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Relative expression of selected immune related genes in larvae of Atlantic cod (*Gadus morhua* L.)

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Declaration

I hereby declare that I am the only author of this thesis and it is the product of my own research.

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Eydís Elva Þórarinsdóttir

It is herby confirmed that this master thesis is satisfactory to M.Sc. – degree in Natural Resource Sciences from the School of Business and Science, faculty of Natural Resource Science at the University of Akureyri.

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Dr. Kristinn P. Magnússon

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Dr. Rannveig Björnsdóttir

Abstract

The growth potential of farmed cod in Iceland is heavily restricted which is partly due to high mortalities during early larval stages. The immune system of cod larvae is poorly developed at hatch and the larvae are therefore dependent upon innate immune parameters for their defence against a wide variety and high numbers of environmental microorganisms. Methods for stimulation of innate immune parameters are therefore needed along with reliable techniques for evaluation of various production approaches.

The aim of the present study was to adapt the RT-qPCR method for quantitative analysis of the expression of selected innate immune related genes, mIgM, g-type lysozyme and hepcidin, during early life stages of cod larvae. Additionally, the effects of probiotic treatment using a mixture of two bacterial isolates, *Arthrobacter* sp. and *Enterococcus* sp., were evaluated through expression analysis of the selected genes.

The results indicate that the RT-qPCR method was successful at monitoring the transcriptional development of the selected genes from hatch to mid metamorphosis. The results furthermore indicate that probiotic treatment may have stimulated the expression of mIgM, as different results were obtained depending on the analytical approach applied. Larval survival and the expression of g-type lysozyme and hepcidin were not affected by the treatment. Further analysis is required to determine the effects of probiotic treatment on mIgM expression of larvae and to study the effects of various treatments and production methods on the innate immune system of cod larvae.

Keywords: RT- qPCR, gene expression, Atlantic cod larvae, immune parameters, probiotics

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Eydís Elva Þórarinsdóttir. 25th of May, 2010

Útdráttur

Þorskeldi er vaxandi atvinnugrein á Íslandi en hefur þó ekki gengið sem skyldi, meðal annars vegna mikilla affalla og slakra gæða lirfa og seiða á fyrstu stigum eldisins. Ónæmiskerfi þorsklirfa er lítt þroskað við klak og þurfa lirfur að reiða sig eingöngu á ósérhæfða ónæmissvörun fyrstu mánuðina, eða þar til sérhæfða ónæmiskerfið nær þroska. Mikilvægt er því að leita leiða til að efla ósérhæft ónæmissvar lirfa sem og að þróa áreiðanlegar aðferðir til að meta hvort örva megi ónæmissvörun lirfa við mismunandi fóðrun, umhverfisaðstæður og meðhöndlun.

Markmið verkefnisins var að aðlaga RT-qPCR aðferðina til magngreiningar á tjáningu valinna gena snemma í eldisferli þorsklirfa, þ.e. IgM, lysósíms (g-gerð) og hepcidin, sem þekkt er að taka þátt í ósérhæfðu ónæmissvari. Einnig var rannsakað hvort meðhöndlun með blöndu tveggja bætibaktería, *Arthrobacter* sp. og *Enterococcus* sp., leiddi til aukinnar tjáningar genanna og hvort hugsanleg örvun á tjáningu leiddi til minni affalla í eldinu.

Niðurstöður verkefnisins benda til þess að RT-qPCR aðferðin sé vel til þess fallin að mæla og fylgjast með tjáningu þessara gena allt frá klaki og fram á mitt myndbreytingarskeið þorsklirfa. Ennfremur kom í ljós að meðhöndlun með bætibakteríunum gæti hafa örvað tjáningu á IgM en aukin tjáning IgM fékkst með annarri af þeim tveimur aðferðum sem notaðar voru við úrvinnslu gagna. Meðhöndlun reyndist ekki hafa áhrif á tjáningu lýsósíms eða hepcidin né heldur á lifun lirfa. Framkvæma þarf frekari rannsóknir á því hvort að bætibakteríumeðhöndlunin sem slík hafi í raun örvandi áhrif á tjáningu IgM í þorsklirfum svo og hvort unnt sé að örva ósérhæfða ónæmissvörun lirfa með breyttum aðferðum við meðhöndlun.

Lykilorð: RT-qPCR, genatjáning, þorsklirfur, ónæmissvar, bætibakteríur

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DNA	deoxyribonucleic acid
gDNA	genomic DNA
RNA	ribonucleic acid
rRNA	ribosomal RNA
mRNA	messenger RNA
tRNA	transfer RNA
cDNA	complementary DNA
dsDNA	double stranded DNA
ssDNA	single stranded DNA
dsRNA	double stranded RNA
ssRNA	single stranded RNA
RQI	RNA quality indicator
PCR	polymerase chain reaction
qPCR	quantitative real-time PCR
RT	Reverse transcription
RT-qPCR	quantitative real-time reverse transcription PCR
C_T	cycle threshold
GOIs	genes of interest
dph	days post hatch
wph	weeks post hatch
IgM	immunoglobulin M
mIgM-H	membrane bound form of Immunoglobulin M heavy chain
sIgM-H	secreted form of Immunoglobulin M heavy chain
AMPs	antimicrobial peptides
LPS	lipopolysaccharides
TLRs	toll-like receptors
EST	expressed sequence tag
ELISA	enzyme linked immunosorbent assay
GI tract	gastrointestinal tract
NTC	no template control
rpm	rounds per minute
S.D.	standard deviation

1 Introduction

Atlantic cod (*Gadus morhua*) has for centuries been regarded as one of the most important species for fishing communities surrounding the North Atlantic Ocean (Rosenlund & Halldorsson, 2007). Cod aquaculture has therefore received increased attention during the last years and Iceland is among countries that have put considerable effort and investments into the development of cod aquaculture as a new industry (Gunnarsson *et al.*, 2007).

The production of cod larvae is the major bottleneck in aquaculture of cod and high mortalities are commonly experienced during the early larval stages, especially around the onset of exogenous feeding (Rosenlund & Halldorsson, 2007; Yúfera & Darias, 2007). At the Icelandic Marine Research Institute hatchery (MRI) at Staður in Grindavík, general survival from larvae to juvenile ranges between 10-20% (Steinarsson, 2004). Environmental factors such as temperature, salinity, light and water-quality factors greatly affect larvae during this crucial time in the development (Kjørsvik *et al.*, 2004). The marine environment is far more hostile environment, in a microbial sence, than life on land and at hatch the poorly developed cod larvae are exposed to an environment which contains high bacterial numbers that might also explain the high mortalities commonly observed (Vadstein *et al.*, 2004). The larvae are furthermore known to ingest bacteria by drinking before active feeding starts and the life prey, offered during the first weeks of exogenous feeding, itself carries high bacterial numbers (Olafsen, 2001) including pathogenic groups (Korsnes *et al.*, 2006). The immune system of the larvae is poorly developed at early production stages and the larvae therefore have to rely on non-specific, innate immune parameters for their defence against pathogens until the specific immune system has fully developed 2-3 months after hatching (Magnadottir *et al.*, 2004; Schrøder *et al.*, 1998).

The innate immune system represents the first line of defence against invading pathogens and is regarded to be well responsive in fish (Ellis, 2001). Knowledge of the innate immune system in cod is increasing and any information about this system and the mechanisms behind it are considered a promising approach for improved survival and disease control under culture conditions (Seppola *et al.*, 2009).

The ontogeny of organs and immune functions in cod has been studied using techniques such as immunohistochemistry (IHC) and RNA-RNA *in situ* hybridization (Lange *et al.*, 2004a; Schrøder *et al.*, 1998). Studying the mRNA expression of innate immune related genes using molecular methods such as quantitative real-time reverse transcription PCR (RT-qPCR), will give more accurate information of the ontogeny of the immune system as well as

being an excellent way for providing quantitative information of how different treatments and/or conditions affect the expression of selected genes.

1.1 Analysis of mRNA gene expression

Changes in mRNA transcription levels are critical in many biological processes and RT-qPCR has become frequently applied to measure the effects of various compounds or experimental conditions as to how these compounds, or altered conditions, affect organisms and cells at the molecular level and to what extent a certain gene is expressed at a certain time (Bustin, 2000; Carey, 2007).

qPCR is one of the most commonly used techniques for quantitatively measuring nucleic acids in samples from various sources and the method is considered a useful tool in biotechnology, molecular medicine, microbiology and diagnostics. RT-qPCR combines reverse transcription with qPCR and is currently the method of choice for amplification and detection of low levels of mRNA gene expression in any biological matrixes and application of the method allows measurement of expression levels in many different samples for a limited number of genes (Bustin, 2000; Bustin *et al.*, 2009; Higuchi *et al.*, 1993)

DNA microarrays is another powerful analytical method that enables simultaneous measurement of gene expression levels for up to tens of thousands of genes. DNA microarrays is mainly used in large-scale gene-expression studies whereas RT-qPCR is often used to validate the microarray results as to analyse the expression of a specific genes of interest (GOIs) (VanGuilder *et al.*, 2008). Before the introduction of the RT-qPCR and DNA microarrays, commonly used methods for RNA detection include Northern blotting, *in situ* hybridisation (Parker & Barnes, 1999) and RNase protection assay (Hod, 1992). The low sensitivity of these assays often makes it hard to estimate the real differences in expression and sometimes the expression of genes that are expressed at low levels cannot be detected at all (Bustin, 2000).

RT-qPCR is regarded to be more reliable, sensitive and accurate method that is less labour intensive and requires less RNA template for detection of specific mRNAs than the methods previously mentioned. The main disavantage of the RT-qPCR method is the cost, whereas the necessary equipment is expensive (Bustin, 2000; Wong & Medreano, 2005). Even though RT-qPCR is the method of choice to quantify mRNA expression it requires a broad understanding of the many procedures involved in gaining high quality RT-qPCR results. This includes an understanding of the PCR theory, different types of detection

chemistries, quantification methods and data normalisation (Wong & Medreano, 2005) which will be discussed in greater details in the following sections. Gene expression studies using the RT-qPCR method require the user to perform several separate steps before obtaining the actual experimental results. The main steps that need to be performed during a typical RT-qPCR experiment are shown in Figure 1.

Figure 1. Flowchart of a typical RT-qPCR experiment

1.1.1 RNA extraction and cDNA synthesis

The first step in any gene expression analysis is sample acquisition and the extraction of RNA, either total RNA or mRNA. The quality of the initial template plays an important role as it can influence the accuracy of the gene expression results. Therefore it is preferable to use RNA of high-quality as a starting point in any RNA-based experiments, as has been reviewed by Fleige and Pfaffl (2006). Total RNA or mRNA can be extracted from any kind of biological samples including blood, tissues and cell cultures, but unlike DNA, RNA is an unstable molecule and highly sensitive to degradation. Degradation may occur through cleavage by RNases as a result of improper handling during sampling, extraction and storage of RNA. It is therefore regarded essential to check the quality and quantity after RNA extraction and before going forward to cDNA synthesis. According to Bustin and Nolan (2004), the RNA template must satisfy the following criteria to be valid for inclusion in a RT-qPCR experiment: it must be of the highest quality (undegraded) for the quantitative results to be relevant, it must be free of genomic DNA and enzymatic inhibitors for RT and PCR reactions, and it must be free of nucleases for extended storage (Bustin *et al.*, 2004). Biological samples may furthermore contain inhibitors that affect RT-qPCR experiments and may therefore compromise the whole experiment. In blood and tissue samples such inhibitors may be haemoglobin, urea, heparin, organic and phenolic compounds, glycogen, fat and calcium as well as contamination from unclean laboratory items such as glove powder, dust, pollen and laboratory plastic ware can also act as inhibitors (Wilson, 2000). A variety of RNA extraction kits are commercially available for extraction of total RNA or mRNA from diverse biological materials.

Quality assessment of RNA preparations is essential before any further steps are taken and one must check the integrity of the RNA in every sample. This can be done by inspecting the two distinct transcriptional products (bands) from 28S and 18S RNA, which are the predominant RNAs. The conventional methods to perform this inspection are for example gel-electrophoresis which is time consuming and requires high amounts of RNA, or spectrometric OD measurements which can be performed at different wavelengths to check quantity, quality and purity (Bustin *et al.*, 2004; Fleige *et al.*, 2006). These conventional methods are, however, not regarded sensitive enough to detect RNA degradation (Imbeaud *et al.*, 2005) and the use of lab-on-chip technologies like micro-fluid capillary electrophoresis systems such as the Experion™ Automated Electrophoresis System (Bio-Rad Laboratories Inc., USA) and Agilent 2100 Bioanalyzer (Aglient Technologies, USA) have gained increased attention for RNA quality and quantity assessments (Bustin, 2002; Imbeaud *et al.*, 2005). The Experion system, for example, allows users to quickly and effectively gain information about the quality and quantity status of the RNA samples and the system provides higher sensitivity and better quantification accuracy to both total RNA and mRNA samples compared to other conventional RNA quantity and quality methods (Urban *et al.*, 2005).

RNA cannot be used as a template for PCR in gene expression studies and therefore the mRNA in the sample needs to be converted into cDNA before any expression of a gene can be measured (Kubista *et al.*, 2006). The reverse transcription is performed by the means of RNA-dependent polymerase that uses ssRNA as a template in the presence of primers to synthesize single-stranded cDNA (Bustin, 2000). The reverse transcription step is regarded an important step towards gaining accurate quantification results, whereas the amounts of produced cDNAs must correctly mirror the input amounts of the mRNAs. This step also introduces more variation into the picture than the following qPCR step, but this variation can be reduced by performing the RT-step with replicates rather than just do PCR replicates (Stahlberg *et al.*, 2004).

RT-qPCR assays can either be performed as a one-step or two-step approach. In the one-step assay the cDNA synthesis and qPCR amplification are performed simultaneously in a single tube (Wong & Medreano, 2005) using RNA- and DNA-dependent polymerase such as the *Tth* polymerase (Cusi *et al.*, 1994), while cDNA synthesis and PCR take place in two separate tubes and reactions in the two-step assays. One-step assays are thought to reduce the analysis time and the risk of contamination but are regarded less sensitive than two-step assays (Vandesompele *et al.*, 2002a; Wong & Medreano, 2005). Two-step assays increase the risk of contamination but are regarded to have more flexibility, sensibility and have the room

for more optimisation than the one-step assays as well as generating stable cDNA samples that can be stored indefinitely (Nolan *et al.*, 2006; Vandesompele *et al.*, 2002a). Two-step assays use RNA-dependent polymerases (reverse transcriptases) in the RT-step and the two most commonly used are avian myeloblastosis virus reverse transcriptase (AMV-RT) and Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Bustin, 2000).

There are three priming strategies available for the RT-step, where the transcript can be primed using random primers such as random hexamers, oligo(dT) primers or gene-specific primers (Figure 2).

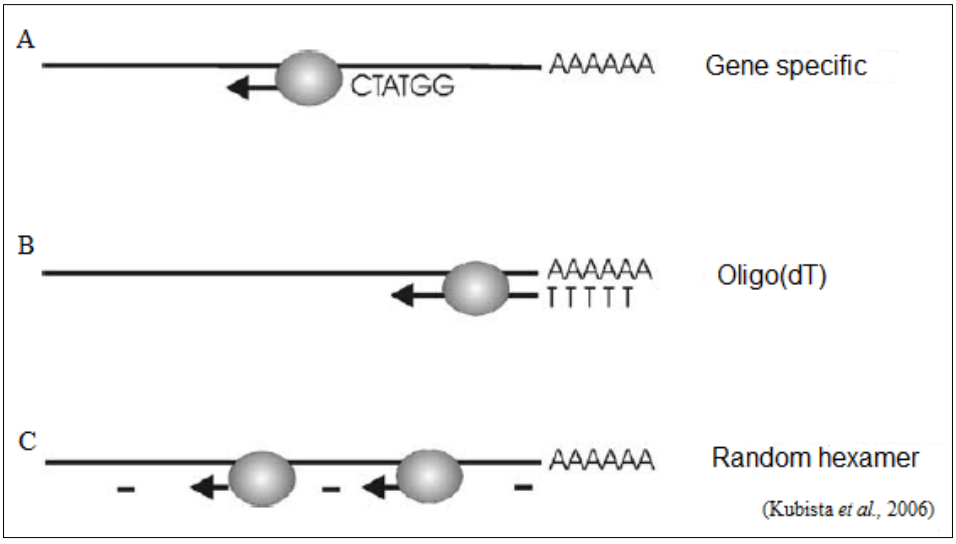


Figure 2. Different priming strategies for cDNA synthesis. A: Gene specific primers B: Oligo(dT) primers C: Random hexamer primers

Random primers are short primers, usually six or nine bases long (hexamers/nonamers) that bind to the RNA transcript at several places. Consequently, random primers produce more than one cDNA per transcript and therefore generate the most cDNA of these priming methods. These primers will copy all RNAs, rRNA, tRNA and mRNA, where most of the cDNA will be derived from rRNA which can be a problem if the target mRNA is in low abundance. Low abundant mRNA may not be primed effectively by these primers which can result in a non-quantitative amplification (Bustin *et al.*, 2004; Kubista *et al.*, 2006). Random primers can vary in length and it has been shown that 15 base pair long random primers yield twice as much cDNA than random hexamers (Stangegaard *et al.*, 2006).

Oligo(dT) primers anneal to the poly(A) tail at the 3´end of the mRNA and will not synthesise cDNA from rRNA or tRNA. These primers are therefore regarded more specific than random primers. The extracted mRNA has to be of good quality whereas the

transcription starts at the 3´end of the mRNA and degraded mRNAs can lack the poly(A) tail and random- or gene-specific primers may therefore be an alternative choice (Kubista *et al.*, 2006).

The use of gene-specific primers for cDNA synthesis is the most specific and efficient priming method. Gene-specific primers are recommended when the RNA sample quantity is not a limited factor and only a few genes are to be analysed at a time, as the main drawback of using gene-specific primers is the requirement that each gene is reversely transcribed in separate cDNA reactions (Bustin *et al.*, 2004; Kubista *et al.*, 2006).

These three priming methods all synthesise cDNA in a different way for the subsequent quantification by the qPCR step. There are pros and cons for each method and that have to be considered carefully, but RT-qPCR results can only be comparable when the exact same priming method and reaction conditions are used (Stahlberg *et al.*, 2004). Also, when producing cDNA with one of these priming methods, it has been recommended to run a regular PCR reaction with the cDNA as a template prior to running a whole expression experiment. The PCR product should then be sequenced to confirm that a correct cDNA has been generated and that the gene of interest has actually been copied (Walker *et al.*, 2002).

1.1.2 Quantitative real-time PCR

qPCR is the next step following cDNA synthesis. qPCR uses fluorescence reporter molecules to monitor the accumulation of the amplified product in “real-time”. By measuring the fluorescence signal emitted by the amplified product and plotting the fluorescence against the cycle number, the real-time PCR instrument generates an amplification plot that represents the accumulation of product over the entire duration of the PCR reaction. The amplification and detection is therefore combined into one step in a closed system and requires no handling of samples after the reaction, opposed to the traditional end-point PCR where detection is usually followed by gel-electrophoresis. This also reduces the risk of cross-contamination (Higuchi *et al.*, 1993; Nolan *et al.*, 2006).

A typical qPCR reaction generally consists of four different kinetic stages (Figure 3). The reaction starts with a linear ground phase or lag phase which usually lasts during the first 10-15 cycles. During this phase, the amplification is just starting but no fluorescence signal above background level is detected and only baseline fluorescence is calculated (Tichopad *et al.*, 2003). The second stage is the early exponential phase where the rise in the fluorescence signal reaches above the background levels, crosses a fixed threshold and gives a defined

value or the C_T. The C_T value is proportional to the starting concentration of the target DNA/cDNA, whereas higher starting concentrations of the target gain lower C_T values. In qPCR data analysis, the C_T value is crucial whereas it is used to calculate all experimental results and therefore serves as the basis for quantification (Bustin *et al.*, 2004; Heid *et al.*, 1996; Higuchi *et al.*, 1993).

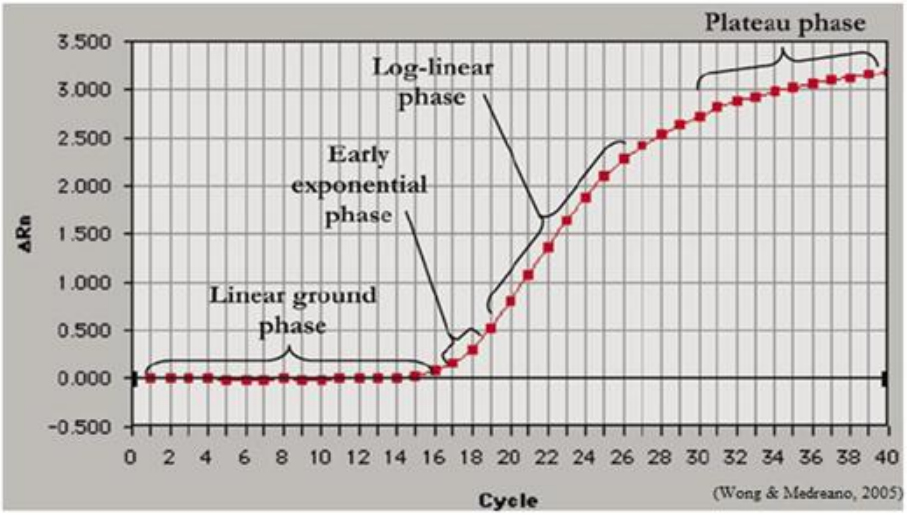


Figure 3. Kinetics of a typical qPCR reaction

An exponential increase of the PCR product occurs during the log-linear phase where the PCR product doubles at every cycle, assuming ideal reaction environment which corresponds to 100% amplification efficiency (E). When reaching the plateau phase, the reaction slows down due to limitations in reaction reagents, followed by a steady state where no more amplicons are produced. C_T values are recorded during the early exponential phase, when the fluorescence reaches above the background fluorescence, and the quantification is therefore not affected by the plateau phase. The threshold level and baseline calculations that define the C_T value can either be set arbitrarily or by using algorithms build within each thermocycler (Bustin, 2000). The setting of threshold is in a way arbitrary and does not affect the differences between C_T values as it rather affects the individual C_T values (Kubista *et al.*, 2006).

The amplification efficiency of the PCR reaction plays an important role when quantifying gene expression. As stated above, an ideal reaction environment corresponds to 100% amplification efficiency where the PCR product doubles during every cycle. This is not always the case and different PCR reactions can have different amplification efficiencies that should therefore be calculated for each PCR assay (amplicon specific efficiency). It is

common that the amplicon specific efficiency in biological samples, such as blood and tissue samples, usually range somewhere between 85-90% (Kubista *et al.*, 2006). PCR efficiency can depend on the assay itself (amplicon specific efficiency) as well as on the sample (sample specific efficiency). It is a matter of dispute how a sample specific efficiency should be calculated, but there is a common agreement on the use of amplicon specific PCR efficiency derived from a series of dilutions of either known or unknown concentrations. The serial dilution can be made from pooled samples from the experiment in order to impersonate as much of the actual samples that are to be measured (Hellemans *et al.*, 2007), from a purified PCR product or a purified plasmid including the target sequence. The C_T values from the serial dilutions are then plotted against the logarithm of the concentration of the sample, number of copies in the sample or the dilution factor (Kubista *et al.*, 2006; Rutledge & Cote, 2003). Using linear regression formulas, the efficiency can be calculated using equations that has been established for this purpose (Radonic *et al.*, 2004).

Small amplicons amplify with higher amplification efficiency and are more lenient towards reaction conditions compared with longer amplicons and the selection of small amplicons is therefore preferable when performing qPCR experiments. Small amplicons are also more likely to become completely denatured during the denaturing step in the PCR reaction, before the primers are allowed to bind to their complementary target sites (Bustin, 2000). Numerous software systems are available for designing primers for PCR and qPCR experiments. The optimal length of primers is regarded to be between 15-20 bp and to maximize specificity, the optimal G/C content should be around 50% but can range between 30-80%. Sequences with high G/C content may reduce the efficiency and form non-specific products (Nolan *et al.*, 2006). The optimal T_m of primers should be between 58-60°C and the difference between forward and reverse primers should not be greater than 1-2°C. Primer concentration can range between 50-400 nM, depending on the detection chemistry used. The concentration needs to be optimised in order to avoid any mispriming and amplification of non-specific products. The risk of non-specific priming can also be reduced by designing primers that only have 1-3 G/C within the last 5 bases at the 3′ end of the primers (Bustin, 2000; Nolan *et al.*, 2006).

1.1.3 Detection chemistries

As stated above, real-time PCR detects and amplifies PCR products with the aid of fluorescence reporter molecules. The fluorescence chemistry can be of a specific or a non-specific character and four different techniques are commonly used. The simplest one is non-specific detection that depends on the use of fluorescence DNA binding dyes like SYBR Green I. The other three are all specific detection assays that depend on the use of fluorescence-labelled amplicon-specific probes that hybridise only to a specific target amplicon (Bustin, 2000; Bustin & Nolan, 2004; Wong & Medreano, 2005). These probe-based specific detection assays are almost exclusively based on fluorescence resonance energy transfer (FRET) and include the use of hybridisation probes, hydrolysis probes such as the TaqMan chemistry or hairpin probes such as molecular beacons or scorpions, as has been reviewed by Wong and Medreano (2005).

DNA binding dyes such as SYBR Green I will bind non-specifically to any dsDNA and emit fluorescence signal. The dye molecules emit very little fluorescence when unbound in the sample but more and more dye molecules will bind directly to the dsDNA produced during the elongation process of the PCR. The accumulation of the amplified product can thereby be monitored whereas the increased fluorescence signal is measured at the end of every elongation step during the cycling process (Bustin, 2000).

The use of DNA binding dyes, such as SYBR Green I, has its advantages and drawbacks just as any other molecular method. The main advantages are that DNA binding dyes are easy to use as they can be used to detect any PCR product and incorporated into any qPCR protocols with reduced running costs since these dyes are relatively cheaper than the probes used in the probe-based methods. The main drawback of DNA binding dyes is their non-specific binding to any dsDNA molecules, including the intended amplicon or any other non-specific product like primer dimers that are created when primers anneal with each other, that may lead to false positives (Bustin *et al.*, 2004).

The specificity can be enhanced by the use of melt curve analysis for identification of specific PCR products following the PCR process. During melt curve analysis, products from the PCR reaction are melted/denatured by slowly increasing the temperature of the sample, resulting in one sharp peak on the melt curve plot represented by the target amplicon. The melting temperature (T_m) of the target amplicon is defined as the temperature where the dsDNA product is denatured into single-strands and therefore depends on the G/C content, length and nucleotide composition of the amplicon. Non-specific products tend to melt at a

lower temperature than the target amplicon in broader peaks and can therefore be distinguished from the target amplicon (Ririe *et al.*, 1997). The melt curve peaks are regarded to be equivalent to the bands from gel-electrophoresis (Nolan *et al.*, 2006).

Another drawback of using DNA binding dyes relies on the fact that binding is dependent on the mass of the double-stranded target amplicon. As many dye molecules bind to every single amplicon, the amount of fluorescence signal depends on the mass of the amplicon and, assuming equal efficiencies, larger amplicons will generate stronger signal compared to shorter amplicons. This can lead to inaccurate quantification (Bustin, 2000). Even though the use of DNA binding dyes depends on non-specific detection is not necessarily less reliable than specific detection assays. DNA binding dye assays need to be well optimised and these assays are practical to quantify mRNA from many different genes because they do not require the investment in expensive gene-specific probes for each target gene (Bustin *et al.*, 2004).

1.1.4 Quantification methods

Levels of gene expression are quantified using one of two different quantification strategies: an absolute or a relative quantification.

Absolute quantification should be used in expression studies where absolute transcript copy numbers are necessary whereas this method determines the input copy number per cell, concentration of total RNA or mass of tissue of the GOI by relating the PCR fluorescence signal to an external standard/calibration curve. For accurate calculations of copy numbers of the GOI, it is essential to use standards of a known concentration that are amplified with the same efficiency as the GOI (Bustin, 2000; Heid *et al.*, 1996).

Relative quantification measures and describes relative changes in expression levels of the GOI. This method of quantification is regarded easier to perform whereas it does not require the use of standard curves with known concentrations. All that is required from the standards is that their relative dilutions are known (Livak & Schmittgen, 2001; Pfaffl, 2004).

In relative quantification, one can measure the mRNA level of expression from one or multiple GOIs over multiple samples and the expression levels are expressed relative to another exogenous or endogenous control. The results are then expressed as a ratio of the GOI and the exo- or endogenous control expressions levels. The gene expression can be relative to one of the following parameters: (i) an endogenous control such as stably expressed reference gene or another GOI, (ii) an exogenous control such as a RNA or DNA control, (iii) a

reference gene index which can consist of multiple averaged endogenous controls or (iv) GOI index consisting of averaged GOIs that are analysed in the same study (Pfaffl, 2006). This is also called normalisation and is described in more detail in section 1.1.5. A second relative parameter can also be chosen and the results then often expressed as relative to an untreated control, time 0 in a time-course study or in some cases healthy individuals. The highest or lowest expression of genes can also be used for this intension (Livak & Schmittgen, 2001).

Several mathematical quantification strategies are available for calculating normalised gene expression levels for relative quantification. The first methods to convert C_T values into normalised relative quantities were reported in 2001 (Livak & Schmittgen, 2001; Pfaffl, 2001). Livak and Schmittgen (2001) introduced the $2^{-\Delta\Delta Ct}$ method which assumes that the GOI and the reference gene both have 100% efficiencies (E = 2 = 100%). The authors recommend that the reference gene is carefully selected and its expression stability should be well validated. Later on, Pfaffl (2001) modified this method by taking amplicon specific efficiencies into account in order to correct for amplicon specific efficiency differences between the GOI and the reference. The relative expression of the GOI is then calculated based on the efficiency of an unknown sample versus a control, and expressed relative to a reference gene. In 2002, the Pfaffl (2001) model was extended by taking into account multiple stably expressed reference genes for normalisation by generating a normalisation factor (NF) which consists of the geometric mean expression of these multiple reference genes. Normalised expression levels of the GOI are then calculated by dividing the relative quantities of the GOI with the appropriate NF and gain normalised GOI expression levels which are the same as relative quantities. Vandesompele and co-workers (2002) then presented *geNorm*, which is a visual basic application for Microsoft Excel, used to evaluate the most stable reference genes in a set of tested genes. The authors were the first to quantify the errors that follow the use of a single, un-validated reference gene which lead to erroneous expression differences of more than 3- and 6-fold in 25% and 10% of the cases they tested, respectively. To account for this, the authors recommend the use of *geNorm* to evaluate the expression stability of selected reference genes and the use of multiple reference genes for normalisation of RT-qPCR data instead of the use of single, un-validated reference gene (Hellemans *et al.*, 2007; Vandesompele *et al.*, 2002b).

