The University of South Bohemia in České Budějovice

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α-GAL immunity in fish and its potential for modulating the effect of parasitic infections

Bachelor thesis

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Annotation

Sphaerospora molnari is a myxozoan parasite causing systemic immune responses and hemolytic anemia in *Cyprinus carpio*. This study aimed to stimulate protective immunity against S. molnari through the administration of a probiotic treatment containing α-GAL-rich Escherichia coli strain (Nissle 1917). Feed enriched with varying doses of lyophilized bacteria was used alongside gavage and intraperitoneal injection methods in six treatment groups. Infection progression was monitored through flow cytometry, qPCR assays, and ELISA tests for antibody titers. Results showed that the E. coli vaccine provided immediate protective immunity, reducing the severity of hemolytic anemia. The findings of this study provide valuable knowledge for future research and development of a probiotic in-feed product against parasitic infections of fish.

Declaration

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

Linz, Austria, 31/07/2023 .....

Manuel Stammler

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First and foremost, I would like to express my sincere gratitude to my supervisor, Dr. Astrid Holzer, for her continuous support, guidance, and encouragement throughout my research journey. Your expert advice, and belief in my capabilities have been instrumental in shaping this thesis. It has been a great privilege to learn from you and work under your supervision. I would also like to thank everyone in the Holzer Lab for warmly welcoming me into their group, particularly Justin Chan for his expertise and the very generous amount of time he gave me- even on Sundays, Joana Pimentel Santos for teaching me how to handle and care for the fish in my experiments and Jovana Majstrovic for her invaluable expertise on lab assays and her understanding and helpful nature throughout the research process.

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Abstract

Sphaerospora molnari is a species belonging to the group of Myxozoa in the phylum Cnidaria. These parasites are evolutionarily and morphologically highly simplified as a result of their endoparasitc lifestyle. *S. molnari* causes a complex systemic immune response in *Cyprinus carpio* and proliferates in the blood before forming spores in the epithelia of gills and skin. During the blood residence the parasite feeds on red blood cells and induces hemolytic anemia, especially when fish are immunosuppressed, which is often the case in aquaculture.

Galactose-alpha-1,3-galactose (α-GAL) is a common carbohydrate expressed in all vertebrates except for humans, fish, and birds. Exposure to pathogens harboring α-GAL can lead to a strong immune response in animals lacking it. In humans, red-meat syndrome is a strong allergic reaction against mammalian meat leading to a production of IgE antibodies and is caused by tick bites. At the same time, the capacity of anti-α-GAL IgM and IgG antibodies to protect humans against parasite pathogens with α-Gal on their surface shows the positive effects α-GAL immunity can have(De La Fuente et al., 2019). The goal of this study was to stimulate the expression of specific antibodies against α-GAL in common carp through a probiotic in-feed treatment and as a result provide protective immunity to the myxozoan *S. molnari* when challenged with the parasite.

Feed enriched with different doses of a particular α-GAL-rich *Escherichia coli* strain (Nissle 1917) was produced by incorporating lyophilized bacteria into commercial feed pellets. An infection trial with six different treatment groups, including the *E. coli* diets as well as gavage and intraperitoneal injection of bacteria was performed to assess the efficacy of a microbial treatment against *S. molnari*. The course of infection was monitored by identifying the degree of anemia and inflammatory cell influx into the blood on a regular basis, using flow cytometry. The presence of *S. molnari* in liver, gills and blood was quantified using a qPCR assay established in our lab. Anti- *S. molnari* antibody titers were determined with Enzyme-linked-immunosorbent-assays (ELISA). We found that a vaccine treatment with *E. coli* provides immediate protective immunity and alleviates the severity of hemolytic anemia caused by proliferative blood stages. An increased number of antibodies against *S. molnari* was detected in vaccinated fish. We also observed that a gavage treatment shortens the duration of *S. molnari* infection, possibly through stimulation of the gut microbiome or innate immune parameters. In-feed treatment as applied in the current study had a negative impact on the infection outcome. We hypothize that continuous treatment with life bacteria in feeds has a negative effect on the host microbiome. More infection trials are necessary to fully understand the effects of a probiotic in-feed treatment on the immune response, potentially involving intermittent low-dose feeding before parasite exposure.

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1. Introduction

1.1. Myxozoa

Myxozoa are a group of endoparasites in the Phylum Cnidaria. Compared to other cnidarians however, their morphology and genetic complexity is highly simplified due to their parasitic lifestyle. This led to them being falsely classified as unicellular protozoans in the past (Kent et al., 1994). It was assumed that there were two classes of myxozoans: Myxosporea, forming spores in vertebrates (mostly fish), and Actinosporea, forming spores in invertebrates (annelid worms). However, research (Wolf & Markiw, 1984) revealed that the two classes represent alternating life forms of each myxozoan species, with actinosporeans being formed in invertebrate hosts and myxosporeans being produced by fish hosts. At present, myxosporeans (2180 species) and malacosporeans (4 species) are recognized as taxonomically and phylogenetically distinct clades of myxozoans. Myxosporeans alternate between fish and annelid hosts while malacosporeans use fish and Bryozoa (class Phylactolaemata) as hosts in their life cycles.

Actinospores typically target epithelial cells in the fish's gill causing damage to gill tissue. This can lead to reduced oxygen uptake, increased susceptibility to infection, and potentially death. Post-invasion infections can spread to other organs and tissues, leading to further complications (Luque, 2004). Despite their massive and likely highly underestimated biodiversity (Okamura et al., 2018) only a few Myxozoa are known to cause severe disease. However, pathogenic species are highly detrimental to natural stocks and aquaculture, causing important economic losses. A few examples for common diseases in infected fish are: salmonid whirling disease caused by *Myxobolus cerebralis* (Gilbert & Jr, 2003), post-mortem myoliquefication caused by *Kudoa thyrsites* in a wide range of fish (Højgaard, 2022), or proliferative kidney disease caused by *Tetracapsuloides bryosalmonae* (Anderson et al., 1999). It is important that several species have shown either an increase in disease severity or a northward expansion due to climate change (Lauringson et al., 2022; Schakau et al., 2019). Therapies or vaccines against myxozoans however are still missing, and there is currently no legal treatment for fish destined for human consumption.

1.1.1. *Sphaerospora molnari*

Myxozoans in the class of *Sphaerospora* typically form spores in the excretory system in both freshwater and marine fish (Patra et al., 2018). *Sphaerospora molnari,* however, is an exception as sporulation takes place in the gills and the skin of common carp(reviewed by Eszterbauer et al., 2013). Importantly, several sphaerosporids have been shown to proliferate in the blood (summarized by Eszterbauer et al., 2013).

Proliferative stages of *S. molnari* in the blood cause hemolytic anemia due to erythrocyte lysis and suggested hemoglobin consumption particularly fish when fish are immunosuppressed (Korytář, Chan, et al., 2019) which is often the case in aquaculture (Gil Barcellos et al., 2004), Necrosis and the release of mature spores from the gills disrupt respiration and osmoregulation. In extreme cases the infection spreads to both skin and branchial cavity (Lom et al., 1983). We have developed laboratory protocols for isolation *of S. molnari* blood stages (Born-Torrijos et al., 2022) and are able to perform controlled infection experiments and to test the effect of treatments on parasite development and host immunology- (Ganeva et al., 2020; Korytář, Wiegertjes, et al., 2019).

1.2. Immune System of Teleostei

Like mammals, the immune system of fish can be divided into two parts: the adaptive, and the innate immune system. The innate immune system provides a first line of defense and includes any means that directly counter an invasion of pathogens: physical barriers, cellular components and humoral factors. (Magnadóttir, 2006) Pattern recognition receptors (PRRs) play an important role in inducing an innate immune response by binding and reacting to pathogen-associated molecular patterns (PAMPs) like lipopolysaccharides and peptidoglycans found on gram-negative bacteria (Boltaña et al., 2011). Cellular components of the innate immune system include white blood cells like macrophages, neutrophils, eosinophils, basophils, and natural killer cells.

The adaptive immune system is represented by a humoral, antibody response component mediated through B cells and a cell-mediated response component (T cells). B cells secrete specific antibodies which bind to antigens on pathogens and mark them for destruction while T cells are capable of detecting infected host cells through familiar antigens presented on their membranes and killing them as a result. (Laing & Hansen, 2011; Schroeder & Cavacini, 2010). The main advantage of the adaptive immune responses is its ability to remember antigens of previous infections and allow for a quick countermeasure upon re-exposure. (Secombes & Wang, 2012) In fish, IgM is the systemic immunoglobulin while IgT has been associated with mucosal surfaces.

Organs involved in the immune response include, but are not limited to spleen, thymus, gut, gills, skin and head kidney(equivalent of the mammalian bone marrow) (Sunyer, 2013). The head

kidney is therefore where most hematopoietic stem cells are found and the site of hematopoiesis which is the process of formatting erythrocytes and leukocytes (Zapata, 1979).

