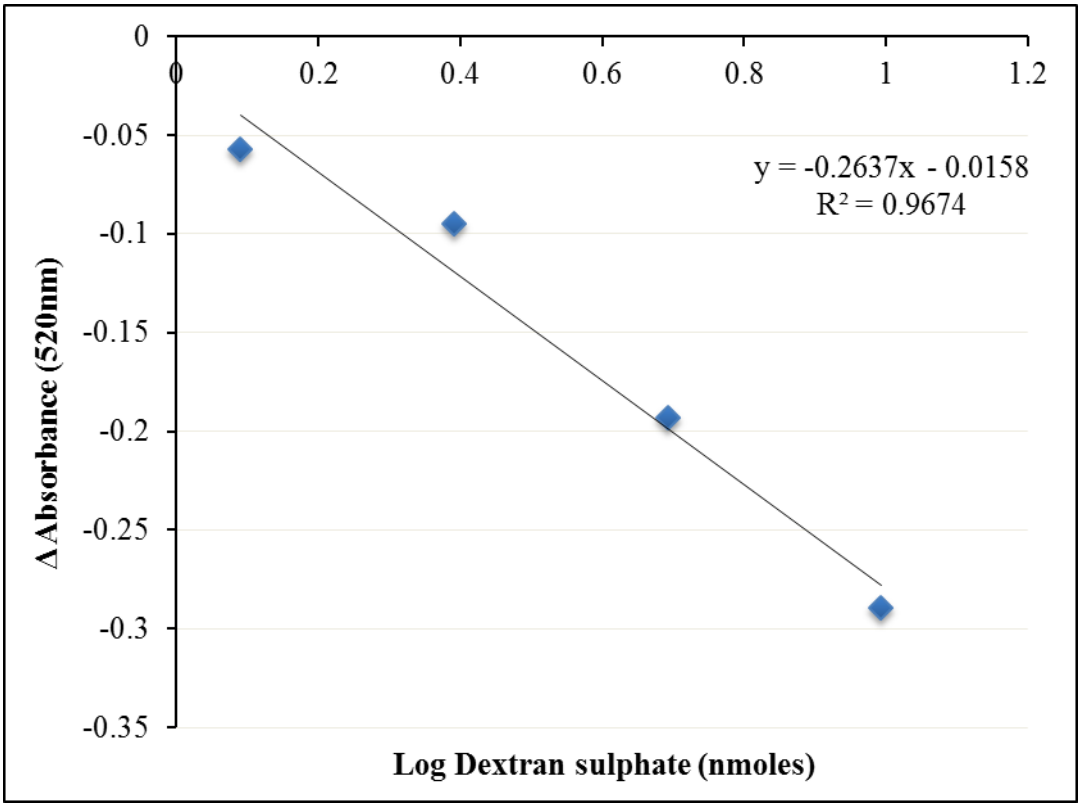


Figure 5-9: Standard curve for calculation of dextran sulphate using the rhodixonate method. Absorbance value is obtained by subtracting absorbance of the sample from absorbance of distilled water.

5.3.5 The effect of Tylose and CHO 1 on CLCs line respiratory burst activity

The comparative respiratory burst activity of Tylose and CHO 1 was establised in CLCs line to confirm the structural and biological changes that happened to tylose was due to the sulphation process (Figure 5-10). This revealed that the levels of cell respiratory burst activity was dependent on the carbohydrate type ($F = 26.24$, $p < 0.0001$), the concentrations of each carbohydrate ($F = 38.23$, $p < 0.0001$) and the interaction between these two parameters ($F = 16.06$, $p < 0.0001$). The Bonferronie test revealed considerable differences between both carbohydrates at concentrations between 25- 250 µg/ml at $p < 0.001$ that suggests the sulphation process affects the structure and biological effect of Tylose.

CHO 1 affected CLCs respiratory burst activity and induced significant differences in comparison to the negative control at concentrations 25- 250 µg/ml, with all p values being less than 0.0001. This results are therefore similar to the previous results obtained in Chapter 4. While Tylose did not induce significant differences ($p \leq 0.05$) in CLCs respiratory burst activity at any concentrations and was there comparable to the negative control.

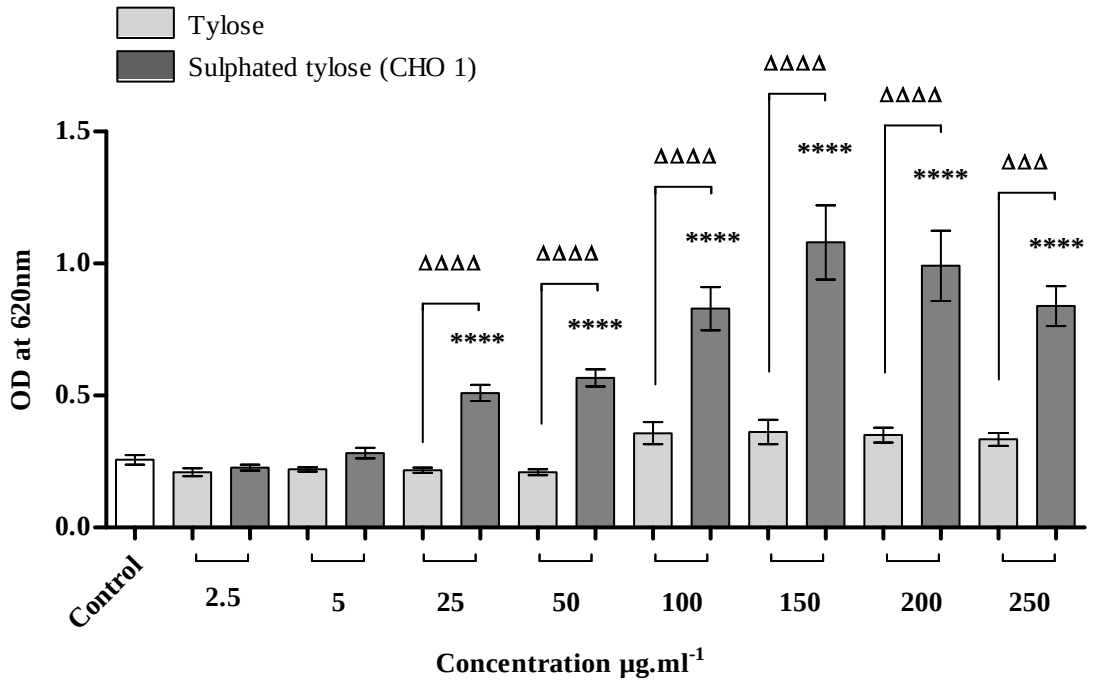


Figure 5-10: The CLCs dose responses to sulphated and non-sulphated CHO 1 determined by NBT assay. The cells were distributed at (2×10^4 cells/well) simulated with rang of tylose and sulphated tylose (CHO 1) at concentrations between 2.5- 250 µg/ml for 24h. Statistic comparison was performed using a two-way ANOVA and Bonferroni post-hoc test at $p \leq 0.05$ and the significant differences between concentrations in comparison to control presented with ****= $p < 0.0001$, also the comparison between the two treatments at each concentration presented with ΔΔΔ= $p < 0.001$ and ΔΔΔΔ= $p < 0.0001$. Data represented the mean ± SEM of 6 wells replicates.

5.3.6 Comparison the effects of different beta glucans and CHO 1 in CLCs line

- **MacroGard® and CHO 1**

Since tylose did not induce any significant effect on cells respiratory burst activity and CHO 1 did, it was interesting to evaluate the stimulatory differences between CHO 1 and different beta glucan sources in the cell line. Therefore, a comparison experiment to establish the concentration dependency effects of both MacroGard® and CHO 1 on CLCs viability and respiratory burst activity was determined (Figure 5-11). The statistical analysis showed the influence of the different carbohydrate treatments ($F = 102.5$, $p < 0.0001$), the concentrations ($F = 20.91$, $p < 0.001$) and the interaction between both factors ($F = 13.62$, $p < 0.001$) on CLCs respiratory burst activity. The post-hoc analysis indicates that these changes are mostly due to the CHO 1 effects and resulted in significant differences ($p < 0.001$) between MacroGard® and CHO 1 at 100, 150, 200 and 250 µg/ml as seen in Figure 5-11 A. Where CHO 1 provoked a significant increases in cell respiratory burst activity at concentrations 50, 100 µg/ml ($p \leq 0.05$) and 150, 200, 250 µg/ml ($p < 0.001$).

The cell viability levels were not affected significantly and no interaction was observed between the used carbohydrates and their concentration ($F = 0.635$, $p = 0.746$). However, a noticeable decrease in MTT optical density can be observed at MacroGard® concentrations 50- 250 µg/ml and at concentration 250 µg/ml of CHO 1 carbohydrate.

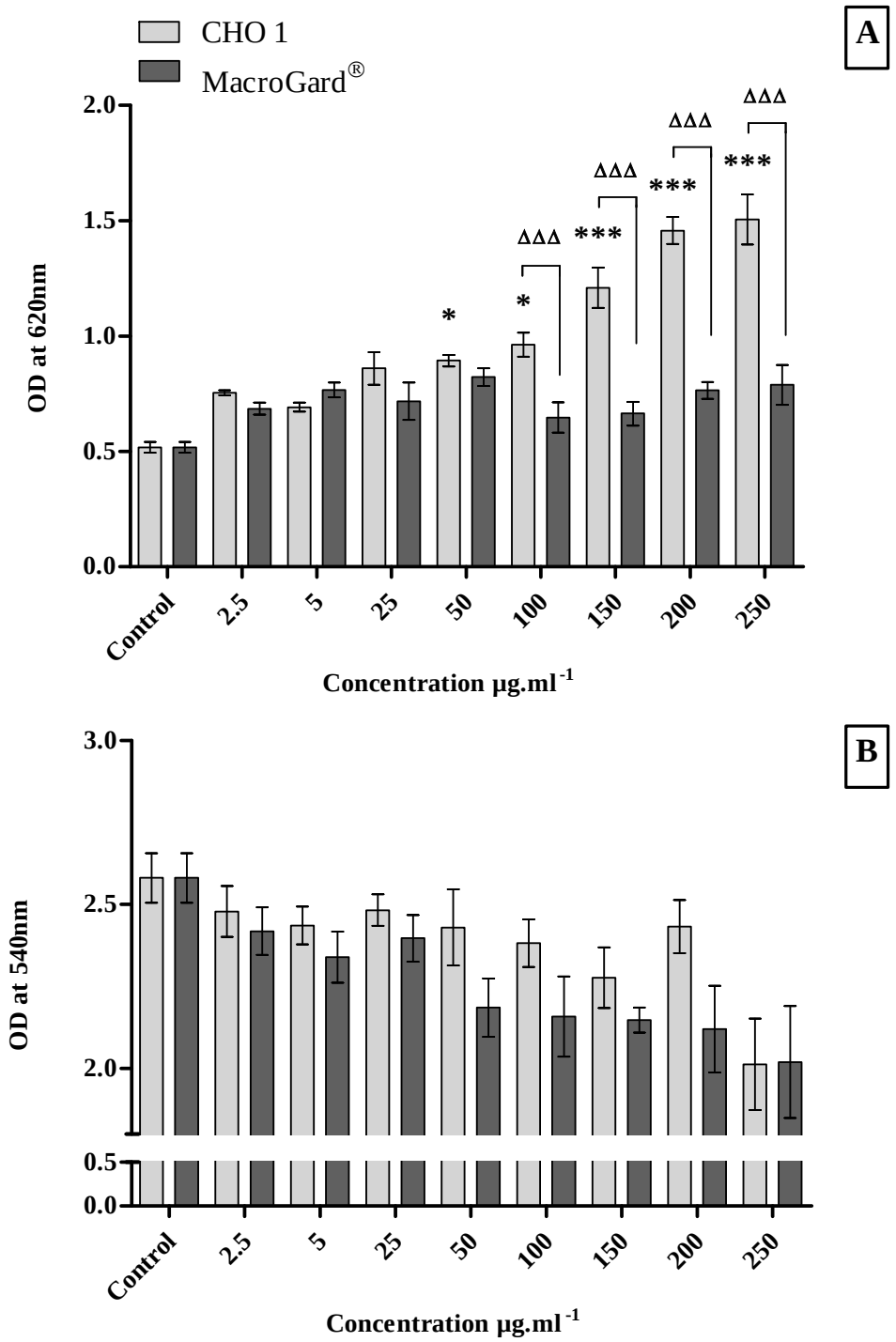


Figure 5-11: Comparison of the CLCs dose responses to MacroGard® and CHO 1 exposure after 24 h. Cells respiratory burst activity and viability were measured with NBT **A** and MTT **B** assay respectively. Cells were distributed at (2×10^4 cells/well) and simulated with either MacroGard® or CHO 1 at concentrations 2.5- 250 µg/ml for 24h. Statistical analysis was performed with a two-way ANOVA and Bonferroni post-hoc test ($p \leq 0.05$) and the significant differences between CHO 1 concentrations in comparison to matched control

performed with *= $p \leq 0.05$ and ***= $p < 0.001$, and the comparison between carbohydrate treatment at each concentration performed with ΔΔΔ= $p < 0.001$. Data represent mean ± SEM of 6 wells replicates.

- **MacroGard®, zymosan and CHO 1**

The same experimental design was repeated to determine the CLCs respiratory burst activity after stimulation with MacroGard®, zymosan or CHO 1 at concentrations 1- 150 µg/ml for 24h. The analysis showed that the carbohydrate treatments ($F = 834.8$, $p < 0.0001$), the concentrations of the treatments ($F = 211.4$, $p < 0.0001$) and the interaction of these two factors ($F = 172.3$, $p < 0.0001$) significantly influenced the reactive oxygen species in CLCs (Figure 5-12). The effect of CHO 1 was highly significant in comparison to MacroGard® and zymosan at concentrations 25, 50, 100 and 150 µg/ml all at $p < 0.0001$. The modified carbohydrate CHO 1 stimulated CLCs respiratory burst activity and verified a significant increase at 25 µg/ml and higher concentrations in comparison to the negative control at p values less than 0.0001.

The previous results indicated the differences in structure and the stimulatory effects of CHO 1 in comparison to Tylose and different beta glucan sources.

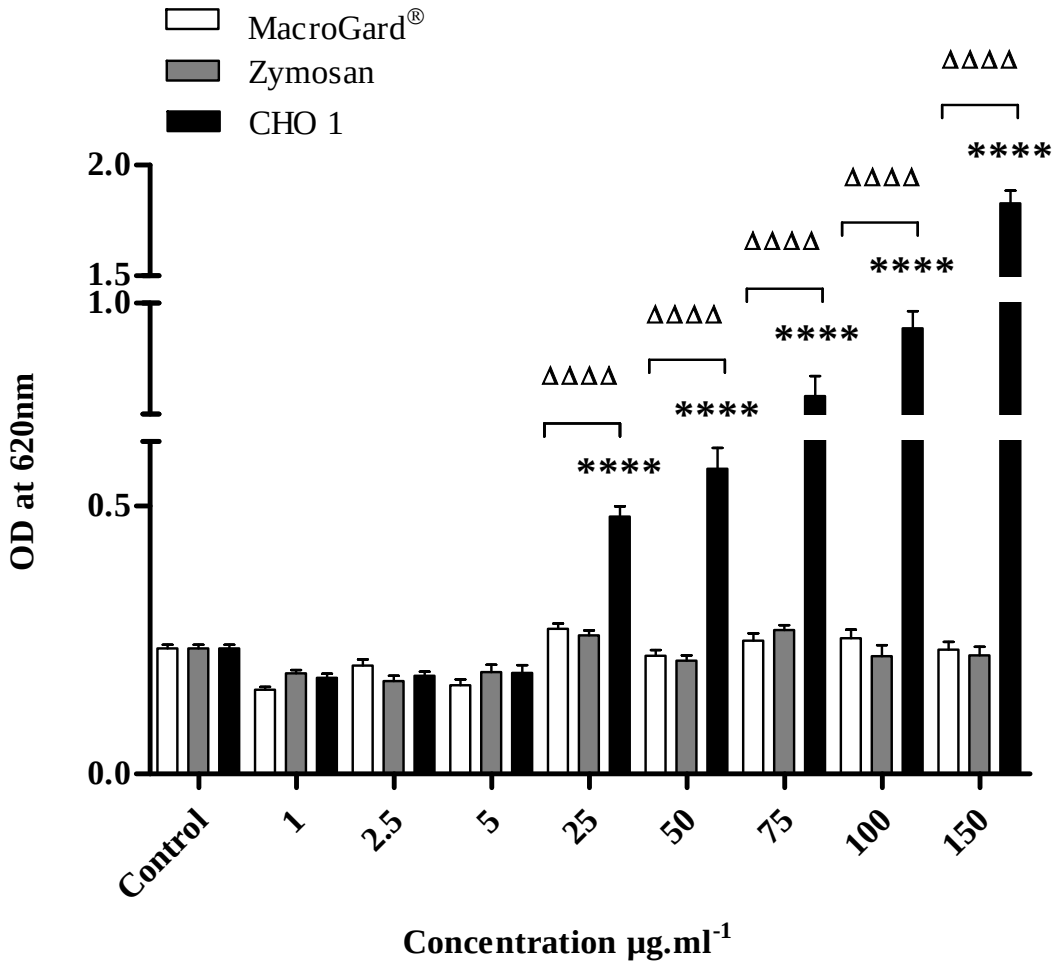


Figure 5-12: Comparison of distinct beta glucan sources and CHO 1 carbohydrate on CLCs respiratory burst activity. Cells respiratory burst activity was measured by NBT assay after cells were distributed at (2×10^4 cells/well) and simulated with either MacroGard®, zymosan or CHO 1 at concentrations 2.5- 150 µg/ml for 24h. Statistical analysis was performed using a two-way ANOVA and Bonferroni post-hoc test ($p \leq 0.05$) and the significant differences between CHO 1 concentrations in comparison to matched control performed with ****= $p \leq 0.0001$, and the comparison between treatments at each concentration performed with ΔΔΔΔ= $p \leq 0.0001$. Data represent mean ± SEM of 6 wells replicates.

5.4 Discussion

5.4.1 Evaluate the biological effect and structure of CHO 1 after sulphation

It was important to determine the effect of the produced CHO 1 after sulphation process to ensure the reproducibility of this modified carbohydrate, therefore the NBT assay was utilised to determine the production of superoxide anion in phagocytic pronephric cells after exposure to different concentrations of CHO 1. After 24 h, a significant increase compared to the control was observed at a concentration equal and higher than 50 µg/ml and reached six fold higher than the control at concentration 250 µg/ml. This effect was comparable to the previous results in Chapter 4 (Figures 4-6 and 4-11) and ascertained the possibility of producing different batches of CHO 1 with the same biological effects. This great and linear increase in phagocytic activity is unusual for an immunostimulant, as most often the effect occurs at certain intermediate concentrations and disappears or even becomes toxic at high concentrations (Kum and Sekkin, 2011).

The sulphation of CHO 1 was confirmed by FTIR spectra (Figure 5-5 and table 5-1), ^{1}H NMR spectrum (Figure 5-6 and 5-7) and double-quantum filtered (^{1}H-^{1}H DQF) COSY spectrum (Figure 5-8). The FTIR spectra for both of Tylose and CHO 1 as in figure 5-5 exhibited absorbtion peaks at 945 cm^{-1} that is a charactaristic for β-D- glucan, similar peaks were found in sulphated beta glucan isolated from *Ganoderma lucidum* (Han et al., 2008). Chang et al. (2006) found in oat beta glucan similar FTIR absorption bands as in CHO 1 at 3431 and 1054 cm^{-1} which were associated with O-H and C-O bonding respectively. In sulphated oat beta glucan, S=O groups vibrate FTIR absorption at 1250 cm^{-1} wavenumber (Chang et al., 2006), however in CHO 1 bands appeared at ~1051 cm^{-1} absorption. The possible peaks of CHO 1 that were associated with a sulphate group shown in table 5-1 was determined with reference to the UNT Digital Library (Tipson, 1968).

At the proton spectroscopy characterisation, the analysis for Tylose was comparable to methylcellulose, the spectrum displayed intense signals at 3.45- 3.44 ppm corresponding to the overlapping of methyl protons at C-2 and C-3, and the proton integrals for carbon 2, 3 methyl group and carbon 6 methyl group were also observed in Tylose spectrum (Nasatto et al., 2015). There are differences in the chemical shifts between Tylose and CHO 1 ^{1}H NMR. The appearance of a strong signal at 4.7 ppm in CHO 1 spectrum might be a characteristic to vinylic group C=C–H that appeared in signals between 4.6-5.9 ppm (Abraham and Mobli, 2008). However, Tylose and CHO 1 show hydrogen proton at carbon 3, 5 signals at 3.27 ppm and methyl group at carbon 6 signal at 3.42 ppm, which both signal types found in ^{1}H NMR spectrum of methylcellulose (Nasatto et al., 2015). Furthermore, the COSY spectrum in Figure 5-8 showed the intense signalling area of Tylose spectra between 3.4- 3.2 ppm and CHO 1 between 4.8- 4.6 ppm.

These previous results indicated the differences between Tylose and CHO 1 and the successful of the sulphation process.

5.4.2 The effect of CHO 1 and different carbohydrates on CLCs line respiratory burst activity

Glucan solubility depends on their degree of polymerisation and branching, also on chemical derivations including sulphation (Chang et al., 2006). Although, there are several methods to induce glucan modifications, sulphation process has the most positive effects on biological functions (Han et al., 2008).

Therefore, the following step was carried to evaluate the biological activities of modified carbohydrate CHO 1 and compare it with the native source (Tylose) and different beta glucans. Interestingly, Tylose, zymosan and MacroGard® had no significant effects on CLCs line respiratory burst activity, while CHO 1 induced a significant increase in CLCs

line respiratory burst activity in comparison to control and previous carbohydrates at a concentration equal to and higher than 25 µg/ml.

These rapid responses to CHO 1 carbohydrate by fish leukocytes might be due to the carbohydrate and protein interaction. Sulphation gives polysaccharides negative charges at the sulphate groups, which may be interacting with positive peptide sequence of proteins (Chang et al., 2006). This is in line with the many biological activities shown in heparin sulphation such as regulation of cellular growth and proliferation, cell adhesion, blood coagulation, cell surface binding of proteins, viral invasion, and tumor metastasis (Rabenstein, 2002). The interaction between heparin and protein is well established and very specific, such as the anticoagulant activity results from binding to antithrombin III of a separate pentasaccharide sequences (Toida et al., 2003).

Furthermore, beta glucans are able to bind to leucocyte cells receptors including: complement receptors 3 and dectin-1 which are pattern recognition receptors that recognise a variety of β 1-3 and/ or β 1-6 linked glucans (Brown and Gordon, 2001).

Many investigations reported that the sulphation process altered the chemical and biological properties of glucans, such as sulphated *Konjac glucomannan*, which induced a high anti- HIV activity in the MT-4 cell line similar to the acquired immune deficiency syndrome (AIDS) drug (Bo et al., 2013). In another investigation, the presence of a sulphate group on the lentinan structure caused significant increases in antioxidant activity (Feng et al., 2010). Furthermore, rice bran beta glucan that was subjected to sulphation had a significant differences in molecular weight, solubility, viscosity and exhibited rat blood anticoagulant activity (Chang et al., 2006).

The immunostimulation effects of sulphated polysaccharides isolated from natural sources was also reported in different studies like the natural sulphated glucan derivate from a marine alga (*Gracilaria verrucosa*) induced respiratory burst activity and phagocytosis in

mice macrophages (Yoshizawa et al., 1996). In addition, carrageenan is a sulphated polysaccharides extract from red algae that possesses potent anti-inflammatory properties in rodents and prime mice leukocytes to produce cytokines (TNFα) in response to LPS (Ogata et al., 1999). The importance of sulphate groups in *Ulva rigida* polysaccharides was also supported in a study conducted by Leiro et al. (2007) on RAW264.7 murine macrophages, where the *Ulva rigida* treatment induced an increase in several chemokine and interleukin expression also nitric oxide production.

Similar result to Chapter 4 was obtained when both of CHO 1 and MacroGard® dose dependant was examined in CLCs, the viability test revealed there was no significant effects on cell cytotoxicity after 24 h incubation in both treatments (see Figure 4-8). However, the optical density level was reduced at the highest concentration (250 µg/ml) of both treatments and this might be due to the threshold concentration of these substance and possible toxicity at certain concentrations or due to the toxic level of reactive oxygen species production in result to cells activities i.e. phagocytosis (Kepka et al., 2014, Miest and Hoole, 2015).

In summary, successful sulphation was demonstrated and results obtained support the hypothesis that the modification (sulphation) is the reason behind this biological effect of CHO 1. Also, the CHO 1 has been validated and will be taken through to *in vivo* system.

Chapter 6. The injection effects of immunostimulant in carp

6.1 Introduction

One of the most effective methods of preventing diseases in aquaculture is the application of vaccines. The development of safe and effective vaccines is extremely desirable for improving fish health and the reduction of other forms of disease control, such as the application of antibiotics. An important component of the vaccine is the addition of adjuvants or immunostimulants to increase vaccine potency and efficiency. Recently there has been considerable interest in the use of natural products such as β-glucan in improving immune status and disease protection in fish (Sakai, 1999). These natural immunostimulants have been studied for their ability to stimulate the nonspecific immune and the specific immune response (Misra et al., 2006b), alone or in combination with vaccine and their effect appears to be dose dependant and may be short lived. The nonspecific immune system in fish is the first line and the main defence system, and macrophages are the core cellular component of this system being associated with massive phagocytic and microbicidal capacity (Brattgjerd et al., 1994). The polysaccharide immunostimulants are recognised as foreign agents by the immune system due to their similarity to the polysaccharides in fungus and Gram negative bacteria. This recognition invoke inflammatory responses that provide effective protection against the possible pathogen (Misra et al., 2006b). The immunostimulant β-glucan has been utilised to enhance resistance against several bacterial diseases such as *Edwardsiella tarda* and *Aeromonas hydrophila* in common carp (Yano et al., 1991, Selvaraj et al., 2005); *Yersinia ruckeri* in Atlantic salmon (Robertsen et al., 1990) and rainbow trout (Jeney and Anderson, 1993a); *Streptococcus sp*. in Yellowtail; *Aeromonas salmonicida* in rainbow trout (Siwicki et al., 1994); *Aeromonas hydrophila* in Catla (Kamilya et al., 2006).

The administration of β-glucan resulted in enhancement and modulation of the defence mechanism through: increasing total leukocytes and differential count (Selvaraj et al., 2005); bacterial killing activity, phagocytosis, production of antimicrobial mediators including superoxide anion (Sakai, 1999, Robertsen, 1999); cytokines synthesis such as interleukin-1β (Secombes et al., 1998, Selvaraj et al., 2006) and complement and lysozyme activity (Engstad et al., 1992).

The use of immunostimulants as adjuvants in vaccine triggers antigen presenting cells APC (such as macrophages, B lymphocytes and dendritic cells) to produce cytokines (e.g. IL1 and IL2) (Raa, 2000). Cytokines induce T helper cells and B cells proliferation, with antibody production occurring when the newly proliferated B cells differentiate into plasma and B memory cells (Raa, 2000) as illustrated below in Figure 6-1.

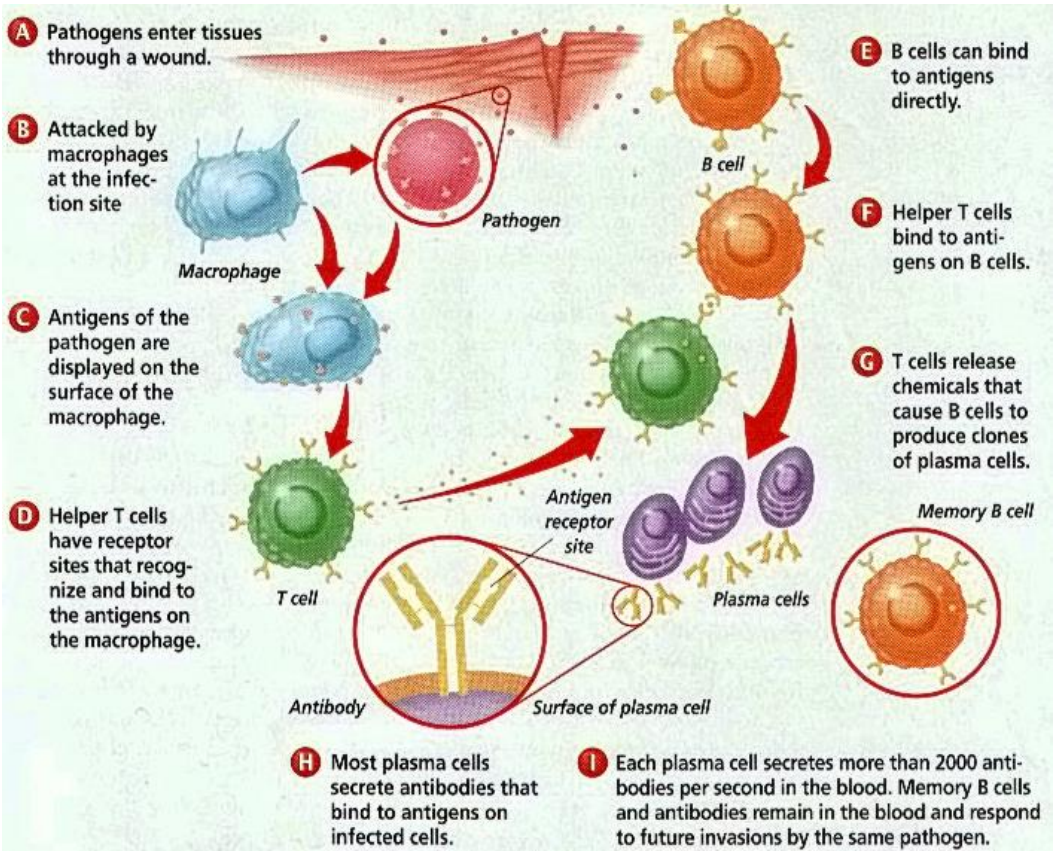


Figure 6-1: Simplified diagram of the phagocytosis and destruction of a bacterial cells in mammals (Kemerer, 2009).

