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Novel insights into *Tetracapsuloides bryosalmonae* virulence genes during proliferative kidney disease in trout through RNA-seq technology

PhD thesis submitted for the fulfilment of the requirements for the degree of
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Submitted by
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The Myxosporea have become important to individuals working in a variety of biological fields. The pathological species are of interest to the aquaculturist. The two-host life cycle has interested the developmental biologist and evolutionist, and the use of Myxozoa as biological tags has made the parasite valuable to the fisheries biologist.

Mike Moser and Michael L. Kent,

Parasitic Protozoa (Second Edition), Volume 8, 1994

Table of Contents

Abbreviations

PKD: Proliferative kidney disease

wpe: Weeks post exposure

ELISA: Enzyme-linked immunosorbent assay

FACS: Fluorescence activated cell sorting

MACS: Magnetic-activated cell sorting

DETs: Differentially expressed transcripts

Publications included in the thesis

1. **Development of fish parasite vaccines in the OMICs Era: Progress and opportunities**
   Saloni Shivam, Mansour El-Matbouli and Gokhlesh Kumar
   Vaccines (Basel), 2021, 9(2): 179.
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2. **Kinetics of Parasite-Specific Antibody and B-Cell-Associated Gene Expression in Brown Trout, *Salmo trutta* during Proliferative Kidney Disease**
   Saloni Shivam, Mansour El-Matbouli and Gokhlesh Kumar
   Biology, 2021, 10(12): 1244.
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3. **Identification of *in vivo* induced antigens of the malacosporean parasite *Tetracapsuloides bryosalmonae* (Cnidaria) using *in vivo* induced antigen technology**
   Gokhlesh Kumar, Arun Sudhagar, Saloni Shivam, Frank Nilsen, Jerri L. Bartholomew and Mansour El-Matbouli
   Frontiers in Cellular and Infection Microbiology, 2022, 12: 1032347.
   Impact factor of the journal (2023): 5.7

4. **Differentially expressed transcripts of *Tetracapsuloides bryosalmonae* (Cnidaria) between carrier and dead-end hosts involved in key biological processes: novel insights from a coupled approach of FACS and RNA sequencing**
   Saloni Shivam, Reinhard Ertl, Mansour El-Matbouli and Gokhlesh Kumar
   Veterinary Research, 2023, 54(1): 51.
   Impact factor of the journal (2023): 4.3

Abstract publications

1. **Expression profile of B cell associated genes in brown trout, *Salmo trutta* during proliferative kidney disease (PKD)**
   Saloni Shivam, Mansour El-Matbouli and Gokhlesh Kumar
   20th International Conference on Diseases of Fish and Shellfish, 20th to 23rd September 2021, Virtual.

2. **Identification of *vivo*-induced antigens recognised by sera of infected brown trout with *Tetracapsuloides bryosalmonae***
   Gokhlesh Kumar, Saloni Shivam and Mansour El-Matbouli
   20th International Conference on Diseases of Fish and Shellfish, 20th to 23rd September 2021, Virtual.

3. **Parasite-specific antibody response in brown trout, *Salmo trutta* during proliferative kidney disease**
   Saloni Shivam, Mansour El-Matbouli and Gokhlesh Kumar
   21st Fish Immunology/Vaccination workshop, 24th to 28th April 2022, Wageningen University and Research, Wageningen, The Netherlands.

4. **Survival strategy of *Tetracapsuloides bryosalmonae*: Adapting gene expression to fish host environment?**
   Saloni Shivam, Reinhard Ertl, Veronika Sexl, Mansour El-Matbouli1 and Gokhlesh Kumar
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Saloni Shivam
October 2023

Declaration

I hereby declare that I have written this PhD thesis entitled “Novel insights into *Tetracapsuloides bryosalmonae* virulence genes during proliferative kidney disease in trout through RNA-seq technology” independently, and that I have followed the rules of good scientific practice to the best of my knowledge.

Saloni Shivam.
October 2023

Summary

Tetracapsuloides bryosalmonae is a malacosporean endoparasite that causes proliferative kidney disease (PKD) in salmonid fishes. This parasite poses a significant threat to both farmed and wild salmonid population in Europe and North America. The life-cycle of this parasite is intricately woven between invertebrate bryozoans and vertebrate fish hosts. Among the fish hosts, brown trout is known to acquire chronic infections while rainbow trout emerges as a dead-end host. A holistic understanding of both the host and the pathogen is the key to effective management strategies including vaccine development. Brown trout, serving as a host where *T. bryosalmonae* can successfully complete its life cycle, was our first point of investigation. We conducted a study to unravel the kinetics of the parasite-specific host's antibody response. In a controlled laboratory setting, brown trout were exposed to the spores of *T. bryosalmonae*. Blood, kidney and spleen were sampled from exposed and unexposed control groups at 2, 4, 6, 8-, 10-, 12- and 17-weeks post exposure (wpe). Samples were processed for indirect ELISA, histopathology, and qRT-PCR. Clinical signs of the disease such as renal hyperplasia, splenomegaly, and pale liver were observed in infected fish from 6 to 12 wpe. Our study showed the presence of *T. bryosalmonae*-specific antibodies from 4 wpe, which persisted until 17 wpe. Furthermore, we observed distinct regulation in the expressions of complement component C4A, and many B cell associated genes (CD34, CD79A, BLNK, CD74, BCL7, and CD22) within the critical immune organs, namely the kidney and spleen. To our knowledge, this marks the first investigation into the time-course of anti-*T. bryosalmonae* antibody response in brown trout. These findings offer valuable insights into the mechanisms governing alterations in B cell development, inflammation, and antibody production throughout the progression of PKD in brown trout. In the subsequent study, we investigated which virulence factors of *T. bryosalmonae* are induced during infection or interactions with host cells. By employing *in vivo* induced antigen technology (IVIAT), we identified 136 *in vivo* induced antigens of *T. bryosalmonae*. These antigenic genes, diverse in function, provided us with an invaluable map of the molecular landscape of this parasite during infection. Rainbow trout, in contrast, acts as a dead-end host, where the development of parasite is seemingly halted. This stark contrast prompted a research question: Does *T. bryosalmonae* adapt to different hosts? To this end, we implemented fluorescent activated cell sorting (FACS) for the isolation of parasites from brown

trout and rainbow trout following experimental infection and then subjected the sorted parasite cells to RNA sequencing. This forms the first report of *T. bryosalmonae* isolation from infected kidney of fish host. This pioneering approach unveiled 1120 differentially expressed parasite transcripts between the two hosts, indicating the parasite's differential adaptations within different host species. Our study forms the first comparative transcriptome analysis of *T. bryosalmonae* between carrier and dead-end host. We observed an intriguing contrast: parasites from brown trout exhibited upregulated transcripts linked to cytoskeleton organization, cell polarity, peptidyl-serine phosphorylation while those from rainbow trout exhibited shifts in translation, protein refolding ribonucleoprotein complex biogenesis and subunit organisation, non-membrane bounded organelle assembly, regulation of protein catabolic process. In this study, we have introduced a novel method involving the use of FACS-based isolation to separate *T. bryosalmonae* cells from the infected kidneys of fish. This approach not only advances research in this field but also enables the identification of parasite transcripts that exhibit differential expression between carrier and dead-end fish hosts for the first time. Our study has generated significant knowledge on anti-*T. bryosalmonae* antibody response, *in vivo* induced genes and differentially expressed transcripts of *T. bryosalmonae* between hosts with contrasting fate for the parasite. The information generated from our study opens up avenues for a better understanding of host-*T. bryosalmonae* interactions, development of diagnostic tools, potential candidates for development of therapeutics and vaccines against *T. bryosalmonae* in salmonids.

Zusammenfassung

Tetracapsuloides bryosalmonae ist ein malacosporischer Endoparasit, der bei Salmoniden eine proliferative Nierenerkrankung (PKD) verursacht. Dieser Parasit stellt eine erhebliche Bedrohung sowohl für die Zucht- als auch für die Wildpopulation in Europa und Nordamerika dar. Der Lebenszyklus dieses Myxozoen-Parasiten wechselt zwischen wirbellosen Bryozoen und Salmoniden. Unter den Fischwirten ist bekannt, dass Bachforellen chronische Infektionen bekommen, während Regenbogenforellen sich als Sackgassenwirte erweisen. Ein ganzheitliches Verständnis sowohl des Wirts als auch des Krankheitserregers ist der Schlüssel zu wirksamen Managementstrategien, einschließlich der Entwicklung von Impfstoffen. Unser erster Untersuchungspunkt war die Bachforelle, die als Wirt dient, in dem *T. bryosalmonae* seinen Lebenszyklus erfolgreich abschließen kann. Wir haben eine Studie durchgeführt, um die Kinetik der Antikörperantwort des parasitenspezifischen Wirts zu entschlüsseln. In einer kontrollierten Laborumgebung Bachforellen wurden im Labor gezüchteten *T. bryosalmonae*-Sporen ausgesetzt. Blut-, Nieren- und Milzproben wurden 2, 4, 6, 8, 10, 12 und 17 Wochen nach der Exposition (wpe) von exponierten und nicht exponierten Kontrollgruppen entnommen. Die Proben wurden für indirekten ELISA, Histopathologie und qRT-PCR verarbeitet. Klinische Anzeichen der Krankheit wie Nierenhyperplasie, Splenomegalie und blasse Leber wurden bei infizierten Fischen im Alter von 6 bis 12 Wochen beobachtet. Unsere Studie zeigte das Vorhandensein von *T. bryosalmonae*-spezifischen Antikörpern ab 4 WPE, die bis 17 WPE bestehen blieben. Darüber hinaus beobachteten wir eine deutliche Regulation der Expression der Komplementkomponente C4A und vieler B-Zell-assoziierter Gene (CD34, CD79A, BLNK, CD74, BCL7 und CD22) in den kritischen Immunorganen, nämlich der Niere und der Milz. Unseres Wissens ist dies die erste Untersuchung des zeitlichen Verlaufs von Anti-T. Bryosalmonae-Antikörperreaktion bei Bachforellen. Diese Ergebnisse bieten wertvolle Einblicke in die Mechanismen, die Veränderungen in der B-Zell-Entwicklung, Entzündung und Antikörperproduktion während des Fortschreitens der PKD bei Bachforellen steuern. In der anschließenden Studie untersuchten wir, welche Virulenzfaktoren von *T. bryosalmonae* bei Infektionen oder Interaktionen mit Wirtszellen induziert werden. Mithilfe der In-vivo-induzierten Antigen-Technologie (IVIAT) identifizierten wir 136 immunogene *T. bryosalmonae*-Proteine. Diese Proteine mit unterschiedlichen Funktionen lieferten uns

eine unschätzbar wertvolle Karte der molekularen Landschaft des Parasiten während der Infektion. Im Gegensatz dazu fungiert die Regenbogenforelle als Sackgassenwirt, bei dem die Entwicklung des Parasiten scheinbar zum Stillstand kommt. Dieser starke Kontrast führte zu einer Forschungsfrage: Passt sich *T. bryosalmonae* an verschiedene Wirte an? Zu diesem Zweck, Wir haben die fluoreszierende aktivierte Zellsortierung (FACS) zur Isolierung von Parasiten aus Bachforellen und Regenbogenforellen nach experimenteller Infektion implementiert und die sortierten Parasitenzellen anschließend einer RNA-Sequenzierung unterzogen. Dies ist der erste Bericht über die Isolierung von T. bryosalmonae aus infizierten Nieren eines Fischwirts. Dieser bahnbrechende Ansatz enthüllte 1120 unterschiedlich exprimierte Parasitentranskripte zwischen den beiden Wirten, was auf die unterschiedlichen Anpassungen des Parasiten innerhalb verschiedener Wirtsarten hinweist. Unsere Studie bildet die erste vergleichende Tranaskriptomanalyse von *T. bryosalmonae* zwischen Träger und Dead-End-Wirt. Wir beobachteten einen faszinierenden Kontrast: Parasiten von Bachforellen zeigten hochregulierte Transkripte, die mit der Organisation des Zytoskeletts, der Zellpolarität und Peptidyl-Serin-Phosphorylierung, während diejenigen von Regenbogenforellen Verschiebungen in der Translation, der Biogenese und Organisation des Ribonukleoprotein-Komplexes bei der Proteinrückfaltung, der nicht membrangebundenen Organellenanordnung und der Regulierung des Proteinkatabolisierungsprozesses aufwiesen. In dieser Studie haben wir eine neuartige Methode vorgestellt, die die Verwendung einer FACS-basierten Isolierung zur Trennung von *T. bryosalmonae*-Zellen aus den infizierten Nieren von Fischen beinhaltet. Dieser Ansatz treibt nicht nur die Forschung auf diesem Gebiet voran, sondern ermöglicht erstmals auch die Identifizierung von Parasitentranskripten, die eine unterschiedliche Expression zwischen Träger- und Dead-End-Fischwirten aufweisen. Unsere Studie hat bedeutende Erkenntnisse über Anti-*T. bryosalmonae*-Antikörperantwort, in vivo induzierte Gene und unterschiedlich exprimierte Transkripte von *T. bryosalmonae* zwischen Wirten mit unterschiedlichem Schicksal für den Parasiten. Die aus unserer Studie gewonnenen Informationen eröffnen Möglichkeiten für ein besseres Verständnis von Host-*T. bryosalmonae*-Wechselwirkungen, Entwicklung diagnostischer Instrumente, potenzielle Kandidaten für die Entwicklung von Therapeutika und Impfstoffen gegen *T. bryosalmonae* bei Salmoniden.

1. General introduction

1.1. Myxozoa as parasitic cnidarians

Myxozoa is a group of metazoan organisms including many known to parasitize cold-blooded vertebrates such as fishes (Kent et al., 2001). The taxonomic position of myxozoa is still debated among the scientific community. Some consider myxozoa as a phylum (Kent et al., 2020) whereas others a class of the phylum Cnidaria (Dezfuli et al., 2021). Presently, more than 2400 myxozoan species belonging to 64 genera have been described (Eszterbauer et al., 2020; Zhang 2011). This group includes morphologically degenerated cnidarians which have a complex life-cycle (Okamura et al., 2015). At present, myxozoa are known to parasitize freshwater, marine and terrestrial hosts. This parasitic group has broadened its vertebrate host range from fish to incorporate amphibians, reptiles, waterfowl and even small mammals (Okamura et al., 2015a). Myxozoans exploit invertebrates as their definitive host and vertebrates as their intermediate host. Infection caused by myxozoan parasites often lead to significant economic losses in aquaculture and fisheries (Shinn et al., 2014).

Myxozoa is further sub divided into myxosporea and malacosporea. While many myxosporean genera are described, only two genera are described from malacosporea. Some notable myxosporean parasites include *Myxobolus cerebralis*, *Enteromyxum leei* and *Ceratonova shasta*. The malacosporeans include members of two genera *Tetracapsuloides and Buddenbrockia.* One of the important myxozoans is *Tetracapsuloides bryosalmonae* that plays a pivotal role of importance both economically (Clifton-Hadley et al., 1986) and ecologically (Ros et al., 2021) as a parasite of salmonids (Canning et al., 2000, 2002).

1.2. *Tetracapsuloides bryosalmonae:* life-cycle, host-range and pathology

Tetracapsuloides bryosalmonae causes an economically important disease Proliferative kidney disease (PKD) in farmed and wild salmonids in Europe and North America (Hedrick et al., 1993). PKD is the suspected cause for declines in wild salmonid populations (Burkhardt-Holm et al., 2005; Sterud et al., 2007) and is considered an emerging disease related to rising anthropogenic challenges of eutrophication and global warming (Okamura et al., 2011).

In common with other myxozoan parasites, the life cycle of *T. bryosalmonae* involves an invertebrate and a vertebrate host. Its primary hosts are bryozoans, which act as reservoirs for parasite spores that infect their secondary hosts, salmonid fish. The parasite transitions through a series of complex developmental changes within both primary (McGurk et al., 2006) and secondary hosts (Morris and Adams 2008). Infected bryozoan colonies release parasite spores, which in turn infect salmonid fish primarily through their gills and then travel through the blood vascular system to the major target organ, the kidney. Inside the kidney of infected fish host, the parasite develops into extrasporogonic and sporogonic stages (Morris and Adams 2008). Subsequently, an intratubular development leads to the formation of spores, which leave the fish host via urine and are infective for the bryozoans (Morris and Adams 2006; Grabner and El-Matbouli 2008). Infected fish show pale and anaemic gills, abdominal swelling, renal proliferation and anaemia within 4–8 weeks after infection at water temperatures above 15 °C (Clifton-Hadley et al., 1986; Ros et al., 2021; Wahli et al., 2002).

T. bryosalmonae infects a wide range of salmonid fish such as brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*), European grayling (*Thymallus thymallus*), Arctic charr (*Salvelinus alpinus*) and European whitefish (*Coregonus lavaretus*), rainbow trout (*Oncorhynchus mykiss*), chinook salmon (*Oncorhynchus tshawytscha*), cutthroat trout (*Oncorhynchus clarkii*), pink salmon (*Oncorhynchus gorbuscha*) and brook trout (*Salvelinus fontinalis*). While these salmonids are susceptible to *T. bryosalmonae* infection exhibiting similar clinical signs and symptoms, at present only brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*) are known to be parasite carriers (Grabner and El-Matbouli 2008). Carrier fish release viable parasite spores that infect the bryozoans. However, in *T. bryosalmonae* infected rainbow trout (dead-end host), similar clinical disease manifestations are observed but parasite spores infective for bryozoans are not shed, and hence the parasite life cycle is terminated (Grabner and El-Matbouli 2008). Currently, we do not have a full understanding of why the life cycle of *T. bryosalmonae* differs in rainbow trout and brown trout. The fact that brown trout can be carriers of the parasite suggests that *T. bryosalmonae* might employ immune evasion tactics or alter host pathways. In contrast, in rainbow trout, the inability of the parasite to complete its life cycle may be due to a strong host response or an ineffective parasite survival strategy. To better comprehend these differences and identify

crucial genes for survival and pathogenesis in the host, conducting comparative transcriptional profiling of *T. bryosalmonae* in rainbow trout and brown trout could be a potential way to gain valuable insights.

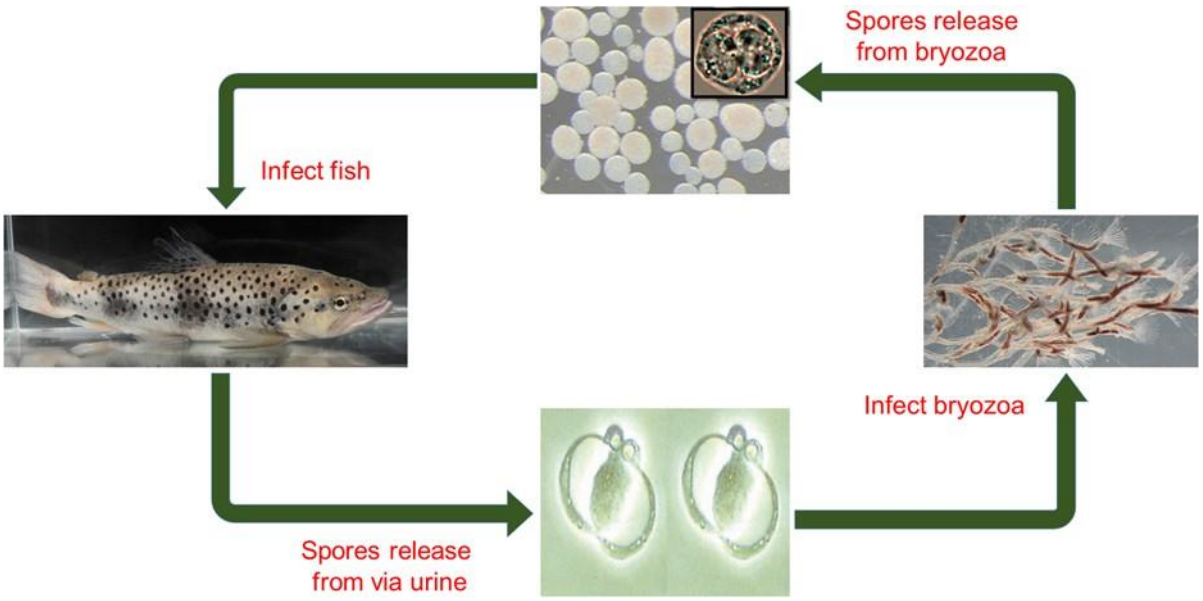

Figure 1. The Life-cycle of *T. bryosalmonae*. *T. bryosalmonae* exploits the invertebrate bryozoans as its definitive host and the vertebrate salmonids as its intermediate host. *T. bryosalmonae* spores develop in the kidney tubules and are finally released via urine to infect bryozoans.

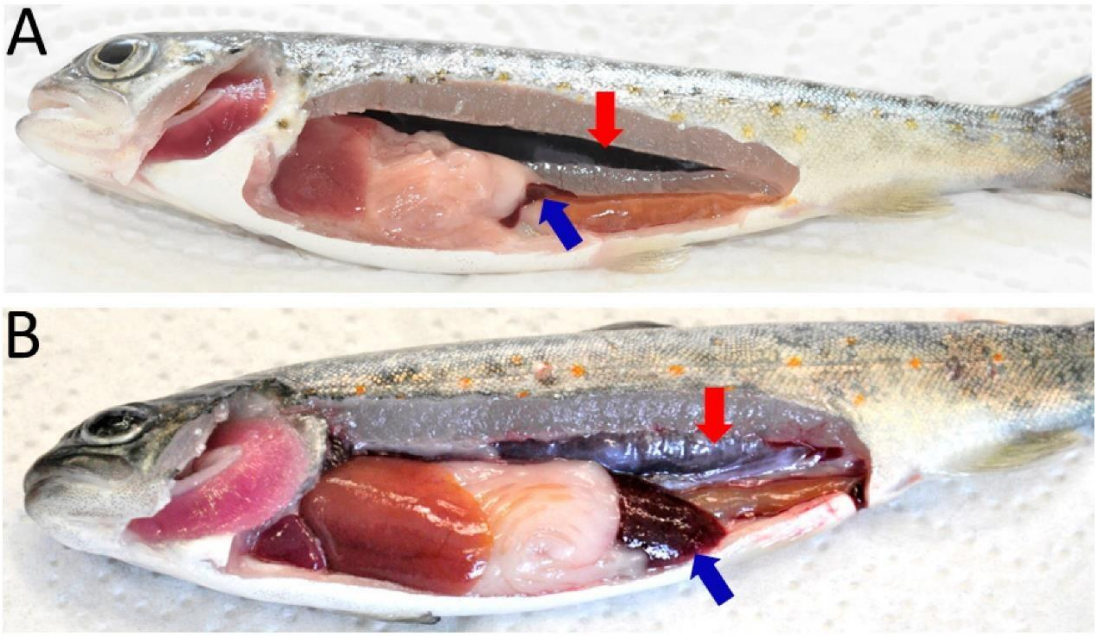

Figure 2. Clinical manifestations of proliferative kidney disease in brown trout. (A) Control fish showing normal kidney and spleen; (B) infected fish showing renal hyperplasia (enlargement of kidney) and splenomegaly (enlargement of spleen). Red arrow: kidney, blue arrow: spleen (Image from Shivam et al., 2021).

