Received: 13 June 2023 | Revised: 24 July 2023 | Accepted: 28 July 2023

DOI: 10.1111/jfd.13846

Journal of Fish Diseases WILEY

RESEARCH ARTICLE

In vitro modelling of the influence of alternative feeds (*Hermetia illucens*, *Arthrospira platensis*) on the resistance of different rainbow trout populations (*Oncorhynchus mykiss*) against the viral haemorrhagic septicaemia virus and *Yersinia ruckeri*

Julia Bauer[1] | Mikolaj Adamek[1] | Anne-Carina Miebach[1] | Jakob Gährken[2] | Stephan Wessels[2] | Jens Tetens[2] | Carsten Dietz[3] | Angela Sünder[3] | Marek Matras[4] | Magdalena Stachnik[4] | Michal Reichert[4] | Dieter Steinhagen[1]

[1]Fish Disease Research Unit, University of Veterinary Medicine Hannover, Hannover, Germany

[2]Aquaculture and Water Ecology, University of Goettingen, Goettingen, Germany

[3]Department of Animal Sciences, Animal Nutrition Physiology, University of Goettingen, Goettingen, Germany

[4]National Veterinary Research Institute, Puławy, Poland

Correspondence
Mikolaj Adamek, Fish Disease Research Unit, Institute for Parasitology, University of Veterinary Medicine Hannover, Buenteweg 17, 30559 Hannover, Germany.
Email: mikolaj.adamek@tiho-hannover.de

Present address
Julia Bauer, Lower Saxony State Office for Consumer Protection and Food Safety, Veterinary Task Force, Hannover, Germany

Funding information
Niedersächsisches Ministerium für Wissenschaft und Kultur; Ministry for Science and Culture in Lower Saxony

Abstract

Replacing fishmeal, a finite resource with high market demand, in the diet of carnivorous rainbow trout with proteins from alternative sources may be a challenge for these fish. Therefore, this study investigated whether replacing fishmeal with protein derived from *Hermetia illucens* or *Arthrospira platensis* could promote disease susceptibility in local trout populations with different growth performance. This was assessed in vitro by measuring susceptibility to infection with the viral haemorrhagic septicaemia virus (VHSV) or the bacterium *Yersinia ruckeri*. Analysis of fin tissue explants and primary cell cultures from scales from the three trout populations infected in vitro with VHSV and gill explants infected with *Y. ruckeri* showed no significant differences in virus replication or bacterial counts. Evaluation of the virucidal or bactericidal effect of skin mucus showed a significant reduction in viral load and bacterial count for all samples with mucus addition, but no significant difference was observed between the experimental groups. This study documents no apparent impairment of innate immune mechanisms in the skin and gills of trout after feeding a diet replacing fishmeal with Arthrospira or Hermetia proteins. This underlines the potential of these alternative protein sources for the further development of sustainable trout aquaculture.

KEYWORDS
Arthrospira platensis, disease resistance, *Hermetia illucens*, in vitro, viral haemorrhagic septicaemia virus, *Yersinia ruckeri*

1 | INTRODUCTION

In the evolution of fish populations, genetic adaptions to new environmental conditions are crucial, but represent a continuous and long-lasting process. In aquaculture facilities, however, sudden changes in nutrition can occur, especially due to changes in feed composition driven by the shortages of certain resources on global markets. In particular, the use of fishmeal and more generally marine resources in the feed of cultured fish can be subjected to reductions due to high competition for this finite resource. Despite advances in stock management due to the common fisheries policy in the European Union, where an increasing number of stocks are managed at the level of maximum sustainable yield, overcapacities and overfishing still constitute a major challenge for marine fish stocks in large parts of our oceans. Therefore, the replacement of fishmeal with alternative protein sources is inevitable and focuses a lot of scientific interest (Luthada-Raswiswi et al., 2021).

The high protein and lipid nutritional requirements of carnivorous fish are often difficult to fulfil without fishmeal. In particular, plant proteins were considered as a cost-effective replacement of fishmeal in aqua feeds (Ghosh et al., 2019). One of the most popular sources for plant proteins in feed for food delivering animals is soybean meal (SBM, *Glycine max*) (Bakke-McKellep et al., 2000). Even though some studies showed that a partial substitution of fishmeal with soy protein had positive effects on growth in large rainbow trout (*Oncorhynchus mykiss*) (Vielma et al., 2000), the inclusion of plant proteins in fish feed had largely negative effects on growth performance and health status of fish (Hemre et al., 2018). A soybean meal-induced enteritis (SBMIE) was confirmed in Atlantic salmon (*Salmo salar*) already at an inclusion rate of 20% (Baeverfjord & Krogdahl, 1996) and other studies suggest an induction of an SBMIE in the gut of rainbow trout as well (Burrells et al., 1999). While in Atlantic salmon (*Salmo salar*), saponins may play a role in the induction of SBMIE (Krogdahl et al., 2015), in rainbow trout, an association was seen between the glycinin and β-conglycinin content of SBM with intestinal pathology (Burrells et al., 1999). Hence, by using low antigen soybean concentrate, in which a large proportion of the anti-nutritive substances have been removed during processing, the risk of SBMIE can be reduced.

In addition to soybean meal, protein from single cell organisms, like the algae *Haematococcus pluvialis* (Ju et al., 2012) or the cyanobacterium *Arthrospira platensis* also known as spirulina (Teimouri et al., 2013) were included in fish feed. In particular, this filamentous, multicellular photoautotrophic cyanobacterium has gained a great deal of attention as a fishmeal substitute and as a functional feed additive in fish and shrimp culture (Ragaza et al., 2020). Due to its high-quality protein content, spirulina was used as partial supplement or complete replacement for proteins from fishmeal in the feed of different fish species including rainbow trout and affected growth performance, carcass quality and immune responses. It was shown that it can limit heavy metal toxicity in several fish species. However, it became obvious that the effect of spirulina is species specific and its potential use needs to be evaluated carefully (Zhang et al., 2020). In addition to plant proteins, further alternative protein sources were investigated, including protein from insects such as the cricket, *Acheta domesticus* (Irungu et al., 2018) or the black soldier fly larvae, *Hermetia illucens* (Irungu et al., 2018; Stadtlander et al., 2017). Altogether, an impact of these fishmeal substitutes on fish health, and differences in the tolerance of carnivorous fish of feed supplemented with these ingredients could be seen in many studies.

An adaptation of fish to the new environmental conditions caused by fishmeal substitutes in the feed has hardly taken place. Fish have high heritabilities of important performance traits and in breeding programmes, selection successes are three to five times higher than in terrestrial animals (Gjedrem, 2012). In a breeding experiment, survival rate and growth of trout fed a plant-based diet could be significantly improved after a single generation of selection (Le Boucher et al., 2012). Therefore, in breeding programmes, the individual variability to adapt to alternative feeds can be used to improve animal welfare and sustainability in trout aquaculture (Le Boucher et al., 2012). Likewise, the potential of locally adapted rainbow trout populations could be explored. However, the effects of fishmeal replacement on disease resistance have been poorly studied. As stated above, an induction of inflammatory responses in the intestine and partial immunosuppression have been observed in various species, including rainbow trout, carp, zebrafish and Atlantic salmon (Burrells et al., 1999; Hedrera et al., 2013; Krogdahl et al., 2003; Uran et al., 2008). In Atlantic salmon, a transcriptome analysis showed significantly altered gene expression of the liver and other tissues in response to the fishmeal content of the ration, but without biometrically measurable differences (Tacchi et al., 2012). The effect of a selection of rainbow trout on the ability to adapt to alternative feeds on disease resistance was not studied.

However, testing the influence of the feed additives or increased utilization of alternative feeds on the resistance to the infections is relatively time consuming, complexed and expensive especially if multiple lines or strains of fish have to be evaluated. Therefore, the development of in vitro methods is an essential goal in modern sciences. Within the '3Rs' principle, the establishment of in vitro alternatives can therefore contribute to reducing animal experiments. In fish research, in vivo experiments are still a major component in investigating viral or bacterial diseases. However, the development of fish cell lines and their use as alternative in vitro methods is advancing fast. With this in mind, this study concentrated on two major diseases in rainbow trout that regularly cause mortality in aquaculture facilities, in order to find a starting point for in vitro infection research for these diseases. Viral haemorrhagic septicaemia (VHS) is caused by the viral haemorrhagic septicaemia virus (VHSV), a single stranded RNA virus from the genus *Novirhabdovirus*, and causes a systemic infection in salmonids with haemorrhages of internal organs, skin and muscle. Acutely infected animals may show high mortality rates and symptoms like apathy, darkening, exophthalmia and ascites (Smail & Snow, 2011). The Enteric red mouth disease (ERM) is caused by the bacterium *Yersinia ruckeri*. A rod-shaped, Gram-negative bacterium

belonging to the family *Enterobacteriaceae*, ERM is characterized by haemorrhages of the skin, often in and around the mouth, throat and fin regions, ascites and haemorrhages of the internal organs. High mortality rates are possible (Barnes, 2011). Both pathogens spread horizontally via water and fish-to-fish contact.

The replication of VHSV in rainbow trout and other fish species was analysed in vivo and in vitro studies. Hereby, a replication of VHSV was proven in gills (Brudeseth et al., 2002) and excised fins (Quillet et al., 2001). VHSV replication was also seen in primary cell cultures of fibroblastic fin cells from ventral fins (Estepa et al., 1993) and in the permanent cell lines like RTG-2 or BF-2 (Lorenzen et al., 1999). Both pathogens enter the fish via mucosal tissues. The causative agent of ERM, *Y. ruckeri* enters the body via skin, gills, stomach and the posterior intestine (Khimmakthong et al., 2013). A basic step for the establishment of an infection is the attachment of the bacterium to epithelial cells at the portal of entry. This process was studied in vitro in a gill perfusion model, where differences between pathogenic and less pathogenic bacterial strains could not be detected (Tobback et al., 2010). The point of entry of VHSV is still debatable. Some studies suggest the entry of Novirhabdoviruses like the infectious haematopoietic necrosis virus, which is closely related to VHSV, via epidermal regions of the fin bases (Harmache et al., 2006). Other studies suggest a fibronectin-mediated cell entry of VHSV via the skin and muscle tissue with high amounts of fibronectin (Bearzotti et al., 1999). Different trout lines proved to have different levels of susceptibility to VHSV infection (Quillet et al., 2001), and in resistant fish, lower virus loads were seen in organs and fin tissue (Dorson et al., 1994). The survival of fish from waterborne infection was significantly correlated with VHSV-replication in excised fin tissue in a VREFT (virus replication in excised fin tissue) assay in vitro with a lower virus load in VREFT-cultures of fish with a lower susceptibility to VHSV infection (Quillet et al., 2007).