1.1.5 Normalisation

When measuring variation in gene expression between subjects it is of great importance to be aware of the fact that the variation mainly rises from two different sources, the biological variation, i.e. the actual differences between individuals, and secondly the non-specific variation or non-biological variation which is often intervened with many different variables induced by the experiment itself (Vandesompele *et al.,* 2009). To attain the best possible gene expression result and eliminating non-biological variation from the biological variation, an accurate method of normalisation has to be chosen. Normalisation is considered one of the biggest problems in generating accurate RT-qPCR results (Dheda *et al.*, 2004; Hellemans *et al.*, 2007; Vandesompele *et al.*, 2002b) and is required to control for any sample specific experimental variation that can arise through various processes during the many steps in the RT-qPCR method. These can include a variation in the amount of starting material between samples, different RNA extraction protocols and differences in both reverse transcription and real-time PCR efficiencies (Bustin, 2000; Vandesompele *et al.*, 2002b).

Various normalisation strategies can be applied to minimize these errors and qPCR data should be normalised according to at least one of the following variables: sample size or tissue volume, total amount of extracted RNA or gDNA, artificial molecules such as RNA *spikes* or using reference gene/s such as rRNAs (18S or 28S) or mRNAs as internal endogenous controls (Huggett *et al.*, 2005; Vandesompele *et al.*, 2002b). Normalisation against sample size or tissue volume is a good way to reduce experimental errors but is rarely used on its own. In some cases sample size or tissue volume may not be so easily detected and/or the sample may not be biologically representative enough. The main drawback of normalisation against total RNA is that the quality of the RNA and the efficiencies of enzymatic reactions are not taken into account. Also, the use of total RNA measures mainly rRNA molecules and is not very representative of the mRNA fraction. Normalisation against gDNA also has its drawbacks, mainly because most RNA extraction protocols are designed to extract and purify RNA, not DNA. Using artificial molecules that are added to the sample in a known concentration at the extraction stage, is sometimes used for normalisation but has the disadvantage that it is not extracted from within the sample, unlike the target mRNA, and may therefore be a problem in some studies. Using rRNAs like 18S or mRNAs as internal endogenous controls remains today the most commonly used strategy for normalisation (Huggett *et al.,* 2005). Normalisation with 18S rRNA should though be well considered due to the fact that 18S rRNA is one of the predominant rRNAs and is found in much higher

abundance than mRNAs. This can lead to different amplification kinetics and misleading results (Vandesompele *et al.*, 2002b).

Using reference genes for normalisation relies on the fact that the ideal reference gene should be expressed at a constant level in all tissues or cell types, at all developmental stages and the most important of all, the reference gene has to be unaffected by any experimental treatment. This means that the reference gene expression should remain stable while the expression of the GOI could be up- or down-regulated. Whereas such a gene is hard to find, it is entirely necessary to validate that the reference gene is stably expressed in each individual experiment before it can be used for normalisation (Bustin, 2002; Stürzenbaum & Kille, 2001).

Over the years, most commonly used reference genes at the human level have included glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, ribosomal genes e.g. 18S and 28S rRNAs, hypoxanthine-guanine phosphoribosyl transferase (HPRT), cyclophilin and elongation factor 1-α (EF1-α) (Bustin, 2000; Huggett *et al.*, 2005; Stürzenbaum & Kille, 2001; Thellin *et al.*, 1999). More and more studies have indicated that expression of these "classic" reference genes is indeed affected by different tissues/cell types or experimental conditions (Radonic *et al.*, 2004; Stürzenbaum & Kille, 2001; Thellin *et al.*, 1999). Hence, it is unacceptable to use randomly chosen or unvalidated reference genes for normalisation, whereas this can greatly influence the gene expression results. Unstable reference genes can greatly increase the noise of the assay as well as making small expression changes of the target gene to be undetected (Huggett *et al.*, 2005). In 1999, only one reference gene was used for normalisation in over 90% of published gene expression studies (Suzuki *et al.*, 2000) but in recent years the golden standard of normalisation has become to use multiple or at least two or three reference genes for normalisation rather than depending only on a single reference gene. This makes it even more possible to detect small changes in gene expression assays (Hellemans *et al.*, 2007; Vandesompele *et al.*, 2002b).

The *geNorm* program is widely used to evaluate expression stabilities of reference genes and whole studies regarding selection of potential reference genes have used *geNorm* for this purpose in studies of various fish species, including the Atlantic cod (Olsvik *et al.*, 2008; Sæle *et al.*, 2009), Atlantic salmon (Jorgensen *et al.*, 2006; Olsvik *et al.*, 2005), and the Atlantic halibut (Fernandes *et al.*, 2008). The program also determines how many genes are needed to calculate normalisation factors (NF), based on the geometric mean expression levels of the most stable genes, for normalisation of the GOIs expression levels (closer details in section 2.5.5). The main drawback of this method for normalisation is that the *geNorm*

program depends on the assumption that none of the genes that are being analysed are co-regulated, whereas the algorithm behind *geNorm* tends to rank co-regulated genes together. It is therefore important for the researcher to be conscious about selecting genes that have broad and different biological functions (Vandesompele *et al.*, 2002b). Furthermore it has been suggested that in some cases it is not practical to use and measure multiple reference genes in small laboratories as the method is reagent intensive or due to the fact that samples are limited (Dheda *et al.*, 2005; Huggett *et al.*, 2005). Keeping that in mind, a two-gene normalisation factor based on the geometric mean of two reference genes has been proposed for normalisation of RT-qPCR data, rather than using three reference genes or, like most commonly practiced, only a single reference gene (Fernandes *et al.*, 2008).

Two studies have been performed to evaluate potential reference genes for use in RT-qPCR gene expression studies in the Atlantic cod. Olsvik *et al*. (2008) used *geNorm* and *NormFinder* programs to evaluate gene expression stability of ten potential reference genes in six tissues of wild population of juvenile Atlantic cod. Overall, the most preferable genes were suggested to be Ubiquitin (Ubi) that is involved in protein degradation and Acidic Ribosomal Protein (ARP) which is a member of the ribosome proteins.

Sæle *et al.* (2009) used also the *geNorm* and *NormFinder* programs in their search for stable reference genes for ontogenic studies on the Atlantic cod. Expression stability of ten reference genes was analysed using the GI tract and whole larvae homogenate samples from 3-97 dph. Overall, Ubiquitin was regarded as the most stable reference gene, but the authors concluded that the ribosomal proteins RPL4, RPL37, RPS9 and ARP could also be used as potential reference genes. The authors also concluded that the use of whole-larvae samples could be used to study relative expression during the early life stages of Atlantic cod, mainly because an accurate dissection of organs in the small larvae is hard and challenging. In both studies, the authors recommended the use of multiple reference genes for improving normalisation data in gene expression studies in the Atlantic cod. Ubiquitin therefore seems to be a good candidate as a reference gene for gene expression studies in Atlantic cod and this gene has also been used as a reference in gene expression studies of other fish species such as the Senegal sole and the Atlantic halibut (Infante *et al.*, 2008).

1.2 Development of cod larvae

Figure 4. Cod larvae at 1 day post hatch. Actual length of the larvae is ~ 4 mm. *Photo by Gregg Arthur from www.thefishsite.com*

For most teleostean species, the basic developmental mechanisms that occur from egg to juvenile stage follow a similar pattern, however, with both species-specific and stage-specific environmental and nutritional differences at the early life stages. The yolk size, environmental conditions and genetic differences greatly affect the development, size at hatch and the time of the shift to exogenous feeding (Kjørsvik *et al.*, 2004). Hatching occurs from relatively small eggs of 1.2–1.4 mm and the length of newly hatched larvae is 4-4.5 mm (Figure 4), corresponding to a dry weight of approximately 0.05 mg. The developmental process of cod larvae from hatch to juvenile involves three distinct stages: a short yolk sac stage of only 4-5 days following hatching, larval stage beginning at the onset of exogenous feeding and metamorphosis when the larval characteristics are lost and the cod transforms into a juvenile (Kjørsvik *et al.*, 2004; van de Meeren, 1991).

The yolk sac contains the necessary nutrients and energy reservoir for normal development and the newly hatched larvae are entirely dependent on the nutrient content of the yolk sac as a food source during this stage (Kjørsvik *et al.*, 2004). The larvae have well developed jaws already at hatch but the mouth and anal opening are closed and the eyes and the digestive tract are fairly undeveloped (Falk-Petersen, 2005). The liver, pancreas and gallbladder are present at hatch and so are the major lymphoid organs represented by the head kidney and spleen. According to Schrøder *et al*. (1998), the thymus cannot be detected until at approximately 28 dph when the larvae have reached 9 mm in length. Most of the organs have started to develop at the end of the yolk sac stage but the lymphoid organs are not fully developed until after metamorphosis (Falk-Petersen, 2005; Schrøder *et al.*, 1998). The mouth, eyes and the larvae´s swimming abilities have become more functional at this stage and the larvae are ready to start to feed on exogenous live prey (Kjørsvik *et al.*, 2004; van de Meeren, 1991).

The larval stage begins when the yolk sac is almost exhausted of nutrients and energy, and exogenous feeding starts. Massive mortalities are associated with this stage, in nature as well as under culture conditions. Any nutrient shortage or inadequate nutrient composition at the onset of exogenous feeding may result in failures in organ structure or physical development which may affect both growth and larval survival. The hunting capability

improves along with larval age and length. The capture of prey is limited by the opening of the mouth and the prey size must be comparable to the mouth gape, however, smaller prey seems to be preferable (Yúfera & Darias, 2007). It has been shown that starvation of cod larvae may result in lost ability to digest food and the larvae have very little chance of survival if starved from the end of the yolk sac stage to 9 dph (Kjørsvik *et al.*, 1991). If, on the other hand, food is abundant at the early life stages, normally developed cod larvae has rapid growth rate and within 48 dph the larvae may have increased their dry body mass about 2000 times (Finn *et al.*, 2002). Tissues and organs have started to develop during the yolk sac stage and undergo further development and increase in size and mass during the larval stage. The digestive system continues to change and develop during the first days of exogenous feeding which is necessary for further digestion and absorption of nutrients. The strength and complexity of the muscles and the myotomes increases with larval growth and age, resulting in increased swimming activity and increased respiratory functionality which relates to both muscle and gill development (Falk-Petersen, 2005).

Anatomical, structural and behaviour changes occur during metamorphosis. It is rather difficult to actually pinpoint the metamorphosis period for cod larvae but it is regarded to start when the median finfold begin to disappear and the development of the median fins starts to develop at a body length of 9-10 mm (Falk-Petersen, 2005; Schrøder *et al.*, 1998). During this stage the skeleton calcifies, the skin thickens and scales begin to appear together with the lateral line, the vision improves, teeth begin to appear and the gills become functional. Organs continue to develop during this stage and the size of the liver, pancreas, kidney and the spleen increase with larval growth (Falk-Petersen, 2005). In the study of Schrøder *et al*. (1998) the immune system was found to become increasingly more developed during metamorphosis. The lymphoid organs were fully developed at a body length of 25 mm but were not regarded fully activated until the juvenile stage was reached.

1.3 Immune functions

The immune system of vertebrates may be divided into two counterparts: innate immunity which acts as a non-specific defence mechanism, and adaptive immunity which is specific and produces a memory response. Various cells and molecules are recruited during activation of the two counterparts that together work towards elimination and neutralisation of the invading pathogen (Goldsby *et al.*, 2003b).

The innate immune system serves as the host´s first line of defence against infection and most of its components are present before the onset of the infection. Cells such as macrophages and neutrophils, barriers such as the skin and mucosal surfaces along with antimicrobial compounds produced by the host are all important components of the innate immune system. The adaptive immune system is activated when foreign antigens encounters the system which then acts with a high degree of specificity towards the antigen and involving a potent memory response. T-lymphocytes and antigen-presenting cells, including B-lymphocytes, macrophages and dendritic cells, are the main cell types involved in adaptive immune responses (Goldsby *et al.*, 2003b).

The immune system of fish shares the main characteristics of that of mammals. Immune functions in fish are, however, more dependent on external parameters and environmental factors such as pollution, temperature, and stress caused by treatments may profoundly affect the defence mechanisms (Khan, 1987; Magnadottir *et al.*, 1999b; Pérez-Casanova *et al.*, 2008). The main physiological differences between teleost fish and mammals is that bone marrow and lymph nodes are absent in fish and the major lymphoid tissues are within organs such as the kidney, spleen, thymus and the gut-associated lymphoid tissues (Ellis, 1989a) (Figure 5). The major lymphoid organ, the head kidney, is nearly exclusively haematopoietic and bearing morphological similarities with the bone marrow in mammals. The thymus is an important site of lymphocyte production, mainly T-lymphocytes, and could therefore be considered a primary lymphoid organ in fish. The spleen acts as a major secondary lymphoid organ in fish and holds diverse cell populations including macrophages and lymphocytes. Along with the head kidney, liver and endothelial cells in the heart, the spleen is responsible for trapping and clearing of foreign substances from the circulation (Press & Evensen, 1999).

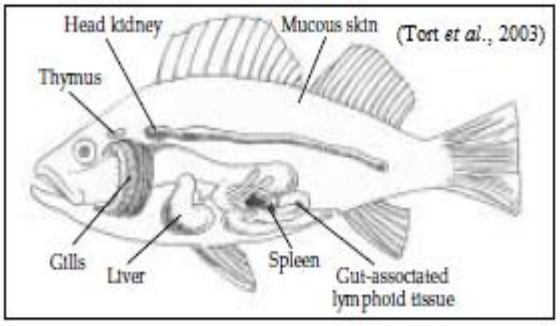


Figure 5. Main organs and structures involved in immune functions in teleost fish.

Another immunological difference between mammals and fish is that mammals produce five different classes of immunoglobulins (IgM, IgG, IgA, IgE and IgD (Goldsby *et al.*, 2003a) but only four different classes have been reported in teleost fish, IgM (Warr, 1995), IgD (Wilson *et al.*, 1997), IgZ (Danilova *et al.*, 2005) and IgT (Hansen *et al.*, 2005). So far only two of these classes have been reported in cod, the main type resembling the

mammalian IgM (Pilström & Bengtén, 1996) and IgD which has been suggested to be mainly expressed as a B-cell receptor (Stenvik & Jørgensen, 2000).

The concentration of IgM in the serum of cod is relatively high (12-16 mg ml^{-1}) compared with other fish species such as for example the Atlantic salmon (<1 mg ml^{-1}) which is capable of producing a strong specific antibody response (Magnadottir, 1998). The immune system of cod, however, fails to generate a strong specific antibody response upon immunization and challenge experiments (Espelid *et al.*, 1991; Magnadottir *et al.*, 2001; Pilström & Peterson, 1991; Pilström *et al.*, 2005; Schrøder *et al.*, 1992). Pilström and his co-workers (2005) suggested that this does not have to be related to deficiencies in the structure, organisation, diversity or expression of IgM, but rather to a deficiency in cod major histocompatibility class II molecules on antigen-presenting cells.

1.3.1 Innate immune parameters

Innate immune parameters are activated by germline-encoded pattern recognition receptors (PRRs) that are able to identify and bind to conserved structural motives present in all major groups of pathogens but that are not present or produced in the host itself. These structural motives are commonly referred to as pathogen-associated molecular patterns (PAMPs) (Medzhitov & Janeway, 2000, 2002) represented by a variety of sugars, proteins, lipid bearing molecules and nucleic acid motives such as peptidoglycans and LPS in the cell walls of bacteria, fungal β1,3-glucan, bacterial DNA and viral double-stranded RNA (Goldsby *et al.*, 2003b; Magnadottir, 2006). The PPRs include the Toll-like receptors (TLRs), which are major components of the innate immune system and are conserved in both vertebrates and invertebrates. Seventeen different TLRs have to date been identified in different fish species (Rebl *et al.*, 2010). In cod, expressed sequence tag (EST) sequences of TLR8, TLR22 and TLR23 have been identified in suppression subtractive hybridization (SSH) cDNA libraries (Feng *et al.*, 2009; Hori *et al.*, 2010)

The components of the innate immune system are commonly divided into three main groups: physical, cellular and humoral parameters (Magnadottir, 2006). Physical parameters such as the skin, gills and the GI tract are protected by mucus consisting mainly of glycoproteins and serving as the primary barrier against the environment and restraining the entry of pathogens. The inside environment of the GI tract is furthermore hostile towards pathogens by the aid of low pH, digestive enzymes and bile (Ellis, 1989b; Shepard, 1994). The mucus furthermore serves as a repository of numerous humoral innate immune factors, as

reviewed by (Whyte, 2007) and mucus contribution to defence against pathogenic microorganisms has been demonstrated in cod (Bergsson *et al.*, 2005).

1.3.1.1 Cellular parameters

Phagocytic activity plays an important role in the antibacterial defence and represents the main cellular defence during early development of fish larvae (Vadstein *et al.*, 2004). There are two cell types that are considered to be specialised phagocytes in teleost fish, the macrophages, derived from monocytes, and the neutrophil/granulocyte or the neutrophils (Ellis, 1989b). Both cell types produce degradative enzymes and antimicrobial peptides with cytotoxic activity towards bacteria and protozoan pathogens (Neumann *et al.*, 2001).

In teleost fish, macrophages are mainly found in the kidney and spleen but are also found widespread in various tissues, particularly in the gills, whereas monocytes are found in the kidney and to a smaller extent in the blood. Even though "macrophage" is often used to characterise a specific division of immune cells, the macrophages include extremely diverse sub-population of cells displaying different characteristics such as cellular functions, localisation in tissues and surface marker expression (Neumann *et al.*, 2001). Macrophages in the head kidney of cod are considered highly phagocytic and are able to maintain high viability for up to 4-7 days when cultured *in vitro* (Sørensen *et al.*, 1997).

A recent study furthermore revealed that B cells in cod and salmon have the capability to behave as semi-professional phagocytes, with 20% of cod B cells from peripheral blood and 30% of cod B cells from the head kidney possessing phagocytic abilities (Øverland *et al.*, 2010).

Neutrophils are present in the kidney, spleen and in the blood, and are commonly found in increased numbers in inflammatory lesions (Ellis, 1989b). It has been suggested that neutrophils in fish have the highest migration activity and these cells are the first to arrive to infected sites with role in hindering the spreading of infection (Matsuyama *et al.*, 1999).

Non-specific cytotoxic cells (NCC) is another important cell type that participates in the innate defence mechanisms in teleost fish and which shows morphological similarities with monocytes (Ellis, 1989b; Evans *et al.*, 2001). The NCCs can directly kill a wide range of infected cells such as tumour cells and virus transformed cells (Evans *et al.*, 1992; Evans *et al.*, 2001). These cells can be recognised by a cell surface protein NCCRP-1 (Jaso-Friedmann *et al.*, 1997) and the NCCRP-1 gene has been identified in the Atlantic cod. Expression

analysis showed that the gene was differently expressed in various organs, with the highest expression observed in the head kidney and the lowest in pylorus (Seppola *et al.*, 2007).

1.3.1.2 Humoral parameters

Innate humoral defence components consist of interferons (INFs), complement components, transferrin, lectins, pentraxins, protease inhibitors, natural antibodies (immunoglobulins, IgM), lysozyme and a variety of antimicrobial peptides (AMPs) (Ellis, 1989b; Gómez *et al.*, 2008).

In vertebrates, IFNs are a family of glycoproteins known as cytokines which can activate an antiviral state within the host's cells and have an important defensive role against virus infections (Samuel, 2001). Interferons are secreted by host cells such as macrophages, lymphocytes, natural killer cells and fibroblasts (Haller *et al.*, 2006).

Complement components are mainly found in the serum of fish but may also be found in mucus secretions and showing many homologies to their counterparts in mammals (Holland & Lambris, 2002). In mammals, activation of the system involves processes such as lysis of cells, bacteria and viruses, phagocytosis, immune complex clearance, inflammatory reactions and antibody production (Goldsby *et al.*, 2003b), but it is not clear whether all these functions also take place in fish (Holland & Lambris, 2002). The complement system is initiated by one or a combination of three activation pathways, the classical, lectin and the alternative pathways, with complement factor 3 (C3) playing a central role in all pathways (Boshra *et al.*, 2006). C3 is regarded an important component of innate immune defence mechanism in cod and a role in the formation and generation of different organs has also been suggested (Lange *et al.*, 2004a).

Transferrin is a glycoprotein found in the serum of fish like other vertebrates. The enzyme has high binding capacity for iron, which is an essential growth element to all living organisms, and is responsible for the transport and delivery of iron to the cells. Transferrin is related to the innate immune system as it is able to bind to and withhold iron, thereby depriving pathogens of iron and creating a hostile environment where only a few pathogenic groups are able to survive (Ellis, 1989b; Gómez *et al.*, 2008).

The C-type lectins are glycoproteins that are able to bind to carbohydrates such as mannose, N-acetyl glucosamine or fucose, resulting in opsonisation, phagocytosis and activation of the complement system (Arason, 1996). The pentraxins, C-reactive protein (CRP) and serum amyloid protein, represent a part of innate defence mechanisms and playing

a central role in acute phase responses (Bayne *et al.*, 2001) as well as aiding with both recognition and the clearance of apoptotic cells (Nauta *et al.*, 2003). Pentraxin homologues have been described in the serum of several fish species including cod (Lund & Olafsen, 1998) and recently two homologues of CRPs have been isolated from the serum of cod by affinity chromatography (Gisladottir *et al.*, 2009).

The serum and other body fluids of fish contain various protease inhibitors (anti-proteases) that possess the ability to delay and inhibit the action of secreted proteolytic enzymes produced by some pathogens for penetration of host barriers (Ellis, 1987; Gómez *et al.*, 2008). In general, the anti-protease activity in cod is high and immunisation or infection do not seem to alter the activity which is highly affected by environmental temperatures (Magnadottir *et al.*, 2002; Magnadottir *et al.*, 1999b; Magnadottir *et al.*, 2001).

1.3.1.2.1 Natural antibodies

Antibodies are considered a part of adaptive immune responses, but natural antibodies (natural IgM) have been classified as a part of innate immunity whereas they provide broad specificity and early protection against pathogens (Carroll *et al.*, 1998). Natural IgM are polyreactive and usually show low affinity towards phylogenetically conserved self epitopes such as single stranded DNA, thyroglobulin myosin and heat shock proteins as well as with haptens which are small molecules that can educe immunological response when attached to larger molecules such as proteins (Casali *et al.*, 1996; Pashov *et al.*, 2002). Natural IgM are found circulating in the serum of normal, non-immunised individuals prior to any infection and are produced in the absence of exogenous antigen stimulation. In mammals, natural IgM are involved in early defence against bacterial infections (Boes, 2000; Boes *et al.*, 1998) as well as participating in early trapping of both bacterial and viral particles in the spleen and through stimulating phagocytosis of spleen macrophages. This indicates that natural IgM are important in preventing dissemination of pathogens to fundamental organs in the body such as the brain (Ochsenbein *et al.*, 1999; Ochsenbein & Zinkernagel, 2000). Natural IgM are also known to be potent activators of the complement system which may lead to direct lysis of bacteria as well as recruiting other immune parameters to the site of infection (Figure 6). Complement activation through natural IgM may furthermore result in stimulation of B cells, including B1 cells that generate natural IgM. Activation of the complement system is therefore important to maintain the magnitude of natural IgM in the circulation. B1 (CD5+) lymphocytes differ from conventional B cells in a way that B1 cells are self-replicating and

generated during both the embryonic and early developmental stages as has been reviewed by Ochsenbein and Zinkernagel (2000) and references therein.

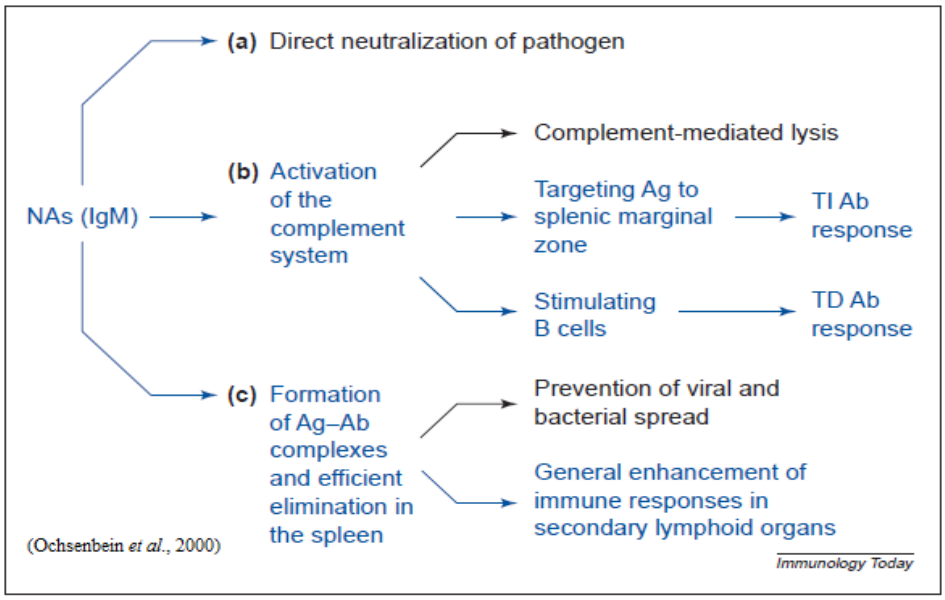


Figure 6. The role of natural antibodies in the fight against infections in mammals. The protection can occur directly or indirectly through the complement system as well as enhancing specific antibody response. Black fonts show the role of natural IgM in the innate immune system while blue fonts indicate the role of natural IgM in the specific antibody response. Ab: antibody, Ag: antigen, NA: natural antibody, TI: T-cell independent, TD: T-cell dependant.

Natural IgM have been identified in various teleost species, with variation in the specificity between fish species observed (Gonzalez *et al.*, 1988). Natural IgM have been known to take part in both viral and bacterial defence (Gonzalez *et al.*, 1989; Sinyakov *et al.*, 2002) and is believed to form a link between innate and adaptive immunity in teleost fish (Carroll *et al.*, 1998; Gonzalez *et al.*, 1988; Sinyakov *et al.*, 2002). Cod serum contains relatively high levels of natural IgM, with increased activity levels observed with age and following infection. The natural IgM activity towards antigens correlated well with high IgM concentration in the serum and it has been speculated if natural IgM response could possibly contribute or compensates for the poor specific antibody response observed in cod (Magnadottir *et al.*, 2002; Magnadottir *et al.*, 2009; Magnadottir *et al.*, 1999a, 1999b; Magnadottir *et al.*, 2001; Pilström & Peterson, 1991).

Immune related parameters may be maternally inherited or transferred, as proteins, mRNA or both (Mulero *et al.*, 2007; Swain & Nayak, 2009). In fish, it has been shown that maternal immunoglobulins are transferred from mother to the offspring and maternal IgM are considered able to contribute to increased survival of larval offspring (Swain & Nayak, 2006). Binding to antigens, aid with phagocytosis and activation of complement during the early

developmental stages are roles suggested for maternal IgM which may also function as a nutritional yolk protein (Magnadottir *et al.*, 2005). Maternal transfer of IgM has, however, not been established in cod (Magnadottir *et al.*, 2004; Seppola *et al.*, 2009).

The basic molecular structure of immunoglobulins in vertebrates consists of two identical polypeptide heavy chains (H) and two identical polypeptide light chains (L) held together by disulfide and non-covalent bonds (Figure 7). The amino terminal part of both heavy and light chains contains highly variable regions (V_H and V_L regions). Within the V-regions are complementary determining regions (CDR) that control the specificity of the antibodies and constitute the antigen binding site. The remaining portion of the molecule is referred to as the constant region (C_H and C_L) (Goldsby *et al.*, 2003b).

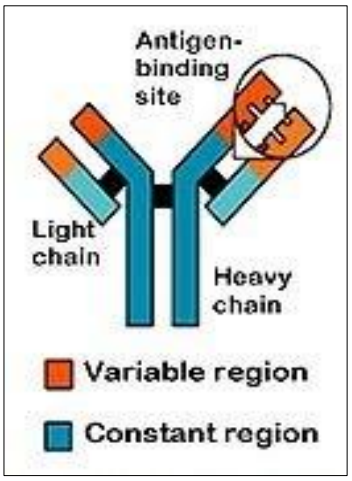


Figure 7. Basic subunit structure of the immunoglobulin molecule
Source: www.wikipedia.org

The IgM molecule shows variations in the polymeric arrangement between different vertebrates, whereas it is pentameric in higher vertebrates and monomeric and tetrameric in teleosts (Acton *et al.*, 1971) including cod (Magnadottir, 1998; Pilström & Bengtén, 1996; Pilström & Peterson, 1991). In teleost fish, B cells functionally resemble mammalian B-1 cells as they can express the membrane IgM as a monomer as well as secreting soluble IgM in a tetrameric form (Miller *et al.*, 1998; Rombout *et al.*, 2005). Consequently there are two forms of H-chains; a membrane bound (mIgM-H) and a secreted (sIgM-H) form which are both encoded by the same gene and mRNA pre-processing determines which form is generated (Pilström & Bengtén, 1996). Bengtén *et al*. (1991) were the first to isolate both these IgM forms in cod and showed that the mRNA splicing pattern was different from mammals. In the membrane bound form, the exon of the transmembrane part splices directly to the end of the C_H3 exon, eliminating the whole C_H4 exon that will only be present in transcripts encoding for the secretory form (Bengtén *et al.*, 1991). This same slicing pattern also occurs in other teleosts and is thought to be a general phenomenon in all teleost species (Hordvik *et al.*, 1992; Lee *et al.*, 1993; Pilström & Bengtén, 1996; Wilson *et al.*, 1990).

1.3.1.2.2 Lysozyme

Lysozyme is found in vertebrates, invertebrates, plants, and microbes as well as bacteriophages and is regarded to be of leucocytic origin (Jollès & Jollès, 1984). Lysosyme is an enzyme (hydrolase) found in secretions such as saliva, mucus, blood, tissues and in cell vacuoles in plants. The enzyme is capable of hydrolysing the β-(1-4) glycosidic bonds between the N-acetyl glucosamine and N-acetyl muramic acid in the peptidoglycan layer of cell walls in Gram-positive bacteria but in Gram-negative bacteria the enzyme only becomes effective when the outer cell wall is disrupted by the complement or other enzymes making the inner peptidoglycan layer exposed. Lysozymes are able to activate the complement system and promote phagocytosis by polymophonuclear leucocytes and macrophages in addition to serving as opsonins. In addition to antibacterial function, lysozyme is known to have antifungal and antiviral activities as well as playing a role in digestion (Jollès *et al.*, 1989; Jollès & Jollès, 1984; Saurabh & Sahoo, 2008).