1.3. Alpha Gal

Alpha-1,3-galactose, better known as alpha-gal (epitope) is a carbohydrate molecule. While present in most vertebrates, humans, birds, and fish do not produce alpha-gal and can induce antibody production when stimulated with the carbohydrate (De La Fuente et al., 2019). Alpha-gal immunity shows great potential for controlling infectious diseases: malaria, leishmaniasis, Chagas disease, granulocytic anaplasmosis, and influenza as pathogens express alpha-gal on their surfaces and enhance the protective immune response against these pathogens (Cabezas-Cruz et al., 2017; Goodrich et al., 2014; Portillo et al., 2019). A single study that focuses on fish demonstrated efficient vaccination with alpha-gal against tuberculosis, while some bacteria can additionally improve the immune response in fishes by probiotic activity. (Pacheco et al., 2021).

2. Aims of the study

In this study, we set out to stimulate anti-alpha-gal immune responses and study the effect of protection against *S. molnari* infections in common carp. Following initial successful trials using gavage, here we aimed to

- Grow *E. coli* Nissle 1917, lyophilize it and integrate bacteria into fish feed at different concentrations.
- Test bacterial survival upon lyophilization.
- Determine the efficacy of mucosal *E. coli* alpha-gal immune stimulation by punctual gavage, long-term in-feed treatment and intraperitoneal vaccination on the infection with *S. molnari* in *Cyprinus carpio* by determining the change in cell composition and the number of parasites.

3. Methods

3.1. Fish

Thirty-six common SPF carp (*Cyprinus carpio*) weighing 9,4g(±2g) were accommodated in groups of 6 fish in aquaria at the animal facility of the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences. The water was exchanged daily with carbon-treated tap water and kept at a temperature of 21(±1)°C. Water was taken from an experimental recirculation system which filtered it both mechanically and biologically. Additionally, the water undergoes treatment with UV light and ozone. Water quality including pH, nitrogen and oxygen levels were monitored regularly. Fish were fed with commercially available carp diet (Skretting, Stavanger, Norway), in re-pelleted form, identical in all groups (see below).

3.2. Bacterial applications

Bacteria (strain *E. coli* Nissle 1917) were grown in LB broth (5g yeast extract, 10g NaCl, 10g Tryptone in 1L distilled water) at 37°C overnight and the amount of colony forming units (CFUs) was measured by photometric absorption at 600nm (10^6CFUs=1OD), using the broth as blank. Fish immunity was stimulated with *E. coli* by two gavages (day 1 and day 21 of experiment), by continuous in-feed treatment, during the whole trial or by vaccination with heat-killed bacteria (for treatment and control groups see table 1). For gavage, bacteria were washed in PBS and using a 100 µL pipette, 10^8 CFU in 200 µl were given by introducing the tip orally into the stomach. For in-feed application bacteria were pelleted by centrifugation at 4000 RPM for 20 min and 10% sucrose were added as a lyoprotectant to the pellet. After 45 min, bacteria were transferred to Florence flasks and frozen by submerging and simultaneously rotating them in liquid nitrogen to coat the inside with a thin layer of bacteria. The flasks were then attached to a lyophilizer and lyophilized overnight. Bacterial growth post-lyophilization was estimated by overnight growth at serial dilutions of 1:10, 1:100, 1:1000, 1:10 000 and 1:100 000 using a bacterial suspension containing $3.4*10^5$ CFUs / m. For feed-integration, commercial feed was ground and re-constituted with a small amount of water to a 2mm pellet size. Some of the ground feed was enriched with *E. coli* at 10^6 CFUs g-1 or 10^4 CFUs g-1. Feeds were dried in a commercial oven at 45°C, for 48 hrs. For vaccination, bacteria were washed in PBS and heat-killed by incubation at 75°C, for an hour. Bacteria were injected intraperitoneally twice (day one and day 21 of experiment). The first vaccination was given at a 1:1 ratio with Freud's incomplete adjuvant.

3.3. Parasite

S. molnari blood stages were taken from a 6+ year-old laboratory line maintained according to standard operating procedures (Born-Torrijos et al., 2022, Holzer and Pimentel-Santos, 2021) at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences. Blood of immunosuppressed donor fish was taken and *S. molnari* blood stages were isolated according to an adapted protocol for DEAE cellulose isolation (Born-Torrijos et al., 2022; Lahnham & Godfrey, 1970) and 100 000 blood stages per fish were injected intraperitoneally into groups B-F, on day 25 of the experiment.

3.4. Treatment groups and experimental timing

Fish were divided into six groups in one tank each (*Figure 2)*. Groups A, B serve as control groups, both being untreated and only B being infected with *S. molnari*. Groups C, D were fed bacteria-enriched diet with different bacterial concentrations. Group E was given two gavages and Group F was vaccinated twice, with Freud's incomplete adjuvant the first time.

GROUP	infected	treatment	bacteria (CFU)	
A	N	none		
B	Y	none		
C	Y	bacteria-enriched diet	10^6/g (throughout trial)	
D	Y	bacteria-enriched diet	10^4/g (throughout trial)	
E	Y	gavage	10^8/fish	x2 (D1,D21)
F	Y	vaccination (+adjuvant)	10^6/fish	x2 (D1,D21)

Table 1 Treatment of Groups

The infection study was conducted over a time period of 81 days (*Figure 1)*. Fish were weighed weekly, and the amount of feed was adjusted according to 2% of total body mass per tank. A blood sample was taken in week one for a baseline measurement of blood cell composition, and to estimate any changes caused by different treatments vs the control group. For Groups A, B, E, F commercial feed was used, groups C and D received the bacterial feed according to *Table 1*. Gavage group (E) and intraperitoneal vaccination group (F) received treatment 15 days after day zero. The fish were challenged with *S. molnari* on day 24, giving time for an immune response to develop from the bacterial treatment. Another blood sample was taken on the day of infection and three more at 31, 35, 42 days post-infection (dpi).

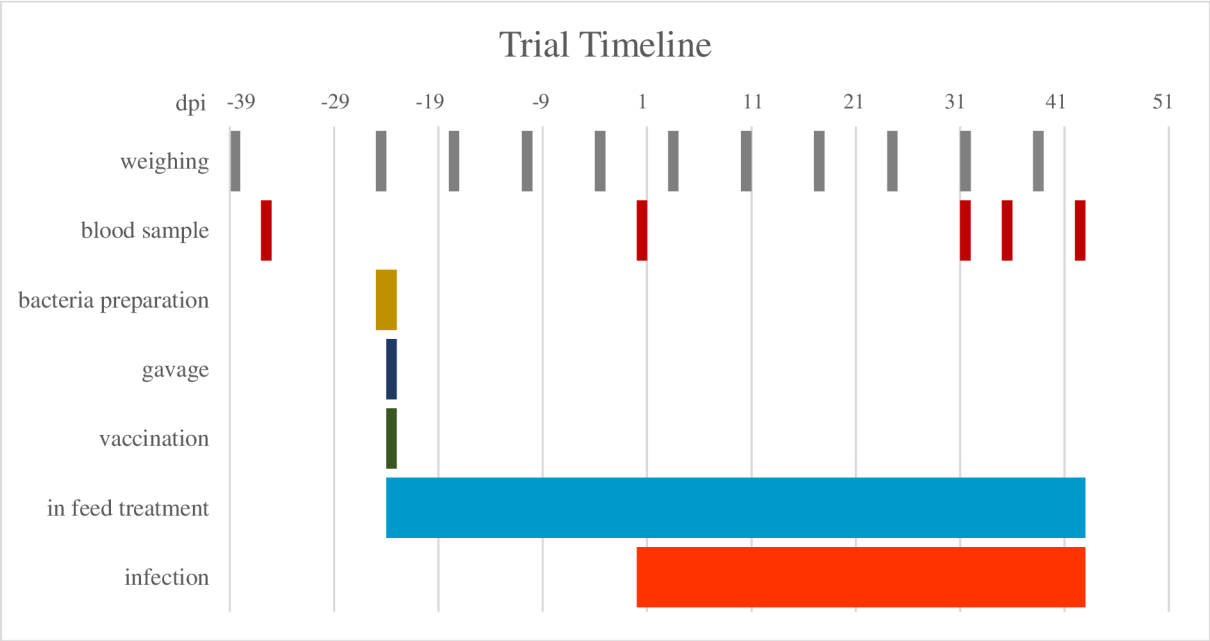

Figure 1 timeline for the experiment. Timescale is in days post-infection.

Figure 2 Experimental treatment group tanks, each containing six fish.

