Optimized encapsulation substances and feeding regimes for administering an oral vaccine in rainbow trout, *Oncorhynchus mykiss* (Walbaum)

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Contents

List of abbreviations

AI	Artificial intelligence
ANOVA	Analysis of variance
ASA	Active surface area
BSA	Bovine serum albumin
CF	Condition factor
DFI	Daily feed intake
GC	Goblet cells
GiT	Gastro-intestinal tract
H/W ratio	Height / width ratio of intestinal villi
HSI	Hepatosomatic index
i.p.	Intra-peritoneal
Ig	Immunoglobulin
IL	Interleukin
MPI	Minimum pellet intake
PEG	Polyethylene-glycol
PIT	Passive integrated transponder
PLGA	Poly lactide-co-glycolide
PM	Protective matrix
RAS	Recirculation aquaculture system
RGC	Relative gene copies
RPS	Relative percent of survival
RR	Retention rate
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SGR	Specific growth rate
SOD	Superoxide dismutase
SSI	Spleen somatic index
TNF	Tumor necrosis factor
VAC	Vacuole
VIE	Visible implant elastomer
VSI	Visceral somatic index
WG	Weight gain

Zusammenfassung

Impfungen bei Tieren sowie bei Menschen sind eine der wertvollsten Errungenschaften der Menschheit, um krankheitsbedingte Herausforderungen einer sich intensiv verändernden Umwelt zu bewältigen. Während Impfstrategien mit Injektions-Impfstoffen für terrestrische domestizierte Tierarten gegenwärtig effektiv entwickelt sind, ist die Impfung von Tierarten in der Aquakultur weltweit bisher keine gängige Praxis. Aufgrund des hohen Arbeitsaufwandes sowie technologischem und finanziellem Aufwand finden injektive Impfungen in der Aquakultur in Entwicklungs- und Schwellenländern kaum Anwendung. Die Anwendung von Oralvakzinen bietet demnach als Alternative zu injektiven Impfungen viele Chancen. Sie können anwendungsbezogenen Probleme bewältigen und dem Tierwohl, der Umwelt, wie auch der Produktionseffizienz zu Gute kommen. Um eine effektive Oralvakzine entwickeln zu können, sind jedoch noch Hürden zu bewältigen, wie die Frage nach der gezielten, oralen Verabreichung und des wirksamen Schutzes vor der Zerstörung der aktiven Vakzine durch das Systemwasser oder durch enzymatische sowie chemische Degeneration.

Die drei Kapitel dieser Dissertation beschäftigen sich mit der Optimierung des Fütterungsregimes, sowie mit der Evaluierung unterschiedlicher Impfstoffträger- / Verkapselungssubstanzen am Beispiel der Zieltierart Regenbogenforelle (*Oncorhynchus mykiss*). Die Ergebnisse dieser Arbeit ermöglichen nicht nur eine kontrollierte und sichere Verabreichung einer Oralvakzine, sondern sind für das Hervorbringen einer effektiven Herdenimmunität nach oraler Impfung unabdingbar.

In Kapitel 1 wurden die Auswirkungen verschiedener Fütterungsregimes auf das individuelle Fressverhalten von juvenilen, in einer Kreislaufanlage (RAS) kultivierten Regenbogenforellen untersucht. Die veränderten Parameter des Fütterungsregimes umfassten Ausnüchterungsperioden, die Anzahl der täglichen Fütterungen und die Anzahl der Futterzugaben pro Fütterung. Diese zeigten einen signifikanten Einfluss auf die Homogenität und Gesamtmenge der Futteraufnahme. Um das Fressverhalten während der verschiedenen Fütterungsszenarien zu bewerten, wurde die minimale Pelletaufnahme (MPI) als Maß für eine garantierte Pelletaufnahme pro Individuum etabliert. Dabei beschreibt die MPI den Anteil an gefütterten Fischen, der eine

Mindestanzahl an Pellets gefressen hat. Im Hinblick auf die Anwendung als Schluckimpfung kann die MPI für die Impfstoffquantifizierung eines Impfpellets verwendet werden. Die Ergebnisse des Ausnüchterungsversuches zeigten, dass die Fresshomogenität bei unveränderter aufgenommener Futtermenge erhöht werden kann, wenn die Tiere keinerlei Ausnüchterungsperiode erfahren. Die Anzahl der täglichen Fütterungen kann genutzt werden, um die Gesamtmenge des aufgenommenen Testfutters bei gleichbleibender Futterhomogenität zu erhöhen. Je nachdem, wie viel Impfstoff bzw. wie viele Pellets pro Fisch intendiert sind, kann die Anzahl der täglichen Fütterungen (ein- oder zweimalige Fütterung pro Tag) die MPI erhöhen, ohne dass dies zulasten der Aufnahmehomogenität geht. Eine zusätzliche dritte Fütterung führte indes zu einer deutlichen Verringerung der Aufnahmehomogenität und wird demzufolge bei der Anwendung einer Schluckimpfung nicht empfohlen. Vorteilhaft für die MPI und die Aufnahmehomogenität ist die Verwendung mehrerer Portionen während eines Fütterungsereignisses. Hier zeigte die Aufteilung auf drei und fünf Portionen pro Fütterung einen deutlichen Vorteil gegenüber nur einer Futterzugabe bei gleicher Gesamtmenge. Insgesamt kann die Optimierung des artspezifischen Fütterungsregimes dazu genutzt werden, die Pelletaufnahme und -homogenität zu erhöhen und eine minimale Pelletaufnahme pro Fisch zu garantieren.

In Kapitel 2 wird die Forschungsfrage untersucht, inwiefern die Substanzen Alginat und Polyethylenglykol (PEG) geeignet sind, um die aktiven Impfstoffkomponenten während der Fütterung vor der Loslösung in das Systemwasser und später vor dem enzymatischen und chemischen Abbau im Fischdarmtrakt zu schützen. Um eine potenziell schädliche Wirkung von Alginat und PEG auf den Verdauungstrakt von Jungforellen auszuschließen, wurden die Testsubstanzen, eingebettet in ein kommerzielles Futter, 22 Tage lang kontinuierlich gefüttert und verschiedene Darmabschnitte der Versuchstiere histomorphologisch ausgewertet. Zudem wurden die Genexpression der proinflammatorischen Gene TNF-α, IL-1β und IL-8 untersucht, um eine eventuell auftretende systemische Entzündung auszuschließen. Die Fütterung beider Substanzen über den festgelegten Testzeitraum führte zu keiner signifikanten intestinalen Beeinträchtigung oder entzündlichen Genreaktion. Die Beimengung von PEG zum Fischfutter führte mitunter zu signifikanten Veränderungen des Darmgewebes, zu

vermindertem Wachstum und zu einem leicht erhöhten Milzgewicht. Beide Substanzen wurden für die Verwendung bei Oralvakzinationen bei einmaliger bzw. bei einer Booster-Immunisierung als unbedenklich eingestuft.

In Kapitel 3 wurden die drei Verkapselungssubstanzen Alginat, PEG, Chitosan sowie ein zusätzliches hydrophobes Öl-Coating auf ihre biophysikalischen Fähigkeiten untersucht, wie sie jeweils ein verkapseltes Modellprotein unter unterschiedlichen pH-Milieus freigeben. Darüber hinaus wurden in situ die Verdauungsgeschwindigkeiten der Testsubstanzen in Verbindung mit einem mit Eisenpuder verabreichten Futtermittel anhand von Röntgenaufnahmen evaluiert. Die Verkapselungsversuche zeigten, dass insbesondere die Verwendung von Alginat mit Chitosan eine ideale Verkapselung und ein optimales Freigabeprofil bei einer modellierten Fütterung darstellten. In der Regel führt das Einbringen der Kapsel in das Systemwasser zu einer starken initialen Freigabe des Modellproteins in das Systemwasser. Dieser „initiale Burst“ kann jedoch durch die Verwendung eines hydrophoben Öl-Coatings stark reduziert werden. Die Beigabe von PEG ermöglicht es, höhere Freigaberaten zu erzielen, jedoch leidet gleichzeitig der Verkapselungsschutz. Die Verwendung einer Alginat-Chitosan-Verkapselung bietet bei einer Schluckimpfung einen signifikanten Schutz gegen Zerstörung durch das Systemwasser und vorzeitige Degeneration im Fischverdauungstrakt. Bei der Evaluierung der Verdauungsgeschwindigkeiten konnten keine signifikanten Unterschiede zwischen den Behandlungen festgestellt werden. Es zeigte sich, dass alle Testfuttermittel den Enddarm nach 16 h erreicht haben und weitere Anpassungen der Verkapselung sollten auf eine maximale Impfstofffreigabe 16 h nach Verabreichung abzielen. Die Ergebnisse erlauben eine gezielte Optimierung der Verkapselungsmatrix beim Schutz der bioaktiven Substanz und der Freigabe am Zielort (Fisch-Enddarm).

Die neuen Erkenntnisse dieser Dissertation tragen zur Lösung aktueller Probleme bei der Entwicklung eines oralen Impfstoffs für die Aquakultur durch die Optimierung der Fütterungsregime und Vakzine-Trägersubstanzen bei. Sie bilden hierbei nicht nur die Grundlage für die optimale Verabreichung und Verkapselung eines oralen Impfstoffes, der für einen effektiven Impfschutz unerlässlich ist, sondern auch für die Verabreichung anderer hochwertiger bioaktiver Substanzen, für die eine definierte Verabreichung von zentraler Bedeutung ist.

Summary

Vaccination in animals as well as humans is one of mankind's most valuable achievements in meeting the challenges of a markedly changing environment. While vaccination strategies using injection vaccines for terrestrial animals are currently effectively developed, vaccination of aquaculture species is not a common practice worldwide, mainly due to difficulties in vaccine delivery. Injectable vaccination in aquaculture involves high labor, technological and financial inputs-, and is therefore less widely used in developing and emerging countries. An oral vaccine could overcome delivery-related problems while benefiting animal welfare, the environment, and production efficiency. However, hurdles remain in the development of an oral vaccine, such as the issue of targeted oral administration and effective protection against destruction of the active vaccine by system water, along with enzymatic and chemical degeneration.

The three chapters of this dissertation aimed to optimize and evaluate vaccine-independent delivery using the target species rainbow trout (*Oncorhynchus mykiss*) as an example. Such optimization and evaluation are essential for effective herd immunity after oral vaccination.

Chapter 1 examined the effects of different feeding regimes on the individual feeding behavior of juvenile rainbow trout cultured in a recirculating aquaculture system (RAS). The parameters of the feeding regimes that were altered included short-term starvation periods, the number of daily feedings, and the number of feed additions per feeding. These showed a significant effect on homogeneity and total amount of feed intake. To evaluate feeding behavior during the different feeding scenarios, minimum pellet intake (MPI) was defined as a measure of guaranteed pellet intake per fish. With respect to its use as an oral vaccine, the MPI can be used for vaccine quantification of a vaccine pellet. Results showed that feeding homogeneity can be increased with no change in the amount of feed ingested if the animals do not experience any short-term starvation period. The number of daily feedings can be used to increase the total amount of test feed consumed, while maintaining feed homogeneity. Depending on the amount of inoculant or pellets intended per fish, the number of daily feedings (one or two feedings

per day) can increase MPI without sacrificing intake homogeneity. However, an additional third feeding resulted in a significant decrease in intake homogeneity and is therefore not recommended when using oral vaccination. Beneficial for MPI and intake homogeneity is the use of multiple portions during a feeding event. Here, three and five portions per feeding showed a clear advantage over only one feed addition. Overall, optimization of the species-specific feeding regime can be used to increase pellet intake and homogeneity and that a specific minimum number of pellets is guaranteed to be consumed by each fish.

The research question posed in Chapter 2 targeted the substances alginate and polyethylene-glycol (PEG), which are used to protect the active inoculant components from dissolution into the system water during feeding and later from enzymatic and chemical degradation in the fish intestinal tract. To rule out a potentially harmful effect of alginate and PEG on the digestive tract of juvenile trout, experimental animals were fed the test compounds for 22 days, continuously, and their different intestinal sections histomorphologically examined. In addition, gene expression of the proinflammatory genes TNF-α, IL-1β, and IL-8 was examined to detect or rule out systemic inflammation. Feeding both substances for the specified test period did not result in significant intestinal impairment or inflammatory gene responses. A significant change in some measured intestinal tissue parameters were detected, with decreased growth and slightly increased spleen weight when PEG was added to the fish diet. Both substances are to be considered suitable for use in oral vaccination delivery over short periods.

Chapter 3 investigated the three encapsulation substances alginate, PEG, chitosan and an additional hydrophobic oil coating for their biophysical abilities to release an encapsulated model protein under different pH environments. In addition, the digestion rates of the test substances were evaluated in conjunction with a feed administered with iron powder using radiographs. The encapsulation experiments showed that especially the use of alginate with chitosan provided an ideal encapsulation and release profile in a modeled feeding. In general, the introduction of the capsule into the system water results in a strong initial release of the model protein into the system water. However, this "initial burst" can be greatly reduced by the use of a hydrophobic oil coating. The addition of PEG allows higher release rates to be achieved, but at the same

time the encapsulation protection is reduced. The use of an alginate-chitosan encapsulation provides significant protection against destruction by system water and premature degradation in the fish digestive tract when swallowed. No significant differences were found when digestion rates were evaluated. All test feeds were found to reach the posterior intestine after 16 h and further adjustments to encapsulation should aim for maximum bioactive release 16 h after administration. The results allow a differentiated application of the encapsulating substances in order to optimize oral delivery of bioactives.

The new findings presented in this dissertation contribute to the solution of current problems in the development of an oral vaccine for aquaculture by optimizing feeding regimes and vaccine carrier substances. In this regard, they form the basis not only for the optimal delivery of an oral vaccine, but also for the delivery of other high-value bioactive substances for which defined delivery is of key importance.

General Introduction

Historical background and topic importance of aquaculture

As stagnating wild catches of fish from marine and freshwater systems failed to meet growing demand, rapidly expanding aquaculture production has contributed an increasing share of total fish production to satisfy the needs of 7.9 billion humans in 2022 (United Nations 2022). While in the 2000s, total global fisheries and aquaculture production was 134.3 Mt, in 2020 that figure reached 177.8 Mt. Thereby the share of aquaculture increased from 32.3 % to 49.2 %, respectively (Figure 1, (FAO 2022)). The contribution of aquaculture to human food applications has exceeded that of fisheries since 2013. This increase of production is due to the intensification of the aquaculture sector, development of new fish species for aquaculture cultivation and rearing methods. In the early years, aquaculture production depended primarily on fishmeal and fish oil. Feed requirements for macro- and micronutrients were determined by empirical experiments with limited fish sizes and under laboratory conditions (Cowey 1992), information from aquaculture industry were left unevaluated (Glencross et al. 2021).

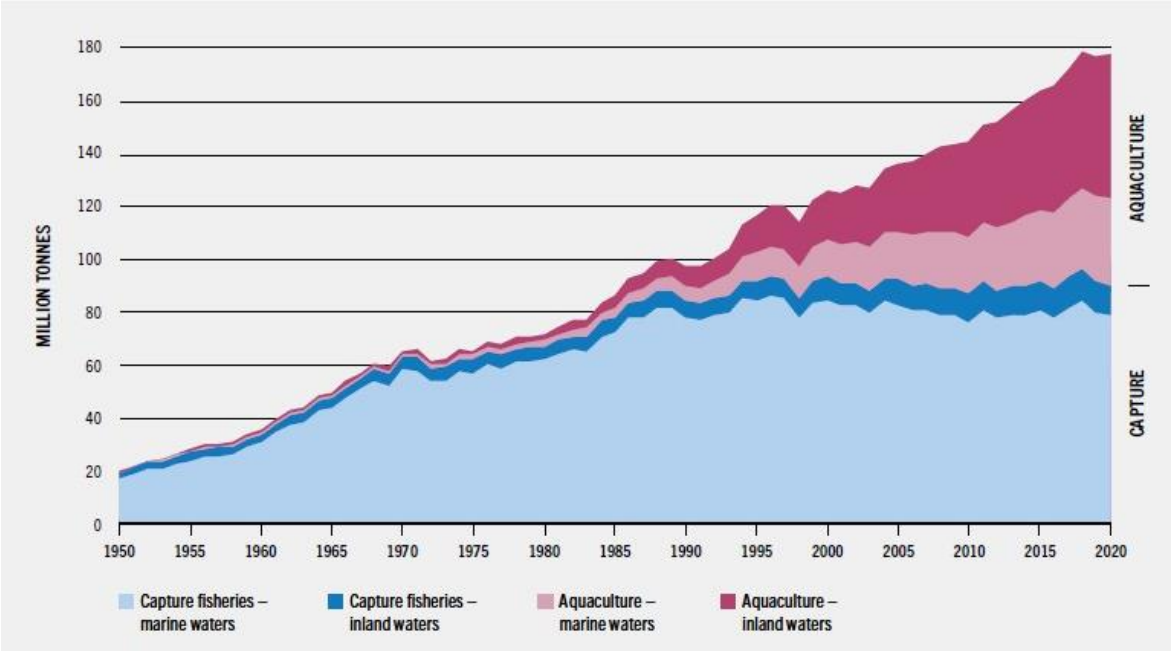

Figure 1 *World capture fisheries and aquaculture production. Not included in the data are aquatic mammals, crocodiles, alligators, caimans and algae. The y-axis presents live weight. Graphic extracted from FAO (2022)*

This lack of information led to aquafeeds with low feed efficiency ratios. This along with rapid expansion of the aquaculture industry led to the marine resource for aquafeeds becoming increasingly exhausted. This problem was addressed through research on feed requirements and alternative feedstocks. Feed efficiency increased, the use of fishmeal and fish oil was significantly decreased with increasing aquaculture production (Naylor et al. 2021). Today, feed requirements for all large cultivated fish in aquaculture are evaluated precisely, with highly accurate information, specifically on the amino acid requirements (Council 2011, Cowey 1992, Glencross et al. 2021) enhancing the sustainability of the products from aquaculture production. The expanding aquaculture sector not only creates higher demand for feedstock, cultivation areas or increasing stock densities (North et al. 2006), it also entails detrimental consequences for the environment such as increased waterway loading (Edwards 2015, Eng et al. 1989) by eutrophication or uncontrolled release of antibiotics / pharmaceuticals. A prominent formative event in European aquaculture was the decimation of salmonid farming in Norway during the 1980s due to bacterial diseases such as vibriosis or coldwater vibriosis, which was mainly treated by the use of antibiotics at the time (Sommerset et al. 2005). As a consequence, the use of antibiotics increased from 3.7 t in 1980 to 48.6 t in 1987 (Grave et al. 1990). Fish losses due the disease “crisis” were up to 90% of cultured fish (Hjerde et al. 2008). To reduce the use of antibiotics and to increase fish health and aquaculture production, immersion vaccines and injective intraperitoneal (i.p.) vaccines were developed and used with great success. While very effective, these methods unfortunately incur high labor, capital and other financial cost and thus, especially for underdeveloped countries, the use of antibiotic as preventive action and disease treatment is still common practice (Henriksson et al. 2018).

Today, licensed vaccines for salmonids are available against infectious hematopoietic necrosis, infectious pancreatic necrosis, infectious salmon anemia, pancreatic disease virus, enteric redmouth disease, vibriosis, furunculosis, bacterial kidney disease, columnaris disease, lactococciosis, salmonid rickettsial septicaemia and wound disease (Ma et al. 2019). Of the available vaccines, only one vaccine against enteric redmouth disease is recommended for oral application. With increased fish vaccinations, outbreaks of diseases with meaningful losses and the use of antibiotics were significantly

reduced. Today, global aquaculture losses due to diseases has been reduced to just 10% of total production. This loss still amounts to more than 10 billion USD annually and illustrates the need for advanced vaccination and health enhancing treatments. The cost efficiency of fish vaccination was described by Lillehaug (1989) as a relation of vaccination cost depending on vaccination method and economical losses due to fish lost. Nonetheless, the labor intensive and thus relative expensive injective immunization showed best cost/efficiency compared to the available immersion vaccination (Lillehaug 1989). This calculation is primarily "tipped" by the lower efficacy of non-injective vaccinations. If immersive or oral vaccinations could obtain an efficacy level similar to injective vaccinations, they would be significantly more economically viable. Their optimization, including optimal delivery, is a pressing challenge for advancing future economic and environmental sustainability of aquaculture.

Vaccination routes in aquaculture

Immersion, injective and oral fish vaccination routes are known. All three come with different advantages and disadvantages. **Immersion vaccination** is suitable for mass vaccination of primarily immunocompetent juvenile fish. Thereby, the antigen is taken up by the skin, gills and the gut to trigger an immune response and hence protect the fish against the pathogen (Bøgwald and Dalmo 2019). Formalin killed bacteria (bacterin) were used in the early immersion vaccine developmental stages, with inconsistent und relatively low protective properties. Recent immersion vaccines such as AQUACAV-COL® use live attenuated viruses, which are licensed and are applied in aquaculture (Shoemaker et al. 2009). Generally, immersion duration and vaccine dosage are of high importance (Du et al. 2017) and immersion vaccines need to be boosted for complete protection. Nevertheless, protective effects are not long lasting (Deshmukh et al. 2012).

The commercially favored use of **injective intra-peritoneal (i.p.) vaccination** comes with higher immunization efficiency and long-lasting effects (Mitchell 1995). Nevertheless, it also results in higher fish stress (Embregts and Forlenza 2016), labor and financial costs and is limited in its application to vulnerable juveniles due to a requirement of a minimal fish size (>20 g) (Plant and LaPatra 2011). Injective vaccination can be performed manually or automatically, whereby the latter requires high levels of capital investment,

is significantly limited in fish size and requires fish anesthetization (Plant and LaPatra 2011). Vaccines for the injective route are multiple and range from bacterin, to attenuated vaccines to DNA vaccines. In most cases adjuvants are needed for a sufficient immune response and therefore entail drawbacks such as inflammation, granuloma and lesions of the site of injection with can reduces flesh quality to the consumer (Ashley 2007). The **oral vaccination route** promises the best prerequisites for effective and low-cost immunization, as it offers a solution as an effective, fish size-flexible method to increase fish health, welfare and pathogen resistance while reducing labor cost and acute stress (Mutoloki et al. 2015). In 1984, Nelson et al. compared the three routes to determine the uptake region of the vaccine. The outcome of the study was not promising as the oral route was not capable of producing any significant response in the affected tissues (Nelson et al. 1985).

Oral vaccines continue to exhibit low effectiveness and lack of lasting protection to this day when compared to injective immunization (Embregts and Forlenza 2016). This is mainly due to vaccine destruction in the acidic fish stomach (Rivas-Aravena et al. 2013) or by enzymatic degradation in the intestine (Jeong et al. 2020). Principally, the induction of an immune response can be initiated in all mucosal tissues of the fish but with different efficiencies. While targeting the mucosal tissues of gills or skin during immersion vaccination only induces short lasting protection as describes above, the intestinal mucosa of fish has the potential to induce a complete and long lasting immunity against the pathogen (Villumsen et al. 2014). The great potential of the intestinal tract in fish to induce a strong immune response have been found in the pyloric caeca (Ballesteros et al. 2013) and the posterior intestine (Rombout et al. 2011, Villumsen et al. 2014). Their study on rainbow trout showed that vaccinating against enteric redmouth disease anally with a low dosage ($1x10^8$ CFU/fish) or a high dosage orally ($5x10^9$ CFU/fish) led to a relative percent of survival (RPS) of 100%, while vaccinating with only one low dosage applied orally resulting in RPS of <53%. A low immune response after oral vaccination might not only be due to the gastric destruction of the vaccine but also be due to the highly tolerogenic gut environment (Joosten et al. 1997, Muñoz-Atienza et al. 2021). Unlike the lymphoid tissue of mammals, the gut associated lymphoid tissue (GALT) of fish lack functional structures such as the Peyer's

patch, in which most of the immune response is induced (Somamoto and Nakanishi 2020). In the lamina propria of all intestinal segments of salmonids, immune responsive active IgM+, T cells, antigen-presenting and mast cells are found and are capable of inducing an immune response post oral vaccination (Bjørgen et al. 2020, Lazado and Caipang 2014). Yet, the transcript levels of immune-related genes of the hindgut had been investigated by Løkka (2014) and show high levels compared to the anterior part of the gastrointestinal tract (Løkka et al. 2014). Hence, the total vaccine uptake is of high importance. To increase the chances of success for an oral-vaccine, research on the protection of the vaccine against the harmful gut milieu has been performed, leading to protection by lipid or alginate microspheres (Rivas-Aravena et al. 2013) or by acidic neutralizers (Adelmann et al. 2008). Further, the way of feeding the oral vaccine plays a significant role in the homogenous uptake and thereby the induction of complete herd immunity.

Protective and encapsulation methods for the administration via the oral route

Research on oral vaccines for aquaculture is of major immunological interest and a multiplicity of laboratory studies / literature is available such as Adelmann et al. (2008), Altun et al. (2010), Ballosteros et al. (2015) or Caruffo et al. (2016). Altun et al. (2010) used bacterin as vaccine for booster and non-booster vaccination and achieved RPS of 62%. The vaccine was immobilized in sodium alginate and poly-co-glycolide (PLGA) to achieve a protective encapsulation and improve the oral administration of the vaccine. With a booster vaccination, RPS increased to 80%. Both encapsulation substances led to a sufficient protection of the vaccine and, due to the lower cost of alginate, the authors recommended alginate as an appropriate encapsulation method. Although an RPS rate of 80% after booster vaccination complies with pharmaceutic standards, an improved RPS will be needed for commercial application. A comparison of orally administered to an injectable administered DNA vaccine was performed by Ballosteros et al. (2015) with the conclusion that oral administration of DNA vaccine required 20 times as much vaccine to induce a significant immune response in rainbow trout compared to the injective route. In that study, alginate was used to protect the vaccine against degradation in the gastrointestinal tract. Several other studies have used alginate to protect a vaccine or other bioactive substance against degradation such as: Rosas-

Ledesma et al. (2012) for the encapsulation of probiotic bacteria, Rodriguez et al. (2018) protection of enzymes (shrimp proteases) for Nile tilapia feeding or Chen et al. (2014) for the encapsulation of an IPNV antigen for the oral vaccination of Atlantic cod. The results of these studies illustrate the development of an encapsulation method with higher protecting rate to induce complete immune protection in fish after oral vaccination.

The use of chitosan in the production of alginate-chitosan microcapsules has been shown to improve the capsule properties for the administration of bioactive components (Wang et al. 2018). The use of chitosan in aquaculture is favored due to its nontoxicity, biodegradability and biocompatibility (Alishahi and Aïder 2012). Chitosan, the deacetylated derivate of chitin has antibacterial and antioxidant properties (Alishahi and Aïder 2012, No et al. 2002) and can increase the transcellular and paracellular transport of macromolecules (Tian et al. 2008). Chitosan was tested as a candidate carrier for a plasmid vaccine for oral immunization of Japanese flounder by Tian et al. (2008). In their study, the chitosan-vaccine pellets were directly released into the fish stomach with a sterile syringe. Administered such, the chitosan pellets exhibited significant protection against degradation due to gastric juices. As the vaccine pellet was directly released into the fish stomach, functional loss of the vaccine due to disintegration into the rearing water during feeding cannot be excluded on the basis of the experimental results. The positive conclusion of the authors for use in aquaculture application needs to be evaluated with caution as a burst release of the encapsulated component is known after the release into the rearing water (Polk et al. 1994). The issue of high initial burst was also demonstrated by Wang et al. (2018) when using a combination of alginate and chitosan to form vaccine pellets for oral vaccination of fish. Concurrent to the chitosan-capsules of Tian et al. (2008) showed the alginate-chitosan pellets by Wang et al. (2018) good protection characteristics against an acidic environment but significantly elevated release under neutral pH.

Chitosan not only functions as an encapsulation component but will potentially increase the immune response as it strongly interacts with the saccharide receptors and surface proteins in the intestine (Yu et al. 2019). A study by Adelmann et al. (2008) used polyethylene-glycol (PEG) as a protective matrix for the administration of an attenuated

virus against viral hemorrhagic septicemia in rainbow trout. Due to its biocompatibility, inert attributes and the trait to increase transepithelial transport (Krugliak et al. 1990, Wood et al. 1998), PEG appears to be a valuable component for oral vaccines. In the study by Adelmann et al. (2008) juvenile rainbow trout were hand-fed manually produced and dark stained oral vaccine particles which led to and RPS rate of 80%. The authors stated that the PEG could have interacted with the hydrophobic mucus layer and thereby amplify the vaccine uptake of the tissue. The use of PEG might further reduce the early release of bioactive substances as shown in the oral delivery of hirudin with chitosan/PEG-alginate microcapsules, which potentially increases the available vaccine in the fish intestine after oral feeding (Chandy et al. 1998).

The current literature presents the great potential of oral vaccines for aquaculture but none of the experiments to date were executed beyond the laboratory stage. The major concern for efficient vaccination is the secure passage of the oral-vaccine through the system water and the harmful milieu of the stomach, into the mucosal immune system in the fish intestine (Villumsen et al. 2014). Alginate, chitosan or PEG all have the potential to overcome these challenges as depicted by the conducted research to date. While the research to date has focused on the induction of immunity or uptake location of the vaccine, no data are available on the effects, these substances can potentially provoke in the fish intestinal tract. Still, these effects need to be evaluated to exclude potential threats as it was present in e.g. research on alternative feedstocks which – in the use of soy – led to enteritis in salmonids and thereby reduce health and growth of fish (Baeverfjord and Krogdahl 1996, Booman et al. 2018).

The optimal feeding regime for the administration of an oral vaccine

A species-specific feeding regime is a key step for a successful oral vaccination is beside an optimized encapsulation method and efficient vaccine the use of an optimal. An optimal feeding regime is characterized by sufficient and appropriately homogeneous vaccine pellet uptake among all fish to induce herd immunity. Caruffo et al. (2016) concluded that the protection offered by an oral vaccine is dependent on the delivery form and the vaccine components. The optimization of the species-specific feeding regime will not only

increase herd immunity but can also increase the efficiency of other feed additives such as pre-/probiotics or other feed additives.

Research on alternative feedstock (Hardy 2010, Kaushik et al. 2004), food efficiency (Okumuş and Mazlum 2002), starvation (Azodi et al. 2015), food deprivation (Nykänen 2006) or rearing densities (McKenzie et al. 2012, Rauw et al. 2017, Yarahmadi et al. 2016) are well presented for rainbow trout and most other prominent aquaculture species. Thereby, the respective parameters were evaluated on the basis of parameters measured across the defined group of experimentally treated fish over time. Existing studies on individual feed intake are rare and, if performed, are not transferable to the optimization of feeding bioactive components. Brännäs and Alanärä (1993) investigated the feeding behavior of Arctic charr at an individual level using automatic feeders. For the trial, fish were marked with passive integrated transponders which enabled a food release to the tank when fish come close to the feeder. Using this method, statements about the feeding hierarchy of the group were made, however, no information about the absolute feed intake could be obtained. A very precise method to determine individual feed intake was developed and applied by Talbot and Higgins (1983), who used an iron marked feed and radiographic spectroscopy to determine feed intake. In fact, not the mass of the ingested food but the marker were determined. For oral vaccination or the administration of other bioactive components this method has the potential for a non-invasive and precise determination of pellet intake for live and sedated or euthanized fish. A downside of this method however, is the increased stress during handling, sedation and imaging. A third option for the determination of individual feed intake over time is visual marking or labelling of fish in combination with video recording. Visible implant elastomer (VIE) tags have been proven in the tracking of fish over time without harmful consequences for the fish (Olsen and Vøllestad 2001). Olsen and Vøllestad (2001) used VIE tags to track juvenile brown trout in a release and catch trial, and this method could be modified to distinguish between fish in a video-recorded feeding trial. Individual pellet intake and thereby individual variations in feed efficiency in Nile tilapia were investigated by De Verdal et al. (2017) in which groups of tilapia were hand-fed pellet-wise. The feeding procedure was recorded and evaluated post-experimentally. This method gave a precise determination of individual feed intake but

ignores common feeding procedures and might alter the feeding behavior of fish. To date, no method is known for a precise determination of feed intake on an individual basis under the application of common feeding practices likely to result in a “natural” or normal feeding response at group and individual levels. The missing method and knowledge could be filled by the combination of VIE tags that allow a higher accuracy of fish determination and video recording as verifiable tool.

Target species: Rainbow trout (*Oncorhynchus mykiss*)

Worldwide production of rainbow trout in 2020 for inland, marine and coastal aquaculture was 0.96Mt or circa. 0.5% of the total aquatic food production of 177.8Mt (FAO 2022). For European aquaculture however, rainbow trout is the main cultured freshwater species with the leading European countries: Italy, Denmark and France (Gutiérrez et al. 2020). German annual production of rainbow trout in 2021 was 5800t and made up 31.3% of total German aquaculture production (Bundesamt 2022).

Rainbow trout are native to North America and Siberia. The early domestic farming of rainbow trout led to the species fast spread around the globe (Figure 2). The high volume of cultivation is due to their high adaptability to different rearing conditions, their high reproductive efficiency and high resistance against disease (Crawford and Muir 2008). While *O. mykiss* can tolerate lower water quality or variations in rearing temperature (13-18°C)(Bear et al. 2007, Valenzuela et al. 2008), fish health and growth depends on highly oxygenated water (Hardy 2002). Due to the early establishment of the animals in aquaculture; many fundamental research questions have been answered in regard to aquaculture production and efficiency. Two phases of feeding activity for juvenile rainbow trout are known, one within 3h of dawn and the other before dusk (Boujard et al. 2002) and different feeding methods were tested. For grow out, demand feeding can

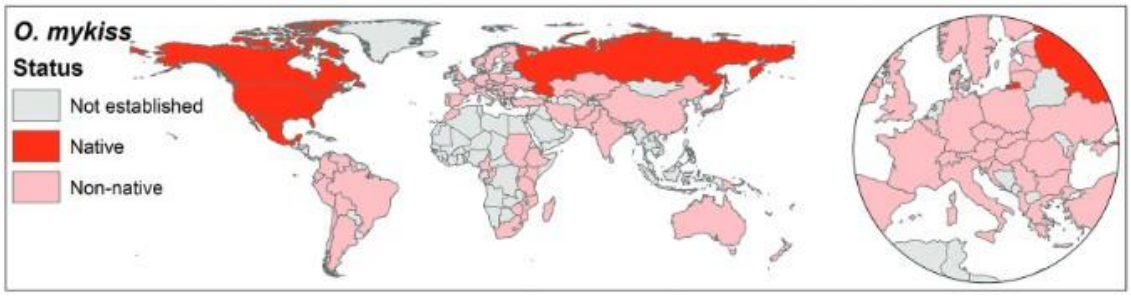

Figure 2 *Distribution of native and non-native rainbow trout from Muhlfeld et al. (2019)*

increased growth performance compared to restricted feeding as food limitations increase fish stress by feed competition which in turn increases metabolic rates and incurs energy loss (Alanärä 1992). The authors of the study further stated that if restricted feeding is used, two feeding events (morning and evening feeding) result in the best growth performance. The feeding frequency of juvenile rainbow trout was verified by (Grayton and Beamish 1977) who tested varying frequencies ranging from 1 meal 2 day^{-1} to 6 meals day^{-1}. Also in this study, maximum daily feed intake was achieved by 2 feeding events to satiation per day. Nevertheless, there are some publications in which no significant differences were found between continuous and restricted feeding of rainbow trout (Hung and Storebakken 1994, Mäkinen 1993).