Lysozymes are classified into six main types, with the chicken (c-type) and goose (g-type) types found in vertebrates and the invertebrate type (i-type) found in invertebrates. The other three types are of phage, bacterial and plant origins. The types differ in their molecular weights, amino acid compositions and enzymatic properties (Grunclová *et al.*, 2003; Prager & Jollès, 1996; Saurabh & Sahoo, 2008).

Both c- and g-type lysozyme have been reported in fish where they are released by leucocytes, mainly neutrophils, monocytes and macrophages. The g-type lysozyme has been reported in several fish species including cod, with lytic activities against known fish pathogens demonstrated (Larsen *et al.*, 2009; Saurabh & Sahoo, 2008). It has been reported that the highest lysozyme levels are found in the leucocyte rich head kidney, the GI tract, spleen, skin mucus, serum, gills, liver and muscle as well as in fertilised eggs (Lie *et al.*, 1989; Yousif *et al.*, 1994). Lysozyme is therefore mainly found at sites with high risk of pathogenic invasion, indicating an important role of the enzyme in the early line of innate defence in fish (Lie *et al.*, 1989). Environmental factors such as water temperature, season, pH and toxic substances in addition to physiological factors such as sex and age, stress and infection are known to affect the lysozyme levels in fish (Saurabh & Sahoo, 2008).

There are mixed reports of lysozyme activity in the serum and mucus of cod. No lysozyme activity was detected in the serum of cod using a plate method (Fänge *et al.*, 1976) nor by using a turbidometric assay (Magnadottir *et al.*, 1999a, 1999b). In contrast to this, King and co-workers (2006) demonstrated lysozyme activity in the serum of juvenile cod using a turbidometric assay (King *et al.*, 2006).

There are also mixed reports about whether the mucus in cod contains lysozyme activity. Epidermal mucus of cod has been studied with respect to lysozyme activity and a comparative study of a number of marine and freshwater fish species revealed twice as much lysozyme activity in mucus of marine as compared to freshwater species (Subramanian *et al.*, 2007). Lysozyme activity in the kidney of cod has, however, been found to be only a small fraction of the activity measured in rainbow trout (Lie *et al.*, 1989). On the other hand, no lysozyme activity was detected in an acidic epidermal mucus extract in cod against *Micrococcus lysodeikticus* using another methodology (Bergsson *et al.*, 2005).

The expression of g-type lysozyme in cod has been studied by transcriptome analysis and Larsen *et al*., (2009) were the first to clone and characterise the g-type lysozyme gene in the cod. The authors demonstrated that the gene is organised in five exons and four introns which is similar to the g-type lysozyme gene in other fish species, indicating that the g-type lysozyme gene is conserved in teleosts. In cod, the gene seems to produce two transcripts which Larsen and co-workers (2009) identified as *codg1* and *codg2*. The *codg1* transcript contained the exon 1a which encodes a putative signal peptide and the transcript *codg2* contained exon 1b which lacks the region which encodes the signal peptide. This may indicate that the transcripts may have different cellular localisations, where the function of *codg1* may be extracellular and the function of *codg2* intracellular. A recombinant g-type *codg2* lysozyme (gLYS) was produced and it showed lysozyme (muramidase) activity towards *Micrococcus luteus* cells. The expression of both the transcripts was analysed using RT-qPCR (TaqMan assay), showing high expression in immune organs and at the site of bacterial injection, indicating that the lysozyme gene has a role in the innate immune system of cod. The up-regulated expression observed following bacterial injection was not statistically significant (Larsen *et al.*, 2009), but significant up-regulation of the g-type lysozyme gene in the blood of cod has been observed following bacterial injection and exposure to stress (Caipang *et al.*, 2008), in the spleen following vaccination using heat-inactivated bacteria (Caipang *et al.*, 2009) and in head kidney leukocytes in response to both live and heat inactivated intestinal bacteria, (Lazado *et al.*, 2010).

G-type lysozyme has been shown to be expressed in unfertilised cod eggs and ovarian fluid which strongly indicates maternal transfer of lysozyme mRNA. Elevated expression was observed following hatching and peaking during metamorphosis. Protein extracts from unfertilised eggs and embryo homogenates showed lysozyme activity that may indicate the role of lysozyme in preventing pathogenic invasion of cod eggs and embryos (Seppola *et al.*, 2009).

1.3.1.2.3 Hepcidin

Antimicrobial peptides (AMPs) are found throughout the animal and plant kingdoms (Zasloff, 2002) and are regarded to have an important role in innate immune functions against bacterial invasion by providing a first line defence at mucosal barriers (Hancock *et al.*, 2000; Park *et al.*, 2001). AMPs are stored in the granules within phagocytes and aid with the killing of engulfed pathogens by disrupting the cell membrane (Zasloff, 2002). They are generally cationic and were initially discovered because of their antimicrobial properties but are considered to have immune modulating functions as well (Brown *et al.*, 2006; Hancock *et al.*, 2000).

One of the AMPs is hepcidin that has also been referred to as LEAP-1 (liver-expressed antimicrobial peptide-1) or HAMP (hepcidin antimicrobial peptide). The peptide was originally recognised because of its antibacterial properties in humans (Krause *et al.*, 2000) and then later on for its role in iron homeostasis (Nicolas *et al.*, 2001). Hepcidin is produced mainly in the liver and is synthesised as a pre-pro peptide with cleavage resulting in a mature antimicrobial hepcidin peptide (Krause *et al.*, 2000; Park *et al.*, 2001). The expression of hepcidin is increased during inflammation, infection and iron overload and often declines under certain conditions like anemia and hypoxia (Ganz, 2007). Iron homeostasis is regulated by hepcidin through mediating absorption of iron and iron recycling by macrophages (Nicolas *et al.*, 2002). Macrophages seek to withhold iron from invading pathogens and thereby hindering their proliferation (Sow *et al.*, 2007).

Hepcidin has been identified in many fish species including the Atlantic cod (Solstad *et al.*, 2008). Antibacterial activity of hepcidin has been demonstrated in fish (Hu *et al.*, 2007) and expression analysis have reported an up-regulation of the hepcidin gene in response to both bacterial and viral infections (Cuesta *et al.*, 2008; Hu *et al.*, 2007).

Solstad *et al*. (2008) identified the hepcidin cDNA sequence in Atlantic cod and performed expression analysis in various tissues using RT-qPCR. The cod's hepcidin gene contains three exons and two introns which is similar to hepcidin genes that have been studied in other fish species and in mammals. The highest expression levels of hepcidin were detected in the liver, leading to the suggestion of the liver as the main production site of cod´s hepcidin. However, the expression seemed to be more inducible in other organs than the liver, such as head kidney and peritoneum, which has also been reported in other fish (Bao *et al.*, 2005; Chen *et al.*, 2005; Kim *et al.*, 2005). The expression of cod hepcidin has also been reported to be up-regulated in both head kidney and spleen following bacterial injection (Feng *et al.*, 2009).

Cod hepcidin possesses antimicrobial activity and hepcidin may therefore be regarded as an important component in the first line defences against pathogenic invasion (Feng *et al.*, 2009; Solstad *et al.*, 2008). According to Seppola *et al.* (2009), hepcidin is not expressed in unfertilised eggs or ovarian fluid, indicating that maternal transfer of hepcidin transcripts does not occur in cod. The onset of hepcidin expression was demonstrated approximately 118 hours post fertilisation and gradually escalated, with a temporary decrease observed following the onset of exogenous feeding. Hepcidin expression in zebrafish has been found to be iron responsive (Fraenkel *et al.*, 2005) and zebrafish and mammalian hepcidin are believed to have similar roles in iron regulation. Cod hepcidin is highly similar to the zebrafish hepcidin and Solstad *et al*. (2008) therefore suggested that cod hepcidin may play an important role in iron regulation, but it´s role as an iron regulator has though yet to be determined.

1.3.1.3 Stimulation of innate immune parameters

Stimulation of innate immune parameters is considered a promising approach for enhanced defence of fish larvae until adaptive immune responses are adequately developed to mount an effective immune response towards pathogens. Such approach might be expected to result in improved survival and overall larval quality (Bricknell *et al.*, 2005).

Probiotics were originally defined as "substances produced by one protozoan that stimulated the growth of another" (Lilly & Stiwell, 1965). In 2001 the definition of probiotics were redefined and are now also applicable to the aquatic environment (Verschuere *et al.*, 2000). Furthermore, it has been suggested that putative probiotic bacteria should preferably be isolated from the host itself, as reviewed by Balcázar *et al*. (2006).

Probiotic bacteria commonly used in aquaculture include a variety of groups, with strain dependent modes of action, as reviewed by Nayak (2010). Some common mechanisms of action have, however, been observed and benefits directly linked to the administering of probiotics to fish have been reported. Enhanced phagocytosis has been reported through feed supplementation using probiotics and oral administration of probiotics have significantly increased the proportion of monocytes, granulocytes and lymphocytes in fish (Irianto & Austin, 2002). Probiotic supplementation may furthermore trigger lysozyme levels in serum, skin and mucus of fish, as reviewed by Nayak (2010). Stimulated B cell production, enhanced complement activity and elevated levels of IgM in serum and mucus have also been reported in fish (Panigrahi *et al.*, 2005; Salinas *et al.*, 2008).

Research on the use of probiotics in cod aquaculture is, however, limited compared to other fish species but has gained more attention during recent years. Enhanced disease resistance and reduced mortality have been reported in cod fed dry feed supplemented with lactic acid bacteria (Gildberg *et al.*, 1998; Gildberg *et al.*, 1997). Antagonistic activity of bacteria isolated from cod, towards fish pathogens has also been reported (Fjellheim *et al.*, 2010; Fjellheim *et al.*, 2007; Ringø *et al.*, 2006). Incubation of heat-inactivated isolates, showing growth inhibition activity towards fish pathogens, with head kidney cells from cod furthermore resulted in different expression of immune-related genes. Increased expression of the g-type lysozyme gene was observed following co-incubation with both live and heat-inactivated form of the bacteria, and the authors therefore suggested that putative probiotic strains isolated from the intestine of cod could have immunomodulary capabilities (Caipang *et al.*, 2010; Lazado *et al.*, 2010).

Lauzon *et al*. (2008) also isolated three putative probionts from healthy cod aquaculture environment which showed antagonistic activities towards fish pathogens *in vitro*. I*n vivo* trials showed that two of the isolates managed to establish in the GI-tract of larvae and one of the strains was associated with increased growth and performance of the larvae (Lauzon *et al.*, 2008; Lauzon *et al.*, 2010).

Proteomic analysis of cod larvae in response to a mixture of probiotic bacteria supplemented into the rearing water, however, showed up-regulation of proteins related to growth and development and down-regulation of proteins related to immune functions. The survival rate in treated larvae was twice as much as in non-treated larvae and the authors therefore speculated whether down-regulation of immune related proteins might be the result of reduced environmental stress through inhibited growth of pathogenic bacteria as a result of the probiotic treatment (Sveinsdottir *et al.*, 2009).

The immunostimulating effects of various compounds and molecules have been studied fish, as adjuvants and as feeding supplements (Pedersen *et al.*, 2004). Immunostimulants are defined as “naturally occurring compounds that modulate the immune system by increasing the host´s resistance against diseases that in most circumstances are caused by pathogens” (Bricknell *et al.*, 2005). Immunostimulants used in aquaculture can be derived from different sources such as bacterial, animal-derived, algae-derived and of nutritional origin as well as hormones/cytokines where the modes of action is mainly associated with increased phagocytic activity. The responses of NCC cells, complement, lysozyme and antibodies may also be stimulated (Sakai, 1999) Various bioactivity has furthermore been demonstrated in proteins and peptides isolated from fish (Kristinsson &

Rasco, 2000), and small peptides may provide nutritional benefits in addition to improved bacterial resistance and immunostimulating effects observed in fish larvae (Hakonardottir *et al.*, 2008; Olafsen, 2001; Pedersen *et al.*, 2004).

Various methods have bee used to detect stimulation of different immunological factors in tissues of fish, including immunohistochemistry and *in situ* hybridisation (Schrøder *et al.*, 1998), immunofluorescence (Hu *et al.*, 2010), flow cytometry (Overland *et al.*, 2010), western blotting (Lange *et al.*, 2004b) and ELISA (Schrøder *et al.*, 2009). All the above mentioned detection methods are qualitative except for ELISA which may be used for quantitative as well as qualitative measurements. In general, there is a lack of *in vivo* gene expression studies describing the effects of probiotics and other immunostimulants on genes involved in innate immunity of fish and to my knowlegde no reports have been published for Atlantic cod larvae.

Stimulation of innate immune parameters in fish using probiotics or any other immunomodulating compound may be expected to affect the expression of genes conducting the production of the particular parameters. Probiotic treatment has been found to result in up- and down-regulation of selected immune related genes in the Atlantic cod (Caipang *et al.*, 2010). Monitoring the expression of selected immune related genes using the RT-qPCR method may therefore represent a promising way to evaluate the effects of treatments using probiotic bacteria, various immunostimulating agents and other manipulation of the environment, with the overall aim to enhance survival and overall quality of intensively produced marine larvae.

1.4 Specific aims of the study

A sound and accurate method is needed for measuring and following transcription of genes of key importance during early development and in response to nutritional and environmental factors that affect the survival and overall quality of marine fish larvae. The specific aims of the present study were therefore to adapt the RT-qPCR method that was then applied for quantitative analysis of the relative expression of immune related genes, mIgM, g-type lysozyme and hepcidin, during the early life stages of intensively reared Atlantic cod larvae. The aims of the study were furthermore to evaluate the effects of probiotic treatment using a mixture of two bacterial isolates.

2 Materials and methods

2.1 Experimental setup

The experiment was performed using commonly practiced production methods at the Icelandic Marine Research Institution hatchery (MRI) (Steinarsson, 2004). 500 ml of fertilised Atlantic cod eggs were placed in 150L silo at an average temperature of 7.5°C. Three days prior to hatching the eggs were sterilised and transferred to a 25L silo. After hatching, 55.000 larvae were divided in two 25L silos, ~30.000 larvae for probiotic treatment (PB) and ~25.000 larvae for untreated control (UC). Two days later, larvae from these two silos were divided into two 150L sibling silos for each treatment, PB1 and PB2, containing ~12.500 larvae/silo and UC1 and UC2, containing ~15.000 larvae/silo. The average temperature was 12.3°C from 10-36 dph. The average temperature was 12.3°C from 10-36 dph. Larvae were fed rotifers (*Brachionus plicatilis*) two times a day from the onset of exogenous feeding (3 dph), 14-30 dph with a mixture of rotifers and *Artemia franciscana* nauplii, 31-48 dph with only *Artemia* and weaning on to dry diet commenced at 49 dph. The samples for the gene expression study were collected between 2-36 dph as explained in Figure 8.

The larvae were collected with small pocket net, placed in a sieve and damped with paper from underneath to withdraw any excess water. 5-10 whole larvae were then sampled with a sterile scalpel knife and placed in 1.5 ml RNase and DNase free Eppendorf® Safe-Lock™ tubes (Eppendorf AG, Hamburg, Germany) containing 1 ml of Tri-Reagent® Solution (Ambion, #AM9738) which is a mono-phasic solution that contains phenol and guanidine thiocyanate that inhibit RNase activity within the sample during cell lysis. Larval samples were collected in triplicate from all four silos, 10 larvae/sample at 2 and 7 dph, and 5 larvae/sample at 26 and 36 dph, placed on ice and then transferred by flight to Akureyri where the samples were frozen at -80°C until RNA extraction.

Two bacterial strains (*Arthrobacter bergerei*, GeneBank accession number AJ609631 and *Enterococcus thailandicus* FP48-3, GeneBank accession number EF197994), isolated from cod farming environment (Lauzon *et al*. 2008), were cultured in the laboratory and freeze-dried preparations of the bacterial isolates were then prepared by the staff at Matís ohf. Akureyri. Probiotic treatment was carried out through bathing (10^{10} bacteria/L) for one hour immediately prior to hatch and at 2 dph. Live feed were also supplemented with the bacterial mixture (10^{10} bacteria/L) for 30 min prior to offering to the larvae at the onset of exogenous feeding and daily during 3-5 dph, 10-11 dph and 18-19 dph.

The gene expression analysis was performed using two different analytical approaches. The first approach included expression analysis of the heavy chain of membrane bound Immunoglobulin M (mIgM) and g-type lysozyme which was performed using three biological replicate samples from the probiotic treatment silos (PB, PB1) and the untreated control silos (UC, UC1). Hereafter this analysis will be addressed as analytical approach 1 for simplification. The second approach included expression analysis of mIgM, g-type lysozyme and hepcidin which was performed by pooling the three biological replicate RNA samples within each individual silo into one sample for each of the sampling days. This pooling step gained one sample per sampling day from all silos, PB, PB1, PB2, UC, UC1 and UC2. This analysis will hereafter be addressed as analytical approach 2.

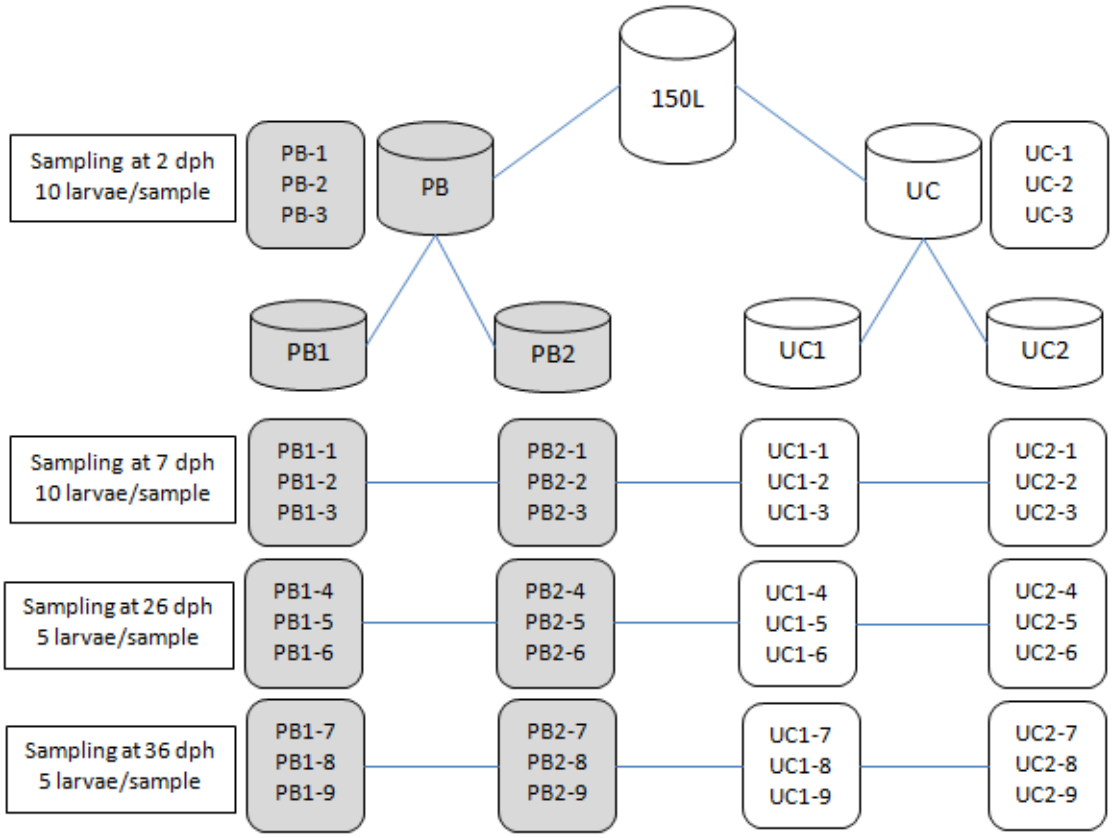


Figure 8. Experimental setup and sampling procedure. Three samples were collected at 2 days post hatch (dph) from silos PB (Probiotic treatment) and UC (Untreated control), at 7 dph from silos (PB1, PB2, UC1 and UC2) repeatedly at 26 and 36 dph. PB1 and PB2 are regarded as sibling silo incubators containing larvae of a common origin, and UC1 and UC2 as well.

2.2 RNA extraction

Prior to any handling and processing of samples to be used for RNA extraction, the lab bench in the fume hood, pipettes and gloves were thoroughly cleaned with RNase*Zap*® Solution (Ambion, # AM9782) and then with distilled water to avoid any RNase contamination during RNA extraction. All pipette tips and eppendorf tubes used were RNase- and DNase free. 75% ethanol was prepared by mixing 250 ml of DEPC-treated (RNase free) water (Ambion, #AM9906) and 750 ml of Absolute Ethanol (Sigma-Aldrich, #32221). The RNA extraction was performed according to the manufacturer protocol with minor adjustments.

Figure 9. Mini-Beadbeater-1
Source: www.biospec.com

The frozen samples, stored in TRI Reagent® Solution, were thawed on ice and then transferred with a pipette into sterile 2 ml Micro tubes (SARSTEDT, Newton, Germany) containing 0.5 ml of 0.1 mm silica beads (BioSpec Products Inc., #11079110z). The samples were then homogenized for 40 seconds at the speed 4800 rounds minute^{-1} using Mini-Beadbeater-1 (Figure 9) (BioSpec Products Inc. Bartlesville, USA) for disruption of the cells and release of the RNA. Immediately following homogenisation, each sample was transferred to a clean eppendorf tube and put on ice. Following homogenization of all samples, the samples were incubated at room temperature for 5 minutes to allow nucleoprotein complexes to completely dissociate. Samples were then centrifuged in an Eppendorf 5417R centrifuge (Eppendorf AG, Hamburg, Germany) at 12.000 x g for 10 minutes at 4°C and the supernatant transferred to fresh tubes. This step is optional and is carried out in order to remove insoluble materials from the homogenates. The centrifugation pellets contain extracellular membranes, polysaccharides and high molecular weight DNA, with RNA left in the supernatant. 200 µl of chloroform (Riedel-de Haën®, #24216) was added to each sample, mixed well by hands for 15 seconds, and the mixture then incubated at room temperature for 10 minutes followed by centrifugation at 12.000 x g for 15 minutes at 4°C. Following centrifugation the RNA remains in the aqueous phase (top phase), DNA in the interphase and proteins in the organic phase (bottom phase). RNA was then transferred with a pipette to a fresh tube, with care taken not to touch the DNA interphase. The chloroform step, centrifugation and RNA transfer was repeated in order to receive higher yields of RNA (pers.comm. Dr. Jorge Fernandes, HIBO, Norway). 500 µl of isopropanol/2-Propanol (Sigma-Aldrich®, #34863) were then added to each sample, vortexed for 5-10 seconds and incubated at room temperature for 10 minutes followed by centrifugation at

12,000 x g for 8 minutes at 4°C. Following this step, the RNA forms white pellet and the supernatant was discarded without disturbing the pellet. To wash the RNA pellets, 1 ml of cold 75% ethanol was added to each sample followed by centrifugation at 7,500 x g for 5 minutes at 4°C. A complete removal of ethanol is necessary for good quality RNA and a brief centrifugation is needed to remove any residual ethanol from the tube using a fine pipette tip. The RNA pellet was allowed to air dry for 3-5 minutes but carefully not allowing it to dry completely as that will significantly reduce its solubility. In the present study, DNase I treatment of samples was carried out immediately following RNA extraction. It is important to treat RNA samples with RNase-free DNase I in order to get rid of any trace of contaminating genomic DNA within the samples, whereas less than 1% DNA in a total RNA sample may be detected by PCR amplification (Dilworth *et al.*, 1992). DNase I treatment was carried out with RNase free DNase I 2,000 units/ml (New England BioLabs® Inc., #M0303S). The DNase I enzyme is supplied with 10X Reaction Buffer which needs to be diluted down to 1X Reaction Buffer. This was done by pipetting 100 µl of the 10X Reaction Buffer into 900 µl of DEPC-treated water (Ambion) and therefore getting 1 ml of 1X DNase I Reaction Buffer (10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, pH 7.6). According to the manufacturer, one unit (U) is defined as the amount of enzyme which will completely degrade 1 µg of pBR322 DNA.

For each RNA sample, the RNA pellet was dissolved in 50 µl of 1X DNase I Reaction Buffer by passing the solution a few times trough a pipette tip. 1 U of the DNase I enzyme was then added to each sample, mixed thoroughly, and incubated at 37°C for 30 minutes followed be a heat inactivation of the enzyme for 5 minutes at 75°C (Huang *et al.*, 1996). Samples were then frozen at -80°C until further analysis

2.3 RNA concentration and quality assessment

RNA concentration and quality assessment was carried out using Experion™ Automated Electrophoresis System (Figure 10) (Bio-Rad Laboratories Inc., Hercules CA, USA), Experion™ RNA StdSens Analysis Kit (Bio-Rad Laboratories Inc., #700-7154) along with Experion™ RNAStdSens chips (Bio-Rad

Figure 10. Experion™ Automated Electrophoresis System
Source: www.bio-rad.com

Laboratories Inc., #700-7153) according to the manufactures protocol. The reagents in the analysis kit were stored at 4°C with the exeption of the RNA ladder that was stored at -20°C. The reagents were allowed to equilibrate at room temperature for 15-20 minutes before use and the RNA ladder allowed to thaw on ice. The RNA stain needs to be protected from light at all times. Right before use the reagents were all vortexed. Lab bench, pipettes and gloves were cleaned with RNase*Zap*® Solution and then by distilled water to avoid any RNase contamination during handling of the RNA samples. All pipette tips and tubes used were RNase- and DNase free. Before and after performing a run in the instrument, a cleaning procedure was performed by cleaning the electrodes with Experion electrode cleaner (Bio-Rad Laboratories Inc., #700-7252) and DEPC-treated (RNase free) water from Ambion. The Experion™ RNA StdSens Analysis Kit instruction manual (Bio-Rad Laboratories Inc., #10000976) can be retrieved from the Bio-Rad website at: http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10000976B%20-%20RNA%20STDSENS%20MANUAL.PDF

The run is finished after approximately 30 minutes and the data can then be analysed. The software detects the fluorescence in each sample and plots the fluorescence intensity vs. time to create an electropherogram of each sample and a virtual gel image of all the samples. After running all the samples, the RNA fragments in each sample were analysed with the use of an internal marker in order to normalise the time between samples in different wells, and using the RNA ladder to determine fragment size and concentration of samples. The internal marker (50 bp) is included in the loading buffer and should therefore appear in all samples (indicated by a pink triangle in the virtual gel image). The first sample analysed is the RNA ladder which contains eight RNA fragments (200-6000 nt) and should therefore show eight peaks following the lower internal marker. The software assigns 18S and 28S rRNA peaks to the samples, based on their size, and then calculates the total concentration as well as the RQI value of each sample. By comparing the area under the electropherogram of each sample with the corrected area under the electropherogram of the RNA ladder, the software uses the known concentration of the ladder (160 ng/µl) to determine the RNA concentration of the sample. To gain the RQI value the software uses an algorithm that compares three regions (pre-18S-, 18S- and 28S peaks area) in the electropherogram of the sample to a series of degradation standards. The RQI value ranges between 1 (highly degraded RNA) and 10 (high quality intact RNA) where values between 7 and 10 are regarded acceptable for further use in RT-qPCR experiments (Denisov *et al.*, 2008). A workflow from cleaning of the electrodes to data analysis is shown in Figure 11.

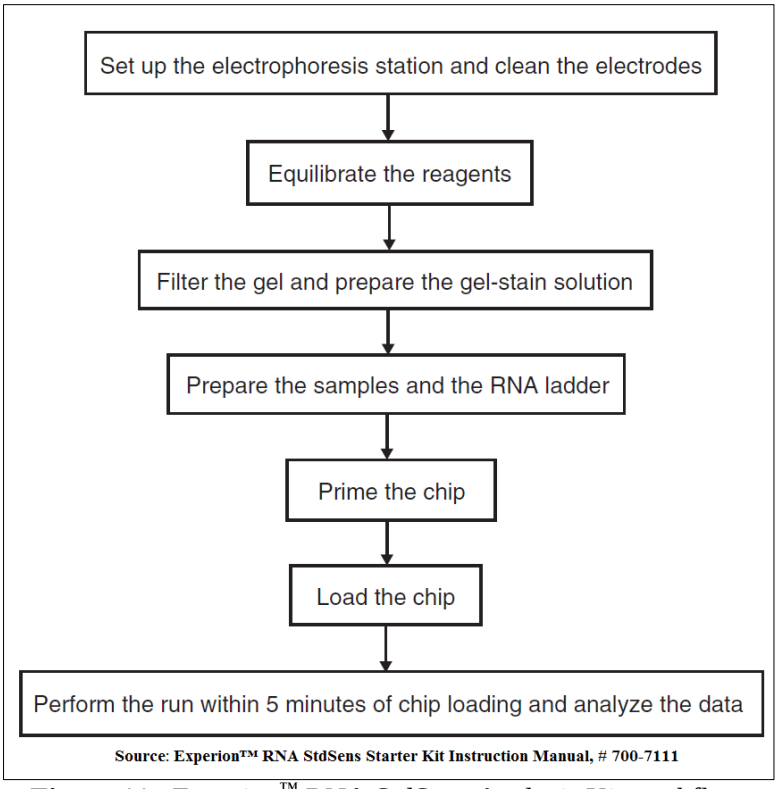


Figure 11. Experion™ RNA StdSens Analysis Kit workflow

2.4 cDNA synthesis

cDNA was synthesized from total RNA samples with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4374966), which includes random primers. The reaction was carried out in 20 µl reaction volumes using 96 well Peltier Thermal Cycler 200 (MJ Research Inc., Massachusetts, USA) according to the manufactures protocol. The High-Capacity cDNA Reverse Transcription kit can convert up to 2 µg of total RNA to cDNA and produce single-stranded cDNA which is suitable for any quantitative PCR applications. The kit was stored at -20°C and its components allowed to thaw on ice before use, except for the reverse transcriptase enzyme which was kept at -20°C at all times. Table 1 lists all the components included in the kit.

Table 1. Components of the High-Capacity cDNA Reverse Transcription kit

Component
10X RT Buffer, 1.0 ml
10X Random Primers, 1.0 ml
25X dNTP Mix (100mM)
Multiscribe™ Reverse Transcriptase, 50 U/µl
RNase Inhibitor, 100 µl

To synthesize cDNA from total RNA, a 2X reverse transcription master mix (2X RT) must be prepared using the kits components. Then total RNA samples must be added to the 2X RT

master mix to create a 1X mix. The volume of the 2X RT master mix is 10 µl and the volume of the total RNA sample is 10 µl which together make the 1X mix of 20 µl per reaction. This preperation must always be performed on ice. Table 2 shows the volumes of each component needed to prepare the 2X master mix for each reaction.