3.5. Flow Cytometry

Syringes were prepared by drawing heparin and dispensing all of it, as the dead volume is sufficient to prevent the blood from clotting. Blood samples were taken with the syringes and transferred to Eppendorf tubes. 2 µl of the blood were added to 195 µl RPMI-1640 (RPMI) on a 96 well plate. The samples were then always kept on ice to avoid clotting. The plate was centrifuged at 500G for 3 minutes and the supernatant was disposed by inverting the plate. The washing step was repeated once and then 100 µl of primary antibody WCI12 solution (diluted 1:200 with RPMI) was added. The solution was pipetted up and down to expose the blood cells to the antibody. This was then incubated for approximately 15 minutes on ice. After centrifugation at 500G for 3 minutes, the cells were washed with 100 µl and centrifuged twice. Then, 100 µl of the secondary antibody anti-mouse IgG-Alexa Fluor™ 647 (diluted 1:500 with RPMI) were added and the samples were again mixed and incubated for 15 minutes on ice. The well was centrifuged at 500G for 3 minutes, 100 µl of DiOC6 solution (1:2000, 1:10000 with RPMI; depending on batch) were added and mixed with the cells. DiOC6 binds to the endoplasmic reticulum and golgi apparatus, which are more prominent in white blood cells when compared to erythrocytes and hence allows for their differentiation. Lastly the cells were centrifuged at 500G for 5 minutes and after 1 final wash, the cells were resuspended in a 195 µl of RPMI. The samples were measured on the FACS Canto II.

3.6. ELISA

To determine the antibody titers in the blood, enzyme-linked immunosorbent assays (ELISA) were performed with blood from fish taken -36, 0-, 31-, 35- and 42-days post-infection. A bicarbonate-carbonate coating buffer was prepared by dissolving 3.03g Na_2CO_3, 6.0g $NaHCO_3$ in 1 L distilled water. *S. molnari* blood stage lysate was diluted in coating buffer to a concentration of 25ug/ml. A 0.1% Tween 20 in PBS solution was used for all washing steps. 50 µL of parasite lysate was added to each well and the plate was left overnight at 4°C. For the blocking solution, 5g dried milk were dissolved in 100 mL PBS to obtain a 5% solution. Subsequently, 100 µL of the blocking solution was added to each well and the blocking process was carried out for 4 hours at room temperature. The plate was dried again by patting it against a paper towel and washed with 100 µL PBS 0.1% Tween 20 (PBS-Tween) solution. It was again dried, and the washing was repeated two more times to remove all remaining blocking solution. Then 50 µL of 1:100 fish blood sera in PBS was added to the appropriate wells. The plate was left to incubate for one hour at ambient temperature. Remaining solution was removed by washing the plate four times with PBS-Tween 20. Mouse anti-carp IgM antibody was added as secondary antibody and the washing step was repeated four times. Lastly 100 µL of goat anti-mouse IgG conjugated to horseradish

peroxidase was added and incubated for one hour at ambient temperature. This time, 5 washing steps were done before adding 50 µL of the 1-Step™ Ultra TMB -ELISA Substrate Solution. The reaction was observed and terminated in a timely manner using a multichannel pipette with 50 µL 2M sulfuric acid to circumvent oversaturation. For quantification, the absorbance was measured at 450nm on a plate reader

3.7. Quantification of *S. molnari* by qPCR

Samples of blood were taken on dpi 28, 35, 42; gills on dpi 35, 42; and liver on dpi 42 (final day of trial). RNA was fixed by suspending tissue samples in Invitrogen RNAlater-stabilization solution (Thermo Fisher scientific). All samples were kept in a freezer for later use. Total RNA was then extracted using a commercial extraction kit (NucleoSpin, Machery Nagel, Düren, Germany) and concentrations were measured using a NanoDrop Spectrophotometer (Thermo Fisher scientific). For quantification 100 ng/µL cDNA samples were prepared with the Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics).

Parasite expression was quantified using a unique Taq-man qPCR assay established by the laboratory, based on the SSU rDNA of *S. molnari* (GenBank accession JX431511) (Korytář, Wiegertjes, et al., 2019). 100ng of the previously prepared cDNA were combined with 0.4 µM forward and reverse primers, 0.2 µM 5' FAM and 4' BHQ1-labelled TaqMan probe and 12.5 µL LightCycler® Probes Master (Roche Diagnostics) resulting in a 25 µL mixture. Accuracy of the measurement was ensured by measuring each sample twice and comparing Ct values with an interplate control sample.

Parallel to the parasite RNA, expression levels of host housekeeping gene *beta-actin* were measured for quantification across samples and organs.

3.8. Statistics:

Data obtained across all methods was visualized using Python and R, on the development environments Pycharm (Jetbrains) and RStudio (Posit, PBC), respectively. For the analysis of flow cytometry results, for each cell count measurement, fish were automatically ranked from highest to lowest. On the consecutive measurement the ranking was repeated, and the cell count was then compared percentage-wise to the measurement with the equivalent ranking to obtain a relative ranked difference (RRD). Then, the absolute difference between the treatment group RRDs and the average negative control RRD was calculated. The mean and standard error of the resulting values were then calculated and visualized in a line plot.

4. Results

The fish were fed on a daily basis, with the amount of food given to each tank determined once a week when fish were weighed. The amount of food was calculated as 2% of the total body mass of all fish per tank. As depicted in *Figure 1* and *Figure 2* a non-significant but clearly visible decrease in growth rate was observed in all infected groups compared to the uninfected control group leading to a weight difference of ~10% 5 weeks post-infection. For the group fed with lower concentration bacterial feed, the decrease in weight was even more pronounced.

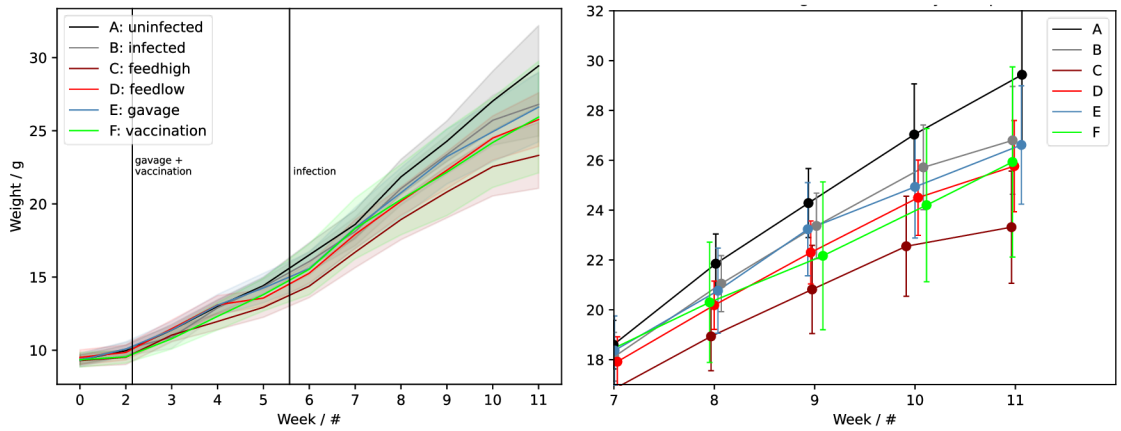

Figure 3 Mean weight of fish per tank over time. Opaque area shows standard error of each group. Black vertical lines indicate date of gavage and vaccination as well as in-feed treatment starting point (first line). Graph on the right shows last three weeks of weight measurements with standard error bars. Most groups are clustered together at roughly 10% less body weight than the negative control.

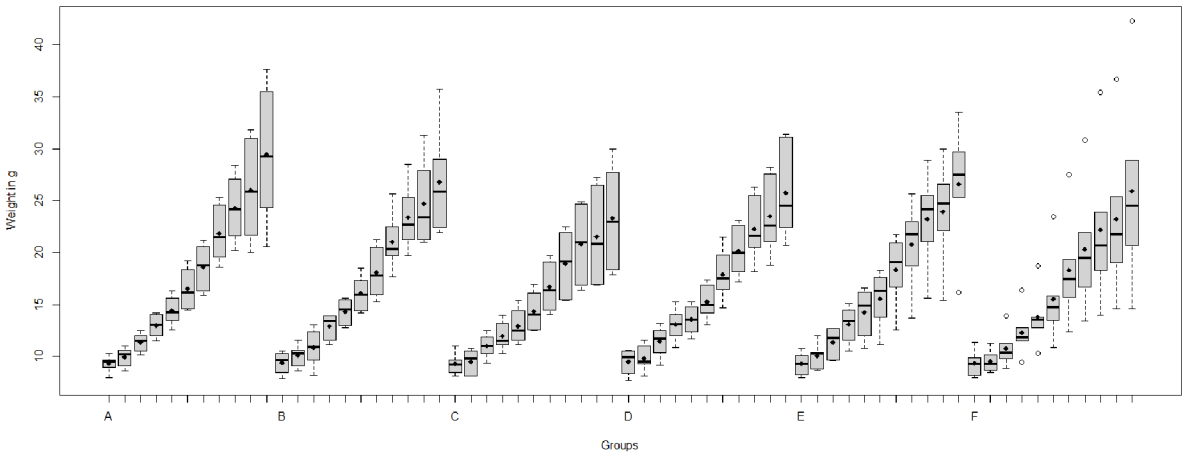

Figure 4 Boxplots of weight of fish for each group over the course of 11 weeks. Mean weights are indicated by black markers. 50% of measured weights around median are shown by boxes, whiskers show upper and lower 25% measurements.