Different levels of food deprivation or starvation have been evaluated for rainbow trout (Dobson and Holmes 1984, Sevgili et al. 2013, Sumpter et al. 1991, Weatherley and Gill 1981). Results show that rainbow trout can compensate growth after longer time periods (21 days) of feed deprivation (Boujard et al. 2000). Boujiard et al. (2000) concluded that feed deprivation leads to an increased in feed efficiency in juvenile rainbow trout and feed restrictions will lead to hyperphagia. For single or multiple administration of bioactive components, this information is valuable as especially hyperphagia has the potential to increase the absolute feed uptake after time restricted feeding. However, information on the homogeneity of uptake is not available. Depending on the focus: total mass of the administered bioactive substances or homogeneity of uptake after different starvation or deprivation feedings might be applied.

In rainbow trout cultivation, rearing densities have significant influence on fish feeding behavior and fish health. Thereby, fish stress due to aggression can be cause in low rearing densities as it promotes territorial behavior (Bagley et al. 1994, Laursen et al. 2015) of fish but also in high rearing scenarios due to limitation in space or competition during feeding (North et al. 2006). As fish welfare is of increasing interest for fish, farmer and consumer; optimal stocking densities need to be used to guarantee high fish quality in aquaculture rearing.

Motivation and thesis outline

Despite the large availability of information on rainbow trout aquaculture rearing, feeding regimes, advances in oral vaccination for aquaculture application and encapsulation methods, there is still a large knowledge gap regarding specific adjustments and optimization of diets and feeding methods for the application for oral vaccines. The impact of aquaculture on environmental, animal and human well-being are continuously increasing and while the early questions of alternative feedstock and their effects have been answered; the urge to increase fish health and reduce impact on the environment continues to increase. The intensification of animal farming has led to disease development and the inevitable use of antibiotics or vaccination; advances towards field-applicable oral vaccines for aquaculture are urgent to reduce the use of antibiotics, as it is still common practice in lower income countries (Bravo and Midtlyng 2007). Immersion or injective vaccines already exist and are administered, however an efficient oral vaccine has the potential for an easy and cost-efficient method for all aquaculture facilities / sites.

The large amount of research regarding optimal trout feeding and growth was primarily performed in studies concentrating on feed uptake and growth among total fish mass / total fed feeds. Variation in pellet consumption have only been evaluated by indirect methods. For oral vaccination, an exact method for quantification of pellet uptake on an individual basis is still lacking. Thus, the Chapter 1 of this dissertation will clarify, how the feeding regime of rainbow trout can be optimized to maximize homogeneous pellet uptake for juvenile rainbow trout and how to guarantee a minimum pellet uptake. In accordance with aquaculture farming practice, adjustable parameters such as starvation scenarios, portion size and number of feed additions will be investigated to optimize the ideal feeding regime for oral vaccines or other bioactive substances for single or booster administration. The homogeneity of uptake and not just the absolute mass of uptake will be the main priority. Within the research question, the applicability for aquaculture farming will be of central interest.

Chapter 2 and 3 of this dissertation will be based on experimental approaches to determine the effects of different encapsulation substances on: Chapter 2 - the fish intestine and potentially inflammatory response and: Chapter 3 - the different release

profiles under varying pH of an encapsulated model protein (bovine serum albumin (BSA)). The novel information obtained in Chapter 2 is intended to close the knowledge gap of the potential harmful effects that the encapsulation substances alginate, chitosan and polyethylene-glycol can have on the intestinal tract of juvenile rainbow trout. Even though these substances were studied on their own and the effects for fish or human application are described, distinct information about the intestinal response are lacking. In the context of an orally applied vaccine, intestinal health is of major importance as only functional tissue will allow for an adequate uptake / interaction with the vaccine and thus induce a sufficient immune response. The experiments performed in Chapter 2 will provide profound intestinal and inflammatory response of juvenile rainbow trout with the aim of excluding any negative effects counterindicating pharmacological use / application. Encapsulation methods and substances as described above will find methodical and practical application to increase the overall efficiency of the encapsulated substance through delivery to immune response. In aquaculture, encapsulations have been used for the administration of probiotic bacteria (Rosas-Ledesma et al. 2012), vaccines (Caruffo et al. 2016) and other bioactive substances (Rodriguez et al. 2018). Different methods and substances were used and there is no direct comparability of the encapsulation practices. In the Chapter 3 of this thesis, the three primarily investigated substances (Chapter 1 and 2) will be used to encapsulate the model protein (BSA) to investigate BSA release under different pH milieus, as occurs during feeding / oral administration in aquaculture. Due to the similar production method, statements can be made to compare the three encapsulation substances and thereby show the potential for their use in oral vaccine encapsulation.

Ultimately, the questions answered in this dissertation are intended to contribute to the momentous development of oral vaccination in aquaculture by optimizing indirect parameters such as the feeding regimes and identifying the effects and protective properties of the three applicable and promising encapsulation matrixes alginate, polyethylene-glycol and chitosan allowing their optimal application in aquaculture feeds.

Author's Contribution of Thesis Chapters

Chapter 1: Video surveillance methods to evaluate individual feeding response in rainbow trout (*Oncorhynchus mykiss*, Walbaum) – implications for feeding regime optimization

Philip N. Just: Conceptualization, methodology, validation, investigation, formal analysis, writing – original draft

Bernd Köllner: Conceptualization and writing – review

Matthew J. Slater: Supervision, methodology review, validation, writing – review & editing

Chapter 2: Effects of two delivery matrix component alginate and polyethylene-glycol on the intestinal tract and inflammatory response of juvenile rainbow trout (*Oncorhynchus mykiss*)

Philip N. Just: Conceptualization, methodology, validation, investigation, formal analysis, writing – original draft.

Matthew J. Slater: Supervision, conceptualization, writing – review & editing

Claudia Müller: Methodology of gene analysis, writing – review.

Bernd Köllner: Methodology of gene analysis, writing – review, formal analysis (gene-expression data), funding acquisition

Chapter 3: Potential of alginate, chitosan and polyethylene-glycol as substances for colloidal drug delivery as determined by protein release and digestion.

Philip N. Just: Conceptualization, methodology, validation, investigation, formal analysis, writing – original draft

Matthew J. Slater: Supervision, writing – review & editing

Chapter 1

Video surveillance methods to evaluate individual feeding response in rainbow trout (*Oncorhynchus mykiss*, Walbaum) – implications for feeding regime optimization

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Video surveillance methods to evaluate individual feeding response in rainbow trout (*Oncorhynchus mykiss*, Walbaum)—implications for feeding regime optimisation

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Abstract
Precisely analysing and optimising feeding regimes is central to salmonid growth performance and delivery of special diets. The current study developed novel video surveillance methods and analysis techniques to assess individual feed intake and minimum pellet intake (MPI) in individually identified juvenile rainbow trout, *Oncorhynchus mykiss*. Three trials were conducted to test the impact of short-term starvation (N=112 [16 tanks, 7 fish per tank], average weight=27.1±3.4g, age= 119 days), portion numbers per feeding (N=105 [15 tanks, 7 fish per tank], average weight=22.8±2.1g, age= 99 days) and varied numbers of daily feeding events (N=84 [12 tanks, 7 fish per tank], average weight=32.4 ±3.3g, age= 133 days). All trails were carried out in a recirculating aquaculture system with 20 tanks held at 15 ± 0.5 °C. All individuals were code-tagged and high quality video images were taken and analysed to identify all feeding interactions. Individual trout feeding activity under different feeding regimes could be precisely analysed with the video methods developed. Moving from one to two daily feeding events doubled pellet intake per fish from 27.4 ± 5.8 to 52.8 ± 11.5 pellets. Pellet intake (58.8 ± 24.2 pellets) did not increase at three daily feeding events but became more variable across fish. MPI nearly doubled to 30 pellets in fish receiving two daily feeding events (MPI_{30}: chi-squared = 8.74, df = 2, p = 0.01). Short-term starvation had no influence on intake (28 ± 8 pellets/fish) or MPI. Increasing portion number from one (27.8 ± 7.4 pellets fish^{-1}) to two (31.1 ± 7.4 pellets fish^{-1}) or more did not significantly increase the number of ingested pellets. Adjusting the feeding regime by increasing daily feeding events to two, possibly combined with multiple portions, can increase pellet intake and reduce the heteroscedasticity of pellet intake. The methods presented in this study are viable for analysing feeding regimes for juvenile rainbow trout and controlled feedstock/supplement delivery. Implications for analyses with other species and for vaccination optimisation are discussed.

Keywords: Salmonid · Video · Feeding regime · Fish welfare · Visual tags

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Abstract

Precisely analyzing and optimizing feeding regimes is central to salmonid growth performance and delivery of special diets. The current study developed novel video surveillance methods and analysis techniques to assess individual feed intake and minimum pellet intake (MPI) in individually identified juvenile rainbow trout, *Oncorhynchus mykiss.* Three trials were conducted to test the impact of short-term starvation (N = 112 [16 tanks, 7 fish per tank], average weight = 27.1 ± 3.4 g, age= 119 d), portion numbers per feeding (N = 105 [15 tanks, 7 fish per tank], average weight=22.8 ± 2.1 g, age = 99 d), and varied numbers of daily feeding events (N=84 [12 tanks, 7 fish per tank], average weight =32.4 ± 3.3 g, age = 133 d). All trails were carried out in a recirculating aquaculture system with 20 tanks held at 15 ± 0.5°C. All individuals were code-tagged and high quality video images were taken and analyzed to identify all feeding interactions. Individual trout feeding activity under different feeding regimes could be precisely analyzed with the video methods developed. Moving from one to two daily feeding events doubled pellet intake per fish from 27.4 ± 5.8 to 52.8 ± 11.5 pellets. Pellet intake (58.8 ± 24.2 pellets) did not increase at three daily feeding events but became more variable across fish. MPI nearly doubled to 30 pellets in fish receiving two daily feeding events (MPI_{30}: chi-squared = 8.74, df = 2, p = 0.01). Short-term starvation had no influence on intake (28 ± 8 pellets $fish^{-1}$) or MPI. Increasing portion number from one (27.8 ± 7.4 pellets $fish^{-1}$) to two (31.1 ± 7.4 pellets $fish^{-1}$) or more did not significantly increase the number of ingested pellets. Adjusting the feeding regime by increasing daily feeding events to two, possibly combined with multiple portions, can increase pellet intake and reduce the heteroscedasticity of pellet intake. The methods presented in this study are viable for analyzing feeding regimes for juvenile rainbow trout and controlled feedstock / supplement delivery. Implications for analyses with other species and for vaccination optimization are discussed.

Keywords salmonid, video, feeding regime, fish welfare, visual tags

1. Introduction

Feed delivery and feeding regime optimization are key factors affecting the performance of fed aquaculture and are of particular importance in salmonid farming (Cho 1992, Guzel and Arvas 2011). Achieving high total diet intake and limiting diet wastage are essential to optimize growth of animals and minimize cost in terms of feed use (Ang and Petrell 1998, Cho and Bureau 2001). Equally important is ensuring the uptake of feed as evenly as possible across the entire fed population in order to reduce uneven growth, development of dominances and the need for grading. Even and sufficient feed intake across the entire fish population is also essential to ensure animals are appropriately dosed when fed special supplementary diets or when receiving oral vaccinations or veterinary treatments. There remains, however, a lack of established methods to measure and analyze feeding regimes at an individual level under replicable and realistic holding conditions.

Most research to date on topics such as feeding methods or alternative feedstocks have been performed using groups of fish, rather than individual feeding, as determinants. Individual feed intake by Arctic charr (*Salvelinus alpinus*) was determined indirectly by Brännäs and Alanärä (1993) using automatic feeders and feeding trigger release as measures for individual feed intake. Ten years earlier, Talbot and Higgins (1983) determined individual feed intake of juvenile Atlantic salmon (*Salmon salar*) using iron-marked pellets and radiographic spectroscopy. Both these methods however have their disadvantages, which include the exclusion of fish interactions and high levels of uncertainty due to indirect counting of feed intake or the impacts of fish euthanasia prior x-ray scans.

Visual determination of video-recorded feeding events can be used to determine direct individual intake in vivo. Drawbacks are the limited holding densities due to overlapping fish during the feeding process and the challenge of determining the identity of individuals within the treatment. De Verdal et al. (2017) used this visual approach to determine the individual feed intake of Nile tilapia (*Oreochromis niloticus*). In their study, pellets were fed to the fish pellet-wise and video records of the feeding event were evaluated post-feeding. The number of fish per tank can be increased by visually marking the fish for better differentiation or location. An example is presented by Olsen

and Vøllestad (2001) who marked 0-age brown trouts (*Salmo trutta*) with visual implant elastomer (VIE) tags. The tag with VIE caused negligible mortality (0.5%) and long lasting visibility. Of all three methods, video is considered the most conservative. Video analyses of feeding may also be hindered in salmonids such as Rainbow trout if the low stocking densities required lead to aggressive fish behavior which can interact with feeding activity (Ellis et al. 2002).

In salmonids, short-term starvation has been investigated as a feeding delivery method to reduce labor costs (Kindschi 1988), increase food efficiency (Azodi et al. 2015) and test for compensatory growth (Weatherley and Gill 1981). Individual pellet intake has not been the main focus in the cited studies. Though, refeeding after starvation periods increases size variation in juvenile rainbow trout, which can be assigned to uneven individual feeding (Kindschi 1988). Furthermore, Kindschi (1988) did not find significant differences in growth performance, but that daily feed intake (DFI) of the deprived fish was higher than the DFI of daily fed fish during refeeding. This was also the case for juvenile rainbow trout exposed to long-term starvation periods. Even starvation phases up to 6 weeks did not lead to changes in final fish weight or cortisol levels (Sumpter et al. 1991). No detailed information is present for the optimal trade-off between maximal DFI and inhomogeneity of individual pellet intake during single feeding.

While starvation experiments can have durations of days and weeks, the term feeding frequency describes the intervals between two feeding sessions mostly within one day. Grayton and Beamish (1977) tested different feeding frequencies (1 meal / 2 d – 6 meals / d) for rainbow trout and found best results, in terms of fish growth, is achieved when applying one or two meals per day. Alanärä (1992) also concluded that increasing numbers of daily feeding events increase fish stress during feeding for rainbow trout fed in net-pens using automatic feeders. Rasmussen et al. (2007) showed that for rainbow trout, an increase in feeding frequency is directly correlated to the degradation of fin condition and thereby fish welfare.

There is a dearth information in the literature on the number of feed portions added, i.e. most studies use single food addition during the feeding procedure. While information on portion size for rainbow trout feeding is a well-reported parameter (Alanärä 1992, Bailey and Alanärä 2006, Hung and Storebakken 1994), information on

the effects of different numbers of portion addition during a feeding event is lacking. If presented it pertains to feeding strategies such as automated versus hand feeding (Alanärä 1992, Azzaydi et al. 1998). Evaluable effects of portion numbers for salmonids are present in a study by Alanärä (1992) who tested different feeding strategies such as time controlled restricted, restricted demand and unrestricted demand feedings which presents one restricted portion, multiple portions with small portion size and multiple portions with large feed amounts. Their results showed that an increased number of portion of small quantities leads to significantly better food conversion rate and less pellet waste.

Individual feed intake under varying feeding regimes is uninvestigated for salmonids. While the general principles and relations of growth and feeding regime are well understood and described based on group performance over extended feeding periods, the literature still lacks detailed experimental results based on the individual feeding response to variations in regime.

The aim of this study is to develop exact methods to observe and quantify rainbow trout feeding response. The combination of visual tagging and high definition video recording analysis are developed to allow quantification of the pellet intake of individual rainbow trout during feeding events. These methods are then applied and validated in a series of controlled feeding experiments.

2. Materials and Methods

2.1 Facility, tanks and water quality

Experiments were conducted in the laboratories and aquaria of the Centre for Aquaculture Research (ZAF) at the Alfred-Wegener-Institute Helmholtz-Centre for Marine and Polar Research, Bremerhaven. The recirculating aquaculture system (RAS) used for all experiments consisted of 20 individual tanks with clear-glass fronts, a cooling element, a moving-bed nitrification bio-filter and a foam sheet filter. Tank dimensions were: 48 x 38 x 49cm (length, width, height). The number of tanks used was modified in accordance with each of the three experimental designs outlined in the following. A photoperiod of 8 h daylight and 16 h darkness was applied at all times and exclusively during the period of video recorded feeding, an additional UV-light (405 nm, SMD5050, 8.4W / tank) was applied.

Water temperature was maintained at 15 ± 0.1 °C, water replacement rate and airflow were adjusted at 4.2 % min^{-1} and 300 L h^{-1} for all tanks to obtain identical experimental conditions. Ammonia, nitrate and nitrite were controlled throughout animal holding and during experiments. Values did not exceed 0.12 mg L^{-1}, 0.2 mg L^{-1} and 50 mg L^{-1}, respectively. Oxygen levels during the trials were 88 ± 6% on average throughout the experimental trials and never fell below 82%.

2.2 Experimental fish and feeds

Rainbow trout fingerlings (*Oncorhynchus mykiss)* were obtained from a commercial fish farm (Die kleine Fischzucht, Geseke, Germany). After arrival, fish were acclimated and were stocked in a 550 L holding tank attached to the system until experiments were carried out. During that period, fish were hand-fed a commercial floating diet (F-1P Classic LT/F 2.5mm, Skretting) twice daily until visual satiation. Mean fish weight and length were 27.1 ± 3.4 g - 13.8 ± 0.5cm, 22.8 ± 2.1 g - 13.7 ± 0.5 cm and 32.4 ± 3.3 g - 14.9 ± 0.5 cm for the starvation, portions and daily feeding events experiments, respectively.

2.3 Sampling, VIE tagging and MPI

For each experiment, fish were taken from the acclimation tank, weight and length measured and fish of adequate size were anesthetized with Tricaine methane-sulphonate (MS-222, 100 mg L^{-1}, 90 s, Sigma Aldrich) for tagging.

Fish were marked behind the eye and alongside the dorsal fin with visual implant elastomer tags (VIE, Northwest Marine Technology, Inc.). Different color combinations were applied to allow each fish to be identified during the video evaluation. Directly after tagging, marked fish were distributed homogenously among the glass tanks in which the trials were performed. Fish were allowed to settle into the glass tanks and recover from any tagging stress for a period of 10 days. Observation and visual estimation of fish behavior was carried out over the first seven days to ensure normal response, also during feeding and further three days was added to ensure full acclimation.

Prior the evaluation feeding, the camera setup (Sony A7sII, 60 fps, ISO4800; Sigma 12-24 @18 mm, f2.8) was arranged in front of the glass tanks and video recording was started approximately 1 min before feeding. After 5 min the video recording was stopped. A preliminary analysis of the video files showed that within the first 100 seconds of feeding, most of the pellet uptake occurred. Hence, for the evaluation of the videos, the first 3 minutes of each feeding event was evaluated by following the tagged fish and counting the consumed pellets for each fish. Pellets that were disgorged by the fish were subtracted from the total pellet intake. When the portions were added to the glass tanks, a wide spread of the pellets over the whole water surface was ensured to present a maximize feeding area. Furthermore, any aggressive behavior amongst fish was recorded.

To classify and evaluate the different feeding scenarios the maximum pellet intake (MPI) was established in this study. While the ingestion rate, which can be calculated directly and indirectly, only shows the overall intake of a cohort or an individual, the MPI represent the ratio of fish that consumed a minimum of x pellets.

2.4 Method Validation Trials

In order to apply and validate the video recording and analysis methods developed above, controlled feeding experiments were established to test the following feed delivery parameters:

1) short-term starvation periods of varying lengths,

2) portion numbers per feeding,

3) number of daily feeding events.

Data in terms of feed pellet intake per individual are presented in the following with the aim of identifying delivery methods to optimize homogeneity of feed intake and increase minimal pellet uptake

2.4.1 Starvation period

In this experiment a total 16 of the maximal 20 tanks and a total of 112 fish were used in the system. Seven VIE tagged fish with an average weight of 27.1 ± 3.4 g (rearing density = 2.3 kg m^{-3}) were acclimated for 10 days in each of the experimental glass tanks. During that period, the seven fish per tank were fed 0.8 % body weight (bw) $fish^{-1}$ twice daily. Fish in four tanks (in quadruplicates) were then starved for 0 (control C), 1 (S1), 2 (S2) or 3 (S3) days before being fed once in a single portion with the standard diet (as above) at a rate of 2% bw $fish^{-1}$ (39 pellets $fish^{-1}$). This feeding event was recorded on video and fish consumption of pellets (uptake, disgorging) and interactions (aggression) was evaluated post-experimentally.

2.4.2 Food portions

In this experiment a total 15 of the maximal 20 tanks and a total of 105 fish were used in the system. Seven VIE tagged fish with an average weight of 22.8 ± 2.1 g (rearing density = 2.0 kg m^{-3}) were acclimated for ten days in the experimental glass tanks. During that period, fish were fed 0.8 % bw $fish^{-1}$ twice daily. At day 11 the video recorded portion feeding was conducted. A total of 2% bw $fish^{-1}$ (33 pellets $fish^{-1}$) was divided into one (PA1), three (PA3) or five (PA5) portions, added every 30 seconds to the respective tanks. Treatments were repeated in five replicate tanks each.

2.4.3 Daily feeding events

In this experiment a total 12 of the maximal 20 tanks and a total of 84 fish were used in the system. Seven VIE tagged fish with an average weight of 32.4 ± 3.3 g (rearing density = 2.8 kg m^{-3}) were acclimated for 14 days in the experimental glass tanks in quadruplicates. During that period, fish were fed 0.8 % bw $fish^{-1}$ twice daily. At day 15, the video recordings of the different feeding scenarios were conducted. Fish of the FE1 treatment were fed once at 9 a.m., fish of the FE2 treatment were fed twice: at 9 a.m. and 3 p.m. and fish of the FE 3 treatment were fed three times: at 9 a.m., 12 p.m. and 3 p.m. Each feeding event, fish were provided with 2% bw $fish^{-1}$ (46 pellets $fish^{-1}$ or approx. 0.65 g $fish^{-1}$) feed.

2.4 Statistical analysis

Pellet consumption data were tested for normal distribution (RSTUDIO, shapiro.test) and variance homogeneity using the Bartlett test. When data were normally distributed and homogeneity of variance was given, a generalized linearized model was used to test for significant differences between treatments. For over-dispersed data, the negative binominal distribution was tested and used (RSTUDIO, glm.nb). When data were normally distributed but variance was not homogeneous among the replicates, a Kruskal-Wallis test was performed and if significant differences were detected the Dunn's post-hoc test was used to show pairwise differences within the groups. The variance of pellet consumption, and all other variance data herein are presented as the standard deviation of the mean. For all tests, a significance level of 0.05 was applied. All statistical tests were conducted using R Version 3.5.0 (R_Core_Team 2018).

3. Results

The VIE tags allowed precise identification of all fish during the video evaluation. Further, videos gave an exact overview of fish behavior during the feeding session. The exact uptake of individual pellets pellet and the disgorging (if any) of pellets was able to be recorded. All aggression events among the fish were also clearly recorded. During the starvation trial one fish in the control treatment was harassed by a conspecific (0.9% of all fish) at the end of the event only, thus aggressive behavior can be excluded from the starvation trial. During the daily feeding events trial, one fish from the control treatment showed aggressive behavior towards the conspecific during the midday feeding event

(1.2% of all fish). However, the fish showing the aggressive behavior showed low pellet intake (16 pellets during the midday feeding). As with the starvation trial, fish aggression can be excluded as a factor in the daily feeding events trial. For the time of video evaluation of the portion trial, one fish from a PA3 and PA5 treatment was harassed by a conspecific 3 and 2 times (1.9% of all fish), respectively. The fish in the PA3 treatment showed reduced pellet uptake with 2 pellets during the specific feeding event. No effect on pellet uptake was present for the fish in the PA5 treatment. This represented less than 3.2 % of all feeding reactions and, as such, aggression can be disregarded for the portion trial.

3.1 Starvation period

Across all treatments, an average of 28 ± 8 pellets were consumed, which is equivalent to 1.45% bw fish^{-1} (Figure 1). There were no significant differences in pellet intake (one-way-ANOVA, $F(3,108) = 0.282$, $p = 0.8$) or pellet intake variance (Bartlett-test, K-squared = 6.6688, df = 3, $p = 0.08$) between animals starved over different time periods. When comparing homogeneity of variance against the control treatment, significant differences of variance are present for control to S2 treatment (Bartlett-test, K-squared = 6.551, df = 1, p-value = 0.01048). Different starvation periods had no impact on the minimum pellet intake (MPI) (Table 1). Generally, the control treatment showed the highest MPI values. While in the control treatment a pellet intake of minimum 10 pellets per fish was present for 100 ± 0 % of fish, only 96.4 ± 7.1 % was achieved for the S1, S2 and S3 treatment. At an MPI set at 20 pellets fish^{-1} the control treatment was 7 % (of total fish) higher than S1 and S3 and 11 % higher than the S2 treatment (Table 1).

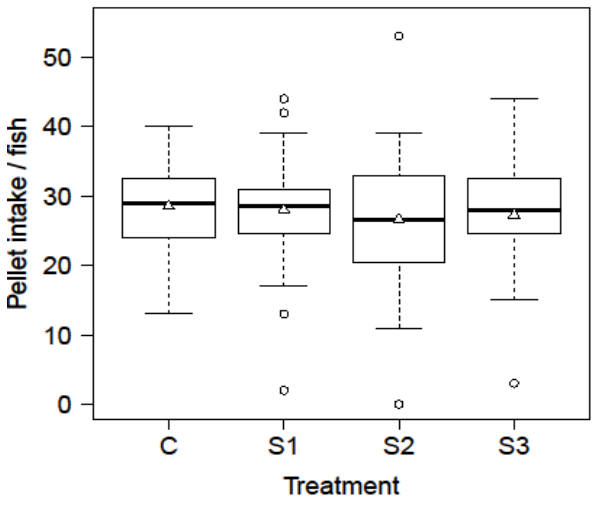

Figure 1 Summary of the starvation experiment, the y-axis presents the ingested pellet fish^{-1}. The triangle presents the treatment mean, circles present outliers

Table 1 *Percent of fish (N = 112) that consumed a minimum of 10, 20 or 30 pellets during the feeding event of the starvation trial.*

MPI (% bw fish^{-1})	C [% fish] ± SD	S1 [% fish] ± SD	S2 [% fish] ± SD	S3 [% fish] ± SD
10 – (0.52%)	100 ± 0	96.4 ± 7.1	96.4 ± 7.1	96.4 ± 7.1
20 – (1.04%)	96.4 ± 7.1	89.3 ± 7.1	85.7 ± 0	89.3 ± 7.1
30 – (1.56%)	42.9 ± 11.7	46.4 ± 29.5	35.7 ± 18.4	35.7 ± 18.4

3.2 Portion trial

There were no significant differences in pellet consumption or variance between fish fed different portion sizes across three treatments (GLM, neg.binom., ϑ = 32.95, Intercept = 3.3238, p = 0.11 OR ANOVA, F(2,98) = 2.281, p = 0.108). During the portion experiment, four of the 105 marked fish (3.8% - consuming zero pellets) showed abnormal swimming behavior and denied feed uptake. These individuals were excluded from the data analysis. It is important to note that the exclusion of these fish do not change the results but reduced the data noise (Figure 2).

An average of 27.8 ± 7.4 pellets was ingested on the addition of one portion (PA1), an average of 31.1 ± 7.4 pellets were ingested in treatments offering three or five portion per feeding event. Consequently, the feed intake per fish is 1.71% bw fish $^{-1}$ for the one portion treatment and 1.91% bw fish $^{-1}$ for all other treatments. Concomitant to the effect of different starvation periods, the portion number has an influence on the MPI. While for all treatments more than 88% of fish ingested a minimum of 20 pellets fish^{-1}, 15% and 17% less fish ingested a minimum of 25 or 30 pellets fish^{-1} when offered only one portion (Table 2).

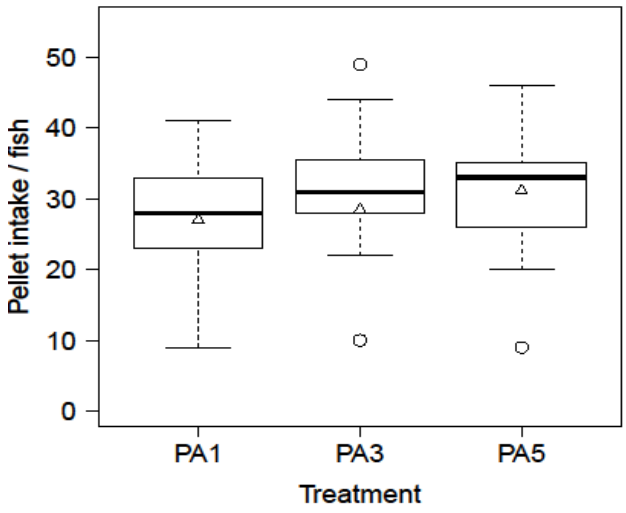

Figure 2 *Summary of the portion experiment, the y-axis shows the ingested pellet of each individual. The triangle presents the treatment mean, circles present outliers*

Table 2 *Percent of fish (N = 105) that consumed a minimum of 10 - 30 pellets during the feeding event of the portion trial.*

MPI (% bw fish^{-1})	PA1 [% fish] ± SD	PA3 [% fish] ± SD	PA5 [% fish] ± SD
10 – (0.62)	94.3 ± 7.8	91.4 ± 7.8	97.1 ± 6.4
15 – (0.93)	91.4 ± 12.8	88.6 ± 12	97.1 ± 6.4
20 – (1.24)	88.6 ± 12	88.6 ± 12	97.1 ± 6.4
25 – (1.54)	65.7 ± 12.8	88.6 ± 12	82.9 ± 12
30 – (1.85)	42.9 ± 14.3	57.1 ± 17.5	60 ± 18.6

3.3 Daily feeding events

During the first feeding event in the morning, fish ingested an average of 28 ± 11 pellets (1.19% bw fish^{-1}) across all treatments. At the end of the feeding trial, fish in the FE1 treatment ingested 27.4 ± 5.8 pellets, fish fed twice daily (FE2) ingested 52.8 ± 11.5 pellets (2.24% bw fish^{-1}) and fish fed three times daily (FE3) ingested 58.8 ± 24.2 pellets (2.5% bw fish^{-1}). A Kruskal-Wallis-test revealed significant differences between the treatments (chi-squared = 46.27, df = 2, $p < 0.01$; Figure 3). Fish receiving only one feeding event per day ingested significantly less diet (Dunn's-test: $p < 0.01$) while no differences of mean pellet intake was present between fish receiving two or three feeding events daily (Dunn's test: $p = 0.92$).

Pellet uptake variance was not homogenous between the treatments (Bartlett-test, K-square = 64.492, df = 11, $p < 0.01$). Furthermore, the inspection of the standard deviation in relation to the mean ingested pellets showed that feeding once or twice daily results in a relative standard deviation of 21.2 and 21.8% of the mean ingested pellets, whereas feeding three times daily leads to a relative standard deviation of 41.2% of the pellet mean intake.

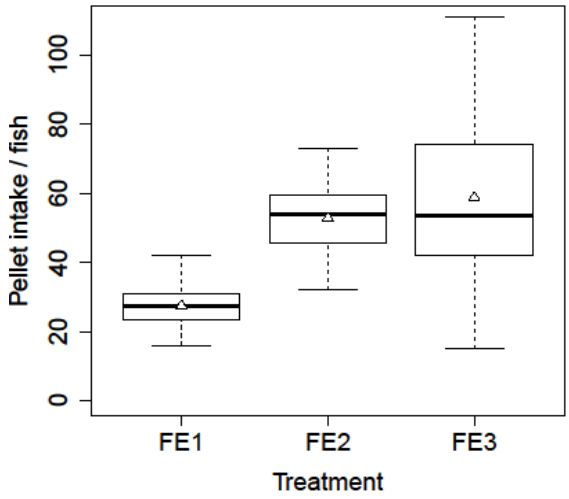

Figure 3 *Summary of the daily feeding event experiment, the y-axis shows the ingested pellet of each individual. The triangle presents the treatment mean, circles present outliers.*

Table 3 *Percent of fish (N = 84) that consumed a minimum of 10 - 40 pellets during the feeding event of the daily feeding event trial. Significant differences within each MPI group are given by an asterisk (** p < 0.01, * p < 0.05)*

MPI (% bw fish^{-1})	FE1 [% fish] ± SD	FE2 [% fish] ± SD	FE3 [% fish] ± SD
10 (0.44)	100 ± 0	100 ± 0	100 ± 0
20 (0.87)	92.9 ± 8.3	100 ± 0	97.1 ± 6.4
30 (1.30)	53.7 ± 18.4**	91.4 ± 19.2	91.4 ± 12.8
40 (1.74)	3.6 ± 7.2*	77.1 ± 16.3	74.3 ± 18.6

Generally, the use of more than one feeding event per day roughly doubles the average pellet intake of fish (Figure 3). Unfortunately, the standard deviation increases concomitantly resulting in higher data noise. At the end of the feeding trial, fish receiving only one feeding event consumed 1.19% bw fish^{-1} d^{-1}, 2.27 bw fish^{-1} d^{-1} and 2.62 bw fish^{-1} d^{-1} for the treatments with two and three daily feeding events, respectively.

The increase in daily feeding events increased the MPI. All fish ingested a minimum of 10 pellets throughout the treatments and fish being fed twice even reached an MPI of 20 pellets for all fish. For both, MPI of 30 and 40 pellets / fish, a significantly reduced number of fish was detected in the FE1 treatment, compared to the FE2 and FE3 treatments (MPI_{30}: chi-squared = 8.735, df = 2, p = 0.01, MPI_{40}: chi-squared = 8.307, df = 2, p = 0.02).

4. Discussion

There is a dearth of reliable and applicable methods to provide information on feed uptake and feeding regimes at an individual level for finfish. There is also a lack of reliable data on individual fish feeding response to different feeding regimes and no quantification of evenness of diet uptake across fed groups of fish. The current study developed and applied a novel methodology to analyze individual diet uptake in Rainbow trout. The video method applied with extremely low-impact individual tagging of fish proved highly effective, although it is a time intensive tool to determine individual food uptake. It allowed the development of direct measures for evenness of diet uptake across a group of individual fish and provides insights into the efficacy of various known feeding regimes / methods considered viable for improving diet uptake.

Portion size, short-term starvation and daily feeding events are among a number of methods previously used as parameters to attempt to influence feed intake among farmed fish. The results in the present study demonstrated that the feeding procedure for a particular feeding event could be optimized by adjusting the food supply methods. While short-term starvation (trial 1) and portion numbers (trial 2) effected no significant differences in feed intake homoscedasticity for rainbow trout, a clear statement of the effect of different feeding regimes and their advantages can be made using MPI.

