The influence of beta-glucans on the relationship between carp (*Cyprinus carpio*) and its associated microbiome

Sarah Jane Harris

Thesis submitted for the degree of Doctor of Philosophy

March 2017

Keele University

Keele University

Declaration Part 1. To be bound in the thesis

SUBMISSION OF THESIS FOR A RESEARCH DEGREE

Part I. DECLARATION by the candidate for a research degree. To be bound in the thesis

Degree for which thesis being submitted **Doctor of Philosophy**

Title of thesis **The influence of beta-glucans on the relationship between carp (*Cyprinus carpio*) and its associated microbiome.**

This thesis contains confidential information and is subject to the protocol set down for the submission and examination of such a thesis.

YES

Date of submission **29th Aug 2016** Original registration date **24th Sept. 2012**
(Date of submission must comply with Regulation 2D)

Name of candidate **Sarah Jane Harris**

Research Institute **Faculty of Natural Sciences**

Name of Lead Supervisor **Professor David Hoole**

I certify that:

(a) The thesis being submitted for examination is my own account of my own research
(b) My research has been conducted ethically. Where relevant a letter from the approving body confirming that ethical approval has been given has been bound in the thesis as an Annex
(c) The data and results presented are the genuine data and results actually obtained by me during the conduct of the research
(d) Where I have drawn on the work, ideas and results of others this has been appropriately acknowledged in the thesis
(e) Where any collaboration has taken place with one or more other researchers, I have included within an 'Acknowledgments' section in the thesis a clear statement of their contributions, in line with the relevant statement in the Code of Practice (see Note overleaf).
(f) The greater portion of the work described in the thesis has been undertaken subsequent to my registration for the higher degree for which I am submitting for examination
(g) Where part of the work described in the thesis has previously been incorporated in another thesis submitted by me for a higher degree (if any), this has been identified and acknowledged in the thesis
(h) The thesis submitted is within the required word limit as specified in the Regulations

Total words in submitted thesis (including text and footnotes, but excluding references and appendices) **50,000**

Signature of candidate……Date ……

Note
Extract from Code of Practice: If the research degree is set within a broader programme of work involving a group of investigators – particularly if this programme of work predates the candidate's registration – the candidate should provide an explicit statement (in an 'Acknowledgments' section) of the respective roles of the candidate and these other individuals in relevant aspects of the work reported in the thesis. For example, it should make clear, where relevant, the candidate's role in designing the study, developing data collection instruments, collecting primary data, analysing such data, and formulating conclusions from the analysis. Others involved in these aspects of the research should be named, and their contributions relative to that of the candidate should be specified (*this does not apply to the ordinary supervision, only if the supervisor or supervisory team has had greater than usual involvement*).

Abstract.

Fish are in contact with microbiota from the moment of hatching. Exterior organs, i.e. skin, gills and intestinal system, are colonised by commensal bacteria populations and a symbiotic relationship is formed. The fish provides a niche and nutrients for the bacteria which stimulate development of the immune response, act as an additional barrier against invading pathogens and, within the gut, aid in digestion.

β-glucans are used within aquaculture as a means of improving fish health and can be applied in various forms, e.g. via diet or injection. Whilst the application of β-glucan is performed to modulate a fish's immune system, it has also been shown to affect the gut microbiota population at concentrations above 1% w/w within the diet which is particularly important to consider when applied orally.

The effect of the commercially available β-glucan MacroGard® upon the gut of common carp (*Cyprinus carpio*) is studied after oral application and injection. Whilst feeding with MacroGard® at 0.1% w/w within the diet does not influence the gut bacteria nor expression of bactericidal innate immune genes, injection (2mg kg^{-1} and 5mg kg^{-1}) resulted in a 90% reduction in bacteria numbers in the gut after 24 hours. Injection of MacroGard® did not significantly alter the expression of CRP, iNOS, bf/C2, IL-1β, ApoA1, HAMP1, LEAP2 and Muc2 within the gut however MSS1, a synthesised β-glucan, significantly increased the gene expression of iNOS, CRP and Muc2. 0.1% MacroGard® in the diet was, however, capable of influencing bacterial species diversity when injection was also performed. This revealed a high proportion of Alphaproteobacteria, which are typically associated with plants rather than gut

systems, and corresponded with a reduction in potential pathogenic bacteria. This showed combining injection and oral application of MacroGard® together is capable of influencing the gut microbiota population within a 2 week period.

Table of Contents.

List of Figures.

List of Tables.

List of abbreviations.

°C: Degrees Celsius.

A: Adenine.

ACP: Alternative Complement Pathway.

AMPs: Antimicrobial peptides.

ApoA: Apolipoprotein.

APP: Acute Phase Protein.

APS: Ammonium persulfate.

AXOS: arabinoxylanoligosaccharides.

BLAST: Basic Local Alignment Search Tool.

C: Cytosine

cDNA: Complementary DNA.

CEV: Carp edema virus.

CFU: Colony forming unit.

CR: Complement receptor.

CRP: C-reactive protein.

CyHV: Cyprinid herpesvirus.

dATP: Deoxyadenosine triphosphate.

dCTP: Deoxycytidine triphosphate.

DGGE: Denaturing Gradient Gel Electrophoresis.

dGTP: Deoxyguanosine triphosphate.

DNA: Deoxyribonucleic acid.

dNTP: Deoxyribonucleotide triphosphate.

dTTP: Deoxythymidine triphosphate.

DTU: Technical University of Denmark, Denmark

EDTA: Ethylenediaminetetraacetic acid.

ENA: European Nucleotide Archive.

FAO: Food and Agriculture Organisation.

FOS: Fructooligosaccharides.

G: Guanine.

genDNA: Genomic DNA.

GOS: Galactooligosaccharides.

HAMP: Hepcidin antimicrobial peptide.

IBD: Irritable bowel disease.

IL: Interleukin.

iNOS: Inducible nitric oxide synthase.

ISAV: Infectious salmon anemia virus.

ITN: International Training Network.

l: Litre.

LAB: Lactic acid bacteria.

LEAP: Liver expressed antimicrobial peptide.

LPS: Lipopolysaccharide.

m: Metre.

M: Mole.

$MgCl_2$: Magnesium Chloride.

MHUE: Miguel Hernandez University of Elche

M-MLV/MuLV: Moloney Murine Leukemia Virus.

MOS: Mannanoligosaccharides.

M.R.S. agar: de Man, Rogosa and Sharpe agar.

Muc: Mucin.

NCBI: National Centre for Biotechnology Information.

NCIMB: National Collection of Industrial and Marine Bacteria.

Nemo: Training network on protective immune modulation in warm water fish by feeding glucans.

nMDS: non metric Multidimensional Scaling ordination.

NO: Nitric oxide.

NOS: Nitric oxide synthase.

OTU: Operational Taxonomic Unit.

PBS: Phosphate Buffer Saline.

PCA: Principle Component Analysis.

PCR: Polymerase Chain Reaction.

PHP: PHP Hypertext Preprocessor.

P.I.: Post injection.

PKD: Proliferative Kidney Disease.

PRRs: Pattern Recognition Receptors.

rDNA: Ribosomal DNA.

RGB: Red Green Blue.

RNA: Riboxynucleic acid.

ROS: Reactive oxygen species.

RT: Reverse Transcriptase.

SA: Serum Amyloid.

SVCV: Spring viremia of carp virus.

T: Thymine.

TAE buffer: Tris-Acetate-EDTA buffer.

TBE buffer: Tris-Borate-EDTA buffer.

TE buffer: Tris EDTA buffer.

TEMED: Tetramethylethylenediamine.

T_H: T-helper cells.

TiHo: Tierärztliche Hochschule (University of Veterinary Medicine), Hanover, Germany.

TNF: Tumor Necrosis Factor.

Tris-HCl: Tris(hydroxymethyl)aminomethane-Hydrocloride.

TLRs: Toll Like Receptors.

UV light: Ultra violet light.

w/v: weight/volume.

w/w: weight/weight.

Acknowledgements.

Firstly, I would like to thank the Fisheries Society of the British Isles for funding my PhD. Without their support, none of this would have been possible.

Secondly, I need to thank the endless support I have had from Professors Dave Hoole and Dieter Steinhagen who have been my supervisors for the past 8 and 6 years respectively. They are both amazing scientists and people, and I have learnt so much under their guidance.

Thirdly, I want to thank all the other people in my life who have ensured I got this far. Their contributions range from simply listening to me whilst I get my thoughts in order to collaborations that allowed my experiments to happen. There are too many to list them all but I will name a few.

I want to thank all my friends from the EU ITN "Nemo". The idea behind my PhD came from my research whilst working with them and their support was paramount in getting my current studies off the ground. I especially want to thank Drs. Joanna Miest, Alberto Falco and Nicolas Pionnier whose work at Keele gave a solid base to my research, and to Dr. Mikolaj Adamek who continues to be both a giant pain and one of my best friends, spending about as much time destroying my work as he does inspiring me and helping me make sense of my data. Thanks to Dr. Dominika Przybylska-Diaz and Patrick Frost for letting me piggy back onto one of their trials which ended up being the basis for the majority of the work featured in my experimental chapters.

As Dave likes to remind me, I started off my PhD with an almost fear of stats so I need to thank Drs. Daniel Bray and Sarah Taylor, and Dave Hulse for helping me to find my footing along the road to becoming someone who gets genuinely excited about good experimental design and statistical analysis as that has been one of my favourite learning curves over the past 3 ½ years. I also wish to thank Dr. Andrew Rutherford and Dr. Paul Warren for their advice on the statistical analysis featured in Chapter 2.

It's become a running joke that if you wish to find the fault in a protocol or piece of kit, the best person to give it to is me so I absolutely have to thank every person who I have gone to when figuring out how to get things working again. I couldn't have done the majority of my work if you folks hadn't been there to help me figure it out. Thanks to all the other people at Keele who have helped me with everything from finding lightbulbs to looking after my fish and all the stuff in between. It's staggering to think how much of my research would probably have failed if it weren't for the help of some really amazing people.

Special thanks to Todd Specht for his ability to take the ideas in my head and translate them into usable tools that have helped me with my research. It is not a common skill to have so thank you for agreeing to collaborate with me and for deciding penguin is a better name than platypus.

Thanks to the guys down at Plymouth University, especially Drs. Dan Merrifield and Ana Rodiles, for their help analysing my samples with NGS and the subsequent data analysis.

My parents and family get an extra special thanks because they are awesome and have provided me with endless amounts of support over the years and continue to do so now.

Finally, I want to thank everyone else who has been integral to me making it through my PhD, especially towards the end. It is a challenge at the best of times but throw in all kinds of housing issues and an annoyingly oversensitive immune system and it becomes something you just flat out cannot do without support. So to everyone who has listened to me complain, stopped me from flailing, acted as a distraction, helped me with house related predicaments, supplied me with tissues for the constant sneezing, laughed with me, cried with me and generally been there for me; it was the best of times, it was the blurst of times. You are my bulwarks – don't ever change.

According to Dr. Jacob Schmidt, the Danish say, "Mange bække små gør en stor å" which he translated as, "many small streams form a big river". This sums up the past 3 1/2 years almost perfectly with many of my data looking utterly nonsensical and unrelated until the final few months in which everything came together in a beautiful crescendo. That being said, I much prefer the google translate offer as to what the phrase means: small hinges swing big doors. Less poetic but just as true in its sentiment.

Chapter 1 – Introduction.

Fish were the earliest vertebrates to evolve and have been present on Earth since the Cambrian explosion, around 540 million years ago. Adaptations to cope with different temperatures, pressures, salinities, diets, availability of light, water qualities and predator/prey dynamics have resulted in highly diverse evolutionary changes resulting in over 40,000 fish species which represents approximately half of all vertebrates on the planet. Fish are prominent in religion, mythology and pop culture, and have a growing economic importance as both a recreational commodity and food source.

Sports fishing represents a huge economic market to many countries, for example the American Sportfishing Association highlighted that $115 billion was spent on recreational fishing in the U.S. in 2011 including equipment and associated touristic costs such as travel, accommodation and spending in local economies (American Sportfishing Association, 2013). In the UK, sport fishing represents a £3 billion industry that is regularly threatened by illegal fish imports to meet the demand for large fish that are not cost effective to produce in the UK's climate (Mewett, 2015). Although public aquaria and marine parks are often maintained as non-profit organisations, their educational value has a huge impact with several, for example Georgia Aquarium and the Sea Life chain (part of Merlin Entertainments), running conservation programs, in addition to simply educating the public on fish health and the health of the seas and oceans (www.georgiaaquarium.org/conserve and www.sealifetrust.org respectively).

The global trade of fish is of vast importance to the economic output in a range of countries including the UK and the USA. The Food and Agriculture Organization of the United Nations report in 2014 stated that approximately 10% of agricultural trade and 1% of all trade worldwide was associated with fish. Aquaculture produced 90.4 million tonnes of fish in 2012 with an economic value of US$144.4 billion, whilst capture fisheries in 2011 generated 93.7 million tonnes of produce which is the second highest ever tonnage recorded (FAO, 2014). Fish are therefore an important and growing source of food highlighted by the fact that in 2010, 16.7% of protein consumed by humans across the globe came from fish (FAO, 2014). Indeed, the Earth Policy Institute (2013) reported that in 2011, the production of meat from beef was overtaken by that produced by fish farms. The per capita consumption of fish globally has almost doubled in the past 50 years (9.9kg to 19.2kg. FAO, 2014) and aquaculture is becoming of increasing importance in meeting the demands to produce a sustainable source of nutrients in the human diet (Figure 1.1). Not only does aquaculture help meet these food demands, it also elevates some of the pressures on wild populations caused by overfishing, enabling stocks to recover. For example the Atlantic tuna (*Thunnus thynnus*), a common fish species farmed for use in sushi and sashimi dishes, currently has a rating of endangered from the International Union for Conservation of Nature and Natural Resources (http://www.iucnredlist.org/details/21860/0) and the European eel (*Anguilla anguilla*), a key ingredient in the English dish of jellied eels, is critically endangered (http://www.iucnredlist.org/details/60344/0). Their red list status, however, has not necessarily translated to human views of fish. TV shows such as the BBC's *MasterChef* have, in the past, received criticism for their use of eels as a main

component of dishes regardless of their endangered status (The Guardian, 1st November 2009).

Figure 1.1: Image taken from the 2014 report published by the FAO: the state of world fisheries and aquaculture. The graph shows the increase in fish production (in million tonnes) over time for both aquaculture (dark blue at the top of the graph) and capture fishing (pale blue at the bottom of the graph). As can be seen, capture production plateaued in the early 90's with aquaculture steadily increasing over the 60 year period shown.

Overfishing can also have a severe impact on human communities in addition to environmental ramifications. Approximately 12% of the world's human population depends on fish for their survival (FA0, 2012). In 2012, it was estimated that over 58 million people worked in the primary sector of fisheries/aquaculture, however this is not uniformly distributed with 84% of these people living in Asia (FAO, 2014). Overfishing has resulted in events such as the crash of the Canadian cod (*Gadus morhua*) population in 1992 where it was reported that 40,000 people lost their jobs in towns along the coast in Newfoundland as a consequence (BBC news, 16th

December 2002). Although over the last 20 years a series of legislative conditions have been introduced, such as maximum fishing limits, the potential impact on smaller communities that depend heavily, if not solely, on capture fishing could still be devastating.

The increase in aquaculture helps to relieve the pressure on capture fishing and reduces the potential for overfishing, however both are equally susceptible to other factors such as global warming, disasters and disease. The increasing international trade of fish is resulting in easier routes for pathogens to travel around the globe. For example, Koi Sleepy Disease, caused by the carp edema virus (CEV), has recently (2014) been detected in koi (*Cyprinus carpio koi*) in Germany and other parts of Europe where it had previously been limited to fish in Japan (Jung-Schroers *et al.* 2015b, Lewisch *et al.* 2015). Mardones *et al.* (2014) cite both local transmission and long distance movement as influencing factors of the infectious salmon anaemia virus (ISAV) epidemic in Chile (2007-2009) in Atlantic salmon, and the spring viremia of carp virus (SVCV) causes mortalities and financial losses globally. The UK, however, has had a SVCV free status since 2010. Taylor *et al.* (2013) describe the pattern of infection in the UK prior to this as a result of international trading rather than an endemic infection spread locally.

Monoculture, often utilised in aquaculture as the preferred practice for fish production, facilitates an optimal environment for the spread of disease. High densities of a single species population that is susceptible to a particular disease can result in significant mortality rates and economic losses. Vaccination programs and the use of antibiotics are employed as means to avoid such catastrophes, however the liberal use of antibiotics and antimicrobial agents throughout aquaculture, agriculture

and for treating human disease have led to a massive increase in drug resistance amongst pathogens (Who, Fact sheet No. 194). The introduction of various legislative policies, such as Regulation (EC) No 1831/2003 which banned the use of antibiotics as growth promotors in animal feeds, restricts the use of a range of chemicals in fish production and as a consequence, a need for alternatives has arisen.

To reduce the dependency on antibiotics and antimicrobials in agriculture, including aquaculture, there is increasing interest in proactively improving the health of animal stocks by increasing resistance to pathogens. Two such approaches include the modulation of the gut microflora populations so that they contain more "good" bacteria species that are beneficial to their host, and the improvement of the animal's ability to defend against pathogenic attack by modulating its immune response prior to possible infection. This thesis studies the effect of β-glucan, a known immunomodulant also capable of influencing the gut microbiota population (Kuhlwein *et al.* 2013, Jung-Schroers *et al.* 2015a), on the health of common carp (*Cyprinus carpio*) in disease free conditions.

1.2 – The importance of the microbiome.

By cell number, humans are actually only 10% *Homo sapiens* (Candela *et al.* 2012) with the remaining 90% being composed of the human microbiome – a collection of commensal bacteria and other microbes that, under normal conditions, live in harmony with us. Whilst it has been known since the 19th century that the microbial population is important for human health (Falk *et al.* 1998), the technology with which to fully study it has only recently become available. Analysis of the

microbiome has become a major line of research with several large scale projects such as the Human Microbiome Project (www.hmpdaac.org), being funded in order to better understand the relationship between host and microbe and how this influences health and incidents of disease. The relationship between a host and its microbiome is symbiotic in nature and begins at birth/hatching (Nayak 2010b). The host provides an environment for the bacteria to live and supplies nutrients, whilst the bacteria play roles in development of the immune system, provides an additional barrier against pathogens and, within the gut, aids in digestion (Nayak 2010a). Studies with gnotobiotic mammals have shown the microbiota is essential for the formation of gut associated lymphoid tissue (GALT) and Immunoglobulin (Ig) A production (Rhee *et al.* 2004, Peterson *et al.* 2007, Nayak 2010a). In germ free zebrafish (*Danio rerio*), a lack of gut epithelium maturation was noted the re-induction of a microbiota population stimulates epithelial growth and maturation (Bates *et al.* 2006). In their study comparing the effects of diet upon gut microbiota populations in rainbow trout (*Oncorhynchus mykiss*) during *Yersinia ruckeri* infection, (Ingerslev *et al.* 2014b) concluded that a "plant diet gave rise to a prebiotic effect favouring the presence of bacterial taxons proving protective in connection to bath challenge by *Y. ruckeri*" highlighting the role of gut microbiota in disease prevention. In his review, Nayak (2010a) stated that the gut microbiota could supply several digestive enzymes including carbohydrases, phosphatases, lipases and proteases, in addition to synthesising essential vitamins and amino acids. Bacterial strains isolated from the gut have been shown to have antibacterial capabilities against pathogenic bacteria and to be a protective barrier against disease (Romero *et al.* 2014). Sugita *et al.* (1998) showed 2.7% of isolates cultured from the gut of Japanese coastal fish

inhibited the growth of *Vibrio vulnificus*, and bacteria species isolated from the gut have also shown antibacterial activity towards multiple *Aeromonas* sp. (Sugita *et al.* 1996). Even if the gut microbiota were to perform only one of these roles, it would still constitute a major asset to a host organism and is clearly vital to survival.

The gut microbiota is however a complex ecosystem that can be divided into two distinct populations: the autochthonous and allochthonous bacteria. The allochthonous bacteria are those that simply pass through the intestinal tract, i.e. the bacteria within the faecal matter, whilst the autochthonous bacteria are those that adhere to the mucosal layer and are considered as "permanent" residents of the intestine (Romero *et al.* 2014).

As previously stated, it has been known since the 1800's that the symbiotic microbiota population is vital to survival (Falk *et al.* 1998), however it is only recently that the technology has become available that allows a full exploration of large microbial communities, and, even more recently, the relationship with the immune response (Gomez and Balcazar 2008, Lazado and Caipang 2014). The gut is constantly exposed to signals from both the commensal microbial population and potentially pathogenic bacteria that exist within the environment. There must therefore be a balance between activation of the immune response and the presence of microbes within the gut, however the study of this balance using ichthyological models is still in its infancy.

Whilst the number of publications considering both immunity and microbiota populations within fish is on the increase, analysis of any relationship between these two components is often missing. Dawood *et al.* (2016) noted an increase in total gut

bacteria population size after feeding red sea bream with the probiotics *Lactobacillus rhamnosus* and *Lactobacillus lactis* in conjunction with a significant increase in alternative complement pathway activity, serum bactericidal activity, mucus lysozyme activity, peroxidase activity and superoxide dismutase production in blood serum. Similarly, Miest *et al.* (2016) observed differences in both the gut microbiota population and immune responses in whole turbot larvae after feeding with MacroGard®, a β-glucan product with immunomodulatory properties. In contrast, however, where Akrami *et al.* (2015) noted a significant increase in leucocyte counts and haemoglobin concentration in juvenile beluga (*Huso huso*) fed with a combination of *Enterococcus faecium* and fructooligosaccharides (FOS), they did not see any differences in lactic acid bacteria (LAB) counts. It is therefore unlikely that there is a functional relationship between leucocyte counts and haemoglobin concentration, and LAB within the gut.

Whilst it is important to look at the overall health of a fish when considering microbiota populations, i.e. any effects on systemic immunity, it is equally important to consider the effects upon local immunity, i.e. gut immunity. Ingerslev *et al.* (2014b) analysed both the gut microbiota and the expression of innate immune genes, including IL-1β and C3, in the gut tissue of rainbow trout and observed that the feeding of a plant based diet, in comparison to a marine based diet, resulted in a lower incidence of infection. Expression of both innate immune genes and stress markers in the gut of hybrid tilapia was studied alongside gut microbiota composition and it was shown that feeding with soybean diet resulted in a decrease in IL-1β expression alongside changes in the gut microbiota population (Zhang *et al.* 2014). What these previous five studies all have in common, however, is a lack of comparison between

the immune parameters analysed and the gut microbiota.

In contrast, Tapia-Paniagua *et al.* (2015) use Principle Component Analysis (PCA) to reveal a relationship between intestinal microbiota populations and expression of CASPASE-6 and NAPDH oxidase in the liver of Senegalese sole (*Solea senegalensis*). To date, this is the only published example of a statistical comparison between the host immune response and the whole gut microbiota population in an ichthyological model, however there are examples of correlation analysis being performed between the immune response and specific pathogens. Pearson's correlation was employed by Gorgoglione *et al.* (2013) to determine if there was a relationship between the presence of *Tetracapsuloides bryosalmonae*, the causative agent of Proliferative Kidney Disease (PKD) in rainbow trout, and the immune status which showed B-cell and antibody response to be linked to PKD pathogenesis. The same statistical means were used to study chemokines in brown trout (*Salmo trutta*) after infection with either *Yersinia ruckeri* or Viral Haemorrhagic Septicaemia Virus (Gorgoglione *et al.* 2016). This showed a predominant trend of significant positive correlations between novel chemokine expression in the kidney and spleen, and both bacterial and viral pathogen loads indicating their role in a pro-inflammatory systemic immune response.

1.3 – Manipulation of the intestinal microbiome.

Manipulation of the gut microbiota population in a way that is beneficial to the host organism is typically performed using pre and probiotics. Prebiotics are oligosaccharides that positively influence the growth and/or activity of "good"

bacteria within the gut (Merrifield *et al.* 2010). Gibson *et al.* (2004) additionally stated that a prebiotic must resist gastric acidity and absorption, and be fermented by the intestinal microbiota. Examples of prebiotics utilised within aquaculture include mannanoligosaccharides (MOS), galactooligosaccharides (GOS) and inulin (Merrifield *et al.* 2010). Probiotics, however, are live bacteria introduced into a population in order to alter the existing population in favour of "good" bacteria. Merrifield *et al.* (2010) highlighted that whilst the textbook definitions of a probiotic, such as that given by Fuller (1989), were written considering mammals and not fish, the differences in typical microbiomes between mammals and fish are great enough that a separate definition as to the characteristics a fish probiotic should be considered and should be different to that of a mammalian probiotic. These characteristics include, but are not limited to, a lack of pathogenicity towards humans and other animals within the local environment, must not contain any plasmids that comprise of antibiotic resistance genes so that transfer to potential pathogens does not occur, must be tolerant of bile salts and low pH as found within the gut, and must be able to thrive within intestinal mucus (Spanggaard *et al.* 2001, Merrifield *et al.* 2010). Balcazar *et al.* (2006) outlined a selection process for how best to identify and test potential probiotics before commercial use.

The manipulation of the gut microbiota has become increasingly popular in aquaculture. A literature search within Web of Science using the key words "gut microbiota fish" identifies 70 research articles, 8 reviews and 1 book that have been published since 2012 (Table 1.1). Between 2000 and 2014, Ringo *et al.* (2014) referenced 53 probiotic trials including research with the commercially important fish species Japanese flounder (*Paralichthys olivaceus*), common carp, tilapia

(*Oreochromis* sp.), grouper (*Epinephelus coioides*), rainbow trout, brown trout, cod, turbot (*Scophthalmus maximus*), sea bass (*Dicentrarchus labrax*), and pollock (*Pollachius* sp.). These authors also noted that LAB are considered to be good potential probiotics with the majority of trials using species of *Lactobacillus* and *Enterococcus*. They are present in multiple fish species naturally (Ringo and Gatesoupe 1998) and feeding with *Lactobacillus* sp. as a probiotic has shown increased body weight and decreased cortisol levels in sea bass and gilthead sea bream larvae (*Sparus aurata*) (Abelli *et al.* 2009), increase the proportion of Firmicutes within the gut of zebrafish (Falcinelli *et al.* 2016), and increase complement and lysozyme activity in red sea bream (*Pagrus major*) (Dawood *et al.* 2016). Several probiotics have been shown to act against different bacterial pathogens, for example *Bacillus* sp. has shown protective abilities against *Aeromonas hydrophila, Edwardsiella ictaluri, Vibrio harveyi* and *Yersinia ruckeri*, and *Enterococcus* sp. has been noted to defend against *E. tarda, V. harveyi* and *V. parahaemolyticus* (Akhter *et al.* 2015).

Table 1.1: (part A) A literature search using the engine Web of Science and the key words "gut microbiota fish" was performed to identify publications studying the effect of probiotics, prebiotics, antibiotics or carbohydrates on the gut microbiota in different fish. The numbers of different publication types by year are given followed by examples of publications over the past 2 years (2014-2016). References are either given alongside details of the trial performed or are included in a list at the end of the table.

Article type	2016 *	2015	2014	2013	2012
Research paper	6	17	11	23	13
Review	1	1	4	1	1
Books			1		
Conference abstract		1	1		
Total	7	19	17	24	14

Fish species	Modulators	Reference
Asian seabass (*Lates calcarifer*)	Hydrolysed wheat gluten	Apper *et al.* 2016
Atlantic salmon (*Salmo salar*)	Fish meal versus pea protein, soy protein, extracted sunflower, hydrolyzed feather meal or poultry biproduct	Hartviksen *et al.* 2014
Beluga (*Huso huso*)	Biomin IMBO	Akrami *et al.* 2015
Black carp (*Mylopharyngodon piceus*)	Intestinal casing meal and yeast of monosodium glutamate	Li *et al.* 2015
Blunt snout bream (*Megalobrama amblycephala*)		
Common carp (*Cyprinus carpio*)	Fructooligosaccharide	Hoseinifar *et al.* 2014
	Mannanoligosaccharide	Momeni-Moghaddam *et al.* 2015
Fathead minnow (*Pimephales promelas*)	Triclosan	Narrowe *et al.* 2015
Gibel carp (*Carassius gibelio*)	Intestinal casing meal and yeast of monosodium glutamate	Li *et al.* 2015
Gilthead seabream (*Sparus aurata*)	Fish meal versus vegetable meal	Estruch *et al.* 2015
	S. putrifaciens	Cordero *et al.* 2015
	Short chain fructooligosaccharides	Guerreiro *et al.* 2016
Goldfish (*Carassius auratus*)	Pentachlorophenol	Kan *et al.* 2015
Grouper (*Epinephelus coioides*)	*B. pumilus*	Yang *et al.* 2014
	Psychrobacter sp.	Sun *et al.* 2014
Hybrid tilapia (*Oreochromis niloticus* ♀ X *O. aureus* ♂)	Completely hydrolyzed feather meal (tradename Aoyouyuan-A)	Zhang *et al.* 2014

Table 1.2: (part B) A literature search using the engine Web of Science and the key words "gut microbiota fish" was performed to identify publications studying the effect of probiotics, prebiotics, antibiotics or carbohydrates on the gut microbiota in different fish species. The number of different publication types by year is given followed by examples of publications over the past 2 years (2014-2016). References are either given alongside details of the trial performed or are included in a list at the end of the table.

Fish species	Modulators	Reference
Jundia (*Rhamdia quelen*)	Multiple carbohydrates: ground corn, wheat, cassava bagasse, broken rice	Pedrotti *et al.* 2015
Rainbow trout (*Oncorhynchus mykiss*)	*Aeromonas* sp., *Bacillus* sp., *C. braakii*, mix of all three	Koca *et al.* 2015
	Different protein: carbohydrate ratios	Geurden *et al.* 2014
	Kocuria and *Rhodococcus* sp.	Sharifuzzaman *et al.* 2014
	Plant based versus marine diet	Ingerslev *et al.* 2014a, Ingerslev *et al.* 2014b
Red sea bream (*Pagrus major*)	*L. rhamnosus* and/or *L. lactis*	Dawood and Koshio 2016
Schizothorax prenanti	Oxidized konjac glucomannan	Zheng *et al.* 2015
Sea bass (*Dicentrarchus labrax*)	β-glucans and essential oil	Carda-Dieguez *et al.* 2014
	Carbohydrates: amylopectin versus high amylose versus fibre	Gatesoupe *et al.* 2014
	Protein hydrolysate	Delcroix *et al.* 2015
Senegalese sole (*Solea senegalensis*)	*Shewanella putrefaciens*	Lobo *et al.* 2014, Tapia-Paniagua *et al.* 2014)
Tilapia (*O. mossambicus*)	Azomite	Musthafa *et al.* 2016
Tilapia (*O. niloticus*)	AquaStar® Growout (*L. reuteri, B. subtilis, E. faecium, P. acidilactici*)	Standen *et al.* 2015, Standen *et al.* 2016
	Baker's yeast	Ran *et al.* 2015
	GroBiotic-A	Peredo *et al.* 2015
	Multiple carbohydrates: ground corn, wheat, cassava bagasse, broken rice	Pedrotti *et al.* 2015
Zebrafish (*Danio rerio*)	*L. rhamnosus*	Falcinelli *et al.* 2015, Falcinelli *et al.* 2016
	N-acyl homoserin lactonase	Cao *et al.* 2014
	Wheat products	Savarese *et al.* 2014

*Jan-March. Additional references counted in literature search: Askarian *et al.* 2012, Cerezuela *et al.* 2012, Desai *et al.* 2012, Geraylou *et al.* 2012, Giannenas *et al.* 2012, He *et al.* 2012, Liu *et al.* 2012, Omar *et al.* 2012, Raggi and Gatlin 2012, Rendueles *et al.* 2012, Sun *et al.* 2012a, Sun *et al.* 2012b, Yang *et al.* 2012, Abid *et al.* 2013, Askarian *et al.* 2013, Bakke *et al.* 2013, Cerezuela *et al.* 2013a, Cerezuela *et al.* 2013b, Del'Duca *et al.* 2013, Geraylou *et al.* 2013a, Geraylou *et al.* 2013b, Gisbert *et al.* 2013, Green *et al.* 2013, He *et al.* 2013a, He *et al.* 2013b, Jaafar *et al.* 2013, Kuhlwein *et al.* 2013, Merrifield *et al.* 2013, Navarrete *et al.* 2013, Nikapitiya 2013, Ramos *et al.* 2013, Respondek *et al.* 2013, Sun *et al.* 2013a, Sun *et al.* 2013b, Zhou *et al.* 2013a, Zhou *et al.* 2013b, Zychowski *et al.* 2013, Merrifield *et al.* 2014, De *et al.* 2014, Lazado and Caipang 2014, Llewellyn *et al.* 2014, Ringo *et al.* 2014, Ghanbari *et al.* 2015, Dawood and Koshio 2016.

There are fewer publications available on the action of prebiotics in comparison to probiotics (Akhter *et al.* 2015), however there are several products, such as β-glucan, that are widely studied yet do not meet all the criteria to be considered a prebiotic. The effect of orally applied FOS and MOS has been shown to increase lysozyme activity in Japanese flounder (Ye *et al.* 2011), and red drum (*Sciaenops ocellatus*) are similarly affected after feeding with inulin and GOS (Zhou *et al.* 2010). Complement activity has also been shown to be increased in gilthead seabream fed with inulin (Cerezuela *et al.* 2013), black amur bream (*Megalobrama terminalis*) fed with FOS (Zhang *et al.* 2015), and Siberian sturgeon (*Acipenser baerii*) fed with arabinoxylanoligosaccharides (AXOS) (Geraylou *et al.* 2013a). FOS has been shown to increase LAB in the gut of carp (Hoseinifar *et al.* 2014) and the relative abundance of *Lactobacillus* sp. and *Lactococcus lactis* increased in the gut of Siberian sturgeon after feeding with AXOS (Geraylou *et al.* 2013b). In gilthead sea bream there was a higher level of bacterial species richness in the gut after the fish were fed with MOS (Dimitroglou *et al.* 2010) however, in contrast in lobsters (*Homarus gammarus*) a decrease in bacterial species richness occurred in the gut after feeding with a combination of MOS and *Bacillus* sp. (Daniels *et al.* 2013).

1.4 – Host immunomodulators: what is a β-glucan?

β-glucans are carbohydrates found, for example, within the cell walls of yeasts and plants. Comprised of β-D-glucose monomers, chains are formed when the hydroxyl group of the carbon (C) 1 within the ring joins with the hydroxyl group on either C3 or C4 resulting in a β-glucose dimer and water (Figure 1.2). β-glucan chains can be any length and can also have branches between the C1 and C6 carbons. β-

glucans differ from α-glucans, such as starch and glycogen, in that the C1 hydroxyl group is in the same plane as the C6. β-glucans from oats are chains with β-1:3 and β-1:4 glycosidic linkages. Laminarin, found in seaweeds such as *Laminaria digitata*, and the cell walls in baker's yeast (*Saccharomyces cerevisiae*) are chains of β-1:3 glycosidic links with β-1:6 branches.

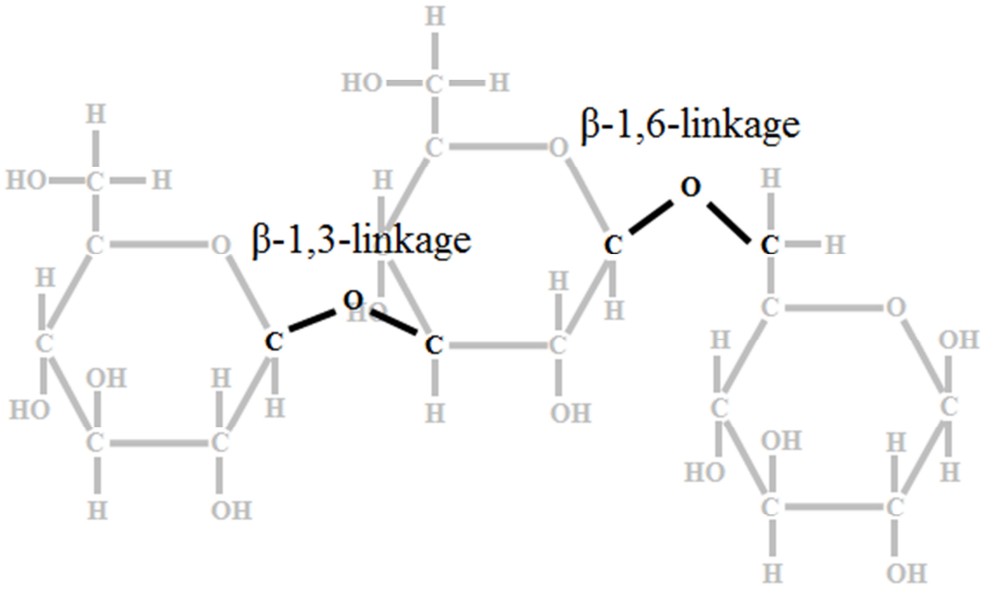


Figure 1.2: Image showing the linkages found in β-D-1,3/1,6-glucan molecules. Taken from Harris 2013.

β-glucans are immunomodulatory molecules which promote an immune response in vertebrates (Dalmo and Bogwald 2008). In mammalian models it has been suggested that the Dectin-1 receptor binds with a β-glucan and activates the immune response (Martin 2012) however to date, this receptor has not been found in fish. Dectin 1 is a C-type lectin (Huysamen and Brown 2009) found on mammalian macrophage cells. In the early 1990's Atlantic salmon macrophages were identified as having a "β-glucan receptor" (Engstad and Robertsen 1993, Engstad and Robertsen 1994) and more recently three C-type lectins (sclra, sclrb and sclrc) and a

complement receptor (CR) 3 have been noted which could be recognising β-glucan molecules (Kiron *et al.* 2016). Although receptors were not identified, Pietretti *et al.* (2013) proposed that multiple receptors were capable of detecting β-glucans in carp macrophages.

Although the mechanism of identification of β-glucans by a fish has yet to be elucidated, it is well documented that β-glucans are able to elicit immune responses in many fish species and reviews by Dalmo and Bogwald (2008), Meena *et al.* (2013), and Vetvicka *et al.* (2013) focus specifically on the use and effects of β-glucans in fish.

1.5 – Immunity in fish.

Immunity can be divided into the innate and adaptive responses. The innate immune response is the immediate defence mechanism found in vertebrates and invertebrates, and responds broadly against pathogen types, i.e. bacteria or viruses, rather than against specific diseases. On the other hand, the adaptive immune response is only found in vertebrates and results in the development of antibodies against pathogens it encounters that result in infection. Immunity can also be divided by location, i.e. a local immune response at the infection site and a systemic response in tissues and/or organs that may not be directly affected.

The importance of local immunity in external organs that come into contact with a wide array of different microbes is highlighted by the increasing number of publications in this area such as gut immunity which are reviewed by Gomez and Balcazar (2008), Nayak (2010a, 2010b), Rombout *et al.* (2011), and Salinas (2015). Gomez and Balcazar (2008) describe the gut as having "tolerogenic mechanisms" that

allow for coexistence with the commensal microbiota whilst still responding to pathogenic microbes. Gut immunity is comprised of both the innate and adaptive immune response and includes, but is not limited to, components such as a layer of mucus covering the external surface, i.e. the inside of the gut, signalling molecules including cytokines, antimicrobial peptides (AMPs), the complement system, and the respiratory burst. The mucosal layer provides both a home for the commensal microbiota population and a cover protecting the epithelial layer underneath from direct exposure to the environment (Gomez and Balcazar 2008). AMPs, complement and the respiratory burst are all defence mechanisms against invading pathogens.

1.5.2 – The role of mucus and mucins.

Mucus is cited as "one of the most important components for fish mucosal immunity" (Koshio 2016). It is involved in osmoregulation, reproduction, and movement, in addition to defence against pathogens and protection, and contains several immune proteins and enzymes such as complement proteins, proteolytic enzymes, AMPs (Koshio 2016) and glycoproteins known as mucins. Mucins are the main component of the mucosal layer with several genes that show differential expression patterns based upon the mucosal membrane. For example, in carp Muc5B expression is found in the skin and gill tissues, but not in the gut and, conversely, Muc2 expression is limited to the gut and not found in the skin or gills of naive fish (Van der Marel *et al.* 2012). The adherence of bacteria to the mucosal surface can be a desirable trait in commensal bacterial species, however the ability of pathogens to adhere is the first step of pathogenesis (Schroers *et al.* 2008).

Studies looking at mucins using ichthyo-models are limited in comparison to mammalian systems. Sloughing of the skin mucosal layers has been seen in carp infected with CyHV-3 as a means of removing pathogens from the surface of the fish (Adamek *et al.* 2013). Chub (*Squalius cephalus*) have been shown to have "excessive yellowish mucus" at the site of infection in the gut with the acanthocephalan *Pomphorhynchus laevis* that was not present at other sites along the intestinal axis nor in parasite free controls (Bosi and Dezfuli 2015). Differences have been seen in the composition of gut mucus in carp such as the amount of and molecular size of glycoproteins were seen after infection with two different strains of *Aeromonas hydrophila* (Schroers *et al.* 2009). Feeding sea bass with MOS for 8 weeks has been shown to increase the number of mucin secreting cells within the gut (Torrecillas *et al.* 2011) and feeding with β-glucans for 2 weeks increases the expression of Muc5B in the skin and gills of carp but a decreases Muc2 gene expression in the gut (Van der Marel *et al.* 2012).

1.5.3 – Communication within the immune system.

Cell communication, which is highly important in maintaining a homeostatic balance, also plays a significant role during activation of the immune response. In the latter, this homeostatic balance is mediated by small molecules known as cytokines. There are three types of cytokines: regulators of the innate immune response, regulators of the adaptive immune response, and stimulators of haematopoiesis (Gomez and Balcazar 2008). Interleukins (ILs) and tumour necrosis factors (TNFs) are two examples of cytokines that are induced by a broad range of pathogen types.

Interleukins can be both instigators and inhibitors of the inflammatory immune response, and are referred to as either being pro- or anti-inflammatory. IL-1β is a pro-inflammatory cytokine that has been found in several fish species including carp, Atlantic salmon, and rainbow trout (Plouffe *et al.* 2005). IL-1β shows differential gene expression patterns in response to pathogens and feeds, in a location dependent manner. Internal organs have been shown to have increased IL-1β gene expression in several fish species after exposure to bacteria/dietary supplements. For example, expression was increased in the liver and spleen of Nile tilapia after 30 days of feeding with dietary acidifiers (Reda *et al.* 2016), the spleen and kidney of tilapia fed with the probiotic *Lactobacillus acidophilus* after 24 hours (Villamil *et al.* 2014), the spleen and head kidney of sea bass infected with *Vibrio anguillarum* (Meloni *et al.* 2015), and in the head kidney of carp infected with *Aeromonas salmonicida* (Falco *et al.* 2012b). The external organs, i.e. the skin, gills and gut, have different expression patterns for IL-1β. The gut was shown to have increased levels of IL-1β gene expression in rainbow trout 5 days post infection with *Yersinia ruckeri* (Ingerslev *et al.* 2014b) and in carp 6 hours post infection with *Aeromonas salmonicida* (Falco *et al.* 2012b). Catfish, however, were shown to have differential expression levels in the gut including up and down regulation upon exposure to LPS from different *Edwardsiella ictaluri* strains 6 hours after treatment (Santander *et al.* 2014). Feeding hybrid tilapia with completely hydrolyzed feather meal (a potential alternative to soybean meal) resulted in a decrease in the gene expression of IL-1β within the gut after 8 weeks (Zhang *et al.* 2014), however expression in the gut of common carp was not influenced by feeding with β-glucans over 14 days (Falco *et al.* 2012b). This differential gene expression seen in external organs could be as a result of constant

exposure to microbes in the environment.

TNFα is another pro-inflammatory marker within the innate immune response and is involved in homeostasis and the induction of apoptosis (Goetz *et al.* 2004). Similar to IL-1β, gene expression of TNFα was shown to be upregulated in internal organs after exposure to pathogens. In turbot infected with *Enteromyxum scophthalmi* there was a significantly higher expression of the TNFα gene expression in the spleen and kidney 6 hours post infection (Ronza *et al.* 2015). Whole zebrafish also showed an increase in expression of the TNFα gene up to 22 hours post inoculation with *Listonella anguillarum* in comparison to non-inoculated controls (Rojo *et al.* 2007), and carp infected with *Aeromonas salmonicida* showed higher expression levels of two TNFα isoforms 6 hours post infection (Falco *et al.* 2012b). Interestingly, feeding carp with β-glucans for 2 weeks did not impact on the gene expression of TNFα-1 in the head kidney, although a decrease in expression of TNFα-2 gene did occur in this organ. This could indicate a sensitivity to signals from live organisms versus extracts. Limited data is currently available on the expression of the TNFα gene in the gut of fish, however there seems to be a trend of decrease in expression. Nile tilapia were shown to have lower expression of this gene after feeding with live yeast as a probiotic for 8 weeks (Ran *et al.* 2015), although in carp fed with β-glucans for 2 weeks there was no effect upon the expression of TNFα-1 gene, but a decrease in expression of TNFα-2 (Falco *et al.* 2012b).

1.5.4 – The Complement system.

The Complement system, which predates adaptive immunity and is thought to

have evolved at least 1,300 million years ago (Holland and Lambris 2002, Nonaka and Kimura 2006), is comprised of four pathways (the Alternative, Classical, Lectin and Cytolytic) centred around the C3 protein. In mammals, the system consists of more than 35 proteins (Sunyer *et al.* 2003, Boshra *et al.* 2006) which are activated by a range of pathogens including viral, bacterial and parasitical, leading to the induction of phagocytosis, cytolysis and inflammation (Holland and Lambris 2002, Sunyer *et al.* 2003, Boshra *et al.* 2006). Figure 1.3 illustrates the three activation pathways within the Complement system: the Classical pathway, the Alternative pathway and the Lectin pathway.

Image removed for copyright reasons.

Figure 1.3: Taken from (Sunyer and Lambris 1998), an illustration of the three main Complement pathways in mammals.

Whilst the complement system of teleosts is similar to that found in mammalian models, there are a few differences. Mammalian complement has evolved

to work best at temperatures found in endothermic organisms, e.g. 37°C in humans, whereas it functions at much lower temperatures (down to 0°C) in teleosts (Sunyer *et al.* 2003). There are also differences in the organs that produce the different complement proteins. In mammals, complement is mainly produced in the liver, whilst in fish, there are a range of production sites including the brain, skin, gills, intestine, kidney and head kidney (Nakao *et al.* 2011). The main difference between mammalian and fish complement, however, is the degree of polymorphism found in each system. On a genetic level, fish are a highly polymorphic vertebrate group and the complement system is subject to several isoforms per component. For example, C3 has been shown to be polymorphic in rainbow trout (Sunyer *et al.* 1996) and gilthead sea bream (Sunyer *et al.* 1997), and that isoforms have different functions within the overall system.

Several studies have highlighted the differential effects that β-glucan can have on the complement system in fish. Ai *et al.* (2007) noted that β-glucan fed yellow croaker (*Pseudosciaena crocea*) showed no differences in serum alternative complement pathway (ACP) activity, whereas in sea bass and rohu (*Labeo rohita*) there was an increased complement activity in the serum (Bagni *et al.* 2000, Misra *et al.* 2006). Duration of feeding period appears to affect spontaneous haemolytic complement activity in channel catfish (*Ictalurus punctatus*) with a decrease in activity occurring after 2 weeks (Welker *et al.* 2012) but not after 4 weeks when fish were fed with β-glucans (Welker *et al.* 2007). In carp, there is conflicting data as to the effect of β-glucan feeding. Selvaraj *et al.* (2006) report no effect on ACP activity after 2 weeks of feeding with β-glucan, however Pionnier *et al.* (2013) noted an increase in the same parameter when carp were fed with β-glucan for 2 weeks.

Pionnier *et al* (2013) also revealed that, whilst there was no effect of feeding with β-glucan on the expression of the genes encoding for bf/C2 (marker for the alternative pathway), MASP2 (marker for the lectin pathway) and C3, there was a significant increase in C1rs gene expression (marker for the classical pathway). Differential expression of genes encoding for 3 different C3 isoforms was shown to occur after rainbow trout were injected with β-glucan however overall, expression of the different isoforms was shown to increase (Lovoll *et al.* 2007). C3 gene expression was also shown to be upregulated in turbot larvae fed with MacroGard® treated rotifers (Miest *et al.* 2016).

1.5.5 – Antimicrobial peptides.

Antimicrobial peptides (AMPs) are short chain polypeptides with the ability to kill invading pathogens. Falco *et al.* (2009) defined AMPs as "gene encoded small cationic peptides" with the ability to act against bacteria, viruses, fungi and parasites. The first AMP was discovered in 1981 in the cecropia moth (*Hyalaphora cecropia*) and since then, over 1200 have been identified (Falco *et al.* 2009, Zhu and Gao 2013). AMPs are considered to work through two different mechanisms: the formation of transmembrane channels in pathogens, or by entering the cell and inhibiting growth and metabolism (Falco *et al.* 2009, Zhu *et al.* 2013). In fish, AMPs are generally produced by immune cells, particularly neutrophils, and tissues that come into contact with external environments such as the skin, digestive system and gills (Noga *et al.* 2011). Although there are more than a thousand known AMPs, those studied in fish include defensins, cathelicidins, piscidins, lysozyme, hepcidin, and apolipoproteins (Noga *et al.* 2011).

In carp, the gene expression of two β-defensin genes was shown to be significantly upregulated in the skin and gills after β-glucan feeding, however expression was not seen in the intestine (Van der Marel *et al.* 2012). In contrast, an increase in expression of the gene encoding cathelicidin occurred in intestinal cells from rainbow trout after feeding with zymosan for 4 weeks (Schmitt *et al.* 2015). Expression of genes encoding LEAP2, two β-defensins and two cathelicidins in the skin of rainbow trout has been shown to be influenced by peptidoglycans within the diet (Casadei *et al.* 2015).

The three antimicrobial peptides analysed within this thesis are Apolipoprotein-I (ApoA-I), hepcidin antimicrobial peptide 1 (HAMP1) and liver expressed antimicrobial peptide 2 (LEAP2). This is the first time the effects of β-glucan upon the expression of these three genes have been studied in fish. ApoA-I proteins from rainbow trout have been shown to inhibit bacterial endotoxins and have antiviral activity (Dietrich *et al.* 2015). HAMP1 gene, which encodes for the protein hepcidin, is a regulator of iron metabolism, and in sea bass has been shown to be influenced by iron overload and anemia, whereas HAMP2 has antimicrobial activity against several bacterial strains (Neves *et al.* 2015). LEAP2 shows antimicrobial activity by disrupting the membranes of pathogens (Li *et al.* 2015). All three genes have been shown to be constitutively expressed in the gut of carp (Dr. Mikolaj Adamek, unpublished data).

1.5.6 – Respiratory burst as a means of defence.

The release of reactive oxygen species during phagocytosis is an innate

immune response known as the respiratory burst (Dahlgren and Karlsson 1999). Hydrogen peroxide and superoxide anion, which are both produced during the respiratory burst, are highly antimicrobial but can also cause damage to the host organism (Dahlgren and Karlsson 1999). The free radical $NO^{\cdot}$ is cytotoxic and produced by nitric oxide synthase (NOS) which has multiple isoforms including endothelial NOS, inducible NOS and neuronal NOS (Aktan 2004) with inducible NOS (iNOS) being part of the innate immune response.

Studies have been performed looking at the effects of naturally derived products in fish diets on iNOS production Expression of the iNOS gene is downregulated in the head kidney and intestine of rohu (*Labeo rohita*) after guava leaves were incorporated into the diet (Giri *et al.* 2015) and in carp, the iNOS gene was upregulated in the head kidney, spleen and intestine after feeding with Chinese foxglove (Wang *et al.* 2015). The expression of iNOS in the gut of carp fed with β-glucans was also shown to be significantly upregulated after 25 days of feeding in comparison to non β-glucan fed carp (Miest *et al.* 2012). iNOS expression has also been associated with infection, for example in rainbow trout larvae this gene was upregulated during infection with *Yersinia ruckeri* (Chettri *et al.* 2012), and in Chinook salmon *(Oncorhynchus tshawytscha*) from two different locations in America, different levels of iNOS gene expression occurred after infection with *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (Metzger *et al.* 2010).

1.5.7 – C-reactive protein.

The Acute Phase Response is described as “the entire array of metabolic and

physiological changes which occur in response to tissue injury of infection" (Bayne and Gerwick 2001), which results in a surge of hormones and leucocytes at the site of injury or infection (Magor and Magor 2001). Those that are considered to be the most responsive are termed Acute Phase Proteins (APPs) which includes molecules such as C-reactive protein (CRP), Serum Amyloid (SA) A and P, and α-2-Macroglobulin (Magor and Magor 2001). Whilst increases in APPs are used as indicators of infections, some APPs will decrease in order to equilibrate the osmotic pressure that builds due to the influx of so called positive APPs to a location (Bayne and Gerwick 2001).

β-glucan feeding, both on its own and in conjunction with *Edwardsiella ictaluri* infection, in the liver of striped catfish showed no effect upon the expression of the CRP gene (Sirimanapong *et al.* 2015). Differential expression patterns of two CRP isoforms were seen in different organs of common carp in response to infection with CyHV-3. A decrease in CRP1 gene expression versus an increase in expression of CRP2 gene in the liver, and an increase in CRP1 expression in the gills was not mirrored by CRP2, which showed a significant increase in expression 1 and 14 days post infection, but a significant decrease in expression 3 and 5 days post infection (Pionnier *et al.* 2014). Infection of tongue sole (*Cynoglossus semilaevis*) with *Vibrio anguillarum* resulted in an increase in CRP gene expression in the kidney, spleen and liver (Li *et al.* 2013). Serum CRP levels in tilapia have also been shown to increase after infection with *Streptococcus iniae* (Gulec and Cengizler 2012). Carp infected with *Aeromonas hydrophila* showed an induction of serum CRP, however carp treated with LPS from *Escherichia coli* did not show a similar induction (MacCarthy *et al.* 2008).

1.6 – The use of vaccination in fish health.

The earliest publication in regards to vaccination describes the concept thus: "no discovery in nature nor in medicine has been more important to the interests of humanity" (Blane 1819). Since its discovery at the end of the 18th century, vaccination has become one of the leading tools in preventing the spread of disease in human medicine, agriculture and aquaculture. Fish vaccinations have been studied since the 1930s (Gudding and Van Muiswinkel 2013) and are available for diseases such as vibriosis, streptococcosis, infectious haematopoietic necrosis virus and viral haemorrhagic septicaemia virus (Lorenzen and LaPatra 2005, Toranzo *et al.* 2005). Vaccines target the adaptive immune response in order to produce antibodies against specific pathogens so that when an organism, for example a fish, comes into contact with these pathogens, it can mount an effective, disease specific immune response much more quickly than if it had not encountered the pathogen before. Vaccinations can be combined with adjuvants which target the innate immune system and research using mammalian models has shown β-glucans to be effective in this role (Bromuro *et al.* 2010, Huang *et al.* 2013, Berner *et al.* 2015). Whilst bathing or oral application of vaccines is considered to be less stressful, injection of vaccines is still the method of application used for the majority of vaccines (Plant and LaPatra 2011). It is therefore important to understand the effects of potential adjuvants, such as β-glucans, both upon the innate immune response, but also their possible effect upon the gut microbiota population when fish are injected in addition to oral application. (Liu *et al.* 2008) showed that whilst LPS was capable of influencing bacterial species richness in the gut of Atlantic salmon 28 days post injection, injection with β-glucan had no effect. Similarly, injection with β-glucan showed no difference in bacterial

species richness within the gut 23 days post injection in carp, however there was a difference 12 days post injection (Harris 2013). Harris also indicated a possible influence upon overall bacterial population size within the gut post injection, however noted that due to the time points analysed, further analysis should be performed to confirm if there was indeed a trend or data was coincidental.

1.7 – Aims and objectives.

The aim of the study presented in this thesis was to determine if there was an influence of β-glucans on the relationship between the common carp and its intestinal microbiota population. To this end, several objectives were devised. Firstly, chapters 2 and 3 outline the optimisation of different methodologies employed within this thesis. Chapter 2 established the effectiveness of RT-qPCR as a method of detecting changes within a microbial population using *in silico* analysis to establish primer specificity towards target and non-target DNA sequences. Chapter 3 compares different methods of analysing *in vitro* bacterial growth including development and optimisation of an image analysis tool, PENGUIN, as a means of accurately measuring bacterial colony sizes. These methods are then employed in Chapter 4 which studies the gut microbiota population in carp fed a diet with and without MacroGard®. Bacteria genus specific qPCR primers analysed using the model presented in Chapter 2 are used to compare the proportions of different bacterial genera within the gut of carp fed with and without MacroGard®, and bacterial isolates taken from the gut of carp fed without MacroGard® were tested for their ability to utilise MacroGard® as a substrate *in vitro* using the methodologies outlined in Chapter 3. This thesis studies the effects of β-glucans upon the immune response in the gut of common carp and the

gut microbiota population when applied orally (Chapter 4) and via intraperitoneal injection (Chapter 5). This includes statistical analysis to determine if there is a correlation between the gut microbiota and immune response. The final experimental chapter looks at the effects of combining both the oral application of MacroGard® and the use of injection focusing on the effects upon the gut microbiota population. To conclude, Chapter 7 gives an overall discussion of all work presented in this thesis and how this can be used to influence current aquaculture practices in order to obtain healthier fish populations.

Chapter 2 – *In silico* analysis of 16S qPCR primers.

The specificity of tools used in research is instrumental in ensuring accurate data is obtained. qPCR assays are now widely used in microbiological analysis, particularly when looking at mixed populations (Thompson *et al.* 2004, Martinez-Puig *et al.* 2007, Desai *et al.* 2009, Himmelheber *et al.* 2009, Bergmark *et al.* 2012) such as those found within the gut of fish (Adamek *et al.* 2013). Genetic markers such as the 16S rDNA gene (also known as 16S rRNA gene) can be very useful when dealing with individual bacteria species or when analysing a mixed bacterial population due to a high level of homogeneity that allows for a single primer pair to successfully anneal to almost all bacteria, whilst analysis of the DNA sequence can identify to a strain specific level. This homogeneity, however, can lead to challenges when looking at taxonomic levels such as genus or family. Genetic differences that allow for species specific identification may prevent primers from annealing, whereas regions that allow for amplification of all bacteria do not distinguish between different families or genera. This can make primer design for qPCR assays a challenge and, as this chapter will show, there are several examples of published primer pairs that amplify non-target DNA sequences from other genera in addition to those they are designed to amplify.

In Chapter 4, genus specific 16S RT-qPCR primers are utilised in order to quantify the changes in the relative proportions of individual bacterial genera in the gut microbiome. Whilst details for this can be found in section 4.3, it is important to note that primer sequences based on previously published work (Thompson *et al.* 2004, Martinez-Puig *et al.* 2007, Adamek *et al.* 2013) for three bacterial genera

(*Aeromonas*, *Vibrio* and *Lactobacillus*) resulted in proportions that represented more than 100% of the overall 16S rDNA expression within each sample as measured using the total 16S rDNA primers designed by (Adamek *et al.* 2013). This discrepancy may be the result of:

- total 16S primers not detecting all the copies of the 16S gene present in the samples.

or,

- individual genus primers amplifying non-target 16S sequences in addition to target sequences.

Prior to their initial use, all primer pairs were analysed using both a standard BLAST search (Basic Local Alignment Search Tool) and a more specific primer BLAST. Neither of these had highlighted the potential for non-target amplification in genus specific primer pairs, nor a lack of target amplification for the total 16S primers, thereby indicating an even more stringent analysis was required. The aims of this chapter are to develop a model to determine the likelihood of amplifying both target and non-target sequences, and to test this model using DNA generated from pure bacterial cultures.

2.2 – The importance of primer/sequence similarity.

The NCBI online primer BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) identifies sequences with at least 1 mismatch in a primer sequence with a maximum of 9 mismatches to the primer

sequence. The program did not, however, identify any sequences with more than 3 mismatches to each primer analysed. Therefore a library of sequences was generated against which primer sequences could be compared. Sequence data were obtained from the European Nucleotide Archive (http://www.ebi.ac.uk/ena) and aligned using the program BioEdit (Thompson *et al.* 1994) by genus to allow for up to 100 sequences to be compared (see Appendix 1 for accession numbers). Sites of primer binding were identified using target sequences and potential binding sites in non-target sequences identified based upon alignments with target sequences. Scores were given based upon the number of "errors" in comparison to the primer nucleotide sequence.

The biggest challenge was in determining the most appropriate way to score errors. Primer design must take into consideration the target DNA sequence, similarities to non-target sequences, similarities to the partner primer and to itself, i.e. the formation of tertiary structures that will inhibit annealing to DNA, and the conditions within the PCR reaction. It is possible to design effective primer pairs that are not 100% homologous to the target DNA site, as happens with degenerate primer pairs, therefore it was first necessary to determine how different to a DNA sequence a primer must be before annealing does not occur. In order to determine how many errors should be analysed, the following points were considered:

- Primer BLAST analysis of primer pairs did not reveal any sequences that may be detected that had more than 3 errors per primer.

- Assuming the total 16S primers were amplifying all 16S sequences, genus specific primers were amplifying non-target sequences that must have at least 3

errors per primer.

So whilst the maximum number of errors that would inhibit annealing is not known, any model developed must take into account that at least 3 errors could occur but successful annealing would not be prevented.

Determining the importance of an error can be based upon several factors. Errors could occur when swapping an adenine (A) for a guanine (G), i.e. substitution of a purine, or a thymine (T) for a cytosine (C), i.e. pyrimidine substitution. This would potentially have less of an impact than if a purine was swapped for a pyrimidine. Although the number of hydrogen bonds holding the primer in place would be different, the distance between the parallel nucleotides would still be the same. In instances where the error results in parallel nucleotides being both either purines or pyrimidines, the distance between the hydrogen atoms is altered which in turn will impact the strength of the bond. Both of these changes, i.e. the number or the strength of the hydrogen bonds, will affect the ability of the primer to remain annealed to the sample nucleotide sequence during PCR (Figure 2.1). Extra nucleotides or deletions will also impact on the ability of a primer to remain annealed to a template sequence and are likely to have a stronger influence than mismatches as they may also influence the binding of the nucleotides on either side of them.

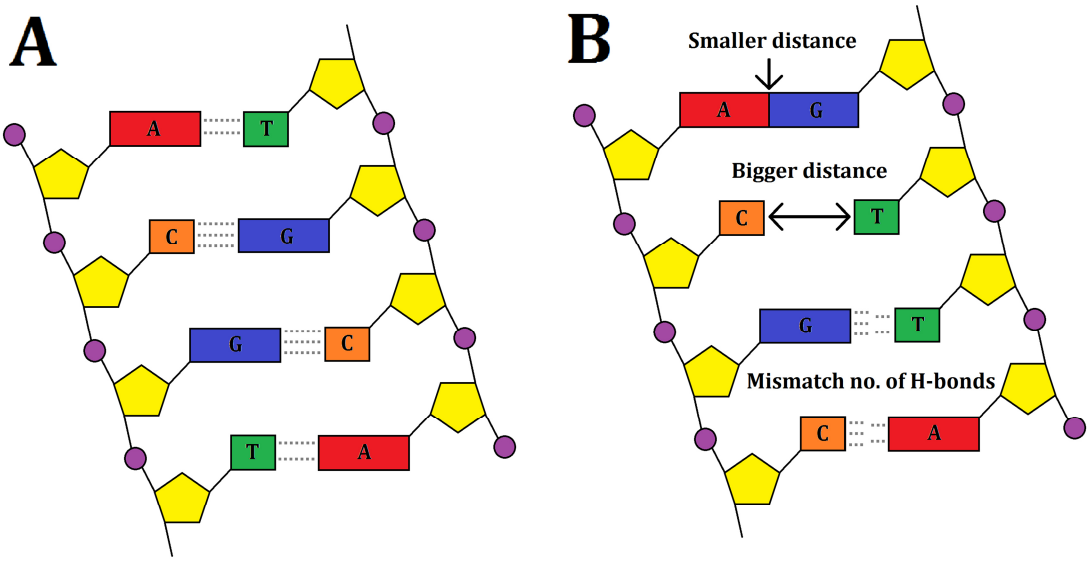


Figure 2.1: Image showing nucleotide bonding of DNA (A) and potential mismatches (B) that can occur between a sequence and a PCR primer. Possible mismatches include the pairing of two purine base pairs (A and G) or two pyrimidine base pairs (T and C) rather than a purine paired with a pyrimidine (A and T or G and C). The altered distance will influence the strength of the hydrogen bonds between the two strands. Mismatches also include swapping an A for a G or a C for a T which leads to differences in the number of bonds between the two strands.

In addition to the type of error, the position of the error must also be considered. Polymerase joins the "spine" of the nucleotide sequence together in the direction of 3' to 5', therefore errors closer to the 3' end of the primer sequence are more likely to inhibit the action of the enzyme due to misaligned nucleotides.

2.3 – Initial considerations of the model.

The model designed had to factor in mismatches and additions/deletions as well as the position of a nucleotide relative to the 1st 3' nucleotide of the primer being analysed. Correct pairings were given a score (S_n) of +2, all types of mismatch were considered together and given a score of -3, and an addition or deletion was given a score of -7. This was then multiplied by a rating (N) relative to the position of

the nucleotide in the sequence. The sum of all of these values was then taken, divided by the sum of all factors multiplied by a correct pairing, i.e. a 100% similar primer and the highest possible score, and given as a percentage. This is expressed by the following equation:

$$Similarity\ score = \frac{\sum_{n=t}(S_n \times N)}{\sum_{n=t}(2N)} \times 100$$

whereby t = the total number of nucleotides in the primer sequence, S_n = the score each nucleotide recieves (+2, -3 or -7) and N is a nucleotides ranking based upon its position relative to the 3' end of the sequence. For example, in a 20 base pair primer the 1st nucleotide at the 3' end of the sequence would be given a factor of 20 and the nucleotide at the 5' end of the primer would be given an N of 1. Therefore:

- A mismatch at nucleotide 14 from the 3' end of the primer would have a result of -3x6, i.e. S_n multiplied by N.
- A mismatch at nucleotide 3 from the 3' end of the primer would have a result of -3x18, i.e. S_n multiplied by N.
- When an addition or deletion occurs, the N is given as that of the closest 3' nucleotide minus 0.5, i.e. the value half way between two nucleotides. This is done so as not to affect the impact of the remaining nucleotides towards the 5' end of the sequence as adding an integer would alter the numerical values of all other nucleotides. An addition/deletion will have a greater impact than a mismatch, therefore the extra N is included in the calculation. However, due to the direction in which polymerase acts, it will be less important than nucleotides closer to the 3' end and more important closer to the 5' end. An

addition/deletion between nucleotides 9 and 10 would therefore have a score of -73.5.

Using the above example, Figure 2.2 gives a diagrammatic representation of how the similarity score is calculated.

The range of similarity scores this model produces, however, showed a high range of "overlap" between different numbers of errors depending upon their position within the primer. For example, three sequential mismatches at the 5' end of the primer gives a score of 92.9% and a single mismatch at the 3' end of the primer gives a score of 76.2%, whereas in reality, one error is less likely to impact annealing than three sequential errors, irrespective of position within the primer. Table 2.1 shows the number of possible combinations of up to 3 errors and the range of possible similarity scores that would be obtained when analysing a 20 base pair primer. As can be seen the score does not necessarily relate to the number of errors. For example, it is possible a sequence with one mismatch error may have a lower score, i.e. be considered as less likely to anneal, than a sequence with 1 mismatch and 2 gaps whereas in reality, a sequence with 3 errors in comparison to the primer will be less likely to anneal than a sequence with only 1 error.

Position		20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1		Total
N		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
Correct pairing score $(N \times 2)$	5'	2	4	6	8	10		14	16	18	20	22	24	26	28	30	32	34		38	40	3'	372
Mismatch score $(N \times -3)$							-18												-54				-72
Addition/deletion score $((N\ of\ 3'nucleotide - 0.5) \times -7)$										-73.5													-73.5
Final score of 100% similarity = 420											Final score of primer similarity = 226.5												Similarity score =53.9%

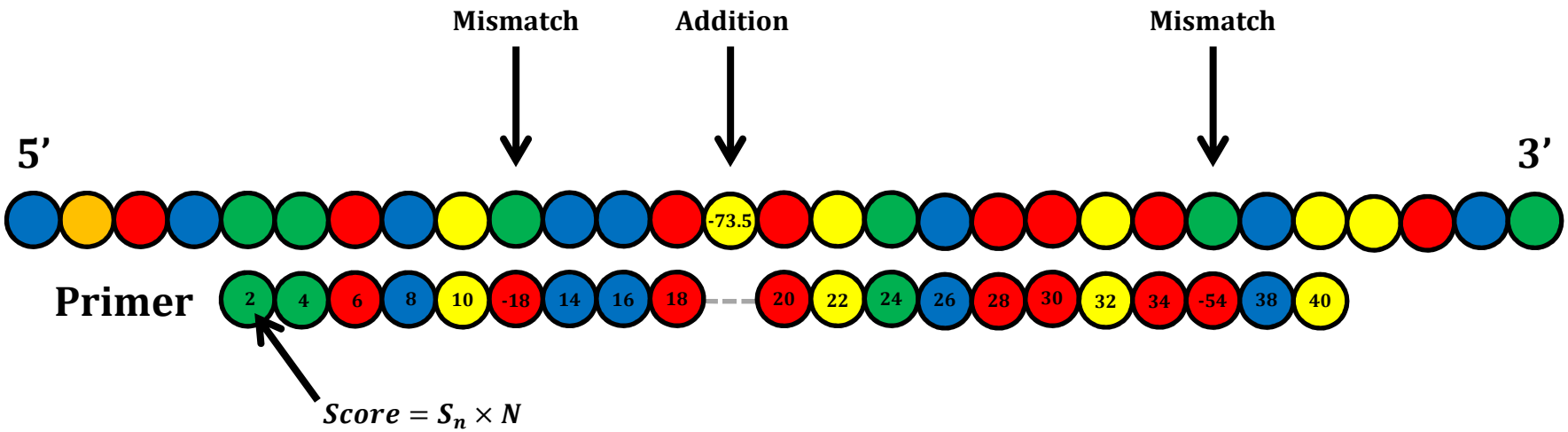


Figure 2.2: Example calculation of a similarity score for a primer that has two mismatches and an addition/deletion between nucleotides 11 and 12 in a hypothetical 20 base pair primer. A hypothetical 20 base pair primer is compared to a DNA sequence and mismatches are found at nucleotides 3 and 15 (counting from the 3' end), and an addition/gap is found between nucleotides 11 and 12. The similarity score is calculated by adding together the score of all correct pairings ($\sum N \times 2 = 372$), mismatches ($\sum N \times -3 = -72$) and addition/deletions ($\sum(N\ of\ 3'nucleotide - 0.5) \times -7 = -73.5$) which totals 226.5. This is divided by the score of a primer with 100% similarity ($\sum N \times 2 = 420$) and multiplied by 100. This gives a similarity score of 53.9%.

Table 2.1: Scoring the similarity of a 20 base pair primer against a target sequence based upon hypothetical errors in different positions along the nucleotide sequence. From the 3' end of the primer, each nucleotide position is assigned a factor (20-1 in descending integers) which is multiplied by a score of either 2, -3 or -7 for a correct pairing, a mismatch pairing or an addition/deletion (A/D) respectively. The similarity score for the primer is calculated as a percentage similarity relative to a score a 100% similar sequence would give.

Type of error	Number of possible combinations across whole primer	Possible similarity score across whole primer (%)	
		Highest	Lowest
1 mismatch	20	98.8	76.2
2 mismatches	190	96.4	53.6
3 mismatches	1150	92.9	32.1
1 A/D	19	97.5	67.5
2 A/D	171	93.3	36.7
3 A/D	969	87.5	7.5
1 mismatch and 1 A/D	397	96.3	43.7
2 mismatches and 1 A/D	3610	93.9	21.1
1 mismatch and 2 A/D	3538	92.1	12.9

Further categorisation was done to reduce the potential overlap range between different types of error by dividing the primer into two parts as shown in Figure 2.3 – the 25% of nucleotides closest to the 3' end and the rest of the primer. Table 2.2 shows the possible similarity scores of a hypothetical 20 base pair primer sequence when the primer is additionally divided into two parts. As can be seen, whilst this does reduce overlap, it is still too broad a range to provide useful information as to whether or not a primer is likely to anneal during PCR.

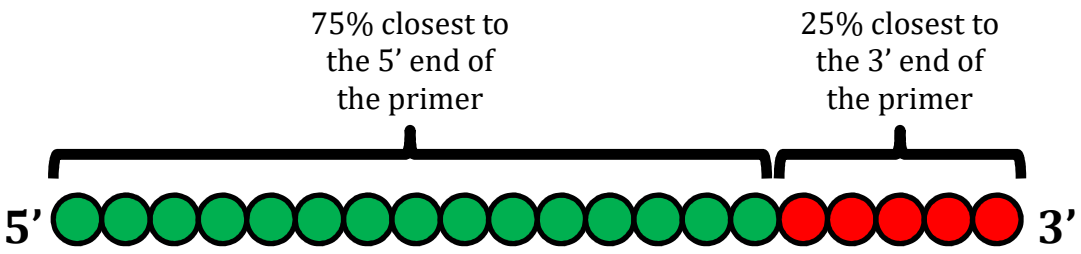


Figure 2.3: Schematic showing the division of sections in a primer as described for use within a model to determine primer specificity based upon location of mismatches and/or deletions in comparison to a potential target nucleotide sequence. As errors closest to the 3' end of a primer sequence are more likely to result in failure to amplify a DNA sequence, the primer was separated with errors being counted either in the 25% of the primer closest to the 3' end or the remaining 75%. Possible combinations based upon number of errors and position of those errors in relation to this split are described in Table 2.2.

Table 2.2: A hypothetical 20 base pair primer was analysed and the range of possible similarity scores calculated for different types of errors that additionally factor in their relative position in comparison to the 3' end of the primer sequence. 5' is defined as the 75% of nucleotides closest to the 5' end, i.e. nucleotides 1-15. 3' is defined as the 25% of nucleotides closest to the 3' end, i.e. nucleotides 16-20. Similarity score is calculated as a percentage similarity relative to a primer that is 100% homologous to the target nucleotide sequence.

Type and location of error	Possible similarity score across whole primer (%)	
	Highest	Lowest
1 mismatch 5'	98.8	82.2
1 mismatch 3'	82.2	76.0
2 mismatch 5'	96.4	65.5
1 mismatch 5' and 1 mismatch 3'	79.8	58.3
2 mismatch 3'	60.7	53.8
3 mismatch 5'	97.5	50.0
2 mismatch 5' and 1 mismatch 3'	77.4	48.9
1 mismatch 5' and 2 mismatch 3'	59.5	35.7
3 mismatch 3'	39.3	32.1
1 A/D 5'	97.5	74.2
1 A/D 3'	72.5	67.5
2 A/D 5'	93.3	50.0
1 A/D 5' and 1 A/D 3'	70.0	41.7
2 A/D 3'	43.3	36.7
3 A/D 5'	87.5	27.5
2 A/D 5' and 1 A/D 3'	65.8	17.5
1 A/D 5' and 2 A/D 3'	40.8	10.8
3 A/D 3'	12.5	7.5
1 mismatch 5' and 1 A/D 5'	96.3	56.3
1 mismatch 5' and 1 A/D 3'	71.3	49.6
1 mismatch 3' and 1 A/D 5'	78.5	55.1
1 mismatch 3' and 1 A/D 3'	53.5	43.7
1 mismatch 5' and 2 A/D 5'	93.9	32.1
1 mismatch 5', 1 A/D 5' and 1 A/D 3'	68.8	23.8
1 mismatch 5' and 2 A/D 3'	42.1	18.8
1 mismatch 3' and 2 A/D 5'	74.3	26.2
1 mismatch 3', 1 A/D 5' and 1 A/D 3'	51.0	17.9
1 mismatch 3' and 2 A/D 3'	24.3	12.9
2 mismatch 5' and 1 A/D 5'	92.1	39.7
2 mismatch 5' and 1 A/D 3'	68.9	33.0
1 mismatch 5', 1 mismatch 3' and 1 A/D 5'	77.3	37.3
1 mismatch 5', 1 mismatch 3' and 1 A/D 3'	51.1	25.8
2 mismatch 3' and 1 A/D 5'	58.2	27.7
2 mismatch 3' and 1 A/D 3'	33.2	21.1

2.4 – Statistical analysis of the primer analysis model.

As shown in section 2.3, using a numerical means of categorising the impact of a nucleotide mismatch, addition or deletion factoring both type and position of an error did not prove successful due to the high amount of overlap in similarity score between sequences that would result in successful amplification and those that would not. Correlation analysis can be used to analyse the similarity between two data sets of ordinal data. Spearman's rank order test is a non-parametric statistical model that is used to determine the correlation between two data sets that produces both a p value of significant linear correlation and a value of ρ (rho) indicating the level of correlation between the two data sets.

Similar to the model outlined in section 2.3, nucleotide position is scored based upon position relative to the 3' end of the primer sequence. Whereas the previous model only considers values "in between" nucleotides when an addition/deletion occurs, in this alternative model these values have been built into the calculation at all possible positions. Primer nucleotides are ranked 1-x (dependent on primer length) starting at the 5' end of the sequence. If the corresponding nucleotide of the target DNA sequence matches this nucleotide an equal score is given, i.e. if the corresponding nucleotide to primer nucleotide 5 is the same, it will also score +5. If the nucleotide is mismatched, however, it will score 0. Each "in between" value will score as if nucleotides match unless next to an addition/deletion. In this instance, the addition/deletion will score 0 as will the "in between" value on either side. For example, if a deletion occurred "opposite" primer nucleotide 15, where the primer nucleotide would score 14, 14.5, 15, 15.5, 16, the target nucleotide would score 14, 0, 0, 0, 16. This is done due to additions and

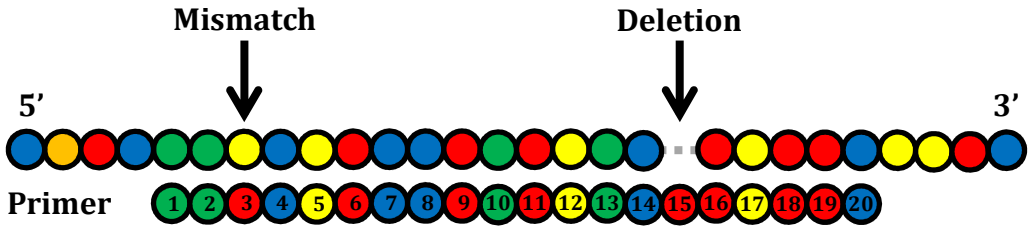
deletions having a stronger biological impact than a mismatch that cannot be directly translated using scores alone. These scores are then compared utilising the following formula:

$$\rho = 1 - \frac{6\sum d_i^2}{n(n^2-1)}$$

where d_i is the difference between each rank, and n is the number of ranks. Using an example of a 20bp primer with 1 mismatch and 1 deletion, Figure 2.4 describes how Spearman's ρ is calculated for this primer.

Using the model that is fully explained in section 2.5, values of ρ were calculated based upon up to 4 errors occurring within a primer. Within this model, the primer is divided into three regions: region A is the 25% of the primer closest to the 3' end, region B is the 50% of the primer closest to the 3' end and includes region A, and region C is the 50% of the primer furthest from the 5' end of the primer. The highest and lowest values of ρ were calculated for errors (either a mismatch or an addition/deletion) in the following combinations: 1 error irrespective of region (category A), 2 errors in region A (B), 1 error in region A and 1 error in region C (C), 2 errors in region B (D), 1 error in region B and 1 error in region C (E), 3 errors in region A (F), 2 errors in region A and 1 error in region C (G), 1 error in region A and 2 errors in region C (H), 3 errors in region B (I), 2 errors in region B and 1 error in region C (J), 1 error in region B and 2 errors in region C (K), 3 errors in region C (L), and 4 or more errors irrespective of region (M). These ranges are shown in Figure 2.5. From this, the more errors there are closer to the 3' end of the primer (e.g. category G), the lower the value of ρ and errors at the 5' end of the primer give higher values of ρ. What this also shows is that a pairing with only 1 error (category B and

deemed within the model to have no impact upon primer annealing) may have a higher score than a pairing with 4 or more errors (category M and deemed within the model to inhibit primer annealing). This may be indicative of issues with the proposed classifications of errors within the model, however this could also be due to an error within the way in which Spearman's ρ is calculated. As discussed within the previous model, how best to weight a mismatch or an addition/deletion is a relative unknown and giving a score of 0 for an error, and weighting additions/deletions 3 fold in comparison to mismatches may be incorrect. Further analysis into the accuracy of using Spearman's correlation as a marker of likelihood of amplification of non-target DNA sequences would require *ex silico* analysis of known DNA sequences for which values of ρ could be calculated and data would be available as to successful amplification or not. It was therefore decided a categorical model should be considered.



Nucleotide	Primer (5' → 3')		Target		d	d2
	Number	Rank order	Number	Rank order		
Match	1	1	1	5	4	16
	1.5	2	1.5	6	4	16
Match	2	3	2	7	4	16
	2.5	4	2.5	8	4	16
Mismatch	3	5	0	2.5	2.5	6.25
	3.5	6	3.5	9	3	9
Match	4	7	4	10	3	9
	4.5	8	4.5	11	3	9
Match	5	9	5	12	3	9
	5.5	10	5.5	13	3	9
Match	6	11	6	14	3	9
	6.5	12	6.5	15	3	9
Match	7	13	7	16	3	9
	7.5	14	7.5	17	3	9
Match	8	15	8	18	3	9
	8.5	16	8.5	19	3	9
Match	9	17	9	20	3	9
	9.5	18	9.5	21	3	9
Match	10	19	10	22	3	9
	10.5	20	10.5	23	3	9
Match	11	21	11	24	3	9
	11.5	22	11.5	25	3	9
Match	12	23	12	26	3	9
	12.5	24	12.5	27	3	9
Match	13	25	13	28	3	9
	13.5	26	13.5	29	3	9
Match	14	27	14	30	3	9
	14.5	28	0	2.5	25.5	650.25
Deletion	15	29	0	2.5	26.5	702.25
	15.5	30	0	2.5	27.5	756.25
Match	16	31	16	31	0	0
	16.5	32	16.5	32	0	0
Match	17	33	17	33	0	0
	17.5	34	17.5	34	0	0
Match	18	35	18	35	0	0
	18.5	36	18.5	36	0	0
Match	19	37	19	37	0	0
	19.5	38	19.5	38	0	0
Match	20	39	20	39	0	0
	-	-	-	-	Total	2377

$$\rho = 1 - \frac{6\sum d^2}{n(n^2-1)} = 1 - \frac{6 \times 2377}{39(39^2-1)} = 1 - \frac{14262}{59280} = 1 - 0.241 = \mathbf{0.759}$$

Figure 2.4: Illustration of how a Spearman's ρ is calculated for a primer sequence in order to determine similarity to a target DNA sequence. Nucleotides are assigned a number based upon position relative to the 3' end of the primer sequence. Additionally, a value of 0.5 is added between each nucleotide in order to distinguish between mismatches and additions/deletions. Biologically, deletions/additions have a larger impact than mismatches. All errors receive a number of 0. Mismatches score a single 0. Additions/deletions score 3 0s, i.e. the position of the nucleotide and the two nearest 0.5 numbers.