The aim of this study was to analyse whether proteins from alternative sources in feed for rainbow trout have the potential to influence the susceptibility of these fish to infection with VHSV or *Y. ruckeri* and whether in vitro models of the main entry tissues for VHSV and *Y. ruckeri* can be used for such an analysis. For this purpose, different regional rainbow trout populations were fed with feed supplemented with alternative protein sources and fin explant and primary cell cultures from scales were established from these rainbow trout populations for an in vitro simulation of a VHSV infection. Additionally, gill explant cultures were established for the in vitro simulation of an infection with *Y. ruckeri*. Differences in fish diets were found to be a main driver of metabolic differences in mucus of fish species (Reverter et al., 2017). Alterations in the composition of fish feed might also change the composition of the skin mucus as well as the expression of immunoactive substances within it. Therefore, in this study, mucus samples of all specimens of the different experimental groups were analysed for their virucidal and bactericidal effects in vitro. In order to verify the results from the in vitro analysis, an in vivo challenge experiment was performed by infecting rainbow trout from different regional populations with VHSV.

2 | MATERIALS AND METHODS

2.1 | Rainbow trout populations and rearing facilities for the feeding trial

Regionally adapted rainbow trout populations were kept on the experimental farm Relliehausen (University of Goettingen, Germany). The broodstocks were kept as closed populations with pedigree matings. All populations were autumn-spawning rainbow trout. In total, 28 full-sib families were produced by artificial reproduction of broodstock from eight populations (R1–R8). The offspring was grown family-wise to a mean weight of 31.2 ± 9.4 g, then specimens were individually tagged by passive integrated transponder tags (PIT-tags) and equally distributed among nine 200 L tanks using a communal testing design. Subsequently, the fish were fed three isoenergetic and isonitrogenous diets containing 20% fishmeal (FM) or, as a replacement of FM, 20% *A. platensis* or *H. illucens* meal (Dietz et al., 2020; Dietz et al., 2023 for composition, see Table S1) at a ratio of 1% body weight per day. The different diets were fed to rainbow trout in three replicate tanks respectively. After 90 days of feeding, family breeding values were estimated for weight gain using individually recorded growth parameters. On the basis of breeding values, families from the R8 population were selected as best performing genotypes and families from the R3 population as least performing genotypes. In a further experiment, growth and feed conversion were analysed in fish from these populations after feeding the diets with a replacement of fishmeal by *Arthrospira* or *Hermetia* proteins (see Table S1). In addition to the R3 and R8 populations, a commercially available rainbow trout strain selected for growth and fish health (C9) was included in the experiment. From all populations or strains, a total of 45 (R3, R8) or 30 (C9) individuals were equally distributed among three replicate tanks and fed the experimental diets at a ratio of 1% body weight per day for 70 days at 15–16°C. Subsequently, all fish were killed and sampled as described below.

All experiments were performed in accordance with national and international regulations and did not require notification or approval by local authorities in accordance with the German Animal Protection Act (Section 7, paragraph 2, clause 3) because there were no interventions before killing. The animals were killed in accordance with Section 4, paragraph 3 of the Animal Protection Act exclusively for the use of their organs or tissues for scientific purposes.

2.2 | Viral and bacterial strain

For all experiments, bacteria from the non-motile strain P3 of *Y. ruckeri* were used, which had been previously isolated from diseased rainbow trout during an ERM outbreak in June 2011 (Huang, Runge et al., 2013). The bacterial isolate was cultured on Columbia sheep blood agar at 15°C for 48 h and growing colonies were suspended using sterile Dulbecco's phosphate buffered saline (PBS, Sigma).

Bacterial concentration of 1.87×10^7 cfu mL^{-1} was estimated by titration on Columbia sheep blood agar plates (Oxoid) and given in colony-forming units (cfu).

The VHSV strain Fi13, belonging to serogroup F1 (Enzmann & Bruchhof, 1989), was provided by the Friedrich Loeffler Institute, Federal Research Institute of Animal Health, Germany. The virus was inoculated into rainbow trout gonad (RTG-2) monolayer cell cultures grown in medium 199, supplemented with 20% foetal calf serum (FCS), 50 IU mL^{-1} penicillin, 50 µg mL^{-1} streptomycin, 120 µg mL^{-1} gentamicin, 3 µg mL^{-1} amphotericin, (Sigma, culture medium) and cultivated at 20°C for 48 h. Then the supernatant medium was collected, filtered through a 0.45 µm filter and the concentration of virus (10^7 $TCID_{50}$ mL^{-1}) was determined by titrating the suspension on RTG-2 cell cultures and the result was given as 50% Tissue Culture Infective Dose ($TCID_{50}$).

2.3 | Preparation of primary skin and gill cultures and subsequent infection with VHSV or *Y. ruckeri*

Fin samples were collected from eight male and eight female parental specimens from eight regionally adapted trout populations after anaesthetizing the fish with clove oil during the annual reproduction procedure.

Additional fin, scale, mucus and gill samples were collected from a total of 81 fish from the regional R3 and R8 populations as well as from the commercial strain C9. After finalizing the second feeding experiment, nine fish from each population or strain and experimental group were stunned by percussive stunning and subsequently killed by exsanguination.

For primary fin cultures, an approximately 1.5 cm piece of the ventral part of the caudal fin was removed with sterile scissors and collected in an ice cold culture medium (medium 199, supplemented with 20% FCS, 50 IU mL^{-1} penicillin, 50 µg mL^{-1} streptomycin, 120 µg mL^{-1} gentamicin and 3 µg mL^{-1} amphotericin [Sigma]). The fins were cut into four small pieces (1 × 0.5 cm) and placed individually in 15 mL tubes filled with 4 mL of fresh culture medium each. A total of 10 µL of a suspension with 10^7 $TCID_{50}$ mL^{-1} tissue culture-derived VHSV was added to three tubes while one tube received medium without the virus and served as negative control. All tubes were incubated at 20°C and 2% CO_2 for 3 days. Directly after infection with VHSV and after 3 days of cultivation, 100 µL of culture medium was collected from each tube and frozen at −80°C for subsequently determining the virus.

Scales were collected by gently scraping the dorsal skin of rainbow trout with a cell culture scraper (Sarstedt) and were directly transferred to culture medium on ice. For preparing cultures, approximately three scales were placed in individual wells of 24-well plates filled with culture medium. The plates were then incubated at 20°C and 2% CO_2 for 4 days and evaluated for epithelial cell growth. Three wells with a monolayer of primary epithelial cells were selected and infected with VHSV by incubating the cells for 1 h with medium containing 10^4 $TCID_{50}$ mL^{-1} of the tissue culture-derived VHS virus. After a medium change, the cells were further incubated at 20°C and 2% CO_2, and 100 µL of the culture medium was collected 2 days post infection for determining the virus concentration.

The virus concentration in samples from skin and scale cultures was determined by titration on RTG-2 cell cultures using the $TCID_{50}$ method.

For primary gill cultures, a gill arch was excised from each individual trout, directly placed in an ice cold cell culture medium (medium 199, supplemented with 20% FCS, 50 IU mL^{-1} penicillin, 50 µg mL^{-1} streptomycin, 120 µg mL^{-1} gentamicin, 3 µg mL^{-1} amphotericin [Sigma]) and incubated for at least 4 h. Then the gill samples were washed with sterile phosphate buffered saline (PBS) in order to eliminate the bacterial microflora and residues of the antimicrobial substances of the collection media from the gill tissue. Subsequently, the gill arches were cut into three pieces of approximately 0.5 cm of gill arch length each and placed into three separate 15 mL tubes filled with 5 mL cell culture medium without antibiotics. Two tubes were infected with 100 µL of a *Y. ruckeri* suspension (1.87×10^7 cfu mL^{-1}). A total of 100 µL of culture medium without bacteria was added as negative control to one tube. All tubes were incubated at 15°C for 24 h. Then, 500 µL of the culture medium was collected, the same amount of veal infusion broth (Oxoid) was added and then frozen at −80°C. In the samples, the bacterial load was determined by titration on Columbia sheep blood agar plates (Oxoid) as described below and results were given in colony-forming units mL^{-1} (cfu).

2.4 | Virucidal and bactericidal effect of mucus on VHSV, *Y. ruckeri* and *Escherichia coli*

After completing the feeding experiment, mucus was collected by gently scraping the dorsal skin of rainbow trout from the R3 and R8 populations as well as from the commercial strain C9 with a cell culture scraper (Sarstedt) and avoiding contamination with scales and faeces. The mucus was transferred to a 2 mL tube and frozen at −80°C until further processing. A crude mucus extract was prepared by mixing 50 µL of mucus with 150 µL of 1 × Tris buffered saline (TBS, Sigma). The mixture was homogenized by vortexing and then aspirated in a syringe and passed through a needle (26G - 0.45 × 25 mm, Braun) approx. 20 times. The homogenate was centrifuged at 18,800 *g* for 10 min and the supernatant was frozen at −80°C until further processing.

For assessing a bactericidal effect of the mucus against *E. coli* and *Y. ruckeri*, 50 µL of mucus extract was incubated with 5 µL of bacterial suspensions (10^3 and 6.73×10^6 cfu mL^{-1} respectively) and 50 µL of veal infusion broth overnight at 20°C. As negative controls, the bacteria were incubated with TBS instead of mucus extract. After incubation, the suspensions were serially diluted with sterile TBS in a range of 1:10 to 1:1,000,000 and 50 µL thereof was plated onto Columbia sheep blood agar plates in duplicate in order to evaluate bacterial growth. Results were given in cfu mL^{-1}.

For assessing a virucidal activity of the mucus against VHSV, 30 µL of mucus extract was incubated with 10 µL of a VHSV suspension

(3.16×10^6 $TCID_{50}$) and 60 μL of minimal essential medium at 20°C and 2% CO_2 overnight. As negative controls, the virus was incubated with 30 μL TBS instead of mucus extract. After incubation, the virus concentration in the samples was determined by titration on RTG-2 cells as described above and given in $TCID_{50}$ mL^{-1}.