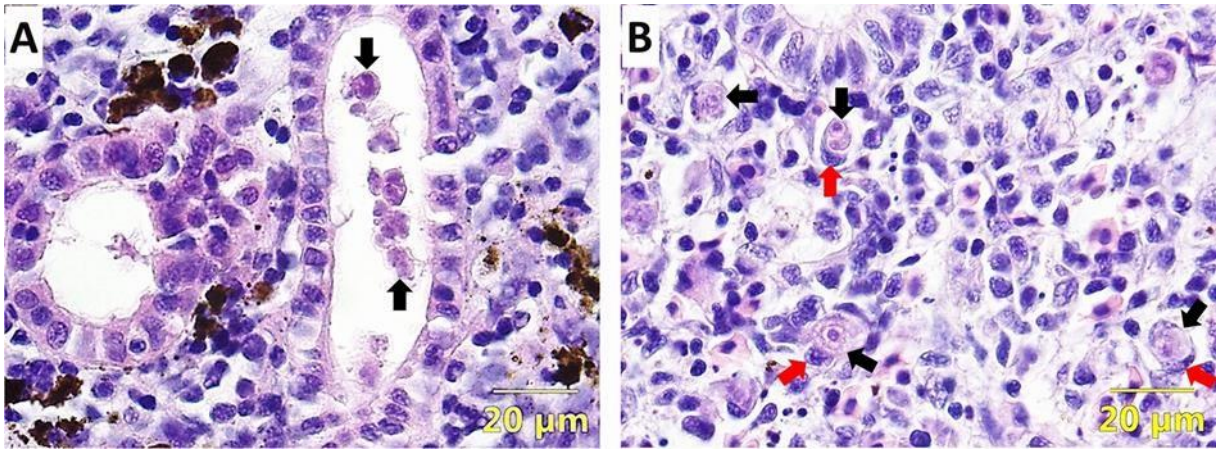

Figure 3. Histological sections of infected kidneys. (A) Brown trout kidney section shows intra-luminal sporogonic stages of *Tetracapsuloides bryosalmonae* (arrows); (B) Rainbow trout kidney section shows interstitial pre-sporogonic stages (black arrows) of *T. bryosalmonae* associated with macrophages (red arrows) (H&E staining) (Image from Shivam et al., 2023).

1.3. Host defense strategies during PKD

During parasitic infections, hosts employ different strategies to counter parasites. The two most important mechanisms include resistance and tolerance. The outcome of employing a resistance strategy, could result in three potential disease scenarios: (1) Complete clearance of the infection by the host, (2) The host maintaining a stable infection, or (3) Host mortality due to the infection (Kutzer and Armitage 2016). While in rainbow trout, *T. bryosalmonae* is completely cleared, in brown trout chronic infection with this parasite occurs (Abd-Elfattah et al., 2014; Soliman et al., 2018).

In the context of parasite infections, B cells and antibodies assume pivotal roles in host immunopathogenesis. B cells not only facilitate antigen presentation but also release cytokines and chemokines, thereby orchestrating various immune response pathways (Lund 2008; Myers 1991). Distinguished by the presence of B-cell receptors (BCR), B cells are indispensable for survival, development, and antibody production. Besides, many studies reported the importance of B cells and antibodies in reducing parasite burden (Umekita et al., 1988; Bermejo et al., 2011; Bickle 2009). During advanced stages of *T. bryosalmonae*-induced PKD pathogenesis, B-cell activity decreases in the dead-end host rainbow trout (Chilmonczyk et al., 2002; Abos et al., 2018; Bailey et al., 2019). There are some reports of immune gene expression studies in the kidneys of brown trout in response to *T. bryosalmonae* (Bailey et al., 2019; Kumar et al., 2014).

Despite these significant advances, there was no report of systematic serum antibody response of brown trout against *T. bryosalmonae*. Besides, dominant B-cell response is reported during PKD in brown trout (Bailey et al., 2019), yet gaps remain in its functional understanding. In the previous study, we identified some B-cell-associated genes with roles in haematopoiesis and B-cell receptor signaling to express variably during PKD pathogenesis (Sudhagar et al., 2019).

1.4. Parasite isolation by FACS in transcriptome analysis

The field of transcriptomics has emerged as a robust tool for deepening our comprehension of interactions between hosts, parasites, and their dynamics (Greenwood et al., 2016, Patino & Ramírez 2017). Recently, RNA-seq has gained widespread adoption for

generating extensive transcriptome data in numerous myxozoan parasites, with the analysis of this data yielding significant insights. With regard to *T. bryosalmonae*, previous research has employed dual RNA-seq to investigate its transcriptome within infected brown trout kidneys (Ahmad et al., 20121), as well as the shared transcriptome between infected rainbow trout kidneys and bryozoan hosts (Faber et al., 2021). Nevertheless, a comparative analysis of the transcriptome of this parasite between infected rainbow trout and brown trout was not done.

A notable challenge in dual RNA sequencing of parasite-infected host samples lies in the dominance of host transcripts, where parasite transcripts may constitute as little as 0.1% (Greenwood et al., 2016). Additionally, the risk of host contamination is a well-documented concern. A viable solution entails the purification of parasites from infected host tissues prior to RNA sequencing. Many cell purification techniques are available that enable the isolation of specific cell types from complex tissues or cultures. Some of the methods of choice include density gradient centrifugation, magnetic-activated cell sorting (MACS) and Fluorescence-Activated Cell Sorting (FACS). Among these techniques, FACS has been demonstrated to allow the selective isolation and collection of *Plasmodium falciparum* trophozoites (Boissière et al., 2012) and *Plasmodium berghei* (Kenthirapalan et al., 2012).

2. Objectives of the thesis

1. To quantify anti-parasite antibody levels in the serum of *T. bryosalmonae*-exposed brown trout at different time points
2. To investigate systematic B-cell-associated gene expression patterns in kidney and spleen of infected brown trout at different time points.
3. To identify *in vivo* induced genes of *T. bryosalmonae* during proliferative kidney disease in brown trout
4. To isolate *T. bryosalmonae* from the main target organ kidney of infected brown trout and rainbow trout
5. To investigate the differentially expressed transcripts of *T. bryosalmonae* between brown trout and rainbow trout

3. Publications

In this thesis one review and three research manuscripts are included. The first manuscript reviews the progress and opportunities in the development of vaccines against fish parasites and the importance of different omics tools. The second manuscript is a research article demonstrating the kinetics of anti-*T. bryosalmone* antibody response in brown trout and also B cell associated gene expression pattern in spleen and kidney. The third publication is another research article and was aimed at elucidating *in vivo* induced genes of *T. bryosalmonae* during PKD in brown trout. The fourth publication is also a research manuscript that analysed the comparative transcriptome of *T. bryosalmonae* between the carrier brown trout and dead-end host rainbow trout.

3.1. Development of fish parasite vaccines in the OMICs Era: Progress and opportunities

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MDPI

Review

Development of Fish Parasite Vaccines in the OMICs Era: Progress and Opportunities

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Abstract: Globally, parasites are increasingly being recognized as catastrophic agents in both aquaculture sector and in the wild aquatic habitats leading to an estimated annual loss between 1.05 billion and 9.58 billion USD. The currently available therapeutic and control measures are accompanied by many limitations. Hence, vaccines are recommended as the "only green and effective solution" to address these concerns and protect fish from pathogens. However, vaccine development warrants a better understanding of host–parasite interaction and parasite biology. Currently, only one commercial parasite vaccine is available against the ectoparasite sea lice. Additionally, only a few trials have reported potential vaccine candidates against endoparasites. Transcriptome, genome, and proteomic data at present are available only for a limited number of aquatic parasites. Omics-based interventions can be significant in the identification of suitable vaccine candidates, finally leading to the development of multivalent vaccines for significant protection against parasitic infections in fish. The present review highlights the progress in the immunobiology of pathogenic parasites and the prospects of vaccine development. Finally, an approach for developing a multivalent vaccine for parasitic diseases is presented. Data sources to prepare this review included Pubmed, google scholar, official reports, and websites.

Keywords: fish parasites; immune response; omics; vaccines

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1. Introduction

Aquaculture continues to be one of the rapid food-producing sectors worldwide. According to an estimate, the latest global aquaculture production was 82 million tons and valued at 250 billion USD in 2018 [1]. However, the sector is frequently hit by several viral, bacterial, and parasitic diseases with devastating consequences [2]. Although viruses and bacteria have been recognized as the leading cause of huge economic losses to the sector, the role of parasites has been realized recently. Growing literature suggests a consider- able increase in parasitic epidemics both in farmed and wild fish populations. Parasites belonging to different groups such as myxozoa, protozoa, crustaceans, monogeneans, and helminths result in heavy losses in aquaculture and consequently to the allied industries. As per a report, the annual global loss of juvenile fish on account of parasitic infections was estimated to vary from 107.31 to 134.14 million USD and loss of marketable size fish from 945.00 million to 9.45 billion USD, the total estimate being 1.05 billion to 9.58 billion USD [3].

The management of parasitic infections in culture facilities involves different strate- gies such as quarantine, disease-free sites, disinfection of water using UV radiation and chemicals, fallowing, and drying of pond bottoms [4]. Instead of ponds, concrete tanks or raceways have been used for fish culture to prevent infection by myxozoan parasites in order to restrict the oligochaetes, which serve as the alternate hosts of these parasites [5]. Nevertheless, early efforts of controlling a parasitic infection in fish relied heavily on the use of chemotherapeutics. Consequently, their relentless use is leading to the emergence of drug

resistance [6,7] and deleterious environmental effects [8,9]. Although phytotherapy-based treatment options are favorable, they suffer from various disadvantages [10–13]. Lately, the use of attractants and traps has been suggested as a promising strategy for certain parasites such as sea lice, by exploiting their chemotactic and phototactic responses [14]. Furthermore, a study highlighted the use of urea and light-based traps for controlling the infection by *Cryptocaryon irritans* and *Neobenedenia girellae* in aquaculture [15]. However, at present, application of this approach in commercial aquaculture is limited due to the unavailability of efficient traps. Vaccination is considered the best method for safeguarding and promoting fish health and welfare against any parasite. Although several commercial vaccines are available for bacterial and viral diseases globally [16], only Chile has a commer- cial parasite vaccine against sea lice [17]. The development of parasite vaccines is limited by several inherent issues. One of the most important factors is the biological complexity of the parasite. The parasite life advances through different developmental stages, which may have a specific antigen profile. Moreover, the life cycle stages alternate between different host species in various parasites [18], thus, interfering with the culturing and maintenance of parasites under laboratory conditions owing to requirements for optimization of several parameters such as temperature and nutritional elements for the alternate host, giving rise to more labor requirement and increased economic costs.

Omics studies are powerful methods for developing vaccines by providing potential vaccine candidates. The suffix "omics" refers to the high-throughput analysis of cellular macromolecules. The most popular omics disciplines include genomics, transcriptomics, and proteomics. The omics era started with genomics, aiming to study the entire gene con- tent (genome) of an organism [19]. The genomic analysis provides abundant information on individual genes, chromosomes, their organization, genetic variants of diseases as well as evolutionary relationships with other phyla and parasites. However, genomics does not provide information on aspects such as gene expression, function and regulation, and structure and characteristics of encoded proteins [20]. These limitations have resulted in the advent of the post-genomic era primarily dominated by transcriptomics and proteomics. A transcriptome comprises all RNA transcripts produced by the genome under a given environmental condition [21]. Transcriptomic profiling provides information on different categories of transcribed RNAs, the transcriptional structure of genes, and the expression of genes [22]. Nonetheless, transcriptomics does not reflect the actual protein complement due to the many events in translation of mRNA transcripts, e.g., post-transcriptional modifications and alternate splicing [23]. Proteomics followed transcriptomics, which is defined as the study of proteomes (the entire set of proteins, that are the key players in biological processes) [21].

Genomic and transcriptomic analyses of certain fish parasites have been performed. In addition, transcriptomics-based studies have been conducted to investigate the host–parasite interaction in some instances [24]. Similar to human and veterinary parasitology, omics data could provide potential therapeutic targets against aquatic parasites.

In this review, we highlight the need and the progress made in developing parasitic vaccines, with particular focus on the immunobiology of fish parasites. Furthermore, we discuss the present status, the prospects of developing successful parasite vaccines in the present era of omics, and an approach for developing multivalent vaccines.

2. Data Sources, Searches and Study Selection

Searches were performed on pubmed, google scholar and google with the keywords either alone or in combination "parasite vaccines", "fish parasites", "transcriptomics", "genomics", and "proteomics". According to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines, peer-reviewed articles were initially selected. The articles were then screened based on title and the abstract. Only full text articles were included in the study. Official reports of FAO (Food and Agriculture Organization of the United Nations), NCBI (National Center for Biotechnology Information) and

OIE (Office International des Epizooties) websites were also referred to. Flow diagram is provided as Supplementary Data (Figure S1).

3. Economically Important Fish Parasites

The majority of the important fish endoparasites belong to the phylum Cnidaria, whereas the ectoparasites belong to phyla Ciliophora and Arthropoda (Table 1). The phylum Platyhelminthes encompasses both endoparasites and ectoparasites. Parasitic diseases in aquaculture cause both direct and indirect losses. Direct losses result from the mortality in farmed fish because of parasite outbreaks. Indirect losses are attributed to the investment made for treating infections, adopting management strategies, reduced growth resulting from infection, and the cost involved from carcass spoilage at harvest [3,18,25].

Table 1. Some economically important fish parasites: Table provides information on mortality and economic loss caused by important parasites of fish. NA: not available, a: mortality, b: economic loss.

Parasite Group	Parasite	Disease	Host	Mortality [a]/ Economic Loss [b]	Reference
		Freshwater Endoparasites			
Cnidaria	*T. bryosalmonae*	Proliferative kidney disease	Rainbow trout	95% [a]	[26]
	M. cerebralis	Whirling disease	Rainbow trout	90% [a]	[27]
	S. molnari	Sphaerosporosis	Carps	NA	[28]
Platyhelminthes	*Bothriocephalus acheilo gnathi*	Ulcer, catarrhal enteritis	Carps	100% [a]	[29]
		Freshwater Ectoparasites			
Ciliophora	*I. multifiliis*	White spot	Rainbow trout, Carps	NA	[30]
Arthropoda	*Argulus*	Argulosis	Indian Major Carps	5.41 million USD [b]	[31]
Platyhelminthes	*Gyrodactylus salaris*	Gyrodactylosis	Atlantic salmon	NA	[32]
		Marine Endoparasites			
Cnidaria	*K. thyrsites*	Soft flesh syndrome	Salmonids	6 million CAD [b]	[33]
	C. shashta	Ceratomyxosis	Salmonids	NA	[34]
	E. leei	Enteromyxosis	Sharpsnout seabream	NA	[35]
Ciliophora	*Uronema marinum, Philasterides dicentrarchi*	Scuticociliatosis	Seabass, Turbot	NA	[36]
		Marine Ectoparasites			
Ciliophora	*C. irritans*	White spot	Greater Amberjack	834 USD [b]	[3]
Arthropoda	*Caligus rogercresseyi, Lepeophtheirus salmonis*	Sea louse disease	Salmonids	100 million USD [b]	[37]

Some notable examples of commercially important myxozoan endoparasites include *Tetracapsuloides bryosalmonae, Myxobolus cerebralis, Sphaerospora molnari, Ceratomyxa shasta*, *Kudoa thyrsites*, and *Enteromyxum* sp. Proliferative kidney disease (PKD), caused by *T. bryosalmonae*, could result in up to 95% mortality in farmed salmonids [26]. *C. shasta* causes ceratomyxosis in chinook and coho salmon [38]. In addition, several other myxo- zoan parasites incur huge direct and indirect economic losses, e.g., *Myxobolus cerebralis* [5] and *Enteromyxum* sp. [39]. Some ectoparasites responsible for significant mortality and thus aquaculture losses include *Ichthyophthirius multifiliis*, *C. irritans*, *Argulus*, *Lepeophtheirus salmonis*, and *Caligus rogercresseyi*. Protozoan parasites *I. multifiliis* and *C. irritans* cause white spot disease in freshwater and marine fishes, respectively [18]. The crustacean parasites *L. salmonis* and *C. rogercresseyi,* commonly referred to as sea lice, cause severe infection

in salmonids [40]. *Argulus* is the freshwater counterpart of sea lice reported from several fish hosts [41]. Furthermore, several parasites have been implicated in the decline of wild fish populations, e.g., *M. cerebralis* [42], *T. bryosalmonae* [43], *C. shasta* [44], *I. multifiliis* [45], *L. salmonis* and *C. rogercresseyi* [40].

Fish species of aquaculture importance differ with countries. Moreover, parasites causing mortality and incurring economic losses may differ. However, certain parasites are of global concern, e.g., *M. cerebralis, I. multifiliis*, and *Argulus* [18]. Despite reports of huge economic losses and fish mortality due to parasitic infections [18], their estimations are either lacking or are specific to locations. For instance, losses in salmonid farming accounted for 6 million Canadian dollars (CAD) in 2015 due to *K. thyrsites* infection in British Columbia, Canada [33]. *Argulus* is reported to cause losses amounting to 5.41 million United States dollars (USD) annually in carp aquaculture in India [31]. Globally, annual indirect and direct losses in salmonid aquaculture due to infestations with the sea louse *L. salmonis* have been estimated to be 500 million to 1 billion USD [46].

4. Progress in Understanding of Host-Parasite Interactions

Teleosts are the lowest vertebrates that elicit both innate and adaptive immune responses against pathogen invasion [47]. However, compared to higher vertebrates, innate immunity plays a major role than the adaptive immune response in aquatic organisms, irrespective of the nature of the pathogen [48]. Remarkably, innate immune reactions are not only the first line of defense but also set the pace for ensuing adaptive immune responses. Thus, the development of an effective vaccine requires a good understanding of immune response during host–parasite interaction and pathogenesis mechanisms. Some progress has been made in understanding the immune response in fish against parasitic diseases. Studies using immunoassays, gene expression, Western blotting, and other techniques have elucidated the expression of different molecules in innate and adaptive immune responses during several parasitic infections. The generation of an elevated immune response upon reinfection in fish, which could survive after previous natural parasite infection, has also been reported. Few examples include endoparasites, e.g., *T. bryosalmonae*, *C. shasta*, *E. scophthalmi*, *E. leei* [49] and ectoparasites, e.g., *I. multifiliis* and *C. irritans* [45,50]. Similar to other vertebrates, the development of immune memory in fish infected with parasites forms the fundamental basis of vaccine development.

4.1. Innate Immune Response during Selected Parasitic Infections

Studies on the immune response generated by parasites during infection and post-immunization, report the involvement of toll-like receptors (TLRs), phagocytes, complement proteins, melanomacrophage centers, proteases, and cytokines. TLRs are type-I transmembrane receptors that recognize pathogen-associated molecular patterns to initiate an immune response [51]. Several TLRs play important roles in the defense of different fish hosts, against various parasites e.g., *T. bryosalmonae* [52], *I. multifiliis* [53], *C. irritans* [54], and *C. rogercresseyi* [55]. TLR1, TLR2, TLR9, TLR19, TLR21, and TLR25 reportedly play an important role in immunity in fish against *I. multifiliis* [53]. However, the concerned parasite epitopes that they recognize are unexplored currently for the majority of TLRs. For example, TLR1, TLR13, and TLR19 are significantly upregulated in the kidney of brown trout during PKD [52], but the moiety of *T. bryosalmonae*, which might be responsible for their activation is unknown. TLR2 on human NK cells are activated by lipophosphoglycan component in the hemoflagellate parasite *Leishmania major*, a phosphoglycan belonging to a family of unique *Leishmania* glycoconjugates [56]. Phagocytic cells such as neutrophils, acidophilic granulocytes, and monocyte-macrophages, are involved in pathogen clearance [57] by producing reactive oxygen intermediates and nitric oxide, resulting in a respiratory burst. Increased phagocytic oxidative burst due to the enhanced capability of phagocytes to produce a higher amount of reactive oxygen species was observed in natural *T. bryosalmonae* infection in rainbow trout [58]. A higher number of NBT-positive cells indicated similar results in fish immunized with either *Sphaerospora dicentrarchi* spores alone or in combi-

nation with an adjuvant on the seventh day post-injection [59]. Respiratory burst and abundant granulocytes were found in vaccinated fish with live *Cryptobia salmositica* [60]. Furthermore, the increased number of thrombocytes and monocytes indicated clearance of *S. molnari* by phagocytosis. Increased thrombocytes acted against inflammation and contributed to wound healing [61]. Moreover, these authors associated the immediately increased expression of cytokines such as interleukin-1β upon peritoneal injection of the parasite with parasite recognition by host TLRs.

Nonspecific cytotoxic cells (NCC) in fish comprise heterogenous leucocyte populations. These are considered as mammalian equivalents of natural killer cells (NK) sharing many characters in common including antiparasitic activity [62]. NCC occur in teleost thymus, kidney, spleen and blood. They are particularly considered important in young fish when specific responses are weak [63]. The antiparasitic activity of NCC is reported against *I. multifiliis* [62] and *Tetrahymena pyriformis* [64]. However, more information on the role of these cells during parasitic infections is required.