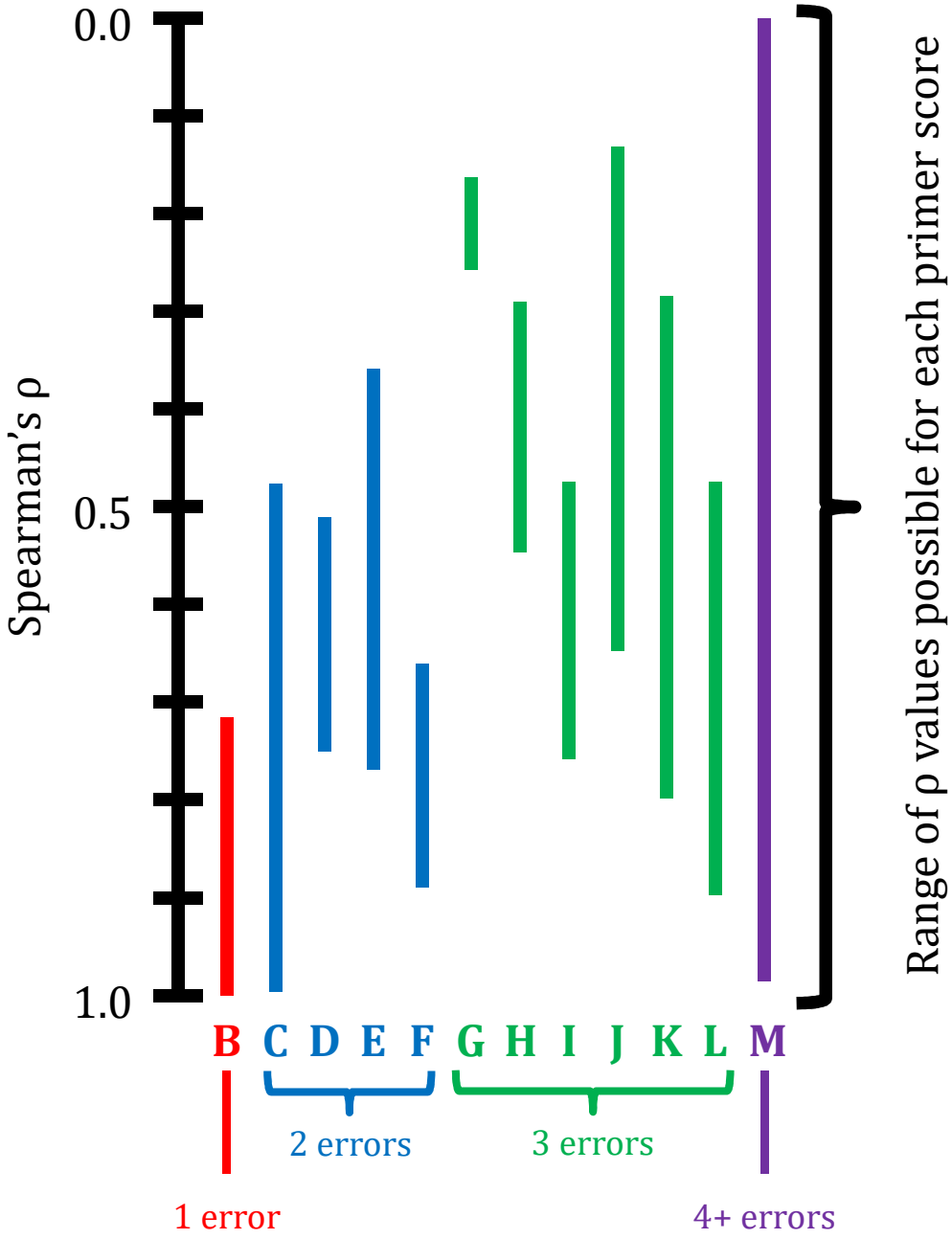


Figure 2.5: The ranges of values of ρ calculated for different numbers of errors (mismatches and additions/deletions) at different locations within the primer based upon the model that is fully elucidated in section Chapter 1. Using a 20bp example, firstly the nucleotides are divided into 2 parts, the 10 nucleotides closest to the 5' end and the 10 nucleotides closest to the 3' end. The 3' section is then further divided by 2. This results in 3 sections: the 25% of the primer closest to the 3' end, the 50% closest to the 3' end (includes the previous section), and the 50% of the primer furthest from the 3' end. The range of possible ρ values for up to 3 errors is calculated for the following location combinations: 1 error irrespective of location (B), 2 errors in the 3' 25% (C), 1 error in the 3' 25% and 1 error in the 5' 50% (D), 2 errors in the 3' 50% (E), 1 error in the 3' 50% and 1 error in the 5' 50% (F), 3 errors in the 3' 25% (G), 2 errors in the 3' 25% and 1 error in the 5' 50% (H), 1 error in the 3' 25% and 2 errors in the 5' 50% (I), 3 errors in the 3' 50% (J), 2 errors in the 3' 50% and 1 error in the 5' 50% (K), 1 error in the 3' 50% and 2 errors in the 5' 50% (L), 4 or more errors irrespective of location (M).

2.5 – Re-evaluation of the model.

As shown in sections 2.3 and 2.4, using a numerical means of categorising the impact of a nucleotide mismatch, addition or deletion factoring both type and position of an error did not prove successful due to the high amount of overlap in similarity score between different numbers of errors. Therefore a simpler approach was considered that focused on a broader definition of position and considered both mismatches and additions/deletions simply as an "error".

Rather than considering the individual position of a nucleotide, primers were divided into three sections. As discussed in section 2.2, the closer an error is to the 3' end of the primer, the more likely it is to negatively impact the ability of polymerase to successfully start connecting the DNA backbone of nucleotides that are next to each other, therefore more emphasis is placed on the 3' end of the primer sequence. Section A is the 50% of the primer closest to the 5' end, Section B is the 50% of the primer closest to the 3' end, and Section C is the 25% of the primer that is closest to the 3' end (Figure 2.6). In this model, up to 3 errors are considered with an assumption that 4 or more errors would result in unsuccessful amplification. The scoring system employed is described in Table 2.3.

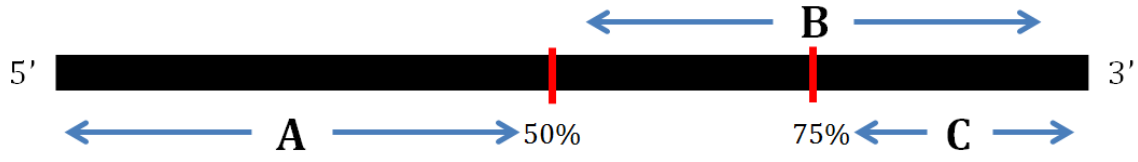


Figure 2.6: Illustration of how a primer is divided into 3 sections. Section A is the 50% of the primer that is closest to the 5' end of the nucleotide sequence, Section B is the 50% of the primer that is closest to the 3' end and Section C is the 25% of the nucleotide closest to the 3' end. This was done in order to emphasise the importance of errors closer to the 3' end of the sequence.

Table 2.3: Scoring of a primer based upon the number of errors (this can be a mismatch, an addition or a deletion of a nucleotide) within 3 different sections as shown in Figure 2.6. Section A is the 50% of the sequence closest to the 5' end of the primer, Section B is the 50% of the sequence closest to the 3' end and Section C is the 25% of the primer closest to the 3' end of the sequence. This is done to emphasise the importance of errors closest to the 3' end of the primer in its ability to anneal to a DNA strand. Up to 3 errors within a primer sequence are considered. Four or more errors within the whole primer are considered to be too many for successful annealing to occur.

Score	Number of errors in		
	Section A	Section B	Section C
A	No errors		
B	One error in whole primer		
C	-	-	2
D	1	-	1
E	-	2	-
F	1	1	-
G	-	-	3
H	1	-	2
I	2	-	1
J	-	3	-
K	1	2	-
L	2	1	-
M	Four or more errors in whole primer		

Once sequences have been scored based upon location of the error as shown in Table 2.3, the likelihood of annealing must then be defined. This, however, can depend upon the conditions within a PCR reaction and can be influenced by the type of polymerase, the primer concentration and $MgCl_2$ concentration. To this end a dual system was proposed for this model. Initial scoring is fixed as it is based upon *in silico* data but likelihood of annealing can be altered to suit the specificity of PCR reaction conditions. The likelihood of annealing was initially divided as follows: scores of A, B, D, F, I and L were considered "likely to anneal", scores of G, J and M were considered "unlikely to anneal", and the remaining scores of C, E, H and K were considered "maybe will anneal".

2.6 – Calculating the probability of a primer pair amplifying a DNA sequence.

As stated in section 2.2, a library of sequence alignments was generated based upon the bacterial 16S rDNA housekeeping gene against which primers could be compared. From this, it was possible to calculate the mathematical probability of a primer pair annealing to a sequence from a specific genus. This was done using the following formula:

$$P(F \cap R) = P(F) \times P(R)$$

whereby $P(F \cap R)$ is the probability that both the forward primer (F) and reverse primer (R) will anneal, $P(F)$ is the probability that the forward primer will anneal and $P(R)$ is the probability that the reverse primer will anneal. The probability of an individual primer annealing is calculated based upon the categorising of "likely to anneal" given in section 2.5 as follows:

$$P(annealing) = (\{A\} + \{B\} + \{D\} + \{F\} + \{I\} + \{L\})$$

probability of each score, the following equation is used:

$$P(score) = \frac{number\ of\ sequences\ with\ a\ specific\ score}{total\ number\ of\ sequences}$$

As an example, a primer pair for the genus *Aeromonas* sp. (Adamek *et al.* 2013) was compared against 1100 *Aeromonas* sp. 16S rDNA sequences. From this, 911 hits were made for the forward primer and 903 hits were made for the reverse primer. All sequences were scored as described in section 2.5 and the number of sequences with each score for each primer was recorded as shown in Table 2.5.

Table 2.4: *Aeromonas* sp. 16S rDNA sequences were scored against a set of Aeromonas sp. PCR primers for the same gene. Scores are based on the analysis model given in section 2.5 and are assigned based upon the number of errors within a primer and their location in relation to the 3' end of the primer sequence. Scores highlighted in green are those expected to result in successful annealing between a primer and a DNA sequence.

Score	A	B	C	D	E	F	G	H	I	J	K	L	M
Forward primer	882	12	0	0	1	2	0	0	0	0	0	1	13
Reverse primer	793	78	0	0	1	1	0	0	0	0	8	10	12

From this data, the probability of each possible outcome for each primer can be calculated as shown in Table 2.5.

Table 2.5: The probability of a 16S rDNA sequence from an *Aeromonas* sp. achieving a score of A-M when compared to a pair of *Aeromonas* sp. 16S rDNA primers. Probabilities are calculated by dividing the number of sequences with a specific score by the total number of sequences analysed for each primer. Scores highlighted in green are those expected to result in successful annealing between a primer and a DNA sequence.

Score	A	B	C	D	E	F	G	H	I	J	K	L	M
P(F)	0.968	0.013	0	0	0.001	0.002	0	0	0	0	0	0.001	0.014
P(R)	0.878	0.086	0	0	0.001	0.001	0	0	0	0	0.009	0.011	0.013

As the probability of a primer annealing to a DNA sequence is not linked to other factors at this stage, it is calculated by summing up the probability of all outcomes that are likely to result in annealing as follows:

$$\boldsymbol{P(F)} = 0.968 + 0.013 + 0 + 0.002 + 0 + 0.001 = \mathbf{0.985}$$

$$\boldsymbol{P(R)} = 0.878 + 0.086 + 0 + 0.001 + 0 + 0.011 = \mathbf{0.977}$$

Unlike the probability of an individual primer annealing to a DNA sequence, the probability that there will be successful amplification in a PCR reaction is dependent upon both primers annealing to a DNA sequence. To this end, in order to calculate this, the probability of both primers annealing must be multiplied as follows:

$$\boldsymbol{P(F \cap R)} = 0.985 \times 0.977 = \mathbf{0.962}$$

From this, based upon the model as stated in section 2.5, the probability of *Aeromonas* sp. 16S rDNA primers amplifying an *Aeromonas* sp. 16S rDNA sequence is 0.962. This indicates that the particular primer pair analysed will successfully anneal to more than 95% of *Aeromonas* sp. sequences within a mixed population. Whilst this information is important it does not ascertain if a primer pair will bind to non-target sequences within a mixed population. In order to resolve this the same primer pair was compared to 16S rDNA sequences from other bacteria genera including *Vibrio* sp., *Pseudomonas* sp., Enterobacteriaceae sp., *Lactobacillus* sp. and *Bacillus* sp.. The probabilities for the primer pairs annealing to sequences from each of these genera are as follows:

$$P(Vibrio\ DNA) = 0$$

$$P(Pseudomonas\ DNA) = 0$$

$$P(Enterobacteriaceae\ DNA) = 0.039$$

$$P(Lactobacillus\ DNA) = 0$$

$$P(Bacillus\ DNA) = 0$$

This appears to be positive for both the model of analysis described in section 2.5 and the use of Adamek *et al.*'s (2013) *Aeromonas* sp. specific 16S rDNA primers within a mixed population. However without confirmation that this translates into a PCR reaction mix, the conclusion at this point is theoretical only.

2.7 – Testing of the model.

In order to test the accuracy of the model outlined in section 2.5, PCRs were

conducted using primer pairs designed to amplify *Pseudomonas* and *Lactobacillus*, i.e. an example of a primer pair that were likely to only amplify target sequences and an example that amplified non-target sequences, in addition to target sequences respectively. cDNA from a range of different bacterial species was prepared and utilised as a template for PCR analysis. Bacteria cultures were donated by Dr. Verena Jung-Schroers (University of Veterinary Medicine, Hanover, Germany) and were grown at 25°C for 24 hours on blood agar plates before a single colony was transferred into 2ml of TriFast reagent and frozen at -80°C. The cultures utilised were as follows: *Aeromonas allosaccharophila* (S39232.2), *Aeromonas hydrophila* (KJ743719.1), *Bacillus cereus* (KJ833790.1), *Bacillus thuringiensis* (KJ722440.1), *Citrobacter freundii* (JX860619.1), *Edwardsiella ictaluri* (KC789872.1), *Vibrio* sp. (GQ359963.1), *Aeromonas hydrophila*, *Aeromonas sobria*, *Citrobacter farmeri*, *Citrobacter youngae*, *Pseudomonas alcaligenes*, *Pseudomonas putida*, and *Pseudomonas fluorescens*. Samples were then shipped to Keele University (UK) on ice where RNA was isolated using a phenol/chloroform isolation protocol as outlined in section 2.7.1. cDNA was synthesised using Moloney Murine Leukemia Virus (MuLV) Reverse Transcriptase (RT) as described in section 2.7.2. PCRs were then performed as outlined in section 2.7.3 with primer sequences being described in Appendix 3.

2.7.1 – RNA isolation.

Bacteria cultures suspended in TriFast were incubated at room temperature for 5 minutes before 0.2ml of chloroform was added and the tubes shaken by hand for 15 seconds. Samples were incubated at room temperature for a further 3 minutes before centrifugation at 12,000 x *g* for 15 minutes at 4°C. The RNA containing

aqueous phase was removed to a fresh tube and mixed with 0.5ml of 100% isopropanol and incubated at room temperature for 10 minutes. Samples were then subject to centrifugation (12,000 x *g*) for 10 minutes at 4°C. The supernatant was removed and the remaining pellet washed with 1ml of 75% ethanol. After a final centrifugation at 7,500 x *g* for 5 minutes at 4°C, the supernatant was discarded and the samples left to air dry for 5 minutes before being resuspended in RNase free water and incubated at 55°C for 10 minutes. Samples were stored at -80°C until used.

2.7.2 – cDNA synthesis.

cDNA was synthesised as outlined in Falco *et al.* (2012b) using M-MuLV RT. Briefly, 500ng of RNA was incubated with a final concentration of 5mM $MgCl_2$, 1X PCR buffer II, 0.5mM dNTPs, 1.25µM random hexamers, 20U RNase Inhibitor and 25U M-MuLV RT at 25°C for 10 minutes, 42°C for 30 minutes and 95°C in a GeneAmp PCR system 9700 thermocycler. Samples were stored at -20°C until further use.

2.7.3 – PCR analysis.

In order to test the two genus specific 16S rDNA primer pairs, it was decided to use the same conditions selected for use in future RT-qPCR analysis of experimental samples. RT-qPCR assays were performed using an ABI Prism® 9000 Sequence Detection System and the SensiFAST™ SYBR® HiROX kit as per the manufacturer's instructions. Assays were performed in a total volume of 20µl with primers utilised at a concentration of 0.2µM each. 2µl of undiluted template cDNA was used in each assay. A thermal profile of an initial 2 minutes at 95°C and 40 cycles

of 5 seconds at 95°C and 30 seconds at 62°C was performed followed by production of a dissociation curve based upon the default thermal settings as defined by version 1.2.3 of the software for the thermocycler. Positive amplification was determined using the dissociation curve rather than based upon a C_t in order to eliminate any false positives produced by primer dimers, and a PCR is described as successful annealing if a peak was obtained in the disassociation curve.

2.7.4 – Results.

Where sequence data was available for the bacteria analysed, analysis was performed using the model described in section 2.5. Successful amplification of DNA was determined based upon presence of a PCR product when analysing the dissociation curve produced at the end of the thermal program. Table 2.6 shows the outcome for each primer pair. The model inaccurately predicted 2 outcomes for the *Pseudomonas* primer pair and only 1 outcome for the *Lactobacillus* primer pair giving the model an overall success rate of 87.5%, i.e. 21/24 predicted outcomes were correct. Based upon this, it was decided to continue using the model in its current format without further adjustment to the scoring system in order to determine the likelihood of qPCR primers amplifying non-target sequences.

Table 2.6: Results of analysis of two primer pairs against cDNA from 14 different bacterial isolates. Where sequence data was available, predictions as to the likelihood of successful PCR were made using the model outlined in section 2.5. Letter/Letter represents the score for the forward and reverse PCR primer. P = successful PCR amplification is expected. NP = successful PCR amplification is not expected. Results highlighted in orange indicate where the outcome of the PCR was not as predicted by the model.

Species of bacteria	Model score for each primer and presence of product			
	Pseudomonas		Lactobacillus	
Aeromonas allosaccharophila S39232.2	M(5)/M(5)	NP	K/D	P
Aeromonas hydrophila KJ743719.1	M(5)/M(5)	NP	K/D	P
Bacillus cereus KJ833790.1	-/M(5)	NP	-/B	P
Bacillus thuringiensis KJ722440.1	M(10)/M(5)	NP	B/B	P
Citrobacter freundii JX860619.1	M(4)/M(5)	NP	K/D	P
Edwardsiella ictaluri KC789872.1	L/M(5)	NP	K/D	P
Vibrio sp. GQ359963.1	K/M(6)	NP	K/D	NP
Aeromonas hydrophila	NP		P	
Aeromonas sobria	NP		P	
Citrobacter farmeri	NP		P	
Citrobacter youngae	P		P	
Pseudomonas alcaligenes	NP		NP	
Pseudomonas putida	P		NP	
Pseudomonas fluorescens	P		P	

2.8 – Using the model to analyse primer pairs.

Primer pairs for the bacterial 16S rDNA gene (Thompson *et al.* 2004, Martinez-Puig *et al.* 2007, Bergmark *et al.* 2012, Adamek *et al.* 2013) were compared against sequences obtained from the ENA database as outlined in section 2.2. The model described in section 2.5 was utilised in order to assess the likelihood of a primer pair amplifying both target and non-target DNA sequences.

The first set of primers analysed were the total 16S rDNA primers (Adamek *et*

al. 2013). The average likelihood of binding was calculated to be 0.969 (Table 2.7) against bacteria that had previously been identified as being present within the gut of carp such as *Aeromonas*, *Pseudomonas*, *Lactobacillus*, Enterobacteriaceae and *Vibrio* (Sugita *et al.* 1996, Jung-Schroers *et al.* 2015).

A pair of 16S primers generated against Enterobacteriaceae sp. (Martinez-Puig *et al.* 2007) were analysed using the model and both forwards and reverse primers had a probability of less than 0.15 of annealing to target sequences therefore further analysis was not performed (Table 2.8) due to the low probability of amplification of target sequences.

From the same paper as the Enterobacteriaceae sp. primers (Martinez-Puig *et al.* 2007), a pair of 16S primers to *Lactobacillus* sp. were also analysed (Table 2.9). From the model, a probability of 0.984 was calculated when the primer pair were compared to target 16S sequences. Although the probability of successful annealing as calculated based upon sequences receiving a score of A, B, D, F, I or L is less than 0.005 for the genera *Aeromonas*, *Vibrio* and the class Enterobacteriaceae, it can clearly be seen in Table 2.9 that many sequences received a score where it was not known if successful annealing would occur (C, E, H or K). Additionally, the primers have a probability of 0.947 of annealing to a 16S sequence from a *Bacillus* sp.. The only genus analysed where amplification will not occur is *Pseudomonas* due to the fact the forward primer scored exclusively M, i.e. 4 or more errors within each primer, for the sequences analysed based upon the model in its current format. If it proves that 4 errors can still result in successful annealing of a primer, due to the fact the reverse primer of 0.951 of successfully annealing, there is a strong chance *Pseudomonas* sp. sequences will also be amplified by this primer pair.

The *Vibrio* sp. 16S primers analysed (Thompson *et al.* 2004) have a probability of 0.978 that they will successfully anneal to target sequences (Table 2.10). The calculated probability of successful annealing as described in section 2.6 is less than 0.001 for all other genera analysed, however the reverse primer has a probability of 0.990 of successfully annealing to a sequence from an *Aeromonas* sp.. Additionally, 99% of the *Aeromonas* sp. sequences against which the forward primer was compared gave a score where it is unknown whether successful annealing is likely to happen (C, E, H or K). In order to accurately determine if this primer pair will amplify *Aeromonas* sp., a more in depth analysis of the annealing capabilities of primers with a "maybe" score (C, E, H or K) must be performed.

Pseudomonas sp. specific 16S primers (Bergmark *et al.* 2012) have a probability of 0.947 of annealing to target sequences (Table 2.11). The probability of these primers successfully annealing to sequences from the genera *Aeromonas*, *Lactobacillus* and *Bacillus* is less than 0.001. This is also the probability for the genus *Vibrio*, however 70% of the *Vibrio* sequences against which the reverse primer was compared gave a score where it is unknown if successful annealing is likely (C, E, H or K). Should these scores prove conducive to successful annealing, this could significantly increase the probability of the primer pair as a whole amplifying *Vibrio* 16S sequences in addition *Pseudomonas*, i.e. the target sequences.

Table 2.7: Analysis of total 16S rDNA primers (Adamek *et al.* 2013) using the model outlined in section 2.5. Primers are compared to 16S rDNA sequences from different bacteria genera and the probability of the primer pair annealing to sequences from each genus is calculated. Green represents a score of A, B, D, F, I or L and is defined as likely to anneal. Blue represents a score of C, E, H or K and is defined as unknown if successful annealing will occur. Red represents a score of G, J or M and is defined as annealing is unlikely to occur.

Total 16S rDNA primers: analysis by genus				
Aeromonas sp.	Forward	469	Reverse	464
	A,B,D,F,I,L	460	A,B,D,F,I,L	464
	C,E,H,K	8	C,E,H,K	0
	G,J,M	1	G,J,M	0
	Probability of successful binding	Forward	$P(F) = 0.981$	
		Reverse	$P(R) = 1.000$	
		Both	$\boldsymbol{P(F \cap R) = 0.981}$	
Bacillus sp.	Forward	654	Reverse	616
	A,B,D,F,I,L	642	A,B,D,F,I,L	610
	C,E,H,K	4	C,E,H,K	2
	G,J,M	8	G,J,M	4
	Probability of successful binding	Forward	$P(F) = 0.982$	
		Reverse	$P(R) = 0.990$	
		Both	$\boldsymbol{P(F \cap R) = 0.972}$	
Enterobacteriaceae sp.	Forward	471	Reverse	409
	A,B,D,F,I,L	452	A,B,D,F,I,L	408
	C,E,H,K	11	C,E,H,K	0
	G,J,M	8	G,J,M	1
	Probability of successful binding	Forward	$P(F) = 0.960$	
		Reverse	$P(R) = 0.998$	
		Both	$\boldsymbol{P(F \cap R) = 0.957}$	
Lactobacillus sp.	Forward	486	Reverse	461
	A,B,D,F,I,L	408	A,B,D,F,I,L	476
	C,E,H,K	0	C,E,H,K	10
	G,J,M	1	G,J,M	0
	Probability of successful binding	Forward	$P(F) = 0.979$	
		Reverse	$P(R) = 0.996$	
		Both	$\boldsymbol{P(F \cap R) = 0.975}$	
Pseudomonas sp.	Forward	305	Reverse	327
	A,B,D,F,I,L	287	A,B,D,F,I,L	326
	C,E,H,K	16	C,E,H,K	0
	G,J,M	2	G,J,M	1
	Probability of successful binding	Forward	$P(F) = 0.941$	
		Reverse	$P(R) = 0.997$	
		Both	$\boldsymbol{P(F \cap R) = 0.938}$	
Vibrio sp.	Forward	338	Reverse	339
	A,B,D,F,I,L	327	A,B,D,F,I,L	339
	C,E,H,K	8	C,E,H,K	0
	G,J,M	1	G,J,M	0
	Probability of successful binding	Forward	$P(F) = 0.967$	
		Reverse	$P(R) = 1.000$	
		Both	$\boldsymbol{P(F \cap R) = 0.967}$	

Table 2.8: Analysis of Enterobacteriaceae sp. primers against 16S rDNA sequences from Enterobacteriaceae sp. and scored as per the model (section 2.5). The probability of successful annealing is calculated. Green represents a score of A, B, D, F, I or L and is defined as likely to anneal. Blue represents a score of C, E, H or K and is defined as unknown if successful annealing will occur. Red represents a score of G, J or M and is defined as annealing is unlikely to occur.

Enterobacteriaceae 16S rDNA primers: analysis by genus				
Enterobacteriaceae sp.	Forward	988	Reverse	1036
	A,B,D,F,I,L	448	A,B,D,F,I,L	341
	C,E,H,K	50	C,E,H,K	127
	G,J,M	490	G,J,M	568
	Probability of successful binding	Forward	$P(F) = 0.453$	
		Reverse	$P(R) = 0.329$	
		Both	$\boldsymbol{P(F \cap R) = 0.149}$	

Table 2.9: Analysis of 16S rDNA primers designed to amplify only bacteria from the genus *Lactobacillus*. Primers are compared against 16S sequences from multiple genera and scored using the model described in section 2.5. The probability of successful annealing is shown. Green represents a score of A, B, D, F, I or L and is defined as likely to anneal. Blue represents a score of C, E, H or K and is defined as unknown if successful annealing will occur. Red represents a score of G, J or M and is defined as annealing is unlikely to occur.

***Lactobacillus* 16S rDNA primers: analysis by genus**				
Aeromonas sp.	Forward	480	Reverse	473
	A,B,D,F,I,L	0	A,B,D,F,I,L	473
	C,E,H,K	477	C,E,H,K	0
	G,J,M	3	G,J,M	0
	Probability of successful binding		Forward	$P(F) = 0.000$
			Reverse	$P(R) = 1.000$
			Both	$\boldsymbol{P(F \cap R) = 0.000}$
Bacillus sp.	Forward	759	Reverse	506
	A,B,D,F,I,L	729	A,B,D,F,I,L	499
	C,E,H,K	25	C,E,H,K	5
	G,J,M	5	G,J,M	2
	Probability of successful binding		Forward	$P(F) = 0.960$
			Reverse	$P(R) = 0.986$
			Both	$\boldsymbol{P(F \cap R) = 0.947}$
Enterobacteriaceae sp.	Forward	1110	Reverse	1020
	A,B,D,F,I,L	0	A,B,D,F,I,L	970
	C,E,H,K	1083	C,E,H,K	39
	G,J,M	27	G,J,M	11
	Probability of successful binding		Forward	$P(F) = 0.000$
			Reverse	$P(R) = 0.951$
			Both	$\boldsymbol{P(F \cap R) = 0.000}$
Lactobacillus sp.	Forward	657	Reverse	493
	A,B,D,F,I,L	653	A,B,D,F,I,L	488
	C,E,H,K	1	C,E,H,K	3
	G,J,M	3	G,J,M	2
	Probability of successful binding		Forward	$P(F) = 0.994$
			Reverse	$P(R) = 0.984$
			Both	$\boldsymbol{P(F \cap R) = 0.990}$
Pseudomonas sp.	Forward	416	Reverse	384
	A,B,D,F,I,L	0	A,B,D,F,I,L	367
	C,E,H,K	0	C,E,H,K	13
	G,J,M	416	G,J,M	4
	Probability of successful binding		Forward	$P(F) = 0.000$
			Reverse	$P(R) = 0.956$
			Both	$\boldsymbol{P(F \cap R) = 0.000}$
Vibrio sp.	Forward	464	Reverse	348
	A,B,D,F,I,L	2	A,B,D,F,I,L	342
	C,E,H,K	455	C,E,H,K	1
	G,J,M	7	G,J,M	5
	Probability of successful binding		Forward	$P(F) = 0.004$
			Reverse	$P(R) = 0.983$
			Both	$\boldsymbol{P(F \cap R) = 0.004}$

Table 2.10: Analysis of 16S rDNA primers designed to amplify only bacteria from the genus *Vibrio*. Primers are compared against 16S sequences from multiple genera and scored using the model described in section 2.5. The probability of successful annealing is shown. Green represents a score of A, B, D, F, I or L and is defined as likely to anneal. Blue represents a score of C, E, H or K and is defined as unknown if successful annealing will occur. Red represents a score of G, J or M and is defined as annealing is unlikely to occur.

***Vibrio* 16S rDNA primers: analysis by genus**				
Aeromonas sp.	Forward	998	Reverse	997
	A,B,D,F,I,L	0	A,B,D,F,I,L	987
	C,E,H,K	992	C,E,H,K	4
	G,J,M	6	G,J,M	6
	Probability of successful binding		Forward	$P(F) = 0.000$
			Reverse	$P(R) = 0.990$
			Both	$\boldsymbol{P(F \cap R) = 0.000}$
Bacillus sp.	Forward	672	Reverse	659
	A,B,D,F,I,L	0	A,B,D,F,I,L	0
	C,E,H,K	256	C,E,H,K	0
	G,J,M	416	G,J,M	659
	Probability of successful binding		Forward	$P(F) = 0.000$
			Reverse	$P(R) = 0.000$
			Both	$\boldsymbol{P(F \cap R) = 0.000}$
Enterobacteriaceae sp.	Forward	345	Reverse	308
	A,B,D,F,I,L	5	A,B,D,F,I,L	289
	C,E,H,K	332	C,E,H,K	9
	G,J,M	8	G,J,M	10
	Probability of successful binding		Forward	$P(F) = 0.014$
			Reverse	$P(R) = 0.938$
			Both	$\boldsymbol{P(F \cap R) = 0.014}$
Lactobacillus sp.	Forward	533	Reverse	506
	A,B,D,F,I,L	0	A,B,D,F,I,L	0
	C,E,H,K	0	C,E,H,K	0
	G,J,M	533	G,J,M	506
	Probability of successful binding		Forward	$P(F) = 0.000$
			Reverse	$P(R) = 0.000$
			Both	$\boldsymbol{P(F \cap R) = 0.000}$
Pseudomonas sp.	Forward	527	Reverse	527
	A,B,D,F,I,L	0	A,B,D,F,I,L	0
	C,E,H,K	0	C,E,H,K	91
	G,J,M	539	G,J,M	436
	Probability of successful binding		Forward	$P(F) = 0.000$
			Reverse	$P(R) = 0.000$
			Both	$\boldsymbol{P(F \cap R) = 0.000}$
Vibrio sp.	Forward	373	Reverse	342
	A,B,D,F,I,L	368	A,B,D,F,I,L	339
	C,E,H,K	1	C,E,H,K	1
	G,J,M	4	G,J,M	2
	Probability of successful binding		Forward	$P(F) = 0.987$
			Reverse	$P(R) = 0.991$
			Both	$\boldsymbol{P(F \cap R) = 0.978}$

Table 2.11: Analysis of 16S rDNA primers designed to amplify only bacteria from the genus *Pseudomonas*. Primers are compared against 16S sequences from multiple genera and scored using the model described in section 2.5. The probability of successful annealing is shown. Green represents a score of A, B, D, F, I or L and is defined as likely to anneal. Blue represents a score of C, E, H or K and is defined as unknown if successful annealing will occur. Red represents a score of G, J or M and is defined as annealing is unlikely to occur.

***Pseudomonas* 16S rDNA primers: analysis by genus**				
Aeromonas sp.	Forward	1060	Reverse	909
	A,B,D,F,I,L	0	A,B,D,F,I,L	0
	C,E,H,K	0	C,E,H,K	0
	G,J,M	1060	G,J,M	909
	Probability of successful binding	Forward	$P(F) = 0.000$	
		Reverse	$P(R) = 0.000$	
		Both	$\boldsymbol{P(F \cap R) = 0.000}$	
Bacillus sp.	Forward	646	Reverse	657
	A,B,D,F,I,L	1	A,B,D,F,I,L	0
	C,E,H,K	0	C,E,H,K	0
	G,J,M	645	G,J,M	657
	Probability of successful binding	Forward	$P(F) = 0.002$	
		Reverse	$P(R) = 0.000$	
		Both	$\boldsymbol{P(F \cap R) = 0.000}$	
Enterobacteriaceae sp.	Forward	1117	Reverse	1028
	A,B,D,F,I,L	72	A,B,D,F,I,L	1
	C,E,H,K	21	C,E,H,K	0
	G,J,M	1024	G,J,M	1027
	Probability of successful binding	Forward	$P(F) = 0.000$	
		Reverse	$P(R) = 0.001$	
		Both	$\boldsymbol{P(F \cap R) = 0.000}$	
Lactobacillus sp.	Forward	649	Reverse	500
	A,B,D,F,I,L	0	A,B,D,F,I,L	0
	C,E,H,K	0	C,E,H,K	0
	G,J,M	649	G,J,M	500
	Probability of successful binding	Forward	$P(F) = 0.000$	
		Reverse	$P(R) = 0.000$	
		Both	$\boldsymbol{P(F \cap R) = 0.000}$	
Pseudomonas sp.	Forward	880	Reverse	976
	A,B,D,F,I,L	856	A,B,D,F,I,L	950
	C,E,H,K	7	C,E,H,K	7
	G,J,M	17	G,J,M	19
	Probability of successful binding	Forward	$P(F) = 0.973$	
		Reverse	$P(R) = 0.956$	
		Both	$\boldsymbol{P(F \cap R) = 0.930}$	
Vibrio sp.	Forward	461	Reverse	350
	A,B,D,F,I,L	32	A,B,D,F,I,L	0
	C,E,H,K	7	C,E,H,K	242
	G,J,M	422	G,J,M	108
	Probability of successful binding	Forward	$P(F) = 0.069$	
		Reverse	$P(R) = 0.000$	
		Both	$\boldsymbol{P(F \cap R) = 0.000}$	

2.9 – Conclusion.

The model and data presented in this chapter highlights the importance of checking primers for specificity, specifically those designed for use with the bacterial 16S rDNA housekeeping gene, before use, even if they are obtained from published journals. Defining the likelihood of a primer pair annealing to non-target DNA sequences is not a common way of considering primers during primer design and, as such, development of a means of analysing a primers ability to amplify non-target DNA needs careful consideration. Testing of primers against DNA templates taken from pure bacterial cultures shows that the final model presented is successful in identifying if a primer pair is likely to successfully amplify non-target DNA sequences before carrying out laboratory work. Although the secondary level of scoring, i.e. if a score based upon the number of errors present is likely to result in annealing, has only been described for one particular set of PCR conditions, this can be adjusted to suit other conditions.

The advantage of using a model such as the one presented is it reduces the long term costs of testing if primers anneal to non-target sequences through laboratory based studies. Primer pairs can be analysed *in silico* and the probability of successful annealing to either target or non-target DNA calculated before any time is spent within a laboratory. This model was utilised to analyse all 16S primer pairs utilised within this thesis.

Chapter 3 – Optimisation of microbiological techniques.

This following chapter presents work pertaining to the optimisation of techniques used during culture based microbiology analysis. Whilst the molecular methodologies employed within this thesis had been previously optimised, those required for analysis of bacteria cultures had not. This chapter also details the design and optimisation of the digital image analysis program PENGUIN which was built in collaboration with web developer Todd Specht (The Woodpecker Project, UK) as a means of accurately measuring differences in bacterial colony size. A list of all chemicals and equipment utilised within the protocols presented in this chapter can be found in Appendix 2.

3.1 – Preparation of MacroGard®.

The aim of all assays presented within this chapter is to establish the best means of measuring the effect of MacroGard® on the rate of bacterial growth. As previously stated, MacroGard® is a β-1/3,1/6-glucan compound produced by Biorigin (Brazil) that contains approximately 60% β-glucan. MacroGard® was prepared as described by the manufacturers as follows: MacroGard® powder was weighed out and mixed with sterile nutrient broth at a concentration of 10% w/v. This was then sonicated in two 30 second bursts using a Sonics Vibra-cell sonicator set on 6 (high). MacroGard® was then incubated at 80°C for 20 minutes and either added to liquid agar before cooling (~50°C) or cooled to 4°C in a fridge before being mixed with nutrient broth.

3.2 – Colourmetric assessment of inhibition of bacterial growth.

A minimum inhibition concentration assay (Mann and Markham 1998) using the dye resazurin was utilised to determine the concentrations of MacroGard® that were toxic to a bacterial suspension (see section 4.2.2 for the details of bacteria utilised). Using a "checkerboard" format on a 96 well plate, concentrations of both bacteria and MacroGard® were analysed as shown in Figure 3.1. Briefly, bacteria were incubated with shaking overnight at 100RPM in 50ml nutrient broth at room temperature (20°C). In order to ascertain the concentration of the bacteria suspension, a colony forming unit (CFU) count was performed. 50µl of a 1:10 serial dilution of inoculated broth was applied to nutrient agar plates (3x per dilution) and incubated at 20°C for 24 hours and the number of CFUs in the undiluted broth was calculated using the following equation:

$$CFU(per\ ml) = (mx + c) \times 20$$

Where m is the slope of the linear plot of CFU versus dilution factor, x is the desired dilution factor, i.e. 1, and c is the point at which the linear trendline intercepts the y axis.

During this time, the original inoculated broth was kept at 4°C to minimise further growth. Once the concentration was determined, suspensions were warmed to room temperature by incubation at 20°C for 20 minutes. MacroGard® was prepared as described in section 3.1 and added to a 96 well plate as shown in Figure 3.1 to produce a final concentration of 0.1% w/v to 0.01% w/v MacroGard® in 100µl of nutrient broth. 100µl of bacteria inoculated nutrient broth was added to each well across a 96 well plate as described in Figure 3.1 giving a final total volume of 200µl

per well. Plates were incubated at room temperature with shaking (MaxQ 4000 incubator, 125RPM) for 18 hours. 10µl of 0.01% w/v resazurin dye was added to each well and the plate incubated for a further 15 minutes at room temperature with shaking (125RPM). In this colorimetric assay, live bacteria metabolise the resazurin dye turning the solution within the well from blue to pink which is visible by eye as illustrated in Figure 3.2. Inhibition of growth is shown by the solution remaining blue after the final incubation period.

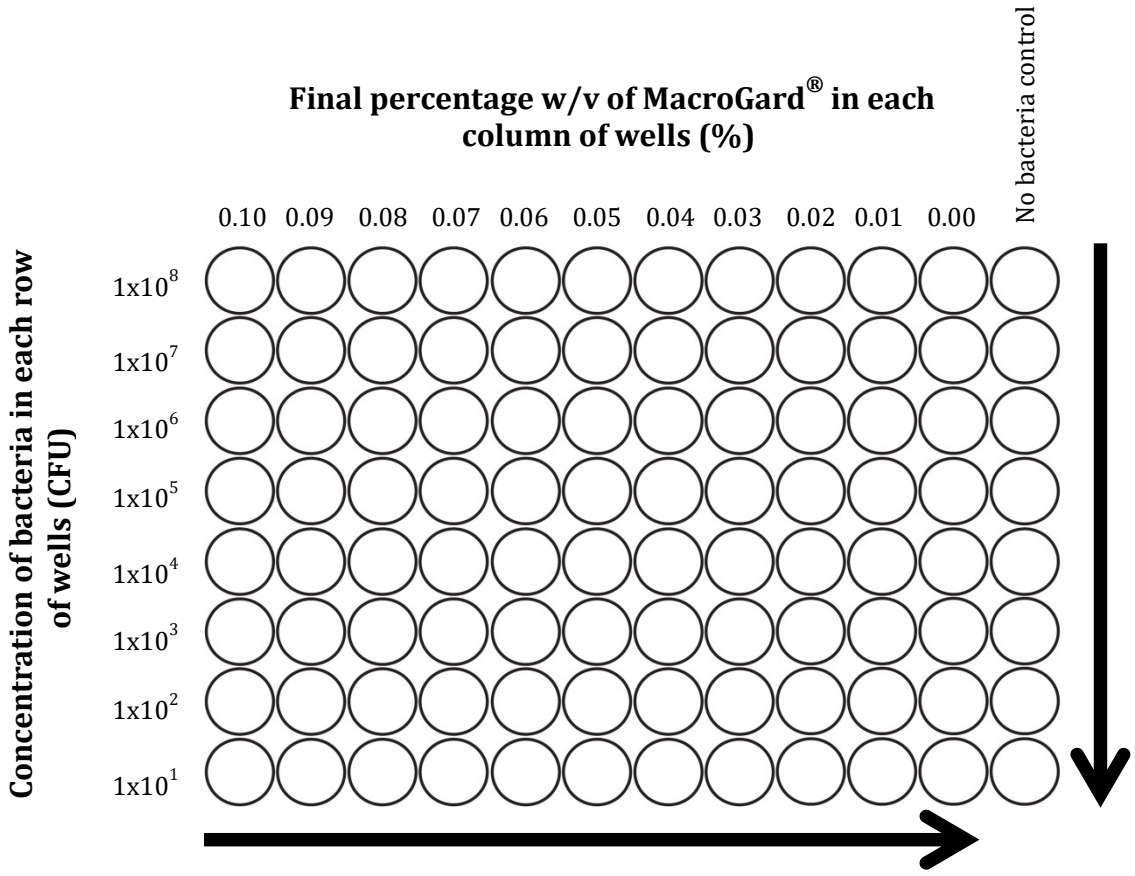


Figure 3.1: Schematic showing the layout of 96 well plates utilised to determine inhibitory concentrations of MacroGard® to bacterial growth. A checkerboard design was applied with columns containing varying MacroGard® concentrations (final concentration of 0.1 – 0.0% w/v) and rows containing a range of initial bacteria concentrations ($1x10^8$ – $1x10^1$ CFU). Column 11 (0.0% MacroGard®) is a MacroGard® negative control. Column 12 is a bacteria negative control. Image adapted from http://www.cellsignet.com/media/plates/96.jpg.

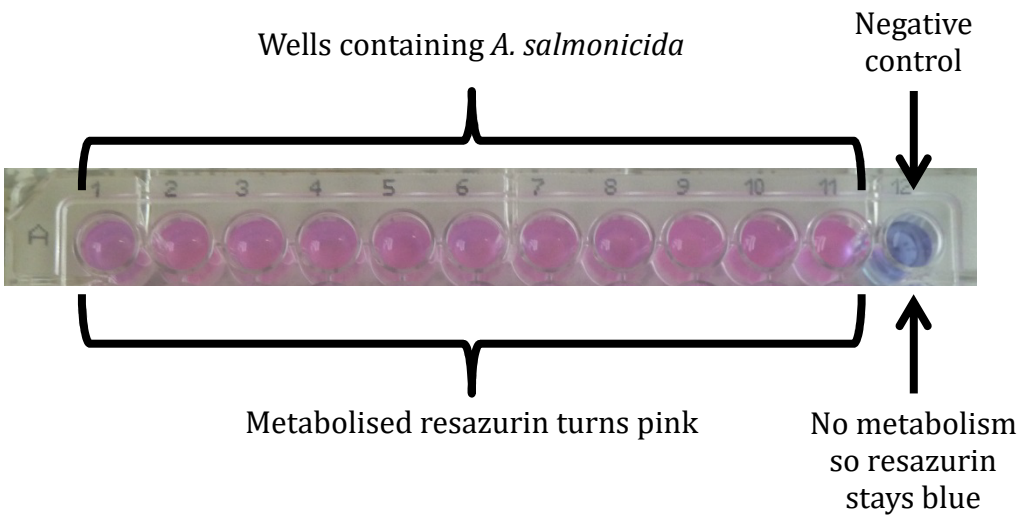


Figure 3.2: Image illustrating the visible by eye colour change that occurs during a resazurin dye based assay. As described in section 3.2, 10µl of resazurin dye (0.01%w/v) is added to an overnight culture and incubated at room temperature (~20°C) for 15 minutes with shaking (MaxQ 4000 incubator, 125RPM). Wells 1-11 were inoculated with *Aeromonas salmonicida* which has metabolised the blue resazurin dye (as seen in well 12 which is a negative control) resulting in a visible colour change to pink.

3.3 – Analysing bacterial growth after incubation in nutrient broth.

Whilst the resazurin assay presented in section 3.2 is an efficient method of analysing a wide range of MacroGard® concentrations in a short space of time, it only shows inhibition and not an increase of growth. Therefore a second broth based assay was considered that allows for identification of both inhibition and promotion of bacterial growth (see section 4.2.2 for details of bacteria utilised).

50ml nutrient broth was inoculated with bacteria and incubated with shaking (100RPM) overnight at room temperature (~20°C). The number of bacteria within the broth after incubation was determined by CFU count as previously described in section 3.2. $1x10^5$CFU (in a volume of 1ml) of bacteria was added to a fresh 50ml nutrient broth preparation, or a 50ml preparation containing 0.1% or 0.01% w/v MacroGard®. These newly inoculated cultures were then incubated for 18 hours at

room temperature after which quantification of CFU was performed based upon a 1:10 serial dilution with counts being taken on plates with less than 300 colonies. To compare differences in average CFU at a specific dilution factor, e.g. $1x10^{-5}$, a 1 way ANOVA was used after the data was checked for normality and homoscedasticity using an Anderson-Darling test and Levene's test respectively.

3.4 – Analysing bacterial growth when MacroGard® is present in a solid agar.

The advantage of the protocol outlined in section 3.3 is that it is possible to detect both inhibition and promotion of bacterial growth, however it is a time consuming protocol taking up to 5 days to complete. As such, ways of reducing this timeframe whilst still having the ability to analyse both inhibition and promotion were considered. The next approach trialled was embedding MacroGard® into a nutrient agar plate. As MacroGard® is not completely soluble, different approaches to achieve an even distribution within the agar were considered.

Firstly, MacroGard®, at a final concentration of 0.1% w/v liquid agar, was added to the agar mix before being autoclaved (121°C for 15 minutes), however the MacroGard® did not disperse evenly within the mix and clumped into visible lumps throughout the plates that could not be broken up through shaking or vortexing the liquid agar (~50°C).

Secondly, MacroGard® was prepared at a concentration of 10% w/v in nutrient broth as described in section 3.1 and added to the nutrient agar (final concentration of 0.1% w/v) after it had been autoclaved. Pouring the MacroGard® into the liquid agar (~50°C) and mixing by hand resulted in an even distribution of MacroGard® by eye which was confirmed by use of the PENGUIN program (see

section 3.5) which showed the same amount of variation between different areas of the same plate and different plates as was present in standard nutrient agar without MacroGard®.

These MacroGard® embedded plates were therefore used to establish the effects of this carbohydrate on bacterial growth. Briefly: bacteria cultures (as detailed in section 4.2.6) were grown overnight at 20°C in 30ml nutrient broth after which they were subject to a 1:100,000 dilution (performed as a 1:10 serial dilution) and 50µl of bacteria was applied to either MacroGard® embedded nutrient agar plates or standard nutrient agar plates (control) in triplicate. Plates were then incubated for up to 72 hours and the number of CFU per plate measured. Statistical analysis was performed as described in section 3.3.

3.5 – Using image analysis to measure bacterial growth rates.

Whilst the previously described methods measure any toxic effect or an increase in bacterial growth, they do not consider if there is an effect upon the speed at which the bacteria growth took place. The following sections described the ideas behind and optimisation of a program that was used to measure bacteria colony size based upon colour changes on an agar plate over time. Using a descriptive moniker, the program has been named "PlatE aNalysis proGram UsINg pixels to measure bacteria colony size", i.e. PENGUIN.

3.5.1 – Concept development.

Vera-Jimenez and Nielsen (2013) describe the use of digital imaging to measure rates of cell growth by transforming photographs of monolayers into a

binary system whereby measurements were taken of pixel change, i.e. a black pixel that was previously white represents growth of the monolayer. Digital imaging has been successfully used to map out changes in size of wounds on the skin of carp and rainbow trout (Przybylska-Diaz *et al.* 2013, Schmidt *et al.* 2016), and it was based on these preliminary observations that the idea of utilising this concept to measure growth rates of bacterial colonies came about.

The use of digital image analysis to measure bacteria cell growth is not new and there is equipment available (e.g. Sorcerer Colony Counter by Perceptive Instruments; Topac Colony Counter) to measure inhibition zones as part of microbiological analysis but, within the scope of this PhD, the costs associated with such products were prohibitory and have the limitation that they cannot be adapted for field based studies.

To this end, a collaboration was established with Todd Specht (The Woodpecker Project, United Kingdom) to generate a tool capable of measuring bacterial colony size without incurring the high costs seen with the commercially available systems. In addition, it was hoped that such a system could also be applied to other aspects of biology, and have some scope to be used in field-based aquaculture. Two approaches were taken to this: 1 – a library of "bacteria colours" would be generated against which single images of colonies could be compared, and 2 –images of bacterial growth would be compared to a base image taken directly after seeding agar plates. In both cases, images are converted into binary, i.e. pixels that are bacteria and pixels that are not bacteria, and from this, growth could be quantified using a pixel count.

3.5.2 – Writing the script for PENGUIN.

All coding work was performed by Todd Specht. The script was written using the language PHP based upon the program php-skindetection which is designed to detect skin/skin tones in images. Initial offline testing of the program showed processing power would be a major restriction therefore a cloud based server was selected to host the program. It is based upon an RGB colour model and can distinguish between 255^3 different colours.

3.5.3 – Single image analysis.

The first approach considered a means of determining pixels that represented bacteria based upon the original use for the script from which PENGUIN was developed. Php-skindetection is a programme designed to identify human skin in photographs and works by calculating the percentage of an image that was "skin" based upon a predetermined library of colours. Any pixel within the image that matched this library was defined as skin.

Transposing this concept to use it to measure bacteria colony size required knowing the colour palette of a bacteria species for analysis. *E. coli* (NCIMB 8277 kindly donated by Nigel Bowers, Keele University, UK) was used during optimisation and images of bacteria colonies on a nutrient agar base were taken under natural light conditions of colony growth after 24 hours against a black background.

An individual colony was chosen from which a range of colours (based upon the RGB colour palette) was selected for testing and a 249x273 pixel image section was analysed. A black and white image was produced whereby black is pixels that are

not within the selected colour range and white pixels are (Figure 3.3).

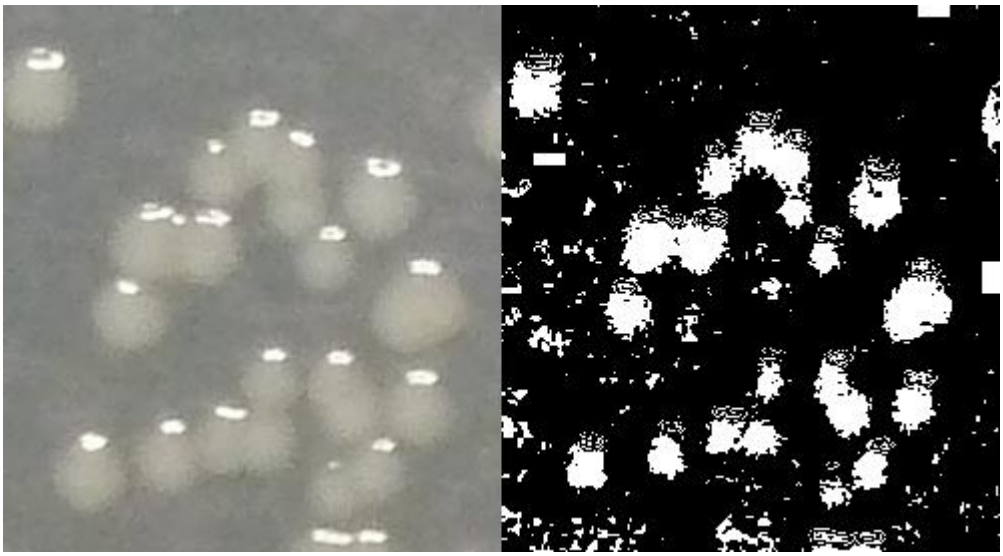


Figure 3.3: Transformation of a multi-coloured digital image into a binary representation of bacteria colonies. Left: *E. coli* colony growth after 24 hours at 37°C on nutrient agar. Picture taken using a Lumix DMC-TZ8 digital camera against a black background (Exposure time – 0.2 sec. ISO speed – ISO-400). Right: Binary image generated using a specific colour range (RGB) that represents the colours present within a colony. Black shows a pixel is not one of these colours and white pixels are.

From this, it was clear that whilst the idea itself showed promise, there was overlap between the colours within the agar and the bacteria colonies which may potentially be a significant problem at the edges of colonies in producing accurate measurements. Additionally, it was noted that whilst variation in the colour palette caused by differences in the conditions under which images were taken could be accounted for, the optimisation process would need to be repeated and a new colour range produced for each bacteria species analysed rather than it being a tool that works across multiple bacteria strains. To this end, a new design was considered based upon measuring the colour palette of the agar instead of the bacteria as this would allow for a wider application of the program.

3.5.4 – Before/after image analysis.

In this second approach to measuring colony size, rather than focusing on the colonies themselves, emphasis is put on the agar on which the bacteria grows. In order to do this, the program was designed to analyse the colour palette of an agar plate immediately after inoculation. This was then used as a base against which images of the same plate after bacteria colonies had grown were compared. The same plate was used in order to minimise any differences in the colour range that may be caused by any variations in the thickness of the agar. Colours that feature only in the secondary image are defined as "new" and therefore represent bacterial growth. From this, the average size of each colony (measured in pixels) can be determined based upon the total number of pixels that are a new colour in an "After" image divided by the number of colonies present in a sample (Figure 3.4).

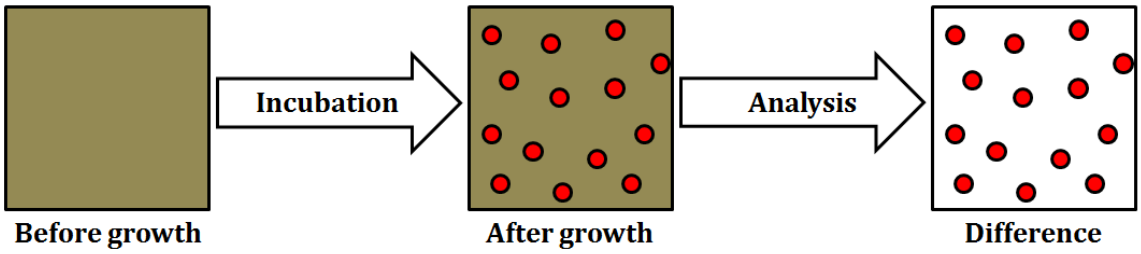
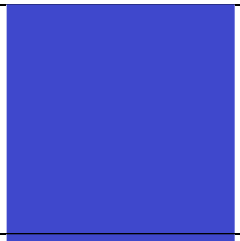
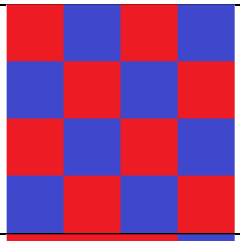
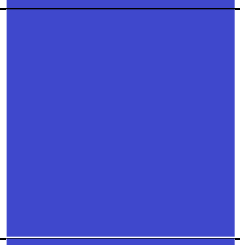
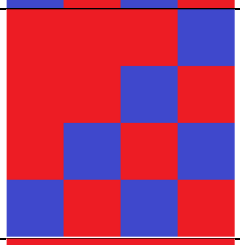
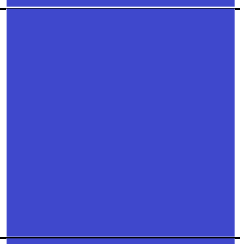
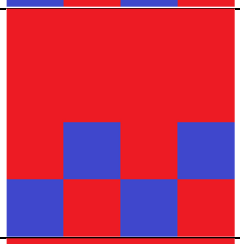
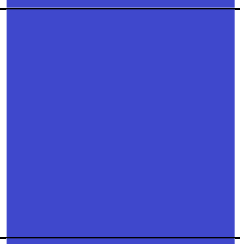
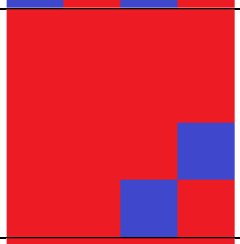
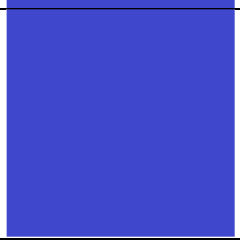
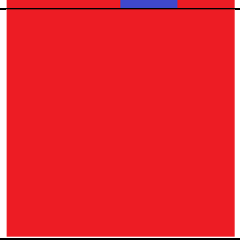


Figure 3.4: Schematic showing the principle behind a before/after approach to quantifying bacterial growth using changes in pixel colour. Images are taken of an agar plate immediately after inoculation (Before growth) and after incubation (After growth). These images are then compared to determine the size of the bacteria colonies measured in pixels. Any colour that appears in both the before growth and after growth image is defined as background. Any colour that features only in the after growth image is defined as bacterial growth. The program then calculates the total number of pixels that are a new colour in the after image. This value can then be divided by the number of colonies within the image to get an average colony size measured in pixels.

3.5.5 – Proving PENGUIN works: "simple" image analysis.

First, it was imperative to determine if the program actually worked and this was done with "simple" images, i.e. images with a limited colour palette and where it is known how many pixels are a specific colour. These images were generated using Microsoft Paint and were 500x500 pixels in size. Firstly, only two colours were used with varying numbers of pixels being changed. These results are shown in Table 3.1. This analysis showed that PENGUIN was able to accurately detect the differences in colour between the two images. Further testing was done including analysing the programs sensitivity by introducing more colours to both "Before" and "After" images, and by reducing the number of pixels that were altered. PENGUIN can accurately determine the difference between each individual colour in the RGB colour palette, i.e. 255^3 different colours, and can detect when only 1 pixel is a new colour in comparison to a "Before" image. Figure 3.5 shows the output when a "Before" image with 100 different colours is compared to an "After" image where only 1 pixel has been changed to a new colour.

Table 3.1: "Simple" images, i.e. images with only two colours, were used to test the program PENGUIN worked. Images were generated using Microsoft Paint and are 500x500 pixels in size. Results are given as the percentage of the After image that are a "new" colour, i.e. a colour that is not found in the Before image. The percentage difference was calculated manually in addition to analysing the images using PENGUIN.

Before	After	Manually calculated percentage difference	PENGUIN calculated percentage difference
		50% different	50% different
		62.5% different	62.5% different
		75% different	75% different
		87.5% different	87.5% different
		100% different	100% different

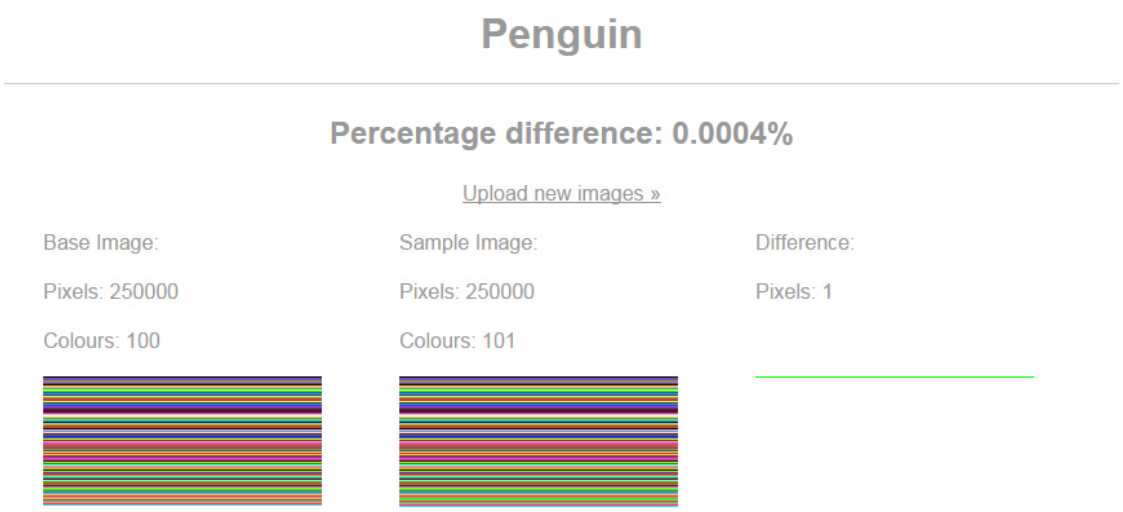


Figure 3.5: The output generated by PENGUIN when a Before image containing 100 different colours (500x500 pixels) is altered by one pixel to a new colour. For the Base Image and Sample Image, i.e. before and after, the number of pixels per image and the number of colours present are given. The final column, Difference, gives the number of pixels that are a colour that is present only in the Sample Image. For all three columns, a visual representation of all colours is given. The Percentage difference given at the top of the output is the percentage of pixels within the Sample Image that are a colour present only in this image, i.e. the pixels identified in the Difference column.

3.5.6 – Optimisation of conditions for "real" images.

Having proven that PENGUIN works, the next step was to optimise the conditions for "real" images, i.e. images of agar plates with and without bacteria colonies. The following aspects were taken into consideration:

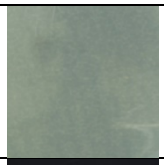
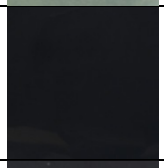
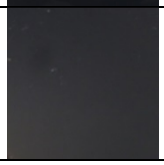
- Length of exposure
- Source of light
- Location of light

Once optimisation of these conditions was performed, PENGUIN was tested using *E. coli* as an example bacteria species.

3.5.6.1 – Source of light.

Light has a significant impact on the colour palette within an image therefore it was of the utmost importance to ensure minimal light variation between "Before" and "After" images. In order to determine the variation of the colour palette produced for "Before" images under different light conditions, 10 images were taken of a nutrient agar plate and the number of colours PENGUIN detected and the average percentage difference between images taken under the same lighting conditions were compared (Table 3.2). Images taken under "natural light" conditions were obtained next to a window on a partially cloudy day. Interestingly, these images had the lowest variability when comparing the average percentage difference, however use of natural light would not be possible if, for example, analysis was to be performed every 12 hours. Images were also taken using a Phillips Master TL4 HE strip light bulb as the light source. This proved to have the highest level of variability when comparing the average percentage difference and an even higher standard deviation. In order to actually detect any differences between "Before" and "After" images, the number of comparisons that would need to be made would be too great to be practically plausible. Finally, using a white tungsten bulb (100W) as a light source was tested. The variation when comparing the average percentage difference is approximately double that of natural light, however where natural light varies based upon the amount of sunlight, the use of a light bulb provides a constant amount of light irrespective of time of day and weather conditions that both affect natural light conditions. Additionally, the number of colours present under tungsten bulb light conditions was the highest indicating these conditions could provide a higher level of sensitivity than the other conditions tested.

Table 3.2: The impact of three different lighting conditions on the variability of images taken of a nutrient agar plate was studied. Average number of colours per image and the difference in the colour range were taken into consideration when deciding which conditions were the most suitable for use. n=10 images per light condition. s.d. = standard deviation.

Light conditions		Average number of colours	Average percentage difference	Considerations
Natural light		935.5 (s.d. ±35.0)	0.29% (s.d. ±0.17)	Smallest variation between images however cannot control e.g. how time of day affects the colour palette.
Strip light		278.4 (s.d. ±40.7)	1.64% (s.d. ±2.87)	High level of variability gives a larger standard deviation than the average percentage difference.
Tungsten bulb		2843.8 (s.d. ±110.5)	0.57% (s.d. ±0.16)	Most reproducible conditions and widest range of colours detected.

3.5.6.2 – Length of exposure.

The time taken for each image to be captured is important for two reasons. Firstly, taking images of a closed plate proved troublesome due to the reflective properties of the lid of the agar plate, therefore it was decided to take images of open plates. This meant the longer a plate was open, the more chance there was of a contamination occurring. Secondly, longer exposure times increased the average percentage difference between images as shown in Figure 3.6. Images were taken of a nutrient agar plate on a black background under a tungsten bulb light source with different exposure times ranging from 0.1 seconds to 1 second. Although there was no statistical difference between the average percentage difference, i.e. the percentage of pixels that are a colour that does not feature in the "Before" image, the smallest standard deviation was seen with an exposure time of 0.2 seconds, therefore this was selected for further image generation.

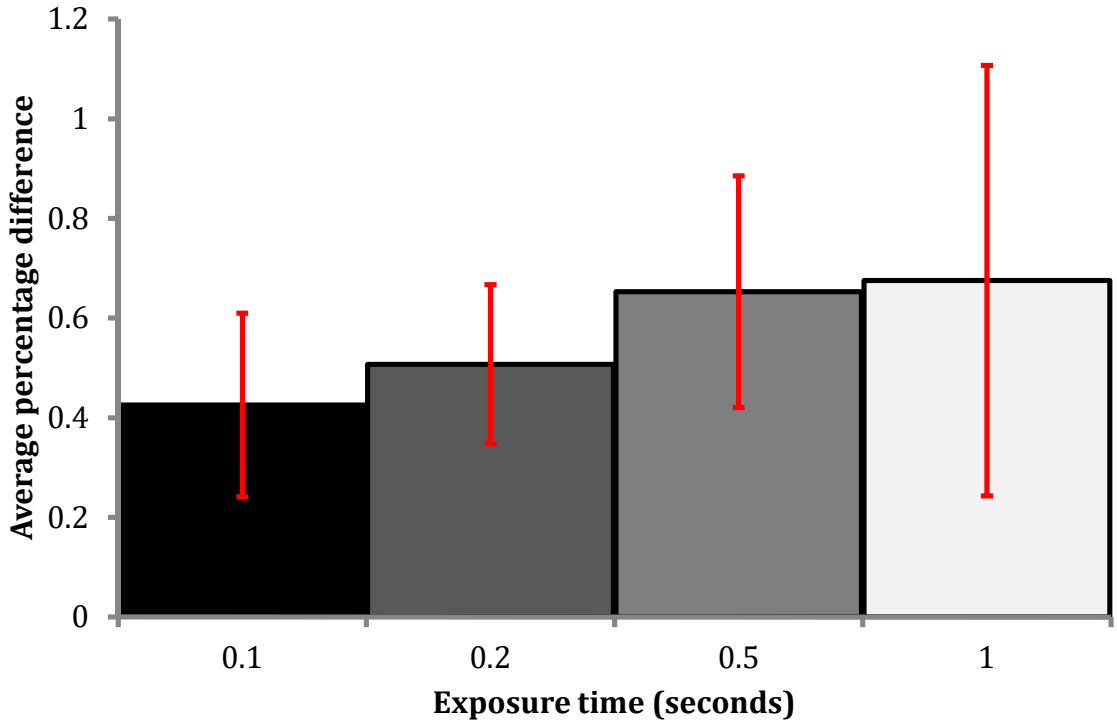


Figure 3.6: The effect of exposure time on the average percentage difference in the colours present within images of a nutrient agar plate was studied. Images (n=10) were taken under using a tungsten bulb as a light source against a black background. Error bars represent standard deviation.

3.5.6.3 – Location of the light source.

Another challenge faced when designing the conditions under which images were captured was the position of the light source. Placing the bulb directly above the plate resulted in both a shadow being produced by the camera and a reflection of the light itself, which obscured colonies within the image (this reflection of light was why using the camera's own flash was not considered in section 3.5.6.1). Having the bulb to the side of the plate resulted in the potential for shadows being cast by the edge of the plate and also from the colonies themselves. Therefore it was suggested that taking multiple images with the light source in different locations could eliminate any impact of shadow on the data produced. This was achieved by rotating the plate rather than moving the equipment in order to ensure the distance of the light source

and the camera from the nutrient agar plate were kept the same at all times. In addition, at this stage of optimisation, a light box (a three sided frame made of white 390mm squares set as a square base with the side where the tripod stood "open") was introduced to minimise the effect of light bouncing off the surfaces of any nearby equipment within the laboratory which could introduce variation between images.

Images were taken of a nutrient agar plate immediately after inoculation with *E. coli* under a tungsten light bulb on a black background with an exposure time of 0.2 seconds. The plate was initially rotated by 90° with two images being taken at each orientation. Images were taken under the same conditions after 24 hours of incubation at 37°C. PENGUIN was used to calculate the percentage difference between images taken at different orientations. A 2-sample t power analysis was performed on this data in order to determine the number of images that should be taken "Before" and "After" incubation to gain an accurate value for how many pixels were a "new" colour, i.e. were bacteria. This data is shown in Table 3.3 and shows that the minimum number of comparisons performed to have a power of 0.8 is 33. This translates to 6 images to be taken "Before" and "After", i.e. 36 comparisons, however in order to accommodate the possibility of a "bad" image (e.g. out of focus) impacting the data, it was decided to take 8 images at each time point. This additionally reduced the "distance" between each orientation, i.e. plates were rotated 45° between each image rather than 60° (360/6).

Table 3.3: Data used to perform a power analysis to determine how many "Before" and "After" images are required to accurately utilise the program PENGUIN. Before images were taken of nutrient agar plates immediately after inoculation with *E. coli* under a tungsten lightbulb on a light background with an exposure time of 0.2 seconds. After images were taken under the same conditions 24 hours after incubation at 37°C. Images were taken in 4 different orientations (rotating 90° between each orientation) with 2 images being taken per orientation. Comparisons were made using PENGUIN between plates with different orientations. A 2-sample t power analysis was performed using the difference between the mean percentage difference of Before and After images and the mean standard deviation. Power was set at 0.8 to obtain the number of replicates required per plate, i.e. the number of comparisons between Before and After images that must be made.

Parameter	Data used in 2-sample t power analysis
Difference between mean number of pixels	0.1476
Mean of standard deviation of each mean	0.2892
Power	0.8
Number of replicates required to obtain power	33

3.5.7 – Final protocol.

The final protocol adopted for use is as follows:

- Images are taken in a dark room using a tungsten bulb as a source of light.
- An exposure time of 0.2 seconds is used for each image.
- Eight images are taken per plate Before and After incubation.
- Plates are rotated 45° between each image.

3.5.8 – Testing PENGUIN against measuring colony sizes by hand.

Whilst it had already been proven that PENGUIN worked on "simple" images (section 3.5.5), it was important to ensure it worked accurately with "real" images, i.e. pictures taken of agar plates. As previously in a single image system, there is the possibility that colours that feature within the bacterial colonies may also feature in the colour of the agar. In order to test this, *E. coli* colony sizes were measured using the optimised protocol for PENGUIN (average number of pixels that are a "new"

colour divided by the number of colonies per image) and "by hand", i.e. drawing round each colony and counting the number of pixels within the area using ImageJ. Figure 3.7 shows there is no difference in the number of pixels counted using each method indicating there are no colours that feature in both the agar and in the bacteria colonies. Whilst this appears to work for *E. coli*, it is noted that this test should be performed for each new bacteria species analysed to confirm the data produced is reproducible.

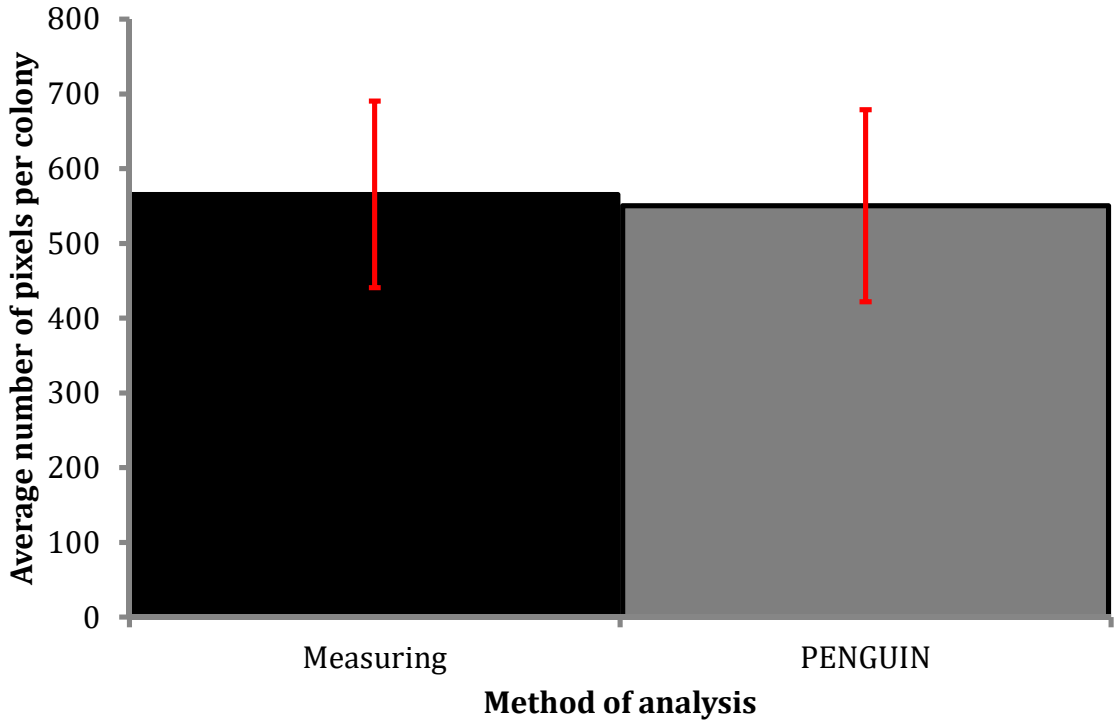


Figure 3.7: The accuracy of PENGUIN was compared against measuring colony size by hand. The average number of pixels per colony was measured using ImageJ (measuring) and by dividing the total number of pixels that are a new colour by the number of colonies per image (PENGUIN). There is no difference in the size of colonies when comparing these two methods.

3.5.9 – Conclusion.

PENGUIN was designed as a low cost method of accurately measuring bacteria colony sizes and the development of the methodologies to achieve this goal are

outlined within section 3.5. As previously stated, the concept of digitally analysing a bacteria colony to measure its size is not new, however the costs involved in acquiring equipment that can do this was prohibitive within the scope of this research project and current instrumentation is not portable. It has been shown here that the program itself works with a very high level of accuracy and that it was possible to generate appropriate conditions under which images could be taken within a limited budget. PENGUIN is used to measure average bacteria colony size during Chapter 3.

Chapter 4 – Analysis of gut microbiota and immune status after feeding with MacroGard®.

Where the previous chapters have focused upon methodologies, in this chapter the effect of MacroGard® upon the immune status and gut health of common carp will be investigated incorporating some of the methodologies established in the previous chapters. As discussed previously, although there is some debate on whether β-glucans can be considered as prebiotics, it is generally accepted within the scientific community that they can have an impact on the microbiome within the gut. Jung-Schroers *et al.* (2015a) and Kühlwein *et al.* (2013) both showed that, particularly at higher concentrations (1%+ w/w inclusion into feed), MacroGard® has an effect on bacterial species richness within the gut of carp when fed for less than a month. Whilst MacroGard® is regularly used as a β-glucan source in many fish feeds, e.g. Tetra GmbH (Germany) and Skretting (Norway), it is usually included at a lower concentration of 0.1% w/w within the feed as higher concentrations make the feed prohibitively expensive.

Manipulation of the gut microbiota population is not, however, the motivation for including MacroGard® within the diet and its primary aim is the immunomodulation of the host. There are a substantial number of publications documenting the immunomodulatory effects of β-glucans in fish (for review see (Dalmo and Bogwald 2008, Vetvicka *et al.* 2013, Akhter *et al.* 2015) and indeed there are several focusing on carp (Van der Marel *et al.* 2012, Przybylska-Diaz *et al.* 2013, Vera-Jimenez *et al.* 2013, Pionnier *et al.* 2014b), yet there still exists a deficit of

knowledge with regards to the relationship between the immune response and gut microbiota using ichthyo-models both in general and more specifically during MacroGard® feeding regimes.

The aim of this chapter is to explore the microbiology and health status of a "normal" carp gut, i.e. under disease free conditions. This comprises three studies:

Firstly, *in vitro* analysis of the gut microbiota in carp that have been maintained upon a 0% MacroGard® diet. Previous studies have shown there are limited differences in bacterial species diversity along the intestinal axis using non culture based methods of analysis (Harris 2013). Therefore, the first study aims ascertain if the same lack of bacterial species diversity along the intestinal axis is seen when different methods are used to access this diversity, i.e. culture based methods.

Secondly, some bacteria are known to be able to use β-glucans as a substrate due to their possessing β-glucanase enzymes (Planas 2000, Beckmann *et al.* 2006, Hattori *et al.* 2013). Isolates procured from the gut of carp and examples of potential fish pathogens and probiotics were tested to ascertain the effects of MacroGard® on *in vitro* growth of individual bacterial species.

Finally, an *in vivo* study looking at the impact of orally applied MacroGard® on the gut of carp will be considered using PCR based means of analysis. In this model the intestinal microbiome is exposed to both MacroGard® and any immunomodulatory affects that occur within the host. Analysis of both aspects of the symbiont, i.e. gut microbiota and carp immune response, were performed to establish if there is any correlation between expression of selected bactericidal innate immune genes within the gut and the bacterial component of the associated microbiome. As

discussed in Chapter 1, whilst in the past few years, research has begun to consider both the immune status of a host fish and the microbiota population (Akrami *et al.* 2015, Dawood *et al.* 2016, Miest *et al.* 2016), there is only one published report on the statistical analysis comparing the overall gut microbiome and the host immune status (Tapia-Paniagua *et al.* 2015). In order to fully elucidate the health promoting abilities of the gut microbiome, such as aiding in disease prevention by outcompeting potential pathogens for space and nutrients (Nayak 2010b), it is imperative to understand the symbiotic relationship between the host and its associated commensal bacterial population. In this chapter, emphasis is put on bactericidal innate immune responses such as antimicrobial peptides (Villarroel *et al.* 2007, Subramanian *et al.* 2008), nitric oxide production (Vera-Jimenez and Nielsen 2013, Wiegertjes *et al.* 2016), and pro-inflammatory cytokines such as IL-1β and TNFα (Secombes *et al.* 2001, Rieger and Barreda 2011) that have been shown to be influenced by feeding with MacroGard® (Miest *et al.* 2012, Falco *et al.* 2013, Pionnier *et al.* 2014b), and how these correlate with bacterial species diversity within the gut.

4.1 – Analysis of the culturable microbiota population from the gut of carp.

Whilst the intestine of carp can be divided into multiple sections, there are limited differences in the physical structure along the intestinal axis in comparison to other fish species and vertebrates (Barrington 1957, Jung-Schroers *et al.* 2015). Carp which are a stomachless fish species possess an intestinal bulb anterior to the oesophagus. Additionally, carp do not possess pyloric caeca, outgrowths that increase the surface area within the gut, and the hindgut is much more primitive in fish species in comparison to higher vertebrates such as humans (Barrington 1957). This

"simplified" gut structure in carp has previously been shown to result in comparable microbiota populations (Harris 2013) however, this was established utilising non culture based methodologies. In this chapter, analysis is performed using culture based techniques in order to obtain bacterial isolates for *in vitro* analysis of the effect of MacroGard® on bacterial growth (section 4.2).

Materials and methods.

A list of all equipment and chemicals used can be found in Appendix 2.

4.1.2 – Fish husbandry.

Four carp, obtained from Hampton Spring Fisheries, UK in September 2013, were maintained on the 0% MacroGard® experimental diet (section 4.1.3) produced by Tetra GmbH at 1% body weight per day for 6 months prior to experimentation. Tanks were 225L in size with Eheim 2227 filter systems and external water chilling units. Water temperature was kept at 15°C and tanks maintained under a 12/12 hour light/dark photoperiod.

4.1.3 – Diet.

A 0% MacroGard® feed was produced by Tetra GmbH (Melle, Germany) comprising of fish protein concentrate (45%), wheat starch (41%), cellulose (2.57%), soybean oil (4.5%), fish oil (4.5%), ethoxyquin (0.02%), vitamin-premix (0.25%), stabilized vitamin C (0.11%) and mineral-premix (2.06%). A second diet was also produced containing 0.1% MacroGard® which was included as a part of the wheat starch proportion of the feed as outlined above.

4.1.4 – Obtaining bacterial isolates from the carp gut.

Four carp were euthanized by submersion in anaesthetic (2-phenoxyethanol, 1ml/5l) before dissection via a ventral incision and the intestines removed. The intestinal tract was divided into four sections: the intestinal bulb, the upper midgut, the lower midgut and the hindgut (Figure 4.1). These sections were then opened lengthways in order to expose the internal gut wall. Any faecal matter was removed by gentle scraping with an inoculation loop with care being taken not to disturb the mucosal layer. Inoculation loops were gently scraped along the intestinal wall to collect bacteria from the mucosal layer which were then washed into 1ml of nutrient broth from the loop. 100µl of inoculated broth was applied to a single agar plate. For each gut section, the following nutrient bases were used: nutrient agar, M.R.S. agar, MacConkey agar or Aeromonas base. Plates were left to grow aerobically except for M.R.S. agar plates which were grown under anaerobic conditions (see below). All plates were incubated at 20°C for 24 hours before subculture. Colony subcultures were maintained on the same agar as they were initially grown.

In order to generate optimum conditions for the growth of facultative anaerobic bacteria, two methods were used: M.R.S. agar set in plastic plates were sealed using Parafilm to prevent entry of oxygen into the plate, or M.R.S. agar set in glass plates were placed in a bell jar and the oxygen content reduced by burning a small flame inside the jar which was sealed to a Perspex base using Vaseline grease.

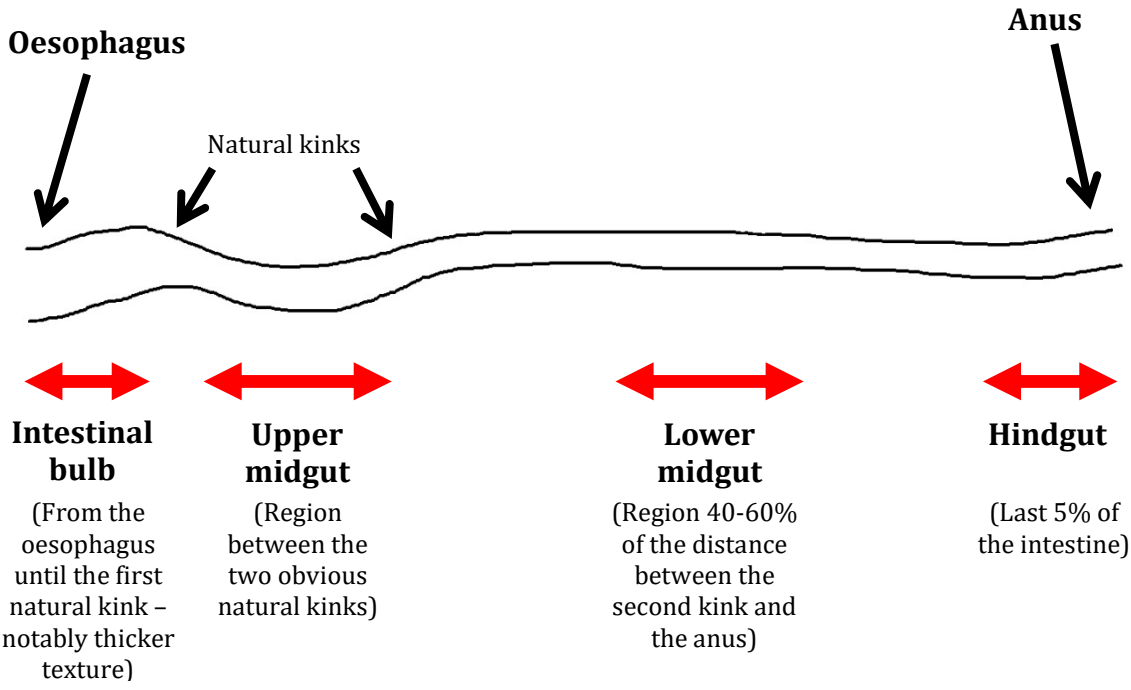


Figure 4.1: Schematic showing the regions of the intestine of the common carp as sampled for isolation of bacteria. The intestine has been divided into 4 sections: the intestinal bulb, the upper midgut, the lower midgut and the hindgut. Following the natural structure of the intestine, the intestinal bulb is defined as the notably thicker in texture section of the intestine at the start (oesophagus end) of the intestine. This region continues until the first natural kink found along the axis. The upper midgut describes the length of intestine found between the two obvious kinks found at this end of the intestine. Next, the lower midgut describes the region 40-60% of the length between the second intestinal kink and the anus end of the intestine. Finally, the hindgut is the last 5% of the intestine. Bacterial swabs were taken from each of these 4 sections with the loop being run over the whole area indicated with a red arrow.