2.5 | In vivo challenge of different trout populations with VHSV

To validate the in vitro model for recording the susceptibility of rainbow trout to VHSV infection, trout from the R3 and R8 populations and the commercial strain C9 were infected with VHSV after completing the feeding experiment. From the R3 and R8 populations, fish from all three experimental feeding groups were included in the experiment, whereas from C9, only rainbow trout fed with the control feed were included. The experiment was conducted at the National Veterinary Research Institute, Puławy, Poland in accordance with international and national regulations for animal experimentation and after approval by the local ethics committee in Lublin, Poland (Approval No. 33/2020). Before infection, the rainbow trout were acclimatized for 24 h, then exposed to 5×10^3 $TCID_{50}$ mL^{-1} of tissue culture-derived VHSV in an aerated water bath for 2 h at 15°C and afterwards distributed among three tanks in mixed groups with three to seven specimens of each population or strain and feed (Figure 1). For negative controls, rainbow trout were exposed to uninfected cell culture medium under the same conditions, and subsequently, these trout were kept separated from infected specimens.

Infected and uninfected rainbow trout were kept for 29 days at 12–15°C. Water quality was maintained by a constant flow-through of well water and the trout were monitored for the development of clinical signs of disease and mortality two to four times per day. Dead fish were collected and severely moribund fish were killed by immersion in a water bath with 500 mg L^{-1} tricaine methane sulphonate, (MS-222, Pharmaq UK) in order to avoid unnecessary suffering in accordance with established humane end points. Approximated mortality (indicating number of dead and killed fish) was documented over 29 days and fin samples were collected at days 2 and 4 post infection from four specimens of each genotype and feed and frozen at −20°C for further analysis.

2.6 | Virus load in fin samples after experimental infection of rainbow trout with VHSV

Total RNA was extracted from fin samples collected from rainbow trout with and without experimental infection with VHSV using Tri-Reagent (Sigma) by following the manufacturer's instructions. Any remaining genomic DNA was removed by digestion with 2 U of DNase I (Thermo Fisher Scientific) following the manufacturer's protocol. Synthesis of cDNA was performed from 300 ng of total RNA using the MaximaTM First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). A non-reverse transcriptase control was included in the analysis of each sample. Prior to RT-qPCR analysis, cDNA samples were diluted 1:20 with nuclease-free water.

Virus load in fin samples was determined by quantitative reverse transcriptase PCR (RT-qPCR). Viral RNA encoding for the VHSV-N-protein and mRNA encoding for elongation factor 1α from host tissue were quantified by SYBR-Green-based RT-qPCR protocols. Primer sequences are listed in Table 1.

The PCR reaction was performed in duplicate with the Maxima SYBR Green 2x Mastermix (Thermo Fisher Scientific) using a StepOne Thermocycler (Applied Biosystems). The reaction mix contained 1x Maxima SYBR Green Mastermix (with 10 nM ROX), 0.2 μM of each primer (Table 1) and 3.0 μL cDNA (1:20 dilution). Nuclease-free water was added to a final volume of 10 μL. The amplification programme consisted of an initial denaturation phase of 10 min at 95°C, 40 identical cycles with denaturation of 30 s at 95°C, annealing at

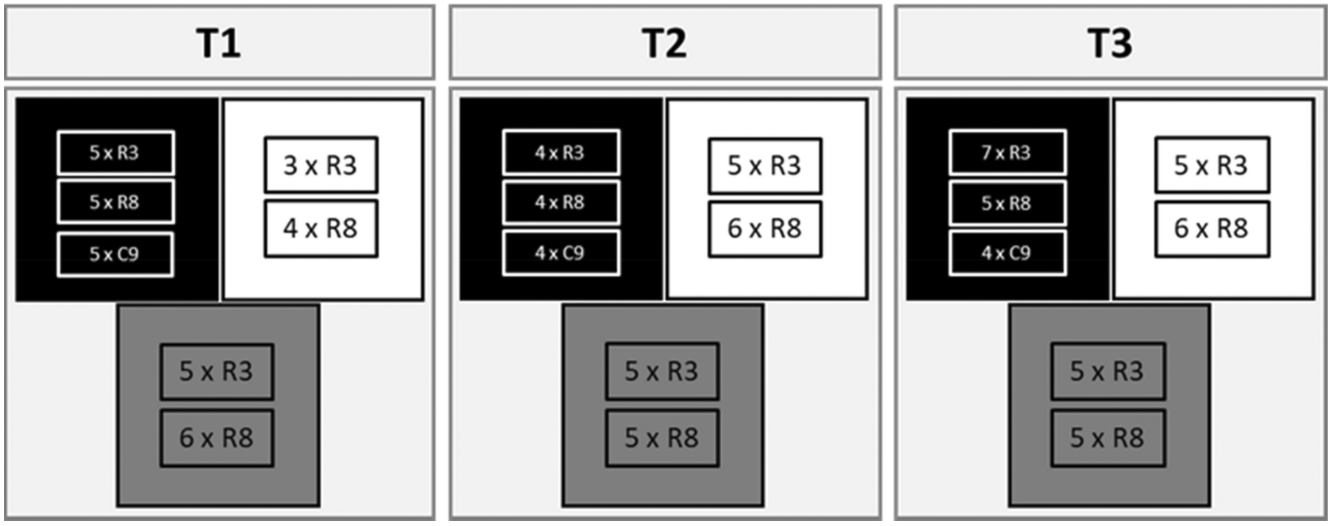

FIGURE 1 Experimental set up of three tanks (T1–3) with the number of trout for each genotype and feed: black = Control; grey = *Hermetia illucens*; white = *Arthrospira platensis* and the R3 and R8 populations as well as the commercial strain C9 under control feed.

TABLE 1 Primer sequences for determining virus load in fin samples.

Gene	Oligo name	Sequence 5′–3′	Reference
VHSV	Onmy-VHSV	F: GAATCCGTGCAGCTTTTTCAGG R: CAAGTGCATCCACGATCACCTTC	Kim et al. (2014)
Elongation factor 1α	Onmy-EL1α	F: TGGGCTGGTTCAAGGGATGG R: CTGGAGGGGCAGACGAAGG	Dietrich et al. (2015)

TABLE 2 Overview of the in vitro assessment of the susceptibility of rainbow trout to VHSV and *Y. ruckeri* infection, and basic data of the in vivo challenge of different populations of rainbow trout (R3 and R8) and the commercial strain (C9) under alternative feeds with *Arthrospira platensis* and *Hermetia illucens* proteins.

	R3	R8	C9
In vitro model (VHSV)			
Fin explant cultures	No significant differences in virus loads		
Primary scale cell cultures	No significant differences in virus loads		
Viruzide effect of mucus	No significant differences in virus loads		
In vitro model (*Y. ruckeri*)			
Gill explant cultures	High values*	Low values*	Intermediate
Bacterizide effect of mucus	No significant differences in virus loads		
In vivo model (VHSV)			
Approximated mortality	59%	15.8%	85%
Fin explants	High values*	Low values*	High values*

Note: Significant differences are indicated (*).
Abbreviation: n.e., not examined.

55°C for 30 s and a final elongation at 72°C for 30 s. Measurements were normalized by determining a housekeeping gene (elongation factor 1α). For quantification, copy numbers of VHSV-specific RNA were normalized against 10^5 copies of the reference gene rainbow trout elongation factor 1α (EF1/100,000).

2.7 | Statistical analysis

Statistical analyses were performed using the Systat Software SigmaPlot 12. Virus load or $TCID_{50}$ data were tested for normal distribution and equality of variances. Significant differences ($p < .05$) between treatments were assessed using a two-way or three-way analysis of variance (ANOVA), with subsequent pairwise multiple comparisons using the Holm–Sidak method when the data showed a normal distribution. When normal distribution was not given, a Kruskal–Wallis one-way ANOVA on ranks was used.

3 | RESULTS

3.1 | In vitro assessment of the susceptibility of trout populations to *Y. ruckeri* infection under alternative and standard feeds

After infecting primary gill cultures of rainbow trout from different populations and feeding regimes and incubation for 1 day, a load of *Y. ruckeri* bacteria was determined in the cell culture medium of between 2.0×10^7 and 6.0×10^7 cfu mL^{-1}. Statistical analysis revealed a significant difference in bacterial load in cultures from different genotypes ($p = .031$), with a significantly lower bacterial content in gill cultures of the R8 population (Table 2). Additionally, in fish from the R8 population fed with *Arthrospira*, the bacterial content was even lower. However, on the basis of feed ($p = .333$) or regarding an interaction between population and feed ($p = .72$), statistically significant differences could not be discerned (Figure 2).

3.2 | Bactericidal effect of mucus on *Y. ruckeri* and *E. coli*

A different bactericidal effect of mucus from rainbow trout from different genotypes or different feeds was tested by incubating *Y. ruckeri* and *E. coli* with mucus samples. In negative control samples, incubated without mucus, a bacterial load was determined in cell culture medium for *Y. ruckeri* and *E. coli* of 6.73×10^6 cfu mL^{-1} and 1.0×10^3 cfu mL^{-1} respectively. The bacterial counts were significantly decreased when adding mucus (Table 2). However, no statistical significant difference was observed between the groups, neither regarding genotype nor feed (line: *Y. ruckeri*: $p = .387$; *E. coli*: $p = .072$; feed: *Y. ruckeri* $p = .369$; *E. coli*: $p = .347$) (Figure 3).