In vitro studies with inactivated serum have reported the function of the classical complement pathway in killing *Philasterides dicentrarchi* [65]. Melanomacrophage centers (MMCs) are aggregation of phagocytic cells in lymphoid organs and involved in pathogen sequestration and antigen presentation [66]. MMCs have been found to contain different myxozoan parasites such as *Enterozoon scophthalami* [67] and *Myxobolus cyprini* [68]. The non-specific cellular innate immune response plays a greater role in the recovery from infection with parasites such as *T. bryosalmonae* [58]. The antiparasitic activity of different fish antiproteases such as α-2 macroglobulin during *E. scophthalmi* infection [69], total serum antiprotease in *E. leei* [67], and serine protein inhibitors [70] have been reported.

Only a few studies have been conducted to understand the susceptibility and resistance to parasites by host fish. For example, the upregulation of IFN-γ was reported both in the resistant Hofer strain and the susceptible US strain of rainbow trout against *M. cerebralis* infection. However, the upregulation of this gene was found to be considerably higher in susceptible trout strains than in the resistant strains after 24 h of pathogen exposure. The higher expression of the gene in susceptible rainbow trout was suggested to be deleterious for the host [71]. Similar observations in susceptible and resistant strains of Chinook salmon to *C. shashta* infection have been reported [72]. IFN-γ has been identified as a key immune effector cytokine with multiple protective roles via enhancing antigen presentation by APCs and initiation of pro-inflammatory responses in coordination with other pathways. Its activity is highly regulated, and its overexpression can damage the host tissue [48]. Among the different genes analyzed, STAT-3 (signal transducer and activator of transcription 3) was the most differentially expressed gene during *M. cerebralis* infection in susceptible and resistant rainbow trouts. In the Hofer strain rainbow trout, the higher expression of STAT-3 gene likely confers resistance against whirling disease. The expression remains unchanged in susceptible rainbow trout during infection [71]. SOCS (suppressor of cytokine signaling) protein family negatively regulates cytokine and growth factor signaling [73]. *T. bryosalmonae* and *M. cerebralis* infection results in the overexpression of SOCS-1 and SOCS-3 genes in rainbow trout during parasite development [74]. The innate and adaptive immune systems are interconnected. Therefore, the knowledge of innate immune effectors in protection, susceptibility, and resistance against parasites in fish is paramount in designing successful vaccines.

Some parasites reportedly evade or suppress the innate immune responses for their continued survival in the fish host. For instance, the ectoparasite *I. multifiliis* invokes a host evasion mechanism by ingestion of neutrophils, thus suppressing further signaling pathways in the immune reactions [75]. *L. salmonis* induces limited innate immune response in one of its most susceptible salmonid host Atlantic salmon which might be an immune suppression approach. Mustafa et al. [76] reported depression in oxidative and phagocytic abilities of host macrophage following *L. salmonis* infection which could be a parasite strategy. Another possible mechanism might be the increased production of proteases by the parasite. *L. salmonis* reportedly produces trypsin-like proteases which

help them to feed and evade host immune responses. Dalvin et al. [77] reported the suppression of complement and nitric oxide in rainbow trout infected with sea lice. Nitric oxide is a signaling molecule leading to an anti-inflammatory response. It modulates the release of different inflammatory mediators from the cells participating in inflammatory responses, e.g., leukocytes, macrophages, mast cells, endothelial cells, and platelets [78]. Thus, suppression of nitric oxide could be a possible parasite strategy to avoid excessive immune response and increase survival of fish host. Additionally, *T. bryosalmonae* causes a severe inflammatory response in the kidney of brown trout which gradually regresses over a period of time and the recovered fish continue to excrete parasite spores [43].

4.2. Adaptive Immune Response during Selected Parasite Infection

Three classes of immunoglobulins have been described in fish, namely IgM, IgD, and IgT [79]. Parasite-specific antibodies have been detected in several parasitic infections, e.g., *T. bryosalmonae* [80,81], *M. cerebralis* [82], *S. dicentriarchi* [83], *C. shasta* [84], *E. scophthalmi* [85], *M. honghuensis* [86], *I. multifiliis* [75] and *L. salmonis* [77]. IgT might be playing important roles against fish parasites. In fish surviving *C. shasta* infection, a higher number of IgT^+ B cells were found than in healthy fish [87]. The authors confirmed the IgT protein abundance and the upregulation of IgT gene. IgT is reportedly the dominant immunoglobulin present in skin and gills of *I. multifiliis*-infected rainbow trout. Abundant IgT^+ B cells were found to occur in the skin epidermis of infected fish. Additionally, IgT was found to cover the parasite and was present in the mucus at high quantities [88]. All three immunoglobulins are formed in response to PKD; however, these might act as an escape mechanism following infection with *T. bryosalmonae*. This study also suggested an important role of IgD in the humoral response to the parasite, based on the appearance of IgD^+ IgM^- B cells, somatic hypermutation, and clonal expansion of some IgD-expressing B cell subsets [89]. During *I. multifiliis* infection, antibodies cross-bind to Iag allowing the parasite to either escape from the host or result in destruction of the parasite [45]. During certain parasitic infections, antibodies were not detected. This response was observed in *C. elongatus*-infected Atlantic salmon because this parasite causes little damage to fish skin leading to decreased contact between louse antigen and the host immune system [90].

T cells are involved in adaptive immunity in all vertebrates. These cells are characterized by the presence of T cell receptors [91]. At present, studies on T cell-mediated immune response in fish parasites are mostly limited to the associated surface markers. Experimental infection by anal intubation of *E. leei*-infected intestinal scraping in gilthead seabream provided evidence for specific T cell response in the head kidney and anterior intestine [35]. In this study, real-time PCR revealed the downregulation of T cell markers such as zap70, cd3, cd4–1, cd4–2, cd8β, and CTL receptors in the head kidney and their upregulation in the anterior intestine. Moreover, FACS analysis provided evidence on the involvement of $CD8^-$ T cells in resistance against *M. cerebralis* infection in the German Hofer strain of rainbow trout [92]. During infection with the intracellular parasite *K. thyrsites*, a Th 1 type response was reported as indicated by the upregulation of il-12 gene in the infected muscles of Atlantic salmon [33]. Currently, T cell responses are measured by ELISPOT assay, which is considered the most sensitive determination method of T cell cytokine production. Additionally, other assays are also used like intracellular staining for cytokines and others markers of T cell activation and function, in vivo CTL assay for measuring the lytic capacity of cytolytic T cells, which provide high sensitivity detection of specific pathogen peptides. These assays are useful in studying the specificity and potency of T cell responses of host and high throughput antigen screening [93]. Adaptive immune response mediated by high antibody titers with decreasing parasitemia was noticed following live vaccination against *C. salmositica* in Atlantic salmon [60]. Both the components of the adaptive immune system, i.e., the humoral response mediated by antibodies and the cellular response mediated by T cells are demonstrated in fish against parasitic infections. The humoral response is important against extracellular parasites, whereas T cell-mediated

immune response is induced against intracellular parasites. Thus, an effective vaccination strategy would stimulate both these arms of the adaptive immune system [94].

5. Parasite Vaccines: Status and Prospects

Currently, there exist 24 commercially available vaccines for viral and bacterial diseases of aquaculture importance [16]. However, only one commercial parasite vaccine, Providean Aquatec Sea Lice produced by Tecnovax S.A. Argentina, is available. As discussed earlier, developing vaccines against aquatic parasites is an extremely challenging task.

Progress towards the development of parasite vaccines for aquaculture has been comparatively slower than those for humans and animals. In addition to the reasons mentioned, several other possibilities exist for this dismal scenario. A possible reason in several countries could be the smaller scale of aquaculture operations resulting in economic impracticality of vaccine production, the recent realization of their importance in causing huge disease losses, and fewer research groups working on this aspect. The available literature suggests that vaccines for parasites infecting fish are confined to a few trials against a limited number of parasites.

5.1. Ectoparasite Vaccine Trials

Vaccines have been attempted for parasites with significant detrimental effects on aquaculture. These include vaccination trials against parasites such as *I. multifiliis*, *C. irritans*, and *L. salmonis* (Table 2). Different vaccine trials comprising live, killed, parasite homogenate, subunit, and DNA have been tested for protection against *I. multifiliis* infection in various fish hosts. The earliest, as well as the most effective method of achieving protection to date remains immunization by live theronts or trophonts [30]. Immunization trials against this parasite with ciliary and whole cell preparation of *I. multifiliis* and *Tetrahymena pyriformis* were used to vaccinate channel catfish. The ciliary antigen of the latter was found to confer effective protection [95]. The lower protection rate obtained using *I. multifiliis* cilia could be attributed to less homogenous antigen preparation, resulting from the lower number of tomites used. The same fine structure of all protozoan cilia [96] and common antigenic determinant [97] provided cross protection. Burkart et al. [98] studied different antigen preparations and vaccination routes for immunizing channel catfish against *I. multifiliis*. The study concluded that intraperitoneal administration of live tomites effectively protected against infection as compared with a surface infection with the parasite. Fish vaccinated with formalin-killed trophonts resulted in a 51% mortality rate. Sonicated and formalin-killed trophont formulations protected rainbow trout ten weeks post-hatch fry. Moreover, this study reported a higher susceptibility to reinfection of bath-treated fish in comparison to IP-injected fish. However, the underlying reasons could not be ascertained [98]. Proteomic screening and in silico analysis were used to test three recombinant proteins (#5, #10, and #11), which were combined in a subunit vaccine and administered intraperitoneally in rainbow trout and resulted in partial protection. Parasite burden was found to be lower with a mean intensity of 1.3 parasites per g/cm fish in the vaccinated group as compared with the uninfected control group (2.8 parasites per g/cm fish).

In addition, specific antibody production was found to be significantly higher than in control fish. The highest antibody response was generated in fish against protein #10 both at four weeks and 13 weeks post-infection, suggesting it as a potential vaccine candidate along with cell and ciliary surface antigens commonly known as immobilization antigen or i-antigen [101]. For the DNA vaccine, the intramuscular administration of i-antigen alone or in combination with cysteine protease of *I. multifiliis* has been used in channel catfish and rainbow trout as a vaccine candidate [99,102]. However, the protection of DNA vaccine was comparatively lower than that of a live vaccine. Both live and killed *C. irritans* theronts conferred protective immunity in grouper, as evident from the high antibody titer

in the mucus of immunized fish and better survival and reduced number of tomonts and trophonts [106].

Table 2. Overview of immunization trials in fish for ectoparasites: Table provides information on immunization trials for important ectoparasites of fish. IM: Intramuscular, IP: Intraperitoneal.

Disease	Parasite	Fish Host	Vaccine Trial Type	Antigen Target	Delivery Method	Reference
White spot disease (Ich)	*Ichthyophthirius multifiliis*	Channel catfish	DNA	(i-antigen) immobilization antigen	IM	[99]
		Channel catfish	Live	Theront	IP	[100]
		Rainbow trout	Subunit	Recombinant proteins (#5, #10, & #11)	IP	[101]
			DNA	(i-antigen) immobilization antigen & Cysteine protease	IM, Needle free injection, Gene gun delivery	[102]
			Live	Theronts	IP	[103]
			Killed	Sonicated formalin killed trophonts	IP	[104]
		Nile Tilapia	Live	Theront and sonicated trophonts	Immersion and IP	[105]
		Channel catfish	Live	Tomites	IP	[98]
			Killed	Trophonts and tomites	IP	
Marine White spot	*Cryptocaryon irritans*	Grouper	Live	Theronts	Bath	[106]
			Killed	Formalin-killed theronts	IP	[107]
Sea louse infestations	*Lepeophtheirus salmonis*	Atlantic salmon	Crude parasite extract	Adult female parasite	IP	

5.2. Endoparasite Vaccine Trials

Currently, the literature on vaccination trials for endoparasites is available only for *T. bryosalmonae*, *Myxobolus koi*, and *Uronema marinum* (Table 3). DNA vaccine targeting a novel micro-exon genes (Tb MEG1) has been reported to elicit Tb-MEG1-specific immune response in rainbow trout [108]. Moreover, this study demonstrated the expression of proteins in and on the surface of parasites using anti-Tb-MEG1 monoclonal antibodies. A study using crude spore proteins reported better survival in *M. koi*-infected gold fish [109]. Intraperitoneal administration of poly D, L-lactic-co-glycolic acid (PLGA)-encapsulated vaccine against *U. marinum* infection significantly reduced the mortality in kelp grouper, *Epinephelus bruneus* [110]. This study reported early enhancement (1 to two weeks post-vaccination) and longer duration (4 weeks post-vaccination) of respiratory burst, complement activity, α2-macroglobulin activity, serum lysozyme, antiprotease activity, and antibody response. Studies on vaccination trials for ecto- and endoparasites report the use of different criteria for determining the efficacy of the tried vaccine, such as percentage mortality, percentage survival, parasite burden, and antibody titer in vaccinated fish as compared with those in the control fish.

Table 3. Overview of immunization trials in fish against parasites: Table provides information on immunization trials for important endoparasites. NA: Not Available, IM: Intramuscular, IP: Intraperitoneal.

Disease	Parasite	Fish Host	Vaccine Type	Antigen Target	Delivery Method	Reference
			Freshwater Endoparasites			
PKD	*Tetracapsuloides bryosalmonae*	Rainbow trout	DNA	Micro-exon gene (TB-MEG1)	Not Available	[108]
Myxobolosis	*Myxobolus koi*	Gold fish	Subunit	Crude protein spore	Immersion	[109]
			Marine Endoparasites			
Scuticociliatosis	*Uronema marinum*	Grouper	Subunit	(i-antigen) immobilization antigen	IP	[110]
	Philasterides dicentrarchi	Turbot	Subunit	Membrane proteins	IP	[111]

6. Perspectives in Fish Parasite Vaccine Development

6.1. Vaccination Strategy

Live, killed, DNA and protein subunit vaccines are being applied in aquaculture to control various pathogenic organisms. The majority of the vaccine trials in fish against parasites have used the live or killed strategy that has proved effective. However, it has certain limitations. Live vaccination is similar to the natural process of infection. This approach employs a controlled infection with a virulent or less virulent or attenuated strain of the parasite. In the case of fish, the attenuated strains of few parasites has been used for immunizing fish. There is an increased risk of mortality with vaccination with live virulent parasites. Another important consideration for both live and killed vaccines is the requirement of numerous parasites of good quality, which currently is difficult to culture in vitro. In addition, live vaccines are not considered safe for use in aquaculture [16]. Although DNA and subunit vaccines are the most promising alternative approaches, both the approaches have conferred partial to moderate protection. This could be attributed to the fact that the parasites present several challenges such as antigenic variability, immune evasion, immunomodulation of effector molecules, and poor immunogenicity of individual antigens. To overcome these issues, the vaccine must target multiple antigens (multi-epitope) simultaneously to be effective. Although a successful example of a multivalent vaccine approach for parasite is not available from fish-based trials, a multivalent DNA vaccine encoding three antigens provided long-lasting protection after mice were challenged with *Leishmania* [112]. Certain factors must be considered while attempting a subunit vaccine, including the technical viability of antigen production, its formulation in suitable adjuvants, and the ease and frequency of delivery [113]. Similarly, the selection of expression vector must ensure the required post-translational processing to retain the immunogenicity of the desired protein.

6.2. Vaccination Routes

Broadly, vaccines can be administered in aquaculture via oral, immersion, and injection routes. In the oral process, antigens encapsulated in the feed are administered to fish. It is the most effective delivery method for aquaculture due to minimal stress, simple administration, and applicability to both large and small fish. However, difficulty in determining the precise dosage received by fish and lack of efficacy limit its application. In addition, antigen degradation is possible during its passage to the stomach before it reaches the hindgut where the antigen is adsorbed [114]. In the immersion process, fish are immersed in water with antigens for a specified period. Immersion can be performed in the form of dip and bath. In the dip method, the fish are kept in water containing antigens at a high dose for some minutes, whereas the bath method involves keeping the fish in water with a low antigen dose for a longer time [115]. The injection route is used to commonly deliver the antigen either intraperitoneally or intramuscularly. The advantages of injection vaccination include precise dosage delivery and longer protection [116]. In most studies, the intraperitoneal route of vaccine administration against parasites was used for live and killed antigen preparations, whereas the intramuscular route has been used for DNA antigens (Tables 2 and 3). Several studies report significant systemic and mucosal antibody production against parasites following the intraperitoneal injection of antigens [98,101]. Certain disadvantages associated with the injection process include stress during handling and anesthetizing. In addition, the method is labor intensive, costly, and impractical for fish below 20 g and for mass vaccination [117].

6.3. Protective Immune Response

The success of any vaccine against a parasite depends on the development of protec- tive immunity in the fish host. The developed protective immune response should mimic as during a naturally occurring parasitic infection. Furthermore, the magnitude of immune response depends on the pathogen, intensity, and the stage of infection [118]. The protective immune responses of fish against some parasites are dependent on the production of spe-

cific antibodies. For example, specific antibodies were found in the sera of recovered and experimentally infected fish, which were confirmed to involve in protective immunity in fish against *Cryptobia salmositica* and Scuticociliates [119,120]. Anti-*T. bryosalmonae* specific antibodies have been detected in sera of experimentally infected brown trout from 4 to 17 weeks post exposure; however, studies on the mechanisms involved in protection are limited (authors own unpublished data). Partial antibody-mediated protection against *I. multifiliis* and *Trypanosoma carassii* was obtained after immunization of fish with recombinant proteins [121,122]. However, in several cases, the presence of specific antibodies were not correlated with protection of fish against parasites.

In the context of fish parasites, information on specific parasite stages that elicit immune response is mostly available for ectoparasites. For example, immunity is directed against the theront and trophont stages of *I. multifiliis* [123] and *C. irritans* [124]. Additionally, from metanauplius larvae to adult *Argulus* stages induce limited host response [18]. With regard to myxozoan endoparasites, the knowledge of host response on intrapiscine development of myxospores exists; however, specific stages capable of eliciting immune response is unknown. In cases of parasites exploiting the mucosal surface to invade the host, such as the myxozoans, the ectoparasites such as *I. multifiliis* and *C. irritans*, the hosts counter the invasion by initiating a mucosal immune response. Therefore, the prophylactic vaccines for such pathogens should be designed to stimulate mucosal immunity. Humoral components such as complement impart protection against *P. dicentrarchi* infection in turbot [125]. Both B cell and T cell responses confer protection in several infections, which depends on both the host and the parasite. Therefore, a vaccine for controlling *P. dicentrarchi* should activate the complement, whereas in the latter case should result in B cell- and T cell-mediated responses. Vaccines for fish parasites, e.g., *E. leei* and *K. thyrsites*, should induce T cell-mediated immune response, using vectors such as plasmid DNA or viruses [126]. Vaccines targeting secretory enzymes have shown promising results against the hookworm *Ancyclostoma caninum* [127] and the helminth *Schistosoma mansoni* [128]. A similar approach can be followed for the crustacean parasites e.g., *L. salmonis*, *C. elongatus* and *Argulus* which use many secretions as their immunesuppression strategy. Moreover, the nature of adjuvants affects the desired immune response. For example, cell-mediated immune response can be achieved by the administration of cytokines along with antigen [129] or by heterologous prime-boost approach, wherein the antigen is delivered sequentially using different vaccine platforms [130]. Oil-based adjuvants generate a high antibody titre in blood [113]. Another important aspect of consideration for a successful vaccination is the vaccine delivery, including the mode of administration, the dosage, and the timing.

6.4. Long-Term Immunity

Immunological memory underpins the concept of long-term immunity and vaccination. Vaccination-induced immune memory provides antibodies continuously and maintains memory cells to allow rapid response on exposure to the pathogen. The existence of immunological memory has been demonstrated in fish [131]. However, studies on its duration in response to parasitic infections in fish are limited. An epidemiological investigation on PKD in rainbow trout reported the presence of immunity in surviving fish during the subsequent year after infection [132]. Following exposure to theronts, channel catfish were found to be immune to re-infection after 3 years due to activation of memory B cells and mobilization of Iag-specific antigen-secreting cells into both systemic and mucosal compartments [133]. T cell-mediated immune response has been demonstrated during certain parasitic infections, e.g., *T. bryosalmonae* [134], *E. leei* [35], and sea lice [135]. In vertebrates, during the natural course of infection, following its control, the pathogen load along with T cell declines. The T cell survivors are responsible for immunological memory. Therefore, the extent of expansion and contraction along with the resulting memory depends on several factors such as the type and amount of antigen, the duration of antigen exposure, the site of antigen introduction, and the ability of the antigen or its co-delivered components to activate innate immune responses [93].

7. Challenges in Vaccine Development

Lack of knowledge of biology and the life cycle of parasites are the biggest challenges in developing vaccines against them. Although the life cycle aspects of a few parasites are relatively well understood [93,135], it is unknown for the majority of the fish parasites. Consequently, the cultivation and maintenance of parasites under laboratory conditions becomes difficult; however, it is a necessity for the preparation of antigens and the production of antibodies for vaccine development [136].

Another limitation is the immune escape mechanism of parasites that allows them to evade the direct interaction with the host's immune system. Fish parasites have devised different strategies to escape the host immune defense during infection. For example, *M. cerebralis* invades the nerves, which is an immunologically privileged site with low host immune response [137]. Antigenic variation is an important feature of protozoan parasites that enables them to evade the host immune response and leading to chronic infections [136]. Antigenic variations of *I. multifiliis* have been confirmed by Northern hybridization, suggesting the expression of genes coding immobilization antigens in different life stages of *I. multifiliis* [138]. In addition, antigenic variations, reflected by nine putative I-antigens, have been reported in *C. irritans* from different life stages (tomont, theront, and trophont) using transcriptomic analysis [124]. Further, vaccine development is challenged by poor information on host–parasite interaction and immune response. As an example, although TLRs are known to play an important role in host defense against pathogens, including parasites, the ligand specificity is not yet determined for the majority of the TLRs. In addition, no information is available on different populations of cells in fish expressing TLRs. Such knowledge holds immense importance in the understanding of the resistance mechanisms in fish against parasites, and the development of novel adjuvants and more effective vaccines [139].