Starvation was ineffective in improving diet uptake. Equally, in this study, the distribution of the first 20 pellets each fish consumed was more homogenous without starvation or with short starvation periods. Research on starvation periods has been linked to compensatory feeding and food efficiency. Information on individual food consumption has not been stated so far or was determined indirectly with automatic feeders (Brännäs and Alanärä 1993). In the current study, one feeding event in the morning during the starvation trial was used for video evaluations. With a notional second food addition in the evening, hyperphagia could have been amplified and differences observable. Similar results were observed by Azodi et al. (2015), who tested refeeding after short-term starvation (1-3 d) on 17 g rainbow trout fingerlings. However, feed intake was determined indirect by feed intake of all fish. No significant effects were found even though daily feed intake of deprived groups (at feeding) tended to be higher than the continuously fed control group. They concluded “that rainbow trout has the ability to achieve full compensatory growth during short-term starvation and re-feeding periods.”

While food deprivation leads to an increase in feed efficiency, food restrictions are better known for inducing hyperphagia (Boujard et al. 2000). In sustainable and equitable aquaculture, good fish welfare is highly beneficial, as poor fish status will lead to a decreased immune response post-vaccination and at the same time decrease resistance against diseases (Yarahmadi et al. 2016). Even though food restriction may have a significantly higher influence on feed intake and intake homoscedasticity as per Boujard et al. (2000), food deprivation as used in trial 1 is an easier method to apply in current aquaculture facilities.

Cho and Buereau (2001) reviewed different diet formulation strategies and feeding systems to reduce feed wastage, stating that the use of demand feeding with restricted portions increases pellet intake of fish and reduces feed leftovers. Moreover, they proposed that fish are given the opportunity in time and space to satisfy their hunger. Both conclusions are integrated and verified in this study. Current results showed that by the addition of only one portion, feed intake is 10% lower than by the addition of 3 or 5 portions. Remarkably, the standard deviation of the 3 and 5 portion treatments did not increase concurrently with the feed intake; leading to relatively better feed distribution among the multiple portion treatments. Even though the increase is not significant, the analysis of the MPI supports the trend that the application of multiple portions during one feeding event is beneficial for homogeneous feed intake. The differences in MPI between one vs. multiple food portions are conspicuous. When fed one portion, approx. 15% less animals which have eaten 30 pellets despite the delivery of exactly the same total amount of feed in all portion treatments.

Under different feeding regimes the majority of fish eat either in the first 2 to 3 hours of light or within two phases; one in the morning and one in the evening (Boujard et al. 2002). These two daily feeding event scenarios match the findings of this study, where the best homoscedasticity of feed intake and MPI was present with 1 or 2 feeding events daily. When higher MPIs are required, the use of two feeding events per day is favorable. An additional feeding event at the end of the day nearly doubles the feed intake and the standard deviation. However, the MPI of 30 pellets is thereby increased from 54% to 100% of all fish from FE1 to FE2 daily. The addition of one more feeding event per day to a total of 3 event did not increase the total feed intake per day and also unfavorably decreased intake homogeneity. It is suggested that this represents simply a further (excess) feeding event which may offer no great feeding benefit but still contributes to increased stress by feeding activity related to avidity (greed) or increased swimming speed, metabolism or competition during the feeding event. Similar feeding behavior was described by Alanärä (1992) when investigating the differences of demand and time restricted feeding. Alanärä (1992) concluded that intensive feeding twice a day in the morning and evening is favorable and that increased numbers of feeding events induces stress and high competition for which might also be the case for this trial. Alternatively,

as Rasmussen et al. (2007) suggested, increased feeding event frequency may lead to more aggressive interactions between fish. However, significant aggression was not observed in the current study.

Increased fish stress can have a variety of sources such as increased rearing densities, aggression or food supply. An easily accessible and representative indicator is the condition of the dorsal fin (Rasmussen et al. 2007). Rasmussen et al. (2007) found increased feeding frequency leads to degraded fin condition so that lower daily feeding frequencies are beneficial for high stock densities. An influence of food supply in terms of fish condition was not found in this study possibly due to low fish density. Rearing a small number of salmonids can cause territorial behavior and aggression and is described in a variety of studies (Alanärä and Brännäs 1996, Laursen et al. 2015, North et al. 2006). However, in the current study negligible feeding or territorial aggressions occurred during the video records. In the current study, it is difficult to predict if increases or decreases in fish numbers would have resulted in different levels of aggression as behavior exhibits high levels of plasticity in behavior under varying densities and holding regimes (Harwood et al. 2002). Greater numbers of fish allow for greater stress in terms of feeding competition interactions at feeding events, however aggression may offer limited benefits to fish when feeding at higher densities (Harwood et al. 2002). Rasmussen et al. (2007) also reported lower levels of aggression at higher densities and suggested aggression may be advantage for feeding strategy at lower densities. In this study decreased territorial behavior may result from higher densities.

Hence, it is tentatively presumed that for higher densities (> 2 kg m^{-3}) or up-scaled experiments, less aggressive behavior will occur due to the above stated knowledge of salmonids reared at high densities. Further development or practice will assumedly allow larger groups of fish to be monitored in controlled feeding using this method. Equally, the advancement of artificial intelligence and computer aided monitoring systems promises to vastly increase the number of fish that can be accurately monitored with these methods.

No significant effects of the different feeding scenarios were present. This is supported by the literature whereby Mäkinen (1993) investigated the feeding rhythm of rainbow trout and neither continuous feeding nor diurnal peak feeding had an advantage on

feeding. However, unlike the current study, a significant tank effect was present in Mäkinen's study. Grayton and Beamish (1977) tested the feed intake of rainbow trout fingerlings feeding from 1 meal per 2 d to 6 meals per d, in experiments exhibiting some similarities to those conducted in this study. Where the current study reports optimal feed intake for 2 meals per day. Grayton and Beamish's (1977) similarly found applying two meals per day ensured fish growth (as an indirect measure of feeding success) was maximized. Similar results have been reported for larger rainbow trout (60-130 g) were 8h feeding cycles are beneficial (Landless 1976). While the use of only one feeding event for rainbow trout (≈ 155 g) is efficient for growth (Başçınar et al. 2007), the application of two daily feeding events decreases feed intake variations, which is highly beneficial for feeding regimes and delivery of special supplement diets or oral vaccines.

The video surveillance and analysis method developed herein allow us to state that MPI can be maximized and homoscedasticity of diet intake increased by feeding unstarved rainbow trout twice daily with multiple portions (3). With this applied feeding regime, the chance that each individual ingests a nominal minimum of 20 pellets (≈1% bw $fish^{-1}$) is maximized to nearly 100%. By this recommendation not only homogeneity and MPI of pellet uptake is maximized but also pellet wastage is minimized, leading to better cost efficiency and water quality. On the basis of the results of the starvation experiment in the current study, it is likely that results of the portion and daily feeding event experiment could be more explicit when applying absolutely no starvation period pre-feeding. Furthermore, results of the daily feeding event trial could be further optimized by the use of multiple portions. These recommendations differ from those of the FAO for homogeneous rainbow trout fingerlings growth which recommended a feeding frequency of 4 feeding events d^{-1} with 1% bw $fish^{-1}$.

In addition to presenting a viable and applicable method for measuring individual feed intake in Rainbow trout, the authors develop the minimum pellet ingestion (MPI) parameter. The MPI is an important tool for the optimization of feeding regimes and can also play a role in application of oral veterinary treatments or vaccines. The MPI measure is extremely useful when not only the quantity of feed intake is important but also minimal treatment or minimal vaccine intake across the entire fish population needs to be determined. To date, research on oral vaccination mainly used individual

handfeeding delivery methods. It is very unlikely that the vaccination approach of e.g. Adelmann et al. (2008) or Ballesteros et al. (2014) can be reproduced at commercial scales to deliver treatments or vaccines and guarantee MPI. Diverse current research on vaccine active components (Adelmann et al. 2008, Bøgwald and Dalmo 2019, Villumsen et al. 2014) and their administration methods (Bowersock et al. 1999, Ellis 1998, Garinot et al. 2007) with different protective mechanisms for the vaccine particles exists. Yet practical vaccine oral delivery methods require fundamental research outputs. This study provides methods and quantitative results related to direct determination and modification of pellet uptake. In doing so it partially bridges the knowledge gap between the active and adjustable components of a vaccine and the fundamental feeding regime required to ensure accurate oral delivery. This lends weight to the value of the methods presented herein to accurately understand and optimize MPI under different feeding regimes to allow accurate veterinary dosing under aquaculture conditions.

Future applications of these methods are planned for other key aquaculture species and in the application and optimization of oral veterinary treatments. Due to the presented method, the examination of e.g. attractants, the acceptance of different transport matrixes for vaccination or supplements of new feed ingredients can be performed fish conservative, specific and fast.

Authors' contributions

Philip N. Just: Conceptualization, methodology, sampling and evaluation, writing of original draft, manuscript reviewing and editing. **Bernd Köllner:** Conceptualization and reviewing. **Matthew James Slater**: Supervision, methodology review, validation, manuscript review and editing.

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Ethical statement

The authors followed all ethical policies of the journal in all methods and actions. Specifically, all experiments were conducted in accordance with the German Animal Protection Act (TierSchG) and the regulations on the protection of animals used for experiments or other scientific purposes (TierSchVersV) in agreement with applicable EU directives and were approved by the Veterinary Authority of Bremen under the administrative number TV148.

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Chapter 2

Effects of two delivery matrix component alginate and polyethylene-glycol on the intestinal tract and inflammatory response of juvenile rainbow trout (*Oncorhynchus mykiss*)

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Abstract

A critical step in the administration of oral vaccines in aquaculture is the effective protection of the vaccine antigens through the delivery route *water to stomach to the mucosal immune system* in the gut. Bioactive substances can be incorporated into protective matrices to ensure safe ingestion and controlled release into the posterior digestive tract. Polyethylene-glycol and alginate are promising and frequently applied substances in the oral administration of pharmaceutical substances, however their direct influence on the intestinal tract or inflammatory response are not well investigated in aquaculture fish. In the current study, these two substances were evaluated, separately and in combination. They were included in a commercial diet for juvenile rainbow trout (18.7 ± 0.1 g) and applied over 22 days in controlled feeding to determine possible gut alterations and inflammatory response. Three different sections of the intestine, the anterior pyloric caeca, the mid intestine and the posterior intestine, were histologically examined and expression of inflammatory genes were measured in each. The inclusion of polyethylene-glycol in the diet led to a significant reduction of villus height in the pyloric caeca and thus a significant reduction in active surface area (ASA). In the mid intestine alginate inclusion resulted in a significant reduction of the mucus secreting goblet cells and a significant increase in villus width. In the posterior intestine, polyethylene-glycol inclusion caused a significant increase in number of large vacuoles present, the addition of alginate buffered this effect. The results of the histological evaluation indicated symptoms which are usually evident in intestinal inflammation which can reduce functions of the respective intestinal segments. No significant increases in pro-inflammatory gene expression TNF-α, IL-8 or IL-1β were present indicating no severe inflammatory response. Based on the observed histological impairments, negative consequences such as reduced efficiency of an oral vaccine or productivity of aquaculture rearing due to hindered nutrient digestion can be considered unlikely.

Keywords Encapsulation matrix, Inflammation, Oral Vaccination, RAS, Fish Health

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1. Introduction

Few oral vaccines are available for aquaculture species despite the potential benefits of oral vaccinations in terms of animal welfare and production performance. There remains a lack of viable delivery and protection matrices for oral delivery of vaccine active ingredients and a dearth of information on the histological effects of matrices used in experiments to date. Immersion and injective immunizations are commonly used in aquaculture but the first cannot provide long lasting protection and the latter causes stress and potential tissue damage during and after vaccination handling along with high labor and capital costs. Effective oral vaccination can offer effective, long-lasting protection while lowering labor cost and fish stress. At present, experimental oral vaccines show efficacy in aquaculture research (Adelmann et al. 2008, Altun et al. 2010, Ballesteros et al. 2015, Caruffo et al. 2016) however, fundamental hurdles need to be overcome including a biocompatible, non-reactive delivery matrix and a defense mechanism to protect the vaccine against the harmful milieu of the fish stomach. Caruffo et al. (2016) concluded that the protection offered by an oral vaccine is dependent on the delivery form and the vaccine components.

Studies to date with oral vaccine active ingredients have applied several diet matrices without considering their histological effects on fish. Alginate, for example, has been used to protect the active components of an oral vaccine with success (Ballesteros et al. 2015, Chen et al. 2014, Joosten et al. 1997, Maurice et al. 2004, Polk et al. 1994, Rodriguez et al. 2018, Romalde et al. 2004, Rosas-Ledesma et al. 2012). In a more recent study by Adelmann et al. (2008) selected polyethylene-glycol as a non-reactive delivery matrix for an oral vaccine. The reasons for selection of these products as matrices are rarely reported (Mohapatra et al. 2019, Weber et al. 2006). The histological effects of these components on aquaculture fish is worthy of investigation and can provide insights on matrix relevance and biocompatibility.

1.1 Polyethylene-glycol

Polyethylene-glycol is a versatile polysaccharide with a broad range of application including pharmaceutical use. It has different properties with its variable molecular weight of 400-40,000 g mol^{-1} (Mishra et al. 2017). The neutrality of polyethylene-glycol has been validated as a reference marker in some mammals (Cooper et al. 1966, Jacobson et al. 1963) and birds (Webling 1966) in intestinal absorption experiments. Due to its eminent biocompatibility and lack of antigenicity and immunogenicity, polyethylene-glycol is considered an optimal delivery

matrix to embed vaccines into oral vaccine pellets for many commercial terrestrial species applications. Experimental aquatic applications include Adelmann et al. (2008) who used polyethylene-glycol-pellets to vaccinate rainbow trout against viral haemorrhagic septicaemia.

Polyethylene-glycol is also known to elevate transepithelial transport / permeability of membranes (Krugliak et al. 1990, Wood et al. 1998). By the conjugation of large molecules such as protein with polyethylene-glycol, a complex is formed that allows transepithelial transport, which is normally not possible for non-pegylated, parent molecules (Bailon and Berthold 1998). In human intestine epithelial cells (M-cells), antigen loaded polyethylene-microparticles induced a complete immune response (Garinot et al. 2007). Garinot et al. (2007) further described the higher stability of nanoparticles, promoted uptake and an increased grafting of molecules by M-cells, however the beneficial effects were not restricted to the cell type. Bailon and Berthold (1998) summarized the function and use of polyethylene-glycol conjugated protein. Generally, pegylated proteins show better thermal stability (Suzuki et al. 1984), protection against susceptibility to enzymatic degradation (Suzuki et al. 1984), increased solubility, enhanced potency and longer in vivo circulating half-life.

Lee et al. (2009) used polyethylene-glycol as a tannin-binding agent to enhance growth and blood antioxidant capacity of grey mullet fed with Sorghum. Their results showed that polyethylene-glycol eliminated the endogenous undesirable growth inhibitory factors and protected against LDL oxidation in blood. Moreover, total antioxidant capacity and cold adaption of grey mullet was improved. Further the polyethylene-glycol allowed slower clearance of protein from the blood and thereby longer acting medicinal effects. Based on the described effects of polyethylene-glycol, the use as delivery matrix for oral vaccination of fish is manifest, however no studies on the effects on histology of fish digestive system or any other tissues are available.

1.2 Alginate

Alginate, a polysaccharide found in the cell walls of brown algae and some bacteria, is commercially extracted from brown seaweeds. Due to its low toxicity, high biocompatibility, gelling properties and comparative low cost (Lee and Mooney 2012), alginate has found broad application in the food industry as well as pharmaceuticals.

In aquaculture, alginate is frequently used as a gelling agent for modification and improvement of feed pellets. In most published research an inclusion rate of 2% alginate is applied. The inclusion of alginate increases the hardness of the pellet, reduces the leaching of water-soluble nutrients and thus avoids reduction in water quality due to leachate (Rodríguez-Miranda et al. 2012). The effect of alginate as an additive in aquafeeds has been examined in a variety of studies with focus on the digestibility of protein, lipids (Spyridakis et al. 1989), macronutrients (Storebakken and Austreng 1987), on feed intake and intestinal passage (Storebakken 1985). Spyridakis et al. (1989) investigated different alginate inclusion levels (2-15%) and in European Seabass showed that high inclusion levels (<8%) can decrease protein and lipid digestibility significantly. Storebakken (1985) and Storebakken and Austeng (1987) tested the digestibility of different alginates and alginate levels in rainbow trout and showed alginates reduce apparent digestibility, pellet passage through the intestinal tract, feed intake and increase moisture content of the feces. No influence was measured for fish mortalities, liver weights or fish condition factors.

Based on these findings, alginate may offer advantageous properties in diets for oral vaccination of fish. A stable pellet represents a better / more efficient delivery to the fish with less vaccine loss to the system water. A slower passage within the fish intestine leaves more time for the pellet to dissolve and release the imbedded vaccine. In any case, less intrusion of lixiviated substances to the water ensures higher fish and environmental health for both open and recirculation aquaculture systems (McHugh 2003).

The described properties of alginate have prompted a variety of researchers to use it to protect diet ingredients from the digestive milieu of the foregut. Rosas-Ledesman et al. (2012) used calcium-alginate to encapsulate probiotic bacteria to pass it to the fish intestine without degradation by gastric juices. Encapsulation with calcium-alginate allowed 80% of the living probiotic bacteria to be transferred to the intestine. Moreover, the calcium-alginate beads showed good stability when stored for a minimum of 30 days without degradation. The protective effects of alginate in combination with chitosan for the oral application of bacterin (vaccine) has been tested by Polk et al. (1994). Within the study, the semi-permeable membranes of the alginate-chitosan encapsulations protected vaccine from degradation by gastric acid and altered time of dissipation and vaccine release to the gastro-intestinal tract. The authors verified the protective effects of alginate however, the substance release of the pellet was significantly reduced and only 35% of the vaccine was released within the first 24h

after feeding. Consequently, 65% of the vaccine remained in the pellet and was excreted, leading to an inefficient and cost intensive oral vaccine pellet.

Besides the gelling effects of alginate, other beneficial effects of administration are reported in the literature. In Nile tilapia (*Oreochromis niloticus*) the inclusion of 1-3% sodium alginate stimulated growth performance, immunity and disease resistance (Van Doan et al. 2016). In sea bream (*Sparus aurata*), the addition of 2% or 5% sodium alginate in the diet led to higher lipid deposition in the flesh resulting in better feed conversion (Peso-Echarri et al. 2012). In juvenile grouper (*Epinephelus fuscoguttatus*), Chiu et al. (2008) found 1 - 2% sodium alginate inclusion in the diet led to significantly increased lysozyme activity, respiratory bursts, superoxide dismutase (SOD) activity and phagocytic activity.

Next to the oral administration of alginate, injective, intraperitoneal (i.p.) administration can enhance the immune capability of fish. After i.p. injection of sodium alginate orange-spotted grouper, showed significantly increased respiratory burst, SOD - and phagocytic activity. Moreover, fish receiving sodium alginate showed significantly increased survival rates after challenge with *Vibrio alginolyticus* compared to control treatments (Cheng et al. 2007). The authors conclude that sodium alginate might increase the non-specific immune response and resistance to bacterial infections. This conclusion is supported by a study by Peddie et al. *(2002)* which investigated the immune stimulating effects of i.p. administered ergosan (0.002% unspecified plant extract, 1% alginate and 99% algal based carrier) to rainbow trout. Likewise, to Cheng et al. (2007), the injection of ergosan led to significantly increased neutrophils, respiratory burst activity and expression of interleukins and thereby increased disease resistance.

While widely applied, polyethylene-glycol and alginate remain unstudied for their own effect on fish gut histology. There is frequent oral use of both substances in aquaculture research, however no data exists of potential alterations to or inflammation of the fish gut. The goal of this study is to assess the effects of polyethylene-glycol and alginate to the digestive tract of rainbow trout in controlled feeding experiments and to draw conclusions about the potential effect on the fish and on the efficacy of delivery of the active oral vaccine ingredients being provided.

2. Materials and Methods

2.1 Experimental fish and design

The experiment was conducted at the Center for Aquaculture Research (ZAF) of the Alfred-Wegener-Institute Helmholtz-Center for Marine and Polar Research, Bremerhaven. Juvenile rainbow trout, *Oncorhynchus mykiss*, Walbaum (18.7 ± 0.1 g) were obtained from a commercial fish farm (Teichwirtschaft Ahlhorn, Emstek, Germany). Fish were acclimated to the experimental conditions (15°C) for 14 days in a circular 0.51 m^3 tank appended to the experimental system before the trial started. During acclimation, fish were hand-fed a commercial diet (F-1P Classic LT / F 2.5 mm, Skretting) twice daily until visual satiation.

Besides the acclimation tank, the recirculating aquaculture system consisted of 16 individual adjustable glass tanks (48 x 38 x 49 cm, length x width x height), two cooling elements (Aquamedic Titan 2000), a foam sheet filter, a moving-bed nitrification bio-filter, a UV-sterilizer (Aquamedic Helix Max 36 W) and a monitoring system (Senect Filter Control with water level and pH / temperature sensor).

Throughout the experimental period, the water temperature was kept at 15 ± 1 °C, aeration and water flow was adjusted and kept at 4.5% min^{-1} and 350 L hr^{-1}, respectively. Additionally, ammonia, nitrate and nitrite were checked regularly and values did not exceed 0.11 mg L^{-1}, 0.2 mg L^{-1} and 45 mg L^{-1}, respectively. Oxygen level was 90 ± 4% and never felt below 85%.

One day before the trial started, 15 juvenile rainbow trout were randomly transferred into each of the 16 tanks (4 treatments and quadruplicates). During the trial, fish were hand-fed the respective experimental diet (***see 2.2***) until visual satiation twice daily (9 a.m. and 5 p.m.). After the feeding sessions, pellet leftovers were siphoned from the tanks to prevent further pellet uptake and to keep water quality high.

2.2 Experimental diets / treatments

Three test diets and a commercial control diet were prepared. The experimental diets were produced by the Technology and Transfer Centre Bremerhaven (TTZ Bremerhaven) using a twin-screw cold extrusion system. For all four treatments a commercial floating trout diet with 2.5mm diameter pellets (F-1P Classic LT / F 2.5mm, Skretting) was used as the base formulation. The base diet contained field bean meal, soybean meal, fish meal, poultry protein, hydrolyzed feather meal, wheat, canola oil, poultry fat, porcine hemoglobin powder, fish oil (Table 1).

Control pellets (C) – To exclude possible effects of the manufacturing process, F-1P pellets for the control diet were crushed and compressed 3 times to produce stable 2.5 mm pellets

Alginate pellets (ALG) – For the production of 100 g ALG pellets, 95 g of F-1P Classic was crushed and mixed with 5 g alginate. Afterwards, the mixture was compressed three times to produced stable 2.5 mm pellets.

Polyethylene-glycol pellets (PEG) – For the production of 100 g PEG pellets, 82 g of F-1P Classic was crushed and compressed with 18 g polyethylene-glycol. The used polyethylene-glycol was a 1:1 mix of polyethylene-glycol 1000 and polyethylene-glycol 1500. Afterwards, the mixture was compressed a further two times to produced stable 2.5 mm pellets.

Alginate/polyethylene-glycol pellets (PEG-ALG) – For the production of 100 g PEG-ALG pellets, 77.9 g crushed F-1P Classic, 17.1 g polyethylene-glycol and 5 g alginate were mixed and compressed and compressed a further two times to produce stable 2.5 mm pellets.

2.3 Sampling

At the beginning of the trial (d0) length and weight of all released fish were measured. Sixteen additional fish from the cohort were euthanized with a combination of a high dosage of tricaine methanesulphonate (MS 222, 300 mg L^{-1}, Sigma-Aldrich) followed by a sharp blow to the head. Directly after, blood was drained from the caudal vein, weight and length measured. Afterwards, the fish abdomen was opened, total entrails, liver and spleen (somatic parameters) were measured and segments of the pyloric caeca, mid- and posterior intestine (intestinal samples) taken. The spleen was removed, transferred to RNAlater (Sigma-Aldrich) and stored at -80°C until mRNA analysis**.** The intestinal samples were collected in embedding cassettes (ROTILABO®) and immediately fixed in Baker's formol for 24 h and transferred to gum sucrose (stored at 4°C) until methacrylate embedding**.** Seven, 14 and 21 days after the trial start, the same samples were taken from four fish of each replicate following the described procedure.

Table 1 Proximate composition of commercial diet

Composition	Inclusion
Protein	47 %
Ash	2.8 %
Lipid	7.5 %
Fiber	1.1 %
Energy	18.5 MJ/kg

2.3.1 Growth and somatic parameters

From the measured weight, length and somatic parameters, the following somatic indices were calculated following:

$$Weight\ gain\ |\text{Specific growth rate}|\ \text{SGR}\ (\%\ \text{body weight}\cdot day^{-1})$$

$$\text{Specific growth rate}|\ \text{SGR}\ (\%\ \text{body weight}\cdot day^{-1}) = \frac{\ln(final\ \text{body}\ weight) - \ln(initial\ \text{body}\ weight)}{total\ duration\ in\ days}\ x\ 100$$

$$= \frac{\ln(final\ \text{body}\ weight) - \ln(initial\ \text{body}\ weight)}{total\ duration\ in\ days}\ x\ 100$$

$$\text{Condition factor}\ |\ \text{CF}\ (\%) = body\ weight\ x\ body\ length^{-3}\ x\ 100$$

$$\text{Hepatosomatic index}\ |\ HSI\ (\%) = \frac{liver\ weight}{total\ body\ weight}\ x\ 100$$

$$\text{Spleen somatic index}\ |\ SSI\ (\%) = \frac{spleen\ weight}{total\ body\ weight}\ x\ 100$$

$$\text{Visceral somatic index}\ |\ VSI\ (\%) = \frac{total\ entrails\ weight}{total\ body\ weight}\ x\ 100$$

2.3.2 Intestinal samples preparation

To prevent fungal damage to samples, the gum sucrose was replaced every 10 days. Tissue embedding: Briefly, after three cleaning rounds with distilled water and four dehydration steps in graded acetone, the samples were washed with methacrylate-monomer overnight and embedded in N,N-dimethylaniline-activated methacrylate the next day. To prevent high temperature and sample damage during polymerization, the filled molds were left in the fridge (7°C) for a minimum of three days. From the polymerized blocks, 3 µm sections were cut with a semi-motorized rotary microtome (Leica RM2145) and mounted on silane coated glass slides. The slides were then stained with hematoxylin and eosin (H & E) for morphological analysis.

2.3.3 Histological analysis

Images of the stained slides were taken with a ZEISS AxioCam with a magnification of x10 and x40. Morphometric evaluation of the samples: The impact of the different fed substances on the intestine was analyzed with the ZEISS Axiovision SE Rel 4.9 software. Slides from the pyloric caeca and mid-intestine were analyzed for goblet cells (number of gc / mm), villus height, villus width, villus height-weight ratio, goblet cell density, active surface area (ASA) and appearance of large vacuoles following scoring by Verdile et al. (2020), Voorhees et al. (2018) and Barnes

et al. (2014) (Table 2). A total of ten folds for each pyloric caeca sample and twelve simple folds for each mid-intestine sample were measured, to represent an adequate number of villi and therefore reduce potential noise (modified from Ferreira (2016)). The measured diameter of the tissue sample was used to correct for segment-size differences.

Due to the complex morphological structure of the posterior intestine (Figure 1), the analyses were less detailed and according to Verdile et al. (2020) only goblet cells and the appearance of large vacuoles of the complex folds were evaluated.

Table 2 *Scoring table for intestine tissue following Voorhees et al. 2018*

Score	Criteria
1	Large vacuoles absent
2	Very few large vacuoles present
3	Increased number of large vacuoles
4	Large vacuoles are numerous
5	Large vacuoles are abundant in most epithelial cells

2.3.3 Inflammatory RNA expression

RNA was isolated and purified from liver and spleen tissue samples as well as PBL using the RNAdvance Tissue Kit (Beckman Coulter) and the RNeasy Mini Kit (Qiagen), respectively, according to manufacturer's instructions. Purified RNA samples were checked for RNA content with a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies) and stored @ - 80°C until expression measurement via RT-qPCR.

Quantitative real-time PCR (RT-qPCR) was performed on a CFX96 Touch™ Detection System (Bio-Rad, Feldkirchen, Germany) to evaluate the expression of inflammatory genes IL-1β, IL-8 and TNF-α (Table 3). SensiFAST SYBR No-Rox Kit (Meridian, Bioline) was used, following the manufacturer's instructions with minor modification. Briefly, for each sample 5 µl SensiFAST SYBR No-Rox One-Step mix, 0.4 µl of the respective forward and reverse primer, 0.1 µl reverse transcriptase and 0.2 µl RiboSafe RNase inhibitor were prepared in each well of the 96-well plate (0.1 ml, white, Steinbrenner SL-PP96-1LWA). In a final step, 10 ng of prepared sample were added and the PCR started. For each run, RNase free water was used as a negative and a confirmed RNA sample was used as a positive control. The following PCR temperature profile was used: an initial step with reverse transcription 10 min @45°C and polymerase activation 2 min @95°C followed by 40 cycles of: denaturation 5 s @95°C, annealing 20 s @56°C for ef1,

TNF-α and IL-8 / @60°C for IL-1β and elongation 5 s @72°C. Resulting ct values were normalized with the elongation factor 1 and relative gene copies calculated following:

$$Relative\ gene\ copies\ (RGC) = 41 - ct$$

To evaluate potential effects of the test substances against the control treatment, RGC data from the control treatment were used to generate a normscore with the respective 25% quantiles, maximum and minimum values (Figure 4).

2.4 Statistical analysis

RStudio (Version 1.2.5033) was used for the statistical evaluation of all parametric data. Data were tested for normal distribution and homogeneity of variance using the Shapiro-Wilk test (*shapiro.test*) and Bartlett test (*bartlett.test*), respectively. For somatic indices data, a log-transformation was performed to obtain a normal distribution. When normal distribution and homogeneity of variance assumptions were true an ANOVA was performed. In case of significant difference, the Tukey HSD post-hoc test (tukeyHSD) was performed to show differences between groups. When assumptions for ANOVA were not given, a Kruskal Wallis test (kruskal.test) was performed to test for significant differences. The Dunn's post-hoc test (*dunnTest*) was used to present significant differences between treatments, additionally the respective effect size (Cohen's d with d = 0.1 small effect, d = 0.3 intermediate effect, d = 0.5 large effect) was calculated (Cohen 1988).

Table 3 RT-qPCR primer sequences, base pairs (BP), reference and annealing temperature (AT)

Primer	Primer sequence	BP	Reference	AT
Elongation factor 1	F: TGCCCCTGGACACAGAGATT R: CCCACACCACCAGCAACAA	90	(Schug et al. 2019)	56°C
Interleukin 1 β	F: TTGGGCCTCTACGATCAGGA R: CAGGGGCGCTTACCACAATA	172	(Wang et al. 2019)	60°C
Interleukin 8	F: ATTGAGACGGAAAGCAGAC R: CTCAGAGTGGCAATGATCTC	101	(Schug et al. 2019)	56°C
Tumor necrosis factor α	F: GTGATGCTGAGTCCGAAAT R: GTCTCAGTCCACAGTTTGTC	97	(Schug et al. 2019)	56°C

3. Results

3.1 Physical and somatic parameters and apparent histological appearance

While growth and somatic parameters did not differ significantly there was a 13% decrease in final fish weight in the PEG treatment compared to the control which equates 30% of weight gain (Table 4). All other parameters did not differ significantly.

3.2 Histomorphological appearance

The histological evaluation led to no apparent abnormalities in the different tissues. The structural organization of the pyloric caeca resemble that of the mid-intestine. These organ samples were characterized by a ring-like arrangement of simple folds in which small and large folds alternated. In contrast to the pyloric caeca, the mid-intestine showed a higher fold density. For the comparability of the statistical evaluation only large villi were assessed. The structure of the posterior intestine had a more complex arrangement compared to the other two sampled tissues. Here, simple folds arose from an inner tissue ring (Figure 3a) making up the complex folds. (Figure 1).

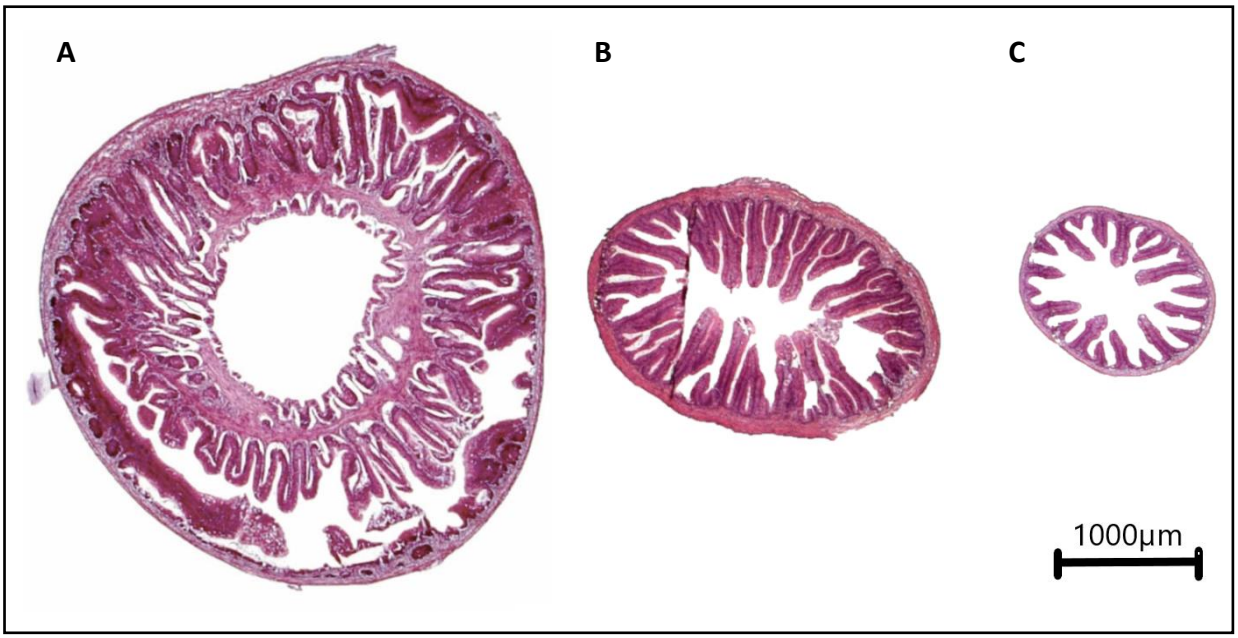

Figure 1 Intestinal cross sections of rainbow trout. A: Cross section of the posterior intestine with simple and complex folds. B: Cross section of the mid intestine. C: Cross section of the pyloric caeca. Images show intestinal sections of the control treatment.