3.3 | In vitro infections with VHSV

3.3.1 | In vitro assessment of VHSV susceptibility of parental specimen from R1 to R8 populations under standard feed

In the culture medium of fin cultures from broodstock rainbow trout, a VHSV concentration of 3.2×10^2 $TCID_{50}$ mL^{-1} was determined on the day of infection. After 3 days of cultivation, the

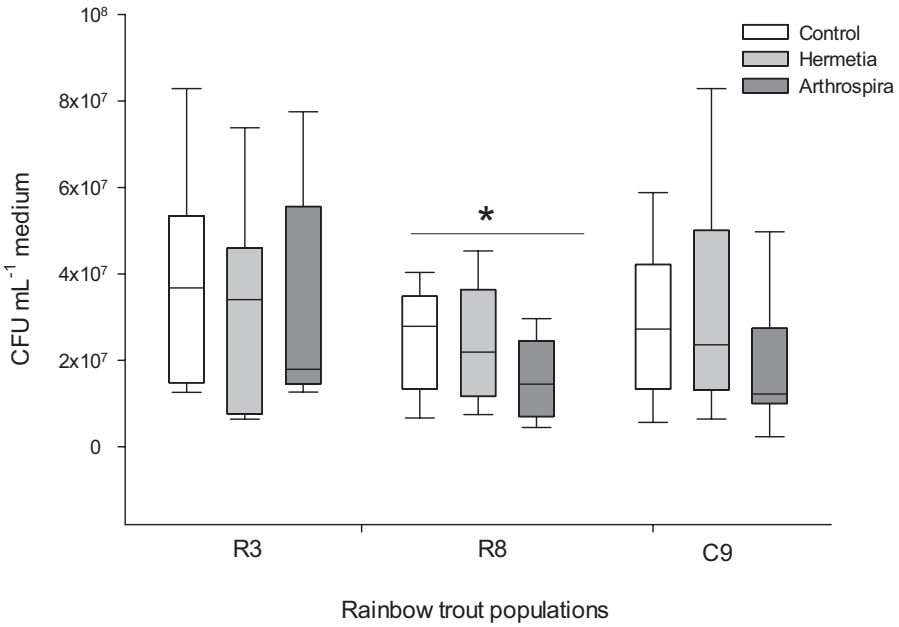

FIGURE 2 In vitro assessment of the susceptibility of rainbow trout from different populations and under alternative feeds to *Yersinia ruckeri*. Depicted is the bacterial load in cell culture media of primary gill cultures from rainbow trout of the R3 and R8 populations and the commercial strain C9, 24 h post infection with *Y. ruckeri*. Shown are means and standard deviations of cfu mL^{-1} from $n = 9$ cultures for each genotype and feed. White: control groups, feed with fishmeal; light grey: fishmeal in feed replaced by proteins from *Hermetia illucens*; dark grey: fishmeal in feed replaced by proteins from *Arthrospira platensis*.

virus load in individual cultures ranged between 1.0×10^3 $TCID_{50}$ mL^{-1} and 3.16×10^6 $TCID_{50}$ mL^{-1}. When the mean value of cultures from different populations was considered, the value from population R4 appeared higher, but this was mainly caused by a high variation in this group and was therefore not statistically significant (Table 2). Overall, no statistically significant different values for virus load were measured between populations ($p = .095$) (Figure 4).

3.3.2 | In vitro assessment of the VHSV susceptibility of different rainbow trout populations after feeding with alternative and standard feeds

After in vitro infection of fin explant cultures of rainbow trout from the R3 and R8 populations and commercial strain C9 with VHSV and 3 days of cultivation, virus loads between 3.16×10^3 and 1.0×10^6 $TCID_{50}$ mL^{-1} were determined in the culture medium. The virus load in individual cultures differed widely. Therefore, the statistical analysis revealed a high standard deviation for cultures from individual genotypes and feeds. Thus, no statistically significant differences were found between feeding groups ($p = .569$; feed: $p = .249$) and genotypes ($p = .089$, Figure 5, Table 2).

In primary cell cultures from scales, the virus load ranged between 1.0×10^5 and 1.0×10^7 $TCID_{50}$ mL^{-1} after 2 days of cultivation. Although mean values of the virus load in cultures from the R8 population were lower than in cultures from the R3 population and commercial strain C9, the variance between the measured values was high, so that no statistically significant differences were discernible between genotypes ($p = .223$) or feeding groups ($p = .639$, population × feed: $p = .979$; Figure 6, Table 2).

3.3.3 | Virucidal effect of mucus from different rainbow trout populations and diets with alternative proteins

In a suspension of tissue culture-derived VHSV incubated with TBS as negative control, a high virus load was determined in the cell culture medium of 3.16×10^6 $TCID_{50}$ mL^{-1} (Figure 7, left column). When the VHSV suspension was incubated in samples from skin mucus from rainbow trout, the tissue culture-infective virus load was significantly decreased. However, a statistically significant difference could not be seen between groups, neither regarding population ($p = .868$) nor feed ($p = .582$) (Figure 7, Table 2).

3.4 | Experimental infection of rainbow trout with VHSV

3.4.1 | Approximated mortality of different rainbow trout populations fed experimental diets exposed to VHSV

The approximated mortality (indicating the number dead and severely diseased fish, which were killed before the death) during a

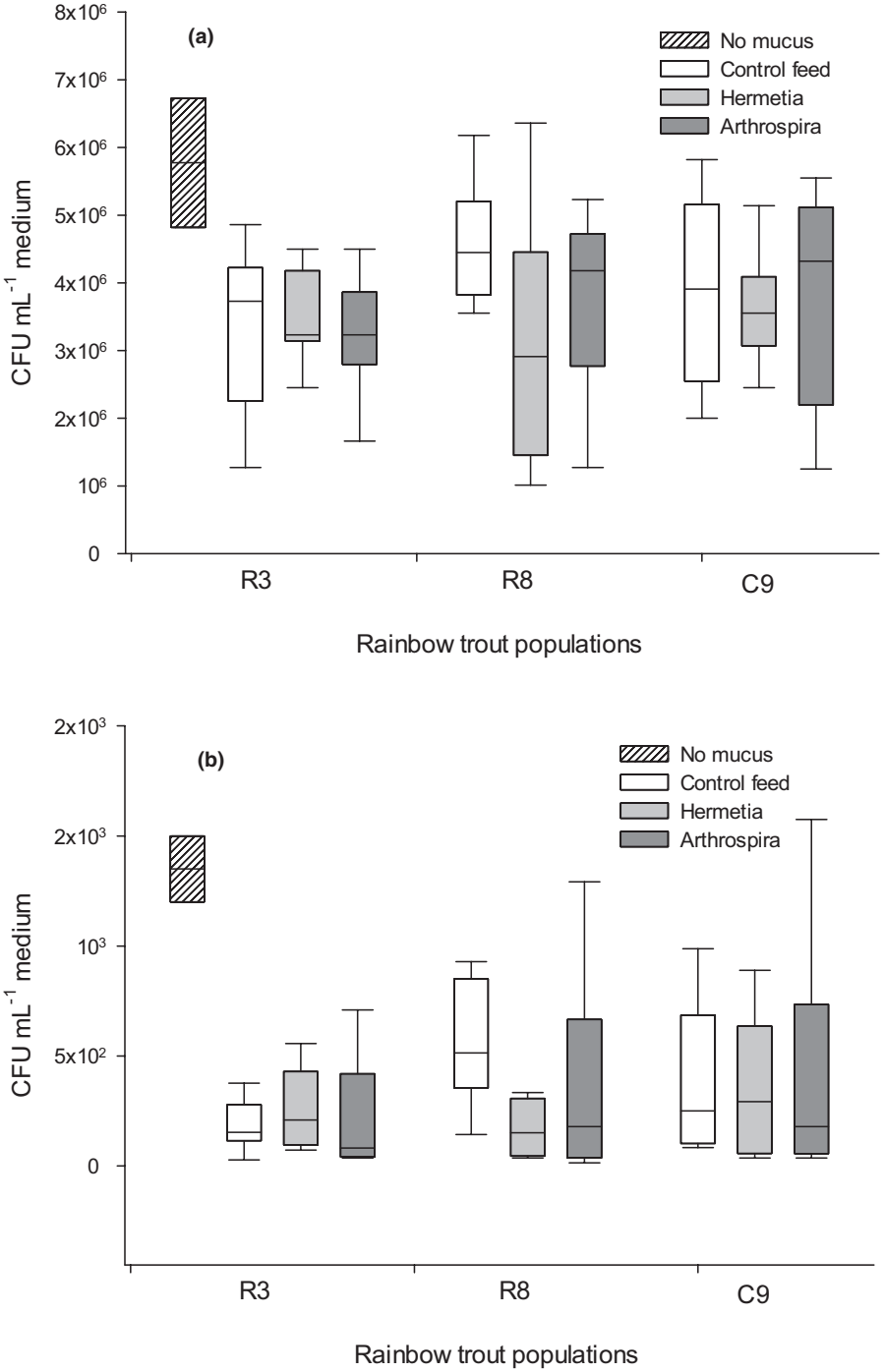

FIGURE 3 Bactericidal effect of mucus from different trout genotypes under alternative feeds against *Y. ruckeri* (a) and *E. coli* (b) 1 day post infection. Shown are means and standard deviations of cfu mL^{-1} in $n = 9$ cultures for each genotype and feed. White: control groups, feed with fishmeal; light grey: fishmeal in feed replaced by proteins from *Hermetia illucens*; dark grey: fishmeal in feed replaced by proteins from *Arthrospira platensis*.

challenge infection with VHSV differed significantly between rainbow trout from different populations. Rainbow trout from the commercial strain C9 and the R3 population suffered highest mortality rates with 85% and 59%, respectively, compared to the rainbow trout R8 population, which showed a mortality of only 15.8% (Figure 8). Statistical analysis revealed no influence of the different diets on the resistance against VHSV ($p = .297$), whereas on the basis of the population genotype, a significant difference could be seen regarding commercial strain C9 and the R3 population compared to the R8 population ($p = <.001$, Table 2). However, there was no statistically significant difference between the R3 population and commercial strain C9 ($p = .057$, Figure 8).

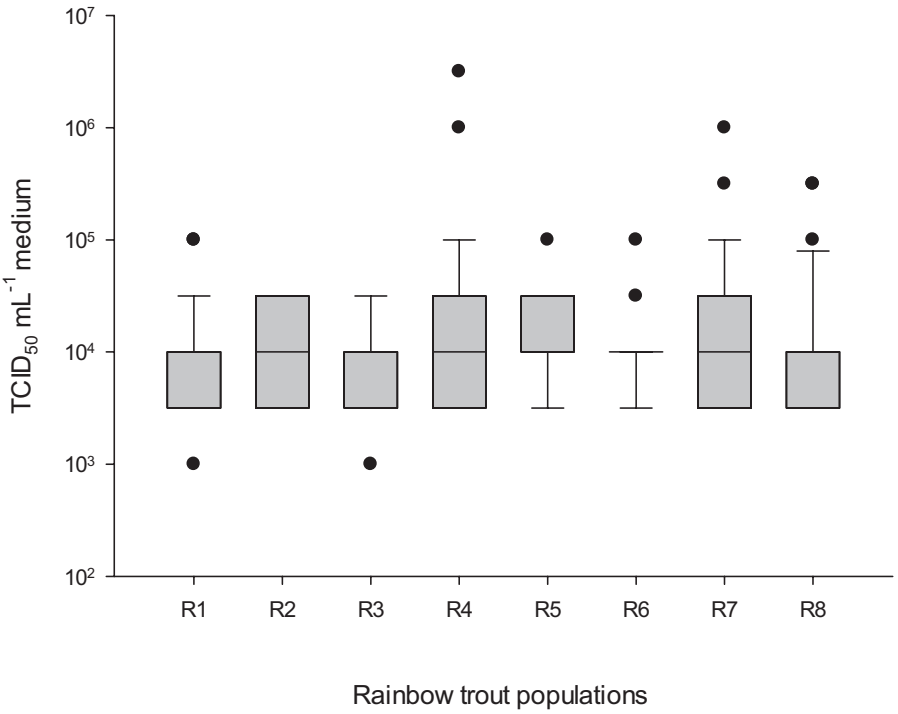

FIGURE 4 In vitro assessment of VHSV susceptibility of rainbow trout from different populations. Depicted is the virus load in $TCID_{50}$ mL^{-1} (means and standard deviations) in fin cultures of eight trout populations, R1–R8; $n = 16$ independent cultures for each population. Individual black dots are indicating outliers.