4.1.5 – Gram staining.

Gram staining of bacteria isolates obtained from the gut of carp was performed. Individual bacterial colonies taken from plate cultures were suspended in sterile water and spread on to a microscope slide. Slides were then dried over a Bunsen flame before staining with crystal violet which was liberally applied across the surface of the slide and incubated for 60 seconds before rinsing with double distilled water. Gram's iodine was then applied and the slide incubated for a further 60 seconds before rinsing with double distilled water. 95% ethanol was applied on a drop by drop basis until the solution ran clear (on average, between 8-10 drops) and

the slide was then rinsed again with double distilled water. Lastly, Gram's saffranin was liberally applied and slides incubated for 30 seconds before a final rinse with double distilled water. Slides were viewed at 400x magnification under oil and bacteria isolates characterized based upon colour, shape and size.

4.1.6 – Testing for catalase activity.

1% hydrogen peroxide, diluted in double distilled water, was applied to bacterial colonies. Isolates that produced bubbles were categorised as catalase positive, whilst isolates that did not produce bubbles were catalase negative (Reiner 2013).

4.1.7 – Testing for oxidase activity.

Bacteria colonies were exposed to oxidase detection strips and categorised as either oxidase positive or negative based respectively upon the strip turning blue or remaining the same colour after 5 seconds of exposure (Gordon and McLeod 1928).

4.1.8 – Comparison of diversity along the intestinal axis.

Bacteria were differentiated based upon Gram stain, cell shape and size, catalase activity and oxidase activity to define distinct colony types which were then utilised as variates for further analysis. As a measure of species diversity, the Shannon-Weiner index and Evenness for each gut segment was calculated (Kuhlwein *et al.* 2013, Jung-Schroers *et al.* 2015a, Standen *et al.* 2015) as follows:

$$H = -\sum(pi * \ln(pi))$$

Where H=Shannon-Weiner index, pi=number of individual species/total

number of species, and ln is the natural log.

$$E = H/H_{max}$$

Where E=Evenness, and H_{max}=maximum amount of diversity possible as calculated by:

$$H_{max} = \ln(N)$$

Where N=species richness, i.e. number of different species present.

The presence of a colony type per gut segment (as a pool of all 4 fish; see section 4.1.4) was counted and the dissimilarity of each segment calculated using the Bray-Curtis dissimilarity index. Briefly, Bray-Curtis dissimilarity is a non-metric index that is highly tolerable of zeros within a data set. In Excel, the following formula (Podani 2000) was used to generate a matrix of dissimilarities:

$$BC_{jk} = \frac{\sum_{i=1}^{n} |x_{ij} - x_{ik}|}{\sum_{i=1}^{n} (x_{ij} + x_{ik})}$$

where j is gut segment 1, k is gut segment 2 and x_i is the number of isolates of a distinct colony type found within a gut segment. This was calculated for all gut segment combinations. Bray-Curtis dissimilarities were then plotted on a non-metric MultiDimensional Scaling ordination (nMDS) using SPSS 21 in order to visually show the "distance" between each gut segment, i.e. the dissimilarity in species diversity.

4.1.9 – Results.

64 plates were inoculated with bacteria from the gut of carp. From these, 157 bacterial samples were produced. Based upon Gram stain colour, bacteria cell shape,

bacteria cell size, catalase activity and oxidase activity, 28 distinct colony types were produced. 39 isolates came from the intestinal bulb, 37 came from the upper midgut, 36 from the lower midgut, and 45 from the hindgut. Only 2 distinct colony types were found in all 4 gut segments and 14 were found in 1 segment only. To assess the similarities between each gut section, the Shannon-Weiner index was calculated for the intestinal bulb, upper midgut, lower midgut and hindgut as 2.42, 2.26, 2.19 and 2.41 respectively. The Evenness was determined for the intestinal bulb as 0.89, upper midgut 0.94, lower midgut 0.91 and hindgut was 0.89. Figure 4.2 shows the Bray-Curtis dissimilarity between all 4 gut segments (as a pool of all fish analysed) plotted as an nMDS ordination. The two midgut sections are the least dissimilar, i.e. they have highly similar species diversity. The hindgut however is the most dissimilar to the other three gut segments, and the intestinal bulb is more dissimilar to the lower midgut than the upper midgut, i.e. there is more similarity between the intestinal bulb and the two sections of the midgut than with any section and the hindgut, and the intestinal bulb shares a more similar level of species diversity with the upper midgut than with the lower midgut.

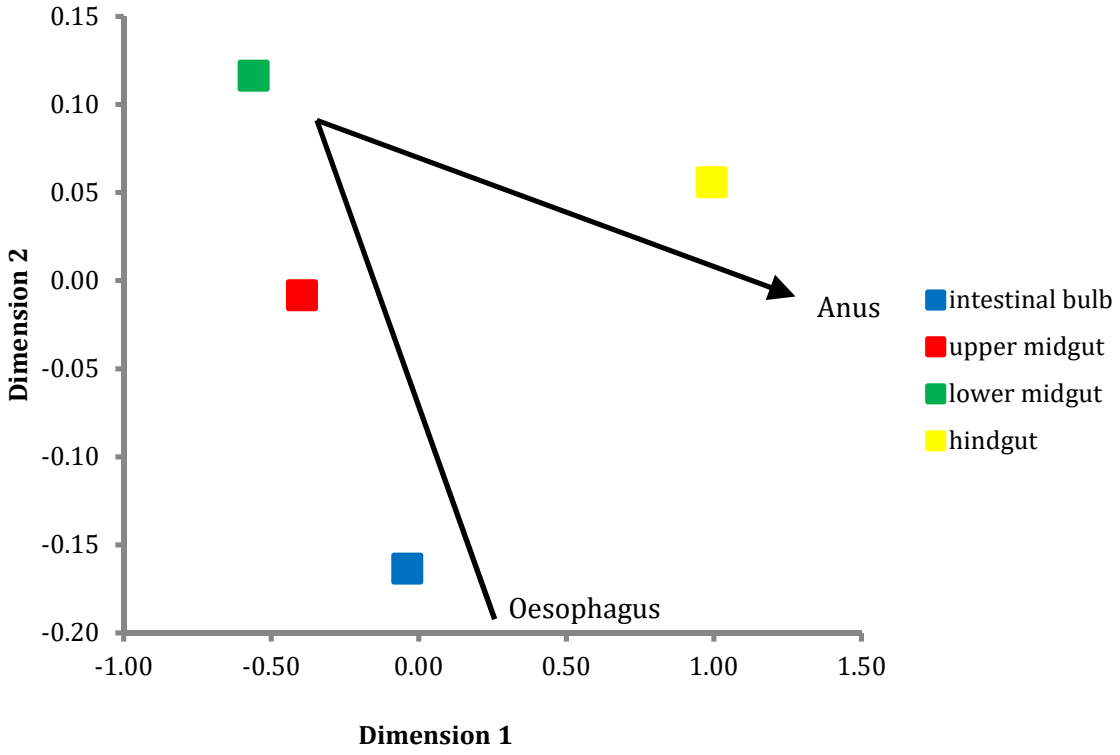


Figure 4.2: Non metric Multidimensional Scaling ordination of the dissimilarities in species diversity between 4 segments of the carp intestine as calculated using a Bray-Curtis dissimilarity index (stress – 0.0005). The intestine of 4 carp was divided into 4 segments following the intestinal axis from oesophagus to anus: the intestinal bulb (between the oesophagus and the first natural kink in the intestine), the upper midgut (from just after the first natural kink to 45% of the intestine length), the lower midgut (the subsequent 45% of the intestine after the upper midgut) and the hindgut (the 2cm of intestine before the anus). Each point represents the total number of isolates found in a pool of 4 carp fed on a 0% MacroGard® diet. Isolates were distinguished as distinct colony types based upon Gram stain, cell shape, cell size, catalase activity and oxidase activity.

4.2 – Analysis of the *in vitro* effect of MacroGard® upon bacterial growth and survival.

In section 4.1, the species diversity of cultured bacteria within the gut of fish fed on 0% MacroGard® was determined. This formed a baseline to establish if MacroGard® had an effect on bacteria species *in vitro*. A resazurin dye assay, incubation with MacroGard® suspended within a broth and MacroGard® embedded into a nutrient agar plate, and a comparison of bacteria colony size after incubation with different concentrations of MacroGard® were used to determine the possible inhibitory and stimulatory effects on growth patterns of 7 bacterial isolates were

studied in the presence and absence of MacroGard®.

Materials and methods.

A list of all equipment and chemicals utilised within this chapter can be found in Appendix 2.

4.2.2 – Bacteria reference strains.

Two reference strains were obtained from the National Collection of Industrial and Marine Bacteria (NCIMB) in October 2014:

NCIMB1102 – *Aeromonas salmonicida* subsp. *salmonicida* batch reference 19/11/1998.

NCIMB8054 – *Bacillus subtilis* subsp. *spizizenii* batch reference 16/04/2013.

Strains were obtained in the form of freeze-dried bacterial culture and grown aerobically in nutrient broth at 20°C (*A. salmonicida*) or 30°C (*B. subtilis*) overnight before being plated onto nutrient agar and incubated at the correct temperatures for each strain as stated by NCIMB (20°C and 30°C respectively). All subsequent subcultures were performed either using nutrient agar plates or nutrient broth.

4.2.3 – Other isolates selected for analysis.

In addition to the two reference strains described in section 4.2.2, a lab strain of *E. coli* (NCIMB8277 courtesy of Nigel Bowers, Keele University, UK) was analysed for effect of MacroGard® upon growth. *E. coli* cultures were grown at 37°C, the temperature for optimum growth of this strain, using either nutrient agar or nutrient

broth.

The growth patterns of four bacterial isolates (342LMB, ISO 20, ISO46 and ISO 60) cultured from the gut of carp were determined. Details as to the identified characteristics of these isolates can be found in Table 4.1. All cultures were grown at 20°C and after initial separation into individual cultures, maintained using either nutrient broth or nutrient agar.

Table 4.1 – Details as to the characteristics for 4 bacterial isolates cultured from the gut of common carp.

Isolate	Gut section	Original agar base	Gram stain	Shape	Size (nm)	Oxidase	Catalase
342LMB	Hindgut	MacConkey agar	Positive	Bacillus	<1	Negative	Negative
ISO 20	Intestinal bulb	Nutrient agar	Negative	Bacillus	3	Negative	Positive
ISO 46	Hindgut	Nutrient agar	Negative	Bacillus	1	Negative	Negative
ISO 60	Upper midgut	Nutrient agar	Negative	Bacillus	1	Positive	Positive

4.2.4 – Analysis of MacroGard® toxicity.

Analysis of the toxicity of a range of MacroGard® concentrations was performed using *A. salmonicida*, *B. subtilis* and *E. coli* as described in section 4.2.2. A range of MacroGard® concentrations from 0.1-0.01% w/v was utilised in conjunction with a range of initial bacteria concentrations of $1x10^8$ to $1x10^1$ CFU per well in a final volume of 200µl of nutrient broth in a checkerboard formation as shown in Figure 3.1. Plates were replicated in triplicate. Incubation was performed at room temperature (25°C) for 18 hours before 15µl 0.01% w/v resazurin dye was added to each well. Plates were left to incubate at room temperature for 15 minutes before noting any colour change from blue to pink within each well.

4.2.5 – Growth after incubation with MacroGard® within a nutrient broth suspension.

Analysis of the effect of growth after incubation with MacroGard® within a nutrient broth suspension was performed using *A. salmonicida*, *B. subtilis* and *E. coli* as described in section 4.2.2. 1ml of $1x10^5$ CFU bacteria was added to either 50ml of 0% MacroGard® nutrient broth, 0.01% MacroGard® nutrient broth or 0.1% MacroGard® nutrient broth and incubated at room temperature for 18 hours. Each broth was then subject to a 1:100,000 dilution and 50µl applied to a nutrient agar plate (0% MacroGard®). Plates were incubated at 20°C for 48 hours before CFU per plate was determined.

4.2.6 – Growth upon MacroGard® embedded plates.

Analysis of bacterial growth upon nutrient agar plates infused with 0.1% w/v MacroGard® was performed using 342LMB, ISO 20, ISO 46 and ISO 60 as described in section 4.2.3. Bacteria cultures were grown for 24 hours in nutrient broth at room temperature before being subjected to a 1:100,000 dilution. 50µl of broth was applied to either nutrient agar plates containing 0% MacroGard® or 0.1% MacroGard®. Plates were incubated at 20°C for 72 hours before CFU per plate was determined.

4.2.7 – Analysis of differences in the rate of colony growth using PENGUIN.

In order to determine if there was a difference in colony size due to incubation with MacroGard®, colonies grown on nutrient agar plates were analysed after incubation in a broth with and without MacroGard® (section 4.2.5), and after

inoculation on nutrient agar plates embedded with MacroGard® (section 4.2.6) using PENGUIN. Immediately after the bacteria was applied to each plate, 8 images were taken as described in section 3.5.7 followed by 8 more images being taken after incubation was complete, i.e. 48 hours for bacteria incubated in a broth and 72 hours for bacteria inoculated onto MacroGard® embedded nutrient agar plates. Images were cropped so that a 1500x1500 pixel section from the centre of the plate remained, i.e. each image was cropped by removing 1250 pixels from the left and right edges and 750 pixels from the top and bottom of the image. This was then resized to 500x500 pixels before analysis using PENGUIN. "After" images, i.e. those taken after incubation, were compared to "Before" images, i.e. those taken immediately after inoculation, resulting in 64 values as to the number of pixels that were a colour that appeared only in the after image. As rotation of the plate resulted in slight variation in the number of bacteria colonies per image, the average number of different coloured pixels for each "After" image against all "Before" images was taken and divided by the number of colonies within that image. The average number of pixels per colony was then taken across all 8 "After" images.

4.2.8 – Results.

Analysis of the 7 bacterial isolates including 2 fish specific reference strains, i.e. *A. salmonicida* and *B. subtilis*, and 4 isolates taken from the gut of carp showed no effect of MacroGard® upon bacterial survival, rate of growth or colony size (Table 4.2). Analysis of survival of *A. salmonicida*, *B. subtilis* and *E. coli* based upon metabolism of resazurin showed no toxic effect of MacroGard® at any concentration. There were significantly more *B. subtilis* CFUs after incubation with 0.01%

MacroGard® in comparison to 0.1% MacroGard® (p=0.0360), however neither concentration resulted in a significant difference in the number of CFUs when compared to the 0% MacroGard® treatment group. Also the number of CFU of *A. salmonicida* and *E. coli* was not different when the bacteria was incubated with MacroGard® in a broth in comparison to controls, i.e. bacteria incubated in nutrient broth with no MacroGard®. Similarly, none of the bacterial isolates taken from the gut of carp had a significant difference in the number of CFUs when grown upon MacroGard® embedded agar in comparison to control agar plates, i.e. nutrient agar plates without MacroGard® embedded into the agar. None of the bacterial species incubated with MacroGard® within a broth had a significant difference in average colony size, however ISO 46 did have significantly (p=0.021) smaller colonies after incubation with MacroGard®. No other isolates showed a difference in colony size.

Table 4.2: (part A) Results of analysis of the effect of MacroGard® upon 7 different bacterial isolates using 4 different analysis techniques. Toxicity was analysed using a resazurin metabolism assay allowing for a wide range of MacroGard® concentrations and initial bacterial counts. CFU counts after incubation with and without MacroGard® within a nutrient broth and also embedded within a nutrient agar plate were performed to assess promotion of growth. On all plates analysed for CFU counts, a secondary analysis of average colony size was performed using the program PENGUIN which calculates average number of pixels per bacteria colony.

Isolate	Concentration of MacroGard®	Number of bacteria	Results			Effect
Resazurin toxicity assay						
A. salmonicida	0.01-0.1% w/v	$1x10^1$ – $1x10^8$ CFU per well at start of incubation	Metabolism of resazurin in all bacteria positive wells			No
B. subtilis			Metabolism of resazurin in all bacteria positive wells			No
E. coli			Metabolism of resazurin in all bacteria positive wells			No
Incubation with MacroGard® in broth						
			Mean CFU ± Standard deviation			
			0% MacroGard®	0.01% MacroGard®	0.1% MacroGard®	
A. salmonicida	0.01% and 0.1% w/v	$1x10^5$ CFU into broth. 50µl of 1:105 dilution onto agar plates	158±35	200±9	187±13	No
B. subtilis			60±3	49±4	87±23	Yes
E. coli	0.1% w/v		287±48	-	348±64	No
Incubation with MacroGard® in agar						
			Mean CFU ± Standard deviation			
			0% MacroGard®		0.1% MacroGard®	
342LMB	0.1% w/v	50µl of 24 hour culture (50ml at RT) diluted $1:10^5$ times	77±9		59±12	No
ISO 20			65±7		34±13	No
ISO 46			33±4		30±4	No
ISO 60			33±7		35±10	No

Table 4.2: (part B) Results of analysis of the effect of MacroGard® upon 7 different bacterial isolates using 4 different analysis techniques. Toxicity was analysed using a resazurin metabolism assay allowing for a wide range of MacroGard® concentrations and initial bacterial counts. CFU counts after incubation with and without MacroGard® within a nutrient broth and also embedded within a nutrient agar plate were performed to assess promotion of growth. On all plates analysed for CFU counts, a secondary analysis of average colony size was performed using the program PENGUIN which calculates average number of pixels per bacteria colony.

Isolate	Concentration of MacroGard®	Number of bacteria	Results		Effect
Difference in colony size using PENGUIN					
			Mean pixels per colony ± Standard deviation		
			0% MacroGard®	0.1% MacroGard®	
A. salmonicida	0.1% w/v	1x10^5 CFU into broth. 50µl of 1:10^5 dilution onto agar plates	40±8	54±11	No
B. subtilis			90±26	46±38	No
E. coli			153±23	118±8	No
354LMB		50µl of 24 hour culture (50ml at RT) diluted 1:10^5 times	49±4	80±27	No
ISO 20			65±7	34±13	Yes
ISO 46			672±23	957±103	No
ISO 60			704±41	569±95	No

4.3 – Oral application of MacroGard® and the effects on the intestinal microbiome over time.

Previous studies have shown that there is a change in species richness of the intestinal microbiome of the common carp after 2-3 weeks (Jung-Schroers *et al.* 2015a) and 4 weeks (Kuhlwein *et al.* 2013) of feeding MacroGard® at a w/w concentration of 1% within the diet. Such concentrations are, however, higher than those typically found in commercial fish feeds as producers limit the inclusion of MacroGard® in their products to 0.1% w/w because of financial constraints. Kühlwein *et al.* (2013) also ascertained that 0.1% w/w MacroGard® in a diet did not affect bacterial species richness in the gut highlighting the possibility that the effect of MacroGard® on bacterial species richness may be dose dependent when it is applied orally. What both of these studies lack, however, is an accompanying comparison with the immune parameters that, whilst studied, were not compared (Kuhlwein *et al.* 2014, Syakuri *et al.* 2014).

In this trial the effect of MacroGard® on the microbiota and innate immune response within the gut of carp will be ascertained and any correlation determined. Gene expression of selected innate immune genes will be assessed using RT-qPCR and analysis of bacterial species richness will be determined using PCR-DGGE. Quantitative analysis of the total bacteria load and the presence of specific bacteria genera within the gut will be determined. Statistical correlation models will be employed to study the relationship between the carp immune status and the profile of the gut microbiota.

Materials and methods.

4.3.2 – Fish husbandry and experimental design.

90 carp obtained from Hampton Spring Fisheries, UK in October 2012, were divided between 6 tanks (15 fish per tank) and maintained as described in section 4.1.2. At the start of this trial, carp had a mean weight of 56.5g (s.d. ±13.5g) and mean length of 14.6cm (s.d. ±1.1cm). Carp were fed at a rate of 1% body weight per day with a diet containing either 0% MacroGard® (3 tanks) or 0.1% MacroGard® (3 tanks) for a period of 7 weeks (see section 4.1.3). Samples of the upper midgut were taken as described in section 4.3.3 at all time points. Sampling was performed at the very start of the trial, i.e. when all fish had only eaten 0% MacroGard® feed (referred to as week 0 in all Tables and Figures), and then 1, 3, 5 and 7 weeks after the start of feeding with the 0.1% MacroGard® diet. At each time point, 3 carp per tank were sampled as shown in Table 4.3.

Table 4.3: Experimental design of 7 week feeding trial analysing the effect of 0.1% w/w MacroGard® in the diet of common carp (*C. carpio*) compared to carp maintained on a 0% MacroGard® diet. Table shows the number of fish sampled at each time point from each tank.

		0% MacroGard® feed			0.1% MacroGard® feed		
		Tank A	**Tank B**	**Tank C**	**Tank D**	**Tank E**	**Tank F**
Time point	**Week 0**	3	3	3	3	3	3
	Week 1	3	3	3	3	3	3
	Week 3	3	3	3	3	3	3
	Week 5	3	3	3	3	3	3
	Week 7	3	3	3	3	3	3

4.3.3 – Tissue sampling of carp.

Carp were euthanized by submersion in anaesthetic (2-phenoxyethanol, 1ml/5L), dissected, the upper midgut (defined as the region after the intestinal bulb)

was removed and stored in RNA*later* at -80°C until further use. Upper midgut sections were divided as follows; total gut (including any faecal matter), gut wall (no faecal matter) and gut contents (faecal matter only) as shown in Figure 4.3.

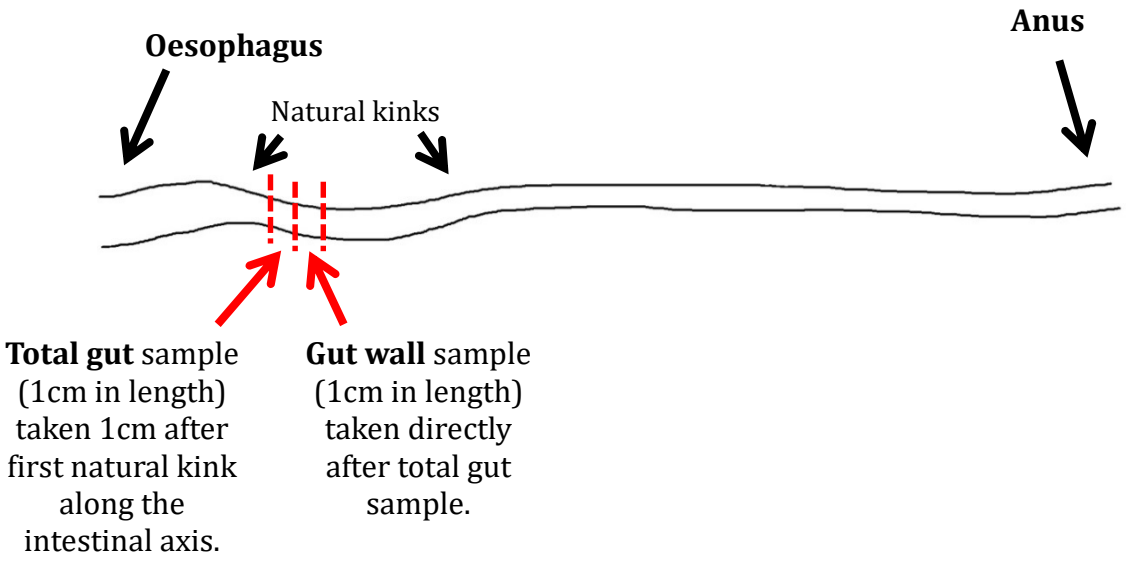


Figure 4.3: Schematic showing the location along the intestinal axis of the common carp at which samples are taken. Total gut is defined as both intestinal tissue and any faecal matter present and includes both the indigenous and transient microbiota populations. Gut wall is defined as intestinal tissue and indigenous microbiota only with faecal matter being removed by gentle squeezing. Both samples were taken from the upper midgut area of the intestine which is defined within this thesis as starting directly after the first natural kink along the gut axis.

4.3.4 – Quantitative analysis of innate immune gene expression and bacterial 16S rDNA expression.

Gene expression analysis was performed on selected innate immune genes in carp and the bacterial 16S rDNA gene. These were as follows: ApoA1, Bf/C2, CRP 2, C1rs, C3, HAMP1, iNOS, IL-1β, the IL-1β receptor, LEAP2, MASP2, muc2, TNFα1 and TNFα2.

4.3.4.1 – RNA isolation.

RNA isolation was performed using the Qiagen RNeasy Mini Kit optimised for bacteria samples as per the manufacturer's instructions. Briefly, approximately 3mm^3 of gut wall tissue was placed into 250µl of PBS solution and subjected to sonication utilising a Bioruptor™ set on high for 10 minutes (pausing after 5 minutes to refresh the ice within the water bath). 500µl of RNAprotect was added to each sample and vortexed before incubating at room temperature for 5 minutes. Samples were pelleted by centrifugation (5,000 x *g* for 10 minutes) and the supernatant removed. 100µl of lysozyme (15mg/ml) suspended in TE buffer (10mM Tris-HCl, 1mM EDTA) was applied to each sample and mixed gently via pipetting to re-suspend the pellet before 10µl of proteinase K (10mg/ml in PCR grade water) was added. Samples were vortexed every 2 minutes in 10 second bursts for 10 minutes before 700µl RLT buffer was added. Samples were then stored overnight at -80°C. After thawing, debris was pelleted by centrifugation (14,000 x *g* for 3 minutes) and 760µl of supernatant added to 590µl of 80% ethanol and mixed by pipetting. Samples were applied to a spin column (two volumes of a maximum of 700µl per column) and the supernatant removed by centrifugation at 8,000 x *g* for 15 seconds. Samples were then washed firstly in 500µl RW1 buffer (centrifugation at 8,000 x *g* for 15 seconds) and then in two washes with 500µl RPE buffer (first wash – centrifugation at 8,000 x *g* for 15 seconds, second wash – centrifugation at 10,000 x *g* for 2 minutes). The washing buffer was then removed by an additional centrifugation step of 8,000 x *g* for 2 minutes. RNA, attached to the spin column membrane, was incubated in 35µl of RNase free water at room temperature for 2 minutes before a final centrifugation step of 8,000 x *g* for 1 minute. Concentrations were determined using a Nanodrop 1000

spectrophotometer and samples stored at -80°C until further use.

4.3.4.2 – DNase treatment of RNA samples.

Prior to cDNA synthesis, RNA samples were treated with DNase to remove any DNA contamination. Briefly, 1µg RNA of sample was suspended in RQ1 buffer (final concentration – 1X), 1ng DNase and 10U RNaseOUT RNase inhibitor in a total volume of 10µl and incubated at 37°C for 30 minutes before the addition of 1µl of RQ1 DNase stop solution. Samples were then incubated at 65°C for 10 minutes.

4.3.4.3 – cDNA synthesis.

500ng of DNase treated RNA was mixed with a final concentration of 2.5µM random hexamers, 1mM dNTP mix (1mM of each dATP, dGTP, dCTP, and dTTP) in a total volume of 12µl. Samples were incubated at 65°C before briefly being chilled on ice and centrifuged to collect any condensation to the bottom of the tube. First Strand Buffer, DTT and RNase Inhibitor (final concentrations – 1X, 0.01M and 40U respectively) were then added to each sample and incubated at 37°C for 2 minutes before 200U of M-MuLV RT was added and the samples incubated in the following conditions: 25°C for 10 minutes, 27°C for 50 minutes, 70°C for 15 minutes. Samples were then stored at -20°C until further use.

4.3.4.4 – RT-qPCR analysis.

In order to quantitatively analyse changes in gene expression levels, RT-qPCR analysis was performed on gut samples. During this feeding trial, the following immune genes were selected for analysis: ApoA1, Bf/C2, CRP2, C1rs, C3, HAMP1,

iNOS, IL-1β, the IL-1β receptor, LEAP2, MASP2, Muc2, TNF α1, and TNFα2. In addition to studying the outlined immune parameters, RT-qPCR analysis of the 16S rDNA gene expression in the bacterial population within the gut was also analysed. Assays were performed to quantify total 16S expression, i.e. an approximation of the overall bacterial population size, and the proportion of the total expression represented by the genera *Aeromonas*, *Pseudomonas*, *Flavobacterium*, *Vibrio* and *Streptococcus*. Primers for all genes can be found in Appendix 3.

Running PCRs for *in vivo* sample analysis.

RT-qPCR assays were performed using either an ABI Prism® 9000 Sequence Detection System or an Mx3000P qPCR System, and the SensiFAST™ SYBR® HiROX kit as per the manufacturer's instructions. Assays were performed in a total volume of 20µl with primers utilised at a concentration of 0.2µM each. 2µl of template cDNA was used in each assay with concentration being determined based upon analysis of a serial dilution of a pool of all samples within an experiment. Individual samples were diluted by a factor of either 1:4 or 1:10 based upon a C_t of 25 from pooled samples. Unless otherwise stated, data was analysed utilising a relative standard generated from a pool of all cDNA samples within a single experiment in a 1:3 serial dilution. Values are calculated as relative expression per 500ng RNA translated to cDNA. For genus specific bacterial assays, an absolute plasmid standard was used for data analysis. Recombinant plasmids were donated by Dr. Mikolaj Adamek at the University of Veterinary Medicine, Hanover, Germany (Adamek *et al.* 2013) and values calculated as absolute copy number.

Statistical analysis of carp immune gene expression data.

Statistical analysis was performed firstly by testing for outliers using Grubbs' test. Outliers, i.e. values that are outside of the normal distribution of a data set, were removed before further analysis. Analysis was performed in Excel by calculating the Z score for each sample using the following formula:

$$Z = (Y_1 - \bar{Y})/s$$

Where Y_1 is the sample, $\bar{Y}$ is the mean of all samples and s is the standard deviation. Z scores that were higher than the critical value (based upon experimental sample size and a critical value of 5%, Grubbs and Beck 1972) were removed from the dataset as outliers before any further analysis was performed.

Analysis of variance was performed using Minitab 14. Data points from each gene were tested for normality using an Anderson-Darling test and homoscedasticity using Levene's test. In cases were a p value of <0.05 was obtained using these tests, data were transformed using a Box-Cox transformation and retested for normality and homoscedasticity. Differences between time points and feeding regimes were compared using either a 2-way nested ANOVA with *post hoc* Tukey's or, in cases were data could not be normalised with a Box-Cox transformation, a 2-way Scheirer-Ray-Hare test was performed. Each figure within this and subsequence chapters includes the results of the Anderson-Darling and Levene's tests to show if data met the conditions required for a parametric test, and values of λ when data was transformed prior to further statistical analysis.

4.3.4.5 – Comparing immune gene expression with 16S rDNA expression.

In order to determine if there was any correlation between immune gene

expression and the 16S expression in the total bacteria population and individual bacterial genera, expression data was analysed using Spearman's rank order test (SPSS 21). All data points including those identified as statistical outliers were included in this comparison.

4.3.5 – Qualitative analysis of bacterial species richness.

In order to study changes in species richness (presence/absence only), the non-culture based method of PCR-DGGE was employed. This section outlines the protocol employed to isolate genDNA from total gut samples and the PCR-DGGE analysis.

4.3.5.1 – genDNA isolation.

GenDNA was isolated utilising the QIAamp DNA Mini Kit optimised for bacteria isolation as per the manufacturer's instructions. Nomenclature for buffers, e.g. AL buffer, was set by the manufacturer and are given within this section as labelled in the kit except for lysis buffer which was produced by the author. Briefly, total gut samples were prepared for genDNA isolation by homogenizing approximately 5mm^3 of tissue suspended in lysis buffer (20mg/ml lysozyme, 20mM Tris-HCl, 2mM EDTA, 1.2% Triton X100) using a TissueLyser II – 20Hz for 2 minutes. Samples were then incubated at 37°C for 1 hour. 200µl of AL buffer and 20µl of proteinase K were added to each sample and pulse vortexed for 15 seconds. Samples were then incubated at 65°C for 30 minutes and 95°C for 15 minutes, mixed with 200µl of absolute ethanol by pulse vortexing before being applied to a DNA spin column. Samples were bound to the membrane and the liquid removed by centrifugation at 6,000 x *g* for 1 minute.

Samples then underwent two washing steps, first with 500µl AW1 buffer (centrifugation at 6,000 x *g* for 1 minute) and secondly with 500µl of AW2 buffer (centrifugation at 20,000 x *g* for 3 minutes) before an additional centrifugation step at 20,000 x *g* for 1 minute to remove any remaining wash solution. They were then incubated in 150µl AE buffer at room temperature for 5 minutes before a final centrifugation step at 6,000 x *g* for 1 minute and stored at -20°C until use.

4.3.5.2 – End point PCR for PCR-DGGE analysis.

PCRs were performed under sterile conditions utilising the following PCR mix (values refer to final concentration): 1X KAPA2G Buffer A, 0.2mM dNTP mix, 10µM each primer in the primer pair DGGE (sequences can be found in Appendix 3), 0.25U Hot Start KAPA2G Robust polymerase, 5µl genDNA (average concentration of 300ng/µl) in a total volume of 25µl. Samples were incubated in an Eppendorf Gradient Mastercycler® with the following touchdown program: 95°C for 5 minutes, 5x cycles of 95°C for 30 seconds, 63-58°C for 30 seconds (-1°C per cycle), 72°C for 1 minute, 35x cycles of 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 1 minute, and a final incubation at 72°C for 7 minutes. Samples were tested for a correct sized product and to confirm no contamination in the template free control on a 1% agarose gel (mixed in 50ml 1X TBE buffer with 2.5µl of Roti®-Safe gel stain for visualisation) which was subject to a voltage of 105V for 20 minutes and visualised under UV light.

4.3.5.3 – DGGE gel preparation and electrophoresis.

PCR samples were pooled by tank and by time point (3 fish per pool) and

mixed with an equal volume of loading buffer (40% glycerol with cresol red) before use. 8% polyacrylamide gels (Rotiphorese® Gel 30 mixed with a final concentration of 1X TAE buffer) containing a 40-60% gradient of urea and formamide (whereby 100% is equivalent to 7M urea and 40% formamide solution) were prepared manually using 40µl of TEMED and 400µl 10% (w/v) APS as setting agents. Gels were prepared and performed using a TV400-DGGE system with a gel size of 16.5x17.5cm. Electrophoresis was performed at 60°C. Samples were initially drawn into the gel at 250V for 2 minutes and then separated at 120V for 820 minutes. Gels were stained using the UV marker SYBRgold by incubation in a 0.01% (v/v) suspension for 30 minutes at room temperature. Samples were visualised under UV light and imaged using a Nikon D3200 Digital SLR with a 55-300mm VR lens and a minimum exposure time of 10 seconds.

4.3.5.4 – PCR-DGGE gel analysis.

After imaging, band patterns were analysed in a binary format, i.e. presence or absence of a band. Bands are arbitrarily ordered from the top of the gel to the bottom with all bands being considered regardless of band pattern. Band 1 represents the band that has undergone the least migration and therefore has the lowest melting temperature. The highest numbered band (gel dependent) within a gel is the band that has migrated the furthest and has the highest melting temperature.

PCR-DGGE analysis works under the assumption that each band within a band pattern represents a single operational taxonomic unit (OTU) with the further assumption that each OTU represents a different bacterial species although this cannot be confirmed without sequence data for each band. Using these assumptions,

however, it is possible to compare how alike each band pattern is in comparison to other band patterns within the same gel. Assumptions of correlation between the presence/absence of bands cannot be made with data alone from a band pattern, therefore metric tests cannot be used. Following the work of Attramadal *et al.* (2014) and Kühlwein *et al.* (2013), Bray-Curtis dissimilarity index followed by plotting dissimilarities using nMDS was performed to compare band patterns within each gel as described in section 4.1.8.

4.3.6 – Results.

The data presented for this experiment is divided into three sections – expression of immune genes (4.3.6.1), analysis of the gut microbiome (4.3.6.2) and statistical analysis to look at any correlation between the size of the microbial population within the gut and expression of the analysed bactericidal immune genes (4.3.6.3).

4.3.6.1 – Expression of immune genes in the gut.

Prior to analysis of individual fish, PCRs were performed using a pool of all samples in order to determine how far cDNA samples should be diluted. This revealed that the expression levels of certain genes did not give a low enough C_t value to analyse expression levels within individual samples and/or limited presence of peaks (i.e. product) when analysing the dissociation curve produced at the end of the PCR cycles. To this end, further analysis of the following genes was not completed: two isoforms of TNFα, and the complement proteins MASP2, C1rs, and bf/C2. The three complement proteins were expressed within the pool of all cDNA samples at lower

levels than required for further analysis within this experiment. There was no expression of either isoform of TNFα in the pool of all samples from this experiment.

Due to a high level of variation in gene expression naturally existing within cohorts of outbred carp it was decided, as standard, to employ a statistical outlier test to all gene expression data before further analysis. Grubbs' outliers test revealed at least one outlying value for each immune gene analysed. These outlying values were not limited to one individual, however the decision was made to remove these data points anyway on the basis that normal distribution and equal variance presumptions were violated, even after a Box-Cox transformation was performed when outliers were included. Whilst a non-parametric 2-way Scheirer-Ray-Hare test could have been used in lieu of the parametric 2-way nested ANOVA in cases were outliers caused the presumptions of normal distribution and equal variance to be violated, *post hoc* analysis is not possible with the Scheirer-Ray-Hare test and it is also the weaker of the two types of analysis, i.e. potentially significant differences that would be highlighted using an ANOVA would not be seen when using the Scheirer-Ray-Hare test. 73.3% of outlier values (15 in total across all genes) were from carp fed with the 0% MacroGard® diet with at least 1 outlier coming from this feed group for all genes analysed except Muc2. Each of these outlier values came from different fish in different tanks at different time points. In comparison, there were only 4 outlier values from carp fed with the 0.1% MacroGard® diet. These were found in the gene expression of iNOS (1 value), Muc2 (1 value) and CRP2 (2 values). As with the outlier data points from the 0% MacroGard® fed fish, these all came from different fish from different tanks and different time points.

A 2-way nested ANOVA revealed that there was no statistically significant

difference in expression levels between fish cohorts, i.e. from different tanks, which received the same feed regime for any of the genes analysed. Levels of C3 expression could not be normalised therefore the non-parametric Scheirer-Ray-Hare test was performed, however, due to limitations of this test, tank effect could not be analysed for this data set.

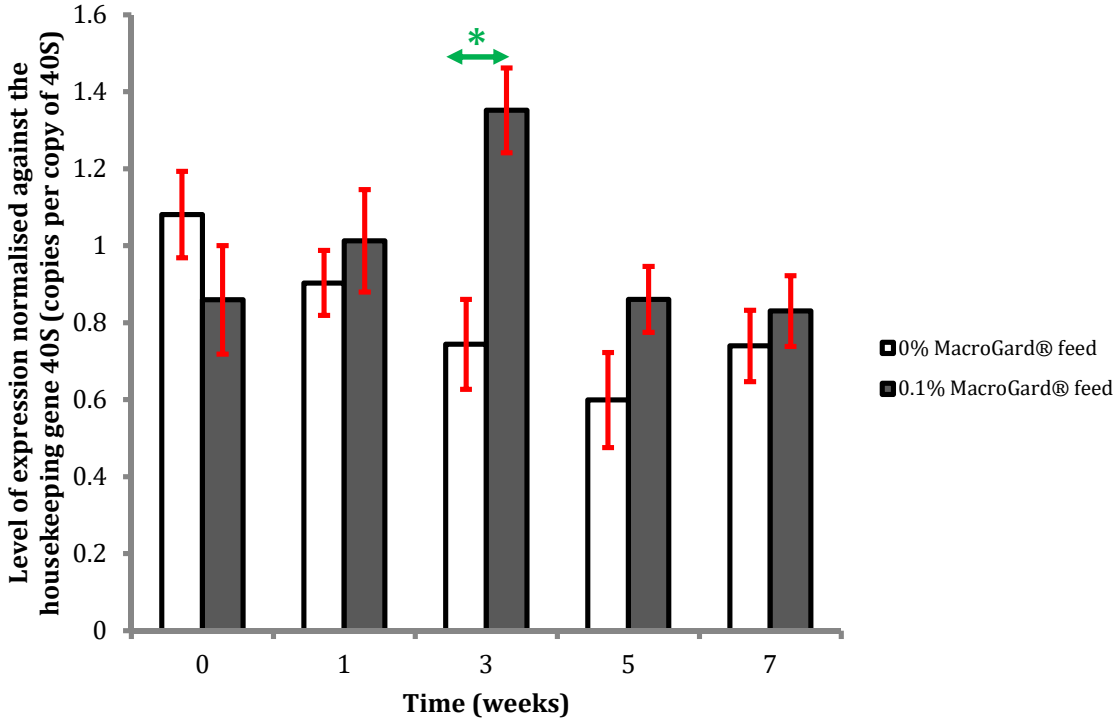
The inclusion of 0.1% MacroGard® in the diet only had a statistically significant overall effect on the expression of the IL-1β receptor ($p=0.019$, Figure 4.4) and IL-1β ($p=0.047$, Figure 4.5). The IL-1β receptor had greater expression levels in carp fed with the 0.1% MacroGard® at all sampling times except for those sampled at the very start of the trial where all fish had only been fed the 0% MacroGard® (week 0 in Figure 4.4). The expression of the IL-1β receptor was only significantly higher in carp fed with the 0.1% MacroGard® diet in comparison to those fed the MacroGard® free diet at week 3 ($p=0.0121$). Inversely, where expression of the IL-1β receptor was higher in MacroGard® fed carp, expression of the IL-1β gene itself was significantly lower in 0.1% MacroGard® fed carp in comparison to those on the 0% MacroGard® diet. *Post hoc* analysis did not, however, reveal any significant differences between the two feed groups at individual time points.

Whereas feed had a minimal impact upon gene expression, time, irrespective of diet, had a significant effect on the expression of the IL-1β receptor, IL-1β, iNOS (Figure 4.6), Muc2 (Figure 4.7) and CRP2 (Figure 4.8) with significance at $p=0.033$, $p=0.001$, $p=0.007$, $p<0.001$ and $p<0.001$ respectively. The IL-1β receptor, whilst showing a significant effect of time overall, did not show any significant differences between each individual time point.

The greatest expression of IL-1β was noted at the very start of the trial before the tanks were divided by diet (described as week 0 in Figure 4.5) and was significantly higher compared to expression seen after 1 week ($p=0.0057$), 3 weeks ($p=0.0016$) and 7 weeks ($p=0.0355$) but not after 5 weeks. In comparison iNOS gene expression was significantly greater at the end of the trial (week 7) compared to those seen at weeks 1 ($p=0.0069$) and 3 ($p=0.0358$). Both Muc2 and CRP2 expression levels are significantly lower at week 5 ($p<0.001$) in comparison to all other time points. There are no significant differences in the expression levels of C3 (Figure 4.9) or the three antimicrobial peptides HAMP1 (Figure 4.10), LEAP2 (Figure 4.11) and ApoA1 (Figure 4.12) over time.

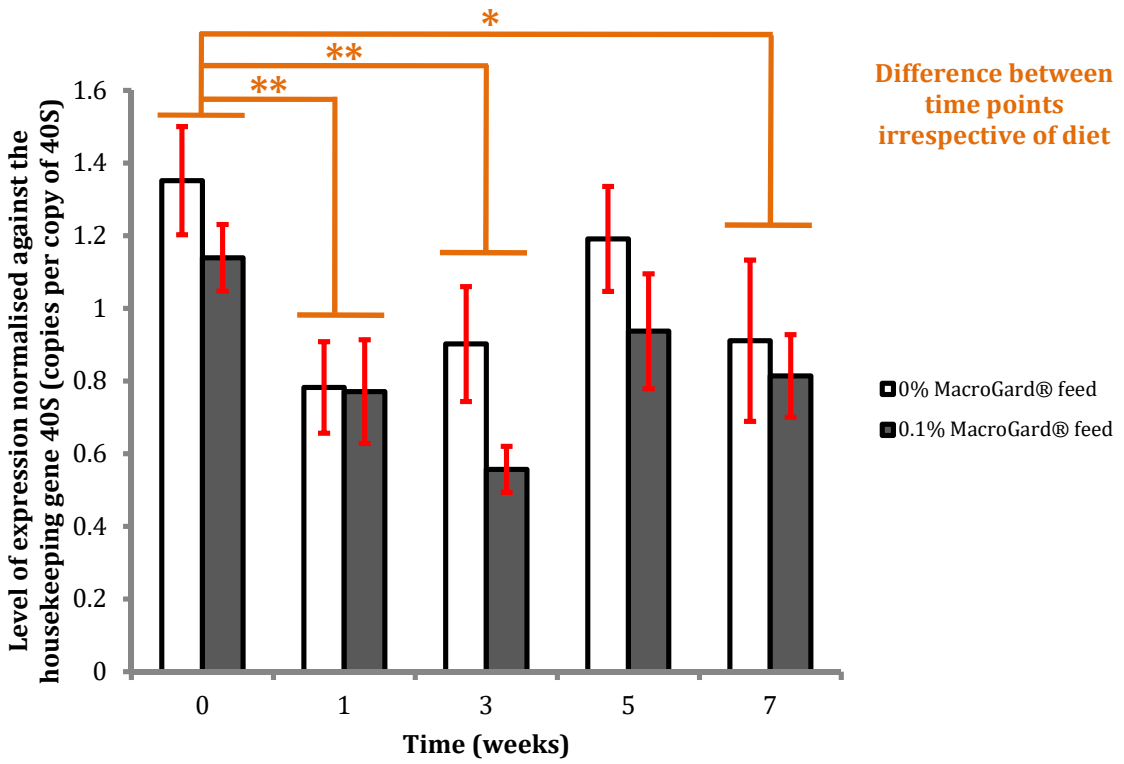
Whilst statistical analysis of data is highly useful in discussing whether an effect is present or simply an artefact of chance, considering the biology is equally as important when interpreting data. The data presented in Figure 4.4 to Figure 4.12 shows that whilst there are statistically significant differences in the levels of gene expression, the magnitude of these differences is minimal. Where expression of the IL-1β receptor gene is significantly greater in carp fed with the 0.1% MacroGard® diet after 3 weeks, the average expression is less than twice (1.8 times) the average expression of carp fed with the 0% MacroGard® diet. Similarly, the higher expression levels of IL-1β seen at the start of the trial (represented in Figure 4.5 as week 0) irrespective of feed are only 1.6, 1.7 and 1.2 times higher than the significantly lower levels of expression after 1, 3 and 7 weeks of feeding. The statistically significant differences in iNOS expression over time irrespective of feed correspond to a 1.6 times higher level of expression at week 1 in comparison to week 7, and a 1.1 times higher level at week 3 in comparison to week 7. Expression of Muc2 was significantly

lower irrespective of feed at week 5 in comparison to all other time points with the largest fold difference (2.0 times higher) in average expression being seen between weeks 5 and 7. The largest fold difference seen for any gene with an associated statistically significant difference is found in CRP2. As with Muc2 expression, this difference is seen between weeks 5 and 7 with expression at week 7 being 3.3 times higher than two weeks previous.



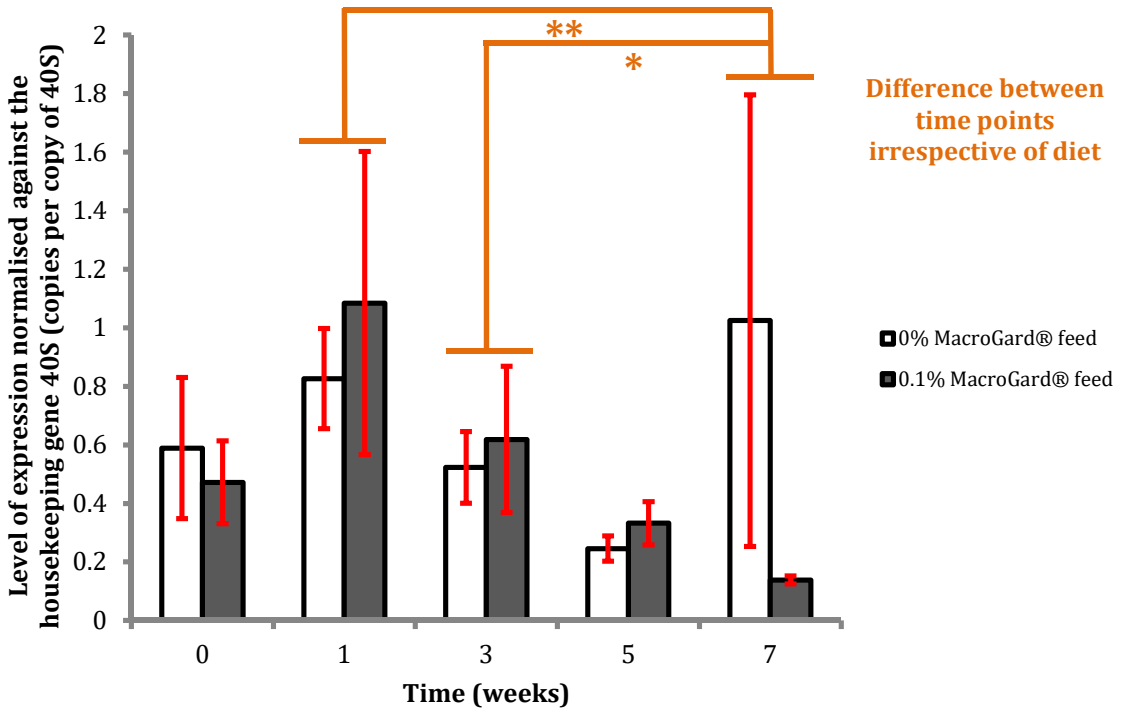
Anderson-Darling test for normality	p=0.418	Levene's test for equal variance		p=0.944
2-way nested (tank within treatment) ANOVA with *post hoc* Tukey's (R^2=30.14%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between feeds	1	5.72	0.019	*
Nested effect of tank variation	4	0.24	0.914	
Overall difference over time	4	2.77	0.033	*
Interaction of feed and time	4	3.54	0.011	*
***Post hoc* analysis**				
Time point: 3 weeks, 0% versus 0.1% MacroGard®		p=0.0121		*

Figure 4.4: Expression of the Interleukin 1β receptor gene in the upper midgut of common carp (*C. carpio*) during a 7 week feeding trial. Carp were fed with either a 0% or 0.1% MacroGard® experimental diet designed by Tetra (GmbH) at a rate of 1% body weight per day. Each feed was given to 3 tanks with 3 carp being sampled per tank per time point (total fish n=90). The bar chart shows the average gene expression for each feed (n=9). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. One fish was categorised as an outlier from tank C (0% MacroGard® feed) at time point 3 weeks. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. A 2-way nested (tank within feed) ANOVA with Tukey's as *post hoc* analysis was utilised to compare differences over time and between feeds. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.



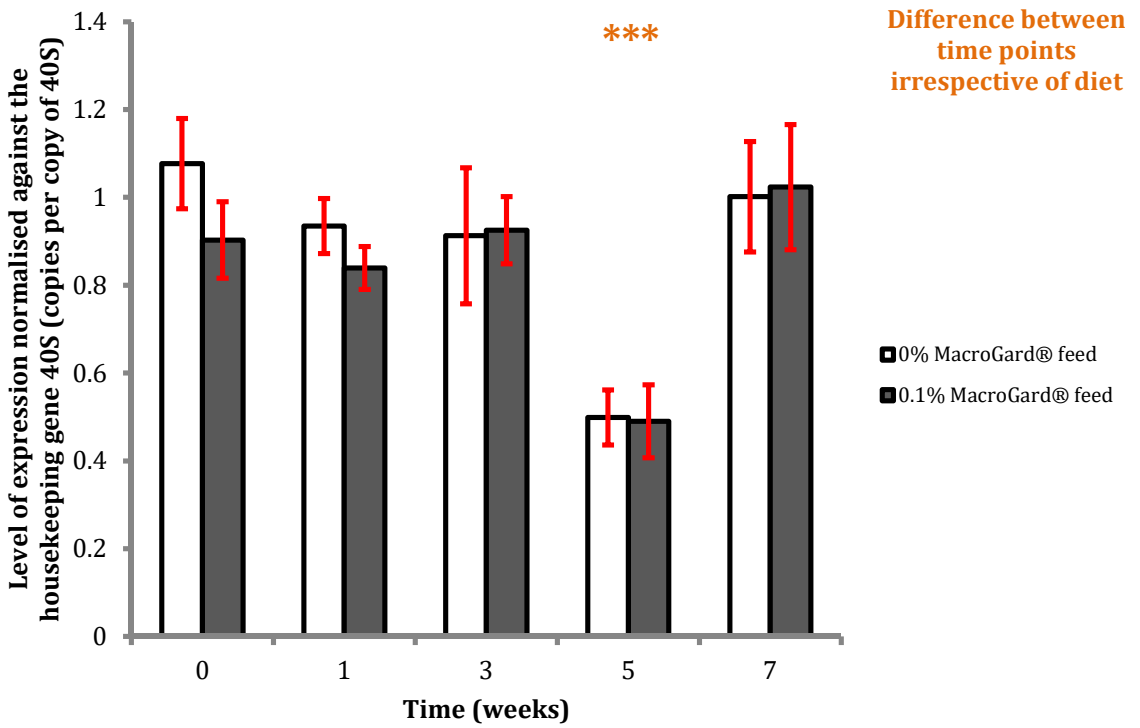
Box Cox transformation		λ=0.32		
Anderson-Darling test for normality	p=0.479	**Levene's test for equal variance**		p=0.993
2-way nested (tank within treatment) ANOVA with *post hoc* Tukey's (R^2=32.67%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between feeds	1	4.06	0.047	*
Nested effect of tank variation	4	2.08	0.091	
Overall difference over time	4	5.42	0.001	**
Interaction of feed and time	4	0.48	0.749	
***Post hoc* analysis**				
Time: 0 weeks versus 1 week		p=0.0057		**
Time: 0 weeks versus 3 weeks		p=0.0016		**
Time: 0 weeks versus 7 weeks		p=0.0355		*

Figure 4.5: Expression of the Interleukin 1β gene in the upper midgut of common carp (*C. carpio*) during a 7 week feeding trial. Carp were fed with either a 0% or 0.1% MacroGard® experimental diet designed by Tetra (GmbH) at a rate of 1% body weight per day. Each feed was given to 3 tanks with 3 carp being sampled per tank per time point (total fish n=90). Bars represent average gene expression for each feed (n=9). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. One fish was categorised as an outlier from tank B (0% MacroGard® feed) at time point 3 weeks. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. A 2-way nested (tank within feed) ANOVA with Tukey's as *post hoc* analysis was utilised to compare differences over time and between feeds. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.



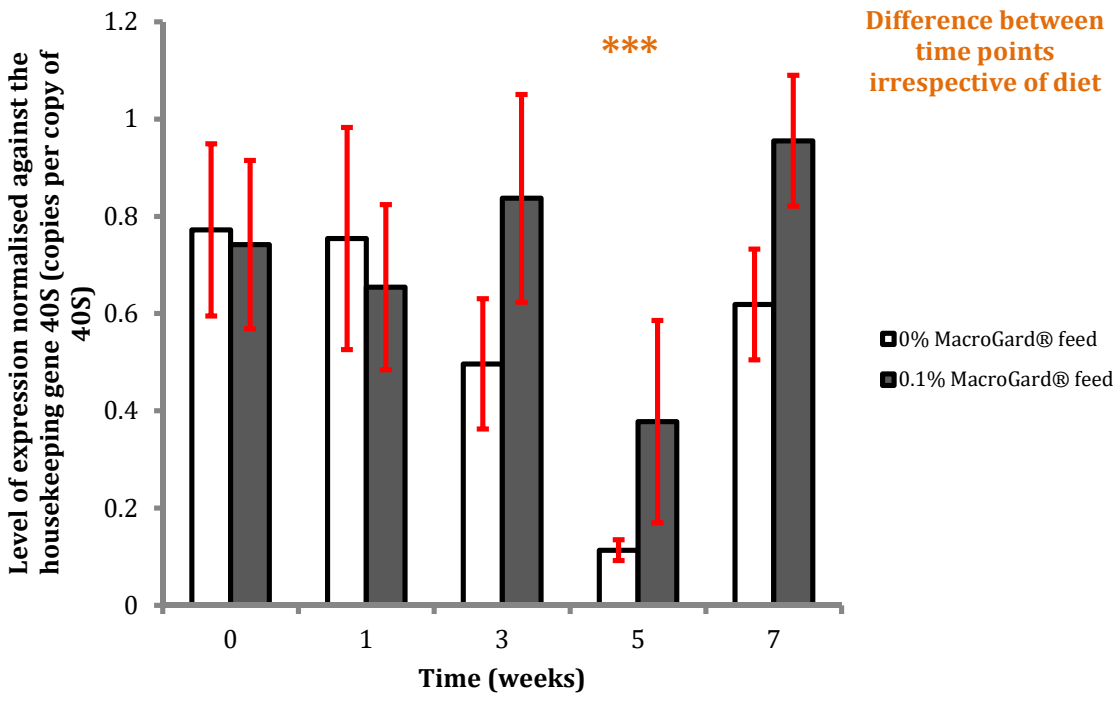
Box Cox transformation		λ=-0.24		
Anderson-Darling test for normality	p=0.255	**Levene's test for equal variance**		p=0.981
2-way nested (tank within treatment) ANOVA with *post hoc* Tukey's (R^2=20.28%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between feeds	1	0.43	0.516	
Nested effect of tank variation	4	0.40	0.811	
Overall difference over time	4	3.80	0.007	**
Interaction of feed and time	4	0.52	0.719	
***Post hoc* analysis**				
Time: 1 week versus 7 weeks		p=0.0069		**
Time: 3 weeks versus 7 weeks		p=0.0358		*

Figure 4.6: Expression of the iNOS gene in the upper midgut of common carp (*C. carpio*) during a 7 week feeding trial. Carp were fed with either a 0% or 0.1% MacroGard® experimental diet designed by Tetra (GmbH) at a rate of 1% body weight per day. Each feed was given to 3 tanks with 3 carp being sampled per tank per time point (total fish n=90). Bars represent average gene expression for each feed (n=9). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. Two fish were categorised as outliers: one from tank C (0% MacroGard® feed) at time point 5 weeks, and one from tank E (0.1% MacroGard® feed) at time point 7. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. A 2-way nested (tank within feed) ANOVA with Tukey's as *post hoc* analysis was utilised to compare differences over time and between feeds. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.



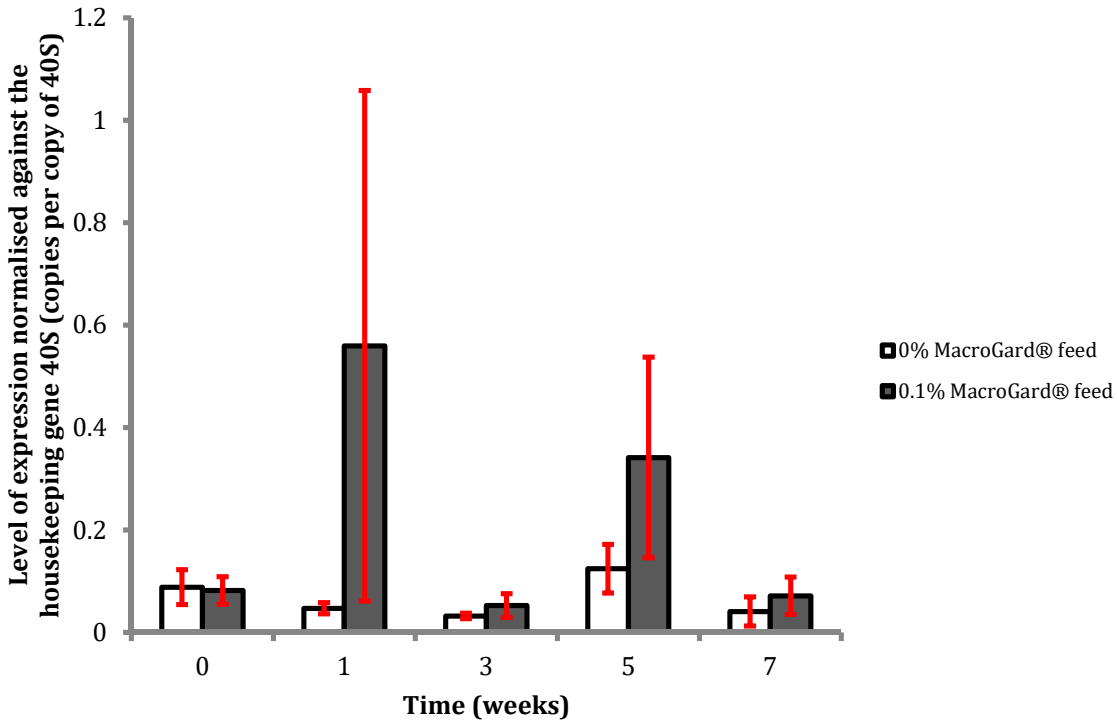
Box Cox transformation		λ=0.16		
Anderson-Darling test for normality	p=0.192	**Levene's test for equal variance**		p=0.929
2-way nested (tank within treatment) ANOVA with *post hoc* Tukey's (R^2=41.09%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between feeds	1	0.36	0.549	
Nested effect of tank variation	4	0.44	0.783	
Overall difference over time	4	12.16	<0.001	***
Interaction of feed and time	4	0.52	0.719	
***Post hoc* analysis**				
Time: 0 weeks versus 5 weeks		p<0.0001		***
Time: 1 week versus 5 weeks		p<0.0001		***
Time: 3 weeks versus 5 weeks		p<0.0001		***
Time: 5 weeks versus 7 weeks		p<0.0001		***

Figure 4.7: Expression of the Muc2 gene in the upper midgut of common carp (*C. carpio*) during a 7 week feeding trial. Carp were fed with either a 0% or 0.1% MacroGard® experimental diet designed by Tetra (GmbH) at a rate of 1% body weight per day. Each feed was given to 3 tanks with 3 carp being sampled per tank per time point (total fish n=90). Bars represent average gene expression for each feed (n=9). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. One fish was categorised as an outlier from tank D (0.1% MacroGard® feed) at time point 0 weeks. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. A 2-way nested (tank within feed) ANOVA with Tukey's as *post hoc* analysis was utilised to compare differences over time and between feeds. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant (p<0.05) *post hoc* comparisons being listed. Both in the graph and table, * signifies p<0.05, ** signifies p<0.01, and *** signifies p<0.001.



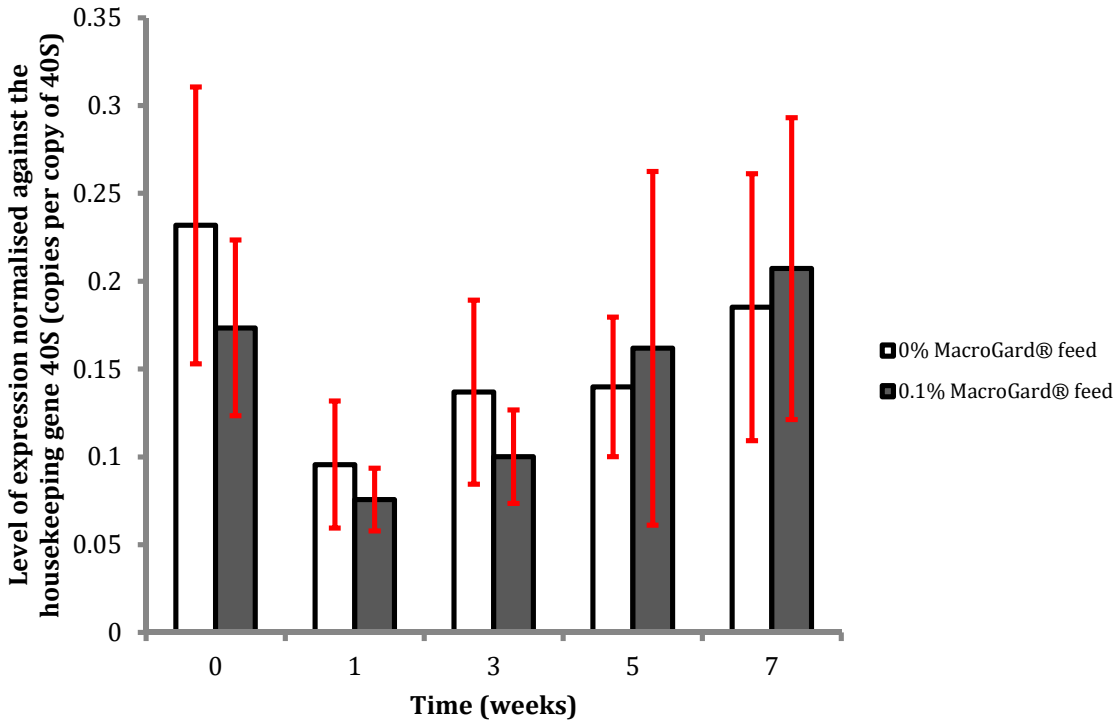
Box Cox transformation		λ=0.22		
Anderson-Darling test for normality	p=0.233	**Levene's test for equal variance**		p=0.946
2-way nested (tank within treatment) ANOVA with *post hoc* Tukey's (R^2=40.56%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between feeds	1	2.80	0.098	
Nested effect of tank variation	4	0.70	0.592	
Overall difference over time	4	10.02	<0.001	***
Interaction of feed and time	4	0.80	0.527	
***Post hoc* analysis**				
Time: 0 weeks versus 5 weeks		p<0.0001		***
Time: 1 week versus 5 weeks		p=0.0002		***
Time: 3 weeks versus 5 weeks		p=0.0004		***
Time: 5 weeks versus 7 weeks		p<0.0001		***

Figure 4.8: Expression of the CRP2 gene in the upper midgut of common carp (*C. carpio*) during a 7 week feeding trial. Carp were fed with either a 0% or 0.1% MacroGard® experimental diet designed by Tetra (GmbH) at a rate of 1% body weight per day. Each feed was given to 3 tanks with 3 carp being sampled per tank per time point (total fish n=90). Bars represent average gene expression for each feed (n=9). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. Three fish was categorised as outliers: one from tank B (0% MacroGard® feed) at time point 3 weeks, one from tank F (0.1% MacroGard® feed) at time point 5 weeks, and one from tank D (0.1% MacroGard® feed) at time point 7 weeks. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. A 2-way nested (tank within feed) ANOVA with Tukey's as *post hoc* analysis was utilised to compare differences over time and between feeds. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.



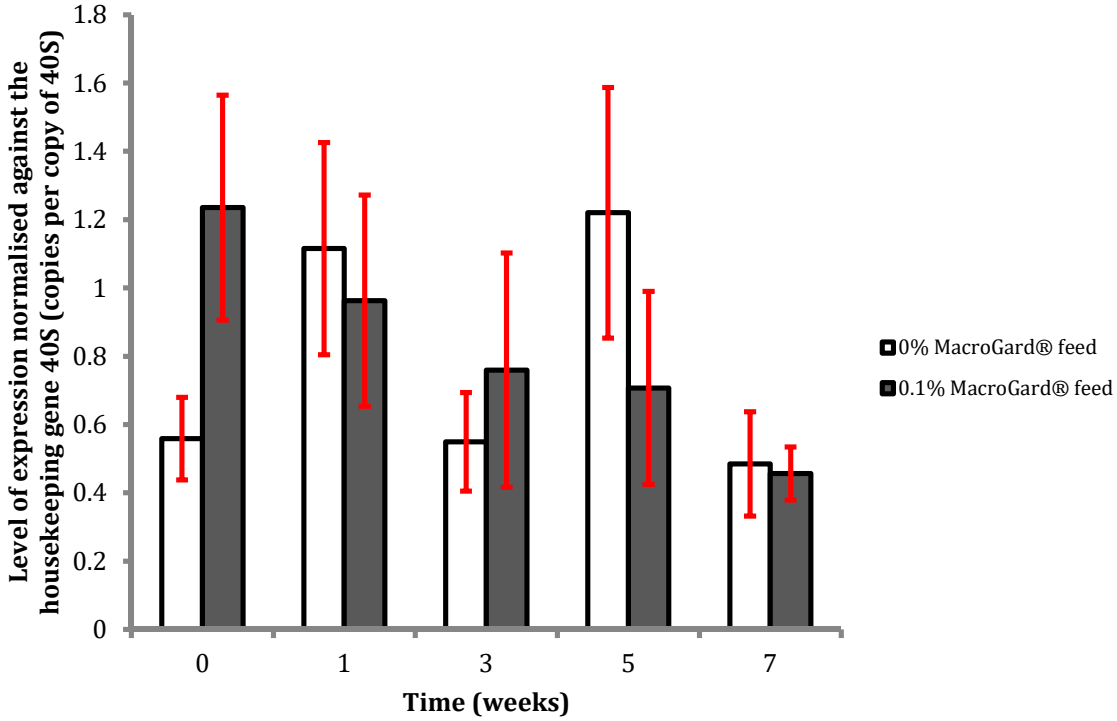
Box Cox transformation		λ>-5		
Anderson-Darling test for normality	p<0.005	**Levene's test for equal variance**		p=0.636
2-way Scheirer-Ray-Hare test (R^2=11.64%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between feeds	1	0.10	0.755	
Overall difference over time	4	2.28	0.067	
Interaction of feed and time	4	0.33	0.860	

Figure 4.9: Expression of the C3 gene in the upper midgut of common carp (*C. carpio*) during a 7 week feeding trial. Carp were fed with either a 0% or 0.1% MacroGard® experimental diet designed by Tetra (GmbH) at a rate of 1% body weight per day. Each feed was given to 3 tanks with 3 carp being sampled per tank per time point (total fish n=90). Bars represent average gene expression for each feed (n=9). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. Two fish were categorised as outliers: one from tank C (0% MacroGard® feed) at time point 1 week, and one from tank A (0% MacroGard® feed) at time point 5 weeks. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed and using a +1 followed by Box Cox transformation showed a value of λ smaller than -5 was required to normalise the data. The non-parametric Scheirer-Ray-Hare test was used for further statistical analysis. Details are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant (p<0.05) *post hoc* comparisons being listed.



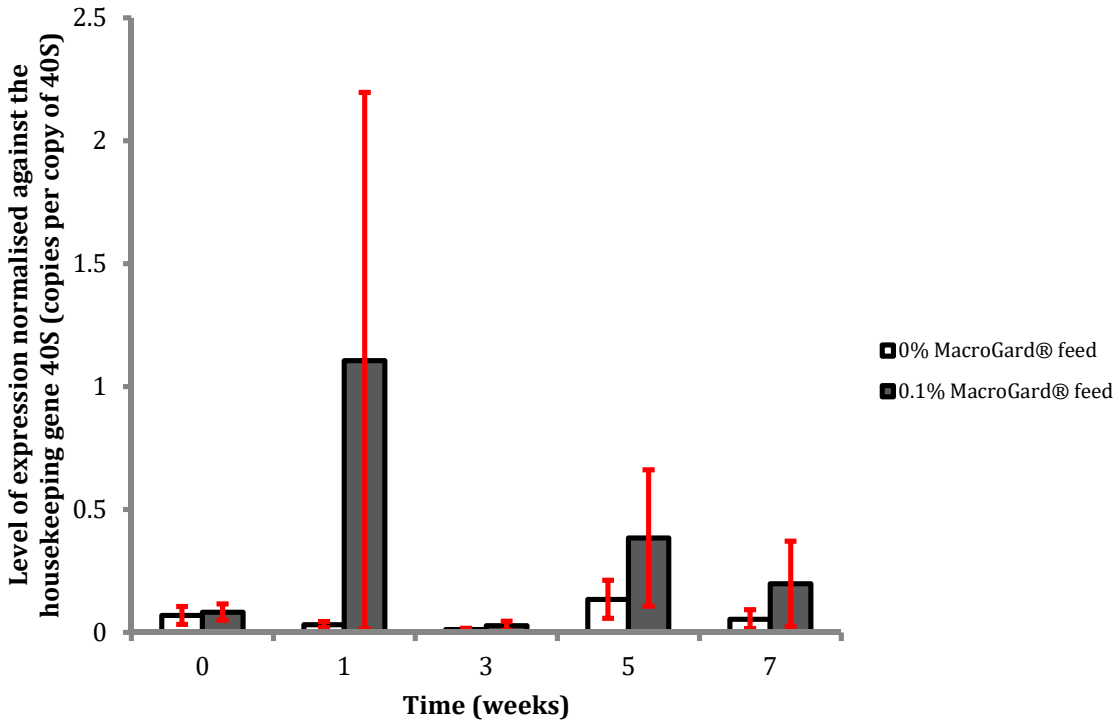
Box Cox transformation		λ=0.01		
Anderson-Darling test for normality	p=0.928	**Levene's test for equal variance**		p=0.914
2-way nested (tank within treatment) ANOVA with *post hoc* Tukey's (R^2=13.54%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between feeds	1	0.08	0.774	
Nested effect of tank variation	4	0.86	0.490	
Overall difference over time	4	1.89	0.122	
Interaction of feed and time	4	0.10	0.980	

Figure 4.10: Expression of the HAMP1 gene in the upper midgut of common carp (*C. carpio*) during a 7 week feeding trial. Carp were fed with either a 0% or 0.1% MacroGard® experimental diet designed by Tetra (GmbH) at a rate of 1% body weight per day. Each feed was given to 3 tanks with 3 carp being sampled per tank per time point (total fish n=90). Bars represent average gene expression for each feed (n=9). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. Three fish were categorised as outliers: one from tank C (0% MacroGard® feed) at time point 1 week, one from tank E (0.1% MacroGard® feed) at time point 1 week, and one from tank A (0% MacroGard® feed) at time point 5 weeks. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. A 2-way nested (tank within feed) ANOVA with Tukey's as *post hoc* analysis was utilised to compare differences over time and between feeds. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed.



Box Cox transformation		λ=-0.01		
Anderson-Darling test for normality	p=0.661	**Levene's test for equal variance**		p>0.999
2-way nested (tank within treatment) ANOVA with *post hoc* Tukey's (R^2=17.04%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between feeds	1	0.00	0.950	
Nested effect of tank variation	4	0.31	0.868	
Overall difference over time	4	1.91	0.117	
Interaction of feed and time	4	1.71	0.157	

Figure 4.11: Expression of the LEAP2 gene in the upper midgut of common carp (*C. carpio*) during a 7 week feeding trial. Carp were fed with either a 0% or 0.1% MacroGard® experimental diet designed by Tetra (GmbH) at a rate of 1% body weight per day. Each feed was given to 3 tanks with 3 carp being sampled per tank per time point (total fish n=90). Bars represent average gene expression for each feed (n=9). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. One fish was categorised as an outliers from tank A (0% MacroGard® feed) at time point 5 weeks. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. A 2-way nested (tank within feed) ANOVA with Tukey's as *post hoc* analysis was utilised to compare differences over time and between feeds. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed.



Box Cox transformation		λ=-0.11		
Anderson-Darling test for normality	p=0.661	**Levene's test for equal variance**		p=0.983
2-way nested (tank within treatment) ANOVA with *post hoc* Tukey's (R^2=17.96%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between feeds	1	0.13	0.716	
Nested effect of tank variation	4	1.16	0.336	
Overall difference over time	4	2.27	0.070	
Interaction of feed and time	4	0.63	0.642	

Figure 4.12: Expression of the ApoA1 gene in the upper midgut of common carp (*C. carpio*) during a 7 week feeding trial. Carp were fed with either a 0% or 0.1% MacroGard® experimental diet designed by Tetra (GmbH) at a rate of 1% body weight per day. Each feed was given to 3 tanks with 3 carp being sampled per tank per time point (total fish n=90). Bars represent average gene expression for each feed (n=9). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. Two fish were categorised as outliers: one from tank C (0% MacroGard® feed) at time point 1 week, and one from tank A (0% MacroGard® feed) at time point 5 weeks. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. A 2-way nested (tank within feed) ANOVA with Tukey's as *post hoc* analysis was utilised to compare differences over time and between feeds. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed.

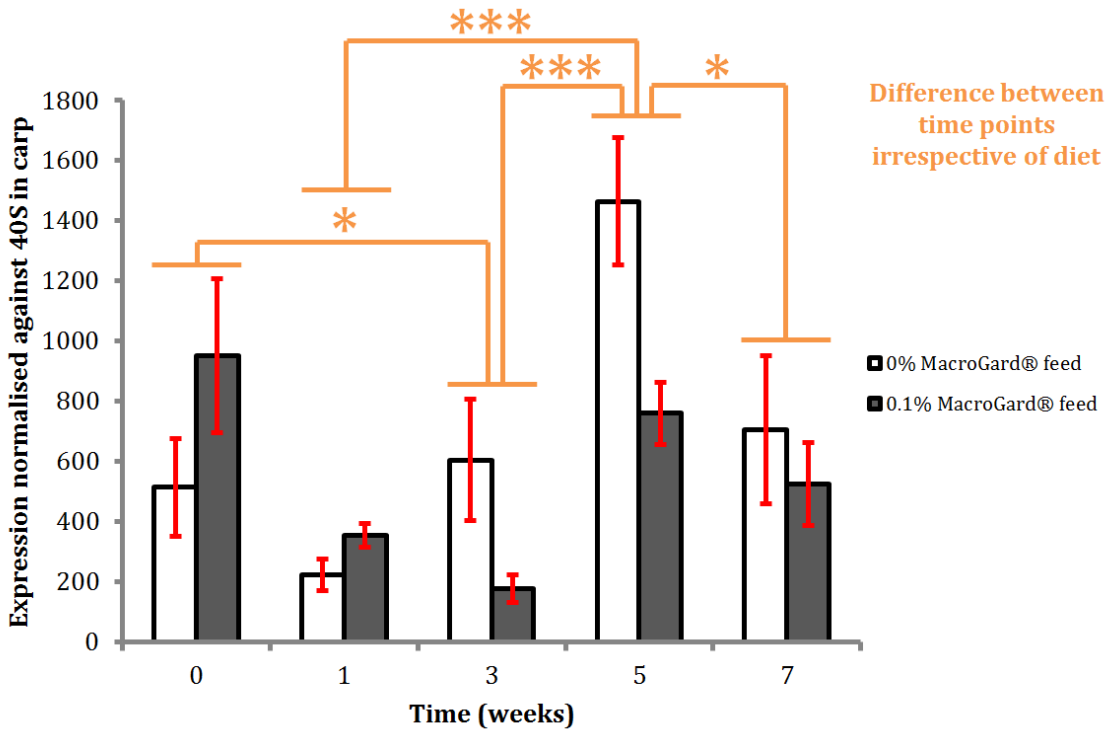
4.3.6.2 – Gut microbiome analysis.

Both quantitative and qualitative analyses were performed on the gut microbiome populations of carp in order to ascertain the impact of MacroGard® consumption on bacterial population sizes and species richness. Quantification of total 16S rDNA expression (Figure 4.13) revealed a significant effect of time, but not diet, on the overall size of the bacterial population in the gut ($p<0.001$). Expression levels at the very start of the trial when all fish had only consumed the 0% MacroGard® diet (described as week 0 in all figures) are significantly higher than those found at week 3 ($p=0.0458$), and week 5 has significantly higher expression levels than weeks 1 ($p<0.0001$), week 3 ($p<0.0001$) and week 7 ($p=0.0201$). Although different mean expression levels are seen when comparing between diets at each time point, *post hoc* analysis revealed no statistical significance between these differences. The largest difference in expression levels over time irrespective of feed is seen between weeks 1 and 5 with the latter being 3.8 times higher than that seen 1 week after the start of the trial. Within each feed over time, the range of average 16S expression was a 6.5 fold difference between the highest and lowest expression levels seen for carp fed with the 0% MacroGard® diet and a 5.3 fold difference for carp fed with the 0.1% MacroGard® diet.

In addition to the analysis of total bacterial population expression of individual genera, i.e. *Aeromonas* sp., *Pseudomonas* sp., *Flavobacterium* sp., *Vibrio* sp., and *Streptococcus* sp., were analysed in fish sampled at the start and end of the trial (labelled weeks 0 and 7 in Figure 4.14). No copies of *Vibrio* sp. or *Streptococcus* sp. 16S rDNA were detected in any of the carp analysed. Figure 4.14 shows the relative proportion of the total microbial population in each fish represented by *Aeromonas*

sp., *Pseudomonas* sp., and *Flavobacterium* sp.. As can be seen there is a large amount of variability both within individual tanks, for example carp from tank E contain proportionally a higher amount of *Aeromonas* sp. than can be seen in carp from tank D, and between the start and end of the trial as can be seen in tank A where *Pseudomonas* sp. and *Flavobacterium* sp. are seen in much higher proportions at the end of the trial than at the start. A 2-way nested ANOVA revealed no statistical differences in the proportions of *Aeromonas* sp. or *Pseudomonas* sp. within the fish gut, however the proportion of *Flavobacterium* sp. was significantly different when comparing fish from different tanks.

Qualitative analysis of species richness was performed based upon PCR-DGGE band patterns. The Bray-Curtis dissimilarity test revealed no difference in the level of dissimilarity between fish from different tanks which received the same feed or between time points, i.e. they were all similar (Figure 4.15), and non-metric Multidimensional Scaling (nMDS) analysis of the data further confirmed this (Figure 4.16). The stress of the nMDS ordination, i.e. how well the data fits within a 2D matrix, is given as 0.1294 which falls within the range described as an acceptable level of stress for 2D graphical representations of data (Podani 2000). The nMDS ordination, constructed from Bray-Curtis dissimilarities from multiple gels and with averaged dissimilarity being used when a comparison occurred on more than one gel (n=1/2 for each comparison), shows that there is no grouping of data points (average level of species richness within a tank at each time point) either by time point or by feed group.



Box Cox transformation		λ=0.22		
Anderson-Darling test for normality	p=0.565	**Levene's test for equal variance**		p=0.927
2-way nested (tank within treatment) ANOVA with *post hoc* Tukey's (R^2=44.87%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between feeds	1	0.90	0.345	
Nested effect of tank variation	4	2.17	0.081	
Overall difference over time	4	9.74	<0.001	***
Interaction of feed and time	4	3.86	0.007	**
***Post hoc* analysis**				
Time: 0 weeks versus 3 weeks		p=0.0458		*
Time: 1 week versus 5 weeks		P<0.0001		***
Time: 3 weeks versus 5 weeks		P<0.0001		***
Time: 5 weeks versus 7 weeks		p=0.0201		*

Figure 4.13: Expression of the bacterial 16S rDNA gene in the upper midgut of common carp (*C. carpio*) during a 7 week feeding trial. Carp were fed with either a 0% or 0.1% MacroGard® experimental diet designed by Tetra (GmbH) at a rate of 1% body weight per day. Each feed was given to 3 tanks with 3 carp being sampled per tank per time point (total fish n=90). Bars represent average gene expression for each feed (n=9). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. One fish was categorised as an outlier from tank A (0% MacroGard® feed) at time point 5 weeks. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. A 2-way nested (tank within feed) ANOVA with Tukey's as *post hoc* analysis was utilised to compare differences over time and between feeds. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.