Table 4 *Somatic indices and growth parameters of juvenile trout fed experimental diets after 22 d. C = Control, ALG = Alginate diet treatment, PEG-ALG = Polyethylene-glycol and Alginate diet treatment, PEG = Polyethylene-glycol diet treatment. CF = Condition factor, VSI = Visceral somatic index, HIS = Hepatosomatic index, SSI = Spleen somatic index. All data presented as mean ± SD, N = 64.*

Parameter	C	ALG	PEG-ALG	PEG
Final length [cm]	13.8 ± 0.9	13.8 ± 0.8	13.7 ± 0.9	13.3 ± 0.9
Final weight [g]	30.0 ± 6.6	29.8 ± 5.6	28.0 ± 6.3	26.6 ± 5.5
Weight gain [g]	11.2 ± 1.8	10.9 ± 1.8	9.4 ± 2.8	7.8 ± 2.0
CF	1.13 ± 0.06	1.12 ± 0.14	1.07 ± 0.07	1.11 ± 0.07
VSI	8.28 ± 1.39	8.51 ± 1.44	9.26 ± 1.47	9.22 ± 1.76
HSI	1.57 ± 0.39	1.39 ± 0.19	1.46 ± 0.24	1.49 ± 0.34
SSI	0.102 ± 0.056	0.109 ± 0.037	0.102 ± 0.038	0.128 ± 0.06

3.3 Alterations of the pyloric caeca

The villus height differed significantly between the PEG and the C / PEG-ALG treatments (Z3.34 p = 0.004, d = 0.2 | Z = 3.6 p = 0.002, d = 0.2) while there were no significant differences between the PEG and the ALG treatment (Z = 1.92 p = 0.22). There were high levels of variation throughout the measured parameters in the pyloric caeca. Results of the active surface area were homologous to the results of the villus height and the ASA of the PEG treatment was significantly lower compared to the control and PEG-ALG treatment (Z = 3.6 p ≤ 0.01, d = 0.2 | R = 3.0 p = 0.2).

Table 5 *Pyloric caeca data of juvenile trout fed experimental diets after 22 d. C = Control, ALG = Alginate diet treatment, PEG-ALG = Polyethylene-glycol and Alginate diet treatment PEG = Polyethylene-glycol diet treatment. ASA = Active surface area, H/W = Height-weight ratio, GC = Goblet cells, VAC = Vacuole Score.*

	C	ALG	PEG-ALG	PEG	Statistical outcome
Height [µm]	279.6±60.7	289.2±79.3	285 ± 72.9	272.8±68.9	
Height / section area	302.9 ± 97	291.1±95.9	301.8±80.9	277.8±78.3	χ^2 =16.43 df03 p<0.001
Width [µm]	90.3 ± 19.7	94.6 ± 20.5	88.4 ± 19.1	88.8 ± 16.7	
Width / section area	98.2 ± 33.1	96.5 ± 31.6	94.4 ± 25.4	90.5 ± 20.6	χ^2=2.23 df=3 p=0.53
ASA [µm]	625 ± 141	641 ± 177	641 ± 181	602 ± 170	
ASA / section area	674 ± 213	651 ± 193	675 ± 190	609 ± 174	χ^2=15 df=3 p=0.002
H/W ratio	3.2 ± 0.9	3.2 ± 1	3.3 ± 1	3.1 ± 0.8	χ^2=4.61 df=3 p=0.2
GC/mm	11.2 ± 4.1[a]	11.9 ± 5.2[a]	8.8 ± 4[b]	8.8 ± 3.4[b]	χ^2=23.2 df=3 p<0.001
VAC	1.78 ± 0.45	1.77 ± 0.43	1.91 ± 0.39	2 ± 0.53	

PEG and ALG did not differ significantly (Z = 1.94 p = 0.21). There were no noticeable changes in villus height - width ratio between treatments. The number of goblet cells was significantly reduced by the inclusion of polyethylene-glycol. Treatments with inclusion of PEG differed significantly from the C and ALG treatment (ALG-PEG-ALG Z = 3.56 p = 0.002 d = 0.2| C-PEG-ALG Z = 3.25 p = 0.005, d = 0.2 | ALG-PEG Z = 3.55 p = 0.002 d = 0.2 | C-PEG Z = 3.24 p = 0.004 d = 0.2). The appearance of large vacuoles (VAC) unaltered (Table 5 / Figure 2). Only in the PEG treatment, some samples showed an increased number of large vacuoles. In comparison to other tested intestine sections, the pyloric caeca were least influenced (Figure 2).

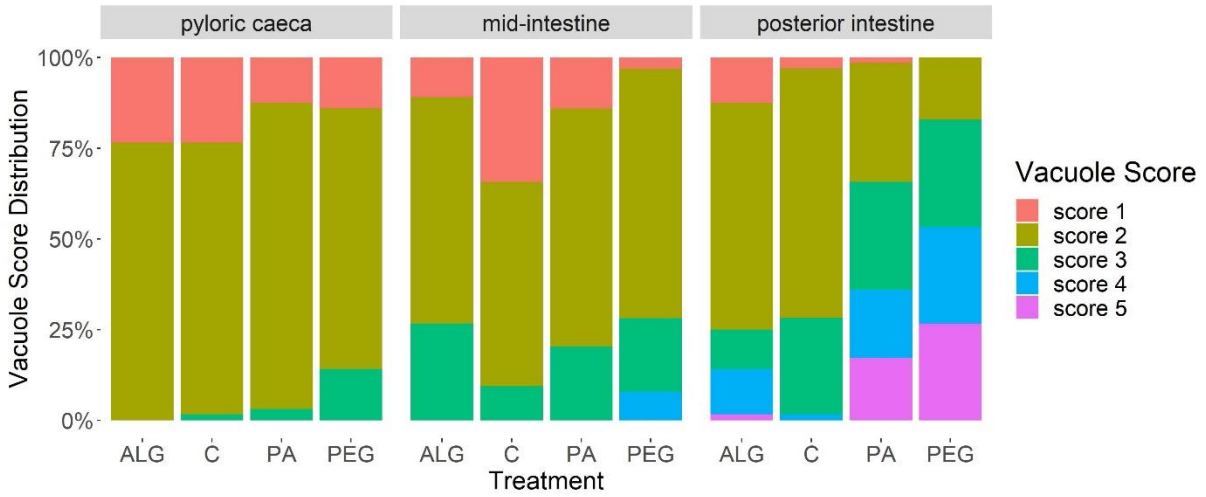

Figure 2 *Relative distribution of the vacuole scores in the pyloric caeca, mid-intestine and posterior intestine.*

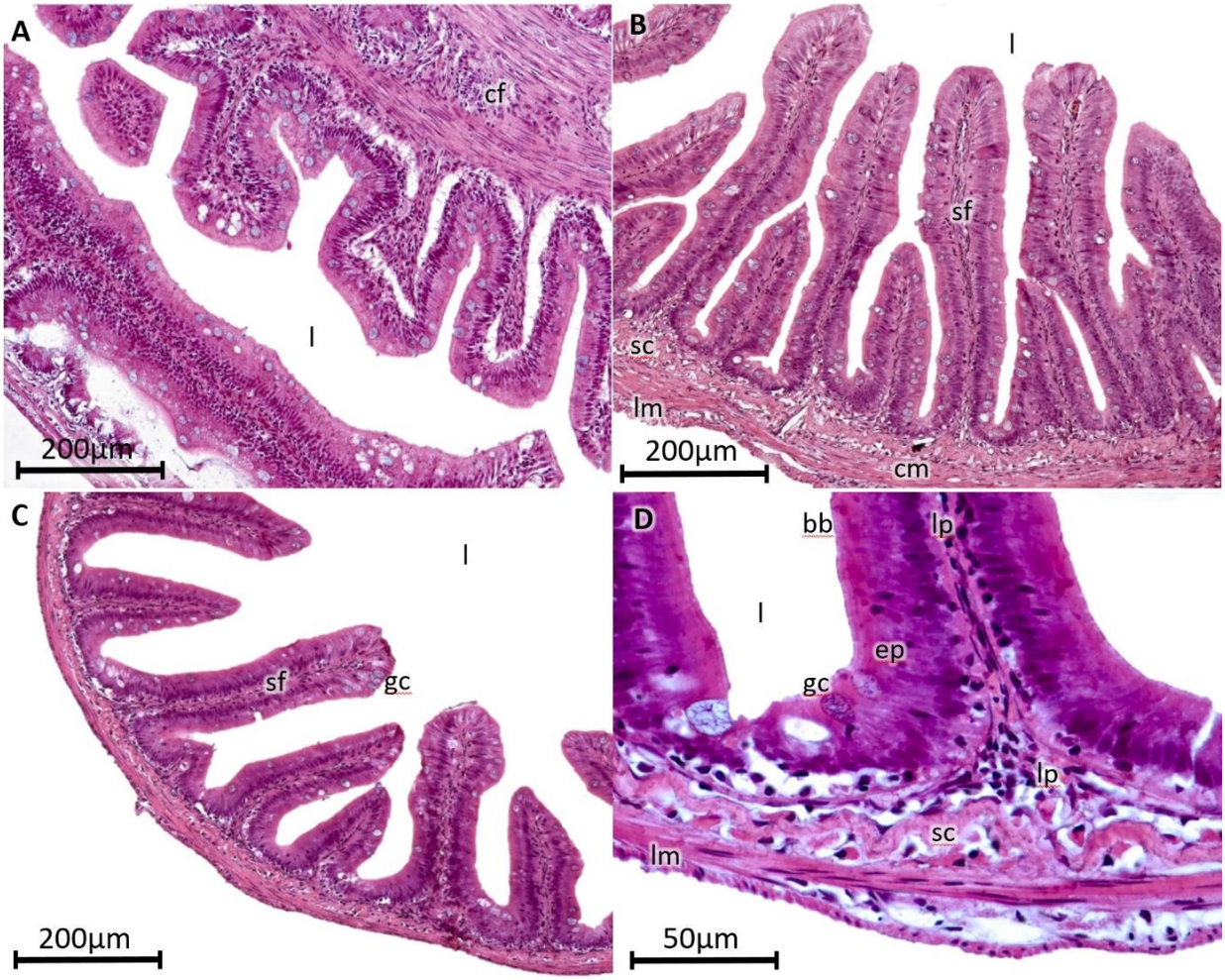

Figure 3 *Detailed morphological structure of rainbow trout intestine stained with hematoxylin and eosin. A. Bottom left section of the posterior intestine with a longitudinal, contorted complex fold (compare Figure 1A). B: section of the mid-intestine, C: section of the pyloric caeca. While the complex folds are characteristic for the posterior intestine, only large and small folds were found in the mid-intestine and pyloric caeca. D: Close up of the base pyloric caeca section. Main distinguishable tissues are labeled: cf – complex folds, sf – simple fold, cm - circular layer of the muscularis, lm – longitudinal layer of the muscularis, ep – epidermis which mainly consist of enterocytes, gc – goblet cells, l – intestinal lumen, lm - lamina propria and sc – stratum compactum*

3.4 Alterations of the mid-intestine

While villus height was not significantly affected by treatment, significant differences between diet treatments were found in villi width and in the derived villi height / weight ratio (H/W ratio) (Table 6). The integration of alginate into the diet led to significantly wider villi and hence significantly lower H/W ratios with a medium effect size (Cohen 1992) (Appendix Table A4 and Table A5). The mid-intestine of fish fed diets containing alginate showed significantly more goblet cells / mm compared to fish that did not receive alginate included diets (C-ALG $Z = 3.5$, $p = 0.002$, $d = 0.2$ PEG-ALG $Z = 5.85$ $p < 0.001$, $d = 0.3$). Further, significant differences for gc/mm existed between the PEG-ALG and PEG treatment ($Z = 4.12$ $p < 0.001$, $d = 0.2$, results of the Post-hoc test are given in the appendix Table A6). No changes however were present in the active surface area of villus in the mid intestine. With the integration of polyethylene glycol, an increased number of large vacuoles was present (Figure 2). While the control and treatments with alginate only showed vacuole score of 1-3, in the PEG treatment, multiple scoring with 4 indicated an increase of large vacuoles in the mid-intestinal tissue.

Table 6 Summary of evaluated parameters in the mid-intestine samples. C = Control, ALG = Alginate diet treatment, PEG-ALG = Polyethylene-glycol and Alginate diet treatment PEG = Polyethylene-glycol diet treatment. Data presented as mean ± SD, $N_{height,\ width,\ ASA,\ HW\ ratio,\ GC} = 768$, $N_{vac} = 256$. *Statistical output presents results of the Kruskal-Test*

	C	ALG	PEG-ALG	PEG	Statistical outcome
Villus height / section area	144.2 ± 38.9	141.7±32.5	137.4±42.3	137.9 ± 32.5	χ^2=5.49 df=3, p=0.14
Villus height	397.8 ± 97.5	377.3±87.4	377 ± 88.4	377.7 ± 87.4	
Width / section area	37.4 ± 10[b]	40.3±10.9[a]	39.2 ± 12.3	37.9 ± 12.5[b]	χ^2=11.49 df=3 p=0.009
Villus width	102.4 ± 20.4	106.5±20.1	107.6±27.9	101.4±24.4	χ^2=10.66 df=3
H/W ratio	4.1 ± 1.4[a]	3.7 ± 1.1	3.8 ± 1.7[b]	3.9 ± 1.2	p=0.014
ASA / section area	323 ± 83.5	316.2±74.7	312.4±98.8	315.5 ± 76.7	χ^2=2.92 df=3 p=0.44
ASA	897.4±221.9	842.9±208	858±216.2	865.8±214.4	χ^2=37.55 df=3
GC/mm	19.4 ± 5.9	21.7 ± 6.5	20.6 ± 7	17.7 ± 5.2	p<0.001
VA (vacuole appearance)	1.75 ± 0.62	2.16 ± 0.6	2.06 ± 0.59	2.14 ± 0.47	

3.5 Alterations of the posterior-intestine

In the posterior intestine, both integrated substances led to significant changes of the appearance of large vacuoles in the tissue (Table 8). While within the control and ALG treatments, the majority of tissue showed small numbers of large vacuoles, in the PEG-ALG treatment 18.8% and 17.2% of the samples had vacuole scores of 4 or 5, respectively. In the PEG treatment, these values were elevated to 26.6% for vacuole scores of 4 and 5 each (Figure 2). Goblet cells per mm surface did not differ significantly between treatments. However, an increased trend of GC/mm was present in the control compared to all other treatments (Table 7).

Table 7 Results of the parameters evaluated in the posterior intestine. C = Control, ALG = Alginate diet treatment, PEG-ALG = Polyethylene-glycol and Alginate diet treatment PEG = Polyethylene-glycol diet treatment. Data presented as mean ± SD, Statistical results present the results of the one-way ANOVA. N = 256

	C	ALG	PEG-ALG	PEG	Statistical outcome
GC/mm	22.1 ± 6.5	20.1 ± 5.9	20.0 ± 6.6	20.0 ± 5.1	F(3,252)= 1.842, p=0.14
VA	2.3 ± 0.5	2.3 ± 0.9	3.2 ± 1.1	3.6 ± 1.1	

3.6 Inflammatory marker analysis (mRNA)

Inflammatory gene markers IL-8, IL-1β and TNF-α did not deviate significantly in levels of expression from the norm value range derived from the control treatment (Figure 4a-c).

Spleen values of IL-1β did not show significant differences among the two early sampling times points t1 = 14 d and t2 = 21 d ($ANOVA_{t1}$ F(3,60) = 0.2, p = 0.9 | $ANOVA_{t2}$ F(3,60) = 2.104, p = 0.1). The average number of relative IL-1β gene copies was 8.3 ± 0.2 after 7 days and 7.3 ± 0.6 after 14 days of continuous feeding of the respective diets. No abnormalities were present (figure 4a). The different dietary treatments resulted in no significant differences for gene expression of TNF-α ($ANOVA_{t1}$ F(3,60) = 2.201, p = 0.1 | $ANOVA_{t2}$ F(3,60) = 1.294, p = 0.29). Mean relative gene copies was 9.6±0.4 for all treatments and evaluated time points (figure 4b). While TNF-α and IL-1β showed strong conformity, small but insignificant differences were present for IL-8 (Figure 4c). Still, values for TNF-α are within the norm value range (Figures 4c, dashed line) and mean relative gene copies were 10.7 ± 0.4 for t1 and 9.6 ± 0.2 for t2 with no significant differences for t1 ($ANOVA_{t1}$: F(3,60) = 2.28, p = 0.09) nor t2 ($ANOVA_{t2}$: F(3,60) = 0.87, p = 0.46). A noticeable decreasing trend was present between t1 and t2, however average cycles decrease was 1.1 cycles and can therefore be neglected.

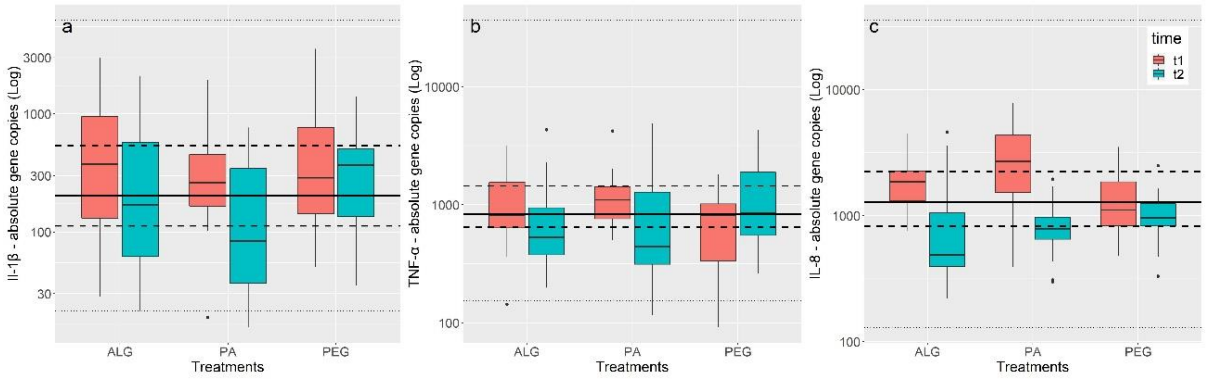

Figure 4 Expression of inflammatory gene markers IL-1β (4a), TNF-α (4b) and IL-8 (4c) as absolute gene copies. The norm value ranges for IL-1β, TNF-α and IL-8 were defined by the 1st and 3rd quantile (dashed line), maximum and minimum values (dotted lines) and the median (straight line) of the control treatments for all time points. N = 48. C = Control, ALG = Alginate diet treatment, PEG-ALG = Polyethylene-glycol and alginate diet treatment PEG = Polyethylene-glycol diet treatment.

4. Discussion

Oral vaccinations promise significant benefits in aquaculture, however suitable diet matrices for vaccine delivery remain poorly developed and untested for possible influences on gut physiology. The current study revealed the influence of orally applied polyethylene-glycol and alginate on the intestinal tract of rainbow trout. None of the test diets led to significant changes in growth parameters or inflammatory gene markers, despite decreases in final fish weight in the PEG treatment. It is unclear if differences were due to reduced digestibility, increased intestinal passage speed due to laxative effects (Schiller et al. 1988), lower pellet uptake during feeding or due to intestinal. The intestinal histological examinations showed symptoms conducive with inflammation after 22 days including a significant reduction in villus height in the pyloric caeca and active surface area (ASA) in trout fed the PEG diet. In the posterior intestine, PEG inclusion also significantly increased large vacuole numbers. In the mid intestine, alginate inclusion significantly reduced mucus secreting goblet cells and increased villus width.

A reduction of the villus height in the pyloric caeca and concomitant reduction of active surface can lead to a reduction in immunological response, as the pyloric caeca are determined as main location for IgM+ and IgT+ B-cell recruitment (Ballesteros et al. 2013). Consequently, less epithelial cells will have space to interact with the intestinal lumen, resulting in a reduced efficiency of orally administered pro- and prebiotics, vaccines or other

pharmaceutical additives. While for most molecules a large epithelial area is of major importance for their uptake, the antigen of an orally administered vaccine is detected by intraepithelial macrophages (Figure 3d) and transported to the secondary lymphatic organs (Embregts and Forlenza 2016). Intestinal damage or alterations of the intestinal folds, as present in the pyloric caeca samples, may increase infiltration of monocytes into the epithelial tissue and consequently increase the detection of the vaccine antigen.

Overall, the results of this study showed that the inclusion of alginate in the PEG treatment decreased potentially unfavorable effects of polyethylene-glycol, as present in mid-intestinal and pyloric caeca width / section area, the pyloric caeca villi height / section area and ASA and mid-intestinal GC/mm (Figure 2). Hence, the posterior intestine was the most negatively affected tissue. These, as well as the alterations of the pyloric caeca, may indicate against the use of PEG as a matrix for oral vaccination as the posterior intestine is the major site for immune response initiation (Villumsen et al. 2014). This may be dependent on the period of application and pathways of immune response stimulation. For the results of this study, no statements can be made about the direct interactions of alginate or polyethylene-glycol regarding the efficiency of an oral vaccine or other pharmaceuticals.

4.1 Changes in large vacuole appearance

Dietary polyethylene-glycol led to significantly increased numbers of high vacuoles scores in the posterior intestine over a feeding period of 22 days. Alterations of the posterior intestine as observed may dampen uptake of macromolecules (oral vaccines or additives) (Ray and Ringo 2014) and effects (Miao et al. 2018) or lead to loss of function (Chikwati et al. 2013). Conversely, these alterations may increase of a vaccine effect / immune response due to local tissue impairments, as it is systematically provoked by adjuvants (Clements and Griffiths 2002). In most vaccines the adjuvants play a crucial role in ensuring sufficient immune response, however tissue damage or different forms of cell death can also have such modulating effects to activate the immune system (Pulendran et al. 2021).

Future research should shed light on the question, whether observed changes by polyethylene-glycol enhance or decrease the effectiveness of an orally applied vaccine. The posterior intestine, as the last part of the intestinal tract, is of high importance for molecule uptake and induction of the immune response as demonstrated by Villumsen et al. (2014). Full protection in rainbow trout against enteric redmouth disease was achieved after anal

vaccination, whereas the protection with the same dosage applied orally was significantly reduced (Villumsen et al. 2014).

Nonetheless, the impacts on growth and diet utilization in commercial aquaculture production must be balanced against any potential positive effect on immune response. In addition to the effect of morphologic changes on the effectiveness of vaccination or other pharmaceutical products, the direct influence of the test-substances on the function of the posterior intestine is likely to be negative. Not only larger proteins or peptides get taken up in the distal intestine (Sire and Vernier 1992) but also digestive enzymes and biological messengers (Bjørgen et al. 2020).

4.2 Changes of villi appearance

A shortening of intestinal villi which indicated an inflammation was explicitly present in the pyloric caeca and was noticeable in the mid intestine, leading to the conclusion that polyethylene-glycol has the potential to reduce the health status of the fish gut significantly when applied over periods of 22 days. The inclusion of alginate in the PEG treatment compensated the negative effects of polyethylene-glycol and the villi height and width was less influenced in the pyloric caeca or mid-intestinal tissue, respectively. The prebiotic effects of alginate were evaluated by Van Doan et al. (2016) in tilapia (*Oreochromis niloticus*) with inclusion of 1-4% in the diet stimulating growth performance, innate immunity and disease resistance.

Conversely, mid-intestine samples in the current did not indicate that alginate reduces inflammation reducing villi width and height. Van Doan et al. (2016) found that increased amounts of alginate reduced beneficial effects. The alginate inclusion levels (5%) in the current study may explain the observed villi widening of the mid-intestine. Widening of the villus can have different causes, and in case of a dietary cause inflammation, the widening is caused by an abundant infiltration of the laminaria propria by immune cells (Bjørgen et al. 2020, Sealey et al. 2009). As described above, harmful alterations of the tissue will lead to a reduced function of the respective tissue. In salmonids, proteins are primarily absorbed in the mid-intestine (Bakke et al. 2010), consequently any deterioration of the mid-intestinal tissue leads to a diminution of protein resorption and thereby reduced growth.

The pyloric caeca did not show significant alterations of the villi width due to alginate but a trend towards reduced villi width in the treatments with polyethylene-glycol inclusion. The

reason for that could be found in the morphological structure of the tissues, as in comparison to the mid-intestine, the contact with the dietary components was lower. Concurrent to the mid-intestine and posterior intestine, signs of inflammation were also present in the pyloric caeca as the villi height was significantly reduced in the treatment with polyethylene-glycol inclusion (without the addition of alginate). Consequently, the active surface area in the pyloric caeca was reduced (Table 5) which can considerably affect the uptake of carbohydrates, lipids and small fatty acids as these substances are taken up in the distal part of the intestine (Bakke-McKellep et al. 2000, Bakke et al. 2010).

4.3 Changes in goblet cells

Significant reductions in goblet cell density were found along the epithelial tissue in the pyloric caeca and mid-intestine in response to polyethylene-glycol diet inclusion. The function of goblet cells is the release of mucin to the intestinal lumen to promote molecule release and uptake and to protect the tissue against shear stress and chemical damage (Colburn et al. 2012, Giorgini et al. 2018). Therefore, inclusion of polyethylene-glycol led to a decrease of mucin production, digestion and resilience. In accordance with the literature, lower numbers of goblet cells were present in the distal region (pyloric caeca) of the intestine (L⊘kka et al. 2013). As described by Baeverfjord (1996) the goblet cell density is linked to starvation, as starved fish have increase number of goblet cells. Santigosa et al. (2008) found a significant decrease of goblet cells in fish under complete substitution of fishmeal by unfavorable plant-based protein. Hence unfavorable diet appears to have decreased the goblet cell density of the fish intestine as presented in this study. We propose that due to elevated transepithelial transport, permeability of membranes (Krugliak et al. 1990, Wood et al. 1998) and the laxative effects of polyethylene-glycol, the need for high amount of mucin and therefore goblet cells were reduced in this study. Interestingly, led the inclusion of alginate to the diet not to an increase of goblet cell density as we have expected due to the beneficial effects of alginate (Chiu et al. 2008, Van Doan et al. 2016). The goblet cell density can be increased by beneficial supplements compared to a commercial diet (Hamidian et al. 2018). Hamidian et al. (2018) revealed that the inclusion of chitosan and zeolite to the diet caused an increase in goblet cell number and function.

4.4 Effects on pro-inflammatory genes

Histological examination showed signs of mild inflammation, but none of the measured inflammatory gene markers showed significantly elevated values. Increased TNF-α, IL-1β and

IL-8 expression are expected if harmful substances were integrated into the respective diet. Significantly increased IL-8 and TNF-α values are described in rainbow trout with soybean meal-induced enteritis (Kumar et al. 2021). Kumar et al. (2021) further showed that probiotics or other beneficial substances will reduce the expression of pro-inflammatory genes. As in this study, sampling points were selected at day 0, 7, 14 and 21 and a strong inflammatory upregulation of the pro-inflammatory genes at the start of the treatment feeding (1-24h post start) as a direct early response cannot be excluded. For a strong ongoing intestinal or systemic inflammation however, the measured genes expression needed to remain elevated due to their functional principles. TNF-α and IL-1β are key components in the innate immune system, being involved in inflammatory events (Jerez-Cepa and Ruiz-Jarabo 2021) and are the best characterized fish cytokines (Jørgensen 2014). As no significant differences in expression of these or IL-8, which is produced by myeloid cells (macrophages and dendritic cells) occurred (Pulendran et al. 2021), significant harmful effects of the test diet to the fish system can be excluded.

5. Conclusion

The current study showed the potential effects of alginate and polyethylene-glycol on the intestine of juvenile rainbow trout. On prolonged application of polyethylene-glycol, intestinal alterations were visible in the respective sections and a small reduction in growth rate was observed in the polyethylene-glycol included treatment. It is very likely that fish under subsequent feeding a commercial diet will compensate their reduced growth given the trial period (Sevgili et al. 2013). As carrier or protective matrixes for a single or booster administration of pharmaceutical active substances with markedly shorter periods of application than the current trial, the observed alterations play no crucial role. The evaluation of the pro-inflammatory cytokines IL-1β, IL-8 and TNF-α showed no signs of ongoing harmful inflammation. The potential beneficial adjuvant effects of polyethylene-glycol should be in focus for future research with consideration for potential growth performance effects and graded application over distinct time periods to appropriately reflect suitable vaccination periods. Alginate and polyethylene-glycol, potentially in combination, remain two basic components for an encapsulated, effective oral vaccine with negligible effects on fish health or growth performance.

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CRediT Authorship contribution statement

Philip N. Just: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – Original Draft. Matthew J. Slater: Supervision, Conceptualization, Writing – Review & Editing. Claudia Müller: Methodology of gene analysis, Writing – Review. Bernd Köllner: Methodology of gene analysis, Writing – Review, Formal analysis of gene-expression data, Funding acquisition

Ethical statement

The authors followed all ethical policies of the journal in all methods and actions. Specifically, all experiments were conducted in accordance with the German Animal Protection Act (TierSchG) and the regulations on the protection of animals used for experiments or other scientific purposes (TierSchVersV) in agreement with applicable EU directives and were approved by the Veterinary Authority of Bremen under the administrative number TV148.

Conflict of interest

The authors declare that they have no conflict of interest

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Appendix

Statistical outcome for pyloric caeca data

Table A1 *Results of the Dunn's Post-hoc test for the analysis for villi height / section area in the pyloric caeca. C = Control, ALG = Alginate diet treatment, PEG-ALG = Polyethylene-glycol and Alginate diet treatment PEG = Polyethylene-glycol diet treatment. Effect size is calculated following Cohen 1988*

Treatment	Z	p adjusted	Effect size d
ALG - C	-1.41	0.31	-
ALG - PEG-ALG	-1.69	0.27	-
C - PEG-ALG	-0.28	0.78	-
ALG - PEG	1.92	0.22	-
C - PEG	3.34	0.004	0.19
PEG-ALG- PEG	3.62	0.002	0.2

Table A2 *ASA/section*

Treatment	Z	p adjusted	Effect size d
ALG - C	-1.0748212	0.56	-
ALG - PEG-ALG	-1.6465475	0.3	-
C - PEG-ALG	-0.5717263	0.57	-
ALG - PEG	1.9449578	0.21	-
C - PEG	3.019779	0.01	0.17
PEG-ALG- PEG	3.5915053	0.002	0.2

Table A3 *gc/mm*

Treatment	Z	p adjusted	Effect size d
ALG - C	0.31	1	-
ALG - PEG-ALG	3.56	0.002	0.2
C - PEG-ALG	3.25	0.005	0.18
ALG - PEG	3.55	0.002	0.2
C - PEG	3.24	0.004	0.18
PEG-ALG- PEG	-0.01	1	-

Statistical outcome for mid-intestine statistic

Table A4 *Summary of Dunn's Post-hoc test for the analysis for mid-intestine width / section area. C = Control, ALG = Alginate diet treatment, PEG-ALG = Polyethylene-glycol and Alginate diet treatment PEG = Polyethylene-glycol diet treatment.*

Variable	Z	p adjusted	Effect size d
ALG - C	2.78	0.03	0.14
ALG- PEG-ALG	0.95	0.69	-
C - PEG-ALG	-1.83	0.27	-
ALG - PEG	2.77	0.03	0.14
C - PEG	-0.01	1	-
PEG-ALG - PEG	1.83	0.2	-

Table A5 *Summary of Dunn's Post-hoc test for the analysis between groups of H/W ratio of the mid intestine. C = Control, ALG = Alginate diet treatment, PEG-ALG = Polyethylene-glycol and Alginate diet treatment PEG = Polyethylene-glycol diet treatment.*

Variable	Z	p adjusted	Effect size d
ALG - C	-2.31	0.1	-
ALG- PEG-ALG	0.64	0.52	-
C - PEG-ALG	2.95	0.02	0.15
ALG - PEG	-1.38	0.5	-
C - PEG	0.93	0.7	-
PEG-ALG - PEG	-2.02	0.2	-

Table A6 *Summary of Dunn's Post-hoc test for the analysis between groups of goblet cells / mm of the mid intestine. C = Control, ALG = Alginate diet treatment, PEG-ALG = Polyethylene-glycol and alginate diet treatment PEG = Polyethylene-glycol diet treatment*.

Variable	Z	p adjusted	Effect size d
ALG - C	3.5	0.002	0.18
ALG- PEG-ALG	1.73	0.08	-
C - PEG-ALG	-1.77	0.15	-
ALG - PEG	5.85	<0.001	0.30
C - PEG	2.35	0.06	-
PEG-ALG - PEG	4.12	<0.001	0.21

Chapter 3

Potential of alginate, chitosan and polyethylene-glycol as substances for colloidal drug delivery as determined by protein release and digestion

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Abstract

Colloidal encapsulations can be applied as protective matrices in aquaculture feeds. They promise an ideal approach to protect bioactive substances such as oral vaccines, pre- or probiotics against degradation due to acidic environments or untimely lixiviation. Alginate, chitosan and polyethylene-glycol (PEG) are substances frequently applied in encapsulations as protective matrices. However, essential information on their direct and comparable characteristics and their effects on digestion speeds after oral application in aquaculture is lacking. The current study evaluated in vitro release and retention profiles of a model protein bovine serum albumin (BSA) after encapsulation with four experimental formulations of protective matrices: ALG – alginate; AC –alginate and chitosan, AP - alginate and PEG and APC – alginate, PEG and chitosan. The iron marked treatment diets were fed to juvenile rainbow trout and digestion speed was investigated using radiographic imaging. Digestion speeds did not differ significantly between treatments (n=4), with all test diets reaching the anterior fish intestine 10 h after feeding. The BSA release under low pH was highest for the alginate-chitosan PM (84.7 ± 5.8%). The inclusion of PEG reduced the release rate in low pH but significantly increased the absolute BSA release (n=3). An oil coating significantly reduced the BSA release during the initial burst for the alginate, alginate-PEG and alginate-chitosan-PEG treatments and significantly reduced retention potential under neutral pH conditions (n=3). The feeding simulation trial showed that an oil-coated diet containing alginate-chitosan as a protective matrix can be used to protect the model protein during feeding (release to the water) and against the harmful milieu of the fish stomach (n=3). Different combinations of the investigated encapsulation substances can be used to achieve optimal encapsulation and protective characteristics depending on the application objective.

1. Introduction

Bioactive components find application in a multiplicity of disciplines such as human health, cosmetics, food production or animal health. Encapsulations present a quick and practical approach for efficient oral delivery of bioactive substances in aquaculture, where significant need exists for suitable solutions for bioactive delivery. Understanding and optimizing encapsulation methods and components is a key challenge for future development of orally applied aquaculture solutions in specialized feeds. Different encapsulation methods such as emulsions, liposomes or microgels are currently known to protect bioactive components against harmful digestive juices and degradation (Perry and McClements 2020). While hard- or softshell capsules are used for delivery to terrestrial organisms. These have their limitation in aquaculture due to fish feeding behavior (Gullapalli and Mazzitelli 2017).