4.1. Bacteria

A cultivation assay was performed to ensure growth of bacteria at low concentrations. Post-lyophilization bacteria (*Figure 6)* were cultivated overnight at serial dilutions from $3.4*10^4$ CFUs / ml to 3.4 CFUs / ml, in a 24-well cell-well plate. All concentrations showed a satisfactory growth (*Figure 5)*. The suspension with the lowest concentration, containing only ~3 CFUs showed no significant difference in growth compared to higher concentrations (Fig. 5).

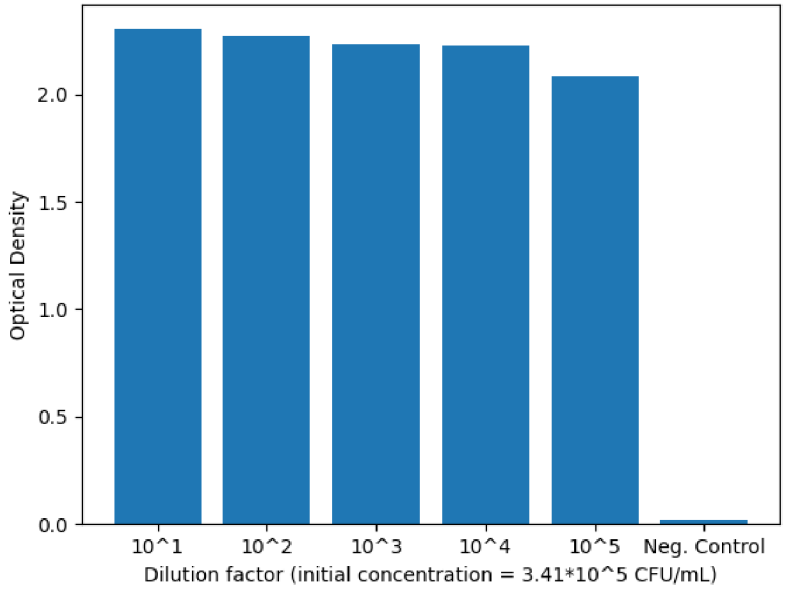

Figure 5 Optical density of bacteria suspensions at different starting concentrations. y-axis shows absorbance measured through a 1cm cuvette; x-axis shows dilution factor of starting concentration with a parent solution containing $3.41*10^5$ CFUs/mL.

Figure 6 Round-bottom flask with bacteria lyophylate coating on inner wall.

4.2. Flow Cytometry

To monitor any changes in cellular composition during the infection, flow cytometry was performed on blood samples of each fish.

Total number of erythrocytes and lymphocytes was determined according to the procedure described in *Figure 7* and *Figure 8.*

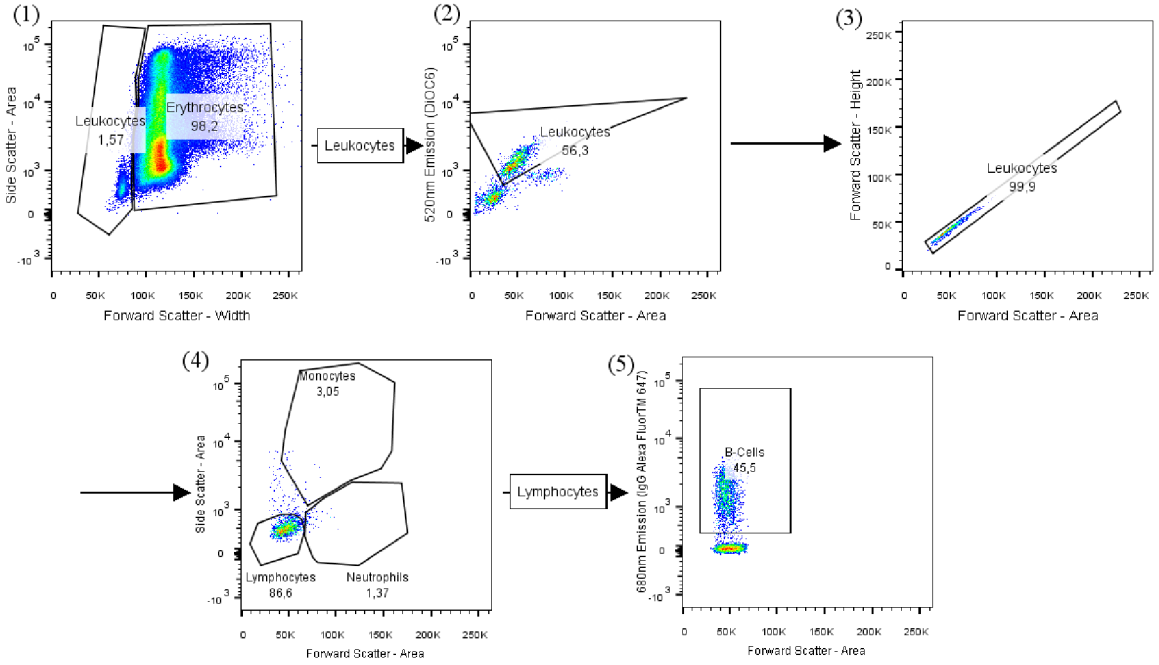

Figure 7: Blood cells of *Cyprinus carpio* 35 days post-infection. (1) gating according to general profile of erythrocytes vs leukocytes with overestimated number of the latter (2) Specification of leukocytes as they show greater fluorescence when stained with DiOC6 due to the presence of expanded endoplasmic reticulum and Golgi apparatus. (3) Exclusion of doublet cells (4) Leukocytes are differentiated by side scatter area which is proportional to cell complexity and forward scatter area which represents size (5) B cells bound to primary antibody WCI12 bound to secondary antibody anti-mouse IgG Alexa Fluor™ 647 fluoresce at 680nm reflects proportion/number of B cells

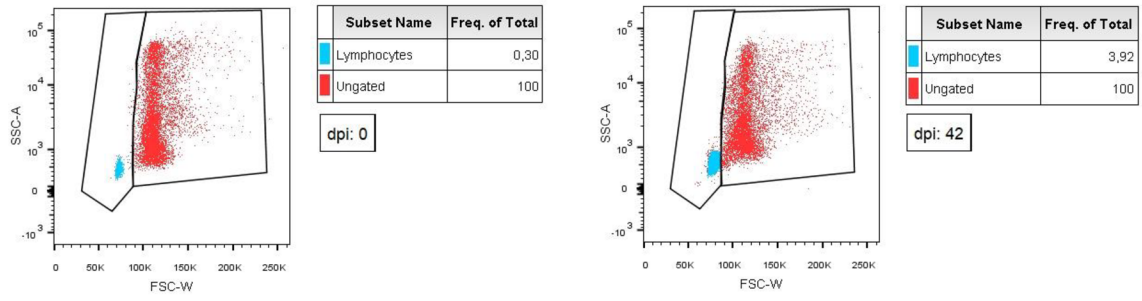

Figure 8: All events recorded vs lymphocytes determined according to Figure 7 for infected positive control group. Fish in this group exhibit up to 13-fold increase in lymphocytes when compared to the control.

4.2.1. Erythrocytes

A ~20% drop of total erythrocytes was observed from 31dpi onwards, likely because a new batch of DiOC6 was used (*Figure 9)*. The observed decrease might therefore be due to a lower binding strength. To exclude this inconsistency and error source, erythrocytes proportional to negative control, as depicted in *Figure 10*, was used instead.

Erythrocyte numbers vary greatly only after infection is induced. Fish in groups C and D show a significant loss of erythrocytes compared to other groups. At a single sampling point (31 dpi), the gavage group shows a higher level of anemia. In contrast to the in-feed groups, the vaccination group indicates an immediate protective effect leading to a significant increase in erythrocytes, 31 dpi and a normalization of red blood cell numbers thereafter, to levels comparable to the uninfected control. A similar normalization was observed in Group E (Figure 10).