Presently, the lack of knowledge of specific antigens of parasites, which trigger the protective immune response is a major constraint. Large-scale production of recombinant proteins that retain the immunological activity similar to the natural parasite protein is an additional roadblock for the development of recombinant vaccines.

8. Role of Omics Technologies in Vaccine Development

Significant research in the study of parasites of human and veterinary importance highlight the potential of omics in the development of vaccines. Analysis of data obtained from omics-studies are an important source of information on SNPs, resistance markers, changes in gene expression, splicing variants, protein modifications, and strain-specificities of parasites. Such information helps in understanding biological attributes of the parasite that may aid in developing disease control strategies [140]. For example, characterization of proteins encoded by the polymorphic loci detected from genomic data of *Plasmodium falciparum* [141], can help in identification of vaccine targets [140]. Hypervariability of parasite antigens is viewed as a major obstacle in developing vaccine against them. Alternative splicing of parasite surface proteins is an important phenomenon that may result in isoforms differing in cell localization, substrate affinities and functions. The structural differences of the isoforms can be large enough to enable the parasite to evade host-immune recognition [142]. RNA-seq based transcriptomic studies have elucidated the role of alternative splicing during cellular differentiation in *Plasmodium berghei* [143]. The majority of the proteins that play pivotal roles in invasion are either stored in the apical secretory organelles or located on the surface of the merozoite, the invasive stage of the *Plasmodium* [144]. Novel secretory organelle proteins and surface-exposed proteins were identified from proteomic analysis of *P. falciparum* [145]. Additionally, an integrated transcriptomic and proteomic approach was applied to describe the *Fasciola hepatica* secretory proteome, thus identifying proteins such as cathepsin, peroxiredoxin, glutathione S-transferase, and fatty acid-binding proteins essential for the design of the first-generation anti-fluke vaccines and flukicidal drugs.

In view of the commonalities existing between mammalian and aquatic parasites (e.g., life cycle with multiple stages, multiple hosts and stage specific antigen profile) and considering the fact that research in mammalian parasitology is far ahead of aquatic parasitology, important lessons need to be learnt from both their failures and successes. The human endoparasites such as *Plasmodium*, *Leishmania* and *Trypanosoma* have been the focus of extensive research for many decades. It has been established by now that early efforts to develop effective vaccines against these parasites have failed as a result of a poor selection of few antigens without the knowledge of antigen repertoire of the parasites [146]. Past efforts have relied on single gene, transcript or protein in vaccine formulations. Likewise, as discussed earlier, vaccination trials against fish parasites have focused on either single or dual target vaccines resulting in limited or suboptimal protection. However, evidences from laboratory experiments [147] and field studies [148] on *Plasmodium* uphold the requirement of a multivalent vaccine wherein a robust immune response towards multiple antigenic determinants can be elicited to provide optimal protection the in host. A multivalent vaccine against human visceral leishmaniasis reportedly elicited significant humoral and cellular responses in pre-clinical trials [149]. Another multivalent vaccine containing stage-specific antigens of *Fasciola hepatica* conferred 83% protection in vaccinated rats [150]. Drawing from these studies from mammalian parasites, it is likely that a multivalent approach might be a better strategy against both endo- and ectoparasites of fish.

In fisheries and aquaculture, the application of omics has just begun, triggered by the advancement as well as cost reduction in NGS technologies [151]. Of the many omics techniques, the application of transcriptomics, genomics and proteomics are emerging in fish parasitology.

8.1. Transcriptomics

Transcriptomics-based studies have widely been used to explore the viral and bacterial pathogen–fish interactions [24]. Similar studies focusing on parasitic diseases in fish are limited. Yet, these restricted works on parasite-induced pathologies have elucidated important phenomena related to the host, such as immune response and concerning the parasite, possible escape strategies and functional biology. Recently, an RNA sequencing-based transcriptome study provided valuable insights into the immune response mounted by brown trout host, as well as modulated host machineries in response to *T. bryosalmonae* infection [152]. Additionally, transcriptome of bryozoan *Fredericella sultana* has been demon- strated, which provides valuable resources for the understanding of the unique biological characteristics and functional transcripts of this important bryozoan species that is the primary host of *T. bryosalmonae* [153]. Transcriptome analysis of *T. bryosalmonae*-infected *F. sultana* revealed 1166 differentially expressed genes with Eukaryotic Initiation Factor 2 signaling as a top canonical pathway and MYCN as a top upstream regulator [154]. Furthermore, the transcriptome of *T. bryosalmonae* from *F. sultana* is found to contain several members of the protease family, e.g., cathepsin L, cysteine protease, zinc metalloprotease, and serine protease [155]. The transcriptome analysis of *E. scophthalmi*-infected turbot suggested the role of IFN-mediated signaling pathways during incipient enteromyxosis as well as the downregulation of complement and acute phase proteins as possible immune evasion mechanisms [156]. De novo assembled transcriptome of *Sphaerospora molnari* blood stages are reported to contain 9436 proteins. This work has provided valuable informa- tion on a proteolytic depot of the parasite consisting 235 putative proteases, mainly of cysteine proteases [157]. Furthermore, genome and transcriptome-based data analysis of *Kudoa iwatai* and *M. cerebralis* was used to study the evolution of endoparasitism in myxozoans [158].

The comparative transcriptomic profile analysis of trophont, tomont and theront stages of *C. irritans* haselucidated the differentially expressed genes with functions in cell division, nutrition analysis and cell growth. Moreover, nine putative immobilization antigen (I-antigen) and protease transcripts, which can be considered as potential vaccine and drug targets were also found [159]. *Argulus siamensis* transcriptome examination

revealed the presence of serine and metalloprotease that are known antigens in many ectoparasites. The study also led to the characterization of the downward signaling molecules of toll pathway [31].

8.2. Proteomics and Genomics

Proteomics and genomics are the other modern high-throughput techniques that have become instrumental in exploring different aspects of both the host and pathogen separately and their interactions at the molecular level. Kumar et al. [160] identified host–parasite protein interaction during proliferative kidney disease using antibody-based purification followed by electrospray ionization mass spectrometry. These identified proteins can be used for understanding the pathogenesis and defense mechanisms of *T. bryosalmonae*. Piriatinskiy et al. [161] identified polar capsule proteins of *C. shasta* using tandem mass spectrometry and suggested that polar capsules and nematocysts are homologous organelles. This study unraveled 112 proteins present in polar capsules of *C. shasta* with diverse functions such as the structural (nematogalectin and minicollagen), protein folding (HSP 70 and isomerases) enzymes involved in poly-γ-glutamate biosynthesis in addition to some novel proteins containing cysteine-rich and proline rich stretches.

Nano-LC ESI MS/MS based proteome analysis of *I. multifiliis* infected fish skin mucus revealed the involvement of innate immune components, e.g., lectins and serpins, in providing protection against the parasite [162]. An iTRAQ (Isobaric tags for relative and absolute quantitation) based quantitative proteomic study identified 2300 proteins in theront and trophont stages of *I. multifiliis*, of which 1520 proteins were differentially expressed in trophonts. These proteins played important roles of binding, catalytic activity, structural molecule activity and transporter activity in the parasite life-cycle [163]. The comparative proteomic analysis of theront, trophont, and tomont of *C. irritans* using 2D-gel electrophoresis and mass spectrophotometry identified different proteins, which could be used as vaccine candidates. Among these, α-tubulin and actin were found to be expressed in all the three developmental stages, whereas enolase was present in theront and trophont and vacuolar ATP synthase (V-ATPases) catalytic subunit α was detected only in theronts [163].

Genome sequencing and analysis of the transcriptionally active macronucleus of *I. multifiliis* has revealed several gene classes functioning in behavior, cellular functions, and host immunogenicity, including protein kinases, membrane transporters, proteases, and surface antigens, providing avenues for selecting vaccine and drug targets [164]. These gene families are identified as lead vaccine targets in many parasites of medical and veterinary importance. For example, members of the protein kinase family, e.g., *Toxoplasma gondii* calcium-dependent protein kinase 2 [165] and proteases [166] are considered promising vaccine candidates against the apicomplexan parasite *Toxoplasma gondii* that infects warm blooded animals [165]. Proteases are excellent shistostome vaccine targets as well. Membrane transport proteins are amongst the most attractive molecular targets of FDA-approved drugs [167].

Overall, the unprecedented wealth of information generated by the analysis of genomic, transcriptomic and proteomic data can better our knowledge of the host as well as the parasite. As discussed, omics can be significant in the identification of suitable antigen candidates of parasites; as such, it is important to note that the efficacy of previously reported vaccines can also be increased. This can be achieved by combining the already reported antigens with the ones selected based on omics data. For example, Jorgensen et al. [75] reported the immunogenicity of the recombinant protein #10; however, it is also noted that in combination with other suitable candidates, it can lead to better protection in fish on vaccination.

Additionally, advanced genome-based techniques can be significant in overcoming many issues concerning complex parasitic organisms, one of the important hurdles being the requirement of parasite culture. This is because maintaining a parasite life-cycle necessitates the maintenance of intermediate and final hosts, which are costly and exhaus-

tive procedures requiring a large amount of time and effort. This is followed by antigen identification, isolation, and purification, which further involves several challenging steps. Besides, omics approaches will possibly be advantageous in cases where many genes and proteins are expressed only during the course of infection and where biomolecules are present in insufficient quantities to be recognized by assays. To date, sequence data are available for a limited number of ectoparasites and endoparasites (Table 4).

Table 4. Available sequence data for parasites: Table enlists the fish parasites for which genome and transcriptome data are available. a: Transcriptome size, b: Genome size, c: Number of contigs, NA: Not Available.

Parasite	Molecular Data Type	Sequencing Platform	Size/No. of Contigs	Accession No.	Reference
		Endoparasite			
Tetracapsuloides bryosalmonae	Transcriptome	NextSeq 550	25908 [c]		[155]
Myxobolus cerebralis	Transcriptome	HiSeq 2000	52972 [c]	GBGI00000000	[158]
Kudoa iwatai	Transcriptome	HiSeq 2000	1637 [c]	GBKL00000000	[158]
Ceratomyxa shasta	Transcriptome	Illumina HiSeq 3000	NA	SRX3741971	[168]
Sphaerospora molnari	Transcriptome	Illumina HiSeq	29560 [c]	PRJNA522909	[157]
Myxobolus squamalis	Transcriptome	Illumina HiSeq 3000	NA	SRX4615721	[169]
		Ectoparasite			
Gyrodactylus salaris	Whole genome	Roche 454 FLX Titanium Illumina GAII	120 Mb [b]	JJOG00000000	[170]
Cryptocaryon irritans	Transcriptome (Trophont)	Illumina HiSeq 2000	2.6 Gb [a]	SRX2417163	[171]
	Transcriptome (Theront)	Illumina HiSeq 2000	3.2 Gb [a]	SRX2417144	[172]
Argulus siamensis	Transcriptome	Illumina HiSeq 2000	46352 [c]	SRX150806	[31]

9. Approach of Multivalent Vaccines

The insights gained from omics data can be invaluable in the development of multivalent vaccines. A multivalent vaccine is a combination of several antigens to elicit a broad protective immune response by the host [173]. The different antigens can be selected from the same parasite, different parasites, parasite strains, and developmental stages. Considering the multiple critical problems associated with the aquatic parasites, particularly the diverse antigenic profile of the developmental stages and parasite strains, a multivalent vaccine, in general, would be an effective strategy against them. For certain parasites like *T. bryosalmonae*, although cell-mediated immune response is speculated to protect fish against infection [58], the exact protective response is not yet defined. Under these circumstances, multiple antigenic candidates can be selected and targeted based on studies on the parasite transcriptome, genome, and proteome along with host–parasite interaction data. Furthermore, a study with inactivated vaccine containing two different isolates of *P. dicentrarchi* did not confer cross-protection in turbot [65]. This could be explained by the fact that the parasite exhibits intraspecific variation both at the morphological and genetic levels [174]. The development of vaccines for such parasites would entail a pan-genomic approach wherein antigens conserved across genomes can be the target for a broad spectrum of protection. A conceptual representation of the multivalent vaccine using an omics dataset is presented in Figure 1.

In designing a multivalent vaccine, genomics, transcriptomics, structural genomics, proteomics, and immunoproteomics can be used to identify a suitable antigen. An approach for the selection of antigenic candidates and the development of vaccines utilizing omics approaches is presented in Figure 2. In this approach, whole-genome sequencing, RNA sequencing, and proteomics data can be used as a starting material of the targeted parasite or the infected host tissue. Further annotation of the data can be carried out using different bioinformatics tools such as Blast, Blast2GO, and UniProt/Swiss-Prot. After annotation,

the gene, RNA transcripts, and proteins can be analyzed for their function as well as the different biological pathways. Vaccine candidates can be administered in fish to check the efficacy and potency of the vaccine.

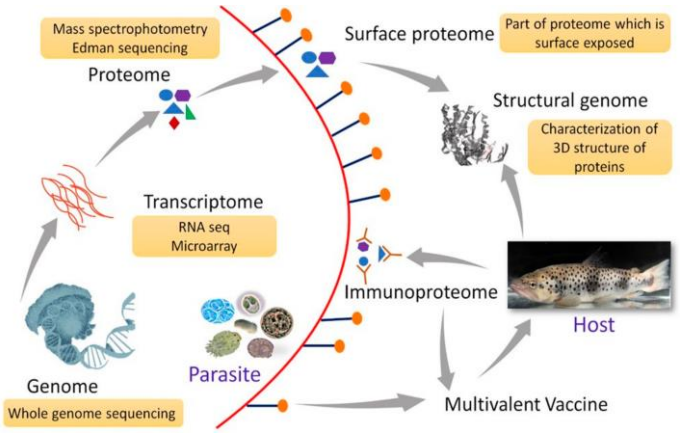

Figure 1. Conceptual representation of multivalent/multiepitope vaccine formulation for fish parasites in the omics era. Analysis of genomic, transcriptomic and proteomic data of parasite and the data obtained from host–parasite interactions enable identifying a suitable antigen as vaccine candidates. Genome sequence contains the entire genetic repertoire of antigens from which novel vaccine targets can be selected. Transcriptome analysis provides insights on the parasite gene expression profile leading to successful establishment and pathology in host. Proteomic analysis provides information on protein expression under specified conditions. It is useful in identification of proteins that are expressed by parasite during infection and the subset of proteins which are present on parasite surface (surface proteome). Surface exposed proteins which are immunogenic in the host can be suitable vaccine candidate. Structural genomics helps to know the three-dimensional structure of proteins produced by an organism and how they interact with antibodies or drugs. Immunoproteomics provides information on the proteins or epitopes which interact with host antibodies.

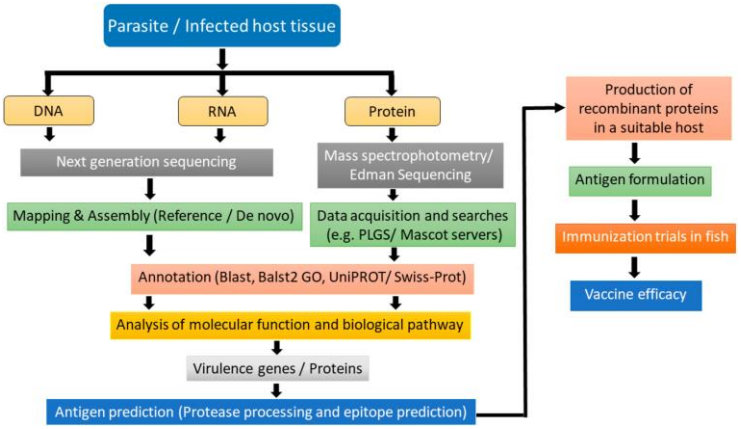

Figure 2. Workflow for identification of vaccine candidates and vaccine formulation using high throughput omics techniques. The material of interest (DNA, RNA or protein) can be extracted from the desired parasite or from-infected host fish tissue. The isolated material serves as sample for next generation sequence analysis (for DNA and RNA) and spectrophotometry and Edman sequencing (for protein). The obtained sequences can be subjected to different bioinformatic tools for analysis and functional annotation of parasite molecules. Based on molecular function and biological pathway analysis, the immunogenic targets (capable of eliciting host immune response) can be selected and their protective epitopes predicted. These molecules can then be produced on a large scale and combined with suitable adjuvants to form vaccine. Subsequently, the vaccine thus produced can be used for conducting trials in suitable fish and its efficacy and appropriate route of administration can be determined.

10. Conclusions

There has been a recent increase in the outbreak of parasitic diseases in farmed and wild fish populations. The general practice for controlling parasitic infestations is to use chemotherapy. Over the past few decades, several concerns have been raised regarding their use, including environmental safety. The experts have recommended vaccines as effective solution to address these issues. Different types of vaccines have shown varying degrees of protection in fish against parasites. However, deeper insights into the host–parasite interaction and parasite's life cycle with different stages are needed to be overcome for the development of successful vaccines. Research on some ectoparasites have been focused with the aim of developing vaccines. However, little attention has been given to the endoparasites. As an example, very little is known about the genes of myxozoans that are induced and expressed in both hosts (invertebrates and vertebrates) and involved in pathogenicity in the fish host during the course of infection. Hence, the identification of the in vivo induced genes of parasites related to disease development is required to improve our understanding of pathogenesis and promote the discovery of novel therapeutic targets. At present, transcriptome, genome, and proteomic data are limited for fish parasites. However, there is a need of large amount of omics data pertaining to more fish parasites for the identification of potential vaccine candidates and designing multivalent vaccines.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-393X/9/2/179/s1, Figure S1: PRISMA flow diagram of study search and selection.

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3.2. Kinetics of Parasite-Specific Antibody and B-Cell-Associated Gene Expression in Brown Trout, *Salmo trutta* during Proliferative Kidney Disease

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Kinetics of Parasite-Specific Antibody and B-Cell-Associated Gene Expression in Brown Trout, *Salmo trutta* during Proliferative Kidney Disease

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Simple Summary: The parasite *Tetracapsuloides bryosalmonae* causes proliferative kidney disease in salmonids. In general, antibodies and B cells play important roles in host defense during chronic infections. In this work, we studied the antibody and B-cell-associated gene expression of brown trout during the course of *T. bryosalmonae* infection. Our results show that antibody responses mounted by brown trout change over time and are maintained at a low level throughout the infection duration. This might be reflective of the host strategy to limit parasite numbers for its survival. Additionally, the expression of genes having important roles in the development, differentiation and signaling of B cells was analysed in the kidney and spleen of infected brown trout from preclinical to post-clinical phases. Our findings indicate that the expression of B-cell-associated genes modulate during the course of parasite development which is suggestive of their critical role for protecting the host against this parasitic invasion. This study brings in important knowledge about the antiparasite antibody and B-cell-associated gene response in infected brown trout, which could be instrumental in developing therapeutic and prophylactic measures against this parasite in future.

Abstract: *Tetracapsuloides bryosalmonae*, a myxozoan endoparasite often causes chronic infection in brown trout. Antiparasite immunity mediated by antibodies and B cells is known as an important determinant of host survival and parasite proliferation during chronic infections. Accordingly, studying their time course during proliferative kidney disease (PKD) might be helpful in improving our understanding of its chronic nature. Therefore, we conducted this study to examine parasite specific serum antibody and B-cell-mediated response in laboratory-infected brown trout at different time points. Brown trout were exposed to the spores of *T. bryosalmonae*, derived from infected bryozoans. Samples were collected at different time points and processed for indirect ELISA, histopathology, and qRT-PCR. *T. bryosalmonae* specific antibody was detected at 4 weeks post exposure (wpe) and it persisted until 17 wpe. Additionally, the expressions of C4A, CD34, CD79A, BL-NK, CD74, BCL7, and CD22 were differentially regulated in the important immune organs, kidney and spleen. To our knowledge, this is the first study addressing anti-*T. bryosalmonae* antibody response in brown trout at different time points. The results from this study provide valuable insights into the processes leading to changes in B cell development, inflammation and antibody production during the course of PKD in brown trout.

Keywords: salmonids; parasite sacs; malacosporean; ELISA; anti-*T. bryosalmonae* antibody; B-cell-mediated response

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1. Introduction

Brown trout (*Salmo trutta*) are a dominant wild fish species in Europe, and are globally introduced into suitable environments. Of late, an increasing number of reports suggest

that wild brown trout populations are declining in European nations such as Austria and Switzerland. Proliferative kidney disease is considered one of the main drivers for their decline, due to its high prevalence in wild brown trout populations [1–5]. The association of this disease with fish stock falloffs stems from the fact that incidences and severity of PKD elevate with elevated water temperatures, resulting in higher mortality. Additionally, infected brown trout transmit the spores of *T. bryosalmonae* to bryozoans [6], and both hosts act as reservoirs for the parasite dispersal in the wild [7].

PKD is widespread among salmonids in Europe and North America [1]. The disease is caused by the myxozoan endoparasite *T. bryosalmonae* and affects susceptible fish of all ages [8]. The major clinical manifestations of this disease in infected fish include renal hyperplasia (kidney swelling) and splenomegaly (spleen enlargement) resulting from intense inflammatory response in these tissues. During advanced pathogenesis, pale gills, indicative of anaemia, are frequently observed [9].

T. bryosalmonae undergo a complex life cycle with the bryozoans as primary and the salmonid fish as secondary host. The spores released from infected bryozoans infect susceptible fish through gills [10]. Subsequently, the parasites migrate to the primary target organ, the kidney, as well as other organs mainly spleen and liver through blood [11]. Interestingly, this parasite can infect and develop clinical symptoms in many salmonids but can complete its life cycle only in some of them, such as brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*) [12]. In rainbow trout, the parasite infection results in severe clinical symptoms. However, infected rainbow trout do not shed viable parasite spores infective for bryozoans, thus halting the parasite life cycle and, consequently, rainbow trout are referred as dead-end hosts [12]. Parasites are transmitted to fish through spores released from infected bryozoans, and in turn, bryozoans are infected by spores released from infected fish, but fish-to-fish transmission of the parasite does not occur [13].