Fish use several immune mechanisms to resist bacterial infection, including both nonspecific and specific immunity. These pre-existing and responsive mechanisms provide protection through preventing pathogen attachment, attack and growth inside or outside the host tissues, however pathogenic bacteria developed several methods to disrupting these defence mechanisms of fish (Ellis, 1999). *Aeromonas hydrophila* is an extremely heterogeneous bacterium, which can infect human and wide range of aquatic and domestic animals especially in stress condition. *Aeromonas hydrophila* is a fermentative, gram negative bacterium which, is a component of intestinal microflora of healthy fish and can cause disease in stressed fish (Ardó et al., 2010). *A. hydrophila* can multiply very rapidly in cultured fish and is associated with tail and fin rot, haemorrhagic septicaemia, epizootic ulcerative syndrome (EUS) and abdominal distension (Ardó et al., 2010). There are several ways to control *A. hydrophila* disease in aquaculture, some antibiotics are described as being effective such as furnace, sulphonamide, chloramphenical, neomycin, sulfamethoxazoletrimethoprim, streptomycin, naladixic acid, oxolinic acid, neomycin, sarafloxacin, rifampicin, oxytetracycline, cephamycins, moxalactam, ciprofloxacin, amoxycillin and enrofloxacin (Poobalane, 2007). Also, some chemotherapeutants such as amino acid-derived hydroximates and hydrogen peroxide (H_2O_2) found to be effective to control *A. hydrophila* (Poobalane, 2007). However, *A. hydrophila* developed a resistance against the chemotherapeutants when was used for long time, and to several antibiotics including: ampicillin, carbenicillin, erythromycin, gentamicin, penicillin, tetracycline, nitrofuradantoin, ormetoprim-sulfadimethoxine, sulfamethoxazole-trimethoprim and triple sulpha (Poobalane, 2007). Vaccination is the most successful strategy to control this disease, although there are no commercial vaccine available for *A. hydrophila*, different vaccines development attempt have been investigated including whole cell (WC), Recombinant outer membrane proteins OMPs, extracellular products ECPs,

Lipopolysaccharide LPS and biofilms. The vaccines have been shown different degrees of increased immunity and protection, however developing an effective safe vaccine for *A. hydrophila* are still the greatest concern. Adjuvants are important ingredients for vaccine efficacy, they mediate the induction of safe, complementary immune responses to those generated during infection (Tafalla et al., 2013). There are several adjuvants available in veterinary and human vaccines that differentiation T helper cells into several T cell lineages, such as Th1, Th2, Th9 and Th17 (Tafalla et al., 2013). Table 6-1 shows list of the commercial and experimental adjuvants.

As mentioned earlier, the use of immunostimulant β-glucan with LPS in the intraperitoneally injection in common carp enhanced the resistance to *A. hydrophila* challenge through several innate and adaptive immune responses (Selvaraj et al., 2006). Several studies have evaluated the use of β-glucan as an adjuvant in fish bacterial vaccines that are injected intraperitoneally. The immune responses to the adjuvant administration is related to the timing of the immunostimulant and vaccine administration, for example high antibody levels were reported when glucan was administrated in combination with a vaccine (Chen and Ainsworth, 1992). In fish the combination of yeast glucan and furunculosis vaccine induced higher protection against furunculosis in Atlantic salmon (Rørstad et al., 1993). When yeast glucan was used as an adjuvant in a *Vibrio damsela* vaccine in turbot, the index and rate of phagocytosis, passive haemolytic plaque numbers, and agglutinating antibody titer was significantly higher than control group when glucan injected with and after the vaccine (Figueras et al., 1998). Mushroom glucan was used as an adjuvant in conjunction with formalin killed *Aeromonas hydrophila* vaccine in Catla, and fish injected with glucan adjuvanted vaccine induced highly significant increase in antigen-specific proliferation, macrophage activating factor (MAF) production and antibody production levels (Kamilya et al., 2006).

Table 6-1: List of adjuvants licensed and experimental adjuvants mainly for human medicine (Tafalla et al., 2013).

Adjuvant	Central immunostimulatory component(s)	PPR/process	Principal immune response elicited
Alum	Aluminium salts	NLRP3 (?)	Ab, Th2 (+Th1 in humans)
MF59 and AS03	Squalene in water emulsions	Tissue inflammation	Ab, Th1 and Th2
AS04	MPL+Alum	TLR4 and NLRP3 (?)	Ab and Th1
Adjuvants in experimental use or in late stage clinical development			
Poly I:C	Synthetic dsRNA		Ab, Th1, CTL
MPL, and in different formulations			Ab, Th1
Flagellin, flagellin-Ag fusion proteins	Recombinant flagellin from bacteria	TLR5	Ab, Th1+Th2
Imiquimods	Imidazoquinoline derivatives	TLR7, TLR8 and both	Ab, Th1, CTL (when conjugated)
CpG, and in different formulations	Synthetic phosphorthioate-linked DNA oligonucleotides with optimised CpG motifs	TLR9	Ab, Th1, CTL (when conjugated)
ISCOMS	Saponins	Not defined	Ab, Th1+Th2, CTL
IFA and montanide formulations	Mineral or paraffin oil+ surfactant	Not defined	Ab, TH1+Th2
CFA	IFA+ peptidoglycan, trehalose dimycolate	NLR, TLR?	Ab, Th1, Th17

MF59 (Novartis proprietary adjuvant MF59 containing squalene, polyoxyethylene sorbitan monooleate and sorbitan trioleate), AS03 (GlaxoSmithKline) contains squalene, DL-a-tocopherol, polysorbate), AS04 (Aluminium hydroxide and monophosphoryl lipid A (MPL), ISCOMs (immune-stimulating complex; nanostructure of cholesterol, phospholipids and Quil-A saponins), IFA (incomplete Freund's adjuvants). Ab: antibodies, ?: not documented yet.

The aim of this chapter was to examine the *in vivo* immunostimulatory effects of the formulated carbohydrate CHO 1 in carp. The solubility of this carbohydrate in water and the poor and inconstant responses of oral vaccines due to antigen destruction in the gut (Sommerset et al., 2005) lead to ascertain the effects of this newly developed carbohydrate as a suitable adjuvant in formalin killed *Aeromonas hydrophila* vaccine in carp. Therefore, two investigations were carried out to achieve this aim.

In the first investigation the influences of CHO 1 and MacroGard® injections on innate immune response in common carp, was examined and monitored by measuring serum lysozyme activity and complement pathway (ACH50), blood smear leukocytes differentiation cell count and immune related gene expression in carp organs.

In the second investigation the adjuvant effect of a formulated carbohydrate (CHO 1) in vaccines against *Aeromonas hydrophila* in common carp was evaluated utilising blood smear leukocytes differentiation cell counts; pronephric cell antigen-specific proliferation and respiratory burst activity; serum complement pathway (ACH50) and antibody titre against *A. hydrophila* vaccine.

6.2 Materials and Methods

6.2.1 Investigation I: The influences of CHO 1 and MacroGard® injections on innate immune response in common carp

6.2.1.1 Source and maintenance of fish

Common carp with average weight 88.02 ± 11.98 g and the head - tail length 15.8 ± 0.8 cm, obtained from Fair Fisheries, Shropshire, UK, were maintained at Keele University as described in (2.1). Fish were kept in 8 tanks each tank each containing 30 individuals which were fed with a 0 % MacroGard® pellets supplied by Tetra (Tetra GmbH, Germany) (see Appendix for the feed composition). Fishes were acclimated to experimental condition at least 3 weeks prior the experimentation.

6.2.1.2 Experimental design

The *in vitro* trials described in both Chapters 4 and 5, which determined CHO 1 concentrations effects on pronephric cells and carp leukocytes cell lines (CLCs) concluded that significant effects occurred at concentrations between 25-150 µg/ml and there was no reduction in viable cells at any of tested concentrations. Therefore, the *in vitro* 50 µg/ml of CHO 1 was used in the *in vivo* trials. This concentration was selected based on two criteria 1) an estimation determined from *in vitro* studies and 2) a review of previous publications where beta glucans were injected into fish.

1) The *in vitro* experiments determined the effect of different concentrations of CHO 1 in 160 µl of cell suspension and thus the concentration in 1 ml of cells suspension could be ascertained. Furthermore, since 1 g of pure water is equal to 1 ml of pure water at 3.98℃ and knowing the average weight of the experimental fish, it was possible to estimate the injection concentration of the modified carbohydrate per standard weight of fish. Thus:

$C_1 \times V_1 = C_2 \times V_2$

Where:

C_1 = Initial concentration or molarity.

V_1 = Initial volume.

C_2 = Final concentration or molarity.

V_2 = Final volume

50µg/ml ×160 µl = C_2 × 1000 µl

Then C_2 = 8 µg/ml for 1 gm fish body weight

Therefore C_2 = 8 mg/ml for 1 kg fish body weight is the possible dose that can be used *in vivo*

2) This estimation of concentration of CHO1 to inject was supported by previous studies. For example, in a study carried out by Selvaraj et al. (2006) carp were injected with either 4, 18 or 36 mg/kg of fish body weight and fishes were sampled after 1, 3 and 5 days post injection. Misra et al. (2006b) also studied the effect of beta glucan injection in *Labeo rohita* fingerlings at concentrations 5, 10 and 15 mg/kg of the body weight and fishes were sampled every two weeks for two months.

Therefore, the chosen dose for the injection with the modified carbohydrate CHO 1 was 5 and 10 mg/kg of fish body weight. MacroGard® at concentration 5 mg/kg was used as a source of non-modified carbohydrate and 100 µl of sterile water (Sigma, W3500) was used as a negative control (see Figure 6-2). Experimental replication comprised 2 experimental tanks for each treatment and at each time point 6 fishes were sampled from both tanks. Water temperature was recorded twice a day during the experimental period.

6.2.1.3 Sample preparation

Fish were sampled from each treatment at 1, 3, 7 and 14 days p.i. Feeding ceased 24 h prior each sampling time point, and was restarted 24 h after sampling. Fish were killed with a lethal dose (~ 0.2 %) of 2-Phenoxyethanol (Sigma, P1126) in aquarium water and blood was collected from the caudal vein using sterile syringes and needles before dissection at each time point. Carp blood was stored at 4 °C overnight, serum was collected after centrifugation for 5 min at 1400 x *g* (Boeco Germany U32-R) and stored at -80 °C. In addition, a blood smear was prepared for each fish and left on a flat surface to air dry, then the blood smear was stained with May- Grünwald's- Giemsa staining as described in Section 6.2.1.4.3.

Fish dissection were carried out under sterile conditions, and the pronephros, liver, spleen and mid gut were removed at each time period post injection, placed in RNA later (see Appendix) and stored at -80℃ freezer. The expression of selected immune genes was determined in sampled organs and comprised: pro-inflammatory cytokines (IL1β and IL6 family), pro-inflammatory chemokines (qCXCα), anti-inflammatory cytokine (IL10), nitric oxide production (iNOS), antiviral cytokines (IFNγ2β), viral resistance protein (Mx), complement system activator (C3), antibacterial activity (Lysozyme-C), and the housekeeping gene (40S). Figure 6-2 shows the experimental design.

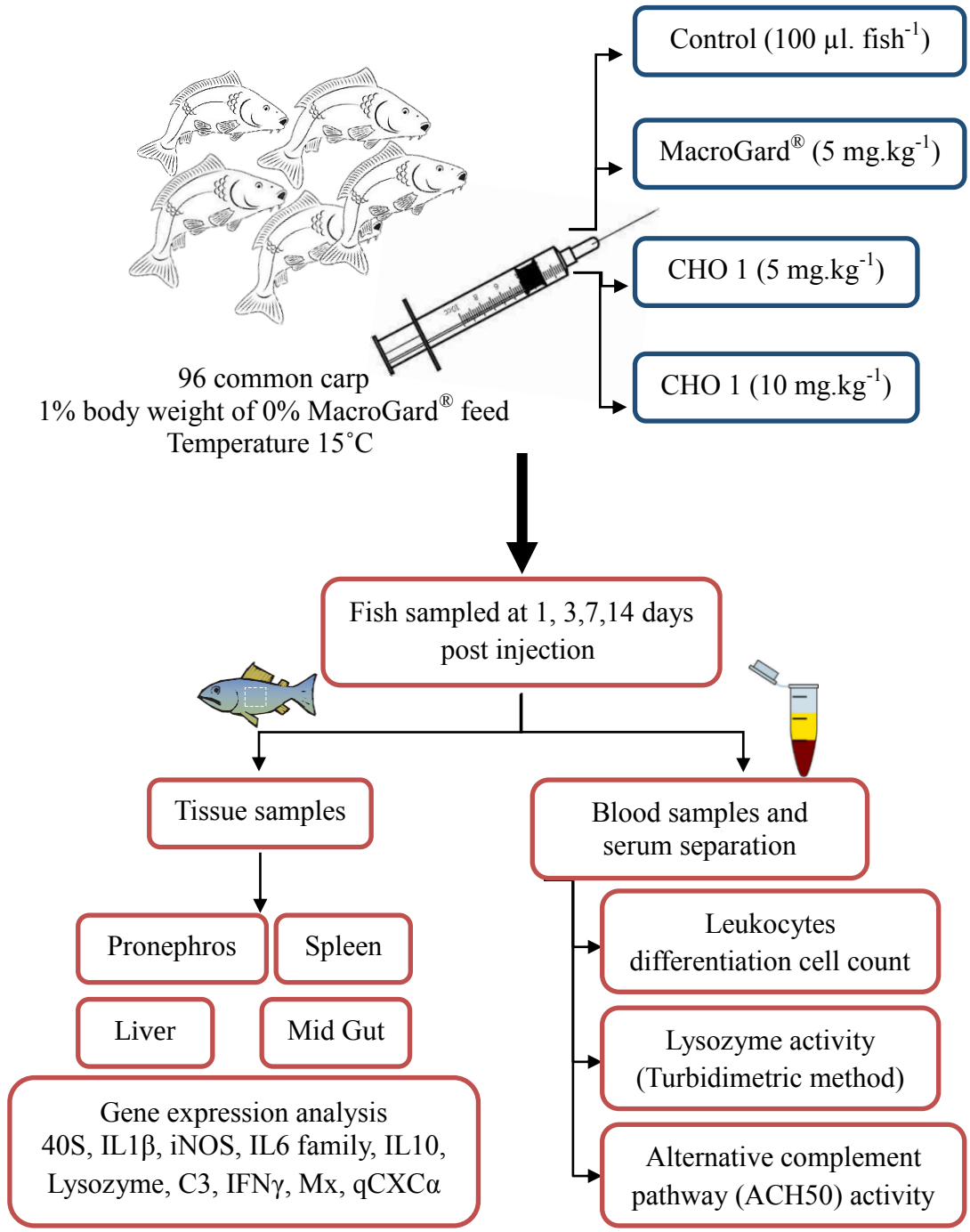


Figure 6-2: The experimental plan for the influences of CHO 1 and MacroGard® injections on innate immune response in common carp.

6.2.1.4 Blood sample analysis

6.2.1.4.1 Lysozyme activity assay

A turbidimetric method was used to measure lysozyme activity in carp serum as described by Wang et al. (2011). Briefly, a suspension of freeze dried *Micrococcus lysodeikticus* (Sigma, M 3770) at 0.2 mg/ml was prepared in 0.05 M phosphate buffer saline (PBS), pH 6.2 (Sigma, P4417). Serum samples from 6 fishes/ treatment/ time point were diluted 1:1 with 0.05 M PBS pH 6.2 and 50 µl of the diluted serum were placed in triplicate in a flat bottom 96 microtitre well plate (Sarstedt, 83.1835.500) and 200 µl of *Micrococcus lysodeikticus* suspension was added to each well. A negative control was prepared by replacing serum with PBS. Plates were read spectrophotometrically at 540 nm and the reduction of the optical density measured from 0 to 5 min at 22℃ in a microplate reader (Multiskan multisoft plate reader, Labsystems, Finland). The lysozyme activity was calculated based on the amount of lysozyme that caused a decrease in the absorbance of 0.001 min^{-1} in 1 ml of serum sample.

6.2.1.4.2 Alternative complement activity

- **Sheep red blood cells preparation**

Two ml of sheep red blood cells (SRBC) in Alsevers (TCS Biosciences) was centrifuged at 400 x *g* for 5 min at 4℃ (Boeco Germany U32-R) and the supernatant discarded. The SRBC pellet was washed with 1.5 ml of 0.85% saline solution, re-centrifuged and the pellet suspended in 1.5 ml gelatin veronol buffer (GVB) (0.1% gelatin, 250 mM NaCl, 50 mM barbitone, 40 mM sodium barbitone, 10 mM $MgCl_2$, 2 mM $CaCl_2$, all from Sigma). Total RBC count per ml was determined using a haemocytometer and calculated as described in Section 2.2.2. The number of SRBC required in the assay was determined by calculating the number of cells required which would give an OD reading of 1 when 100%

haemolysis occurred (see Figure 6-3) and as a result, a standard working concentration of 2.5×10^7 cells/ml in GVB were used in the assay.

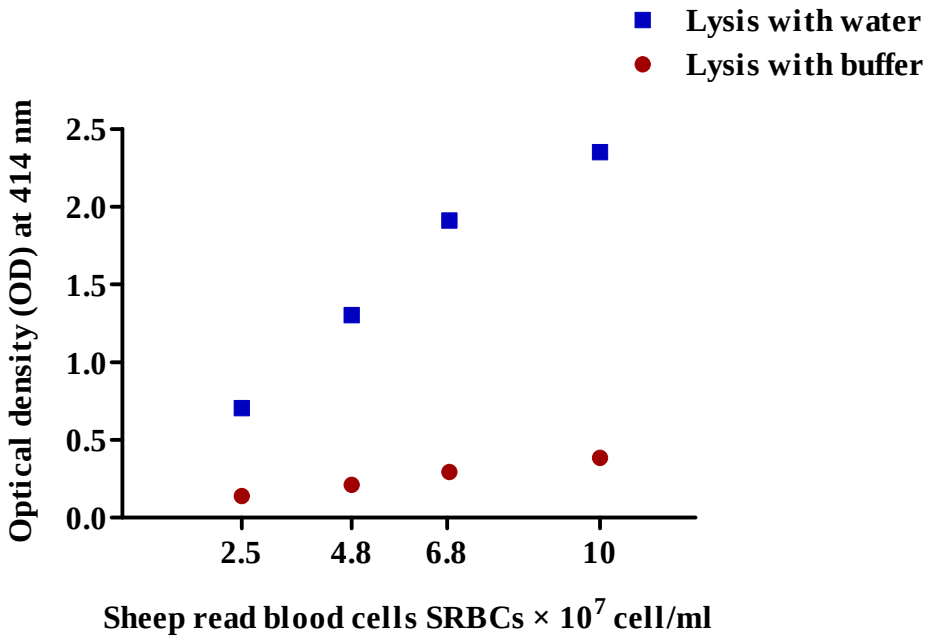


Figure 6-3: The number of SRBC required in the alternative complement pathway assay. The assay optimisation was determined by calculating the number of cells required which would give an OD reading of 1 when 100% haemolysis occurred.

- **Serum collection**

Experimental fishes were sacrificed as described in Section 6.2.1.3, 6 fishes for each treatment group were used and a blood sample was collected by exsanguination from each fish as mentioned in Section 6.2.1.3. Carp blood was stored in 4 °C overnight and serum was collected after centrifugation for 5 min at 1400 x *g* (Thermo Scientific Heraeus Pico and Fresco 21) and stored at -80 °C.

- **Alternative complement activity assay**

Complement activity was determined as described by (Pionnier et al., 2013). Briefly, a serial dilution of carp serum from 0.5% to 7% were established in 1.5 ml centrifuge tubes, each dilution had a positive control which represented 100% haemolysis in distilled water. This comprised an appropriate volume of serum, 100 µl of each of distilled water and SRBC. However, the sample tests comprise the same amount of serum as in the positive control, 100 µl of each of 10 mM EGTA-GVB and SRBC. Both of positive controls and test samples were incubated 1 hour at 19±1℃ (20 ± 2℃) with a shaking step after 30 min. Samples were centrifuged at 400 x *g* for 5 min at 4℃ and placed on ice for 5 min to stop the lytic reaction. The samples were read spectrophotometrically by placing 200 µl of supernatant into a flat bottom 96 microtitre well plate (Sarstedt, 83.1835.500) and the haemoglobin release optical density (OD) was read at 414 nm (Multiskan multisoft plate reader, Labsystems, Finland). Haemolysis was determined for each serum concentration by dividing the OD value for the test sample by the OD value for the positive control. A graph of log (x= serum dilution) against log (y= ratio of SRBC haemolysis) was plotted to create calibration curve, the graph equation was used to calculate the estimating volume of serum giving 50% SRBC haemolysis i.e. ACH50 (Pionnier et al., 2013).

6.2.1.4.3 Leukocytes differentiation cell count

May- Grünwald's- Giemsa staining is one of the most common staining methods to detect and differentiate blood cells type in fish. The staining method is established on the electrostatic interaction between dye and target molecules. The May Grünwald's solution, consisting of eosin-methylene blue, and Giemsa solution, a complex consisting of methylene blue chloride, eosin-methylene blue and azure II. The basic dye (methylene blue and azures) stains nuclei, granules of basophil granulocytes and RNA molecules in the

cytoplasm of white blood cells, a blue colour. The acid dye (eosin) stains red blood cells and granules of eosinophil granulocytes in a red colour.

Blood smears were prepared as described in session 6.2.1.3, fixed with 100% methanol (Fisher, 11976961) for 30 sec, left to dry, and the slide then flooded with 20-30 drop of May- Grünwald's solution (VWR, 352622M) for 3 min. An equal volume of distilled water was added for 1 min, the stain was decanted and then placed in a coplin jar filled with diluted Giemsa solution (BDH, Gurr, 35014) at a 1:10 dilution with distilled water for 20 min. The smear was washed with distilled water and left to dry before microscopic observation (Schaperclaus, 1991).

Differential leukocytes counts was determined microscopically (Olympus CH2) at 100 x oil immersion magnification. 100 leukocytes were counted and the percentage of different types of leukocytes was calculated as described at Selvaraj et al. (2005).

6.2.1.5 Gene expression in carp tissues

The expression of a range of immune genes was determined in the pronephros, liver, spleen, and mid gut at each time point during the experiment. Tissues were prepared as described in Section 6.2.1.3 and the RNA was extracted using the RNeasy kit (Qiagen) as detailed in Chapter 2 Section 2.7.1. The complementary DNA (cDNA) was synthesised from the extracted RNA as described in Chapter 2 Section 2.7.2 and expression determined using qPCR as mentioned in Chapter 2 Section 2.7.5.

6.2.2 Investigation II: Adjuvant effect of a formulated carbohydrate (CHO 1) in vaccines against *Aeromonas hydrophila* in common carp (*Cyprinus carpio*).

6.2.2.1 Source and maintenance of fish

Common carp with average weight 109.56 ± 25.0 g and the head - tail length 17.24 ± 1.3 cm, obtained from Fair Fisheries, Shropshire, UK were maintained at Keele University as described in (2.1). Fish were kept in 4 tanks each tank contained 32 fished and fed with a 0 % MacroGard® pellets suppiled by Tetra (Tetra GmbH, Germany) during the experiment (see Appendix for the feed composition). Fishes were acclimated to experimental conditions for at least 3 weeks prior the experiment.

6.2.2.2 Experimental design

The previous *in vivo* trial in which the dose responses of carp to two concentrations 5 and 10 mg/kg of CHO 1 was determined, revealed enhancement of innate immune parameters i.e. complement pathway (ACH50), lysozyme activity and the increased expression of some immune genes. This study has been extended to establish the role of CHO 1 as an adjuvant in formalin killed *Aeromonas hydrophila* vaccine NCIMB 9240 in carp. The vaccine was kindly provided by Professor Alexandra Adams (Institute of Aquaculture, University of Stirling, United Kingdom) at administered concentration of 1×10^9 cell/ml with dose recommendation of 100 µl/ fish. Carp were injected intraperitoneally with either: 1) CHO 1 5 mg/kg, 2) formalin killed *Aeromonas hydrophila* vaccine 1×10^9 cell/ml, 3) a combination of CHO 1 5 mg/kg and formalin killed *Aeromonas hydrophila* vaccine 1×10^9 cell/ml or 4) PBS as a control (see Figure 6-4). The experimental replication comprised 8 fishes at each time point and water temperature was recorded twice every day during the experiment.

6.2.2.3 Sample preparation

The samples was taken weekly from each treatment tank and 8 fishes were sacrificed with a lethal dose (~ 0.2 %) of 2-Phenoxyethanol (Sigma, P1126) at 7, 14, 21 and 28 days p.i., and the tissue and blood sampling was carried out as described in Section 6.2.1.3. To determine the Stimulation Index (SI) of pronephric leukocytes, a small piece of pronephros from each fish was kept in 1.5 ml microcentrifuge containing 1 ml of complete medium (RPMI+), stored on ice and analysed after 4 h post extraction. In addition, a blood smear was prepared for each fish and the blood smear was stained as described in Section 6.2.1.4.3. The collected serum samples were stored in -80℃ freezer for later alternative complement pathway analysis (Section 6.2.1.4.2) and antibody titer (Section 6.2.2.6.2). Fish dissection were carried out under sterile conditions, and the pronephros, liver, spleen and mid gut were removed at each time period post injection, placed in RNA later (see Appendix for the recipe) and stored in -80℃ freezer for future determination of the expression immune related gene (Figure 6-4).

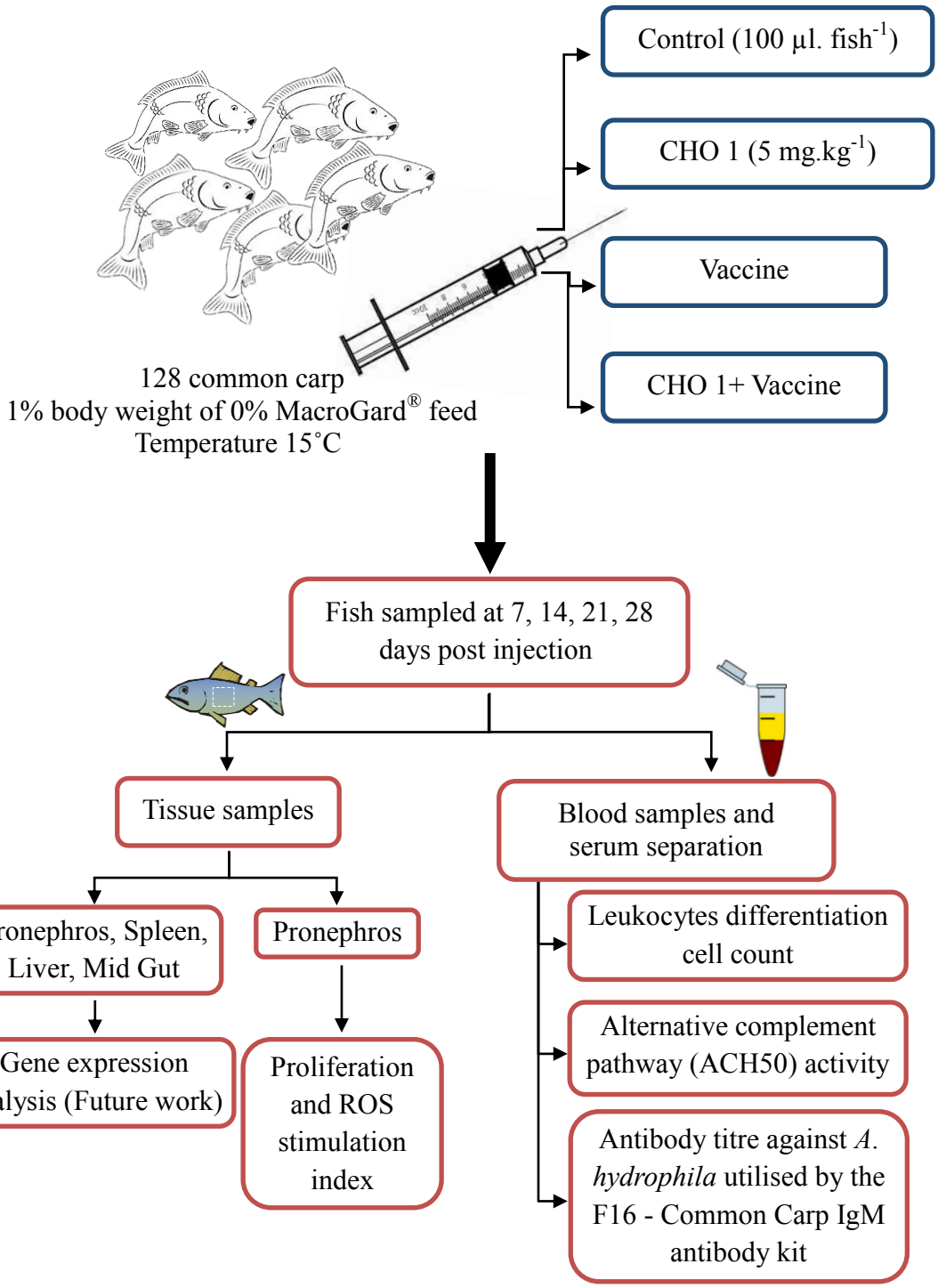


Figure 6-4: The experimental plan for the influences of CHO 1 as an adjuvant in vaccines against *Aeromonas hydrophila* in common carp.

6.2.2.4 Blood sample analysis

6.2.2.4.1 Leukocytes differentiation cell count

The May- Grünwald's- Giemsa stain was used to stain cells as detailed in Section 6.2.1.4.3, and percentage of lymphocytes, granulocytes and monocytes/ macrophages was calculated as described at Selvaraj et al. (2005).

6.2.2.5 Pronephric leukocytes activities

6.2.2.5.1 Antigen-specific proliferation

The colorimetric MTT assay utilised to determine the proliferation of pronephric leukocytes of experimental carp in responses of to *A. hydrophila* antigen specificity as described at Kamilya et al. (2006). For this purpose a pronephric cell suspension was prepared for each fish as explained in Section 2.2.1, aliquots of 100 µl cell suspension at density 2×10^6 cells/ml were added to 96-well flat bottom plates (Corning, 3596) in 6 wells replicates. Three of these wells was in the presence of a further 100 µl of heat-killed *A. hydrophila* at concentration 1×10^7 cells/ml while the remaining 3 wells were without additive. The plate was incubated for 24 h at 27℃ and 5% CO_2 incubator (L11738 Heto Cellhouse 170). After incubation, 20 µl of MTT solution (dissolved in PBS at a concentration of 5 mg MTT/ml) were added to all the wells and the plate was incubated at 27℃ for 4 h. After incubation, the supernatant was discarded and cells solubilised in 200 µl of DMSO Dimethyl sulfoxide (Fisher, BP 231) and measured spectrophotometrically on a microplate reader (BioTek EL800) at 540 nm. The results of the proliferation assay were expressed with and without the antigen specific and also as a Proliferation Index, which was calculated by dividing the mean OD of stimulated cultures by the OD of the non-stimulated cultures.

6.2.2.5.2 Respiratory burst activity

The respiratory burst activity of pronephric leukocytes was measured by the nitro blue tetrazolium salt (NBT) reduction assay after cell isolation, following the method described by Bastardo et al. (2012) and Sirimanapong et al. (2014) with slight modification. Briefly, pronephric leukocytes at density 1×10^6 cells/well were incubated in 96-well flat bottom plates (Corning, 3596) at 27 C° in 5% CO_2 incubator for 2-3 h until the phagocytic cells attached to the well. The supernatant was discarded and cells were washed with phenol red free Hank's balanced salt solution (HBSS) (Sigma, H8264) and 100 µl of modified RPMI+ containing NBT with concentration 1 mg/ml (Sigma,N6876) were add to each well. Six replicate wells of leukocytes were prepared for each fish. Half of the wells were stimulated with 100 µl of heat-killed *A. hydrophila* at a concentration 1×10^7 cells/ml, while the cells in the remaining 3 wells were not stimulated. After 24 h incubation at 27℃ and 5% CO_2, the remaining steps of NBT assay was performed as detailed at Section 2.6.1 and data was presented as the respiratory burst of experimental fishes with and without antigen specific stimulation, also as a ROS stimulation index that was calculated as follows: SI = (antigen specific stimulation ROS [OD]) / (unstimulated ROS [OD]).