Table 2. Components of the 2X reverse transcription master mix and the volume in µl for one reaction

Component	Volume per reaction (µl)
10X RT Buffer	2.0
10X Random Primers	0.8
25X dNTP Mix (100mM)	2.0
Multiscribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
DEPC H_2O	3.2
Total volume/reaction	10.0

It is necessary to prepare a few extra reactions to account for pipetting losses of the reagents. After preparing the 2X RT master mix, 10 µl of the mix were pipetted into each reaction tube and 10 µl of the RNA sample then added, followed by mixing the sample well by pipetting up and down a few times and then sealing the tubes properly. When preparing the total RNA samples and before adding to the 2X master mix, the samples needed to be diluted using DEPC-treated water (Ambion) according to their concentration in order to get identical final concentration in all samples. The reaction tubes and caps used were RNase-free 0.2 ml MicroAmp® Optical 8-Tube Strips (Applied Biosystems, #4316567) and MicroAmp® Optical 8-Cap Strips (Applied Biosystems, #4323032). After adding the samples to each tube, the tubes were briefly centrifuged to remove any air bubbles from the samples.

The thermal cycler program used to perform the reverse transcription reactions is shown in Table 3. The conditions in the reaction were optimized for use with the High-Capacity cDNA Reverse Transcription kit.

Table 3. Reverse transcription thermal cycler program

	Step 1	Step 2	Step 3	Step 4
°C	25	37	85	4
Time	10 min	120 min	5 sec	forever

2.4.1 cDNA synthesis – analytical approach 1

The cDNA synthesis was performed as described above (section 2.4). Three RNA samples were used from each sampling day (2, 7, 26 and 36 dph), collected from one probiotic treatment silo (PB and PB1) and one control silo (UC and UC1), a total of 24 samples. Additionally, one sample from each sampling day and control/treatment silo were used to generate cDNA for standard curves in the qPCR for calculating the real-time PCR efficiency (8 samples in total). All samples used are listed in Table 4.

cDNA was synthesized from 1500 ng of total RNA from each sample as well as generating a negative control sample (RT-) which consisted of a sample without adding the Multiscribe™ Reverse Transcriptase to the reaction. All 33 samples were reverse transcribed in the same run. The samples were then diluted 10X with ddH_2O, generating 200 µl of cDNA with concentration of 7.5 ng/µl per sample, except for the samples used to generate the standard curves which were not diluted at all. Additionally, 5 µl of each diluted cDNA sample were pooled together in one sample to use as a positive plate control in the qPCR analysis. The 8 samples used for generating the standard curves were pooled together in one sample of 160 µl with concentration of 75 ng/µl. After the reverse transcription, the cDNA samples were stored at -20°C until qPCR analysis.

Table 4. All samples used for cDNA synthesis in analytical approach 1

	Sample	**dph**		**Sample**	**dph**
Control group	UC-1	2	**Probiotic group**	PB-1	2
	UC-2			PB-2	
	UC-3			PB-3	
	UC1-1	7		PB1-1	7
	UC1-2			PB1-2	
	UC1-3			PB1-3	
	UC1-4	26		PB1-4	26
	UC1-5			PB1-5	
	UC1-6			PB1-6	
	UC1-7	36		PB1-7	36
	UC1-8			PB1-8	
	UC1-9			PB1-9	
Samples for standard curves					
	Sample	**dph**		**Sample**	**dph**
UC	UC-2	2	PB	PB-2	2
	UC1-1	7		PB1-3	7
	UC1-6	26		PB1-4	26
	UC1-8	36		PB1-7	36

2.4.2 cDNA synthesis – analytical approach 2

The cDNA synthesis was performed as described above (section 2.4). The three replicate RNA samples from each sampling day (2, 7, 26 and 36 dph) were pooled together using 10 µl of each sample for preparation of one sample for each treatment at each sampling day (Table 5).

Samples PB2-1 and UC2-1 showed signs of degraded RNA in the Experion analysis and were therefore excluded from the analysis. The pooled samples from day 7 in silos PB2 and UC2 were therefore pooled from only two samples at the respective sampling days.

Table 5. Pooling of samples for cDNA synthesis in analytical approach 2.

Probiotic groups		**Control groups**	
Before pooling	**After pooling**	**Before pooling**	**After pooling**
PB-1 PB-2 PB-3	PB (2 dph)	UC-1 UC-2 UC-3	UC (2dph)
PB1-1 PB1-2 PB1-3	PB1 (7 dph)	UC1-1 UC1-2 UC1-3	UC1 (7 dph)
PB1-4 PB1-5 PB1-6	PB1 (26 dph)	UC1-4 UC1-5 UC1-6	UC1 (26 dph)
PB1-7 PB1-8 PB1-9	PB1 (36 dph)	UC1-7 UC1-8 UC1-9	UC1 (36 dph)
- PB2-2 PB2-3	PB2 (7 dph)	- UC2-2 UC2-3	UC2 (7 dph)
PB2-4 PB2-5 PB2-6	PB2 (26 dph)	UC2-4 UC2-5 UC2-6	UC2 (26 dph)
PB2-7 PB2-8 PB2-9	PB2 (36 dph)	UC2-7 UC2-8 UC2-9	UC2 (36 dph)

After pooling the samples, assessment of RNA quality was performed by electrophoresis on a 1.2% agarose gel (Sigma-Aldrich, #A9539) containing SYBR Safe DNA gel stain *10.000X concentrate in DSMO (Invitrogen, #S33102). 1.2 g of agarose powder was then dissolved in 100 ml of 0.5 TBE buffer and heated in a microwave oven until the solution was clear. The agarose was cooled down and 30 ml then poured into a 100 ml beaker. 2 µl of SYBR Safe

DNA gel stain was pipetted into the beaker and mixed carefully. The agarose was then poured into the gel tray, 15 well comb inserted, and the gel allowed to polymerize for 30 min while preparing the RNA samples. 2 µl of each sample were then transferred to 0.2 ml MicroAmp® reaction tubes together with 8 µl of DEPC water and 2 µl of 6X loading dye (Fermentas, #R0611). Samples were then heated in the PCR machine at 65°C for 5 minutes and 10 µl of each prepared sample loaded into each well and electrophoresis run on 115 V for 45 minutes. The gel was then analysed under UV light using Syngene InGenius LHR gel documentation system (Synoptics Ltd., Cambridge, England) and a picture recorded with GeneSnap software that is included in the system.

The RNA concentration, in the pooled samples, was measured using the Quant-iT™ RNA Broad Range Assay Kit (Invitrogen, #Q10213) with the Qubit® Fluorometer (Invitrogen, Oregon, USA) according to the manufactures protocol. The kits components were stored at 4°C but were allowed to equilibrate at room temperature for 30 minutes before use with the except of the rRNA ladders, which were kept at 4°C. The components of the assay kit, and their concentration, are listed in Table 6.

Table 6. Components of the Quant-iT™ RNA Broad Range Assay Kit and their concentration

Components	Concentration
Quant-iT™ RNA BR reagent	200X in DSMO
E. coli rRNA standard 1	0 ng/µl
E. coli rRNA standards 2	10 ng/µl
Quant-iT™ RNA BR buffer	

Quant-iT working solution was prepared by diluting the Quant-iT™ RNA BR reagent 1:200 in Quant-iT™ RNA BR buffer. The standards are made from 190 µl of the working solution and adding 10 µl of each rRNA standard gained 200 µl of standard 1 and 200 µl of standard 2. For each of the samples, 199 µl of working solution was added to the sample tube along with 1 µl of the total RNA sample. The assay tubes were then vortexed followed by incubation at room temperature for 2 minutes. The Qubit fluorometer was then calibrated, first with standard 1 and then with standard 2 after which the samples can be read in the fluorometer providing sample concentration in ng/µl. The reaction tubes used were 0.5 ml clear Qubit assay tubes (Invitrogen, #Q32856). Each sample was measured twice in the Qubit fluorometer and the average concentration then calculated.

Following concentration calculations, the samples were submitted to DNase I treatment before the cDNA synthesis. cDNA was synthesised from all 14 pooled samples in

duplicate using 1100 ng of total RNA from each sample as well as generating negative control sample (RT-). All 29 samples were reverse transcribed in the same run. After the reverse transcription, 14 samples and the RT- sample were diluted 10X with ddH_2O, generating 200 µl of cDNA with concentration of 5.5 ng/µl per sample. Additionally, 5 µl of each cDNA sample were pooled together to be used as a positive plate control in qPCR. The remaining 14 cDNA samples were intended for generating standard curves in qPCR. 10 µl of each sample (not diluted) were pooled together in one sample that contained in total 140 µl of cDNA with the concentration of 55 ng/µl. All cDNA samples were stored in -20°C until qPCR analysis.

2.5 qPCR analysis

All qPCR reactions were run on the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, USA) (Figure 12) with SYBR® Green I chemistry using Power SYBR® Green PCR Master Mix (Applied Biosystems, #4367659), containing ROX as a reference dye, in 15 µl reactions. Samples were prepared and run in MicroAmp® Fast Optical 48-Well Reaction Plates (Applied Biosystems, #43758) or in clear 0.2 ml MicroAmp® Optical 8-Tube Strips (Applied Biosystems, #4316567) and sealed with MicroAmp® Optical 8-Cap Strips (Applied Biosystems, #4323032). All qPCR reactions were amplified using the same amplification program (Table 7). Fluorescence data were read and collected at the end of each annealing/extension step. A melt curve analysis was performed after the last cycle, in order to investigate the specificity of the qPCR reaction and the presence of primer dimers, using a temperature gradient from 60-95°C and a ramp speed of 0.3°C/sec. During melt curve analysis the fluorescence readings were acquired every 0.3°C.

Figure 12. StepOne™ Real-Time PCR System.
Source: www.appliedbiosystems.com

Table 7. qPCR amplification program and the program for melt curve analysis

Step	**AmpliTaq Gold® Polymerase activation**	**PCR**		**Melt curve analysis**
		40 cycles		Denature at 95°C for 1 minute
		Denature	**Anneal/Extension**	
	Hold	**Cycle**		Annealing at 60°C for 1 minute
°C / time	95 °C 10 minutes	95 °C 15 seconds	60°C 1 minute	0.3°C temperature rise / sec from 60-95°C
Volume	**15 µl**			

All samples were run in duplicate along with one RT- sample and one NTC sample. A positive plate control sample (pooled cDNA sample) was furthermore included in every reaction plate as an inter-plate calibration sample and the same primers were used for this sample in all the plates in each experiment. The run was considered to be successful if a difference between plates was within 0.5 C_T for the positive plate control sample. If the difference was greater than 0.5 C_T, the plate needed to be rerun (pers.comm. Dr. Jorge Fernandes, HIBO, Norway). Standard curves were run in triplicate for calculating amplicon specific efficiency for each gene with the slope of a linear regression model (Pfaffl, 2001). Real-time PCR efficiencies were calculated according to the equation: $E = (10^{-1/m} - 1) * 100$, where m is the slope of the linear regression model fitted over log-transformed data of the input cDNA concentrations versus C_T values (Radonic *et al.,* 2004). Gene expression levels were listed as C_T values that match the number of cycles where the fluorescence signal was detected above the threshold line which was manually and arbitrary set to a specific threshold for all genes. The threshold was set above the background fluorescence and below the plateau but within the exponential phase of the amplification curve, as recommended by Applied Biosystems.

Direct sequencing of cDNA PCR products was performed using BigDye terminator cycle sequencing kit, to verify that the amplified RT qPCR product matched the GOIs using ABI PRISM® 377 DNA Sequencer (Applied Biosystems, Foster City, USA) according to the instructions of the manufacturer and grouped with 98% limits in the Sequencer programme. Sequencing was performed in both directions using corresponding gene-specific GOIs primers that were used in the qPCR part. The BLASTn alignment program (Altschul *et al.*, 1997) was then used to match these sequences with known entries in GenBank. All sequencing work were carried out by Matis-Prokaria Ltd.

2.5.1 Primers

The primers that were used were ordered from Sigma-Aldrich® and had annealing temperature between 58-60°C (Table 8). All stock primers were dissolved in TE buffer to 100 µM and then diluted to 5 µM by pipetting 2.5 µl of stock solution into 47.5 µl of ddH_2O before use. The final concentration of primers in the reactions was 300 nM for all primers except for ARP3 were the final concentration was 200 nM. Melt curve analysis was used to identify whether the primers did amplify a single product and to check whether primer dimers were present.

Primers for mIgM-H (Seppola *et al.*, 2009), hepcidin (Solstad *et al.*, 2008), EF1-α (Pérez-Casanova *et al.*, 2008) and RPL4 (Olsvik *et al.*, 2008; Sæle *et al.*, 2009) were used according to their origins but the primers LysTR1, Ubiq and ARP3 were designed using sequences with the following accession numbers in GenBank database; EU377606 (Larsen *et al.*, 2009), EX735613 and EX741373 (Olsvik *et al.*, 2008; Sæle *et al.*, 2009), respectively. These primers were designed with Primer Express 3.0.0 Applied Biosystems software using the following settings: 100 - 150 bp as optimal sequence length, optimal T_m from 58-60°C and GC% content between 30 and 80%.

The mIgM primers, designed by Seppola *et al.* (2009), were designed with the forward primer in the CH3 exon and the reverse primer in the exon-exon border between CH3 and TM1. The LysTR1 primers designed in this study, were designed to amplify a 100 basepair sequence from the *codg1* mRNA transcript (Larsen *et al.*, 2009).

Table 8. Primers for the three genes of interest and four reference genes with their GenBank accession numbers, corresponding primer sequences and the length of the amplified product provided

Genes	Primers	Accession number	Forward primer sequence 5´-3´ Reverse primer sequence 5´-3´	Amplicon length (bp)
Heavy chain of membrane bound Immunoglobulin M	mIgM	AJ87128 (X58871)	AAGGAATGAAGTGGTTCTGTGAGG TTCAGTCAGGACAAGAAACGCAT	100
g-type lysozyme	LysTR1	EU377606	CCGGGAACGTCATCTTCAAC CTCCTCTCGGTTCGTGGTATCT	102
Hepcidin	Hep	EU334514	CCAGAGCTGCGGATCGA AAGGCGAGCACGAGTGTCA	100
Ubiquitin	Ubiq	EX735613	GAGGTCGAGCCCAGTGACA GCTTGCCAGCGAAGATCAG	100
Acidic ribosomal protein	ARP3	EX741373	TAGGCATCCGACGTCCAAAC CTCATCGTCGTGGAGGATCA	100
Elongation factor 1-α	EF1-α	CO541820	GATGCACCACGAGTCTCTGA GGGTGGTTCAGGATGATGAC	171
Ribosomal protein L4	RPL4	EX725959	GGTGCCATACAGCTGATCCA CCAGGCATCACACTGCAGAA	126

2.5.2 Reaction setup

PCR master mix was made for each GOI and reference gene using the components listed in Table 9. The appropriate amounts of the real-time PCR master mix were pipetted to each reaction well following addition of cDNA template. The sample preperation was always performed on ice. Few extra preaparations were made of the PCR master mix to account for pipetting losses. Power SYBR® Green PCR master mix was stored at 4°C between use.

Table 9. Components of the real-time PCR master mix and their final concentration per reaction

Components	Final concentration
Power SYBR® Green PCR Master Mix (2X)	1X
Forward primer	200-300 nM*
Reverse primer	200-300 nM*
ddH_2O	

* Final primer concentration for genes of interests (GOIs) and reference genes

Before performing the gene expression analysis, estimation was made for appropriate reaction volume to use in qPCR analysis. StepOne™ Real-Time PCR System supports reaction volumes ranging from 10-30 µl so three different reaction volumes were analysed to find out which volume gave the lowest C_T values for the genes to cross over the threshold. Approximately 20 ng of cDNA from one sample, PB1, was used as a template using primers for the GOIs (mIgM and g-type lysozyme) along with one reference gene (Ubiquitin) in reactions containing 15, 20 and 30 µl total volume. In one reaction, half of the reaction volume contains Power SYBR® Green PCR master mix and the other half contains the gene specific primers, ddH_2O and cDNA template.

Standard curves were made for each experiment for calculation of real-time PCR efficiencies. Five-point standard curves of 5-fold dilution series (1:1 – 1:625) were prepared for samples used in analytical approach 1 and 4-fold dilution series (1:1 – 1:256) for samples used in analytical approach 2. Appropriate volume of each dilution was then added as a cDNA template, in triplicate, for each gene in the reaction plates. The plates were then sealed and centrifuged at 4500 rpm for 3 minutes with Universal 32 (Hettich Zentrifugen, Tuttlingen, Germany) for eliminating any air bubbles within the samples. The plates were then placed in the StepOne™ Real-Time PCR System for amplification and melt curve analysis.

2.5.3 qPCR – analytical approach 1

The cDNA samples from section 2.4.1 were used as templates in the qPCR analysis. This analytical approach measures relative gene expression levels of mIgM-H (mIgM) and g-type lysozyme (LysTR1) along with three reference genes, Acidic ribosomal protein (ARP3), Ribosomal protein L4 (RPL4) and Elongation factor 1α (EF1-α). Two of the reference genes were then selected for normalisation after *geNorm* analysis.

Gene expression levels were listed as C_T values that match the number of cycles where the fluorescence signal was detected above the threshold line which was manually and arbitrary set on 0.5 for all genes. For each sample of GOIs and reference genes, a PCR master mix was made according to Table 9. Appropiate amount of the PCR master mix was pipetted to each reaction well following 3 µl addition of cDNA template (22.5 ng). Five-point standard curves of 5-fold dilution series (1:1 – 1:625) were prepared from eight pooled cDNA samples, (Table 4) for real-time PCR efficiencies calculations as described in 2.5.2. The dilutions series were prepared as follows: 150 µl of the eight pooled cDNA samples were diluted with 150 µl of ddH_2O and vortexed well (1:1 dilution). 60 µl of the 1:1 dilution was transferred into 240 µl of ddH_2O and vortexed well (1:5 dilution). This dilution step was then repeated three times, making up 1:25, 1:125 and 1:625 dilution series. 3 µl of each dilution were then added as a cDNA template, in triplicate, for each gene in the reaction plates.

This analysis included 24 samples in total, which along with the standard curve samples needed to be spread over approximately 1 ½ reaction plate for each gene. Therefore a positive plate control sample (pooled cDNA from every sample) was placed in duplicate on every plate as a form of inter-plate calibration. mIgM primers were used for this sample in all the plates. The plate setup and all C_T values are provided in appendix A. After running all samples and standard curves for all genes, mean C_T values were calculated from each technical replicate samples and two reference gene chosen following evaluation by *geNorm* software (see section 2.5.5). Normalisation factor was generated for each sample and relative quantities were calculated for each sample day for both GOIs, where the sample with the highest expression was used as a calibrator sample and the transcriptional levels were set to value 1 according to the *geNorm* manual. C_T values from each of the three biological replicate samples were averaged after normalisation with the appropriate normalisation factors. Expression levels were calculated as mean values ± S.D.

2.5.4 qPCR – analytical approach 2

The cDNA samples from section 2.4.2 were used as templates in the qPCR analysis. This analytical approach measures relative gene expression levels of mIgM-H (mIgM), g-type lysozyme (LysTR1) and hepcidin (Hep) along with three reference genes, Ubiquitin (Ubiq), Acidic ribosomal protein (ARP3) and Ribosomal protein L4 (RPL4). Two of three reference genes were then selected for normalisation after *geNorm* analysis.

PCR master mix were made for the GOIs and reference genes according to Table 9 and the appropriate amounts of master mix pipetted to each reaction well following 4 µl addition of cDNA template (22 ng). Five-point standard curves of 4-fold dilution series (1:1 – 1:256) were prepared from pooled cDNA samples for real-time PCR efficiencies calculations. The dilutions series were prepared as follows: 140 µl of pooled cDNA samples were diluted with 140 µl of ddH_2O and vortexed well (1:1 dilution). 70 µl of the 1:1 dilution were pipetted into 210 µl of ddH_2O and vortexed well (1:4 dilution). This dilution step was then repeated three times, making up 1:16, 1:64 and 1:256 dilution series. 4 µl of each dilution were then added as a cDNA template, in triplicate, for each gene in the reaction plates. The threshold line was manually and arbitrary set on 0.3 for all genes.

This analysis included 14 samples in total and all samples and the standard curve samples for each gene were run in one reaction plate. A positive plate control sample (pooled cDNA from every sample), NTC sample and RT- sample were included in every plate. LysTR1 primers were used for the positive plate control sample in all plates. The plate setup and all C_T values are provided in appendix B. After running all samples and standard curves for all genes, mean C_T values were averaged from each technical replicate samples and two reference gene chosen following evaluation by *geNorm* software (see section 2.5.5). Normalisation factor was generated for each sample and relative quantities were calculated for each sample day for the GOIs where the sample with the highest expression was used a calibrator sample and the transcriptional levels were set to value 1 according to the *geNorm* manual. Expression levels were calculated as mean values ± S.D.

2.5.5 *geNorm* analysis and calculation of normalised expression levels

The *geNorm* program calculates the gene expression stability value (*M*) for a potential reference gene as well as an average pairwise variation value (*V*) for that gene and for all reference genes tested. Following calculation of the *M* value, the program then ranks the genes in such order that the most stable genes receives the lowest *M* value which increases the suitability of that particular gene as a reference gene. Stepwise elimination of the highest *M* values leads to a combination of two reference genes that have the most stable expression across the samples tested. The program also determines the optimal number of genes for an accurate normalisation by calculating the pairwise variation ($V_{(n/n+1)}$) between the two normalisation factors NF_n and NF_{n+1}, where each normalisation factor is based on the geometric mean expression of the "n" most stable genes. Vandesompele and co-workers (2002) proposed the cut-off value to be 0.15, where a variation above 0.15 means that the added gene has significant effect and should indeed be included to gain the appropriate normalisation factor. The authors recommend the minimal use of the three most stable reference genes for calculation of the normalisation factor (NF_3) and a stepwise inclusion of more reference genes until the n^{th} gene has no significant effect on the normalisation factor. Following calculation of the appropriate normalisation factor for all samples, the normalised expression levels of GOIs can be calculated by dividing GOI raw quantities for each sample by the appropriate normalisation factor. The authors also recommend that the genes should be quantified on the same batch of cDNA to minimize experimental errors which may be in large parts due to cDNA synthesis (Vandesompele *et al.*, 2002b).

In order to use the *geNorm* software according to Vandesompele *et al.* (2002), mean C_T values from technical replicates were transformed into raw quantities using the standard curves for each gene. The following equation was used to transform C_T values to quantities: $10^{(\text{mean CT value} - b)}/m$, where b stands for y-intercept of the standard curve line and m for the slope of the linear regression model that was fitted over log-transformed data of the input cDNA concentrations versus the C_T values. After gaining raw quantities for each reference gene an input file needs to be created to perform the *geNorm* analysis. Highest relative quantities of each reference gene were set to 1 by dividing raw quantities of each sample by the highest quantity of each gene. This input file was loaded into the *geNorm* excel file and the program then calculated the *M* and *V* values.

Out of the three reference genes, the one gene that had the highest *M* value was excluded and the two other reference genes were used to calculate normalisation factors for

each sample analysed. The normalisation factor calculated by *geNorm* is equal to manually calculate the geometric mean of the two reference genes expression levels. The normalised GOI expression levels were then calculated by dividing the GOI raw quantities for each sample by the appropriate normalisation factor. By performing this procedure, normalised GOI expression levels are the same as relative quantities, calibrated with the sample which has the highest expression level. This is described in more details in the *geNorm* manual that can be retrieved from:
http://medgen.ugent.be/~jvdesomp/genorm/geNorm_manual.pdf.

2.6 Statistical analysis

Statistical analysis was performed using SigmaStat® release 3.5 (Systat Software Inc., CA 94804-2028, USA). Kolmogorov-Smirnov test was used to analyse the normality of the data distribution. The effects of the expression of GOIs in the probiotic treated compared to untreated control was analysed with a *t*-test and Mann-Whitney Rank Sum test was used when equal variance test failed.

For analytical approach 1, the mean ± S.D. expression levels of three samples for each GOI in both experimental groups at all sampling days were calculated.

For analytical approach 2, the mean ± S.D. expression levels of all GOIs in two samples at all sampling days were calculated.

Differences were regarded statistically significant when $p<0.05$.

3 Results

3.1 RNA concentration and quality assessment

All samples used in the gene expression studies were analysed by Experion software and the virtual gel images produced by the software are shown in Figures 13 and 14. The virtual gel image reveals the two dominant RNA fragments, the 18S (lower band) and 28S (upper band) rRNAs. The RNA concentration in ng/µl and the RQI numbers for all samples is shown in Table 10.

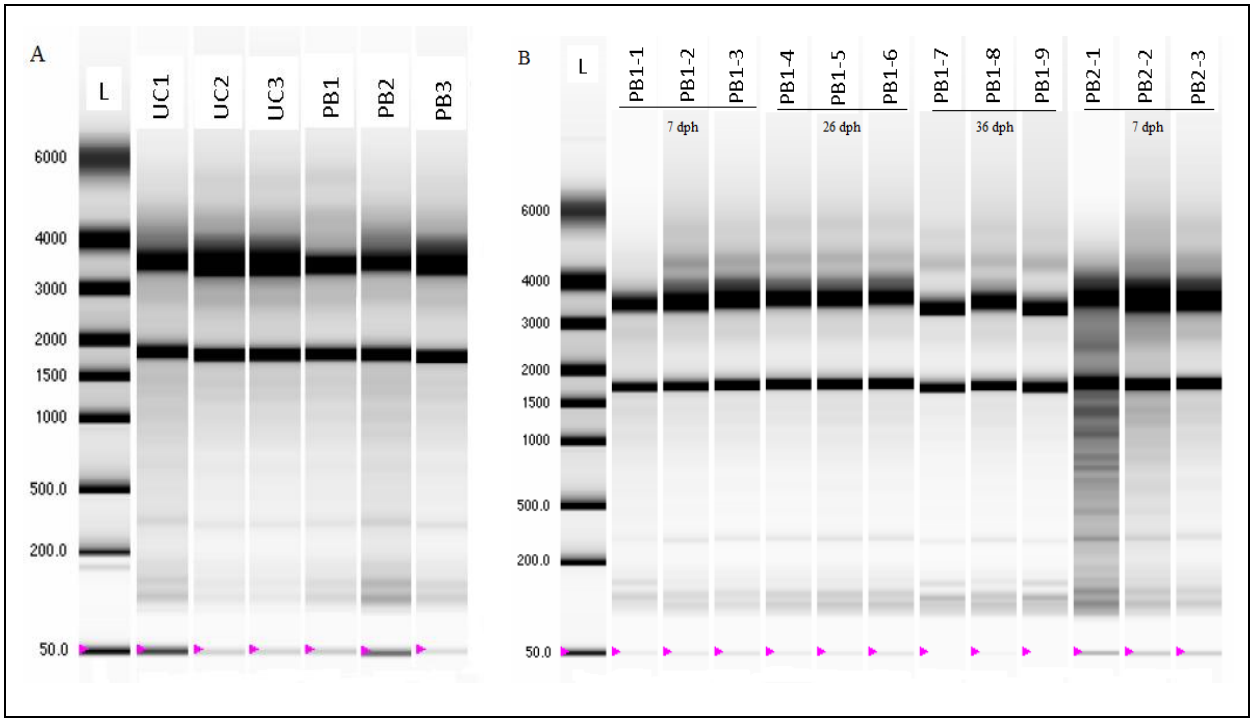


Figure 13. Virtual gel images of RNA samples. A: RNA samples of larvae from untreated (UC) and probiotic treated (PB) silos on day 2 post hatch. B: RNA samples of larvae from the two probiotic treated silos (PB1 and PB2) at various days post hatch. L: RNA ladder.

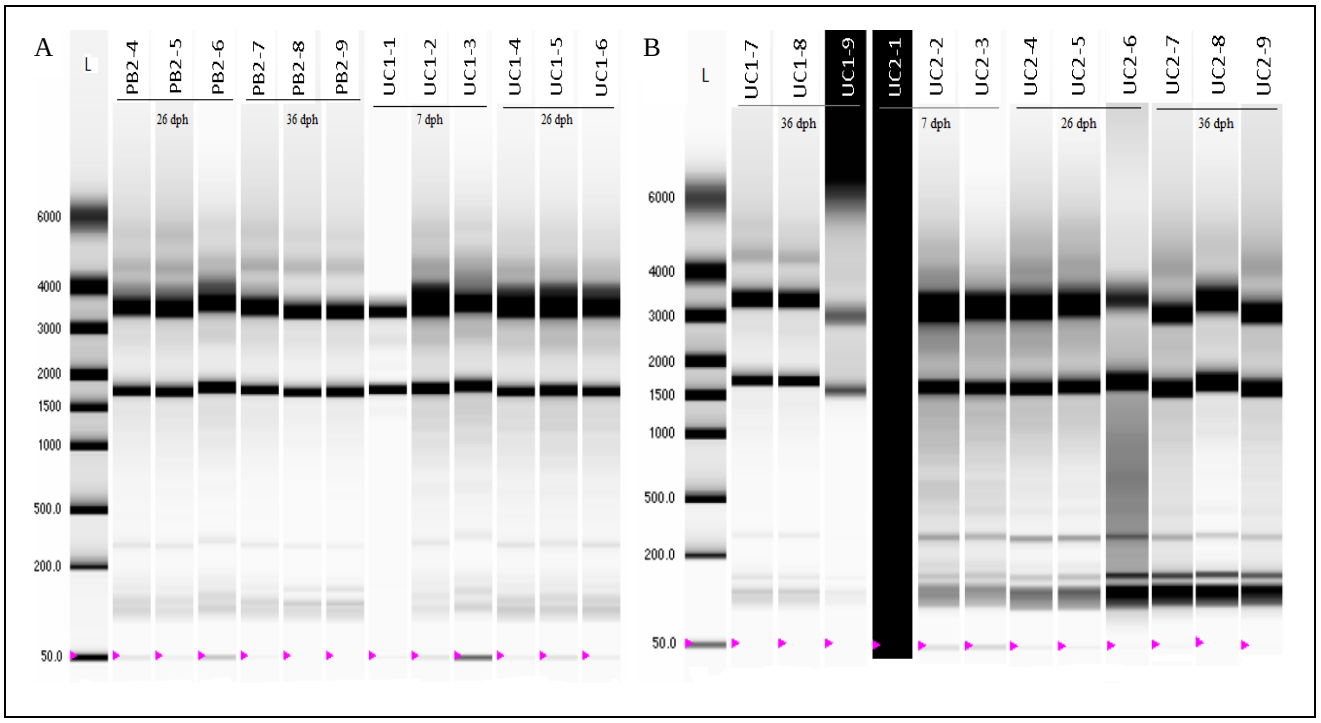


Figure 14. Virtual gel images of RNA samples. A: RNA samples of larvae from the probiotic treated (PB2) and untreated (UC1) silos at various dph. B: RNA samples of larvae from the untreated silos (UC1 and UC2) at various dph. L: RNA ladder.