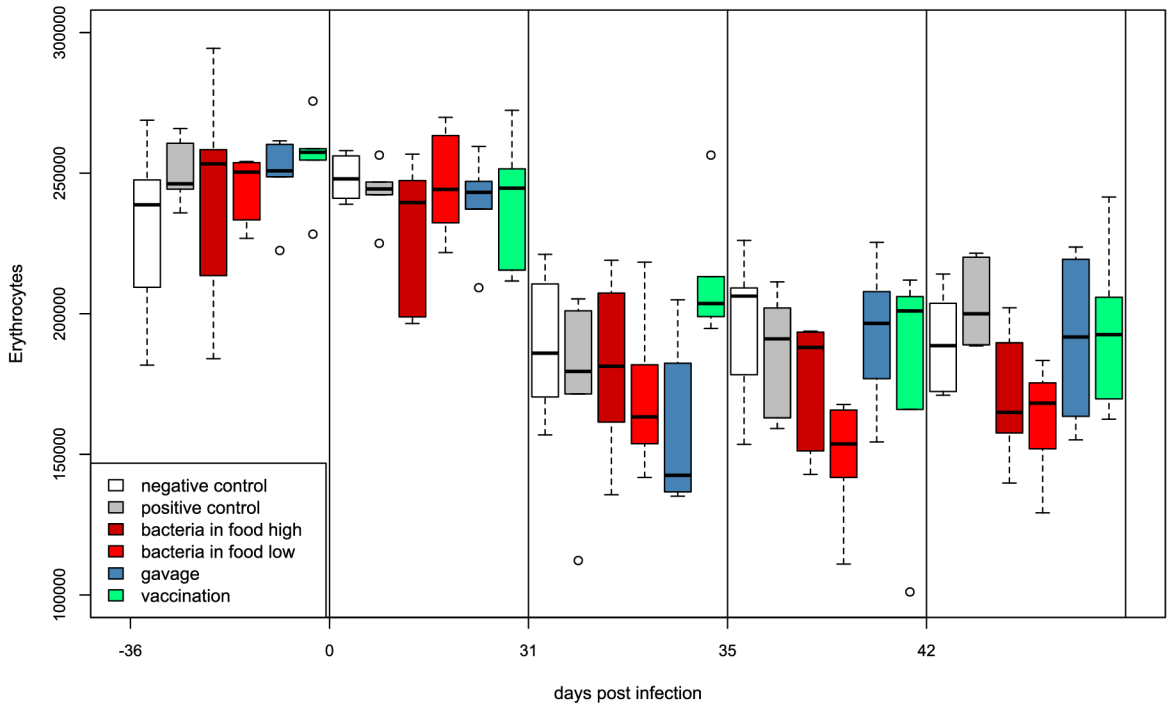

Figure 9 Erythrocyte count in each treatment group at different days during treatment and prior to and after infection with *S. molnari*. Each section, divided by vertical lines, displays the results for one day of sample measurements. x-axis represents the dpi at the time of taking the sample, while y-axis indicates the total number of erythrocytes counted. Note artificial drop in erythrocyte number from day 31 onwards, due to a different lot of DiOC6 used in the diagnostic procedure.

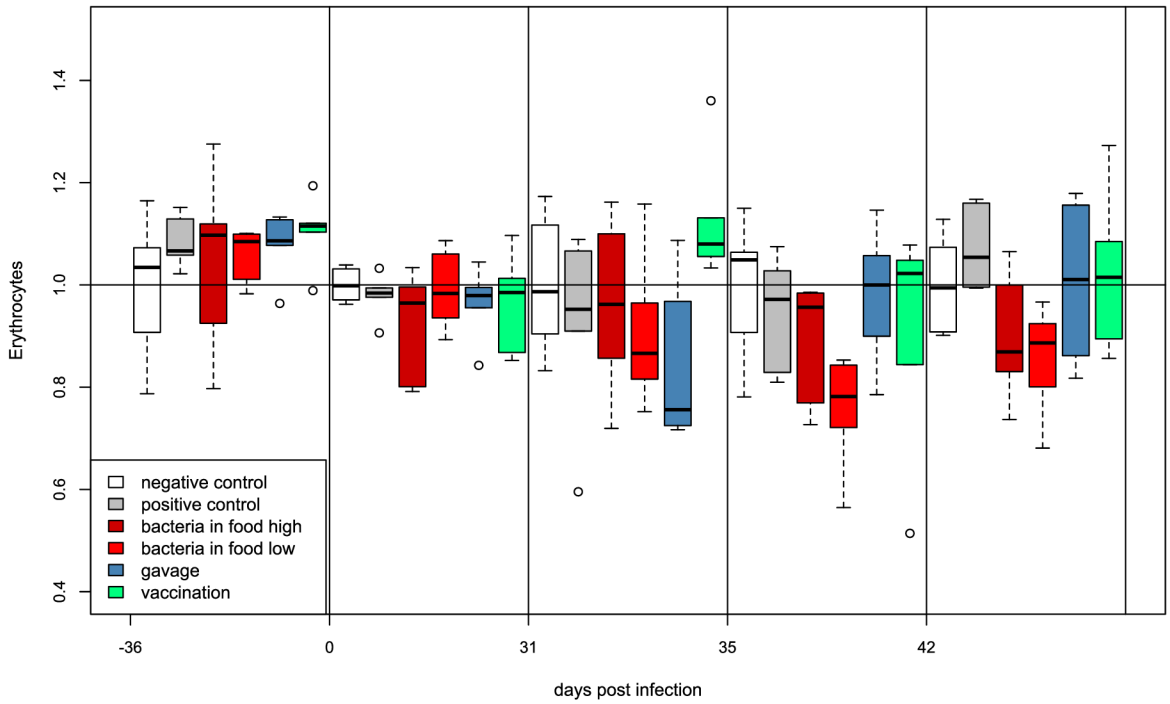

Figure 10 Relative erythrocyte Count (data from Fig. 10 proportional to the negative control – no treatment, no infection). The horizontal black line indicates the average number of erythrocytes in the negative control for each day. Y-axis represents the relative quantity of erythrocytes, while x-axis shows dpi at the time of taking the sample.

To visualize changes in blood cell composition, each fish was ranked according to cell number and then compared with the fish with the same ranking on the consecutive measurement. The difference between average percental change per group and average percental change to the negative control group was then plotted in *Figure 11*.

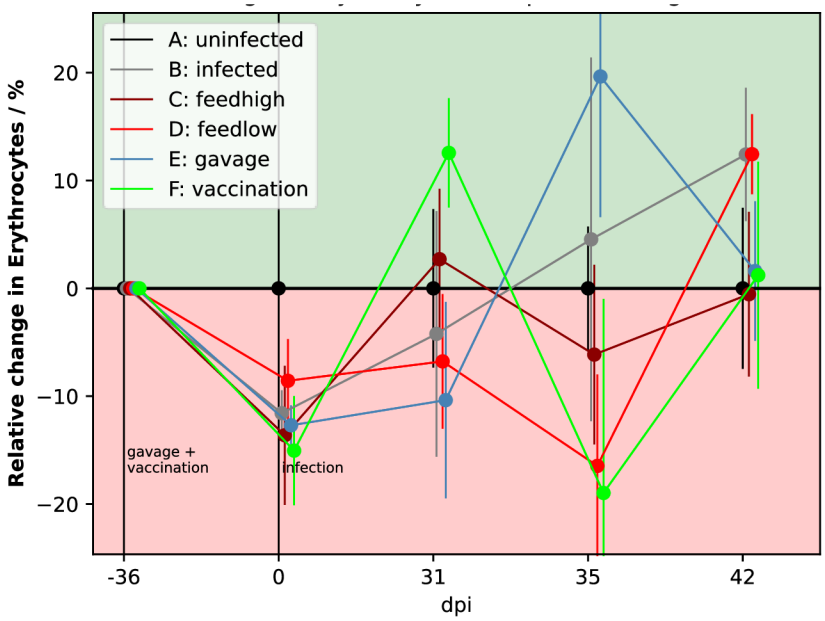

Figure 11 Relative change in erythrocyte numbers compared to negative control, including standard error bars. Each dot represents the average change in erythrocyte count, compared to the previous measurement. y-axis shows percental difference of erythrocytes compared to average negative control (black line). x-axis shows dpi of measurement.

4.2.2. Leukocytes

Changes in Leukocyte counts are depicted in *Figure 12.*

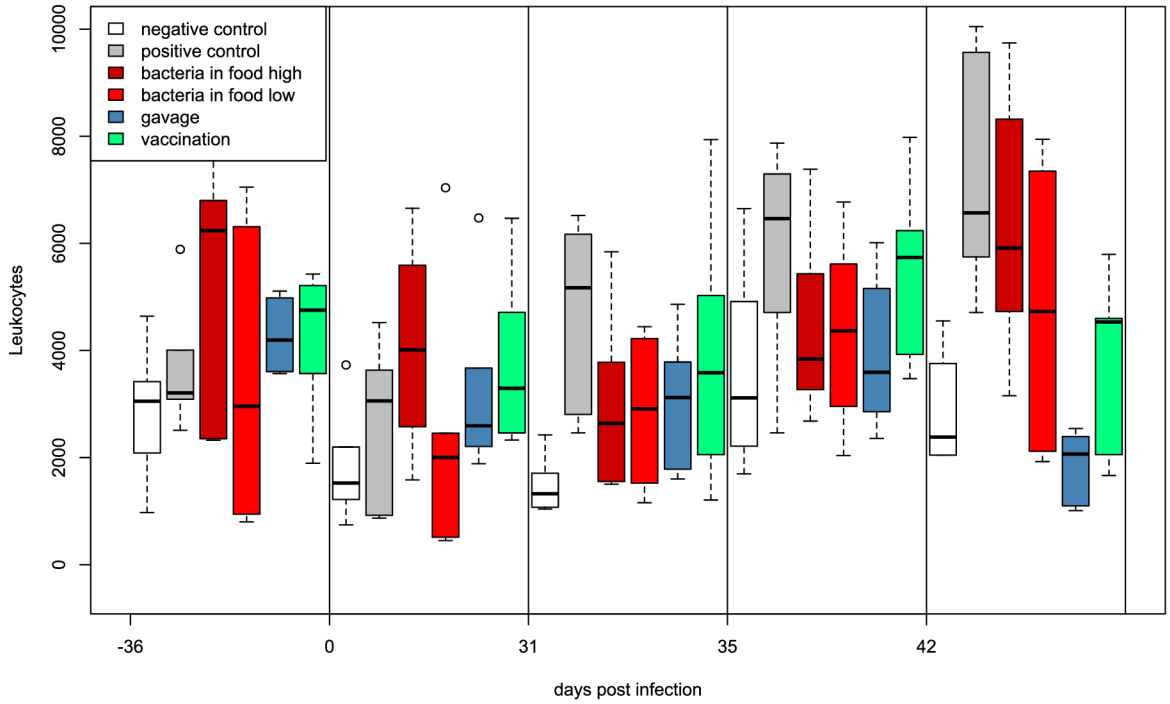

Figure 12 Leukocyte counts per day and treatment groups. y-axis shows total leukocyte count, x-axis shows dpi at the time of sampling.