6.2.2.6 Serum activity

6.2.2.6.1 Alternative complement activity assay

Sheep red blood cells haemolytic activity of complement level in experimental fish serum was utilised as described in Section 6.2.1.4.2.

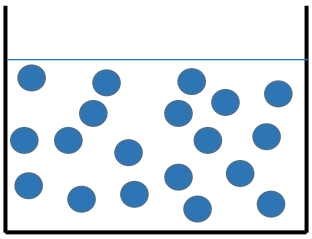
6.2.2.6.2 Antibody titre against *A. hydrophila*

In order to determine the antibody level against *A. hydrophila* in serum samples from treated fishes, the anti-Carp/ Koi Carp (*Cyprinus carpio*) IgM monoclonal antibody F16 kit from Aquatic Diagnostic Company, Stirling was used. The manufacture's protocol was followed to determine antibody levels of antigen- induced IgM, however it was important to ensure the validity of the protocol to the experimental conditions utilised. Therefore a several experiments were carried out to optimise the ELISA assay.

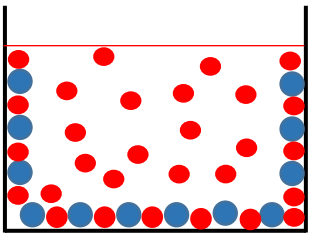
The protocol steps are illustrated in Figure 6-5. Briefly, a 96 well microplate flat bottom (Greiner, 655061) were coated with 100 µl/well 0.05% (w/v) poly-L-lysine (Sigma, P8920) in coating buffer (see Appendix for the recipe) and incubated for 60 min at 22℃. Plates were washed twice with low salt washing buffer (see Appendix) and then 100 µl/well of heat killed *A. hydrophila* at density 1×10^8 cells/ml was add to each well and incubated overnight at 4℃. 50 µl/well of 0.05% (v/v) glutaraldehyde (Sigma, 340855) in PBS was add to the antigen which was incubated at 22℃ for 20 min, and then washed three times with low salt buffer. To block the nonspecific binding sites, 250 µl/well of 1% (w/v) of bovine serum albumin (BSA) in PBS was add and incubated at 22℃ for 2 h. Plates were then washed three times with low salt washing buffer and 100 µl/well of 1:2 serially diluted serum samples in PBS were added and incubated overnight at 4℃. Dilutions of serum samples used ranged from 1/100 to 1/204800, with control wells containing PBS alone. Plates were washed five times with high salt washing buffer (see Appendix for the recipe), then 100 µl/well of the reconstituted anti-carp IgM monoclonal antibody F16 added and incubated for 1 hour at 22℃. Plates were washed five times with high salt wash buffer incubating for five min on the last wash, before adding 100 µl/well goat anti-mouse IgG-HRP (BIO-RAD, 172-1011) diluted 1/1000 in conjugate buffer (as

detailed in Appendix). After one hour incubation at 22℃, plates were again washed five times with high salt wash buffer incubating for five min on the last wash. The detecting reaction was carried out by adding 100 µl/well of 1-Step™ Ultra TMB-ELISA Substrate Solution (Fisher scientific, 34028) for 10 min at 22℃, and then the assay was stopped by the addition of 50 µl/well of 2 M sulphuric acid (H_2SO_4) (Fisher Scientific, 10558620) (Figure 6-5). The optical density of the reaction was measured with a microplate reader at 450nm (GloMax-Multi Microplate Reader, Promega, US). All the washing steps were performed using a microplate washer (ATLANTIS, UK) and plates were tapped onto paper tissue.

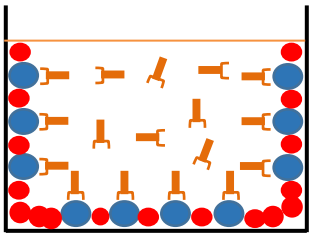
The cut-off point titre of the antibody response was defined as the reciprocal of the dilution showing an absorbance at least two times greater than the negative control (PBS). The data was presented as the mean of the logarithm to the base 10 of the dilutions and compared to the experimental control group i.e. fishes injected with PBS.



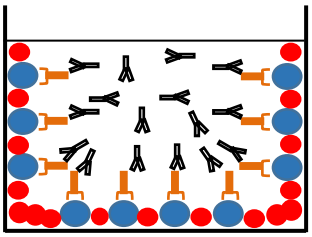
1. Incubation of *A. hydrophila* antigen ( ) in wells overnight at 4℃.



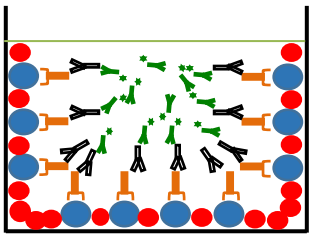
2. Blocking ( ) of remaining binding sites with 3% BSA for 2 h at 22℃.



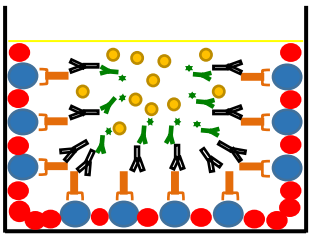
3. Antibody source (serum) ( ) incubation for 3 h at 22℃.



4. Anti-fish specific IgM monoclonal antibody ( ) incubated for 60 min at 22℃.



5. Conjugate anti-mouse HRP ( ) incubated for 60 min at 22℃.



6. Substrate buffer TMB ( ) is added and colour developed for 10 min and reaction is stopped using H_2SO_4.

Figure 6-5: Antibody titer against *A. hydrophila* procedure: ELISA procedure for measuring antibody level of antigen-induced IgM.

- **ELISA optimisation**

The protocol steps that described above (Figure 6-5) were carried out on non-experimental fish serum to check for specificity and cross reaction of each component of the protocol could be serial eliminated. The testing and optimisation procedures were carried out in 96 wells ELISA plate (Greiner, 655061) and comprised three replicates per condition. The conditions comprised wells: 1. Absence of *A. hydrophila* antigen to ensure the blocking was effective and there is no non-specific binding of the antibodies in the serum sample; 2. Absence of serum sample to check the nonspecific binding of the primary antibody (anti-carp IgM) to the plate; 3. Absence of the primary antibody to test the specific binding of the secondary to the primary antibody; and 4. Absence of secondary antibody, to ensure the reaction did not occur without the enzyme that is conjugate with the secondary antibody. The optimisation showed nonspecific colour development occurred when the serum, primary and secondary antibody were eliminated from the ELISA plate. However, the specificity of the secondary antibody was successfully proved (see Figure 6-6).

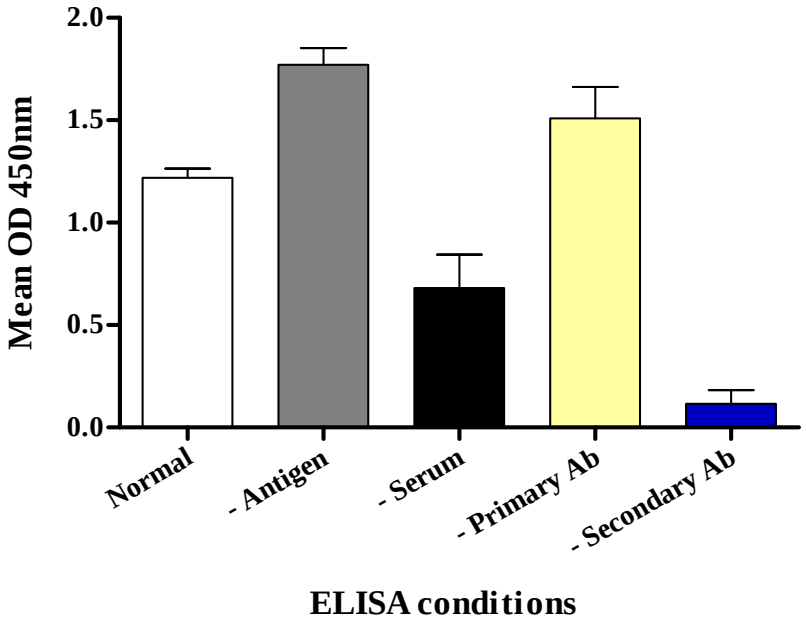


Figure 6-6: Optimisation the condition of ELISA steps. Bars represent mean ± SEM of 3 wells.

The next optimisation step was to increase the blocking solution concentration. Briefly, the concentration of blocking solution, BSA, was increased to 3% and compared to semi skimmed dried milk powder DMP (Marvel) at concentrations 3 and 5% (w/v) in PBS. Similar ELISA conditions as described above in Section 6.2.2.6.2 were utilised. As seen in figure 6-7, 3% BSA reduced background colour in to values comparable to 1% BSA (see Figure 6-6) and both concentrations of DMP. In addition the nonspecific binding of the primary antibody was eliminated as the result of increasing the blocking solution concentration with both BSA and DMP. However, it appeared that the nonspecific binding of serum antibody to the blocking products still occurred but blocking with 3% BSA produced less colour intensity in comparison to DMP at both concentrations (see Figure 6-7). Therefore, the concentration of blocking solution utilised in the analysis was 3% (w/v) BSA in PBS.

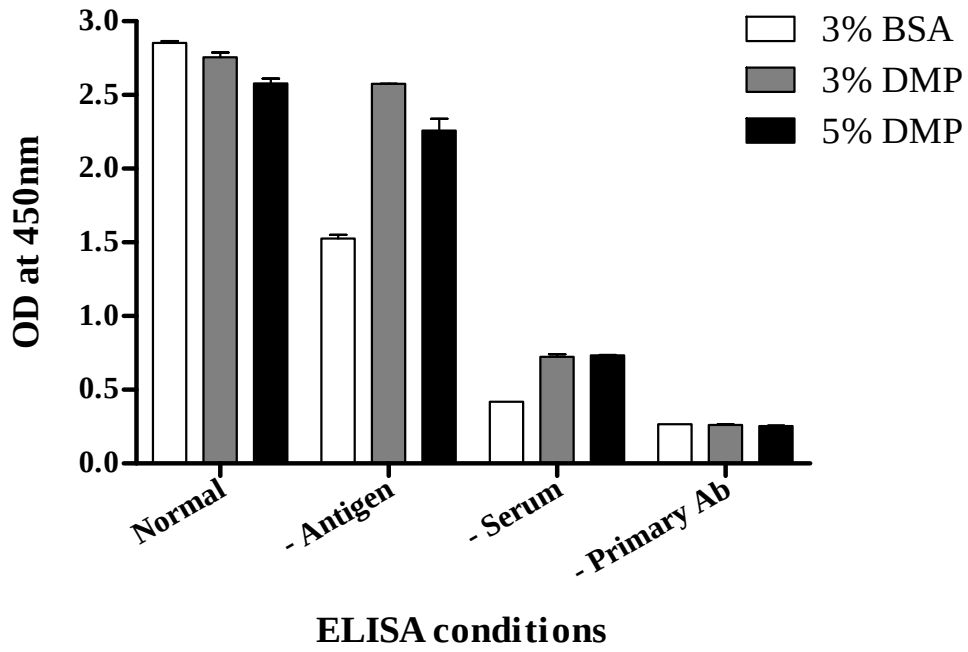


Figure 6-7: Optimisation of type and concentration of blocking solution utilised in the ELISA assay. Bars represent mean ± SEM of 3 wells.

During the optimisation process, on some occasions dark brown sediments were observed in the wells after adding the substrate (TMB) that might cause nonspecific and high colour development in all the ELISA conditions. Therefore, it was important to consider other factors that might affect the background noise and the produced sediments at the ELISA assay. The concentration of the secondary antibody affects the colour development of the assay, thus the third optimisation was to test the ELISA condition at two dilutions of the anti-mouse IgG HRP i.e. 1/1000 and 1/3000 (manufacture recommendation) and the incubation period of the substrate (Figure 6-8). The ELISA protocol was repeated as described in Section 6.2.2.6.2 with two wells of the ELISA plate with and without serum samples for different incubation periods with TMB i.e. 10, 15 and 20 min. At the ELISA condition without serum, the manufacture recommendation 1:3000 dilution factor of the antibody dilution produced consistent background colour at all the TMB incubation periods without any sediments at the bottom of the wells (Figure 6-8 A), while the 1:1000 dilution group caused increases in colour development within incubation period and a precipitate was produced at all incubation periods (Figure 6-8 B). Whilst, the colour development of ELISA condition with serum sample increased with time in the wells that had been incubated with 1:3000 dilution of the secondary antibody (Figure 6-8A), the 1:1000 dilution group developed a reaction colour which increased within the incubation period forming a precipitate. Therefore the 1:3000 dilution with a 10 min substrate incubation period was used in the following ELISA assays.

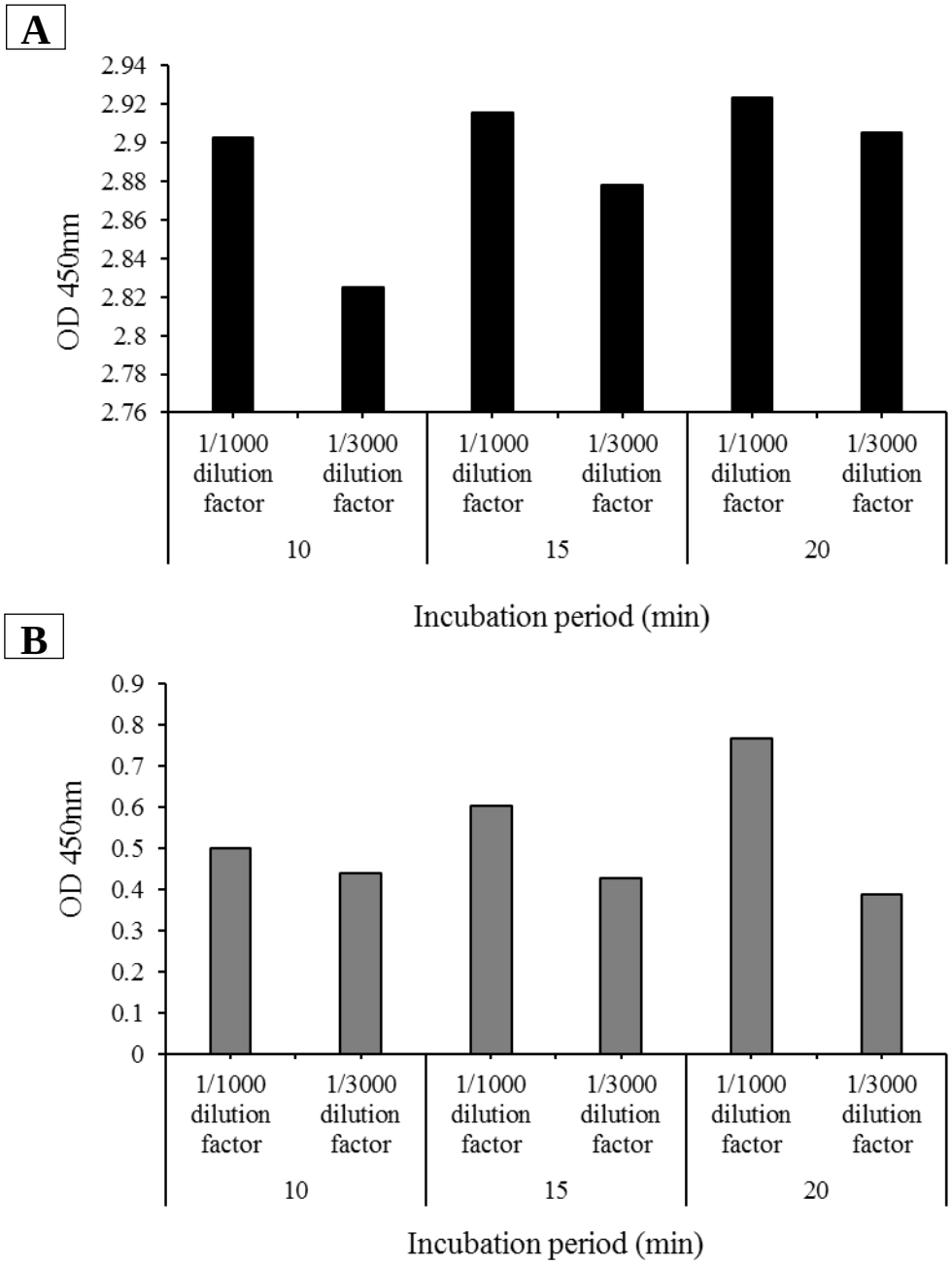


Figure 6-8: Optimisation the secondary antibody concentration and incubation period of the ELISA. The ELISA condition was determined with non-experimental serum sample A and without serum as a negative control for ELISA assay B. The substrate TMB was incubated at three incubation periods 10, 15 and 20 min.

6.2.3 Data analysis and statistics

The data were tested for normality and equal distribution of variance. Data with unequal distribution of variance were normalised with log-transformation or arc-sin in case of percentage data. The data of each of the lysozyme activity, alternative complement pathway and antibody titre against *A. hydrophila* were analysed with a two way ANOVA and a two post hoc analysis. The Bonferroni Post-hoc analysis was carried out to test for differences between treatments to control at each time point. While Tukey's Post-hoc analysis used to compare the treatment differences within different time periods.

The data on differential leukocytes count, gene expression, pronephric cell proliferation and cell respiratory burst activity were analysed with a two way ANOVA and a Bonferroni Post-hoc test. All the significance was defined as $p \leq 0.05$ and graphs show mean ± SEM.

6.3 Results

6.3.1 Investigation I: The influences of CHO 1 and MacroGard® injections on innate immune response in common carp

6.3.1.1 Determine lysozyme activity in serum

Serum lysozyme activity was monitored by turbidimetric assay at different days post injection. At each time point, the effect of the different carbohydrates were compared to the negative control (Figure 6-9). The data analysis revealed that the different carbohydrates ($F = 24.789$, $p < 0.0001$); the exposure time ($F = 22.295$, $p < 0.0001$) and the interaction between both factors ($F = 6.026$, $p < 0.0001$) had a significant effect on lysozyme level in serum implying that the effect of different carbohydrates was dependent on the exposure duration (Figure 6-9). There was no significant difference detected in lysozyme levels at all the time points of the negative control group (see Figure 6-9 B). In contrast, the modified carbohydrate CHO 1 at 5 mg per kg fish body weight induced a significant increase in lysozyme activity after one day p.i. ($p < 0.01$). This level subsequently dropped at three days p.i. and was significantly ($p < 0.05$) less than levels in control fish after seven days p.i. (Figure 6-9 A). The higher concentration of CHO 1 group (10 mg/kg) also caused significant reduction in lysozyme level at one ($p < 0.05$) and three ($p < 0.01$) days p.i. (Figure 6-9 A).

In addition, MacroGard® (5 mg/kg) also caused a reduction in serum lysozyme levels and had a significant effect at one ($p < 0.01$), three ($p < 0.01$) and seven ($p < 0.001$) days p.i. (Figure 6-9 A). In contrast, at 14 days p.i. there was no significant differences in all of the injected groups compared to the negative control.

To test the statistical rigour the data was also subjected to post-hoc analysis using Tukey's Figure (6-9 B). It can be seen that MacroGard® caused a decrease in lysozyme level in

serum at 1, 3 and 7 days p.i., however this level increased significantly after 14 days p.i. The modified carbohydrate CHO 1 at 5 mg/kg fish body weight induced a significant increase in lysozyme activity at 1 and 14 days p.i. whilst CHO 1 at 10 mg/kg fish body weight caused significant decrease in lysozyme level only at three days p.i. compared to other time periods.

Overall it can be concluded that the modified carbohydrate CHO 1 at 5 mg/kg induced an early i.e. after 24 h p.i. significant increase in lysozyme activity compared to control fish and the lysozyme activity of the injected fishes exposed to the different stimulant carbohydrates had returned to control levels after 14 days p.i.

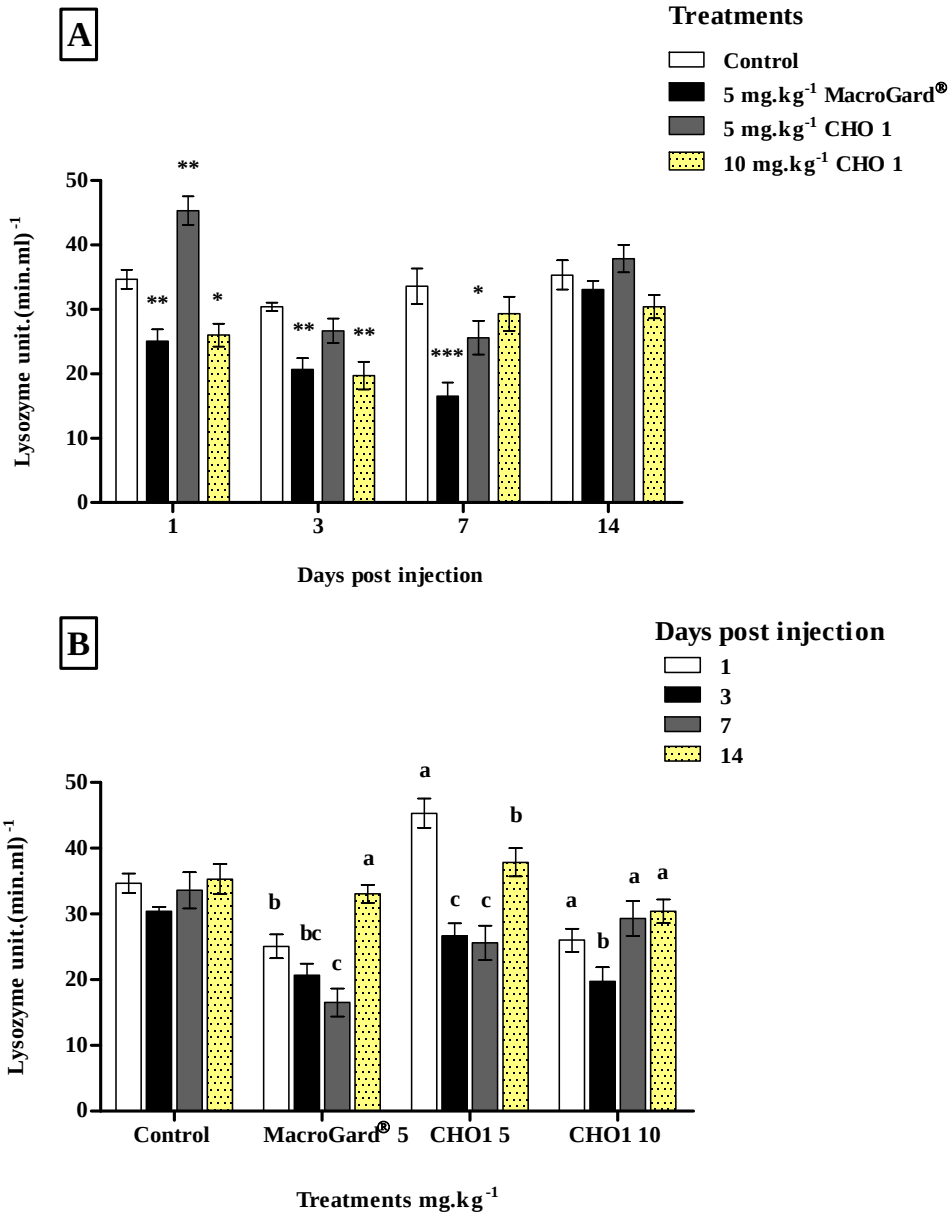


Figure 6-9: Serum lysozyme activity of carp injected with different carbohydrates. Carp were injected with 100µl water, MacroGard® (5 mg/kg), CHO 1 (5 mg/kg) or (10 mg/kg) and serum was collected at 1, 3, 7 and 14 days post injection. Serum lysozyme level was utilised by turbidimetric assay. Graphs show mean ± SEM of 6 fishes. Two ways ANOVA used to analyse the data, Bonferroni post-hoc analysis used to compare treatment to control at each time point (Figure A) and Tukey's post-hoc analysis used to compare the treatment differences within time periods (Figure B). In A the treatment differences in comparison to control performed are indicated thus * = $p \leq 0.05$ ** = $p \leq 0.01$ and *** = $p \leq 0.001$. In B the significant difference ($p \leq 0.05$) between time points at each treatment are indicated with different letter.

6.3.1.2 Determination of alternative complement pathway activity in serum

From the same serum samples described above, the alternative complement level was determined at different days post injection. A comparison carried out between the effect of different carbohydrates and the negative control (100µl water) in six fishes at each time point post injection (Figure 6-10 A). The statistical analysis incorporated two way ANOVA and Bonferroni post-hoc analysis. Whilst the sampling points post injection with different carbohydrates was significantly different ($F = 9.1$, $p < 0.00003$), the treatments effect ($F = 0.83$, $p = 0.4813$) and the interaction between both factors ($F = 0.7673$, $p = 0.6466$) had no significant effects on complement level in serum. The modified carbohydrate CHO 1 at 5 mg/kg fish body weight induced a significant increase ($p \leq 0.047$) in alternative complement pathway activity at 3 days p.i. (Figure 6-10 A). There was however no significant differences detected in complement levels in control group at all-time points post injection (Figure 6-10 B). Both MacroGard® (5 mg/kg) and CHO 1 (10 mg/kg) affected serum complement levels in the same way i.e. there was no significant differences in complement level at day 1 and 3 p.i. but the level dropped at 7 and 14 days p.i. (Figure 6-10 B). In contrast, CHO 1 at 5 mg/kg induced a significant increase ($p < 0.05$) in complement levels at 3 days p.i. compared to 1 ($p < 0.013$), 7 ($p < 0.003$) and 14 ($p < 0.011$) days p.i. (Figure 6-10 B).

In conclusion CHO 1 (5 mg/kg) caused an increase in complement level in serum at 3 days p.i.

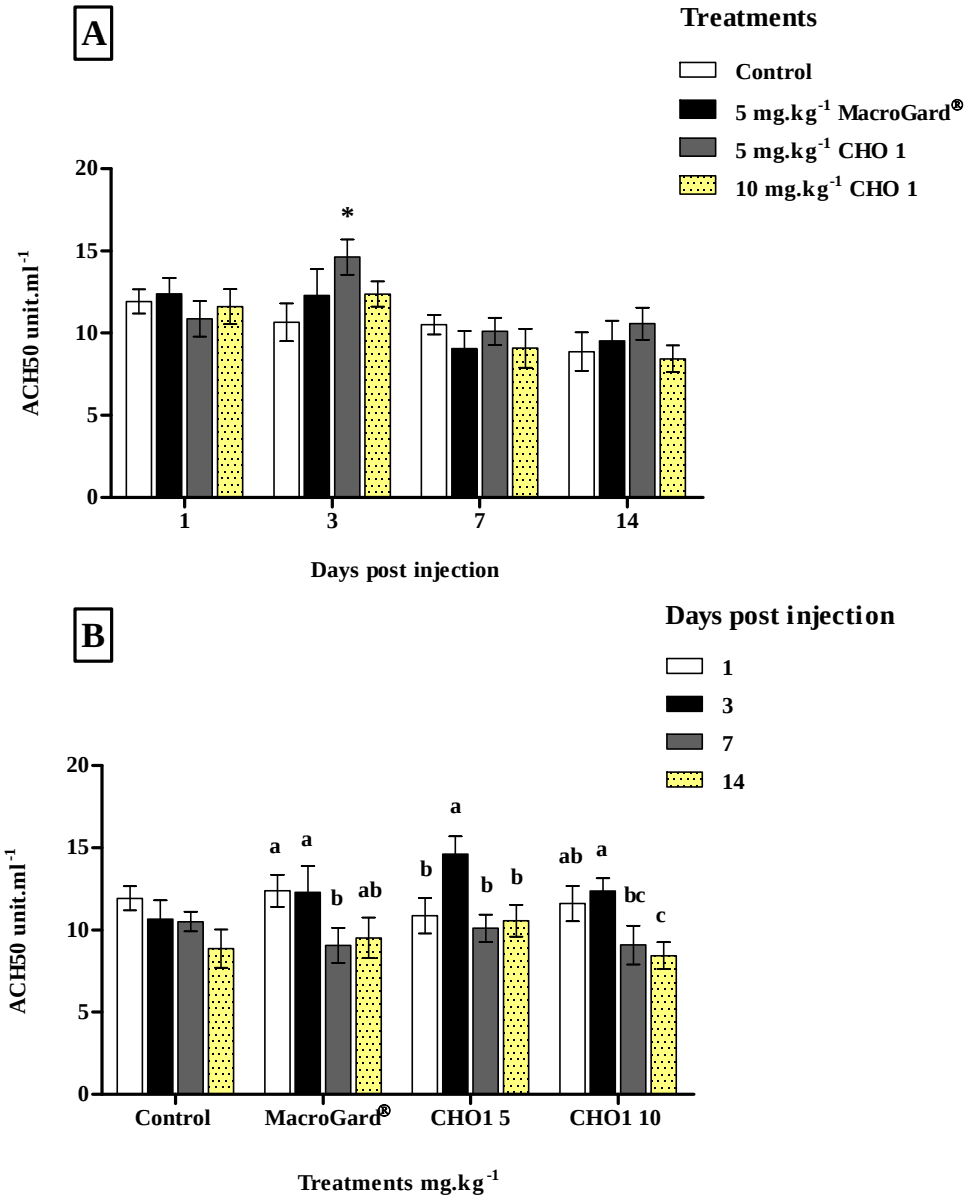


Figure 6-10: Alternative complement pathway activity of carp injected with different carbohydrates. Injected fishes with either 100µl water, MacroGard® (5 mg/kg), CHO 1 (5 mg/kg) or (10 mg/kg) were executed and serum collected at 1, 3, 7 and 14 days post injection. The complement level was utilised by the haemolytic activity of sheep red blood cells. Two ways ANOVA used to analyse the data, Bonferroni post-hoc analysis used to compare treatment to control at each time point (Figure A) and Tukey's post-hoc analysis used to compare the treatment differences within time periods (Figure B). The treatment differences in comparison to control is indicated thus * = $p \leq 0.05$. The significant differences ($p \leq 0.05$) between time points at each treatment is signified by different letter. Bars represent mean ± SEM of 6 fishes.

6.3.1.3 Leukocytes differentiation cell count

A blood smear was prepared at each time point for 5 fishes and the May- Grünwald's-Giemsa staining used to differentiate blood cells types according to Sirimanapong et al. (2014), Valdebenito et al. (2011) and Ranzani-Paiva et al. (2003) i.e. thrombocytes, lymphocytes, granulocytes and monocytes/ macrophages (Figures 6-11).