3.4.2 | Virus load in fin samples from rainbow trout under experimental infection with VHSV

The virus load in fin tissue of rainbow trout measured 2 and 4 days post experimental infection with VHSV differed significantly in samples from different populations (Figure 9). In detail, a significant difference was observed in all feeding groups between fin samples from the R3 and R8 populations ($p < .001$) as well as from the commercial strain C9 compared to R8 ($p < .001$) without considering the feed. At day 2, a significant difference was observed in all feeding groups between the R3 and R8 populations without considering the feed ($p = .006$) and between the commercial strain C9 compared to R8 ($p = .002$). At day 4, a significant difference was observed in all feeding groups between fin samples from the commercial strain C9 and the R8 population ($p = <.001$). No significant difference was observed between sampling days (overall: $p = .233$; R3: $p = .683$; R8: $p = .338$; C9 $p = .448$).

A comparison of samples from rainbow trout fed the control feed showed significantly lower virus loads in fin samples from R8 population when compared to samples from the commercial strain C9 ($p < .001$) when all sampling days were considered. At day 2, a significant difference was observed among samples from the control groups of the R3 and R8 populations ($p = .038$) as well as between the commercial strain C9 and the R8 population ($p = .033$), whereas at day 4, a significant difference was only observed between samples from the commercial strain C9 and the R8 population ($p = .006$). In contrast to this, there were no significant differences in the virus load in fin samples collected from individual populations/strains regarding the sampling day (overall: $p = .535$; R3: $p = .997$; R8: $p = .883$; C9: $p = .355$).

When the different diets were considered, lowest virus loads were found in *Arthrospira*-fed groups, whereas highest values were found in groups fed the control diet. Hence, across all populations/strains, a significant difference was observed between control and *Arthrospira*-fed groups ($p < .001$). A significant difference was particularly observed between control and *Arthrospira*-fed rainbow trout from the R3 population ($p = .042$). In addition, a significantly different virus load was detected between trout fed the control diet ($p = .015$) and the diet supplemented by *Hermetia* proteins ($p = .007$) from the R3 and R8 populations.

4 | DISCUSSION

Due to the persisting challenges in capture fisheries and sustainable management of many marine fish stocks, the constant development towards a more sustainable production of fish in aquaculture is necessary. For this, a partial or complete substitution of fishmeal in standard fish feed formulations will be needed (Luthada-Raswiswi et al., 2021). This study was designed to evaluate the influence of the ability of rainbow trout to adapt to fishmeal substitutes *A. platensis* and *H. illucens* in the diet on the disease susceptibility using mostly in vitro methods.

Although the complex interaction between pathogens with its host can best be studied using in vivo infection experiments, the in vitro methods are constantly developed and improved and can

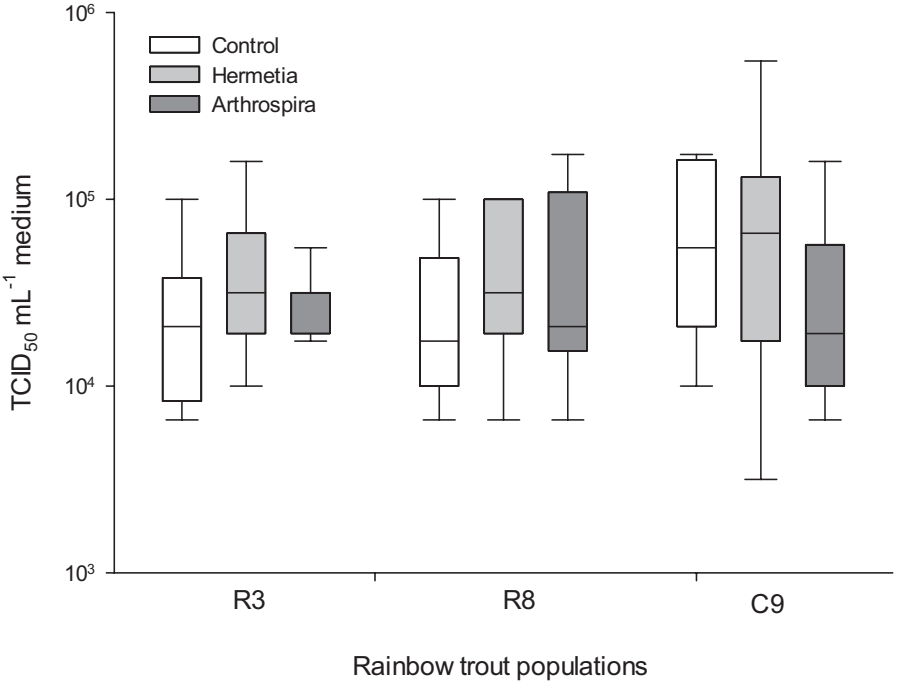

FIGURE 5 In vitro assessment of VHSV susceptibility of different rainbow trout populations after feeding proteins from alternative sources. Depicted is virus load in cell culture media in $TCID_{50}$ mL^{-1} in fin cultures of rainbow trout from the R3 and R8 populations and the commercial strain C9, 3 days post infection; $n = 9$ cultures for each genotype and feed. White: control groups, feed with fishmeal; light grey: fishmeal in feed replaced by proteins from *Hermetia illucens*; dark grey: fishmeal in feed replaced by proteins from *Arthrospira platensis*.

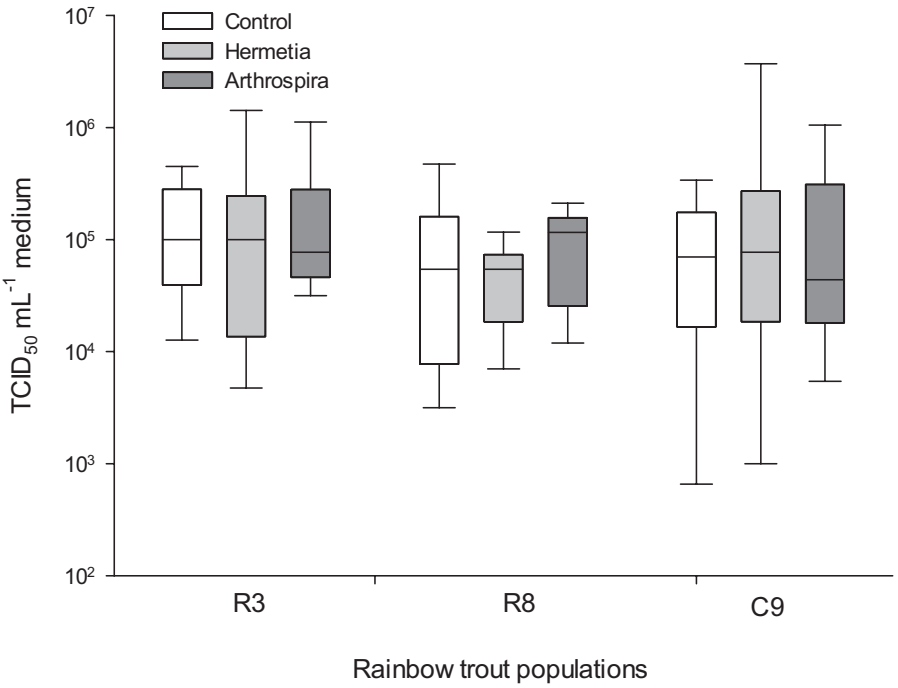

FIGURE 6 Virus loads in cell culture media of primary scale cultures monolayers, 2 days post infection with VHSV. Shown are means and standard deviations of virus load in $TCID_{50}$ mL^{-1} in scale cultures from the R3 and R8 rainbow trout populations and commercial strain C9. $n = 9$ for cultures for each genotype and feed. White: control groups, feed with fishmeal; light grey: fishmeal in feed replaced by proteins from *Hermetia illucens*; dark grey: fishmeal in feed replaced by proteins from *Arthrospira platensis*.

support the findings from animal experimentations. Previous studies using in vivo experiments showed that the susceptibility to VHSV infection differed significantly between rainbow trout lines (Quillet et al., 2001) and resistant fish harboured significantly lower virus loads in internal organs and also in fin tissue (Dorson et al., 1994). Our studies also revealed differences in VHS-resistance between

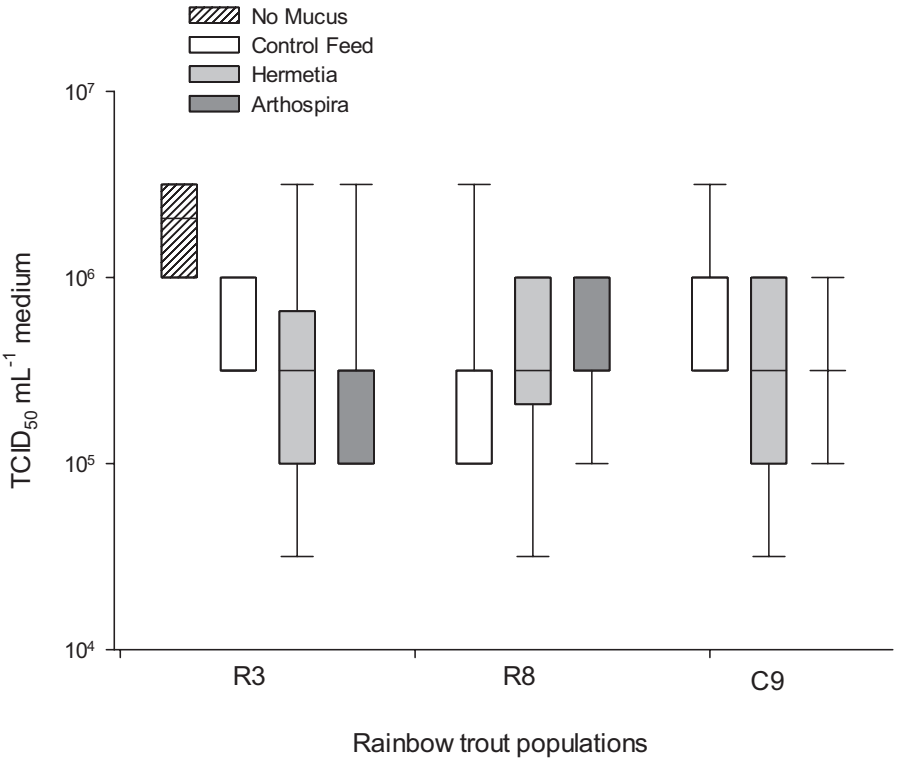

FIGURE 7 Virucidal effect of skin mucus of rainbow trout from different populations fed alternative feeds. Depicted is the virus load after overnight incubation of VHSV suspensions with mucus samples from the rainbow trout R3 and R8 populations and commercial strain C9. Shown are means and standard deviations of tissue culture-infective virus content $TCID_{50}$ mL^{-1}; in $n = 9$ samples after incubation with mucus from each population and feed. White: control groups, feed with fishmeal; light grey: fishmeal in feed replaced by proteins from *Hermetia illucens*; dark grey: fishmeal in feed replaced by proteins from *Arthrospira platensis*.