Fluctuations between measuring days were visualized according to the same procedure as for the erythrocyte numbers, visible in *Figure 13.*

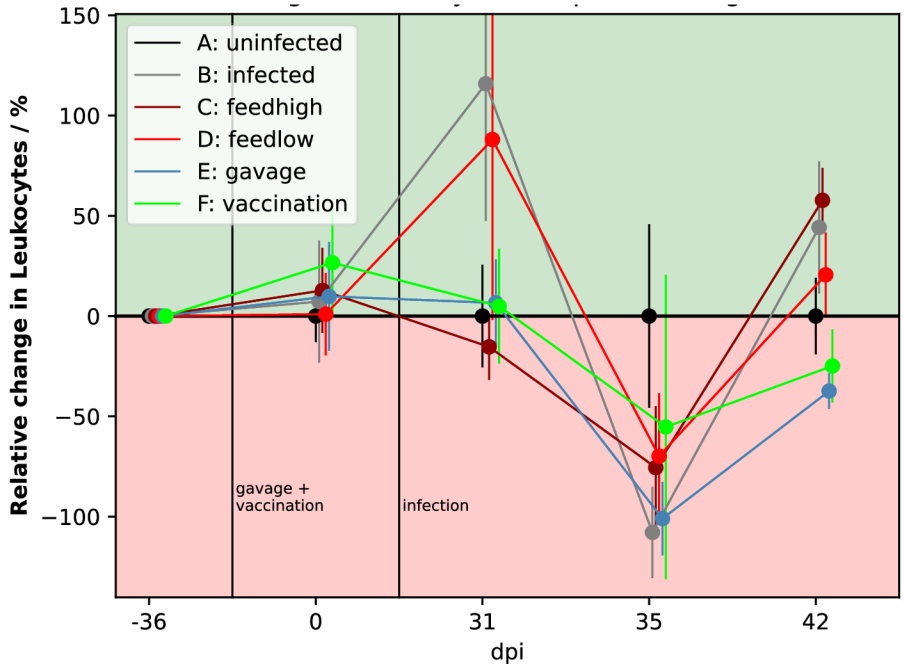

Figure 13 Relative change in leukocyte numbers compared to negative control, including standard error bars. Each dot represents the average change in leukocyte count, compared to the previous measurement. y-axis shows percental difference of leukocytes compared to average negative control (black line). x-axis shows dpi.

4.2.3. B cells

B cell counts are depicted in *Figure 14* as boxplots per group and in *Figure 15* according to fluctuation data transformation.

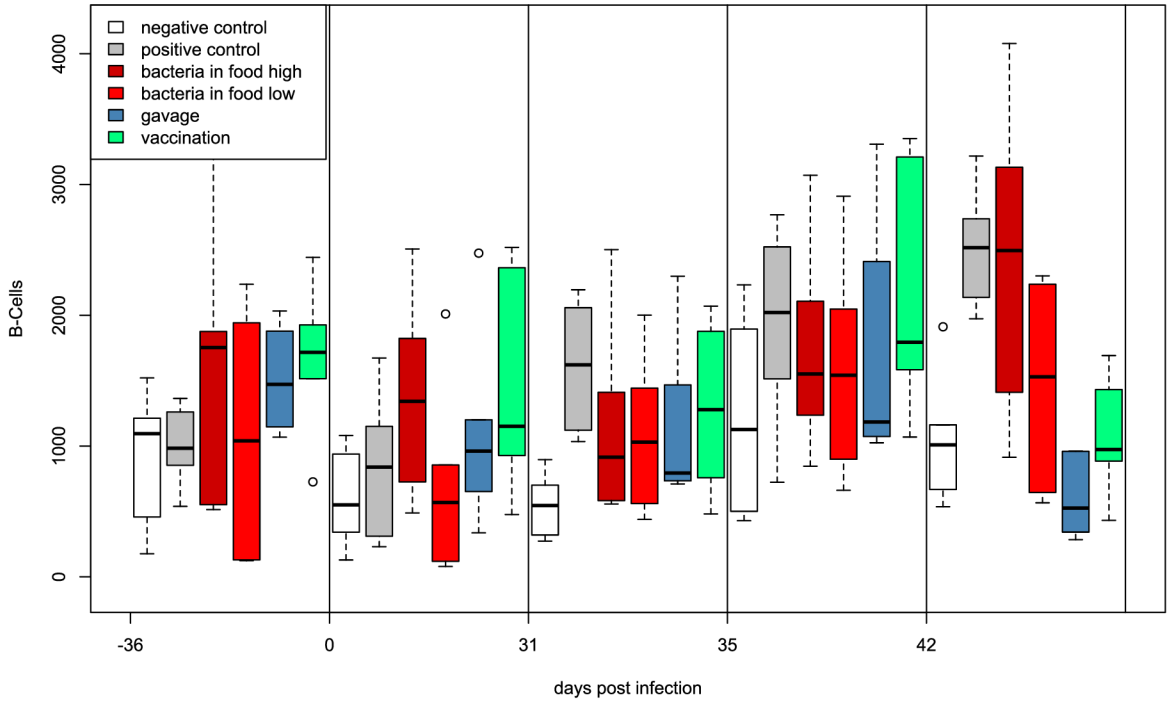

Figure 14 B cell counts per day and treatment group. y-axis shows total B cell count, x-axis shows dpi at the time of sampling.

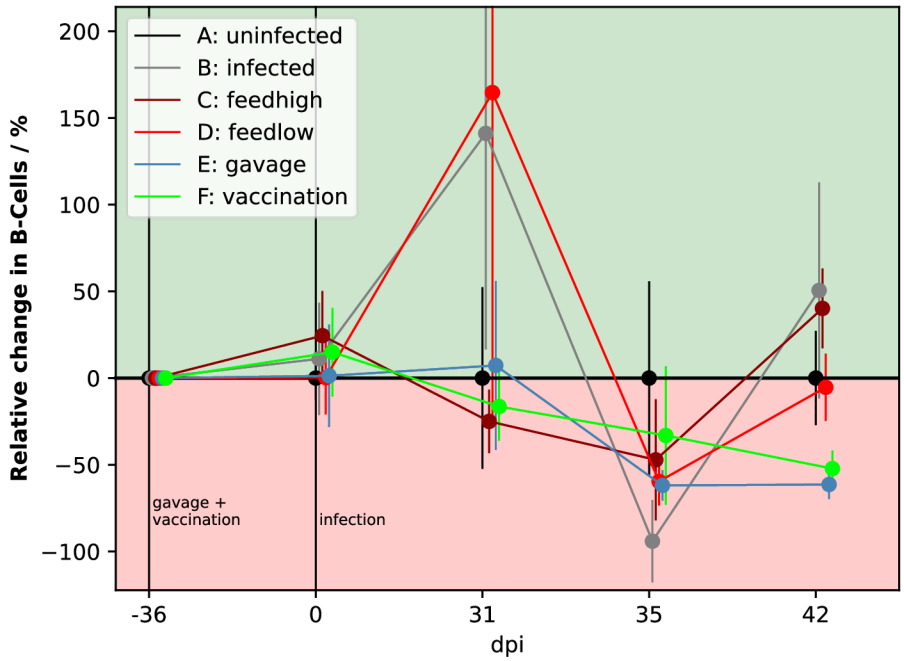

Figure 15 Relative change in B cell count compared to negative control, including standard error bars. Each dot represents the average change in B cell count, compared to the previous measurement. y-axis shows percental difference of erythrocytes compared to average negative control (black line). X-axis shows dpi of measurement.

4.3. ELISA

To determine antibody titers at all stages throughout the infection, blood serum was isolated from blood samples and incubated with parasite lysate coated onto a 96-well plate. ELISAs were performed using sera from experimental fish and an anti-carp IgM antibody.

For each group of samples, a calibration curve was generated using blood of strongly infected fish with confirmed affinity towards *S. molnari* lysate antigen. A serial two-fold dilution of the sample, starting at 1:50 to 1: 6400 was prepared. A linear regression analysis as depicted in *Figure 16* was conducted on the relationship between the logarithm of the dilution factor and the logarithm of the absorbance values. The purpose of such a curve is to account for inter-assay variation between different plates, days, and readings of the experiment.