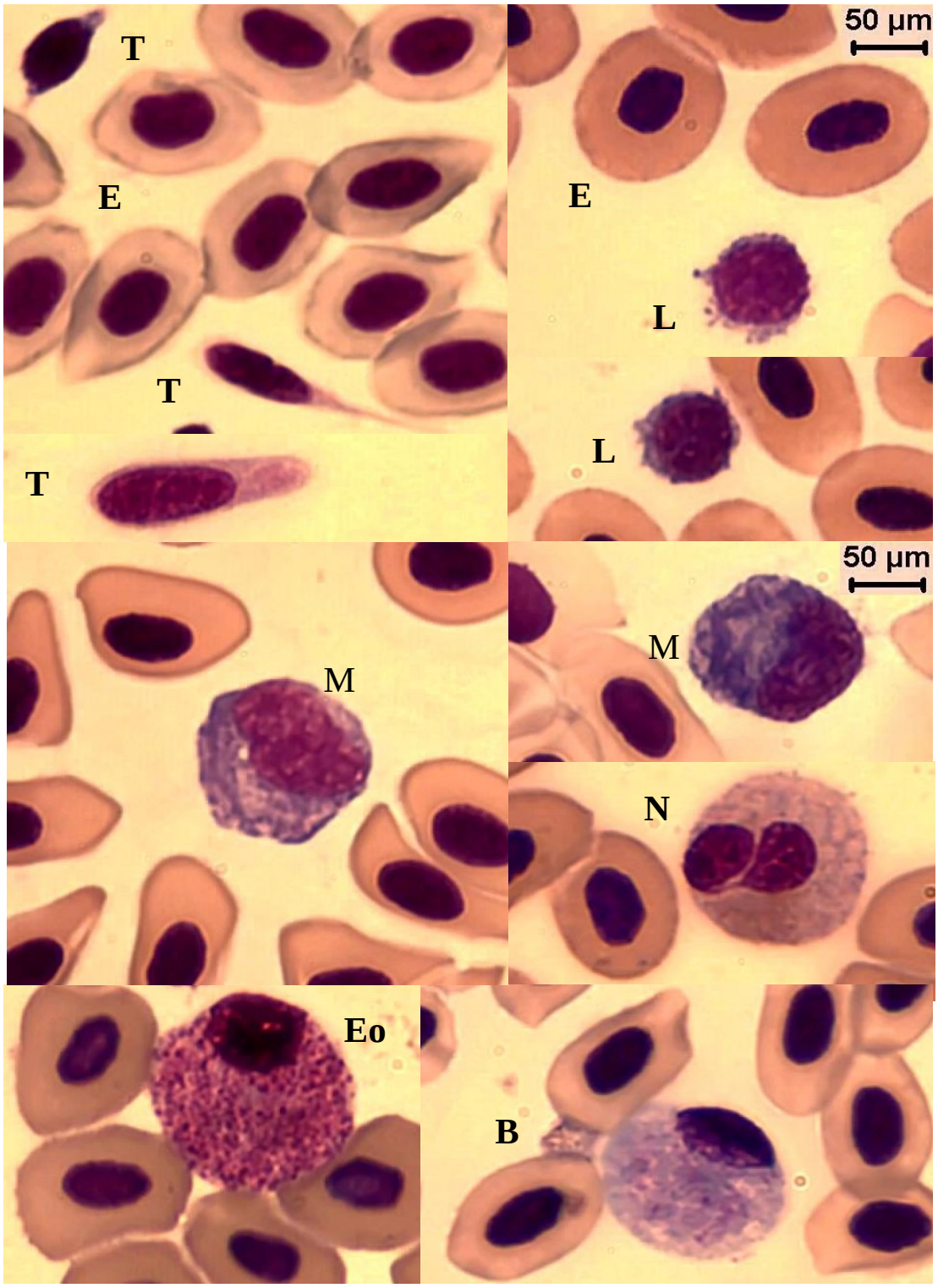


Figure 6-11 A: Different stained thrombocytes forms of carps injected with different carbohydrates. Injected fishes with either 100µl water, MacroGard® (5 mg/kg), CHO 1 (5 mg/kg) or (10 mg/kg) were killed and smears was taken from caudal puncture at 1, 3 and 7 days post injection. Dried slides was stained with May Gründwald-Giemsa. Images were observed at 1000x under oil, (T) Thrombocytes, (E) Erythrocyte, (L) Lymphocytes, (M) Monocytes, (N) Neutrophils, (Eo) Eosinophils and (B) Basophils.

The statistical analysis revealed that the exposure time had a significant effect on the percentage of thrombocytes (F = 3.691, p = 0.033), granulocytes (F = 3.998, p = 0.026) and monocytes/macrophage (F = 6.728, p = 0.003) population in blood (Table 6-2). While different carbohydrate treatments had no effects (Table 6-2). The modified carbohydrate CHO 1 at 5 mg/kg and 10 mg/kg concentrations induced a significant increase (p = 0.01 and 0.018 respectively) in the percentage of granulocytes after 1 day p.i. (Figure 6-12). However there were no significant differences in comparison to the control at 3 and 7 days p.i.

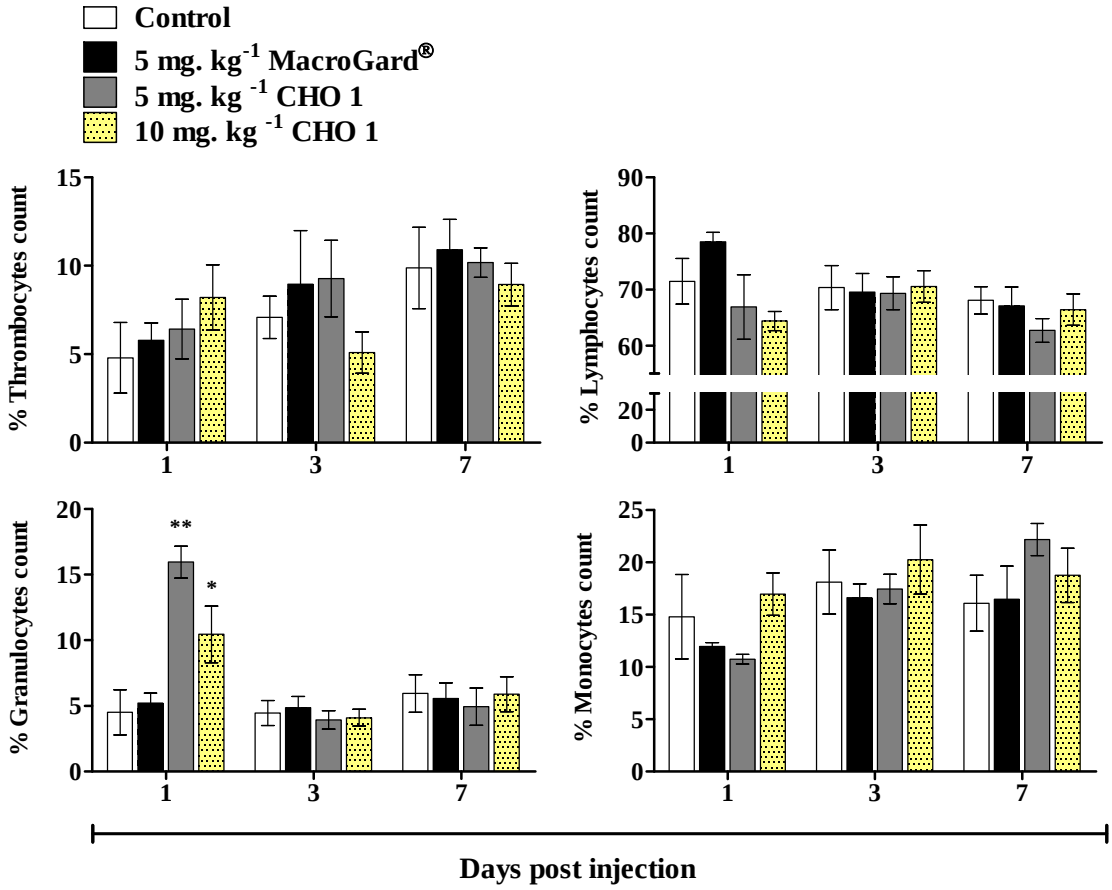


Figure 6-12: The percentage of differential leukocytes count of carp injected with various carbohydrates. Injected fishes with either 100µl water, MacroGard® (5 mg/kg), CHO 1 (5 mg/kg) or (10 mg/kg) were killed and blood smears prepared from circulating blood at 1, 3 and 7 days post injection. Cells were stained with May Gründwald-Giemsa.Two ways ANOVA was used to analyse the data and Bonferroni post-hoc analysis compared

treatment to control at each time point. The differences in comparison to matched control performed are indicated with * = $p < 0.05$ ** = $p \leq 0.01$. Bars represent mean ± SEM of 5 fishes.

Table 6-2: The two way ANOVA analysis of pronephric cell proliferation activity in carp post injection*.

Leukocytes type	Time		Treatment		Interaction	
	F	P	F	P	F	P
Thrombocytes	3.691	0.033	0.267	0.849	0.741	0.62
Granulocytes	3.998	0.026	0.877	0.461	1.638	0.161
Monocytes/ Macrophages	6.728	0.003	1.571	0.211	1.244	0.304
Lymphocytes	2.061	0.14	1.620	0.199	1.146	0.353

*The grey background highlights the significant effects at $p \leq 0.05$.

6.3.1.4 Gene expression in carp tissues post injection with different carbohydrates

6.3.1.4.1 The expression of pro-inflammatory cytokines

- **Interleukin -1 beta (IL1β)**

As shown in table 6-3 in both the head kidney (F= 8.117, $p < 0.0001$) and spleen (F= 11.429, $p < 0.0001$) IL1β expression was affected by the experimental duration, whilst treatments also significantly altered the expression of this cytokine in liver and mid gut (F=2.845, $p = 0.043$; F= 3.449, $p = 0.02$ respectively). Furthermore, in the liver a dependency of the treatment effect on the sampling time point was observed (F= 2.713, $p = 0.009$).

The expression of IL1β gene post injection with MacroGard® and CHO 1 at 1, 3, 7 and 14 days is presented in Figure 6-13. In the liver, MacroGard® increased the expression of IL1β gene at 24 h p.i. ($p = 0.013$), whilst CHO 1 at the same concentration (5 mg/kg)

induced significant (p = 0.002) the expression of IL1β gene after 14 days p.i. However, increasing the concentration of CHO 1 to 10 mg/kg induced an earlier significant (p = 0.01) up-regulation of IL1 β expression after 3 days p.i. This dose effect was however not noted in the mid gut, whilst injection with CHO 1 at both concentrations 5 and 10 mg/kg stimulated significant (p = 0.012 and 0.001 respectively) up-regulation of IL1β gene expression after 14 days p.i. (Figure 6-13).

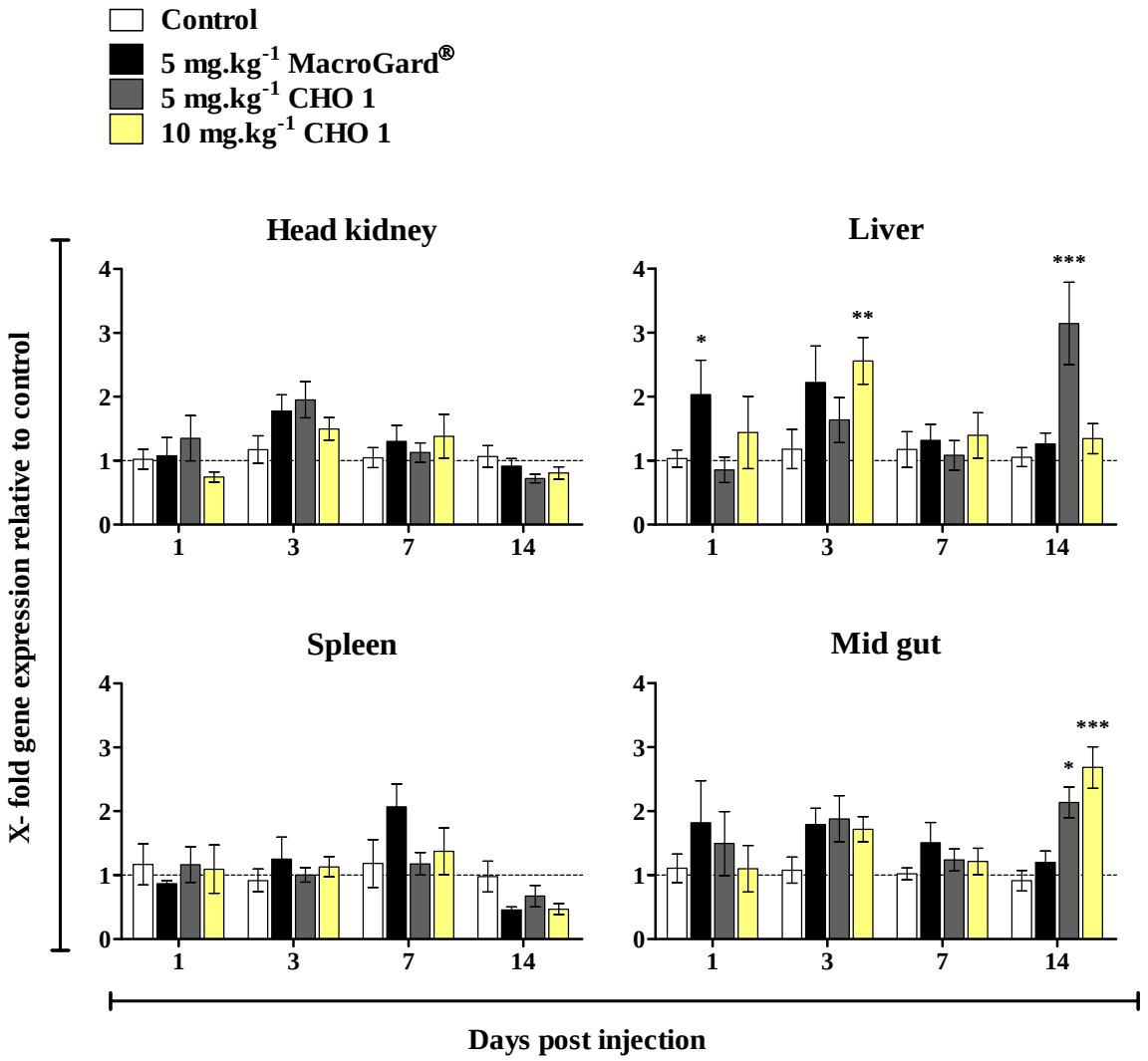


Figure 6-13: Interleukin one beta (IL1β) gene expression in different carp tissues after injection with different carbohydrates. Carp were injected with either 100µl water, MacroGard® (5 mg/kg), CHO 1 (5 mg/kg) or (10 mg/kg). Fish were sampled after 1, 3, 7 and 14 days post injection. Graphs show mean ± SEM of the x-fold gene expression

relative to the control injection group of the day from 6 fishes (dashed line at y-value equal to 1 indicating expression equal to control). Two way ANOVA was used to analyse the data, Bonferroni post-hoc analysis compared treatment to control at each time point. The differences in comparison to matched control are indicated with * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

Table 6-3: The two way ANOVA analysis of x-fold IL1β gene expression in carp organs post injection with different carbohydrates*.

IL1β Gene						
Tissue	Time		Treatment		Interaction	
	F	P	F	P	F	P
Head Kidney	8.117	<0.0001	1.259	0.294	0.956	0.483
Spleen	11.429	<0.0001	0.115	0.951	1.356	0.223
Liver	2.009	0.12	2.845	0.043	2.713	0.009
Mid gut	2.863	0.042	3.449	0.02	1.661	0.113

*The grey background highlights the significant effects at $p \leq 0.05$.

- **Interleukin six (IL6)**

There was a significant effect of exposure time on the expression of IL6 in the pronephros (F= 5.844, $p = 0.0013$) and spleen (F= 9.957, $p < 0.0001$). Also IL6 expression differed significantly between treatments in liver (F= 2.794, $p = 0.042$) and mid gut (F= 2.942, $p = 0.039$) tissues (see table 6-4).

MacroGard® (5 mg/kg) induced significant up-regulation in IL6 expression in liver ($p \leq 0.001$) after 24 h p.i. and in spleen ($p \leq 0.05$) after one week p.i. The only significant ($p = 0.012$) down regulation of this cytokine was noted at the high concentration of CHO 1 (10 mg/kg) in spleen after 14 days p.i. (Figure 6-14).

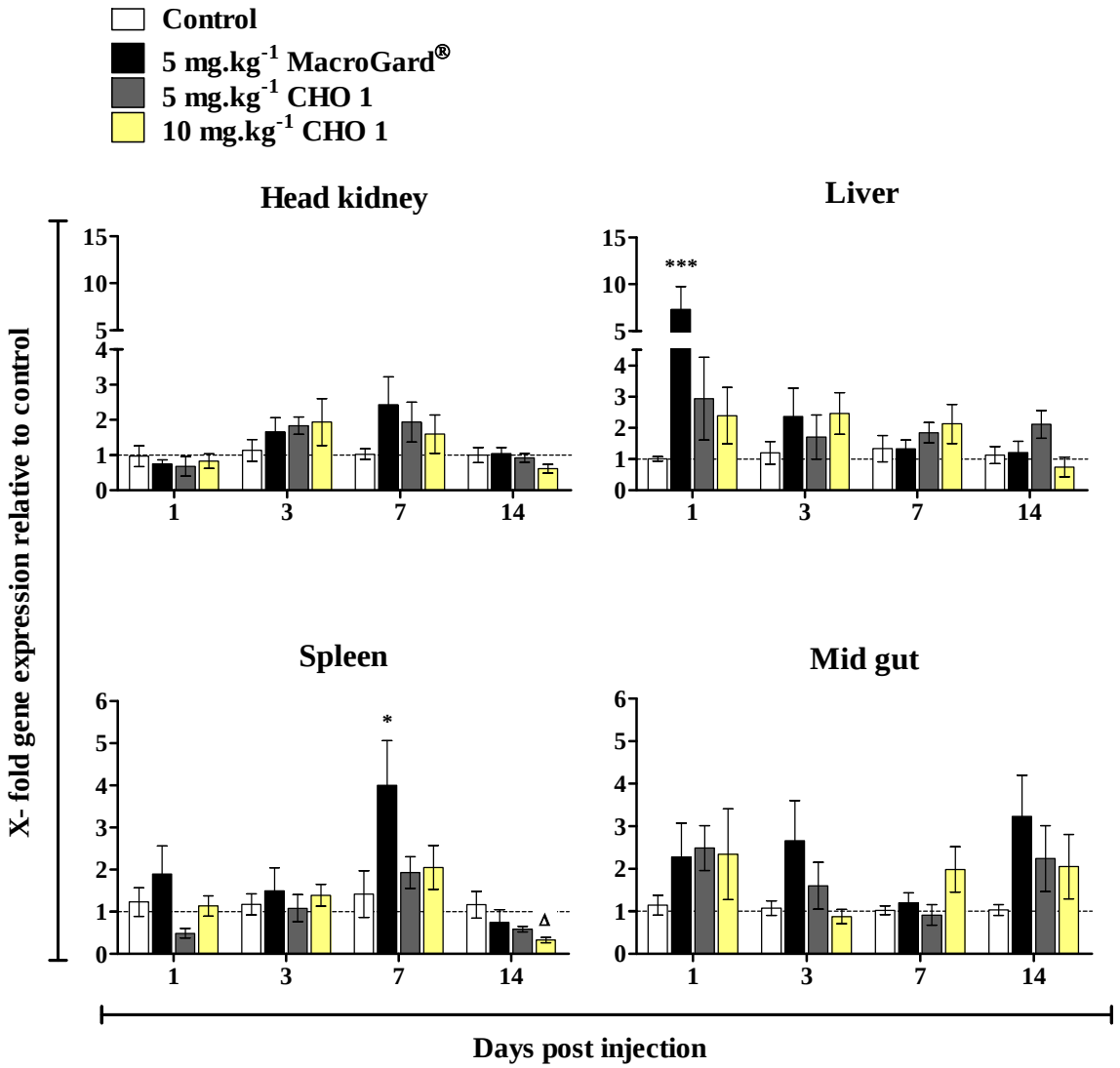


Figure 6-14: Interleukin six expression in different carp tissues after injection with different carbohydrates. Carp were injected with either 100µl water, MacroGard® (5 mg/kg), CHO 1 (5 mg/kg) or (10 mg/kg). Fish were sampled after 1, 3, 7 and 14 days post injection. Graphs shows mean ± SEM of the x-fold gene expression relative to the control injection group of the day from 6 fishes (dashed line at y-value equal to 1 indicating expression equal to control). Two ways ANOVA used to analyse the data and Bonferroni post-hoc analysis was used to compare treatment to control at each time point. The differences in comparison to matched control performed are indicated thus *= $p \leq 0.05$, *** = $p \leq 0.001$ for up-regulation and Δ= $p \leq 0.05$ for down-regulation.

Table 6-4: The two way ANOVA analysis of x-fold IL6 gene expression in carp organs post injection with different carbohydrates*.

IL6 Gene						
Tissue	Time		Treatment		Interaction	
	F	P	F	P	F	P
Head Kidney	5.844	0.0013	0.468	0.705	0.968	0.478
Spleen	9.957	<0.0001	1.514	0.217	1.231	0.289
Liver	2.324	0.082	2.794	0.042	1.802	0.083
Mid gut	1.684	0.178	2.942	0.039	0.845	0.578

*The grey background highlights the significant effects at $p \leq 0.05$.

- **The expression of pro-inflammatory chemokine (CXC)**

The expression of chemokine CXC gene in the pronephros, spleen and liver (Table 6-5) was significantly affected by experimental duration e.g. F= 3.705, p= 0.015; F= 5.838, p= 0.001; F= 3.471, p= 0.02 respectively. In addition, there was a significant effect of treatment on CXC expression in the spleen (F= 3.02, p= 0.035) and mid gut (F= 4.576, p= 0.005), whilst a significant effect of the interaction between these two parameters was only noted in the liver (F= 2.702, p= 0.009).

Figure 6-15 shows that the modified carbohydrate CHO 1 significantly up regulated the gene expression of CXC in the liver and mid gut. Whilst in the latter organ 5 mg/kg induced significant up-regulation at 1 day and 3 days p.i. (p = 0.007, 0.004), in the liver up regulation only occurred at these two time periods at 10 mg/kg concentration i.e. 1 day p = 0.036; 3 days p = 0.02. A significant down regulation of CXC expression was only noted in the spleen at both concentrations of CHO 1 after 14 days p.i. (p< 0.05).

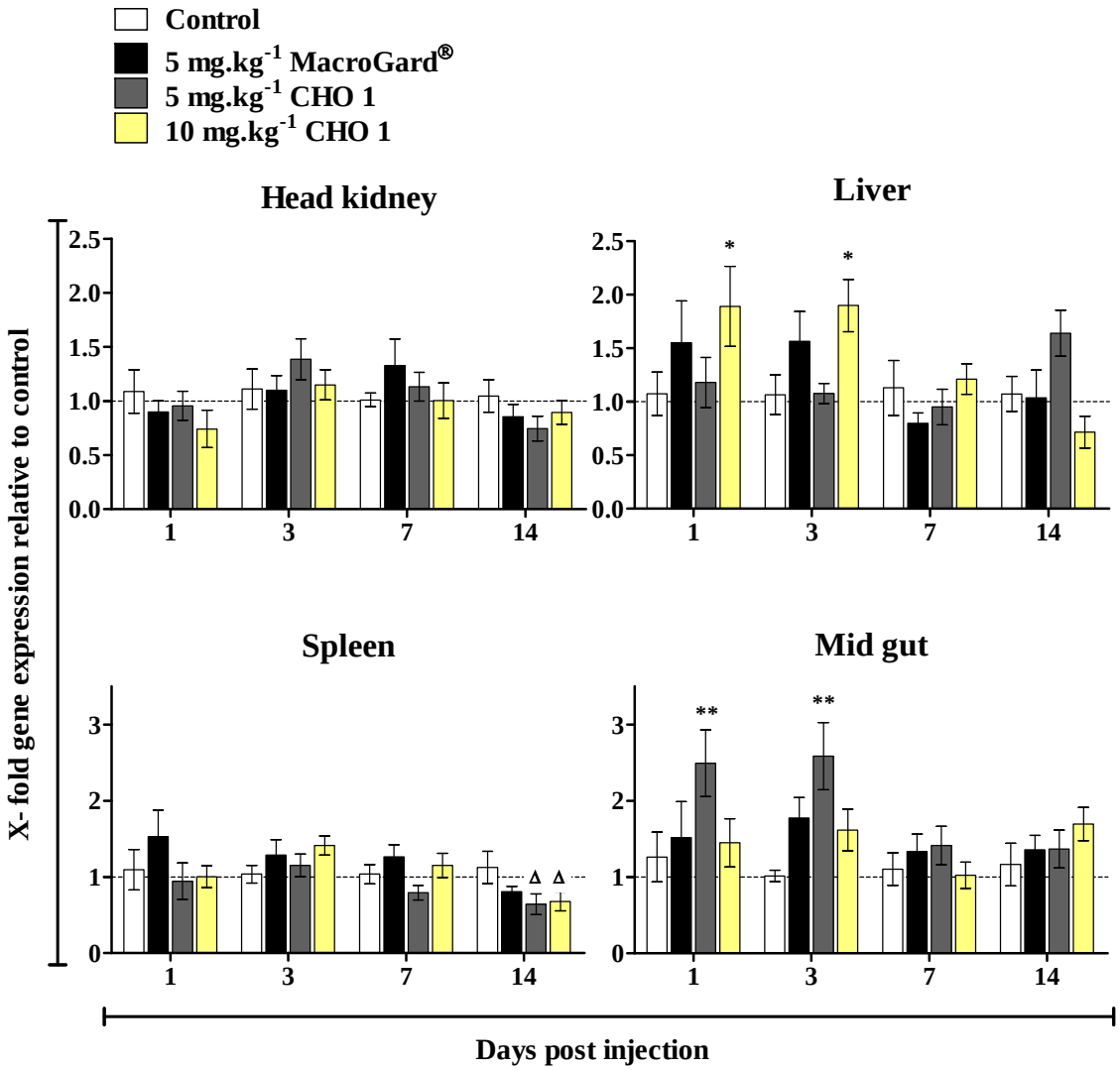


Figure 6-15: CXC gene expression in different carp tissues after injection with different carbohydrates. Carp were injected with either 100µl water, MacroGard® (5 mg/kg), CHO 1 (5 mg/kg) or (10 mg/kg). Fish were sampled after 1, 3, 7 and 14 days post injection. Graphs shows mean ± SEM of the x-fold gene expression relative to the control injection group of the day from 6 fishes (dashed line at y-value equal to 1 indicating expression equal to control). Two ways ANOVA used to analyse the data and Bonferroni post-hoc analysis was used to compare treatment to control at each time point. The differences in comparison to matched control are indicated with * = $p \leq 0.05$, ** = $p \leq 0.01$ for up-regulation and Δ= $p \leq 0.05$ for down-regulation.

Table 6-5: The two way ANOVA analysis of x-fold CXC gene expression in carp organs post injection with different carbohydrates*.

CXC Gene						
Tissue	Time		Treatment		Interaction	
	F	P	F	P	F	P
Head Kidney	3.705	0.015	0.546	0.652	0.989	0.456
Spleen	5.838	0.001	3.02	0.035	1.032	0.422
Liver	3.471	0.02	1.049	0.376	2.702	0.009
Mid gut	1.806	0.153	4.576	0.005	0.917	0.515

*The grey background highlights the significant effects at $p \leq 0.05$.

6.3.1.4.2 The expression of anti-inflammatory cytokine IL10

Table 6-6 shows the statistical analysis for IL10 expression in carp organs at 1, 3, 7 and 14 days p.i. The expression of this cytokine was significantly dependant on experimental duration in both the pronephros (F= 15.769, $p < 0.0001$) and spleen (F= 3.92, $p = 0.012$), whilst a significant interaction was only noted in the pronephros (F= 2.93, $p = 0.005$). However in both of liver and mid gut, no significant effect was observed in IL10 expression.

The effect of injecting fish with different carbohydrates was compared to the negative control which had received 100µl water/ fish (Figure 6-16). Whilst there was a significant down regulation of IL10 expression in the pronephros of fish injected with CHO 1 (5 mg/kg) after 1 ($p = 0.032$) and 14 ($p = 0.014$) days, this concentration of modified carbohydrate CHO 1 induced a significant ($p = 0.024$) up regulation of IL10 expression in the pronephros after 3 days p.i. In contrast, MacroGard® only induced a significant ($p = 0.022$) up regulation of IL10 expression after 7 days suggesting that CHO 1 induced the

IL10 expression one week earlier than MacroGard®. At a higher concentration of CHO 1 (10 mg/kg) a significant (p = 0.002) down regulation of IL10 expression was noted in the pronephros after 1 day p.i. This higher concentration i.e. 10 mg/kg of CHO 1 (p = 0.025) and MacroGard® (p = 0.038) significantly affected an increase in IL10 expression in liver at 3 days p.i.

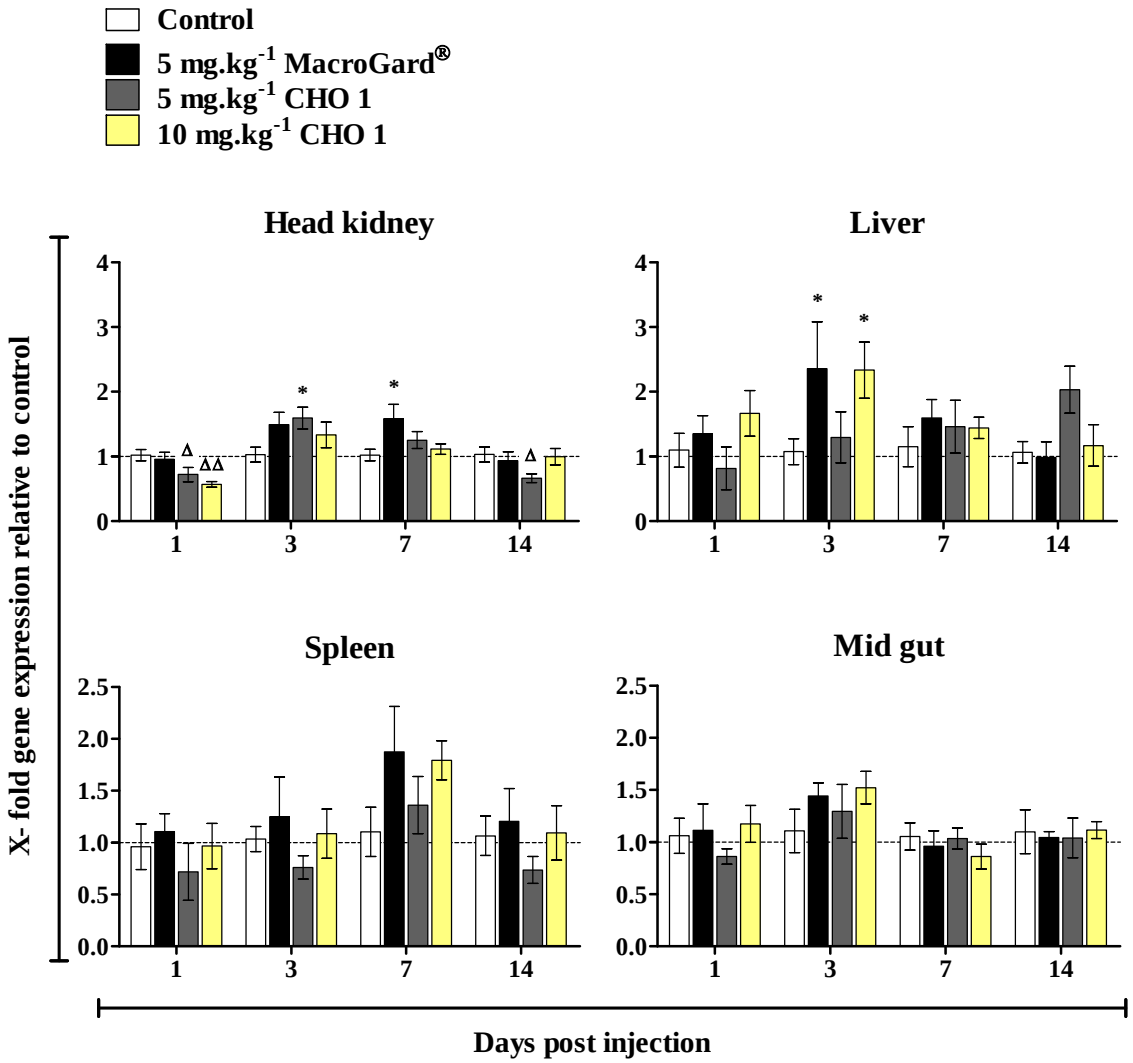


Figure 6-16: Interleukin ten expression in different carp tissues after injection with different carbohydrates. Carp were injected with either 100µl water, MacroGard® (5 mg/kg), CHO 1 (5 mg/kg) or (10 mg/kg). Fish were sampled after 1, 3, 7 and 14 days post injection. Graphs show mean ± SEM of the x-fold gene expression relative to the control injection group of the day from 6 fishes (dashed line at y-value equal to 1 indicating expression equal to control). Two ways ANOVA used to analyse the data, Bonferroni post-hoc analysis used to compare treatment to control at each time point. The differences in comparison to matched control performed with * = $p \leq 0.05$ for up-regulation and Δ= $p \leq 0.05$, ΔΔ= $p \leq 0.01$ for down-regulation.