The colloidal system is considered the most suitable and applicable for oral administration of water-in-oil produced micropellets (<100 µm) in aquaculture. Colloid micropellets can be used to protect the bioactive components such as probiotics, prebiotics, antibiotic or vaccines against degradation due to the harmful environment of the gastro-intestinal tract (GiT) and dissociating effect of water. Encapsulation techniques using alginate (Ghosh et al. 2015), chitosan (Alexakis et al. 1995) or PLGA (Fredriksen and Grip 2012) showed promising results in terms of encapsulated active component efficiency and efficacy. Several scientific papers describe alginate encapsulation e.g. Ballesteros et al. (2015), Tian et al. (2008), Yu et al. (2019), Polk et al. (1994), Zhang et al. (2016), Alexakis et al. (1995), Mandal et al. (2006), Ghosh et al. (2015) or Liu et al. (1997). The encapsulation methods can be divided into spray application / dropping into $CaCl_2$ or water-in-oil emulsification. There is, however a large amount of methodological variation between previous studies, even those with the same encapsulation approach, therefore there is limited comparability between existing data.

The use of alginate-chitosan as a protective barrier against the gastric destruction (pH 1.5) of the bioactive components in human applications was investigated by Yu et al. (2019). Thereby, the components were used as coating to protect the original bioactive-substance carrier (layered double hydroxide nanocomposites). When

considering transfer of this method to aquaculture application, it appears this approach lacks sufficient protection against the initial release of the test-substance into the tank water. Especially for aquatic application, colloidal systems can provide extended protection for varying pH during animal administration. Wang et al. (2018) used colloidal alginate-chitosan microspheres to vaccinate channel catfish with a recombinant protein of *Streptococcus iniae*. Beside the release profile evaluation for pH 2 and pH 9 individually, a digestion stimulation of a 6h initial release at pH 2 and a consecutive release at pH 9 was executed in vitro. The approach however ignored the initial phase of pellet interaction with the rearing water at neutral pH. Still, the use of the micropellets increase the relative percent of survival (RPS) of fish from 35% of unprotected vaccine to 60% for the colloidal encapsulated vaccine by alginate-chitosan.

Slightly increased RPS were achieved by the use of polyethylene-glycol (PEG) as a protective matrix for the vaccination of juvenile rainbow trout against viral hemorrhagic septicemia (Adelmann et al. 2008). Before the PEG pellet was formed, salts were added to the PEG to later neutralize the acidic stomach environment of the orally vaccinated fish. The approach led to a RPS of 85% in challenge tests, representing the huge potential of PEG as a protective matrix substance; even though no clear information about the release profile under acidic (present in the fish stomach) or neutral (present in rearing water and fish intestine) pH conditions were available.

The aim of this study is to give insights into how the three encapsulation components alginate, chitosan and PEG affect the digestion rate in juvenile rainbow trout (*Oncorhynchus mykiss*), how these components affect the release rates of the encapsulated model-protein bovine serum albumin (BSA) under acidic and neutral pH conditions and if an additional hydrophobic oil coating can reduce the initial burst of BSA. Finally, the applicability of the micropellets were evaluated in a trial by simulating the four key-steps during a practical feed administration 1 into system water (pH8), into stomach (pH 3), into anterior intestine (pH 8) and into posterior intestine (pH 8).

2. Materials and Methods

2.1 Protective matrix (PM) production

Each PM was produced by the water-in-oil emulsification method following the procedure modified from Ghosh et al. (2015) and Liu et al. (1997). A total of four PM treatments were formulated and produced:

ALG with alginate,

AC with alginate and chitosan,

AP with alginate and polyethylene-glycol and

APC with alginate, polyethylene-glycol and chitosan

Briefly, for the aqueous phase of alginate PM (Treatment: ALG), 1 g medium-viscose sodium alginate and 100 mg bovine albumin serum (BSA) where dissolved in 100 ml distilled water. For the oil phase 15 m L Span-80 where added to 180ml octane, under stirring with a shear mixer, the aqueous phase was introduced into the oil phase. After 90 seconds of stirring 15ml Tween-80 was added and the mixture was adjusted to pH 3 using HCl. For micropellet hardening, under magnetic stirring a 8% $CaCl_2$ solution (w / v) was added slowly. The solution was broken with 2-propanol for 25 min. Afterwards the micropellets were centrifuge and washed twice, frozen to -80°C and lyophilized prior to experimental use. For the production of the alginate-chitosan pellet (Treatment AC), 0.5% chitosan was added to the $CaCl_2$ solution and continued as described above. For the production of the PMs containing PEG (Alginate - PEG: AP; alginate - chitosan - PEG: APC) 10% PEG_{1000} was added to the aqueous phase prior the emulsification process. The morphological appearance was evaluated by scanning electron microscopy. Therefore, the PM powder of the respective treatment was mounted on stubs, sputter coated with gold-palladium (Emscope SC500; Ashford, UK) and images were taken at 10 kV under a scanning electron microscope (FEI Quanta FEG200; Eindhoven, the Netherlands).

2.2 Protective matrix (PM) digestion trial

2.2.1 Facility, fish and rearing conditions

The digestion trial was conducted at the Center for Aquaculture Research (ZAF) of the Alfred-Wegener-Institute Helmholtz-Center for Marine and Polar Research, Bremerhaven applying the recirculating aquaculture system holding conditions describe in detail in Just et al. (2022). During acclimation and execution phase the water temperature was kept at 15 ± 1 °C, aeration and water flow was at 4.5 % min^{-1} and 350 L h^{-1}, respectively. Oxygen level was above 90% throughout the experimental rearing.

Juvenile rainbow trout, *Oncorhynchus mykiss*, Walbaum (14.7 ± 4.5 g) were obtained from a commercial fish farm (Die kleine Fischzucht, Geseke, Germany) and 15 fish were randomly distributed to the respective rearing tanks. Fish were acclimated to the experimental conditions (15°C) for 10 days before the experimental feeding started. During acclimation, fish were hand-fed a commercial diet (F-1P Classic LT / F 2.5mm, Skretting) twice daily until visual satiation. Pellet leftovers were siphoned to maintain high water quality.

2.2.2 Experimental diets

Three test diets ALG – alginate as PM, CHT – Chitosan as PM, PEG: Polyethylene-glycol as PM and a control diet (control: CTR), each containing inert iron powder were tested. The diets were produced by the Technology Transfer Centre Bremerhaven (TTZ Bremerhaven) using a twin-screw cold extrusion system. The commercial diet (F-1P Classic LT / F 2.5 mm, Skretting) was used as basal pellet component. For the three test treatments, 8 g of crushed F-1P Classic LT / F was mixed with 0.6 g iron power plus 4 g PMs as outlined in 2.1 above. For the control treatment 12 g of crushed F-1P Classic LT / F was mixed with 0.6 g iron power. The mixtures were compressed three times to form stable 2.5 mm pellets.

2.2.3 Trial execution

To evaluate the different digestion rates of the four treatments, fish in each tank were fed 1% of the total fish weight in each of the four tank replicates. The time point of food addition was defined as t_0. 15 min, 30 min and each full hour until 10h after t_0, each 2h until 16h after t_0, 20 h and 24h after t_0 fish were removed from the tanks. After removal

from the tank, fish were anesthetized with MS-222, killed by a sharp blow to the head and body weight was noted. Euthanized animals were kept at 5°C until the radiographic evaluation was finished. The total experimental period was thus 24 h

2.2.4 Radiographic Evaluation

Radiographic images of the chilled animals were taken at Hanover University of Veterinary Medicine Foundation, Department of Fish Diseases and Fish Husbandry. After the images were taken, fish were stored at - 80°C in case any further analysis or verifications were needed.

The intestinal tract was subdivided into 7 sections in the radiographic images (Figure 1), following the definition of Weinreb and Bilstad (1955). Assessment of pellet position was differentiated to the included iron particles in the test diets. In case of unclear evaluation via radiographic images, the respective stored animal was defrosted and pellet position determined by dissection.

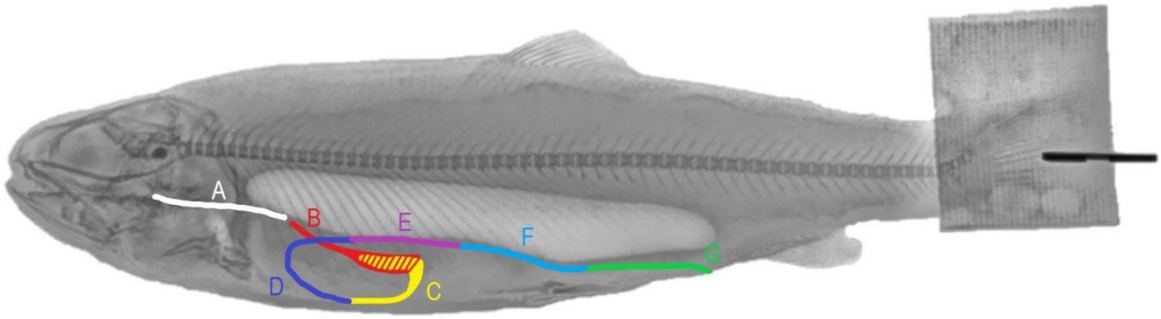


Figure 3 Radiographic image of an juvenile rainbow trout. The intestine sectioned were divided as follow: A – esophagus and stomach, B – Stomach, C – Stomach and pyloric caeca, D – pyloric caeca and anterior intestine, E – Anterior intestine, F – mid intestine, G – posterior intestine / rectum

2.3 PM protein release trials

2.3.1 Trial 1 pH dependency and trial 2 oil coating effects

To evaluate BSA retention / release of the different PMs under laboratory conditions, 10mg of the respective lyophilized PM (see section 2.1) was added to reaction tubes (Eppendorf Safe-Lock Tubes) in triplicate. After preparation of all sample tubes, 1.9 mL of H_2O (pH 3 or pH 8) was added to each test tube and tubes were gently rotated (test-tube-rotator 34528, Snijders Scientific). After 15 min, 30 min, 12 h and each full hour tubes were centrifuged (10 min, 16,000 g) and samples were taken and stored at 7°C until protein determination. For the oil coating trial, 50µl fish oil (provided by the TTZ, Bremerhaven) was added to the test tube and PM / oil mixtures were left for 5 min for the oil to soak all the PM. Afterwards the trial was executed.

2.3.2 Trial 3 Simulated PM digestion

The simulation trial was carried out to investigate BSA release of the different PM formulations in varying pH milieus. The feeding process was simulated as follows: 1 Pellet feeding – introducing to fish tanks with exposure to system water at pH8. 2 Pellet ingestion – stomach with exposure at pH 3. 3 Pass-through to anterior intestine with exposure at pH8 and 4 pass-through to posterior intestine with exposure at pH 8.

For each treatment, 10 ± 0.3 mg of coated and uncoated PM was filled into the sample tubes (Eppendorf Safe-Lock Tubes). Oil-coated treatments contained pellets coated with 30 µl oil. The experiment was carried out in quadruplicate. After the oil-coating process was finished, (1) 1 ml of pH8 water was added to all samples and placed on the rotator. After 5 minutes, tubes were centrifuged (10 min, 16,000 g) and supernatant was removed, labelled and stored for protein determination. The same procedure was repeated with (2) 1 ml of pH 3 water and 50 min reaction time, (3) 1 ml of pH 8 water and 50min reaction time and (4) pH 8 water and 5:50 hour reaction time on the same PM probe. When all samples were collected, protein concentration was determined.

2.4 Protein determination

Protein concentration of the stored samples (7°C) were determined with the Pierce™ Coomassie protein-assay-kit (Thermo Scientific™) following the manufacturer's instructions.

2.5 Statistical analysis

Data from protein release trials were tested for normality and homogeneity of variance. Dependent on the distribution and heteroscedasticity an ANOVA or GLM (Method=inverse.Gaussian) was performed. The TukeyHSD or Holm-Sidak test was used to show differences between and within the treatments. A significance level of $p<0.05$ was used for all tests.

$$Retention\ rate = 100 - \frac{protein\ concentration\ pH8_{sampletime}}{Protein\ concentration\ pH3_{sampletime}} * 100$$

3. Results

3.1 Digestion rate analysis

The PM digestion trial showed that a ≈ 30% dietary inclusion of alginate, PEG or chitosan had no significant effect on digestion rates of juvenile rainbow trout. After 16 hours, all pellets reached the posterior intestine / rectum. The passage into the anterior intestine (Figure 3, GiS E) was reached after 10-12 hours in all diet formulations. The fastest digestion rate (non-significant) in the intestinal tract (GiS D-G) was present in the control treatment which had no inclusion of any PM test substances.

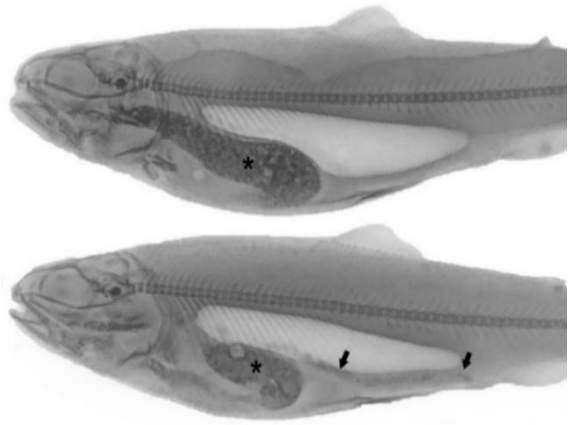
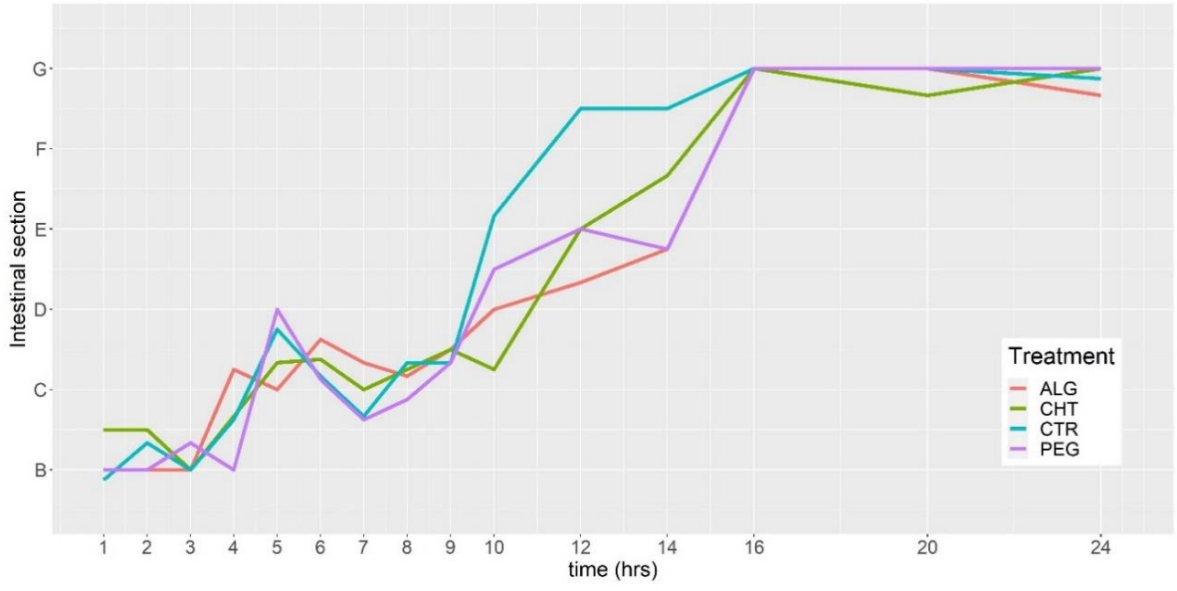


Figure 2 *Inverted radiographic image of juvenile rainbow trout post feeding with the iron marked treatment. The upper image was taken 1h post feeding. Iron marked feed is visible in the gut (asterisk). The lower picture shows a fish 20 h post feeding. The iron marked feed is still present in the stomach (asterisk) and mid- and posterior part of the intestine (arrow)*

Figure 3 *Intestinal passage rate of juvenile rainbow trout. Fish intestine was divided into following gastrointestinal sections (GiS): A – esophagus and stomach. B – Stomach. C – Stomach and pyloric caeca. D – pyloric caeca and anterior intestine. E – Anterior intestine. F – mid intestine. G – posterior intestine / rectum. Four different feed formulations were used, containing a commercial diet as main component (approx. 66%) and the test substance (33%): ALG – alginate PM, CHT – Chitosan PM, PEG: Polyethylene-glycol PM or not test substance (control: CTR). n=4*



3.2 pH dependency of basal PM

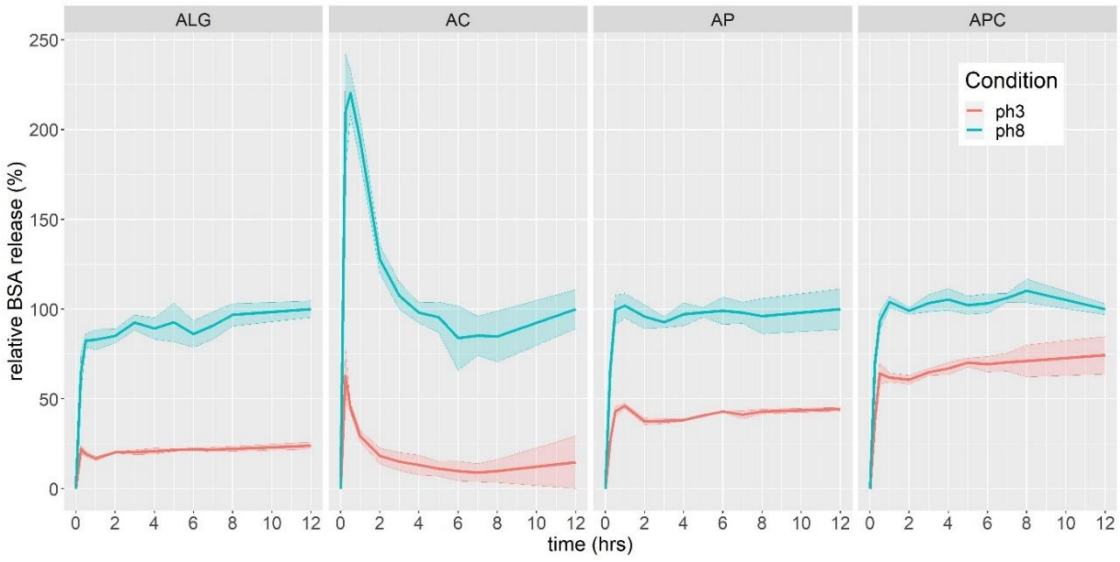


Figure 4 *Relative release profile of basal formulations under different pH conditions (ALG – PM with alginate, AC – PM with alginate and chitosan, AP – PM with alginate and polyethylene-glycol and APC - PM with alginate, polyethylene-glycol and chitosan). Each sub-graph shows the two pH conditions for each treatment. Sample means are given by the centered line. The area presents the respective standard deviation, n=4.*

The relative release rates of the different PM treatments differ significantly between the initial burst (IB) (t = 0.5 h) and final release potential (RP) (t = 24 h | $F(1,32) = 39.02$, $p < 0.001$), the different PM formulations ($F(3,32) = 55.8$, $p < 0.001$) and the two pH conditions (pH 3 and pH 8 | $F(1,32) = 1037$, $p < 0.001$), Figure 4).

After the IB phase (t0.25 - 1 h) a positive trend in relative BSA release was present in the ALG, AP and APC treatment. Only the AC treatment showed a reduction of free BSA in the condition media (pH 3 / 8 water) for the first 8h. No significant increase in relative BSA release was observed (GLM, inverse.gaussian p = 0.8) after IB and RP was handled as final release potential / rate. Highest retention rates were present in the AC and ALG formulations with 84.7 ± 5.8 % and 75.7 ± 3.7 %, respectively, followed by the AP formulation with 57.8 ± 2.1 % and APC formulation with 35.3 ± 4.3 %.

Besides the relative release rate, the absolute values differed significantly across the pH conditions and PM formulations (3-way-ANOVA $F(3,32) = 34.6$, $p < 0.001$). The lowest BSA loading was present in the AC treatment and highest in the PM formulations with PEG inclusion (Table 2).

Table 2 Summary of Initial burst rate (IB) and release potential (RP) for the four treatments (ALG – PM with alginate, AC – PM with alginate and chitosan, AP – PM with alginate and polyethylene-glycol and APC - PM with alginate, polyethylene-glycol and chitosan) under the two tested release conditions. Values present the absolute release values in mg BSA / g PM ± S.D. Differences were tested for significance by a 2-way-ANOVA. The Holm-Sidak method was used to present differences within the conditions. A significance level of α < 0.01 was used. N=3

	Condition	ALG	AC	AP	APC	ANOVA results
IB	pH3	17.4 ± 1.4a	7.9 ± 0.4a	70.5 ± 4.7b	112.7 ± 10.2c	F(3,8)=222.3 p<0.001
	pH8	74.1 ± 3.3a	38.5 ± 2.2b	163 ± 13.8c	163.9 ± 8.4c	F(3,8)=174.5 p<0.001
RP	pH3	21.6 ± 1.8a	2.6 ± 2.6b	72.7 ± 1.8c	130.5 ± 18.4d	F(3,8)=112.1 p<0.001
	pH8	89.8 ± 4.2a	17.5 ± 1.9b	163.8 ± 18.5c	175.4 ± 5.3c	F(3,8)=164.6 p<0.001

3.3 Effects of oil coating as hydrophobic barrier

The PM processing with fish oil as hydrophobic coating led to significant differences in release profiles among the treatments (3-way ANOVA $F(3,32) = 38.9$, $p < 0.01$). While the release rates were not significantly reduced in the ALG, AP and APC treatment, the release in the oil coated AC was significantly higher ($t = 11.7$, $p < 0.01$) during IB. For all treatments but AC, the BSA release for the initial burst coated PM was less than that of uncoated PM.

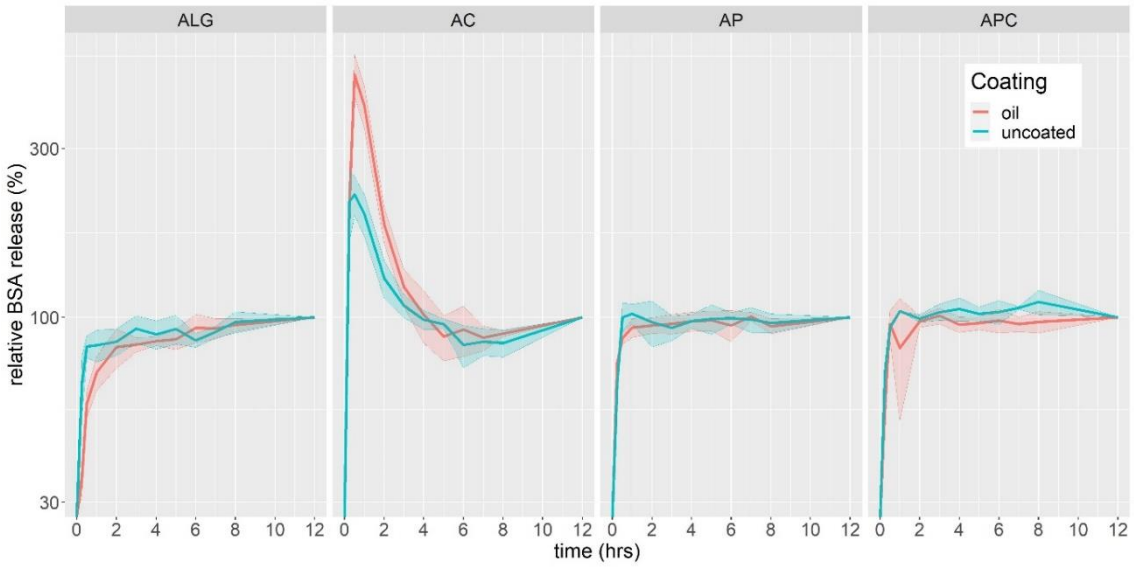


Figure 5 Relative release profile of basal and oil coated PM formulations under pH 8 conditions (ALG – PM with alginate, AC – PM with alginate and chitosan, AP – PM with alginate and polyethylene-glycol and APC - PM with alginate, polyethylene-glycol and chitosan).Each facet shows the coated and uncoated PM for the respective treatment. Sample means are given by the centered line. The area presents the respective standard deviation. The y-axis is giving in a logarithmic scale n=4.

Absolute release profiles showed strongly reduced release rate at IB and RP (3-way-ANOVA$_{treat\ x\ coating}$ $F(3,32) = 278.7$, $p < 0.001$). The retention rate (RR) of the oil treatment differed significantly between the different PM formulations ($\chi^2 = 36.3$, $df = 3$, $p < 0.001$). Significant differences within the different PM treatments were found between APC – AC ($q = 7.2$, $p < 0.05$), APC – ALG ($q = 7$, $p < 0.05$), AP-AC ($q = 4.2$, $p < 0.05$) and AP-ALG ($q = 4.2$, $p < 0.05$). PM formulations with the inclusion of chitosan showed an increased initial burst when directly compared to the ALG and AP treatment. In both of these formulations (ALG and AP) the initial BSA release peak as present in the uncoated formulations (Figure 5, blue lines) was reduced and a continuous release of BSA was observed.

Table 3 Retention ratios of the oil coating trial for the initial burst (IB) and release potential (RP). ALG – PM with alginate, AC – PM with alginate and chitosan, AP – PM with alginate and polyethylene-glycol and APC - PM with alginate, polyethylene-glycol and chitosan. Values present percentages ± s.d. Differences were tested for significance with a 2-way-ANOVA. The Holm-Sidak method was used to present differences within the conditions. A significance level of α<0.01 was used. N=3

	Coating	ALG	AC	AP	APC	ANOVA results
IB	-	82.6 ± 5.9^a	38.5 ± 2.2^b	163 ± 13.8^c	163.9 ± 1.3^c	$F(3,8)=174.5$, $p<0.001$
	Oil	20.2 ± 1	29 ± 2.1	26.5 ± 2.1	20.2 ± 7.1	$F(3,8)=4$, $p=0.05$
RP	-	89.8 ± 4.2^a	17.5 ± 1.9^b	163.8 ± 18.5^c	175.4 ± 5.3^c	$F(3,8)=164.6$, $p<0.001$
	Oil	35.6 ± 1.4^a	6 ± 0.4^b	30.4 ± 1.7^c	25.2 ± 1.3^d	$F(3,8)=286.2$, $p<0.001$

3.4 Digestion stimulation

Besides the significant differences among the different coated and uncoated PM formulations for each pH condition (Figure 6), the oil coating led to significantly different BSA releases for the same PM formulation. Oil coating led to significantly reduced BSA release in tank condition for AC-PM formulation (Holm-Sidak $t = 5.2$ $p < 0.001$) and a consequential significantly higher BSA release of the oil coated AC-PM formulation in the stomach (Holm-Sidak $t = 26.4$ $p < 0.001$), anterior intestine (Holm-Sidak $t = 7.3$ $p < 0.001$) and posterior intestine (Holm-Sidak $t = 5.5$ $p < 0.001$) pH conditions. Further, the oil coating significantly decreased the release in the ALG-PM formulation (Holm-Sidak $t=3.5$ $p < 0.01$) in the anterior intestine pH condition and significantly increased the BSA release for the AP-PM formulation (Holm-Sidak $t = 3.1$ $p < 0.01$) in the stomach pH condition.

Table 4 Absolute BSA release for water release and organic uptake locations (stomach. anterior and posterior intestine). RR – Retention rate, RA – Release advantage of coated treatments. ALG – PM with alginate, AC – PM with alginate and chitosan, AP – PM with alginate and polyethylene-glycol and APC - PM with alginate, polyethylene-glycol and chitosan. Differences were tested for significance with a 1-way-ANOVA. The Holm-Sidak method was used to present differences within the treatments. A significance level of α<0.05 was used

Location	Coating	ALG	AP	AC	APC	ANOVA
Initial burst (System water)	uncoat	138.3 ± 8.4 [a]	270 ± 11.9 [b]	87.5 ± 11.8 [c]	285.9 ± 6 [d]	F(3,12)=393.6 p<0.001
	coated	90.3 ± 14.1 [a]	255.3 ± 19.6 [bd]	32.6 ± 5.5 [c]	257.4 ± 18.4 [d]	F(3,12)=222.4 p<0.001
	RR (%)	34.7	5.5	62.8	10.0	
Organism release	uncoat	2.4 ± 0.9	1.9 ± 0.9	4.7 ± 1.6 [a]	1.3 ± 0.1	F(3,12)=7.8 p<0.01
	coated	2.9 ± 2.6 [a]	12 ± 6 [b]	20.4 ± 1.8 [c]	4.9 ± 2.3 [a.d]	F(3,12)=19.8 p<0.001
	RA (%)	21.4	526.6	336.1	280.6	

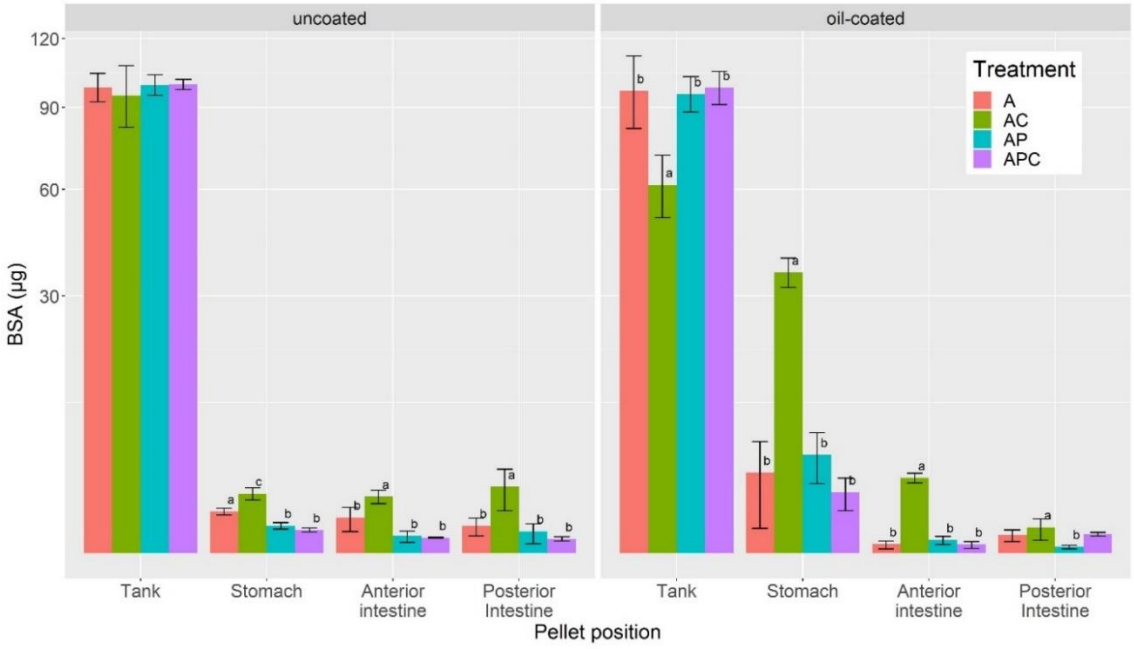


Figure 6 BSA release during intake and digestion simulation. Data were normalized to 100µg / treatment. Data were tested position-wise with an ANOVA. When data were significantly different, the Tukey HSD test was used to show differences between the different PM formulations, ALG – PM with alginate, AC – PM with alginate and chitosan, AP – PM with alginate and polyethylene-glycol and APC - PM with alginate, polyethylene-glycol and chitosan. A significance level of α < 0.05 was used. y-axis is presenting a square-root scale.

Although the AC treatment showed the lowest cumulative absolute release of BSA, the absolute BSA release in the organism simulation (stomach, anterior & posterior intestine) was the highest among all treatments for the coated and uncoated AC treatment (Table 3).

3.5 Morphological appearance of micropellets

The inclusion of PEG to the micropellet formulation led to a deformation of the circular micropellets (Figure 7). Micropellets without the use of PEG showed less adherence to each other. The use of alginate or alginate with chitosan led to the formation of circular micropellets.

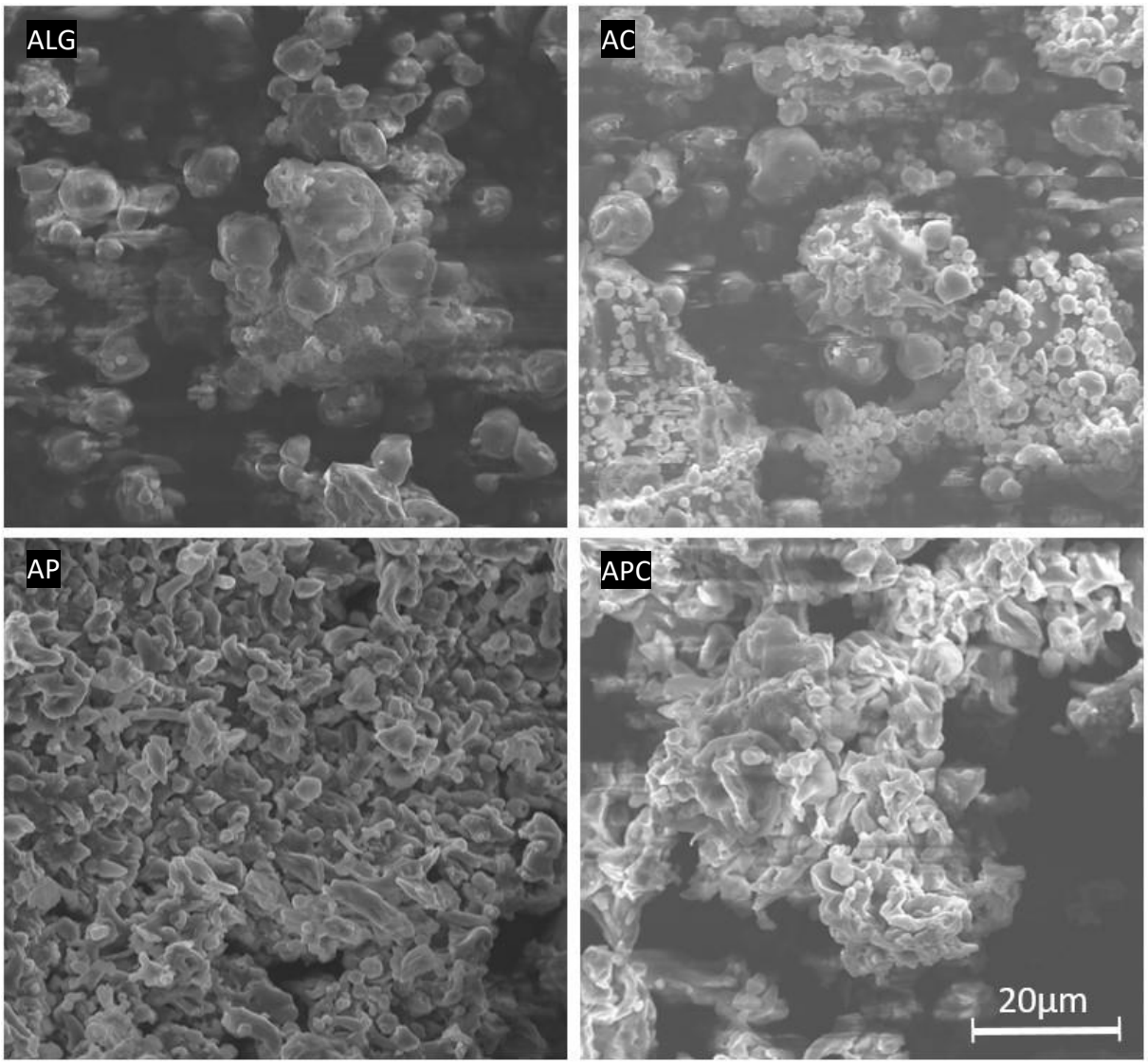


Figure 7 Morphological appearance of lyophilized micropellets. ALG – PM with alginate, AC – PM with alginate and chitosan, AP – PM with alginate and polyethylene-glycol and APC - PM with alginate, polyethylene-glycol and chitosan Magnification: 3000x

4. Discussion

Encapsulations or protective matrices (PMs) are key to effective delivery of bioactive components in aquaculture specialized diets or oral vaccinations. Understanding protection, digestion and release of bioactives under application of various PMs is key to choosing and applying the right PM in specialized diets to obtain optimal bioactive protection and delivery. In the following study, multiple proposed and applied PMs were compared in controlled feeding experiments and laboratory release experiments applying different pH conditions reflecting the digestive system/milieu and with different pellet coatings. The results provide clear indications for optimization of coatings as related to the water and gut milieu, along with timing and completeness of bioactive component release. Digestion times were unaffected by matrices and the highest release protection in low pH conditions and best release rates under simulated conditions were achieved by the AC treatment with alginate and chitosan as PM. The additional oil coating significantly reduced all absolute release rates for the tested PMs.