the studies populations. Rainbow trout from the population R3 and the commercial strain C9 under infection with VHSV suffered mortality rates from 59% to 85%, with a virus load of about 1×10^7 normalized copies, while rainbow trout from the R8 population experienced a mortality of only 15.8% with a virus load of about 1×10^5 genome copies. However, in vivo infection experiments require large numbers of fish because the host responses may widely vary between individuals. In order to reduce the number of fish used in such experiments, which might also be exposed to considerable suffering, the use of in vitro studies should be explored. In an earlier study on the susceptibility of different rainbow trout genotypes to VHSV, virus replication in excised fin tissue (VREFT) was correlated to the survival of the trout from experimental infection (Quillet et al., 2001, 2007). In these studies, a lower virus load was measured in VREFT-cultures from rainbow trout with a lower susceptibility of VHSV infection (Quillet et al., 2007). Furthermore, it was previously found that size and weight of individual fish had no influence on virus replication in trout of the same age (Quillet et al., 2001). Thus, a comparison of virus replication in trout of the same age but with differences in growth performance should not have an influence on the results of the current in vitro studies.

While VREFT cultures of parental specimens from eight trout populations as well as from offspring derived from the R3 and R8 populations and individuals from commercial line C9 were not significantly different, the analysis of VHSV replication in primary cell cultures from scales harboured lower virus loads in media of cultures with cells from R8 specimens when compared to cultures from R3 and C9 specimens. It has to be noted, that specimens from both C9 and R8 populations with a large difference in VHS-resistance had a high ability to utilize the alternative diets. In addition, in gill cultures from the R8 population, with a good growth performance on alternative diets, the load of *Y. ruckeri* was significantly lower compared to cultures from R3 and C9. Due to the high individual variation, the difference in VHSV load was not statistically significant, but the in vitro virus loads in media of primary cell cultures from scales correlated with the virus load measured in vivo in fin tissue of rainbow trout from these lines after experimental infection with VHSV, whereas in vitro virus loads in VREFT cultures did not. However, this data shows, that the ability of rainbow trout populations to utilize the fish meal substitute *Arthrospira* or *Hermetia* in the diet was not correlated with susceptibility to VHSV or *Y. ruckeri* infection.

The experimental VHSV infection of rainbow trout from the R3 and R8 populations and the commercial strain C9 revealed a significant influence of the population genotype on disease susceptibility but not regarding the feed/diet. The disease development and approximated mortality rates were highest in the commercial strain C9 and the R3 population and lowest in the R8 population. The virus load in fin tissue correlated with the approximated mortality. While in fin tissue of the commercial strain C9 and the R3

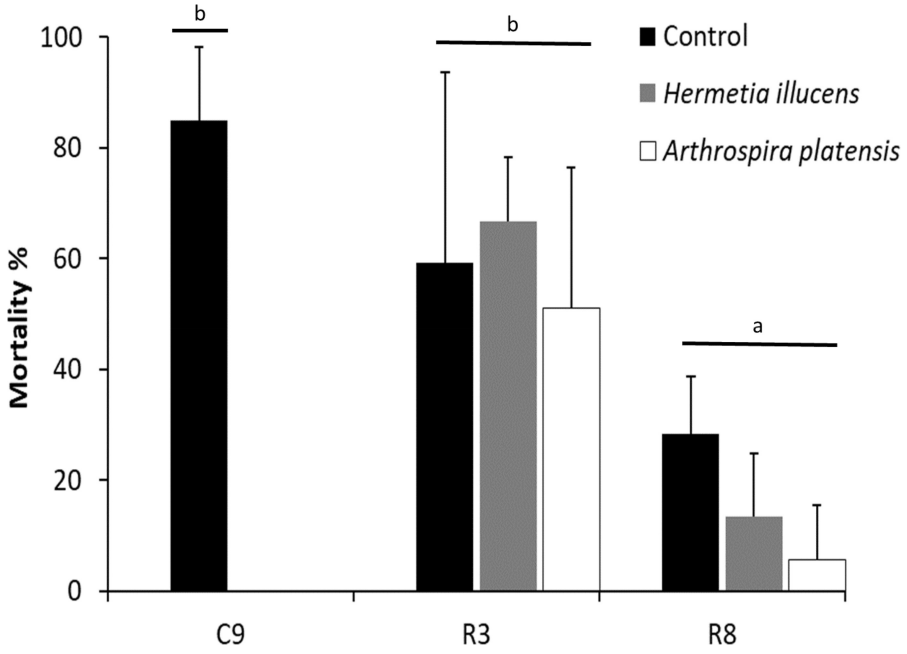

FIGURE 8 Approximated cumulative mortality in rainbow trout from different populations fed different diets during a 29-day period after experimental infection with VHSV. Shown are mean and standard deviation of the approximated cumulative mortality in $n = 3$ groups of rainbow trout from the R3 and R8 populations under alternative feeds and commercial strain C9 under standard feed. Black: control groups, feed with fishmeal; grey: fishmeal in feed replaced by proteins from *Hermetia illucens*; white: fishmeal in feed replaced by proteins from *Arthrospira platensis*. Letters 'a' and 'b' indicate statistically significant difference between the groups at $p < .05$.

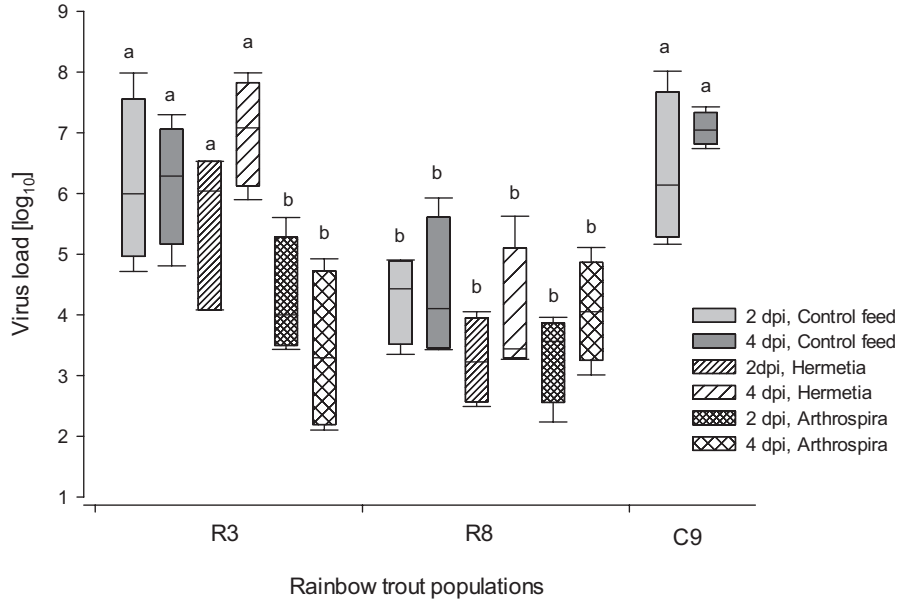

FIGURE 9 Virus load in fin tissue of rainbow trout experimentally infected with VHSV (mean and standard deviation) from R3 and R8 populations R3 and the commercial strain C9 at day 2 and 4 post infection (d.p.i.). Letters 'a' and 'b' indicate statistically significant difference between the groups at $p < .05$.

population, high virus loads were found, the virus load was significantly lower in R8. This suggests that the difference of rainbow trout populations regarding the susceptibility to VHSV can already be seen when mucosal immune responses are active and the amount of virus infiltrating fin tissue might be lower than in more susceptible genotypes. It was previously shown that the first virus replication takes place in dermal cells, mainly at the fin bases (Harmache et al., 2006; Montero et al., 2011), whereas epidermal cells were shown to block the replication of the virus by mucosal immune responses. In addition, the importance of skin mucus in the defence barrier of fish against external pathogens has been emphasized previously (Cabillon & Lazado, 2019; Reverter et al., 2018; Tiralongo et al., 2020). Fish diet was identified as a main driver for metabolic differences in gill mucus (Reverter et al., 2017). The fatty acid composition of skin mucus changed in gilthead sea bream (*Sparus aurata*) fed high or low fishmeal diets, with a higher content of polar lipids in the skin mucus of fish fed the high fishmeal diet (Torrecillas et al., 2019). The higher content of polar lipids was considered to increase mucus viscosity and rigidity and this might influence its protective role in a parasitic environment (Rahman et al., 2012). With the replacement of fishmeal proteins by plant or insect resources, the composition of

metabolites in skin mucus changed (Ekman et al., 2015; Roques et al., 2020), which is suspected to influence the protective role of mucus as well (Ekman et al., 2015; Roques et al., 2020). There are several reports on antimicrobial activity of skin mucus in fish (Palaksha et al., 2008; Subramanian et al., 2008) as well as an activity against viral infection (Raj et al., 2011). In this study, the incubation of *VHSV*, *Y. ruckeri* or *E. coli* with skin mucus preparations from rainbow trout clearly revealed protective effects of the skin mucus from rainbow trout. However, in this study, an influence of trout genotype or diet composition could not be detected which suggest that the replacement of fishmeal did not have an adverse effect of the resistance of rainbow trout to both pathogens. This was further supported by the results from in vitro cell cultures, which showed no dietary influence on the development of VHSV or *Y. ruckeri* load in fin, scale or gill cultures. In the in vivo experiment, however, some influence of the replacement of fishmeal proteins by algal or insect proteins on the susceptibility of rainbow trout from different populations to a VHSV infection could be discerned. In this experiment, spirulina feeding was associated with lower virus load in fins in the susceptible population of rainbow trout (R3) but this was not reflected in the final outcome of the infection and mortality. This putative beneficial effect of spirulina is in line with results of others where the inclusion of spirulina in the diet was shown to spur the immune defence of common carp to bacterial pathogens (Watanuki et al., 2006) and increase the antioxidant capacity of rainbow trout (Teimouri et al., 2019). However, this effect seems not to be strong enough to fully protect from a highly virulent VHSV.