Table 6-6: The two way ANOVA analysis of x-fold IL10 gene expression in carp organs post injection with different carbohydrates*.

IL10 Gene						
Tissue	Time		Treatment		Interaction	
	F	P	F	P	F	P
Head Kidney	15.769	<0.0001	2.27	0.087	2.93	0.005
Spleen	3.92	0.012	2.516	0.064	0.541	0.84
Liver	1.606	0.195	1.813	0.152	1.91	0.06
Mid gut	1.6	0.146	1.298	0.281	0.95	0.487

*The grey background highlights the significant effects at $p \leq 0.05$.

6.3.1.4.3 Nitric oxide production

The analysis revealed that treatments affected the expression of iNOS (F= 5.107, p= 0.003) in the pronephros (Table 6-7), and that was primarily associated with injection of MacroGard® after 7 days p.i. (p= 0.035; Figure 6-17). In contrast, in both of the spleen and liver, the expression of iNOS was influenced significantly by treatment (F= 6.115, p= 0.001; F= 10.53, p< 0.0001 respectively) and by time i.e. in the spleen (F= 9.503, p < 0.0001) and in the liver (F= 11.273 and 5.485, p < 0.0001). In addition, only the spleen was influenced significantly (F= 2.211, p= 0.03) by the interaction of both parameters. Further analysis revealed that this was primarily due to MacroGard® injection which caused a significant up-regulation in iNOS expression at 1 day and 7 days (p = 0.002) post injection in spleen, and in liver at 1 day (p < 0.0001) and 3 days (p = 0.0002) post injection (Figure 6-17).

Treatment (F= 5.99, p = 0.001) and time (F= 4.382, p = 0.007) affected significantly iNOS expression in mid gut, however the expression was not influenced by the interaction of both these parameters. Furthermore, after 3 days p.i., both the modified carbohydrate CHO

1 and MacroGard® at concentration 5 mg/kg induced up-regulation in iNOS expression i.e. p = 0.001 and 0.008 respectively (Figure 6-17).

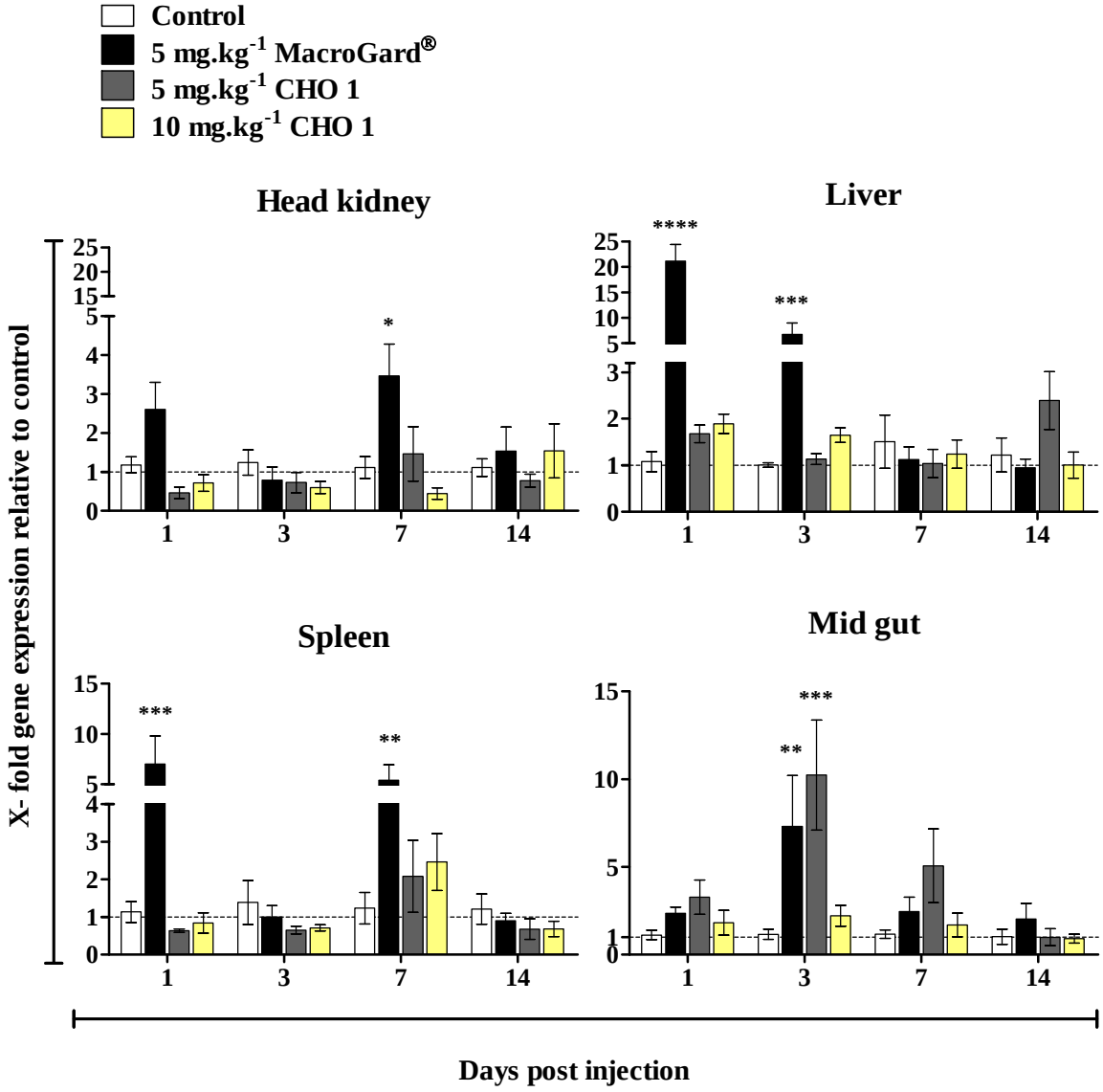


Figure 6-17: iNOS expression in different carp tissues after injection with different carbohydrates. Carp were injected with either 100µl water, MacroGard® (5 mg/kg), CHO 1 (5 mg/kg) or (10 mg/kg). Fish were sampled after 1, 3, 7 and 14 days post injection. Graphs show mean ± SEM of the x-fold gene expression relative to the control injection group of the day from 6 fishes (dashed line at y-value equal to 1 indicating expression equal to control). Two way ANOVA was used to analyse the data with Bonferroni post-hoc analysis being used to compare treatment to control at each time point. The differences in comparison to matched control are shown thus * = $p \leq 0.05$ ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

Table 6-7: The two way ANOVA analysis of x-fold iNOS gene expression in carp organs post injection with different carbohydrates*.

iNOS Gene						
Tissue	Time		Treatment		Interaction	
	F	P	F	P	F	P
Head Kidney	1.801	0.154	5.107	0.003	1.454	0.181
Spleen	9.503	<0.0001	6.115	0.001	2.211	0.03
Liver	11.273	<0.0001	10.53	<0.0001	5.485	<0.0001
Mid gut	5.99	0.001	4.382	0.007	1.259	0.273

*The grey background highlights the significant effects at $p \leq 0.05$.

6.3.1.4.4 Anti-viral cytokines and protein

- **Interferon gamma (IFN-γ2β)**

The expression of interferon gamma was determined post injection in all tissues and analyses statistically (Table 6-8). The experimental duration affected significantly IFN-γ2β expression (F= 6.718, p= 0.0004) in spleen and the effect occurred after 7 days p.i. in fish injected with MacroGard® (p = 0.02; Figure 6-18). In liver, the expression of IFN-γ2β was influenced significantly by treatment (F= 2.743, p = 0.049), experimental duration (F= 7.914, p < 0.0001) and the interaction between both factors (F= 2.3, p = 0.025). The modified carbohydrate CHO 1 (10 mg/kg) stimulated a highly significant up-regulation IFN-γ2β expression only at day 3 p.i. (p < 0.0001), whilst MacroGard® at 5 mg/kg induced significant effects at day 1 (p = 0.033) and day 3 (p = 0.06) post injection (Figure 6-18). In addition, the modified carbohydrate at 10 mg/kg concentration induced a significant up-regulation (p = 0.032) in the pronephros after 3 days p.i. There was no effect observed in mid gut tissue during the experiment.

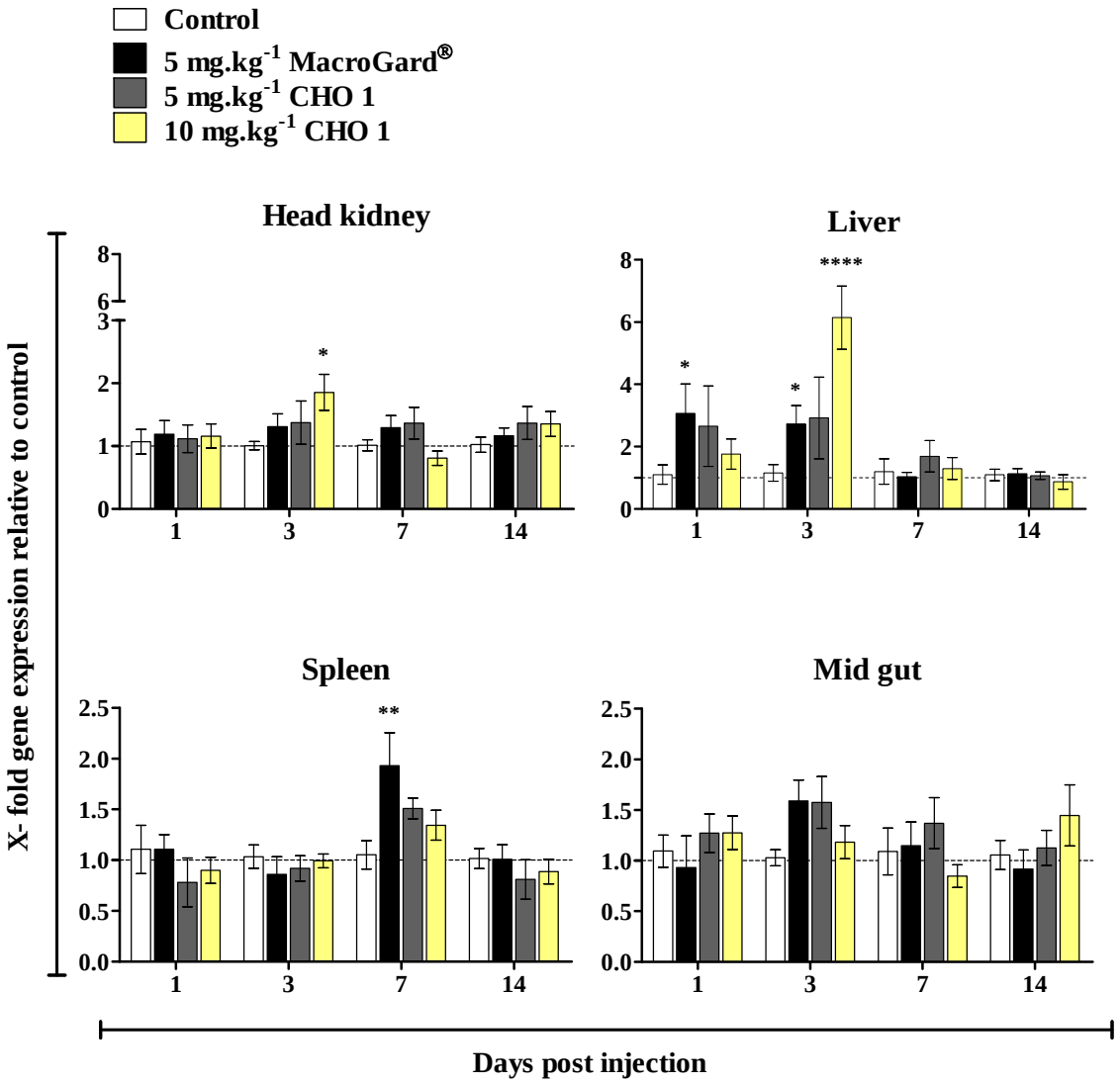


Figure 6-18: Interferon gamma expression in different carp tissues after injection with different carbohydrates. Carp were injected with either 100µl water, MacroGard® (5 mg/kg), CHO 1 (5 mg/kg) or (10 mg/kg). Fish were sampled after 1, 3, 7 and 14 days post injection. Graphs show mean ± SEM of the x-fold gene expression relative to the control injection group of the day from 6 fishes (dashed line at y-value equal to 1 indicating expression equal to control). Two way ANOVA was used to analyse the data with Bonferroni post-hoc analysis to compare treatment to control at each time point. The differences in comparison to matched control are indicated thus * = $p \leq 0.05$, ** = $p \leq 0.01$, **** = $p \leq 0.0001$.

Table 6-8: The two way ANOVA analysis of x-fold IFN-γ2β gene expression in carp organs post injection with different carbohydrates*.

IFN-γ2β Gene						
Tissue	Time		Treatment		Interaction	
	F	P	F	P	F	P
Head Kidney	1.047	0.377	0.74	0.532	1.11	0.367
Spleen	6.718	0.0004	1.299	0.281	1.384	0.21
Liver	7.914	<0.0001	2.743	0.049	2.3	0.025
Mid gut	1.299	0.281	1.242	0.3	1.132	0.351

*The grey background highlights the significant effects at $p \leq 0.05$.

- **Carp Mx**

There was a significant effect of experimental duration on Mx gene expression in all studied organs. This was primarily due to effects in the pronephros and spleen. In the pronephros, the modified carbohydrate at 10 mg/kg induced down-regulation in Mx expression (p = 0.036) after 24 h p.i. and a similar effect (p = 0.001) was observed in spleen at the same time period (Figure 6-19).

Statistical analysis also revealed a significant effect of interaction in the spleen (F= 2.894, p = 0.005) and liver (F= 2.074, p = 0.041). Fish injected with 5 mg/kg CHO 1 had a highly significant up-regulation in Mx expression after 14 days p.i. in the liver (Figure 6-19). Although, exposure time affected Mx expression in mid gut tissues (F= 4.855, p = 0.004) there was no significant differences between treatment in comparison to control at all-time points (Table 6-9).

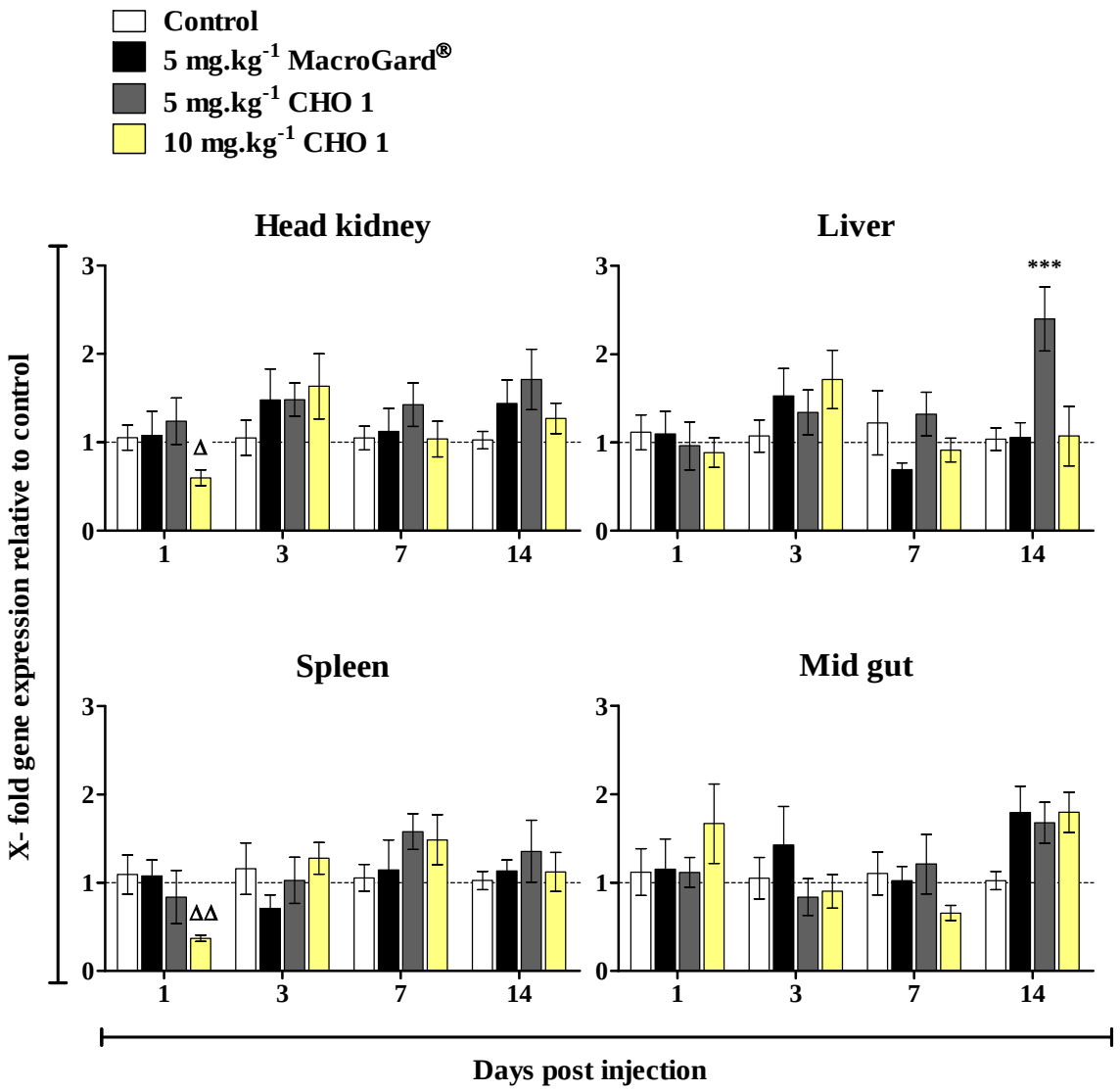


Figure 6-19: Mx gene expression in different carp tissues after injection with different carbohydrates. Carp were injected with either 100µl water, MacroGard® (5 mg/kg), CHO 1 (5 mg/kg) or (10 mg/kg). Fish were sampled after 1, 3, 7 and 14 days post injection. Graphs show mean ± SEM of the x-fold gene expression relative to the control injection group of the day from 6 fishes (dashed line at y-value equal to 1 indicating expression equal to control). Two way ANOVA used to analyse the data with Bonferroni post-hoc analysis used to compare treatment to control at each time point. The differences in comparison to matched control are shown thus *** = $p \leq 0.001$ for up-regulation and Δ= $p \leq 0.05$, ΔΔ= $p \leq 0.01$ for down-regulation.

Table 6-9: The two way ANOVA analysis of x-fold Mx gene expression in carp organs post injection with different carbohydrates*.

Mx Gene						
Tissue	Time		Treatment		Interaction	
	F	P	F	P	F	P
Head Kidney	4.298	0.007	1.817	0.151	0.744	0.667
Spleen	5.349	0.002	0.498	0.684	2.894	0.005
Liver	3.461	0.02	2.011	0.119	2.074	0.041
Mid gut	4.855	0.004	0.34	0.817	1.302	0.25

*The grey background highlights the significant effects at $p \leq 0.05$.

6.3.1.4.5 Complement system activator (C3)

The experimental duration caused a significant effect on complement system activator C3 expression in pronephros and spleen (F= 9.137, $p < 0.0001$; F= 4.164, $p = 0.009$ respectively) in injected fishes (Table 6-10). After 3 days p.i. both MacroGard® and CHO 1 at a concentration of 5 mg/kg induced a significant down-regulation of C3 expression ($p = 0.032$, 0.007 respectively) in the pronephros (Figure 6-20). In contrast, in the liver both treatment and exposure duration affected significantly (F= 2.825, $p = 0.044$; F= 6.211, $p = 0.001$ respectively) C3 expression (Table 6-10). Furthermore, an early enhancement in C3 expression was observed in fish injected with CHO 1 (10 mg/kg) after 24 h ($p = 0.016$). In contrast, only after 3 days post injection, was there a significant C3 expression in comparison to the control with MacroGard® ($p = 0.005$) and CHO 1 at 5 mg/kg ($p = 0.019$) and 10 mg/kg ($p = 0.009$; Figure 6-20). No effect was observed in mid gut tissue after injection with different treatment at all-time points.

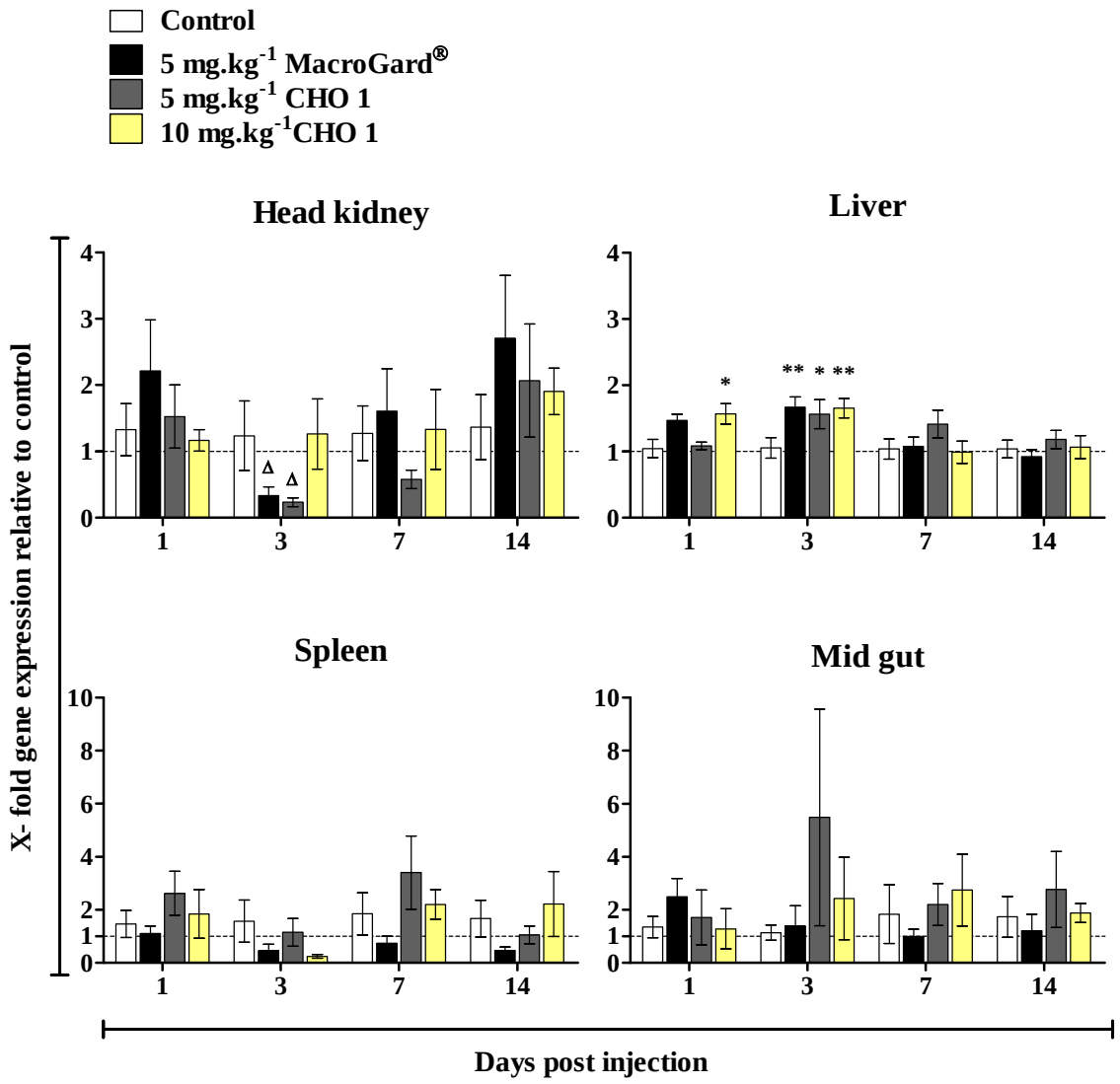


Figure 6-20: Complement C3 gene expression in different carp tissues after injection with different carbohydrates. Carp were injected with either 100µl water, MacroGard® (5 mg/kg), CHO 1 (5 mg/kg) or (10 mg/kg). Fish were sampled after 1, 3, 7 and 14 days post injection. Graphs show mean ± SEM of the x-fold gene expression relative to the control injection group of the day from 6 fishes (dashed line at y-value equal to 1 indicating expression equal to control). Two ways ANOVA was used to analyse the data and Bonferroni post-hoc analysis was used to compare treatment to control at each time point. The differences in comparison to matched control are shown with * = $p \leq 0.05$, ** = $p \leq 0.01$ for up-regulation and Δ= $p \leq 0.05$ for down-regulation.

Table 6-10: The two way ANOVA analysis of x-fold C3 gene expression in carp organs post injection with different carbohydrates*.

C3 Gene						
Tissue	Time		Treatment		Interaction	
	F	P	F	P	F	P
Head Kidney	9.137	<0.0001	0.819	0.487	1.625	0.124
Spleen	4.164	0.009	1.748	0.164	0.567	0.82
Liver	6.211	0.001	2.825	0.044	1.931	0.059
Mid gut	0.105	0.957	1.121	0.346	0.964	0.476

*The grey background highlights the significant effects at $p \leq 0.05$.

6.3.1.4.6 Antibacterial activity (Lysozyme-C) gene

The statistical analysis of the antibacterial activity (lysozyme-C) is presented in table 6-11. The experimental parameters affected lysozyme expression differently in the sampled organs. The lysozyme expression in the pronephros differed significantly between sampling point (F= 4.669, p = 0.005), where the CHO 1 at 10 mg/kg induced down-regulation in lysozyme expression after 1 day (p = 0.034) and 7 days (p = 0.043) post injection (Figure 6-21). In contrast, whilst in the spleen, treatment also affected significantly lysozyme expression (F= 6.641, p = 0.0005), both CHO 1 at 10 mg/kg after 3 days and MacroGard® after 7 days caused a down-regulation in lysozyme expression (p = 0.007 and 0.005 respectively) (Figure 6-21).

The experimental duration and the interaction between both factors caused significant effect on lysozyme expression in the liver. This was primarily due to the effects of CHO 1, where 10 mg/kg induced a significant up-regulation in comparison to control (p = 0.004) after 3 days post injection (Figure 6-21). However, after 24 h the lower concentration of

CHO 1 (5 mg/kg) induced a significant down-regulation in lysozyme expression (p = 0.016).

Lysozyme expression was also affected significantly by the treatments in mid gut tissue (F= 5.562, p = 0.002), where the CHO 1 at concentration 5 mg/kg induced significant up-regulation in lysozyme expression after 3 days (p = 0.021) and 7 days (p = 0.04) post injection. Furthermore, the higher concentration of CHO 1 also up-regulated lysozyme expression after 3 days (p = 0.031) and 14 days (p = 0.01) post injection (Figure 6-21).