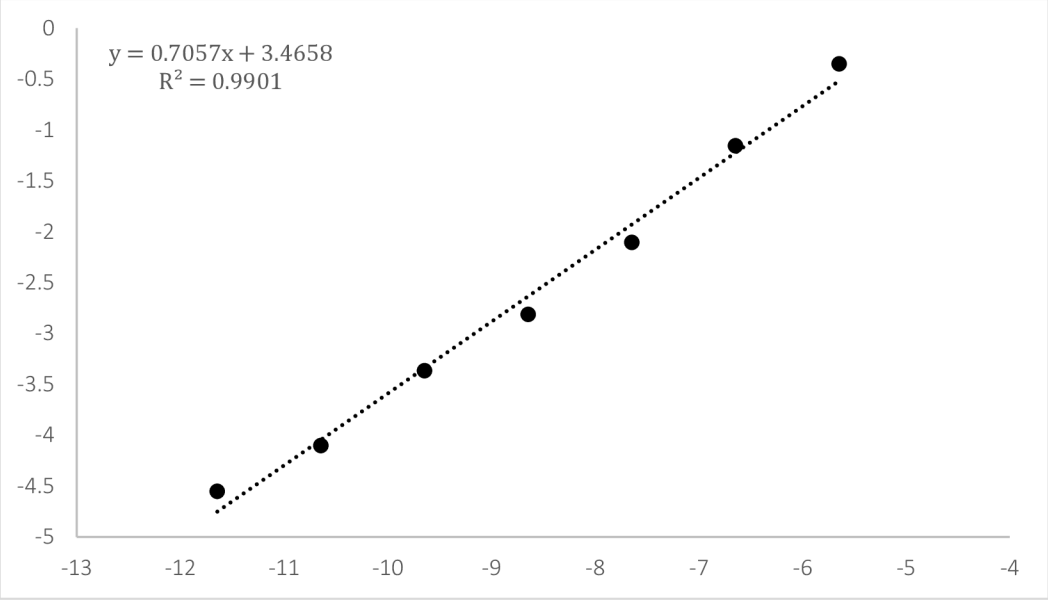

Figure 16 ELISA calibration curve generated at dpi -12. negative logarithm of dilution factor on x-axis vs logarithm of absorbance on y-axis. Not all values were included due to non-linearity in low and high concentrations.

Before measuring the blood samples from experimental fish, fish from both water systems in the facility (one holding infected and one holding uninfected fish) were checked for background contamination levels (potentially produced by floating myxospores) by taking blood samples of uninfected fish and performing ELISA according to the same protocol. Levels were found to be consistent across both systems (*Figure 17)*.

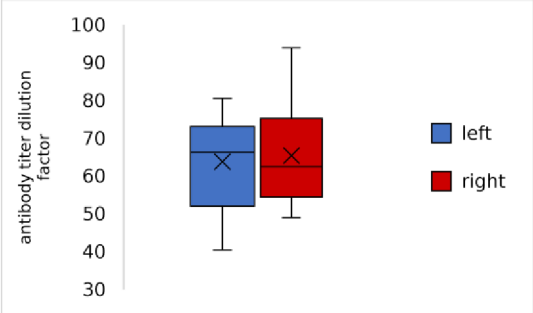

Figure 17 Background antibody titer levels measured in each of the water systems. No significant difference was observed.

The absorbance values measured for the left-right comparison were then used as a negative control, and the negative cut-off value was calculated according to *Equation 1* (Sacks & Gillette, 1988).

$$Cutoff_{abs} = a \cdot \bar{X} + f \cdot SD \quad (1)$$

a ..... mean multiplier, set to 1

$\bar{X}$ ..... arithmetic mean

f ..... standard deviation multiplier, set to 2

SD ..... standard deviation

The resulting absorbance of 0.083015 was below the measured absorbance for the calibration dilution of 1/800 which was determined to be the antibody titer of the positive control used in *Equation 2*. The log transformed dilution factor of

$$\left(2\text{^}(-6.64385619 - \frac{\log_2(ABS_{sample}) - d}{k}) * Titer_{dil}\right)^{-1} \quad (2)$$

ABS_{sample} .... absorbance for sample

d ..... calibration curve y-axis intercept

k ..... calibration curve slope

$Titer_{dil}$ ..... positive cut-off dilution factor, 1/800

Absorbance values were measured, and titers were calculated according to *Equation 2*. The results varied greatly, and antibody titers were generally low across all groups. Still, as shown in *Figure 18* as well as the average values in *Figure 19,* antibody levels were highest in the vaccine group.

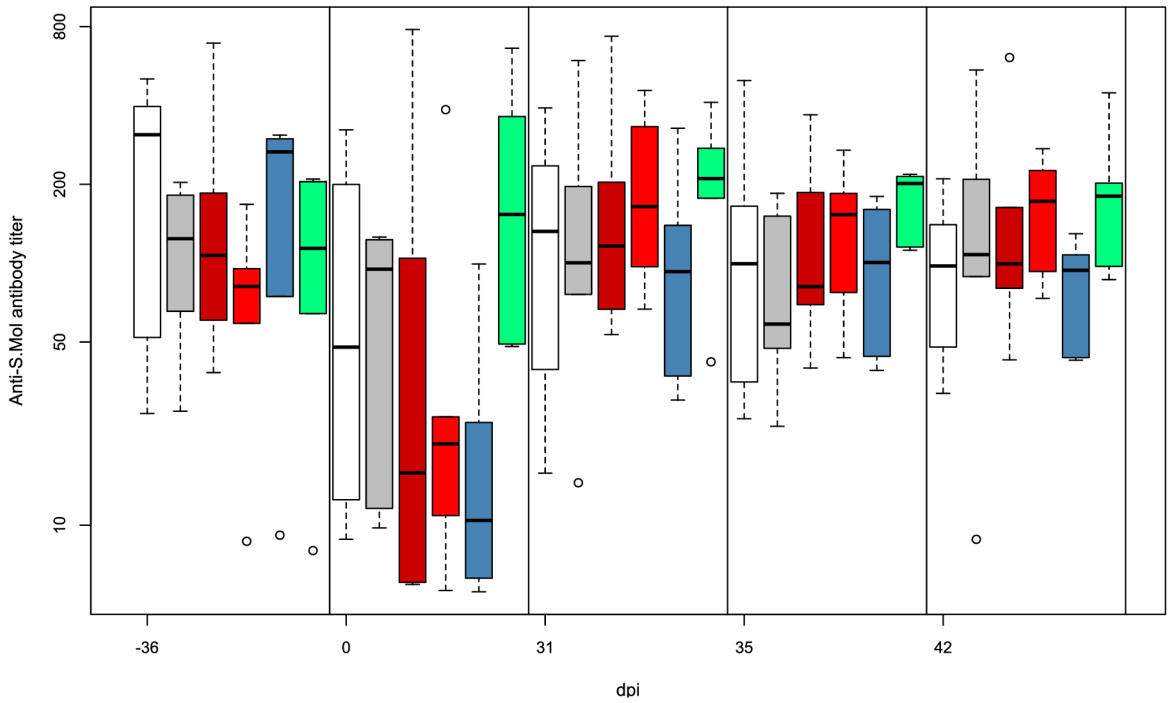

Figure 18 Results for ELISA with parasite lysate coating to measure anti-*S. molnari* antibody titers of blood sera. Y-axis represents dilution titer calculated using the linear regression calibration curve and *Equation 2*. Boxplots are grouped together by days post-infection (dpi). From left to right: dpi -36: pre-bacterial treatment, pre-*S. molnari* infection; dpi 0 post-bacteria treatment, pre-*S. molnari* infection (day of infection); remaining boxes are at dpi 31, 35, 42.

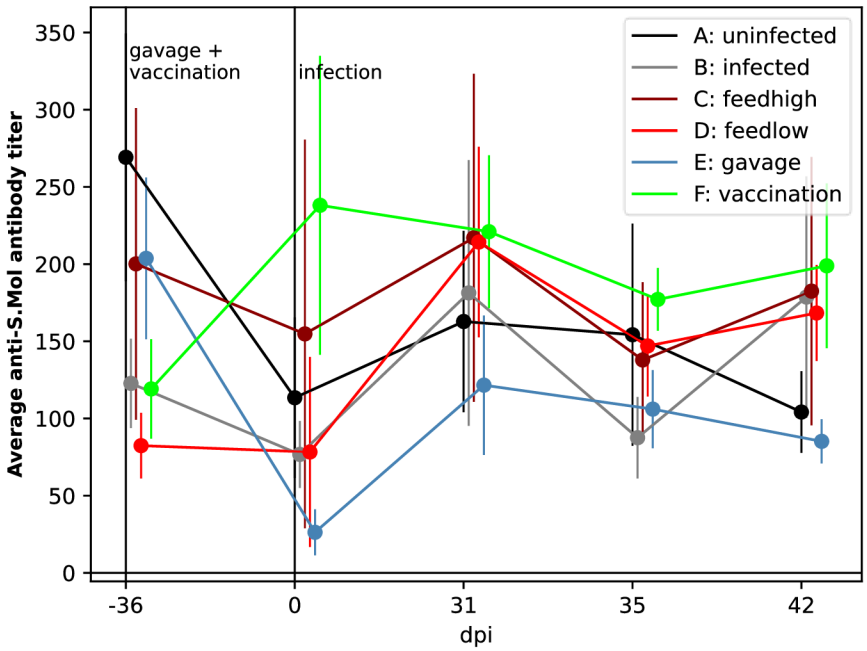

Figure 19 Average results per group for ELISA with lysed parasite coating to measure anti-*S. molnari* antibody titers of blood sera. Y-axis shows titer calculated according to linear regression calibration curve and *Equation 2*.