B cells and antibodies are central to immunopathogenesis in hosts during parasitic infections. In addition to producing antibodies, B cells are required for antigen presentation and secretion of cytokines and chemokines, which mediate many pathways in the immune response [14,15]. B cells are characterised by the presence of B-cell receptors (BCR) which are required for B-cell survival and development, as well as antibody production [16]. The important roles of antibodies are well demonstrated during mammalian parasitic infections such as *Plasmodium* [17], *Trypanosoma cruzi* [18], *T. brucei gambiense* [19] and *Necator americanus* [20]. B cells are essential for the development of Th2 cell response for protection as demonstrated against *Leishmania* [21] and helminths infections [22]. Besides, many studies reported the importance of B cells and antibodies in reducing parasite burden [23–26].

While in fish hosts, protective immune responses following infections are reported against several parasites [27], unlike mammals, information on precise functions of antibodies and B cells is rather restricted. Involvement of antibodies and B cells has been demonstrated in some infections such as *Enteromyxum leei* [28], *Enteromyxum scophthalmi* [29] and *Ceratomyxa shasta* [30]. During advanced stages of *T. bryosalmonae*-induced PKD pathogenesis, B-cell activity decreases in the dead-end host rainbow trout [31–33]. In brown trout, this parasite develops chronic infections [34], and the infected fish are capable of releasing viable spores for several years [7]. There are some reports of gene expression studies in the kidneys of brown trout in response to *T. bryosalmonae* [33,35]. Recently, a global transcriptome analysis of the posterior kidney of brown trout during PKD was performed to visualise a broader picture during host–parasite interaction [36]. Despite these significant advances, there is no report of systematic serum antibody response of brown trout against *T. bryosalmonae*. Besides, dominant B-cell response is reported during PKD in brown trout [33], yet gaps remain in its functional understanding. In the previous study, we identified some B-cell-associated genes with roles in haematopoiesis and B-cell receptor signalling to express variably during PKD pathogenesis [36]. This study was thus designed to quantify antiparasite antibody levels in the blood of *T. bryosalmonae*-exposed

brown trout using indirect ELISA and to investigate systematic B-cell-associated gene expression in kidney and spleen at different time points.

2. Materials and Methods

2.1. Fish Sampling

The details of the experimental exposure of brown trout to the *T. bryosalmonae* have been described in our previous study [36]. Briefly, brown trout (n = 69, 12 ± 2 cm) were exposed to the spores of *T. bryosalmonae* released from the laboratory-infected bryozoans [6]. Fish were maintained in 100 litre capacity aquaria with continuous water flow through system at 15 ± 1 °C with sufficient feeding. Blood, posterior kidney and spleen were collected from exposed and unexposed control fish, individually at 2, 4, 6, 8, 10, 12, and 17 weeks post exposure (wpe). Approximately 0.6–0.8 mL blood was drawn from the caudal vein of individual fish, and allowed to clot at room temperature for 1 h and then at 4 °C overnight. Blood samples were centrifuged at 2000× g for 5 min. Sera were collected and frozen at −80 °C for further use. Individual tissue samples were fixed in RNAlater (Sigma-Aldrich, Steinheim, Germany) overnight at 4 °C and stored at −20 °C until further processing for RNA isolation.

2.2. Parasite Detection

Blood smears were prepared on clean microscopic slides, stained with Diff-Quick stain (Labor+ Technik, Goerzallee, Berlin, Germany) and examined by light microscopy. Additionally, the parasite presence in blood was confirmed with nested PCR as described earlier [6]. Furthermore, histology was performed following the method by Kumar et al. [6]. Briefly, tissues were fixed in 10% neutral buffered formalin followed by washing, dehydration and embedding in paraffin wax. Tissue sections of 5 µm were prepared and either stained with Haematoxylin and Eosin or further processed for immunostaining. Immunostaining was carried out using *Tetracapsuloides bryosalmonae* monoclonal antibody P01 (Aquatic Diagnostics, Stirling, UK) following the manufacturer's recommendations. A VECTASTAIN® ABC HRP Kit (Vector laboratories, Burlingame, CA, USA) was used to visualise the antibody–antigen reaction. Afterwards, sections were counterstained with haematoxylin, mounted, and examined.

2.3. Parasite Antigen Preparation

To optimise the indirect enzyme-linked immunosorbent assay (ELISA), parasite antigen was prepared. For this purpose, a large number of parasite sacs (n = 10,000) was collected from the laboratory infected bryozoan *Fredericella sultana*. The parasite sacs were pelleted by centrifugation at 4500× g for 5 min and resuspended in 800 µL of TE buffer. Sample was then homogenised using tissue lyser II (Qiagen) for 2 min at 25 Hz. Subsequently, the sample was freeze–thawed in liquid nitrogen (6X) followed by six rounds of sonication on ice for 10 s at 10 Hz (40% power). Later, the sample was centrifuged at 18,000× g for 5 min at 4 °C. The soluble lysate was collected and stored at −80 °C until use.

2.4. Immunoassay

The level of specific anti-*T. bryosalmonae* antibody in each infected brown trout serum (n = 6) was determined at different time points using indirect enzyme-linked immunosorbent assay (ELISA). The optimisation of the ELISA was accomplished with *T. bryosalmonae* antigen ranging from 1.0 to 10.0 µg/mL, serum dilutions from 1:20–1:800, rabbit anti-salmonid Ig antibody (Bio-Rad, product code: AHP761) dilutions from 1:1500–1:9000, and peroxidase-conjugated antirabbit IgG (whole molecule) antibody (Sigma-Aldrich) dilutions from 1:1500–1:12,000.

A 96-well microtiter plate (MaxiSorp™, Nunc, ThermoFisher Scientific, Roskilde, Denmark) was coated with 100 µL/well parasite antigen (2.0 µg/mL) in bicarbonate coating buffer (pH 9.6, Sigma-Aldrich) and incubated overnight at 4 °C. The wells were washed 3 times with phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 (PBS-T) and

once with PBS and then blocked with 2% bovine serum albumin (BSA, Sigma-Aldrich) in PBS for 1 h at 37 °C. The plate was washed and then 100 µL/well of 1:40 fish serum diluted in 1% BSA was added. After incubating for 1 h at 37 °C, the plate was washed again and rabbit anti-salmonid Ig antibody (1:3000) was added to each well and incubated for 1 h at 37 °C. After the washing, 100 µL peroxidase-conjugated antirabbit IgG antibody (1:6000) were added to each well and incubated for 1 h at 37 °C. After the last washing, TMB peroxidase substrate (Sigma-Aldrich) was added and incubated at room temperature for 15 min. The reaction was stopped with stop reagent (Sigma-Aldrich), and the plate was read at 450 nm with plate reader. Wells with all antibodies and substrates were included as negative controls for the ELISA. The test on each serum sample was conducted in triplicate. The mean values of antibody levels (n = 6) and parasite intensity (as measured below in Section 2.7) in infected brown trout were log2 transformed at each time point. Further, correlations between antibody levels in serum and parasite intensity in organs were evaluated using Pearson's product–moment correlation coefficient (R) in R (version 1.2.5033).

2.5. RNA Extraction

The RNeasy Mini Kit (Qiagen, Hilden, Germany) with an on-column DNase digestion step was used to extract total RNA from the posterior kidney samples of exposed (n = 6) and unexposed control (n = 6) brown trout according to manufacturer's protocol. Initially, individual tissue samples (10 mg) were lysed in RLT buffer containing β-mercaptoethanol. Following this, steel beads were added to the sample and homogenised using TissueLyser II (Qiagen) for 2 min at 20 Hz. Finally, the quality and amount of total RNA extracted were determined by NanoDrop 2000 spectrophotometer and by 4200 TapeStation (Agilent, Santa Clara, CA, USA). Afterwards, reverse transcription was performed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) on one µg of total RNA isolated from infected and control posterior kidney samples.

2.6. Primer Designing

Based on the kidney transcriptome data (NCBI Bioproject ID PRJNA542491) [36], we selected some B-cell-associated genes that showed differential expression (Table 1) to understand the serum antibody/B-cell-associated response against *T. bryosalmonae*.

The genes analysed for their expression in this study include C4A (Complement component 4); CD34 (Haematopoietic progenitor cell antigen CD34-like); CD79A (B-cell antigen receptor complex-associated protein alpha chain-like); CD74 (H-2 class II histocompatibility antigen gamma chain-like); BLNK (B-cell linker protein-like); BCL7(B-cell CLL/lymphoma 7 protein family member B-A-like) and CD22 (B-cell receptor CD22-like). Specific primers were designed for these genes using Primer blast tool from NCBI. The optimum annealing temperature of the designed primers was determined by gradient PCR and primer efficiency of each gene was checked using serial dilutions. Further, unique amplicon of each primer set was sequenced and BLAST analysed to ensure their specificity and sensitivity.

2.7. Reverse Transcription-Quantitative Real Time PCR (RT-qPCR) Analysis

The real-time PCR assay for every gene was performed in a total volume of 10 µL containing 5 µL of 2× SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad), 0.5 µL of forward and reverse primer, 1 µL of nuclease free water and 3 µL of 1:20 dilution of cDNA samples. The real-time qPCR cycling conditions included initial denaturation at 95 °C for 5 min, followed by 37 cycles of final denaturation at 95 °C for 30 s, annealing at 60, 64 and 66 °C for 30 s (annealing temperature was different for primers, presented in Table 1) and elongation at 72 °C. Final elongation was performed at 95 °C for 30 s. Melting curve analysis was carried at 60–90 °C with an increment of 0.5 °C per 10 s. The real-time PCR was performed on CFX96 Touch Real-Time PCR detection system (Bio-Rad, München, Germany). The relative parasite intensity was measured using the mean Cq values of six individual samples at each time point by qPCR using *T. bryosalmonae* 60S ribosomal protein

L7 (RPL7) gene. The expression of host target genes and parasite gene was normalised to the geometric mean of both reference genes: elongation factor alpha and β-actin [35]. The relative gene expression of host genes between exposed and control groups was calculated using $2^{-\Delta\Delta Ct}$ method [37].

Table 1. List of quantitative qRT-PCR primers used in this study. F: forward; R: reverse; bp: base pairs.

Code	Sequence	Size (bp)	Annealing (°C)	Primer Efficiency
C4A F	CTGCCCACTCTGTGTCCTTA	161	64	93.1
C4A R	GGCAACTGAAGGGAAAGACC			
CD34 F	GTGTGTGCGTCAGCTATACA	195	60	93.0
CD34 R	GATCTGGGTTCAGCTTGCAG			
CD79A F	GAGTGGACCGGAGAGACAAC	185	66	96.0
CD79A R	GTAGACATGCAGGAAGGTGC			
CD74 F	ACGAAAAGACTCCCATGACG	144	60	95.0
CD74 R	TCCATCTGTCTCTTCAGGCT			
CD22 F	GTCCAACTCTCCTAACCGCT	191	60	90.0
CD22 R	CAGCAGGTAGGGCTCTAGTC			
BCL7 F	GAAGGTCATGGCGGTCATTG	196	60	95.0
BCL7 R	GTGTGGGTTTTCTGAGGCTG			
BLNK F	TATCATTGGCACTTTGCCCAG	188	60	93.0
BLNK R	GGCTGAACATGCCTTACACC			
RPL7 F	GATTAGGATATCCCCAAGCAACG	152	60	92.5
RPL7 R	AGGTATTCCTCATGTACCTCCAA			
EF alpha F	AGACAGCAAAAACGACCCCC	167	60	90.3
EF alpha R	AACGACGGTCGATCTTCTCC			
β-actin F	CAGGCATCAGGGAGTGATG	127	60	96.5
β-actin R	GTCCCAGTTGGTGACGATG			

The two-tailed unpaired Student's t-test with Welch's correction was employed for analysing the statistical significance of the difference between the groups. Relative host gene expression levels were Log2 transformed and its correlations with *T. bryosalmonae* intensity was analysed using Pearson product–moment correlation coefficient (R) in R (version 1.2.5033).

3. Results

3.1. Clinical Signs

Typical clinical symptoms of PKD such as renal hyperplasia, splenomegaly and pale liver were observed at 6, 8, 10, and 12 wpe (Figure 1). However, none of the exposed fish exhibited clinical signs at 2, 4, and 17 wpe. No clinical symptoms were observed in unexposed control fish.

3.2. Parasite Detection

T. bryosalmonae was detected in blood smears from 2 to 6 wpe (Figure 2A), which was confirmed by nested PCR (data not shown). Furthermore, histological examination showed the presence of presporogonic parasite stages in the kidney (Figure 2B,C). The degeneration of kidney tubules, necrosis and reduction of melanomacrophages in the kidney were evident from histological examination of exposed brown trout mainly from 6 to 12 wpe.

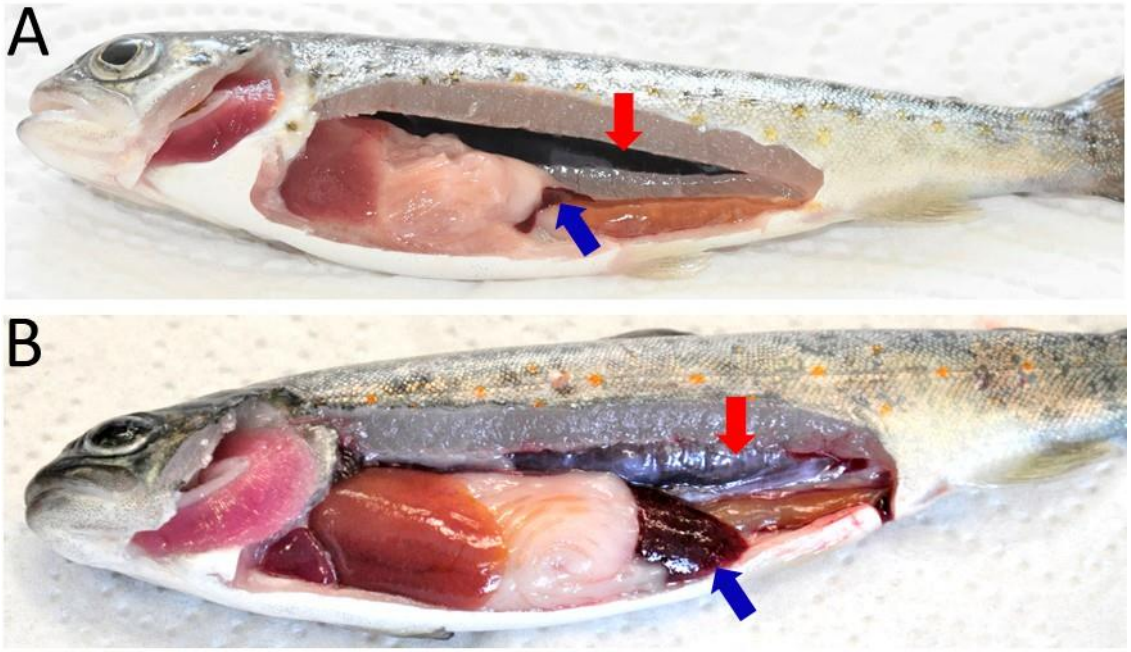

Figure 1. Clinical manifestations of proliferative kidney disease in brown trout. (**A**) Control fish showing normal kidney and spleen; (**B**) infected fish showing renal hyperplasia (enlargement of kidney) and splenomegaly (enlargement of spleen). Red arrow: kidney, blue arrow: spleen.

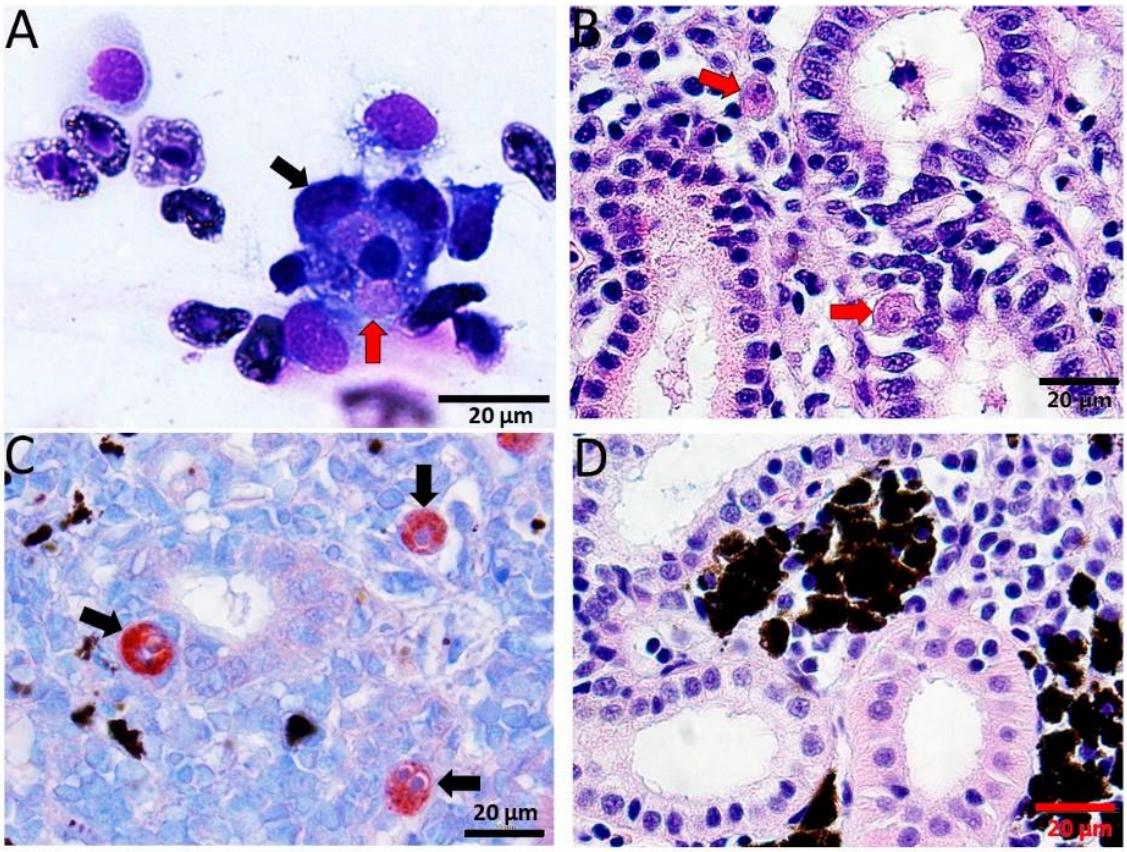

Figure 2. Diff-Quick stained blood smear and histological sections of kidney of infected brown trout. (**A**) Macrophages (black arrow) attached to the *T. bryosalmonae* (red arrow); (**B**) H&E stained sections showing presporogonic stages of the parasite (red arrows) in the renal interstitium and renal tubule; (**C**) immunostained sections showing parasitic stages in renal interstitium and tubular epithelium (black arrows); (**D**) H&E stained sections from control kidney showing normal renal structure.

Immunohistochemical staining of kidney sections revealed *T. bryosalmonae* stages from 4–12 wpe (Figure 2D).

3.3. Anti-T. bryosalmonae Antibody Response

The kinetics of serum antibody response to *T. bryosalmonae* antigen is shown in Figure 3A. First anti-*T. bryosalmonae* antibody response was detected at 4 wpe (OD = 0.82). Thereafter a progressive increase was observed and the peak was observed at 8 wpe (OD = 1.49). At 10 wpe, the antibody levels declined (OD = 0.89) followed by a transient increase before decreasing at 17 wpe (OD = 0.66).

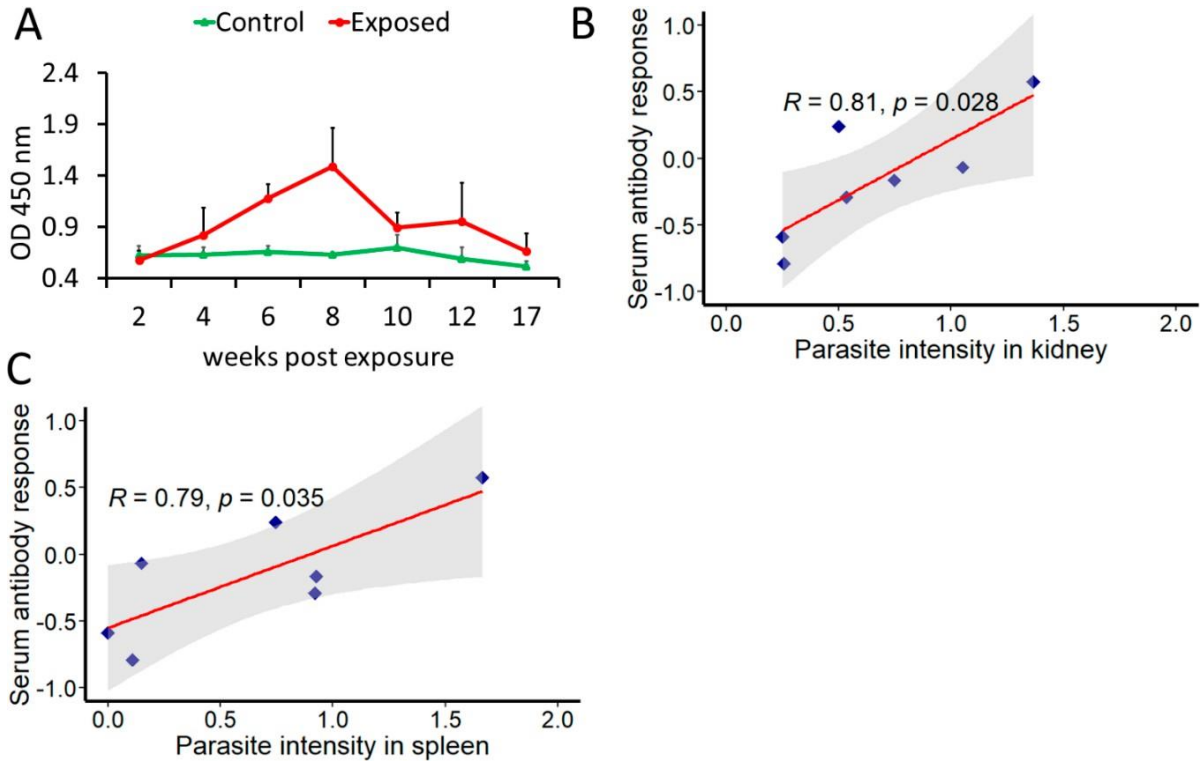

Figure 3. Determination of anti-*T. bryosalmonae* antibody and its correlation with parasite intensity. The mean values of antibody levels and parasite intensity in infected brown trout (n = 6) were log2 transformed (**A**) Kinetics of anti-*T. bryosalmonae* antibody in brown trout using indirect ELISA at different time points; (**B**) correlation between serum antibody levels and parasite intensity in kidney; (**C**) correlation between serum antibody levels and parasite intensity in spleen. For correlation plots, values on x and y axes are log2 transformed. Pearson product-moment correlation coefficient (R) was calculated and statistical significance was tested at p = 0.05. Shaded region represents 95% confidence intervals.