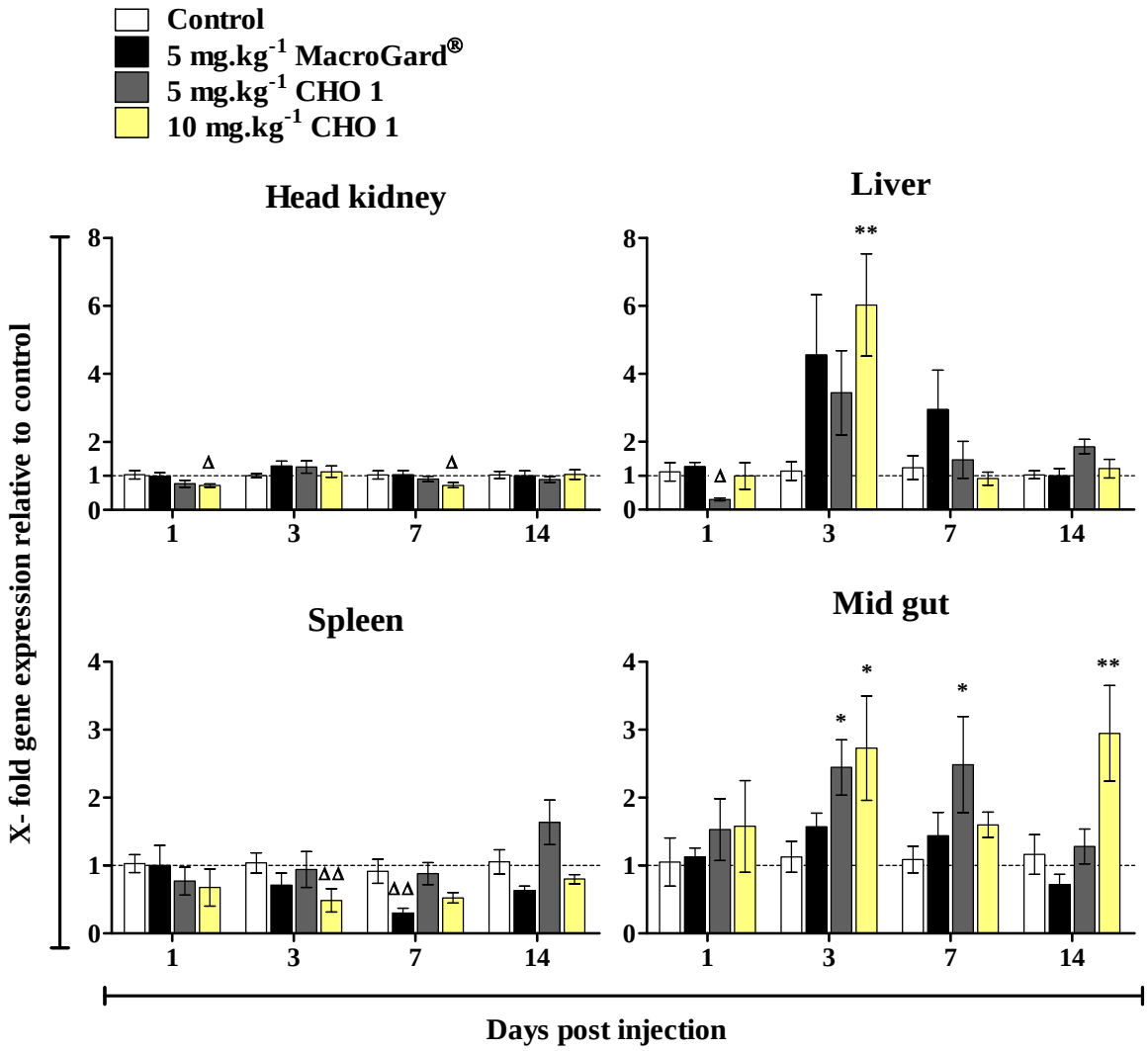


Figure 6-21: Lysozyme gene expression in different carp tissues after injection with different carbohydrates. Carp were injected with either 100µl water, MacroGard® (5 mg/kg), CHO 1 (5 mg/kg) or (10 mg/kg). Fish were sampled after 1, 3, 7 and 14 days post injection. Graphs show mean ± SEM of the x-fold gene expression relative to the control injection group of the day from 6 fishes (dashed line at y-value equal to 1 indicating expression equal to control). Two ways ANOVA used to analyse the data with Bonferroni post-hoc analysis to compare treatment to control at each time point. The differences in comparison to matched control are shown thus * = $p \leq 0.05$, ** = $p \leq 0.01$ for up-regulation and Δ= $p \leq 0.05$, ΔΔ= $p \leq 0.01$ for down-regulation.

Table 6-11: The two way ANOVA analysis of x-fold Lysozyme-C gene expression in carp organs post injection with different carbohydrates*.

Lysozyme-C Gene						
Tissue	Time		Treatment		Interaction	
	F	P	F	P	F	P
Head Kidney	4.669	0.005	2.151	0.101	1.07	0.395
Spleen	3.434	0.21	6.641	0.0005	1.383	0.212
Liver	7.663	<0.0001	1.026	0.386	2.551	0.013
Mid gut	2.3	0.084	5.562	0.002	1.243	0.283

*The grey background highlights the significant effects at $p \leq 0.05$.

To summarise the previous immune related gene expression Table 6-12 shows the significant using Bonferroni post-hoc analysis to compare treatment to control at each time point.

Table 6-12: Summary of the significant effects of MacroGard® 5mg/kg, CHO 1 at concentration 5 and 10 mg/kg injection on immune related gene expression in carp. The significant up regulation presented with ▲ = $p \leq 0.05$, ▲▲ = $p \leq 0.01$ and ▲▲▲ = $p \leq 0.001$ for up-regulation and ● = $p \leq 0.05$, ●● = $p \leq 0.01$ for down-regulation.

Tissue	Gene	Treatment	Days post injection			
			1	3	7	14
Head kidney	iNOS	MacroGard®			▲	
	IFNγ	CHO 1 10mg/kg		▲		
	IL10	MacroGard®			▲	
		CHO 1 5mg/kg	●	▲		●
		CHO 1 10mg/kg	●●			
	Lysozyme	CHO 1 10mg/kg	●		●	
	C3	MacroGard®		●		
		CHO 1 5mg/kg		●		
	Mx	CHO 1 10mg/kg	●			
Spleen	iNOS	MacroGard®	▲▲▲		▲▲	
	IFNγ	MacroGard®			▲▲	
	IL6	MacroGard®			▲	
		CHO 1 10mg/kg				●
	Lysozyme	MacroGard®			●●	
		CHO 1 10mg/kg		●●		
	Mx	CHO 1 10mg/kg	●●			
	CXC	CHO 1 5mg/kg				●
		CHO 1 10mg/kg				●

Tissue	Gene	Treatment	Days post injection			
			1	3	7	14
Liver	iNOS	MacroGard®	▲▲▲	▲▲▲		
	IFNγ	MacroGard®	▲	▲		
		CHO 1 10mg/kg		▲▲▲		
	IL1β	MacroGard®	▲			
		CHO 1 5mg/kg				▲▲▲
		CHO 1 10mg/kg		▲▲		
	IL6	MacroGard®	▲▲▲			
	IL10	MacroGard®		▲		
		CHO 1 10mg/kg		▲		
	Lysozyme	CHO 1 5mg/kg	●			
		CHO 1 10mg/kg		▲▲		
	C3	MacroGard®		▲▲		
		CHO 1 5mg/kg		▲		
		CHO 1 10mg/kg	▲	▲▲		
	Mx	CHO 1 5mg/kg				▲▲▲
	CXC	CHO 1 10mg/kg	▲	▲		
Mid gut	iNOS	MacroGard®		▲▲		
		CHO 1 5mg/kg		▲▲▲		
	IL1β	CHO 1 5mg/kg				▲
		CHO 1 10mg/kg				▲▲
	IL10	CHO 1 5mg/kg				▲
		CHO 1 10mg/kg				▲▲
	Lysozyme	CHO 1 5mg/kg		▲	▲	
		CHO 1 10mg/kg		▲		▲▲
	CXC	CHO 1 5mg/kg	▲▲	▲▲		

6.3.2 Investigation II : Adjuvant effect of a formulated carbohydrate (CHO 1) in vaccines against *Aeromonas hydrophila* in common carp (*Cyprinus carpio*)

6.3.2.1 Differential Leukocytes count

The May- Grünwald's- Giemsa staining method was used to determine the differential blood leukocytes count. Cells were differentiated as described by Sirimanapong et al. (2014), Valdebenito et al. (2011) and Ranzani-Paiva et al. (2003). Only three groups of leukocytes populations were counted i.e. lymphocytes, granulocytes and monocytes/ macrophages (see Section 6.2.1.4.3 and Figures 6-11).

At each time point, the effect of treatments were compared to the negative control (100µl PBS) in six fishes (Figure 6-22) utilising the Bonferroni post-hoc analysis. The two ways ANOVA analysis (Table 6-13) revealed a significant interaction between treatment and experimental duration on the percentage of leukocytes count i.e. lymphocytes ($F = 2.685$, $p = 0.008$); granulocytes ($F = 3.827$, $p < 0.0001$) and monocytes/ macrophages ($F = 2.671$, $p = 0.008$). However there were no significant effects observed in the individual factors i.e. treatments and time points on any of the different leukocytes types. The modified carbohydrate CHO 1 increased significantly ($p = 0.026$) the percentage of granulocytes at 7 days p.i. and the combination of the vaccine and CHO 1 group induced the highest values in granulocytes ($p < 0.0001$) in comparison to the time matched control and the combination of the vaccine and CHO 1 group at the other time points (Figure 6-22).

Significant differences in the percentage of monocytes/ macrophages were observed at 14 days p.i. with either CHO 1 ($p = 0.017$), vaccine ($p = 0.007$) or the combination group of CHO 1 and vaccine ($p = 0.017$) in comparison to control. However, the vaccine group induced a significant reduction ($p = 0.02$) in monocyte/ macrophage population after 21

days p.i., while the combination group of CHO 1 and vaccine group had similar cells number in comparison to the control.

The combination group of CHO 1 and vaccine also induced a significant reduction in the percentage of lymphocytes after 7 days p.i. (p = 0.00016), whilst CHO 1 induced a significant (p = 0.003) decrease after 14 days post injection (Figure 6-22).

In summary, the results show an early enhancement of the percentage of granulocyte at 7 days p.i. and monocytes/ macrophages at 14 days p.i. with the combination of CHO 1 and the vaccine, and the CHO 1 alone. *A. hydrophila* vaccine induced an increase in monocytes/ macrophages population at 14 days p.i.

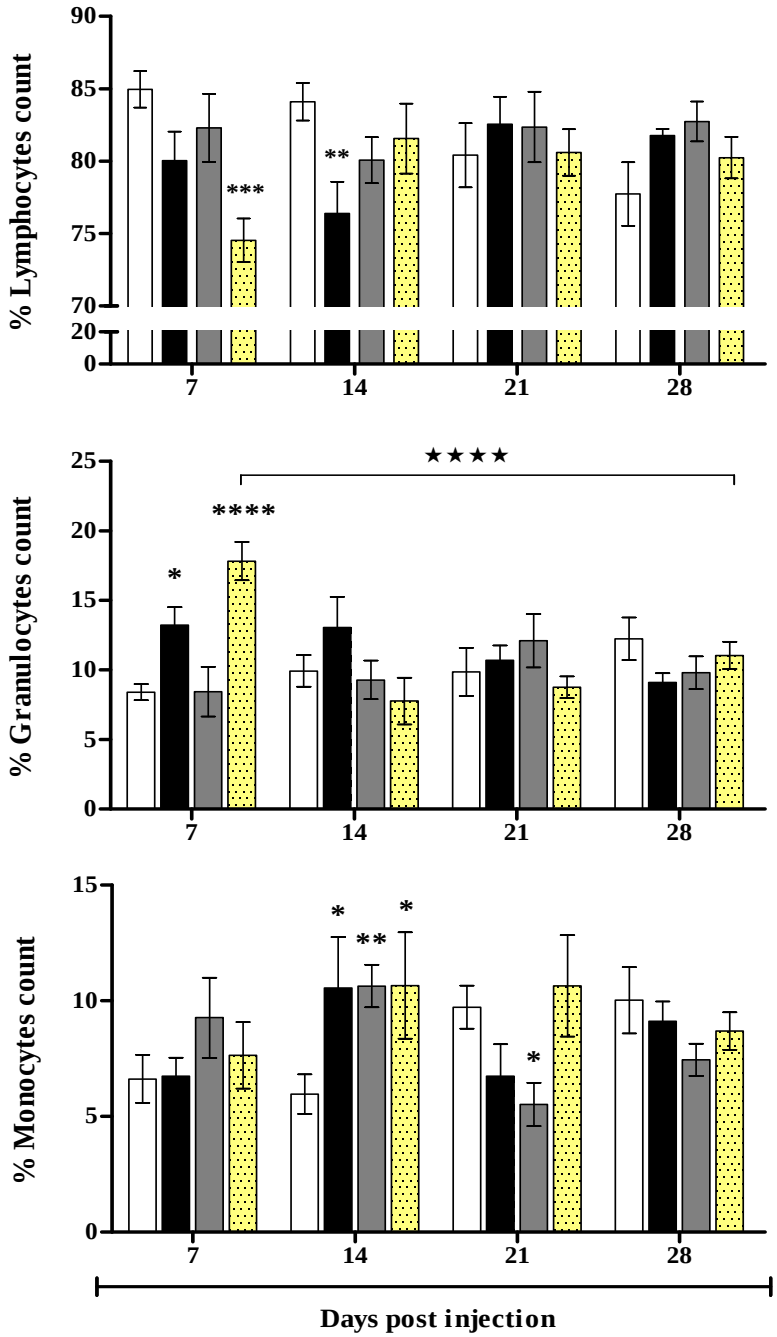


Figure 6-22: The percentage of differential leukocytes count in vaccinated carp. Fishes were injected with either 100µl PBS□; CHO1 (5 mg/kg)■; *Aeromonas hydrophila* vaccine (1×109 cfu/ml) ■ or *A. hydrophila* (1×109 cfu/ml) mixed with CHO1 (5 mg/kg)■. Fishes were killed each week for one month and blood smears prepared from caudal puncture. Cells were stained with May Gründwald-Giemsa. Two ways ANOVA was used to analyse the data, with Bonferroni post-hoc analysis to compare treatment to control at each time point. Differences as signified thus * = $p \leq 0.05$, ** = $p \leq 0.01$ *** = $p \leq 0.001$,

**** = p ≤ 0.0001. The comparison between different time point with in one treatment performed with ⬟ ⬟ ⬟ ⬟ = p ≤ 0.0001. Bars represent mean ± SEM of 6 fishes.

Table 6-13: The two way ANOVA analysis of Leukocytes differentiation cell count in carp blood smear post injection*.

Leukocytes type	Time		Treatment		Interaction	
	F	P	F	P	F	P
Lymphocytes	0.241	0.868	1.982	0.122	2.685	0.008
Granulocytes	1.334	0.268	1.262	0.292	3.827	<0.0001
Monocytes/ Macrophages	1.75	0.162	0.483	0.695	2.671	0.008

*The grey background highlights the significant effects at $p \leq 0.05$.

6.3.2.2 Pronephric leukocytes activities

6.3.2.2.1 Antigen-specific proliferation

Pronephric cell proliferation in response to *A. hydrophila* antigen was determined in carp post injection with PBS, CHO 1, vaccine and combination of CHO 1 and vaccine. Cell proliferation was analysed after 24h incubation, with and without the presence of heat killed *A. hydrophila* antigen, using the MTT assay as described in (Kamilya et al., 2006). Although, the proliferation assay might need longer incubation period but these results showed the possibility of pronephros cells to respond to the antigen availability in the assay. Statistical analysis (Table 6-14) revealed that there was a significant effect of experimental duration ($F = 26.675$, $p < 0.0001$), treatment ($F = 5.788$, $p = 0.001$) and the interaction of both parameters ($F = 3.771$, $p = 0.0003$) on pronephric cell proliferation incubated with *A. hydrophila* antigen. This effect was associated with the significant

increase in cell proliferation at 14 days p.i. in fish injected with CHO 1 (p = 0.001), however pronephric cell proliferation decreased significantly (p = 0.041) after 28 days p.i. in the same group. (See Figure 6-23 A).

Pronephric cell proliferation was also affected significantly by time (F = 21.904, p < 0.0001) and was dependant on treatments effects (F = 3.782, p = 0.0003). The modified carbohydrate induced a significant proliferation at 14 days p.i. (p = 0.001), although after 28 days p.i. a significant (p = 0.012) reduction in cell proliferation was revealed in comparison to control. In comparison, after 2 weeks p.i. the combination group (CHO 1 + vaccine) caused reduction in cells (p = 0.036) comparable to control (Figure 6-23 B).

The dependency of both studied factors i.e. time and treatments (F = 2.123, p = 0.033) and treatment (F = 5.781, p = 0.001) affected significantly proliferation index values that was primarily associated with the highly significant increase in proliferation index in fish that had received the vaccine alone after one week p.i. (p = 0.0003) as seen in Figure 6-23 C.

In conclusion pronephric cells from the vaccine alone group recognised the *A. hydrophila* antigen of the assay and induced leukocytes proliferation index after 1 week p.i. Moreover, the CHO 1 group stimulated leukocytes proliferation after 14 days in both incubation conditions i.e. with and without *A. hydrophila* antigen of the assay.

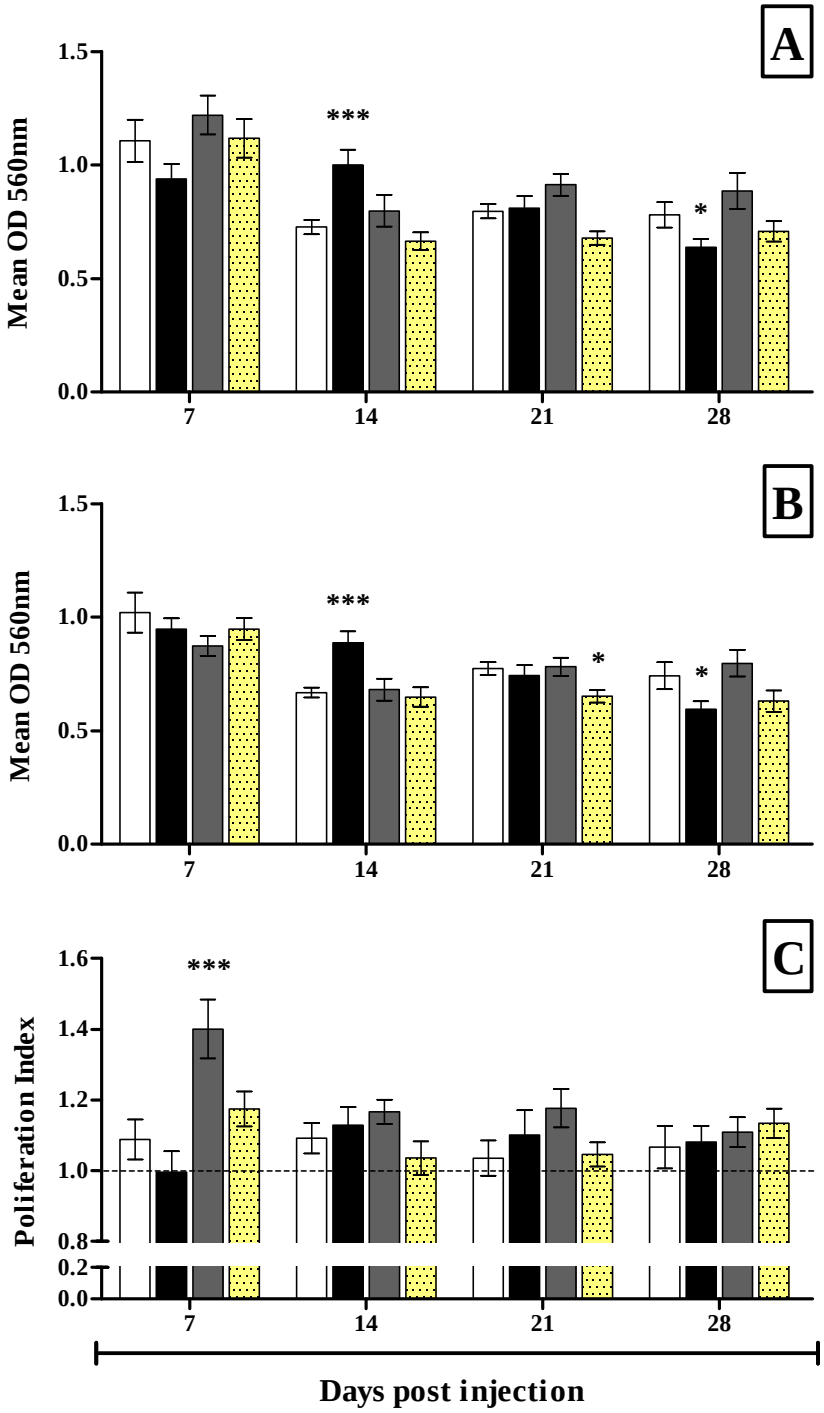


Figure 6-23: Pronephric leukocytes proliferation in vaccinated carps. The pronephros of injected fishes with either 100µl PBS □; CHO1 (5 mg/kg) ■; *A. hydrophila* vaccine (1×10^9 cfu/ml) ■ or *A. hydrophila* (1×109 cfu/ml) mixed with CHO1 (5 mg/kg) □ were isolated every week for a month. The MTT assay was utilised to determine (2×10^6 cell/ml) pronephric proliferation incubated for 24h:A) with PMA; B) without PMA; and C) proliferation index. Two ways ANOVA was used to analyse the data with Bonferroni post-hoc analysis to compare treatment to control at each time point. The differences in

comparison to matched control are indicated thus: * = p ≤ 0.05, *** = p ≤ 0.001. Bars represent mean ± SEM of 8 fishes and the dashed line at y-value equal to 1 shows the non-stimulation level.

Table 6-14: The two way ANOVA analysis of pronephros cells proliferation activity in carp post injection*.

Pronephros proliferation activity	Time		Treatment		Interaction	
	F	P	F	P	F	P
Cell with antigen	26.675	<0.0001	5.788	0.001	3.771	0.0003
Cell without antigen	21.904	<0.0001	2.685	0.05	3.782	0.0003
Proliferation index	1.117	0.346	5.781	0.001	2.123	0.033

*The grey background highlights the significant effects at $p \leq 0.05$.

6.3.2.2.2 Respiratory burst activity

The respiratory burst activity of carp pronephric cells was determined after injection with PBS, CHO 1, vaccine and combination of CHO 1 and vaccine, and isolated cells exposed to heat killed *A. hydrophila* antigen or incubated without this antigen.

Statistical analysis is presented in table 6-15. Regardless to the incubation with antigen, the respiratory burst of pronephric cells were significantly different between treatments (F= 8.074-14.745, $p < 0.0001$) and between sampling points (F= 23.608- 29.09, $p < 0.0001$), in addition a dependency of treatments effect on the sampling point was observed (F= 3.25-5.051, $p < 0.001$). The reactive oxygen species (ROS) index was calculated as described in Section 6.2.2.5.2, and analysis revealed the significant dependency of the treatments effect on experimental duration (F= 2.124, $p = 0.033$).

The respiratory burst activity of pronephric cells isolated from vaccine alone group shows a highly significant difference compared to control ($p < 0.0001$) after one week p.i. and irrespective to the availability of *A. hydrophila* antigen in the incubation (Figure 6-24 A, B). In comparison, all the treatments induced a slight significant increase ($p < 0.05$) in pronephric cell reactive oxygen activity when incubated with *A. hydrophila* antigen after two weeks p.i. compared to control (Figure 6-24 A). This led to significant increase in ROS index after 14 days p.i. in all treatments i.e. CHO 1 ($p = 0.02$), vaccine ($p = 0.001$) and the combination of CHO 1 and vaccine ($p = 0.011$) (Figure 6-24 C).

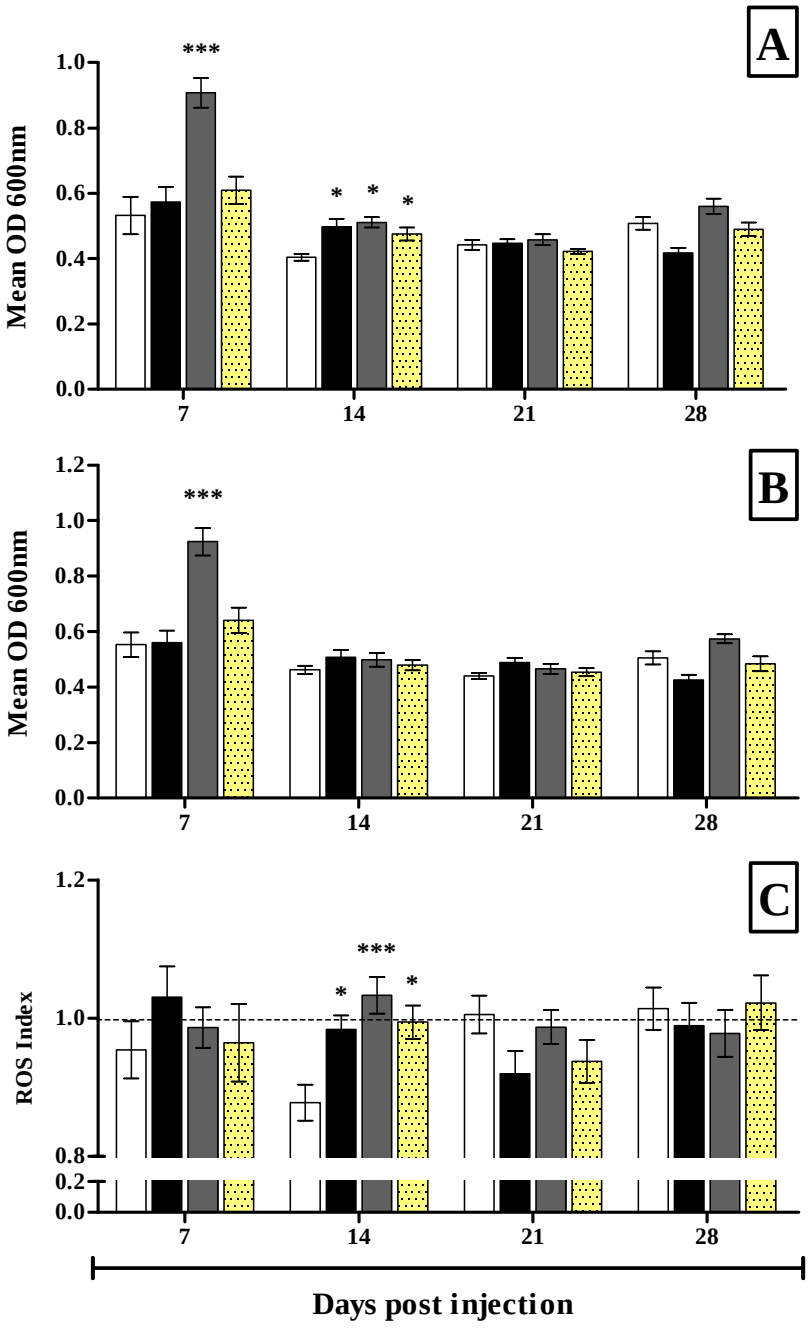


Figure 6-24: Pronephric leukocytes respiratory burst activity in injected carps. The pronephros of injected fishes with either 100µl PBS □; CHO1 (5 mg/kg) ■; *A. hydrophila* vaccine (1×10^9 cfu/ml) ■ or *A. hydrophila* (1×10^9 cfu/ml) mixed with CHO1 (5 mg/kg) □ were isolated every week for a month. The NBT assay was utilised to determine (2×10^6 cell/ml) pronephric respiratory burst activity incubated for 24h: A) with PMA; B) without PMA; and C) reactive oxygen species (ROS) stimulation index. Two way ANOVA was used to analyse the data incorporating and Bonferroni post-hoc analysis used to compare treatment to control at each time point. The differences in comparison to matched control

are indicated thus: * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001. Bars represent mean ± SEM of 8 fishes and the dashed line at y-value equal to 1 show the non-stimulation level.

Table 6-15: The two way ANOVA analysis of pronephros cells respiratory burst activity in carp post injection*.

Pronephros activity	Time		Treatment		Interaction	
	F	P	F	P	F	P
Cell with antigen	29.09	<0.0001	14.745	<0.0001	5.051	<0.0001
Cell	23.608	<0.0001	8.076	<0.0001	3.25	0.0003
ROS index	0.9	0.444	0.741	0.53	2.124	0.033

*The grey background highlights the significant effects at $p \leq 0.05$.

6.3.2.3 Determine alternative complement pathway activity in serum

Serum alternative complement level was determined in carp after injection with either 100 µl/ fish PBS, CHO 1, vaccine and combination of CHO 1 and vaccine. Blood samples were taken every week for one month. At each time point, the effect of different treatments was compared to the negative control (100µl PBS) in eight fishes (Figure 6-25) and analysed with two way ANOVA with Bonferroni post-hoc analysis used to calculate differences between treatments. The data analysis revealed the significant differences between experimental factors i.e. treatments ($F = 5.047$, $p = 0.003$), the exposure time ($F = 8.736$, $p < 0.0001$) and the interaction between both factors ($F = 2.633$, $p = 0.01$) that showed the effect of treatments was dependent on the exposure duration (Figure 6-25).

The previous significant effects were associated with the differences between all treatments i.e. CHO 1 ($p = 0.003$), vaccine and combination of CHO 1 and vaccine ($p <$

0.0001) in comparison to control after 7 days p.i. (Figure 6-25 A). No significant effects were observed between treatments at the other time points.

In contrast, the alternative complement level after 1 week p.i. in the control group was significantly lower than the rest of time points ($p < 0.0001$). There was however no significant differences between sampling points in each treatment group.

The pervious results concluded that the reduction in control group after 1 week p.i. led to the significant differences in comparison to all treatment groups.

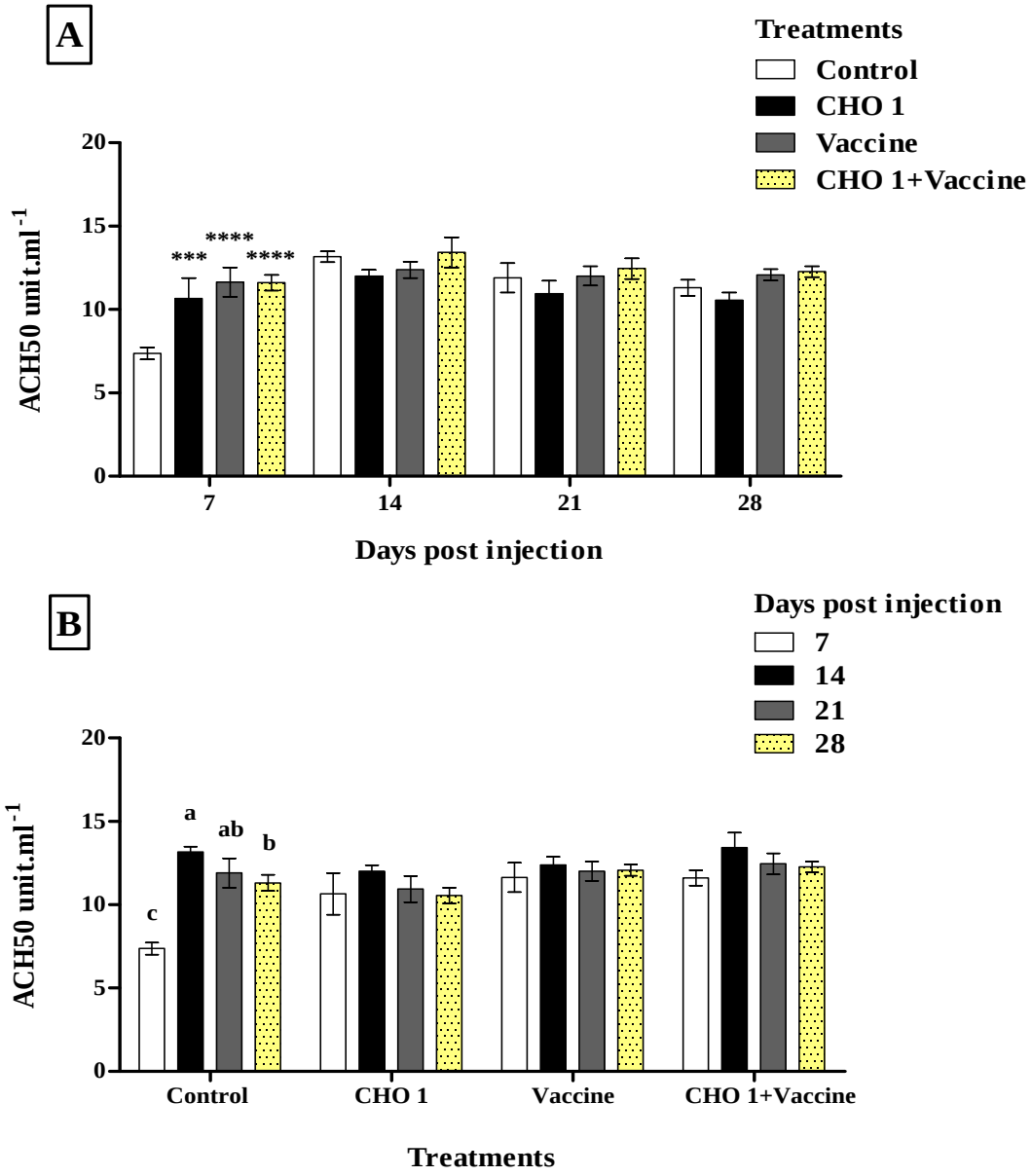


Figure 6-25: Alternative complement pathway activity of vaccinated carps. Fishes were injected with either 100µl PBS, CHO1 (5 mg/kg), *A. hydrophila* vaccine (1×10^9 cfu/ml) or *A. hydrophila* (1×10^9 cfu/ml) mixed with CHO1 (5 mg/kg). Fishes were killed and serum collected each week for one month. The complement level was determined by measuring the hemolytic activity on sheep red blood cells. Two way ANOVA was used to analyse the data with Bonferroni post-hoc analysis to compare treatment to control at each time point (Figure A) and Tukey's post-hoc analysis to compare the treatment differences within time periods (Figure B). The treatment differences in comparison to control are indicated thus *** = $p \leq 0.001$, **** = $p \leq 0.0001$, whilst significant differences between time points at each treatment are depicted by different letters. Bars represent mean ± SEM of 8 fishes.