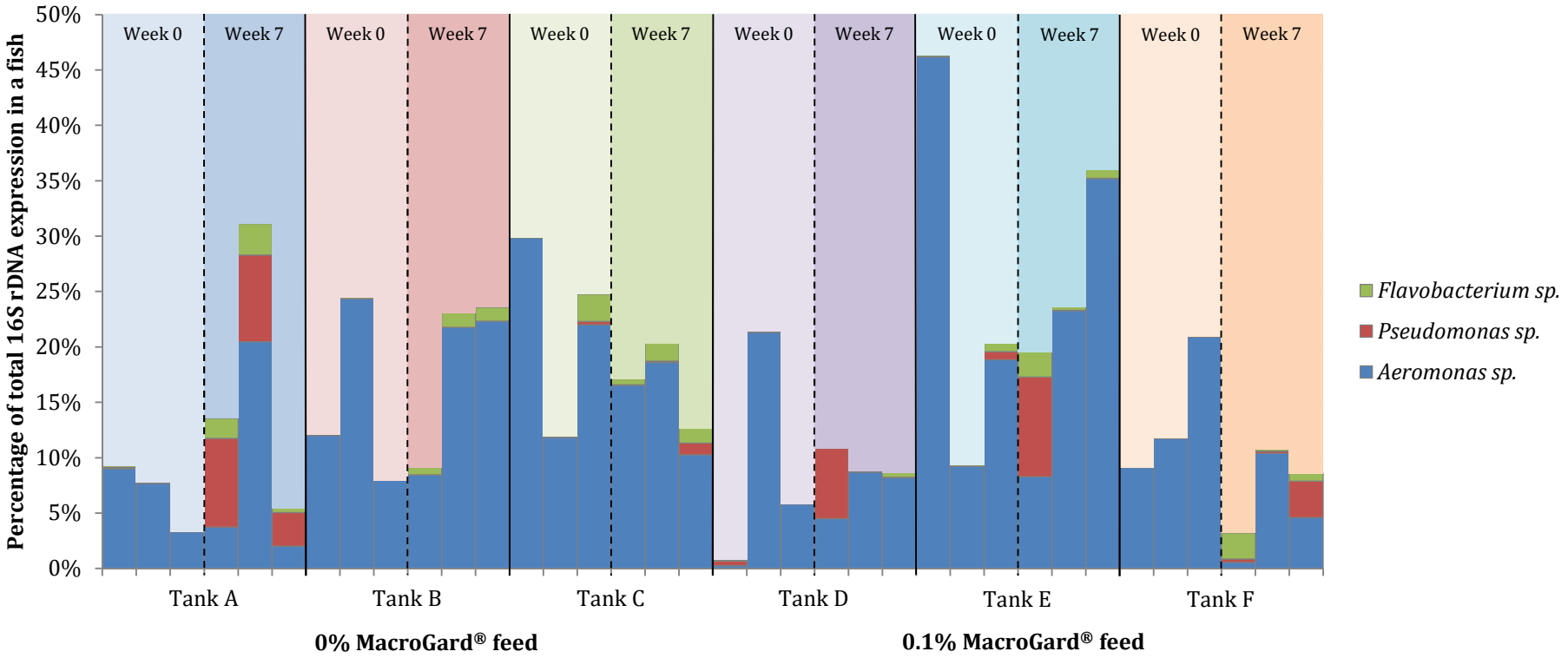


Figure 4.14: (part A) Expression of the bacterial 16S rDNA gene for three different bacterial genera in the upper midgut of common carp (*C. carpio*) at week 0 and week 7 of a 7 week feeding trial (part A). Carp were fed with either a 0% or 0.1% MacroGard® experimental diet designed by Tetra (GmbH) at a rate of 1% body weight per day. Each feed was given to 3 tanks with 3 carp being sampled per tank per time point (total fish n=36). Graph shows the relative proportion of 100% of total 16S rDNA expression for each fish within each group. Pastel colours represent the remaining bacteria genera in each fish. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. A 2-way nested (tank within feed) ANOVA with Tukey's as *post hoc* analysis was utilised to compare differences over time and between feeds for each of the analysed bacterial genera. Details are shown in part 2 of this figure. Degrees of freedom, F distribution and P values for overall significance are given.

***Aeromonas* sp.**	Box Cox transformation	λ=-0.01	**2-way nested ANOVA (tank within feed)**				
			(R^2=34.40%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
	Anderson-Darling test for normality	p=0.134	Overall difference between feeds	1	1.58	0.220	
			Overall effect of tank	4	2.53	0.063	
	Levene's test for equal variance	p=0.862	Overall difference over time	1	2.60	0.118	
			Interaction of feed and time	1	0.39	0.537	
***Pseudomonas* sp.**	Box Cox transformation	λ=-0.00	**2-way nested ANOVA (tank within feed)**				
			(R^2=15.19%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
	Anderson-Darling test for normality	P=0.097	Overall difference between feeds	1	1.81	0.189	
			Overall effect of tank	4	0.41	0.803	
	Levene's test for equal variance	p=0.605	Overall difference over time	1	0.76	0.391	
			Interaction of feed and time	1	0.82	0.374	
***Flavobacterium* sp.**	Box Cox transformation	λ=0.01	**2-way nested ANOVA (tank within feed)**				
			(R^2=34.70%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
	Anderson-Darling test for normality	p=0.795	Overall difference between feeds	1	2.08	0.161	
			Overall effect of tank	4	2.92	0.039	*
	Levene's test for equal variance	p=0.951	Overall difference over time	1	1.08	0.308	
			Interaction of feed and time	1	0.06	0.802	

Figure 4.14: (part B) Expression of the bacterial 16S rDNA gene for three different bacterial genera in the upper midgut of common carp (*C. carpio*) at week 0 and week 7 of a 7 week feeding trial (part B). Carp were fed with either a 0% or 0.1% MacroGard® experimental diet designed by Tetra (GmbH) at a rate of 1% body weight per day. Each feed was given to 3 tanks with 3 carp being sampled per tank per time point (total fish n=36). Graph shows the relative proportion of 100% of total 16S rDNA expression for each fish within each group. Pastel colours represent the remaining bacteria genera in each fish. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. A 2-way nested (tank within feed) ANOVA with Tukey's as *post hoc* analysis was utilised to compare differences over time and between feeds for each of the analysed bacterial genera. Details are shown in part 2 of this figure. Degrees of freedom, F distribution and P values for overall significance are given.

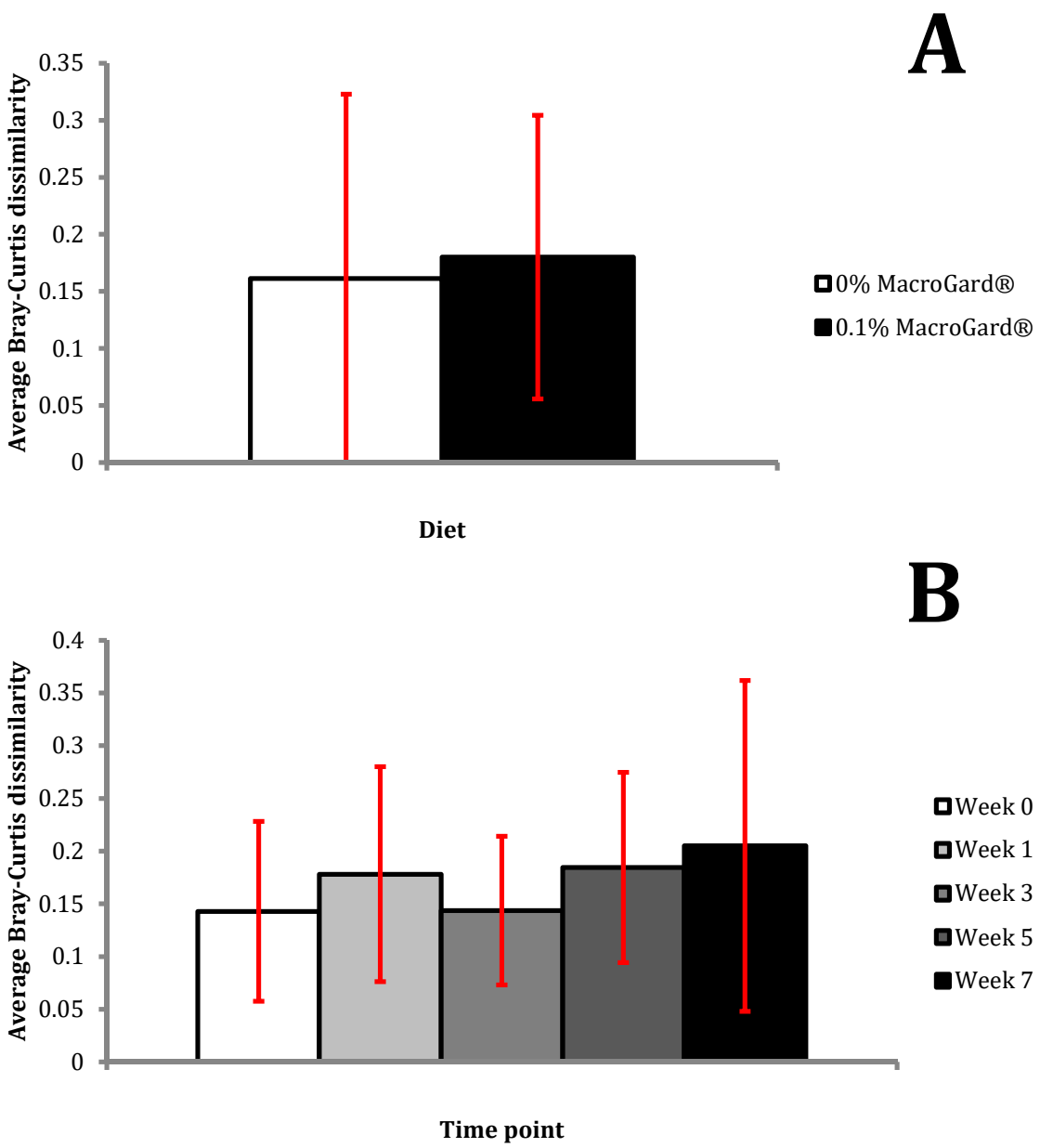


Figure 4.15: Average Bray-Curtis dissimilarities for carp fed with either a 0% or 0.1% MacroGard® diet for up to 7 weeks. Error bars represent standard deviation. A dissimilarity of 0 indicates two samples are identical. A shows the average dissimilarity between feed groups. B shows the average dissimilarity between time points.

Grouping	Marker
0% MacroGard® fed fish	Square
0.1% MacroGard® fed fish	Diamond
Week 0	Black
Week 1	Blue
Week 3	Red
Week 5	Green
Week 7	Orange

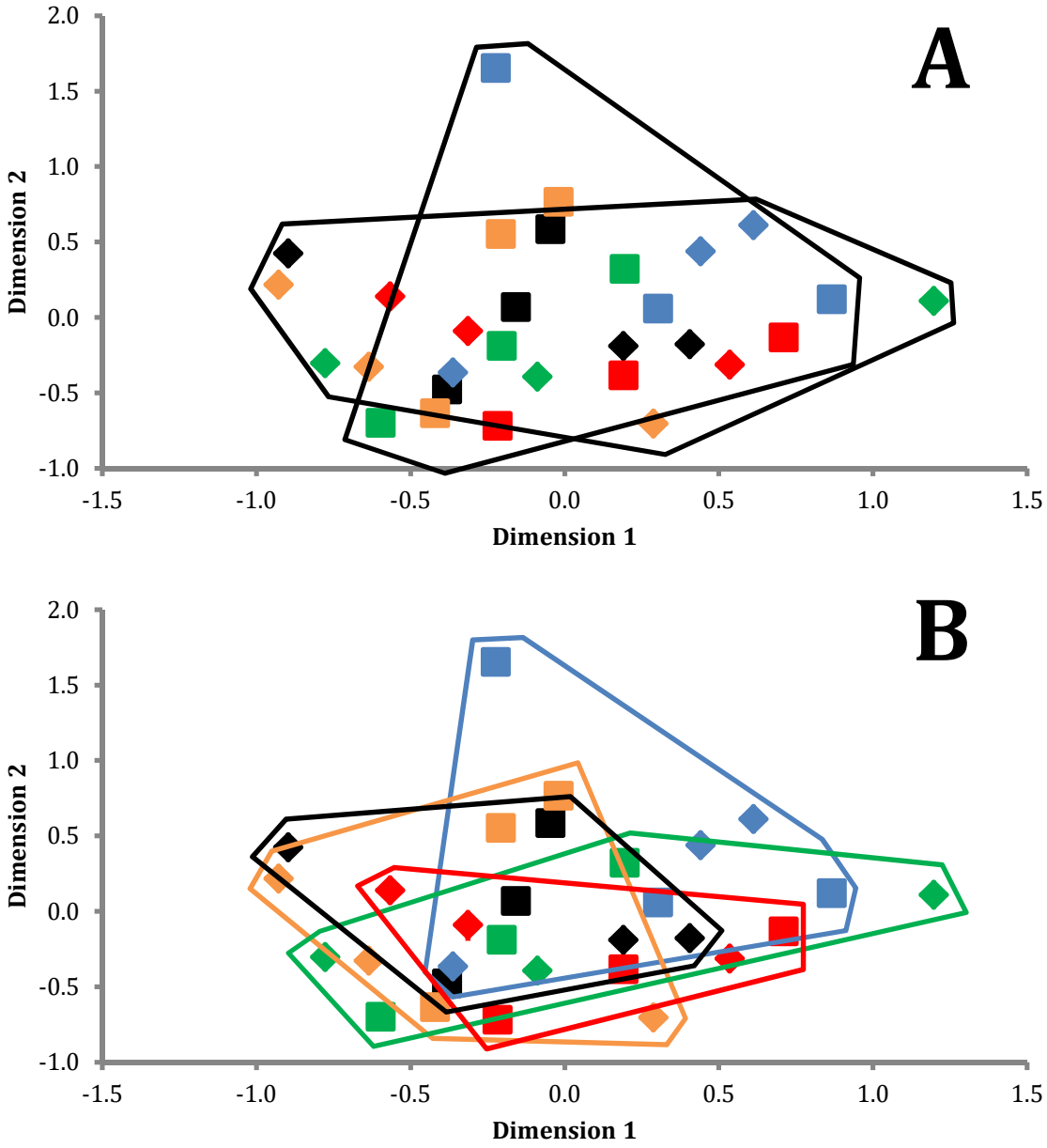


Figure 4.16: Non metric Multidimensional Scaling (nMDS) ordination (stress: 0.1294) of Bray-Curtis dissimilarities looking at the differences in species richness based on PCR-DGGE band patterns of carp that had been fed with either a 0% or 0.1% MacroGard® diet. Data is shown twice to easily visualise groupings by feed (A) and by time point (B). Where a comparison between two band patterns was performed on multiple gels, an average was used to generate the nMDS ordination (n=1/2).

4.3.6.3 – Comparing the immune response of carp and the gut microflora population.

In sections 4.3.6.1 and 4.3.6.2, the expression of innate immune genes and quantitative analysis of both the overall bacteria population size and amount of selected bacterial genera were performed. In this section this data is compared in order to study the relationship between these genes and the associated microflora within the gut and correlation is assigned based upon the classifications defined by Fowler *et al.* (1998). As shown in Table 4.4, the expression of the IL-1β receptor, IL-1β and CRP2 show a significant, modest correlation to total 16S rDNA expression ($p<0.001$). Both the IL-1β receptor and CRP2 were negatively correlated whereas IL-1β expression was positively correlated with 16S rDNA expression. There was no correlation between the expression of iNOS, C3, or the antimicrobial peptides HAMP1, LEAP2 and ApoA1 when compared against total 16S rDNA expression. Similarly, the expression of these genes at weeks 0 and 7 did not correlate with the expression of the 16S gene from the genera *Aeromonas*, *Pseudomonas* or *Flavobacterium* (see Table 4.5). The strongest correlation coefficient seen was between LEAP2 and the amount of Pseudomonas in the gut (positive correlation), however this is still only considered to be a weak correlation.

Table 4.4: The probability of a linear relationship between each of the immune genes analysed in the gut of common carp during a 7 week feeding trial and the size of the bacterial population within the gut as measured by total 16S rDNA expression (n=90). As data was not normally distributed, Spearman's test was used to calculate the correlation coefficient, i.e. how linear a correlation is, and the statistical significance. A rating as to how strong the correlation is (as described by Fowler *et al.* 1998) is also given with a coefficient of 0.00-0.19 being a very weak correlation, 0.20-0.39 being a weak correlation, 0.40-0.69 being a modest correlation, 0.70-0.89 being a strong correlation and 0.90-1.00 being a very strong correlation.

Gene	Correlation coefficient	P value	Correlation
IL-1β receptor	-0.498	<0.001	Modest negative
IL-1β	0.488	<0.001	Modest positive
iNOS	-0.135	0.205	Very weak
C3	0.022	0.838	Very weak
Muc2	-0.344	0.001	Weak negative
CRP2	-0.479	<0.001	Modest negative
HAMP1	0.075	0.479	Very weak
LEAP2	0.065	0.544	Very weak
ApoA1	0.080	0.451	Very weak

Table 4.5: The probability of a linear relationship between each of the immune genes analysed in weeks 0 and 7 of a 7 week feeding trial and the presence of the genera Aeromonas, Pseudomonas and Flavobacterium as measured by 16S rDNA expression. As data was not normally distributed, Spearman's test was used to calculate the correlation coefficient, i.e. how linear a correlation is, and the statistical significance. A rating as to how strong the correlation is (as described by Fowler *et al.* 1998) is also given with a coefficient of 0.00-0.19 being a very weak correlation, 0.20-0.39 being a weak correlation, 0.40-0.69 being a modest correlation, 0.70-0.89 being a strong correlation and 0.90-1.00 being a very strong correlation.

Gene	Correlation coefficient	P value	Correlation
***Aeromonas* sp.**			
IL-1β receptor	0.216	0.206	Weak
IL-1β	0.014	0.937	Very weak
iNOS	0.140	0.417	Very weak
C3	0.018	0.916	Very weak
Muc2	0.180	0.293	Very weak
CRP2	0.090	0.293	Very weak
HAMP1	-0.005	0.997	Very weak
LEAP2	0.225	0.185	Weak
ApoA1	0.007	0.185	Very weak
***Pseudomonas* sp.**			
IL-1β receptor	0.250	0.142	Weak
IL-1β	-0.047	0.787	Very weak
iNOS	0.141	0.411	Very weak
C3	0.033	0.847	Very weak
Muc2	0.134	0.435	Very weak
CRP2	0.262	0.122	Weak
HAMP1	-0.118	0.492	Very weak
LEAP2	0.308	0.068	Weak
ApoA1	-0.003	0.988	Very weak
***Flavobacterium* sp.**			
IL-1β receptor	0.203	0.234	Weak
IL-1β	-0.125	0.467	Very weak
iNOS	0.065	0.707	Very weak
C3	0.092	0.592	Very weak
Muc2	0.076	0.660	Very weak
CRP2	0.098	0.571	Very weak
HAMP1	-0.132	0.442	Very weak
LEAP2	0.232	0.172	Weak
ApoA1	0.060	0.727	Very weak

4.4 – Discussion.

Analysis of the cultured microbiota population within the gut of 0% MacroGard® fed carp revealed that the diversity observed was similar to that seen in other studies. Jung-Schroers *et al.* (2015) and Kuhlwein *et al.* (2013) both performed comparable trials containing groups of carp that received a 0% MacroGard® diet. The level of bacterial species diversity noted in this thesis falls between that described by Jung-Schroers *et al.* (2015) who reported a slightly lower level of diversity whereas Kühlwein *et al.* (2013) report a slightly higher level. As the carp in all three of these trials come from different locations and from a mix of outdoor and indoor reared fish, this indicates a comparable level of cultured bacterial diversity irrespective of environment.

Comparing dissimilarities between the different gut segments indicates differences in species diversity that are potentially geographically comparable to their location along the intestinal axis, i.e. the segments are more dissimilar the further away from each other they are. Whilst this is the opposite of previous studies looking at species richness along the carp intestinal axis (Harris 2013), the differences in methods employed to study the microbiota could contribute to the differences noted. Site specific microbial profiles are found in, for example, humans (Methe *et al.* 2012, Weinstock 2012), therefore it is not surprising to see a difference along the intestinal axis in fish. However it does highlight the interesting question that if non culture based methodologies do not elucidate differences along the axis and culture based methods do, are the differences seen using culture based techniques truly differences at all? It has been widely accepted that only a limited proportion of microbiota populations can be studied using culture based techniques

owing to the fact many bacterial species cannot be successfully grown under laboratory conditions (for review: Nayak 2010b) and as technology has evolved there is much less reliance upon more classic techniques in favour of methodologies such as PCR-DGGE and high throughput sequencing (Muyzer and Smalla 1998, Methe *et al.* 2012). This does not, however, discount culture based analysis as a viable means of studying gut ecology, and indeed allows for the procurement of samples for *in vitro* trials such as those performed within this thesis to study the effect of MacroGard® upon bacteria growth and survival separately from the influence of host immunity.

That MacroGard® did not influence the growth of *A. salmonicida* can be viewed as a positive outcome. *A. salmonicida* is the causative agent of furunculosis in salmonid species (Romer Villumsen *et al.* 2015) and has also been utilised as a pathogenic agent in experimental infection trials in carp (Falco *et al.* 2012b, Pionnier *et al.* 2013). Whilst it would be advantageous if MacroGard® was toxic to *A. salmonicida*, particularly when applied orally, that it does not promote growth of a potential pathogen is still a positive result. The lack of toxicity also indicates that greater survival rates seen after β-glucan application against furunculosis is possibly due to immunomodulation of the host rather than simply a reduction in pathogen number (Siwicki *et al.* 1994).

There are several studies on the use of *Bacillus* sp. as potential probiotics for fish (for review see Wang *et al.* 2008, Nayak 2010a), in addition to the use of probiotics and prebiotics in a combined approach to positively influence the gut microbiota population (Saad *et al.* 2013, Dawood and Koshio 2016). It is therefore unfortunate that MacroGard® did not promote increased growth of this potential bacterial probiotic, however this does not rule out other carbohydrates as potential

prebiotics that could promote the growth of *Bacillus* species (Abhari *et al.* 2015, Tamamdusturi *et al.* 2015). Application of probiotic and synbiotic diets of *B. coagulans* and inulin to rats lead to an increase in lactic acid bacteria, and significant decrease in Enterobacteriaceae within the GI tract (Abhari *et al.* 2015). In iridescent sharks (*Pangasianodon hypophthalmus*), a combination of *Bacillus* sp. NP5 and MOS resulted in a higher specific growth rate and food conversion rate than either *Bacillus* sp. or MOS feeding alone, and a higher CFU count was recovered from sharks fed with the synbiotic diet in comparison to those fed with the probiotic alone (Tamamdusturi *et al.* 2015). The absence of toxic effects on *B. subtilis* could still lead to combined feeding regimes with an immunomodulative effect of β-glucan directly upon the host and a probiotic effect from the bacteria itself.

The study of the effect of prebiotics and other carbohydrates such as β-glucan on the intestinal microbial population has become a highly popular area of research during the past 10 years with the development of technology allowing for in depth analysis of *in vivo* systems (for review see Ghanbari *et al.* 2015, Dawood and Koshio 2016). In comparison, the relationship between the microbiota and its host, remains relatively unknown due in part to the vast number of potential interactions, although studies, particularly in mammals, are highlighting possible interactions are involved in diseases such as irritable bowel disease (IBD) (Alipour *et al.* 2015, Jones-Hall *et al.* 2015, Peterson *et al.* 2015). With MacroGard® having been shown previously to be able to influence the intestinal microbiota of carp when orally applied (Kuhlwein *et al.* 2013, Jung-Schroers *et al.* 2015a), it is important to understand whether this is due to the immunomodulative capabilities of β-glucans or due to a prebiotic effect directly upon the microbiota population itself. As only some bacteria possess β-glucanases

(Planas 2000, Hattori *et al.* 2013), it should therefore not be surprising that none of the isolates selected for analysis showed an increase in growth after incubation with MacroGard® as isolates were taken from carp maintained on a 0% MacroGard® diet, i.e. an environment that would not necessarily selectively favour bacterial species that possess β-glucanases based solely upon this characteristic. It is interesting, however, that MacroGard® appeared to reduce the colony sizes, but not number of colonies of ISO20. This implies there is not a toxic effect, which logically would have resulted in fewer colonies, but there could be an impact upon the rate at which the bacteria replicates, i.e. how quickly the colonies grow. A retardation of growth of a bacterial species could influence the overall species diversity of a mixed population depending upon what proportion that species represented. This implies any prebiotic effect MacroGard® may have upon the microbiota population within the gut is likely to be subtle or on a smaller proportion of the species present which has an impact on the larger population overall.

In *in vivo* studies, tank or pond affect can be of major concern in aquaculture and experimental trials due to pseudoreplication, i.e. individual fish are replicates within a single tank (Riley and Edwards 1998). The use of a design and statistical model utilised in this thesis, which took the concept of pseudoreplication into consideration, showed there was no effect of tank on immune parameters within the feeding trial. Overall, feeding carp with a diet containing 0.1% MacroGard® did not result in any differences in the expression of selected immune genes in the gut when compared to carp fed with a 0% MacroGard® diet. Activation of the immune system without an association of a pathogen involvement is typically associated with asthma and allergies (Lewis 2002, McLoughlin and Mills 2011), therefore it can be seen as a

positive outcome as the fish during this trial were apparently healthy, were not experimentally exposed to potential pathogens, and additionally did not experience an alteration of immune status.

There are publications that also analyse the expression of innate immune parameters in carp that contradict the data shown in this thesis. Pionnier *et al.* (2013) detailed the expression of key components of the three different complement pathways, in addition to the central protein, C3, within the gut of carp after 14 days of feeding with MacroGard®. Interestingly, where the data presented by Pionnier *et al.* (2013) show expression of C1rs, bf/C2 and MASP2 in the gut of individual carp, expression levels within pooled samples within this thesis were too low to continue with further analysis. It is, however, unknown as to what level of expression for each gene Pionnier *et al.* (2013) saw as their data is presented as relative to negative controls i.e. expression seen in 0.1% MacroGard® fed carp relative to those fed with a 0% MacroGard® diet. Whilst the primers for analysis of expression levels of these genes were the same in both Pionnier *et al.*'s (2013) study and the data presented here, the PCR conditions, i.e. the polymerase utilised, were different. C3 expression is shown to be significantly higher 11 days after hatching in turbot (*Scophthalmus maximus*) fed with MacroGard® treated rotifers in comparison to non-treated rotifers (Miest *et al.* 2016). The majority of studies, however, looking at the effect of β-glucans upon complement activity focus on analysis of serum complement.

Results presented in this thesis do not support the observations made by (Falco *et al.* 2012b) who showed that expression of both TNFα isoforms occurred in both non MacroGard® fed and MacroGard® fed carp. The lack of expression seen in my studies could be related to the fish utilised. Different carp strains have been

shown to have different survival rates against viral pathogens such as Cyprinid herpesvirus (CyHV) 3 (Shapira *et al.* 2005, Piackova *et al.* 2013, Adamek *et al.* 2014) and although the effect of fish strain on the mechanisms of the immune response is still being elucidated, it has been shown that strains of carp and crossbreeding can have an effect on serum complement levels (Nath *et al.* 2014) and TNFα in crossbred cattle showed differential expression patterns based upon polymorphisms which affected their susceptibility to mastitis (Ranjan *et al.* 2015).

In addition to there being no observed effect upon the expression of the immune genes analysed, there was also no difference in overall bacterial population size within the gut between 0% and 0.1% MacroGard® fed carp. In contrast, time seems to be a highly influential factor in 16S expression. Kuhlwein *et al.* (2013) showed a decrease over time in culturable aerobic heterotrophic autochthonous bacteria analysed using culture based methods. This indicates variation in gut microbiota population size over time could occur naturally irrespective of feed regime. Whilst Miest *et al.* (2016) analysed total 16S expression in turbot larvae during a feeding trial, they presented their data as the relative percentage of specific genera of total expression therefore it is unknown if diet influences the overall gut microbiota population size within the gut.

In contrast to Jung-Schroers *et al.* (2015a), there was no change in bacterial species richness within the gut due to MacroGard® feeding. Studies carried out by Kuhlwein *et al.* (2013) however, may explain the difference in these findings. Where Jung-Schroers *et al.* (2015) fed MacroGard® at an inclusion rate of 1% w/w within the diet, in my investigation MacroGard® was only included at 0.1% w/w. Kuhlwein *et al.* (2013) utilised both concentrations of MacroGard® i.e. 0.1% and 1% which, together

with the data presented by Jung-Schroers *et al.* (2015) and within this thesis, suggests the effect on species richness is dependent on the dose of MacroGard® utilised. This makes sense in that bacteria capable of utilising β-glucans as a substrate would be more prolific when there is a larger food source available and would ultimately outcompete species that could not use β-glucan. This data indicates the concentration of MacroGard® required to influence bacterial species richness within the gut is greater than that found in commercial food products, i.e. greater than 0.1% w/w within the feed.

Whilst qualitative analysis showed no differences between feeds or time points, quantitative analysis of the genera *Aeromonas*, *Pseudomonas* and *Flavobacterium* revealed that there was a large amount of variation and the lack of any significance between both time points and feeding regimes is linked to a small sample size. There is too much variation when comparing individual fish, i.e. a standard deviation that is almost equal to the mean copy number of 16S for each genus, to make any discernible conclusions as to what can be considered as a "normal" amount of each bacterial genera to be present within the gut under standard rearing conditions.

Flavobacterium sp. are the causative agents of multiple diseases including bacterial cold water disease (Sugahara and Eguchi 2012) and bacterial gill disease (Sink and Lochmann 2008). As the presence of *Flavobacterium* sp. was only significantly influenced by tank and not by time or feeding with MacroGard®, this highlights the importance of taking tank effect into consideration based upon the analysis to be performed (Riley and Edwards 1998). Infection of *Flavobacterium* has also been shown to cause secondary bacterial infections after preliminary viral

infections in carp (Adamek *et al.* 2013) however if a potential pathogen is only present in some tanks within an experimental design, this could have implications in how any resultant data is analysed, i.e. if a secondary bacterial infection were to occur in only a proportion of fish analysed but all fish within a single tank showed symptoms of infection, reporting data irrespective of tank would not be appropriate.

In summary, culture based analysis of the intestinal microbiota population showed differences in species diversity along the intestinal axis in carp fed with a 0% MacroGard® diet, although previous studies have shown no difference when using non culture based methods of analysis (Harris 2013). *In vitro* analysis of bacteria isolates have revealed a limited effect of MacroGard® upon growth and survival of bacteria, however it did not promote growth of known potential pathogens which can be considered as a positive effect. The oral application of MacroGard® did not influence either the immune status of carp nor the microbiota population which is in contrast to the data presented by Jung-Schroers *et al.* (2015) who showed, in association with this investigation and work carried out by Kuhlwein *et al.* (2013), that there could be a dose effect of MacroGard® with higher concentrations being able to influence the microbiota population. These concentrations are, however, beyond those that are present in commercial feeds indicating any protective effects seen in other β-glucan trials and in the field are due to the immunomodulatory effect rather than due to a biproduct prebiotic effect.

Chapter 5 – Analysis of the effect of injection upon the gut microbiota and immune status.

In Chapter 4 the effect of oral application of MacroGard® on the bacterial fauna in and immune status of the gut in carp was described. Since β-glucan has previously been shown to also act as an immunomodulant when applied via injection (Selvaraj *et al.* 2005, Selvaraj *et al.* 2006), the effect of intraperitoneal injection of MacroGard® on the immune status of carp and gut bacterial fauna was elucidated.

To date, only two published papers (Liu *et al.* 2008, Liu *et al.* 2015) and one masters thesis (Harris 2013) have addressed the impact of injection of β-glucans upon the intestinal microbiome in fish. Liu *et al.* (2008) noted that whilst LPS was able to influence the bacterial species richness in the microbiome within the gut of Atlantic salmon (*Salmo salar*) 28 days after injection, β-glucan did not. Unfortunately, analysis on the total size of the bacterial population was not performed. Liu *et al.* (2015) similarly did not analyse the total size of the gut bacteria population in their trial which studied the effect of bath vaccination of grass carp (*Ctenopharyngodon idella*) against *Aeromonas hydrophila* infection. Analysis of species richness 10 days post injection with a pathogenic strain of the bacterial species did however show a decrease in *Aeromonas* sp. within the gut microbial population. In addition to studying bacterial species richness, the author, in her previous studies (Harris 2013), compared gut bacteria population size in carp before and after injection with MacroGard®. Bacterial species richness (as measured using PCR-DGGE) 23 days post injection with MacroGard® was similar to that found in non-injected controls however the richness was notably different 12 days post injection to both non

injected controls and at day 23. In addition to a difference in species richness, total 16S rDNA expression revealed the overall bacterial population size was lower at day 12 post injection with MacroGard®. Based upon this evidence, it was suggested that injection with MacroGard® reduced the bacterial population size within the gut and that species richness was affected as the population "returned to normal", i.e. species richness and population size seen in non-injected controls.

β-glucans are macromolecules based upon β-D-glucose monomers and, as such, chain length, degree of branching and solubility can vary between molecules. β-glucan products are typically defined by type of linkage between individual monomers, i.e. carbon 1 linking to either carbon 3, 4 or 6 of the following unit, but they are heterogeneous mixes within this description. It is known that size can influence the effect of β-glucan upon the innate immune system (Przybylska-Diaz *et al.* 2013), but the mechanisms as to how and why require elucidation.

MacroGard® is a heterogeneous mix of β-1/3,1/6-glucans and whilst it is extensively used within aquaculture, results vary on its effect on immune parameters. For example, Selvaraj *et al.* (2005) showed no difference in ACP activity between control (β-glucan free) and β-glucan fed carp, whereas Pionnier *et al.* (2014) noted an increase in ACP activity in β-glucan fed carp in comparison to carp fed a β-glucan free diet. This could be due to structural differences in the β-glucan such as different chain lengths and number of branches, both of which are affected by processing methodologies, or even concentration of a particular β-glucan structure within a heterogeneous mix. Research at Keele University is generating a library of different purified β-1/3,1/6-glucans to test their individual activities as opposed to their effect within a heterogeneous mix. One of these β-glucans, MSS1, has been shown to be

more effective than MacroGard® at eliciting an immune response during *in vitro* trials (Nawroz Kareem, unpublished data).

The aim of this chapter is therefore to establish if injection of MacroGard® and the specifically formulated MSS1 affected the immune response of the gut and the gut microbiome when injected intraperitoneally into carp.

An initial small scale injection trial focused on total 16S rDNA expression and species richness of the microbiome in the gut of carp. In addition, the expression of bactericidal innate immune genes (iNOS and C3) and immune associated signalling genes (TNFα and IL-1β), known to be significantly affected by orally applied MacroGard® during infection trials (Falco *et al.* 2012b, Miest *et al.* 2012, Pionnier *et al.* 2014), was established and correlated against bacterial 16S rDNA expression as a measure of the gut microbiota population size. In the second trial, carp were injected separately with MacroGard® and MSS1 and the effects on the expression of selected bactericidal innate immune genes in the gut and the intestinal microbiota population ascertained. As discussed in Chapter 4, there are limited studies that consider both the immune response and the intestinal microbiota and how they may interact with each other. Statistical analysis will therefore be used to determine if there is any correlation between innate gene expression and the size of the bacteria population within the gut.

5.1 Materials and methods.

5.1.1 – Small injection trial: fish husbandry and experimental design.

35 carp, obtained from Hampton Spring fisheries, UK, in October 2012, were maintained (section 4.1.2) in a single tank and fed on 0% MacroGard® feed (section 4.1.3) prior to and during the experiment at a rate of 1% body weight per day. Carp had an average weight of 49.4g (s.d. ±11.2g) and an average length of 127mm (s.d. ±10mm) at the start of the trial. Carp were divided into 4 treatment groups as outlined in Table 5.1 and received either no injection, injection with PBS, injection with LPS (4mg kg^{-1}) or injection with MacroGard® (2mg kg^{-1}). Injections were prepared as described in sections 5.1.3, 5.1.5, and 5.1.6 respectively. Carp (n=5) that did not receive any injection were sampled at the start of the trial and are described as 0 days post injection. Fish were briefly submerged in 2-phenoxyethanol (1ml/5L) for approximately 1 minute before receiving their injection in the mid ventral aspect and a small cut to their tail fin to identify treatment group. These fish were then observed for 10 minutes to ensure no immediate adverse effects of handling/injection before being returned to the experimental tank. From each treatment group, 5 carp were sampled 1 day and 4 days post injection. Before sampling, fish were euthanized by submersion in 2-phenoxyethanol (1ml/5L), sections of total gut, gut wall and gut content taken from the upper midgut as illustrated in Figure 4.3 and were stored in RNA*later* at -80°C until further analysis.

Table 5.1: Experimental design of intraperitoneal injection pilot trial. Table shows time points and treatments. Each group consists of n = 5 fish.

		Treatment			
		No injection	PBS	LPS	MacroGard®
Time point	0 days	5	-	-	-
	1 day	-	5	5	5
	4 days	-	5	5	5

5.1.2 – Large injection trial: fish husbandry and experimental design.

120 carp were acquired in September 2013 and maintained in 8 tanks (n=15 fish per tank) on a 0% MacroGard® diet for 4 months prior to analysis and during the experimental period at a feed rate of 1% body weight per day (see sections 4.1.2 and 4.1.3 for fish husbandry and feed details). These fish were treated with Banish Fish Ulcer, Parasite and White Spot Treatment 3 weeks after entering the aquarium due to an outbreak of *Ichthyophthirius multifiliis* and were not utilised for experimental use until 3 months after the final treatment.

Carp had an average weight of 91.4g (s.d. ±12.4g) and an average length of 162mm (s.d. ±8mm). Treatment groups were divided as described in Table 5.2 with 30 carp in each treatment group (2 tanks with 15 carp per tank). Fish that received an injection of MacroGard® were injected with 10mg kg^{-1} (section 5.1.6) and those that received MSS1 did so at a concentration of either 5mg kg^{-1} or 10mg kg^{-1} (section 5.1.7). Both substances were suspended in sterile double distilled water and carp that were part of the injected negative control group were injected with sterile double distilled water (section 5.1.3). Before injection, carp were sedated in 2-phenoxyethanol (1ml/5L) and monitored for 10 minutes before being returned to their original tank for the remainder of the trial. From each tank, 3 carp which did not receive any injection were sampled at the very start of the trial (described in all

figures and tables at time point 0 days post injection). Carp were euthanized by submersion in 2-phenoxyethanol (section 4.3.3) and segments of the total gut, gut wall and gut content were stored in RNA*later* at -80°C until further use. Carp (n=3 per tank) were sampled 1, 3, 7 and 14 days post injection.

Table 5.2: Experimental design of injection trial utilising MacroGard® and MSS1, a novel β-glucan isoform, to modulate the immune system of common carp via intraperitoneal injection. Each treatment group consists of two tanks and n=3 fish per tank. Carp samples on day 0 received no injection and acted as a treatment negative control.

Treatment		Water		MacroGard®		MSS1 5mg kg-1		MSS1 10mg kg-1	
Tank		A	B	C	D	E	F	G	H
Time point (days post injection)	0	3	3	3	3	3	3	3	3
	1	3	3	3	3	3	3	3	3
	3	3	3	3	3	3	3	3	3
	7	3	3	3	3	3	3	3	3
	14	3	3	3	3	3	3	3	3

5.1.3 – Preparation of injections and fish identification.

All injections within this chapter were performed by Professor David Hoole (Keele University, United Kingdom. PPL 40/3532). In both trials, carp were injected with 100µl of liquid regardless of size, experiment or treatment. Treatments were suspended in either phosphate buffer saline (PBS) or sterile water for injection. Negative control groups were injected with eluent alone. All syringes were prepared within the 24 hours prior to injection and kept at 4°C during the interim period between preparation and use. Prior to injection carp were, as previously described, anaesthetised in 2-phenoxyethanol (1ml/5L) for approximately 1 minute before receiving an intraperitoneal injection through the ventral body wall between the pelvic and pectoral fins. Fish were placed in fresh water and observed for up to 10 minutes to ensure a full recovery.

5.1.4 – Identification of treatment groups within a single tank – small injection trial only.

As carp were maintained in the same tank to reduce variation in the water microbial population during this trial, they were marked via clipping of the tail fin in order to identify different treatment groups. This was performed at the same time as anaesthetisation.

5.1.5 – Preparation of LPS for injection (small injection trial only).

Lipopolysaccharide (LPS) from *E. coli* (O55:B5) was utilised as a positive control for immunostimulation of innate immune gene expression in the gut of common carp (Falco *et al.* 2012b). LPS was prepared as per the manufacturer's instructions. 5mg of LPS was dissolved in 1ml of PCR grade water and the concentration adjusted accordingly using sterile PBS solution (negative control used within this trial). LPS preparation was performed in the 6 hours prior to injection and stored at 4°C until use.

5.1.6 – MacroGard® preparation (both trials).

MacroGard® was prepared at a concentration of 10% w/v PBS solution or sterile water as described in section 3.1. The concentration was adjusted to 1% w/v, mixed by inversion before incubation at 80°C for 20 minutes and then held at 4°C until use on the same day of preparation.

5.1.7 – MSS1 preparation (large injection trial only).

MSS1 was prepared by Nawroz Kareem as follows. MacroGard® (500g) was

added to pre-chilled dry pyridine in advance of the addition of chlorosulfonic acid (1:16 v/v). The mixture was incubated at 95°C for 2 hours prior to cooling with the assistance of an ice-bath. Sodium hydroxide (10M) was added to the mixture with stirring until precipitation occurred. The contents were subsequently transferred to ice cold ethanol that had been pre-saturated with sodium acetate. The precipitate was washed extensively before dissolution in and dialysis against double distilled water. The dialysed solution was frozen and lyophilised before size exclusion chromatography was performed using HPLC grade water and a pre-packed PD-10 column, as per the manufacturer's instructions. The carbohydrate was then suspended in 100µl of double distilled water at concentrations corresponding to 5mg kg^{-1} and 10mg kg^{-1}.

5.1.8 – Taking of tissue samples from carp.

Samples of the gut wall, gut content and total gut were taken as described in section 4.3.3 and stored in RNA*later* at -80°C until further use.

5.1.9 – Quantitative analysis of innate immune gene expression and total 16S rDNA expression.

For both trials within this chapter, very similar techniques were employed during analysis as outlined for the feeding trial described in Chapter 4. RNA was isolated from gut wall samples as outlined in section 0. Prior to cDNA synthesis, RNA was treated with DNase to remove any DNA contamination. This was performed as outlined in section 4.3.4.2. Although the same Moloney Murine Leukemia Virus Reverse Transcriptase (M-MuLV RT) kit was used for cDNA synthesis, a change in the

protocol by the manufacturer, Invitrogen, unavoidably resulted in two slightly different protocols being employed within this chapter. cDNA synthesis from RNA in the small injection trial was performed as described in section 2.7.2 whereas cDNA in the large injection trial was synthesised as described in section 4.3.4.3. RT-qPCR analysis was performed as described in section 4.3.4.4. The following genes were analysed in each trial (sequences are given in Appendix 3) with the 40S housekeeping gene being used as a baseline:

Small injection trial: 16S_uniBact, C3, IL-1β, iNOS TNFα isoforms 1 and 2.

Large injection trial: 16S_uniBact, ApoA1, Bf/C2, CRP2, C1rs, HAMP1, iNOS, LEAP2, MASP2 and Muc2.

5.1.10 – Comparing immune gene expression with 16S rDNA expression.

Correlation analysis of the expression of the 16S rDNA gene, i.e. an approximation of the overall bacteria population size within the gut, against each of the immune genes studied was performed as described in section 4.3.4.5.

5.1.11 – Qualitative analysis of bacterial species richness.

genDNA isolation and PCR-DGGE analysis of total gut samples from both trials were performed as described in section 4.3.5.

5.2 – Results: initial small injection trial.

The results for this experiment are divided into three sections: analysis of innate immune parameters (section 5.2.1), gut microbiota population (section 5.2.2) and statistical analysis comparing these two components (section 5.2.3).

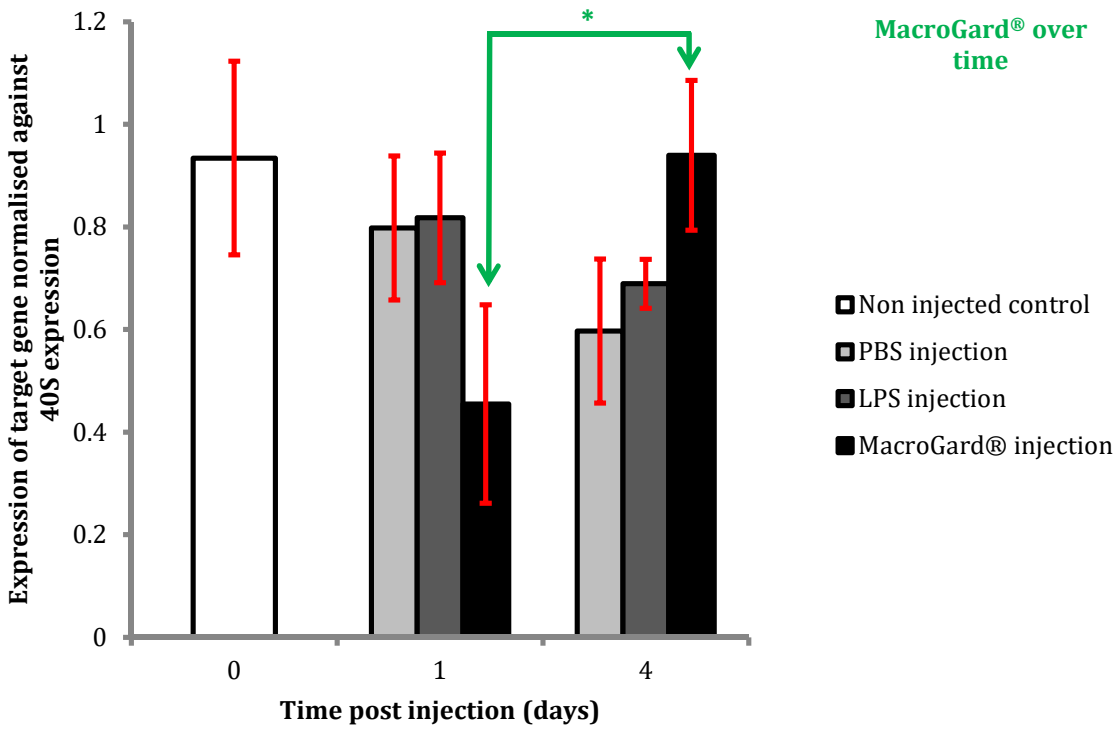
5.2.1 – Bactericidal innate immune parameters within the gut.

The expression levels of the innate immune genes IL-1β (Figure 5.1), iNOS (Figure 5.2), TNFα 1 & 2 (Figure 5.3 and Figure 5.4 respectively) and C3 (Figure 5.5) were analysed. As with the feeding trial presented in section 4.3, Grubbs' test was employed before further statistical analysis to eliminate outliers within the data. One outlier was found for each gene analysed. Four out of five outliers were found in either the non-injected control group (iNOS) or the PBS injected group (both isoforms of TNFα and C3) with the outlier for IL-1β appearing in the LPS injected group 4 days post injection.

There was no significant effect of either time or treatment on expression of IL-1β gene, however expression was significantly higher (2 fold increase) in MacroGard® injected carp 4 days post injection than 1 day post injection ($p=0.0354$). There was also no significant difference within each time point between expression in MacroGard® injected carp and those that received PBS only. In contrast, whilst iNOS expression did not show any significant differences when comparing treatments within time points, expression was significantly higher in LPS injected carp than the control group ($p=0.0087$). At both time points, average expression was at least 5 times higher in LPS injected carp (5.0 times higher 1 day post injection, 5.6 times higher 4 days post injection). MacroGard® injection had no significant effect upon iNOS gene expression.

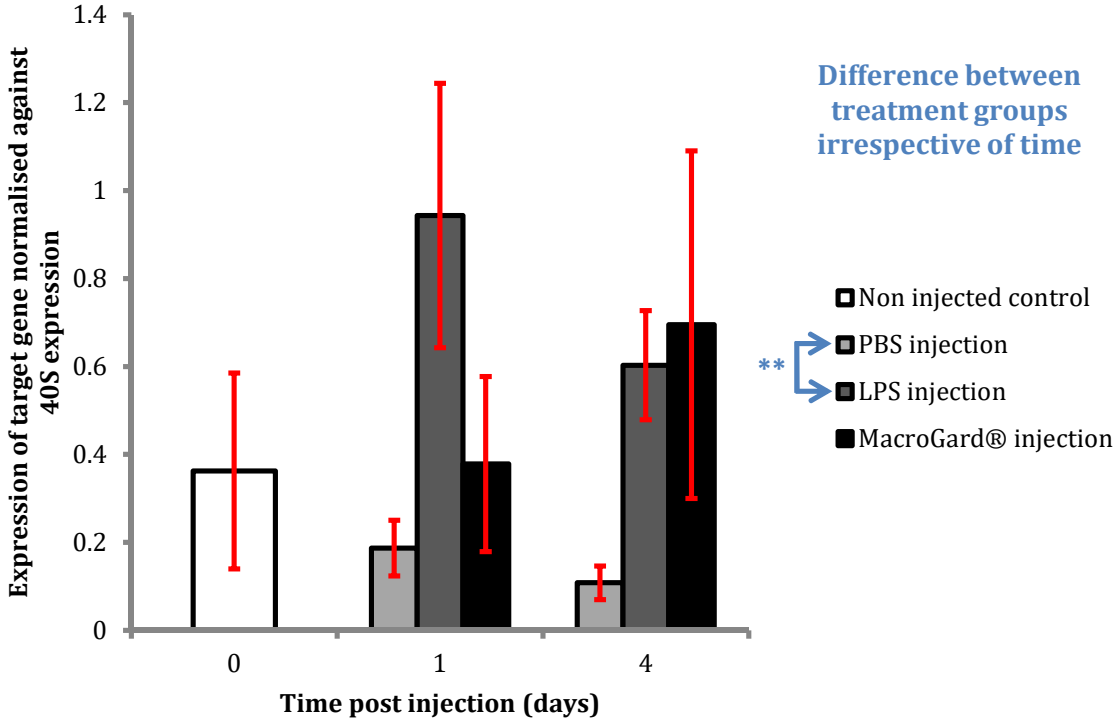
A similar expression profile occurred in both isoforms of the TNFα gene. The average expression for each treatment group within each time point was lower than that seen in the non-injected control carp, however there was no significant effect of

time or treatment seen for either isoform. Expression of the C3 gene was significant different when comparing treatment and time point (1 and 4 days post injection only, p=0.041), however *post hoc* analysis revealed no further significance between treatments at each time point. Expression levels of carp sampled 1 day after injection were similar to those in non-injected controls.



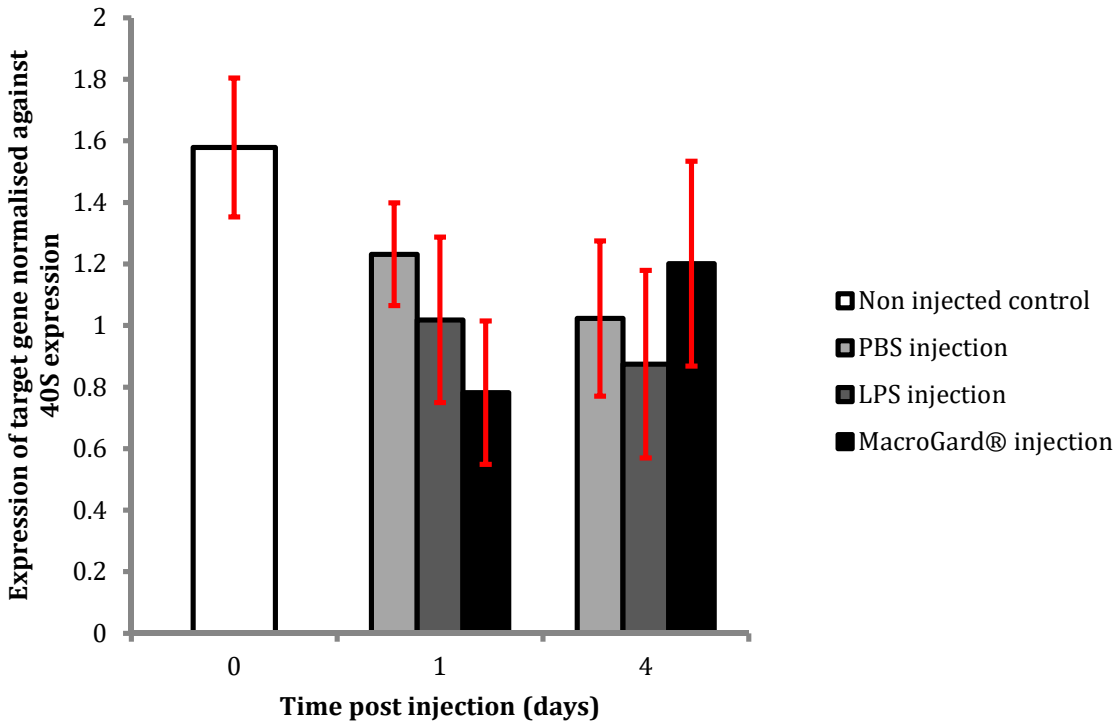
Box Cox transformation		λ=0.06		
Anderson-Darling test for normality	p=0.157	**Levene's test for equal variance**		p=0.918
2-way ANOVA with *post hoc* Tukey's (R^2=37.82%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between treatments	2	0.81	0.456	
Overall difference over time	1	0.82	0.374	
Interaction of treatment and time	2	5.65	0.010	*
***Post hoc* analysis**				
Time within treatment: MacroGard® day 1 vs day 4		p=0.0354		*

Figure 5.1: Expression of the IL-1β gene in the gut of common carp (*C. carpio*) within a small scale injection trial (n=35). Carp were divided into 4 treatment groups: non injected control fish were sampled 0 days post injection, PBS, LPS and MacroGard® injected carp were sampled 1 and 4 days post injection. Bars represent average gene expression for each treatment group (n=5). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers using all data points which were removed before any further statistical analysis. One outlier was found in the LPS treatment group at time point 4 days. Further statistical analysis did not include data from time point 0. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. A 2-way ANOVA with Tukey's as *post hoc* analysis was utilised to compare differences over time and between treatment groups. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.



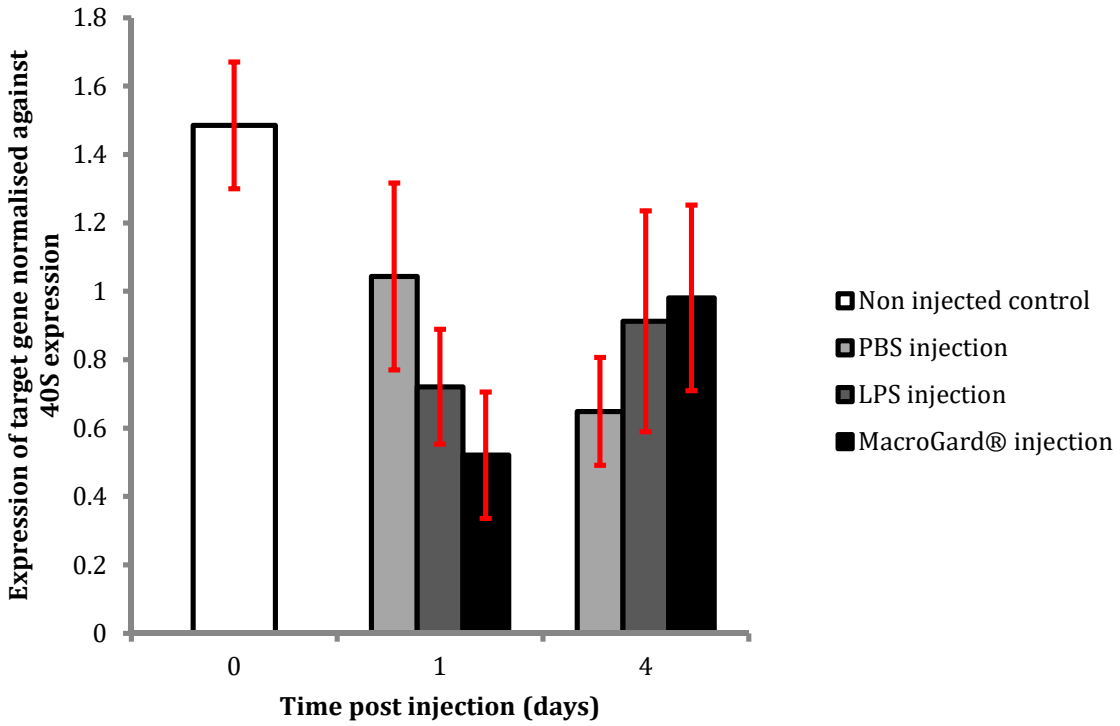
Box Cox transformation		λ=0.19		
Anderson-Darling test for normality	p=0.428	**Levene's test for equal variance**		p=0.282
2-way ANOVA with *post hoc* Tukey's (R^2=25.89%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between treatments	2	5.49	0.011	*
Overall difference over time	1	0.09	0.768	
Interaction of treatment and time	2	0.27	0.762	
***Post hoc* analysis**				
Treatment: PBS versus LPS		p=0.0087		**

Figure 5.2: Expression of the iNOS gene in the gut of common carp (*C. carpio*) within a small scale injection trial (n=35). Carp were divided into 4 treatment groups: non injected control fish were sampled 0 days post injection, PBS, LPS and MacroGard® injected carp were sampled 1 and 4 days post injection. Bars represent average gene expression for each treatment group (n=5). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers using all data points which were removed before any further statistical analysis. One outlier was found in the non-injected control group at time point 0. Further statistical analysis did not include data from time point 0. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. A 2-way ANOVA with Tukey's as *post hoc* analysis was utilised to compare differences over time and between treatment groups. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.



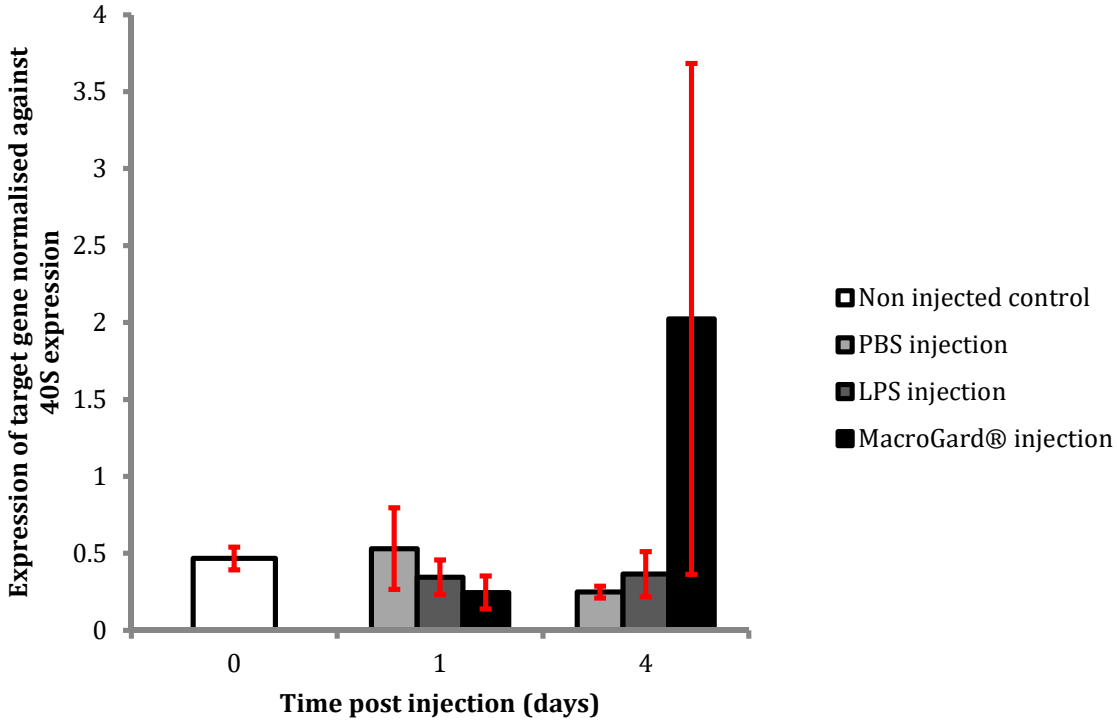
Anderson-Darling test for normality	p=0.485	Levene's test for equal variance		p=0.965
2-way ANOVA with *post hoc* Tukey's (R^2=8.19%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between treatments	2	0.23	0.796	
Overall different over time	1	0.01	0.921	
Interaction of treatment and time	2	0.81	0.455	

Figure 5.3: Expression of the TNFα-1 gene in the gut of common carp (*C. carpio*) within a small scale injection trial (n=35). Carp were divided into 4 treatment groups: non injected control fish were sampled 0 days post injection, PBS, LPS and MacroGard® injected carp were sampled 1 and 4 days post injection. Bars represent average gene expression for each treatment group (n=5). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers using all data points which were removed before any further statistical analysis. One outlier was identified within PBS injected fish at time point 1 day. Further statistical analysis did not include data from time point 0. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. A 2-way with Tukey's as *post hoc* analysis was utilised to compare differences over time and between treatment groups. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed.



Anderson-Darling test for normality	p=0.130	Levene's test for equal variance		p=0.820
2-way ANOVA with *post hoc* Tukey's (R^2=13.88%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between treatments	2	0.08	0.919	
Overall different over time	1	0.20	0.662	
Interaction of treatment and time	2	1.65	0.214	

Figure 5.4: Expression of the TNFα-2 gene in the gut of common carp (*C. carpio*) within a small scale injection trial (n=35). Carp were divided into 4 treatment groups: non injected control fish were sampled 0 days post injection, PBS, LPS and MacroGard® injected carp were sampled 1 and 4 days post injection. Bars represent average gene expression for each treatment group (n=5). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. Further statistical analysis did not include data from time point 0. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. A 2-way ANOVA with Tukey's as *post hoc* analysis was utilised to compare differences over time and between treatment groups. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed.

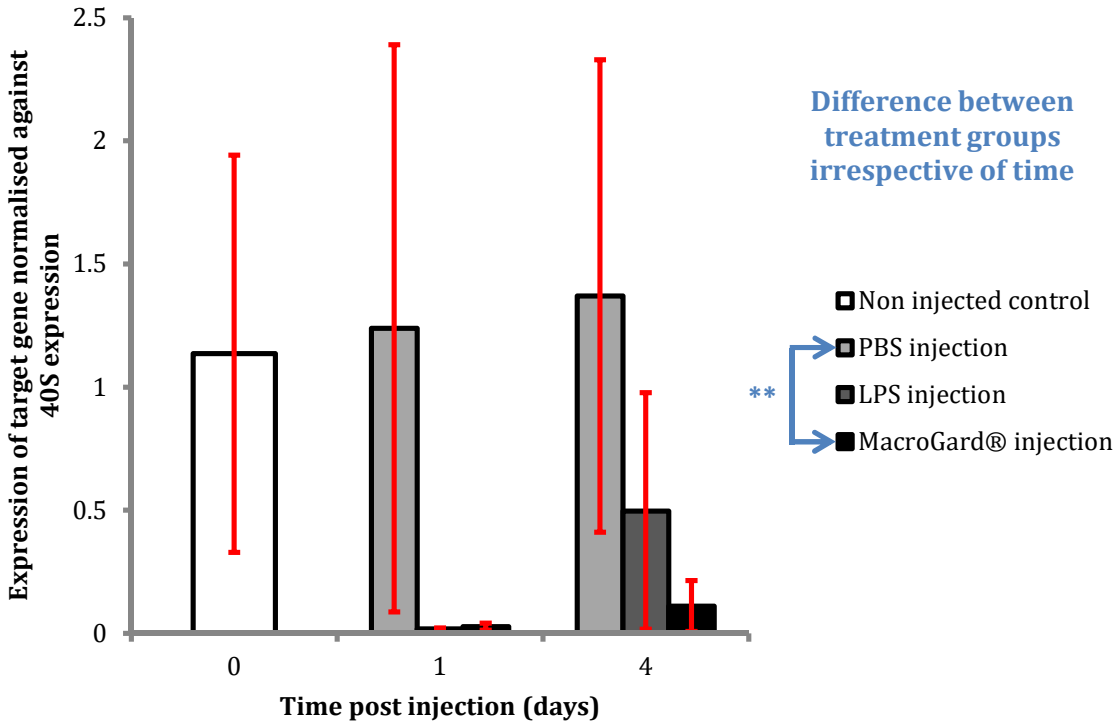


Box Cox transformation		λ=-0.11		
Anderson-Darling test for normality	p=0.118	**Levene's test for equal variance**		p=0.625
2-way ANOVA with *post hoc* Tukey's (R^2=26.29%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between treatments	2	0.15	0.865	
Overall difference over time	1	0.45	0.507	
Interaction of treatment and time	2	3.69	0.041	*

Figure 5.5: Expression of the C3 gene in the gut of common carp (*C. carpio*) within a small scale injection trial (n=35). Carp were divided into 4 treatment groups: non injected control fish were sampled 0 days post injection, PBS, LPS and MacroGard® injected carp were sampled 1 and 4 days post injection. Bars represent average gene expression for each treatment group (n=5). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. One outlier was found at time point 1, treatment PBS injection. Further statistical analysis did not include data from time point 0. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. A 2-way ANOVA with Tukey's as *post hoc* analysis was utilised to compare differences over time and between treatment groups. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.

5.2.2 – Analysis of the gut microbiota.

Total 16S rDNA expression was measured as an approximation of overall bacterial population size within the gut of common carp (Figure 5.6). Expression levels in PBS injected carp at both time points post injection, i.e. day 1 and 4 were comparable to those in non-injected control fish sampled at the time of injection (day 0). There was a significant overall effect of treatment as shown with a 2-way ANOVA ($p=0.005$) with *post hoc* analysis revealing MacroGard® injected carp had significantly lower levels of 16S rDNA expression than PBS injected carp ($p=0.0041$). The mean expression level in LPS and MacroGard® injected carp were 2.37% and 2.18% of the expression levels of non-injected control fish respectively. For PCR-DGGE analysis, samples were pooled at each time point by treatment, i.e. each band pattern is representative of the microbiota from 5 fish. A comparison of species richness using the Bray-Curtis dissimilarity index plotted using nMDS (Figure 5.7) showed differences between treatment and time points. There was no overlap between circles drawn round the different time points. There was however an overlap between treatments with the microbiota in PBS and LPS injected carp on day 4 having a dissimilarity score of 0, i.e. they were identical. It should, however, be noted that the sample size for this trial is small and is only between 7 sets of data.



Box Cox transformation		λ=-0.29		
Anderson-Darling test for normality	p=0.476	**Levene's test for equal variance**		p=0.709
2-way ANOVA with *post hoc* Tukey's (R^2=39.88%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between treatments	2	6.84	0.005	**
Overall difference over time	1	0.06	0.805	
Interaction of treatment and time	2	0.70	0.505	
***Post hoc* analysis**				
Treatment: PBS vs MacroGard®		p=0.0041		**

Figure 5.6: Expression of the bacterial 16S rDNA gene in the gut of common carp (*C. carpio*) within a small scale injection trial (n=35). Carp were divided into 4 treatment groups: non injected control fish were sampled 0 days post injection, PBS, LPS and MacroGard® injected carp were sampled 1 and 4 days post injection. Bars represent average gene expression for each treatment group (n=5). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. One outlier was identified in the PBS group at time point 1 day post injection. Further statistical analysis did not include data from time point 0. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. A 2-way ANOVA with Tukey's as *post hoc* analysis was utilised to compare differences over time and between treatment groups. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.

Grouping	Marker
No injection control	Diamond
PBS injection	Square
LPS injection	Triangle
MacroGard® injection	Circle
Day 0	White
Day 1	Black
Day 4	Red

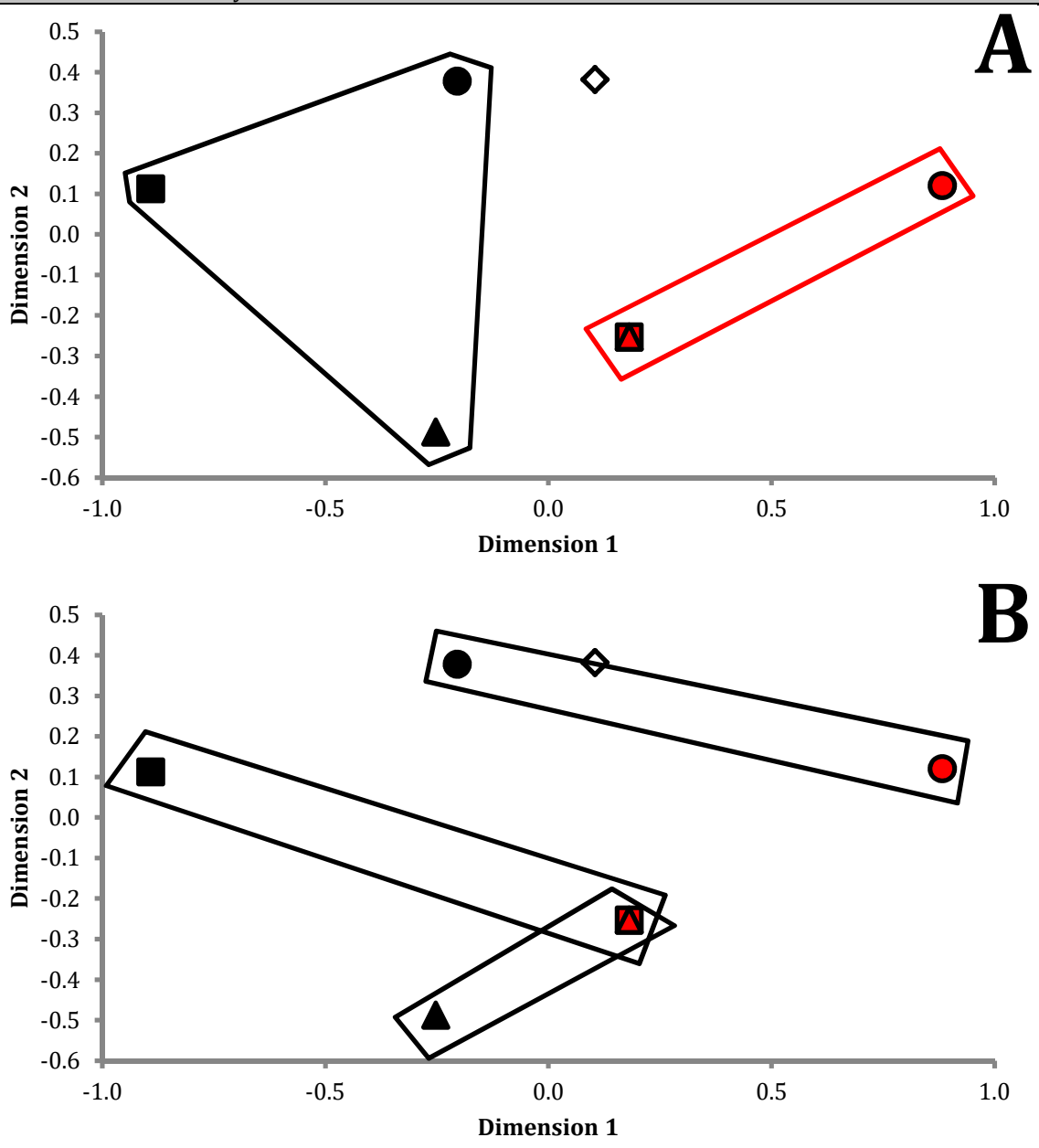


Figure 5.7: Non metric Multidimensional Scaling (nMDS) ordination (stress: 0.1872) of Bray-Curtis dissimilarities looking at the differences in species richness based on PCR-DGGE band patterns of carp within a small scale injection trial. Carp were divided into 4 treatment groups: non injected control fish were sampled 0 days post injection, PBS, LPS and MacroGard® injected carp were sampled 1 and 4 days post injection. Data is shown twice to easily visualise groupings by time point (A) and by treatment (B).

5.2.3 – A comparison of innate immune gene expression against gut microbiota size.

The expression levels of each of the innate immune genes analysed were compared to the 16S rDNA gene found in the bacteria within the gut using Spearman's rank correlation. Table 5.3 shows there was no correlation between these genes and the overall size of the bacterial gut population.

Table 5.3: The probability of a linear relationship between each of the immune genes analysed in the gut of common carp during a 4 day injection trial and the size of the bacterial population within the gut as measured by total 16s rDNA expression (n=35). Carp were divided into 4 treatment groups: non injected control fish were sampled 0 days post injection, PBS, LPS and MacroGard® injected carp were sampled 1 and 4 days post injection. As data was not normally distributed, Spearman's test was used to calculate the correlation coefficient, i.e. how linear a correlation is, and the statistical significance. A rating as to how strong the correlation is (as described by Fowler *et al.* (Fowler, Cohen *et al.* 1998)) is also given with a coefficient of 0.00-0.19 being a very weak correlation, 0.20-0.39 being a weak correlation, 0.40-0.69 being a modest correlation, 0.70-0.89 being a strong correlation and 0.90-1.00 being a very strong correlation.

Gene	Correlation coefficient	P value	Correlation
IL-1β	-0.030	0.863	Very weak
iNOS	-0.088	0.614	Very weak
TNFα-1	0.128	0.465	Very weak
TNFα-2	0.041	0.814	Very weak
C3	0.120	0.492	Very weak

5.3 – Results: large injection trial.

As with the feeding trial and initial small scale injection trial described in Chapter 4 and section 5.2 respectively, this section considers the effect of exposure to MacroGard®, via injection, on innate immune gene expression in the gut (section 5.3.1) the intestinal microbiota population (section 5.3.2) and a statistical comparison of gene expression levels of the innate immune parameters to the total 16S rDNA gene expression, i.e. an approximation of the overall size of the bacteria population within the gut (section 5.3.3).

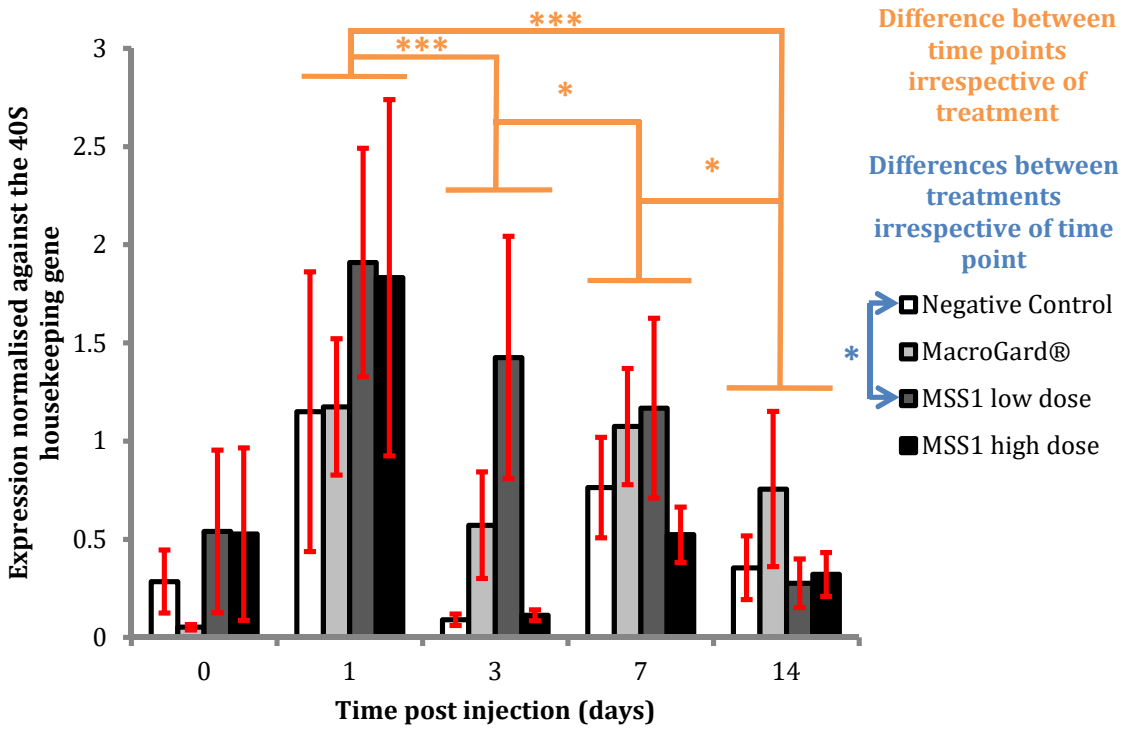
5.3.1 – Bactericidal innate immune gene expression.

As multiple tanks were utilised for each treatment, where possible, a nested 2-way ANOVA was utilised to compare data. None of the expression levels of the genes analysed were shown to be significantly affected by tank, however the expression of CRP2 (Figure 5.9) had a p value of 0.05. (Lew 2012) discusses the concept of misunderstanding p values based upon a hybridisation of the definition of "significance" as defined by Fisher (1925) and Neyman-Pearson (1933). Where Fisher defines p values as an "index of evidence against the null hypothesis" (Lew 2012), the Neyman-Pearson consider the rate of false positive conclusions (type I errors) and false negative conclusions (type II errors). In this thesis, the tolerance for false positives (α) is set at 0.05, and p values lower than this are thus considered statistically significantly different, however the use of the p value as set out by Fisher requires more fluidity. Where the p value for the effect of tank upon the expression of CRP2 was 0.05, i.e. on the threshold of being statistically significantly different expression levels between tanks based upon the Neyman-Pearson interpretation of

"significant", further statistical analysis was performed to determine if tank had an effect upon CRP2 expression. A 1-way nested ANOVA comparing CRP2 expression in non-injected negative controls showed no variation between tanks (p=0.931).

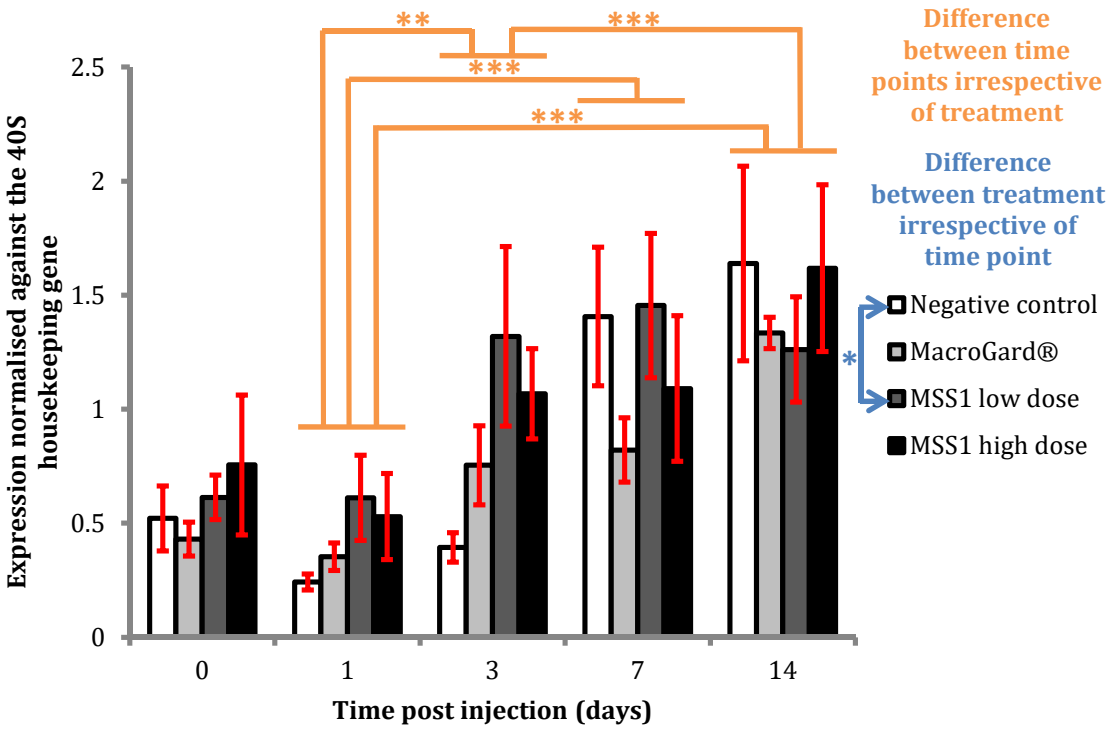
There was an overall significant effect of treatment on the expression of iNOS (Figure 5.8 – p=0.019), CRP2 (Figure 5.9 – p=0.033), Muc2 (Figure 5.11 – p=0.038) and LEAP2 (Figure 5.13 – p=0.032). *Post hoc* analysis revealed that carp injected with MSS1 at the low dose (5mg kg^{-1}) had significantly higher expression levels of iNOS, CRP2 and Muc2 genes than the water injected control carp. Expression of these genes was, however, not significantly different in carp injected with either MacroGard® or the high dose of MSS1 (10mg kg^{-1}) compared to negative controls. Overall expression, however, of iNOS at day 1 and 7 was significantly higher than those on days 3 and 14 irrespective of treatment. In contrast, expression of CRP2 gene, irrespective of treatment, was significantly higher at days 3 and 7 in comparison to day 1, and significantly higher again at day 14 in comparison to day 3. Similar to CRP2, Muc2 has lower expression levels 1 day post injection irrespective of treatment with days 7 and 14 being significantly higher than day 1. Analysis of the genes expressing the antimicrobial peptides revealed differential responses. There was no significant effect of treatment on ApoA1 (Figure 5.12) and HAMP1 (Figure 5.14) expression, however there were significant differences in expression of these genes over time, for example, ApoA1 expression was significantly lower 3 days post injection in comparison to 14 days post injection (p=0.0181). Expression of ApoA1 in control carp, i.e. injection with water, was highly variable and had a large standard error of the mean, in comparison to other genes analysed. In comparison, expression of the other antimicrobial peptide, HAMP1, is highest at day 1 post injection (significantly higher than 3 and 7 days,

$p<0.0001$ and $p=0.0001$ respectively) which declines during days 3 and 7 before increasing again after 14 days (significantly higher than day 3, $p=0.0317$) irrespective of treatment. Neither treatment nor time had an effect on expression of bf/C2 (Figure 5.10).