A positive correlation between parasite intensity and antibody levels was found in both kidney (R = 0.81, p = 0.028) and spleen (R = 0.79, p = 0.035) (Figure 3B,C).

3.4. Gene Expression in Kidney and Spleen

The expression profiles of the seven genes (complement cascade gene C4A and B-cell-associated genes) in posterior kidney and spleen tissues of infected and control brown trout are represented as relative fold change (Figures 4 and 5). C4A was upregulated in the kidney at all time points from 2–17 wpe. The highest upregulation occurred at 4 wpe (22.8 folds). On the contrary, in spleen C4A was downregulated at 4 wpe (−2.8 folds) and 8 wpe (−2.21 folds) and no significant change in expression occurred at all other times.

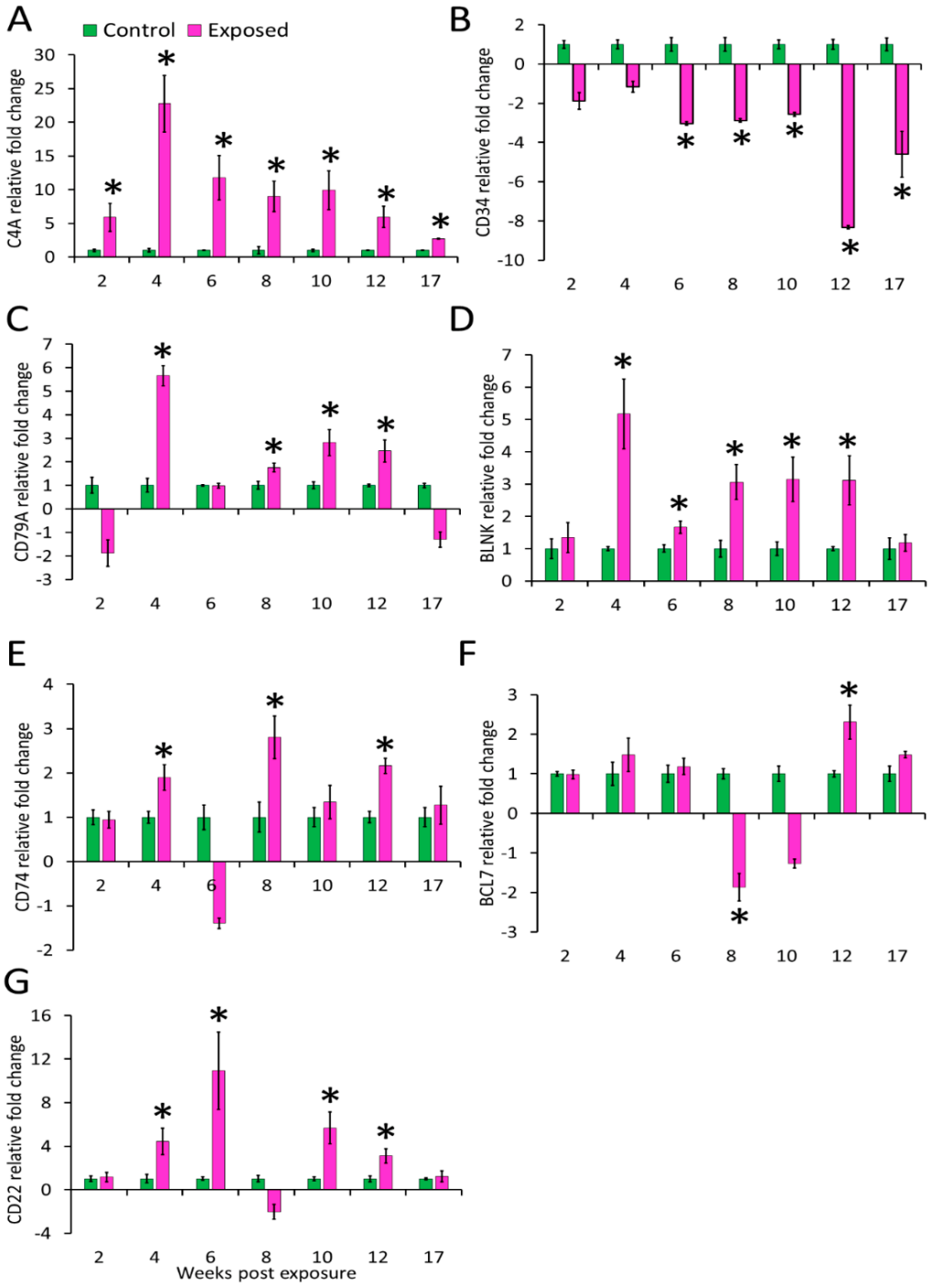

Figure 4. Relative expression of immune genes in kidney. (**A**) C4A; (**B**) CD34; (**C**) CD79A; (**D**) BLNK; (**E**) CD74; (**F**) BCL7; and (**G**) CD22. The relative fold change was first normalised to EF-1 and β-actin, and then represented as a fold change relative to control fish gene expression levels. Asterisks (*) denote the significant difference in relative fold change expression. Each bar shows the mean ± SEM (n = 6).

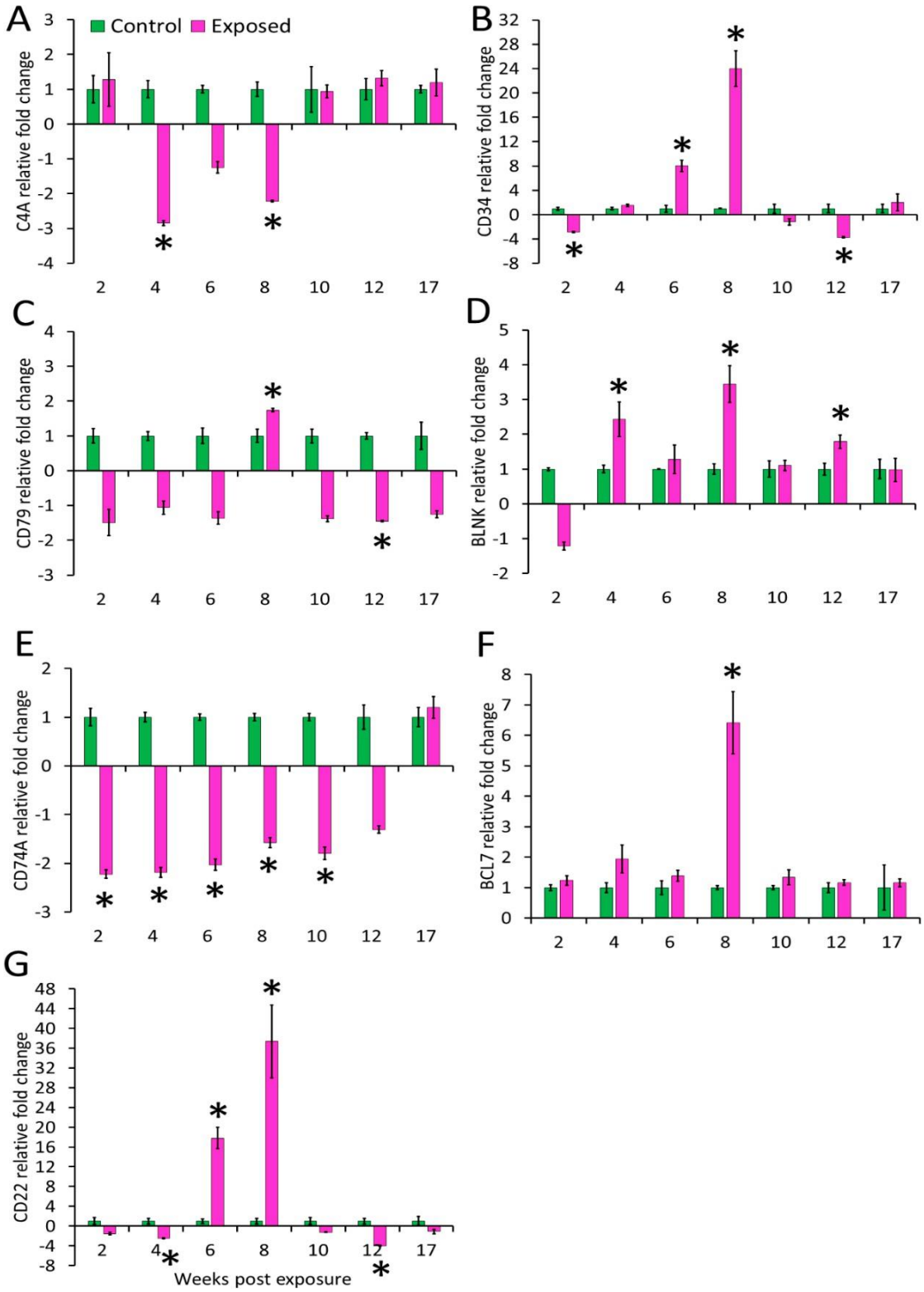

Figure 5. Relative expression of immune genes in spleen. (**A**) C4A; (**B**) CD34; (**C**) CD79A; (**D**) BLNK; (**E**) CD74; (**F**) BCL7, and (**G**) CD22. The relative fold change was first normalised to EF-1 and β-actin, and then represented as a fold change relative to control fish gene expression levels. Asterisks (*) denote the significant difference in relative fold change expression. Each bar shows the mean ± SEM (n = 6).

The expression of CD34 in kidney was decreased at all time points from 6–17 wpe with the highest decrease at 12 wpe (−8.32 folds) and no significant difference in expression was observed at 2 and 4 wpe. In the spleen its expression was increased at 6 wpe (8 folds) and

8 wpe (24 folds) and decreased at 2 (−2.9 folds) and 12 wpe (−3.7 folds) while expression did not alter at rest of the time points. CD79A was upregulated at majority of the time- points (4, 8, 10, and 12 wpe) in the kidney and at other time points, change in expression was not significant. The highest expression of CD79A (6 folds) occurred at 4 wpe. In the spleen, CD79A was found to be significantly upregulated at 8 wpe (1.74 folds) and downregulated at 12 wpe (−1.5 folds). However, the expression of CD79A was not significant at 2, 4, 6, 10, and 17 wpe. CD74 showed a comparable increase at 4, 8, and 12 wpe in the kidney whereas in the spleen its expression was decreased from 2 to 10 wpe.

The B-cell signaling pathway genes were variably expressed during the course of infection. Among the studied genes, BCL7 expression decreased at 8 wpe (−1.9 folds) and increased at 12 wpe (2 folds) in the kidney. However, in the spleen this gene significantly expressed only at 8 wpe (6 folds). BLNK was upregulated from 4 to 12 wpe in kidney and at 4, 8, and 12 wpe in the spleen of exposed brown trout.

In the kidney of exposed group, CD22 was upregulated at 4, 6, 10, and 12 wpe and downregulated at 8 wpe. While in spleen, its expression was decreased at 4 and 12 wpe and increased at 6 and 8 wpe.

Further, correlations were analysed between the parasite intensity and expression of immune genes in both kidney and spleen at different time points (Figure 6). In kidney, significant correlation was observed with parasite intensity only for the expression of CD74 (R = 0.78). In spleen, genes found to be correlated include CD79A (R = 0.8) and BCL7 (R = 0.86). All correlations were considered significant at $p < 0.05$.

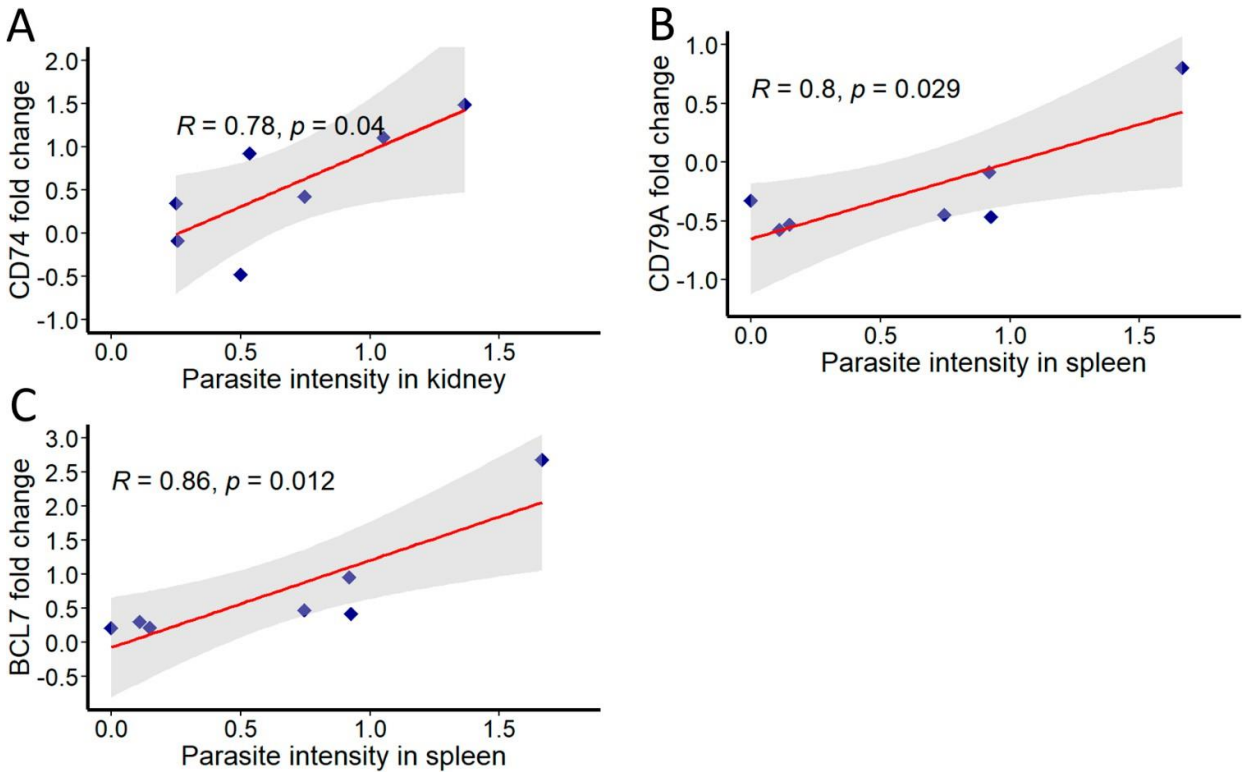

Figure 6. Correlation of *T. bryosalmonae* intensity and immune genes in infected brown trout kidney and spleen. The mean values of fold change and parasite intensity in infected brown trout ($n = 6$) were log2 transformed and correlation coefficient (R) was determined using Pearson product-moment at each time point. Pearson product-moment correlation was calculated and statistical significance was tested at $p = 0.05$. Shaded region represents 95% confidence intervals. (**A**) CD74, (**B**) CD79A and (**C**) BCL7.

4. Discussion

T. bryosalmonae invade fish primarily through the gills and, are later transported to the main target organ kidney through the blood [10]. Earlier studies reported the parasite in blood of rainbow trout at 4 wpe [11] and in wild brown trout [38]. In our study, parasite presence was detected in blood by smears and nested PCR from 2 to 6 wpe (preclinical phase) but not from 8 to 10 wpe (clinical phase) suggesting that the parasites migrate from blood to the organs such as kidney and spleen. Additionally, in the blood of infected brown trout, the parasite was detected by PCR at later time points such as 104 wpe, when there were no clinical signs of the disease [12]. This indicates that the parasite might be present in the blood only during the pre- and post-clinical phases of the disease. The presence of parasite during the post-clinical phase may be due to parasite replication. However, precisely the underlying reason is not yet clear. Further studies focused on in vivo imaging techniques might play a key role in delineating the route of parasite migration from the time of entry in fish and, also for exploring other aspects of interaction between host and parasite. Similar in vivo imaging technology led to the discovery of novel sites of infection with *Trypanosoma cruzi* in murine model [39].

Kidney being the major target organ of *T. bryosalmonae* presents the most prominent disease manifestation. However, *T. bryosalmonae* reportedly invade spleen and other organs as well. In line with earlier reports, we observed swelling in the kidney and spleen from 6 to 12 wpe in the exposed brown trout and hence our study focused on these two major target organs. Previous studies have reported clinical signs mostly from 6 to 10 wpe [12], and 8 to 17 wpe in brown trout [6], and in rainbow trout from 6 wpe [47 days post exposure (dpe)] to 10 wpe (75 dpe) [40], and 8 to 14 wpe [6]. The parasite proliferation triggers intense granulomatous cellular reaction, finally causing swelling in these organs [41,42]. After resolution of clinical symptoms in terms of normal organ structure restoration is confirmed in surviving brown trout [7,12,43] and rainbow trout [33]. In the present work, we did not observe clinical signs at 17 wpe, indicating that the fish had recovered and the damage to the affected organs reversed. In contrast to rainbow trout, where parasite clearance is reported, the brown trout continue to release viable spores probably throughout their lives. However, the mechanism of recovery in both hosts as well as parasite clearance in rainbow trout is not understood entirely. Bailey et al. [44] proposed a few possible explanations for recovery from clinical disease and parasite clearance in rainbow trout. One proposition by the authors was regarding the role of B cells in parasite clearance and recovery of clinical symptoms via restoration of B-cell homeostasis, with the other being the degradation of the parasite, resulting in termination of life cycle in rainbow trout.

Vertebrate hosts respond to parasitic infections by generating antibodies, which are effector molecules of humoral immunity. Antibody-mediated immune response plays a critical role during many parasitic infections to protect, clear parasite or to confer resistance to fish [45]. Antiparasite antibodies have been reported in fish during certain parasitic infections such as *Bothriocephalus acheilognathi* [46], *Trypanosoma sp*. [47] and *Neobenedenia melleni* [48]. Anti-*T. bryosalmonae* antibodies have been detected in the sera of rainbow trout at six wpe by indirect immunofluorescence testing [49], however, this test does not provide quantitative measurement of antiparasite antibody. In the present study, we found the anti-*T. bryosalmonae* antibody levels in the serum of exposed brown trout from 4 to 17 wpe. While in the early phase of infection antibodies progressively increased, they declined in the late phase. The persistence of antibodies even at 17 wpe probably indicates a continuous stimulation of immune system due to parasite presence. Additionally, antiparasitic antibodies were also found to have a positive correlation with parasite intensity in the kidney and spleen. This suggests that antiparasite antibody response during the course of *T. bryosalmonae* infection might be useful for profiling PKD-associated clinical manifestations and developing vaccines, as well as for indicating if the host has mounted an effective humoral response. In fish, three isotypes or classes of antibodies (IgM, IgD, and IgT), while in mammals, five isotypes namely IgM, IgG, IgD, IgT, and IgA are known [50]. Considerable progress has occurred in the field of fish immunology and

much of the knowledge derives from analogy to higher vertebrates. In many mammals, the production of antibody isotypes depends on the mode of B-cell activation and the inflammatory environment surrounding the B-cell subsets is established [51]. For example, during *Plasmodium* infection, the proinflammatory Th1 environment primarily promotes isotype flipping to cytophilic or opsonising Abs, such as IgG1 and IgG3 in humans [52] and IgG2a and IgG2b in mice [53]. Research has elucidated significant upregulation of Th1-like cytokines during PKD in brown trout at day 50 post exposure to parasite [33]. In the present study, maximum antibody titre was observed around the same time, i.e., at 8 weeks post exposure. It is noteworthy that fish do not possess IgG though IgM is known for opsonising pathogens [54]. Similar to mammals, fish antibody isotypes are known to play specific roles, however, not much has been elucidated. In this context, the confirmation of the type of immunoglobulin present at this time point could help in gaining insights into the generated immune response.

While our study chronicles the systematic antiparasite antibody response in *T. bryosalmonae*-exposed brown trout, it has a potential caveat. As previously stated, we used antigens prepared from parasite sacs developed in bryozoans, which are infective for fish. The stages of *T. bryosalmonae* have distinct attributes in primary (bryozoan) and secondary (salmonid) hosts. For instance, *T. bryosalmonae* spores have one amoeboid cell and two polar capsules in bryozoans and two amoeboid cells and four polar capsules in fish [55]. Inside the fish, *T. bryosalmonae* undergo developmental transition through presporogonic and sporogonic stages. Consequently, the response of antibodies targeting presporogonic and sporogonic parasite stages derived from fish might differ, relative to the infective stages derived from bryozoans, due to their different antigenic determinants. Thus, it is plausible that the antibody levels observed may differ little from the parasite antigens derived from kidney in infected brown trout. Saulnier and de Kinkelin [56] demonstrated the antigenicity of parasite antigens prepared from infected kidney towards sera of *T. bryosalmonae* infected rainbow trout during the development of monoclonal antibodies against *T. bryosalmonae* but they did not examine the systematic antiparasite antibody response in infected fish. Testing the sera against homogenates of severely infected fish tissues could have been one approach to address this possibility. Additionally, further study is needed to investigate the extent of antigenic differences present between parasite derived from bryozoans and parasite derived from kidney.