6.3.2.4 Antibody titre against *A. hydrophila*

The specific serum antibody response of carp to *A. hydrophila* was determined post injection. Blood samples was taken from 8 fishes/ treatments group weekly for one month. The two way ANOVA revealed highly significant effects ($p < 0.0001$) of treatment (F= 43.64), sampling point (F= 23.316) and interaction (F= 9.806) on antibody level in carp.

The specific antibody (IgM) titre against *A. hydrophila* was significantly different from the control (fish injected with 100 µl PBS) at 14 days p.i. for fishes injected with either vaccine ($p = 0.00013$) or the combination of CHO 1 and vaccine ($p < 0.0001$) (Figure 6-26 A). The antibody level increased every week in both injected groups and after 21 days p.i. the antibody level in vaccine group ($p = 0.031$) and the combination of CHO 1 and vaccine ($p < 0.0001$) were significantly higher than control, whilst CHO 1 induced significant reduction in antibody level ($p = 0.009$). Furthermore, after 28 days p.i. both injection groups i.e. vaccine and the combination of CHO 1 and vaccine ($p < 0.0001$) stimulated a highly significant antibody response in comparison to control (Figure 6-26 A).

The differences in antibody level between experimental times points were analysed with Tukey's post-hoc analysis for each injection treatments (Figure 6-26 B). The control group showed a varying antibody level at 7 days p.i., and the antibody level during the experiment was comparable to PBS injected carp in Selvaraj et al. (2006). Whilst the CHO 1 group showed a slow decrease each week in antibody level with the greatest level of antibody occurring at 7 days p.i., both the combination (vaccine and CHO 1) and the vaccine alone groups showed a steep weekly increase in antibody level. Whilst the vaccine group stimulated the highest antibody level after 28 days p.i., interestingly the combination group induced the highest antibody level a week earlier i.e. after 21 days suggesting that CHO1 reduced the time to when a significant antibody level was evoked.

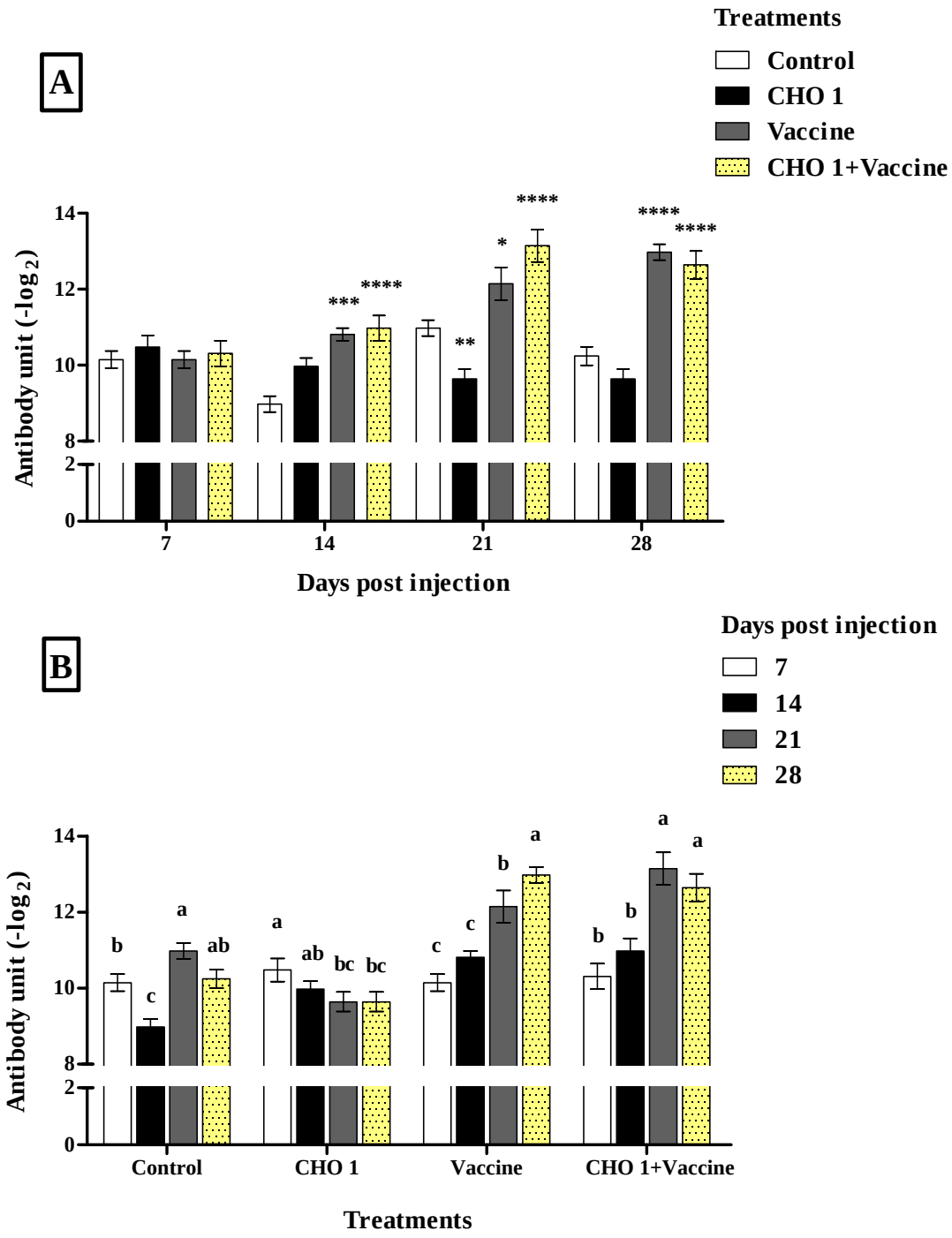


Figure 6-26: The antibody titre against *Aeromonas hydrophila* in carp serum. Fishes were injected with either 100µl PBS, CHO1 (5 mg/kg), *A. hydrophila* vaccine (1×10^9 cfu/ml) or *A. hydrophila* (1×10^9 cfu/ml) mixed with CHO1 (5 mg/kg). Carp serum of each of the fishes were used to detect antibodies ($\log_2$ titre) against *Aeromonas hydrophila* using the F16 - Common Carp/Koi carp (*Cyprinus carpio*) IgM antibody kit from Aquatic Diagnostics. Two way ANOVA was utilised with Bonferroni post-hoc analysis to compare

treatment to control at each time point (Figure A) and Tukey's post-hoc analysis to compare the treatment differences within time periods (Figure B). The treatment differences in comparison to control are shown thus: performed with * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$ and the significant differences between time points at each treatments are indicated with different letter. Bars represent mean ± SEM of 8 fishes.

6.4 Discussion

6.4.1 Evaluation of the effect of immunostimulant on the expression of immune related genes in carp organs

The structure and function of cells are dictate by proteins, the production of these functional proteins are encoded by the expressed genes during mRNA synthesis. In this study the effect of injecting carp with different immunostimulants i.e. MacroGard® at 5 mg/kg and CHO 1 at concentrations 5 mg/kg, 10 mg/kg on a range of immune related genes i.e. pro-inflammatory cytokines (IL1β, IL6 family) and chemokines (qCXCα), antiviral cytokines (IFNγ2b), viral resistance protein (carp Mx), anti-inflammatory cytokine (IL10), nitric oxide production (iNOS), antibacterial activity (lysozyme-C) and complement system activator (C3) was determined in pronephros, spleen, liver and mid gut tissues. Results indicate varied effects on the expression on a range of inflammatory cytokines.

Pro-inflammatory cytokines

IL1β, an essential and early response cytokine to infections and tissue injury, has a pleiotropic activity and provokes a cascade of effects leading to activation of several genes or modification of proteins. IL1β is expressed by monocytes/ macrophages, dendritic cells, endothelial and epithelial cells (Wang et al., 2011). Functionally, IL1β triggers leukocytes migration to inflammation site through the induction of chemokine expression and up-regulation of cell adhesion molecules (Chadzinska et al., 2008). In carp, IL1β is expressed in the pronephros, spleen, liver, gill, brain and pituitary tissues (Bridle et al., 2002).

In my study CHO1 at both concentrations, 5 and 10 mg/kg, induced up-regulation of IL1β expression after 3 and 14 days p.i. respectively in the liver, whilst in mid gut tissue up-regulation only occurred at 14 days p.i. However, MacroGard® stimulated IL1β up-

regulation after 24 h p.i. in the liver. The IL1β expression in response to glucan administration by injection reported in carp pronephros macrophages, IL1β transcription was higher in glucan injected carp and the expression peaked after 48 h p.i. with 1000 µg of glucan/ fish (Selvaraj et al., 2005). In addition, the effect of glucan was dose dependant (Selvaraj et al., 2005). Fujiki et al. (2000) reported that injected carp with sodium alginate (2 mg/100 g) and scleroglucan (1 mg/100 g) induced IL1β expression in pronephros after 48 h p.i. Glucan activity appeared to be mediated through changes in the gene expression of pro-inflammatory cytokine and chemokines (Falco et al., 2012, Biswas et al., 2012). The increase in IL1β expression in both of liver and mid gut are secondary lymphoid organs which have a larger number of macrophages, NK cells and lymphocytes. Since IL1 secretion serves as a starting point for a number of cascade reactions, including effects on IL2 release from stimulated T-cells and NK cell activity, it is possible that the increase in IL1β expression seen here could have many downstream effects in fish (Biswas et al., 2012). Increased phagocyte activity may also result from an increased IL1β production. Thus both specific and nonspecific immune responses could be affected by this marked increase in IL-1β transcription (Low et al., 2003).

Another pro-inflammatory cytokine that also has anti-inflammatory properties is the interleukin 6 family (IL6 family), which have a major role in many biological functions including tumorigenesis, inflammation, immune regulation and hematopoiesis (Wang and Secombes, 2009). It is also reported to be associated with immunoglobulin production, lymphocyte and monocyte differentiation, chemokine secretion and migration of leukocytes to inflammation sites. The IL6 family of cytokines is thought to be produced by a range of cells including T lymphocytes, macrophages, fibroblasts, neurons, endothelial and glial cells (Reyes-Cerpa et al., 2012).

The expression of the IL6 gene was significantly up-regulated in carp liver 24 h after injection with MacroGard®, whilst in spleen the effect occurred at 7 days p.i. The expression of IL6 gene was in line to IL1β expression, with a significant up-regulation in liver and the high level of expression in spleen at a similar time points to IL6. In contrast, CHO 1 did not induce significant increases in IL6 expression in all studied organs but at a 10 mg/kg concentration induced significant down-regulation 14 days p.i. Several studies have reported similar effects of β-glucan on modulation of the pro-inflammatory cytokine genes expression profile. The intraperitoneal injection with β-glucan in rainbow trout induced increases in IL1β and IL6 expression in liver, pronephros and spleen after 48 h p.i. (Lovoll et al., 2007). Similar up-regulation of IL1β and IL6 genes has been noted in *in vitro* experiments when trout pronephric macrophages were exposed to 10 mg/ml β-glucan for 48 h (Lovoll et al., 2007). Chettri et al. (2011) studied the *in vitro* effects of β-glucan treatment in trout pronephric leukocytes and reported up-regulation of IL1β, IL6, IL10 and tumor necrosis factor α (TNFα) and decreased transforming growth factor β (TGFβ) at the transcript level.

These significant increases might be due to the increase in phagocytic cell activity and proliferation, and indeed, a study in rainbow trout suggested that IL6 production during inflammation promoted macrophage proliferation locally (Costa et al., 2011). In addition, the expression of IL-6 reported as a potent inducer of antimicrobial peptide gene expression in fish (Secombes et al., 2011).

Chemokines

The effect of MacroGard® and CHO1 on the pro-inflammatory chemokine expression qCXCα gene was determined. Chemokines are small proteins which have chemotactic cytokine properties and play an important role in the pro-inflammatory phase of the immune response. Their function is to recruitment of leukocytes to the inflammation site

and participate in cell migration in normal tissue maintenance and development (Reyes-Cerpa et al., 2012). Some CXC chemokines has been characterised as potent mediators of neutrophil chemotaxis, induce neutrophils to leave the bloodstream and enter surrounding tissue, while others direct lymphocytes to the CXC formation source (Huising et al., 2004, Gonzalez et al., 2007). The analysis of this pro-inflammatory chemokine in carp revealed a significant up-regulation of qCXCα expression in mid gut tissue at 1 and 3 days p.i. with CHO1 at 5 mg/kg and in liver the higher dose of CHO 1 (10 mg/kg) had the same effects at the same sampling points. However, in the spleen both CHO1 doses induced a significant down-regulation of CXC expression after 14 days p.i. These results supported by Chadzinska et al. (2008) reported that the intraperitoneal injection of carp with zymosan induced peritoneal and pronephric IL1β, TNFα, CXCα and iNOS after 24 h p.i., suggesting that stimulation carp phagocytic leukocytes with phorbol myristate acetate (PMA) up-regulate CXCα and CXCβ to induce leukocytes chemotaxis (Huising et al., 2003). In my investigation, the up-regulation of qCXCα gene occurred after 24 h p.i. with CHO 1 and lasted until 3 days p.i., suggesting an influx and migration of phagocytes cells, most probably neutrophilic granulocytes from circulation towards the liver and mid gut tissues since circulating leukocytes do not express CXC (Huising et al., 2003, Gonzalez et al., 2007).

Anti-inflammatory cytokines

Interleukin 10 (IL10) is an anti-inflammatory cytokine with immunosuppressive functions regulating the inflammatory response, inhibiting the activation of macrophages and monocytes, also has a stimulatory effects on different types of leukocytes such as a factor for B cells survival and differentiation (Gonzalez et al., 2007). It is produced by activated monocytes, T cells (Th2) and other cell types like keratinocytes (Reyes-Cerpa et al., 2012). In this study, both of MacroGard® and CHO1 at 10 mg/kg induced up-regulation in IL10

expression at 3 days p.i. in the liver, whilst in the pronephros, CHO1 at 5 mg/kg induced the IL10 expression earlier (3 days p.i.) in comparison to MacroGard® at 7 days p.i. However, a significant down-regulation in IL10 expression observed in pronephros, after 24 h p.i. in both CHO1 concentrations and at 14 days p.i. in CHO1 at concentration 5 mg/kg. In a study on carp skin inflammatory responses, Gonzalez et al. (2007) suggested that up-regulation of IL10 expression happens post the up-regulation of pro-inflammatory cytokines, and the IL10 expression expected to occur after 24 h post stimulation (Gonzalez et al., 2007). Similar effects have been found after injection carp with zymosan, IL10 up-regulated after 2 days p.i. and maximum expression was noted 4 days p.i. the peritoneal and at 7 days p.i. in the pronephros (Chadzinska et al., 2008). However in my investigation no down-regulation in IL1β expression was observed at the same time point when of IL10 up-regulation occurred in liver and pronephros, thus it seems unlikely that this up-regulation of IL10 could have an effect on IL1β expression, this is in lined with other studies carried out on carp by Gonzalez et al. (2007). In addition, the up-regulation of IL10 expression might be happened due to the activation and differentiation of leukocytes in pronephros and liver, this was explained that depending on the stimulation type and the production of cytokines, macrophages differentiate to the alternative activated macrophages that release anti-inflammatory cytokines i.e. IL10 and TFG-β (Joerink et al., 2006).

Antiviral activity

Antiviral cytokines play an important role in immune defence in vertebrates for example interferon gamma (IFN γ), is the only member of interferon type II, which mediates cellular resistance against intracellular viral and bacterial replication directly and by modulating innate and adaptive immune systems. It is also able to activate macrophages, provoke class II major histocompatibility complex (MHCII) expression and induces

apoptosis, especially during viral infection, also inhibits cell proliferation (Zou and Secombes, 2011). IFN γ is produced by T lymphocytes (CD4+ Th1) and Natural Killer cells (NK cells) in response to interleukin-12 and 18, mitogens or antigens (Zou and Secombes, 2011, Reyes-Cerpa et al., 2012). IFN can induce host cells to produce proteins that are able to inhibit the translation of viral mRNA, such as 2′, 5′-oligoadenylate synthetase, protein kinase and Mx protein (Ellis, 2001). Mx is intracellular mediators of viral resistance, expressed in cells exposed to double-stranded RNA or virus infection (Leong et al., 1998).

The intraperitoneal injection with MacroGard® induced significant increase in IFNγ2b expression after 1, 3 days p.i. in the liver and 7 days in spleen, while no effects were detected on carp Mx expression in all sampled tissues. Injected carp with CHO1 at 10 mg/kg induced significant up-regulation of IFNγ2b expression at 3 days p.i. in the pronephros and liver. However, the effect of CHO1 at 5 mg/kg occurred after 2 weeks p.i. in the liver and induced up-regulation in carp Mx expression. Similar early enhancement of IFNγ expression of kidney tissue was reported in zebrafish pre-injected with β-glucan at concentration 5 mg/ml and challenged with *Aeromonas hydrophila*, the IFNγ expression peaked at 4 h post infection while PBS injection and infected with bacteria enhanced the expression levels at 6 h post infection (Rodriguez et al., 2009). These results suggested the possibility of β-glucan being able to modulate the expression of IFNγ and as a result activate macrophages and the T helper (Th1) cells immune responses during infections thus, the immune cells could be more active and react rapidly in short period against a challenge (Rodriguez et al., 2009). Furthermore, feeding trial in carp also confirmed the early up-regulation in IFNγ2 expression after feeding with 5 mg per fish of commercial baker's yeast extract, a rapid expression reported after 1 day post treatment suggesting that

IFN-γ expression mediated intracellular pathogen killing ability of leukocytes in carp (Biswas et al., 2012).

There was no corresponding in Mx expression to IFNγ2b up-regulation in both of MacroGard® and CHO 1 injection group, results which are not in line with an *in vitro* study performed by Kitao et al. (2009). Correspondingly up-regulation was found in both IFN and Mx expression in carp pronephric leukocytes after 1 h post treatment with poly I:C and lasted for 48 h suggesting the IFN direct stimulation to the Mx gene expression.

It is possible that MacroGard® and CHO 1 may modulate the expression of IFNγ2b and the immune cells could be more active and have a reaction time faster during inflammation. This may be the IFN capability to respond during early stages of viral infection through mediate antiviral defence mechanism, supplying certain protection until the specific immune defences are able to respond (Ellis, 2001).

Nitric oxide production

Nitric oxide synthases are a family of enzymes catalysing the production of nitric oxide (NO) from L-arginine. NO has the ability to regulate and effect molecules in different biological functions, such as an inhibitor of cell proliferation, a mediator of antitumor and mediating resistance against different pathogens. Also, NO has been suggested to mediate inflammation caused by infection, and to be immunosuppressive. The inducible isoform iNOS is produced by many cell types in response to cytokine production, tumour growth, invaders such as parasites and bacteria (Saeij et al., 2000).

In this investigation injecting with MacroGard® induced a rapid response in iNOS expression in liver after 1 and 3 days p.i.; in spleen at 1 and 7 days p.i. and in the pronephros at 7 days p.i. However in mid gut both of MacroGard® and CHO1 at 5 mg/kg induced significant up-regulation of iNOS expression after 3 days p.i. The increases of iNOS expression suggested an increased level of nitric oxidative species (NO) in the

studied organs that might be due to enhancement in macrophage proliferation or activity which is one of the important non-specific defence mechanism in fish (Ellis, 1999). This supports the previous studies which also suggest that β-glucan induces a significant up-regulation in iNOS expression after 25 days of feeding common carp with 0.1% MacroGard® supplemented feed, (Miest et al., 2012) and intraperitoneal injection with β-glucan enhanced the bactericidal activity of rainbow trout macrophages after 2 weeks p.i. (Jorgensen et al., 1993).

Antibacterial activity

Lysozyme is also an important component of the innate immune defence system and in fishes is thought to destroy both Gram-positive and negative bacteria. Lysozyme has been found in pronephros, skin, digestive tract, gills and eggs, particularly in the yolk. Lysozyme is thought to be located within neutrophils, monocytes/ macrophages, and eosinophilic granular cells of freshwater and marine fish (Erik and Judith, 2008). The effect of CHO1 injection on lysozyme expression was found in mid gut tissue, where the injection with 5 mg/kg induced a significant up-regulation after 3 and 7 days p.i., while 10 mg/kg concentration stimulated significant increases at 3 and 14 days p.i.. In addition, CHO1 at 10 mg/kg up-regulated lysozyme expression only after 3 days p.i. The effect of injected carbohydrate CHO1 might be mediated through an enhancement of leukocytes (macrophage) proliferation and differentiation stage, mainly in tissues rich with macrophage and neutrophils like kidney and intestine (Uribe et al., 2011). In Atlantic salmon, the effect of β-glucan on pronephric macrophages revealed an increase in lysozyme level in cell culture supernatant after 3 days post treatment and the effect lasted till 5 days post treatment (Paulsen et al., 2001). In present investigation, the lysozyme expression did not mirror the lysozyme activity that detected in serum, which comprised

the significant increase in lysozyme level after 24 h p.i with CHO1. This delay in protein levels compared to gene expression was observed with dietary 1% β-glucan supplemented feed in rainbow trout, where lysozyme expression was up-regulated 6.3 fold by 13 days of the onset glucan feeding while there was an enhancement of lysozyme activity in plasma after 59 days post glucan feeding (3 days post challenge with *Yerisina ruckeri*) (Skov et al., 2012). This is perhaps not unexpected and represents the time for translational processes to occur between gene expression and the yield of a measureable amount of protein in the serum.

Complement system activator

The C3 gene encodes for the complement component 3 (or C3) protein of the complement system and plays an important role in humoral immune response. In contrast to mammals, in teleost multiple isoforms of the individual complement components exist for example, multiple C3 forms have been described for carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata*) (Erik and Judith, 2008). C3 gene was up-regulated significantly after 24 h p.i. with CHO1 at 10 mg/kg concentration also injection with MacroGard® and both dose of CHO1 after 3 days p.i. in liver. However, C3 was down-regulated significantly after 3 days exposure to MacroGard® and CHO1 at 5 mg/kg in the pronephros. This up-regulation primarily in the liver might be because the liver hepatocytes are thought to be one of the main production sites of C3, which is essential to all three activation pathways of the complement system and is the major opsonin of the complement system and essential for the generation of the membrane attack complex (Lovoll et al., 2007). My observations also corroborate other studies which have indicated that immunostimulants such as LPS and β-glucan have the ability to activate the complement cascade directly via alternative complement pathway or indirectly through

lectins and C-reactive protein (Secombes et al., 1996). In the present study, the stimulation with MacroGard® and CHO1 significantly increased the expression of C3 gene after 3 days p.i. in the liver, suggesting that C3 is equally stimulated by MacroGard® and CHO1. In addition, this result was correlated to the significant increase of serum alternative complement pathway activity at 3 days p.i. with CHO1 at concentration 5 mg/kg. However, the early stimulation with CHO1 at 10 mg/kg concentration significantly up-regulated C3 expression after 24h p.i. and showed the rapid effect of CHO1 on complement system, suggesting that this carbohydrate may lead to an increase in the ability of complement system to destroy invading pathogens through lysing cell wall or by enhancing the opsonic system to increasing the uptake by phagocytic cells (Erik and Judith, 2008). Studies on rainbow trout also revealed the effect of glucan on C3 where intraperitoneally injected with β-glucan at 20 mg/kg for 48 h differentially regulated C3 subtypes in studied tissues (Lovoll et al., 2007).

The expression of immune related genes were more prominent in fishes injected with the modified carbohydrate group CHO 1, particularly in liver, mid gut and spleen were the most affected organs in the present investigation. It might be due to the differential involvement of different immune organs over time (Deshmukh et al., 2013). In addition, Geist et al. (2007) also showed that different tissues in striped bass exhibited different quality and quantity of mRNA transcription after exposure to stress factors (Geist et al., 2007).

6.4.2 Evaluate the biological effect of immunostimulant on carp leukocytes

Fish leukocytes play an important role in innate and adaptive immunity of fish during inflammation and their count is considered as an indicator of its health status (Secombes, 1997). Leukocytes comprise each of lymphocytes including T and B cells, cytotoxic cells

(similar to natural killer cells), monocytes/ macrophages and granulocytes they are cellular components of innate and adaptive immune system (see table 6-16) involved in maintaining internal homeostasis by removing dead or unwanted cells (Erik and Judith, 2008). Differentiation leukocytes cells count was carried out to determine the population of each leukocytes type after injection with immunostimulants.

Table 6-16: Fish nonspecific immune cells and their functional characteristics (Shoemaker et al., 2001).

Cell type	Functional characteristics
Monocyte/macrophage	Phagocytosis, cytokine production, intracellular killing, antigen processing and presentation
Neutrophil/granulocyte	Inflammation, cytokine production, extracellular killing, phagocytosis
Nonspecific cytotoxic cells/ natural killer cells	Recognition and target cell lysis
Lymphocytes: cytotoxic T cells, helper T (Th) cells and B cells	Eliminate the antigen through specific recognition, releasing antibodies (B cells), cytotoxic granules (cytotoxic T cells), cytokine production and signalling to other cells of the immune system (helper T cells), proliferation and differentiation of antigen-specific lymphocytes clones and creating memory for effective immune responses for subsequent antigen exposure.

Granulocytes increased significantly after 24 h p.i. with the modified carbohydrate, CHO 1, at both concentration used i.e. 5 and 10 mg/kg and reached three fold higher than control in fishes injected with 5 mg/kg. Previous studies have also revealed that intraperitoneal

injection with yeast β-glucan at 1 mg per fish induced significant increase in total leukocyte counts and also increased the proportion of neutrophil and monocyte in carp blood (Selvaraj et al., 2005). Neutrophils, the most abundant granulocytes in kidney teleost, are the first leukocytes that migrate to an inflammatory site where they have potent antimicrobial responses against invading pathogens (Havixbeck and Barreda, 2015). Activation of teleost neutrophils is via recognition receptors of pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular pattern molecules (DAMPs). On activation the cells are capable of phagocytosing and destroying pathogens through the toxic intracellular granules, the increase in reactive oxygen species (ROS) and reactive nitrogen species (NOS), and the production of neutrophil extracellular traps (NETs) (Havixbeck and Barreda, 2015).

In my second investigation when the CHO 1 was injected as adjuvant with *A. hydrophila* vaccine, after 7 days p.i. there was a significant increase in the percentage of granulocytes observed. While the percentage of monocytes/ macrophages was significantly higher after 14 days p.i. in fish injected with adjuvant alone, vaccine alone and the combination of adjuvant and vaccine. Selvaraj et al. (2006) also reported an increase in neutrophils and monocytes in blood of carp injected with combination of 1000 µg β-glucan+100 µg LPS/ fish after 16 days p.i. In my first investigation, injecting carp with CHO 1 induced significant increases in the percentage of granulocytes after 24 h p.i. and there was no significant effect after 7 days p.i. In contrast, when used as adjuvant with vaccine the effect lasted till 7 days p.i. despite the non-significant effect of vaccine group alone. This might be due to the migration of mature granulocytes, especially neutrophils, from the hematopoietic tissues to the circulation as they are rapidly recruited from the blood stream to the inflammation sites by chemotactic signals derived from stimulated immune organ with immunostimulant (Havixbeck and Barreda, 2015).

The effect of injecting the immunostimulants varied between leukocytes types, for example a significant decrease in lymphocyte proportion was observed after 7 days p.i. in the combination of adjuvant and vaccine group, also after 14 days p.i. in adjuvant alone group. There was also a reduction recorded in the percentage of monocytes after 21 days p.i. in the vaccine alone group. This phenomenon has also noted in association with pathogen where decreases in quantity of circulating leukocytes, in particular lymphocytes and/or suppress their activity (Harikrishnan et al., 2011).

The pronephros, is the most important site of blood cell formation and immune functions, and contains reticular cells, macrophages, lymphocytes and plasma cells which are involved in antigen trapping, phagocytosis and play a role in immunologic memory (Secombes et al., 1982, Tort et al., 2003). Antigen specific proliferation of pronephric leukocytes was determined in the vaccination trial and highlighted the higher proliferative responses occurred in fish injected with vaccine alone at 7 days p.i. Similar antigen-specific proliferation has been reported in trout blood leukocytes in response to *Aeromonas salmonicida* antigens (Marsden et al., 1995, Marsden and Secombes, 1997), also in Catla pronephric leukocytes exposed to *A. hydrophila* antigens (Kamilya et al., 2006). However, in results presented in this thesis based on the MTT assay reveal that leukocytes proliferation occurred in CHO 1 injected fish with and without the presence of the *A. hydrophila* antigen with in the assay at 14 day p.i. This suggests that CHO 1 non-specifically induced leukocytes proliferation. Therefore, the significant increases in leukocytes population in pronephros after injection with CHO 1 reflects the importance of this organ as a site of blood cell formation and immune functions (Hoole et al., 2001).

The ability of antigen presenting cells APC i.e. macrophages, dendritic cells, B-lymphocytes and epithelial cells to recognise and phagocyte pathogens through its receptors is the most important mechanism of protection (Misra et al., 2006b, Selvaraj et

al., 2005). In my study the high significant enhancement of the ROS activity of pronephric leukocytes was observed after 7 days p.i. with *A. hydrophila* vaccine alone, this response was not however specific to the availability of *A. hydrophila* antigen in the assay, it was correlated to the significant increase in pronephric proliferation index of vaccine group after 7 days p.i.