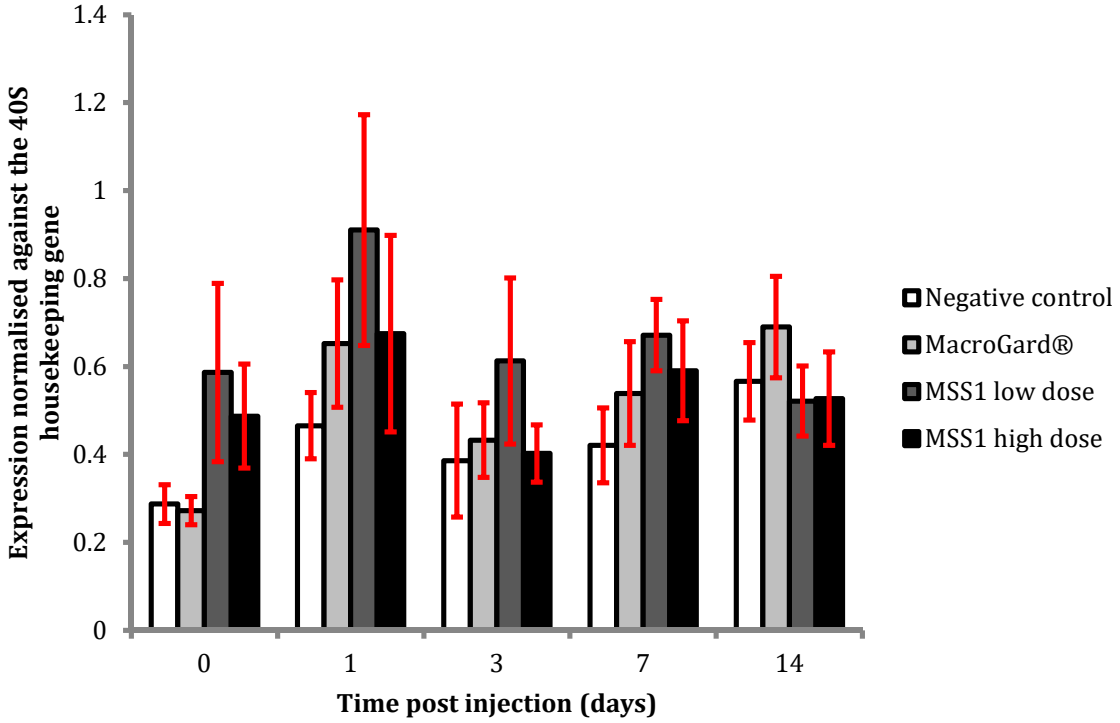
Box Cox transformation		λ=0.00		
Anderson-Darling test for normality	p=0.854	**Levene's test for equal variance**		p=0.942
2-way nested (tank within treatment) ANOVA with *post hoc* Tukey's (R^2=45.07%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between treatments	3	3.53	0.019	*
Nested effect of tank variation	4	1.04	0.394	
Overall difference over time	3	9.65	<0.001	***
Interaction of treatment and time	9	1.91	0.063	
***Post hoc* analysis**				
Treatment: negative control vs MSS1 low dose		p=0.0366		*
Time: day 1 vs day 3		p=0.0002		***
Time: day 1 vs day 14		p=0.0004		***
Time: day 3 vs day 7		p=0.0133		*
Time: day 7 vs day 14		p=0.0248		*

Figure 5.8: Expression of the iNOS gene in the gut of common carp (*C. carpio*) within an injection trial (n=120). Carp were divided into 5 treatment groups: non injected control fish were sampled 0 days post injection, negative control (water), MacroGard®, MSS1 low dose (5mg kg^{-1}) and MSS1 high dose (10mg kg^{-1}) injected carp were sampled 1, 3, 7 and 14 days post injection. Bars represent average gene expression for each treatment group (n=6). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant (p<0.05) *post hoc* comparisons being listed. Both in the graph and table, * signifies p<0.05, ** signifies p<0.01, and *** signifies p<0.001.



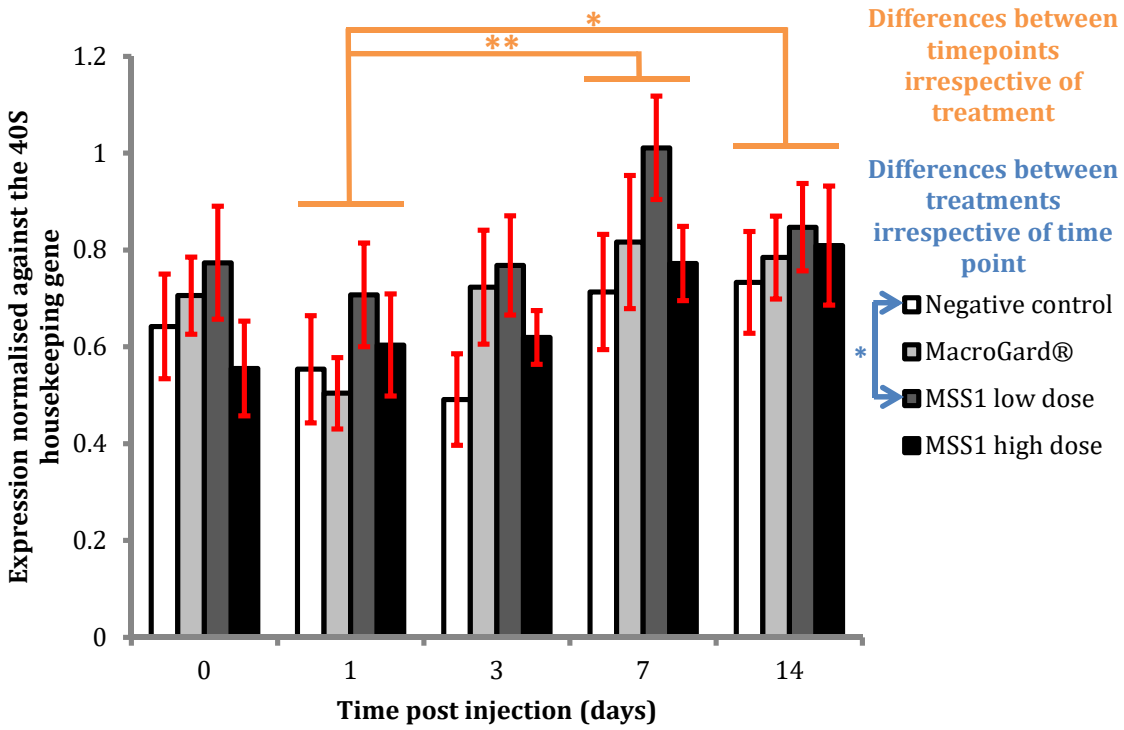
Box Cox transformation		λ=-0.04		
Anderson-Darling test for normality	p=0.680	**Levene's test for equal variance**		p=0.994
2-way nested (tank within treatment) ANOVA with *post hoc* Tukey's (R^2=45.07%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between treatments	3	3.06	0.033	*
Nested effect of tank variation	4	2.50	0.050	
Overall difference over time	3	26.15	<0.001	***
Interaction of treatment and time	9	1.96	0.056	
***Post hoc* analysis**				
Treatment: negative control vs MSS1 low dose		p=0.0451		*
Time: day 1 vs day 3		p=0.0003		***
Time: day 1 vs day 7		p<0.0001		***
Time: day 1 vs day 14		p<0.0001		***
Time: day 3 vs day 14		p=0.0007		***

Figure 5.9: Expression of the CRP2 gene in the gut of common carp (*C. carpio*) within an injection trial (n=120). Carp were divided into 5 treatment groups: non injected control fish were sampled 0 days post injection, negative control (water), MacroGard®, MSS1 low dose (5mg kg^{-1}) and MSS1 high dose (10mg kg^{-1}) injected carp were sampled 1, 3, 7 and 14 days post injection. Bars represent average gene expression for each treatment group (n=6). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. Two outliers were identified at time point 14 days, one from the negative control and one from the MacroGard® injection groups. Further statistical analysis did not include data from time point 0. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant (p<0.05) *post hoc* comparisons being listed. Both in the graph and table, * signifies p<0.05, ** signifies p<0.01, and *** signifies p<0.001.



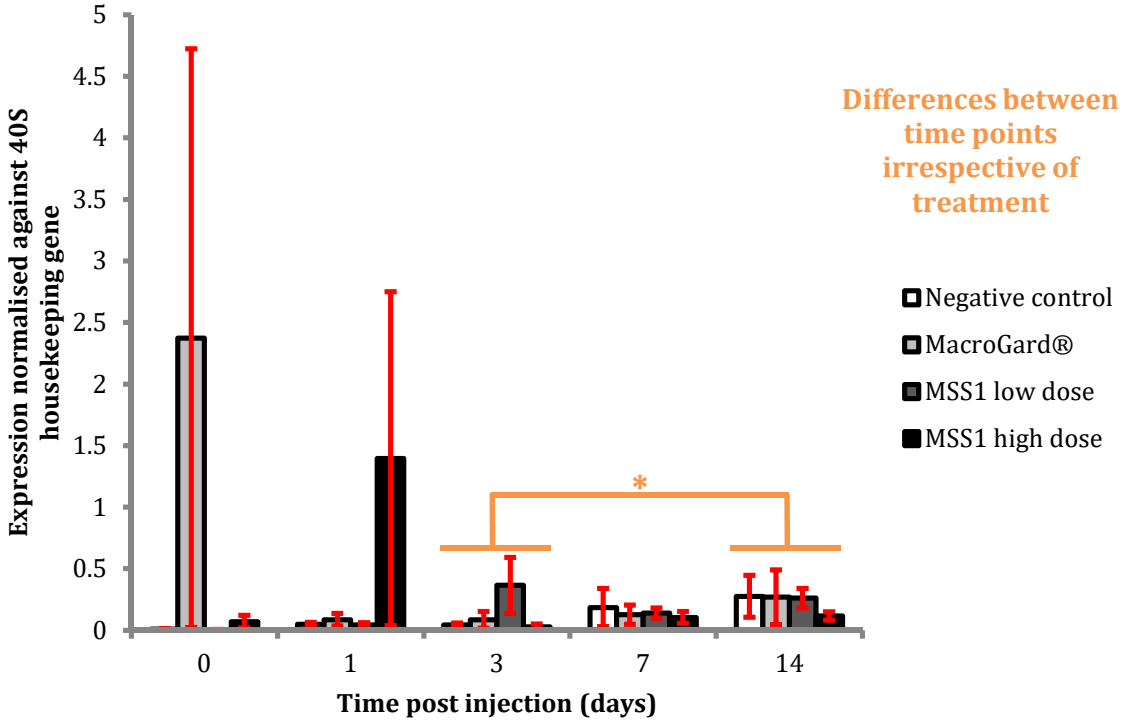
Box Cox transformation		λ=-0.16		
Anderson-Darling test for normality	p=0.228	**Levene's test for equal variance**		p=0.990
2-way nested (tank within treatment) ANOVA with *post hoc* Tukey's (R^2=20.00%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between treatments	3	1.99	0.122	
Nested effect of tank variation	4	0.64	0.638	
Overall difference over time	3	2.14	0.098	
Interaction of treatment and time	9	0.47	0.891	

Figure 5.10: Expression of the bf/C2 gene in the gut of common carp (*C. carpio*) within an injection trial (n=120). Carp were divided into 5 treatment groups: non injected control fish were sampled 0 days post injection, negative control (water), MacroGard®, MSS1 low dose (5mg kg^{-1}) and MSS1 high dose (10mg kg^{-1}) injected carp were sampled 1, 3, 7 and 14 days post injection. Bars represent average gene expression for each treatment group (n=6). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. One outlier was identified from the treatment group MSS1 low dose at time point 1 day. Further statistical analysis did not include data from time point day 0. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed.



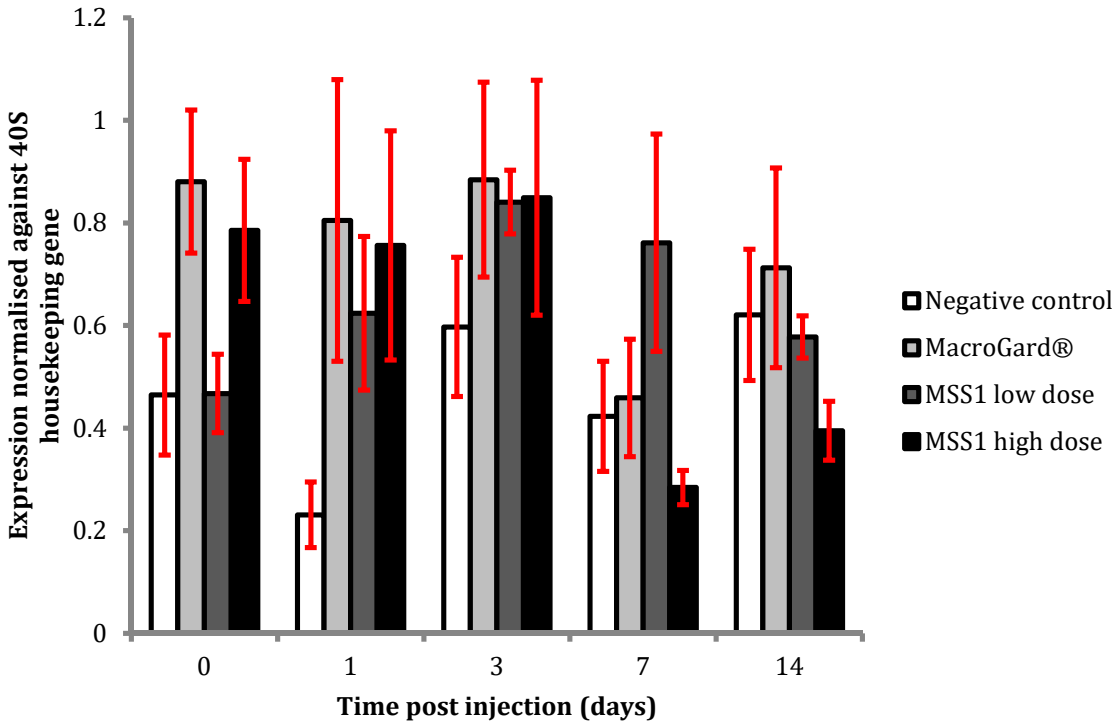
Anderson-Darling test for normality	p=0.232	Levene's test for equal variance		p>0.999
2-way nested (tank within treatment) ANOVA with *post hoc* Tukey's (R^2=30.25%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between treatments	3	2.96	0.038	*
Nested effect of tank variation	4	1.49	0.214	
Overall difference over time	3	4.95	0.003	**
Interaction of treatment and time	9	0.37	0.948	
***Post hoc* analysis**				
Treatment: negative control vs MSS1 low dose		p=0.0222		*
Time: day 1 vs day 7		p=0.0082		**
Time: day 1 vs day 14		p=0.0318		*

Figure 5.11: Expression of the Muc2 gene in the gut of common carp within an injection trial (n=120). Carp were divided into 5 treatment groups: non injected control fish were sampled 0 days post injection, negative control (water), MacroGard®, MSS1 low dose (5mg kg^{-1}) and MSS1 high dose (10mg kg^{-1}) injected carp were sampled 1, 3, 7 and 14 days post injection. Bars represent average gene expression for each treatment group (n=6). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. No outliers were found in this data set. Further statistical analysis did not include time point 0 days. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.



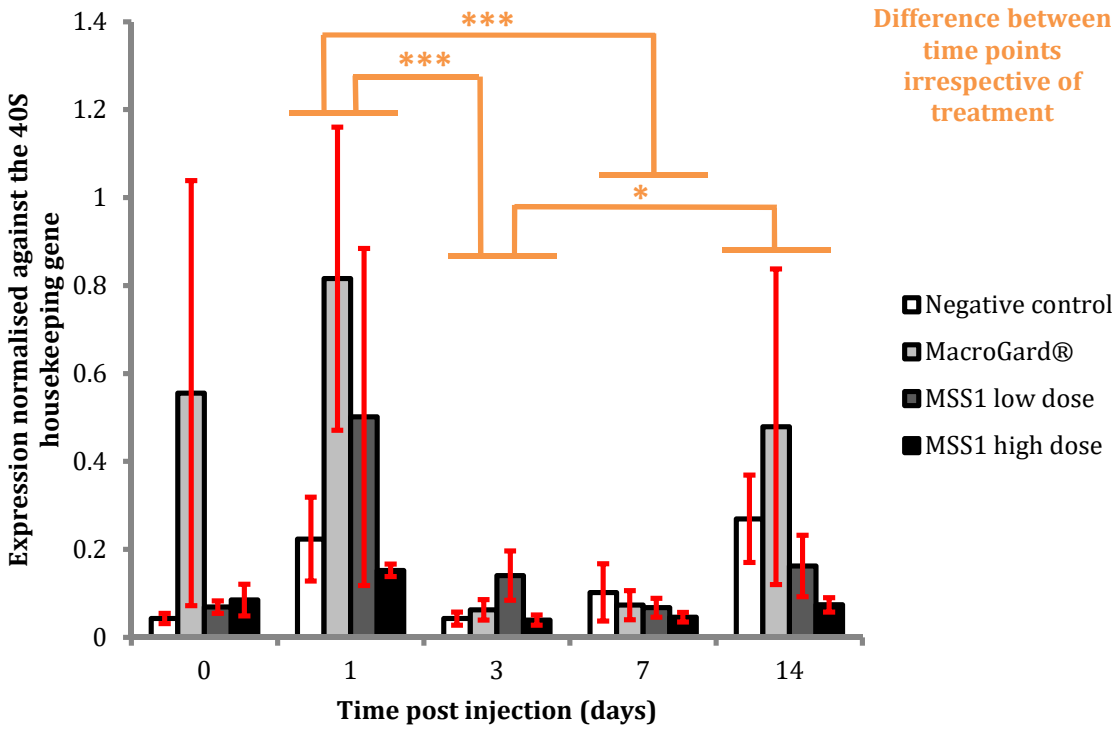
Box Cox transformation		λ=-0.04		
Anderson-Darling test for normality	p=0.348	**Levene's test for equal variance**		p=0.996
2-way nested (tank within treatment) ANOVA with *post hoc* Tukey's (R^2=45.07%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between treatments	3	0.90	0.445	
Nested effect of tank variation	4	1.13	0.347	
Overall difference over time	3	3.18	0.029	*
Interaction of treatment and time	9	1.08	0.391	
***Post hoc* analysis**				
Time: day 3 vs day 14		p=0.0181		*

Figure 5.12: Expression of the ApoA1 gene in the gut of common carp (*C. carpio*) within an injection trial (n=120). Carp were divided into 5 treatment groups: non injected control fish were sampled 0 days post injection, negative control (water), MacroGard®, MSS1 low dose (5mg kg^{-1}) and MSS1 high dose (10mg kg^{-1}) injected carp were sampled 1, 3, 7 and 14 days post injection. Bars represent average gene expression for each treatment group (n=6). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. One outlier was identified at time point 14 days from the negative control treatment group. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.



Box Cox transformation		λ=-0.01		
Anderson-Darling test for normality	p=0.375	**Levene's test for equal variance**		p=0.974
2-way Scheirer-Ray-Hare test (R^2=27.51%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between treatments	3	3.07	0.032	*
Overall difference over time	3	3.35	0.023	*
Interaction of treatment and time	9	1.23	0.287	

Figure 5.13: Expression of the LEAP2 gene in the gut of common carp (*C. carpio*) within an injection trial (n=120). Carp were divided into 5 treatment groups: non injected control fish were sampled 0 days post injection, water, MacroGard®, MSS1 low dose (5mg kg^{-1}) and MSS1 high dose (10mg kg^{-1}) injected carp were sampled 1, 3, 7 and 14 days post injection. Bars represent average gene expression for each treatment group (n=6). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.



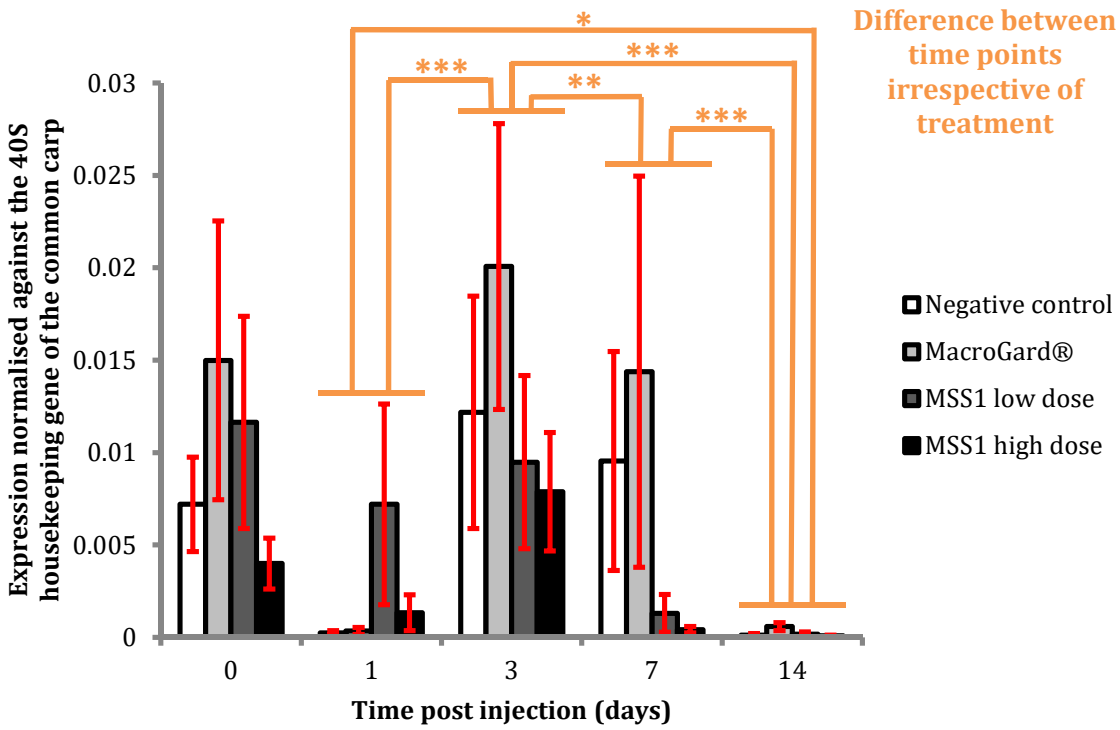
Box Cox transformation		λ=-0.04		
Anderson-Darling test for normality	p=0.271	**Levene's test for equal variance**		p=0.873
2-way nested (tank within treatment) ANOVA with *post hoc* Tukey's (R^2=39.05%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between treatments	3	1.38	0.256	
Nested effect of tank variation	4	0.25	0.906	
Overall difference over time	3	10.94	<0.001	***
Interaction of treatment and time	9	0.99	0.452	
***Post hoc* analysis**				
Time: day 1 vs day 3		p<0.0001		***
Time: day 1 vs day 7		p=0.0001		***
Time: day 3 vs day 14		p=0.0317		*

Figure 5.14: Expression of the HAMP1 gene in the gut of common carp (*C. carpio*) within an injection trial (n=120). Carp were divided into 5 treatment groups: non injected control fish were sampled 0 days post injection, water, MacroGard®, MSS1 low dose (5mg kg^{-1}) and MSS1 high dose (10mg kg^{-1}) injected carp were sampled 1, 3, 7 and 14 days post injection. Bars represent average gene expression for each treatment group (n=6). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant (p<0.05) *post hoc* comparisons being listed. Both in the graph and table, * signifies p<0.05, ** signifies p<0.01, and *** signifies p<0.001.

5.3.2 – Analysis of the intestinal microbiota population.

Expression of the bacterial 16S rDNA gene in the gut of carp (Figure 5.15) during this trial was not effected by tank. There was a significant overall effect of time ($p<0.001$) and a significant interaction of time and treatment ($p=0.040$), however *post hoc* analysis revealed no further significant effects. Expression of the 16S rDNA gene was significantly higher 3 days post injection irrespective of treatment in comparison to all other time points in injected fish, and day 14 is significantly lower than all other time points in injected fish. There were differences in the mean expression of control injected carp with 1 and 14 days post injection being lower than non-injected control carp from the same tanks as well as lower than the mean at 3 and 7 days post injection. The average expression of the 16S rDNA gene 1 day post injection in carp that received the control and MacroGard® treatments were 2.27% and 3.43% respectively of the expression in non-injected carp from the same tanks samples 24 hours previously. Injection with MSS1 at both the low and high dose showed a lower mean expression relative to non-injected controls from the same tank (61.88% and 33.28% respectively).

Qualitative analysis of bacterial species richness during this trial was performed by comparing PCR-DGGE band patterns of pooled samples (by treatment at each time point). Bray-Curtis dissimilarity comparisons were made between all samples and data is presented on an nMDS plot in Figure 5.16 with groupings highlighted by treatment and by time point. No effect of treatment or time point can be seen during this trial with all four treatments and all five time points overlapping at the centre of the plot.



Box Cox transformation		λ=0.07		
Anderson-Darling test for normality	p=0.414	**Levene's test for equal variance**		p=0.894
2-way nested (tank within treatment) ANOVA with *post hoc* Tukey's (R^2=55.01%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall difference between treatments	3	1.17	0.326	
Nested effect of tank variation	4	1.89	0.122	
Overall difference over time	3	19.18	<0.001	***
Interaction of treatment and time	9	2.11	0.040	*
***Post hoc* analysis**				
Time: day 1 vs day 3		p=0.0001		***
Time: day 1 vs day 14		p=0.0418		*
Time: day 3 vs day 7		p=0.0078		**
Time: day 3 vs day 14		p<0.0001		***
Time: day 7 vs day 14		p=0.0008		***

Figure 5.15: Expression of the bacterial 16S rDNA gene in the gut of common carp (*C. carpio*) within an injection trial (n=120). Carp were divided into 5 treatment groups: non injected control fish were sampled 0 days post injection, water, MacroGard®, MSS1 low dose (5mg kg^{-1}) and MSS1 high dose (10mg kg^{-1}) injected carp were sampled 1, 3, 7 and 14 days post injection. Bars represent average gene expression for each treatment group (n=6). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant (p<0.05) *post hoc* comparisons being listed. Both in the graph and table, * signifies p<0.05, ** signifies p<0.01, and *** signifies p<0.001.

Grouping	Marker
Water injected control	Diamond
MacroGard® injection	Square
MSS1 5mg injection	Triangle
MSS1 10mg injection	Circle
Day 0	Blue
Day 1	Green
Day 3	Red
Day 7	Orange
Day 14	Black

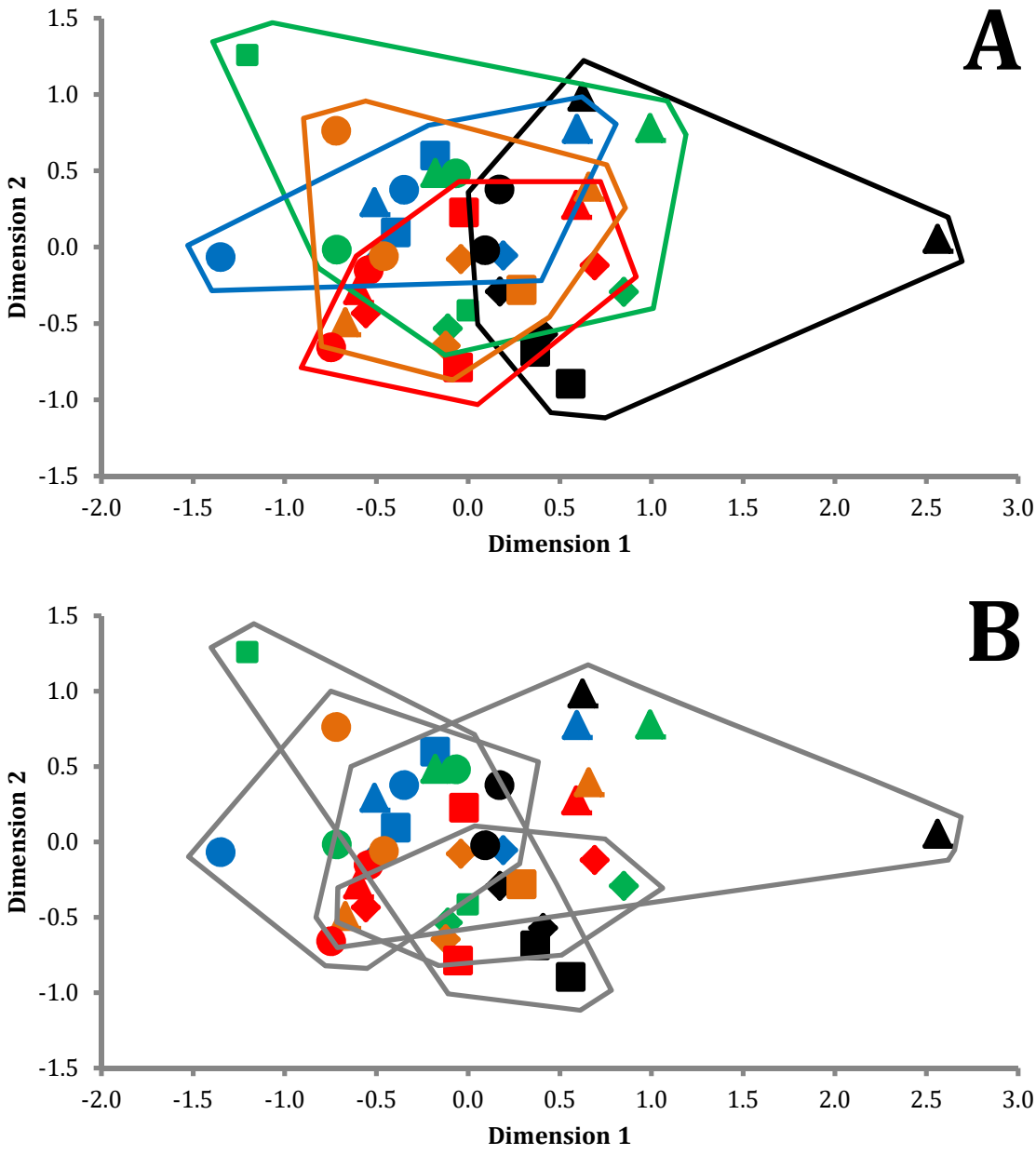


Figure 5.16: Non metric Multidimensional Scaling (nMDS) ordination (stress: 0.0809) of Bray-Curtis dissimilarities looking at the differences in species richness based on PCR-DGGE band patterns of carp within a large injection trial. Carp were divided into 5 treatment groups: non injected control fish were sampled 0 days post injection, water, MacroGard® MSS1 low dose (5mg kg^{-1}) and MSS1 low (10mg kg^{-1}) injected carp were sampled 1, 3, 7 and 14 days post injection. Data is shown twice to easily visualise groupings by time point (A) and by treatment (B).

5.3.3 – Statistical analysis of innate immune gene expression and overall bacteria population size.

Expression levels of each of the immune genes analysed was compared to expression of the 16S rDNA gene using the non-parametric Spearman's rank order correlation test (Table 5.4). This revealed a weak negative correlation between the 16S rDNA gene and bf/C2 (ρ=-0.228, p=0.014), ApoA1 (ρ=-0.383, p<0.001) and HAMP1 expression (ρ=-0.223, p=0.016). There was no correlation with expression of iNOS, CRP2, Muc2 or LEAP2.

Table 5.4: The probability of a linear relationship between each of the immune genes analysed in the gut of common carp during a 14 day injection trial and the size of the bacterial population within the gut as measured by total 16s rDNA expression (n=116). Carp were divided into 5 treatment groups: non injected control fish were sampled 0 days post injection, water, MacroGard®, MSS1 low dose (5mg kg^{-1}) and MSS1 high dose (10mg kg^{-1}) injected carp were sampled 1, 3, 7 and 14 days post injection. As data was not normally distributed, Spearman's test was used to calculate the correlation coefficient, i.e. how linear a correlation is, and the statistical significance. A rating as to how strong the correlation is (as described by Fowler *et al.* (Fowler, Cohen *et al.* 1998)) is also given with a coefficient of 0.00-0.19 being a very weak correlation, 0.20-0.39 being a weak correlation, 0.40-0.69 being a modest correlation, 0.70-0.89 being a strong correlation and 0.90-1.00 being a very strong correlation.

Gene	Correlation coefficient (ρ)	P value	Correlation
iNOS	-0.129	0.168	Very weak
CRP2	-0.127	0.175	Very weak
bf/C2	-0.228	0.014	Weak (negative)
Muc2	-0.074	0.432	Very weak
ApoA1	-0.383	<0.001	Weak (negative)
LEAP2	0.159	0.087	Very weak
HAMP1	-0.223	0.016	Weak (negative)

5.4 – Discussion.

This chapter aimed to ascertain if injection with MacroGard® caused a decrease in the amount of bacteria within the gut. The data presented revealed a significant effect on overall gut microbiota population size and indicates there may be an impact upon species richness however, interpretation must at this stage be cautious due to the relatively low sample size. The differences seen indicate that LPS and MacroGard® have a limited effect on the gene expression of the selected innate immune parameters. A lack of upregulation of the inflammation markers, IL-1β and TNFα, suggests a systemic immune response did not occur within the gut. Additionally, C3, the central marker for an activation of the complement pathways, was not affected by injection with LPS or MacroGard®, however an upregulation of iNOS was seen for LPS injected carp. In contrast however, there was more than a 95% reduction in overall gut bacteria population size after LPS and MacroGard® injection. A lack of correlation between the decrease in bacteria population size and iNOS expression, however, does not preclude NO production as having an impact upon the gut microbiota population. For example, whilst Pijanowski *et al.* (2015) showed higher expression of the iNOS gene in stressed carp neutrophils, this did not correlate with production of superoxide anion as determined by NBT assay. Both Falco *et al.* (2012b) and Syakuri *et al.* (2013) showed IL-1β expression to be increased within the gut within the first 24 hours after injection (*A. salmonicida* and CyHV-3 respectively) however the levels of expression return to similar to control levels by 24 hours. IL-1β and TNFα are signals that induce immune cascades rather than directly act upon microbes and therefore the lack of correlation with bacterial population size does not exclude their role in the reduction of gut bacteria population size. Indeed, studies in

other systems have noted a possible link between gut microflora and these immune signals, for example Irritable Bowel Disease (IBD) in humans is a disharmony between the intestinal immune response and the commensal bacteria population within the gut. TNFα blockers are used as a treatment for the symptoms of this disease, possibly indicating a role of signalling molecules, e.g. TNFα, in dysbiosis between host and microbiota (de Bie *et al.* 2012, Jones-Hall *et al.* 2015, Tursi *et al.* 2015).

In this chapter the immunomodulatory properties of a formulated β-glucan, MSS1, on gut immune status was also investigated. As with the initial smaller trial, only a limited effect of MacroGard® injection was seen upon the immune responses analysed, however the lower dose of MSS1 had a significant effect upon several of the immune genes studied. MSS1 was selected out of a range of β-glucans generated from MacroGard® for its stimulation of cell proliferation/lack of toxicity in *in vitro* studies working with carp leucocyte cells (CLCs) and head kidney cells (Nawroz Kareem, unpublished data). Where MSS1 is a singular β-glucan structure rather than a mix of different chain lengths, degrees of branching and solubility, MacroGard® is a heterogenous mix. The initial carbohydrate material from which MSS1 was generated was MacroGard® therefore it is likely that MacroGard® will contain some of the same β-glucan structures as MSS1, i.e. the same chain lengths, degrees of branching and solubility. The data presented in this chapter shows that the particular β-glucan structure of MSS1 has a stronger immunomodulatory capability than MacroGard®. That MSS1 did not have the same affect at the higher concentration (10mg kg^{-1} versus 5mg kg^{-1}), however, is intriguing. This indicates that there is an optimal concentration at which MSS1 has an effect. The expression levels of the genes analysed in carp

injected with the higher concentration of MSS1 (10mg kg^{-1}) are generally lower than expression levels seen in carp injected with the lower concentration (5mg kg^{-1}), therefore there could be a potentially inhibitory affect above a certain concentration. A dose dependent immune response has also been seen after the oral application of lyophilised whole yeast cells (*S. cerevisiae*) to gilthead seabream (Ortuno *et al.* 2002) with an increase in phagocytic ability/capacity of head kidney leucocytes as the concentration of yeast cells within the diet increased. The data presented by Ortuno *et al.* (2002), however, did not indicate a maximum concentration at which the yeast was effective in inducing a greater immune response. Chitin, a β-1,4 linked N-acetyl-D-glucosamine polymer, shows different dose dependent responses in Catla (*Catla catla*) when comparing between *in vitro* and *in vivo* trials (Sangma and Kamilya 2015). When catla were fed diets containing chitin, production of superoxide anion by head kidney leucocytes increased as the concentration of chitin increased, however *in vitro* studies showed the lowest concentration of chitin (0.01mg/ml) showed the highest increase with a dose dependent decrease in production as the concentration of chitin was increased (Sangma and Kamilya 2015). The concept of dose dependent inhibition of activity has been considered since the 1980s in relation to anti-cancer drugs as reviewed by (Powis 1983) however studies looking at MacroGard® have only previously shown a dose dependent increase in activity at higher concentrations of the carbohydrate (Kuhlwein *et al.* 2013, Vera-Jimenez *et al.* 2013).

The decrease in gut bacterial population size was greater in MacroGard® injected carp than those that received MSS1. This is interesting considering the significantly higher levels of gene expression of the immune parameters seen within this trial. The limited correlation between immune parameters and bacterial numbers

seen within the gut indicates that, if indeed the immune response is directly responsible for the decrease in bacteria population size, it is not via the pathways considered within this study or it is simply not detectable on the level of gene expression. Further studies in determining what causes the reduction in bacteria numbers could focus on Pattern Recognition Receptors (PRRs) such as Toll Like Receptors (TLRs) e.g. TLR4 which recognises LPS, a bacteria associated endotoxin (Swain *et al.* 2008) rather than bactericidal activity.

Whilst Tapia-Paniagua *et al.* (2015) used Principle Component Analysis (PCA) as a statistical means of comparing immune activity in the liver of Senegalese sole with the intestinal microbiota, immune genes in the studies presented in this thesis were compared individually to total 16S rDNA expression. This was done due to data not being normally distributed which is an assumption of PCA. Whilst Spearman's rank order correlation revealed slight correlation between expression of the three antimicrobial peptides analysed and the bacteria population size, biologically it is most likely that it is a combination of multiple immune parameters working together that are affecting the gut microbiota rather than one individual parameter. Examples of different immune pathways working together to reduce infections in carp models include infection with Cyprinid herpesvirus (CyHV) 3 and the parasite *Trypanoplasma borreli*. During CyHV-3 infection, there is an increase in expression of genes associated with the complement pathway, iNOS, interferon, CRP, lysozyme and a decrease in defensin B (Adamek *et al.* 2013, Pionnier *et al.* 2014), and nitric oxide, Immunoglobulin M and complement are indicated to work together resulting in lysis during *T. borreli* infection (Forlenza *et al.* 2009b).

As previously stated, this is the first trial in which quantification of the gut

microbiota population size has been compared against immune gene expression in the gut in an ichthyo-model. As has been shown through studying gnotobiotic models, the presence of bacteria is highly important for the development of the immune system from hatching and, in zebrafish (*Danio rerio*), the species diversity of the gut microbiota population is thought to stabilise by the time the fish are considered as juveniles (Rombout *et al.* 2011). Examples of dysbiosis within the symbiont, i.e. an instability in bacterial species diversity and over activation of the host immune response, are found in disease conditions such as Ulcerative Colitis in which a loss of α-diversity has been seen (Alipour *et al.* 2015). The lack of variation in bacterial species richness seen in the trials presented here, however, implies this may not be a suitable comparison due to IBDs being chronic conditions, whereas the model presented here could be considered acute, i.e. a singular event rather than continuous modulation of the immune response due to the lack of differences in species richness between injected (irrespective of treatment) and non-injected carp.

Due to there being no strong correlation between any of the immune parameters analysed and overall gut microbiota population size, alternative means as to how the reduction in gut bacteria population size occurred were considered. Carp have been shown to slough their mucosal layer within the gut as a means of ejecting pathogens during CyHV-3 infection trials (Adamek *et al.* 2013) which, if a sloughing event occurred during the trial presented here, this would give an explanation as to the lower number of bacteria within the mucus layer 1 day post injection. Analysis of Muc2, the gene expressed in the gut that encodes for mucin, the backbone of the peptoglycan molecules that are the main constituents of mucus (Van der Marel *et al.* 2012), did not show any difference either between treatment groups or over time,

nor was there any correlation with total 16S expression. Unfortunately, histological analysis of the gut was not performed to determine if any change in the thickness of the mucosal membrane occurred, however due to the lack of change in gene expression, it is likely there was no effect as an increase in Muc2 gene expression would be expected in order to replenish the mucosal membrane.

Chapter 6 – Studying the effect of combining different methods of applying MacroGard® on the gut microbiota population.

The modulation of the intestinal microbiome in favour of so called "good" bacteria, i.e. species which promote health of the host organism, is currently receiving a lot of attention as an alternative means of reducing incidents of pathogenic infection in aquaculture (for reviews, see Gatesoupe 1999, Wang *et al.* 2008, Nayak 2010b, Perez *et al.* 2010, Saad *et al.* 2013, Merrifield *et al.* 2014). Whilst the oral application of MacroGard® is capable of influencing bacterial species diversity within the intestine of carp at higher concentrations (Kuhlwein *et al.* 2013, Jung-Schroers *et al.* 2015a), this is not the primary reason for including it into the diet of commercial fish species and, indeed, the data presented in Chapter 4 shows there is no effect upon bacterial species richness when fish are fed MacroGard® at a comparable concentration to that found in commercial diets.

Chapter 5, however, revealed that injection with MacroGard® was capable of reducing the population size of the gut microbiota by more than 95% (as measured by 16S copy number). Research using mammalian models has also shown that β-glucans to be effective adjuvants in combination with vaccines (Bromuro *et al.* 2010, Huang *et al.* 2013, Berner *et al.* 2015). Although the majority of research as to the immunomodulatory properties of β-glucans in ichthyo-models focuses on oral application, there are examples of its use as an adjuvant in fish species (Midtlyng and Lillehaug 1998, Guselle *et al.* 2006, Kubilay *et al.* 2008). The adjuvant concept is important as injection has also been cited as the application method of choice used

for the majority of vaccines in aquaculture (Plant and LaPatra 2011).

The influence of orally and injected application of β-glucan bacterial species diversity within the gut, could have potential uses within aquaculture, i.e. timing of injection based vaccination programs with β-glucan based feeding regimes in order achieve both immune protection against specific pathogens through vaccination and an overall "better" gut microbiota that promotes health within the host organism and can outcompete potential pathogens from colonising the gut.

Considering the concepts of orally applied MacroGard® influencing gut microbiota species diversity at higher concentrations and injection of MacroGard® reducing the gut microbiota population size, the aim of this chapter is to establish if a combination of orally applied and injected MacroGard® can influence gut bacterial species diversity, so as to improve overall health of the fish by injection and maintaining or improving a diverse gut microbiome. To achieve this a combination feeding and injection trial where carp will be maintained on either a 0% or 0.1% MacroGard® diet and fish from both feed groups will receive an injection of MacroGard® will be undertaken. Assessment of the impact of this β-glucan exposure regime expression of a range of immune related genes will be undertaken. IL-1β and C3 gene expression has been previously shown to be effected by orally applied MacroGard® during infection trials (Falco *et al.* 2012b, Miest *et al.* 2012, Pionnier *et al.* 2014), and the iNOS gene was shown to have higher expression levels 1 day post injection with a β-glucan in chapter 5. Therefore these three genes were selected as markers of host immunomodulation. The bacterial 16S rDNA gene was used as a measure of gut microbiota population size and next generation sequencing techniques were utilised in order to ascertain if orally applied MacroGard® was

capable of influencing bacterial species diversity within the gut after carp were additionally injected with MacroGard®.

During the development of experimental design from the injection trials described in Chapter 5 to the combination feeding and injection trial presented here, the decision was made to alter the way in which the non-injected negative controls were handled. As shown in Chapter 4, significant changes in gut microbiota population size would occur over a 2 week period, and it was concluded that fish should be compared to non-injected controls sampled at the same time point rather than comparing them against fish that were sampled up to 2 weeks previous, as would occur if non injected controls were only taken at the time of injection. This, however, brought up logistical complications in terms of experimental design, and in particular, if handling fish i.e. netting, submersion in anaesthetic and tail clipping affected the gut microbiota population size. An initial trial was therefore undertaken to investigate this.

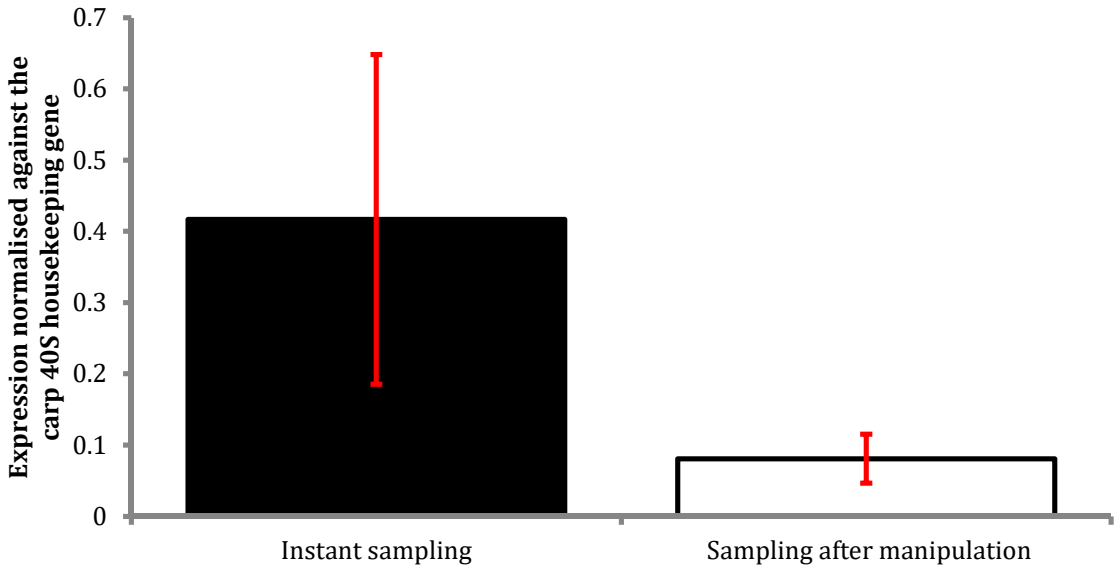
6.1 – Comparison of gut microbiota population size when different handling techniques for non-injected negative controls are used.

6.1.1 Materials and Methods.

Ten carp were selected from the stock population acquired from Hampton Spring Fisheries, UK, in October 2014 and were moved to a single tank and maintained as described in section 4.1.2 until the start of the trial and fed at a rate of 1% body weight per day on a 0% MacroGard® diet (section 4.1.3). All carp were removed from the tank simultaneously with 5 being subject to manipulation, i.e. fish dipped briefly (approximately 30 seconds) to anaesthetise in 2-phenoxyethanol (1ml/5L) before their tails were cut and then returned back to their tank. Five carp were euthanized straight away by submersion in 2-phenoxyethanol followed by destruction of the brain before dissection. Gut samples were taken as described in section 4.3.3 with samples of total gut, gut wall and gut contents being stored in RNA*later* at -80°C until further use. 24 hours after the first group of carp were sampled, the 5 fish that were subject to manipulation were euthanized and gut samples taken for analysis. Tail cuts were performed by Professor Dave Hoole (Keele University, UK). RNA was isolated from gut wall samples as outlined in section 0 and treated with DNase before being translated to cDNA as described in sections 4.3.4.2 and 4.3.4.3. RT-qPCR analysis was performed as outlined in section 4.3.4.4 with the primer pairs 40S (carp) and uniBact_16S (bacteria). Primer sequences are listed in Appendix 3. Statistical analysis was performed using a 1-way ANOVA after a Box-Cox transformation in order to ensure data did not violate the assumptions of normality and homoscadicity.

6.1.2 Results.

Although there was a decrease in 16S expression (relative to expression of the 40S gene) when comparing the two methods of handling, this difference was not significant (Figure 6.1). It was therefore concluded that non injected negative controls within the combination feeding and injection trial would be handled in the same way as injected fish.



Box Cox transformation		λ=0.13		
Anderson-Darling for normality	p=0.380	**Levene's test for equal variance**	p=0.982	
One way ANOVA (R^2=25.26%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall effect of handling	1	2.37	0.168	

Figure 6.1: Expression of the bacterial 16S rDNA gene in carp (*C. carpio*) that were either sampled straight after removal from the tank or were briefly dipped in anaesthetic, had their tails cut and and were returned to the tank for 24 hours before sampling as a comparison of the two different methods employed for handling non injected negative controls (total n=10). Bars represent average gene expression for each treatment group (n=5). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. One outlier was identified from the group of carp that were sampled after manipulation. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. A one way ANOVA was performed to determine statistical significance. Details of the statistical analysis are shown in the table below the graph. Degrees of freedom, F distribution and P values for overall significance are given.

6.2 – Analysing the effect of orally applied MacroGard® in combination with application via intraperitoneal injection.

6.2.1 – Materials and methods.

120 Carp were acquired from Hampton Spring Fisheries, UK, in October 2014 and divided between two tanks maintained at 18°C. All fish were fed on a 0% MacroGard® diet prior to the start of the trial at a rate of 1% body weight per day for 4 months (see sections 4.1.2 and 4.1.3 for details on fish husbandry and feed composition). During the trial carp were either fed with a 0% MacroGard® diet (n=60) or a 0.1% MacroGard® diet (n=60) at a rate of 1% body weight per day for 3 weeks prior to injection and for the duration of the experiment. Carp were anaesthetised (30 seconds in 2-phenoxyethanol at 1ml/5l) before receiving an injection and a cut to their tail fin for identification of treatment groups. Fish were observed for 10 minutes before being returned to their original tank. Carp received either no injection, PBS injection, MacroGard® 2mg kg^{-1}, or inactivated *Aeromonas salmonicida* sp. *salmonicida* (reference strain NCIMB 1102, see section 4.2.2. 10^5 CFU per fish) with details for the preparation of each injection being found in section 6.2.1.1. 30 fish received each treatment as described in Table 6.1. Samples were taken 1, 8 and 15 days post injection as described in section 4.3.3. Carp were euthanized by submersion in 2-phenoxyethanol (1ml/5L) and samples of the total gut, gut wall and gut content were stored in RNA*later* at -80°C until further use.

Table 6.1: Experimental design for a trial studying the effect of combining both oral application of MacroGard® and intraperitoneal injection with either PBS, MacroGard® (2mg kg^{-1}) or heat inactivated *Aeromonas salmonicida* subsp. *salmonicida* (10^5 CFU per fish) to common carp (*C. carpio*). Carp were split into 2 tanks, one of which received the 0% MacroGard® experimental feed and the other was maintained on the 0.1% MacroGard®. All fish were fed at a rate of 1% body weight per day for a 3 week period on each diet before injection and for the remainder of the trial. In order to distinguish between treatment groups, fish were marked with an incision in the tail fin.

Feed	Injection	Time point (days)		
		1	8	15
0% MacroGard®	No injection	5	5	5
	PBS	5	5	5
	MacroGard®	5	5	5
	A. salmonicida	5	5	5
0.1% MacroGard®	No injection	5	5	5
	PBS	5	5	5
	MacroGard®	5	5	5
	A. salmonicida	5	5	5

6.2.1.1 – Preparation of MacroGard® and inactivated *Aeromonas salmonicida* subsp. *salmonicida* for injection.

MacroGard® was prepared at a final concentration of 2mg kg^{-1} per fish in 100µl of PBS as described in section 3.1.

Aeromonas salmonicida subsp. *salmonicida* (NCIMB 1102, see section 4.2.2 for details of this strain) was incubated for 18 hours in 50ml nutrient broth at room temperature (approximately 25°C). Undiluted broth was heat inactivated by autoclaving at 121°C for 15 minutes. Before the bacteria was autoclaved, a serial 1:10 dilution ($1x10^{-1}$ to $1x10^{-10}$) was performed using 50µl nutrient broth applied to a nutrient agar plate (in triplicate for each dilution) and incubated at 20°C for 24 hours. The number of bacteria present within the undiluted broth was calculated based upon colony forming unit (CFU) count. Confirmation of successful heat inactivation was performed in triplicate by applying 50µl of undiluted autoclaved bacteria to nutrient agar plates which were incubated at 20°C for 72 hours after which no colonies were produced.

6.2.1.2 – Quantitative analysis of innate immune gene expression and total 16S rDNA expression in the gut.

Gut wall, gut content and total gut samples were taken as described in section 4.3.3 and stored in RNA*later* at -80°C until further use. RNA was isolated as outlined in section 0, treated with DNase before being reverse transcribed to cDNA as described in sections 4.3.4.2 and 4.3.4.3. RT-qPCR analysis was performed as outlined in section 4.3.4.4 to establish gene expression for C3, IL-1β, iNOS and the bacterial 16S rDNA gene. Correlation analysis of gene expression of the 16S rDNA gene against the analysed immune parameters was performed as described in section 4.3.4.5.

6.2.1.3 – Next generation sequencing analysis of species diversity in MacroGard® injected carp from both feed groups.

Carp sampled 8 and 15 days post injection that received an injection with MacroGard® irrespective of feed were analysed by the Fish Health and Nutrition group at the University of Plymouth (United Kingdom) using next generation sequencing analysis. genDNA from gut wall samples was isolated and OTU identification was performed to the genus level. genDNA isolation was performed as outlined in section 4.3.5.1.

Next Generation Sequencing Analysis was performed by Dr. Ana Rodiles of the University of Plymouth (United Kingdom) and Appendix 4 gives the full report including methodologies provided by Dr. Daniel Merrifield. Briefly, the V1-V2 region of the 16S rDNA gene was amplified in a reaction mix consisting of the primer pair 16S_seq (see Appendix 3 for sequences) at a final concentration of 0.568µM, 50µl of MyTaqTM, and 2µl of genDNA in a total volume of 88µl. PCR was performed using the

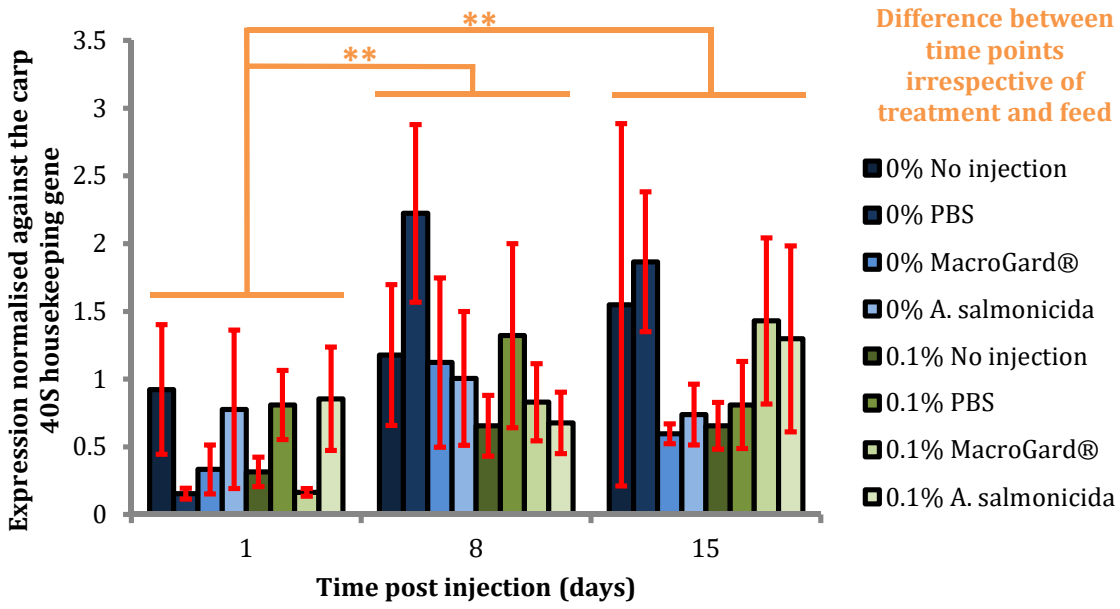
following protocol and a TC-512 thermal cycler: initial denaturation at 94°C for 7 min, then 10 cycles at 94°C for 30 sec, followed by a touchdown of 1°C per cycle from 62 - 53°C for 30 sec and 72°C for 30 sec. A further 20 cycles were performed at 94°C for 30 sec, 53°C for 30 sec and 72°C for 30 sec before a final extension for 7 min at 72°C. PCR products were subject to electrophoresis using an agarose gel and bands of the correct expected size (300bp) isolated and cleaned using a QIAquick Gel Extraction Kit as per the manufacturer's instructions. Samples were then adjusted to a concentration of 26pM before being prepared using an Ion PGMTM Template OT2 kit. Sequencing was performed using an Ion Torrent Personal Genome Machine.

6.2.2 – Results

As with the previous chapters results will be considered by analysis of the expression of 3 innate immune genes utilised, followed by analysis of the overall gut microbiota size in all samples and NGS analysis of the species richness within the gut. Finally, a comparison of innate immune gene expression and the overall size of the microbiota population was carried out.

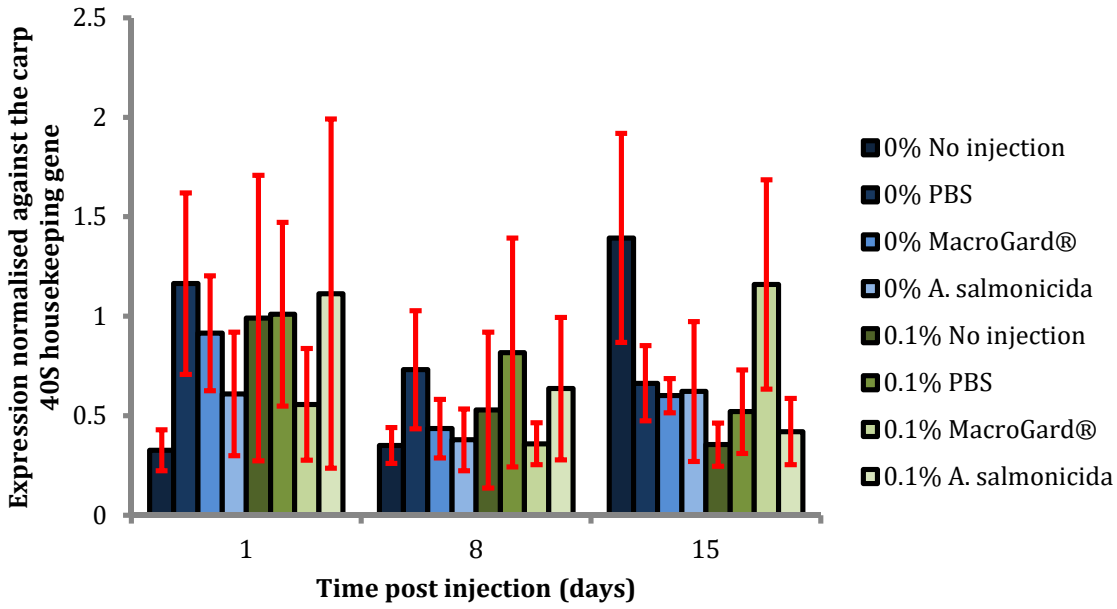
6.2.2.1 – Innate immune gene expression.

Although expression of iNOS gene was significantly influenced by time ($p=0.001$), treatment or feed regime did not appear the affect the expression of iNOS (Figure 6.2). Expression 1 day post injection was significantly lower than expression at 8 ($p=0.0037$) and 15 ($p=0.0025$) days post injection. In contrast, C3 gene expression was not significantly different over time, between injection or between feed groups (Figure 6.3). Whilst IL-1β expression (Figure 6.4) was not significantly affected by injection, there was a significant difference between feed groups ($p=0.001$) and time point within feed groups ($p=0.024$). Gene expression for IL-1β in fish fed 0.1% MacroGard® was significantly higher 1 and 8 days post injection ($p=0.0146$ and $p=0.0433$ respectively) compared to 0% MacroGard® fed fish.



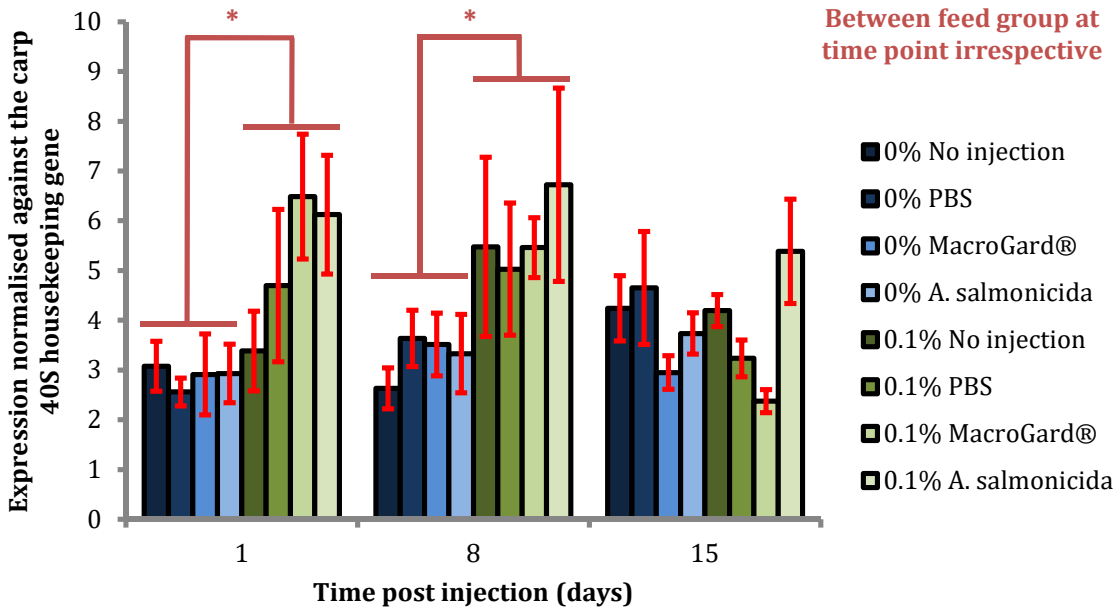
Box Cox transformation		λ=0.01		
Anderson-Darling test for normality	p=0.953	**Levene's test for equal variance**		p=0.626
3-way ANOVA with *post hoc* Tukey's (R^2=25.68%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall effect of time	2	7.64	0.001	**
Overall effect of injection	3	1.04	0.380	
Overall effect of feed	1	0.11	0.739	
Interaction between time and injection	6	0.81	0.563	
Interaction between time and feed	2	0.36	0.700	
Interaction between injection and feed	3	0.16	0.922	
Interaction between all variables	6	1.35	0.244	
***Post hoc* analysis**				
Time: day 1 vs day 8		p=0.0037		**
Time: day 1 vs day 15		p=0.0025		**

Figure 6.2: Expression of iNOS in the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=120). Carp were fed either a 0% or 0.1% MacroGard® diet and were further divided into one of 4 treatment groups: no injection, PBS injection, MacroGard® injection, inactivated *A. salmonicida* injection. Carp were sampled 1, 8 and 15 days post injection (n=5). Bars represent average gene expression for each treatment group (n=6). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. One outlier was identified from the non-injected group of carp that received the 0% MacroGard® diet at time point 15 days. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.



Box Cox transformation		λ=-1.98		
Anderson-Darling test for normality	p=0.007	**Levene's test for equal variance**		p=0.998
3-way ANOVA with *post hoc* Tukey's (R^2=25.68%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall effect of time	2	1.41	0.249	
Overall effect of injection	3	1.74	0.164	
Overall effect of feed	1	1.06	0.307	
Interaction between time and injection	6	0.94	0.473	
Interaction between time and feed	2	0.05	0.947	
Interaction between injection and feed	3	0.67	0.574	
Interaction between all variables	6	0.86	0.525	

Figure 6.3: Expression of C3 in the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=120). Carp were fed either a 0% or 0.1% MacroGard® diet and were further divided into one of 4 treatment groups: no injection, PBS injection, MacroGard® injection, inactivated *A. salmonicida* injection. Carp were sampled 1, 8 and 15 days post injection (n=5). Bars represent average gene expression for each treatment group (n=6). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. Four statistical outliers were identified: inactivated A. salmonicida injection 0.1% MacroGard® feed at time point day 1, PBS injection 0% MacroGard® feed at time point day 8, MacroGard® injection 0% MacroGard® feed at time point day 8, and PBS injection 0.1% MacroGard® feed at time point day 8. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a +1 followed by Box Cox transformation before further analysis. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.



Box Cox transformation		λ=0.09		
Anderson-Darling test for normality	p=0.305	**Levene's test for equal variance**		p=0.769
3-way ANOVA with *post hoc* Tukey's (R^2=29.03%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall effect of time	2	0.69	0.505	
Overall effect of injection	3	0.67	0.575	
Overall effect of feed	1	12.30	0.001	**
Interaction between time and injection	6	1.23	0.298	
Interaction between time and feed	2	3.87	0.024	*
Interaction between injection and feed	3	0.93	0.432	
Interaction between all variables	6	0.67	0.675	
***Post hoc* analysis**				
Time and feed: day 1 between feeds		p=0.0146		*
Time and feed: day 8 between feeds		p=0.0433		*

Figure 6.4: Expression of IL-1β in the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=120). Carp were fed either a 0% or 0.1% MacroGard® diet and were further divided into one of 4 treatment groups: no injection, PBS injection, MacroGard® injection, inactivated *A. salmonicida* injection. Carp were sampled 1, 8 and 15 days post injection (n=5). Bars represent average gene expression for each treatment group (n=6). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. Details of the statistical analysis are shown beneath the graph. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.

6.2.2.2 – Analysis of the gut microbiota population.

Analysis of total 16S rDNA expression.

Expression of the bacterial 16S rDNA gene (Figure 6.5) was significantly affected by time ($p<0.001$) and a combination of time and feed group ($p=0.001$), but not by injection group. 16S expression levels 1 day post injection were significantly lower than expression on days 8 and 15 post injection irrespective of feed group and injection treatment ($p<0.0001$ in both cases). For all 4 injection treatment groups, there is a general trend of an increase in total 16S expression over time. Non injected negative controls showed a non-significant increase in 16S expression for the duration of the trial irrespective of diet. Similarly, PBS injected negative controls also showed a trend of increased 16S expression for the duration of the trial irrespective of diet, however expression in carp fed with the 0% MacroGard® diet was significantly higher ($p=0.0006$) 15 days post injection compared to 1 day post injection. There was no significant difference in 16S expression between time points for carp that received the 0.1% MacroGard® diet and PBS injection. For both MacroGard® and inactivated *A. salmonicida* injected carp that were fed the 0.1% MacroGard® diet, 16S expression followed the same trend as both negative controls, i.e. an increase in expression over time. This increase was not significant for either injection treatment. For 0% MacroGard® fed carp that received an injection of either MacroGard® or *A. salmoncida*, however, a significant increase in expression was seen 8 days post injection in comparison to 1 day post injection (MacroGard® $p=0.0017$, *A. salmonicida* $p=0.0059$). Expression levels then decreased by 15 days post injection in both injection groups. Expression at day 15 in MacroGard® injected carp was still significantly higher than 1 day post injection ($p=0.0306$).

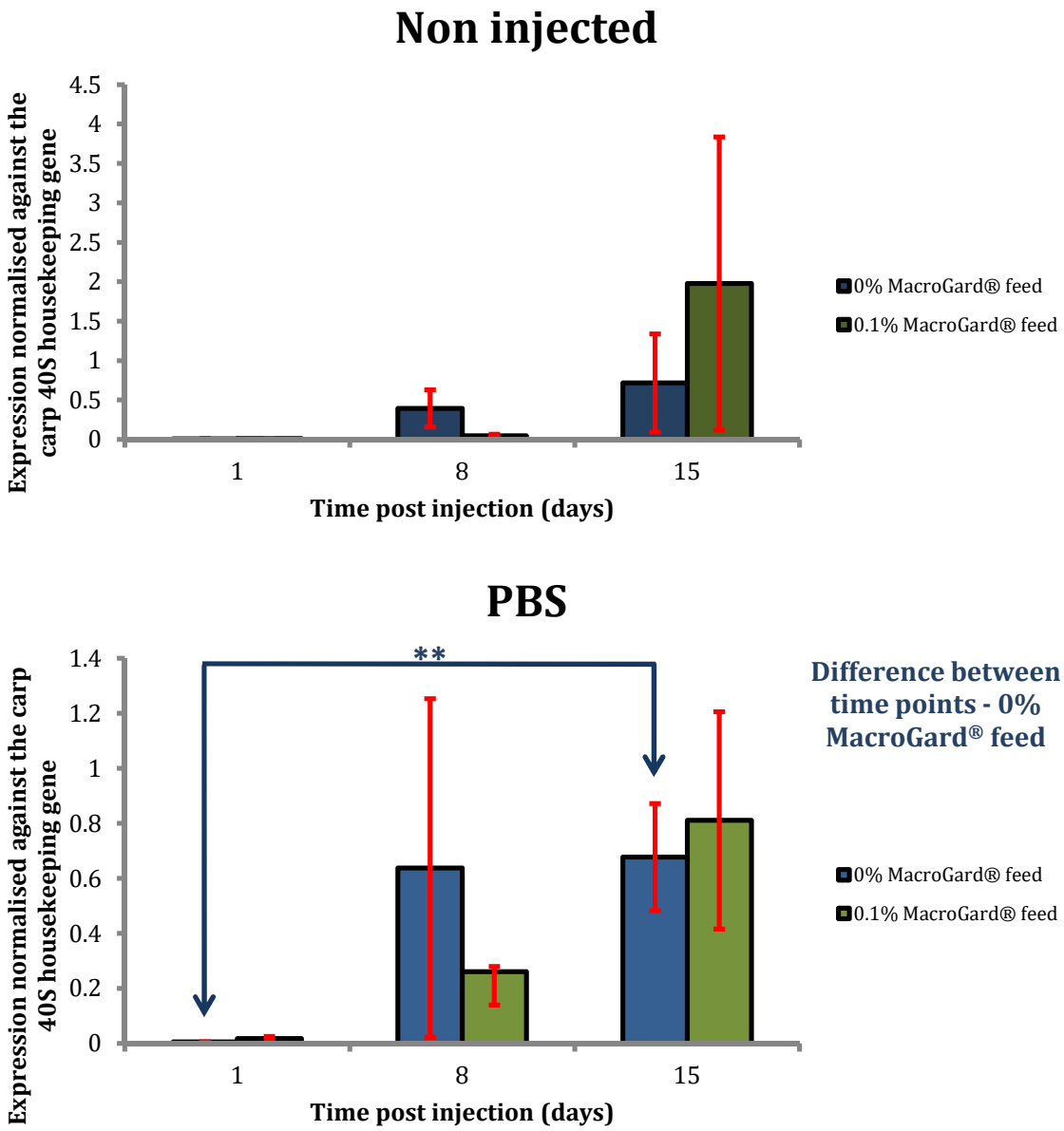


Figure 6.5: (part A) Expression of the bacterial 16S rDNA gene in the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=120). Carp were fed either a 0% or 0.1% MacroGard® diet and were further divided into one of 4 treatment groups: no injection, PBS injection, MacroGard® injection, inactivated *A. salmonicida* injection. Carp were sampled 1, 8 and 15 days post injection (n=5). Bars represent average gene expression for each treatment group (n=6). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. Details of the statistical analysis are shown in part C of this figure. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) *post hoc* comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.

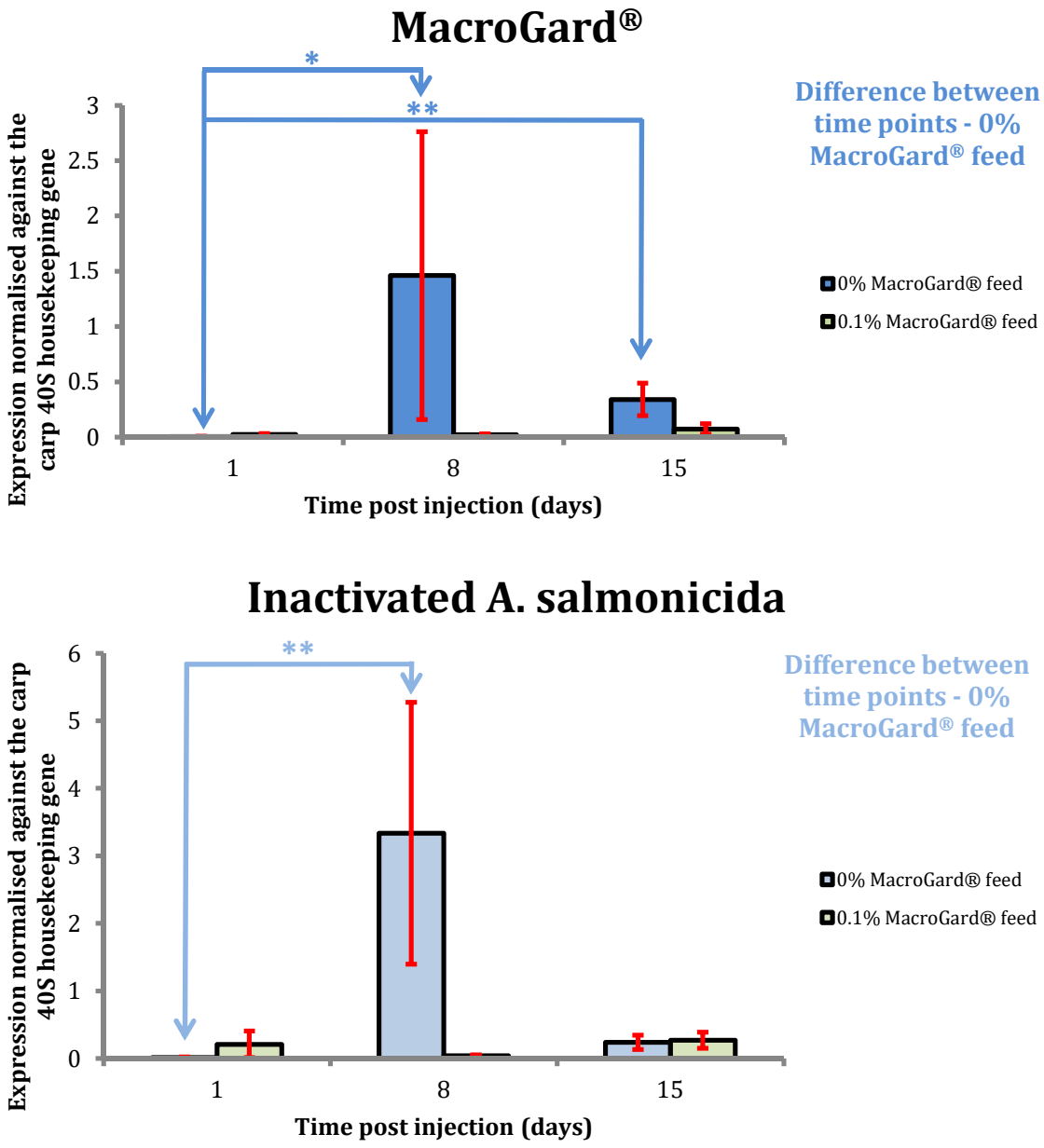


Figure 6.5: (part B) Expression of the bacterial 16S rDNA gene in the gut of common carp (*Cyprinus carpio*) within a combined feeding and injection trial (n=120). Carp were fed either a 0% or 0.1% MacroGard® diet and were further divided into one of 4 treatment groups: no injection, PBS injection, MacroGard® injection, inactivated A. salmonicida injection. Carp were sampled 1, 8 and 15 days post injection (n=5). Bars represent average gene expression for each treatment group (n=6). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. Details of the statistical analysis are shown in part C of this figure. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) post hoc comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.

Box Cox transformation		λ=-0.24		
Anderson-Darling test for normality	p=0.538	**Levene's test for equal variance**		p=0.959
3-way ANOVA with *post hoc* Tukey's (R2=56.19%)	**Degrees of freedom**	**F distribution**	**P value**	**Significance**
Overall effect of time	2	44.73	p<0.001	***
Overall effect of injection	3	0.68	0.565	
Overall effect of feed	1	0.00	0.968	
Interaction between time and injection	6	0.34	0.916	
Interaction between time and feed	2	7.47	0.001	**
Interaction between injection and feed	3	0.74	0.529	
Interaction between all variables	6	1.28	0.276	
***Post hoc* analysis**				
Time: day 1 vs day 8			p<0.0001	***
Time: day 1 vs day 15			p<0.0001	***
Time and feed: day 0, 0% versus 0.1%			p=0.0346	*
0% feed: PBS day 1 vs PBS day 15			p=0.0006	***
0% feed: MacroGard® day 1 vs MacroGard® day 8			p=0.0306	*
0% feed: MacroGard® day 1 vs MacroGard® day 15			p=0.0017	**
0% feed: A. salmonicida day 1 vs A. salmonicida day 8			p=0.0059	**

Figure 6.5: (part C) Expression of the bacterial 16S rDNA gene in the gut of common carp (*Cyprinus carpio*) within a combined feeding and injection trial (n=120). Carp were fed either a 0% or 0.1% MacroGard® diet and were further divided into one of 4 treatment groups: no injection, PBS injection, MacroGard® injection, inactivated A. salmonicida injection. Carp were sampled 1, 8 and 15 days post injection (n=5). Bars represent average gene expression for each treatment group (n=6). Error bars are given as standard error of the mean. Grubbs test was utilised to identify any statistical outliers which were removed before any further statistical analysis. Data was checked for normality using the Anderson-Darling test on both raw data and residual data, and Levene's test for equal variance. Raw data was not normally distributed therefore was subject to a Box Cox transformation before further analysis. Details of the statistical analysis are shown in part C of this figure. Degrees of freedom, F distribution and P values for overall significance are given with significant ($p<0.05$) post hoc comparisons being listed. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.

Bacterial species richness and diversity in carp injected with MacroGard®.

Analysis of the bacterial species richness of the autochthonous population in the gut, i.e. gut wall samples, from both feed groups that were injected with MacroGard® at time points 8 and 15 days post injection (n=20), was performed using next generation sequencing technology. In total, 3 011 088 OTUs were obtained for all samples analysed and taxonomic identification was performed to the species level based upon 97% sequence similarity and a 0.8 confidence threshold. Cyanobacteria (2,736 sequences) and Propionibacteriaceae (1,111 sequences) sequences were discarded as contaminants as recommended by Dr. Ana Rodiles of Plymouth University (UK). This left a total of 1 288 902 identified OTUs. Data was analysed based upon overall presence/absence and percentage of sequences obtained per fish as an approximation of relative abundance.

Proteobacteria (61 genera) accounted for 70.1% of identified OTUs followed by Fusobacteria (27.2% - 3 genera) and Firmicutes (2.2% - 39 genera). The remaining genera belonged to the phylum Actinobacteria (17 genera), Bacteroidetes (9 genera), Spirochaetes (1 genus), Verrucomicrobia (1 genus) and 4 different OTUs that were unidentified at the phylum level. Time did not have an effect on the percentage relative abundance of any phylum, however feed had a significant effect on the presence of Fusobacteria and Proteobacteria. Fusobacteria was significantly lower ($p=0.03$) in 0.1% MacroGard® fed carp in comparison to those fed with the 0% MacroGard® diet. In contrast, there was a significantly higher proportion of Proteobacteria ($p=0.03$) in carp fed with the 0.1% MacroGard® diet.

At the class level within the Proteobacteria, the percentage relative abundance

of Alphaproteobacteria is significantly higher (p=0.016) in 0.1% MacroGard® fed carp whereas the proportion of Gammaproteobacteria is significantly lower (p=0.009) in these fish.

Table 6.2 lists each genus identified and the number of carp per group (by feed and time point) in which it is present at more than 0.1% of the OTUs within a fish. 25 OTUs were present in all fish analysed, however only 3 of these (*Cetobacterium*, *Phyllobacterium* and unknown Rhizobiales genus) were present in all fish with a relative abundance greater than 0.1%. 26 OTUs appeared only in the guts of carp fed with the 0% diet 8 days post injection, however only 5 of these (*Akkermansia*, unknown Acidimicrobiales, unknown Actinobacteria, unknown Deltaproteobacteria and unknown Firmicutes) were present with a relative abundance greater than 0.1%. No OTUs were found exclusively 15 days post injection in carp fed the 0% MacroGard® diet. For carp fed with the 0.1% MacroGard® diet, 1 OTU was found at each time point (unknown Sphingobacteriales at day 8 and unknown Lactobacillales at day 15). Neither of these were more than 0.1% of the relative abundance and both only appeared in 1 fish. Two OTUs were time point specific irrespective of feed (unknown Procabacteriaceae at 8 days post injection and an unidentified OTU 15 days post injection). In both cases, the OTU was found in 4 fish per time point (2 per treatment), however only 1 fish (0.1% feed, 15 days post injection) was shown to have greater than 0.1% relative abundance of the unidentified OTU. No OTU was present at both time points for carp fed with the 0% MacroGard® diet, however one OTU (unknown Actinomycetales) was found at both time points for 0.1% MacroGard® fed carp (1 fish per time point) and represented less than 0.1% of the OTUs identified per fish. 129 unique OTUs were found in at least 1 fish fed with the 0% MacroGard® 8

days post injection, 86 were found in carp fed the 0.1% MacroGard® diet 8 days post injection, 88 were found in carp fed with the 0% MacroGard® diet 15 days post injection, and finally 106 were found in carp fed the 0.1% MacroGard® diet 15 days post injection. Of these, 45%, 62%, 65% and 54% respectively were found in all 5 fish within each treatment group.

Table 6.2: (part A) Bacterial species richness within the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=20). Carp were fed either a 0% or 0.1% MacroGard® diet and, after 3 weeks of feeding, received an intraperitoneal injection of MacroGard®. Carp were sampled 8 and 15 days post injection (n=5) and gut samples analysed using High Throughput Sequencing. OTUs were analysed using the Greengenes database (DeSantis, Hugenholtz *et al.* 2006) and taxonomic identification was made at the genus level based upon 97% sequence similarity and a 0.8 confidence threshold. Data was first analysed based upon presence/absence and, secondly, presence/absence whereby presence is defined as representing more than 0.1% of OTUs obtained, i.e. relative abundance. When a genus is present in all carp within a treatment group, this is highlighted in bold red. When further analysis of a specific genus has been performed, the genus is highlighted in bold.

	Number of fish where genus is present				**Number of fish where genus represents >0.1% of sequences analysed (relative abundance)**			
Time point	**Day 8**		**Day 15**		**Day 8**		**Day 15**	
Percentage MacroGard® in diet	**0%**	**0.1%**	**0%**	**0.1%**	**0%**	**0.1%**	**0%**	**0.1%**
Unknown bacteria	2	3	1	4	1	-	-	-
Unknown Microthrixaceae	1	-	-	-	-	-	-	-
Unknown Acidimicrobiales	1	-	-	-	1	-	-	-
Unknown Actinomycetales	-	1	-	1	-	-	-	-
Corynebacterium	4	4	2	4	-	-	1	-
Cryocola	2	2	2	4	-	-	-	-
Microbacterium	**5**	**5**	**5**	4	1	1	-	-
Unknown Microbacteriaceae	1	-	-	1	1	-	-	1
Mycobacterium	4	3	3	4	-	-	-	-
Rhodococcus	1	**5**	2	3	-	-	-	-
Propionicimonas	1	-	-	1	-	-	-	-
Unknown Nocardioidaceae	1	-	-	1	-	-	-	-
Unknown Pseudonocardiaceae	1	-	-	-	-	-	-	-
Unknown Actinomycetales	4	**5**	**5**	**5**	1	-	-	1
Bifidobacterium	1	-	-	-	-	-	-	-
Collinsella	1	-	-	-	-	-	-	-
Patulibacter	**5**	**5**	**5**	**5**	1	**5**	2	4

Table 6.2: (part B) Bacterial species richness within the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=20). Carp were fed either a 0% or 0.1% MacroGard® diet and, after 3 weeks of feeding, received an intraperitoneal injection of MacroGard®. Carp were sampled 8 and 15 days post injection (n=5) and gut samples analysed using High Throughput Sequencing. OTUs were analysed using the Greengenes database (DeSantis, Hugenholtz *et al.* 2006) and taxonomic identification was made at the genus level based upon 97% sequence similarity and a 0.8 confidence threshold. Data was first analysed based upon presence/absence and, secondly, presence/absence whereby presence is defined as representing more than 0.1% of OTUs obtained, i.e. relative abundance. When a genus is present in all carp within a treatment group, this is highlighted in bold red. When further analysis of a specific genus has been performed, the genus is highlighted in bold.