In rainbow trout, the gastric pH is influenced by the stomach filling after feeding and strongly acidic (pH below 3) stomach milieu is only present prior to a meal or 48h after a meal or later (Bucking and Wood 2009). Successful targeted administration of active components require a homogeneous uptake or a minimum pellet uptake for each individual animal. In juvenile rainbow trout, feeding a single dose without a starvation period improves uptake homogeneity and minimum pellet uptake (Just et al. 2021).

Based on this knowledge, a modification of protective mechanism should be directed to reduce the loss of bioactive component to the system water as this implies a loss of ≈ 98 ± 2 % of BSA loading as present in this study for the uncoated PMs (Figure 6).

Further, the stability of the bioactive pellet plays a significant role in uptake and pass-through through the stomach into the GiT. Better attraction, pellet uptake and pass-through are known for pellets with low disintegration stability (Bogevik et al. 2021) and the inclusion of PEG into the pellet could be beneficial as it increases the “water attraction” and consequently increases the soaking of a pellet. Different apparent properties which were noted during the handling as distinct, definable spheres were validated by the microscopic investigation. Due to the less distinct and round spheres

for the control and AC treatments, showed the PMs with inclusion of PEG increased agglomerations of the lyophilized PM and resuspended pellets. The digestion trial showed that the inclusion of the test PMs into a commercial pellet did not differ the digestion speeds significantly. The anterior and posterior intestine were reached 10h and 16h post feeding respectively for all formulations (Figure 3). Expected increasing digestion speed based on the laxative effects of PEG (Di Palma et al. 2002) were not found in this study. When attempting oral application of fragile and bioactive components, protective mechanisms such as encapsulation should be effective for at least 4 hours to move the bioactive component safely and unharmed through the system water and stomach into the intestine. Coating diet pellets with a hydrophobic substance such as fish oil can protect the encapsulation matrix, as in this study with its main component alginate. Coatings decrease the initial burst in the system water and consequently increase the amount of encapsulated substance entering the target species. Oral vaccination by an uncoated alginate encapsulated vaccine was performed by Ballesteros et al. (2015) and significantly higher amounts of vaccine were needed compared to injective vaccination to induce an adequate immune response. Based on the results of this study it is very likely that vaccination trials with the inclusion of PEG to the oral vaccine formulation and a hydrophobic top-coating will increase the efficiency of such vaccination trials.

Alginate can interfere with the polar group of BSA, leading to interaction with the encapsulation materials. Fundamentally, amphiphilic molecules can react with the polar groups to form stable foams, emulsions or suspensions (Dickinson 1999). Blocher McTigue and Perry (2019) evaluated the different electrostatic effect of BSA and showed coacervation with encapsulation polymers and presents the main dependency between pH and encapsulation success. The PEG used in this study for the encapsulation process has been utilized as surfactant to increase the water – oil interactions in previous studies, however beneficial effects on intestinal uptake of the target bioactive molecules are expected, as PEG can alter the barrier function of the epithelial tissue (D'souza and Shegokar 2016).

Reduced BSA loading in the chitosan containing PM formulations in this study might be based on the positive electrical potential of chitosan in comparison to alginate (Perry

and McClements 2020). In the encapsulation process with the inclusion of chitosan, low pH was used to form microcapsules that have higher retention potential at low pH (Liu et al. 1997), whereas higher encapsulation efficiency was achieved when the encapsulation media was set to pH 8 (McTigue and Perry 2019). This inverse dependency may explain the significantly reduced absolute BSA release rates in the alginate- chitosan treatments in all trials. During the production process, the acidic chitosan solution may negatively interfere with the positive charged BSA at low pH. At a pH below 5.5 the BSA charge will become positive and thereby repels from the positive charged chitosan. The mechanism of alginate repelling under different pH has been demonstrated by Zhang et al. (2016). The inclusion of chitosan in this study led to the highest initial BSA burst and at the same time to the highest BSA release in the simulated fish intestine. The addition of PEG to the alginate-chitosan treatment reduced the beneficial effects of an oil coating, compared to the non-chitosan treatments (Figure 6).

The previously described initial burst of BSA into the condition media was present for all PM formulations. From the different basal encapsulation methods: alginate, alginate-chitosan, alginate-PEG and the combination of both showed that under unchanged production methods, the omission of chitosan and the inclusion of PEG and top oil coating will increase the total release while improving the retention rate against tank water and the gastric juices of the fish stomach during feeding.

Further studies should focus on the effect of different encapsulation sizes with modified surface – volume ratio to reduce the unwanted initial burst. As described by Polk et al. (1994) significantly reduced BSA release rates by the use of chitosan did not apply for nanocapsules (diameter ≈ 5 nm).

The radiographic method applied in this paper provided a quick and direct tool to determine the digestion times of different feeds. With regard to the application and administration of an oral vaccine or other bioactive substance, the exact determination of the location of the respective substance in the animal is of major importance. Protective matrices should be specifically modified based on the results of this trial, to guarantee an unharmed passage of bioactives through the stomach into the intestine of rainbow trout in aquaculture applications. The method offers a precise determination of gastric, mid- and posterior intestine contents, however the precision to determine

progress along the intestine for the anterior part (Figure 1 and 3, GiS: D and E) was hindered, due to the overlapping loop structure of the gastrointestinal tract (GiT, Figure 1). The radiographic method was first described by Molnár and Tölg (1960) with iron powder by Talbot and Higgins (1983). The advantages of this non-invasive method (Talbot and Higgins 1983) could be further used and modified for a live digestion evaluation based on improved technology and rearing techniques. To increase the precision of pellet determination and therefor minimize uncertainties, increased iron powder grain size is also recommended.

5. Conclusion

The substances tested for the encapsulation of bioactive substances as applied in this study have high potential to increase the efficiency and success of those bioactive substances for oral application. In contrast to encapsulation methods for human or other terrestrial animal application, oral use in aquatic application is linked to protection against the initial burst in the system water. Based on the results of the current study, chitosan in combination with alginate and a hydrophobic oil coating thus presents the best method to increase the bioactive component release into the target species. Even though the inclusion of PEG decreased the release profile quality of the model-protein BSA, higher total releases were present and potential beneficial interactions with the intestinal epithelial cells are expected and should be investigated in future research. The radiographic evaluation method should be further modified to improve precision. Nonetheless, it can be used for live and euthanized fish species and varying sizes to determine not only digestion speeds of modified or new feedstock and feed additives, but also to determine residual times for the intestinal areas of choice.

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Appendix - Statistical results

Trial 1 – pH dependency of uncoated PM formulations

Kruskal-wallis test for release potential t=8h (χ^2 = 21.36, df=7, p=0.003)
Dunn (1964) Kruskal-Wallis multiple comparison for t=8

Treatment	Z	p	p (adjusted)
ALGpH3 - ALGpH8	-2.252	**0.024**	0.56
ACpH3 - ACpH8	-2.194	0.028	0.59
APpH3 - APpH8	-1.732	0.083	1
APCpH3 - APCpH8	-1.905	0.057	1
ALGpH3 - APpH3	-0.52	0.603	1
ACpH3 - APCpH3	-1.674	0.094	1
ACpH3 - ALGpH3	-0.52	0.603	1
ACpH3 - APpH3	-1.039	0.299	1
APCpH3 - APpH3	0.635	0.525	1
ACpH8 - ALGpH8	-0.577	0.564	1
ACpH8 - APCpH8	-1.386	0.166	1
ALGpH8 - APCpH8	-0.808	0.419	1
ACpH8 - APpH8	-0.577	0.564	1
ALGpH8 - APpH8	0	1	1
APCpH8 - APpH8	0.808	0.419	1

Kruskal-wallis test for release potential t=12h (χ^2 = 19.92, df=7, p<0.01)
Dunn (1964) Kruskal-Wallis multiple comparison p-values adjusted with the Holm method.

Treatment	Z	p	p (adjusted)
ALGpH3 - ALGpH8	-2.656	0.008	0.21
ACpH3 - ACpH8	-2.483	0.013	0.3
APpH3 - APpH8	-1.732	0.083	1
APCpH3 - APCpH8	-1.443	0.149	1
ALGpH3 - APpH3	-0.693	0.488	1
ACpH3 - APCpH3	-1.386	0.166	1
ACpH3 - ALGpH3	-0.173	0.862	1
ACpH3 - APpH3	-0.866	0.386	1
APCpH3 - APpH3	0.52	0.603	1
ACpH8 - ALGpH8	-0.346	0.729	1
ACpH8 - APCpH8	-0.346	0.729	1
ALGpH8 - APCpH8	0	1	1
ACpH8 - APpH8	-0.115	0.908	1
ALGpH8 - APpH8	0.231	0.817	1
APCpH8 - APpH8	0.231	0.817	1

Trial 2 – influence of oil coating

Test for relative c_mg

Kruskal-wallis test for oil coating effect t=15min (χ^2 = 19.88, df=7, p<0.01)

Comparison	Z	p	p (adjusted)
ACc - ACo	-0.058	0.954	0.954
ALGc - ALGo	1.443	0.149	1
APc - APo	-1.097	0.273	1
APCc - APCo	1.097	0.273	1

Kruskal-wallis test for oil coating effect t=30min (χ^2 = 20.493, df=7, p<0.01)

Comparison	Z	p	p (adjusted)
ACc – Aco	-0.52	0.603	1
ALGc - ALGo	0.693	0.488	1
APc – APo	1.097	0.273	1
APCc - APCo	0	1	1

Kruskal-wallis test for oil coating effect t=1h (χ^2 = 19.747, df=7, p<0.01)

Comparison	Z	p	p (adjusted)
ACc – Aco	-0.52	0.603	1
ALGc - ALGo	0.52	0.603	1
APCc - APCo	1.27	0.204	1
APc - APo	0.808	0.419	1

Kruskal-wallis test for oil coating effect t=2h (χ^2 = 17.76, df=7, p=0.01)

Comparison	Z	p	p (adjusted)
ACc – Aco	-0.577	0.564	1
ALGc - ALGo	0.115	0.908	1
APCc – APCo	0.289	0.773	1
APc – APo	-0.058	0.954	0.954

Kruskal-wallis test for oil coating effect t=3h (χ^2 = 16.667, df=7, p=0.02)

Comparison	Z	p	p (adjusted)
ACc - ACo	-0.693	0.488	1
ALGc - ALGo	1.155	0.248	1
APCc – APCo	0.404	0.686	1
APc - APo	-0.404	0.686	1

Kruskal-wallis test for oil coating effect t=4h (χ^2 = 11.6, df=7, p=0.1)

Kruskal-wallis test for oil coating effect t=5h (χ^2 = 7.75, df=7, p=0.4)

Kruskal-wallis test for oil coating effect t=6h (χ^2 = 10.29, df=7, p=0.2)

Kruskal-wallis test for oil coating effect t=7h (χ^2 = 15.47. df=7. p=0.03)

Comparison	Z	p	p (adjusted)
ACc - ACo	-0.289	0.773	1
ALGc – ALGo	-0.289	0.773	1
APCc – APCo	1.501	0.133	1
APc - APo	-0.462	0.644	1

Kruskal-wallis test for oil coating effect t=8h (χ^2 = 14.25, df=7, p<0.05)

Comparison	Z	p	p (adjusted)
ACc – ACo	-0.635	0.525	1
ALGc - ALGo	0.058	0.954	1
APCc - APCo	1.328	0.184	1
APc - APo	0.289	0.773	1

Test t2 pH – Factor time (no differences)

*Outcome for GLM (glm(formula = rel_rep ~ treat * mod + time, family = inverse.gaussian)*
Deviance Residuals:

Min	1st Quantile	Median	3rd Quantile	Max
-0.277335	-0.008036	-0.000249	0.007197	0.18205

Coefficients:

	Estimate	S.D.	t value	p
(Intercept)	6.53E-03	4.55E-04	14.359	<0.001
treatALG	-4.34E-03	4.97E-04	-8.745	<0.001
treatAP	-5.92E-03	4.61E-04	-12.847	<0.001
treatAPC	-6.32E-03	4.56E-04	-13.87	<0.001
modpH8	-6.43E-03	4.55E-04	-14.147	<0.001
time	1.14E-06	4.88E-06	0.234	0.816
treatALG:modpH8	4.36E-03	4.98E-04	8.762	<0.001
treatAP:modpH8	5.92E-03	4.62E-04	12.825	<0.001
treatAPC:modpH8	6.31E-03	4.57E-04	13.816	<0.001

Null deviance: 2.25720 on 167 degrees of freedom
Residual deviance: 0.40929 on 159 degrees of freedom
AIC: 1480.6 | Number of Fisher Scoring iterations: 5

Analysis: Effects of oil coating

Outcome for GLM glm(formula = c_rel_mg ~ mod * time * coat, family = inverse.gaussian)

Deviance Residuals:

Min	1st Quantile	Median	3rd Quantile	Max
-0.092764	-0.007878	0.000556	0.008632	0.039545

Coefficients:	Estimate	S.D.	t value	p
(Intercept)	1.99E-05	4.42E-06	4.492	<0.001
modALG	1.36E-04	1.31E-05	10.382	<0.001
modAP	1.01E-04	1.15E-05	8.762	<0.001
modAPC	9.36E-05	1.09E-05	8.564	<0.001
time	1.40E-05	1.54E-06	9.099	<0.001
coatoil	-1.83E-05	4.79E-06	-3.833	<0.001
modALG:time	-1.94E-05	2.45E-06	-7.913	<0.001
modAP:time	-1.62E-05	2.34E-06	-6.954	<0.001
modAPC:time	-1.68E-05	2.23E-06	-7.506	<0.001
modALG:coatoil	9.12E-05	2.00E-05	4.567	<0.001
modAP:coatoil	2.58E-05	1.60E-05	1.614	0.11
modAPC:coatoil	4.33E-05	1.59E-05	2.727	0.007
time:coatoil	8.16E-07	1.96E-06	0.417	0.68
modALG:time:coatoil	-8.94E-06	3.33E-06	-2.683	0.008
modAP:time:coatoil	-1.54E-06	3.18E-06	-0.483	0.63
modAPC:time:coatoil	-2.41E-06	3.10E-06	-0.776	0.44
Null deviance: 0.342824 on 263 degrees of freedom				
Residual deviance: 0.086504 on 248 degrees of freedom				
AIC: 2303.5 | Number of Fisher Scoring iterations: 6				

Kruskal-wallis test for retention rate (trial 2, oil coating effects) ($\chi^2 = 36.3$, df=3, $p<0.001$)

Analysis of Simulation trial (Table 1)

Holm Sidak test for System water uncoated analysis (absolute values)

Comparison	Diff of Means	t	P
APC vs. AP	198.360	28.475	<0.001
AC vs. AP	182.469	26.194	<0.001
APC vs. A	147.592	21.187	<0.001
AC vs. A	131.702	18.906	<0.001
A vs. AP	50.767	7.288	<0.001
APC vs. AC	15.890	2.281	0.042

Holm Sidak test for System water oil coated analysis (absolute values)

Comparison	Diff of Means	t	P
APC vs. AP	224.836	20.623	<0.001
AC vs. AP	222.703	20.427	<0.001
APC vs. A	167.066	15.324	<0.001
AC vs. A	164.933	15.128	<0.001
A vs. AP	57.770	5.299	<0.001
APC vs. AC	2.134	0.196	0.848

Holm Sidak test for Organism release uncoated analysis (absolute values)

Comparison	Diff of Means	t	P
AP vs. APC	3.387	4.531	0.004
AP vs. AC	2.770	3.705	0.015
AP vs. A	2.262	3.025	0.042
A vs. APC	1.125	1.505	0.403
AC vs. APC	0.617	0.825	0.670
A vs. AC	0.509	0.680	0.509

Holm Sidak test for Organism release oil coated analysis (absolute values)

Comparison	Diff of Means	t	P
AP vs. A	17.498	6.943	<0.001
AP vs. APC	15.495	6.148	<0.001
AC vs. A	9.065	3.597	0.015
AP vs. AC	8.433	3.346	0.017
AC vs. APC	7.063	2.802	0.032
APC vs. A	2.003	0.795	0.442

Simulation Graph Statistics (Figure 2)

Results of ANOVA

Results of treatment analysis per pellet position

Coating	Position	F Statistic	p
oil caoted	tank	F(3,12)=11.4	<0.001
	stomach	F(3,12)=161.9	<0.001
	anterior int	F(3,12)=213.1	<0.001
	posterior int	F(3,12)=3.5	0.049
uncoated	tank	F(3,12)=0.33	0.8
	stomach	F(3,12)=49.01	<0.001
	anterior int	F(3,12)=24.31	<0.001
	posterior int	F(3,12)=8.67	0.002

Results of Post-Hoc TukeyHSD test Multiple comparisons of means with 95% family-wise confidence level

TukeyHSD(Tank, oil coated)

	difference	lower	upper	p adjusted
AC - A	0.04276346	-0.3192916	0.4048185	0.984
AP - A	2.53071866	2.1686636	2.8927737	<0.001
APC - A	-0.0023296	-0.3643847	0.3597254	1
AP - AC	2.4879552	2.1259002	2.8500102	<0.001
APC - AC	-0.0450931	-0.4071482	0.3169619	0.982
APC - AP	-2.5330483	-2.8951034	-2.1709933	<0.0010

TukeyHSD(stomach intestine, oil coated)

	difference	lower	upper	p adjusted
AC - A	1.430254	-3.973737	6.834245	0.859
AP - A	32.731849	27.327858	38.135841	<0.001
APC - A	-1.277309	-6.6813	4.126682	0.894
AP - AC	31.301595	25.897604	36.705587	<0.001
APC - AC	-2.707563	-8.111554	2.696428	0.474
APC - AP	-34.009159	-39.41315	-28.605167	<0.001

TukeyHSD(anterior intestine, oil coated)

	difference	lower	upper	p adjusted
AC - A	0.04276346	-0.3192916	0.4048185	0.984
AP - A	2.53071866	2.1686636	2.8927737	<0.001
APC - A	-0.0023296	-0.3643847	0.3597254	1
AP - AC	2.4879552	2.1259002	2.8500102	<0.001
APC - AC	-0.0450931	-0.4071482	0.3169619	0.982
APC - AP	-2.5330483	-2.8951034	-2.1709933	<0.001

TukeyHSD(posterior intestine, oil coated)

	difference	lower	upper	p adjusted
AC - A	-0.137	-0.4	0.126	0.445
AP - A	0.151	-0.112	0.414	0.365
APC - A	0.008	-0.255	0.271	1
AP - AC	0.287	0.024	0.55	0.031
APC - AC	0.145	-0.118	0.408	0.396
APC - AP	-0.142	-0.405	0.121	0.412

TukeyHSD(Tank, uncoated)

	difference	lower	upper	p adjusted
AC - A	-0.44	-0.973	0.093	0.119
AP - A	0.872	0.339	1.405	0.002
APC - A	-0.471	-1.004	0.062	0.09
AP - AC	1.312	0.779	1.845	<0.001
APC - AC	-0.031	-0.564	0.502	0.998
APC - AP	-1.343	-1.876	-0.81	<0.001

TukeyHSD(Stomach, uncoated)

	difference	lower	upper	p adjusted
AC - A	-0.448	-0.824	-0.073	0.018
AP - A	0.827	0.452	1.203	<0.001
APC - A	-0.545	-0.92	-0.169	0.005
AP - AC	1.276	0.9	1.651	<0.001
APC - AC	-0.097	-0.472	0.279	0.869
APC - AP	-1.372	-1.748	-0.997	<0.001

TukeyHSD(Anterior intestine, uncoated)

	difference	lower	upper	p adjusted
AC - A	-0.44	-0.973	0.093	0.119
AP - A	0.872	0.339	1.405	0.002
APC - A	-0.471	-1.004	0.062	0.09
AP - AC	1.312	0.779	1.845	<0.001
APC - AC	-0.031	-0.564	0.502	0.998
APC - AP	-1.343	-1.876	-0.81	<0.001

TukeyHSD(Posterior intestine, uncoated)

	difference	lower	upper	p adjusted
AC - A	-0.13	-1.413	1.153	0.99
AP - A	1.66	0.376	2.943	0.011
APC - A	-0.255	-1.538	1.029	0.934
AP - AC	1.79	0.506	3.073	0.006
APC - AC	-0.125	-1.408	1.159	0.991
APC - AP	-1.914	-3.198	-0.631	0.004

TukeyHSD Results for simulation trial Figure 5

Coating	Comparison	Difference of Ranks	q	p
oil coated	tank-ant	631	8.473	<0.05
	tank-post	620	8.325	<0.05
	tank-stom	285	3.827	<0.05
	stom-ant	346	4.646	<0.05
	stom-post	335	4.498	<0.05
	post-ant	11	0.148	>0.05
uncoated	tank-post	552	7.412	<0.05
	tank-ant	551	7.398	<0.05
	tank-stom	433	5.814	<0.05
	stom-post	119	1.598	>0.05
	stom-ant	118	1.584	>0.05
	ant-post	1	0.0134	>0.05

Synopsis

Summarizing Statements

The overarching aim of this dissertation was to better understand rainbow trout, *Oncorhynchus mykiss*, feeding behavior, response to potential vaccine carriers and the release of proteins from carrier matrices in order to enhance and optimize the prerequisites for efficient oral vaccination in aquaculture. As part of the collaborative EU project: *Modular orally-applicable multi-vaccine – A principle solution* the novel information presented in this thesis contributed to the unspecific optimization of oral vaccine administration. The findings of this study help to successfully administer pro- and prebiotics or other fragile aquaculture food additives, without functional losses during the feeding procedure, ingestion and digestion of the fish. The Chapters of this dissertation present novel data on the following fundamental and adjustable parameters / principles for of oral vaccine administration, asking:

1 – How can feeding regimes be optimized to obtain homogeneous and guaranteed pellet uptake among fish?

2 – What are the potential side effects of the vaccine carrier / protective matrix substances on the intestinal tract? What are potential benefits or pitfalls of the respective substances regarding the intestinal immune induction?

3 – How can different applied protective matrix substances be used to adjust and optimize the release of a model-protein (substitutional for an active vaccine) for the specific application of an oral fish vaccine?

Each of the three Chapters discusses the novel information regarding their direct implication for application and their importance. When considering the information of the presented studies as a whole it can be argued that significant advances in terms of knowledge facilitating oral vaccination of fish have been made. Results of Chapter 1 clearly showed that the adjustment of the feeding regime allowed a homogeneous and guaranteed minimum uptake of an oral substance. For aquaculture application and scientific field studies, which are highly necessary, feed distribution during vaccine feeding, as well as the portion size and a defined starvation period prior to the vaccination feeding can be easily implemented. These optimizations set a realistic

baseline for successful oral vaccination development and use data based on repeated novel experimental methodologies.

The main hurdle of vaccine destruction in the water milieu or the fish gut has been addressed within Chapter 2 and Chapter 3. Trials of Chapter 2 targeted the question what are the effects of alginate and polyethylene-glycol on the intestine and if the substances can lead to a systemic inflammatory response in juvenile rainbow trout. The results of Chapter 2 showed that none of the tested substances led to significant alterations or increase inflammatory responses. Thereby the safe and harmless use of both substances in the delivery and encapsulation of oral vaccines is ensured. Finally, Chapter 3 focused on the protective mechanisms of the three encapsulation substances alginate, chitosan and polyethylene-glycol. Results in Chapter 3 showed that the applied substances can be used to modify the encapsulation characteristics of an oral vaccine to increase the release of the active component at the location of immune induction of fish.

The following section of the synopsis discusses the implication of the novel information of this dissertation beyond the discussions of the respective Chapters and highlights tasks that need further investigations for the consistent improvements for oral vaccines and their administration characteristics.

Immune status of fish

The basis for an efficient immune response after vaccination in general is the immune-competence of fish. Thereby, age but also the fish weight can be used to determine the earliest suitable vaccination time point (Tort et al. 2003). During early life stages, when the adaptive immune system is not yet fully developed, the innate immune system of rainbow trout presents a sufficient protection against bacterial infections (Chettri et al. 2012) but vaccination will not further enhance immunological protection, independent of the route of vaccination. First B cell development in rainbow trout was detected 10 days post fertilization, sufficient adaptive immune-competence for rainbow trout was reported for fish with 2-5g (Bøgwald and Dalmo 2019). Keystones of the development of the trout immune system were summarized by Hansen (1997) and first thymus plants were found 20d post fertilization. After hatching at day 26, tissue and function of the

thymus enlarges and the spleen develops lymphocytes. After 85 days, the spleen and thymus are fully developed and represent the most important lymphoid organ. The early fry stage (0.8 g) showed already similar cytokine and acute phase protein expression as it was found for later fingerlings (4-6 g), however the response was modest and less sophisticated systemic reaction was present compared to fish larger than 6 g (Chettri et al. 2012). Concurrent to injective vaccinations of fish earliest when fish reach 3 g, this weight based on the development of the immune system is also valid and recommended for fish vaccination via the oral route (Brudeseth et al. 2013). Oral vaccinations, if effective as argued herein, are clearly more practical for application on fish of this size, reducing fish loss/injures due to handling stress and injection procedures.

Immunosuppression based on fish stress can play a significant role in limiting vaccine efficacy, as can alterations of the immune system due to circadian (Takahashi 2015), circalunar (Raible et al. 2017) and circannual cycles (MacMurray et al. 1983, Montero et al. 2022). Our investigations on the seasonal clock of juvenile rainbow trout showed that a mild immunosuppression during the winter month was present (Appendix). For efficient immune stimulation by oral vaccination, the immune status of fish has major importance and a suppressed immune status of fish during the winter months could lead to a reduced immune response and therefore hampers the success of an efficient immune protection after vaccination. Understanding this seasonality allows timely

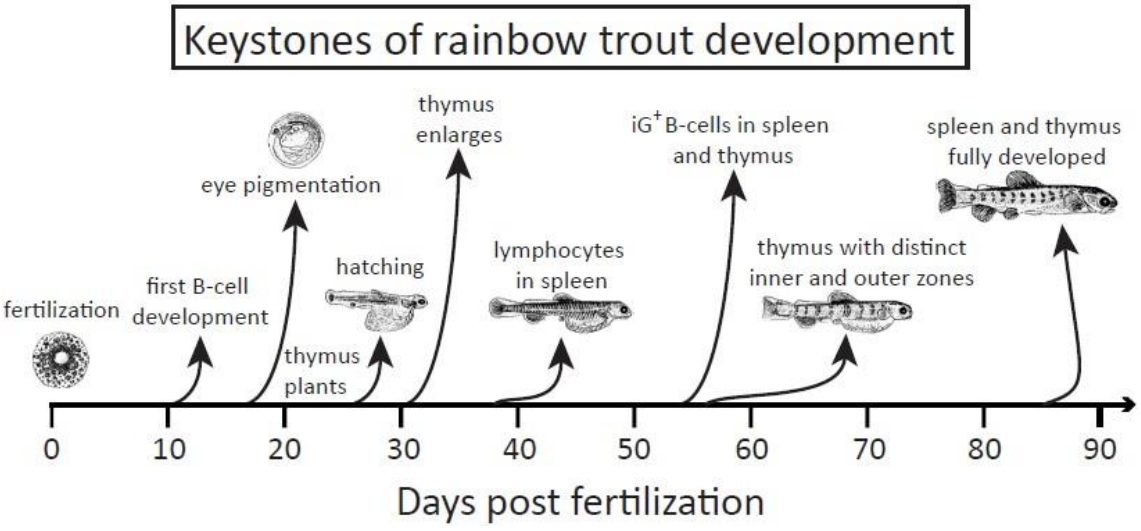

Figure 1 *Keystones of rainbow trout immune system development. Data and images were summarized following Hansen (1997), Hoitsy* et al. *(2012) and Razquin* et al. *(1990)*

application of oral vaccines to ensure optimal immune response, building on optimal delivery as a specialized diet and further contributing to their viable application in aquaculture.

The current research presented herein on the seasonal variation showed that during summertime the mucosal mediated immunoglobulin (IgT^+ B cells) were elevated in comparison to winter. IgT^+ B cells play an important role in the mucosal immunity of teleost fish (Wang et al. 2022) especially during the early developmental stages of trout (Chettri et al. 2012). For instance, vital fish that survived infection with a gut parasite showed significantly elevated numbers of IgT^+ B cells, whereas fish that died from the disease exhibited no such increased numbers (Zhang et al. 2010).

According to the present results, oral vaccination of juvenile rainbow trout is recommended to be performed during the summer month when fish possess the highest immunocompetent status, resulting in a maximal immune response and immune protection after vaccination. Only when practical limitations or urgent immunological or operative needs dictate otherwise, vaccinations in other seasons may be considered necessary if not optimal.

Advances of oral vaccination in aquaculture application

Fish diseases due to bacterial and viral infections are persistent threats to aquaculture that increase with farming density (Stentiford et al. 2012). Injective immunization has been used to overcome early and recent disease outbreaks with severe fish and financial losses (Sommerset et al. 2005). A methodical benefit of oral vaccination is the reduction of stress due to crowding, netting, manual handling and injection which negatively affects the fish immune systems on its own (Ashley 2007, Sakai 1999). Research on oral vaccines for aquaculture application has mainly focused on the induction of sufficient immune response by single protective methods for active vaccine ingredients. Alginate, for example, as a biocompatible and inexpensive polysaccharide (Jain and Bar-Shalom 2014) has proven its use for encapsulation of probiotics (Rosas-Ledesma et al. 2012), prebiotics (Ashouri et al. 2020) or oral vaccines (Romalde et al. 2004). The continuous admixture of alginate to commercial diets of juvenile rainbow trout as examined in this thesis is shown to increase fish welfare, health and improve the immune status. The

morphological negative alterations of the intestinal villi in the pyloric caeca of juvenile rainbow trout in the dietary treatments with polyethylene-glycol (PEG) were prevented by the addition of alginate to the diet (Chapter 2).

Advanced encapsulation techniques by alginate, PEG and chitosan in combination with a palatable commercial pellet as presented herein will allow the group feeding as required for practical oral vaccine application. These encapsulations concurrently present a protection barrier against lixiviation into the system water and destruction by the gastric environment (Chapter 3). When fully developed, vaccines can lead to full protection against different bacterial and viral infections (Mondal and Thomas 2022). New findings of this dissertation focused on unsolved issues of oral application of vaccines including the optimal uptake of vaccine particles, which is inevitable for a successful, functional oral vaccine.

In addition to use in primary vaccination, oral vaccination can be successfully used in booster vaccination of fish. Booster vaccination refreshes and enhances the duration and protection primarily vaccinated fish and is mostly linked to the humoral immune responses (Newaj-Fyzul and Austin 2015). Most vaccination trials against bacterial infections such as *Vibrio salmonicida* (Steine et al. 2001), *Flavobacterium columnare* (Leal et al. 2010) or *Vibrio anguillarum* (Vervarcke et al. 2004) led to significant elevated antibody titers, however, no strict correlation between antibody titers and resistance against infection was found (Leal et al. 2010). Due to the oral route of immune induction, the immunogenicity of the vaccine can be reduced and cause immunosuppression (Mondal and Thomas 2022, Somamoto and Nakanishi 2020). The addition of adjuvants can help to increase the immunogenicity by bypassing the mucosal tolerance (Muñoz-Atienza et al. 2021). Adjuvants can be toxin-based, immune-stimulatory, particulated or microbiota-derived (Muñoz-Atienza et al. 2021). Depending on the used adjuvant, fish flesh can be affected negatively and involve reduced product quality for human consumption by lesion in the flesh or adjuvant residuals in the fish (Koppang et al. 2005). Alginate and PEG offer biocompatibility and potential adjuvant attributes that may supersede the use of harmful classical adjuvants.

The use of boosters after initial vaccine administration in aquaculture can induce significant protection among the fish group. To maximize the homogeneity in vaccine

pellet uptake during commercial oral-vaccine administration, the use of a single oral-vaccine addition during initial or booster vaccination is highly beneficial if one event can provide the fish with adequate dosing of the vaccine per fish. The current research results indicate that, instead of a single addition, the total oral vaccine mass should be fed in multiple portions, or if possible continuously added over a time of 3-5 minutes. The suggested method increases homogeneity of pellet uptake among the group and can guarantee a minimal pellet uptake with the required doses for each fish and therefore maximize the chance for a complete herd immunity. The efficiency quantification of vaccination trials by Ballesteros et al. (2015) or Adelmann et al. (2008) should be repeated with this study's new information on feeding regimes and encapsulation methods to adjust the feeding procedure to more commercially applied feeding method in aquaculture. The oral vaccination trial against viral hemorrhagic septicemia by Adelmann et al. (2008) used PEG as a vaccine carrier and the inclusion of salts functioned as neutralizer for the acidic stomach milieu. Even though significant immune protection was induced by the oral vaccine applied by Adelmann et al. (2008), the feeding method excludes field application of the vaccine, as during the feeding session, the oral vaccine was added pellet wise to ensure pellet uptake. For oral vaccination of rainbow trout against hematopoietic necrosis virus by Ballesteros et al. (2015), fish were anaesthetized and the alginate encapsulated oral vaccine was directly introduced into the fish mouth with an automatic pipette. It is very likely, that this invasive oral vaccination method hindered more pronounced immune protection by the fish, resulting in relatively low relative percent of survival of 56% compared to other studies. A consecutive step for the studies of Adelmann et al. (2008) and Ballesteros et al. (2015) would be the imbedding of the oral vaccine into a vaccine feeding pellet with similarities to the original feed pellet to allow a feeding procedure applied in commercial aquacultures.