In addition to leukocytes proliferation, the respiratory burst activity index was significantly higher than the control at all the injection treatments after 14 days p.i. and fish injected with vaccine alone induced higher stimulation index. The activation of this important killing mechanism might be involved in the destruction of *A. hydrophila* up to 2 weeks giving enough time for the adaptive immunity to be activated, and indeed several studies have reported the enhancement of bacterial killing activity when glucan was used as adjuvant in Atlantic Salmon (Jorgensen and Robertsen, 1995), Carp (Selvaraj et al., 2005) and Catla (Kamilya et al., 2006). However, Chen and Ainsworth (1992) and also Selvaraj et al. (2005) concluded that the activation induced by glucans only lasted for a short period of time i.e. 7 days post administration and might need more than one administration to maintain the disease protection.

6.4.3 Evaluation of the biological effects of immunostimulant on soluble immune mechanism in carp serum

The nonspecific defence mechanisms include, cellular and non-cellular (soluble) factors. The soluble components comprise for example, complement, acute-phase proteins, lysozyme and cytokines (i.e., interferons, interleukins, tumor necrosis factors, and transforming growth factors) which are involved in the regulation of both innate and adaptive immune responses (Erik and Judith, 2008). Lysozymes are involved in a range of defence mechanism such as bacteriolysis, opsonisation, immune response potentiation,

restricted anti-viral and antineoplastic activity (Lie et al., 1989). In my investigation the lysozyme activity was determined in carp serum injected with MacroGard® and CHO 1, after 24 h p.i. It was revealed that CHO 1 at 5 mg/kg induced significant increases in lysozyme activity which correlates with the significant increases in granulocytes percentage after 24 h p.i. This might have been expected as lysozyme has been recorded with the intracellular granular proteins of neutrophils, monocytes/ macrophages, and eosinophilic granular cells (Erik and Judith, 2008, Saurabh and Sahoo, 2008). Indeed, several studies have noted that glucan administration can affect lysozyme activity in fish, for example the multi injection of Rohu with β-glucan at concentration 10 mg/kg induced significant increases in serum lysozyme activity at all-time points between 14-56 days p.i. (Misra et al., 2006b), whilst in Atlantic salmon, the intraperitoneal injection with β-glucan increased plasma lysozyme activity, after 7 days p.i. and reached a peak at 14 days p.i. (Engstad et al., 1992). These previous studies revealing the time dependency of lysozyme production. Therefore, this might be the reason for lysozyme level reduction in fishes injected with glucan (5 mg/kg MacroGard®) after 1, 3 and 7 days p.i. and normalised of the level to control after 14 days p.i.

Complement is a series of proteins that helps the ability of antibodies and cells to attack pathogens (Hoole et al., 2001). Thus the complement system is an important soluble defence mechanism, which can lyse the cell wall of the pathogen and/ or opsonise the invaders cell surface to enhance the uptake by phagocytic cells (Shoemaker et al., 2001). My investigation has revealed that the intraperitoneal injection with CHO 1 at concentration 5 mg/kg provoked a significant increase in complement activity after 3 days p.i. and although no effects was observed at 1, 7 and 14 days p.i., this may be involved in an early enhancement of innate immune defence (Erik and Judith, 2008). In contrast, in Atlantic salmon, glucan injection caused significant increase in complement level between

14-56 days p.i. (Engstad et al., 1992), and at a similar sampling point in Rohu fish, the multi injection of glucan enhanced complement level (Misra et al., 2006b).

Injecting fish with CHO 1 alone and as adjuvant with *A. hydrophila* vaccine induced significant increase in complement activity at 7 days p.i., which might be related of the significant reduction in complement level at control group after 7 days p.i. in comparison to the other time points. Injection of glucan alone and as adjuvant with bacterin did not induce complement activity in carp serum (Selvaraj et al., 2005, Selvaraj et al., 2006).

Antibodies are plasma proteins that identify and neutralize pathogens; the IgM class being the major immunoglobulin in teleosts (Uribe et al., 2011). Several studies have reported a positive correlation between antibody titer and protection level in vaccinated fish, such as in formalin-killed *Aeromonas hydrophila* vaccine with mushroom glucan adjuvant in catla (Kamilya et al., 2006), formalin-killed *Aeromonas salmonicida* vaccine in rainbow trout (Villumsen et al., 2012), *Yersinia ruckeri* bacterin vaccine with Montanide ISA 763 A VG adjuvant in rainbow trout (Jaafar et al., 2015). In my investigation serum IgM levels were significantly induced in fish injected with vaccine or the combination of vaccine and adjuvant compared with control at 14, 21 and 28 days p.i. Furthermore fish injected with vaccine adjuvant combination had higher significant level in comparison to the vaccine alone after 21 days p.i. and enhanced fish protection one week earlier comparable to the vaccine level at 28 days p.i. The adjuvant effect of glucan was reported previously in several studies, for example, Catla injected with *Aeromonas hydrophila* vaccine (Kamilya et al., 2006), catfish with vaccine against *Edwardsiella ictaluri* (Chen and Ainsworth, 1992), turbot in vaccine against *Vibrio damsela* (Figueras et al., 1998), and in carp injected with 100 µg β-glucan and 10 µg LPS/ fish induced significant antibody titer against *Aeromonas hydrophila* (Selvaraj et al., 2006). These significant increases in antibody levels might be associated with the adjuvant's stimulation effect to B cells that leads to

phagocytosis of glucans and subsequent antigen binding MCH class II on their surface, which leads to activate T helper cells after the binding to MCH class II and produce cytokines IL2. The exposed IL2 induce B cells proliferation, increases in antibody production when the newly proliferated B cells differentiate to plasma and B memory cells high production of IgM (Raa, 2000).

The evaluation of vaccine efficiency is dependent on the protection and the specific immune response to the vaccine such as antibody level (Ellis, 1988). Therefore, the observation that combination of adjuvant and vaccine induces an early antibody response against *A. hydrophila* than vaccine alone, may be a significant finding as it implies that this modified carbohydrate, CHO 1, would lead to an earlier protection in vaccinated fish.

In conclusion this study has highlighted the potential of using this novel carbohydrate CHO1 as an effective immune-adjuvant in fish vaccines due to the enhancement and modulation range of innate and adaptive immune parameters, including humoral immunity i.e. serum lysozyme and complement activity, antibody level and immune related genes (Table 6-12) and cellular immunity including the differentiation cell count, proliferation and respiratory burst activity in pronephros cells. Further studies are in progress to determine the immune related gene in the present vaccination trial. Also, more research is needed to evaluate the effective dose, administration time and induction of immune protection against pathogen challenge.

Chapter 7. General Discussion

Over many years there have been extensive studies on a variety of mechanisms used to control infection in fish. Whilst initial studies utilised a range of treatments such as antibiotics, the development of antibiotic resistance and the introduction of legislation on the use of certain antibiotics, such as chloramphenicol and fluoroquinolones, and antimicrobial compounds such as malachite green have limited their use on fishes used for human consumption (Rintamaki-Kinnunen et al., 2005, Romero et al., 2012). Therefore, this has driven an increased interest in other control strategies such as stimulation of the immune response to increase disease resistance.

Whilst there have been extensive studies on vaccine development, for example against spring viraemia of carp virus (SVCV) in ornamental koi (*Cyprinus carpio koi*) (Emmenegger and Kurath, 2008) and koi herpes virus (KHV) in carp (Schmid et al., 2016), which target the specific immune responses, immunostimulants which primarily enhance the non-specific immune parameters have received relatively little attention.

Recent investigations, for example the use of polyunsaturated fatty acids (PUFAs) in Atlantic salmon (*Salmo salar* L.) (Thompson et al., 1996); Freund's complete adjuvant (FCA) in Yellowtail (Kawakami et al., 1998); vitamin C in rohu (Tewary and Patra, 2008) and β-glucan in carp (*Cyprinus carpio*) (Selvaraj et al., 2005), rohu (*Labeo rohita*) (Misra et al., 2006b) and in Striped catfish (*Pangasianodon hypophthalmus*) (Sirimanapong et al., 2015b) have revealed that immunostimulants are important substances in aquaculture and can increase protection against range of pathogens such as *Aeromonas salmonicida, Vibrio anguillarum, Pasteurella piscicida, Aeromonas hydrophila, Edwardsilla tarda* and *Edwardsiella ictaluri.*

All of these general immunostimulants tend to be obtained from natural sources where quality assurance can be unpredictable. This is highlighted for example with the natural β-glucans which exhibit various immune activities dependent on their different molecular weights, general structure, frequencies of branching of the carbohydrate chain and solubility (Li et al., 2013). This variation leads to difficulties in predicting the immune activity of β-glucans resulting in unpredictable consequences when such immunostimulants are utilised as feed supplements or as adjuvants.

Therefore in this thesis, it was hypothesised that the immune capability of immunostimulants such as β-glucan can be enhanced by modifying the glucan structure and producing a novel carbohydrate that is characterised by greater immunomodulatory properties in carp. An approach recognised in the pharmaceutical industry in drug development e.g. in the modification of the anti-malaria and anti-cancer Artemisinin from qinghao (*Artemisia annua L.*) (Chen et al., 2015), but until the study presented in this thesis, has been not been used in aquaculture.

In order to determine the effects of a wide range of modified carbohydrates, it was necessary to establish an *in vitro* system to screen the immune capability and test the cytotoxicity of modified carbohydrates.

The cell culture type is the most important factor in an *in vitro* system. Primary cell culture is driven from fish tissues characterised by short life span that last for only a few days and slow growth rate in comparable to the cell line. Although the cell line is derived from primary cells, it has advantages over the primary cultures. For example, cell lines are suitable source for *in vitro* studies because they are quite homogeneous, easy to reproduce once it established and provide an unlimited supply of cells (Bols et al., 2005). Therefore, the CLCs line was used instead of the pronephros as a main cell type of the *in vitro* system in the carbohydrates screening process.

The immune system recognises immunostimulants by the presence of pathogen recognition receptors (PRRs) that are present on the cell wall of the immune cells. This recognition leads to activation of the immune cell and enhancement of their responses, which usually comprises an increase in their bactericidal activities, which includes the stimulation of phagocytosis, leukocytes migration and the production of cytokines (e.g. IL-1, TNFα), nitric oxide (NO) and reactive oxygen species (Sakai, 1999). Therefore in this thesis, the intracellular reduction of the nitroblue tetrazolium (NBT) assay was selected to determine the respiratory burst activity in stimulated immune cells, which is one of the early defence mechanisms of the innate immune system. In addition, several studies have highlighted the dose effects of β-glucans on cell cytotoxicity/ viability, for example in an investigation carried out on common carp, a significant increase in apoptosis occurred when pronephric cells were stimulated with β-glucans at concentrations 500 µg/ml and higher for 6 h incubation (Miest and Hoole, 2015). Thus, the tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) assay was utilised to determine cells viability post treatment with different glucans.

The effect of β-glucans was dependant on its structure, dose and period of exposure and supports previous *in vitro* findings such as the effect of MacroGard® on carp pronephric cells (Miest and Hoole, 2015), zymosan on carp neutrophils (Kepka et al., 2014), mushroom β-glucans on liver cells of Nile tilapia (*Oreochromis niloticus*) (Hsiao et al., 2015), MacroGard® and zymosan on carp macrophages (Pietretti et al., 2013), yeast β-glucan on Atlantic salmon (*Salmo salar L.*) macrophages (Jorgensen and Robertsen, 1995), yeast and fungus β-glucans on turbot (*Psetta maxima*) and gilthead seabream (*Sparus aurata*) phagocytes (Castro et al., 1999), and MacroGard® and zymosan β-glucans on carp pronephric cells (Vera-Jimenez and Nielsen, 2013).

Therefore, whilst it was expected that the modified carbohydrates used in this study would have dose dependent effects, screening for a dose response of all the 75 modified carbohydrates would be extremely time consuming and expensive. For these reasons, the dose response obtained for MacroGard® and sulphated MacroGard was utilised to determine the effect of the modified carbohydrates. In Chapter 3, MacroGard® and sulphated MacroGard at concentrations between 2.5-150 µg/ml did not induce a significant reduction in pronephric cell viability after 24 h incubation, however, a non-significant decrease in viability level was observed at concentrations higher than 50 µg/ml. This observation supported studies in cells from porcine peripheral blood, where a decrease in lymphocyte proliferation occurred at β-glucan (MacroGard®) concentrations higher than 50 µg/ml (Sonck et al., 2010).

The established *in vitro* system, which comprised utilising NBT assay to determine the respiratory burst of carp leukocytes cell lines (CLC) after exposure to 50 µg/ml of modified carbohydrates for 24 h, was successfully applied. The most effective carbohydrate was selected for its dose responses on CLC respiratory burst activity and viability. The modified cellulose drive carbohydrate (Tylose) induced a significant exponential increase in the respiratory burst in both CLC and carp pronephric cells after 24 h incubation.

Cellulose is linear (1→4)-β-D-glucans found in the cell wall of plants and it is derived from materials such as wood or fibres (Synytsya and Novak, 2014). In laboratory conditions, tylose is methyl-hydroxyethyl cellulose ethers (MHEC) that is synthesised from cellulose after heating with caustic substances such as sodium hydroxide. My results presented in Chapter 4 appear to suggest that modification through sulphation causes an increase in respiratory burst in the immune cell. It is of interest that in previous literature, the role of the sulphation process in several polysaccharides has been reported to be

associated with a different biological activity like anticoagulant, antitumor and resistant against HIV infection (Chang et al., 2006).

Thus, different *in vitro* comparisons was carried out between a modified carbohydrate (CHO 1) and tylose (the native source) and MacroGard® and zymosan, which are different sources of β-glucan to ascertain the effect of the modification of carbohydrate structure. The dose dependant effect of CHO 1 induced a substantial increase in respiratory burst in the CLC line at all the comparisons after 24 h. This biological alteration might result from the negative charge on the sulphated carbohydrate which interacts with regions of positive charge in proteins (Chang et al., 2006) or because of the variation of glucan recognition by leukocytes receptors (Brown and Gordon, 2001). The sulphation process is known to change the chemical and biological properties of different glucans, such as in lentinan, where sulphation caused significant increase in antioxidant activity in leukaemia cells (Feng et al., 2010). In addition, the sulphation process has been observed to affect the biological properties of carbohydrates in mammals. For example, sulphated rice bran beta glucan exhibited rat blood anticoagulant activity (Chang et al., 2006); natural sulphated glucan derivate from a marine alga (*Gracilaria verrucosa*) induced respiratory burst activity and phagocytosis in mice macrophages (Yoshizawa et al., 1996); natural sulphated polysaccharides from red algae possesses potent anti-inflammatory in mice leukocytes to produce cytokines (TNFα) in response to LPS (Ogata et al., 1999) and sulphate groups in *Ulva rigida* polysaccharides induced an increase in several chemokine and interleukin expression also nitric oxide production murine macrophages (Leiro et al., 2007).

Another important biological observation of CHO 1 was its lack of toxicity on viable cells at concentrations up to 150 µg/ml. In fact, there was a significant increase in viable CLCs at concentrations 1 and 2.5 µg/ml as measured by the MTT assay and at 2.5 µg/ml utilising trypan blue viable cell count. In contrast, other sources of β-glucan such as MacroGard®

caused a decrease in CLC viability level at doses higher than 50 µg/ml in this present study, a result that supports the previous study in carp pronephric cells (Miest and Hoole, 2015).

This modified carbohydrate CHO 1 enhanced a range of cellular and humoral immune responses when injected alone or as an adjuvant in carp, for example both doses of CHO 1 alone at concentrations 5 and 10 mg/kg of carp body weight induced a significant increase in the percentage of granulocytes after 1 day post injection perhaps indicating an enhancement of the nonspecific immune mechanism because of high mobility of these cells and involvement in phagocytosis, respiratory burst activity and chemotaxis (Secombes, 1997). In addition, the possible migration of these granulocytes to the liver and mid gut can be concluded due to the significant increase in chemokine CXC expression in both organs after 1 and 3 days post injection with CHO1. The ability of granulocytes to produce cytokines, which recruit immune cells to the inflammation site (Shoemaker et al., 2001) might explain the effective expression of immune related gene in both of the liver and mid gut in comparison to pronephros and spleen. For instance the up-regulation of lysozyme and inducible nitric oxide synthase (iNOS) expression in mid gut, and the significant up-regulation of Interleukins (IL1β, IL10) interferon gamma (IFNγ), lysozyme, complement receptors (C3), and iNOS in liver after injection carp with CHO 1.

Furthermore, this increase of different cytokines expression leads to enhancement of the immune mechanism through involvement in innate immune response, production of cytotoxic T cells and antibodies. For example, the increase of IL1β after 3 days post injection may provoke the acute phase proteins including complement factor C3 after 3 days post injection with CHO 1. The activation of T helper cells (CD4+) may also occur as there is a significant up-regulation of IFNγ and IL10 observed in pronephros and liver after 3 days post injection with CHO 1. The increase in IFNγ and IL 10 may also suggest an

effect on T cell proliferation and differentiation possibly via the effect of the immunostimulant (CHO 1) on antigen presenting cells (APCs) and the presentation of antigen by MHC II, leading to activation of T helper cells to proliferate and differentiate to Th1 (produce IFNγ)/ Th2 (produce IL10) (Faisal, 2015).

The biological variation in the gene expression increases in fish tissues due to the heterogeneous cell population. Therefore, the effect of specific treatment on particular cell type may be varied between the studied tissues depending on that cell type ratio in the tissues. That affects the level of any transcript in the RNA sample from that tissue, because the change is hidden by the expression of the gene in the unaffected cells. Also, the individual genotype might cause further variation in the expressed gene. This problem can be overcome through pooling the RNA from the individuals and increasing the experimental replicates (Logan et al., 2009).

The enhancement of humoral innate immune mechanism i.e. lysozyme and ACH50 was also observed after 24 h and 3 days respectively post injection with CHO 1 at 5 mg/kg of carp body weight. Complement and lysozyme mediate protection against pathogens via lysing cell wall peptidoglycan and promoting phagocytosis by direct activation of neutrophils and macrophages, or indirectly by opsonic effect (Saurabh and Sahoo, 2008).

The incorporation of an adjuvant in vaccine, enhances the cellular and humoral immune responses and affords better protection against the vaccine antigen. Antibody level was another humoral component that was enhanced when CHO 1 was incorporated as an adjuvant in *A. hydrophila* vaccine injected into carp. It is well established that adjuvants alone induce the innate immune responses and with vaccine antigens they increase the protection level against a range of pathogens (Jaafar et al., 2015). The combination of CHO 1 with the vaccine provoked the antibody level after 14 days till 28 days post injection and it was higher than the vaccine alone group at 21 days post injection. In

addition, there was no increase in antibody level detected at the CHO 1 alone injected fish. This could possibly be explained by the observations made by Li et al. (2013) when an enhancement of cytotoxic T cells and T helper 1 cells responses was recorded in mice injected with the combination of synthetic β-glucan and DNA vaccine, suggesting that the type and presence of antigens affected the immunomodulatory response of β-glucan that was used as an adjuvant.

The availability of adjuvant in vaccines to increase the uptake of the vaccine antigen by the APCs is thought to be an important aspect of adjuvant action in mammals (Smith et al., 2013). The adjuvant size determines the ability and speed to diffuse inside tissues and reach the immune organ. At sizes less than 40 nm transmission is more rapid than the large size adjuvant 100 nm such occurs in polysaccharides, which are transport from the injection site by dendritic cells to the immune organs (Smith et al., 2013) (see Figure 7-1). The CHO 1 might activate the MHC I and MHC II molecules due to the stimulation of the APCs and dendritic cells activation at the injection site thus leading to priming both of CD4+ and CD8+ T cells (Smith et al., 2013).

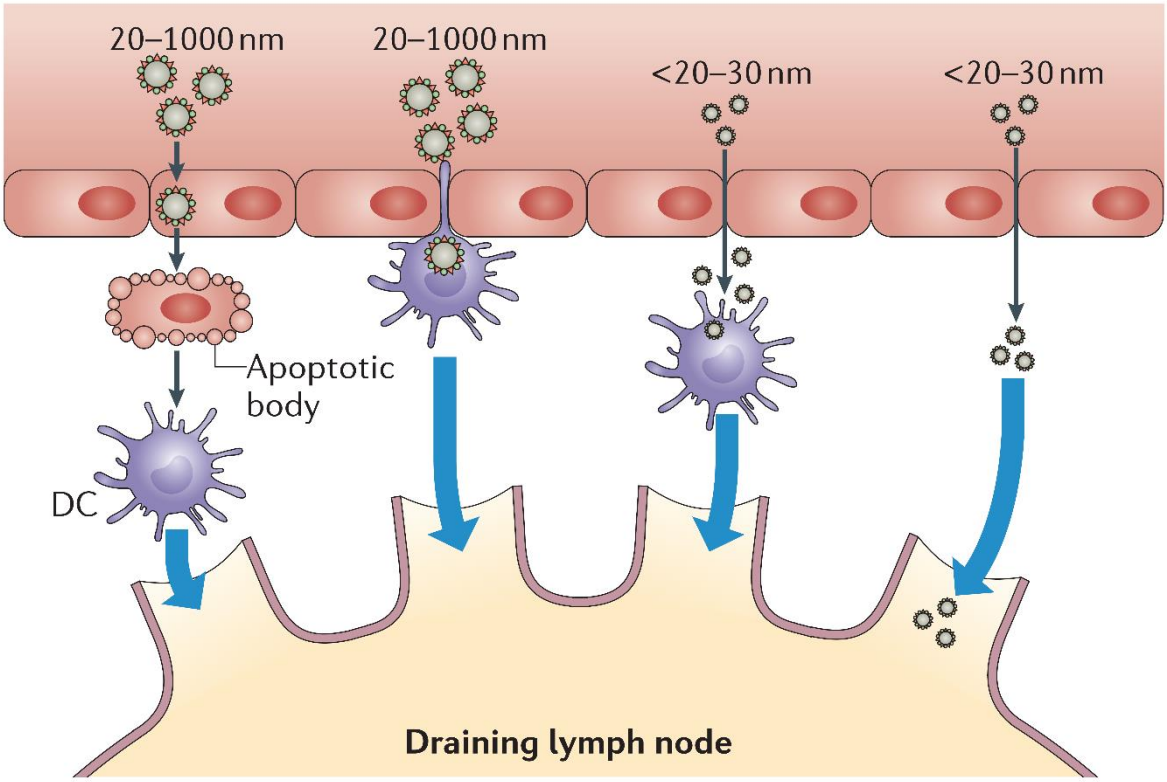


Figure 7-1: The nanoparticles stimulation mechanisms of the immune responses in mammals. The immunostimulatory activity of nanoscale materials has been attributed to diverse mechanisms: the delivery of antigens, including particle size-dependent tissue penetration and access to the lymphatics (Smith et al., 2013).

In Chapter 5, the sulphation of CHO 1 was confirmed successfully through utilising several analytical techniques such as FTIR spectra, ^{1}H NMR spectrum and double-quantum filtered (^{1}H-^{1}H DQF) COSY spectrum. The chemical shifts and differences between the analysis of tylose and CHO 1 revealed the replacement of sulphite group (SO_3^{-2}) at substitution position. However, the degree of substitution that is between 0-3 affects the valuable medicobiological properties such as antibacterial, anticoagulant, antitumor in the sulphated carbohydrates (Rakhmanova et al., 2009). When the degree of sulphation is high there is an increase in the anticoagulant activity of the modified carbohydrate, therefore, highly sulphated carbohydrates such as Dextran (degree of sulphation was 5.25 moles/µg

of disaccharides) has a high anticoagulant activity in blood (Yoshida et al., 1995) However, the degree of CHO 1 sulphation was 1.74 moles/µg of disaccharides that showed the sulphation was enough to induce an important biological activities without causing side-effect (e.g. mortality) when used in carp.

The CHO 1 was able to trigger several protection mechanisms i.e. the respiratory burst activity, increase leukocytes cells count, serum alternative complement pathway and lysozyme level, production of inflammatory cytokines and chemokines, the gene expression of anti-viral proteins, antibacterial activity and complement system activator, and initiates the development of adaptive immunity through antibody production. Therefore, these immune responses elucidate the signalling pathways involved in CHO 1 recognition in carp. The pattern recognition receptors (PRRs) that are involved in β-glucans recognition include Toll-like receptors TLRs, Dectin-1 (β-glucan receptor (βGR)), mannose receptor, complement receptors CR3, scavenger receptors and lactosylceramide (Gantner et al., 2003, Herre et al., 2004). The variation of glucan structure and the associated recognition by receptors is thought to be linked with their differences in structure, source, molecular weight and solubility hence different forms do not induce the immune response equally (Li et al., 2013). In addition, the association of the NF-κB pathway with various innate immune responses such as the enhancement of neutrophil oxidative burst and antimicrobial functions (Meena et al., 2012) and its important role in inflammation, immune response, apoptosis, cell growth, and differentiation (Correa et al., 2004) suggests that CHO 1 involvement in NF-κB activation and cytokine production. However, the involvement of CHO 1 in the cell signalling pathways requires further investigation.

In conclusion, the results presented in this thesis make a significant contribution to the development of the use of carbohydrates as immunostimulants in fish. This represents the

first attempt to combine synthetic biochemical approaches with carbohydrate design to produce a novel carbohydrate that modulates the immune system at the cellular, humoral and molecular level. The potential use of this modified carbohydrate and the concept of producing synthetic carbohydrates with clearly designed structures that maximise their role as adjuvants for vaccine development or as an immunostimulant in anti-infection therapies have been established. These studies have industrial implications and Innovation Impact funding has been awarded by Keele University to develop the concept further. The expression of a range of immune gene markers in the head kidney organ will be analysed from the last vaccination trial detailed in Section 6.2.2. These genes include: T cell Marker (CD4, CD8β), T helper cell markers (IL12, IFNγ for T helper 1 and IL4, IL1β for T helper 2) and B cells marker (IgM). Following on, the next step will be to produce different structural forms of this formulated carbohydrate CHO 1 and expand the formulation process through controlling the degree of sulphation and the position of the sulphite group on the carbohydrate molecules. Further experiments are also required to take this concept forward to commercialisation, such as dose response determination and evaluation of the adjuvant potency in a challenge study to determine the protection level against *Aeromonas hydrophila*. The technology described here can be applied in the future to other important bacterial diseases in the aquaculture industry.

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Appendixes

1. Shows the composition of the basic feed provided by Tetra GmbH

Feed Formula	%
Fish Protein Concentrate	45.000
Wheat starch	41.000
Cellulose	2.565
Soybean oil	4.500
Fish oil	4.500
Ethoxyquin	0.015
Vitamin-Premix	0.250
Stabilized Vitamin C	0.110
Mineral-Premix	2.060
TOTAL	100.00

2. RNALater composition

To make up a 1 L of RNA later:

1. 935 ml of autoclaved distilled water.
2. 700 g Ammonium sulphate (Sigma, A4418).

Stir on a hot plate stirrer on low heat until it dissolved

3. 25 ml of 1 M sodium citrate (Sigma, S 1804).
4. 40 ml of 0.5M EDTA (Sigma, E 5134).
5. Adjust to pH 5.2 using H_2SO_4 (Sigma, 339741) (about 20 drop= 1 ml).
6. Store at 19±1℃.

3. 10x TAE buffer

To make up a one litter of 10x TAE buffer:

1. 48.4 g Tris Base (Sigma, T8524)
2. 11.4 ml glacial acetic acid (Sigma, 695092)
3. 3.7 g of EDTA (Sigma, E 5134), disodium salt

Dissolve the above component in 800 ml of deionized water then bringing the final volume up to 1 L.

4. Sulphate determination assay with sodium rhodizonate

- $BaCl_2$ buffer:

1. 5ml 2M acetic acid (fisher A/0400/PB17 lot 1156637)
2. 1ml 5mM $BaCl_2$ (Sigma 342920 lot BP979OV)
3. 4ml 20mM $NaHCO_3$ (Fisher S/4240/60 lot 1354921)

Make up to 50ml with absolute ethanol.

- Sodium rhodizonate

1. 2.5mg sodium rhodizonate (Acros 132340050 lot A0353258) in 10ml water
2. 50mg L-ascorbic acid (Acros 105021000 lot A0353343)

Dissolve fully and make up to 50ml with absolute ethanol.

Can be used after 30 min and is stable for almost 2 days max.

4. Alternative complement activity assay

- GV buffer

1. 0.5 g Gelatin (Sigma, 48723)
2. 4.25 g Sodium chloride NaCl (Sigma, S9888)
3. 0.288 g Barbiton (5.5 diethylbarbituric acide) (Sigma, B0375)
4. 0.188 g Sodium barbiton (5,5-Diethylbarbituric acid sodium) (Sigma, B0500)
5. 0.051 g Magnesium chloride $MgCl_2$ (Sigma, M8266)
6. 0.016 g Calcium chloride $CaCl_2$ (Sigma, C1016)

Dissolved in 500 ml of distilled water, stir on a hot plate stirrer until it dissolved

- GV buffer +EGTA mM

Prepare from the GV buffer stock solution

1. 0.19g EGTA (Sigma, 03780)
2. 50 ml of GV buffer, stir on a hot plate stirrer until it dissolved

5. Antibody titre against *A. hydrophila* (ELISA assay)

- Antibody buffer

Add 1 g of BSA (Sigma, 05470) to 100 ml of PBS (i.e. 1 % BSA solution)

- Conjugate buffer

Add 1g of BSA to 100 ml of low salt wash buffer

- Substrate buffer (Sodium acetate/ citric acid buffer)

21.0 g Citric acid (Timstar laboratory, CI2132)

8.2 g Sodium acetate (Sigma, S 8750)

Dissolved in one litre of distilled water. Adjusted to pH 5.4 with 1 M NaOH. Add 5µl of H_2O_2 to 15 ml substrate buffer

- Stop reagent

2M H_2SO_4 in distilled water (Fisher Scientific, 10558620).

- Coating buffer (carbonate-bicarbonate solution)

1.56 g Sodium carbonate Na_2CO_3 (Sigma, S2127)

2.93 g Sodium bicarbonate $NaHCO_3$ (Sigma, S6014)

Dissolved in one litre of distilled water. Adjust to pH 9.6

Prepare fresh coating buffer on each occasion

- Phosphate buffer saline PBS

0.02M phosphate, 0.15M NaCl

0.876 g Sodium dihydrogen phosphate $NaH_2PO_4.2H_2O$ (Sigma, S8750)

2.56 g Disodium hydrogen phosphate dihydrate, $Na_2HPO_4.2H_2O$ (BDH, 102494)

8.77 g Sodium chloride NaCl (Sigma, S9888)

Dissolved in one litre of distilled water. Adjust to pH 7.3 with concentrated HCl.

- Wash buffer (X10) low salt

24.2 g Trisma base (Sigma, T8524)

222.2 g Sodium chloride NaCl (Sigma, S9888)

1 g Merthiolate (Thimerosal) (Sigma, T5125)

5 ml Tween 20 (Sigma, P1379)

Dissolved in one litre of distilled water. Adjust to pH 7.3 with concentrated HCl.

- Wash buffer (X10) high salt

24.2 g Trisma base (Sigma, T8524)

292.2 g Sodium chloride NaCl (Sigma, S9888)

1 g Merthiolate (Thimerosal) (Sigma, T5125)

20 ml Tween 20 (Sigma, P1379)

Dissolved in one litre of distilled water. Adjust to pH 7.7 with concentrated HCl.