	Number of fish where genus is present				**Number of fish where genus represents >0.1% of sequences analysed (relative abundance)**			
Time point	**Day 8**		**Day 15**		**Day 8**		**Day 15**	
Percentage MacroGard® in diet	**0%**	**0.1%**	**0%**	**0.1%**	**0%**	**0.1%**	**0%**	**0.1%**
Unknown Actinobacteria	1	-	-	-	1	-	-	-
Sediminibacterium	2	-	1	1	-	-	-	-
Paludibacter	1	1	2	2	-	-	-	1
Unknown Rikenellaceae	3	-	-	-	-	-	-	-
Unknown Rikenellaceae	2	-	-	-	-	-	-	-
Chryseobacterium	**5**	**5**	**5**	**5**	-	-	-	-
Unknown Weeksellaceae	3	4	4	4	-	-	-	-
Flavobacterium	**5**	**5**	**5**	**5**	2	2	-	3
Unknown Sphingobacteriales	-	1	-	-	-	-	-	-
Sphingobacterium	2	-	-	3	-	-	-	1
Bacillus	**5**	4	4	4	-	-	-	-
Unknown Bacillaceae	1	-	-	1	-	-	-	-
Brevibacillus	2	3	4	**5**	-	-	-	-
Staphylococcus	**5**	4	**5**	**5**	-	-	-	-
Unknown Bacillales	3	1	3	2	-	-	-	-
Unknown Gemellales	1	-	-	1	-	-	-	-
Aerococcus	1	-	2	2	-	-	-	-

Table 6.2: (part C) Bacterial species richness within the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=20). Carp were fed either a 0% or 0.1% MacroGard® diet and, after 3 weeks of feeding, received an intraperitoneal injection of MacroGard®. Carp were sampled 8 and 15 days post injection (n=5) and gut samples analysed using High Throughput Sequencing. OTUs were analysed using the Greengenes database (DeSantis, Hugenholtz *et al.* 2006) and taxonomic identification was made at the genus level based upon 97% sequence similarity and a 0.8 confidence threshold. Data was first analysed based upon presence/absence and, secondly, presence/absence whereby presence is defined as representing more than 0.1% of OTUs obtained, i.e. relative abundance. When a genus is present in all carp within a treatment group, this is highlighted in bold red. When further analysis of a specific genus has been performed, the genus is highlighted in bold.

	Number of fish where genus is present				**Number of fish where genus represents >0.1% of sequences analysed (relative abundance)**			
Time point	**Day 8**		**Day 15**		**Day 8**		**Day 15**	
Percentage MacroGard® in diet	**0%**	**0.1%**	**0%**	**0.1%**	**0%**	**0.1%**	**0%**	**0.1%**
Carnobacterium	1	-	-	1	-	-	-	-
Enterococcus	4	4	4	**5**	1	-	-	-
Vagococcus	1	-	-	1	-	-	-	-
Unknown Enterococcaceae	4	2	4	3	-	-	-	-
Unknown Lactobacillaceae	1	2	-	1	-	-	-	1
Lactobacillus	**5**	**5**	**5**	**5**	1	-	3	2
Pediococcus	1	-	-	2	-	-	-	1
Unknown Leuconostocaceae	**5**	**5**	**5**	**5**	1	-	-	-
Leuconostoc	**5**	**5**	**5**	**5**	1	-	3	2
Weissella	**5**	**5**	**5**	**5**	1	-	-	1
Unknown Leuconostocaceae	1	-	-	-	-	-	-	-
Lactococcus	4	4	**5**	**5**	-	-	-	1
Streptococcus	**5**	**5**	**5**	**5**	2	-	4	2
Unknown Streptococcaceae	**5**	**5**	**5**	4	-	-	-	-
Unknown Lactobacillales	-	-	-	1	-	-	-	-
Unknown Bacilli	4	4	**5**	4	-	1	1	-
Unknown Clostridiales	1	-	-	1	1	-	-	-

Table 6.2: (part D) Bacterial species richness within the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=20). Carp were fed either a 0% or 0.1% MacroGard® diet and, after 3 weeks of feeding, received an intraperitoneal injection of MacroGard®. Carp were sampled 8 and 15 days post injection (n=5) and gut samples analysed using High Throughput Sequencing. OTUs were analysed using the Greengenes database (DeSantis, Hugenholtz *et al.* 2006) and taxonomic identification was made at the genus level based upon 97% sequence similarity and a 0.8 confidence threshold. Data was first analysed based upon presence/absence and, secondly, presence/absence whereby presence is defined as representing more than 0.1% of OTUs obtained, i.e. relative abundance. When a genus is present in all carp within a treatment group, this is highlighted in bold red. When further analysis of a specific genus has been performed, the genus is highlighted in bold.

	Number of fish where genus is present				**Number of fish where genus represents >0.1% of sequences analysed (relative abundance)**			
Time point	**Day 8**		**Day 15**		**Day 8**		**Day 15**	
Percentage MacroGard® in diet	**0%**	**0.1%**	**0%**	**0.1%**	**0%**	**0.1%**	**0%**	**0.1%**
Unknown Mogibacteriaceae	3	-	-	-	1	-	-	-
Finegoldia	2	1	4	4	-	-	-	1
Unknown Christensenellaceae	1	-	-	-	-	-	-	-
Christensenella	1	-	-	-	-	-	-	-
Clostridium	4	**5**	**5**	**5**	-	-	-	-
Unknown Clostridiaceae	**5**	**5**	**5**	**5**	-	-	1	1
Epulopiscium	3	2	4	3	-	-	-	-
Unknown Lachnospiraceae	**5**	3	4	**5**	2	-	-	-
Oscillospira	2	-	-	1	1	-	-	-
Ruminococcus	3	-	-	-	1	-	-	-
Unknown Ruminococcaceae	2	-	-	-	1	-	-	-
Unknown Clostridiales	**5**	**5**	**5**	**5**	3	1	3	2
Eubacterium	1	-	-	-	-	-	-	-
Catenibacterium	1	-	-	-	-	-	-	-
Unknown Firmicutes	1	-	-	-	1	-	-	-
Cetobacterium	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Psychrilyobacter	3	-	-	1	-	-	-	-

Table 6.2: (part E) Bacterial species richness within the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=20). Carp were fed either a 0% or 0.1% MacroGard® diet and, after 3 weeks of feeding, received an intraperitoneal injection of MacroGard®. Carp were sampled 8 and 15 days post injection (n=5) and gut samples analysed using High Throughput Sequencing. OTUs were analysed using the Greengenes database (DeSantis, Hugenholtz *et al.* 2006) and taxonomic identification was made at the genus level based upon 97% sequence similarity and a 0.8 confidence threshold. Data was first analysed based upon presence/absence and, secondly, presence/absence whereby presence is defined as representing more than 0.1% of OTUs obtained, i.e. relative abundance. When a genus is present in all carp within a treatment group, this is highlighted in bold red. When further analysis of a specific genus has been performed, the genus is highlighted in bold.

	Number of fish where genus is present				**Number of fish where genus represents >0.1% of sequences analysed (relative abundance)**			
Time point	**Day 8**		**Day 15**		**Day 8**		**Day 15**	
Percentage MacroGard® in diet	**0%**	**0.1%**	**0%**	**0.1%**	**0%**	**0.1%**	**0%**	**0.1%**
Unknown Fusobacteriaceae	4	1	3	1	1	-	-	-
Unknown bacteria	-	-	2	2	-	-	-	1
Unknown Caulobacteraceae	4	**5**	**5**	**5**	-	-	-	-
Mycoplana	2	3	**5**	4	-	-	-	-
Phenylobacterium	1	-	-	-	-	-	-	-
Unknown Caulobacteraceae	4	**5**	**5**	**5**	-	2	1	2
Afipia	3	4	**5**	4	-	-	-	-
Bradyrhizobium	**5**	**5**	**5**	**5**	4	**5**	**5**	**5**
Unknown Bradyrhizobiaceae	4	**5**	**5**	**5**	1	3	1	3
Unknown Methylobacteriaceae	4	**5**	4	**5**	-	1	1	1
Unknown Methylobacteriaceae	3	4	4	2	-	-	-	-
Mesorhizobium	**5**	**5**	**5**	**5**	2	**5**	3	4
Phyllobacterium	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Unknown Phyllobacteriaceae	**5**	**5**	**5**	**5**	4	**5**	**5**	**5**
Labrys	4	4	3	3	-	-	-	-
Unknown Rhizobiales	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Amaricoccus	1	-	-	-	-	-	-	-

Table 6.2: (part F) Bacterial species richness within the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=20). Carp were fed either a 0% or 0.1% MacroGard® diet and, after 3 weeks of feeding, received an intraperitoneal injection of MacroGard®. Carp were sampled 8 and 15 days post injection (n=5) and gut samples analysed using High Throughput Sequencing. OTUs were analysed using the Greengenes database (DeSantis, Hugenholtz *et al.* 2006) and taxonomic identification was made at the genus level based upon 97% sequence similarity and a 0.8 confidence threshold. Data was first analysed based upon presence/absence and, secondly, presence/absence whereby presence is defined as representing more than 0.1% of OTUs obtained, i.e. relative abundance. When a genus is present in all carp within a treatment group, this is highlighted in bold red. When further analysis of a specific genus has been performed, the genus is highlighted in bold.

	Number of fish where genus is present				**Number of fish where genus represents >0.1% of sequences analysed (relative abundance)**			
Time point	**Day 8**		**Day 15**		**Day 8**		**Day 15**	
Percentage MacroGard® in diet	**0%**	**0.1%**	**0%**	**0.1%**	**0%**	**0.1%**	**0%**	**0.1%**
Paracoccus	1	-	-	1	1	-	-	-
Phaeobacter	1	-	-	-	-	-	-	-
Rhodobacter	3	4	2	4	1	-	-	-
Ruegeria	1	-	-	-	-	-	-	-
Unknown Rhodobacteraceae	2	3	3	4	-	-	-	-
Unknown Rhodospirillaceae	**5**	**5**	**5**	**5**	4	**5**	**5**	**5**
Unknown Pelagibacteraceae	2	2	**5**	2	-	-	-	-
Novosphingobium	1	1	2	2	-	-	1	-
Sphingobium	**5**	**5**	**5**	4	1	1	1	-
Sphingomonas	1	2	3	3	-	-	1	1
Unknown Sphingomonadaceae	**5**	**5**	**5**	**5**	-	2	2	2
Unknown Alcaligenaceae	2	-	1	1	-	-	-	-
Unknown Alcaligenaceae	2	-	2	1	-	-	-	-
Burkholderia	3	3	4	3	-	-	-	-
Acidovorax	3	4	**5**	**5**	-	-	-	-
Alicycliphilus	1	3	4	2	-	-	-	-
Delftia	1	1	2	2	-	-	-	-

Table 6.2: (part G) Bacterial species richness within the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=20). Carp were fed either a 0% or 0.1% MacroGard® diet and, after 3 weeks of feeding, received an intraperitoneal injection of MacroGard®. Carp were sampled 8 and 15 days post injection (n=5) and gut samples analysed using High Throughput Sequencing. OTUs were analysed using the Greengenes database (DeSantis, Hugenholtz *et al.* 2006) and taxonomic identification was made at the genus level based upon 97% sequence similarity and a 0.8 confidence threshold. Data was first analysed based upon presence/absence and, secondly, presence/absence whereby presence is defined as representing more than 0.1% of OTUs obtained, i.e. relative abundance. When a genus is present in all carp within a treatment group, this is highlighted in bold red. When further analysis of a specific genus has been performed, the genus is highlighted in bold.

	Number of fish where genus is present				**Number of fish where genus represents >0.1% of sequences analysed (relative abundance)**			
Time point	**Day 8**		**Day 15**		**Day 8**		**Day 15**	
Percentage MacroGard® in diet	**0%**	**0.1%**	**0%**	**0.1%**	**0%**	**0.1%**	**0%**	**0.1%**
Limnohabitans	-	2	1	1	-	-	-	-
Unknown Comamonadaceae	4	**5**	**5**	4	-	-	-	-
Polynucleobacter	**5**	**5**	**5**	**5**	2	2	-	3
Unknown Oxalobacteraceae	4	**5**	4	2	2	-	-	-
Unknown Burkholderiales	1	-	2	3	-	-	-	-
Unknown Procabacteriaceae	2	2	-	-	-	-	-	-
Unknown Deltaproteobacteria	2	-	2	1	-	-	-	-
Unknown Desulfovibrionaceae	3	-	-	-	1	-	-	-
Unknown Deltaproteobacteria	1	-	-	-	1	-	-	-
Arcobacter	2	-	-	-	1	-	-	-
Unknown Campylobacteraceae	1	-	-	-	-	-	-	-
Unknown Aeromonadales	**5**	**5**	**5**	**5**	**5**	1	4	2
Shewanella	**5**	**5**	**5**	3	1	-	2	-
Escherichia	1	-	-	1	-	-	-	1
Pantoea	2	3	4	2	-	-	-	-
Plesiomonas	3	3	4	4	-	-	-	-
Trabulsiella	3	**5**	**5**	4	-	-	-	-

Table 6.2: (part H) Bacterial species richness within the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=20). Carp were fed either a 0% or 0.1% MacroGard® diet and, after 3 weeks of feeding, received an intraperitoneal injection of MacroGard®. Carp were sampled 8 and 15 days post injection (n=5) and gut samples analysed using High Throughput Sequencing. OTUs were analysed using the Greengenes database (DeSantis, Hugenholtz *et al.* 2006) and taxonomic identification was made at the genus level based upon 97% sequence similarity and a 0.8 confidence threshold. Data was first analysed based upon presence/absence and, secondly, presence/absence whereby presence is defined as representing more than 0.1% of OTUs obtained, i.e. relative abundance. When a genus is present in all carp within a treatment group, this is highlighted in bold red. When further analysis of a specific genus has been performed, the genus is highlighted in bold.

	Number of fish where genus is present				**Number of fish where genus represents >0.1% of sequences analysed (relative abundance)**			
Time point	**Day 8**		**Day 15**		**Day 8**		**Day 15**	
Percentage MacroGard® in diet	**0%**	**0.1%**	**0%**	**0.1%**	**0%**	**0.1%**	**0%**	**0.1%**
Unknown Enterobacteriaceae	**5**	**5**	**5**	**5**	1	1	4	3
Acinetobacter	2	4	4	4	-	-	-	-
Enhydrobacter	**5**	**5**	**5**	**5**	2	**5**	**5**	4
Psychrobacter	-	1	1	-	-	-	-	-
Pseudomonas	4	**5**	**5**	**5**	-	-	1	-
Pseudoalteromonas	1	3	-	2	-	-	-	-
Vibrio	**5**	**5**	**5**	**5**	**5**	4	**5**	4
Unknown Vibrionales	**5**	**5**	**5**	**5**	2	-	4	3
Unknown Sinobacteraceae	**5**	4	4	4	-	-	-	-
Nevskia	1	2	2	1	-	1	-	-
Stenotrophomonas	**5**	**5**	**5**	**5**	2	4	2	4
Unknown Gammaproteobacteria	3	3	3	4	-	-	-	-
Unknown Brevinemataceae	3	4	**5**	2	-	-	-	-
Unknown bacteria	3	1	1	1	-	-	-	-
Unknown bacteria	1	-	-	-	-	-	-	-
Akkermansia	1	-	-	-	1	-	-	-

Statistical analysis of percentage relative abundance data at different taxonomic levels.

Statistical analysis was performed on percentage relative abundance data on the taxonomic levels of class, order, family and genus when a classification was present in all 20 fish analysed (Table 6.3). For example, the class Gammaproteobacteria was found in all 20 carp therefore statistical analysis was performed, however the genus *Escherichia* was only present in 2 fish therefore statistical analysis was not performed. A 2-way ANOVA was performed when data was or could be normalised. In cases where this was not possible, the Scheirer-Ray-Hare test was utilised.

Out of the 48 data sets analysed across the taxonomic levels of class to genus, 22 showed statistically significant differences. 15 were due to an effect of diet, 4 were due to an effect of time, and 3 showed an interaction between diet and time point. Out of 9 classes, 3 were shown to be significantly different and in each case, based upon the data shown in Table 6.3, diet was determined to be the influential factor. The percentage relative expression of both Thermoleophilia and Alphaproteobacteria was significantly higher in 0.1% MacroGard® fed carp ($p=0.032$ and $p=0.016$ respectively) whereas in contrast, Gammoproteobacteria was significantly lower in 0.1% MacroGard® fed carp ($p=0.009$). Only 1 Thermoleophilia OTU was detected, however 26 unique OTUs were identified as Alphaproteobacteria and 18 unique OTUs were identified as Gammaproteobacteria.

The 26 Alphaproteobacteria OTUs were divided between 6 different orders of which 4 were present in all fish analysed. Of these 4, the percentage relative abundance of Rhizobiales and Rhodospirillales was both shown to be significantly

greater in carp fed with the 0.1% MacroGard® diet in comparison to carp fed with the 0% MacroGard® diet (p=0.016 and 0.032 respectively).

Within the order Rhizobiales, 5 families were detected but only 2 of these were found to be present in all fish. The relative abundance of the family Bradyrhizobiaceae was not influenced by diet, however there was a significant difference when comparing over time with a higher percentage relative abundance found 15 days post injection in comparison to 8 days post injection (p=0.006). 3 genera within this family were detected with only 1 genus being present in all 20 fish. As with Bradyrhizobiaceae, there was significantly more *Bradyrhizobium* present in samples taken on day 15 than on day 8 (p=0.005). The relative abundance of this genus was not significantly affected by diet. The other family present in all 20 fish within the order of Rhizobiales was Phyllobacteriaceae. Unlike Bradyrhizobiaceae, the percentage relative abundance was not affected by time but by diet (p=0.016). 3 different OTUs were detected at the genus level and all 3 showed a statistically significantly higher percentage relative expression in 0.1% MacroGard® in comparison to those fed with the 0% MacroGard® diet (*Mesorhizobium* p=0.0.23, *Phyllobacterium* p=0.015 and an unknown Phyllobacteriaceae p=0.018).

Within the class of Gammaproteobacteria, 18 OTUs were identified. This revealed 7 orders of which 5 were present in all carp analysed and 4 of these showed statistically significant differences. 1 genus belonging to the order Aeromonadales was identified and this was present in significantly lower amounts in carp fed with the 0.1% MacroGard® diet in comparison to those on the 0% MacroGard® diet (p=0.002). Within the order Enterobacteriales, only 1 family was identified (Enterobacteriaceae) in which 5 genera were found. Overall, there was a significantly

larger proportion of Enterobacteriales 15 days post injection in comparison to 8 days post injection (p=0.019) with no effect of diet. There was a significant difference in the percentage relative abundance of Pseudomonadales (2 families) with a p value of 0.029 for effect of feed and a value of 0.031 for the effect of an interaction between time and feed. Whilst there was no difference in the mean percentage relative abundance between feeds at day 15, there was significantly more in 0.1% MacroGard® fed carp 8 days post injection. Within the order of Pseudomonadales, the family Moraxellaceae showed the same pattern with a significant effect of feed (p=0.025) and an interaction between time and feed (p=0.029). Out of 3 genera within the Moraxellaceae family, only *Enhydrobacter* was present in all fish analysed. As with its order and family, the same pattern of percentage relative abundance was seen (feed p=0.026, interaction p=0.033). Finally, the order of Vibrionales, composing of 3 families, was significantly lower (p=0.003) in carp fed with the 0.1% MacroGard® diet in comparison to those fed with the 0% MacroGard® diet. Within this order, 3 families were identified, each containing 1 genus. The family Vibrionaceae and an unidentified Vibrionales were present in all fish analysed. The percentage relative abundance of both Vibrionaceae and the unknown Vibrionales was significantly lower (p=0.002 and p=0.025 respectively) in carp fed with the 0.1% MacroGard® diet in comparison to those on the 0% MacroGard® diet.

Table 6.3: (part A) Bacterial species diversity within the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=20). Carp were fed either a 0% or 0.1% MacroGard® diet and, after 3 weeks of feeding, received an intraperitoneal injection of MacroGard®. Carp were sampled 8 and 15 days post injection (n=5) and gut samples analysed using High Throughput Sequencing. OTUs were analysed using the Greengenes database (DeSantis, Hugenholtz *et al.* 2006) and taxonomic identification was made to the genus level based upon 97% sequence similarity and a 0.8 confidence threshold. Percentage abundance of each OTU relative to the total number of OTUs per fish was calculated at the levels of class, order, family and genus. Statistical analysis was performed when a level was present in all 20 carp. Data was tested for normality and homoscedasticity using the Anderson-Darling and Levene's test respectively. Where data was not normally distributed, a BOX-COX transformation was performed (values of λ are given) and statistical differences analysed using a 2-way ANOVA with post hoc Tukeys. In instances were transformed data was still not normally distributed, data was analysed using the Scheirer-Ray-Hare test. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.

Bacteria			BOX-COX (λ)	Statistical test	R^2	Significant differences			
Taxonomic level	**Name**	**Sublevels**				**Time**	**Feed**	**Interaction**	**Difference**
Class	Actinobacteria	2 orders	-0.09	2-way ANOVA	4.00%				
Order	Actinomycetales	8 families	-0.09	2-way ANOVA	4.10%				
Family	Microbacteriaceae	3 genera	0.11	2-way ANOVA	1.93%				
Class	Thermoleophilia	1 genus	0.05	2-way ANOVA	40.56%		p=0.032		↑ 0.1%
Class	Flavobacteriia	1 order	0.35	2-way ANOVA	12.69%				
Family	Flavobacteriaceae	1 genus	0.25	2-way ANOVA	20.69%				
Family	Weeksellaceae	2 genera		2-way ANOVA	31.84%				
Genus	*Chryseobacterium*			2-way ANOVA	33.32%	p=0.049			↑ day 15
Class	Bacilli	4 orders	-0.27	2-way ANOVA	25.60%				
Order	Lactobacillales	7 families	-0.29	2-way ANOVA	35.04%				

Table 6.3: (part B) Bacterial species diversity within the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=20). Carp were fed either a 0% or 0.1% MacroGard® diet and, after 3 weeks of feeding, received an intraperitoneal injection of MacroGard®. Carp were sampled 8 and 15 days post injection (n=5) and gut samples analysed using High Throughput Sequencing. OTUs were analysed using the Greengenes database (DeSantis, Hugenholtz *et al.* 2006) and taxonomic identification was made to the genus level based upon 97% sequence similarity and a 0.8 confidence threshold. Percentage abundance of each OTU relative to the total number of OTUs per fish was calculated at the levels of class, order, family and genus. Statistical analysis was performed when a level was present in all 20 carp. Data was tested for normality and homoscedasticity using the Anderson-Darling and Levene's test respectively. Where data was not normally distributed, a BOX-COX transformation was performed (values of λ are given) and statistical differences analysed using a 2-way ANOVA with *post hoc* Tukeys. In instances were transformed data was still not normally distributed, data was analysed using the Scheirer-Ray-Hare test. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.

Bacteria			BOX-COX (λ)	Statistical test	R^2	Significant differences			
Taxonomic level	**Name**	**Sublevels**				**Time**	**Feed**	**Interaction**	**Difference**
Family	Lactobacillaceae	3 genera	-0.25	2-way ANOVA	21.77%				
Genus	*Lactobacillus*		-0.25	2-way ANOVA	23.34%				
Family	Leuconostocaceae	4 genera	-0.18	2-way ANOVA	27.34%				
Genus	*Leuconostoc*			2-way ANOVA	24.16%				
Genus	*Weissella*		-0.09	2-way ANOVA	26.58%				
Family	Streptococcaceae	3 genera	-0.25	2-way ANOVA	29.17%				
Genus	*Streptococcus*			2-way ANOVA	28.18%				
Class	Clostridia	1 order	-0.11	2-way ANOVA	29.76%				
Order	Clostridiales	8 families	-0.11	2-way ANOVA	17.52%				
Family	Clostridiaceae	2 genera		2-way ANOVA	22.79%				

Table 6.3: (part C) Bacterial species diversity within the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=20). Carp were fed either a 0% or 0.1% MacroGard® diet and, after 3 weeks of feeding, received an intraperitoneal injection of MacroGard®. Carp were sampled 8 and 15 days post injection (n=5) and gut samples analysed using High Throughput Sequencing. OTUs were analysed using the Greengenes database (DeSantis, Hugenholtz *et al.* 2006) and taxonomic identification was made to the genus level based upon 97% sequence similarity and a 0.8 confidence threshold. Percentage abundance of each OTU relative to the total number of OTUs per fish was calculated at the levels of class, order, family and genus. Statistical analysis was performed when a level was present in all 20 carp. Data was tested for normality and homoscedasticity using the Anderson-Darling and Levene's test respectively. Where data was not normally distributed, a BOX-COX transformation was performed (values of λ are given) and statistical differences analysed using a 2-way ANOVA with *post hoc* Tukeys. In instances were transformed data was still not normally distributed, data was analysed using the Scheirer-Ray-Hare test. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.

Bacteria			BOX-COX (λ)	Statistical test	R^2	Significant differences			
Taxonomic level	**Name**	**Sublevels**				**Time**	**Feed**	**Interaction**	**Difference**
Family	Fusobacteriaceae	3 genera		2-way ANOVA	29.76%		p=0.030		↓ 0.1%
Genus	*Cetobacterium*			2-way ANOVA	29.52%		p=0.023		↓ 0.1%
Class	Alphaproteobacteria	6 orders		2-way ANOVA	33.89%		p=0.016		↑ 0.1%
Order	Caulobacterales	1 family 4 genera		2-way ANOVA	20.18%				
Order	Rhizobiales	5 families		2-way ANOVA	33.98%		p=0.016		↑ 0.1%
Family	Bradyrhizobiaceae	3 genera		2-way ANOVA	38.19%	p=0.006			↑ day 15
Genus	*Bradyrhizobium*			2-way ANOVA	40.18%	p=0.005			↑ day 15
Family	Phyllobacteriaceae	3 genera		2-way ANOVA	34.38%		p=0.016		↑ 0.1%
Genus	*Mesorhizobium*			2-way ANOVA	28.43%		p=0.023		↑ 0.1%
Genus	*Phyllobacterium*			2-way ANOVA	34.41%		p=0.015		↑ 0.1%

Table 6.3: (part D) Bacterial species diversity within the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=20). Carp were fed either a 0% or 0.1% MacroGard® diet and, after 3 weeks of feeding, received an intraperitoneal injection of MacroGard®. Carp were sampled 8 and 15 days post injection (n=5) and gut samples analysed using High Throughput Sequencing. OTUs were analysed using the Greengenes database (DeSantis, Hugenholtz *et al.* 2006) and taxonomic identification was made to the genus level based upon 97% sequence similarity and a 0.8 confidence threshold. Percentage abundance of each OTU relative to the total number of OTUs per fish was calculated at the levels of class, order, family and genus. Statistical analysis was performed when a level was present in all 20 carp. Data was tested for normality and homoscedasticity using the Anderson-Darling and Levene's test respectively. Where data was not normally distributed, a BOX-COX transformation was performed (values of λ are given) and statistical differences analysed using a 2-way ANOVA with *post hoc* Tukeys. In instances were transformed data was still not normally distributed, data was analysed using the Scheirer-Ray-Hare test. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.

Bacteria			BOX-COX (λ)	Statistical test	R^2	Significant differences			
Taxonomic level	**Name**	**Sublevels**				**Time**	**Feed**	**Interaction**	**Difference**
Genus	Unknown Phyllobacteriaceae			2-way ANOVA	34.10%		p=0.018		↑ 0.1%
Order	Rhodospirillales	1 family		2-way ANOVA	27.32%		p=0.032		↑ 0.1%
Order	Sphingomonadales	1 family 4 genera	0.06	2-way ANOVA	19.25%				
Class	Betaproteobacteria	2 orders		Scheirer-Ray-Hare	2.68%				
Order	Burkholderiales	5 families		Scheirer-Ray-Hare	2.68%				
Family	Oxalobacteraceae	2 genera	-0.09	2-way ANOVA	12.81%				
Genus	*Polynucleobacter*		-0.11	2-way ANOVA	8.01%				
Class	Gammaproteobacteria	7 orders	-0.31	2-way ANOVA	39.41%		p=0.009		↓ 0.1%
Order	Aeromonadales	1 genus		2-way ANOVA	48.84%		p=0.002		↓ 0.1%
Order	Enterobacteriales	1 family 5 genera		2-way ANOVA	33.82%	p=0.019			↑ day 15

Table 6.3: (part E) Bacterial species diversity within the gut of common carp (*C. carpio*) within a combined feeding and injection trial (n=20). Carp were fed either a 0% or 0.1% MacroGard® diet and, after 3 weeks of feeding, received an intraperitoneal injection of MacroGard®. Carp were sampled 8 and 15 days post injection (n=5) and gut samples analysed using High Throughput Sequencing. OTUs were analysed using the Greengenes database (DeSantis, Hugenholtz *et al.* 2006) and taxonomic identification was made to the genus level based upon 97% sequence similarity and a 0.8 confidence threshold. Percentage abundance of each OTU relative to the total number of OTUs per fish was calculated at the levels of class, order, family and genus. Statistical analysis was performed when a level was present in all 20 carp. Data was tested for normality and homoscedasticity using the Anderson-Darling and Levene's test respectively. Where data was not normally distributed, a BOX-COX transformation was performed (values of λ are given) and statistical differences analysed using a 2-way ANOVA with *post hoc* Tukeys. In instances were transformed data was still not normally distributed, data was analysed using the Scheirer-Ray-Hare test. Both in the graph and table, * signifies $p<0.05$, ** signifies $p<0.01$, and *** signifies $p<0.001$.

Bacteria			BOX-COX (λ)	Statistical test	R^2	Significant differences			
Taxonomic level	Name	Sublevels				Time	Feed	Interaction	Difference
Order	Pseudomonadales	1 genus	0.11	2-way ANOVA	45.96%		p=0.029	p=0.031	↑ 0.1% day 8
Family	Moraxellaceae	3 genera	0.07	2-way ANOVA	46.38%		p=0.025	p=0.029	↑ 0.1% day 8
Genus	Enhydrobacter		0.07	2-way ANOVA	45.61%		p=0.026	p=0.033	↑ 0.1% day 8
Order	Vibrionales	3 families	-0.16	2-way ANOVA	43.52%		p=0.003		↓ 0.1%
Family	Vibrionaceae	1 genus	-0.17	2-way ANOVA	45.01%		p=0.002		↓ 0.1%
Family	Unknown Vibrionales	1 genus	-0.02	2-way ANOVA	40.15%		P=0.025		↓ 0.1%
Order	Xanthomonadales	2 families		2-way ANOVA	22.50%				
Family	Xanthomonadaceae	1 genus		2-way ANOVA	20.49%				

Correlation between innate immune gene expression and size of the gut microbiota population.

As with the previous trials, expression of carp innate immune genes was compared to expression of the bacterial 16S rDNA gene, i.e. the total size of the bacteria population within the gut. Spearman's rank test (Table 6.4) showed a significant but weak positive correlation between the 16S rDNA gene and both iNOS (ρ=0.252, p=0.003) and IL-1β gene expression (ρ=0.284, p=0.001). There was no correlation between 16S gene expression and C3 gene expression.

Table 6.4: The probability of a linear relationship between each of the immune genes analysed in the gut of common carp during a combination feeding and injection trial and the size of the bacterial population within the gut as measured by total 16s rDNA expression (n=120). Carp were fed either a 0% or 0.1% MacroGard® diet and were further divided into one of 4 treatment groups: no injection, PBS injection, MacroGard® injection, inactivated A. salmonicida injection. Carp were sampled 1, 8 and 15 days post injection (n=5). As data was not normally distributed, Spearman's test was used to calculate the correlation coefficient, i.e. how linear a correlation is, and the statistical significance. A rating as to how strong the correlation is (as described by Fowler *et al.* 1998) is also given with a coefficient of 0.00-0.19 being a very weak correlation, 0.20-0.39 being a weak correlation, 0.40-0.69 being a modest correlation, 0.70-0.89 being a strong correlation and 0.90-1.00 being a very strong correlation.

Gene	Correlation coefficient	P value	Correlation
iNOS	0.252	0.003	Weak (positive)
C3	-0.036	0.346	Very weak
IL-1β	0.284	0.001	Weak (positive)

6.3 – Discussion.

The aim of this chapter was to ascertain if a combination of oral application and injection of MacroGard® would affect bacterial species diversity within the gut of carp. As shown in Chapter 4, the inclusion of MacroGard® into the diet at 0.1% w/w did not have any influence on bacterial species richness, however Chapter 5 showed injection of MacroGard® was capable of reducing the overall gut bacteria population size by more than 95% relative to non-injected controls. Kuhlwein *et al.* (2013) and Jung-Schroers *et al.* (2015) both showed that influencing the bacterial species richness within the gut is possible at higher concentrations of MacroGard®. It was hypothesised by the author that by reducing the overall bacteria population size, the lower concentration of orally applied MacroGard® may be great enough to influence species diversity, i.e. a reduction in population size should have a similar impact upon bacterial species diversity as an increase in MacroGard® concentration within the food. In addition to analysing bacteria population size and species diversity within the gut, iNOS, IL-1β and C3 gene expression were analysed as markers of an immune response within the gut. iNOS gene expression was shown in chapter 5 to be increased after injection with MacroGard®, and IL-1β and C3 gene expression have both been shown to be significantly influenced by MacroGard® feeding during bacterial infection conditions (Falco *et al.* 2012b, Pionnier *et al.* 2013).

iNOS gene expression in the gut did not appear to be influenced either by diet or by injection which supports the observations of (Miest *et al.* 2012) who noted a similar response in the gut of carp injected with live *A. salmonicida* 1 and 3 days post injection. However, they did show significant differences in the iNOS gene expression in the liver and spleen, which may indicate differential organ response, but also

highlighting a systemic response was present. Injection of zebrafish with a pathogenic strain of *A. hydrophila* did not induce an increase in iNOS gene expression in the gut in the first 6 hours post injection irrespective of whether the bacteria was alive or dead (Rodriguez *et al.* 2008). In a review by Wiegertjes *et al.* (2016) iNOS gene expression was proposed as a marker of the presence of M1 type macrophages which directly produce Reactive Oxygen Species (ROS), Nitric Oxide (NO) and Interferon (IFN)-γ, and activated the production of TH1 cells, all of which are involved in defence against bacterial infection. It is feasible, therefore, that the subsequent immune response caused by an increase in M1 cells, as indicated by iNOS gene expression, may have an impact upon the gut microbiota population. Whilst the lack of iNOS expression seen within the trial presented here does not exclude ROS, IFN-γ and T_H1 cell production from having occurred and having been involved in the reduction of the number of bacteria within the gut, it does indicate M1 cells may not have been involved. Analysis such as fluorescence-activated cell sorting (FACS) should be performed in order to determine if M1 cell presence alters within the gut after injection with MacroGard®, and to ascertain there is a correlation between a specific immune cell type and size of the bacterial population within the gut.

Feed, treatment or time did not appear to have an effect on C3 gene expression. Whilst Gomez *et al.* (2013) noted that there is a distinct lack of publications relating to C3 gene expression at mucosal sites, previous studies at Keele University have shown the complement system may be affected by β-glucan feeding (Pionnier *et al.* 2013, Pionnier *et al.* 2014). In contrast to the work presented here, Pionnier *et al.* (2013) showed a significant increase in C3 expression 3 and 5 days post injection with A. salmonicida and a difference in expression between the PBS

injected controls fed with both a 0% and 0.1% MacroGard® diet. Their 2014 paper also revealed a significant increase in C3 gene expression after LPS injection in non MacroGard® fed carp and a significant decrease in expression of this gene in fish fed with MacroGard®. Both these studies contrast with the data presented in this chapter and as discussed in Chapter 4, may result from different strains of carp (Shapira *et al.* 2005, Piackova *et al.* 2013, Adamek *et al.* 2014, Nath *et al.* 2014). Analysis of the effect of different potential probiotics in gnotobiotic cod larvae also revealed that C3 gene expression is not only influenced by the different species of probiotic, but also whether the bacteria are alive or dead (Forberg *et al.* 2012). Changes have been seen in both serum complement levels and the autochthonous gut microbiota population in hybrid tilapia after feeding with DVAQUA® although it is not known if they were correlated (He *et al.* 2009).

The higher levels of IL-1β gene expression seen in all 0.1% MacroGard® fed carp at 1 and 8 days post injection suggest that MacroGard® feeding influences expression during a systemic immune response irrespective of injection treatment. In contrast, (Falco *et al.* 2012b) did not observe an effect of feeding MacroGard® supplemented diet to carp injected with *A. salmonicida* however, there was a significant increase in IL-1β gene expression in 0% MacroGard® fed carp that were infected 6 hours post injection. In contrast to the data presented here, Lee *et al.* (2014) observed a decrease in IL-1β expression in mice after oral application in Irritable Bowel Disease (IBD) models. Lee *et al.* (2014) attribute the recovery of the gut after artificial induction of IBD to the upregulation of anti-inflammatory genes such as IL-10 after β-glucan feeding. This could indicate the involvement of an inflammatory response in the reduction of the gut bacteria population size however,

the increase of IL-1β expression seen in MacroGard® fed fish did not correlate with a smaller number of bacteria. Further studies should be performed analysing a wider range of pro-inflammatory and also anti-inflammatory markers to determine if it is indeed an inflammation response that is having an impact upon the gut bacteria population size.

Next generation sequencing analysis revealed an increase in Alphaproteobacteria which coincided with a decrease in Gammaproteobacteria in 0.1% MacroGard® fed fish. The presence of Alphaproteobacteria is interesting as they are typically associated with plant roots and soil rather than with gut environments. Three genera of the Alphaproteobacteria, *Bradyrhizobium*, *Phyllobacterium* and *Mesorhizobium*, are examples of nitrogen fixing bacteria which do not have any obvious biological role within the gut of fish. Wu *et al.* (2012) have identified a single *Phyllobacterium* sequence within a clone library of the gut of yellow catfish (*Pelteobagrus fulvidraco*), and *Bradyrhizobium* was identified through pyrosequencing in the gut of European sea bass (*Dicentrarchus labrax*), their function in the gut was however not ascertained (Carda-Dieguez *et al.* 2014). Alphaproteobacteria have also been found in the gut of a limited number of invertebrates such as earthworms, queen European honey bees (*Apis mellifera*) and land snails (*Helix pomatia*) (Depkat-Jakob *et al.* 2010, Nicolai *et al.* 2015, Tarpy *et al.* 2015). If analysis of mixed bacteria populations was still solely reliant upon culture based methodologies, a lack of identification of Alphaproteobacteria within the gut of fish species could be explained by the logic that media selected for the isolation of cultures would favour expected bacteria genera such as *Pseudomonas*, *Vibrio* or *Aeromonas*, and if it was not expected to find Alphaproteobacteria, selection media

specifically for these bacteria would not be used. Advances in molecular biology, however, eliminate the need for specific growth conditions and that identification of Alphaproteobacteria is still limited to 2 publications (Wu *et al.* 2012, Carda-Dieguez *et al.* 2014) which indicates their presence within the intestinal system of ichthyo-models is not common. It is therefore likely that the abundance with which they are present within the gut of these carp is opportunistic rather than functional.

Further research into Alphaproteobacteria within the ENA database revealed over 250 sequences encoding for β-glucanases in genera such as *Phyllobacterium*, *Bradyrhizobium* and *Mesorhizobium*. This could account for the significantly higher predominance of *Phyllobacterium* and *Mesorhizobium* OTUs in 0.1% MacroGard® fed carp as the immunomodulant, being a carbohydrate (β-glucan), could be an additional food source.

The decrease in Gammaproteobacteria noted may result of being outcompeted for space (Moons *et al.* 2009) by the Alphaproteobacteria which represent the largest proportion of the bacteria identified within the gut samples. The Gammaproteobacteria comprise several pathogenic bacteria such as *A. salmonicida*, *Pseudomonas anguilliseptica* and *Vibrio anguillarium*, therefore a decrease in the overall presence of Gammaproteobacteria may be beneficial irrespective of whether this is a direct or indirect effect of MacroGard® itself.

As previously stated, the genera of *Phyllobacterium* and *Bradyrhizobium* have only been reported in the gut of fish by Wu *et al.* (2012) and Carda-Dieguez *et al.* (2014) and there are no published examples of *Mesorhizobium* sp. in association with a fish species. This therefore leads to an interesting speculation on the source of these

bacteria. The carp used within this investigation were originally reared in outdoor ponds before being transferred into indoor aquaria. Carp are a naturally omnivorous species and eat plant debris within their environment (Barrington 1957), and thus *Phyllobacterium* sp. and *Mesorhizobium* sp. may enter the gut as part of the normal feeding process. The original body of water hosting the carp therefore needs to be analysed for the presence of these bacterial genera, although it should be noted that carp were maintained in indoor aquaria for 3 months before the start of the trial.

To conclude, the aim of this chapter was to ascertain if it was possible to influence the gut microbiota population within the gut of carp with a commercially viable concentration of MacroGard® in the feed, by combining oral application with injection of MacroGard®. The data presented here showed the bacterial species diversity within the gut of carp that received the 0.1% MacroGard® was indeed different to that found in 0% MacroGard® fed carp. An increase in the relative abundance of Alphaproteobacteria was seen in 0.1% MacroGard® fed carp which corresponded with a decrease in Gammaproteobacteria. Examples of Gammaproteobacteria include the fish pathogens *A. salmonicida*, *P. anguilliseptica* and *V. anguillarium*, therefore it was considered beneficial to have an overall smaller proportion of Gammaproteobacteria within the gut. This research highlights the potential benefits of combining injection and oral application of MacroGard® and future work should include ascertaining if it is possible to preferentially promote the growth of, for example, probiotic strains of bacteria such as *Lactobacillus* and *Bacillus* sp..

Chapter 7 – General discussion.

The aim of the study presented in this thesis was to determine if there was an influence of β-glucans on the relationship between the gut immune response of the common carp and its intestinal microbiota population. To this end, the results presented in chapters 4, 5 and 6 show that modulation of the intestinal microbiota population by MacroGard®, a commercially available β-1/3,1/6-glucan, varies based upon method of application. Chapter 4 reveals that the oral application of MacroGard® at a concentration of 0.1% w/w within the feed does not alter the overall bacteria population size within the gut, nor does it have an effect upon bacterial species richness. Injection of MacroGard®, however, has the ability to drastically reduce the bacterial population size within the gut 24 hours post injection, yet when carp are additionally maintained on a 0% MacroGard® diet, there is no effect seen on bacterial species richness (Chapter 5). Interestingly, bacterial species diversity within the gut is influenced when MacroGard® is applied in a combination of both oral application and intraperitoneal injection (Chapter 6). There is, however, only a limited amount of correlation seen between the expression of some of the immune genes analysed, i.e. the IL-1β receptor, IL-1β, bf/C2, ApoA1, HAMP1 and iNOS and the overall gut bacteria population size. It was essential prior to undertaking the experimentation to meet the whole thesis aim that optimisation of different methods of analysing bacteria, both *in vivo* and *in vitro* was carried out.

One of the major challenges faced when designing genus specific qPCR assays as a means of quantifying individual bacteria genera within a mixed population, such as those found within the gut (Jung-Schroers *et al.* 2015), is the amplification of non-

target DNA sequences. The specificity of genus specific qPCR primers that had been previously published (Thompson *et al.* 2004, Martinez-Puig *et al.* 2007, Adamek *et al.* 2013) was questioned when analysis of *Aeromonas*, *Vibrio* and *Lactobacillus* within a mixed population resulted in more than 100% of the total 16S expression as measured by "universal" primers, i.e. those designed to amplify all bacterial 16S sequences. Prior to initial use, all primer pairs were subject to analysis to determine specificity, which showed primer pairs would only amplify target sequences. This indicated the use of the primer BLAST search was not necessarily appropriate for the bacterial 16S rDNA gene.

The advantage of considering each nucleotide individually within a primer is that the effect of distance from the 3' end of the sequence can be taken into consideration. However, translating this into a numerical output and factoring in the different types of errors, i.e. mismatches or additions/deletions, in order to compare between different numbers of errors, types of errors and location of errors highlighted challenges with assigning values. The use of a score, such as ρ as calculated using Spearman's rank order correlation, would allow for further analysis to determine if the similarity of non-target sequences to PCR primers was statistically different from that between target sequences and primers. However, in addition to determining the most correct means of calculating ρ, i.e. accurate emphasis on mismatches and additions/deletions based upon *ex silico* data, any analysis must also factor in that both primers within a primer pair must successfully anneal to allow amplification. This is something that the initial models considered in Chapter 2 do not do, i.e. they consider each primer separately rather than as a pair. Desai *et al.* (2009) presented genus specific primers for 4 different bacterial genera (*Alcaligenes,*

Bacillus, Stentrophomonas and *Enterococcus*) where, whilst having 4 different forward primers, each assay uses the same "universal" reverse primer. It is especially important to ensure the forward primers only amplify target DNA sequence as the reverse primer will not distinguish between different bacterial genera. It highlights that primers must be considered as a pair rather than individually, as whilst one primer may anneal to many non-target sequences, the other primer within the pair must also anneal to the same sequence to result in amplification.

The final model presented in Chapter 2 is less sophisticated than the previous models in that it does not distinguish between types of error and the position of an error is based upon sections of the primer rather than an individual position. However comparison with *ex silico* data showed 21/24 predictions as to annealing to be accurate. Not all scores within the model were given a definitive classification of successful/unsuccessful annealing, i.e. a score of C, E, H or K was classified as unknown if successful annealing will occur. In order to increase the accuracy of the model, *ex silico* analysis should be performed using template DNA with scores of either C, E, H or K therefore allowing determination of whether these scores result in successful annealing. This will further improve the accuracy of the model before future use. As only one set of PCR conditions were utilised within this research, the effectiveness of the model, i.e. ability to accurately predict successful amplification of a DNA sequence, was only compared against this one set of conditions. Successful amplification, however, is not solely dependent upon the similarity of the primer to the template DNA. Temperature, concentration of template, $MgCl_2$, primer, and type of polymerase can all influence successful annealing (Harris 2013). Future work to improve this model should include testing accuracy under different PCR conditions. It

may be prudent to develop a second layer to the model, i.e. where the scoring of a primer based upon its similarity to a DNA sequence is fixed (number of errors in a specific location), whether or not the likelihood of a score resulting in successful amplification can be varied based upon the PCR conditions being used.

There are both advantages and disadvantages to the use of probability as a means of predicting the successful amplification of a DNA sequence that considers both primers within a pair. The probability of two separate events occurring simultaneously can be calculated which allows for the likelihood of both primers within a primer pair annealing to a DNA sequence being more easily determined. In comparison, the use of Spearman's ρ, as performed within this thesis, considers primers individually and did not take into consideration the fact that both primers must anneal in order for successful amplification to occur. The main disadvantage to probability, however, is that only a small proportion of the sequence data available through databases, such as the ENA database, were analysed. Where statistical analysis gives a more fluid result, i.e. at a confidence level of 0.05, if you were to repeat a trial 20 times with 20 independent data sets, you would expect the same outcome in 19 trials (95%). Probability, however, considers data more rigidly and any additional data analysed would inevitably alter the probability value obtained. Whilst this may not appear to strongly impact the outcome when determining the probability of an individual primer successfully annealing, this could have a larger effect upon the overall probability of annealing when both primers are considered together. Further refinement of the model should take both approaches, i.e. based upon probability and statistical analysis, into consideration.

The overall conclusion from Chapter 2, however, is that the model worked as a

good predictor as to primer specificity, i.e. annealing only to target DNA sequences. Total 16S and genus specific 16S PCR primers used throughout Chapters 4, 5 and 6 were analysed using this model and where initial trials showed the relative proportion of only 3 bacterial genera to be greater than 100%, the summation of the individual genera analysed in Chapter 4 did not exceed 50% of the total 16S expression.

The aim of Chapter 3 was to explore different methods of analysing bacterial growth using *in vitro* models with all methods analysed being subsequently utilised in Chapter 4. Whilst equipment is available to measure bacteria colony sizes, the prohibitive costs of these lead to the development of the image analysis program PENGUIN. This proved to be a cheap, accurate method of measuring bacteria colony sizes.

Whilst PENGUIN was shown to be highly accurate and reliable, the main challenge faced with this method of analysis was ensuring the consistency of the conditions used for analysis. Lighting proved the most difficult factor to control with even different models of the same light bulb resulted in different values of power, i.e. different numbers of comparisons between “Before” and “After” images. Further work in developing this method of analysis should take into consideration variations in lighting thereby allowing its use in a broader range of conditions.

Chapter 4 is the first of three experimental chapters and discussed the impact of direct exposure of the gut microbiota population to MacroGard® through *in vitro* studies and through an oral application *in vivo* trial. Although some bacteria possess β-glucanases and are capable of utilising β-glucans as a substrate (Planas 2000),

MacroGard® showed only a slight impact upon bacterial growth *in vitro*. The number of colonies of *B. subtilis* produced after incubation within a nutrient broth containing 0.01% MacroGard® and the size of ISO 20 colonies after incubation on MacroGard® embedded nutrient agar plates were both reduced indicating a reduction in bacterial survival. Feeding with MacroGard® had no effect upon bacterial species richness or overall bacteria population size within the gut of carp. This can be considered as a positive as this means there was no increase in different potential pathogens, i.e. feeding MacroGard® is unlikely influence the gut microbiota in favour of bacterial disease conditions.

Similarly, whilst injection resulted in changes in the overall bacterial population size, there were no differences in bacterial species richness between the different injection treatments, although a cause of the reduction of the gut microbial population was not apparent. Where the initial trial showed injection with LPS and MacroGard® drastically reduced the bacterial population size, carp injected with PBS did not show this same reduction when compared to non-injected controls. Similarly, the large injection trial revealed the same reduction in gut bacteria population size in MacroGard® injected carp in comparison to non-injected controls. However in contrast, within the combination feeding and injection trial, there was no difference in gut bacteria population size when comparing MacroGard® and PBS injected carp. This is the first known report of the effect of injection of any compound upon bacterial population size within the gut of a fish with previous studies only comparing bacterial species richness (Liu *et al.* 2008, Liu *et al.* 2015). Additionally, no reports of comparable studies could be found in mammalian models therefore more research must be performed to fully elucidate the cause of the reduction in bacterial numbers.

The results of the combination feeding and injection trial initially indicated that handling may have had an effect upon the gut bacterial population, i.e. non handled controls showed the same smaller overall population size on day 1 in comparison to 8 and 15 days post injection as occurred in all injected treatment groups. Stress caused by handling is known to affect immune responses in fish including inhibition of pro-inflammatory cytokines and phagocytosis, lymphocyte proliferation and the production of anti-inflammatory cytokines caused by increase in corticosteroids including cortisol (Tort 2011). Indeed, feeding fish with immunomodulants during periods of stress, i.e. grading, movement between fresh and saltwater, and vaccination, has been shown to be effective in reducing outbreaks of disease (Bricknell and Dalmo 2005). From this, it was considered that handling stress may be involved in causing the much smaller gut bacteria population sizes seen in carp that were injected in comparison to non-injected fish. However, a direct comparison of the two methods used, i.e. instantly sampling a fish versus handling, returning to the tank overnight and sampling after 24 hours, showed no statistical difference in gut bacteria population size. This lead to the conclusion that the reduction in gut bacteria population size may not be as a result of handling stress.

A much more comprehensive method of analysis of the gut microbiota population was employed in Chapter 6 (Next Generation Sequencing) to study the bacteria within the gut of carp than used in Chapter 5 (PCR-Denaturing Gradient Gel Electrophoresis). Interestingly, even though the sensitivity of NGS is much greater than that of PCR-DGGE, a similar number of OTUs were identified as representing >0.1% of the percentage relative abundance with this method as were detected by PCR-DGGE which has a higher detection limit of 1% percentage relative abundance

(Harris 2013). The obvious advantages NGS has over PCR-DGGE, however, are that identification of the OTUs was performed whereas only identification of different OTUs was performed with the method of PCR-DGGE, and quantitative analysis was performed where only qualitative analysis was possible when using PCR-DGGE. This allowed for the identification of both potential pathogens and their proportion within the gut bacterial population, and revealed the presence of Alphaproteobacteria in proportions that have never before been reported in fish. At this point, it is only possible to speculate as to the presence of *Phyllobacterium*, *Mesorhizobium* and other Alphaproteobacteria within the gut of carp injected with MacroGard® in the combination feeding and injection trial. To date, there are only two examples of Alphaproteobacteria being found in the gut of fish. Wu *et al.* (2012) found Bradyrhizobiaceae and Phyllobacteriaceae in the gut of yellow catfish from Niushan Lake in Central China, and Carda-Dieguez *et al.* (2014) observed Bradyrhizobiaceae in the gut of sea bass fed diets containing β-glucans. Given Alphaproteobacteria such as *Phyllobacterium*, *Mesorhizobium* and *Bradyrhizobium* are typically associated with plant systems and soil (Vacheron *et al.* 2013, Laranjo *et al.* 2014), it would make sense that if they were a common component of the gut microbiota of fish, they would not have historically been identified using culture based methods of analysis which will favour the bacterial species expected to be found (Mackie and McCartney, 1956). Recent advances in technology, however, eliminate this need for favouritism (Nayak 2010) and, as such, if Alphaproteobacteria were a common feature in intestinal gut microbiota, logically more examples of their presence would be documented. As this is not the case, this indicates the presence of Alphaproteobacteria seen within the combination feeding and injection trial is likely to be opportunistic rather than

typical. As discussed in Chapter 6, Alphaproteobacteria are able to utilise β-glucans as a potential food source, i.e. they have the sequences for β-glucanases within their genome (ENA database). The significantly higher proportion of Alphaproteobacteria found in carp fed with the 0.1% MacroGard® diet is likely due to a higher availability of food that only bacteria with β-glucanases can utilise in comparison to carp fed with the 0% MacroGard® diet, i.e. a food source that is unique to these bacteria. Similarly, Carda-Dieguez *et al.* (2014) observed a higher proportion of Bradyrhizobiaceae in diets containing β-glucan in comparison to the diet that had no β-glucan. Due to the limited number of examples of the presence of Alphaproteobacteria within the gut of fish, there is no data available indicating if there is a direct contribution towards fish health. In fish, probiotic species such as *Lactobacillus* sp. and *Bacillus* sp. are associated with good gut health and improved disease resistance (Merrifield *et al.* 2010, Romero *et al.* 2014) however in human models, *Bifidobacterium* sp. are considered to be the promotors of gut health (Nayak 2010a). Further analysis will be required to determine if there are health benefits to having Alphaproteobacteria within the fish gut microbiota population, i.e. are they capable of positively influencing the immune response, defending against invading pathogens and do they aid digestion? The reduction in potential pathogenic bacterial genera seen in conjunction with a higher presence of Alphaproteobacteria after feeding with MacroGard®, however, indicates they were able to outcompete other bacteria in a way that was potentially beneficial to the carp.

One of the aspects of this thesis was to study the relationship between the gut microbiota population and the immune response of the gut of common carp. The increased interest in the immune capability of the gut of fish (Rombout *et al.* 2011,

Gomez *et al.* 2013) has been driven by a need to find alternative means of improving fish health and resistance to disease to the liberal application of antibiotics. The gut microbiota is essential in the initial development of the host's immune response (Nayak 2010a) and several studies have shown that the immune status of the gut of carp can be affected by infection, e.g. *Aeromonas salmonicida* and CyHV-3 infections have both been shown to influence cytokine and iNOS expression (Falco *et al.* 2012b, Syakuri *et al.* 2013), and *Aeromonas salmoncida* additionally influenced the expression of c1rs, bf/C2, MASP2, i.e. markers of three of the complement pathways, and C3 (Pionnier *et al.* 2013).

It is however only recently that the involvement of the gut microbiome has been considered in conjunction with the immune status of the gut, and there are a limited number of studies of how the whole system i.e. microbiome and gut immune responses interact in either a positive or negative way. The effects of oral application of different dietary ingredients upon the immune response and the microbiota population have been investigated in turbot (β-glucan), red sea bream (*Lactobacillus rhamnosus* and *Lactobacillus lactis*), juvenile beluga sturgeon (*Enterococcus faecium* and FOS), rainbow trout (plant and marine based diets) and hybrid tilapia (soybean meal), however none of these compare immunity against the microbiota population (Ingerslev *et al.* 2014, Zhang *et al.* 2014, Akrami *et al.* 2015, Dawood *et al.* 2016a, Miest *et al.* 2016). Systemic immunity and the gut microbiota of both turbot (Miest *et al.* 2016) and red sea bream (Dawood *et al.* 2016a) were shown to be influenced by their respective diets, however it is not known if this is directly related. In contrast, whilst the number of LAB within the gut of beluga sturgeon did not change after feeding with *Enterococcus faecium* and FOS, the number of leucocytes and

haemoglobin concentration in the blood was increased indicating there is no relationship between LAB counts and these two immune parameters (Akrami *et al.* 2015). The studies in rainbow trout (Ingerslev *et al.* 2014) and hybrid tilapia (Zhang *et al.* 2014) both focused upon gut immunity and, similar to turbot and red sea bream, changes were seen in both the gut microbiota and the immune response yet, again, it cannot be said if these are directly related to each other.

In contrast, Tapia-Paniagua *et al.* (2015) performed a direct comparison of immune genes in the liver and the whole gut microbiota of Senegalese sole using Principle Component Analysis, and showed a negative correlation between the gut microbiota and expression of CASPASE-6 and NAPDH oxidase. To date, this is the only published example of a statistical comparison of an immune response and whole gut microbiota population in a fish model. The data presented in this thesis was, however, analysed considering each gene individually, similar to the studies presented by Gorgoglione *et al.* (2013, 2016) who compare specific pathogen load against immunity. Statistically, it was more appropriate to perform analysis on individual genes in this thesis due to the assumption of PCA as defined by Dytham (2003), i.e. "[data] is continuous and normally distributed", being violated and raw data required different transformations in order to achieve normality. Any conclusions drawn from PCA analysis would not be accurately substantiated.

Gorgoglione *et al.* (2013, 2016) showed that correlations occurred between specific pathogen load and immune gene expression in *Tetracapsuloides bryosalmonae* versus AMP and pro-inflammatory cytokine expression in rainbow trout, and viral haemorrhagic septicaemia virus or *Yersinia ruckeri* versus novel chemokine expression in brown trout respectively. These correlations were of a

greater magnitude than those observed in this thesis between total bacteria population size and the IL-1β receptor, IL-1β, iNOS, ApoA1, and HAMP2. All these studies, however, show varying levels of correlation of the different immune genes against the pathogenic/microbiota component. This highlights that whilst it may be prudent to consider the immune response as a whole, as done by Tapia-Paniagua *et al.* (2015) through their use of PCA, focusing on individual immune responses can be as important. The genes selected for study within this thesis were done so based upon their activity against specifically bacteria, therefore it was expected that a greater level of correlation between their expression and the gut microbiota population as a whole would be seen than actually occurred. Additionally, the lack of consistency in the correlations between the microbiota and individual immune genes when comparing the different trials indicates the interaction between the overall gut immune response and the microbiota is much more complex than linear relationships with individual genes. Further analysis in regards to studying the relationship within the holobiont, i.e. carp and its associated microbiota population, should consider a much larger range of bactericidal immune genes and statistical analysis should, if possible, be performed on both an individual gene and overall immune response level.

What Gorgoglione *et al.* (2013, 2016) and Tapia-Paniagua *et al.* (2015) show with their statistical analysis is the functional relationship between the immune response and the pathogenic/microbial counterpart in their studies. Tapia-Paniagua *et al.* (2015) describe lower expression of NADPH, which is involved in the production of ROS, within the liver of Senegalese sole treated with the antibiotic oxytetracycline (OTC) combined with the probiotic *Shewanella putrefaciens*, in

comparison to OTC alone. Their conclusion is that the probiotic "might exert a protective effect on the hepatocytes" as ROS are capable of destroying host cells in addition to invading pathogens (Dahlgren and Karlsson 1999). Inversely, Gorgoglione *et al.* (2013, 2016) show positive correlations between the pro-inflammatory immune response and the presence of pathogens. Within this thesis, overall gut bacteria population size most commonly negatively correlates with gene expression, i.e. with the IL-1β receptor, Muc2, and CRP2 in the feeding trial, and bf/C2, ApoA1 and HAMP2 within the large injection trial. This higher level of immune gene expression correlating with lower bacteria numbers within the gut does indicate these genes are important in determining the overall gut bacteria population size, however the lower values of ρ for all of these genes implies they may not be individually responsible, but likely work together. Similarly to Gorgoglione *et al.* (2013), who identified a positive correlation between the presence of Tetracapsuloides bryosalmonae and IL-1β expression in brown trout, a positive correlation between gut microbiota population size and IL-1β expression in both the feeding trial and the combination feeding and injection trial presented in this thesis. In all cases, however, correlation was relatively low.

The overall conclusion of this thesis is that MacroGard® is capable of influencing both the expression of innate immune genes within the gut of carp and also the gut microbiota population however the method of application is critical in this modulation. Oral application of MacroGard® at a concentration of 0.1% within the feed alone did not show an effect upon the gut microbiota or immune gene expression yet injection was capable of influencing both. Combining both of these methods of application was able to influence bacterial species diversity within the gut

where only injection of MacroGard® did not. It is not currently known if the significant increase in the presence of Alphaproteobacteria within the gut is directly beneficial to the health of the carp, however the corresponding decrease in the presence of genera such as *Aeromonas* and *Vibrio*, both of which contain examples of fish pathogens (Siwicki *et al.* 1994, Xie *et al.* 2007) can be seen as a positive effect of modulation. Influencing the gut microbiota in favour of a healthier host is a growing field of research and practice (Merrifield *et al.* 2010, 2014), however, many studies as to the effects of pre and probiotics last upwards of 8 weeks (Hartviksen *et al.* 2014, Sun *et al.* 2014, Estruch *et al.* 2015) which may have financial implications when translated to field trials or use within aquaculture, i.e. the cost of feeding specialist diets for long periods of time could become inhibitory when the volume required is scaled up to meet the demands of fisheries. Chapter 6, however, shows modulation of the gut microbiota occurred within a time frame of 15 days, i.e. a much shorter time span than seen in other studies. Further work must be performed in order to determine the stability of these changes in the microbiota and, indeed, as to the effect of Alphaproteobacteria within the fish gut. As an initial study, however, it points in the direction of utilising pre-existing vaccination programs within aquaculture in conjunction with pre and probiotic feeding regimes in order to decrease the time required to generate a "healthy" microbiota population. This could result in a positive economic impact if shorter feeding periods are required to achieve a healthy microbiota, i.e. a reduction in the amount of a specific feed ingredient required which would reduce feeding costs.

References.

Abelli, L., Randelli, E., Carnevali, O., and Picchietti, S. (2009). "Stimulation of gut immune system by early administration of probiotic strains in *Dicentrarchus labrax* and *Sparus aurata*." *Trends in Comparative Endocrinology and Neurobiology* **1163**: 340-342.

Abhari, K., Shekarforoush, S. S., Sajedianfard, J., Hosseinzadeh, S., and Nazifi, S. (2015). "The effects of probiotic, prebiotic and synbiotic diets containing *Bacillus coagulans* and inulin on rat intestinal microbiota." *Iranian Journal of Veterinary Research* **16**(3): 267-273.

Abid, A., Davies, S. J., Waines, R., Emery, M., Castex, M., Gioacchini, G., Carnevali, O., Bickerdike, R., Romero, J. and Merrifield, D. L. (2013). "Dietary synbiotic application modulates Atlantic salmon (*Salmo salar*) intestinal microbial communities and intestinal immunity." *Fish & Shellfish Immunology* **35**(6): 1948-1956.

Adamek, M., Rakus, K. L., Brogden, G., Matras, M., Chyb, J., Hirono, I., Kondo, H., Aoki, T., Irnazarow, I. and Steinhagen D. (2014). "Interaction between type I interferon and cyprinid herpesvirus 3 in two genetic lines of common carp *Cyprinus carpio*." *Diseases of Aquatic Organisms* **111**(2): 107.

Adamek, M., Syakuri, H., Harris, S., Rakus, K. L., Brogden, G., Matras, M., Irnazarow, I. and Steinhagen D. (2013). "Cyprinid herpesvirus 3 infection disrupts the skin barrier of common carp (*Cyprinus carpio* L.)." *Veterinary Microbiology* **162**(2-4): 456-470.

Ai, Q. H., Mai, K. S., Zhang, L., Tan, B. P., Zhang, W. B., Xu, W. and Li H. T. (2007). "Effects of dietary β-1,3-glucan on innate immune response of large yellow croaker, *Pseudosciaena crocea*." *Fish & Shellfish Immunology* **22**(4): 394-402.

Akhter, N., Wu, B., Memon, A. M. and Mohsin M. (2015). "Probiotics and prebiotics associated with aquaculture: A review." *Fish & Shellfish Immunology* **45**(2): 733-741.

Akrami, R., Nasri-Tajan, M., Jahedi, A., Jahedi, M., Razeghi Mansour, M. and Jafarpour, S. A. (2015). "Effects of dietary synbiotic on growth, survival, *Lactobacillus* bacterial count, blood indices and immunity of beluga (*Huso huso* Linnaeus, 1754) juvenile." *Aquaculture Nutrition* **21**(6): 952-959.

Aktan, F. (2004). "iNOS-mediated nitric oxide production and its regulation." *Life Sciences* **75**(6): 639-653.

Alipour, M., Zaidi, D., Valcheva, R., Jovel, J., Martinez, I., Sergi, C., Walter, J., Mason, A. L., Wong, G. K., Dieleman, L. A., Carroll, M. W., Huynh, H. Q. and Wine, E. (2015). "Mucosal barrier depletion and loss of bacterial diversity are primary abnormalities in paediatric ulcerative colitis." *Journal of Crohn's & Colitis* **10**(4): 462-471.

American Sportfishing Association (2013). "Comparing NOAA's recreational and commercial fishing economic data."

Askarian, F., Sperstad, S., Merrifield, D. L., Ray, A. K. and Ringo, E. (2013). "The effect of different feeding regimes on enzyme activities of gut microbiota in Atlantic cod (*Gadus morhua* L.)." *Aquaculture Research* **44**(5): 841-846.

Askarian, F., Zhou, Z., Olsen, R. E., Sperstad, S. and Ringo, E. (2012). "Culturable autochthonous gut bacteria in Atlantic salmon (*Salmo salar* L.) fed diets with or without chitin. Characterization by 16S rRNA gene sequencing, ability to produce enzymes and *in vitro* growth inhibition of four fish pathogens." *Aquaculture* **326**: 1-8.

Attramadal, K. J. K., Truong, T. M. H., Bakke, I., Skjermo, J., Olsen, Y. and Vadstein, O. (2014). "RAS and microbial maturation as tools for K-selection of microbial communities improve survival in cod larvae." *Aquaculture* **432**: 483-490.

Bagni, M., Archetti, L., Amadori, M. and Marino G. (2000). "Effect of long-term oral administration of an immunostimulant diet on innate immunity in sea bass (*Dicentrarchus labrax*)." *Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health* **47**(10): 745-751.

Bakke, I., Skjermo, J., Tu Anh, V. and Vadstein, O. (2013). "Live feed is not a major determinant of the microbiota associated with cod larvae (*Gadus morhua*)." *Environmental Microbiology Reports* **5**(4): 537-548.

Balcazar, J. L., de Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D. and Muzquiz, J. L. (2006). "The role of probiotics in aquaculture." *Veterinary Microbiology* **114**(3-4): 173-186.

Barrington, E. J. W. (1957). "The alimentary canal and digestion". In *The Physiology of Fishes* (M. E. Brown, ed), pp. 109-161. Academic Press, New York.

Bates, J. M., Mittge, E., Kuhlman, J., Baden, K. N., Cheesman, S. E. and Guillemin, K. (2006). "Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation." *Developmental Biology* **297**(2): 374-386.

Bayne, C. J. and Gerwick, L. (2001). "The acute phase response and innate immunity of fish." *Developmental and Comparative Immunology* **25**(8-9): 725-743.

Beckmann, L., Simon, O. and Vahjen, W. (2006). "Isolation and identification of mixed linked β-glucan degrading bacteria in the intestine of broiler chickens and partial characterization of respective 1,3-1,4-β-glucanase activities." *Journal of Basic Microbiology* **46**(3): 175-185.

Bergmark, L., Poulsen, P. H. B., Abu Al-Soud, W., Norman, A., Hansen, L. H.and Sorensen, S. J. (2012). "Assessment of the specificity of *Burkholderia* and *Pseudomonas* qPCR assays for detection of these genera in soil using 454 pyrosequencing." *Fems Microbiology Letters* **333**(1): 77-84.

Berner, V. K., duPre, S. A., Redelman, D. and Hunter, K. W. (2015). "Microparticulate beta-glucan vaccine conjugates phagocytized by dendritic cells activate both naive CD4 and CD8 T cells *in vitro*." *Cellular Immunology* **298**(1-2): 104-114.

Blane, G. (1819). "A Statement of Facts tending to establish an Estimate of the true Value and present State of Vaccination." *Medico-chirurgical transactions* **10**(Pt 2): 315-338.

Boshra, H., Li, J.and Sunyer, J. O. (2006). "Recent advances on the complement system of teleost fish." *Fish & Shellfish Immunology* **20**(2): 239-262.

Bosi, G. and Dezfuli, B. S. (2015). "Responses of *Squalius cephalus* intestinal mucous cells to *Pomphorhynchus laevis*." *Parasitology International* **64**(2): 167-172.

Bromuro, C., Romano, M., Chiani, P., Berti, F., Tontini, M., Proietti, D., Mori, E., Torosantucci, A., Costantino, P., Rappuoli, R. and Cassone, A. (2010). "β-glucan-CRM197 conjugates as candidates antifungal vaccines." *Vaccine* **28**(14): 2615-2623.

Candela, M., Biagi, E., Maccaferri, S., Turroni, S. and Brigidi, P. (2012). "Intestinal microbiota is a plastic factor responding to environmental changes." *Trends in Microbiology* **20**(8): 385-391.

Cao, Y. A., Liu, Y. C., Mao, W., Chen, R. D., He, S. X., Gao, X. H., Zhou, Z. G. and Yao, B. (2014). "Effect of Dietary N-acyl Homoserin Lactonase on the Immune Response and the Gut Microbiota of Zebrafish, *Danio rerio*, Infected with *Aeromonas hydrophila*." *Journal of the World Aquaculture Society* **45**(2): 149-162.

Carda-Dieguez, M., Mira, A. and Fouz, B. (2014). "Pyrosequencing survey of intestinal microbiota diversity in cultured sea bass (*Dicentrarchus labrax*) fed functional diets." *Fems Microbiology Ecology* **87**(2): 451-459.

Casadei, E., Bird, S., Wadsworth, S., Vecino, J. L. G. and Secombes, C. J. (2015). "The longevity of the antimicrobial response in rainbow trout (*Oncorhynchus mykiss*) fed a peptidoglycan (PG)

supplemented diet." *Fish & Shellfish Immunology* **44**(1): 316-320.

Cerezuela, R., Fumanal, M., Tapia-Paniagua, S. T., Meseguer, J., Morinigo M. A. and Esteban, M. A. (2012). "Histological alterations and microbial ecology of the intestine in gilthead seabream (*Sparus aurata* L.) fed dietary probiotics and microalgae." *Cell and Tissue Research* **350**(3): 477-489.

Cerezuela, R., Fumanal, M., Tapia-Paniagua S. T., Meseguer, J., Morinigo, M. A. and Esteban, M. A. (2013a). "Changes in intestinal morphology and microbiota caused by dietary administration of inulin and *Bacillus subtilis* in gilthead sea bream (*Sparus aurata* L.) specimens." *Fish & Shellfish Immunology* **34**(5): 1063-1070.

Cerezuela, R., Meseguer, J. and Esteban M. A. (2013b). "Effects of dietary inulin, *Bacillus subtilis* and microalgae on intestinal gene expression in gilthead seabream (*Sparus aurata* L.)." *Fish & Shellfish Immunology* **34**(3): 843-848.

Chettri, J. K., Raida, M. K., Kania, P. W. and Buchmann, K. (2012). "Differential immune response of rainbow trout (*Oncorhynchus mykiss*) at early developmental stages (larvae and fry) against the bacterial pathogen *Yersinia ruckerie*." *Developmental and Comparative Immunology* **36**(2): 463-474.

Dahlgren, C. and Karlsson, A. (1999). "Respiratory burst in human neutrophils." *Journal of Immunological Methods* **232**(1-2): 3-14.

Dalmo, R. A. and Bogwald, J. (2008). "β-glucans as conductors of immune symphonies." *Fish & Shellfish Immunology* **25**(4): 384-396.

Daniels, C. L., Merrifield, D. L., Ringo, E. and Davies, S. J. (2013). "Probiotic, prebiotic and synbiotic applications for the improvement of larval European lobster (*Homarus gammarus*) culture." *Aquaculture* **416**: 396-406.

Dawood, M. A. O. and Koshio, S. (2016). "Recent advances in the role of probiotics and prebiotics in carp aquaculture: A review." *Aquaculture* **454**: 243-251.

Dawood, M. A. O., Koshio, S., Ishikawa, M., Yokoyama, S., El Basuini, M. F., Hossain, M. S., Nhu, T. H., Dossou, S. and Moss, A. S. (2016). "Effects of dietary supplementation of *Lactobacillus rhamnosus* or/and *Lactococcus lactis* on the growth, gut microbiota and immune responses of red sea bream, *Pagrus major*." *Fish & Shellfish Immunology* **49**: 275-285.

De, B. C., Meena, D. K., Behera, B. K., Das, P., Das Mohapatra, P. K. and Sharma, A. P. (2014). "Probiotics in fish and shellfish culture: immunomodulatory and ecophysiological responses." *Fish Physiology and Biochemistry* **40**(3): 921-971.

de Bie, C. I., Escher, J. C. and de Ridder, L. (2012). "Antitumor necrosis factor treatment for pediatric inflammatory bowel disease." *Inflammatory Bowel Diseases* **18**(5): 985-1002.

Del'Duca, A., Cesar, D. E., Diniz, C. G. and Abreu, P. C. (2013). "Evaluation of the presence and efficiency of potential probiotic bacteria in the gut of tilapia (*Oreochromis niloticus*) using the fluorescent *in situ* hybridization technique." *Aquaculture* **388**: 115-121.

Depkat-Jakob, P. S., Hilgarth, M., Horn, M. A. and Drake, H. L. (2010). "Effect of earthworm feeding guilds on ingested dissimilatory nitrate reducers and denitrifiers in the alimentary canal of the earthworm." *Applied and Environmental Microbiology* **76**(18): 6205-6214.

Desai, A. R., Links, M. G., Collins, S. A., Mansfield, G. S., Drew, M. D., Van Kessel, A. G. and Hill, J. E. (2012). "Effects of plant-based diets on the distal gut microbiome of rainbow trout (*Oncorhynchus mykiss*)." *Aquaculture* **350**: 134-142.

Desai, C., Jain, K., Patel, B. and Madamwar, D. (2009). "Efficacy of bacterial consortium-AIE2 for contemporaneous Cr(VI) and azo dye bioremediation in batch and continuous bioreactor systems, monitoring steady-state bacterial dynamics using qPCR assays." *Biodegradation* **20**(6): 813-826.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P. and Andersen, G. L. (2006). "Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB." *Applied and Environmental Microbiology* **72**(7): 5069-5072.

Dietrich, M. A., Adamek, M., Bilinska, B., Hejmej, A., Steinhagen, D. and Ciereszko, A. (2014). "Characterization, expression and antibacterial properties of apolipoproteins A from carp (*Cyprinus carpio* L.) seminal plasma." *Fish & Shellfish Immunology* **41**(2): 389-401.

Dietrich, M. A., Nynca, J., Adamek, M., Steinhagen, D., Karol, H. and Ciereszko, A. (2015). "Expression of apolipoprotein A-I and A-II in rainbow trout reproductive tract and their possible role in antibacterial defence." *Fish & Shellfish Immunology* **45**(2): 750-756.

Dimitroglou, A., Merrifield, D. L., Spring, P., Sweetman, J., Moate, R. and Davies, S. J. (2010). "Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed utilisation, intestinal histology and gut microbiota of gilthead sea bream (*Sparus aurata*)." *Aquaculture* **300**(1-4): 182-188.

Engstad, R. E. and Robertsen B. (1993). "Recognition of yeast-cell wall glucan by Atlantic Salmon (*Salmo Salar* L) macrophages." *Developmental and Comparative Immunology* **17**(4): 319-330.

Engstad, R. E. and Robertsen, B. (1994). "Specificity of a β-glucan receptor on macrophages from Atlantic salmon (*Salmo salar* L)." *Developmental and Comparative Immunology* **18**(5): 397-408.

Falcinelli, S., Rodiles, A., Unniappan, S., Picchietti, S., Gioacchini, G., Merrifield, D. L. and Carnevali, O. (2016). "Probiotic treatment reduces appetite and glucose level in the zebrafish model." *Scientific Reports* **6**: 13.

Falco, A., Cartwright J. R., Wiegertjes G. F. and Hoole D. (2012a). "Molecular characterization and expression analysis of two new C-reactive protein genes from common carp (*Cyprinus carpio*)." *Developmental and Comparative Immunology* **37**(1): 127-138.

Falco, A., Frost, P., Miest, J., Pionnier, N., Irnazarow, I. and Hoole, D. (2012b). "Reduced inflammatory response to *Aeromonas salmonicida* infection in common carp (*Cyprinus carpio* L.) fed with β-glucan supplements." *Fish & Shellfish Immunology* **32**(6): 1051-1057.

Falco, A., Miest, J., Pionnier, N., Pietretti, D., Forlenza, M., Wiegertjes, G. F. and Hoole, D. (2013). "β-glucan supplemented diets induce high and broad expression levels of TLR3 what explains protection conferred by these additives against viral infections in fish." *Fish & Shellfish Immunology* **34**(6): 1706-1706.

Falco, A., Ortega-Villaizan, M., Chico, V., Brocal, I., Perez, L., Coll, J. M. and Estepa, A. (2009). "Antimicrobial peptides as model molecules for the development of novel antiviral agents in aquaculture." *Mini-Reviews in Medicinal Chemistry* **9**(10): 1159-1164.

Falk, P. G., Hooper, L. V., Midtvedt, T. and Gordon, J. I. (1998). "Creating and maintaining the gastrointestinal ecosystem: What we know and need to know from gnotobiology." *Microbiology and Molecular Biology Reviews* **62**(4): 1157.

FAO (2012) "The state of world fisheries and aquaculture." Rome, FAO 2012.

FAO (2014) "The state of world fisheries and aquaculture." Rome, FAO 2014.

Fisher, R. A. (1925). "Statistical Methods for Research Workers." Oliver and Boyd: Edinburgh. http://psychclassics.yorku.ca/Fisher/Methods/

Forberg, T., Vestrum, R. I., Arukwe, A. and Vadstein, O. (2012). "Bacterial composition and activity determines host gene-expression responses in gnotobiotic Atlantic cod (*Gadus morhua*) larvae." *Veterinary Microbiology* **157**(3-4): 420-427.

Forlenza, M., Magez, S., Scharsack, J. P., Westphal, A., Savelkoul, H. F. J. and Wiegertjes, G. F. (2009a). "Receptor-mediated and lectin-like activities of carp (*Cyprinus carpio*) TNF-alpha." *Journal of*

Immunology **183**(8): 5319-5332.

Forlenza, M., Nakao, M., Wibowo, I., Joerink, M., Arts, J. A. J., Savelkoul, H. F. J. and Wiegertjes, G. F. (2009b). "Nitric oxide hinders antibody clearance from the surface of *Trypanoplasma borreli* and increases susceptibility to complement-mediated lysis." *Molecular Immunology* **46**(16): 3188-3197.

Fowler, J., Cohen, L. and Jarvis, P. (1998). "Practical statistics for field biology. Second edition." *Practical statistics for field biology. Second edition*. i-xi, 1-259.

Fuller, R. (1989). "Probiotics in man and animals." *Journal of Applied Bacteriology* **66**(5): 365-378.

Gatesoupe, F. J. (1999). "The use of probiotics in aquaculture." *Aquaculture* **180**(1-2): 147-165.

Gatesoupe, F. J., Huelvan, C., Le Bayon, N., Severe, A., Aasen, I. M., Degnes, K. F., Mazurais, D., Panserat, S., Zambonino-Infante, J. L. and Kaushik, S. J. (2014). "The effects of dietary carbohydrate sources and forms on metabolic response and intestinal microbiota in sea bass juveniles, *Dicentrarchus labrax*." *Aquaculture* **422**: 47-53.

Geraylou, Z., Souffreau, C., Rurangwa, E., D'Hondt, S., Callewaert, L., Courtin, C. M., Delcour, J. A., Buyse, J. and Ollevier, F. (2012). "Effects of arabinoxylan-oligosaccharides (AXOS) on juvenile Siberian sturgeon (*Acipenser baerii*) performance, immune responses and gastrointestinal microbial community." *Fish & Shellfish Immunology* **33**(4): 718-724.

Geraylou, Z., Souffreau, C., Rurangwa, E., De Meester, L., Courtin, C. M., Delcour, J. A., Buyse, J. and Ollevier, F. (2013a). "Effects of dietary arabinoxylan-oligosaccharides (AXOS) and endogenous probiotics on the growth performance, non-specific immunity and gut microbiota of juvenile Siberian sturgeon (*Acipenser baerii*)." *Fish & Shellfish Immunology* **35**(3): 766-775.

Geraylou, Z., Souffreau, C., Rurangwa, E., Maes, G. E., Spanier, K. I., Courtin, C. M., Delcour, J. A., Buyse, J. and Ollevier, F. (2013b). "Prebiotic effects of arabinoxylan oligosaccharides on juvenile Siberian sturgeon (*Acipenser baerii*) with emphasis on the modulation of the gut microbiota using 454 pyrosequencing." *Fems Microbiology Ecology* **86**(2): 357-371.

Geurden, I., Mennigen, J., Plagnes-Juan, E., Veron, V., Cerezo, T., Mazurais, D., Zambonino-Infante, J., Gatesoupe, J., Skiba-Cassy, S. and Panserat, S. (2014). "High or low dietary carbohydrate: protein ratios during first-feeding affect glucose metabolism and intestinal microbiota in juvenile rainbow trout." *Journal of Experimental Biology* **217**(19): 3396-3406.

Ghanbari, M., Kneifel, W. and Domig, K. J. (2015). "A new view of the fish gut microbiome: Advances from next-generation sequencing." *Aquaculture* **448**: 464-475.

Giannenas, I., Triantafillou, E., Stavrakakis, S., Margaroni, M., Mavridis, S., Steiner, T. and Karagouni, E. (2012). "Assessment of dietary supplementation with carvacrol or thymol containing feed additives on performance, intestinal microbiota and antioxidant status of rainbow trout (*Oncorhynchus mykiss*)." *Aquaculture* **350**: 26-32.

Gibson, G. R., Probert, H. M., Van Loo, J., Rastall, R. A. and Roberfroid, M. B. (2004). "Dietary modulation of the human colonic microbiota: updating the concept of prebiotics." *Nutrition Research Reviews* **17**(2): 259-275.

Giri, S. S., Sen, S. S., Chi, C., Kim, H. J., Yun, S., Park, S. C. and Sukumaran, V. (2015). "Effect of guava leaves on the growth performance and cytokine gene expression of *Labeo rohita* and its susceptibility to *Aeromonas hydrophila* infection." *Fish & Shellfish Immunology* **46**(2): 217-224.