Table 10. RNA quality indicator numbers (RQI) and the calculated RNA concentration in all samples

	Sample	dph	RQI number	[RNA] ng/µl		Sample	dph	RQI number	[RNA] ng/µl
Probiotic groups	PB-1	2	8.8	450.4	Control groups	UC-1	2	7.3	157.4
	PB-2		8.9	779.4		UC-2		9.1	614.4
	PB-3		8.7	644.5		UC-3		9.1	594.9
	PB1-1	7	8.2	814.9		UC1-1	7	8.8	398.9
	PB1-2		9.3	977.9		UC1-2		9.3	1075.7
	PB1-3		8.7	712.8		UC1-3		8.9	190.2
	PB1-4	26	8.5	808.4		UC1-4	26	9.4	1447.7
	PB1-5		8.7	805.8		UC1-5		9.1	874.2
	PB1-6		8.1	623		UC1-6		9.1	1262
	PB1-7	36	9.4	2730.1		UC1-7	36	9.5	2044.6
	PB1-8		9.1	1121.5		UC1-8		9.5	2233.8
	PB1-9		8.7	3178.7		UC1-9		9.5	3850.7
	PB2-1	7	* 6.5	423.1		UC2-1	7	* 0	0
	PB2-2		8.3	435.4		UC2-2		8.5	663
	PB2-3		9	339.8		UC2-3		8.7	523.2
	PB2-4	26	9.1	1117.6		UC2-4	26	9	1789
	PB2-5		9.4	1811.4		UC2-5		9.1	1926.5
	PB2-6		8.3	525.7		UC2-6		9.2	2052.5
	PB2-7	36	9.5	2122.6		UC2-7	36	7.5	2354.1
	PB2-8		9.5	3607.8		UC2-8		8.8	2661.2
	PB2-9		9.5	4329.1		UC2-9		8.2	2849.8

* marked are samples exluded from the analysis

As shown in Table 10, most of the samples received RNA quality indicator (RQI) numbers ranging between 7-9.5 which the Experion software regards appropriate for any downstream applications, including RT-qPCR. Two samples contained degraded RNA and were excluded

from further analysis. The PB2-1 sample received a RQI number of 6.5 which indicates that the sample was partly degraded whereas the UC2-1 sample was completely degraded and was not detected by the Experion software.

The sequencing results of 100 bp cDNA amplicons from each GOI are shown in Table 11. The results from the best BLASTn hits verify that the primers used in the qPCR did amplify the products targeted. Sequencing of the mIgM with the reverse primer was however not successful. The accession number (X58871) for the mIgM that was sequenced with the forward mIgM primer, refers to the 559 bp mRNA sequence of *Gadus morhua* transmembrane immunoglobulin heavy chain which is generated by splicing of the CH3 exon into TM1 exon.

Table 11. Sequencing results for the amplicon of the genes of interest, their accession numbers in GenBank, best BLASTn hit, % identity and E-values of the best BLASTn hit

Sequence	Accession nr.	BLASTn hit	% ID	E-value
mIgM_f	X58871	*G. morhua* TM IgM-H mRNA	39/39 (100%)	4e-12
Lysozyme_f	EU377606	*G. morhua* g-type lysozyme 1 mRNA	55/56 (98%)	5e-18
Lysozyme_r	EU377606	*G. morhua* g-type lysozyme 1 mRNA	77/78 (98%)	2e-29
Hepcidin_f	EU334514	*G. morhua* hepcidin precursor mRNA	53/55 (96%)	8e-16
Hepcidin_r	EU334514	*G. morhua* hepcidin precursor mRNA	49/50 (98%)	2e-16

f: sequenced with the corresponding gene-specific forward primer
r: sequenced with the corresponding gene-specific reverse primer
%ID: % identity over the aligned region
E-value: Expectation value (significance of the best BLASTn hit)
TM IgM-H: transmembrane immunoglobulin heavy chain

3.2 Estimation of appropriate reaction volumes for qPCR

The StepOne™ Real-Time PCR System supports reaction volumes ranging between 10-30 µl and three different reaction volumes were chosen for the analysis; 15, 20 and 30 µl total volumes. Half of the total reaction volume contained Power SYBR® Green PCR master mix and the other half contained gene specific primers, ddH_2O and a cDNA template. After running each sample in duplicate for three different genes (mIgM, LysTR1 and Ubiq) and in three different reaction volumes, the C_T values were averaged. The 15 µl reaction volume, containing 7.5 µl of the Power SYBR® Green PCR master mix, produced the lowest C_T values for all genes, with results for randomly selected samples shown in Table 12. Based on this information and another study, that used 15 µl reaction with ABI SYBR® Green PCR

master mix (Inami *et al.*, 2009), a decision was made to use 15 µl as a total reaction volume in all further qPCR runs.

Table 12. Averaged C_T values for the three selected genes, for decision of total reaction volumes in qPCR

Sample	Gene	Reaction volume		
		15 µl	20 µl	30 µl
		C_T value		
PB1-2	Ubiq	18.1	19.2	19.6
PB1-2	mIgM	33.2	33.5	34.1
PB1-2	LysTR1	28.7	30.1	30.3

3.3 Results for analytical approach 1

Analytical approach 1 included expression analysis of the heavy chain of membrane bound IgM (mIgM) and g-type lysozyme (LysTR1). The analysis was performed using the three biological replicate samples from the probiotic treatment silos (PB and PB1) and untreated control silos (UC and UC1). The reference genes analysed were RPL4, ARP3 and EF1-α. Samples from these silos were chosen because, according to the virtual gel images of all samples (Figures 13 and 14), they appeared to produce cleaner rRNA bands compared with the samples from the PB2 and UC2 silos, even though similar RQI numbers were obtained for the samples.

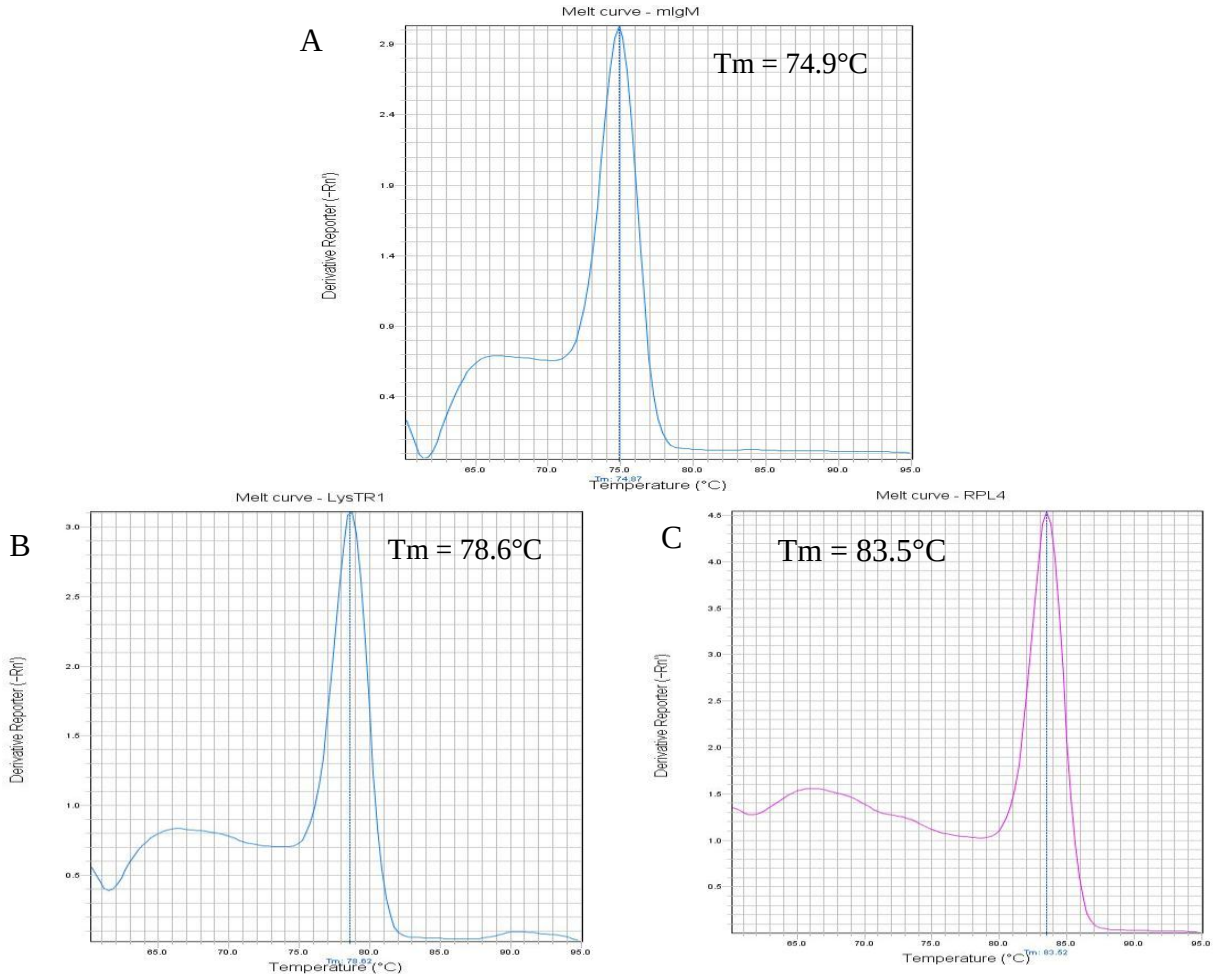
As described in section 1.5.3, the samples and standard curves were spread over approximately 1.5 reaction plates for each gene (8 plates in total). A positive plate control sample was placed in duplicate on every plate, serving as an inter-plate calibration sample. mIgM primers were used for this sample in all the plates. The C_T values of the positive plate control sample on reaction plates never exceeded 0.4 C_T and the results therefore regarded to be comparable between the plates. The C_T values for the plates are listed in Table 13.

Table 13. C_T values for the inter-plate calibration sample on every reaction plate in analytical approach 1

	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Plate 7	Plate 8
C_T	29.6	29.6	29.7	29.8	30.0	29.9	29.8	29.8

3.3.1 Primer specificity

The specificity of all primers was evaluated by melt curve analysis, showing a single amplified product for all genes and verifying that the primers did not generate any unspecific products. The StepOne™ Real-Time PCR System automatically records a second melting temperature if it detects any other amplified product beside the specific amplicon. The melt curves for GOIs amplicons, generated with mIgM and LysTR1 primers, and the reference gene amplicons, generated with RPL4, ARP3 and EF1-α primers are shown in Figure 15. The melting temperature of the amplified products were; 74.9°C for mIgM, 78.6°C for LysTR1, 83.5°C for RPL4, 78.6°C for ARP3 and 81.6°C for EF1-α. The same sample (PB1-9) was arbitrary chosen and used to generate the melt curves representing each gene product.



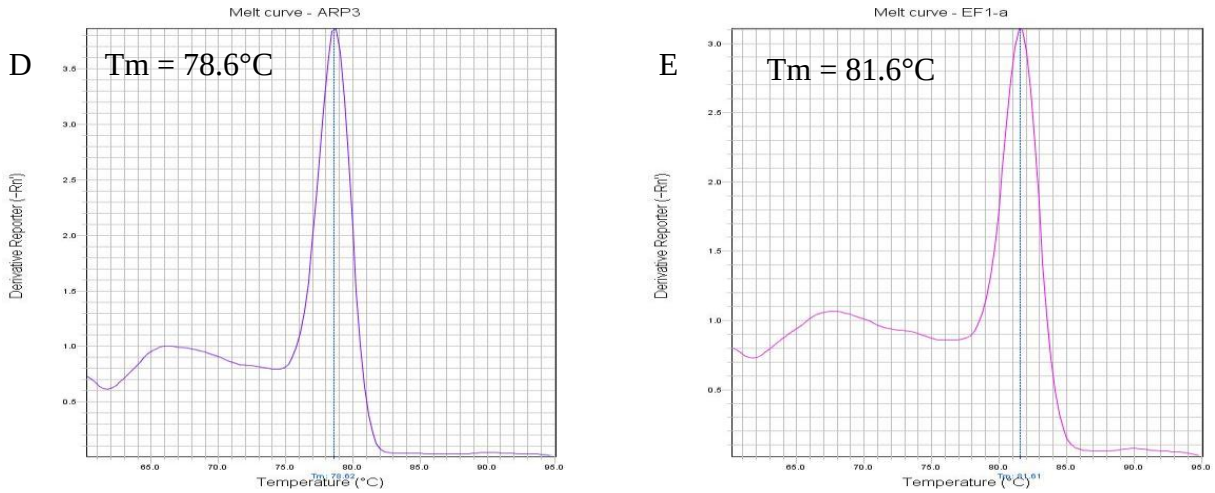


Figure 15. Melt curves for all amplicons generated with primers used in analytical approach . Tm: melting temperature of the amplified product. A: mIgM B: LysTR1 C: RPL4 D: ARP3 and E: EF1-α.

3.3.2 Standard curves and PCR amplification efficiencies

Real-time PCR reaction efficiencies were determined using five-point standard curves of 5-fold dilution series (1:1–1:625) for both GOIs as well as for the reference genes. Standard curves were generated in triplicate and the technical replicate dilutions then averaged to gain the C_T value. The standard curve for mIgM only contained four dilution points as the last dilution point (1:625) was undetected in qPCR. The primers, PCR reaction efficiencies and correlation coefficient (r^2) for all genes are listed in Table 14.

Table 14. Reaction efficiency of selected primers in analytical approach 1

Primers	PCR reaction efficiency	r^2
mIgM	77.9%	0.996
LysTR1	87.0%	0.997
RPL4	88.2%	0.998
ARP3	90.0%	0.997
EF1-α	86.7%	0.995

3.3.3 Stability evaluation of reference genes – *geNorm* analysis

The *geNorm* evaluated RPL4 and EF1-α to be more stably expressed than ARP3 which received a considerably higher *M* value of 0.349 compared with the *M* value obtained for RPL4 and EF1-α (0.199). RPL4 and EF1-α genes were therefore used for calculating the normalisation factors (NF) for all samples (Table 15).

Table 15. Normalisation factors for all samples at all sampling days from the untreated group (UC and UC1) and the probiotic group (PB and PB1). Also presented is the average expression stability (*M* values) for the reference genes (RPL4 and EF1-α)

geNorm geNorm

	RPL4	EF1-α	Normalisation Factor		RPL4	EF1-α	Normalisation Factor
UC-1	0.515	0.670	**0.5992**	PB-1	0.293	0.335	**0.3134**
UC-2	0.327	0.386	**0.3550**	PB-2	0.576	0.636	**0.6053**
UC-3	0.514	0.606	**0.5579**	PB-3	0.732	0.840	**0.7838**
UC1-1	0.426	0.357	**0.3901**	PB1-1	0.481	0.462	**0.4714**
UC1-2	0.198	0.266	**0.2295**	PB1-2	0.889	0.902	**0.8952**
UC1-3	0.334	0.481	**0.4010**	PB1-3	0.482	0.520	**0.5002**
UC1-4	0.192	0.219	**0.2051**	PB1-4	0.803	0.872	**0.8370**
UC1-5	0.356	0.521	**0.4307**	PB1-5	0.230	0.262	**0.2455**
UC1-6	0.281	0.406	**0.3378**	PB1-6	0.877	1.000	**0.9367**
UC1-7	0.613	0.697	**0.6534**	PB1-7	0.544	0.536	**0.5398**
UC1-8	0.633	0.667	**0.6501**	PB1-8	1.000	0.959	**0.9788**
UC1-9	0.402	0.456	**0.4280**	PB1-9	0.533	0.566	**0.5491**
		M < 1.5	RPL4 (M) = **0.199** EF1-α (M) = **0.199**				

The pairwise variation value (*V*) for the two more stable reference genes was 0.139 which is below the cut-off value of 0.15 and as recommended by *geNorm*, the two reference genes should therefore be used for calculations of appropriate normalisation factors for all samples analysed.

3.3.4 Relative expression levels for mIgM and g-type lysozyme

Normalised expression levels of mIgM at all sampling days are shown in Figure 16, revealing no significant difference between the group's expression levels prior to the onset of exogenous feeding of larvae at 2 dph ($p = 0.542$). Figure 16 furthermore shows that five days later, approximately four days after the onset of exogenous feeding, no significant difference in expression levels was observed between the groups ($p = 0.946$), with expression levels similar to those observed at 2 dph. At the onset of metamorphosis at 26 dph, significant difference was observed between the untreated control (UC) and probiotic treated (PB) group ($p = 0.028$), with expression levels of 0.58 and 0.84 observed for the UC and PB groups, respectively. A significant increase ($p = 0.035$) in the expression levels of mIgM was observed in the PB group between 7 and 26 dph, while the expression levels in the UC group were still around the same level as at 2 and 7 dph.

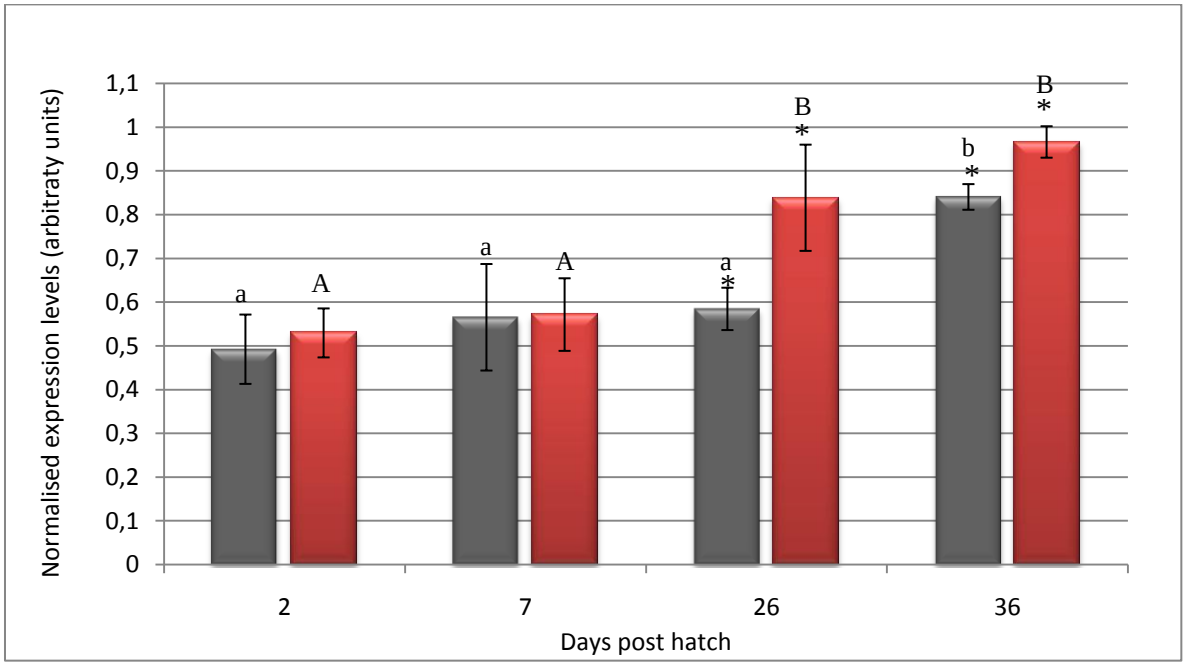


Figure 16. Expression levels of mIgM in cod larvae on days 2, 7, 26 and 36 post hatch. Grey columns represent samples from the untreated control silos (UC and UC1) and red columns larval samples from silos receiving probiotic treatment (PB and PB1). The expression levels were normalised to RPL4 and EF1-α and calibrated with respect to the sample showing the highest expression. Expression levels were obtained from three pooled biological replicate samples and the mean ± S.D. calculated. Bars marked with an asterisk (*) indicate statistical difference ($p<0.05$) between untreated control and probiotic treated larvae on that particular day post hatch. Bars marked with "ab" indicate statistical difference ($p<0.05$) between sampling days in the UC group and "AB" between sampling days in the PB group.

A significant difference ($p = 0.009$) between the groups was also observed during metamorphosis at 36 dph, where the expression levels for the UC and PB group were 0.84 and 0.97, respectively. A significant ($p = 0.001$) increase in the expression levels for the UC group appeared between the last two sampling days, with values increasing from 0.58 at 26 dph to 0.84 at 36 dph. The expression levels of mIgM in the PB group increased from 0.84 to 0.97 between days 26 and 36 post hatch but the difference was not statistically significant ($p = 0.156$).

Normalised expression levels for g-type lysozyme in both untreated control (UC) and probiotic treatment (PB) groups at all sampling days are shown in Figure 17. Prior to the onset of exogenous feeding at 2 dph, the expression levels commenced around 0.3, with no significant difference observed between the groups ($p = 0.698$).

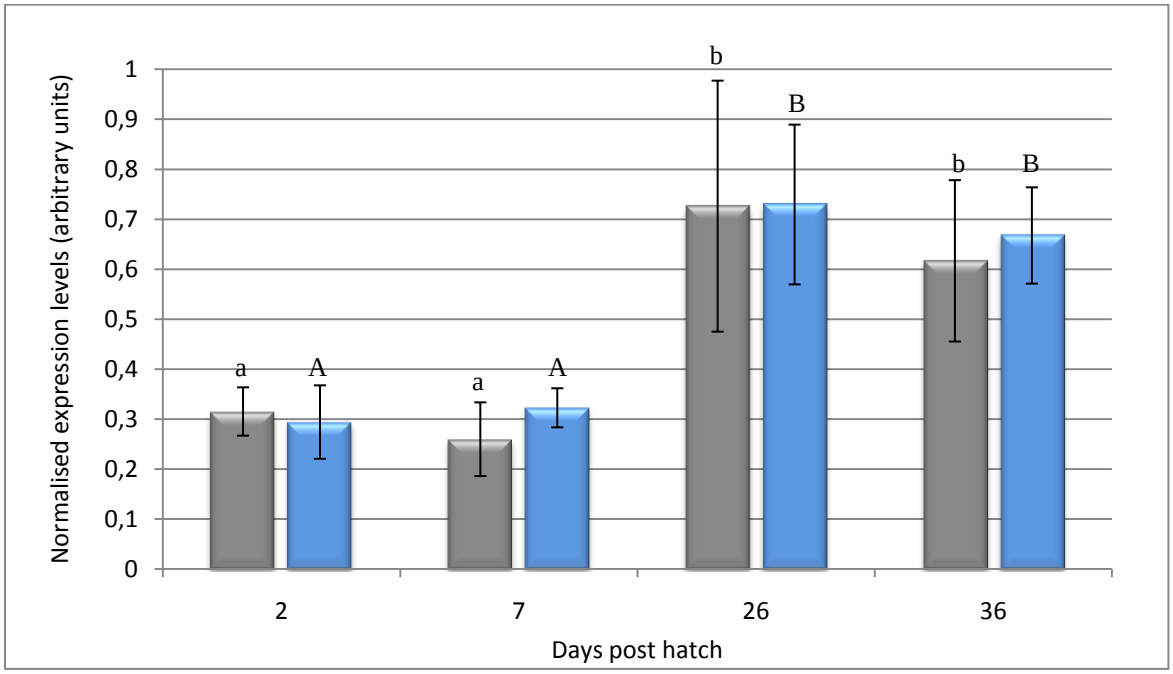


Figure 17. Expression levels of g-type lysozyme in cod larvae on days 2, 7, 26 and 36 post hatch. Grey columns represent samples from the untreated control silos (UC and UC1) and blue columns samples of larvae receiving probiotic treatment (PB and PB1). The expression levels were normalised to RPL4 and EF1-α and calibrated with respect to the sample showing the highest expression. Expression levels were obtained from three pooled biological replicate samples and the mean ± S.D. calculated. Bars marked with "ab" indicate statistical difference ($p<0.05$) between sampling days in the UC group and "AB" between sampling days in the PB group.

From 2 dph and to the next sampling at 7 dph, four days after the onset of exogenous feeding, the expression levels in the UC group decreased from 0.32 to 0.26 whereas a slight increase was observed between the sampling days for the PB group (from 0.29 to 0.32). The increase was, however, not found to be significant ($p = 0.584$) and no significant difference ($p = 0.262$) was observed between the groups at 7 dph. A considerable increase in the expression levels of g-type lysozyme occurred between 7 and 26 dph, corresponding to early metamorphosis. The increased expression was significant in both groups ($p = 0.037$ and 0.013 for the UC and PB groups, respectively). No significant difference was observed between the groups ($p = 0.986$). During metamorphosis at 36 dph, the expression levels in both groups decreased to 0.62 and 0.67 for UC and PB groups, respectively. The difference between the groups was not significant at this time point ($p = 0.665$).

3.4 Results for analytical approach 2

Analytical approach 2 included relative gene expression mIgM, g-type lysozyme and hepcidin in pooled RNA samples from untreated control silos (UC, UC1 and UC2), and silos which were subjected to probiotic treatment (PB, PB1 and PB2). One sample was therefore gained from each individual silo at the individual sampling days. The reference genes analysed and used in this experiment included Ubiq, ARP3 and RPL4. After gaining the expression levels for each sample, expression levels from the two sibling silos in each treatment group were averaged and the mean expression levels at the individual sampling days then calculated.

The samples and standard curve dilutions were spread over one reaction plate for each gene (6 plates in total). The same positive plate control sample was added to all plates using LysTR1 primers. The C_T values of the positive plate control sample on plates are listed in Table 16 and the difference between plates never exceeded 0.3 C_T.

Table 16. C_T values for the positive plate control sample on every reaction plate in analytical approach 2

	Plate 1	**Plate 2**	**Plate 3**	**Plate 4**	**Plate 5**	**Plate 6**
C_T	26.8	27.0	27.0	26.9	27.0	27.1

3.4.1 RNA concentration and quality assessment for pooled RNA samples

After pooling the samples (see section 2.4.2), RNA concentration was measured using the Qubit® fluorometer. Each sample was measured twice and average concentration in ng/µl then calculated (Table 17).

Table 17. Average RNA concentration in pooled samples. Each sample was measured twice (M1, M2) and average RNA concentration in ng/µl then calculated. Shown is the RNA concentration in samples from untreated larvae (UC) larvae subjected to probiotic treatment (PB) at the individual sampling days.

	Sample	dph	M1	M2	Average [RNA] ng/µl		Sample	dph	M1	M2	Average [RNA] ng/µl
Control groups	UC (1,2,3)	2	115	116	115.5	probiotic groups	PB (1,2,3)	2	264	264	264
	UC1 (1,2,3)	7	230	228	229		PB1 (1,2,3)	7	598	592	595
	UC1 (4,5,6)	26	670	670	670		PB1 (4,5,6)	26	3380	3360	3370
	UC1 (7,8,9)	36	1480	1475	1477.5		PB1 (7,8,9)	36	1680	1665	1672.5
	UC2 (2,3)	7	322	320	321		PB2 (2,3)	7	303	302	302.5
	UC2 (4,5,6)	26	1605	1590	1597.5		PB2 (4,5,6)	26	630	630	630
	UC2 (7,8,9)	36	4040	3955	3997.5		PB2 (7,8,9)	36	2960	2920	2940
M1 = measurement 1 M2 = measurement 2											

After measuring and calculating the average RNA concentration in the samples, each sample was treated with two units of DNase I for complete removal of any DNA contamination. Following the treatment, the samples were submitted to agarose gel electrophoresis for determination of the quality of each sample (Figure 18). The image shows that each sample contains the predominant 18S and 28S rRNAs. Samples from 2 and 7 dph showed weaker 18S and 28S rRNA bands than samples from 26 and 36 dph, which is in accordance with the concentration of the samples (see Table 17). Samples from 2 and 7 dph furthermore contained lower RNA concentrations compared with samples from 26 and 36 dph.

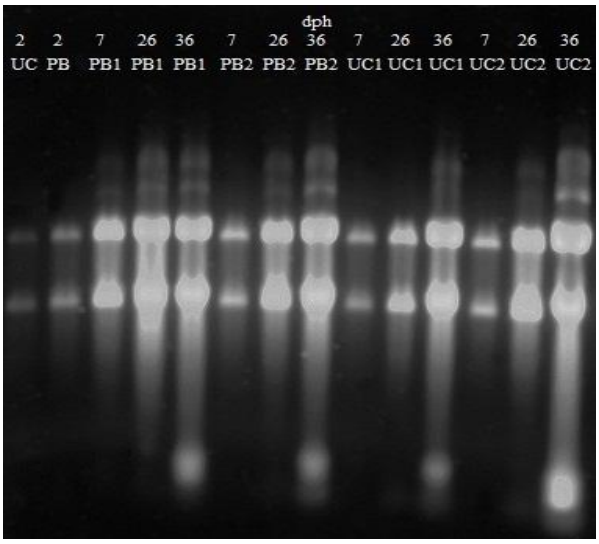


Figure 18. Agarose gel image of pooled RNA samples from all silos at the different sampling days. From left to right, the first two lanes represent samples collected from the untreated silo (UC) and probiotic treated (PB) silo at 2 days post hatch (dph). The following lanes contain samples collected from PB1, PB2, UC1 and UC2 silos at 7, 26 and 36 dph.

3.4.2 Primer specificity

Like in analytical approach 1, the specificity of the primers was analysed by melt curve analysis and all melt curves showed a single amplified product for all genes, verifying the absence of unspecific products. Figure 19 demonstrates the melt curves for the amplicons generated with the Hep and Ubiq primers. Melt curves for the amplicons generated with mIgM, LysTR1, ARP3 and RPL4 primers are not shown as they generated the same melt curves as in analytical approach 1 (Figure 15).

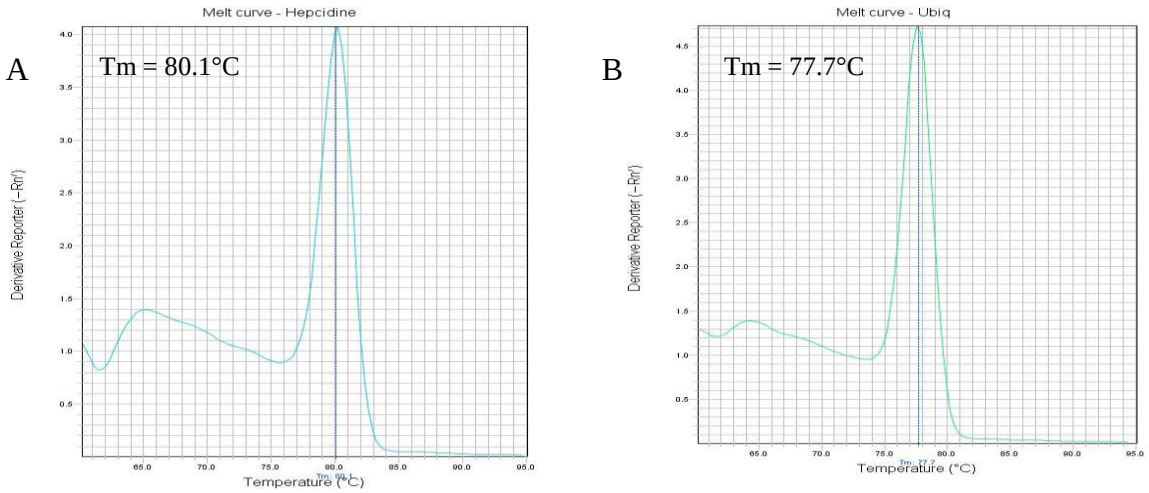


Figure 19. Melt curves for amplicons generated with Hep and Ubiq primers. Tm: melting temperature of the amplified product. A: Hepcidin, B: Ubiquitin

3.4.3 Standard curves and PCR amplification efficiencies

Real-time PCR reaction efficiencies were determined using five-point standard curves of 4-fold dilution series (1:1 – 1:265) for the GOIs and reference genes. Standard curves were generated in triplicate and the average values for each dilution then calculated to gain the C_T value. The primers, PCR reaction efficiencies and correlation coefficient (r^2) for all genes are listed in Table 18.