Alginate and polyethylene-glycol as adjuvant and immune enhancers

Using alginate as an encapsulation substance, the vaccine is protected against the harmful gastric juices in the stomach and its immune-stimulation effects may later increase the immune response in the fish intestine. Improved immunity by the integration of alginate is based on the enhanced expression of different immune-related genes such as Toll-like receptor 3 (TLR3) which, along with other TLRs plays a significant role in pathogen recognition to induce innate immune response. The activation of TLR3 leads to the activation of cytokine: interleukin-1β (IL-1β) and interleukin 8 (IL-8). While IL-1β highly regulates the immune and inflammatory responses in mammals and salmonids (Ingerslev et al. 2006) and functions as a key mediator in response to tissue impairments (Gioacchini et al. 2010). IL-8 is produced by myeloid cells (macrophages and dendritic cells). Dendritic cells stimulate antigen-specific T and B cell responses and thereby can have adjuvant effects (Pulendran et al. 2021).

Besides the beneficial effects of alginate on the intestinal health of juvenile rainbow trout or the encapsulating and therefore protective effects, alginate has the potential to stimulate non-specific humoral immunity and non-specific cellular immune ability of fish (Cheng et al. 2007, Peddie et al. 2002). These effects were reported for the injective administration of alginate and it is unclear if the reported immunity boost is also valid for oral administration. As the effects were described for the unspecific immunity, it is likely, that the mucosal response can be boosted likewise. It can be argued that any oral vaccine administration using alginate-based delivery matrix as tested in the current study will benefit from this response and ultimately results in improved overall immune response and protection post oral vaccination. Depending on the content of alginate's mannuronic acid, the local cytokine stimulating effects in the intestinal mucosa can be beneficial. For the interaction of alginate with human monocytes, increase expression of TNF-α, IL-6 and IL-1β was reported (Otterlei et al. 1991). Homologous interactions are expected for fish monocytes. "IL-1β has also been shown to enhance antibody production when administered with bacterial vaccines, suggesting that it may be exploited as an immune-adjuvant for improving vaccine efficacy" (Zou and Secombes 2016). Overall, the investigated substances showed high potential for the successful production and administration of an oral vaccine for aquaculture production.

The use of PEG as a delivery matrix has the potential to increase the immunogenicity of the vaccine by increasing the interaction with the mucosal surface (Minato et al. 2003) and additionally functions as barrier against enzymatic degradation (Suzuki et al. 1984), thereby functioning as adjuvant. Especially for vaccines encapsulated in liposome, PEG can be used to prolong the outflow pattern of the liposome and thereby delay the release of the vaccine pellet irrespective of any additional encapsulation method (Minato et al. 2003). The PEG-modified liposome of Minato et al (2003) showed 2.3-fold longer intestinal transit times which stand in contrast to the expected increased digestion speed due to the laxative effects of PEG. Concurrent to the results of the Chapter 3, model-protein release of the PEG-liposomes was increased compared to the unmodified liposome; which illustrates the beneficial effects of PEG for the quantitative increased release profiles in the optimal region for uptake as shown by the in vitro trials of Chapter 3. The use of PEG further improves antigen presentation to T cells and enhances the draining to the lymphatic system and thereby can potentially improve the humoral and cell-mediated immune response (Garinot et al. 2007, Sekiya et al. 2017).

Unfavorable effects on oral vaccination efficacy were reported for the use of PEG in human application. Conjugations of PEG with e.g. the adenoviral vaccine reduced the interaction with endothelial cells and immune cells which dampens the adaptive and innate immune response (Weaver et al.). For the administration of vector vaccines this results in protection against degradation; for the oral application this can potentially reduce the immunogenicity of the oral vaccine and lead to less pronounce immune responses post oral vaccination. A second potential downside of PEG use in oral vaccination is the reduced uptake of liposome-protected / delivered vaccines by macrophages. However, there were no information if this only affects liposomal uptake or vaccines without a liposomal carrier (Minato et al. 2003).

Results herein regarding the effect of PEG on the gastrointestinal tract of juvenile rainbow trout showed no elevated inflammatory response, if the use of PEG in an oral vaccine formulation will lead to an enhanced immune response in the respective intestinal uptake regions needs further investigations. Still, alterations of the intestinal tissues were present and intestinal health were maintained by the addition of alginate or original feedstock to any vaccine diet containing PEG (Chapter 2). The results of

Chapter 2 raised the possibility that the use of PEG in combination with alginates may combine the advantages of both substances and thus demonstrate not only optimal delivery, but also adjuvant effects. Even though no reliable statements on the direct immunological effects of PEG for oral vaccination of fish can be made by the herein executed trials, harmful effects on the intestinal tract after oral application can be considered biologically insignificant.

The true adjuvant effects of alginate or PEG for oral vaccination however, must be clarified in trials focusing on the direct fish immune parameters post oral vaccination as conducted by Caruffo et al. (2016), Ballesteros et al. (2015) or Adelmann et al. (2008) but with improved vaccine pellets which are capable for the administration in commercial aquacultures.

Video-evaluation as precise approach to determine fish individual feeding behavior

Detailed information on individual feed intake of juvenile rainbow trout as provided in Chapter 1 are lacking for most cultured fish species and if information is available, the methods used to obtain the data were highly invasive, possibly resulting in non-representative responses by stressed animals (McCarthy et al. 1993). To date, information on individually reared and fed fish are available for cod (*Gadus morhua*) (Houlihan et al. 1989) or minnow (*Phxinus phoxinus*) (Cui and Wootton 1989). The determination of individual feed intake and digestion for species reared in groups were performed by using marked feeds and radiographic spectroscopy (Talbot and Higgins 1983) as well as through chemical indicators (Gudmundsson et al. 1995, Storebakken et al. 1981). However, no information on directly measured individual feed uptake has been published.

With increasing aquaculture production and cultivation intensity, the efficient use of the finite feedstock for aquaculture and the maintenance of fish health are assigned an increasingly important role (Stentiford et al. 2012). The determination of individual feeding behavior is of major interest for the continuous improvement of efficient pellet uptake for all aquaculture relevant species. The method presented in Chapter 1 can be used not only for the determination of feed intake of juvenile rainbow trout but also for most cultured species to illustrate intraspecies variations in feeding behavior. Analysis

of the feeding behavior in a self-feeding system of European sea bass (*Dicentrarchus labrax)* (Covès et al. 2006) and rainbow trout (Azzaydi et al. 1998) showed that intraspecies feeding behavior were present for the tested species. Therefore, Covès et al. (2006) used passive integrated transponders (PIT) to track the feeding machine activation by the individually marked fish and the results showed that ≈25% of fish did not activate the automated feeder, concluding significant differences in feeding behavior. PIT tags generally show lower errors due to the machine read out especially for low underwater visibility, however do not offer information on fish behavior in general. A videographic approach could further clarify the preferences of fish, to optimize feeding methods for aquaculture cultivation but also present information for dedicated feeding events such as feeding of oral vaccines. Visual tags, such as visual implant elastomer tags (VIE) as used in the conducted experiments help to differentiate between ostensibly identical individuals.

The basis for a successful oral vaccination or administration of other bioactive substances is the sufficient and ideally homogenous uptake of the substance. The effect of individually feeding behavior for different feeding regimes within the first Chapter of this dissertation was evaluated with a videographic approach. The trials on starvation, number of portions and daily feeding events showed that all parameters affect not only total pellet uptake but also uptake homogeneity.

Although the determination of individual feed intake through video evaluation was proven as a reliable tool in this thesis, the manual evaluation of the video footage showed the potential limitation of the direct video-evaluation due to overlapping fish during the energetic feeding process. This can be a reason why today, the method is scarcely applied even though high quality data can be achieved. Research on computer based evaluation software already focused on the issue of overlapping by the use of a multi-module setting (Yu et al. 2022). Video monitoring for experimental evaluation of fish growth performance in aquaculture cultivation, might be a significant step in the progression of aquaculture rearing methods, further increasing fish health, growth performance and optimized feeding methods. Video evaluation itself provides a precise tool to determine fish behavior and health, the implementation of artificial intelligence (AI) can help to significantly develop automated evaluation systems for laboratory and

field use. Since the past 30 years, aquaculture rearing methods only showed small improvements which are mainly dedicated to manual methods, however technology and especially AI have been developed significantly and today, could provide excellent complementary technique (Chang et al. 2021). For instance, by the use of AI and the implemented sensor type, an automated determination and evaluation of different aquaculture parameters such as total biomass, animal size and numbers or different water parameters is possible (e.g. https://monitorfish.com, Mustapha et al. (2021) or Yu et al. (2022)). The use of multiple camera-fusion for 3D estimates, in combination with AI can be used for automated tracking of fish as performed in the trials of Chapter 1 and therefore helps to reduce the high time demanding video evaluation (Dockstader and Tekalp 2001). The methodical error such as overlapping movements of a one-camera-evaluation as used in Chapter 1 could be minimized by the technically advanced approach fused-multiple cameras (Dockstader and Tekalp 2001). To date, intelligent aquaculture is still experience based and a shift towards scientific data acquisition in combination with modern technology is highly needed (Li and Li 2020). Unfortunately, unmanned, intelligent and high precision technology as seen in agriculture are still missing in the aquaculture mainly due to the surprisingly little urge of the quantitatively fast growing aquaculture sector (Lakshmi and Corbett 2020, Li and Li 2020).

Nonetheless, recent increases in aquaculture outputs are dependent on technical improvements including effective non-invasive vaccinations (Hegde et al. 2022). The expanding aquaculture sector will make vaccination for fish in the future indispensable; therefore, information for individual feed uptake and the guarantee for a minimal pellet uptake will be needed for all common aquaculture species. Adaptations of the presented video-graphic approach are highly beneficial to determine the species specific prerequisites.

Besides the importance of fish health and welfare, the video approach can be used to optimize feeding methods of expensive, high-valuable finishing feeds, to reduce waste and increase efficiency and uptake homogeneity. Moreover, advances in feeding regimes can be used to thwart the reduction of fish quality in regard to the consumers' needs / claims as it happened by the sustainable substitution of fish meal and fish oil with plant derived alternatives (Rosenlund et al. 2010). Yıldız et al. (2018) has proven

that a plant based and with fish oil enriched finishing-feeds significantly increase the nutritional amino acid profile of fish and thereby can enhance the fish quality for human consumption.

Future Prospect

To finally develop and optimize a functional oral vaccine for aquaculture application, research should be performed regarding the optimal delivery matrixes. Furthermore, immunological studies should be performed to clarify the expected adjuvant effects of the encapsulation substances. If the optimization of the three keystones for oral vaccination: **1** optimal feeding regime / delivery method, **2** efficient and potent vaccine and **3** the optimal protection of the bioactive component until transit to the immune-active tissue are clearly defined for species and size, oral vaccination of fish will significantly increase fish welfare and producers profits by reducing labor cost and fish loss and diminish the use of antibiotics and the contamination of the environment in open water systems.

The first chapter of this dissertation provides information on different feeding regime parameters such as starvation periods, portion sizes and daily feeding session based on individual fish feeding behavior. Special attention should be directed towards the in Chapter 1 defined minimum pellet intake (MPI) as by that, a threshold uptake of e.g. an oral vaccine for aquaculture species can be guaranteed and therefore lead to an efficient immune response. Consequently, data of the MPI should be collected for all major aquaculture species to tackle the challenges future intensification. Hence, the increasing demand of aquafeeds for the continuously increasing aquaculture sector can be used more efficient and further increase the sustainability of modern, highly needed aquaculture facilities

It is reassuring that the biocompatible substances PEG and alginate, which have been positively used so far, have not led to any serious changes in the intestine of juvenile rainbow trout within the trials of Chapter 2. These substances can and should be further used to modify aquafeeds or to improve different encapsulations approaches without direct harmful, organic side effects as it was performed in Chapter 3.

The novel information about digestion speed and location of pellets in the digestive tract presented in Chapter 3 of this thesis showed that release profiles of vaccine encapsulations should be optimized to have a peak vaccine release earliest after ≈ 10h post administration of juvenile rainbow trout, reared at 15°C. Digestion speeds vary along species, size, diet composition and temperature (Aas et al. 2017, He and Wurtsbaugh 1993, Storebakken 1985) and the evaluation for each prominent aquaculture species is mandatory for a successful oral vaccine development and subsequent administration. For instance, gut evacuation rate of brown trout (1.2 kg) increases exponentially with temperature and consequently, falsely adjusted encapsulations of oral vaccines would lose their effects if administered under unknown conditions (He and Wurtsbaugh 1993).

Independent of the used and encapsulated oral vaccine, these optimizations help to protect the vaccine or bioactive substance against degradation and therefore increases the quantity of the bioactive substance at the location of immune induction.

Administration manual for the oral vaccination of juvenile rainbow trout

Oral vaccination of juvenile rainbow trout should be performed only for fish with 3 g or more and without starvation periods prior oral vaccine feeding. The procedure can be therefore integrated into the daily feeding routine of aquaculture farming. To obtain maximal uptake homogeneity and a minimum vaccine pellet uptake for each fish, the vaccine pellet administration should be performed during a 5 min feeding session. Thereby, the addition of vaccine pellet should be performed in repetitive small portions over the 5 minutes time span. Pellets should be spread across the complete water surface. The color of the vaccination pellets should resemble the original diet. Unpublished feeding trials with different feeding pellets showed that variations in color, especially bright pellet tints irritate fish and lead to poor pellet uptake.

Differences in pellet size if in a commercial used range will not tremendously alter the feeding behavior of fish, however the use of larger pellets will reduce the digestion of the pellets and small pellets are potentially at higher risk for lixiviation. Consequently will lead a wrong or bad choice of pellet size to insufficient feeding, feed utilization and poor water quality.

To enhance the vaccine efficiency and to decrease lixiviation of the vaccine into the rearing water, the vaccine encapsulation into alginate is highly beneficial. The encapsulation process however, depends on the used vaccines, production process and vaccine quantity needed for an adequate fish vaccination. If applicable in the production process, an extruded and therefore floating pellet is beneficial to monitor the feeding process. Floating and slow sinking pellets allow fish to feed on a prolonged period and especially for fish with low hierarchy enhances pellet uptake and therefore herd immunity after vaccination.

Closing remarks

Efficient and sustainable use of resources has never been more important than today and scientific models expect the world's population and wealth to continue to grow. The intensification of food production, including aquaculture, is essential to satisfy the hunger of the world's population. In contrast to land-based food production, industrial aquaculture has a shorter history and a shorter period of intensification. This may also be a reason for the large potential, the aquaculture sector still has.

With the introduction of injective immunization, the impact on wildlife and the environment has been significantly reduced. However, this technique requires high technological standard, labor and often capital costs that are prohibitive in many countries or cannot be met irrespective of cost, especially in developing countries. The development of an orally administrable vaccine represents an ideal opportunity to prevent disease-related aquaculture losses, increase fish health, sustainability, efficiency and reduce the harmful impact on nature.

This dissertation answered important question in the progress of oral vaccine development for aquaculture with specification for rainbow trout. A viable method for the determination of the species specific feeding behavior was demonstrated which should be applied for most common aquaculture species. Next to the vaccine specific feeding behavior, generic information on the compatibility and biomechanical characteristics of vaccine delivery substances are provided. Still, advances in vaccine research is needed to elaborate a functional vaccine that will be eaten in adequate quantities and then provide sufficient and long-lasting vaccine protection. The continuative research for oral vaccines and their delivery methods is inevitable to solve the aquaculture and mankind problems of the 21st century.

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Appendix

Variations in rainbow trout immune responses against *A. salmonicida*: Evidence of an internal seasonal clock in *Oncorhynchus mykiss*

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Variations in Rainbow Trout Immune Responses against *A. salmonicida*: Evidence of an Internal Seasonal Clock in *Oncorhynchus mykiss*

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Simple Summary: Our bodies run on an internal schedule or clock, telling us when to rest, sleep, or digest, and when to wake up, be active, or burn calories. That's why we experience jetlag because we may well set our watches forward or backward, but our bodies haven't yet. Imagine a seasonal clock that helps get us through the year, not just through the day. We set out to prove that such a clock exists in fish just like it does in humans. We exposed rainbow trout to bacteria to imitate natural encounters. We raised fish in the laboratory under the same light and temperature all year long. When we tested them in summer and winter, the fish consequently experienced days that were artificially longer/shorter or warmer/colder. Nonetheless, certain fish white blood cells didn't react or see the bacteria as a threat in winter unlike in summer. They were probably behaving based on the time of year, or season and not on their immediate environment, just like how a jetlagged individual behaves based on an internal clock, not on what it's like outside. Immunity and other processes are regulated differently between seasons, making animals less or more vulnerable in summer or winter.

Abstract: In poikilothermic vertebrates, seasonality influences different immunological parameters such as leukocyte numbers, phagocytic activity, and antibody titers. This phenomenon has been described in different teleost species, with immunological parameters peaking during warmer months and decreased levels during winter. In this study, the cellular immune responses of rainbow trout *(Oncorhynchus mykiss)* kept under constant photoperiod and water temperature against intraperitoneally injected *Aeromonas salmonicida* during the summer and winter were investigated. The kinetics of different leukocyte subpopulations from peritoneal cavity, spleen, and head kidney in response to the bacteria was measured by flow cytometry. Furthermore, the kinetics of induced *A. salmonicida*-specific antibodies was evaluated by ELISA. Despite maintaining the photoperiod and water temperature as constant, different cell baselines were detected in all organs analyzed. During the winter months, B- and T-cell responses were decreased, contrary to what was observed during

summer months. However, the specific antibody titers were similar between the two seasons. Natural antibodies, however, were greatly increased 12 h post-injection only during the wintertime. Altogether, our results suggest a bias toward innate immune responses and potential lymphoid immunosuppression in the wintertime in trout. These seasonal differences, despite photoperiod and water temperature being kept constant, suggest an internal inter-seasonal or circannual clock controlling the immune system and physiology of this teleost fish.

Keywords: seasonality; immune responses; trout; fish inner rhythms

1. Introduction

All species on our planet have co-evolved with their environment, responding to exogenous stimuli and rhythms, and have developed intrinsic clocks that allow them to anticipate periodic changes and respond accordingly [1]. Different species are influenced by several cycles, such as daily and circadian rhythms; tidal, lunar, and semi-lunar cycles; and seasonality (circannual rhythms) [2]. Circadian rhythms are well studied in mammals and have been characterized at transcriptomic levels [3,4]. This rhythmicity influences physiological parameters such as immune cell levels and composition in the bloodstream or in the organs throughout 24 h cycles [5].

Aeromonas salmonicida is the causative agent of furunculosis, an infectious disease affecting wild and farmed salmonids. This bacterium has been widely used as a stimulation and infection model in salmonids [6]. Previously, we described that rainbow trout (*Oncorhynchus mykiss*) exhibit daily diurnal fluctuations in their immune cell composition and respond differently when injected intraperitoneally with *A. salmonicida* at different times of the day [7]. We hypothesize that the immune response against a pathogen could be influenced not only by the time of day but by the time of the year or seasons, as fish also possess circannual rhythms.

Immune functions and the influence of seasonality have been studied in a diverse range of species, including rodents, birds, reptiles [8], and humans [9]. Different approaches have been used, including cell counting between seasons, measurements of organ size and hematological parameters, determination of lysozyme and phagocytic activities, and antibody titers [8]. Recently, researchers have profiled the transcriptomes of human blood samples [10] and, interestingly enough, of the teleost fish three-spined stickleback (*Gasterosteus aculeatus*) [11,12]. Brown et al. [11] showed a strong circannual oscillation of immune-related genes in the stickleback, measuring the expression of sets of genes that are winter- or summer-biased. Among the summer-biased transcripts, they found genes related to adaptive immune responses (*rag1*, *rag2*, *zap-70*, *cd8 a*, *foxp3b*, *il4*, *igh*), while the winter-skewed ones had higher levels of innate immune (*il1* and non-classical complement pathways) and lymphocyte immunosuppressive genes.

Specifically in rainbow trout, leukocyte numbers peak during summertime and are lower in the winter [13–15]. Morgan et al. broadened the seasonal evaluation of parameters and measured, for 12 months, the white and red blood cells counts, plasma lysozyme activity, and respiratory burst of head kidney cells [16]. Erythrocytes showed a peak in warm months (June and July), and total white blood cells were significantly higher in the summer months than in the winter. Plasma lysozyme activity also followed this pattern, and no clear seasonal trend was observed for respiratory burst activity. Considering this, it is hypothesized that in general, all parameters are diminished in winter and increased in summer [17]. Water temperature and photoperiod are key factors that influence the seasonal variation of immune parameters [14]. However, some studies have shown changes in immune parameters even when temperature and photoperiod were constant, suggesting that endogenous rhythms may also be involved [18]. Despite this, to our knowledge, there are no studies evaluating differences in the composition of leukocyte subsets in warm or cold seasons, nor studies examining the capacity to respond to the

antigenic challenge among seasons, nor studies investigating if the inner clocks of fish regulate their immune responses circannually while maintaining the same water temperature and photoperiod.

In this study, we answer some of these questions by stimulating rainbow trout intraperitoneally (i.p.) with inactivated *A. salmonicida* while keeping water temperature and photoperiod constant over the winter and summer months. We measured how immune cell populations responded to the bacterial stimulus by flow cytometry, and additionally, we evaluated the antibody response in both conditions.

2. Materials and Methods

2.1. Ethics Statement

The experiment was approved by the State Office for Agriculture, Food Safety and Fisheries (approval number LALLF 7221.3-2-042/17), according to the German and European guidelines on animal welfare (Tierschutzgesetz, Tierschutz-Versuchstierverordnung, Directive 2010/63/EU).

2.2. A. salmonicida for Stimulation Experiments

An aliquot of the *A. salmonicida ssp. salmonicida* highly virulent strain JF 5505 from stock cryo-preserved batches—previously checked for purity by Gram staining, cell morphology, and motility—was cultivated in tryptic soy broth media (TSB, Becton Dickinson, Heidelberg, Germany) at 15 °C for 24 h. The bacterial suspension was inactivated in 1.5% paraformaldehyde (PFA) for 1.5 h at 4 °C. Inactivated bacteria were plated out on TSB agar plates to verify that no bacteria grew after inactivation. The PFA was washed away twice with TSB media, each time centrifuging at 4000 *g* for 10 min at 4 °C. The pellet was resuspended in TSB 25% glycerol at a concentration of 1.5×10^8 bacteria/mL. For intraperitoneal immunization, the bacteria were washed once with PBS and set to a concentration of 1×10^7 bacteria/mL. Injections were prepared under aseptic conditions with sterile 1× phosphate-buffered saline (PBS).

2.3. Fish

The Born strain of rainbow trout (*O. mykiss)* was bred and purchased in Germany, with no gender selection, from the commercial trout breeding farm Forellenzucht Uthoff GmbH, Neubrandenburg (Germany). The fish were kept in 300 L glass aquaria in a partially recirculating water system, at constant 12 ± 0.2 °C and constant 12 h light: 12 h dark period for both summer and winter experiments. Dissolved oxygen (11 ± 0.5) and pH (7.1 ± 0.1) were monitored daily. Ammonia levels never surpassed 0.1 mg/L. Summer experiments were performed in September; winter experiments were performed in January, European time zone. Fish were fed twice per day with commercial dry food pellets (Aller Futura, Aller Aqua GmbH Golssen, Germany). A total of 130 fish randomly distributed through the groups were used for the experiments, without special criteria for bias. Fish weighed 26.4 ± 12.7 g at the beginning of the trial and weighed 36.4 ± 21.3 g at the end of the trial (group 28 days post-i.p. injection). Five fish were used for the cell baselines in summer and five fish for the cell baselines in winter (ten in total). A total of 120 fish were used for sampling at the different time points for both control PBS-injected fish and for the bacteria-injected fish.

The fish were separated according to the sampling time points and received a single i.p. injection, containing 100 µL of 1×10^7 bacteria/mL or 100 µL of PBS (control fish). After i.p. injection, control and stimulated fish were kept in separated tanks. The tanks were divided into two areas (one per time point) using a plastic mesh, in order to keep fish separated. Five fish were used at each time point per condition (12 h, 24 h, 48 h, 72 h, 14 d, and 28 d post-i.p. stimulation with inactivated bacteria or injection with PBS) for a total of 60 per season.

2.4. Sampling and Leukocyte Preparation

On the day of sampling, fish were sacrificed with an overdose of benzocaine (Sigma, Gernsheim, Germany). Blood was taken from the caudal vein, collected in EDTA-containing tubes (Sarstedt AF & Co., Nümbrecht, Germany) and kept on ice until processing. Thymus, spleen, head kidney, and peritoneal cells were collected. All cell processing was performed on ice and all reagents were ice-cold. Blood volume, peritoneal lavage volume, and fish and organ weights were measured and recorded for each animal.

Peritoneal leukocytes (PELs) were obtained via lavage with 5 mL of ice-cold 5 mM EDTA-PBS. Leukocytes from the other organs were obtained after homogenization in 5 mL of 1% newborn calf serum (NCS)-PBS buffer (FACS Buffer, FB). Blood was washed with 5 mL of FB. All cell suspensions were centrifuged at 4 °C for 5 min at 290 *g* and then resuspended in 1 mL of FB. A hypotonic lysis protocol for erythrocytes was modified from Crippen et al. [19] and optimized for each organ sampled. Briefly, erythrocytes were disrupted by adding 8 mL of ice-cold Milli-Q water and mixing by inversion for 5 s for the PELs and 10 s for spleen and head kidney; 1 mL of 10× PBS was added to restore the isotonicity. The cell suspension was placed on ice for 5 min, allowing debris to clump and precipitate. Next, the leukocytes were filtered through a 100 μm mesh and then centrifuged. The pellet was finally resuspended in 1 mL of FB and then the living cells were counted by trypan blue exclusion (Gibco, Thermo Fisher, Bremen, Germany).

The cell composition of the above-mentioned tissues was analyzed using flow cytometry. Additionally, the kinetics of the *A. salmonicida*-specific antibodies were measured by Enzyme-Linked Immunosorbent Assay (ELISA).

2.5. Flow Cytometry

A multicolor flow cytometry approach was used to analyze the immune cell kinetics and distribution in the different tissues. All monoclonal antibodies were previously validated [20]. Briefly, a first blocking step was made prior to antibody staining, incubating 4 × 10^5 cells per tube with FB for 30 min at 4 °C. The cells were then pelleted by centrifugation at 290 *g* for 5 min at 4 °C, and the pellet was resuspended in 200 μL of antibody solution containing anti-pan T-cell monoclonal antibody D30 (mAb D30) and anti-trout CD8α. Cells were incubated for 30 min at 4 °C and then washed with 700 μL of FB by centrifugation as described above. The cell pellet was resuspended in 200 μL of secondary antibody solution (anti-mouse IgG1 405 (Jackson Immuno Research, Biozol, Eching, Germany; anti-rat IgG-Alexa 647 (Invitrogen, Fisher Scientific GmbH, Schwerte, Germany)). After an incubation of 30 min at 4 °C, a washing step was performed as described. The cells were resuspended in 200 μL of a mixture of directly labeled monoclonal antibodies: anti-Igμ chain monoclonal antibody 1.14 (mAb 1.14), anti-light chain monoclonal antibody N2 (mAb N2), anti-myeloid cell monoclonal antibody 21 (mAb 21), and anti-thrombocyte monoclonal antibody 42 (mAb 42, to exclude this population from the analysis). Cells were incubated for 30 min at 4 °C, washed, and finally incubated with the viability dye Zombie Aqua (BioLegend, San Diego, CA, USA) at a dilution of 1:1000 in 1× PBS for 20 min at 4 °C. After a final wash step, the cell pellet was resuspended in 300 μL of FB. Cells were analyzed by the Cell Analyzer BD LSR Fortessa (Becton Dickinson, Germany), recording a minimum of 30,000 events for each sample. Doublet discrimination was performed in dot plots using the parameters FSC-H vs. FSC-A (forward scatter height versus forward scatter area) and SSC-H vs. SSC-A (side scatter height versus side scatter area). Lymphoid (FSC^{low} SSC^{low}) and myeloid cells (FSC^{hi} SSC^{hi}) were distinguished by their scatter profiles. The cytometric analysis was made in the BD FACSDIVA software (BD Biosciences, Heidelberg, Germany).

2.6. ELISA

To measure specific anti-*A. salmonicida* IgM antibodies, high-binding ELISA plates (Greiner, Sigma, Germany) were coated with 1 μg per well of inactivated *A. salmonicida* in

0.2 M Carbonate buffer pH 9.6 overnight at room temperature (RT) and then blocked with 150 µL of ROTI®Block solution (Carl Roth, Karlsruhe, Germany) for 1.5 h at RT. A total of 100 µL of a two-fold dilution series of 1:10 pre-diluted fish sera from 1:80 to 1:81,920 was incubated for 1 h at RT. After three washing steps with PBS 0.05% Tween 20 (PBST), 200 ng per well of purified anti-trout IgM antibody 4C10 was incubated for 1 h at RT, followed by three washing steps with PBST. Finally, the secondary antibody goat anti-mouse IgG1 HRP conjugated 1:5000 (mouse IgG2a, IgG2b, IgG3, IgM, IgA; human, bovine, and horse serum proteins cross-adsorbed, Southern Biotech, Biozol, Eching, Germany) was incubated for 1 h at RT. After three washing steps with PBST, the wells were developed by a 10-min incubation at RT with 50 µL of TMB/E Horseradish Peroxidase Substrate (Merck, Darmstadt, Germany). Finally, an additional 50 µL of H_2SO_4 was added to stop the reaction, and the optical density of each well was measured at 450 nm in the Tecan ELISA plate reader (Tecan Sales Austria GmbH, Grödig, Austria). To measure "natural antibodies", plates were coated with 100 ng of 2,4-Dinitrophenyl-Keyhole Limpet Hemocyanin (DNP-KLH, Merck, Darmstadt, Germany). The ELISA protocol used was identical to the one above.

3. Results

3.1. Seasonal Cell Composition of Unstimulated Fish

Figure 1a shows the stepwise gating strategy we used to define the lymphocyte and myeloid gates. Figure 1b shows representative morphometric plots (forward scatter versus side scatter, or size versus intracellular complexity in other words) of leukocytes in each organ analyzed as well as the respective lymphoid and myeloid gates that we defined. Analyzing the number of leukocytes in the peritoneum (Figure 1c), significantly higher numbers of myeloid cells were detected in the wintertime, whereas no statistically significant difference was measured for the lymphoid population. In the spleen, head kidney, and thymus, significantly higher numbers of lymphocytes were detected during the summer season. Among these tissues, we measured a decrease in the number of myeloid cells only in the thymus ($p > 0.05$). Thus, under identical experimental photoperiod and water temperature, we nonetheless measured inter-seasonal variation in lymphoid cell numbers in all lymphoid organs tested as well as in myeloid cell numbers in the peritoneum and thymus.

3.2. Seasonal Cell Composition of the Peritoneal Cavities of A. salmonicida-Stimulated Fish

The differences in the cell numbers in the different lymphoid organs between the two seasons led us to test if this influences the cellular kinetics in response to a pathogen. For this purpose, we stimulated fish i.p. with fixed *A. salmonicida* strain JF5505 and we followed up the cell kinetics locally (peritoneal cavity) and systemically (spleen and head kidney) after 12 h, 24 h, 48 h, 72 h, 14 d, and 28 d post-stimulation.

In the peritoneal cavity in the summer (Figure 2a, upper panels), during the first 24 h post-stimulation (hps) with the bacteria, the intraperitoneal stimulation induced a clear switch in the predominant local cell type (Figure 2a); the myeloid population increased starting at 12 hps and peaked at 24 hps. As the myeloid population started to drop down 48 hps, the lymphoid population started increasing at this time point, reaching a peak at 72 hps. In the late response phase, i.e., 14 and 28 days post-stimulation (dps), the cell levels in the peritoneal cavity returned to baseline and were comparable to those of the PBS-injected group. In the winter (Figure 2a, lower panels), we instead observed a simultaneous increase in both myeloid and lymphoid cells, starting at 12 hps and reaching a peak at 48 hps. Afterwards, the myeloid fraction dropped down faster than the lymphoid one, which remained slightly higher at 72 hps, but not statistically significantly. As in the summer, at 14 and 28 dps, both levels were back to baseline.

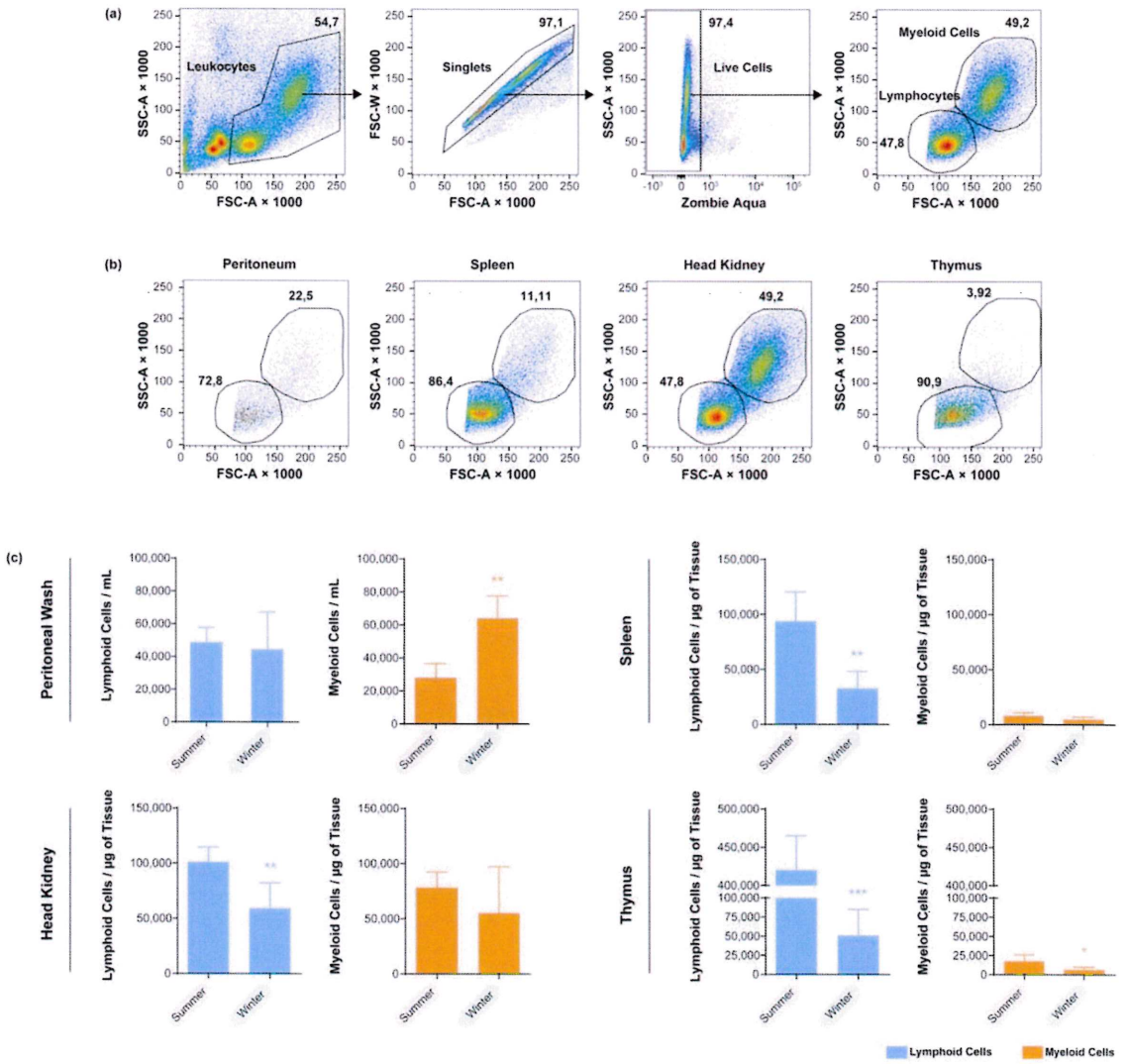

Figure 1. Unstimulated rainbow trout demonstrate different lymphoid and myeloid cell counts between the summer and winter seasons in the peritoneal cavity and lymphoid organs. (**a**) Gating strategy used for the flow cytometric analysis. (**b**) Representative morphometric plots of each organ analyzed as well as the gates established to distinguish lymphoid from myeloid lineage cells. (**c**) Lymphoid and myeloid cell counts. Bars are color-coded to represent either lymphoid (blue) or myeloid (orange) cells. Note the decrease in lymphocytes in spleen, head kidney, and thymus during winter. Any statistical significance was determined with Student's *t*-tests; $n = 5$ per group per season. * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$. The data are presented as mean values ± standard deviation (SD).