Although antibodies by themselves can act on parasites through multiple mechanisms, complement activation enhances their activity. Considering this, we investigated the expression of the complement cascade gene C4A. As expected, the upregulation of this gene was evident at all the studied time points in the kidney. The constant upregulation of C4A probably reflects the importance of a complementary pathway in *T. bryosalmonae* infection. Together with C3A and C5A, C4A leads to complement pathway activation [57]. Com- plement is amongst the first line of defence in fish hosts and is involved in inflammatory response and pathogen killing through opsonisation. Activation of the complement cascade stimulates degranulation of basophils and mast cells and increases vascular permeability. In the present study, expression pattern of C4A points toward its possible role in persistent vascular permeability for the continued infiltration of immune cells into the kidney during chronic infection. On the contrary, in the spleen significant downregulation occurred at 4 and 8 wpe, whereas at all other time points no significant difference was observed between exposed and control fish.

We also investigated the expression of CD34, which is a marker of haematopoietic stem cells (HSCs). This gene was predominantly downregulated (6 to 17 wpe) in the kidney whereas in the spleen downregulation was noticed during early (2 wpe) and late stage (12 wpe). CD34 was upregulated at 6 and 8 wpe with highest expression (24 folds) at 8 wpe in the spleen. CD34 plays an important role in differentiation of haematopoietic stem and progenitor cells. The downregulation of this gene promotes the differentiation of HSCs into granulocytes and megakaryocyte lineages and suppresses erythroid lineages [58]. This

might be the underlying reason for the presence of inflammatory cells in the posterior kidney of exposed brown trout as also reported in previous studies.

Expression of genes associated with B cell was analysed to understand B-cell-mediated immune response during PKD. B lymphocytes are key players in the immune response against pathogens, particularly through their role in antigen presentation, secretion of cytokines and antibody production in all vertebrates, including fish. Upon antigen contact, BCR are activated and initiate a cascade of processes leading to antibody production. B cells are characterised by the presence of surface markers CD34, CD79 and CD22. Recently, CD79 and CD34 were identified as B-cell markers in salmon [59]. CD79A is a component of the CD79 transmembrane protein. Along with surface immunoglobulins, CD79 forms the signalling unit of B-cell antigen receptor complex for B-cell growth, differentiation and proliferation [60]. In our study, CD79A was upregulated in the kidney at most time points, whereas in spleen upregulation was observed at 8 wpe and downregulation at 12 wpe. Similar CD79A upregulation in fish kidney at early stages and in spleen at later stage is reported during *C. irritans* infection, possibly an indication of B-cell differentiation in the kidney and maturation in the spleen [61].

Many cells including B and T-cells, macrophages, epithelial and endothelial cells express CD74 (MHC class II invariant chain), which is a type II transmembrane glycoprotein [62]. It has been reported from many teleost fish and supposedly functions similar to that in mammals [63]. In this study, CD74 was upregulated in the kidney whereas it was downregulated in the spleen of exposed brown trout. Among the multitude of functions, CD74 serves an important role in inflammation and tissue repair in mammals. CD74 is the receptor of the inflammatory cytokine MIF (macrophage migration inhibitory factor) which regulates the movement of activated immune cells to the site of inflammation [64]. Furthermore in mammals, another member of MIF family, MIF-2, is described, which binds to CD74 and initiates signalling pathways promoting tubular cell regeneration and helps in recovery of injured kidney tissue [65]. Besides, MIF is also known to engage other chemokine receptors including CXCR2 [66], which was significantly upregulated from grade 1 to grade 3 swollen kidneys in PKD-infected rainbow trout [67]. The upregulation of CD74 during the clinical phase of PKD in brown trout, marked by intense inflammatory response, followed by recovery of the kidney tissue might be indicative of a similar role in this fish host. Additionally, CD74 expression in cells increases in response to IFN-γ [68]. The upregulation of interferon-γ in the posterior kidney of brown trout during *T. bryosalmonae* proliferation is reported [33–36]. Nevertheless, more studies are warranted to confirm the role of CD74 during PKD in exposed fish.

B-cell CLL/lymphoma 7 protein family member B-A-like gene (bcl7ba) is a member of the BCL7 gene family. Although this gene has been characterised from many fish species including trout, which is evident from the sequences available in the NCBI database, functional studies are lacking. In *Caenorhabditis elegans,* BCL-7 regulates terminal cell differentiation in somatic stem-like cells, whereas in human gastric cancer cells it functions to positively regulate apoptosis by inhibiting the expression of antiapoptotic factors [69]. Interestingly, in our study, this gene was significantly downregulated at 8 wpe (−1.9 folds) and upregulated at 12 wpe (2.3 folds) in the kidney. A probable explanation for this effect, could be that this gene has a role in tissue regeneration during recovery following *T. bryosalmonae* infection [41]. In the spleen, it was found to be highly upregulated (6.4 folds) only at 8 wpe in the spleen. The genes of BCL7-family members share a unique N-terminal domain that has been evolutionarily conserved in the animal kingdom, but the remaining sequences lack homology [70]. The results from this study are indicative of an important role of BCL7 family genes during PKD pathogenesis and hence should be studied in greater depth.

The adaptor protein, BLNK, functions in the BCR signalling pathway [71]. This protein is involved in the activation of B-cell receptor-associated kinase to downstream signalling pathways, affecting B-cell activation [60]. We observed significant upregulation of this gene in the kidney from 4 to 12 wpe and in spleen at 4, 8, and 12 wpe. BLNK regulation

in different organs was reported following *C. irritans* infection. Mo et al. [72] reported the differential regulation of BLNK in head kidney, spleen, skin and gill tissues. They suggested proliferation and differentiation of B cells and macrophages in the primary haematopoietic organ, followed by migration to infected sites.

Significant upregulation of CD22 was evident at 4, 6, 10, and 12 wpe in infected kidney. In the spleen at early (4 wpe) and late time points (12 wpe) it was significantly downregulated whereas at 6 and 8 wpe it showed upregulation. CD22 is a lectin-like member of the Ig superfamily expressed exclusively by mature B cells. CD22 acts as an antagonist to B cell activation most likely by enhancing the threshold of BCR-induced signals [73]. Upregulation of CD22 has been demonstrated in skin during *C. irritans* infection in *Epinephelus coides* [74].

Given the important role of B cells in modulating the fate of *T. bryosalmonae* and its pathogenesis in the host, we examined the correlation between the parasite intensity and the expression of the above-discussed B-cell-associated genes in kidney and spleen. Overall, parasite intensity in kidney significantly correlated only with CD74 expression. Likewise, the expressions of CD79A and BCL7 showed a positive association with parasite intensity in spleen. As discussed previously, the positive correlation of CD74 probably reflects its important role in controlling parasite burden by mediating inflammatory response in the kidney. Similarly, the positive associations of CD79A and BCL7 indicate that these genes play a crucial role in the defence against the parasite in spleen.

5. Conclusions

Our study demonstrates the kinetics of anti-*T. bryosalmonae* antibody response against parasite sac antigens in chronically infected brown trout for the first time. The observed trend of parasite-specific antibodies in the sera and presence of parasites in the kidney of infected fish point towards a sustained activation of the immune system. Though this study generates significant information, there could be a possible extension to our work, wherein antibodies specific to various developmental stages of *T. bryosalmonae* in fish can be studied to gain enhanced understanding of the specific humoral response against this parasite of economic and ecological relevance. The kinetics of a parasite-specific antibody response may serve as markers of protection and thus may be important for vaccine development. We also investigated the expression pattern of complement cascade gene C4A and some B-cell-associated genes for the first time during PKD pathogenesis, which provides valuable insights into the observed inflammation, host survival and recovery. The antibody and B-cell-mediated response observed in this study reflect a strategy of parasite persistence and host survival. Although the differential modulation of these genes in kidney and spleen in course of the infection suggest an important role of these genes in immunopathogenesis, more studies are required to define the role of B cells which might be helpful in developing therapeutic strategies for controlling *T. bryosalmonae* infections.

Author Contributions: Conceptualisation, G.K. and M.E.-M.; methodology, G.K. and S.S.; software, validation, formal analysis and investigation S.S. and G.K.; supervision, G.K.; writing—original draft preparation, S.S.; writing—review and editing, G.K. and M.E.-M.; project administration and funding acquisition G.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was approved by the institutional ethics committee of the University of Veterinary Medicine Vienna and the national authority, according to §26 of the Austrian Law for Animal Experiments, Tierversuchsgesetz 2012 under approval number BMWFW-68.205/0181-WF/V/3b/2017. All experiments were performed in accordance with relevant guidelines and regulations.

Informed Consent Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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3.3 Identification of *vivo*-induced antigens recognised by sera of infected brown trout with *Tetracapsuloides bryosalmonae*

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Identification of *in vivo* induced antigens of the malacosporean parasite *Tetracapsuloides bryosalmonae* (Cnidaria) using *in vivo* induced antigen technology

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Tetracapsuloides bryosalmonae is a malacosporean endoparasite that causes proliferative kidney disease (PKD) in wild and farmed salmonids in Europe and North America. The life cycle of *T. bryosalmonae* completes between invertebrate bryozoan and vertebrate fish hosts. Inside the fish, virulence factors of *T. bryosalmonae* are induced during infection or interactions with host cells. *T. bryosalmonae* genes expressed *in vivo* are likely to be important in fish pathogenesis. Herein, we identify *in vivo* induced antigens of *T. bryosalmonae* during infection in brown trout (*Salmo trutta*) using *in vivo* induced antigen technology (IVIAT). Brown trout were exposed to the spores of *T. bryosalmonae* and were sampled at different time points. The pooled sera were first pre-adsorbed with antigens to remove false positive results. Subsequently, adsorbed sera were used to screen a *T. bryosalmonae* cDNA phage expression library. Immunoscreening analysis revealed 136 immunogenic
T. bryosalmonae proteins induced in brown trout during parasite development. They are involved in signal transduction, transport, metabolism, ion-protein binding, protein folding, and also include hypothetical proteins, of so far unknown functions. The identified *in vivo* induced antigens will be useful in the understanding of *T. bryosalmonae* pathogenesis during infection in susceptible hosts. Some of the antigens found may have significant implications for the discovery of candidate molecules for the development of potential therapies and preventive measures against *T. bryosalmonae* in salmonids.

KEYWORDS

myxozoa, proliferative kidney disease (PKD), *Tetracapsuloides bryosalmonae*, antigen, *in vivo* induced antigens (IVIAT), host-parasite interaction, salmonids

Introduction

Tetracapsuloides bryosalmonae is an endoparasitic myxozoan that completes its life cycle between an invertebrate (bryozoan) and a vertebrate (salmonid fish) host. The parasite causes proliferative kidney disease (PKD) in various species of salmonids (Anderson et al., 1999; Feist et al., 2001) and is reported in Europe and North America, leading to severe losses in trout farms (Hedrick et al., 1993). While the economic impact of the disease makes it an important factor for aquaculture (Clifton-Hadley et al., 1986), PKD is also suspected to be a factor contributing to the decline of wild brown trout and salmonid populations in Europe (Wahli et al., 2002; Sterud et al., 2007; Okamura et al., 2011; Skovgaard and Buchmann, 2012; Dash and Vasemägi, 2014; Vasemägi et al., 2017; Waldner et al., 2020). In Austria, a 92% overall prevalence of *T. bryosalmonae* was detected among wild brown trout sampled from the river Wulka, indicating a possible reason for their decline in this river. The findings suggest that wild brown trout might soon be extinct in the river Wulka of Austria (Waldner et al., 2020). In North America, *T. bryosalmonae* killed thousands of mountain whitefish (*Prosopium williamsoni*) in Montana's Yellowstone River in August 2016, with a 90% mortality rate, which suggested *T. bryosalmonae* is endemic in Montana (Hutchins et al., 2021) and can cause impacts on native salmonid populations.

The ability of a pathogen to cause disease depends primarily on its ability to sense and adapt to a variety of host environmental signals. A parasite, while invading its host, senses the *in vivo* environment by inducing or repressing the expression of specific genes. A repetitive cycle of cell invasion and replication stimulates the expression of these parasite genes, resulting in disease. Thus, parasite genes expressed *in vivo* are likely to be important virulence mechanisms and potential therapeutic targets. For example, serine and cysteine protease genes of the myxozoan *Myxobolus cerebralis* induced in fish during parasite development are involved in host tissue invasion, virulence, and the initiation of sporogenesis (Kelley et al., 2003; Kelley et al., 2004; Dörfler and El-Matbouli, 2007). In *Ceratonova shasta*, genes associated with parasite adhesion and migration are found to be responsible for the enhancement of virulence in the parasite (Alama-Bermejo et al., 2019). In addition, single nucleotide polymorphism in the motility and protease genes of *C. shasta* affects the outcome of virulence of the parasite in the salmonid host (Alama-Bermejo et al., 2020). Microneme protein MIC11, dense granule protein 5, and calmodulin of *Toxoplasma gondii* are involved in cell division and parasitophorous vacuole maintenance/parasite survival after cellular invasion and pathogenesis in pigs (Tao et al., 2014). The identification of *T. bryosalmonae* virulence genes would improve our understanding of *T. bryosalmonae* infection and promote the discovery of novel therapeutic targets, as well as provide insights into the infection process of the parasite. For this reason, in this study we aimed to identify *T. bryosalmonae* genes that are induced in brown trout during the course of infection. This was achieved using an *in vivo* induced antigen technology (IVIAT), an immunoscreening technique that identifies parasite antigens expressed during infection of the fish host. This approach used pooled serum from brown trout infected with the *T. bryosalmonae* to identify *in vivo* induced (IVI) genes expressed during infection.

Materials and methods

T. bryosalmonae collection

Our laboratory maintains the life cycle of *T. bryosalmonae* between fish and bryozoan hosts according to Kumar et al. (2013), where we regularly hatch statoblasts and grow bryozoan colonies, harvest parasite sacs and infect brown trout, and cohabitate specific pathogen free *Fredericella sultana* colonies with infected brown trout. For this study, a large number of *T. bryosalmonae* sacs (n ≈ 20,000) were collected from the laboratory-infected colonies by manual microdissection under a stereomicroscope, then transferred to a Petri plate filled with water. The clean isolated parasite sacs were pipetted into 2 ml Eppendorf tubes and centrifuged at 5000 X g for 5 min. The parasite pellets were stored at -80°C for antigen preparation or directly resuspended in an RLT buffer containing b-mercaptoethanol for RNA extraction. Similarly, *T. bryosalmonae* infected *F. sultana* zooids were collected in RNAlater and stored at -20°C for further molecular studies.

T. bryosalmonae expression library

The *in vivo* induced genes of *T. bryosalmonae* in brown trout host were identified using IVIAT and the steps used in this study are presented in a flowchart (Figure 1). Total RNA was extracted from the frozen parasite sacs using the RNeasy mini kit (Qiagen, Hilden, Germany). An on-column DNase (Qiagen) digestion step was included according to the manufacturer's protocol. RNA integrity was measured on the 4200 TapeStation using the RNA Screen Tape assay (Agilent Technologies, USA). Messenger RNA was purified from the extracted RNA (18 µg) sample using the Oligotex mRNA kit (Qiagen) according to the manufacturer's protocol.

One hundred nanograms of mRNA were synthesized into first-strand cDNA using the SMART cDNA synthesis kit (Clontech Laboratories, USA), then into second strand cDNA by using an LD-PCR protocol. Subsequently, digestion and fractionation steps were performed. The resulting cDNA library was ligated into lTriplEx2 vector (Clontech Laboratories) and then packed in a high-efficiency

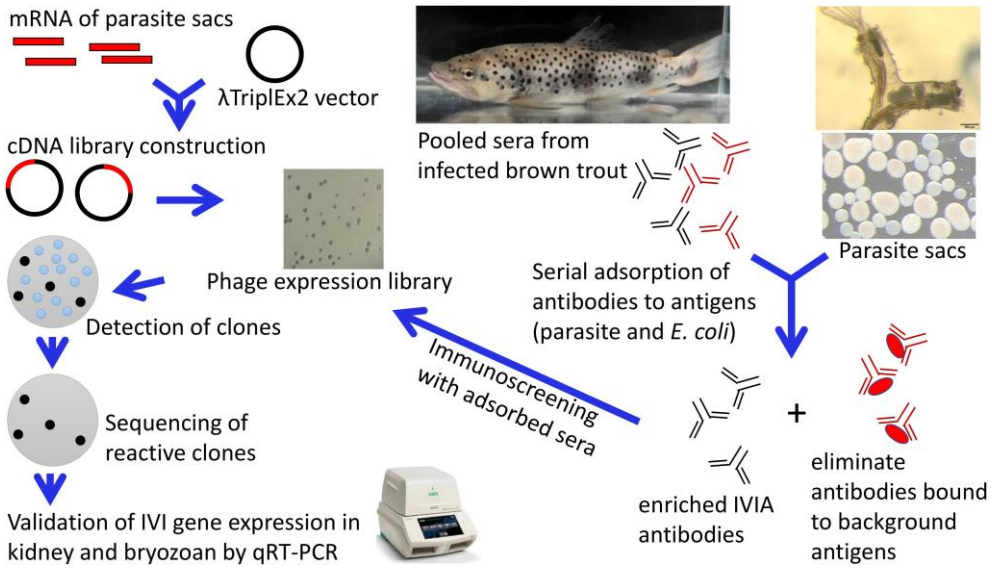

FIGURE 1
In vivo-induced antigen technology applied to *Tetracapsuloides bryosalmonae*. An expression library of *T. bryosalmonae* was constructed into the lTriplEx2 vector. Adsorption of pooled sera were performed to remove antibodies against three forms of parasite sacs and *E. coli* XL1-Blue antigens (native, ultrasonic, and heat-denatured). This process eliminates antibodies in the sera that react to antigens constitutively expressed by *T. bryosalmonae* in the bryozoan body cavity and eliminates background that may arise from the *E. coli*. Immunoscreening of expression cDNA library was performed with adsorbed pooled sera. Positive clones were sequenced, subcloned and validated in infected kidney of brown trout and infected bryozoan by qRT-PCR.

system using Gigapack III Gold Packaging Extract (Agilent Technologies, USA) according to the manufacturer's instructions. Resulting library was amplified and titered. To determine the percentage of recombinant clones, blue–white screening was performed on LB/MgSO4 agar plates containing IPTG and X-gal. Thirty clones were randomly picked and tested by PCR analysis using the vector primers (F 5` CTCGGGAAGCGCGCCATTG TGTTGGT 3` and R 5` TAATACGACTCACTATAGGGCG 3`). PCR amplicons were sequenced at the LGC Genomics GmbH (Berlin, Germany) to determine the presence of an insert.

Sera samples

The sera were previously collected from brown trout infected with *T. bryosalmonae* at 2, 4, 6, 8, 10, 12, and 17 weeks post exposure (wpe) (Sudhagar et al., 2019). Kidney samples were also collected at the same timepoints in RNAlater and stored at -20 °C for molecular studies. Individual serum samples were tested for the presence of *T. bryosalmonae* specific antibodies using indirect ELISA (Shivam et al., 2021). Anti-*T. bryosalmonae* antibody was first detected at 4 wpe and persisted until 17 wpe, thus, these sera were selected for this study. For use in IVIAT, equal volumes of each serum sample were pooled and adsorbed with different antigens as described below. Serum samples from specific pathogen free brown trout that were negative for *T. bryosalmonae* were used to verify the specificity of selected clones. All the serum samples were stored at -80°C until use.

Preparation of antigens

Three forms of parasite sacs and *E. coli* XL1-Blue antigens were prepared: native, heat-denatured, and non-heat-denatured cell lysates. Parasite sacs and *E. coli* XL1-Blue cells were suspended in Tris-HCl EDTA buffer containing complete protease inhibitor cocktails (Roche, Germany). Both suspensions were divided into three portions. To prepare the non-heat-denatured antigens, parasite sacs and *E. coli* XL1-Blue cells were freeze-thawed six times in liquid nitrogen followed by six rounds of sonication on ice for 10 seconds at 10 Hz. To prepare the heat-denatured antigens, both parasite sacs and *E. coli* XL1-Blue cells were placed at 95°C for 10 minutes.

Adsorption of sera

Adsorption of pooled sera was performed to remove antibodies against parasite sacs and *E. coli* XL1-Blue. This procedure removes antibodies in the sera that react to antigens constitutively expressed by *T. bryosalmonae* in the bryozoan body cavity and eliminates any background or false positives that

may arise from the *E. coli* host strain provided with the SMART cDNA library construction kit. The pooled sera from infected brown trout were adsorbed with native, heat-denatured, and non-heat-denatured cell lysates. Native parasite sacs and *E. coli* XL1-Blue cells were resuspended in pooled sera with the addition of 10 mM EDTA and a complete cocktail of protease inhibitors. The sera-parasite sacs suspension was mixed overnight with gentle rocking (10 rpm) at 4°C. The absorbed sera were recovered by centrifugation at 5,000 x g for 10 minutes at 4°C. Afterwards, heat-denatured and non-heat denatured cell lysates of parasite sacs and *E. coli* XL1-Blue were immobilized on Protran nitrocellulose membranes 0.45 mm (Sigma Aldrich, Germany) overnight at 4°C on a rocking table. Membranes were dried on Whatman papers to remove the excess buffer. Subsequently, membranes were rinsed 5 times in 10 ml of Tris- Buffered Saline (TBS) composed of 10 mM Tris–HCl (pH 7.5) and 150 mM NaCl containing Tween-20 (0.05%). The membranes were blocked by immersion for one hour in 10 ml of 5% immunoblot blocking reagent (Sigma Aldrich) in TBS-T at room temperature on a rocking table. Then membranes were rinsed three times with 10 ml of TBS-T. The pooled sera were incubated on top of the lysate-coated membranes at 4°C overnight with gentle rocking (10 rpm). The same adsorption processes were performed in parallel with pooled sera from non- infected control brown trout. Each adsorption procedure was repeated three times. The final adsorbed sera were centrifuged at 5,000 x g for 10 minutes at 4°C and absorbed sera were recovered by aspiration. A 100 µl aliquot of absorbed sera was collected after the adsorption step to evaluate the efficacy of the process. The remaining adsorbed sera were aliquoted and stored at -80°C. The efficiency of the adsorbed sera from each step were tested by indirect ELISA according to the previously optimized protocol (Shivam et al., 2021).