4.4. qPCR

Blood samples were taken three times post-infection and since blood stage peak times can vary and are short-lived, additionally, gill and liver samples were extracted on the last day of the experiment. As shown in *Figure 20* Ct values for blood were mostly between 30-40, indicating non-peak sampling, while gills and liver showed much higher concentrations of *S. molnari*. Lowest parasite numbers across all sample types were observed in the gavage group indicating a lower level of infection. Groups with bacterial in-feed treatment show the highest *S. molnari* numbers when compared to all other groups indicating presence of a larger amount of parasite in gills, liver, and blood. Trends are reflected in all samples of the relevant groups, though differences are not statistically significant.

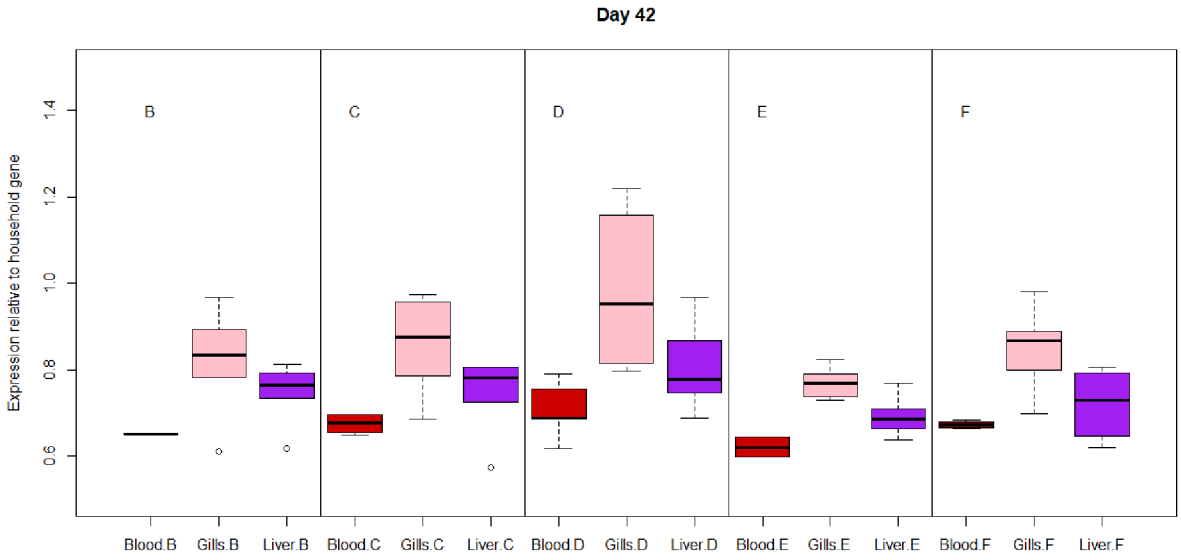

Figure 20 Quantification of *S. molnari* relative to carp housekeeping gene *beta-actin* at dpi 42. Each section shows expression per treatment groups, colors represent sample origin (red = blood, pink = gills, purple = liver).

5. Discussion

In this study, we aimed to determine the efficacy of mucosal immune stimulation with probiotic measures in *Cyprinus carpio* and develop a potential in-feed product for fish in aquacultures to treat or reduce symptoms caused by *Sphaerospora Molnari* infection. We established a method of incorporating *E. coli* Nissle 1917 into a commercial feed product and determined states of infection for thirty-six fish over 80 days using enzyme-linked immunosorbent assay, flow cytometry and quantitative PCR. Our results showed that vaccination provided the strongest protection against the infection, while oral gavage also yielded promising results. These findings contribute valuable knowledge about *S.molnari's* interaction with *Cyprinus carpio* and inform the development of immunostimulatory treatments.

5.1. Vaccination

There are currently no available vaccines against parasitic infections, primarily due to high costs of obtaining sufficient parasite lysate (Sommerset et al., 2005), confirming the need for alternative treatment options, but killed bacterial vaccines have shown to be efficacious against several diseases in fish (Muktar & Tesfaye, 2016).

Our results showed that vaccination had an immediate protective effect on erythrocytes while fish fed with low and high concentrations of bacteria-enriched diet had a significant loss of erythrocytes compared to other groups. Furthermore, a significant increase in antibody titer was observed for the vaccine group, further confirming the efficacy of the treatment.

The fact that intraperitoneal injection produced more antibodies suggests that the vaccine's effects on erythrocyte count may be due to differences in absorption compared to other treatments.

5.2. Gavage

An increased growth rate in the gavage group was observed, which is in line with previous research (Boonthai et al., 2011). Additionally, a promising recovery in erythrocytes despite low antibody levels might indicate a component of innate immunity rather than just antibody-mediated immunity. These observations imply that while intraperitoneal vaccination is effective, oral administration can also yield beneficial results. However, the degree of degradation of bacterial antigens in the stomach poses a challenge for oral administration (Mutoloki et al., 2015). Furthermore since gut microbial flora strongly influences the immune system (Talwar et al., 2018), it remains unclear whether these effects result from direct immune stimulation or gut microbiome stimulation.

5.3. In-feed

The beneficial effects of probiotic in-feed products on growth (Kennedy et al., 1998), immunostimulation through other polysaccharides like β-1,3 glucan (Ai et al., 2007) and antagonism against pathogens (Robertson et al., 2000) have been proven extensively.

In this trial, when comparing low and high bacterial concentrations in feed, high concentrations appear to offer better protection against the parasite but at the cost of reduced growth rates. This finding may indicate that lower doses provide optimal stimulation, while higher concentration could result in settlement of treatment bacteria and disruption of natural microbiome. The fact that higher concentrations might lead to inferior protection has been confirmed in previous studies (Irianto & Austin, 2002).

5.4. Summary

The significant loss of erythrocytes in Groups C and D, coupled with the immediate protective effect observed in the vaccination group, indicates that administering bacteria concentrate peritoneally may be more effective than providing it through diet, however oral administration still showed promising results in the gavage group suggesting that a refinement of bacterial feed concentration and feeding intervals might yield similar results when only administering the in-feed treatment, as probiotic, in-feed treatment with high α-Gal content has shown protective effects against unrelated pathgogens (Pacheco et al., 2021). The bacterium used in this trial, *E.Coli* was shown to express α-Gal in previous research (Yilmaz et al., 2014).

The generally low antibody response across all groups might be due to the utilization of an S. molnari batch with lower infectivity or an insufficient number of parasites per dose.

Our findings suggest that vaccination is the most effective method among those evaluated to protect fish against *S. molnari* infection, potentially making it a viable option for controlling the disease in aquaculture settings. Furthermore, these results contribute valuable knowledge about *S. molnari's* interaction with *Cyprinus carpio* and shed light on the different treatment approaches' effectiveness.

Future studies should consider extending the duration of the in-feed treatment prior to parasitic exposure, which may prove advantageous by allowing the fish to address initial inflammation before encountering the parasite. To gain a more comprehensive understanding of the immune response, assays focusing on the immune system's reaction to pure alpha-gal could be performed because we have only indirectly measured reaction to alpha-gal and cannot confirm its levels in the parasite lysate.

Investigating changes in microbiome composition within feces or water in the tank after feeding could provide additional insights into how bacterial treatments impact gut microbiota and subsequently influence fish immunity. Furthermore, addressing outliers related to certain fish potentially grabbing more food than others due to size differences could enhance experimental accuracy and ensure that all subjects receive consistent treatment doses. Generally a trial employing several different concentrations and intervals for bacterial feeding might yield advantageous results, due to the major influence of dosage on the immunological effects (Mutoloki et al., 2015).

Lastly, incorporating a control group with no parasitic challenge but only bacteria administration would allow to better understand the effects of bacterial treatments on fish health and immunity independent of infection.

6. Conclusion

Our findings suggest that alpha-gal rich bacteria represent a good prophylaxis for alpha-gal positive parasites in alpha-gal negative fish. intraperitoneal vaccination offers immediate protection against *S. molnari* and hemolytic anemia and induces a strong immune response, while oral gavage also presents promising results, particularly with respect to growth rate and lower parasite numbers possibly due to a form of adaptive immune system response. It remains unclear why the in-feed treatment did not lead to a similar immune stimulation as observed in the vaccine or the gavage group. Possible causes might be a settlement of E-Coli Nissle 1917 or an inflammation due to continuous stimulation, potentially weakening the immune system. Further research should focus on understanding the role of innate immunity and gut microbial flora in the observed responses, as well as refining treatment strategies using intermittent feeding to maximize efficacy without compromising growth rates.

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