Table 18. Reaction efficiency of selected primers in analytical approach 2

Primers	PCR reaction efficiency	r^2
mIgM	95.1%	0.983
LysTR1	87.1%	0.999
Hep	93.9%	0.999
RPL4	90.7%	0.993
ARP3	93.2%	0.994
Ubiq	90.4%	0.990

3.4.4 Stability evaluation of reference genes – *geNorm* analysis

The *geNorm* evaluated Ubiq and ARP3 to be more stably expressed than RPL4 and these two reference genes were therefore used to calculate the normalisation factors (NF) for all samples (Table 19).

Table 19. Normalisation factors for all samples at all sampling days from both untreated groups (UC,UC1 and UC2) and both probiotic groups (PB, PB1 and PB2). Also presented is the expression stability (*M* values) for the reference genes (Ubiq and ARP3)

geNorm geNorm

	Ubiq	ARP3	Normalisation Factor		Ubiq	ARP3	Normalisation Factor
UC 2 dph	0.3120	0.3280	0.6166	PB 2 dph	0.4860	0.5740	1.0162
UC1 7 dph	0.4730	0.4630	0.9009	PB1 7 dph	0.6020	0.6220	1.1782
UC1 26 dph	0.4210	0.4850	0.8705	PB1 26 dph	0.1660	0.2780	0.4134
UC1 36 dph	0.7620	0.7240	1.4299	PB1 36 dph	1.0000	1.0000	1.9253
UC2 7 dph	0.3880	0.4980	0.8464	PB2 7 dph	0.4290	0.5500	0.9349
UC2 26 dph	0.5100	0.6000	1.0654	PB2 26 dph	0.5830	0.8430	1.3491
UC2 36 dph	0.6250	0.6000	1.1792	PB2 36 dph	0.5070	0.6820	1.1318
			M < 1.5	Ubiq (M) = **0.246**	ARP3 (M) = **0.246**		

RPL4 received a considerably higher *M* value of 0.412 than the *M* value obtained for Ubiq and ARP3 (0.246). The *V value* for Ubiq and ARP3 was 0.152 and only slightly over the cut-off limits of 0.150 proposed by Vandesompele and co-workers (2002). A third reference gene should be added for calculation of appropriate normalisation factors. The *geNorm* analysis was, however, mainly intended for selection of two out of three more stably expressed reference genes for normalisation and the third reference gene was therefore not added. Furthermore, only a small difference between the cut-off value (0.150) and obtained *V* value (0.152) was observed.

3.4.5 Relative expression levels for mIgM, g-type lysozyme and hepcidin

Normalised expression levels of mIgM are presented in Figure 20, showing no significant difference between the groups at any sampling day ($p > 0.05$).

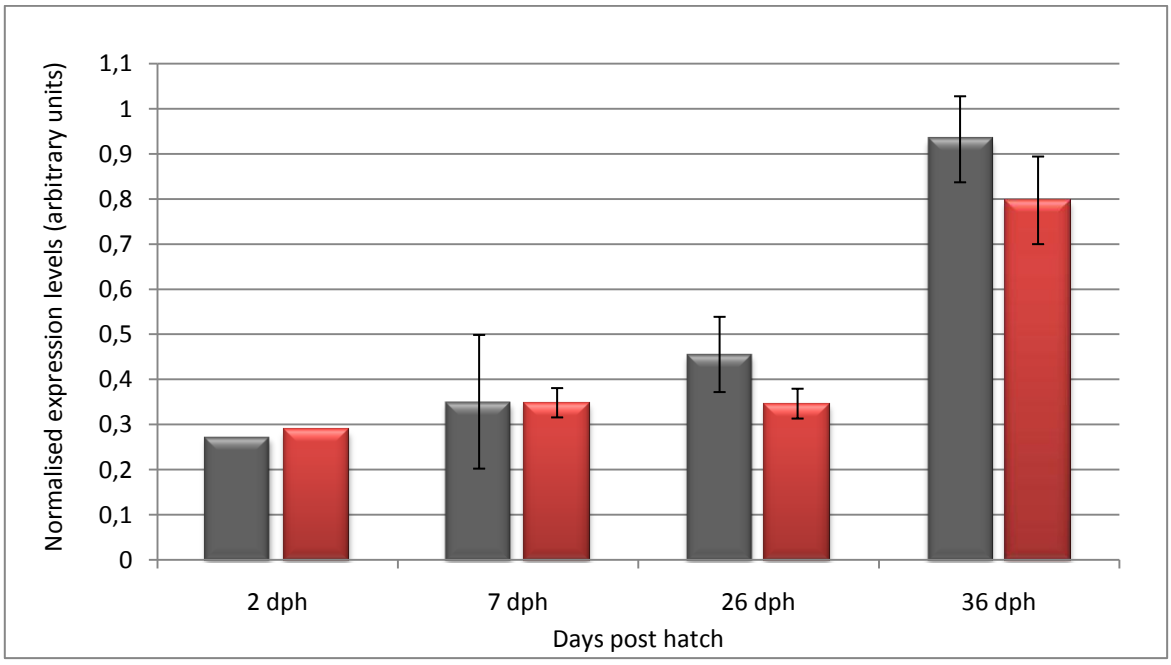


Figure 20. Expression levels of mIgM in cod larvae on days 2, 7, 26 and 36 post hatch. Grey columns represent samples from the untreated control silos (UC, UC1 and UC2) and red columns samples from silos receiving probiotic treatment (PB, PB1and PB2). The expression levels were normalised to Ubiq and ARP3 and calibrated with respect to the sample showing the highest expression. Expression levels were obtained from two samples, one from each silo from each treatment group at individual sampling days and the mean values ± S.D. then calculated.

Similar expression levels were observed for both groups at 2, 7 and 26 dph. At the last sampling day, collected during metamorphosis (36 dph), the results showed a sudden increase in expression from the earlier sampling days in both groups. Higher expression levels were observed in the UC group compared with the PB, but the difference between the two groups was not significant ($p > 0.05$).

Normalised expression levels of g-type lysozyme are shown in Figure 21, where no significant difference between the groups at any sampling day or between sampling days was observed ($p > 0.05$). The expression levels were similar between the groups prior to and after the onset of exogenous feeding at 2 and 7 dph. Increased expression was observed in both groups after onset of metamorphosis at 26 dph as well as at 36 dph.

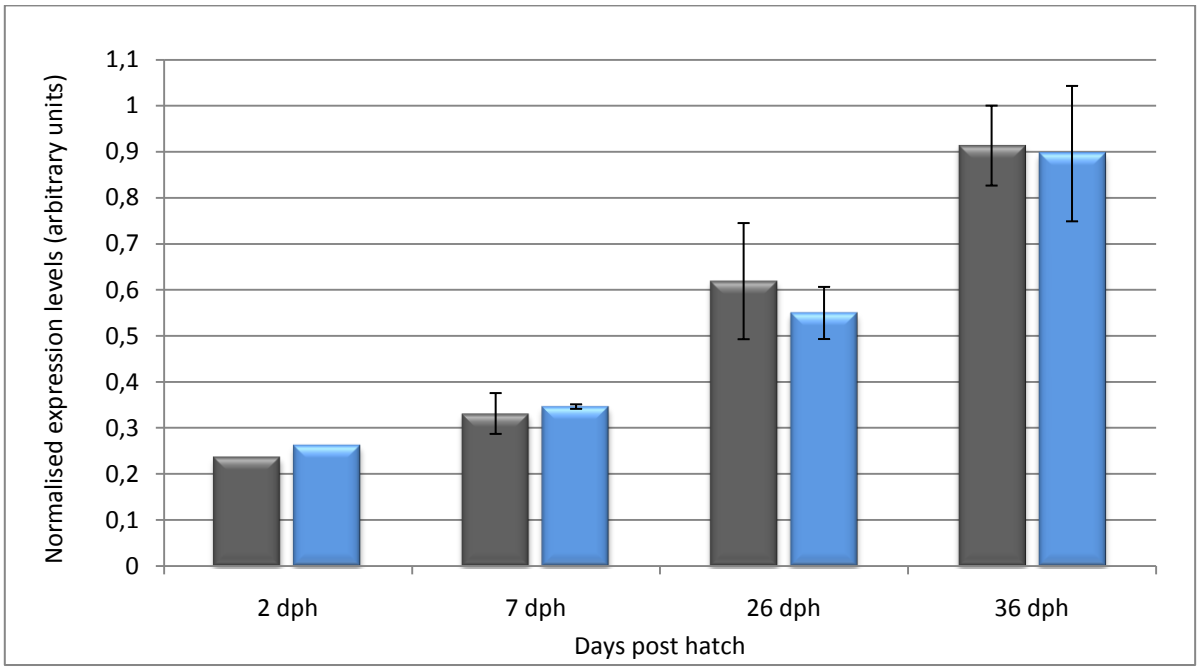


Figure 21. Expression levels of g-type lysozyme in cod larvae on days 2, 7, 26 and 36 post hatch (dph). Grey columns represent samples from the untreated control silos (UC, UC1 and UC2) and blue columns samples from silos receiving probiotic treatment (PB, PB1and PB2). The expression levels were normalised to Ubiq and ARP3 and calibrated with respect to the sample showing the highest expression. Expression levels were obtained from two samples, one from each silo from each treatment group at individual sampling days and the mean values ± S.D. then calculated.

Normalised expression levels of hepcidin are shown in Figure 22. No significant difference was detected between the groups at any sampling day or between sampling days ($p>0.05$). At 2 dph the expression levels of hepcidin, in both groups, commenced at a higher expression level than observed for both mIgM and lysozyme. The highest expression levels were observed in both groups at 7 dph whereas decreased expression was observed in both groups at the onset and during metamorphosis at 26 and 36 dph. Relatively lower expression levels were observed in the UC group compared with the PB group, but the difference between the groups was not significant.

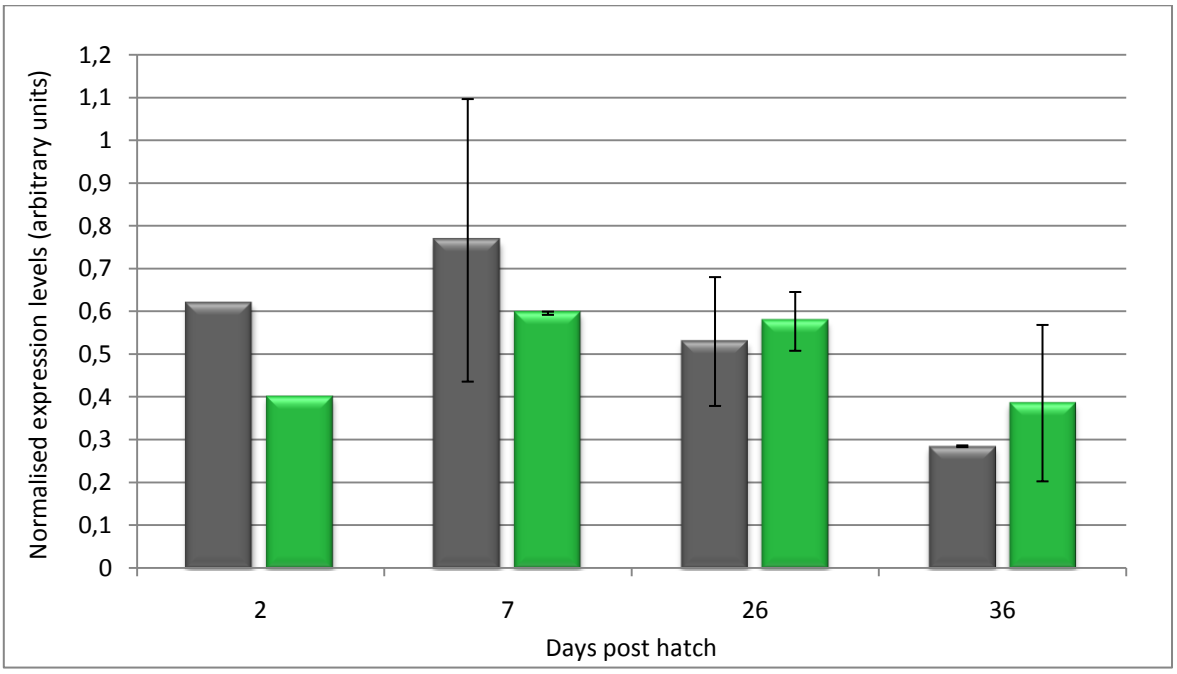


Figure 22. Expression levels of hepcidin in cod larvae on days 2, 7, 26 and 36 post hatch. Grey columns represent samples from the untreated control silos (UC, UC1 and UC2) and green columns samples from silos receiving probiotic treatment (PB, PB1and PB2). The expression levels were normalised to Ubiq and ARP3 and calibrated with respect to the sample showing the highest expression. Expression levels were obtained from two samples, one from each silo from each treatment group at individual sampling days and the mean values ± S.D. then calculated.

4 Discussion

The present study describes the adaptation of the RT-qPCR method for measuring expression of selected immune related genes during early development of Atlantic cod larvae. The method was also used to evaluate if probiotic treatment might affect the expression of the GOIs. During the present study, the expression profiles of the selected and other genes in normally developed cod larvae were published by Seppola *et al*. (2009), but no reports on the effects of probiotic or other treatment on expression of the selected genes in cod larvae have yet been published.

The immune system of cod larvae is poorly developed at hatch and the larvae have to rely on innate immune parameters for their defence against microorganisms before the adaptive immune system is fully developed (Schrøder *et al.*, 1998). High mortalities, commonly observed at the onset and after exogenous feeding, in cod larvae are often regarded to be due to diseases and infections caused by opportunistic pathogenic bacteria (Vadstein *et al.*, 2004). Immunostimulation by probiotics, or other immunomodulating compounds, is considered being a promising way to counteract these high mortalities and to strengthen the immune system of cod larvae (Bricknell *et al.*, 2005; Verschuere *et al.*, 2000)

The gene expression analysis was carried out according to Vandesompele *et al*. (2002), where normalisation of the gene expression data was performed based on mean expression of two reference genes. This approach is regarded to be more reliable as opposed to the commonly used method of using one reference gene for normalisation (Livak & Schmittgen, 2001).

The selected GOIs included mIgM, g-type lysozyme and hepcidin. IgM is regarded to form a link between innate and adaptive immune responses (Carroll *et al.*, 1998) as the IgM molecules can either be natural IgMs that provide early and broad protection, without prior exposure to antigens (innate/non-specific), or produced after exposure to antigens, resulting in an antigen-specific response (adaptive/specific) G-type lysozyme and hepcidin are important parameters in the innate immune system, acting as the first line of defence against bacterial invasion (Larsen *et al.*, 2009; Solstad *et al.*, 2008).

Prior to the present study and the results from Seppolat *et al*. 2009, it was assumed that production of IgM in cod larvae did not start until 58 dph, when IgM positive B cells were detected based on *in situ* hybridisation and immunostaining (Schrøder *et al.*, 1998). In the study of Hakonardottir and Hrolfsdottir (2008), IgM was, however, detected at 28 dph, using immunostaining with specific antibodies against IgM. Seppola *et al*. (2009) concluded that

mIgM expression starts about 1 week prior to hatch and that IgM positive cells are present at an earlier stage than Schrøder *et al.* (1998) maintained or already at 4 wph. This apparant difference in expression of mIgM, may be explained by the higher sensitivity of RT-qPCR method compared with *in situ* hybridisation or immunostaining. The results of Seppola *et al*. (2009) are furthermore in agreement with mIgM expression in larvae of the closly related species, haddock, where low expression levels were detected from fertilisation and a pronounced increase in the expression observed around 26-29 dph (Corripio-Miyar *et al.*, 2007). In other fish species, the expression of mIgM heavy chain transcripts have been detected before the appearance of IgM positive B cells, as reviewed by Rombout *et al*. (2005).

The expression profile of hepcidin in this study differed to some extent from the expression profile of hepcidin observed in the study of Seppola *et al*. (2009) with increased expression reported after the onset of first feeding and highest expression observed during metamorphosis (35 dph). The present study showed that the expression of hepcidin was highest at 7 dph. The reason for decreased hepcidin expression between 7-36 dph is not known but it may be postulated that other immune related parameters have started to provide the protection against further exposure of pathogens such as g-type lysozyme or mIgM where the expression of these genes seems to increase after 7 dph.

The expression levels and patterns of mIgM and g-type lysozyme varied, depending on which analytical approach was used. The expression levels observed at all samling days using analytical approach 2 were consistantly lower as compared to analytical approach 1. Increased expression of mIgM was not observed until at the last sampling day at 36 dph using analytical approach 2 compared with at 26 dph using analytical approach 1 and a gradual increase in expression profiles of g-type lysozyme was observed during 2-36 dph using analytical approach 2 while an earlier peak was observed at 26 dph using analytical approach 1.

The expression of mIgM using analytical approach 1 followed a similar profile as in the study of Seppola *et al*. (2009), with a marked increase in the expression observed at the onset of and during metamorphosis. In the present study, the expression of mIgM had started already at 2 dph, indicating that the ontogeny of B cells has already started, as pointed out by Seppola *et al*. (2009). The expression profile of g-type lysozyme followed a similiar trend as it did in the study of Seppola *et al*. (2009) showing low expression levels prior to and after the onset of first feeding with a marked increased expression after the onset of metamorphosis at 26 dph with a slight decreased expression at 36 dph. Seppola *et al*. (2009) also observed decreased expression of g-type lysozyme between 35 and 42 dph.

In analytical approach 2 the biological replicate RNA samples within each silo and sampling day were pooled together in one sample by mixing equal volumes of each sample. By collecting equal volumes from all samples, the RNA concentrations in individual samples were ignored. A better approach would have been to mix the samples together according to their RNA concentration. Furthermore, the pooled samples had been repeatedly thawed and refrozen prior to being submitted to a second DNase I treatment, with the possibility of RNA degradation that may explain the lower expression levels observed in analytical approach 2 compared with approach 1. It is well known that RNA is sensitive to degradation and all handling and storing of the samples may lead to degradation of the RNA, as reviewed by Fleige and Pfaffl (2006). Degradation of the RNA, caused by freeze-thaw processes, can easily be prevented by dividing the original RNA sample into smaller aliquots.

A significant difference in expression levels of mIgM in analytical approach 1 was observed between probiotic treated and untreated control 26 and 36 dph, with higher expression levels observed in the probiotic treated compared with the untreated group. Furthermore, probiotic treatment may have accelerated the expression of mIgM whereas the expression levels in the probiotic treated group at 26 dph were at the same levels as in the untreated group 10 days later or at 36 dph. The results therefore suggest that the probiotic treatment resulted in stimulation of mIgM expression in the larvae and it could be concluded that probiotic treatment may have stimulated the proliferation of B cells in the larvae, as has been suggested by Nayak (2010) and references therein. Probiotic treatment did not affect the expression levels of mIgM when using analytical approach 2, nor did it affect the expression of g-type lysozyme or hepcidin genes in larvae. The reason for this lack of stimulatory effects is not known, but possible reasons may include poor orverall larval survival. The survival in the present study was only 6% in both groups compared with 10-20% average survival at the MRI hatchery (Steinarsson, 2004). Poor egg quality has been correlated with low survival rates and it has been observed that fish larvae, hatching from poor quality egg groups, show lower viability and a lower degree of normal development up to the juvenile stage compared with larvae that hatch from good quality egg groups (Kjørsvik *et al.*, 2003). The larvae may therfore not have developed normally and their immune system not functioning optimally which may explain the lack of immunostimulation following probiotic treatment.

Furthermore, the mode of delivery of the probionts may affect the functionality of the bacteria. In this study the probiotic bacterial isolates were in a freeze-dried form as opposed to fresh bacterial suspension used by Lauzon *et al*. (2010) which was considered a highly unpractical mode of delivery. Freeze-dried preparations of bacteria have been considered an

efficient mode of delivery of probiotic bacteria in aquaculture (Panigrahi *et al.*, 2005). The present results, however, may suggest that the use of fresh bacterial suspensions represent a better form for delivery of the respective bacterial isolates compared to the freeze-dried form as Lauzon *et al*. (2010) observed increased larval survival in treated groups.

Selection of qRT-PCR reference genes for this study was done by handpicking the most promishing candidates from two studies where expression of dozens of reference genes in cod had been evaluated (Olsvik *et al.*, 2008; Sæle *et al.*, 2009). The reference genes used in the present study included Ubiq, ARP3, RPL4 and EF1-α. Amplification was detected in the RT- control of Ubiq during verification of the gene specificity prior to carrying out the analysis only in approach 1 and different reference genes were therefore used for normalisation in the two analytical approaches. The results from the two analyses are therefore not fully comparable. Interestingly, *geNorm* ranked RPL4 and EF1-α to be more stable than ARP3 using analytical approach 1 and Ubiq and ARP3 to be more stable than RPL4 using analytical approach 2. The main drawback of *geNorm* is considered to be that it tends to favour genes that are co-regulated (Vandesompele *et al.*, 2002b). This may represent a potential problem, since RPL4 and ARP3 both belong to the group of ribosomal proteins. That was however not the case during the present studies, since *geNorm* did not rank RPL4 and ARP3 together in either of the *geNorm* analysis. *geNorm* determines the most stable reference genes from a set of tested genes in a given cDNA sample panel so it should not be surprising that different reference genes are chosen in the experiments since the main difference between analytical approach 1 and 2 was indeed the samples and how they were approached (biological replicates vs. pooled samples).

To summarise, the present study demonstrates the feasibility of using RT-qPCR expression analysis as a mean to monitor relative gene expression profiles in cod larvae. The adaptation of the RT-qPCR method proved to be successful and the method was successfully applied for measuring changes in transcription of several genes in cod larvae between 2-36 dph. The lower expression levels observed when using analytical approach 2 may indicate that the "pooled sample approach" may not be as efficient way to perform gene expression analysis compared with the sample approach used in analytical approach 1. Using biological replicate samples, normalise each sample individually and average the normalised samples to gain normalised expression value, as was done in analytical approach 1, seems to be a more efficient approach for detecting differences such as in untreated compared to probiotic treated larvae and will furthermore give the analysis more statistical power. In addition, the study indicates that probiotic treatment during early stages may have stimulated the expression of

mIgM in the larvae, but neither the expression of g-type lysozyme nor hepcidin. Further analysis is required to determine whether the expression of mIgM in larvae can actually be stimulated by treatment using the putative probionts.

Future steps include further adaptation of the RT-qPCR method developed in the present studies for expression analysis of selected immune related genes following various treatments and environmental conditions. Offering peptide-enriched live prey to larvae has previously been related to increased production of IgM in cod larvae (Hakonardottir & Hrolfsdottir, 2008) and C3 and lysozyme in halibut larvae (Hermannsdottir *et al.*, 2009). In this context it is of interest to analyse whether treatment using pollock protein hydrolysates and using live prey animals as vectors, may be an effective way to stimulate selected immune related genes in the early life stages of cod larvae with the overall aim to increase survival and improve the quality of larvae.

Furthermore, it is of interest to compare the two different delivery approaches using fresh as compared to freeze dried preparations and analysing the expression of the selected immune related genes used in this study. It would also be of interest to analyse whether other immune related genes respond differently to the probiotic treatment than the genes selected here. A possible candidate could be the gene that encodes for the complement factor 3 (C3) that is regarded to be a central complement component, taking part in all known activation pathways of the complement system (Holland & Lambris, 2002). The transcription of C3 has been found to increase significantly following hatching of larvae challenged with LPS (Wang *et al.*, 2008). It would thus be interesting to see if probiotic treatment stimulated the expression of C3 in cod larvae, not least due to the suggestion that activation of the complement system may lead to stimulation of B cells that generate IgM (Ochsenbein & Zinkernagel, 2000).

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6 Appendices

A Original data and plate setup for analytical approach 1

Plate 1

Well	Sample	Gene	Task	Cт	Cт Mean	Cт SD
A1	1:1	IgM	STANDARD	27.0095	26.9751	0.0395
A2	1:1	IgM	STANDARD	26.9320	26.9751	0.0395
A3	1:1	IgM	STANDARD	26.9838	26.9751	0.0395
A4	1:5	IgM	STANDARD	29.2262	29.6531	0.3953
A5	1:5	IgM	STANDARD	29.7264	29.6531	0.3953
A6	1:5	IgM	STANDARD	30.0066	29.6531	0.3953
A7	1:25	IgM	STANDARD	31.9355	32.1597	0.3006
A8	1:25	IgM	STANDARD	32.5013	32.1597	0.3006
B1	1:25	IgM	STANDARD	32.0423	32.1597	0.3006
B2	1:125	IgM	STANDARD	35.3370	35.4605	0.1154
B3	1:125	IgM	STANDARD	35.5657	35.4605	0.1154
B4	1:125	IgM	STANDARD	35.4788	35.4605	0.1154
B5	1:625	IgM	STANDARD	Undetermined		
B6	1:625	IgM	STANDARD	Undetermined		
B7	1:625	IgM	STANDARD	Undetermined		
C1	PB1	IgM	UNKNOWN	31.5476	31.1650	0.5411
C2	PB1	IgM	UNKNOWN	30.7824	31.1650	0.5411
C3	PB2	IgM	UNKNOWN	30.2085	29.9889	0.3106
C4	PB2	IgM	UNKNOWN	29.7692	29.9889	0.3106
C5	PB3	IgM	UNKNOWN	29.0673	29.2490	0.2570
C6	PB3	IgM	UNKNOWN	29.4307	29.2490	0.2570
C7	PB1-1	IgM	UNKNOWN	30.3546	30.4288	0.1050
C8	PB1-1	IgM	UNKNOWN	30.5031	30.4288	0.1050
D1	PB1-2	IgM	UNKNOWN	29.0767	29.1475	0.1001
D2	PB1-2	IgM	UNKNOWN	29.2183	29.1475	0.1001
D3	PB1-3	IgM	UNKNOWN	29.7751	29.8387	0.0900
D4	PB1-3	IgM	UNKNOWN	29.9023	29.8387	0.0900
D5	PB1-4	IgM	UNKNOWN	28.4161	28.4891	0.1033
D6	PB1-4	IgM	UNKNOWN	28.5622	28.4891	0.1033
D7	PB1-5	IgM	UNKNOWN	30.2318	30.4552	0.3159
D8	PB1-5	IgM	UNKNOWN	30.6786	30.4552	0.3159
E1	PB1-6	IgM	UNKNOWN	28.7535	28.6371	0.1647
E2	PB1-6	IgM	UNKNOWN	28.5206	28.6371	0.1647
E3	PB1-7	IgM	UNKNOWN	28.7228	28.9928	0.3818
E4	PB1-7	IgM	UNKNOWN	29.2628	28.9928	0.3818
E5	PB1-8	IgM	UNKNOWN	28.0921	28.0115	0.1140
E6	PB1-8	IgM	UNKNOWN	27.9309	28.0115	0.1140
E7	PB1-9	IgM	UNKNOWN	29.2307	29.0920	0.1961
E8	PB1-9	IgM	UNKNOWN	28.9534	29.0920	0.1961
F5		IgM	NTC	Undetermined		
F6		IgM	RT-	Undetermined		
F7	Pooled cDNA	IgM	UNKNOWN	29.5643	**29.5724**	0.0114
F8	Pooled cDNA	IgM	UNKNOWN	29.5805	**29.5724**	0.0114

Plate 2

Well	Sample	Gene	Task	Cт	Cт Mean	Cт SD
A1	UC1	IgM	UNKNOWN	30.2808	30.1712	0.1550
A2	UC1	IgM	UNKNOWN	30.0617	30.1712	0.1550
A3	UC2	IgM	UNKNOWN	31.2950	31.1563	0.1962
A4	UC2	IgM	UNKNOWN	31.0175	31.1563	0.1962
A5	UC3	IgM	UNKNOWN	29.8464	29.8713	0.0352
A6	UC3	IgM	UNKNOWN	29.8961	29.8713	0.0352
A7	UC1-1	IgM	UNKNOWN	30.7424	30.5001	0.3428
A8	UC1-1	IgM	UNKNOWN	30.2577	30.5001	0.3428
B1	UC1-2	IgM	UNKNOWN	32.0392	31.9146	0.1762
B2	UC1-2	IgM	UNKNOWN	31.7900	31.9146	0.1762
B3	UC1-3	IgM	UNKNOWN	30.0050	30.1805	0.2481
B4	UC1-3	IgM	UNKNOWN	30.3559	30.1805	0.2481
B5	UC1-4	IgM	UNKNOWN	31.6287	31.5059	0.1736
B6	UC1-4	IgM	UNKNOWN	31.3832	31.5059	0.1736
B7	UC1-5	IgM	UNKNOWN	30.6276	30.4891	0.1958
B8	UC1-5	IgM	UNKNOWN	30.3506	30.4891	0.1958
C1	UC1-6	IgM	UNKNOWN	30.5832	30.6753	0.1303
C2	UC1-6	IgM	UNKNOWN	30.7674	30.6753	0.1303
C3	UC1-7	IgM	UNKNOWN	29.0181	29.0252	0.0101
C4	UC1-7	IgM	UNKNOWN	29.0324	29.0252	0.0101
C5	UC1-8	IgM	UNKNOWN	29.0444	28.9693	0.1061
C6	UC1-8	IgM	UNKNOWN	28.8943	28.9693	0.1061
C7	UC1-9	IgM	UNKNOWN	29.6029	29.6383	0.0499
C8	UC1-9	IgM	UNKNOWN	29.6736	29.6383	0.0499
D1		IgM	NTC	Undetermined		
D2		IgM	RT-	Undetermined		
D3	1:1	LysTR1	STANDARD	23.1296	23.1648	0.0309
D4	1:1	LysTR1	STANDARD	23.1770	23.1648	0.0309
D5	1:1	LysTR1	STANDARD	23.1877	23.1648	0.0309
D6	1:5	LysTR1	STANDARD	25.9856	26.0687	0.0968
D7	1:5	LysTR1	STANDARD	26.1749	26.0687	0.0968
D8	1:5	LysTR1	STANDARD	26.0457	26.0687	0.0968
E1	1:25	LysTR1	STANDARD	28.7241	28.8570	0.1352
E2	1:25	LysTR1	STANDARD	28.9943	28.8570	0.1352
E3	1:25	LysTR1	STANDARD	28.8525	28.8570	0.1352
E4	1:125	LysTR1	STANDARD	31.1391	31.0225	0.1032
E5	1:125	LysTR1	STANDARD	30.9851	31.0225	0.1032
E6	1:125	LysTR1	STANDARD	30.9431	31.0225	0.1032
E7	1:625	LysTR1	STANDARD	33.4724	33.5373	0.0607
E8	1:625	LysTR1	STANDARD	33.5927	33.5373	0.0607
F1	1:625	LysTR1	STANDARD	33.5467	33.5373	0.0607
F2		LysTR1	NTC	Undetermined		
F3		LysTR1	RT-	Undetermined		
F4	PB1	LysTR1	UNKNOWN	26.8410	26.8323	0.0123
F5	PB1	LysTR1	UNKNOWN	26.8236	26.8323	0.0123
F7	Pooled cDNA	IgM	UNKNOWN	29.5685	**29.6465**	0.1103
F8	Pooled cDNA	IgM	UNKNOWN	29.7245	**29.6465**	0.1103