5 | CONCLUSION

This study aimed at monitoring the influence of the ability of rainbow trout populations to utilize fishmeal substitutes on disease resistance of rainbow trout by mainly in vitro methods. In primary cell cultures of skin cells from scales of rainbow trout from a population with low susceptibility to VHSV, this virus replicated at a lower rate than in cell cultures from trout from susceptible populations. Likewise, in gill explant cultures of rainbow trout from the same population, the bacterial pathogen *Y. ruckeri* multiplied to a lesser extent than in gill cultures from the other studied populations supporting the hypothesis of mostly genetic factors influencing the resistance. Important however, all applied in vivo and in vitro assays gave no indication of a negative impact of the inclusion of the fishmeal substitutes *H. illucens* and *A. platensis* in the diet of rainbow trout on the susceptibility of rainbow trout to VHSV and *Y. ruckeri*. There was also no correlation between the ability of rainbow trout populations to adapt to a diet with proteins of insects or spirulina and pathogen resistance. This implicates that *H. illucens* and *A. platensis* might be valuable protein sources for reducing the use of fishmeal in trout feed without jeopardizing immunity and natural resistance of certain rainbow trout lines to bacterial and viral pathogens.

AUTHOR CONTRIBUTIONS

Mikolaj Adamek: Writing – original draft; investigation; conceptualization; methodology; supervision. **Julia Bauer:** Conceptualization; methodology; data curation; investigation; writing – original draft. **Anne-Carina Miebach:** Investigation. **Jakob Gährken:** Investigation; resources. **Stephan Wessels:** Conceptualization; investigation; resources; project administration. **Jens Tetens:** Formal analysis; resources. **Carsten Dietz:** Conceptualization; investigation. **Angela Sünder:** Conceptualization; investigation. **Marek Matras:** Investigation; validation; formal analysis; methodology. **Magdalena Stachnik:** Investigation; validation; formal analysis; methodology. **Michal Reichert:** Investigation; validation; formal analysis. **Dieter Steinhagen:** Funding acquisition; validation; visualization; project administration; writing – original draft; supervision; conceptualization; data curation.

ACKNOWLEDGEMENTS

This project was coordinated by the University of Goettingen, Germany and funded by the Ministry for Science and Culture in Lower Saxony, Hannover, Germany (Niedersächsisches Ministerium für Wissenschaft und Kultur). Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Mikolaj Adamek https://orcid.org/0000-0003-4890-3164
Marek Matras https://orcid.org/0000-0002-9075-4062

REFERENCES

Baeverfjord, G., & Krogdahl, A. (1996). Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L., distal intestine: A comparison with the intestines of fasted fish. *Journal of Fish Diseases*, *19*(5), 375–387. https://doi.org/10.1046/j.1365-2761.1996.d01-92.x

Bakke-McKellep, A. M., Press, C. M., Baeverfjord, G., Krogdahl, Å., & Landsverk, T. (2000). Changes in immune and enzyme histochemical phenotypes of cells in the intestinal mucosa of Atlantic salmon, *Salmo salar* L., with soybean meal-induced enteritis. *Journal of Fish Diseases*, *23*(2), 115–127. https://doi.org/10.1046/j.1365-2761.2000.00218.x

Barnes, A. (2011). Enteric Redmouth Disease (ERM) (*Yersinia ruckeri*). In P. T. K. Woo & D. W. Bruno (Eds.), *Fish diseases and disorders: Viral, bacterial and fungal infections* (pp. 484–511). CABI Publishing.

Bearzotti, M., Delmas, B., Lamoureux, A., Loustau, A. M., Chilmonczyk, S., & Bremont, M. (1999). Fish rhabdovirus cell entry is mediated by fibronectin. *Journal of Virology*, *73*(9), 7703–7709. https://doi.org/10.1128/jvi.73.9.7703-7709.1999

Brudeseth, B. E., Castric, J., & Evensen, Ø. (2002). Studies on pathogenesis following single and double infection with viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus in rainbow trout (*Oncorhynchus mykiss*). *Veterinary Pathology*, *39*(2), 180–189. https://doi.org/10.1354/vp.39-2-180

Burrells, C., Williams, P. D., Southgate, P. J., & Crampton, V. O. (1999). Immunological, physiological and pathological responses of rainbow trout (*Oncorhynchus mykiss*) to increasing dietary concentrations of soybean proteins. *Veterinary Immunology and Immunopathology*, *72*(3), 277–288. https://doi.org/10.1016/S0165-2427(99)00143-9

Cabillon, N. A. R., & Lazado, C. C. (2019). Mucosal barrier functions of fish under changing environmental conditions. *Fishes*, *4*(1), 2. https://doi.org/10.3390/fishes4010002

Dietrich, M. A., Nynca, J., Adamek, M., Steinhagen, D., Karol, H., & Ciereszko, A. (2015). Expression of apolipoprotein A-I and A-II in rainbow trout reproductive tract and their possible role in antibacterial defence. *Fish & Shellfish Immunology*, *45*(2), 750–756. https://doi.org/10.1016/j.fsi.2015.05.048

Dietz, Sünder, A., & Liebert, F. (2020). "Does genetic background of rainbow trout impact on growth and feed utilization following fshmeal substitution by partly defatted insect meal (*Hermetia illucens*) or microalgae powder (*Arthrospira platensis*)?". *Proceedings of the Society of Nutrition Physi-ologyvol. 29*, (p. 38). DLG-Verlag GmbH.

Dietz, C., Wessels, S., Sünder, A., Sharifi, R., Gährken, J., & Liebert, F. (2023). Does Genetic Background of Rainbow Trout Impact Growth and Feed Utilisation following Fishmeal Substitution by Partly Defatted Insect Meal (*Hermetia illucens*) or Microalgae Powder (*Arthrospira platensis*)? *Aquaculture Research*, *2023*, 4774048.

Dorson, M., Torhy, C., & de Kinkelin, P. (1994). Viral haemorrhagic septicaemia virus multiplication and interferon production in rainbow trout and in rainbow trout × brook trout hybrids. *Fish & Shellfish Immunology*, *4*(5), 369–381. https://doi.org/10.1006/fsim.1994.1032

Ekman, D. R., Skelton, D. M., Davis, J. M., Villeneuve, D. L., Cavallin, J. E., Schroeder, A., Jensen, K. M., Ankley, G. T., & Collette, T. W. (2015). Metabolite profiling of fish skin mucus: A novel approach for minimally-invasive environmental exposure monitoring and surveillance. *Environmental Science & Technology*, *49*(5), 3091–3100. https://doi.org/10.1021/es505054f

Estepa, A., Frias, D., & Coll, J. (1993). In vitro susceptibility of rainbow trout fin cells to viral haemorrhagic septicaemia virus. *Diseases of Aquatic Organisms*, *15*, 35–39. https://doi.org/10.3354/dao015035

Enzmann, P. J., & Bruchhof, B. (1989). Comparative studies on viral haemorrhagic septicaemia viruses and infectious hematopoietic necrosis virus. An attempt to demonstrate an immunological relationship. In K. Lillelund & H. Rosenthal (Eds.), *Fish health protection stategies* (pp. 107–120). Bundesministerium fiir Forschung und Technologie, Bonn.

Ghosh, K., Ray, A. K., & Ringø, E. (2019). Applications of plant ingredients for tropical and subtropical freshwater finfish: Possibilities and challenges. *Reviews in Aquaculture*, *11*(3), 793–815. https://doi.org/10.1111/raq.12258

Gjedrem, T. (2012). Genetic improvement for the development of efficient global aquaculture: A personal opinion review. *Aquaculture*, *344–349*, 12–22. https://doi.org/10.1016/j.aquaculture.2012.03.003

Harmache, A., LeBerre, M., Droineau, S., Giovannini, M., & Brémont, M. (2006). Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for *Novirhabdovirus*. *Journal of Virology*, *80*(7), 3655–3659. https://doi.org/10.1128/JVI.80.7.3655-3659.2006

Hedrera, M. I., Galdames, J. A., Jimenez-Reyes, M. F., Reyes, A. E., Avendaño-Herrera, R., Romero, J., & Feijóo, C. G. (2013). Soybean meal induces intestinal inflammation in zebrafish larvae. *PLoS One*, *8*(7), e69983. https://doi.org/10.1371/journal.pone.0069983

Hemre, G. I., Amlund, H., Aursand, M., Bakke, A. M., Olsen, R., Ringoe, E., … Torrissen, O. (2018). Criteria for safe use of plant ingredients in diets for aquacultured fish. *European Journal of Nutrition & Food Safety*, *8*, 240–242. https://doi.org/10.9734/EJNFS/2018/43861

Huang, Y., Runge, M., Michael, G. B., Schwarz, S., Jung, A., & Steinhagen, D. (2013). Biochemical and molecular heterogeneity among isolates of Yersinia ruckeri from rainbow trout (Oncorhynchus mykiss, Walbaum) in north west Germany. *BMC veterinary research*, *9*, 215.