Gisbert, E., Castillo, M., Skalli, A., Andree, K. B. and Badiola, I. (2013). "*Bacillus cereus* var. *toyoi* promotes growth, affects the histological organization and microbiota of the intestinal mucosa in rainbow trout fingerlings." *Journal of Animal Science* **91**(6): 2766-2774.

Goetz, F. W., Planas, J. V. and MacKenzie, S. (2004). "Tumor necrosis factors." *Developmental and Comparative Immunology* **28**(5): 487-497.

Gomez, D., Sunyer, J. O. and Salinas, I. (2013). "The mucosal immune system of fish: The evolution of tolerating commensals while fighting pathogens." *Fish & Shellfish Immunology* **35**(6): 1729-1739.

Gomez, G. D. and Balcazar, J. L. (2008). "A review on the interactions between gut microbiota and innate immunity of fish." *Fems Immunology and Medical Microbiology* **52**(2): 145-154.

Gordon, J. and McLeod, J. W. (1928) "The practical application of the direct oxidase reaction in bacteriology." *The Journal of Pathology* **31**: 185-190.

Gorgoglione, B., Wang, T. H., Secombes, C. J. and Holland, J. W. (2013). "Immune gene expression profiling of Proliferative Kidney Disease in rainbow trout *Oncorhynchus mykiss* reveals a dominance of anti-inflammatory, antibody and T helper cell-like activities." *Veterinary Research* **44**: 16.

Gorgoglione, B., Zahran, E., Taylor, N. G. H., Feist, S. W., Zou, J. and Secombes, C. J. (2016). "Comparative study of CXC chemokines modulation in brown trout (*Salmo trutta*) following infection with a bacterial or viral pathogen." *Molecular immunology* **71**: 64-77.

Green, T. J., Smullen, R. and Barnes, A. C. (2013). "Dietary soybean protein concentrate-induced intestinal disorder in marine farmed Atlantic salmon, *Salmo salar* is associated with alterations in gut microbiota." *Veterinary Microbiology* **166**(1-2): 286-292.

Grubbs, F. E. and Beck, G. (1972). "Extension of sample sizes and percentage points for significance tests of outlying observations." *Technometrics* **14**(4): 847.

Gudding, R. and Van Muiswinkel, W. B. (2013). "A history of fish vaccination Science-based disease prevention in aquaculture." *Fish & Shellfish Immunology* **35**(6): 1683-1688.

Gulec, A. K. and Cengizler, I. (2012). "Determination of acute phase proteins aft er experimental *Streptococcus iniae* infection in tilapia (*Oreochromis niloticus* L.)." *Turkish Journal of Veterinary & Animal Sciences* **36**(4): 380-387.

Guselle, N. J., Markham, R. J. F. and Speare, D. J. (2006). "Intraperitoneal administration of β-1,3/1,6-glucan to rainbow trout, *Oncorhynchus mykiss* (Walbaum), protects against *Loma salmonae*." *Journal of Fish Diseases* **29**(6): 375-381.

Harris (2013). "Denaturing gradient gel electrophoresis as a non-culture based method of analysing fish-associated microflora." Keele University, UK.

Hartviksen, M., Vecino, J. L. G., Ringo, E., Bakke, A. M., Wadsworth, S., Krogdahl, A., Ruohonen, K. and Kettunen, A. (2014). "Alternative dietary protein sources for Atlantic salmon (*Salmo salar* L.) effect on intestinal microbiota, intestinal and liver histology and growth." *Aquaculture Nutrition* **20**(4): 381-398.

Hattori, T., Kato, Y., Uno, S. and Usui, T. (2013). "Mode of action of a β-1/6-glucanase from *Penicillium multicolor*." *Carbohydrate Research* **366**: 6-16.

He, S., Wu, Z., Liu, Y., Wu, N., Tao, Y., Xu, L., Zhou, Z., Yao, B. and Ringo, E. (2013a). "Effects of dietary 60 g kg^{-1} dried distiller's grains in least-cost practical diets on production and gut allochthonous bacterial composition of cage-cultured fish: comparison among fish species with different natural food habits." *Aquaculture Nutrition* **19**(5): 765-772.

He, S., Zhang, Y., Xu, L., Yang, Y., Marubashi, T., Zhou, Z. and Yao, B. (2013b). "Effects of dietary *Bacillus subtilis* C-3102 on the production, intestinal cytokine expression and autochthonous bacteria of hybrid tilapia *Oreochromis niloticus* female x *Oreochromis aureus* male." *Aquaculture* **412**: 125-130.

He, S., Zhou, Z., Liu, Y., Cao, Y., Meng, K., Shi, P., Yao, B. and Ringo, E. (2012). "Do dietary betaine and the antibiotic florfenicol influence the intestinal autochthonous bacterial community in hybrid tilapia (*Oreochromis niloticus* a™ Euro x *O. aureus* a™)?" *World Journal of Microbiology & Biotechnology* **28**(3): 785-791.

He, S. X., Zhou, Z. G., Liu, Y. C., Shi, P. J., Yao, B., Ringo, E. and Yoon, I. (2009). "Effects of dietary

Saccharomyces cerevisiae fermentation product (DVAQUA®) on growth performance, intestinal autochthonous bacterial community and non-specific immunity of hybrid tilapia (*Oreochromis niloticus* female x *O. aureus* male) cultured in cages." *Aquaculture* **294**(1-2): 99-107.

Himmelheber, D. W., Thomas, S. H., Loffler, F. E., Taillefert, M. and Hughes, J. B. (2009). "Microbial colonization of an *in situ* sediment cap and correlation to stratified redox zones." *Environmental Science & Technology* **43**(1): 66-74.

Holland, M. C. H. and Lambris, J. D. (2002). "The complement system in teleosts." *Fish & Shellfish Immunology* **12**(5): 399-420.

Hoseinifar, S. H., Soleimani, N. and Ringo, E. (2014). "Effects of dietary fructo-oligosaccharide supplementation on the growth performance, haemato-immunological parameters, gut microbiota and stress resistance of common carp (*Cyprinus carpio*) fry." *British Journal of Nutrition* **112**(8): 1296-1302.

Huang, H., Ostroff, G. R., Lee, C. K., Specht, C. A. and Levitz, S. M. (2013). "Characterization and optimization of the glucan particle-based vaccine platform." *Clinical and Vaccine Immunology* **20**(10): 1585-1591.

Huttenhuis, H. B. T., Taverne-Thiele, A. J., Grou, C. P. O., Bergsma, J., Saeij, J. P. J., Nakayasu, C. and Rombout, J. (2006). "Ontogeny of the common carp (*Cyprinus carpio* L.) innate immune system." *Developmental and Comparative Immunology* **30**(6): 557-574.

Huysamen, C. and Brown, G. D. (2009). "The fungal pattern recognition receptor, Dectin-1, and the associated cluster of C-type lectin-like receptors." *Fems Microbiology Letters* **290**(2): 121-128.

Ingerslev, H. C., Jorgensen, L. V., Strube, M. L., Larsen, N., Dalsgaard, I., Boye, M. and Madsen, L. (2014a). "The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type." *Aquaculture* **424**: 24-34.

Ingerslev, H. C., Strube, M. L., Jorgensen, L. V., Dalsgaard, I., Boye, M. and Madsen, L. (2014b). "Diet type dictates the gut microbiota and the immune response against *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*)." *Fish & Shellfish Immunology* **40**(2): 624-633.

Jaafar, R. M., Kania, P. W., Larsen, A. H., Nielsen, D. S., Fouz, B., Browdy, C. and Buchmann, K. (2013). "Gut microbiota changes in rainbow trout, *Oncorhynchus mykiss* (Walbaum), during organic acid feed supplementation and *Yersinia ruckeri* infection." *Journal of Fish Diseases* **36**(6): 599-606.

Jones-Hall, Y. L., Kozik, A. and Nakatsu, C. (2015). "Ablation of Tumor Necrosis Factor is associated with decreased inflammation and alterations of the microbiota in a mouse model of inflammatory bowel disease." *Plos One* **10**(3): 17.

Jung-Schroers, V., Adamek, M., Jung, A., Harris, S., Doza, O.-S., Baumer, A.and Steinhagen, D. (2015a). "Feeding of β-1,3/1,6-glucan increases the diversity of the intestinal microflora of carp (*Cyprinus carpio*)" *Aquaculture Nutrition* DOI: 10.1111/anu.12320.

Jung-Schroers, V., Adamek, M., Teitge, F., Hellmann, J., Bergmann, S. M., Schuetze, H., Kleingeld, D. W., Way, K., Stone, D., Runge, M., Keller, B., Hesami, S., Waltzek, T. and Steinhagen, D. (2015b). "Another potential carp killer?: Carp Edema Virus disease in Germany." *BMC Veterinary Research* **11**: 114.

Kiron, V., Kulkarni, A., Dahle, D., Vasanth, G., Lokesh, J. and Elvebo, O. (2016). "Recognition of purified β 1,3/1,6 glucan and molecular signalling in the intestine of Atlantic salmon." *Developmental and Comparative Immunology* **56**: 57-66.

Koshio, S. (2016). "Immunotherapies targeting fish mucosal immunity - current knowledge and future perspectives." *Frontiers in Immunology* **6**: 1-4.

Kubilay, A., Altun, S., Ulukoy, G., Ekici, S. and Diler, O. (2008). "Immunization of rainbow trout (*Oncorhynchus mykiss*) against *Lactococcus garvieae* using vaccine mixtures." *Israeli Journal of*

Aquaculture-Bamidgeh **60**(4): 268-273.

Kuhlwein, H., Emery, M. J., Rawling, M. D., Harper, G. M., Merrifield, D. L. and Davies, S. J. (2013). "Effects of a dietary β-(1,3)(1,6)-D-glucan supplementation on intestinal microbial communities and intestinal ultrastructure of mirror carp (*Cyprinus carpio* L.)." *Journal of Applied Microbiology* **115**(5): 1091-1106.

Kuhlwein, H., Merrifield, D. L., Rawling, M. D., Foey, A. D. and Davies, S. J. (2014). "Effects of dietary β-(1,3)(1,6)-D-glucan supplementation on growth performance, intestinal morphology and haemato-immunological profile of mirror carp (*Cyprinus carpio* L.)." *Journal of Animal Physiology and Animal Nutrition* **98**(2): 279-289.

Lazado, C. C. and Caipang, C. M. A. (2014). "Mucosal immunity and probiotics in fish." *Fish & Shellfish Immunology* **39**(1): 78-89.

Lee, K. H., Park, M., Ji, K. Y., Lee, H. Y., Jang, J. H., Yoon, I. J., Oh, S. S., Kim, S. M., Jeong, Y. H., Yun, C. H., Kim, M. K., Lee, I. Y., Choi, H. R., Ko, K. S. and Kang, H. S. (2014). "Bacterial β-(1,3)-glucan prevents DSS-induced IBD by restoring the reduced population of regulatory T cells." *Immunobiology* **219**(10): 802-812.

Lew, M. J. (2012). "Bad statistical practice in pharmacology (and other basic biomedical disciplines): you probably don't know P." *British Journal of Pharmacology* **166**(5): 1559-1567.

Lewis, D. B. (2002). "Allergy immunotherapy and inhibition of Th2 immune responses: a sufficient strategy?" *Current Opinion in Immunology* **14**(5): 644-651.

Lewisch, E., Gorgoglione, B., Way, K. and El-Matbouli, M. (2015). "Carp Edema Virus/Koi Sleepy Disease: An Emerging Disease in Central-East Europe." *Transboundary and Emerging Diseases* **62**(1): 6-12.

Li, H. X., Lu, X. J., Li, C. H. and Chen, J. (2015). "Molecular characterization of the liver-expressed antimicrobial peptide 2 (LEAP-2) in a teleost fish, *Plecoglossus altivelis*: Antimicrobial activity and molecular mechanism." *Molecular Immunology* **65**(2): 406-415.

Li, M. F., Chen, C., Li, J. and Sun, L. (2013). "The C-reactive protein of tongue sole *Cynoglossus semilaevis* is an acute phase protein that interacts with bacterial pathogens and stimulates the antibacterial activity of peripheral blood leukocytes." *Fish & Shellfish Immunology* **34**(2): 623-631.

Liu, L., Gong, Y. X., Zhu, B., Liu, G. L., G. X. Wang and Ling, F. (2015). "Effect of a new recombinant *Aeromonas hydrophila* vaccine on the grass carp intestinal microbiota and correlations with immunological responses." *Fish & Shellfish Immunology* **45**(1): 175-183.

Liu, Y., Zhou, Z., Yao, B., Shi, P., He, S., Holvold, L. B.and Ringo, E. (2008). "Effect of intraperitoneal injection of immunostimulatory substances on allochthonous gut microbiota of Atlantic salmon (*Salmo salar* L.) determined using denaturing gradient gel electrophoresis." *Aquaculture Research* **39**(6): 635-646.

Liu, Y. C., Zhou, Z. G., Wu, N., Tao, Y., Xu, L., Cao, Y. N., Zhang, Y. T. and Yao, B. (2012). "Gibel carp *Carassius auratus* gut microbiota after oral administration of trimethoprim/sulfamethoxazole." *Diseases of Aquatic Organisms* **99**(3): 207-213.

Llewellyn, M. S., Boutin, S., Hoseinifar, S. H. and Derome, N. (2014). "Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries." *Frontiers in Microbiology* **5**: 17.

Lobo, C., Tapia-Paniagua, S., Moreno-Ventas, X., Alarcon, F. J., Rodriguez, C., Balebona, M. C., Morinigo, M. A. and de la Banda, I. G. (2014). "Benefits of probiotic administration on growth and performance along metamorphosis and weaning of Senegalese sole (*Solea senegalensis*)." *Aquaculture* **433**: 183-195.

Lorenzen, N. and LaPatra, S. E. (2005). "DNA vaccines for aquacultured fish." *Revue Scientifique Et Technique-Office International Des Epizooties* **24**(1): 201-213.

Lovoll, M., Fischer, U., Mathisen, G. S., Bogwald, J., Ototake, M. and Dalmo, R. A. (2007). "The C3 subtypes are differentially regulated after immunostimulation in rainbow trout, but head kidney macrophages do not contribute to C3 transcription." *Veterinary Immunology and Immunopathology* **117**(3-4): 284-295.

MacCarthy, E. M., Burns, I., Irnazarow, I., Polwart, A., Greenhough, T. J., Shrive, A. K. and Hoole, D. (2008). "Serum CRP-like protein profile in common carp *Cyprinus carpio* challenged with *Aeromonas hydrophila* and *Escherichia coli* lipopolysaccharide." *Developmental and Comparative Immunology* **32**(11): 1281-1289.

Mackie, T. J. and McCartney, J. E. (1956) "Handbook of practical bacteriology. A guide to bacteriological laboratory work." Ninth Edition. *E. & S. Livingstone LTD.* Edinburgh and London.

Magor, B. G. and Magor, K. E. (2001). "Evolution of effectors and receptors of innate immunity." *Developmental and Comparative Immunology* **25**(8-9): 651-682.

Mann, C. M. and Markham, J. L. (1998). "A new method for determining the minimum inhibitory concentration of essential oils." *Journal of Applied Microbiology* **84**(4): 538-544.

Mardones, F. O., Martinez-Lopez, B., Valdes-Donoso, P., Carpenter, T. E. and Perez, A. M. (2014). "The role of fish movements and the spread of infectious salmon anemia virus (ISAV) in Chile, 2007-2009." *Preventive Veterinary Medicine* **114**(1): 37-46.

Martin, K. R. (2012). "β-glucans: going through GM-CSF to get to dectin." *Journal of Leukocyte Biology* **91**(4): 521-524.

Martinez-Puig, D., Castillo, M., Nofrarias, M., Creus, E. and Perez, J. F. (2007). "Long-term effects on the digestive tract of feeding large amounts of resistant starch: A study in pigs." *Journal of the Science of Food and Agriculture* **87**(11): 1991-1999.

McLoughlin, R. M. and Mills, K. H. G. (2011). "Influence of gastrointestinal commensal bacteria on the immune responses that mediate allergy and asthma." *Journal of Allergy and Clinical Immunology* **127**(5): 1097-1107.

Meena, D. K., Das, P., Kumar, S., Mandal, S. C., Prusty, A. K., Singh, S. K., Akhtar, M. S., Behera, B. K., Kumar, K., Pal, A. K. and Mukherjee, S. C. (2013). "β-glucan: an ideal immunostimulant in aquaculture (a review)." *Fish Physiology and Biochemistry* **39**(3): 431-457.

Meloni, M., Candusso, S., Galeotti, M. and Volpatti, D. (2015). "Preliminary study on expression of antimicrobial peptides in European sea bass (*Dicentrarchus labrax*) following *in vivo* infection with *Vibrio anguillarum*. A time course experiment." *Fish & Shellfish Immunology* **43**(1): 82-90.

Merrifield, D. L., Dimitroglou, A., Foey, A., Davies, S. J., Baker, R. T. M., Bogwald, J., Castex, M. and Ringo, E. (2010). "The current status and future focus of probiotic and prebiotic applications for salmonids." *Aquaculture* **302**(1-2): 1-18.

Merrifield, D. L. and Ringo, E. (2014). "Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics." *Wiley-Blackwell*.

Merrifield, D. L., Shaw, B. J., Harper, G. M., Saoud, I. P., Davies, S. J., Handy, R. D. and Henry, T. B. (2013). "Ingestion of metal-nanoparticle contaminated food disrupts endogenous microbiota in zebrafish (*Danio rerio*)." *Environmental Pollution* **174**: 157-163.

Methe, B. A., Nelson, K. E., Pop, M., Creasy, H. H., Giglio, M. G., Huttenhower, C., Gevers, D., Petrosino, J. F., Abubucker, S., Badger, J. H., Chinwalla, A. T., Earl, A. M., FitzGerald, M. G., Fulton, R. S., Hallsworth-Pepin, K., Lobos, E. A., Madupu, R., Magrini, V., Martin, J. C., Mitreva, M., Muzny, D. M., Sodergren, E. J., Versalovic, J., Wollam, A. M., Worley, K. C., Wortman, J. R., Young, S. K., Zeng, Q., Aagaard, K. M., Abolude, O. O., Allen-Vercoe, E., Alm, E. J., Alvarado, L., Andersen, G. L., Anderson, S., Appelbaum, E., Arachchi, H. M., Armitage, G., Arze, C. A., Ayvaz, T., Baker, C. C., Begg, L., Belachew, T., Bhonagiri, V., Bihan, M., Blaser, M. J., Bloom, T., Bonazzi, V. R., Brooks, P., Buck, G., Buhay, C. J., Busam, D. A., Campbell, J. L., Canon, S. R.,

Cantarel, B. L., Chain, P. S., Chen, I. M. A., Chen, L., Chhibba, S., Chu, K., Ciulla, D. M., Clemente, J. C., Clifton, S. W., Conlan, S., Crabtree, J., Cutting, M. A., Davidovics, N. J., Davis, C. C., DeSantis, T. Z., Deal, C., Delehaunty, K. D., Dewhisrst, F. E., Deych, E., Ding, Y., Dooling, D. J., Dugan, S. P., Dunne, W. M., Durkin, A. S., Edgar, R. C., Erlich, R. L., Farmer, C. N., Farrell, R. M., Faust, K., Feldgarden, M., Felix, V. M., Fisher, S., Fodor, A. A., Forney, L., Foster, L., Di Francesco, V., Friedman, J., Friedrich, D. C., Fronick, C. C., Fulton, L. L., Gao, H., Garcia, N., Giannoukos, G., Giblin, C., Giovanni, M. Y., Goldberg, J. M., Goll, J., Gonzalez, A., Griggs, A., Gujja, S., Haas, B. J., Hamilton, H. A., Harris, E. L., Hepburn, T. A., Herter, B., Hoffmann, D. E., Holder, M. E., Howarth, C., Huang, K. H., Huse, S. M., Izard, J., Jansson, J. K., Jiang, H. Y., Jordan, C., Joshi, V., Katancik, J., Keitel, W., Kelley, S. T., Kells, C., Kinder-Haake, S., King, N. B., Knight, R., Knights, D., Kong, H. H., Koren, O., Koren, S., Kota, K. C., Kovar, C. L., Kyrpides, N. C., La Rosa, P. S., Lee, S. L., Lemon, K. P., Lennon, N., Lewis, C. M., Lewis, L., Ley, R. E., Li, K., Liolios, K., Liu, B., Liu, Y., Lo, C. C., Lozupone, C. A., Lunsford, R. D., Madden, T., Mahurkar, A. A., Mannon, P. J., Mardis, E. R., Markowitz, V. M., Mavrommatis, K., McCorrison, J. M., McDonald, D., McEwen, J., McGuire, A. L., McInnes, P., Mehta, T., Mihindukulasuriya, K. A.,. Miller, J. R, Minx, P. J., Newsham, I., Nusbaum, C., O'Laughlin, M., Orvis, J., Pagani, I., Palaniappan, K., Patel, S. M., Pearson, M., Peterson, J., Podar, M., Pohl, C., Pollard, K. S., Priest, M. E., Proctor, L. M., Qin, X., Raes, J., Ravel, J., Reid, J. G., Rho, M., Rhodes, R., Riehle, K. P., Rivera, M. C., Rodriguez-Mueller, B., Rogers, Y. H., Ross, M. C., Russ, C., Sanka, R. K., Sankar, P., Sathirapongsasuti, J. F., Schloss, J. A., Schloss, P. D., Schmidt, T. M., Scholz, M., Schriml, L., Schubert, A. M., Segata, N., Segre, J. A., Shannon, W. D., Sharp, R. R., Sharpton, T. J., Shenoy, N., Sheth, N. U., Simone, G. A., Singh, I., Smillie, C. S., Sobel, J. D., Sommer, D. D., Spicer, P., Sutton, G. G., Sykes, S. M., Tabbaa, D. G., Thiagarajan, M., Tomlinson, C. M., Torralba, M., Treangen, T. J., Truty, R. M., Vishnivetskaya, T. A., Walker, J., Wang, L., Wang, Z., Ward, D. V., Warren, W., Watson, M. A., Wellington, C., Wetterstrand, K. A., White, J. R., Wilczek-Boney, K., Wu, Y. Q., Wylie, K. M., Wylie, T., Yandava, C., Ye, L., Ye, Y., Yooseph, S., Youmans, B. P., Zhang, L., Zhou, Y. J., Zhu, Y. M., Zoloth, L., Zucker, J. D., Birren, B. W., Gibbs, R. A., Highlander, S. K., Weinstock, G. M., Wilson, R. K., White, O. and Human Microbiome Project (2012). "A framework for human microbiome research." *Nature* **486**(7402): 215-221.

Metzger, D. C., Elliott, D. G., Wargo, A., Park, L. K. and Purcell, M. K. (2010). "Pathological and immunological responses associated with differential survival of Chinook salmon following *Renibacterium salmoninarum* challenge." *Diseases of Aquatic Organisms* **90**(1): 31-41.

Mewett, J. (2015) "The threat of illegal fish movements on national fish health status." *17th EAFP International Conference on Diseases of Fish and Shellfish, September 2015* **O-203**.

Midtlyng, P. J. and Lillehaug, A. (1998). "Growth of Atlantic salmon *Salmo salar* after intraperitoneal administration of vaccines containing adjuvants." *Diseases of Aquatic Organisms* **32**(2): 91-97.

Miest, J. J., Arndt, C., Adamek, M., Steinhagen, D. and Reusch, T. B. H. (2016). "Dietary beta-glucan (MacroGard®) enhances survival of first feeding turbot (*Scophthalmus maximus*) larvae by altering immunity, metabolism and microbiota." *Fish & Shellfish Immunology* **48**: 94-104.

Miest, J. J., A. Falco, N. P. M. Pionnier, P. Frost, I. Irnazarow, G. T. Williams and D. Hoole (2012). "The influence of dietary beta-glucan, PAMP exposure and Aeromonas salmonicida on apoptosis modulation in common carp (Cyprinus carpio)." Fish & Shellfish Immunology **33**(4): 846-856.

Misra, C. K., Das, B. K., Mukherjee, S. C. and Pattnaik, P. (2006). "Effect of long term administration of dietary β-glucan on immunity, growth and survival of *Labeo rohita* fingerlings." *Aquaculture* **255**(1-4): 82-94.

Moons, P., Michiels, C. W. and Aertsen, A. (2009). "Bacterial interactions in biofilms." *Critical Reviews in Microbiology* **35**(3): 157-168.

Muyzer, G., Dewaal, E. C. and Uitterlinden, A. G. (1993). "Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S ribsosomal RNA." *Applied and Environmental Microbiology* **59**(3): 695-700.

Muyzer, G. and Smalla, K. (1998). "Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology." *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **73**(1): 127-141.

Nakao, M., Tsujikura, M., Ichiki, S., Vo, T. K. and Somamoto, T. (2011). "The complement system in teleost fish: Progress of post-homolog-hunting researches." *Developmental & Comparative Immunology* **35**(12): 1296-1308.

Nath, M., Singh, B. P. and Saxena, V. K. (2014). "Estimation of crossbreeding parameters for humoral response in broiler." *Livestock Science* **170**: 8-15.

Navarrete, P., Fuentes, P., De la Fuente, L., Barros, L., Magne, F., Opazo, R., Ibacache, C., Espejo, R. and Romero, J. (2013). "Short-term effects of dietary soybean meal and lactic acid bacteria on the intestinal morphology and microbiota of Atlantic salmon (*Salmo salar*)." *Aquaculture Nutrition* **19**(5): 827-836.

Nayak, S. K. (2010a). "Probiotics and immunity: A fish perspective." *Fish & Shellfish Immunology* **29**(1): 2-14.

Nayak, S. K. (2010b). "Role of gastrointestinal microbiota in fish." *Aquaculture Research* **41**(11): 1553-1573.

Neves, J. V., Caldas, C., Vieira, I., Ramos, M. F. and Rodrigues, P. N. S. (2015). "Multiple Hepcidins in a Teleost Fish, *Dicentrarchus labrax*: Different Hepcidins for Different Roles." *Journal of Immunology* **195**(6): 2696-2709.

Neyman, J. and Pearson, E. S. (1933). "On the problem of the most efficient tests of statistical hypotheses." *Philos Trans R Soc Lond A* **231**: 289–337.

Nicolai, A., Rouland-Lefevre, C., Ansart, A., Filser, J., Lenz, R., Pando, A. and Charrier M. (2015). "Inter-population differences and seasonal dynamic of the bacterial gut community in the endangered land snail *Helix pomatia* (Gastropoda: helicidae)." *Malacologia* **59**(1): 177-190.

Nikapitiya, C. (2013). "Marine bacteria as probiotics and their applications in aquaculture." *Marine Microbiology: Bioactive Compounds and Biotechnological Applications*: 97-126.

Noga, E. J., Ullal, A. J., Corrales, J. and Fernandes, J. M. O. (2011). "Application of antimicrobial polypeptide host defenses to aquaculture: Exploitation of downregulation and upregulation responses." *Comparative Biochemistry and Physiology D-Genomics & Proteomics* **6**(1): 44-54.

Nonaka, M. and Kimura, A. (2006). "Genomic view of the evolution of the complement system." *Immunogenetics* **58**(9): 701-713.

Omar, S. S., Merrifield, D. L., Kuehlwein, H., Williams, P. E. V. and Davies, S. J. (2012). "Biofuel derived yeast protein concentrate (YPC) as a novel feed ingredient in carp diets." *Aquaculture* **330**: 54-62.

Ortuno, J., Cuesta, A., Rodriguez, A., Esteban, M. A. and Meseguer, J. (2002). "Oral administration of yeast, *Saccharomyces cerevisiae*, enhances the cellular innate immune response of gilthead seabream (*Sparus aurata* L.)." *Veterinary Immunology and Immunopathology* **85**(1-2): 41-50.

Perez, T., Balcazar, J. L., Ruiz-Zarzuela, I., Halaihel, N., Vendrell, D., de Blas, I. and Muzquiz, J. L. (2010). "Host-microbiota interactions within the fish intestinal ecosystem." *Mucosal Immunology* **3**(4): 355-360.

Peterson, C. T., Sharma, V., Elmen, L. and Peterson, S. N. (2015). "Immune homeostasis, dysbiosis and therapeutic modulation of the gut microbiota." *Clinical and experimental immunology* **179**(3): 363-377.

Peterson, D. A., McNulty, N. P., Guruge, J. L. and Gordon, J. I. (2007). "IgA response to symbiotic bacteria as a mediator of gut homeostasis." *Cell Host & Microbe* **2**(5): 328-339.

Piackova, V., Flajshans, M., Pokorova, D., Reschova, S., Gela, D., Cizek, A. and Vesely, T. (2013). "Sensitivity of common carp, *Cyprinus carpio* L., strains and crossbreeds reared in the Czech Republic to infection by cyprinid herpesvirus 3 (CyHV-3; KHV)." *Journal of Fish Diseases* **36**(1): 75-80.

Pietretti, D., Vera-Jimenez, N. I., Hoole, D. and Wiegertjes, G. F. (2013). "Oxidative burst and nitric oxide

responses in carp macrophages induced by zymosan, MacroGard® and selective dectin-1 agonists suggest recognition by multiple pattern recognition receptors." *Fish & Shellfish Immunology* **35**(3): 847-857.

Pijanowski, L., Verburg-van Kemenade, B. M. L., Irnazarow, I. and Chadzinska, M. (2015). "Stress-induced adaptation of neutrophilic granulocyte activity in K and R3 carp lines." *Fish & Shellfish Immunology* **47**(2): 886-892.

Pionnier, N., Adamek, M., Miest, J. J., Harris, S. J., Matras, M., Rakus, K. L., Irnazarow, I. and Hoole, D. (2014a). "C-reactive protein and complement as acute phase reactants in common carp *Cyprinus carpio* during CyHV-3 infection." *Diseases of Aquatic Organisms* **109**(3): 187-199.

Pionnier, N., Falco, A., Miest, J., Frost, P., Irnazarow, I., Shrive, A. and Hoole, D. (2013). "Dietary beta-glucan stimulate complement and C-reactive protein acute phase responses in common carp (*Cyprinus carpio*) during an *Aeromonas salmonicida* infection." *Fish & Shellfish Immunology* **34**(3): 819-831.

Pionnier, N., Falco, A., Miest, J. J., Shrive, A. K. and Hoole, D. (2014b). "Feeding common carp *Cyprinus carpio* with β-glucan supplemented diet stimulates C-reactive protein and complement immune acute phase responses following PAMPs injection." *Fish & Shellfish Immunology* **39**(2): 285-295.

Planas, N. (2000). "Bacterial 1,3-1,4-beta-glucanases: structure, function and protein engineering." *Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology* **1543**(2): 361-382.

Plant, K. P. and LaPatra, S. E. (2011). "Advances in fish vaccine delivery." *Developmental and Comparative Immunology* **35**(12): 1256-1262.

Plouffe, D. A., Hanington, P. C., Walsh, J. G., Wilson, E. C. and Belosevic, M. (2005). "Comparison of select innate immune mechanisms of fish and mammals." *Xenotransplantation* **12**(4): 266-277.

Podani, J. (2000). "Introduction to the exploration of multivariate biological data." *Introduction to the exploration of multivariate biological data*: i.

Powis, G. (1983). "Dose-dependent metabolism, therapeutic effect, and toxicity of anticancer drugs in man." *Drug Metab Rev* **14**(6): 1145-1163.

Przybylska-Diaz, D. A., Schmidt, J. G., Vera-Jimenez, N. I., Steinhagen, D. and Nielsen, M. E. (2013). "β-glucan enriched bath directly stimulates the wound healing process in common carp (*Cyprinus carpio* L.)." *Fish & Shellfish Immunology* **35**(3): 998-1006.

Raggi, T. and Gatlin, III, D. M. (2012). "Prebiotics Have Limited Effects on Nutrient Digestibility of a Diet Based on Fish Meal and Soybean Meal in Goldfish." *North American Journal of Aquaculture* **74**(3): 400-407.

Ramos, M. A., Weber, B., Goncalves, J. F., Santos, G. A., Rema, P. and Ozorio, R. O. A. (2013). "Dietary probiotic supplementation modulated gut microbiota and improved growth of juvenile rainbow trout (*Oncorhynchus mykiss*)." *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology* **166**(2): 302-307.

Ran, C., Huang, L., Liu, Z., Xu, L., Yang, Y. L., Tacon, P., Auclair, E. and Zhou, Z. G. (2015). "A Comparison of the Beneficial Effects of Live and Heat-Inactivated Baker's Yeast on Nile Tilapia: Suggestions on the Role and Function of the Secretory Metabolites Released from the Yeast." *Plos One* **10**(12): 16.

Ranjan, S., Bhushan, B., Panigrahi, M., Kumar, A., Deb, R., Kumar, P. and Sharma, D. (2015). "Association and Expression Analysis of Single Nucleotide Polymorphisms of Partial Tumor Necrosis Factor α Gene with Mastitis in Crossbred Cattle." *Animal Biotechnology* **26**(2): 98-104.

Reda, R. M., Mahmoud, R., Selim, K. M. and El-Araby, I. E. (2016). "Effects of dietary acidifiers on growth, hematology, immune response and disease resistance of Nile tilapia, *Oreochromis niloticus*." *Fish & Shellfish Immunology* **50**: 255-262.

Rendueles, O., Ferrieres, L., Fretaud, M., Begaud, E., Herbomel, P., Levraud, J. P. and Ghigo, J. M. (2012). "A New Zebrafish Model of Oro-Intestinal Pathogen Colonization Reveals a Key Role for Adhesion in Protection by Probiotic Bacteria." *Plos Pathogens* **8**(7).

Respondek, F., Gerard, P., Bossis, M., Boschat, L., Bruneau, A., Rabot, S., Wagner, A. and Martin, J. C. (2013). "Short-Chain Fructo-Oligosaccharides Modulate Intestinal Microbiota and Metabolic Parameters of Humanized Gnotobiotic Diet Induced Obesity Mice." *Plos One* **8**(8).

Rhee, K. J., Sethupathi, P., Driks, A., Lanning, D. K. and Knight, K. L. (2004). "Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire." *Journal of Immunology* **172**(2): 1118-1124.

Rieger, A. M. and Barreda, D. R. (2011). "Antimicrobial mechanisms of fish leukocytes." *Developmental and Comparative Immunology* **35**(12): 1238-1245.

Riley, J. and Edwards, P. (1998). "Statistical aspects of aquaculture research: pond variability and pseudoreplication." *Aquaculture Research* **29**(4): 281-288.

Ringo, E. and Gatesoupe, F. J. (1998). "Lactic acid bacteria in fish: a review." *Aquaculture* **160**(3-4): 177-203.

Ringo, E., Olsen, R. E., Jensen, I., Romero, J. and Lauzon, H. L. (2014). "Application of vaccines and dietary supplements in aquaculture: possibilities and challenges." *Reviews in Fish Biology and Fisheries* **24**(4): 1005-1032.

Rodriguez, I., Novoa, B. and Figueras, A. (2008). "Immune response of zebrafish (*Danio rerio*) against a newly isolated bacterial pathogen *Aeromonas hydrophila*." *Fish & Shellfish Immunology* **25**(3): 239-249.

Rojo, I., de Ilarduya, O. M., Estonba, A. and Pardo, M. A. (2007). "Innate immune gene expression in individual zebrafish after *Listonella anguillarum* inoculation." *Fish & Shellfish Immunology* **23**(6): 1285-1293.

Rombout, J., Abelli, L., Picchietti, S., Scapigliati, G. and Kiron, V. (2011). "Teleost intestinal immunology." *Fish & Shellfish Immunology* **31**(5): 616-626.

Romer Villumsen, K., Koppang, E. O. and Raida, M. K. (2015). "Adverse and long-term protective effects following oil-adjuvanted vaccination against *Aeromonas salmonicida* in rainbow trout." *Fish & shellfish immunology* **42**(1): 193-203.

Romero, J., Ringo, E. and Merrifield, D. L. (2014). "The Gut Microbiota of Fish." *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics* 75-100.

Ronza, P., Bermudez, R., Losada, A. P., Sitja-Bobadilla, A., Pardo, B. G. and Quiroga, M. I. (2015). "Immunohistochemical detection and gene expression of TNFα in turbot (*Scophthalmus maximus*) enteromyxosis." *Fish & Shellfish Immunology* **47**(1): 368-376.

Saad, N., Delattre, C., Urdaci, M., Schmitter, J. M. and Bressollier, P. (2013). "An overview of the last advances in probiotic and prebiotic field." *Lwt-Food Science and Technology* **50**(1): 1-16.

Salinas, I. (2015). "The Mucosal Immune System of Teleost Fish." *Biology-Basel* **4**(3): 525-539.

Sangma, T. and Kamilya, D. (2015). "*In vitro* and dietary effects of chitin on cellular and humoral immune parameters of catla, *Catla catla* (Hamilton)." *Journal of the World Aquaculture Society* **46**(6): 617-623.

Santander, J., Kilbourne, J., Park, J. Y., Martin, T., Loh, A., Diaz, I., Rojas, R., Segovia, C., DeNardo, D. and Curtis, R. (2014). "Inflammatory effects of *Edwardsiella ictaluri* lipopolysaccharide modifications in catfish gut." *Infection and Immunity* **82**(8): 3394-3404.

Savarese, M., Tousignant, K. D. and Uno, J. (2014). "The effect of wheat products on the colonization of

the microbiota in the intestines of zebrafish." *Faseb Journal* **28**(1): 1.

Schmidt, J. G., Andersen, E. W., Ersboll, B. K. and Nielsen, M. E. (2016). "Muscle wound healing in rainbow trout (*Oncorhynchus mykiss*)." *Fish & Shellfish Immunology* **48**: 273-284.

Schmitt, P., Wacyk, J., Morales-Lange, B., Rojas, V., Guzman, F., Dixon, B. and Mercado, L. (2015). "Immunomodulatory effect of cathelicidins in response to a β-glucan in intestinal epithelial cells from rainbow trout." *Developmental and Comparative Immunology* **51**(1): 160-169.

Schroers, V., Van der Marel, M., Neuhaus, H. and Steinhagen, D. (2009). "Changes of intestinal mucus glycoproteins after peroral application of *Aeromonas hydrophila* to common carp (*Cyprinus carpio*)." *Aquaculture* **288**(3-4): 184-189.

Schroers, V., Van der Marel, M. and Steinhagen, D. (2008). "Influence of carp intestinal mucus molecular size and glycosylation on bacterial adhesion." *Diseases of Aquatic Organisms* **81**(2): 135-142.

Secombes, C. J., Wang, T., Hong, S., Peddie, S., Crampe, M., Laing, K. J., Cunningham, C. and Zou, J. (2001). "Cytokines and innate immunity of fish." *Developmental & Comparative Immunology* **25**(8–9): 713-723.

Selvaraj, V., Sampath, K. and Sekar, V. (2005). "Administration of yeast glucan enhances survival and some non-specific and specific immune parameters in carp (*Cyprinus carpio*) infected with *Aeromonas hydrophila*." *Fish & Shellfish Immunology* **19**(4): 293-306.

Selvaraj, V., Sampath, K. and Sekar, V. (2006). "Adjuvant and immunostimulatory effects of β-glucan administration in combination with lipopolysaccharide enhances survival and some immune parameters in carp challenged with *Aeromonas hydrophila*." *Veterinary Immunology and Immunopathology* **114**(1-2): 15-24.

Shapira, Y., Magen, Y., Zak, T., Koder, M., Hulata, G. and Levavi-Sivan, B. (2005). "Differential resistance to koi herpes virus (KHV)/carp interstitial nephritis and gill necrosis virus (CNGV) among common carp (*Cyprinus carpio* L.) strains and crossbreds." *Aquaculture* **245**(1-4): 1-11.

Sharifuzzaman, S. M., Al-Harbi, A. H. and Austin, B. (2014). "Characteristics of growth, digestive system functionality, and stress factors of rainbow trout fed probiotics *Kocuria* SM1 and *Rhodococcus* SM2." *Aquaculture* **418**: 55-61.

Sink, T. D. and Lochmann, R. T. (2008). "Preliminary observations of mortality reduction in stressed, *Flavobacterium columnare* challenged golden shiners after treatment with a dairy-yeast prebiotic." *North American Journal of Aquaculture* **70**(2): 192-194.

Sirimanapong, W., Thompson, K. D., Ooi, E. L., Bekaert, M., Collet, B., Taggart, J. B., Bron, J. E., Green, D. M., Shinn, A. P., Adams, A. and Leaver, M. J. (2015). "The effects of feeding β-glucan to *Pangasianodon hypophthalmus* on immune gene expression and resistance to *Edwardsiella ictaluri*." *Fish & Shellfish Immunology* **47**(1): 595-605.

Siwicki, A. K., Anderson, D. P. and Rumsey, G. L. (1994). "Dietary intake of immunostimulants by rainbow trout affects nonspecific immunity and protection against furunculosis." *Veterinary Immunology and Immunopathology* **41**(1-2).

Spanggaard, B., Huber, I., Nielsen, J., Sick, E. B., Pipper, C. B., Martinussen, T., Slierendrecht, W. J. and Gram, L. (2001). "The probiotic potential against vibriosis of the indigenous microflora of rainbow trout." *Environmental Microbiology* **3**(12): 755-765.

Standen, B. T., Rodiles, A., Peggs, D. L., Davies, S. J., Santos, G. A. and Merrifield, D. L. (2015). "Modulation of the intestinal microbiota and morphology of tilapia, *Oreochromis niloticus*, following the application of a multi-species probiotic." *Applied Microbiology and Biotechnology* **99**(20): 8403-8417.

Steinum, T., Sjastad, K., Falk, K., Kvellestad, A. and Colquhoun, D. J. (2009). "An RT PCR-DGGE survey of gill-associated bacteria in Norwegian seawater-reared Atlantic salmon suffering proliferative gill inflammation." *Aquaculture* **293**(3-4): 172-179.

Subramanian, S., Ross, N. W. and MacKinnon, S. L. (2008). "Comparison of antimicrobial activity in the epidermal mucus extracts of fish." *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **150**(1): 85-92.

Sugahara, K. and Eguchi, M. (2012). "The use of warmed water treatment to induce protective immunity against the bacterial cold-water disease pathogen Flavobacterium psychrophilum in ayu (*Plecoglossus altivelis*)." *Fish & Shellfish Immunology* **32**(3): 489-493.

Sugita, H., Hirose, Y., Matsuo, N. and Deguchi, Y. (1998). "Production of the antibacterial substance by *Bacillus* sp. strain NM 12, an intestinal bacterium of Japanese coastal fish." *Aquaculture* **165**(3-4): 269-280.

Sugita, H., Shibuya, K., Shimooka, H. and Deguchi, Y. (1996). "Antibacterial abilities of intestinal bacteria in freshwater cultured fish." *Aquaculture* **145**(1-4): 195-203.

Sun, H., Jami, E., Harpaz, S. and Mizrahi, I. (2013a). "Involvement of dietary salt in shaping bacterial communities in European sea bass (*Dicentrarchus labrax*)." *Scientific Reports* **3**.

Sun, Y. Z., Yang, H. L., Huang, K. P., Ye, J. D. and Zhang, C. X. (2013b). "Application of autochthonous *Bacillus* bioencapsulated in copepod to grouper *Epinephelus coioides* larvae." *Aquaculture* **392**: 44-50.

Sun, Y. Z., Yang, H. L., Ma, R. L. and Zhai, S. W. (2012a). "Does dietary administration of *Lactococcus lactis* modulate the gut microbiota of grouper, *Epinephelus coioides*." *Journal of the World Aquaculture Society* **43**(2): 198-207.

Sun, Y. Z., Xia, H. Q., Yang, H. L., Wang, Y. L. and Zou, W. C. (2014). "TLR2 signaling may play a key role in the probiotic modulation of intestinal microbiota in grouper *Epinephelus coioides*." *Aquaculture* **430**: 50-56.

Sun, Y. Z., Yang, H. L., Ma, R. L., Huang, K. P. and Ye, J. D. (2012b). "Culture-independent characterization of the autochthonous gut microbiota of grouper *Epinephelus coioides* following the administration of probiotic *Enterococcus faecium*." *Aquaculture International* **20**(4): 791-801.

Sunyer, J. O., Boshra, H., Lorenzo, G., Parra, D., Freedman, B. and Bosch, N. (2003). "Evolution of complement as an effector system in innate and adaptive immunity." *Immunologic Research* **27**(2-3): 549-564.

Sunyer, J. O., Tort, L. and Lambris, J. D. (1997). "Structural C3 diversity in fish - Characterization of five forms of C3 in the diploid fish *Sparus aurata*." *Journal of Immunology* **158**(6): 2813-2821.

Sunyer, J. O., Zarkadis, I. K., Sahu, A. and Lambris, J. D. (1996). "Multiple forms of complement C3 in trout that differ in binding to complement activators." *Proceedings of the National Academy of Sciences of the United States of America* **93**(16): 8546-8551.

Swain, P., Nayak, S. K., Nanda, P. K. and Dash, S. (2008). "Biological effects of bacterial lipopolysaccharide (endotoxin) in fish: A review." *Fish & Shellfish Immunology* **25**(3): 191-201.

Syakuri, H., Adamek, M., Brogden, G., Rakus, K. L., Matras, M., Irnazarow, I. and Steinhagen, D. (2013). "Intestinal barrier of carp (*Cyprinus carpio* L.) during a cyprinid herpesvirus 3-infection: Molecular identification and regulation of the mRNA expression of claudin encoding genes." *Fish & Shellfish Immunology* **34**(1): 305-314.

Syakuri, H., Jung-Schroers, V., Adamek, M., Brogden, G., Irnazarow, I. and Steinhagen, D. (2014). "β-glucan feeding differentiated the regulation of mRNA expression of claudin genes and prevented an intestinal inflammatory response post Aeromonas hydrophila intubation in common carp, *Cyprinus carpio* L." *Journal of Fish Diseases* **37**(2): 149-156.

Tamamdusturi, R., Widanarni and Yuhana, M. (2015). "Administration of Microencapsulated Probiotic *Bacillus* sp NP5 and Prebiotic Mannan Oligosaccharide for Prevention of *Aeromonas hydrophila* Infection on *Pangasianodon hypophthalmus*." *Journal of Fisheries and Aquatic Science* **11**(1): 67-76.

Tapia-Paniagua, S., Lobo, C., Moreno-Ventas, X., de la Banda, I. G., Morinigo, M. A. and Balebona, M. C. (2014). "Probiotic Supplementation Influences the Diversity of the Intestinal Microbiota During Early Stages of Farmed Senegalese Sole (*Solea senegalensis*, Kaup 1858)." *Marine Biotechnology* **16**(6): 716-728.

Tapia-Paniagua, S. T., Vida, S., Lobo, C., de la Banda, I. G., Esteban, M. A., Balebona, M. C. and Morinigo, M. A. (2015). "Dietary administration of the probiotic SpPdp11: Effects on the intestinal microbiota and immune-related gene expression of farmed *Solea senegalensis* treated with oxytetracycline." *Fish & Shellfish Immunology* **46**(2): 449-458.

Tarpy, D. R., Mattila, H. R. and Newton, I. L. G. (2015). "Development of the Honey Bee Gut Microbiome throughout the Queen-Rearing Process." *Applied and Environmental Microbiology* **81**(9): 3182-3191.

Taylor, N. G. H., Peeler, E. J., Denham, K. L., Crane, C. N., Thrush, M. A., Dixon, P. F., Stone, D. M., Way, K. and Oidtmann, B. C. (2013). "Spring viraemia of carp (SVC) in the UK: The road to freedom." *Preventive Veterinary Medicine* **111**(1-2): 156-164.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). "Clustal-W – improving the sensitivity of progressive multiple sequence alignment through weighting, position-specific gap penalties and weight matrix choice." *Nucleic Acids Research* **22**(22): 4673-4680.

Thompson, J. R., Randa, M. A., Marcelino, L. A., Tomita-Mitchell, A., Lim, E. and Polz, M. F. (2004). "Diversity and dynamics of a north Atlantic coastal *Vibrio* community." *Applied and Environmental Microbiology* **70**(7): 4103-4110.

Toranzo, A. E., Magarinos, B. and Romalde, J. L. (2005). "A review of the main bacterial fish diseases in mariculture systems." *Aquaculture* **246**(1-4): 37-61.

Torrecillas, S., Makol, A., Caballero, M. J., Montero, D., Gines, R., Sweetman, J. and Izquierdo, M. (2011). "Improved feed utilization, intestinal mucus production and immune parameters in sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides (MOS)." *Aquaculture Nutrition* **17**(2): 223-233.

Tursi, A., Elisei, W., Picchio, M., Giorgetti, G. and Brandimarte, G. (2015). "Accuracy of rapid fecal calprotectin test in monitoring inflammatory bowel diseases under treatment with TNFα antagonists." *Digestive Diseases and Sciences* **60**(5): 1406-1413.

Van der Marel, M., Adamek, M., Gonzalez, S. F., Frost, P., Rombout, J., Wiegertjes, G. F., Savelkoul, H. F. J. and Steinhagen, D. (2012). "Molecular cloning and expression of two β-defensin and two mucin genes in common carp (*Cyprinus carpio* L.) and their up-regulation after β-glucan feeding." *Fish & Shellfish Immunology* **32**(3): 494-501.

Vera-Jimenez, N. I., Pierretti, D., Wiegertjes, G. F. and Nielsen, M. E. (2013). "Comparative study of β-glucan induced respiratory burst measured by nitroblue tetrazolium assay and real-time luminol-enhanced chemiluminescence assay in common carp (*Cyprinus carpio* L.)." *Fish & Shellfish Immunology* **34**(5): 1216-1222.

Vera-Jimenez, N. J. and Nielsen, M. E. (2013). "Carp head kidney leukocytes display different patterns of oxygen radical production after stimulation with PAMPs and DAMPs." *Molecular Immunology* **55**(3-4): 231-236.

Vetvicka, V., Vannucci, L. and Sima, P. (2013). "The effects of β-glucan on fish immunity." *North American journal of medical sciences* **5**(10): 580-588.

Villamil, L., Reyes, C. and Martinez-Silva, M. A. (2014). "*In vivo* and *in vitro* assessment of *Lactobacillus acidophilus* as probiotic for tilapia (*Oreochromis niloticus*, Perciformes:Cichlidae) culture improvement." *Aquaculture Research* **45**(7): 1116-1125.

Villarroel, F., Bastias, A., Casado, A., Amthauer, R. and Concha, M. I. (2007). "Apolipoprotein A-I, an antimicrobial protein in *Oncorhynchus mykiss*: Evaluation of its expression in primary defence barriers and plasma levels in sick and healthy fish." *Fish & Shellfish Immunology* **23**(1): 197-209.

Wang, J. L., Meng, X. L., Lu, R. H., Wu, C., Luo, Y. T., Yan, X., Li, X. J., Kong, X. H. and Nie, G. X. (2015). "Effects of *Rehmannia glutinosa* on growth performance, immunological parameters and disease resistance to *Aeromonas hydrophila* in common carp (*Cyprinus carpio* L.)." *Aquaculture* **435**: 293-300.

Wang, Y. B., Li, J. R. and Lin, J. D. (2008). "Probiotics in aquaculture: Challenges and outlook." *Aquaculture* **281**(1-4): 1-4.

Weinstock, G. M. (2012). "Genomic approaches to studying the human microbiota." *Nature* **489**(7415): 250-256.

Welker, T. L., Lim, C., Yildirim-Aksoy, M. and Klesius, P. H. (2012). "Effect of short-term feeding duration of diets containing commercial whole-cell yeast or yeast subcomponents on immune function and disease resistance in channel catfish, *Ictalurus punctatus*." *Journal of Animal Physiology and Animal Nutrition* **96**(2): 159-171.

Welker, T. L., Lim, C., Yildirim-Aksoy, M., Shelby, R. and Klesius, P. H. (2007). "Immune response and resistance to stress and *Edwardsiella ictaluri* challenge in channel catfish, *Ictalurus punctatus*, fed diets containing commercial whole-cell yeast or yeast subcomponents." *Journal of the World Aquaculture Society* **38**(1): 24-35.

Wiegertjes, G. F., Wentzel, A. S., Spaink, H. P., Elks, P. M. and Fink, I. R. (2016). "Polarization of immune responses in fish: The 'macrophages first' point of view." *Molecular Immunology* **69**: 146-156.

Wu, S. G., Tian, J. Y., Wang, G. T., Li, W. X. and Zou, H. (2012a). "Characterization of bacterial community in the stomach of yellow catfish (*Pelteobagrus fulvidraco*)." *World Journal of Microbiology & Biotechnology* **28**(5): 2165-2174.

Wu, S. G., Wang, G. T., Angert, E. R., Wang, W. W., Li, W. X. and Zou, H. (2012b). "Composition, diversity, and origin of the bacterial community in grass carp intestine." *Plos One* **7**(2): 11.

Yang, H. L., Sun, Y. Z., Ma, R. L. and Ye, J. D. (2012). "PCR-DGGE analysis of the autochthonous gut microbiota of grouper *Epinephelus coioides* following probiotic *Bacillus clausii* administration." *Aquaculture Research* **43**(4): 489-497.

Yang, H. L., Xia, H. Q., Ye, Y. D., Zou, W. C. and Sun, Y. Z. (2014). "Probiotic *Bacillus pumilus* SE5 shapes the intestinal microbiota and mucosal immunity in grouper *Epinephelus coioides*." *Diseases of Aquatic Organisms* **111**(2): 119-127.

Ye, J. D., Wang, K., Li, F. D. and Sun, Y. Z. (2011). "Single or combined effects of fructo- and mannan oligosaccharide supplements and *Bacillus clausii* on the growth, feed utilization, body composition, digestive enzyme activity, innate immune response and lipid metabolism of the Japanese flounder *Paralichthys olivaceus*." *Aquaculture Nutrition* **17**(4): E902-E911.

Zhang, C. N., Li, X. F., Xu, W. N., Zhang, D. D., Lu, K. L., Wang, L. N., Tian, H. Y. and Liu, W. B. (2015). "Combined effects of dietary fructooligosaccharide and *Bacillus licheniformis* on growth performance, body composition, intestinal enzymes activities and gut histology of triangular bream (*Megalobrama terminalis*)." *Aquaculture Nutrition* **21**(5): 755-766.

Zhang, Z., Xu, L., Liu, W. S., Yang, Y. L., Du, Z. Y. and Zhou, Z. G. (2014). "Effects of partially replacing dietary soybean meal or cottonseed meal with completely hydrolyzed feather meal (defatted rice bran as the carrier) on production, cytokines, adhesive gut bacteria, and disease resistance in hybrid tilapia (*Oreochromis niloticus* female x *Oreochromis aureus* male)." *Fish & Shellfish Immunology* **41**(2): 517-525.

Zhou, Q. C., Buentello, J. A. and Gatlin, D. M. (2010). "Effects of dietary prebiotics on growth performance, immune response and intestinal morphology of red drum (*Sciaenops ocellatus*)." *Aquaculture* **309**(1-4): 253-257.

Zhou, Y., Yuan, X. C., Liang, X. F., Fang, L., Li, J., Guo, X. Z., Bai, X. L. and He, S. (2013a). "Enhancement of growth and intestinal flora in grass carp: The effect of exogenous cellulase." *Aquaculture* **416**: 1-7.

Zhou, Z., Karlsen, O., He, S., Olsen, R. E., Yao, B. and Ringo, E. (2013b). "The effect of dietary chitin on the autochthonous gut bacteria of Atlantic cod (*Gadus morhua* L.)." *Aquaculture Research* **44**(12): 1889-1900.

Zhu, L. Y., Nie, L., Zhu, G., Xiang, L. X. and Shaom J. Z. (2013). "Advances in research of fish immune-relevant genes: A comparative overview of innate and adaptive immunity in teleosts." *Developmental and Comparative Immunology* **39**(1-2): 39-62.

Zhu, S. Y. and Gao, B. (2013). "Evolutionary origin of β-defensins." *Developmental and Comparative Immunology* **39**(1-2): 79-84.

Zychowski, K. E., Pohlenz, C., Mays, T., Romoser, A., Hume, M., Buentello, A., Gatlin III, D. M. and Phillips, T. D. (2013). "The effect of NovaSil dietary supplementation on the growth and health performance of Nile tilapia (*Oreochromis niloticus*) fed aflatoxin-B1 contaminated feed." *Aquaculture* **376**: 117-123.

WHO fact sheet no. 194. http://www.who.int/mediacentre/factsheets/fs194/en/ (accessed 14.07.2015)

http://www.theguardian.com/lifeandstyle/2009/nov/01/eels-on-tv-menu-protest (accessed 09.07.2015)

http://news.bbc.co.uk/1/hi/sci/tech/2580733.stm (accessed 09.07.2015)

http://www.bbc.co.uk/news/world-asia-31724554 (accessed 09.07.2015)

Appendix 1.

Accession numbers for the 16S rDNA sequences analysed in Chapter 2 from different bacterial families and genera.

Aeromonas sp.

AY532690 AY532691 AY987746 AY987747 AY987776 AY987777 FJ599747 AY987723 AY987725 Y987726 AY987727 AY987728 AY987730 AY987732 AY987733 AY987734 AY987735 AY987755 AY987756 AY987757 AY987758 AY987759 AY987760 AY987761 AY987772 AY987773 AY987731 AY987738 AY987740 AY987743 AY987752 AY987753 AY987764 DQ207728 AY987765 AY987766 AY987767 AY987768 AY987769 AY987770 AY987771 AY987774 AY987775 AY987724 AY987729 AY987744 AY987751 AY987762 AY987763 AY987778 GQ426312 AB027541 AB027542 AB027543 AM179869 AM179870 AM179874 AM179877 AM179878 AM179893 AM179898 AB027005 AB027006 AB027545 AB027546 AB034759 AB034760 AB034762 AB034763 AJ223181 AJ224308 X60407 AB034761 X60404 X60405 X60406 X60408 X60409 X60410 X60411 X60412 X60417 AJ491712 AJ491714 AJ491716 AJ585221 X60413 X60415 X60416 AB028881 AJ223179 AJ318898 AJ318899 AJ416907 AM184217 AM184242 AM184246 AM184262 AM184282 AM184287 AM184292 AM184293 AJ458408 AM184306 AM263528 AM263529 AM263531 AJ458409 AJ458411 AJ458414 AJ458415 AJ458416 AJ536820 AJ223182 AJ536821 AM262153 AM262155 AM262156 AM397652 AM397653 AF418209 AF418210 AF418211 AF418212 AF418213 AF418214 AF418215 AF418216 AF418217 X60414 AB027544 AF418218 AF418219 AJ009859 AJ458402 AM296501 AM296502 AM296504 AM296506 AM296507 AF472489 AF472490 AF472491 AF472492 AF472493 AM262151 AM296508 AM296509 AM296510 AF472494 AF472495 AF472496 AF472497 AF472498 AF472499 AF472500 AF472501 AF472502 AF472503 AF472504 AF472505 AF472506 EU488681 EU488682 EU488683 EU488684 EU488685 EU488686 EU488687 EU488688 EU488689 EU488690 EU488691 EU488692 EU488693 EU488694 EU488695 EU488696 EU488697 EU488698 EU488699 FJ168769 FJ168770 GQ891129 HQ832414 HQ832415 HQ832416 HQ832417 JN559379 FJ168771 FJ168772 FJ168773 FJ168774 FJ168775 FJ168776 FJ168777 KC210757 KC210758 KC210759 KC210760 KC210761 KC210777 KC210787 KC210788 KC210789 KC210790 KC210791 KC210792 KC210794 KC210795 KC210796 KC210797 KC210798 KC210799 KC210800 KC210801 KC210802 KC210803 GU174503 GU174504 GU174505 GU187060 GU205190 GU205191 KC210804 KC210805 KC210806 KC210807 GU205192 GU205193 GU205194 GU205195 GU205196 GU205197 GU205198 GU205199 GU205200 GU205201 FJ940793 FJ940794 FJ940795 FJ940796 FJ940797 FJ940798 FJ940799 FJ940800 FJ940801 JX308270 FJ940802 FJ940803 FJ940804 FJ940805 FJ940806 FJ940807 FJ940808 FJ940809 FJ940810 FJ940811 FJ940812 FJ940813 FJ940814 FJ940815 FJ940816 FJ940817 FJ940818 FJ940819 FJ940820 FJ940821 FJ940822 FJ940823 FJ940824 FJ940825 FJ940826 FJ940827 FJ940828 FJ940829 FJ940830 FJ940831 FJ940832 FJ940833 FJ940834 FJ940835 FJ940836 FJ940837 FJ940838 FJ940839 FJ940840 FJ940841 FJ940842 FJ940843 FJ940844 FJ940845 FJ940846 FJ940847 FJ940848 FJ940849 FJ940850 AF468056 AJ278105 DQ979324 EU770270 EF681112 EF681113 FN432809 GQ466170 HE610109 EU770271 EU770272 EU770273 FJ966347 FJ966348 JN412503 JQ004789 JX286501 JX987090 EU770274 EU770275 EU770276 EU770277 EU770278 EU770279 EU770280 EU770281 EU770282 EU770283 EU770284 EU770285 EU770286 EU770287 EU770288 EU770289 EU770290 EU770291 EU770292 EU770293 EU770294 EU770295 EU770296 EU770297 EU770298 EU770299 EU770300 EU770301 EU770302 EU770303 EU770304 EU770305 EU770306 EU770307 EU770308 EU770309 FJ233856 FJ233857 FJ233859 AY538658 FJ233861 FJ233866 FJ407187 FJ462702 GQ844300 GQ844301 GQ844302 GQ844303 AB472906 GQ845450 GQ845451 GQ845452 GQ845453 KC776583 KC776584 KC776585 KC776586 KC776587 AB472919 AB472920 AB472921 AB472922 AB472923 AB472924 AB472925 AB472926 AB472927 AB472928 AB472929 AB472930 AB472931 AB472932 AB472933 AB472934 AB472935 AB472936 AB472937 AB472938 AB472939 AB472940 AB472941 AB472955 AB472956 AB472959 AB472969 AB472970 AB472971 AB472972 AB472973 AB472975 AB472977 AB472983 AB472984 AB472985 AB472986 AB473021 AB473022 AB473023 AB473024 AB473025 AB473026 AB473027 AB473028 AB473029 AB473030 AB473031 AB473032 AB473033 AB473034 AB473035 AB473036 AB473037 AB473038 AB473039 AB473040 AB473041 AB473042 AB473043 AB270590 AF200329 HM032146 HM044908 X74675 EU254223

Accession numbers for the 16S rDNA sequences analysed in Chapter 2 from different bacterial families and genera.

Aeromonas sp.

EU254224 HM044909 HM044910 HM044911 HM044912 HQ124005 EU254225 EU254226 EU254227 EU368681 EU368682 EU368683 EU368684 EU678635 GQ184148 GQ262778 GQ334329 JX888463 KC252599 KC252600 KC254647 KC254648 KC254649 KC254650 KC473947 KC507819 KC549803 KC549804 HQ407423 HQ453345 KC549805 KC549806 KC549807 KC549808 JF920473 JF920474 JF920475 JF920476 JF920477 JF920478 JF920479 JF920480 JF920481 JF920482 JF920483 JF920484 JF920485 JF920486 JF920487 JF920488 JF920489 JF920490 JF920491 JF920492 JF920494 JF920495 JF920496 JF920497 JF920498 JF920499 JF920500 JF920501 JF920502 JF920503 JF920504 JF920505 JF920506 JF920509 JF920510 JF920513 JF920514 JF920515 JF920516 JF920517 JF920518 JF920519 JF920520 JF920533 JF920534 JF920535 JF920536 JF920537 JF920538 JF920539 JF920540 JF920541 JF920542 JF920543 JF920544 JF920545 JF920546 JF920547 JF920548 JF920549 JF920550 JF920551 JF920552 JF920553 JF920554 JF920555 JF920557 JF920559 JF920560 JF920561 JF920563 AF079299 AF079300 AF079301 GQ983052 GU169711 GU296671 JF920507 JF920508 JF920511 JF920512 EF077527 GU296672 GU296673 JX122726 JX155398 JX390650 JX390651 KC130961 KC130962 GU596499 GU722154 KC130963 KC130964 KC130965 KC130966 KC130967 JN051353 JN120259 JN120260 JN120261 JN120263 JN120264 JN120265 JN120269 JN120271 JN120272 JN120273 JN120280 JN120281 JN120283 JN120284 JN120290 JN120292 JN120293 JN120295 JN120296 JN120297 JN120298 JN120299 JN120302 JN120303 JN120304 JN120306 JN120308 JN120309 JN400039 JN400040 JN400041 JN400042 JN400046 JQ040101 JQ040102 JQ040103 JQ040104 JQ040105 JQ040106 JQ040107 JQ040108 JQ040109 JQ040110 JQ040111 JQ040112 JQ040113 JQ040114 JQ040115 JQ040116 JQ040117 JQ040118 JQ040119 JQ040120 JQ040121 JQ040122 JQ040123 JQ040124 JQ045713 FJ026735 FJ202054 FJ515776 FJ515777 FJ599747 FJ607409 FJ607412 FJ660445 FJ660446 JQ599381 FJ660447 FJ660448 FJ660449 FJ660453 AY422755 AY827493 AY827494 AY987746 AY987747 AY987776 FJ660458 FJ660459 AY987777 EU913838 EU913839 EU913840 EU913841 EU913842 EU913843 EU913844 EU913845 EU913846 AJ508765 EU913849 EU913850 EU913851 EU913854 EU913855 EU913856 EU913858 EU913859 EU913860 AM296503 AM296505 EU696781 GQ401237 HM240294 JN120266 FJ998415 FJ998416 FJ998417 KC150866 KC776588 KC776589 KC776590 AJ133636 AJ308468 AM181660 AM913921 AY928476 AY928481 FJ666317 AJ458403 AJ458404 AJ489337 AJ489339 AM184259 AM235169 AM269519 AJ876662 FJ562211 AJ876663 AJ876679 AJ876681 GQ259885 U88662 KC210762 KC210763 KC210764 KC210765 KC210766 KC210767 KC210768 KC210769 KC210770 KC210771 KC210772 KC210773 KC210774 KC210775 KC210776 KC210778 KC210779 KC210780 KC210781 KC210782 AF468055 EF681114 FJ936134 GQ470995 GQ470996 JX262991 KC210783 KC210784 KC210785 KC210786 AM689939 GQ304779 GU563992 JX262992 DQ217638 JN561162 AB472902 AB472903 AB472904 AB472905 AB472942 AB472943 AB472944 AB472945 AB472946 AB472947 AB472948 AB472950 AB472952 AB472998 AB472999 AB473000 AB473001 AB473002 AB473003 AB473004 AB473005 AB473006 AB473007 AB473008 AB473009 AB473010 AB473011 AB473012 AB473013 AB473014 AB473015 AB473016 AB473017 AB473018 AB473019 AB473020 KC884665 KC884669 KC884670 KC884671 DQ822699 KC884672 KC884673 KC884674 DQ822746 DQ822759 X74676 A. X74677 X74678 X74679 X74680 X74681 X74682 X74683 X74684 EF645798 EF645799 EF669478 EF669480 EU254233 GQ141871 AY130991 AY130992 GQ205446 GQ232759 GQ860944 HQ541164 HQ541165 HQ609947 JF490061 JF490062 JF490063 JF490064 JF490065 JF490066 JF490067 JF490068 JF490069 JF490070 JN019024 DQ133170 GU563993 GU563994 GU563995 JX294914 JN083778 JN836327 JN836328 JN836329 JN836330 JN836331 JN836332 JN836333 JN836334 JQ319029 AY987723 AY987725 AY987726 AY987727 AY987728 AY987730 AY987732 AY987733 AY987734 AY987735 AY987736 AY987737 AY987739 AY987741 AY987742 AY987745 AY987748 AY987749 AY987750 AY987754 AY987755 AY987756 AY987757 AY987758 AY987759 AY987760 AY987761 AY987772 AY987757 AY987758 AY987759 AY987760 AY987761 AY987772 AY987773 GQ141871 GQ205446 GQ232759 AB181995 AB181997 AB181998 AB181999 GU563993 GU563994 GU563995 JX294914 AB182000 AB182003 AB182004 AB182005 AB182007 AB182008 AB182009 AB182010 AB182011 AB182012 AB182013 AB182014 AB182015 AB182016 AB182017 AB182018 AB182019 AB368776 AB182020 AB182021 AB182022 AB182023 AB182024 AB182026

Accession numbers for the 16S rDNA sequences analysed in Chapter 2 from different bacterial families and genera.

Aeromonas sp.

AB182027 AB182028 AB182029 AB182030 AB182031 AB182032 AB182033 AB182034 AB182036 AB182037 AB182042 AB182044 AB182045 AB182048 AB182049 AB182050 AB182051 AB182052 AB182054 AB182055 AB182056 AB182063 AB182073 AB182081 AB182082 AB182083 AB182084 AB182085 AB182086 AB182089 AB182090 AB182091 AB182092 AB182093 AB182094 AB182095 AB182096 AB182097 AB182098 AB182099 AB182100 AB182164 AB182168 AB182169 AB182171 AB182172 AB182173 AB182176 AB182183 AB182193 AB182196 AB182197 AB182198 AB182210 AB182212 AB182217 AB182219 AB182220 AB182225 AB182226 AB182232 AB182237 AB182238 AB182239 GQ860945 HQ189118 HQ189119 EU932930 EU932932 EU932935 EU932936 EU932937 EU932941 EU932944 EU932947 HQ189120 HQ189121 EU932948 EU932949 EU932950 EU932952 EU932953 EU932954 EU932956 EU932957 EU932958 FJ230076 FJ230077 FJ464567 FJ464568 FJ464569 FJ464570 FJ464572 FJ653620 FJ464573 FJ464578 FJ464580 FJ464581 FJ464582 FJ464584 AJ458405 AJ458406 AJ458407 AJ458410 AJ458412 AJ458413 AJ786806 AM236305 DQ095911 DQ127823 FJ422569 AF118387 AF118388 AF157690 AF509473 JN038331 JN038335 JN038336 JN106432 JN106434 JN106435 JN108034 JN108035 JN129493 JN561149 JN561697 JN621033 JN621034 JN644057 JN644058 JN644059 JN644060 JN644061 JN644062 JN644063 JN644064 JN644065 JN644542 JN644562 JN644579 JN644601 JN644602 JN703730 JN796925 JQ246785 JQ301790 JQ301791 JQ315431 JQ389574 JQ389577 JQ389578 JQ389579 JQ389587 JQ389588 JQ389594 AY136078 AY136084 AY264937 AY297782 GQ983054 GU003810 GU003818 JQ389667 JQ511594 JQ511641 GU003826 GU003829 GU003836 GU013470 GU227144 GU295963 GU296111 GU319978 FJ794069 FJ808727 FJ947060 FJ976606 FM208196 FM208200 FM209206 FM875883 EF579776 EF634207 EF634214 EF634217 EF634218 EF634219 EF634220 EF634223 EF634228 EU082830 EU082831 EU085557

Bacillus sp.

AJ316309 EU360724 EU360725 JX512716 KF632441 KF699134 KF700084 KF700242 KF840793 HQ834723 KF578076 KF636527 KF636528 KF636529 KF636530 KF669646 KF673102 KF673349 KF751643 JQ323103 KF673350 KF715621 KF715622 KF717600 KF850148 KF914405 KF914406 KF914407 JQ806056 KC152050 KF658192 KF668669 KF914412 KF914413 KF939332 JQ806052 JQ806053 KF668670 KF668671 KF668672 KF668673 KF668674 KF683170 KF683171 KF815527 HG794253 JQ806054 KC634259 KC634265 KC634272 KC634275 KC634279 KC634280 KF447399 KF447429 KF515654 KF641792 KF641807 KF641812 KF641820 KF641821 KF641824 KF641825 KF641826 KF641827 KF641830 KF641831 KF641832 KF641833 KF641834 KF641836 KF641852 KF656779 KF672748 KF675197 KF687005 KF687006 KF687008 KF687009 KF687014 KF687015 KF687022 KF687024 KF687025 KF687028 KF687031 KF687032 KF687036 KF687037 KF687038 KF687039 KF687040 KF687041 KF687042 KF687043 KF687045 KF687046 KF687047 KF687048 KF687049 KF687051 KF687052 KF687054 KF687055 KF687057 KF687058 KF687059 KF687061 KF687062 KF687064 KF687065 KF687066 KF687067 KF687068 KF687071 KF687073 KF687074 KF687075 KF687076 KF687077 KF687078 KF687079 KF687080 KF687081 KF687083 KF687085 KF687086 KF687087 KF687088 KF687089 KF687091 KF687092 KF687093 KF687094 KF687095 KF687096 KF717367 KF725719 KF751576 KF751578 KF831379 KF831381 KF831382 KF831388 KF831390 KF831393 KF831395 KF831396 KF831398 JN084160 JN644484 JX984634 JX984639 KF831399 KF831401 KF831402 KF892538 KF951357 KF951358 FJ867919 FJ867921 HF584764 HF584770 HF584771 HF584780 HF584790 HF584795 HF584799 JX984640 HF584801 HF584802 HF584803 HF584808 HF584810 HF584812 HF584814 HF584819 HF584826 HF584829 HF584833 HF584834 HF584839 HF584841 HF584843 HF584845 HF584848 HF584854 HF584877 HF584901 HF584905 HF584906 HF584908 HF584910 HF584911 HF584912 HF584916 HF584917 HF584923 HF584925 HF584928 JX274438 HF584935 HF584946 HF584947 HF584998 HF585005 HF585007 HF585010 HF585012 HF585017 HF585020 KC223570 KC223571 KC223572 KC223573 KC223574

Accession numbers for the 16S rDNA sequences analysed in Chapter 2 from different bacterial families and genera.

Bacillus sp.

KF725636 AB669589 HG794254 KC223575 KC223576 KC414931 KC634250 KC634261 KC634292 KC634308 KF179185 KF179188 KF179189 KF447403 KF447406 KF447420 KF447426 KF515652 KF515657 KF515665 KF560310 KF560311 KF560312 KF564276 KF564277 KF574822 KF624694 KF640224 KF641789 KF641790 KF641791 KF641793 KF641794 KF641795 KF641798 KF641799 KF641801 KF641802 KF641803 KF641804 KF641806 KF641809 KF641810 KF641811 KF641813 KF641814 KF641815 KF641817 KF641818 KF641819 KF641835 KF641839 KF641841 KF641846 KF641848 KF644462 KF668238 KF668617 KF680995 KF680996 KF686781 KF687013 KF687027 KF687090 KF696627 KF704144 KF704747 KF709956 KF709958 KF709960 KF710009 KF710010 KF710015 KF710016 KF710017 KF710018 KF710019 KF710020 KF710021 KF710022 KF710024 KF710026 KF710028 KF710031 KF726124 KF710033 KF712534 KF717365 KF717366 KF717499 KF717500 KF717514 KF717517 KF717518 KF717519 KF717520 KF718837 KF724028 KF724029 KF724906 KF728603 KF735116 KF737353 KF737354 KF737861 KF747359 KF766113 KF800760 KF800766 KF800768 KF800774 KF800778 KF800780 KF800786 KF800788 KF800789 KF800794 KF803996 KF831365 KF831366 KF831367 KF831368 KF831369 KF831370 KF831371 KF831372 KF831377 KF831383 KF831384 KF831392 KF831397 KF831400 KF835724 KF835728 KF835731 KF835812 KF835815 KF835820 KF835822 KF835829 KF835839 KF861580 KF861581 KF861583KF861585 KF861588 KF861589 KF861590 KF861592 KF861594 KF861595 KF861596 KF861597 KF861601 KF861603 KF861604 KF861605 KF861606 KF861607 KF861608 KF861611 KF861612 KF861615 KF861617 KF861618 KF861776 KF861777 KF892535 KF900213 KF900214 KF934439 KF939126 KF939127 KF939128 KF939131 KF951359 KF951360 KF957865 KF957997 KF958869 KF958870 HQ224517 HQ284888 HQ433452 HQ433466 HQ433471 JN084129 JN084137 JN084146 JN084155 JN084158 JN644485 JN644487 JN644507 JN644518 JN644540 JN644541 JN644554 JN644556 JN644557 JN644572 JN644573 JN644612 JN644613 JQ905061 JQ905068 JQ905069 JQ905070 JQ905071 JQ905072 JQ905073 JQ905074 JQ905075 JQ905077 JQ905078 JQ905079 JQ905080 JQ905081 JQ905083 JQ905084 JQ905085 JQ905092 JQ905096 JX239694 JX281763 JX281764 JX281765 JX281767 JX281768 JX281772 JX281774 JX281782 JX281783 JX281786 JX281790 JX984632 JX984641 FJ867920 FJ867925 GU322908 JX984645 JX984646 JX987716 KC110837 KC315897 KC315899 KC315907 GU583651 HF562877 HF562888 HF562894 HF584760 HF584761 HF584765 HF584766 HF584767 HF584768 HF584769 HF584772 HF584773 HF584774 HF584775 HF584776 HF584777 HF584779 HF584781 HF584782 HF584783 HF584789 HF584794 HF584797 HF584807 HF584809 HF584811 HF584813 HF584816 HF584817 HF584818 HF584821 HF584822 HF584823 HF584824 HF584827 HF584828 KC311558 HF584831 HF584832 HF584835 HF584836 HF584837 HF584838 HF584840 HF584842 HF584846 HF584847 HF584849 HF584850 HF584851 HF584853 HF584855 HF584858 HF584862 HF584865 HF584866 HF584867 HF584868 HF584869 HF584870 HF584871 HF584875 HF584878 HF584879 HF584883 HF584887 HF584889 HF584890 HF584891 HF584892 HF584898 HF584899 HF584900 HF584903 HF584907 HF584913 HF584914 HF584915 HF584919 HF584920 HF584922 HF584926 HF584927 HF584929 HF584931 HF584932 HF584933 HF584936 HF584939 HF584941 HF584942 HF584943 HF584944 HF584945 HF584949 HF584950 HF584958 HF584959 HF584970 HF584976 HF584978 HF584982 HF584983 HF584992 HF584995 HF585003 HF585004 HF585006 HF585009 HF585014 HF585015 HF585016 HF585021 HF585023 HF585027 HF585028 HF585029 HF585032 HF585033 HF585035 HF585036 HF585038 HF585069 HF585082 KF646674 KF646693 KF646703 KF672703 KF712896 KF779071 KF779076 KF815538 KF815545 KF944304 JQ388737 JX994096 JX994097 JX994107 JX994115 JX994125 JX994144 JX994146 JX994147 KF944307 JX994148 KC435130 KC435131 KC435132 KC435133 KC435134 KC435135 KC435136 KC435137 KC435138 KC435139 KC435140 KC435141 KC435142 KC435143 KC435144 KC435145 KC435146 KC435147 KC435148 KC435149 KC435150 KC435151 KC435152 KC435153 KC435154 KC435155 KC435156 KC435157 KC435158 GU171377 GU171378 GU171381 KC435159 KC435160 KC435161 KC435162 KC435163 KC435164 KF578077 JN392971 JQ246449 KC311559 KF415293 KF533727 KF543097 KF578078 KF578079 KF730662 KF559322 KF585035 KF585036 KF585037 KF646673 KF646675 KF646679 KF646682 KF646683 KF646684 KF646689 KF646690 KF646691 KF646692 KF646695 KF646700 KF649246

Accession numbers for the 16S rDNA sequences analysed in Chapter 2 from different bacterial families and genera.

Bacillus sp.

KF650698 KF650699 KF650700 KF662318 KF672705 KF672706 KF672707 KF672720 KF672725 KF688211 KF688217 KF688218 KF702308 KF702310 KF702317 KF702383 KF706412 KF706413 KF706414 KF706415 KF712895 KF712912 KF712913 KF718836 KF720922 KF722848 KF725773 KF722855 KF725774 KF737352 KF737355 KF771911 KF771912 KF771915 KF797800 KF797801 KF814106 KF815530 KF815531 KF815532 KF815535 KF815536 KF815552 KF848488 KF848501 KF853106 KF853107 KF853108 KF853109 KF853110 KF853111 KF853112 KF853114 KF853116 KF853118 KF853119 KF853120 KF853121 KF853122 KF853123 KF853126 KF853128 KF853129 KF853130 KF853131 KF853132 KF900211 HM035089 HM054468 HM054473 HM054474 KF900212 KF933349 KF944305 KF944308 KF957839 HM460885 HQ834292 HQ834296 HQ834298 HQ834299 HQ834300 HQ834301 HQ834302 HQ834303 JN036548 JX232168 JX994091 JX994092 JX994099 JX994100 JX994104 JX994106 JX994112 AB873004 AB889607 GQ925365 GU434676 JX994117 JX994124 JX994128 JX994129 JX994130 JX994131 AB894827 HG529983 HG794239 HG794256 HG794257 HG794258 HG794263 HG794264 HG794265 KC847112 KF053357 KF113576 KF147865 KF220427 KF367460 KF447395 KF447396 KF447404 KF447407 KF466000 KF515660 KF515664

Enterobacteriaceae sp.

FM178865 AM179868 AM179871 AM179876 AM179880 AM179886 AM179900 AJ302154 AJ302155 AM403596 AM403598 AM403607 AM403612 AM403613 AM403629 AB523726 AJ620950 AM403639 AM403641 AM403649 HQ660391 JN662937 JX880249 AB714422 AB714423 AB714424 AB714427 AB714429 AB714430 AB714431 JX880250 JX880251 AB714432 AB714433 AB714434 AB714435 AB714436 AB714437 AB714441 AB714446 AB714449 AB714450 AB714451 AB714453 AB714455 AB714456 AB714457 AB714458 AB714459 AB715425 DQ231044 EU622576 EU622577 DQ436917 DQ812974 EF581856 AB703078 AB703079 AB703080 AB703081 AB703082 AB703083 AB703084 AB703085 AB703086 AB703087 AB368822 AB368823 AB368824 AB368825 AB368826 AB368827 AB368828 AB368829 AB703088 AB703089 AB368830 AB368831 AB368832 AB368833 AB368834 AB368835 AB368836 AB368837 AB368838 AB368839 AF451273 AF513468 AF513469 DQ226208 JN050952 JN050953 JN050954 JN050961 JN392785 JN392811 JN392823 JN392824 JN392826 JN392830 JN392831 JN392836 JN392840 JN392844 JN392846 JN392850 JN392851 JN392855 JN558594 JN613161 JN613165 JN613167 JN613169 JN872509 JN872510 JQ072087 JQ072088 JQ266301 JQ314007 JQ314009 JQ314014 JQ314024 JQ314030 JQ314031 JQ314032 JQ314034 JQ314045 JQ314053 JQ314056 JQ314062 JQ314073 JQ314084 JQ512966 JQ595463 GQ915095 GQ915098 GQ915099 GU138678 GU213133 GU223213 GU237034 GU237035 GU361689 GU381803 EF605610 EF605613 EF605614 FJ887888 FJ908094 FJ965841 EU036757 EU040283 EU102275 EU260287 EU260328 EU341310 EU341313 EU348748 EU360121 EU447445 EU626739 EU626744 EU626746 EU626747 EU626748 EU626750 EU626751 EU626753 EU626762 EU626777 EU626779 EU626780 EU626781 EU626782 FR693319 JX987136 KC108998 KC119218 KC119219 KC119220 KC153267 KC153280 KC153294 KC153296 KC310831 KC310839 KC310841 KC407618 KC407637 KC410779 KC441057 KC510034 KC560019 KC855287 KC855288 HE604343 HE604344 HQ224621 KF021738 KF021761 KF057960 KF453765 KF453775 HQ224632 HQ259699 HQ259700 HQ259701 HQ259702 HQ259703 HQ259704 HQ283408 HQ284818 HQ284819 HQ284821 HQ284822 HQ284823 HQ284824 HQ284825 HQ284826 HQ284828 HQ284829 HQ284830 HQ284831 HQ284891 HQ284892 HQ284893 HQ284894 HQ284895 HQ284896 HQ284898 HQ284899 HQ284900 HQ284901 HQ284902 HQ284903 HQ284904 HQ284905 HQ284906 HQ284948 HQ284949 HQ284950 HQ292694 HQ824844 HQ824845 HQ824846 HQ824881 HQ825020 JF262584 JF262588 JF495478 JF683277 JF683279 AB174799 JF683285 JF683287 JF683292 JF683303 JF683306 AB174805 AB174819 AB174820 AB174830 AB174831 AB174835 AB275371 AB275372 AB304396 AY538694 AY579198 AY618905 AY633492 GQ152398 EU724058 EU724059 EU863187 EU881982 EU887701 HE805090 EU887702 EU977298 FJ013300 FJ013301 FJ013302 FJ013304 FJ013333 FJ013344 FJ348017 FJ348018

Accession numbers for the 16S rDNA sequences analysed in Chapter 2 from different bacterial families and genera.