Coloured fields indicate samples from probiotic treated larvae

Plate 3

Well	Sample	Gene	Task	Cт	Cт Mean	Cт SD
A1	PB2	LysTR1	UNKNOWN	26.3206	26.3173	0.0046
A2	PB2	LysTR1	UNKNOWN	26.3141	26.3173	0.0046
A3	PB3	LysTR1	UNKNOWN	26.0985	26.1135	0.0212
A4	PB3	LysTR1	UNKNOWN	26.1285	26.1135	0.0212
A5	PB1-1	LysTR1	UNKNOWN	26.3038	26.2791	0.0349
A6	PB1-1	LysTR1	UNKNOWN	26.2545	26.2791	0.0349
A7	PB1-2	LysTR1	UNKNOWN	25.2452	25.3432	0.1386
A8	PB1-2	LysTR1	UNKNOWN	25.4412	25.3432	0.1386
B1	PB1-3	LysTR1	UNKNOWN	26.6336	26.5661	0.0955
B2	PB1-3	LysTR1	UNKNOWN	26.4986	26.5661	0.0955
B3	PB1-4	LysTR1	UNKNOWN	24.4115	24.3525	0.0835
B4	PB1-4	LysTR1	UNKNOWN	24.2934	24.3525	0.0835
B5	PB1-5	LysTR1	UNKNOWN	25.8450	25.8110	0.0480
B6	PB1-5	LysTR1	UNKNOWN	25.7771	25.8110	0.0480
B7	PB1-6	LysTR1	UNKNOWN	24.2681	24.3101	0.0593
B8	PB1-6	LysTR1	UNKNOWN	24.3520	24.3101	0.0593
C1	PB1-7	LysTR1	UNKNOWN	25.0893	25.0433	0.0650
C2	PB1-7	LysTR1	UNKNOWN	24.9973	25.0433	0.0650
C3	PB1-8	LysTR1	UNKNOWN	24.4090	24.3515	0.0813
C4	PB1-8	LysTR1	UNKNOWN	24.2940	24.3515	0.0813
C5	PB1-9	LysTR1	UNKNOWN	24.7766	24.8098	0.0469
C6	PB1-9	LysTR1	UNKNOWN	24.8430	24.8098	0.0469
C7	UC1	LysTR1	UNKNOWN	25.8013	25.8912	0.1272
C8	UC1	LysTR1	UNKNOWN	25.9811	25.8912	0.1272
D1	UC2	LysTR1	UNKNOWN	27.1841	27.2166	0.0460
D2	UC2	LysTR1	UNKNOWN	27.2491	27.2166	0.0460
D3	UC3	LysTR1	UNKNOWN	26.1196	26.1268	0.0102
D4	UC3	LysTR1	UNKNOWN	26.1340	26.1268	0.0102
D5	UC1-1	LysTR1	UNKNOWN	27.6742	27.6833	0.0129
D6	UC1-1	LysTR1	UNKNOWN	27.6924	27.6833	0.0129
D7	UC1-2	LysTR1	UNKNOWN	27.2985	27.5888	0.4105
D8	UC1-2	LysTR1	UNKNOWN	27.8790	27.5888	0.4105
E1	UC1-3	LysTR1	UNKNOWN	26.8353	26.9059	0.0998
E2	UC1-3	LysTR1	UNKNOWN	26.9765	26.9059	0.0998
E3	UC1-4	LysTR1	UNKNOWN	25.7890	25.9504	0.2283
E4	UC1-4	LysTR1	UNKNOWN	26.1118	25.9504	0.2283
E5	UC1-5	LysTR1	UNKNOWN	25.9438	25.8530	0.1284
E6	UC1-5	LysTR1	UNKNOWN	25.7622	25.8530	0.1284
E7	UC1-6	LysTR1	UNKNOWN	25.7815	25.7861	0.0065
E8	UC1-6	LysTR1	UNKNOWN	25.7907	25.7861	0.0065
F1	UC1-7	LysTR1	UNKNOWN	24.4947	24.4902	0.0064
F2	UC1-7	LysTR1	UNKNOWN	24.4857	24.4902	0.0064
F3	UC1-8	LysTR1	UNKNOWN	24.9551	25.3474	0.5547
F4	UC1-8	LysTR1	UNKNOWN	25.7396	25.3474	0.5547
F5		LysTR1	NTC	Undetermined		
F6		LysTR1	RT-	Undetermined		
F7	Pooled cDNA	IgM	UNKNOWN	29.7575	**29.6522**	0.1488
F8	Pooled cDNA	IgM	UNKNOWN	29.5470	**29.6522**	0.1488

Plate 4

Well	Sample	Gene	Task	Cт	Cт Mean	Cт SD
A1	UC1-9	LysTR1	UNKNOWN	25.5234	25.5708	0.0672
A2	UC1-9	LysTR1	UNKNOWN	25.6183	25.5708	0.0672
A3		LysTR1	NTC	Undetermined		
A4		LysTR1	RT-	Undetermined		
A5	1:1	RPL4	STANDARD	13.6375	13.7371	0.0876
A6	1:1	RPL4	STANDARD	13.8021	13.7371	0.0876
A7	1:1	RPL4	STANDARD	13.7717	13.7371	0.0876
A8	1:5	RPL4	STANDARD	16.3101	16.2908	0.0181
B1	1:5	RPL4	STANDARD	16.2742	16.2908	0.0181
B2	1:5	RPL4	STANDARD	16.2882	16.2908	0.0181
B3	1:25	RPL4	STANDARD	19.1893	19.1823	0.0300
B4	1:25	RPL4	STANDARD	19.1494	19.1823	0.0300
B5	1:25	RPL4	STANDARD	19.2082	19.1823	0.0300
B6	1:125	RPL4	STANDARD	21.6035	21.5977	0.0067
B7	1:125	RPL4	STANDARD	21.5992	21.5977	0.0067
B8	1:125	RPL4	STANDARD	21.5903	21.5977	0.0067
C1	1:625	RPL4	STANDARD	23.8065	23.8074	0.0172
C2	1:625	RPL4	STANDARD	23.7907	23.8074	0.0172
C3	1:625	RPL4	STANDARD	23.8251	23.8074	0.0017
C4	PB1	RPL4	UNKNOWN	16.7962	16.7470	0.0697
C5	PB1	RPL4	UNKNOWN	16.6977	16.7470	0.0697
C6	PB2	RPL4	UNKNOWN	15.7126	15.6800	0.0461
C7	PB2	RPL4	UNKNOWN	15.6474	15.6800	0.0461
C8	PB3	RPL4	UNKNOWN	15.3682	15.3016	0.0941
D1	PB3	RPL4	UNKNOWN	15.2351	15.3016	0.0941
D2	PB1-1	RPL4	UNKNOWN	15.9443	15.9660	0.0307
D3	PB1-1	RPL4	UNKNOWN	15.9877	15.9660	0.0307
D4	PB1-2	RPL4	UNKNOWN	14.9366	14.9941	0.0812
D5	PB1-2	RPL4	UNKNOWN	15.0515	14.9941	0.0812
D6	PB1-3	RPL4	UNKNOWN	15.9314	15.9641	0.0463
D7	PB1-3	RPL4	UNKNOWN	15.9968	15.9641	0.0463
D8	PB1-4	RPL4	UNKNOWN	15.1583	15.1536	0.0066
E1	PB1-4	RPL4	UNKNOWN	15.1489	15.1536	0.0066
E2	PB1-5	RPL4	UNKNOWN	17.1258	17.1280	0.0032
E3	PB1-5	RPL4	UNKNOWN	17.1303	17.1280	0.0032
E4	PB1-6	RPL4	UNKNOWN	14.9691	15.0145	0.0641
E5	PB1-6	RPL4	UNKNOWN	15.0598	15.0145	0.0641
E6	PB1-7	RPL4	UNKNOWN	15.7603	15.7707	0.0148
E7	PB1-7	RPL4	UNKNOWN	15.7812	15.7707	0.0148
E8	PB1-8	RPL4	UNKNOWN	14.7695	14.8076	0.0539
F1	PB1-8	RPL4	UNKNOWN	14.8457	14.8076	0.0539
F2	PB1-9	RPL4	UNKNOWN	15.8460	15.8025	0.0615
F3	PB1-9	RPL4	UNKNOWN	15.7590	15.8025	0.0615
F4		RPL4	NTC	Undetermined		
F5		RPL4	RT-	Undetermined		
F7	Pooled cDNA	IgM	UNKNOWN	29.8733988	**29.8063**	0.0949
F8	Pooled cDNA	IgM	UNKNOWN	29.7392406	**29.8063**	0.0949

Coloured fields indicate samples from probiotic treated larvae

Plate 5

Well	Sample	Gene	Task	Ct	Ct Mean	Ct SD
A1	UC1	RPL4	UNKNOWN	15.9910	15.8559	0.1912
A2	UC1	RPL4	UNKNOWN	15.7207	15.8559	0.1912
A3	UC2	RPL4	UNKNOWN	16.6203	16.5772	0.0610
A4	UC2	RPL4	UNKNOWN	16.5341	16.5772	0.0610
A5	UC3	RPL4	UNKNOWN	15.8784	15.8607	0.0250
A6	UC3	RPL4	UNKNOWN	15.8431	15.8607	0.0250
A7	UC1-1	RPL4	UNKNOWN	16.0938	16.1553	0.0869
A8	UC1-1	RPL4	UNKNOWN	16.2168	16.1553	0.0869
B1	UC1-2	RPL4	UNKNOWN	17.3368	17.3652	0.0402
B2	UC1-2	RPL4	UNKNOWN	17.3936	17.3652	0.0402
B3	UC1-3	RPL4	UNKNOWN	16.5971	16.5407	0.0797
B4	UC1-3	RPL4	UNKNOWN	16.4843	16.5407	0.0797
B5	UC1-4	RPL4	UNKNOWN	17.3982	17.4156	0.0246
B6	UC1-4	RPL4	UNKNOWN	17.4330	17.4156	0.0246
B7	UC1-5	RPL4	UNKNOWN	16.4561	16.4412	0.0210
B8	UC1-5	RPL4	UNKNOWN	16.4264	16.4412	0.0210
C1	UC1-6	RPL4	UNKNOWN	16.9241	16.8151	0.1543
C2	UC1-6	RPL4	UNKNOWN	16.7060	16.8151	0.1543
C3	UC1-7	RPL4	UNKNOWN	15.6461	15.5817	0.0911
C4	UC1-7	RPL4	UNKNOWN	15.5173	15.5817	0.0911
C5	UC1-8	RPL4	UNKNOWN	15.5049	15.5294	0.0346
C6	UC1-8	RPL4	UNKNOWN	15.5538	15.5294	0.0346
C7	UC1-9	RPL4	UNKNOWN	16.2299	16.2491	0.0272
C8	UC1-9	RPL4	UNKNOWN	16.2684	16.2491	0.0272
D1		RPL4	NTC	Undetermined		
D2		RPL4	RT-	Undetermined		
D3	1:1	ARP3	STANDARD	14.6998	14.6572	0.0548
D4	1:1	ARP3	STANDARD	14.6764	14.6572	0.0548
D5	1:1	ARP3	STANDARD	14.5954	14.6572	0.0548
D6	1:5	ARP3	STANDARD	17.0158	16.9969	0.0619
D7	1:5	ARP3	STANDARD	16.9277	16.9969	0.0619
D8	1:5	ARP3	STANDARD	17.0471	16.9969	0.0619
E2	1:25	ARP3	STANDARD	19.9596	19.9967	0.0648
E3	1:25	ARP3	STANDARD	19.9589	19.9967	0.0648
E4	1:25	ARP3	STANDARD	20.0715	19.9967	0.0648
E5	1:125	ARP3	STANDARD	22.3883	22.4194	0.0323
E6	1:125	ARP3	STANDARD	22.4528	22.4194	0.0323
E7	1:125	ARP3	STANDARD	22.4170	22.4194	0.0323
E8	1:625	ARP3	STANDARD	24.3516	24.5321	0.1568
F1	1:625	ARP3	STANDARD	24.6337	24.5321	0.1568
F2	1:625	ARP3	STANDARD	24.6111	24.5321	0.1568
F3	PB1	ARP3	UNKNOWN	16.8847	16.8616	0.0328
F4	PB1	ARP3	UNKNOWN	16.8384	16.8616	0.0328
F5		ARP3	NTC	Undetermined		
F6		ARP3	RT-	Undetermined		
F7	Pooled cDNA	IgM	UNKNOWN	29.9389	**29.9787**	0.0563
F8	Pooled cDNA	IgM	UNKNOWN	30.0184	**29.9787**	0.0563

Plate 6

Well	Sample	Gene	Task	Ct	Ct Mean	Ct SD
A1	PB2	ARP3	UNKNOWN	15.9823	15.9185	0.0903
A2	PB2	ARP3	UNKNOWN	15.8546	15.9185	0.0903
A3	PB3	ARP3	UNKNOWN	16.1746	16.0001	0.2468
A4	PB3	ARP3	UNKNOWN	15.8256	16.0001	0.2468
A5	PB1-1	ARP3	UNKNOWN	17.3618	17.3621	0.0005
A6	PB1-1	ARP3	UNKNOWN	17.3625	17.3621	0.0005
A7	PB1-2	ARP3	UNKNOWN	15.5544	15.5722	0.0251
A8	PB1-2	ARP3	UNKNOWN	15.5900	15.5722	0.0251
B1	PB1-3	ARP3	UNKNOWN	16.6848	16.6980	0.0186
B2	PB1-3	ARP3	UNKNOWN	16.7112	16.6980	0.0186
B3	PB1-4	ARP3	UNKNOWN	15.8091	15.8053	0.0053
B4	PB1-4	ARP3	UNKNOWN	15.8015	15.8053	0.0053
B5	PB1-5	ARP3	UNKNOWN	17.0896	17.0394	0.0710
B6	PB1-5	ARP3	UNKNOWN	16.9891	17.0394	0.0710
B7	PB1-6	ARP3	UNKNOWN	15.6708	15.6426	0.0399
B8	PB1-6	ARP3	UNKNOWN	15.6143	15.6426	0.0399
C1	PB1-7	ARP3	UNKNOWN	17.0880	17.0376	0.0712
C2	PB1-7	ARP3	UNKNOWN	16.9873	17.0376	0.0712
C3	PB1-8	ARP3	UNKNOWN	15.8245	15.8396	0.0213
C4	PB1-8	ARP3	UNKNOWN	15.8546	15.8396	0.0213
C5	PB1-9	ARP3	UNKNOWN	16.7713	16.7686	0.0038
C6	PB1-9	ARP3	UNKNOWN	16.7659	16.7686	0.0038
C7	UC1	ARP3	UNKNOWN	16.1682	16.1754	0.0102
C8	UC1	ARP3	UNKNOWN	16.1826	16.1754	0.0102
D1	UC2	ARP3	UNKNOWN	16.8745	16.9360	0.0871
D2	UC2	ARP3	UNKNOWN	16.9976	16.9360	0.0871
D3	UC3	ARP3	UNKNOWN	16.3098	16.2873	0.0319
D4	UC3	ARP3	UNKNOWN	16.2647	16.2873	0.0319
D5	UC1-1	ARP3	UNKNOWN	18.3094	18.3109	0.0021
D6	UC1-1	ARP3	UNKNOWN	18.3124	18.3109	0.0021
D7	UC1-2	ARP3	UNKNOWN	17.5959	17.5512	0.0632
D8	UC1-2	ARP3	UNKNOWN	17.5065	17.5512	0.0632
E1	UC1-3	ARP3	UNKNOWN	17.1125	17.0533	0.0838
E2	UC1-3	ARP3	UNKNOWN	16.9940	17.0533	0.0838
E3	UC1-4	ARP3	UNKNOWN	17.5683	17.7746	0.2917
E4	UC1-4	ARP3	UNKNOWN	17.9809	17.7746	0.2917
E5	UC1-5	ARP3	UNKNOWN	16.8738	16.7920	0.1157
E6	UC1-5	ARP3	UNKNOWN	16.7102	16.7920	0.1157
E7	UC1-6	ARP3	UNKNOWN	17.1475	16.8660	0.3980
E8	UC1-6	ARP3	UNKNOWN	16.5846	16.8660	0.3980
F1	UC1-7	ARP3	UNKNOWN	16.7861	16.8279	0.0591
F2	UC1-7	ARP3	UNKNOWN	16.8697	16.8279	0.0591
F3	UC1-8	ARP3	UNKNOWN	16.8825	16.7563	0.1785
F4	UC1-8	ARP3	UNKNOWN	16.6300	16.7563	0.1785
F5		ARP3	NTC	Undetermined		
F6		ARP3	RT-	Undetermined		
F7	Pooled cDNA	IgM	UNKNOWN	29.8374	**29.8881**	0.0718
F8	Pooled cDNA	IgM	UNKNOWN	29.9389	**29.8881**	0.0718

Coloured fields indicate samples from probiotic treated larvae

Plate 7

Well	Sample	Gene	Task	Cт	Cт Mean	Cт SD
A1	UC1-9	ARP3	UNKNOWN	17.4736	17.4202	0.0755
A2	UC1-9	ARP3	UNKNOWN	17.3668	17.4202	0.0755
A3		ARP3	NTC	Undetermined		
A4		ARP3	RT-	Undetermined		
A5	1:1	EF1-a	STANDARD	12.9334	12.9147	0.0624
A6	1:1	EF1-a	STANDARD	12.8450	12.9147	0.0624
A7	1:1	EF1-a	STANDARD	12.9656	12.9147	0.0624
A8	1:5	EF1-a	STANDARD	15.0004	14.9311	0.0709
B1	1:5	EF1-a	STANDARD	14.8587	14.9311	0.0709
B2	1:5	EF1-a	STANDARD	14.9341	14.9311	0.0709
B3	1:25	EF1-a	STANDARD	18.0606	18.0461	0.0186
B4	1:25	EF1-a	STANDARD	18.0252	18.0461	0.0186
B5	1:25	EF1-a	STANDARD	18.0525	18.0461	0.0186
B6	1:125	EF1-a	STANDARD	20.8828	20.8074	0.0692
B7	1:125	EF1-a	STANDARD	20.7467	20.8074	0.0692
B8	1:125	EF1-a	STANDARD	20.7927	20.8074	0.0692
C1	1:625	EF1-a	STANDARD	22.9541	22.8645	0.0781
C2	1:625	EF1-a	STANDARD	22.8107	22.8645	0.0781
C3	1:625	EF1-a	STANDARD	22.8288	22.8645	0.0781
C4	PB1	EF1-a	UNKNOWN	15.6135	15.6862	0.1029
C5	PB1	EF1-a	UNKNOWN	15.7589	15.6862	0.1029
C6	PB2	EF1-a	UNKNOWN	14.4649	14.6588	0.2742
C7	PB2	EF1-a	UNKNOWN	14.8527	14.6588	0.2742
C8	PB3	EF1-a	UNKNOWN	14.1778	14.2142	0.0516
D1	PB3	EF1-a	UNKNOWN	14.2507	14.2142	0.0516
D2	PB1-1	EF1-a	UNKNOWN	15.1545	15.1701	0.0220
D3	PB1-1	EF1-a	UNKNOWN	15.1857	15.1701	0.0220
D4	PB1-2	EF1-a	UNKNOWN	14.2742	14.1000	0.2463
D5	PB1-2	EF1-a	UNKNOWN	13.9258	14.1000	0.2463
D6	PB1-3	EF1-a	UNKNOWN	15.0630	14.9816	0.1151
D7	PB1-3	EF1-a	UNKNOWN	14.9003	14.9816	0.1151
D8	PB1-4	EF1-a	UNKNOWN	14.0929	14.1539	0.0862
E1	PB1-4	EF1-a	UNKNOWN	14.2149	14.1539	0.0862
E2	PB1-5	EF1-a	UNKNOWN	16.0938	16.0822	0.0164
E3	PB1-5	EF1-a	UNKNOWN	16.0706	16.0822	0.0164
E4	PB1-6	EF1-a	UNKNOWN	13.9394	13.9344	0.0070
E5	PB1-6	EF1-a	UNKNOWN	13.9295	13.9344	0.0070
E6	PB1-7	EF1-a	UNKNOWN	14.9741	14.9338	0.0570
E7	PB1-7	EF1-a	UNKNOWN	14.8935	14.9338	0.0570
E8	PB1-8	EF1-a	UNKNOWN	14.0330	14.0029	0.0425
F1	PB1-8	EF1-a	UNKNOWN	13.9729	14.0029	0.0425
F2	PB1-9	EF1-a	UNKNOWN	14.8111	14.8467	0.0503
F3	PB1-9	EF1-a	UNKNOWN	14.8823	14.8467	0.0503
F4		EF1-a	NTC	Undetermined		
F5		EF1-a	RT-	Undetermined		
F7	Pooled cDNA	IgM	UNKNOWN	29.8658	**29.7764**	0.1263
F8	Pooled cDNA	IgM	UNKNOWN	29.6871	**29.7764**	0.1263

Coloured fields indicate samples from probiotic treated larvae

Plate 8

Well	Sample	Gene	Task	Cт	Cт Mean	Cт SD
A1	UC1	EF1-a	UNKNOWN	14.5553	14.5131	0.0597
A2	UC1	EF1-a	UNKNOWN	14.4709	14.5131	0.0597
A3	UC2	EF1-a	UNKNOWN	15.4920	15.4595	0.0460
A4	UC2	EF1-a	UNKNOWN	15.4270	15.4595	0.0460
A5	UC3	EF1-a	UNKNOWN	14.7766	14.7366	0.0566
A6	UC3	EF1-a	UNKNOWN	14.6966	14.7366	0.0566
A7	UC1-1	EF1-a	UNKNOWN	16.0506	15.5848	0.6587
A8	UC1-1	EF1-a	UNKNOWN	15.1190	15.5848	0.6587
B1	UC1-2	EF1-a	UNKNOWN	15.9774	16.0578	0.1136
B2	UC1-2	EF1-a	UNKNOWN	16.1381	16.0578	0.1136
B3	UC1-3	EF1-a	UNKNOWN	15.0209	15.1057	0.1199
B4	UC1-3	EF1-a	UNKNOWN	15.1905	15.1057	0.1199
B5	UC1-4	EF1-a	UNKNOWN	16.2615	16.3664	0.1484
B6	UC1-4	EF1-a	UNKNOWN	16.4713	16.3664	0.1484
B7	UC1-5	EF1-a	UNKNOWN	14.9904	14.9776	0.0181
B8	UC1-5	EF1-a	UNKNOWN	14.9648	14.9776	0.0181
C1	UC1-6	EF1-a	UNKNOWN	15.3776	15.3771	0.0007
C2	UC1-6	EF1-a	UNKNOWN	15.3766	15.3771	0.0007
C3	UC1-7	EF1-a	UNKNOWN	14.4781	14.5134	0.0498
C4	UC1-7	EF1-a	UNKNOWN	14.5486	14.5134	0.0498
C5	UC1-8	EF1-a	UNKNOWN	14.5383	14.5827	0.0629
C6	UC1-8	EF1-a	UNKNOWN	14.6272	14.5827	0.0629
C7	UC1-9	EF1-a	UNKNOWN	15.1191	15.1922	0.1033
C8	UC1-9	EF1-a	UNKNOWN	15.2652	15.1922	0.1033
D1		EF1-a	NTC	Undetermined		
D2		EF1-a	RT-	Undetermined		
D3	Pooled cDNA	IgM	UNKNOWN	29.7936	**29.7986**	0.0071
D4	Pooled cDNA	IgM	UNKNOWN	29.8036	**29.7986**	0.0071

A.1 Calculations of raw quantities and *geNorm* input file

	Raw quantities						geNorm input			
	Sample	mIgM	LysTR1	RPL4	ARP3	EF1-α	Sample	RPL4	ARP3	EF1-α
2 dph	UC1	149.188	209.0372	248.2169	385.8744	334.2186	UC1	0.515285	0.679985	0.696734
	UC2	84.5814	91.20001	157.2813	237.2684	185.0999	UC2	0.326508	0.418112	0.385871
	UC3	177.333	180.3836	247.4543	359.248	290.6914	UC3	0.513702	0.633064	0.605994
7 dph	UC1-1	123.442	68.10081	205.3877	98.51028	171.1672	UC1-1	0.426374	0.173594	0.356826
	UC1-2	54.6434	72.25045	95.54623	160.1143	127.4007	UC1-2	0.198349	0.282152	0.265588
	UC1-3	148.397	110.7756	160.9545	220.1336	230.8617	UC1-3	0.334133	0.387918	0.481269
26 dph	UC1-4	69.1488	201.4341	92.55176	138.8033	105.0692	UC1-4	0.192133	0.244598	0.219034
	UC1-5	124.225	214.0974	171.4081	260.1607	250.0786	UC1-5	0.355834	0.458453	0.52133
	UC1-6	111.589	223.2474	135.3145	248.1307	194.8681	UC1-6	0.280906	0.437254	0.406235
36 dph	UC1-7	288.714	502.3333	295.2124	254.2521	334.1725	UC1-7	0.612845	0.448041	0.696637
	UC1-8	298.166	293.7827	305.1508	266.1696	320.0041	UC1-8	0.633477	0.469042	0.667101
	UC1-9	202.81	255.4428	193.5515	174.1008	218.725	UC1-9	0.401803	0.306799	0.455968
2 dph	PB1	84.1567	115.9924	141.2692	248.8422	160.6683	PB1	0.293267	0.438508	0.334939
	PB2	165.715	160.1054	277.4208	454.7803	305.156	PB2	0.575911	0.80141	0.636148
	PB3	253.798	181.8885	352.4279	431.6492	402.7912	PB3	0.731622	0.760649	0.839684
7 dph	PB1-1	128.612	163.9802	231.5153	180.6888	221.7607	PB1-1	0.480613	0.318408	0.462297
	PB1-2	269.082	294.5545	428.1194	567.4751	432.5791	PB1-2	0.888753	1	0.901782
	PB1-3	180.693	137.0242	231.7939	276.2805	249.4491	PB1-3	0.481192	0.486859	0.520018
26 dph	PB1-4	393.195	547.5596	387.0154	488.9026	418.259	PB1-4	0.803424	0.86154	0.871929
	PB1-5	126.676	219.7921	111.0111	222.1026	125.4687	PB1-5	0.230453	0.391387	0.26156
	PB1-6	361.071	562.2876	422.628	542.5157	479.6937	PB1-6	0.877353	0.956017	1
36 dph	PB1-7	294.162	355.3655	261.9502	222.3492	257.0104	PB1-7	0.543795	0.391822	0.53578
	PB1-8	517.746	547.8943	481.7078	478.3108	459.606	PB1-8	1	0.842875	0.958124
	PB1-9	277.818	411.2772	256.7374	264.0844	271.3853	PB1-9	0.532973	0.465367	0.565747

A.1.1 Calculation of normalised quantities / normalised expression levels for mIgM and g-type lysozyme

	NF	Sample	Normalised quantities (NQ) mIgM	NQ relative to highest expression	Average NQ	SD	NF	Sample	Normalised quantities (NQ) LysTR1	NQ relative to highest expression	Average NQ	SD
2 dph	0.5992	UC1	248.9794315	0.456888455			0.5992	UC1	348.8605603	0.355209416		
	0.3550	UC2	238.2573393	0.437212934	0.492462	0.079267	0.3550	UC2	256.9014261	0.261576732	0.315332	0.048334
	0.5579	UC3	317.8582522	0.583284189			0.5579	UC3	323.3261168	0.329210275		
7 dph	0.3901	UC1-1	316.4361956	0.580674651			0.3901	UC1-1	174.5726964	0.177749716		
	0.2295	UC1-2	238.0975777	0.436919764	0.565562	0.121791	0.2295	UC1-2	314.8167629	0.320546061	0.259857	0.073768
	0.4010	UC1-3	370.0684492	0.679092249			0.4010	UC1-3	276.2484867	0.281275887		
26 dph	0.2051	UC1-4	337.1469216	0.618679765			0.2051	UC1-4	982.1264441	1		
	0.4307	UC1-5	288.4252113	0.529273235	0.584714	0.048418	0.4307	UC1-5	497.0916905	0.506138179	0.726351	0.25123
	0.3378	UC1-6	330.3407288	0.606190095			0.3378	UC1-6	660.8864219	0.672913784		
36 dph	0.6534	UC1-7	441.8637615	0.810839876			0.6534	UC1-7	768.7991244	0.782790372		
	0.6501	UC1-8	458.6462528	0.841636502	0.840674	0.029365	0.6501	UC1-8	451.9038632	0.460127986	0.61687	0.161527
	0.4280	UC1-9	473.8551024	0.869545425			0.4280	UC1-9	596.828884	0.607690474		
2 dph	0.3134	PB-1	268.5282188	0.492761359			0.3134	PB-1	370.1098469	0.376845414		
	0.6053	PB-2	273.7727759	0.502385357	0.529781	0.055992	0.6053	PB-2	264.5058104	0.269319508	0.294149	0.073497
	0.7838	PB-3	323.8046452	0.594196088			0.7838	PB-3	232.0598204	0.236283038		
7 dph	0.4714	PB1-1	272.830728	0.500656657			0.4714	PB1-1	347.8579517	0.354188561		
	0.8952	PB1-2	300.5831134	0.551583532	0.571711	0.08297	0.8952	PB1-2	329.037646	0.335025747	0.322713	0.039114
	0.5002	PB1-3	361.2407965	0.662893109			0.5002	PB1-3	273.9387876	0.278924154		
26 dph	0.8370	PB1-4	469.7665738	0.862042791			0.8370	PB1-4	654.1930244	0.666098575		
	0.2455	PB1-5	515.9933991	0.946871095	0.838757	0.121443	0.2455	PB1-5	895.2833929	0.911576506	0.729628	0.159944
	0.9367	PB1-6	385.4713174	0.707357205			0.9367	PB1-6	600.2856841	0.611210183		
36 dph	0.5398	PB1-7	544.9457712	1			0.5398	PB1-7	658.3280337	0.670308836		
	0.9788	PB1-8	528.9604432	0.970666204	0.96637	0.035971	0.9788	PB1-8	559.7612453	0.569948247	0.66763	0.09637
	0.5491	PB1-9	505.9512766	0.928443349			0.5491	PB1-9	749.0023616	0.762633331		