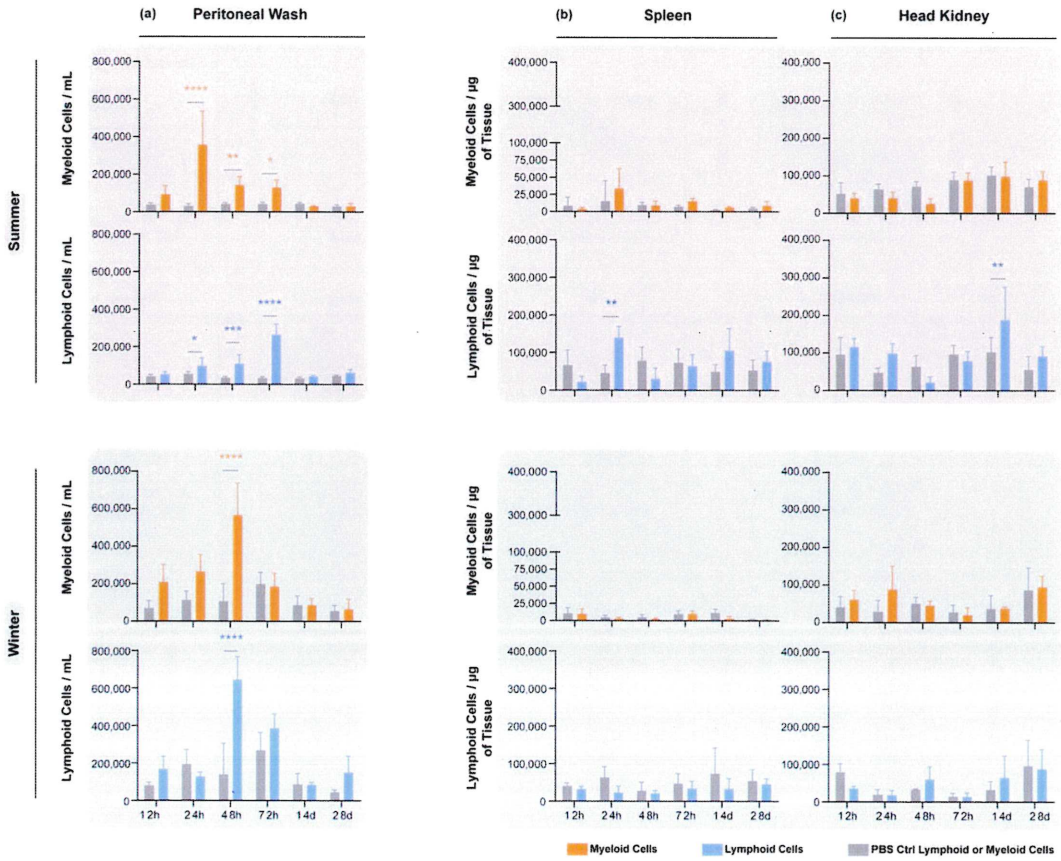

Figure 2. Myeloid and lymphoid cells at different time points after stimulation with *A. salmonicida*. Gray bars represent control fish injected with PBS. (**a**) Peritoneum, (**b**) spleen, (**c**) head kidney stimulated in summer (two upper rows) and winter (two lower rows) under constant water temperature and photoperiod. The local peritoneal response in summer was composed of an orchestrated response, with a myeloid-dominant phase in the first 24 hps, switching to a lymphoid phase at 48 hps. In winter, the peritoneal leukocytes increased, jointly reaching a peak at 48 hps. The spleen responded faster than the head kidney in the summertime; however, in the winter, no response was observed in both organs. Y-axes depict the number of cells per mL of peritoneal wash or the number of cells normalized to the amount of tissue collected (for the lymphoid organs), whereas the x-axes represent the number of days after administration of either *A. salmonicida* or PBS. Bars are color-coded to represent either lymphoid (blue) or myeloid (yellow) cell counts after stimulation, or (gray) their corresponding groups of lymphoid or myeloid cells from fish injected with PBS. Statistical significance was calculated with a two-way ANOVA multiple comparison test, with a Bonferroni post hoc correction test. $n = 5$ for each time point. * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$; **** indicates $p < 0.0001$. The data are presented as mean values ± standard deviation (SD).

3.3. Seasonal Cell Composition in the Spleens and Head Kidneys of A. salmonicida-Stimulated Fish

In the spleen during the summer (Figure 2b, upper panels), no statistically significant variation was observed in the myeloid cell population. However, a significant increase in the lymphoid population was observed in the spleen at a single time point (24 hps) before returning to baseline levels. In the wintertime (Figure 2b, bottom panels), no response was observed for the lymphoid nor myeloid cells at any time point.

In the head kidney (Figure 2c, upper panels), the lymphoid cell numbers increased significantly only at a single time point, as in the spleen, except it was very much delayed to 14 dps. Just like in the spleen, no significant changes were measured for the myeloid

population (Figure 2c, top panel) nor any changes in these populations during the wintertime (Figure 2c, bottom panels).

3.4. Response of Leukocyte Subpopulations in the Peritoneal Cavities of Stimulated Fish

To determine which subpopulation was responsible for the observed changes in the response of lymphoid and myeloid cells, the leukocytes were labeled with different leukocyte population-specific antibodies and analyzed by flow cytometry.

The response of B-cell populations in the peritoneal cavity to *A. salmonicida* stimulation was different between the summer and winter. In the summer, an increase in the IgM^- (IgT^+) B-cell population between 24 h and 72 h post-injection was observed, and the same occurred with the IgM^+ lymphocytes (Figure 3a,b, left column). In comparison, in the wintertime, we observed a peak in IgM^+ lymphocytes only at 48 h post-injection, whereas no IgT response was observed (Figure 3a,b, right column).

For the $CD8^+$ T-cell population, delayed responses were measured at 72 hps in the summer and maintained on day 14 post-stimulation, returning to baseline levels thereafter (Figure 3c). In contrast, no significant change was observed in winter for the $CD8^+$ T cells nor among $CD8^-$ T cells (that are likely $CD4^+$ T cells) at any time point or in any season (data not shown).

During the summer, the local response of the myeloid population (Figure 3d, left) showed an early increase in the cell numbers at 12 hps, reaching a peak in cellularity at 24 hps. At 48 hps, the response already began decreasing while remaining higher than the baseline and remained so until 72 hps. In winter, the response observed in the first 72 h was stronger than the one observed in summer (Figure 3d right). A higher early increase in cell numbers at 12 hps was observed and increased gradually, peaking at 48 hps. Thus, in response to *A. salmonicida* stimulation, the myeloid compartment expanded and peaked rapidly in the summer, decreasing gradually, whereas the change in the season led to a gradual expansion and peak, with a sudden drop at 72 hps.

Our results matched those presented in Figure 2 for the peritoneal cavity, with cellular kinetics suggesting the phenomenon of an initial myeloid phase response during the first 24 h in the summer, followed by a delayed lymphoid phase response. In winter, only a synchronized cell increase between myeloid and lymphoid cells (predominantly IgM^+ lymphocytes) was observed, peaking at 48 hps.

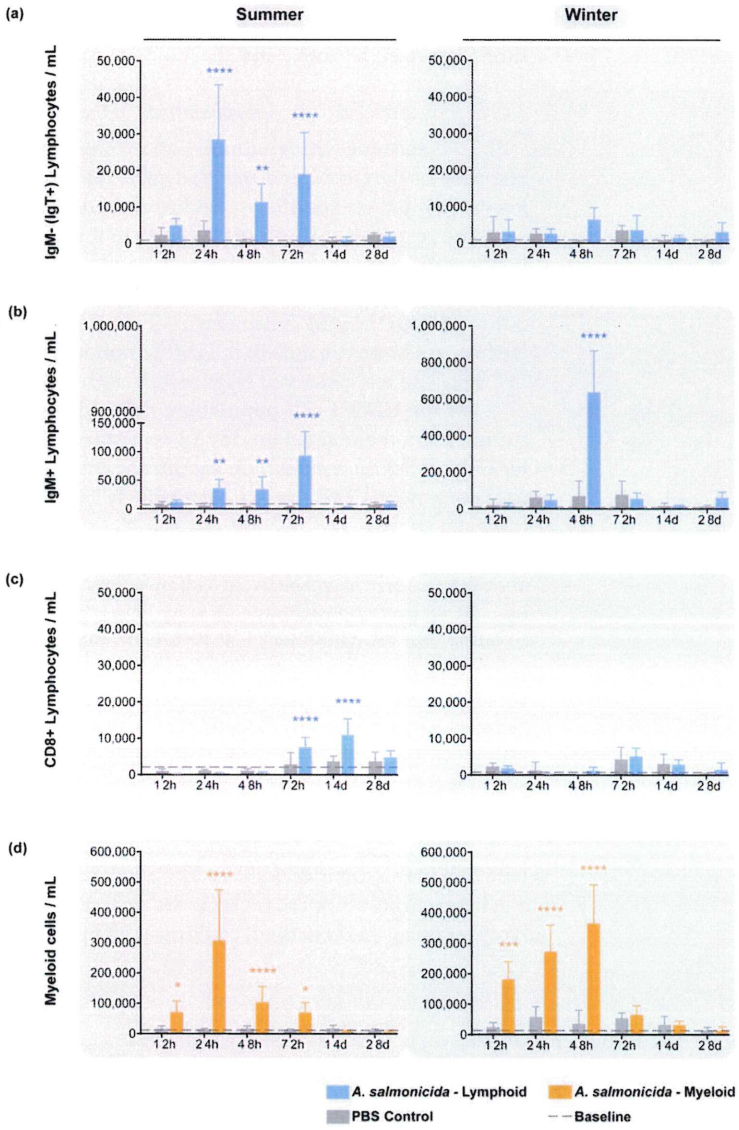

Figure 3. Response of peritoneal cells after stimulation with *A. salmonicida* analyzed by flow cytometry. (**a**) IgM^- lymphoyctes/mL; (**b**) IgM^+ lymphoyctes/mL; (**c**) $CD8^+$ lymphocytes/mL; (**d**) myeloid cells/mL in summer (**left**) and in winter (**right**). Whereas IgM^+ B cells responded in both seasons, the IgM^- (IgT^+) cells increased after stimulation in the summer only. $CD8^+$ T cells also responded exclusively in the summer. In the summertime, myeloid cells responded as early as 12 hps, whereas in the winter, the recruitment peaked at 48 hps and dramatically decreased after this time point. Baseline levels of the cells are from 5 naïve fish. Y-axes depict the number of cells per mL of peritoneal wash, whereas the x-axes represent the number of days after administration of either *A. salmonicida* or PBS. Bars are color-coded to represent either lymphoid (blue) or myeloid (yellow) cell numbers from fish stimulated with the bacterium. Gray bars are the corresponding lymphoid or myeloid cell counts from fish injected with PBS. Statistical significance was calculated with a two-way ANOVA multiple comparisons test, with a Bonferroni post hoc correction test. $n = 5$ for each time point. * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$; **** indicates $p < 0.0001$. The data are presented as mean values ± standard deviation (SD).

3.5. Response of Leukocyte Subpopulations in the Spleens of Stimulated Fish

Shifting our attention to the spleen after fish were stimulated with *A. salmonicida*, a rapid, significant, and short-lived increase in the number of cells per μg of tissue was observed in the summer at 24 hps in both B-cell populations (Figure 4a,b, left column) relative to PBS-injected individuals. On the contrary, no trend or statistically significant change was observed during the wintertime (Figure 4a,b, right column).

Myeloid cells (labeled with mAb 21) increased at 24 hps during the summer (Figure 4c, left panel) and mirrored the lymphocyte compartment with this change or any change being completely absent in winter.

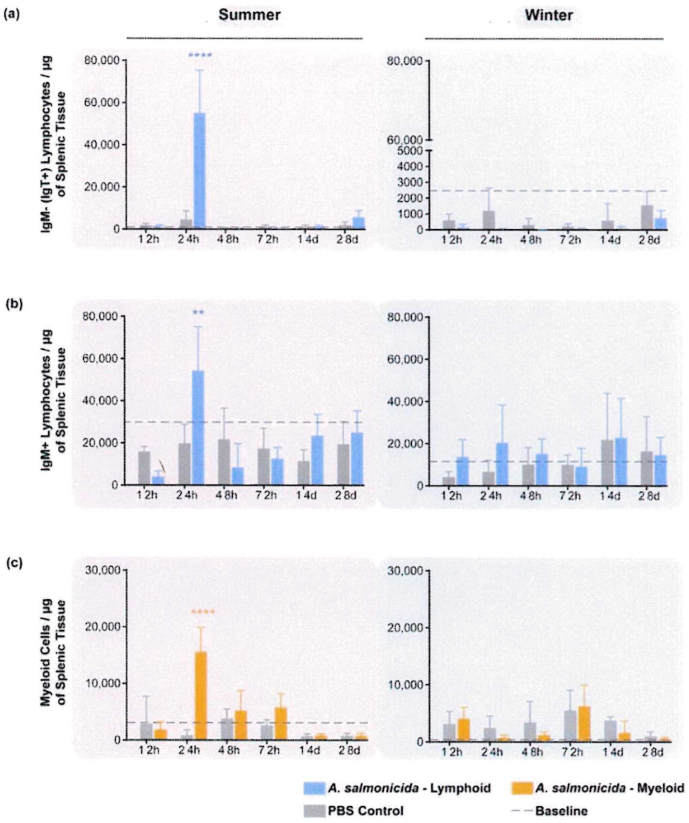

Figure 4. Cell response in the spleen after stimulation with *A. salmonicida* analyzed by flow cytometry. (**a**) IgM^- lymphoyctes/mL; (**b**) IgM^+ lymphoyctes/mL; (**c**) myeloid cells/mL in summer (**left**) and in winter (**right**). An increase in the number of Ig^+ cells was only observed during summer (at 24 hps) for both Ig isotypes. Myeloid cell numbers peaked at 24 hps in summer. No statistically significant peak of response was observed during the wintertime. Baseline levels of the cells are from 5 naïve fish. The bars are colored blue to represent the number of lymphoid cells from *A. salmonicida*-exposed animals or the number of myeloid cells (yellow) from the same animals. Y-axes represent the number of cells per μg of splenic tissue, whereas the x-axes represent the number of days since fish were injected. Gray bars represent the corresponding lymphoid or myeloid cells from groups of fish injected with PBS. Statistical significance was calculated with a two-way ANOVA multiple comparisons test, with a Bonferroni post hoc correction test. $n = 5$ for each time point. ** indicates $p < 0.01$; **** indicates $p < 0.0001$. The data are presented as mean values ± standard deviation (SD).

3.6. Response of Leukocyte Subpopulations in the Head Kidneys of Stimulated Fish

In the head kidney, there were variable increases in the number of IgT^+ and IgM^+ lymphocytes during the summer at 12 and 24 hps and at 14 and 28 dps (Figure 5a,b, left column). Meanwhile, in the winter, no statistically significant responses were measured at any time point for either of these compartments. (Figure 5a,b, right column).

On the other hand, higher cell numbers of $CD8^-$ T cells ($CD4^+$ T cells) were observed at 28 dps only during summer (Figure 5c, left); no trend nor difference between PBS- and *A. salmonicida*-injected fish were noted during winter within this cell population (Figure 5c, right panel).

Myeloid cells showed no change at any time point regardless of the season or the stimulus (Figure 5d).

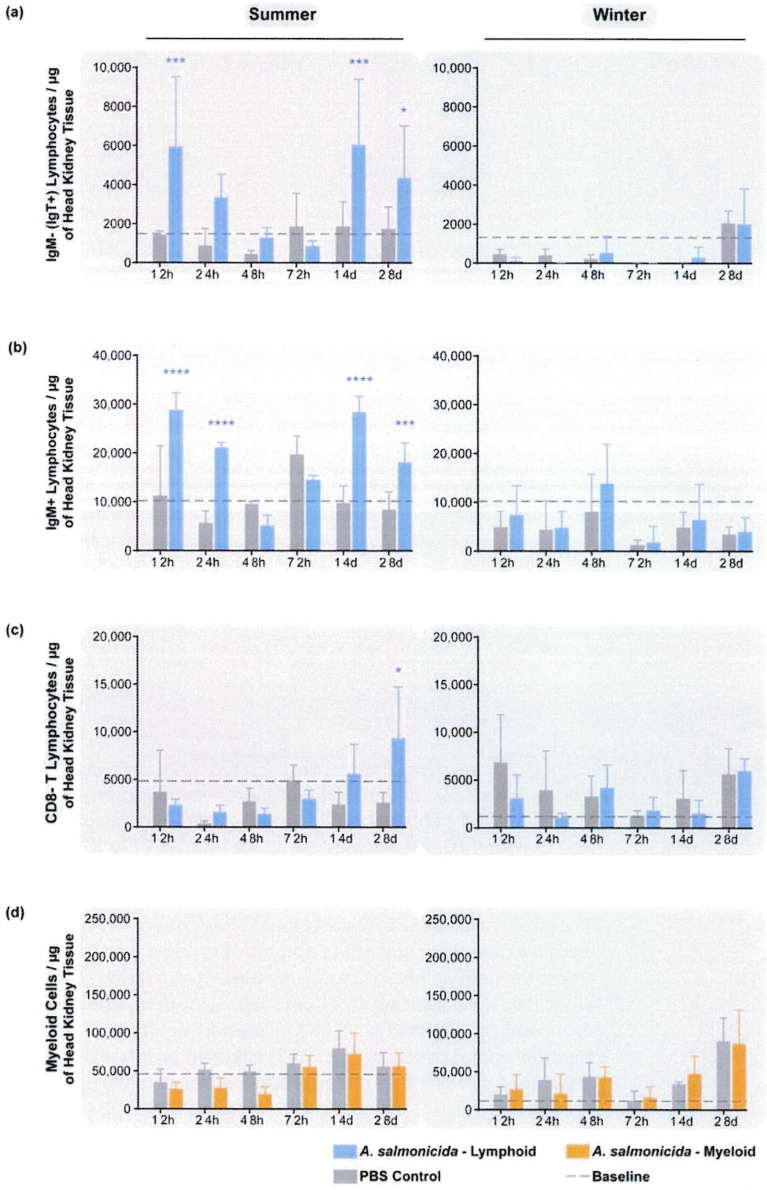

Figure 5. Cell response in the head kidney after stimulation with *A. salmonicida* analyzed by flow cytometry. (**a**) IgM^- lymphoyctes/mL; (**b**) IgM^+ lymphoyctes/mL; (**c**) $CD8^-$ lymphocytes/mL; (**d**) myeloid cells/mL in summer (**left**) and in winter (**right**). An increase in the number of IgM^- IgT^+ B cells and IgM^+ B cells was observed sporadically early (12 hps for both populations and also 24 hps for IgM^+ B cells) and at 14 and 28 dps during summer. $CD8^-$ T cells ("CD4") were higher at 28 dps. No statistically significant trend was observed in the myelocytic response in both seasons. Baseline levels of the cells are from 5 naïve fish. Y-axes represent the number of cells adjusted to the µg of head kidney tissue collected. X-axes represent the days since injection of either the bacterium or PBS. Bars are color-coded according to whether they show the lymphoid (blue) or myeloid (yellow) cell populations after stimulation. The gray bars are data for the corresponding myeloid or lymphoid cells from fish exposed to PBS only. Statistical significance was calculated with a two-way ANOVA multiple comparisons test, with a Bonferroni post hoc correction test. $n = 5$ for each time point. * indicates $p < 0.05$; *** indicates $p < 0.001$; **** indicates $p < 0.0001$. The data are presented as mean values ± standard deviation (SD).

3.7. Specific and Non-Specific Antibody Titers in Fish Stimulated with A. salmonicida

Due to the significant changes in the B-cell compartment and the expected contribution of B cells to the response against the bacteria, we evaluated specific and non-specific antibody production by ELISA across all the time points used.

During the summertime (Figure 6, left graph), minimal non-specific antibodies were detected during the earliest time points evaluated (12–72 hps). A slight increase was observed at 14 dps (titer 1:160) and 28 dps; the titer increased to 1:320. With regard to the specific antibodies, they were not detected until day 14 post-stimulation, with a titer of 1:640. The titer remained at 1:640 by day 28 post-stimulation.

During the wintertime (Figure 6, graph on the right) at 12 hps, an unusually high titer of non-specific antibodies was present in the serum of fish injected with the bacteria (1:5120). After 24 h, the titer rapidly decreased to 1:320 and remained so until 48 hps. Only in the wintertime during the first 12 h after the injection with the bacteria was this exponentially larger non-specific response observed. The specific antibodies were detected at 14 dps with a titer of 1:640, remaining in that range until 28 dps, identical to what we observed in the summertime.

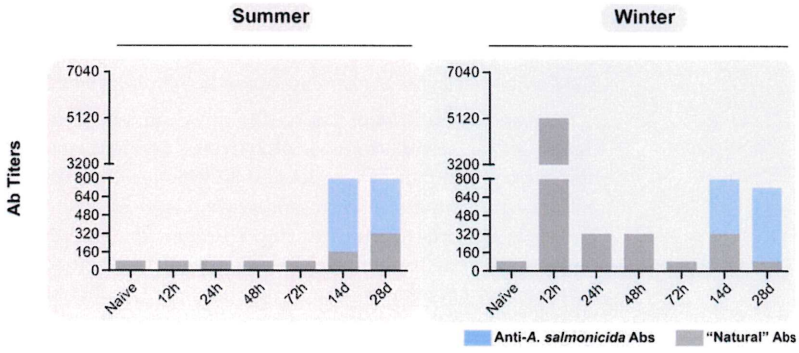

Figure 6. Antibody titers specific for *A. salmonicida* (in blue) compared to "natural" antibodies reactive against DNP-KLH (in gray) in sera from rainbow trout stimulated with *A.salmonicida*. Specific antibodies against *A. salmonicida* were detected in both seasons starting at 14 dps. A spike in "natural" antibodies was measured in winter at 12 hps; afterward, the "natural" antibody titer decreased over time. The y-axes represent antibody titer, whereas the x-axes represent the hours or days since animals were stimulated with *A. salmonicida*. The serum of 5 fish individually was used to measure the antibody titer for each time point.

4. Discussion

As animals do not control their environment, they develop strategies to anticipate and cope with seasonal shifts. This way, they are prepared to respond accordingly at the

molecular, cellular, and metabolic levels, favoring survival and maintaining organism fitness [21]. We observed interseasonal differences in how fish responded or did not respond to stimuli. Although these experiments need to be repeated and expanded upon with more fish and more photoperiod/temperature conditions, it nonetheless warrants discussion and speculation as to how our findings compare to the very limited number of publications on the effect of seasonality on fish immunity, what specific cell subsets are most affected between seasons, and the mechanisms behind a potential circannual clock.

4.1. Seasonal Immune Status: Immune Parameters Influenced by the Season

A study in the three-spined stickleback (*Gasterosteus aculeatus*) reported that immune markers were winter- or summer-biased, observing suppression of adaptive immune and lymphocyte proliferation genes during winter [11]. In *O. mykiss*, changes have been reported in the baselines of immune cells, lysozyme and phagocytic activities, and antibody titers between winter and summer [16]. Additionally, it was reported that erythrocyte and leukocyte numbers peak during summer and are the lowest during winter [13–15].

Our initial analysis of the cell baselines showed that during summer, spleen, head kidney, and thymus had more lymphocytes than in winter. This suggests that lymphocytes are actively being produced during this season and are home to organs such as the spleen and head kidney. Fish lack lymph nodes; therefore, these two organs are probably where the T-cell and antigen-presenting cell (APC) interactions happen [22]. Interestingly, in the peritoneum, lymphoid cell numbers were similar during both seasons; however, the myeloid cell population was increased during the wintertime. The decreased number of lymphoid cells in the winter suggests reduced hematopoietic activities in the thymus and head kidney and potentially a limited proliferative activity in the spleen. Fish thymuses involute during winter months because of their season-dependent activity, meaning thymus thickness decreases, has low lymphocyte numbers, and has more adipose tissue and collagenous fibers [23–26]. Considering this, we would expect less T lymphocytes circulating during these months, contributing to a long-term reduced immune response activity in poikilothermic fish. Myeloid cells are rapid and first responders limiting the spread of invading pathogens. The higher number of myeloid cells in the peritoneal cavity might be an adaption to wintertime. Perhaps resident myeloid cells are preferred over constantly recirculating cells for this season.

4.2. Peritoneal Cavity: Biphasic Response in Summer, Joint Response in Winter

From the peritoneal cavity, the development of a biphasic leukocyte response was clear after i.p. stimulation: a switch from a myeloid- to a lymphoid-dominant phase at 48 hps during summer. These leukocyte kinetics correspond with what has already been published by others using a stimulation and infection model with *A. salmonicida* [27]. Thus, we hypothesize that during summer, the lymphocytes residing in the peritoneal cavity contribute to the immune response upon the bacterial stimulus. They secrete pro-inflammatory cytokines that, within the first 24 h, help to recruit myeloid cells to the cavity [28,29]. In winter, a different scenario was evident. A higher simultaneous increase in lymphoid and myeloid cells was observed until 48 hps. In this situation, pathogen detection and cytokine release by the two populations were probably concomitant and increased in the winter. Consequently, much higher cell numbers/mL of peritoneal wash were reached, suggesting a stronger inflammatory process occurred from which fish should recover afterwards.

4.3. Immune Response Skewed toward Innate Immune Responses in Winter in Trout

T and B lymphocytes are described as the main resident populations in the peritoneum of mice; one resident B cell subpopulation is B1 B cells [30]. They sense pathogen-associated molecular patterns (PAMPs) through TLRs, act as APCs and phagocytes, secrete cytokines for cell recruitment, and secrete high amounts of low-affinity poly-reactive

antibodies [31,32]. Even though B1 B cells are not fully described in fish, the first hints of their presence in zebrafish [33] and rainbow trout [34] were already reported. Considering the immune functions of B1 B cells, it would be reasonable to hypothesize that B1 B cells reside in the peritoneal cavity of fish.

From our results, we can observe a strong peritoneal IgM^+ response in winter, maybe due to the presence of a higher number of B1 B-like cells during this season, since this subset expresses high IgM on their membranes [35]. As these cells are innate-like and we also observed more myeloid cells present during winter, we hypothesize that during the winter there is a shift toward an innate status of this cavity to combat pathogens. This could be due to the need to manage metabolism and the trade-offs needed to balance investment in immunity versus other physiological processes to promote survival [36]. Colder months are already challenging for fish, with limited nutrient and oxygen availability among others, such that fish need to compensate by, for example, limited activity and lower heart rate. From an immunological perspective, these types of survival decisions have been reported in different species, including birds [37] and humans [38]. In mice, it has been shown that the initial generation of plasmablasts against malaria is metabolically taxing, to the point of compromising the generation of memory B cells and favoring the progression of the parasite life cycle [39]. Innate responses require lower initial energetic demand and are not energetically costly to maintain, which is why they are favored in winter months. Additionally, they constitute a fast and effective responder system against new pathogens [38]. Overall, this suggests a regulation of adaptive immunity during the winter, rather than a loss of function.

4.4. Seasonal and Circannual Rhythms Modulate the Immune Response of Trout

In lower ectothermic vertebrates, including fish, lower peripheral blood lymphocyte counts during the winter and higher numbers during the summer are general trends, except in *Salmo trutta*, in which the parameters were reversed [18]. In teleosts, three different isotypes—IgM, IgD, and IgT—have been described [40]. In this study, we analyzed IgM^+ cells and IgM^-, mostly and likely IgT^+ cells [41]. We observed a mixed response between these two B-cell subsets only during summer, which suggests that immune fitness and response capacity are higher during the summer months and that the mucosal response mediated by IgT is suppressed in winter. Moreover, only in summer, the head kidney showed an increase in the IgT^+ population 14 and 28 days after stimulation. Since we lack antibodies against IgT, we could not measure IgT levels in serum to at least have an indication of whether plasma cells could also play a role in this response.

4.5. Temperature and Photoperiod: Key Parameters Influencing Immune Seasonal Variations in Trout

On the one hand, it is known that temperature affects B cells and antibody secretion [42]. In this study, water temperature was constant at 12 °C in both seasons, and the titer of specific IgM antibodies against *A. salmonicida* were similar in the two periods. However, interestingly, non-specific antibodies were highly increased 12 h after the bacterial stimulation and gradually decreased afterwards. Together, this also suggests that more B1 B cells could be present during winter in the peritoneal cavity, secreting natural non-specific antibodies to support the immune response against pathogens, since fish are not in an optimal immune state.

On the other hand, we observed that despite maintaining the water temperature and photoperiod constant, different cellular immune responses were observed in the two seasons. This has been reported previously for mice [43,44] and also for lower vertebrates [18,45], suggesting that neuroendocrine and other endogenous rhythms are involved in regulating these responses. It is known that adrenocortical activity is modulated on a circannual basis, having a higher corticosteroid secretion in winter and a lower one during summer, directly affecting immune parameters. Glucocorticoids suppress cellular immune function, affecting T cells to a greater extent. Thus, when glucocorticoids are elevated, T cell activity is suppressed, and B cell activity is elevated [46]. In fish, it was

described that cortisol, the primary corticosteroid produced in teleosts, exerts strong anti-inflammatory effects and inhibits pro-inflammatory cytokines [47]. In vitro studies made in gilthead seabream [48], sea bass, and rainbow trout [49] showed that the cortisol effect is immunosuppressive and may enhance disease susceptibility. In rainbow trout, cortisol reduced the number of circulating lymphocytes; decreased lymphocyte proliferation, antibody production, and phagocytosis; and increased apoptosis [50]. This is in line with what we observed during winter, where T cell responses were dramatically decreased. However, the effects of hormones/cortisol are not fully depleting but rather immunosuppressive for B cell lymphopoiesis, sparing cortisol-resistant long-lived memory effector B cell populations. Probably, this effect is to favor the survival of spawning fish upon return to their natal streams. Thus, similarly, the immunosuppressive effects of cortisol in winter may not directly impact the survival of fish if neither their compartment of differentiated, highly selected effector memory cells nor the antibodies they produce are compromised, with only less (excessive) energy expanded on lymphopoiesis [51].

4.6. Indirect Evidence of a Potential Internal Circannual Clock in Trout

Marine animals, including teleost fish, are highly regulated by circadian, circalunar, and circannual rhythms [52,53]. Cell-autonomous, long-term timekeeping systems (circannual rhythms) are endogenous calendars that animals have maintained throughout evolution to track physiological and behavioral cycles and to anticipate and sense environmental variations that are crucial for their survival [54–57]. How these annual clocks work is still unknown. It has been proposed that circannual rhythm generation depends on tissue-autonomous cell regulation, repetitive cell division cycles, functional cell differentiation, and cell death [58]. In rainbow trout, circadian rhythm-related genes such as *Clock1a, Bmal1, and Per1* have been described in the retina and hypothalamus [59]. Thus, as environmental cues are not necessary to modulate this clock, the circannual biological pacemakers run untethered, even if stimuli such as light or temperature are experimentally altered. As we observed in our experimental conditions, even keeping photoperiod and water temperatures constant resulted in different cell baselines and a different immune response against the bacterial pathogen. Further studies to elucidate which transcription factor(s) modulate the circannual modulation of the immune response should be performed and, using these markers, tissue specificity should be investigated.

5. Conclusions

Altogether, this work shows a difference in the immune response against a bacterial stimulus between two seasons, despite environmental cues such as water temperature and photoperiod remaining unchanged. It provides information suggesting rainbow trout may have circannual clocks that modulate physiological parameters, including immune cell levels, that may impact immune responses directly. Moreover, we only measured mixed innate and adaptive cell responses during the summer. These important findings should be considered in three main contexts: (a) to update "when" and under which conditions we perform our laboratory experiments in order to improve reproducibility; (b) to optimize "when" we apply stimuli to fish (if we consider vaccination as a stimulus, based on our results, we should vaccinate fish during summer months, coinciding with fish being at their most immunocompetent status); and (c) to improve fish health when they are raised under artificial conditions. Recirculating aquaculture systems frequently use unnatural photoperiod regimens such as 24 h of light and 0 h of darkness (24L:0D) or 16L:8D for extended periods. Although the reasoning behind this is to improve the growth rate, it is thought that fish could also be immunologically protected, as summer conditions are mimicked. However, our results indicate that despite the attempt to "manipulate" the physiology of fish, this may not be possible due to their inner clocks. Finally, relevant topics remain to be investigated further, such as elucidating how different photoperiod regimens and their durations influence the immune status and immune responses of fish, to promote immune protection and decrease susceptibility to pathogens.

Author Contributions: The individual contributions are as follows: R.M., M.Ø., T.K., K.M. and B.K. conceptualized the study. R.M. designed the experiments and also drafted, wrote, prepared figures for, reviewed, and edited this manuscript. J.T.H.C. prepared figures and edited and reviewed this manuscript. C.M. and P.N.J. performed experiments and reviewed the manuscript. S.O. performed experiments. M.Ø., T.K., K.M. and B.K. reviewed and edited the manuscript. M.Ø., T.K., K.M. and B.K. provided supervision. R.M. and B.K. obtained funding for and administrated the project. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the State Office for Agriculture, Food Safety and Fisheries (approval number LALLF 7221.3-2-042/17), according to the German and Euro-pean guidelines on animal welfare (Tierschutzgesetz, Tierschutz-Versuchstierverordnung, Directive 2010/63/EU.

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