Irungu, F. G., Mutungi, C. M., Faraj, A. K., Affognon, H., Tanga, C., Ekesi, S., Nakimbugwe, D., & Fiaboe, K. K. M. (2018). Minerals content of extruded fish feeds containing cricket (*Acheta domesticus*) and black soldier fly larvae (*Hermetia illucens*) fractions. *International Aquatic Research*, *10*(2), 101–113. https://doi.org/10.1007/s40071-018-0191-8

Ju, Z. Y., Deng, D.-F., & Dominy, W. (2012). A defatted microalgae (*Haematococcus pluvialis*) meal as a protein ingredient to partially replace fishmeal in diets of Pacific white shrimp (*Litopenaeus vannamei*, Boone, 1931). *Aquaculture*, *354-355*, 50–55. https://doi.org/10.1016/j.aquaculture.2012.04.028

Khimmakthong, U., Deshmukh, S., Chettri, J. K., Bojesen, A. M., Kania, P. W., Dalsgaard, I., & Buchmann, K. (2013). Tissue specific uptake of inactivated and live *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*): Visualization by immunohistochemistry and in situ hybridization. *Microbial Pathogenesis*, *59–60*, 33–41. https://doi.org/10.1016/j.micpath.2013.03.001

Kim, J. O., Kim, W. S., Kim, S. W., Han, H. J., Kim, J. W., Park, M. A., & Oh, M. J. (2014). Development and application of quantitative detection method for viral hemorrhagic septicemia virus (VHSV) genogroup IVa. *Viruses*, *6*(5), 2204–2213. https://doi.org/10.3390/v6052204

Krogdahl, Å., Bakke-McKellep, A. M., & Baeverfjord, G. (2003). Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar* L.). *Aquaculture Nutrition*, *9*(6), 361–371. https://doi.org/10.1046/j.1365-2095.2003.00264.x

Krogdahl, Å., Gajardo, K., Kortner, T. M., Penn, M., Gu, M., Berge, G. M., & Bakke, A. M. (2015). Soya saponins induce enteritis in Atlantic Salmon (*Salmo salar* L.). *Journal of Agricultural and Food Chemistry*, *63*(15), 3887–3902. https://doi.org/10.1021/jf506242t

le Boucher, R., Dupont-Nivet, M., Vandeputte, M., Kerneïs, T., Goardon, L., Labbé, L., Chatain, B., Bothaire, M. J., Larroquet, L., Médale, F., & Quillet, E. (2012). Selection for adaptation to dietary shifts: Towards sustainable breeding of carnivorous fish. *PLoS One*, *7*(9), e44898. https://doi.org/10.1371/journal.pone.0044898

Lorenzen, E., Carstensen, B., & Olesen, N. J. (1999). Inter-laboratory comparison of cell lines for susceptibility to three viruses: VHSV, IHNV and IPNV. *Diseases of Aquatic Organisms*, *37*(2), 81–88. https://doi.org/10.3354/dao037081

Luthada-Raswiswi, R., Mukaratirwa, S., & O'Brien, G. (2021). Animal protein sources as a substitute for fishmeal in aquaculture diets: A systematic review and meta-analysis. *Applied Sciences*, *11*(9), 3854.

Montero, J., Garcia, J., Ordas, M. C., Casanova, I., Gonzalez, A., Villena, A., Coll, J., & Tafalla, C. (2011). Specific regulation of the chemokine response to viral hemorrhagic septicemia virus at the entry site. *Journal of Virology*, *85*(9), 4046–4056. https://doi.org/10.1128/jvi.02519-10

Palaksha, K. J., Shin, G.-W., Kim, Y.-R., & Jung, T.-S. (2008). Evaluation of non-specific immune components from the skin mucus of olive flounder (*Paralichthys olivaceus*). *Fish & Shellfish Immunology*, *24*(4), 479–488. https://doi.org/10.1016/j.fsi.2008.01.005

Quillet, E., Dorson, M., Aubard, G., & Torhy, C. (2001). In vitro viral haemorrhagic septicaemia virus replication in excised fins of rainbow trout: Correlation with resistance to waterborne challenge and genetic variation. *Diseases of Aquatic Organisms*, *45*(3), 171–182. https://doi.org/10.3354/dao045171

Quillet, E., Dorson, M., Aubard, G., & Torhy, C. (2007). In vitro assay to select rainbow trout with variable resistance/susceptibility to viral haemorrhagic septicaemia virus. *Diseases of Aquatic Organisms*, *76*(1), 7–16. https://doi.org/10.3354/dao076007

Ragaza, J. A., Hossain, M. S., Meiler, K. A., Velasquez, S. F., & Kumar, V. (2020). A review on spirulina: Alternative media for cultivation and nutritive value as an aquafeed. *Reviews in Aquaculture*, *12*(4), 2371–2395. https://doi.org/10.1111/raq.12439

Rahman, M., Arshad, R., Shaharom, F., & Ariffin, N. A. (2012). Amino acid and fatty acid profile in epidermal mucus of bluestreak cleaner wrasse (*Labroides dimidiatus*): Possible role as defense mechanism against pathogens. *Journal of Life Sciences*, *6*, 1371–1377.

Raj, V. S., Fournier, G., Rakus, K., Ronsmans, M., Ouyang, P., Michel, B., Delforges, C., Costes, B., Farnir, F., Leroy, B., Wattiez, R., Melard, C., Mast, J., Lieffrig, F., & Vanderplasschen, A. (2011). Skin mucus of *Cyprinus carpio* inhibits cyprinid herpesvirus 3 binding to epidermal cells. *Veterinary Research*, *42*, 92. https://doi.org/10.1186/1297-9716-42-92

Reverter, M., Sasal, P., Banaigs, B., Lecchini, D., Lecellier, G., & Tapissier-Bontemps, N. (2017). Fish mucus metabolome reveals fish life-history traits. *Coral Reefs*, *36*(2), 463–475. https://doi.org/10.1007/s00338-017-1554-0

Reverter, M., Tapissier-Bontemps, N., Lecchini, D., Banaigs, B., & Sasal, P. (2018). Biological and ecological roles of external fish mucus: A review. *Fishes*, *3*(4), 41.

Roques, S., Deborde, C., Richard, N., Skiba-Cassy, S., Moing, A., & Fauconneau, B. (2020). Metabolomics and fish nutrition: A review in the context of sustainable feed development. *Reviews in Aquaculture*, *12*(1), 261–282. https://doi.org/10.1111/raq.12316

Smail, D. A., & Snow, M. (2011). Viral haemorrhagic septicaemia. In P. T. K. Woo & D. W. Bruno (Eds.), *Fish diseases and disorders: Viral, bacterial and fungal infections* (pp. 110–142). CABI Publishing.

Stadtlander, T., Stamer, A., Buser, A., Wohlfahrt, J., Leiber, F., & Sandrock, C. (2017). *Hermetia illucens* meal as fish meal replacement for rainbow trout on farm. *Journal of Insects as Food and Feed*, *3*, 165–175. https://doi.org/10.3920/JIFF2016.0056

Subramanian, S., Ross, N. W., & MacKinnon, S. L. (2008). Comparison of antimicrobial activity in the epidermal mucus extracts of fish. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *150*(1), 85–92. https://doi.org/10.1016/j.cbpb.2008.01.011

Tacchi, L., Secombes, C. J., Bickerdike, R., Adler, M. A., Venegas, C., Takle, H., & Martin, S. A. M. (2012). Transcriptomic and physiological responses to fishmeal substitution with plant proteins in formulated feed in farmed Atlantic salmon (*Salmo salar*). *BMC Genomics*, *13*(1), 363. https://doi.org/10.1186/1471-2164-13-363

Teimouri, M., Amirkolaie, A. K., & Yeganeh, S. (2013). The effects of *Spirulina platensis* meal as a feed supplement on growth performance and pigmentation of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *396-399*, 14–19. https://doi.org/10.1016/j.aquaculture.2013.02.009

Teimouri, M., Yeganeh, S., Mianji, G. R., Najafi, M., & Mahjoub, S. (2019). The effect of *Spirulina platensis* meal on antioxidant gene expression, total antioxidant capacity, and lipid peroxidation of rainbow trout (*Oncorhynchus mykiss*). *Fish Physiology and Biochemistry*, *45*(3), 977–986. https://doi.org/10.1007/s10695-019-0608-3

Tiralongo, F., Messina, G., Lombardo, B., Longhitano, L., Li Volti, G., & Tibullo, D. (2020). Skin mucus of marine fish as a source for the development of antimicrobial agents. *Frontiers in Marine Science*, *7*, 541853. https://doi.org/10.3389/fmars.2020.541853

Tobback, E., Hermans, K., Decostere, A., Van den Broeck, W., Haesebrouck, F., & Chiers, K. (2010). Interactions of virulent and avirulent *Yersinia ruckeri* strains with isolated gill arches and intestinal explants of rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms*, *90*(3), 175–179. https://doi.org/10.3354/dao02230

Torrecillas, S., Montero, D., Domínguez, D., Robaina, L., & Izquierdo, M. (2019). Skin mucus fatty acid composition of Gilthead Sea bream (*Sparus aurata*): A descriptive study in fish fed low and high fish meal diets. *Fishes*, *4*(1), 15.

Uran, P. A., Aydin, R., Schrama, J. W., Verreth, J. A. J., & Rombout, J. H. W. M. (2008). Soybean meal-induced uptake block in Atlantic salmon *Salmo salar* distal enterocytes. *Journal of Fish Biology*, *73*(10), 2571–2579. https://doi.org/10.1111/j.1095-8649.2008.02091.x

Vielma, J., Mäkinen, T., Ekholm, P., & Koskela, J. (2000). Influence of dietary soy and phytase levels on performance and body composition of large rainbow trout (*Oncorhynchus mykiss*) and algal availability of phosphorus load. *Aquaculture*, *183*(3), 349–362. https://doi.org/10.1016/S0044-8486(99)00299-9

Watanuki, H., Ota, K., Tassakka, A. R., Kato, T., & Sakai, M. (2006). Immunostimulant effects of dietary *Spirulina platensis* on carp, *Cyprinus carpio*. *Aquaculture*, *258*, 157–163. https://doi.org/10.1016/j.aquaculture.2006.05.003

Zhang, F., Man, Y. B., Mo, W. Y., & Wong, M. H. (2020). Application of spirulina in aquaculture: A review on wastewater treatment and fish growth. *Reviews in Aquaculture*, *12*(2), 582–599. https://doi.org/10.1111/raq.12341

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Bauer, J., Adamek, M., Miebach, A.-C., Gährken, J., Wessels, S., Tetens, J., Dietz, C., Sünder, A., Matras, M., Stachnik, M., Reichert, M., & Steinhagen, D. (2023). In vitro modelling of the influence of alternative feeds (*Hermetia illucens*, *Arthrospira platensis*) on the resistance of different rainbow trout populations (*Oncorhynchus mykiss*) against the viral haemorrhagic septicaemia virus and *Yersinia ruckeri*. *Journal of Fish Diseases*, *00*, 1–15. https://doi.org/10.1111/jfd.13846