B Original data and plate setup for analytical approach 2

Plate 1

Well	Sample	Gene	Task	Cт	Cт Mean	Cт SD
A1	1:1	LysTR1	STANDARD	23.3999	23.9158	0.7691
A2	1:1	LysTR1	STANDARD	24.7997	23.9158	0.7691
A3	1:1	LysTR1	STANDARD	23.5477	23.9158	0.7691
A4	1:4	LysTR1	STANDARD	26.2330	26.1937	0.0473
A5	1:4	LysTR1	STANDARD	26.2070	26.1937	0.0473
A6	1:4	LysTR1	STANDARD	26.1412	26.1937	0.0473
A7	1:16	LysTR1	STANDARD	28.4432	28.3139	0.1136
A8	1:16	LysTR1	STANDARD	28.2684	28.3139	0.1136
B1	1:16	LysTR1	STANDARD	28.2301	28.3139	0.1136
B2	1:64	LysTR1	STANDARD	30.4365	30.5905	0.1880
B3	1:64	LysTR1	STANDARD	30.5348	30.5905	0.1880
B4	1:64	LysTR1	STANDARD	30.8001	30.5905	0.1880
B5	1:256	LysTR1	STANDARD	32.9553	32.7853	0.1489
B6	1:256	LysTR1	STANDARD	32.6781	32.7853	0.1489
B7	1:256	LysTR1	STANDARD	32.7223	32.7853	0.1489
B8	UC 2 dph	LysTR1	UNKNOWN	28.7486	28.7376	0.0157
C1	UC 2 dph	LysTR1	UNKNOWN	28.7265	28.7376	0.0157
C2	PB 2 dph	LysTR1	UNKNOWN	27.7609	27.7774	0.0234
C3	PB 2 dph	LysTR1	UNKNOWN	27.7940	27.7774	0.0234
C4	PB1 7 dph	LysTR1	UNKNOWN	27.1743	27.1179	0.0797
C5	PB1 7 dph	LysTR1	UNKNOWN	27.0616	27.1179	0.0797
C6	PB1 26 dph	LysTR1	UNKNOWN	27.8796	27.9232	0.0615
C7	PB1 26 dph	LysTR1	UNKNOWN	27.9667	27.9232	0.0615
C8	PB1 36 dph	LysTR1	UNKNOWN	24.9678	24.9967	0.0408
D1	PB1 36 dph	LysTR1	UNKNOWN	25.0256	24.9967	0.0408
D2	PB2 7 dph	LysTR1	UNKNOWN	27.5136	27.4550	0.0829
D3	PB2 7 dph	LysTR1	UNKNOWN	27.3963	27.4550	0.0829
D4	PB2 26 dph	LysTR1	UNKNOWN	26.2633	26.2682	0.0070
D5	PB2 26 dph	LysTR1	UNKNOWN	26.2731	26.2682	0.0070
D6	PB2 36 dph	LysTR1	UNKNOWN	25.5163	25.4724	0.0621
D7	PB2 36 dph	LysTR1	UNKNOWN	25.4285	25.4724	0.0621
D8	UC1 7 dph	LysTR1	UNKNOWN	27.2979	27.4561	0.2238
E1	UC1 7 dph	LysTR1	UNKNOWN	27.6144	27.4561	0.2238
E2	UC1 26 dph	LysTR1	UNKNOWN	27.2303	26.9061	0.4585
E3	UC1 26 dph	LysTR1	UNKNOWN	26.5819	26.9061	0.4585
E4	UC1 36 dph	LysTR1	UNKNOWN	25.0089	25.1399	0.1852
E5	UC1 36 dph	LysTR1	UNKNOWN	25.2708	25.1399	0.1852
E6	UC2 7 dph	LysTR1	UNKNOWN	27.8596	27.8592	0.0005
E7	UC2 7 dph	LysTR1	UNKNOWN	27.8588	27.8592	0.0005
E8	UC2 26 dph	LysTR1	UNKNOWN	26.0606	26.1198	0.0838
F1	UC2 26 dph	LysTR1	UNKNOWN	26.1791	26.1198	0.0838
F2	UC2 36 dph	LysTR1	UNKNOWN	25.5630	25.6626	0.1409
F3	UC2 36 dph	LysTR1	UNKNOWN	25.7622	25.6626	0.1409
F4		LysTR1	NTC	Undetermined		
F5		LysTR1	RT-	Undetermined		
F6						
F7	Pooled cDNA	LysTR1	UNKNOWN	26.7185	**26.7943**	0.1071
F8	Pooled cDNA	LysTR1	UNKNOWN	26.8700	**26.7943**	0.1071

Plate 2

Well	Sample	Gene	Task	Cт	Cт Mean	Cт SD
A1	1:1	IgM	STANDARD	27.5624	28.3039	1.1566
A2	1:1	IgM	STANDARD	27.7128	28.3039	1.1566
A3	1:1	IgM	STANDARD	29.6366	28.3039	1.1566
A4	1:4	IgM	STANDARD	30.6041	30.4046	0.1779
A5	1:4	IgM	STANDARD	30.3474	30.4046	0.1779
A6	1:4	IgM	STANDARD	30.2624	30.4046	0.1779
A7	1:16	IgM	STANDARD	32.4770	32.8970	0.3644
A8	1:16	IgM	STANDARD	33.0863	32.8970	0.3644
B1	1:16	IgM	STANDARD	33.1278	32.8970	0.3644
B2	1:64	IgM	STANDARD	35.1873	35.2673	0.6775
B3	1:64	IgM	STANDARD	35.9813	35.2673	0.6775
B4	1:64	IgM	STANDARD	34.6334	35.2673	0.6775
B5	1:256	IgM	STANDARD	35.9533	36.2470	0.3590
B6	1:256	IgM	STANDARD	36.6472	36.2470	0.3590
B7	1:256	IgM	STANDARD	36.1407	36.2470	0.3590
B8	UC 2 dph	IgM	UNKNOWN	32.8313	32.8507	0.0275
C1	UC 2 dph	IgM	UNKNOWN	32.8701	32.8507	0.0275
C2	PB 2 dph	IgM	UNKNOWN	32.0750	32.0284	0.0659
C3	PB 2 dph	IgM	UNKNOWN	31.9818	32.0284	0.0659
C4	PB1 7 dph	IgM	UNKNOWN	31.6478	31.4240	0.3165
C5	PB1 7 dph	IgM	UNKNOWN	31.2002	31.4240	0.3165
C6	PB1 26 dph	IgM	UNKNOWN	33.5561	33.1994	0.5044
C7	PB1 26 dph	IgM	UNKNOWN	32.8427	33.1994	0.5044
C8	PB1 36 dph	IgM	UNKNOWN	29.5698	29.6817	0.1583
D1	PB1 36 dph	IgM	UNKNOWN	29.7937	29.6817	0.1583
D2	PB2 7 dph	IgM	UNKNOWN	31.7906	31.9676	0.2502
D3	PB2 7 dph	IgM	UNKNOWN	32.1445	31.9676	0.2502
D4	PB2 26 dph	IgM	UNKNOWN	31.2308	31.2277	0.0044
D5	PB2 26 dph	IgM	UNKNOWN	31.2246	31.2277	0.0044
D6	PB2 36 dph	IgM	UNKNOWN	30.0160	30.2182	0.2860
D7	PB2 36 dph	IgM	UNKNOWN	30.4205	30.2182	0.2860
D8	UC1 7 dph	IgM	UNKNOWN	31.3207	31.5209	0.2831
E1	UC1 7 dph	IgM	UNKNOWN	31.7210	31.5209	0.2831
E2	UC1 26 dph	IgM	UNKNOWN	31.6287	31.7788	0.2123
E3	UC1 26 dph	IgM	UNKNOWN	31.9290	31.7788	0.2123
E4	UC1 36 dph	IgM	UNKNOWN	29.9331	29.8698	0.0895
E5	UC1 36 dph	IgM	UNKNOWN	29.8065	29.8698	0.0895
E6	UC2 7 dph	IgM	UNKNOWN	32.4804	32.5360	0.0786
E7	UC2 7 dph	IgM	UNKNOWN	32.5916	32.5360	0.0786
E8	UC2 26 dph	IgM	UNKNOWN	30.9708	31.0872	0.1647
F1	UC2 26 dph	IgM	UNKNOWN	31.2037	31.0872	0.1647
F2	UC2 36 dph	IgM	UNKNOWN	29.9242	29.9414	0.0244
F3	UC2 36 dph	IgM	UNKNOWN	29.9587	29.9414	0.0244
F4		IgM	NTC	Undetermined		
F5		IgM	RT-	Undetermined		
F7	Pooled cDNA	LysTR1	UNKNOWN	26.9964	**26.9653**	0.0439
F8	Pooled cDNA	LysTR1	UNKNOWN	26.9343	**26.9653**	0.0439

Coloured fields indicate samples from probiotic treated larvae

Plate 3

Well	Sample	Gene	Task	Cт	Cт Mean	Cт SD
A1	1:1	Hep	STANDARD	20.0078	20.0501	0.0980
A2	1:1	Hep	STANDARD	20.1621	20.0501	0.0980
A3	1:1	Hep	STANDARD	19.9803	20.0501	0.0980
A4	1:4	Hep	STANDARD	21.9834	21.9745	0.0183
A5	1:4	Hep	STANDARD	21.9534	21.9745	0.0183
A6	1:4	Hep	STANDARD	21.9867	21.9745	0.0183
A7	1:16	Hep	STANDARD	24.0473	24.0392	0.0415
A8	1:16	Hep	STANDARD	24.0760	24.0392	0.0415
B1	1:16	Hep	STANDARD	23.9942	24.0392	0.0415
B2	1:64	Hep	STANDARD	26.4254	26.3363	0.0967
B3	1:64	Hep	STANDARD	26.2334	26.3363	0.0967
B4	1:64	Hep	STANDARD	26.3501	26.3363	0.0967
B5	1:256	Hep	STANDARD	28.1031	28.3366	0.5416
B6	1:256	Hep	STANDARD	27.9509	28.3366	0.5416
B7	1:256	Hep	STANDARD	28.9558	28.3366	0.5416
B8	UC 2 dph	Hep	UNKNOWN	22.8756	22.8696	0.0084
C1	UC 2 dph	Hep	UNKNOWN	22.8637	22.8696	0.0084
C2	PB 2 dph	Hep	UNKNOWN	22.9222	22.7732	0.2108
C3	PB 2 dph	Hep	UNKNOWN	22.6242	22.7732	0.2108
C4	PB1 7 dph	Hep	UNKNOWN	21.9742	21.9571	0.0243
C5	PB1 7 dph	Hep	UNKNOWN	21.9399	21.9571	0.0243
C6	PB1 26 dph	Hep	UNKNOWN	23.6903	23.7133	0.0326
C7	PB1 26 dph	Hep	UNKNOWN	23.7364	23.7133	0.0326
C8	PB1 36 dph	Hep	UNKNOWN	21.5423	21.4292	0.1600
D1	PB1 36 dph	Hep	UNKNOWN	21.3161	21.4292	0.1600
D2	PB2 7 dph	Hep	UNKNOWN	22.2533	22.2927	0.0557
D3	PB2 7 dph	Hep	UNKNOWN	22.3320	22.2927	0.0557
D4	PB2 26 dph	Hep	UNKNOWN	21.6689	21.6727	0.0054
D5	PB2 26 dph	Hep	UNKNOWN	21.6766	21.6727	0.0054
D6	PB2 36 dph	Hep	UNKNOWN	23.2280	23.2848	0.0803
D7	PB2 36 dph	Hep	UNKNOWN	23.3416	23.2848	0.0803
D8	UC1 7 dph	Hep	UNKNOWN	21.4719	21.5737	0.1439
E1	UC1 7 dph	Hep	UNKNOWN	21.6755	21.5737	0.1439
E2	UC1 26 dph	Hep	UNKNOWN	22.3203	22.3081	0.0172
E3	UC1 26 dph	Hep	UNKNOWN	22.2960	22.3081	0.0172
E4	UC1 36 dph	Hep	UNKNOWN	22.6559	22.7651	0.1544
E5	UC1 36 dph	Hep	UNKNOWN	22.8743	22.7651	0.1544
E6	UC2 7 dph	Hep	UNKNOWN	22.5796	22.6187	0.0552
E7	UC2 7 dph	Hep	UNKNOWN	22.6577	22.6187	0.0552
E8	UC2 26 dph	Hep	UNKNOWN	22.6907	22.6189	0.1016
F1	UC2 26 dph	Hep	UNKNOWN	22.5470	22.6189	0.1016
F2	UC2 36 dph	Hep	UNKNOWN	23.0313	23.0711	0.0563
F3	UC2 36 dph	Hep	UNKNOWN	23.1109	23.0711	0.0563
F4			NTC	Undetermined		
F5			RT-	Undetermined		
F7	Pooled cDNA	Lystr1	UNKNOWN	26.9643	27.0075	0.0611
F8	Pooled cDNA	Lystr1	UNKNOWN	27.0507	27.0075	0.0611

Plate 4

Well	Sample	Gene	Task	Cт	Cт Mean	Cт SD
A1	1:1	Ubiq	STANDARD	13.5972	13.5511	0.0540
A2	1:1	Ubiq	STANDARD	13.4918	13.5511	0.0540
A3	1:1	Ubiq	STANDARD	13.5644	13.5511	0.0540
A4	1:4	Ubiq	STANDARD	15.5801	15.5941	0.0143
A5	1:4	Ubiq	STANDARD	15.6087	15.5941	0.0143
A6	1:4	Ubiq	STANDARD	15.5934	15.5941	0.0143
A7	1:16	Ubiq	STANDARD	17.5718	17.6058	0.0652
A8	1:16	Ubiq	STANDARD	17.5645	17.6058	0.0652
B1	1:16	Ubiq	STANDARD	17.6810	17.6058	0.0652
B2	1:64	Ubiq	STANDARD	20.5268	20.5434	0.1365
B3	1:64	Ubiq	STANDARD	20.6875	20.5434	0.1365
B4	1:64	Ubiq	STANDARD	20.4160	20.5434	0.1365
B5	1:256	Ubiq	STANDARD	21.6592	21.8438	0.2187
B6	1:256	Ubiq	STANDARD	21.7869	21.8438	0.2187
B7	1:256	Ubiq	STANDARD	22.0854	21.8438	0.2187
B8	UC 2 dph	Ubiq	UNKNOWN	16.4454	16.5364	0.1287
C1	UC 2 dph	Ubiq	UNKNOWN	16.6274	16.5364	0.1287
C2	PB 2 dph	Ubiq	UNKNOWN	15.9593	15.8507	0.1536
C3	PB 2 dph	Ubiq	UNKNOWN	15.7421	15.8507	0.1536
C4	PB1 7 dph	Ubiq	UNKNOWN	15.4465	15.5173	0.1002
C5	PB1 7 dph	Ubiq	UNKNOWN	15.5881	15.5173	0.1002
C6	PB1 26 dph	Ubiq	UNKNOWN	17.6291	17.5213	0.1524
C7	PB1 26 dph	Ubiq	UNKNOWN	17.4136	17.5213	0.1524
C8	PB1 36 dph	Ubiq	UNKNOWN	14.7446	14.7289	0.0221
D1	PB1 36 dph	Ubiq	UNKNOWN	14.7133	14.7289	0.0221
D2	PB2 7 dph	Ubiq	UNKNOWN	16.0282	16.0448	0.0235
D3	PB2 7 dph	Ubiq	UNKNOWN	16.0613	16.0448	0.0235
D4	PB2 26 dph	Ubiq	UNKNOWN	15.5022	15.5678	0.0928
D5	PB2 26 dph	Ubiq	UNKNOWN	15.6334	15.5678	0.0928
D6	PB2 36 dph	Ubiq	UNKNOWN	15.8562	15.7840	0.1021
D7	PB2 36 dph	Ubiq	UNKNOWN	15.7118	15.7840	0.1021
D8	UC1 7 dph	Ubiq	UNKNOWN	15.8312	15.8925	0.0867
E1	UC1 7 dph	Ubiq	UNKNOWN	15.9538	15.8925	0.0867
E2	UC1 26 dph	Ubiq	UNKNOWN	16.1198	16.0706	0.0696
E3	UC1 26 dph	Ubiq	UNKNOWN	16.0214	16.0706	0.0696
E4	UC1 36 dph	Ubiq	UNKNOWN	15.0887	15.1504	0.0872
E5	UC1 36 dph	Ubiq	UNKNOWN	15.2120	15.1504	0.0872
E6	UC2 7 dph	Ubiq	UNKNOWN	16.3470	16.1990	0.2093
E7	UC2 7 dph	Ubiq	UNKNOWN	16.0510	16.1990	0.2093
E8	UC2 26 dph	Ubiq	UNKNOWN	15.7924	15.7734	0.0269
F1	UC2 26 dph	Ubiq	UNKNOWN	15.7543	15.7734	0.0269
F2	UC2 36 dph	Ubiq	UNKNOWN	15.4981	15.4581	0.0565
F3	UC2 36 dph	Ubiq	UNKNOWN	15.4182	15.4581	0.0565
F4		Ubiq	NTC	Undetermined		
F5		Ubiq	NTC	Undetermined		
F7	Pooled cDNA	LysTR1	UNKNOWN	26.8990	26.8638	0.0497
F8	Pooled cDNA	LysTR1	UNKNOWN	26.8287	26.8638	0.0497

Coloured fields indicate samples from probiotic treated larvae

Plate 5

Well	Sample	Gene	Task	Cт	Cт Mean	Cт SD
A1	1:1	ARP3	STANDARD	14.4642	14.4852	0.0357
A2	1:1	ARP3	STANDARD	14.5264	14.4852	0.0357
A3	1:1	ARP3	STANDARD	14.4651	14.4852	0.0357
A4	1:4	ARP3	STANDARD	16.2436	16.5806	0.4231
A5	1:4	ARP3	STANDARD	16.4427	16.5806	0.4231
A6	1:4	ARP3	STANDARD	17.0554	16.5806	0.4231
A7	1:16	ARP3	STANDARD	18.6105	18.6626	0.0462
A8	1:16	ARP3	STANDARD	18.6787	18.6626	0.0462
B1	1:16	ARP3	STANDARD	18.6987	18.6626	0.0462
B2	1:64	ARP3	STANDARD	21.2073	21.2848	0.0736
B3	1:64	ARP3	STANDARD	21.2935	21.2848	0.0736
B4	1:64	ARP3	STANDARD	21.3537	21.2848	0.0736
B5	1:256	ARP3	STANDARD	22.6889	22.6639	0.0984
B6	1:256	ARP3	STANDARD	22.7474	22.6639	0.0984
B7	1:256	ARP3	STANDARD	22.5553	22.6639	0.0984
B8	UC 2 dph	ARP3	UNKNOWN	17.6629	17.6525	0.0148
C1	UC 2 dph	ARP3	UNKNOWN	17.6421	17.6525	0.0148
C2	PB 2 dph	ARP3	UNKNOWN	16.7340	16.8054	0.1009
C3	PB 2 dph	ARP3	UNKNOWN	16.8767	16.8054	0.1009
C4	PB1 7 dph	ARP3	UNKNOWN	16.6828	16.6820	0.0012
C5	PB1 7 dph	ARP3	UNKNOWN	16.6811	16.6820	0.0012
C6	PB1 26 dph	ARP3	UNKNOWN	17.8945	17.9038	0.0131
C7	PB1 26 dph	ARP3	UNKNOWN	17.9131	17.9038	0.0131
C8	PB1 36 dph	ARP3	UNKNOWN	15.9583	15.9614	0.0045
D1	PB1 36 dph	ARP3	UNKNOWN	15.9646	15.9614	0.0045
D2	PB2 7 dph	ARP3	UNKNOWN	16.8837	16.8690	0.0207
D3	PB2 7 dph	ARP3	UNKNOWN	16.8543	16.8690	0.0207
D4	PB2 26 dph	ARP3	UNKNOWN	16.2105	16.2212	0.0152
D5	PB2 26 dph	ARP3	UNKNOWN	16.2319	16.2212	0.0152
D6	PB2 36 dph	ARP3	UNKNOWN	16.5702	16.5433	0.0381
D7	PB2 36 dph	ARP3	UNKNOWN	16.5163	16.5433	0.0381
D8	UC1 7 dph	ARP3	UNKNOWN	17.1074	17.1304	0.0325
E1	UC1 7 dph	ARP3	UNKNOWN	17.1534	17.1304	0.0325
E2	UC1 26 dph	ARP3	UNKNOWN	17.2661	17.0603	0.2911
E3	UC1 26 dph	ARP3	UNKNOWN	16.8545	17.0603	0.2911
E4	UC1 36 dph	ARP3	UNKNOWN	16.3968	16.4529	0.0793
E5	UC1 36 dph	ARP3	UNKNOWN	16.5090	16.4529	0.0793
E6	UC2 7 dph	ARP3	UNKNOWN	17.0269	17.0200	0.0098
E7	UC2 7 dph	ARP3	UNKNOWN	17.0131	17.0200	0.0098
E8	UC2 26 dph	ARP3	UNKNOWN	16.4896	16.7373	0.2151
F1	UC2 26 dph	ARP3	UNKNOWN	16.6276	16.7373	0.2151
F2	UC2 36 dph	ARP3	UNKNOWN	16.9442	16.7373	0.2151
F3	UC2 36 dph	ARP3	UNKNOWN	16.8877	16.7373	0.2151
F4		ARP3	NTC	Undetermined		
F5		ARP3	RT-	Undetermined		
F7	Pooled cDNA	LysTR1	UNKNOWN	26.9655	**26.9838**	0.0259
F8	Pooled cDNA	LysTR1	UNKNOWN	27.0022	**26.9838**	0.0259

Plate 6

Well	Sample	Gene	Task	Cт	Cт Mean	Cт SD
A1	1:1	RPL4	STANDARD	13.4459	13.4478	0.0032
A2	1:1	RPL4	STANDARD	13.4515	13.4478	0.0032
A3	1:1	RPL4	STANDARD	13.4459	13.4478	0.0032
A4	1:4	RPL4	STANDARD	15.3992	15.5339	0.1239
A5	1:4	RPL4	STANDARD	15.6430	15.5339	0.1239
A6	1:4	RPL4	STANDARD	15.5595	15.5339	0.1239
A7	1:16	RPL4	STANDARD	17.6377	17.7132	0.0687
A8	1:16	RPL4	STANDARD	17.7297	17.7132	0.0687
B1	1:16	RPL4	STANDARD	17.7721	17.7132	0.0687
B2	1:64	RPL4	STANDARD	20.4468	20.3913	0.1651
B3	1:64	RPL4	STANDARD	20.5215	20.3913	0.1651
B4	1:64	RPL4	STANDARD	20.2057	20.3913	0.1651
B5	1:256	RPL4	STANDARD	21.8223	21.7626	0.0664
B6	1:256	RPL4	STANDARD	21.7744	21.7626	0.0664
B7	1:256	RPL4	STANDARD	21.6911	21.7626	0.0664
B8	UC 2 dph	RPL4	UNKNOWN	17.9320	17.9739	0.0594
C1	UC 2 dph	RPL4	UNKNOWN	18.0159	17.9739	0.0594
C2	PB 2 dph	RPL4	UNKNOWN	16.6255	16.6477	0.0314
C3	PB 2 dph	RPL4	UNKNOWN	16.6699	16.6477	0.0314
C4	PB1 7 dph	RPL4	UNKNOWN	15.1279	15.2109	0.1174
C5	PB1 7 dph	RPL4	UNKNOWN	15.2939	15.2109	0.1174
C6	PB1 26 dph	RPL4	UNKNOWN	17.4658	17.4702	0.0061
C7	PB1 26 dph	RPL4	UNKNOWN	17.4745	17.4702	0.0061
C8	PB1 36 dph	RPL4	UNKNOWN	14.4368	14.4703	0.0474
D1	PB1 36 dph	RPL4	UNKNOWN	14.5038	14.4703	0.0474
D2	PB2 7 dph	RPL4	UNKNOWN	16.4154	16.4399	0.0346
D3	PB2 7 dph	RPL4	UNKNOWN	16.4644	16.4399	0.0346
D4	PB2 26 dph	RPL4	UNKNOWN	15.6154	15.6657	0.0711
D5	PB2 26 dph	RPL4	UNKNOWN	15.7160	15.6657	0.0711
D6	PB2 36 dph	RPL4	UNKNOWN	15.5815	15.5581	0.0332
D7	PB2 36 dph	RPL4	UNKNOWN	15.5346	15.5581	0.0332
D8	UC1 7 dph	RPL4	UNKNOWN	15.6697	15.7196	0.0705
E1	UC1 7 dph	RPL4	UNKNOWN	15.7694	15.7196	0.0705
E2	UC1 26 dph	RPL4	UNKNOWN	16.3045	16.3044	0.0002
E3	UC1 26 dph	RPL4	UNKNOWN	16.3042	16.3044	0.0002
E4	UC1 36 dph	RPL4	UNKNOWN	15.0905	15.0767	0.0195
E5	UC1 36 dph	RPL4	UNKNOWN	15.0629	15.0767	0.0195
E6	UC2 7 dph	RPL4	UNKNOWN	16.3090	16.3865	0.1096
E7	UC2 7 dph	RPL4	UNKNOWN	16.4640	16.3865	0.1096
E8	UC2 26 dph	RPL4	UNKNOWN	15.6655	15.6269	0.0546
F1	UC2 26 dph	RPL4	UNKNOWN	15.5884	15.6269	0.0546
F2	UC2 36 dph	RPL4	UNKNOWN	15.4098	15.4230	0.0188
F3	UC2 36 dph	RPL4	UNKNOWN	15.4363	15.4230	0.0188
F4		RPL4	NTC	Undetermined		
F5		RPL4	RT-	Undetermined		
F7		LysTR1	UNKNOWN	27.0632	**27.1225**	0.0838
F8		LysTR1	UNKNOWN	27.1818	**27.1225**	0.0838

Coloured fields indicate samples from probiotic treated larvae

B.1 Calculations of raw quantities and *geNorm* input file

Raw quantities							geNorm input			
Sample	mIgM	LysTR1	Hep	Ubiq	ARP3	RPL4	Sample	Ubiq	ARP3	RPL4
UC dph	53.6769	49.2864	145.6233	143.4386	127.4417	54.7506	UC dph	0.3123	0.3284	0.1042
UC1 7 dph	130.5555	109.9959	343.5962	217.1407	179.7240	234.5687	UC1 7 dph	0.4727	0.4632	0.4465
UC1 26 dph	109.8794	155.2480	211.2332	193.6114	188.2183	160.8284	UC1 26 dph	0.4215	0.4850	0.3062
UC1 36 dph	393.5965	469.4414	156.0583	350.1796	280.7629	355.1992	UC1 36 dph	0.7623	0.7235	0.6762
UC2 7 dph	66.2426	85.4490	171.9559	178.2482	193.2761	152.5226	UC2 7 dph	0.3880	0.4981	0.2903
UC2 26 dph	174.4482	254.0687	171.9346	234.4584	232.8200	249.0212	UC2 26 dph	0.5104	0.6000	0.4740
UC2 36 dph	375.1926	338.3444	127.4266	287.2271	232.8200	284.0479	UC2 36 dph	0.6253	0.6000	0.5407
PB 2 dph	92.9996	89.9397	155.2278	223.0662	222.6174	128.8635	PB 2 dph	0.4856	0.5737	0.2453
PB1 7 dph	139.2904	135.9533	266.5386	276.4850	241.4577	325.7250	PB1 7 dph	0.6019	0.6222	0.6200
PB1 26 dph	42.5174	82.0933	83.2727	76.0712	108.0113	75.7876	PB1 26 dph	0.1656	0.2783	0.1443
PB1 36 dph	446.3148	513.4835	378.1071	459.3583	388.0436	525.3255	PB1 36 dph	1.0000	1.0000	1.0000
PB2 7 dph	96.8573	110.0746	213.4050	196.8639	213.4818	147.3569	PB2 7 dph	0.4286	0.5501	0.2805
PB2 26 dph	158.8170	231.5165	321.7757	267.6353	327.0412	242.8670	PB2 26 dph	0.5826	0.8428	0.4623
PB2 36 dph	311.8206	381.1667	110.6047	232.8555	264.5445	260.3374	PB2 36 dph	0.5069	0.6817	0.4956

B.1.1 Calculation of normalised quantities / normalised expression levels for mIgM, g-type lysozyme and hepcidin

NF	Sample	Normalised quantities (NQ) mIgM	NQ relative to highest expression	Normalised quantities (NQ) LysTR1	NQ relative to highest expression	Normalised quantities (NQ) Hep	NQ relative to highest expression
0.6166	UC dph	87.0531	0.2736	79.9325	0.2373	236.1714	0.6192
0.9009	UC1 7 dph	144.9168	0.4555	122.0955	0.3625	381.3921	1.0000
0.8705	UC1 26 dph	126.2256	0.3967	178.3435	0.5296	242.6574	0.6362
1.4299	UC1 36 dph	275.2615	0.8651	328.3037	0.9748	109.1393	0.2862
0.8464	UC2 7 dph	78.2640	0.2460	100.9559	0.2998	203.1615	0.5327
1.0654	UC2 26 dph	163.7397	0.5146	238.4726	0.7081	161.3803	0.4231
1.1792	UC2 36 dph	318.1755	1.0000	286.9271	0.8520	108.0619	0.2833
1.0162	PB 2 dph	91.5171	0.2876	88.5059	0.2628	152.7532	0.4005
1.1782	PB1 7 dph	118.2231	0.3716	115.3907	0.3426	226.2252	0.5932
0.4134	PB1 26 dph	102.8481	0.3232	198.5809	0.5896	201.4337	0.5282
1.9253	PB1 36 dph	231.8157	0.7286	266.7031	0.7919	196.3887	0.5149
0.9349	PB2 7 dph	103.6018	0.3256	117.7395	0.3496	228.2651	0.5985
1.3491	PB2 26 dph	117.7207	0.3700	171.6081	0.5096	238.5114	0.6254
1.1318	PB2 36 dph	275.5086	0.8659	336.7792	1.0000	97.7246	0.2562