Enterobacteriaceae sp.

FJ348019 FJ348020 FJ348021 FJ348023 FJ357818 FJ357823 FJ357827 FJ477673 FJ486216 FJ664513 JQ638280 JX067682 JQ680474 JQ638281 JQ638282 JQ638283 JQ638303 JQ638308 JQ638310 JQ638312 JQ638319 JQ638324 JQ638330 JQ638338 JQ638346 JQ638359 JQ680831 JQ680886 JQ680888 JQ680895 JQ680896 JX067664 JX067684 JX067699 JX067700 JX067710 JX067714 JX067721 JX162032 JX162033 JX162034 JX162035 JX162036 JX162037 JX162038 JX162039 JX162040 JX162041 JX162042 JX162043 JX162045 JX162046 JX162047 JX162048 JX162049 JX162050 JX162051 JX162057 JX162058 JX162059 JX162060 JX162061 JX162062 JX162063 JX162064 JX162065 JX162066 JX162067 JX162070 JX162071 JX162072 JX162079 JX162080 JX162081 JX162085 JX162086 DQ288160 DQ313392 DQ520801 DQ837038 DQ837039 DQ837041 DQ837042 DQ837043 DQ837052 JX485634 EF151985 EF212936 EF212937 EF212938 EF212939 EF212940 EF212941 EF212942 EF212943 EF212944 EF212945 EF212946 EF212947 EF212948 EF212950 EF212951 EF212952 EF212953 EF212955 EF212956 EF212957 EF212958 EF428984 GU459203 GU935754 EU530451 HM235482 GU935774 GU935780 HM142759 HM235484 HM235485 HM235486 HM235490 HM235492 HM235493 HM235494 HM235499 HM235500 HM245069 HM245087 HM245089 HM245128 HM245153 HM245154 HF947094 HM245159 HM245165 HM245171 HM245177 HM245183 HM245189 HM245195 HM245222 EU530394 Uncultured EU530455 EU530459 EU530460 HF953101 EU530462 EU530464 EU530465 EU530469 EU530470 EU530471 EU530472 EU530473 EU530474 EU530475 EU530476 EU530479 GQ464391 JX568242 JX568248 JX568250 JX568253 JX568255 JX568299 AB460996 HM054416 HQ912166 JF268887 JX568326 JX568339 JX568620 JX568697 JX568748 AB461591 AB461609 AB461621 AB461622 AB461626 AB461643 AB461644 AB461645 AB461647 AB461668 AB461677 AB461741 AB461744 AB461745 AB461748 AB461749 AB461750 AB461764 AB461789 AB461793 AB461795 AB461797 AB461798 AB461800 AB461809 AB461810 AB461821 JQ595503 AB438072 AB480765 AB480768 AB480770 AB480772 GU361685 GU361688 HE802771 KC310833 AB114621 AB114622 AB194673 AB745443 FN298290 JQ958875 AB745444 EU639760 JX966472 KC011135 KC188063 KC283047 KC283051 KC283055 EF562123 EF562210 EF562241 EF562276 EU640691 EU641035 EU641703 DQ453465 DQ453466 DQ453472 EF645302 HM526962 JQ961613 JX104053 JX104054 GU075021 GU075022 GU075038 GU075039 GU075040 HM592563 HM597893 JQ818206 JQ818207 JQ515704 JQ516273 JQ516305 JQ516317 JQ516318 JQ516354 JQ818208 JQ818209 JQ818210 JQ516356 JQ516357 JQ516385 JQ516386 JQ516387 JQ516388 JQ516389 JQ516395 JQ516396 JQ516404 JQ516407 JQ516408 JQ516409 JQ516412 JQ516414 JQ516416 JQ516418 JQ516419 JQ516421 JQ516423 JQ516429 JQ516439 JQ516441 JQ516445 JQ516456 JQ516472 JQ516474 JQ516477 JQ516481 JQ516483 JQ516487 JQ516488 JQ516489 JQ516499 JQ516502 JQ516506 JQ516515 JQ516519 JQ516520 JQ516523 JQ516524 JQ516529 JQ516537 JQ516540 JQ516554 JQ516561 JQ516574 JQ516577 JQ516578 JQ516581 JQ516590 JQ683531 JQ683536 JQ683537 FJ485060 FJ485358 JQ726780 JQ726781 JQ726815 JQ763381 JN420387 JN420396 JN420397 JN420398 JN420399 JN420401 JN420402 JN420404 JN420406 JN420407 JN420411 JN420412 JN420413 JN420414 JN420415 JN420416 JN420418 JN420424 JN420425 JN420426 JN420430 JN420431 JN420432 JN420433 JN420434 JN420438 JN420440 JN420441 JN420446 JN420448 JN420449 JN420450 JN420459 JN420470 JN420471 JN420472 JN420475 Uncultured JN420485 JN420500 JN420501 JN420502 JN420503 JN420504 JN420505 JN420506 JN420507 JN420508 JN420511 JN420512 JN420513 JN420514 JN420515 JN420516 JN420517 JN420518 JN420519 JN420520 JN420521 JN420522 JN420523 JN420524 JN420525 JN420526 JN420527 JN420528 JN420529 JN420530 JN420533 JN420534 JN420535 JN420536 JN420537 JN420538 JN420539 JN420540 JN420542 JN420543 JN420544 JN420546 JN420547 JN420548 JN420549 JN420551 JN420581 JN420620 JN420635 JN420638 JN420651 JN421150 JN421151 JN421153 JN421154 JN421155 JN421156 JN421157 JN421158 JN421160 JN421162 JN421163 JN421164 JN421165 JN421166 JN421167 JN421168 JN421169 JN421170 JN421171 JN421172 JN421175 JN421176 JN421178 JN421179 JN421181 JN421182 JN421183 JN421184 JN421185 JN421186 JN421187 JN421188 JN421189 JN421190 JN421191 JN421192 JN421193 JN421194 JN421195 JN421196 JN421197 JN421201 JN421202 JN421204 JN421205 JN421206 JN421207 JN421208 JN421209 JN421211

Accession numbers for the 16S rDNA sequences analysed in Chapter 2 from different bacterial families and genera.

Enterobacteriaceae sp.

JN421212 JN421213 JN421214 JN421215 JN421216 JN421218 JN421219 JN421220 JN421221
JN421222 JN421223 JN421224 JN421225 JN421226 JN421229 JN421230 JN421232 JN421233
JN421234 JN421235 JN421236 JN421237 JN421238 JN421239 JN421241 JN421242 JN421244
JN421245 JN421246 JN421248 JN421249 JN421250 JN421251 JN421252 JN421253 JN421254
JN421255 JN421256 JN421257 JN421259 JN421260 JN421261 JN421262 JN421264 JN421266
JN421267 JN421268 JN421269 JN421271 JN421272 JN421273 JN421274 JN421275 JN421276
JN421277 JN421278 JN421279 JN421280 JN421281 JN421282 JN421283 JN421284 JN421285
JN421287 JN421288 JN421292 JN421293 JN421294 JN421295 JN421296 JN421297 JN421298
JN421299 JN421300 JN421301 JN421305 JN421306 JN421307 JN421308 JN421310 JN421311
JN421312 JN421314 JN421315 JN421316 JN421317 JN421318 JN421319 JN421326 JN421404
JN421405 JN421410 JN421411 JN421413 JN421416 JN421417 JN421418 JN421419 JN421420
JN421422 JN421424 JN421426 JN421429 JN421430 JN421431 JN421433 JN421434 JN421435
JN421437 JN421467 JN421486 JN421511 JN421589 JN421590 JN421597 JN421598 JN421599
JN421602 JN421603 JN421605 JN421609 JN421615 JN421616 JN421618 JN421622 JN421624
JN421625 JN421627 JN421634 JN421636 JN421640 JN421646 JN421648 JN421662 JN421671
JN421721 JN421722 JN421723 JN421724 JN421726 JN421727 JN421728 JN421729 JN421731
JN421732 JN421733 JN421734 JN421736 JN421737 JN421739 JN421740 JN421741 JN421743
JN421744 JN421745 JN421746 JN421748 JN421749 JN421750 JN421751 JN421752 JN421753
JN421754 JN421755 JN421756 JN421757 JN421758 JN421759 JN421760 JN421761 JN421762
JN421763 JN421766 JN421767 JN421768 JN421770 JN421771 JN421772 JN421773 JN421775
JN421776 JN421777 JN421778 JN421779 JN421780 JN421781 JN421782 JN421783 JN421784
JN421786 JN421787 JN421788 JN421789 JN421790 JN421791 JN421792 JN421793 JN421794
JN421796 JN421797 JN421798 JN421799 JN421800 JN421801 JN421802 JN421803 JN421804
JN421805 JN421806 JN421807 JN421808 JN421809 JN421810 JN421811 JN421812 JN421813
JN421814 JN421815 JN421816 JN421817 JN421818 JN421819 JN421820 JN421821 JN421822
JN421823 JN421824 JN421825 JN421826 JN421827 JN421828 JN421829 JN421830 JN421831
JN421832 JN421833 JN421834 JN421835 JN421836 JN421837 JN421838 JN421839 JN421840
JN421841 JN421842 JN421844 JN421845 JN421846 JN421847 JN421848 JN421849 JN421850
JN421851 JN421852 JN421853 JN421854 JN421855 JN421856 JN421857 JN421858 JN421859
JN421860 JN421861 JN421862 JN421863 JN421864 JN421865 JN421866 JN421867 JN421868
JN421869 JN421870 JN421871 JN421873 JN421874 JN421875 JN421876 JN421877 JN421878
JN421879 JN421880 JN421881 JN421882 JN421883 JN421884 JN421885 JN421886 JN421887
JN421888 JN421889 JN421890 JN421891 JN421892 JN421893 JN421894 JN421895 JN421896
JN421897 JN421898 JN421900 JN421902 JN421903 JN421904 JN421905 JN421906 JN421907
JN421908 JN421909 JN421910 JN421911 JN421912 JN421913 JN421914 JN421915 JN421916
JN421917 JN421922 JN421923 JN421924 JN421925 JN421928 JN421929 JN421930 JN421934
JN421935 JN421936 JN421937 JN421939 JN421941 JN421942 JN421943 JN421944 JN421945
JN421946 JN421947 JN421948 JN421949 JN421950 JN421951 JN421952 JN421953 JN421954
JN421958 JN421959 JN421960 JN421961 JN421962 JN421964 JN421965 JN421966 JN421967
JN421968 JN421969 JN421972 JN421974 JN421975 JN421976 JN421977 JN421978 JN421979
JN421980 JN421981 JN421982 JN421983 JN421985 JN421986 JN421987 JN421989 JN421990
JN421991 JN421992 JN421993 JN421994 JN421996 JN421997 JN421998 JN421999 JN422000
JN422001 JN422002 JN422003 JN422004 JN422005 JN422006 JN422007 JN422008 JN422009
JN422010 JN422011 JN422013 JN422014 JN422015 JN422016 JN422017 JN422018 JN422019
JN422020 JN422022 JN422024 JN422025 JN422026 JN422027 JN422028 JN422029 JN422030
JN422031 JN422032 JN422033 JN422034 JN422035 JN422036 JN422037 JN422039 JN422040
JN422041 JN422042JN422043 JN422044 JN422045 JN422046 JN422047 JN422048 JN422049
JN422050 JN422051 JN422052 JN422053 JN422055 JN422056 JN422057 JN422058 JN422059
JN422060 JN422061 JN422062 JN422063 JN422065 JN422069 JN422070 JN422071 JN422072
JN422073 JN422077 JN422078 JN422079 JN422083 JN422084 JN422086 JN422087 JN422088
JN422089 JN422090 JN422091 JN422093 JN422094 JN422095

Accession numbers for the 16S rDNA sequences analysed in Chapter 2 from different bacterial families and genera.

Lactobacillus sp.

HE573915 HE573916 HE573917 HE573918 HE600693 HM036120 HM070024 HM070025 HM101282 HM101283 HM101284 HM101285 HM101286 HM101287 HM101288 HM101289 HM101290 HM101291 HM101292 HM101293 HM101294 HM101295 HM101296 HM101297 HM101298 HM101300 HM101301 HM101302 HM101303 HM101304 HM101305 HM101306 HM101307 HM101309 HM101310 HM101311 HM101312 HM101313 HM101315 HM101316 HM101317 HM101318 HM101319 HM101320 HM101321 HM101322 HM101323 HM101324 HM101325 HM101326 HM101327 HM101328 HM101329 HM101330 HM101331 HM101332 HM125048 HM125049 HM125050 HM162410 HM162411 HM162412 HM162413 HM162414 HM162416 HM162417 HM162418 HM162419 HM162420 HM162424 HM162425 HM448901 HM585368 EU194344 EU194349 HM623785 HM641232 HM641233 HM800504 HQ010403 HQ117896 HQ117897 AB366387 AB366390 AB366391 AB366394 AB366395 AB366396 E17066 EU350220 AB366399 AB429369 AB429372 AB429373 AB063479 AB326301 EF426247 EF426248 EF426249 EF426250 FJ542295 FJ542296 FJ542297 FJ542298 FJ542299 FJ542300 FJ542301 FJ542302 FJ542303 FJ542304 FJ581418 FJ595943 JQ775393 JX020702 JX112898 JX112899 JX185493 JX185494 JX185495 JX185496 JX185497 JX406745 JX406746 JX440372 JX440373 JX440374 JX440375 JX440376 JX440377 JX440378 JX440379 DQ316398 DQ317562 DQ317604 DQ317605 DQ333457 DQ444477 DQ480531 JX490159 JX490160 JX501236 DQ644524 DQ644525 DQ644526 DQ644527 DQ644528 DQ644529 DQ644530 DQ644531 DQ644532 DQ644533 DQ644534 EF053510 EF053511 EF053512 EF113958 EF113959 EF113960 EF113961 EF113962 EF113963 EF113964 EF113965 EF113966 EF120368 EF120369 EF120370 EF120371 EF120372 EF120373 EF120374 EF120375 EF120376 EF375889 EF375890 EF375891 EF442276 EF442277 EF442278 EF442279 EF442280 EF442281 EF442282 EF442288 EF442289 EF442290 EF442291 EF442292 EF442293 EF442294 EF442295 EF442296 EF445113 GU591883 GU591884 GU591885 HE573913 HE573914 FN252881 FN557015 EF113958 EF053510 EF053511 EF053512 EF113959 EF113960 EF113961 EF113962 EF113963 EF113964 EF113965 EF113966 EF120368 EF120369 EF120370 EF120371 EF120372 EF120373 EF120374 EF120375 EF120376 EF375889 EF375890 EF375891 EF442276 EF442277 EF442278 EF442279 EF442280 EF442281 EF442282 EF442288 EF442289 EF442290 EF442291 EF442292 EF442293 EF442294 EF442295 EF442296 EF445113 EF534204 JQ775393 JX020702 JX112898 AB107637 HQ449670 HQ650232 HQ726794 HQ853454 JF763842 JF923643 JF923644 AB547127 AY379071 AY383631 AY522567 AY590769 AY590770 AY590771 AY590772 AY590773 AY590774 AY590776 AY681131 AY852248 GQ141805 GQ141806 GQ141807 GQ141809 GQ141810 GQ141811 GQ141812 GQ141813 GQ141814 GQ141815 GQ141816 GQ141817 GQ141818 GQ141819 GQ141828 GQ141829 GQ202837 GQ202838 GQ202839 GQ202840 GQ303169 GQ337858 GQ455406 AF090328 EU722291 FJ386571 FJ455518 FJ455519 HF545640 FJ542289 FJ542291 FJ542292 FJ542293 FJ542294 AY522567 AY590769 AY590770 AY590771 AY590772 AY590773 AY590774 AY590776 AY681131 AY735405 AY852248 GQ141806 GQ141807 GQ141809 GQ141810 GQ141811 GQ141812 GQ141813 GQ141814 GQ141815 GQ141816 GQ141817 GQ141818 GQ141819 GQ141828 GQ141829 GQ202836 GQ202837 GQ202838 GQ202839 GQ202840 GQ303169 GQ337858 GQ455406 AF090328 EU722291 EU867793 FJ386571 FJ455518 FJ455519 HF545640 FJ542288 FJ542289 FJ542290 FJ542291 FJ542292 FJ542293 FJ542294 FJ542295 FJ542296 FJ542297 FJ542298 FJ542299 FJ542300 FJ542301 FJ542302 FJ542303 FJ542304 FJ581418 FJ595943 JQ775393 JX020702 JX112898 JX112899 JX185493 JX185494 JX185495 JX185496 JX185497 JX406745 JX406746 JX440372 JX440373 JX440374 JX440375 JX440376 JX440377 JX440378 JX440379 DQ316398 DQ317562 DQ317604 DQ317605 DQ333457 DQ444477 DQ480531 JX490159 JX490160 JX501236 DQ644524 DQ644525 DQ644526 DQ644527 DQ644528 DQ644529 DQ644530 DQ644531 DQ644532 DQ644533 DQ644534 JF923644 JN012220 JN012221 JN012222 JN012223 JN012227 AF090328 GU086364 GU138143 GU138144 GU138145 GU138146 GU138147 GU138148 GU173834 GU173836 GU173837 GU173838 GU173839 GU173840 GU173841 GU173844 GU191837 DQ316398 DQ317562 DQ317604 DQ317605 DQ333457 DQ444477 GU237039 GU237040 GU237041 GU290217 DQ480531 DQ644524 DQ644525 DQ644526 DQ644527 DQ644528 DQ644529 DQ644530 DQ644531 DQ644532 DQ644533 DQ644534 EF053510 EF053511 EF053512 EF113958 EF113959 EF113960 EF113961

Accession numbers for the 16S rDNA sequences analysed in Chapter 2 from different bacterial families and genera.

Lactobacillus sp.

EF113962 EF113963 EF113964 EF113965 EF113966 EF120368 EF120369 EF120370 EF120371 EF120372 EF120373 EF120374 EF120375 EF120376 EF375889 EF375890 EF375891 EF442276 EF442277 EF442278 EF442279 EF442280 EF442281 EF442282 EF442288 EF442289 EF442290 EF442291 EF442292 EF442293 EF442294 EF442295 EF442296 EF445113 EF534204 JQ775393 JX020702 JX112898 AB107637 HQ449670 HQ650232 HQ726794 HQ853454 JF763842 JF923643 JF923644 AB547127 AY379071 AY383631 FN252881 FN557015 EU380191 EU380192 EU380193 EU381121 EU381122 EU381123 EU381124 EU381125 EU381126 EU381128 EU381129 EU547296 EU547297 EU547298 EU547299 EU547300 EU547301 EU547302 EU547303 EU547304 EU547305 EU547307 EU547308 EU547309 EU547310 EU547311 EU547312 EU547313 EU555174 EU621848 EU621849 EU621850 EU621851 EU688975 EU688976 EU722291 GQ141805 GQ141806 GQ141807 HE663132 GQ141809 GQ141810 GQ141811 GQ141812 GQ141813 GQ141814 GQ141815 GQ141816 GQ141817 GQ141818 GQ141819 GQ141828 GQ141829 GQ202836 GQ202837 GQ202838 GQ202839 GQ202840 AB107637 GQ303169 GQ337858 GQ455406 AB547127 AY082883 AY082884 AY196968 AY196970 AY196977 FR681901 AY230219 AY230220 AY230221 AY230222 AY230223 AY230224 AY230225 AY230226 AY230227 AY230228 AY230229 AY230230 AY318799 AY318825 AY379071 AY383631 KC145829 KC155629 KC700038 KC700040 HQ292207 HQ449670 HQ650232 HQ726794 KC857461 KC857465 KC857466 JF763842 JF923643 AF275311 AB703604 AB703605 AF404708 AF302116 AF335475 AF382389 EU194344 EU194349 EU350220 AF382390 AF382391 AF385770 HM036120 HM070024 HM070025 HM101282 HM101283 HM101284 HM101285 HM101286 HM101287 HM101288 HM101289 HM101290 HM101291 HM101292 HM101293 HM101294 HM101295 HM101296 HM101297 HM101298 HM101300 HM101301 HM101302 HM101303 HM101304 HM101305 HM101306 HM101307 HM101309 HM101310 HM101311 HM101312 HM101313 HM101315 HM101316 HM101317 HM101318 HM101319 HM101320 HM101321 HM101322 HM101323 HM101324 HM101325 HM101326 HM101327 HM101328 HM101329 HM101330 HM101331 HM101332 HM125048 HM125049 HM125050 HM125051 HM162410 HM162411 HM162412 HM162413 HM162414 HM162416 HM162417 HM162418 HM162419 HM162420 HM162424 HM162425 HM448901 HM585368 HM623785 HM641232 HM641233 AM920327 HM800504 HQ010403 HQ117896 HQ117897 HQ214673 HQ259238 HQ259243 AM920328 AM920329 AM920330 EU187503 EU377823 EU377824 EU380190

Pseudomonas sp.

D84010 AB049747 AB049749 AB049750 AM179865 AM179872 AM179883 D84012 D84009 D84013 D85991 D85996 D85997 Y11150 D84014 D84020 D84022 D84027 AJ012712 AJ249451 AJ308298 AJ308302 L28676 AJ308303 AJ308304 AJ308305 AJ308306 AJ308307 AJ308309 AJ308311 AJ308312 AJ308313 AJ308315 AJ308320 AJ310536 D83788 D85992 AJ537601 AJ537602 AJ537603 D85993 D85994 D85995 D85998 D85999 D86000 AB030583 AJ492826 AJ492827 AJ492828 AJ492829 AJ492831 D87104 AJ310393 AJ344082 AJ970164 AM088475 AJ970171 AJ970172 AJ970173 AJ970174 AM262973 AM419153 D87098 D87099 D87102 D87108 AJ319662 AJ410871 AJ410872 AJ633553 AJ633554 AJ633555 AJ633556 AJ633557 AJ633558 AJ633559 AJ633560 AJ633561 AJ633562 AJ633563 AJ633564 AJ876736 X99540 AJ006103 AJ006107 AJ270451 AJ270452 AJ270453 X99541 AJ270454 AJ270455 AJ270456 AJ270457 AJ270458 AJ292426 AJ312156 AJ312157 AJ312159 AJ312160 AJ312161 AJ312162 AJ312163 AJ312164 AJ312165 AJ312166 AJ312167 AJ312168 AJ312169 AJ312170 AJ312171 AJ312172 AJ312173 AJ312175 AJ312176 AM114525 AM114526 AM905853 AM905855 AM905856 AM905857 AM905858 AM905859 AB009457 AB031277 AM114527 AM114532 AM114533 AM114534 AJ344226 AJ550469 AJ585226 AJ833919 AJ011331 AJ132993 AJ278814 AJ288286 AJ288293 AJ288301 AJ288304 AJ288305 AJ288148 AJ288151 AJ288306 AJ288309 AJ310484 AM062695 AM263483 AM263484 AM293356 AM293357 AM293365 AM293366 AM263487 AM263488 AM263489 AM263490 AM263492 AM263493 AM263495

Accession numbers for the 16S rDNA sequences analysed in Chapter 2 from different bacterial families and genera.

Pseudomonas sp.

AJ006104 AJ309500 AJ583501 AM263498 AM263499 AM905852 AM111049 AM262071 AM293568 AM495258 X06684 Z79594 AM293678 AM293679 AM404437 AM410088 AM410615 AM410616 AM410620 AM410631 AM411058 AM411059 AM411067 AM411071 AM411185 AM411211 AM419154 AM419155 AM421016 AM491070 AM707022 AM711587 AJ344483 AJ413199 AJ417072 AJ491835 AJ508696 AM711588 AM711596 AJ509809 AM398412 AM398413 AM398414 AM398415 AM398416 AM398417 AM398418 AM398419 AM398420 AM398421 AM398422 AM398425 AM398426 AM398427 AM398428 AM398429 AM398430 AM398431 AM398432 AM398433 AM398434 AM773555 AM773556 AM773557 AM773558 AM773559 AM773560 AM773561 AM773562 AM773563 AM773564 AM773565 AM773567 AM773568 AM773573 AM773575 AM773576 AM773577 AM773578 AM773580 AM773583 AB028924 AM773588 AM773589 AJ512378 AJ512380 AJ512381 AM399034 AM399035 AJ512385 AJ512386 AJ512387 AJ512389 AJ512390 AJ512391 AJ512392 AJ512394 AJ512395 AJ512396 AJ512397 AJ512398 AJ512399 AJ512400 AJ512401 AJ512402 AJ512403 AJ512404 AJ512405 AJ512406 AJ512407 AJ512408 AJ517396 AJ517397 AJ517398 AJ517399 AJ517400 AJ517401 AJ517402 AJ517403 AJ517404 AJ517405 AJ517406 AJ517407 AJ517408 AJ517409 AJ517410 AJ292381 AJ293858 AJ293859 AJ544239 AJ544240 AJ864394 AJ890253 AM041055 AM085309 AJ007526 AJ007527 AJ007533 AJ007535 AJ007536 AM403529 AJ458198 AJ519791 AJ550465 AJ623285 AJ845183 AJ846267 AM745260 AJ846268 AJ846270 AJ846274 AJ846276 AJ846278 AJ846279 AJ846280 AJ846281 AJ846282 AJ846284 AJ846285 AJ846286 AJ846287 AJ846290 AJ868441 AM110955 AM110968 AM110993 AM111004 AM111013 AM111015 AM111025 AM111029 AM111035 AM111036 AM111037 AM111039 AM111041 AM111042 AM111043 AM111044 AM111045 AM111046 AM111047 AJ243603 AJ243606 AJ278107 AM111052 AM111063 AM111077 AM111089 AM111092 AJ288143 AM076674 AM158279 AM265390 AM265391 AM265392 Z76666 AB038140 Z76652 Z76653 Z76654 Z76658 AJ631287 Z76663 Z76671 AJ631288 AJ631289 AJ631290 AJ631291 AJ631292 AJ631293 AJ631294 AJ631295 AJ631296 AJ631297 AJ404575 AJ404580 AJ404603 AJ492830 AJ578066 AJ617689 AJ970167 AM293370 AM293375 AJ970168 AJ970169 AJ970170 D87100 FN395007 AM422559 D84004 D87103 D87105 D87107 AB004241 AJ535755 AM396914 AM396933 AM396934 AM397051 AM398216 AM402949 AM403657 AM410086 AM410089 AM410090 AM410614 AM410617 AM410619 AM410621 AM410622 AM410623 AM410625 AM410626 AM410627 AM410628 AM410629 AM410630 AM411057 AM411060 AM411062 AM411063 AM411065 AM411069 AM411619 AM411620 AM411621 AM411992 AM411994 AM411997 AM411999 AM412215 AM418387 AM421975 AM421976 AM421981 AM421982 AM489694 AM491058 AM491059 AM491060 AM491061 AM491463 AM491464 AM491465 AM491466 AM691617 AM691618 AM691619 AM691622 AM691623 AM691630 AM707021 AM709775 AM746975 AM746976 AJ318913 AJ318918 AJ410281 AJ410283 AJ410285 AJ413198 AJ417068 AJ417069 AJ417070 AJ417074 AJ417370 AJ419672 AJ419673 AJ419674 AJ419675 AJ489332 AJ489334 AJ489342 AJ489344 AJ489346 AJ489348 AJ534859 AJ534869 AJ539228 AJ634921 AJ634922 AJ634927 AJ634940 AJ634949 AJ704794 AJ785569 AJ864849 AJ864859 AJ867217 AJ870968 AJ871943 AJ871944 AJ876660 AJ884889 AJ884890 AJ884891 AJ884892 AJ889841 AJ009710 AM905940 AM905941 AM905942 AM905943 AM913883 AM913885 AM913888 AM913891 AM913892 AM913893 AM913894 AM913895 AM913896 AM913897 AM913898 AM913900 AM913903 AM913905 AM913906 AM913915 AM913943 AM913961 AM937256 AJ551097 AJ551146 AJ551153 AJ551158 AM937258 AM937261 AJ551160 AJ551161 AM050098 AM050101 AM084013 AM084017 AM084021 AM084027 AM084028 AM084037 AM084071 AM084105 AM084159 AM084173 AM086250 AM110075 AM158919 AM180745 AM184269 AM184301 AM231084 AM232729 AM237088 AM237089 AM237090 AM237092 AM260197 AM260540 AM267085 AM269470 AM269522 AM284989 AM285005 AM285021 AM285023 AJ002801 AJ002813 AJ132994 AJ237965 AM113740 AM113741 AM286272 U26414 Y18235 AM689940 U26417 U26418 U26419 U26420 AM689985 AM690033 AB073312 AF058286 EF426771 EU224277 U63901 U63902 U63903 U63904 AJ306832 AJ306834 U63905 U63906 U63907 U63908 U63909 AJ548920 AJ567594 AJ576114 AJ576116 AJ577093 AM167976 AM421033 AM421034 AM421035 AM421036 AM421037

Accession numbers for the 16S rDNA sequences analysed in Chapter 2 from different bacterial families and genera.

Pseudomonas sp.

AM421136 AM421137 AM421138 AM421139 AM421144 AM421145 AM421156 AM421157 AM421158 AM421159 AM421160 AM421161 AM421162 AM421163 AM421164 AM421165 AM421180 AM421181 AM421182 AM421183 AM421196 AM421197 AM421198 AM421199 AM421200 AM421201 AM421202 AM421203 AM421204 AM421205 AM421206 AM421207 AM421208 AM421209 AM421210 AM421211 AM421212 AM421213 AM421214 AM421215 AM421217 AM421218 AM421219 AM421220 AM421221 AM421222 AM421223 AM421224 AM421225 AM421226 AM421227 AM421231 AM421235 AM421238 AM421239 AM421240 AM489702 AM747115 AM747118 AM747119 AM779870 AM889172 Z36532 EF418612 AF448349 AF448350 AF448351 AF448352 AB257323 AY063234 AY063235 AY074894 AM403600 AM403604 AM403615 AM403625 AM412165 AJ968720 AJ516053 AJ536421 AM710608 AJ842221 AJ842224 AJ842238 AJ842243 AJ842244 AJ842246 AJ842249 AJ842250 AJ842251 AJ842253 AM000005 AM000006 AM000020 AJ291839 AJ291840 AJ291841 AJ291844 AJ291845 AJ344482 AJ344484 AJ344485 AJ344486 AJ387903 AJ007005 AJ007006 AJ387904 AJ864722 AB271010 AB576190 AB621592 AF143245 AJ271413 AY091527 KC428669 AB665551 EU579530 FJ587079 KF317822 KF478199 U26415 U26416 AF064460 AY623816 DQ286456 GQ327971 KC206029 KC206030 KC206031 KF055856 U22426 U22427 EU043322 FM881781 KF146882 U25280 U25431 U25432 U26261 U26262 AB013844 AB062598 AB062599 AB088754 AB204716 EU043323 EU043325 EU043329 EU043330 AB247185 AB247188 AB247189 AB247194 AB247195 AB247196 AB247197 AB247198 AB247199 AB247200 AB247201 AB247202 AB247203 AB247204 AB247218 AB302401 AB302402 AB494443 AB494444 AB494445 AB543806 DQ071557 DQ071559 DQ073449 DQ073450 DQ073451 DQ073452 DQ073453 DQ073454 DQ133506 DQ140381 DQ140382 DQ140383 JX915743 JX970974 JX970975 JX970976 EU714901 FN650142 JX970977 JX970978 JX970979 KC333649 KC662503 KC710974 FJ577676 FR714937 HM151878 HQ141340 HQ454496 HQ680964 HQ680980 HQ840718 HQ848377 HQ874650 HQ880245 KC845571 FJ705888 FM203408 FN996012 FR820588 FR820589 KC893551 KC893552 KF195926 KF539786 KF591451 AF494091 EF530571 EF530572 EF538425 EF552367 EF552368 EU037096 EU194235 EU194236 EU194237 EU194238 EU194239 EU257454 EU257455 EU350370 EU534410 EU599569 EU661864 JQ669958 JQ691692 JQ691693 JQ691694 JQ691695 JQ691696 JQ691697 JQ691698 JQ691699 JQ691700 JQ691701 JQ691702 JQ691704 JQ691705 JQ691707 JQ691708 JQ691709 JQ839149 JX010738 GU220068 HF545840 HF545842 HF545843 JX010739 JX204836 DQ211696 DQ356904 DQ864462 DQ864463 HF545845 HF545846 AY121976 AY264292 EF488968 EF488969 JF951725 JN002065 JN051364 JN099687 JN123466 JN389433 JN600615 JN674083 JN836325 JQ027335 JX913784 JX913785 AF067960 AF468448 AF468449 AF495871 EU344794 HM217131 JX913786 JX913787 JX913788 JX913789 AY486350 AY486353 AY486355 AY486356 AY486357 JQ904623 JX104229 AY486358 AY486359 AY486360 AY486361 AY486362 AY486363 AY486364 AY486365 AY486366 AY486367 AY486368 AY486369 AY486370 AY486371 AY486384 AY486385 AY486386 GQ870338 GQ870339 GQ870340 AB189452 AJ583805 AJ966856 AM403308 AM489671 AB504737 AB060131 AB060132 AB060133 AB060134 AB060135 AB087853 AB117953 AB119535 AB127967 AB060136 AB060137 AB334768 FJ665502 KC119195 KC428662 KC428666 KC428670 KC428673 AB056120 AB091837 AB440177 DQ358054 EF026153 EF487999 FJ905913 GQ160904 GQ916542 DQ073039 DQ083947 DQ095878 DQ095879 DQ095880 DQ095881 DQ095882 DQ095883 DQ095890 DQ095891 DQ095892 DQ095893 DQ095894 DQ095895 DQ095896 DQ095914 DQ095915 DQ181650 DQ181651 JX271040 JX313019 JX477649 JX678983 JX885767 JX885769 JX905208 JX915832 JX915833 JX915834 JX915835 JX915836 JX915837 KC013301 KC189961 KC495567 KC495568 KC495569 KC495570 KC495571 KC495572 KC505184 KC570343 KC602116 KC631644 KC633744 KC660988 KC663615 KC663616 KC663617 KC688876 KC699534 KC699535 KC699536 KC699537 KC699538 KC699539 KC699540 KC699541 KC699542 KC699543 KC762216 AF038653 AF074383 EU834943 EU849119 EU930815 FN599522 KC762217 EU930816 FJ001818 FJ004920 FJ422406 FJ422810 FJ472580 FJ496659 FJ534557 FJ534640 FJ556919 FJ605510 HM014234 HM036358 HM036359 HM101170 HM150717 HM190218 FR668235 FR695882 FR695883 FR695884 HM190219 HM190220 HM190221 HM190222 HM190223 HM190224 FR695885 FR695886 HM224410 HM245963 HM481449 HM582425 KC83432

Accession numbers for the 16S rDNA sequences analysed in Chapter 2 from different bacterial families and genera.

Pseudomonas sp.

HM582426 HQ123430 HQ123431 HQ271083 HQ271084 HQ283403 HQ324110 HQ660081 HQ697262 HQ896494 HQ995498 JF261631 GU447237 GU447238 GU475123 GU480532 GU951516 HE585219 JF422069 JF495456 KC794741 KC796784 KC796785 KC796786 KC796787 KC796788 KC796789 KC796790 KC796791 KC796792 KC796793 KC796794 KC820813 KC834302 KC834304 KC834305 KC834315 KC834317 KC834322

Vibrio sp.

X99762 L05178 AJ582807 AJ582810 AB038030 AJ316187 AJ515218 AJ515219 AJ515220 AJ515221 AJ515222 AJ515223 AJ515224 AJ515225 AJ515226 AJ515227 AJ515228 AJ582809 FM162399 AB013297 FM162401 FM162402 FM162404 AB010811 AJ630202 AJ630203 X97987 X97988 X97989 X97990 AJ515229 AJ515230 AJ318954 AJ414114 AJ414116 AJ414118 AJ414121 AJ421444 AJ421445 AJ491832 AJ554204 AJ582808 AJ630102 AJ630103 AJ845012 AJ845014 AJ845016 AJ845018 AJ845020 AJ845022 AJ874352 AJ874353 AJ874354 AJ874359 AJ874363 AJ874364 AJ874367 AJ885017 AJ885024 AJ885034 AJ885035 AJ885036 AJ885041 AJ885044 AJ885045 AJ293802 AJ310647 AJ310648 AM048781 AM181657 AM181658 AB016271 AJ278426 AJ876732 AM777383 AM902263 AJ316174 AJ316182 AJ316184 AJ316185 AJ316188 D11214 AF134581 AJ609638 AM921804 HE795132 HE795133 HE795134 HE795135 HE795136 HE795137 HE795138 HE795139 HE795140 HE795141 HE795142 HE795143 HE795144 HE795145 HE795146 HE795147 HE795148 HE795149 HE795150 HE795151 AJ345065 HE795152 HE795153 HE795154 HE795155 HE795156 HE795157 HE795158 JQ409383 AF493805 AJ582803 AJ582804 AJ582805 AJ582806 KC954165 KC954166 KC954167 KC954168 AF493809 AB038023 AB038024 AB038025 AB038026 AB038029 AM401583 AM495249 AM495250 AM495251 AM709736 AJ414125 AM747235 AJ784124 AJ784128 AJ784131 AJ784135 AJ784136 AJ784137 AJ784139 AJ784140 AJ866938 AJ885012 AJ885030 AJ936940 AM778454 AM778456 AM778457 AM778458 AM778459 AM778460 AM778461 AM778462 AM778463 AM884367 AM913884 AM913887 AM157655 AM162595 AM913925 AM913929 AF426805 AF426806 AF426811 AF426814 AF426824 KC740490 AF426825 FM204836 FM204837 FM204838 FM204839 FM204840 FM204845 FM204846 FM204847 FM204848 FM204849 FM204850 FM204851 FM204852 FM204853 FM204854 FM204855 FM204856 FM204857 FM204858 FM204859 FM204860 FM204865 FM204866 FM204867 FM204868 FM204869 FM204870 AF537959 EU579452 HQ694831 GU727812 HQ694832 HQ694833 HQ849470 JF260912 JF264469 JF264470 JF264472 JF264473 JF330909 GU727813 GU974342 KC912685 KC954171 KF158717 KF158718 KF158719 KF158720 KF158721 KF158722 KF158723 KF158724 KF158725 KF158726 KF158727 KF158728 KF158729 KF158730 KF158731 KF158732 KF158733 KF158734 KF158735 KF158736 KF158737 KF158738 KF158739 KF158740 KF158741 KF158742 KF158743 KF158744 KF158745 KF158746 KF158747 KF158748 KF158749 KF158750 KF158751 KF158752 KF158753 KF158754 KF158755 KF158756 KF158757 KF158758 KF158759 KF158760 KF158761 KF158762 KF158763 KF158764 KF158765 KF158766 KF158767 KF158768 KF158769 KF158770 KF158771 KF158772 KF158773 KF158774 KF158775 KF158776 KF158777 KF158778 KF158779 KF158780 KF158781 KF158782 KF158783 KF158784 KF158785 KF158786 KF158787 KF158788 KF158789 KF158790 KF158791 KF158792 FJ752498 FJ824663 FJ906747 FJ906748 FJ906749 FJ906750 FJ906751 U37801 U37802 EF684899 EF684900 EF684901 EF684902 EF684903 EF684904 EF684905 EU031646 FR797810 EU143769 EU204961 EU652246 EU652252 FN432778 JQ307093 JQ307094 AY456924 AY494842 AY494843 AY562192 JQ307095 JQ307096 JQ307097 JQ904784 JQ958596 AY628645 AY628646 AY800101 AY827492 AY863432 HF541921 HF541922 HF541923 HF541924 HF541925 HF541926 HF541927 HF541928 HF541929 HF541931 HF541932 HF541933 HF541934 HF541935 HF541936 HF541937 HF541938 HF541939 HF541940 HF541941 HF541942 HF541943 HF541944 HF541945 HF541946 HF541947 HF541948 HF541949 HF541950 HF541951 HF541952 HF541953 HF541954 HF541955 HF541956 HF541957 HF541958 HF541959 HF541960 HF541961 HF541962

Accession numbers for the 16S rDNA sequences analysed in Chapter 2 from different bacterial families and genera.

Vibrio sp.

HF541963 HF541964 DQ298045 DQ298046 DQ298047 DQ298048 DQ304558 HF541965 HF541966 HF541967 HF541968 DQ440933 DQ440936 DQ440937 DQ440938 DQ440939 DQ440940 DQ440941 DQ440943 DQ440945 DQ440947 DQ440948 DQ440949 DQ440950 DQ440951 DQ440953 DQ440956 DQ440959 EF467290 DQ440966 DQ440968 DQ440969 DQ440970 DQ440972 DQ440973 DQ440976 DQ923054 DQ985231 EF178477 EF178478 EF178479 EF178480 EF178481 EF178482 EF178483 EF178484 EF178485 EF178486 HE795129 AY264936 AY332565 HE795130 HE795131 JF731344 JF779826 JF779827 JF779828 JF779830 JF779831 JF779833 JF779834 JF779835 JF779836 JF779837 JF779838 JF779839 JF779840 JF779841 JF907569 JF907572 JN003627 JN108879 JN188401 JN188402 JN188403 JN188404 JN188405 JN188408 JN188409 JN188411 JN188413 JN188414 JN188415 JN188416 JN188417 JN188418 JN188419 JN188420 JN188421 JN188422 JN188423 JN188424 GQ180184 GQ180185 GQ180186 GQ249053 GQ260160 GQ260161 GQ260162 GQ260163 GQ372983 HE584769 HE584771 HE584774 HE584775 HE584776 HE584777 HE584778 HE584779 HE584780 HE584782 HE584784 HE584786 HE584787 HE584788 HE584789 HE584790 HE584792 HE584794 HE584795 HE584797 HQ449744 HQ449745 HQ449746 KC185413 KC185414 KC185415 HQ449747 FJ906812 HQ449748 HQ449749 HQ449750 HQ449751 HQ449752 HQ449753 HQ449754 HQ449755 HQ449756 HQ449757 HQ449758 HQ449759 HQ449760 HQ449761 HQ449762 HQ449763 HQ449764 HQ449765 HQ449766 HQ449767 HQ449768 HQ449769 HQ449770 HQ449771 HQ449772 HQ449773 HQ449774 HQ449775 HQ449776 HQ449777 HQ449778 JQ409372 JX221044 JX221045 KC794715 KC794716 GU064357 GU064358 GU064361 GU064369 GU064371 GU064372 GU064373 GU064375 GU064376 GQ332279 GQ332282 GQ332283 GU064377 GU064378 GU262992 GQ332284 GQ332285 GQ332286 GQ332287 GQ332288 GQ332289 GQ332290 GQ332291 GQ332292 GQ332293 FJ404761 FJ404762 FJ404763 FJ404764 GQ332294 GQ332295 GQ332296 GQ332297 GQ332298 GQ332299 FJ404765 HM996960 HM996961 HM996962 HM996963 HM996964 HM996965 HM996966 HM996967 HM996968 HM996969 HM996970 HM996971 HM996972 HM996973 KC954162 KC954163 KC954164 EU091326 EU091331 EU091332 EU091333 EU091334 EU091335 EU091337 GQ487487 KF150774 KF150776

Appendix 2.

Table outlining all chemicals and equipment utilised within this thesis.

Product	Supplier	Catalogue number
0% MacroGard® experimental feed	Tetra GmbH	N/A
0.1% MacroGard® experimental feed	Tetra GmbH	N/A
2-phenoxyethanol	Sigma	P1126
96 well plate	Sarstedt	83.3924.500
ABI Prism® 9000 Sequence Detection System	Applied Biosystems	N/A
Aeromonas Isolation Agar	Fluka Analytical	17118-500G
Aeromonas Selective Supplement	Fluka Analytical	17119-5VL
Aeromonas salmonicida subsp. *salmonicida*	NCIMB	NCIMB1102
Ammonium persulfate (APS)	Sigma	A3678
Bacillus subtilis subsp. *spizizenii*	NCIMB	NCIMB8054
Bioruptor™ UCD-200	Diagenode	N/A
Bermuda Banish Fish Ulcer, Parasite and White Spot Treatment	Glovers Aquatics	N/A
Crystal violet	Pro-lab Diagnostics	PL7001
Dissolved Oxygen Meter	Hanna	H19142
dNTPs	Invitrogen	10297
EconoSpin™ Spin column for DNA (silica membrane)	Epoch Life Sciences	1910-250
EconoSpin™ Spin column for RNA (silica membrane)	Epoch Life Sciences	1940-250
Eheim 2227 filter system	Eheim	N/A
Eppendorf Gradient Mastercycler®	Eppendorf	950000015
Ethidium bromide	Sigma	E8751

Table outlining all chemicals and equipment utilised within this thesis.

Product	Supplier	Catalogue number
Formamide	Sigma	F9037
GeneAmp PCR system 9700 thermocycler	Applied Biosystems	N/A
GoTaq® G2 Flexi DNA polymerase	Promega	M7801
Gram's iodine	Pro-lab Diagnostics	PL7004
"Hi-Pure" Low EEO Agarose	BioGene	300-300
Hot Start KAPA2G Robust Polymerase: 5X KAPA2G buffer A, Polymerase, PCR grade Water	Kapabiosystems	KK5511
Hydrogen peroxide	Fisher	H/1862/15
LPS from *E. coli* O55:B5	Invivogen	LPS-B5
Loading buffer	Made in house by Dr. Adamek (TiHo)	
Lysozyme from chicken egg white	Sigma	L6876
MacConkey agar	Oxoid	CM0109
MacroGard®	Biorigin	Batch number: 250813
Magnesium Chloride	Promega	A3511
MaxQ 4000 E-class incubator	Barnstead/Labline	N/A
Moloney Murine Leukemia Virus Reverse Transcriptase (M-MuLV RT).	Invitrogen	No longer available

Table outlining all chemicals and equipment utilised within this thesis.

Product	Supplier	Catalogue number
M-MLV Reverse Transcriptase: M-MLV RT, 5X First Strand Buffer, 0.1M DTT	Invitrogen	28025-013
Microsoft Excel	Microsoft	N/A
Minitab 14	Minitab	N/A
M.R.S. agar (de Man, Rogosa and Sharpe)	Oxoid	CM0361
Mx3000P qPCR System	Stratagene	N/A
Nanodrop 1000	Thermo Scientific	N/A
Nikon AF-S DX NIKKOR 55-300mm f/4.5-5.6G VR Lens	Amazon.co.uk	N/A
Nikon D3200 Digital SLR Camera with 18-55mm VR Lens Kit - Black (24.2MP) 3 inch LCD	Amazon.co.uk	N/A
Nutrient agar	Oxoid	CM0003
Nutrient broth	Oxoid	CM0001
Oxidase detection strips	Oxoid	MB0226
PBS	Sigma	P-4417
PCR Buffer II	Invitrogen	4379878
peqGOLD TriFast	Peqlab	30-2010
Primers	Eurofins	N/A
Proteinase K	Peqlab	0706-1G
QIAamp DNA Mini Kit: AL buffer, AW1 buffer, AW2 buffer, AE buffer	Qiagen	51304
Random hexamers	Invitrogen	N8080127

Table outlining all chemicals and equipment utilised within this thesis.

Product	Supplier	Catalogue number
Resazurin	Sigma	R7017-1G
RNaseOUT™ RNase inhibitor	Invitrogen	10777-019
RNAprotect	Qiagen	76506
RNeasy Mini kit : RLT buffer, RW1 buffer, RPE buffer, RNase free water	Qiagen	74104
Roti®-Safe Gel Stain	Carl Roth	3865.1
Rotiphorese® Gel 30 (37.5:1)	Carl Roth	3029.1
RQ1 DNase 10X Reaction Buffer	Promega	M198A
RQ1 DNase Stop Solution	Promega	M199A
RQ1 RNase-Free DNase	Promega	M610A
Safranin	Pro-Lab Diagnostics	PL7013
SeKem® LE Agarose	Lonza	50004
SensiFAST™ SYBR® HiROX kit	Bioline	BIO-92020
SPSS 21	SPSS	N/A
SYBR® Gold Nucleic Acid Gel Stain (10,000X concentrate in DMSO)	Life Technologies	S-11494
Tea light candle	Morrisons	N/A
TEMED	Sigma	T9281
TissueLyser II	Qiagen	85300
TV400-DGGE system (gel size 16.5x17.5cm)	Biostep	TV400-DGGE
Urea	Sigma	U5378
Vaseline	Sainsburys	N/A
Water chiller unit HC300A	Hailea	N/A
Whatman Nucleopore Polycarbonate 47mm, 3µm	Sigma	111112

Appendix 3.

A list of all of the PCR primers used within this thesis. Primer sequences, target, and their intended use is given.

Primer	Sequence (5' → 3')	Target	Reference	Use
16S_seq_27F	AGAGTTTGATCMTGGCTCAG	V1-V2 region of 16S rDNA gene for high throughput sequencing	See appendix 4.	Plymouth University
16S_seq_338R	GCWGCCWCCCGTAGGWGT			
16S_uniBact_fw	AGGATTAGATACCCTGGAGTCCA	Approximation of total bacteria activity – 16S rDNA	Adamek *et al.* 2013	RT-qPCR Plasmids
16S_uniBact_rv	CATGCTCCACCGCTTGTGC			
40S_fw	CCGTGGGTGACATCGTTACA	Carp – 40S housekeeping gene	Huttenhuis *et al.* 2006	RT-qPCR
40S_rv	TCAGGACATTGAACCTCACTGTCT			
Aero16S_fw	GCGAAGGCGGCCCCCTGGACAAAGA	*Aeromonas* sp. activity – 16S rDNA	Adamek *et al.* 2013	RT-qPCR Plasmids
Aero16S_rv	CCACGTCTCAAGGACACAGCCTCCAAATC			
ApoA1_fw	CCATCTCCGCCTCCTTTC	Carp – Apolipoprotein 1	Dietrich *et al.* 2014	RT-qPCR
ApoA1_rv	ATGTGTTAGTGTGTGTGCTTC			
Bergmark_16S_fw	ACTTTAAGTTGGGAGGAAGGG	*Pseudomonas* sp. activity – 16S rDNA	Bergmark *et al.* 2012	RT-qPCR
Bergmark_16S_rv	ACACAGGAAATTCCACCACCC			
Bf/C2_fw	CGGTCATGGGAAAAAGCATTGAGA	Carp – Complement pathway	Forlenza *et al.* 2009	RT-qPCR
Bf/C2_rv	GATATCTTTAGCATTTGTCGCAG			
CRP1_fw	AGCAATGCAACATTTTTCCGTC	Carp – C-reactive protein isoform	Falco *et al.* 2012a	RT-qPCR
CRP1_rv	ACTTGCGTCAAAGCCACCCAC			
CRP2_fw	GATGCTGCAGCATTTTTCAGTC	Carp – C-reactive protein isoform	Falco *et al.* 2012a	RT-qPCR
CRP2_rv	CTCCGCATCAAAGTTGCTCAAAT			
C1rs	CAAGCCCATCTTGGCTCCTGG	Carp – Complement pathway	Forlenza *et al.* 2009b	RT-qPCR
C1rs	GTCCAGATCAAGCGGGGACGT			
C3_fw	GGTTATCAAGGGGAGTTGAGCTAT	Carp – Complement pathway	Forlenza *et al.* 2009b	RT-qPCR
C3_rv	TGCTGCTTTGGGTGGATGGGT			
DGGE_fw	CGCCCGCCGCGCGCGGCGGCGGGCGGGGCGGGGGCA CGGGGGGCCTACGGGAGGCAGCAG	Qualitative analysis of bacteria diversity – 16S rDNA	Muyzer *et al.* 1993	PCR-DGGE
DGGE_rv	ATMTCTACGCATTTCACCGCTAC		Steinum *et al.* 2009	
Flav16S_fw	GGGATAGCCCAGAGAAATTTGGAT	*Flavobacterium* sp. activity – 16S rDNA	Adamek *et al.* 2013	RT-qPCR Plasmids
Flav16S_rv	AGTCTTGGTAAGCCGTTACCTT			

A list of all of the PCR primers used within this thesis. Primer sequences, target, and their intended use is given.

Primer	Sequence (5' → 3')	Target	Reference	Use
HAMP1_fw	TGGAGAGTGAGGCACACCAGGAG	Carp – Hepcidin antimicrobial peptide 1	Designed by Dr. Adamek (TiHo)	RT-qPCR
HAMP1_rv	TGCCAGGGGATTGGTTTG			
IL1β_fw	AAGGAGGCCAGTGGCTCTGT	Carp – Interleukin 1β	Huttenhuis *et al.* 2006	RT-qPCR
IL1β_rv	CCTGAAGAAGAGGAGGCTGTCA			
IL1β_rec_fw	ACGCCACCAAGAGCCTTTTA	Carp – Interleukin 1β receptor	Designed by Dr. Falco (MHUE)	RT-qPCR
IL1β_rec_rv	GCAGCCCATATTTGGTCAGA			
iNOS_fw	AACAGGTCTGAAAGGGAATCCA	Carp – Inducible nitric oxidase	Forlenza *et al.* 2009b	RT-qPCR
iNOS_rv	CATTATCTCTCATGTCCAGAGTCTCTTCT			
LEAP2_fw	GGATCGTGGGCACTAAACCTC	Carp – Liver expressed antimicrobial peptide 2	Designed by Dr. Adamek (TiHo)	RT-qPCR
LEAP2_rv	GCCTTTCCTGCATATTCCTGTC			
Martinez_16S_fw	GCAGGCCTAACACATGCAAGTC	*Lactobacillus* sp. activity – 16S rDNA	Martinez-Puig *et al.* 2007	RT-qPCR
Martinez_16S_rv	CTGCTGCCTCCCGTAGGAGT			
MASP2_fw	CAAGCTGTCCAAGGTGATTG	Carp – Complement pathway	Forlenza *et al.* 2009b	RT-qPCR
MASP2_rv	AGCAGTGAGGACCCAGTTGT			
Muc2_fw	TGACTGCCAAAGCCTCATTC	Carp – Mucin isoform	Van der Marel *et al.* 2012	RT-qPCR
Muc2_rv	CCATTGACTACGACCTGTTTCTC			
Pseud16S_fw	TGCCTAGGAATCTGCCTGGTAGT	*Pseudomonas* sp. activity – 16S rDNA	Designed by Dr. Adamek (TiHo)	RT-qPCR Plasmids
Pseud16S_rv	AATCCGACCTAGGCTCATCTGATAGCG			
Strept16S_fw	CGGTAACTAACCAGAAAGGGA	*Streptococcus* sp. activity – 16S rDNA	Designed by Dr. Adamek (TiHo)	RT-qPCR Plasmids
Strept16S_rv	ATAAATCCGGACAACGCTCGRAGA			
TNFα1_fw	GAGCTTCACGAGGACTAATAGACAGT	Carp – Tumor necrosis factor α isoform	Forlenza *et al.* 2009a	RT-qPCR
TNFα1_rv	CTGCGGTAAGGGCAGCAATC			
TNFα2_fw	CGGCACGAGGAGAAACCGAGC	Carp – Tumor necrosis factor α isoform	Forlenza *et al.* 2009a	RT-qPCR
TNFα2_rv	CATCGTTGTGTCTGTTAGTAAGTTC			
Vib16S_fw	GTTTGCCAGCGAGTAATGTC	*Vibrio* sp. activity – 16S rDNA	Designed by Dr. Adamek (TiHo)	RT-qPCR Plasmids
Vib16S_rv	TAGCTTGCTGCCCTCTGTATGCG			

Appendix 4.

Report produced by the University of Plymouth detailing the methodologies used for Next Generation Sequencing.

AQUACULTURE WITH PLYMOUTH UNIVERSITY

16S rRNA barcode sequencing analysis of carp intestinal samples

January 2016

School of Biological Sciences.
Plymouth University

1.1. Samples

Twenty DNA extractions were received from Keele University. The samples were divided into two groups: 8MGW and 15MGW, and each group contained 10 replicates. Sample details are listed in Appendices 1 and 2.

1.2. PCR Amplification

The 16S rRNA V1-V2 region was amplified from the DNA extractions using the primers 338R (GCW GCC WCC CGT AGG WGT) and 27F (5' - AGA GTT TGA TCM TGG CTC AG – 3'). The following reagents were included in each PCR tube: 1 µL of primer 338R and 1 µL of primer 27F (each 50 pmol μL^{-1}; Eurofins MWG, Ebersberg, Germany), 2 µL of DNA template, 50 µL of MyTaq™ (Bioline, London, UK) and 34 µL of PCR grade water. Thermal cycling was conducted using a TC-512 thermal cycler (Techne, Staffordshire, UK) under the following conditions: initial denaturation at 94°C for 7 min, then 10 cycles at 94°C for 30 s, followed by a touchdown of 1°C per cycle from 62 -53°C for 30 s and 72°C for 30 s. A further 20 cycles were performed at 94°C for 30 s, 53°C for 30 s and 72°C for 30s before a final extension for 7 min at 72°C.

Agarose gel electrophoresis revealed multiple bands after PCR amplification (Figure 1). PCR reactions were therefore cleaned using a QIAquick Gel Extraction Kit (QIAGEN) following the manufacturer's instructions.

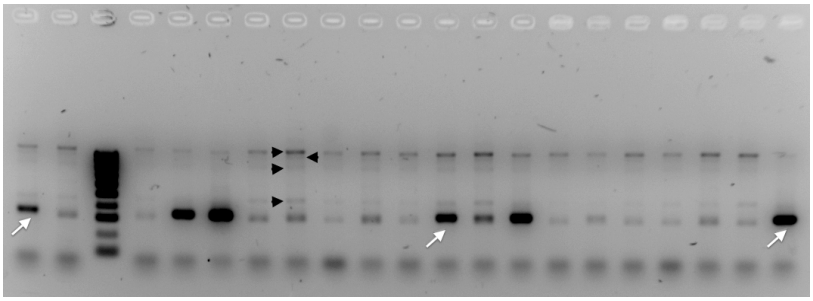


Figure 1. Agarose gel of PCR amplicons. Note several bands (black arrow head) larger than 350 bp (V1-2 region) (white arrows).

1.3. High-throughput sequencing and bioinformatics

Prior to sequencing the amplicons were assessed for fragment concentration using an Ion Library Quantitation Kit (LifeTechnologies™, USA), and concentrations were then adjusted to 26 pM. Amplicons were attached to Ion Sphere Particles (ISPs) using Ion PGM™ Template OT2 400 kit (LifeTechnologies™, USA) according to the manufacturer's instructions. Multiplexed sequencing was conducted using Ion Xpress™ Barcode Adapters (LifeTechnologies™) and a 318™ chip (LifeTechnologies™) on an Ion Torrent Personal Genome Machine (LifeTechnologies™) at the Systems Biology Centre in

Plymouth University (UK). Sequences were binned by sample and filtered within the PGM software to remove low quality reads. Data were then exported as FastQ files.

Taxonomic analyses of sequence reads were performance after the removal of low quality scores (Q score <20 at 80% probability) with FASTX-Toolkit (Hannon Lab, USA). Sequences were concatenated and sorted by sequence similarity into a single fasta file. Sequences were denoised and analyzed with QIIME (Caporaso *et al*., 2010). Briefly, OTU mapping was performed using the USEARH quality filter pipeline (Edgar, 2010), to remove putatively erroneous reads (chimeras), then OTU picking was achieved with a minimum pairwise identity of 97%. The most abundant sequence in each OTU were selected to assign a taxonomic classification based on the Greengenes database (DeSantis *et al*., 2006) using the RDP classifier (Wang *et al*., 2007), clustering the sequences at 97% similarity with a 0.80 confidence threshold. PyNast was used to create a multiple alignment of the representative sequences for each OTU (Caporaso *et al*., 2009) with minimum sequence length threshold of 150 base pairs and 95% identification. Sequences were filtered to remove outliers and filter positions with gaps (0.95) and singletons.

Alpha diversity metrics were calculated on rarefied OTU tables with QIIME to asses sampling depth coverage using observed species, Chao1, Shannon's diversity index and Good's coverage. QIIME was also used to calculate Beta diversity metrics among samples using unweighted and weighted Unifrac distances (Lozupone *et al*., 2007) and Bray-Curtis similarity (Bray and Curtis, 1957). The distance matrices were represented by two dimensional principal coordinates analysis (PCoA) plots. Reads assigned to the *Cyanobacteria* phylum (after Wong et al., 2013) and the *Propionibacteriaceae* Family were considered as contaminants, and thus were removed from downstream anlyses.

1.4. Statistics

To test for significant differences among intestinal microbiome data a non-parametric t-test was performed to compare OTUs abundance using STAMP and alpha diversity metrics. Vegan and ape packages of R were used to analyse the beta diversity of the groups. Statistical significance was accepted at the $P < 0.05$ level.

2. Results and Discussion

2.1. High-throughput sequencing analysis

High-throughput sequencing libraries from samples generated 3,011,088 sequences. After trimming, QC and removal of *Cyanobacteria* (2,736 reads) and *Propionibacteriaceae* (1,111 reads), a total of 1,288,902 reads were retained. Good's coverage rarefaction curves for all individual samples reached a plateau close to 1 (i.e. 0.9988-0.9989) (Figure 2; Table 1), thus the microbiome of the samples were fully sampled.

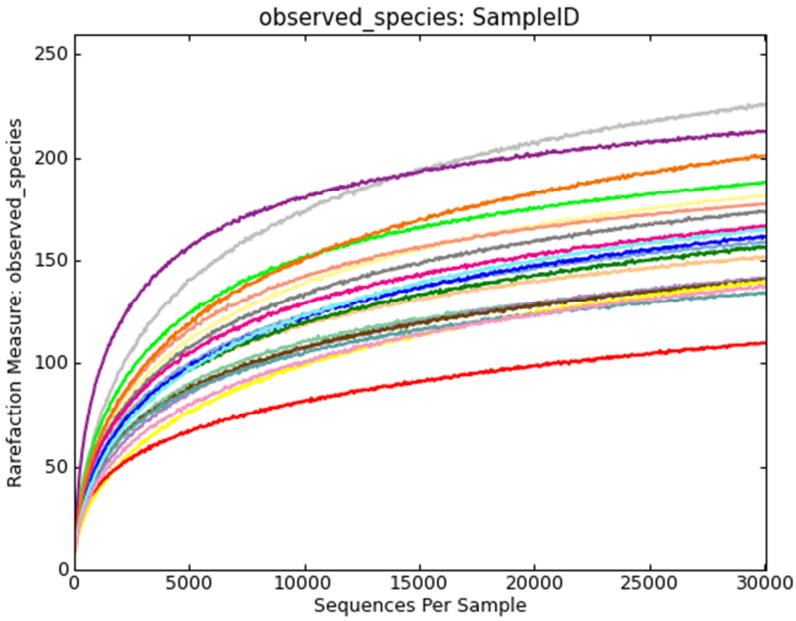


Figure 2. Good's coverage rarefaction curves of the intestine samples.

Table 1. Good's coverage estimations by treatment.

Sample code	Good's coverage
8MGW	0.9988 ± 0.0001
15MGW	0.9989 ± 0.0001

The majority of reads derived from the mucosa samples belonged to members of *Proteobacteria* (accounting for 70.1% of the reads), *Fusobacteria* (27.2%), *Firmicutes* (2.2%) and to a lesser extent *Actinobacteria*, *Bacteriodetes*, *GN02, Verrucomicrobia* were also observed (Table 2 and Figure 3). There were no significant differences in the phyla composition between the treatments.

Table 2. Abundance of the OTUs present in the treatments at the phylum level.

Taxon	8MGW	15MGW
Proteobacteria	69.85 ± 34.25	70.27 ± 27.01
Fusobacteria	26.83 ± 30.33	27.54 ± 27.57
Firmicutes	2.66 ± 6.84	1.65 ± 2.62
Actinobacteria	0.37 ± 0.37	0.34 ± 0.28
Bacteroidetes	0.16 ± 0.11	0.15 ± 0.11
GN02	0.00 ± 0.00	0.02 ± 0.05
Verrucomicrobia	0.04 ± 0.14	0.00 ± 0.00
Bacteria (Other)	0.05 ± 0.15	0.00 ± 0.00

Data are represented by mean ± SD.

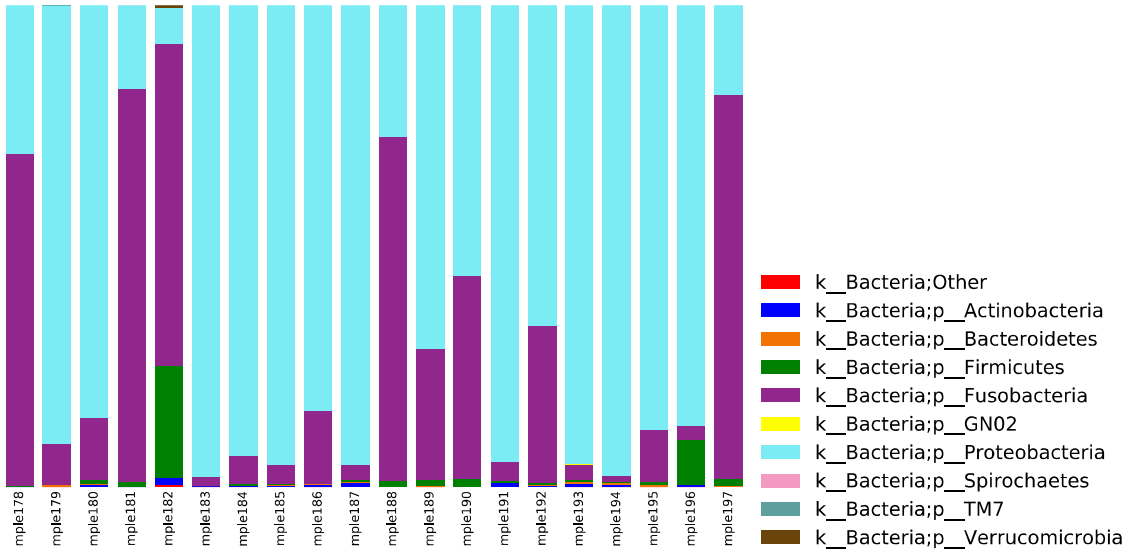


Figure 3. The relative abundance (100% stack bars) of the assigned at the phylum level.

Table 3 and Figure 4 show the most abundant genera per treatment and per sample, respectively. The OTUs composition of the samples was very similar, but some significant differences were observed for OTUs present at relatively low abundances. For example, the abundance of *Bradyrhizobium,* Enterobacteriaceae, *Leuconostoc, Acidovorax, Clostridium, Trabulsiella* was significantly higher in the 15 treatment than in the 8 group ($P < 0.05$).

At the species level the samples of the treatment 15 contained significantly higher abundance of *Bradyrhizobium* sp., *Clostridium butyricum*, *Leuconostoc* sp*., Lactobacillus zeae, Trabulsiella* sp., Acidovorax sp., *Brevibacillus reuszeri* and some unknown species of the Genus *Pseudomonas* and *Bradyrhizobium* and unknown species from the Family *Enterobacteriaceae* and *Caulobacteraceae* ($P < 0.05$). Data at the species level is provided in a separate excel file.

Table 3. Abundance of the OTUs at the genus level (where possible). Data represented are means ± SD of the genera with abundance accounting for >0.1% of the total reads.

	8MGW	**15MGW**
Phyllobacterium	55.72 ± 31.82	52.22 ± 27.69
Cetobacterium	26.61 ± 30.06	27.54 ± 27.57
Family Phyllobacteriaceae (Other)	5.28 ± 3.03	5.27 ± 2.64
Vibrio	2.18 ± 3.15	5.00 ± 10.27
Family Rhodospirillaceae (Other)	1.86 ± 1.15	1.79 ± 1.16
Bradyrhizobium	0.71 ± 0.45[a]	1.57 ± 0.59[b]
Order Rhizobiales (Other)	1.22 ± 0.67	1.35 ± 0.80
Enhydrobacter	0.50 ± 0.65	0.62 ± 0.60
Order Aeromonadales (Other)	0.29 ± 0.28	0.43 ± 0.46
Lactobacillus	0.03 ± 0.04	0.42 ± 1.00
Order Vibrionales (Other)	0.13 ± 0.21	0.39 ± 0.68
Mesorhizobium	0.33 ± 0.21	0.33 ± 0.21
Patulibacter	0.14 ± 0.12	0.22 ± 0.21
Pediococcus	0.00 ± 0.00	0.21 ± 0.65
Lactococcus	0.01 ± 0.01	0.18 ± 0.46
Stenotrophomonas	0.11 ± 0.08	0.16 ± 0.11
Order Clostridiales (Other)	0.88 ± 2.38	0.16 ± 0.14
Enterobacteriaceae (Other)	0.06 ± 0.04[a]	0.14 ± 0.09[b]
Family Bradyrhizobiaceae (Other)	0.12 ± 0.14	0.14 ± 0.11
Streptococcus	0.25 ± 0.62	0.12 ± 0.12
Family Sphingomonadaceae (Other)	0.06 ± 0.09	0.12 ± 0.14
Polynucleobacter	0.40 ± 0.95	0.11 ± 0.15
Leuconostoc	0.02 ± 0.03[a]	0.09 ± 0.08[b]
Flavobacterium	0.11 ± 0.12	0.08 ± 0.07
Acidovorax	0.01 ± 0.01[a]	0.04 ± 0.03[b]
Clostridium	0.00 ± 0.00[a]	0.02 ± 0.01[b]
Family Lachnospiraceae (Other)	0.74 ± 2.13	0.01 ± 0.01
Trabulsiella	0.00 ± 0.00[a]	0.01 ± 0.01[b]
Family Fusobacteriaceae (Other)	0.20 ± 0.59	0.00 ± 0.00
Ruminococcus	0.28 ± 0.82	0.00 ± 0.00
Family Desulfovibrionaceae (Other)	0.22 ± 0.65	0.00 ± 0.00
Others	1.38 ± 1.91	1.11 ± 0.43

[a,b] different superscripts denote significant differences ($P < 0.05$)

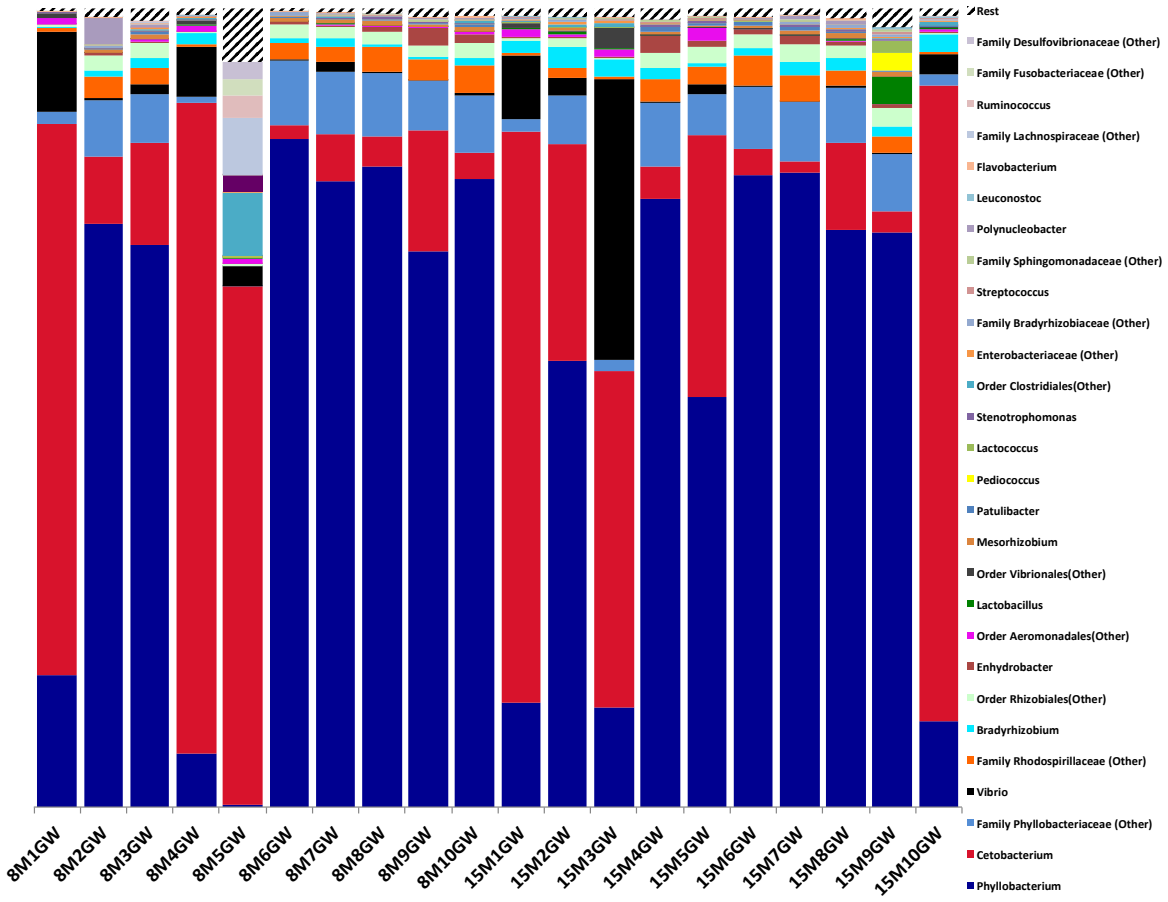


Figure 4. The relative abundance (100% stack bars) of the reads from individual samples, assigned at the genus level. Data shown are those accounting for >0.1% of the total sequence reads.

The alpha diversity parameters are displayed in the Table 4. No significant differences were detected between the treatments.

Table 4. Alpha diversity parameters.

Sample code	Chao1	Observed species	Phylogenetic tree	Shannon
8MGW	189.42 ± 30.26	155.73 ± 29.69	6.48 ± 1.2	2.53 ± 0.61
15MGW	199.4 ± 27.01	170.53 ± 24.11	6.72 ± 0.59	2.78 ± 0.48
t stat	-0.738	-1.16	-0.528	-0.949
P-value	0.463	0.268	0.623	0.353

Data are represented by mean ± SD.

Figures 5 shows the beta diversity of the samples through PCoA plots (unconstrained). Figure 6 shows Distance-based ReDundancy Analysis (dbRDA) when the data are constrained by the treatments. No significant differences were observed between the treatments in any of the beta rarefactions statistics.

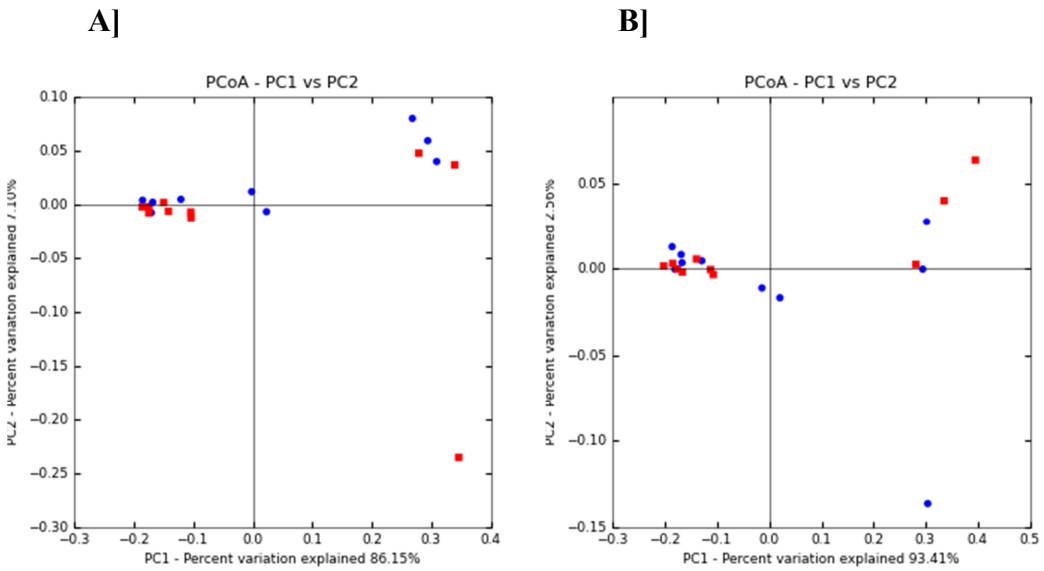


Figure 5. Beta rarefaction PCoA plots of samples using Bray-Curtis (A) and Weighted (B) approaches. 8MGW: red; 15MGW: blue.

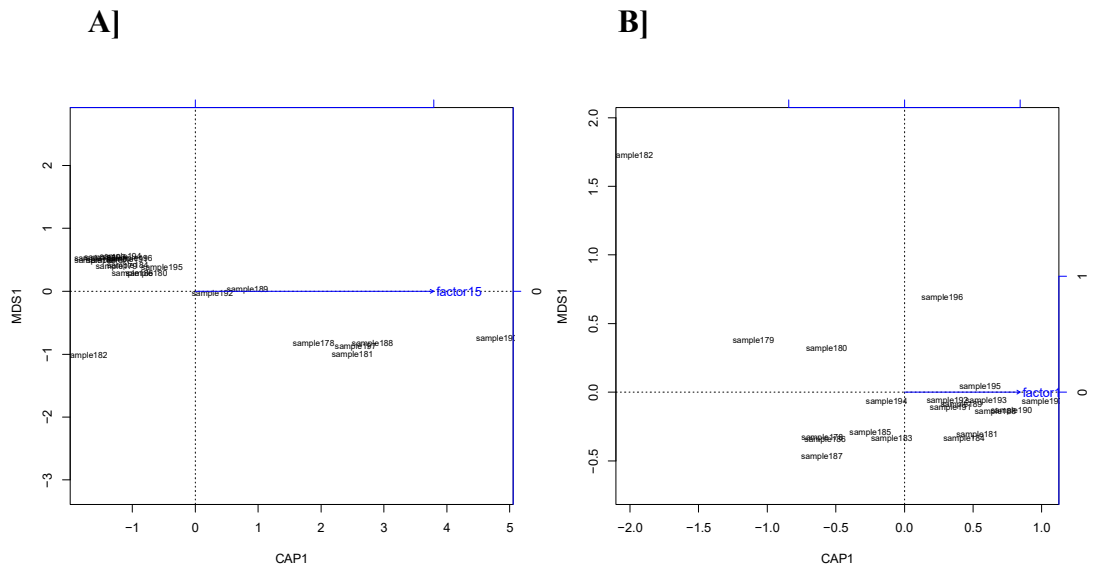


Figure 6. Distance-based ReDundancy Analysis (dbRDA). Beta rarefaction plots of intestines samples following Bray-Curtis (A) and Unweighted (B).

References

Austin B. & Austin D.A. (2007) Bacterial Fish Pathogens: Disease in Farmed and Wild Fish. Springer-Praxis, Goldalming.

Austin B. & Austin D.A. (2012) Bacterial Fish Pathogens: Disease of Farmed and Wild Fish, Springer-Praxis, Goldalming.

Balcazar, J.L. de Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Girones, G. & Muzquiz J.L (2007) Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout (*Oncorhynchus mykiss*). FEMS Immunol Med Microbiol 51, 185–193.

Bray JR, Curtis JT (1957). An ordination of the upland forest communities of southern Wisconsin. *Ecological monographs* **27:** 325-349.

Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* **26:** 266-267.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK (2010b). QIIME allows analysis of high-throughput community sequencing data. *Nature methods* **7:** 335-336.

Dimitroglou, A., Merrifield, DL., Spring, P., Sweetman, J., Moate, R. & Davies, S. J. 2010. Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed utilisation, intestinal histology and gut microbiota of gilthead sea bream (*Sparus aurata*). Aquaculture*,* 300**,** 182-188.

Edgar RC (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26:** 2460-2461.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology* **72:** 5069-5072.

Dimitroglou A, Merrifield DL, Carnevali O, Picchietti S, Avella M, Daniels C. (2011). Microbial manipulations to improve fish health and production–a Mediterranean perspective. *Fish & Shellfish Immunology* **30:** 1-16.

Faith DP (1992). Conservation evaluation and phylogenetic diversity. *Biological Conservation* **61:** 1-10.

Lozupone CA, Hamady M, Kelley ST, Knight R (2007). Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Applied and Environmental Microbiology* **73:** 1576-1585.

Merrifield D. L. & Ringø E. (2014). Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics (2014) ISBN: 978-0-470-67271-6. Wiley-Blackwell.

Tsuchiya, C., Sakata, T. & Sugita H. (2008) Novel ecological niche of *Cetobacterium somerae*, an anaerobic bacterium in the intestinal tracts of freshwater fish. Letters in Applied Microbiology 46, 43–48.

Pérez-Sánchez, T, Balcázar, J.L., García, Y., Halaihel , N., Vendrell, D. , de Blas, I., Merrifield, D. L. & Ruiz-Zarzuela I.(2011) Identification and characterization of lactic acid bacteria isolated from rainbow trout *Oncorhynchus mykiss* (Walbaum) with inhibitory activity against *Lactococcus garvieae*. *Journal Fish Diseases.* 34, 499-507.

Vendrell, D., Balcazar, J.L., de Blas, I., Ruiz-Zarzuela, I., Girones, O. & Muzquiz J.L. (2008) Protection of rainbow trout (*Oncorhynchus mykiss*) from lactococcosis by probiotic bacteria. Comparative Immunology, Microbiology & Infectious Diseases 31 (2008) 337–345

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73:** 5261-5267.

Wang Y, Qian P-Y (2009). Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *PloS one* **4:** e7401.

Wong, S., Waldrop, T., Summerfelt, S., Davidson, J., Barrows, F., Kenney, P.B., Welch, T., Wiens, G.D., Snekvik, K. & Rawls, J.F. (2013) Aquacultured rainbow trout (*Oncorhynchus mykiss*) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. Applied and environmental microbiology, 79, 4974-4984.

Appendix 1. Samples provided.

Molecular microbiology analysis			
Group 1 - Samples to analyse (n=10)			
DNA extraction	8M1GW	DNA extraction	8M6GW
DNA extraction	8M2GW	DNA extraction	8M7GW
DNA extraction	8M3GW	DNA extraction	8M8GW
DNA extraction	8M4GW	DNA extraction	8M9GW
DNA extraction	8M5GW	DNA extraction	8M10GW
Group 2 - Samples to analyse (n=10)			
DNA extraction	15M1GW	DNA extraction	15M6GW
DNA extraction	15M2GW	DNA extraction	15M7GW
DNA extraction	15M3GW	DNA extraction	15M8GW
DNA extraction	15M4GW	DNA extraction	15M9GW
DNA extraction	15M5GW	DNA extraction	15M10GW

Appendix 2. Identification of samples used in microbiological analyses and number of sequence reads per sample.

Sample code	**Sample ID**	**Number of reads**
sample178	8M1GW	49739
sample179	8M2GW	64042
sample180	8M3GW	47995
sample181	8M4GW	54252
sample182	8M5GW	113940
sample183	8M6GW	89023
sample184	8M7GW	88378
sample185	8M8GW	65637
sample186	8M9GW	103753
sample187	8M10GW	55412
sample188	15M1GW	43512
sample189	15M2GW	36247
sample190	15M3GW	31854
sample191	15M4GW	65047
sample192	15M5GW	57941
sample193	15M6GW	67808
sample194	15M7GW	74304
sample195	15M8GW	63196
sample196	15M9GW	55336
sample197	15M10GW	61486