Immunoscreening of *T. bryosalmonae* cDNA library

For primary screening, 10 µl of 1:10,000 diluted cDNA library was mixed with 500 µl of overnight culture of *E. coli* XL1-Blue suspended in 7.5 ml of 10mM $MgSO_4$ and incubated at 37°C for 15 min. Five hundred microlitre melted (45°C) LB/ MgSO4 top agar was added to the phage-bacteria mixture. Subsequently, the mixture was poured immediately onto a pre-warmed (37°C) LB/MgSO4 agar plate (150 mm size) and swirled until the surface of the plate was covered. The plates were incubated at 37°C overnight. The next day, plates were overlaid with Protran nitrocellulose membranes (132 mm size) pre-soaked in IPTG (10 mM), which were left for 5 hours at 37°C to transfer phage particles to the membranes. Afterwards, membranes were rinsed 5 times with TBS-T and blocked with 5% immunoblot blocking reagent for 2 hours at room temperature before being incubated with the pooled optimized adsorbed sera (1:100, diluted in 1% BSA - Dulbecco′s phosphate buffered saline) overnight at 4°C with gentle rocking. Subsequently, membranes were washed with TBS-T five times for 5 min and then incubated with rabbit anti-salmonid polyclonal antibody, Bio-Rad (1:5,000) for 1 hour at 37°C. After washing, peroxidase-conjugated anti-rabbit IgG (whole molecule) antibody, Sigma Aldrich (1:8,000) was applied for 1 hour at 37°C and washed.

The immunoblots were developed using membrane substrate (Sigma Aldrich) according to the user manual. Reactive clones were identified manually by comparing with control and negative control sera. Positive plaques were removed from the gel as a plug, using a cut-off end of a 200 ml pipette tip and then placed into 500 ml of SM buffer containing 3% chloroform. Phages were allowed to diffuse into SM buffer overnight at 4°C. After centrifugation, the supernatants were transferred to new tubes and stored at 4°C. Each positive plaque was screened at least two additional times to confirm that it reacts with the adsorbed sera. *In vivo* excision was performed to convert the recombinant phages to recombinant plasmids according to the user manual. The inserts of *T. bryosalmonae* were determined by PCR amplification using the vector primers (F and R). Sequencing was performed at the LGC Genomics GmbH, Berlin, Germany. The obtained sequences were analysed using the BLASTX search against NCBI's non-redundant protein database and the *T. bryosalmonae* transcriptome assembly (PRJNA680464, Kumar et al., 2021).

Gene ontology

The sequences identified by IVIAT were subjected to gene ontology (GO) analysis for biological process, molecular function, and cellular components. GO analysis was performed using Blast2GO version 5.2 software (Gotz et al., 2008). Briefly, the FASTA sequences were subjected to BLASTX in the NCBI non-redundant database with a threshold of E-value 1.0E−3. Subsequently, mapping was done to retrieve the GO terms of the BLAST results. Simultaneously, InterPro annotation was done to retrieve the information regarding protein domain/motif and the obtained information was merged with the already available GO terms. Subsequently, the GO terms were annotated for their functional characteristics.

Validation of *in vivo*-induced genes

Fourteen genes were selected for validation analysis based on various criteria such as function, involvement in virulence factors and importance in cellular activities. The expression patterns of the genes were validated in infected kidney and infected bryozoan by quantitative real time PCR (qRT-PCR). RNA (n = 6) was extracted from the posterior kidneys of the

experimentally infected brown trout (12 wpe) and infected bryozoan *F. sultana* colonies using an RNeasy mini kit (Qiagen) including DNase treatment and was then reverse transcribed to cDNA using iScript cDNA synthesis kit (Bio- Rad). Primers specific for each target gene of *T. bryosalmonae* (Supplementary Table 1) were designed using NCBI Primer BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). qRT-PCR was optimized using a temperature gradient. The PCR amplicon of each specific primer was sequenced, and BLAST analysed to ensure their specificity and sensitivity. A CFX96 Touch Real-Time PCR detection system (Bio-Rad) was used to assess the *in vivo* expression of the target genes. qRT-PCR had a final volume of 10 µl, which contained 3 µl of 1:20 fold diluted cDNA, 0.4 mM of each primer, 1X SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad), and sterile DEPC-treated distilled water. qRT-PCR included initial denaturation at 95°C for 5 minutes, followed by 37 cycles of denaturation at 95°C for 30 seconds, annealing at 60-64°C for 30 seconds, and elongation at 72°C. Final elongation was performed at 95°C for 30 seconds. A melting-point curve analysis was carried from 60–95°C with an increment of 0.5°C per 10 seconds to detect any non-specific PCR products. All samples were analysed in triplicate. The expression level of each tested gene was normalized to the reference genes: 60S ribosomal protein L18 (Gorgoglione et al., 2013) and NADH dehydrogenase. The fold change of *in vivo* induced genes in the infected kidney relative to its infected bryozoan was calculated by the 2^{-DDCt} method (Livak and Schmittgen, 2001). The statistical difference between infected kidney and infected bryozoan samples was determined using the two-tailed unpaired Student's t-test with Welch's correction using IBM SPSS software version 25.0.

Results

Adsorption of pooled sera

Most of the reductions in OD values of pooled sera were observed after adsorption steps with the parasite sac antigens. OD values from 0.914 to 0.076 were detected for pooled sera diluted 1:100, which was the optimal dilution used for the immunoscreening of the cDNA library. As shown in Figure 2A, there was a significant reduction in reactivity of the pooled sera after the parasite sac lysates adsorption step. In contrast, there was considerably less elimination of antibodies recognizing *E. coli* XL1-Blue antigens (Figure 2B), suggesting that the adsorption with *E. coli* antigens had no effect on the antibodies against *T. bryosalmonae* in the sera of brown trout.

In vivo induced genes of *T. bryosalmonae*

The phage cDNA library (1.89 x 10^6 pfu/ml) had a recombination efficiency of 97.88% (Figure 3). About 40,000 clones were screened from *T. bryosalmonae* phage cDNA library using the adsorbed pooled positive sera from infected brown trout. A total of 136 clones were identified as immune-reactive;

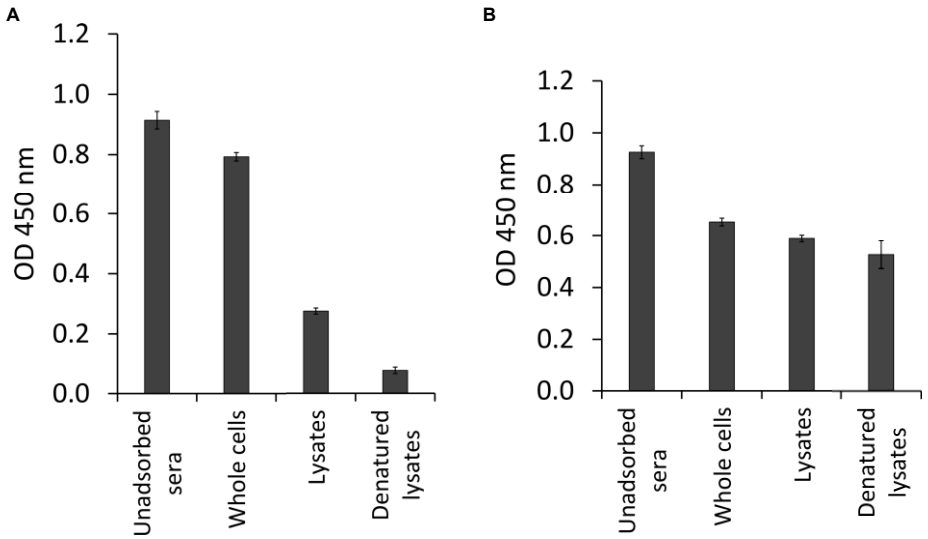

FIGURE 2
Antibody determination of pooled sera from infected brown trout after sequential adsorption steps by indirect ELISA. (A) pooled sera adsorbed by three different forms of *T. bryosalmonae* (native parasite sac whole cells, ultrasonic lysates, and heat-denatured lysates), and (B) pooled sera adsorbed by three different forms of *E. coli* XL1-Blue (native bacterial whole cells, ultrasonic lysates, and heat-denatured lysates).

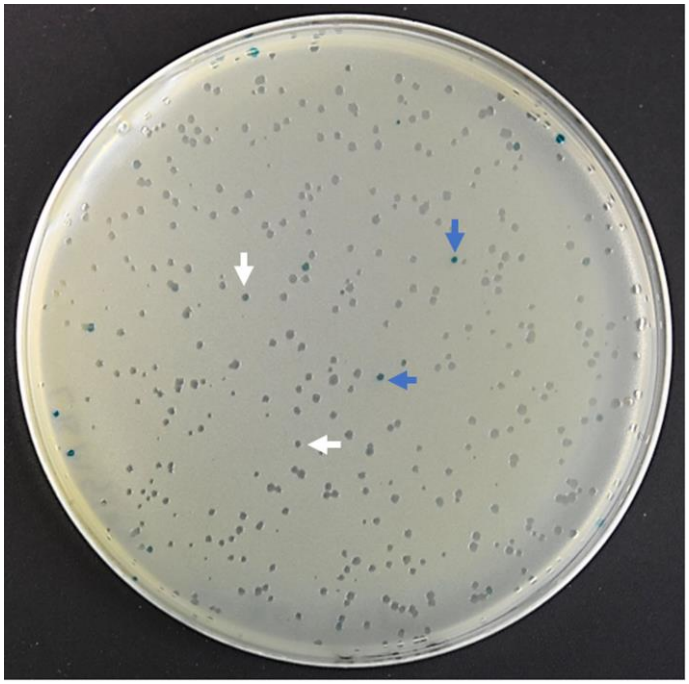

FIGURE 3
The efficiency of recombinant phage cDNA library. To determine the percentage of recombinant clones, blue–white screening was performed on LB/MgSO4 agar plates containing IPTG and X-gal. White plaque indicates recombinant plaque and blue plaque indicates nonrecombinant plaque.

these were then isolated and confirmed by sequencing and NCBI BLAST analysis. The complete list of identified IVI genes by the immunoscreening is presented in Supplementary Table 2. Additionally, the functions and locations of some of the identified IVI genes are provided in Table 1. PCR products indicated variation in the size of inserts in the cDNA library (Supplementary Figure 1).

Gene ontology

In vivo induced genes were categorised into biological process, molecular function, and cellular components (Figure 4). Majority of biological process were associated with the cellular, metabolic, and transport processes. In terms of molecular function, genes were enriched in catabolic activity, binding, and structural molecular activity. Cellular components of genes were abundant in organelle, cytoskeleton, membrane, and ribosome.

Validation of IVI genes

The expression patterns of the fourteen IVI genes in *T. bryosalmonae* dwelling in the infected brown trout kidney and infected bryozoans are shown in Figure 5. Five IVI genes were significantly ($p < 0.05$) upregulated: Ras-related protein Rab-35, calmodulin, transmembrane emp24 domain-containing protein 7, F-actin-capping protein, and vesicle-associated membrane protein 3. Four IVI genes were significantly ($p < 0.05$) downregulated in *T. bryosalmonae* from infected kidney, compared to their expression in infected bryozoans: zinc finger CCHC domain-containing protein 10, gamma-aminobutyric acid receptor-associated protein, CD63 antigen, and casein kinase I. Additionally, non-significant ($p = 0.40$) upregulation of five IVI genes (14-3-3-like protein, iron-sulfur cluster assembly scaffold protein, peptidyl-prolyl cis-trans isomerase, S-methyl-5'-thioadenosine phosphorylase, and desumoylating isopeptidase 2) was also observed in the *T. bryosalmonae* from infected kidney compared to infected bryozoans.

Discussion

The cnidarian parasite *T. bryosalmonae* develops in the kidney of salmonid fishes and can cause devastating disease. The anterior kidney is a major lymphoid organ in fish and has hemopoietic functions. Moreover, both anterior and posterior kidney serve as immunocompetent organs in fish (Kum and Sekki, 2011). In spite of the immune functions of the kidney,

TABLE 1 Identified antigenic genes of *Tetracapsuloides bryosalmonae*.

In vivo induced antigens	Function	Location
Ras-related protein Rab-35	Rab protein signal transduction	Plasma membrane/cytoplasm/nucleous
Calmodulin	Calcium-mediated signaling	Plasma membrane/cytoskeleton
14-3-3-like protein	Signal transduction	Plasma membrane/cytoplasm/nucleous
Transmembrane emp24 domain-containing protein 7	Protein transport	Membrane
Vesicle-associated membrane protein 3-like	Vesicular transport	Plasma membrane
Zinc transporter 1	Zinc transport	Plasma membrane
F-actin-capping protein subunit alpha-2	Actin cytoskeleton organization	Cytosol/extracellular region or secreted/cytoskeleton
Cell division control protein 42 homolog	Actin cytoskeleton organization	Plasma membrane
Endophilin-B1-like	Membrane dynamics	Mitochondrion/Golgi apparatus
Peptidyl-prolyl cis-trans isomerase-like	Protein folding	Cytoplasm
Desumoylating isopeptidase 2	Protein deubiquitination	Cytoplasm
Iron-sulfur cluster assembly enzyme ISCU	Iron-sulfur clusters	Cytoplasm/mitochondrion
Heat shock protein 90	Protein folding	Cytoplasm
COP9 signalosome complex subunit 5	Cellular and developmental processes	Cytoplasm
S-methyl-5'-thioadenosine phosphorylase-like	Purine ribonucleoside salvage	Cytoplasm
Enolase-like	Glycolysis	Cytoplasm
Ceramide synthase 1-like	Sphingolipid biosynthetic pathway	Membrane
Hydroxyacyl-coenzyme A dehydrogenase	Beta-oxidation pathway	Cytoplasm/mitochondrion

In vivo induced antigens were identified by immunoscreening of phage cDNA library using pooled adsorbed sera of infected brown trout. Full table is presented in Supplementary Table 2.

T. bryosalmonae can successfully undergo sporogony in the kidney of the affected fish and be released in the urine (Sudhagar et al., 2019; Sudhagar et al., 2020) suggesting that this parasite has evolved to survive in an extremely hostile environment by molecular adaption. Most of the previous experiments on brown trout - *T. bryosalmonae* interactions were conducted to understand the host (brown trout) response (Kumar et al., 2014; Kumar et al., 2015; Bailey et al., 2019; Sudhagar et al., 2019; Sudhagar et al., 2020; Shivam et al., 2021; Sudhagar et al., 2022), while only a few studies focused on generating molecular information on *T. bryosalmonae* (Ahmad et al., 2021; Faber et al., 2021; Kumar et al., 2021). Identification of *in vivo* induced antigens or genes of the parasite during host- pathogen interactions, particularly during its development host, will provide valuable insight into the molecular adaption of the parasite. IVIAT has been successfully used to identify antigenic proteins of pathogens expressed during host-pathogen interactions, by using antisera from the infected host (Amerizadeh et al., 2013a; Amerizadeh et al., 2013b; Tao et al., 2014; Ron et al., 2015). In our study, 136 *in vivo* induced genes of *T. bryosalmonae* in brown trout were identified using IVIAT (Supplementary Table 2). These genes are involved in a variety of functions critical for the parasite including signal transduction, actin cytoskeleton organization, transport, metal ion binding, transcription, mitochondrial organization, translation, protein folding, metabolic process, cell division, and DNA repair. Additional genes encode for hypothetical proteins, and other genes are uncharacterized. The *in vivo* induced genes identified in this study are antigenic in nature and could potentially be used for the development of vaccines or utilized as drug targets for treating PKD in salmonids. Although there are limitations in the IVIAT technique such as a requirement for cultivable pathogens and an antibody response in the host, it has led to the identification of previously unknown immunogenic antigens of many pathogenic organisms (Rollins et al., 2005).

Genes involved in signal transduction

We identified several IVI genes involved in signal transduction activity such as ras-related proteins (Rab-35, Rap-1A, Rab-2B, and Ral-B), ras-related and estrogen-regulated growth inhibitor, and ran-specific GTPase-activating protein. Signal transduction is the process by which a cell receives a signal and transmits it to another part of the cell. This can be initiated by either a cell-surface receptor or an intracellular receptor (Bradshaw and Dennis, 2010). During host-parasite interaction; signal transduction mechanisms aid in the molecular cross-talk between the host and the parasite (Lopes-Junior et al., 2022). Rab proteins have an important function in cellular signal transduction and autophagy. Additionally, they are essential for cytokinesis (Kouranti et al., 2006) and act as molecular switches in intracellular membrane trafficking (Li and Marlin, 2015). In the present study, Rab35 was identified as an *in vivo* induced antigen of *T. bryosalmonae*. Gene expression analysis showed *T. bryosalmonae* Rab35 to be

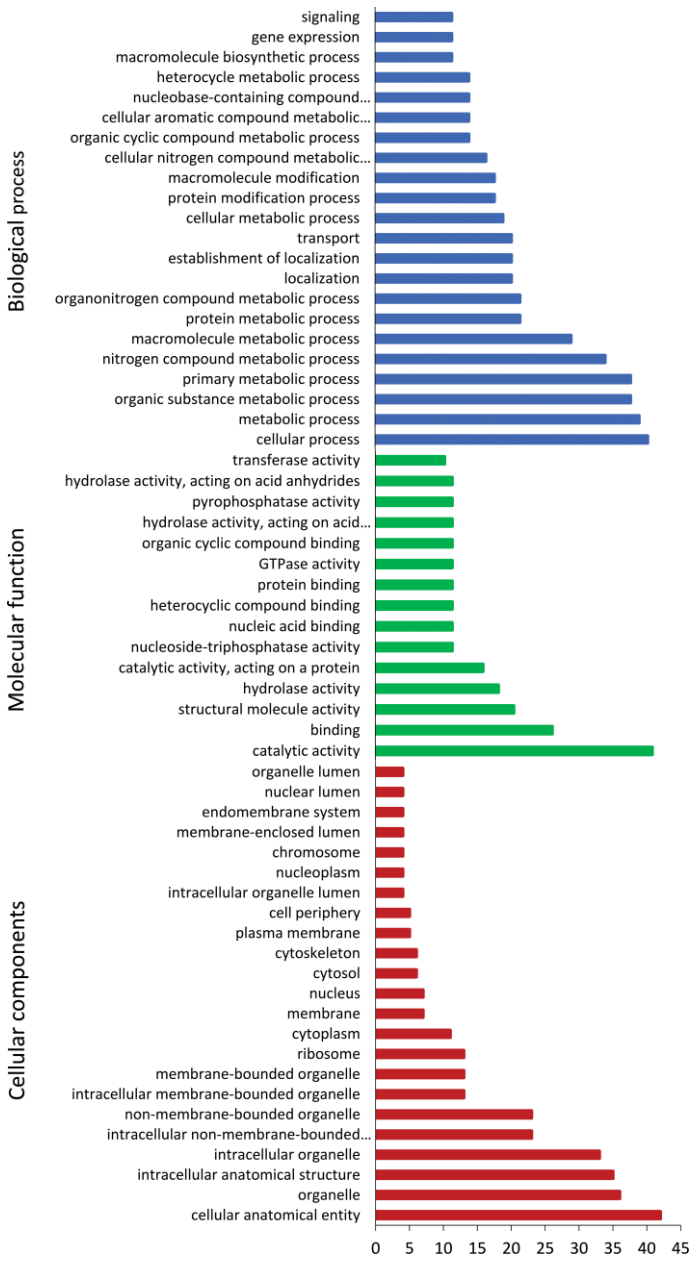

FIGURE 4
Gene ontology annotation of *Tetracapsuloides bryosalmonae in vivo*-induced genes. Most frequent level 2 GO terms in *T. bryosalmonae* IVI genes, separated for the GO domains; biological process, molecular function, and cellular component.

upregulated (4.39 fold) in the kidney of infected brown trout. In humans, amoebic colitis is caused by an anaerobic parasitic amoebozoan, *Entamoeba histolytica*. This parasite phagocytoses hosts blood cells and microbiota during pathogenesis. Rab35 is known to regulate erythrophagocytosis of *E. histolytica* in the cup formation and phagosomal maturation (Verma and Datta, 2017). Similarly, developmental stages of *T. bryosalmonae* in the kidney interstitium are commonly found engulfed by host phagocytes (Kent and Hedrick, 1986). The latter researchers found evidence of endocytosis of host material by the parasites migrating to the lumen of the kidney tubules. In addition, during mitotic division of the parasite primary cell, cytokinesis has been demonstrated by ultrastructural analysis (Morris and Adams, 2008). Therefore, we hypothesize a potential role of Rab35 in the cellular processes of endocytosis and cytokinesis in *T. bryosalmonae*.

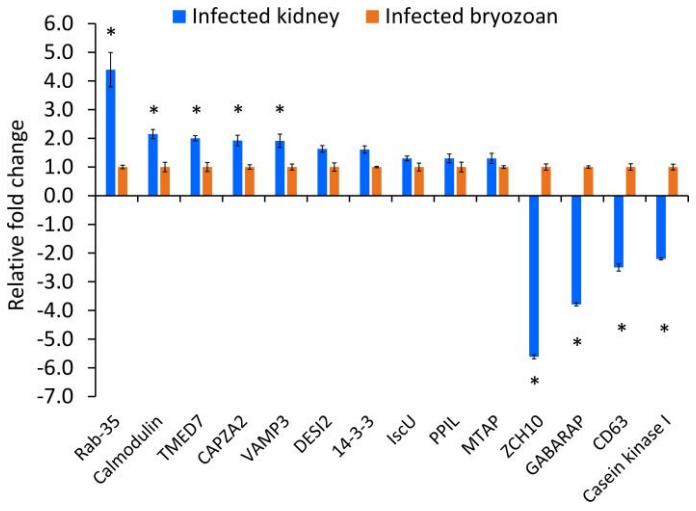

FIGURE 5
Relative expression of *Tetracapsuloides bryosalmonae in vivo*-induced genes. Assessment of expression of IVI genes in infected kidney of brown trout compared to their expression in infected bryozoan. qRT-PCR data were normalized with reference genes, and represented as a fold change relative to infected bryozoan expression levels. Significant differences ($p < 0.05$) between infected kidney and infected bryozoan of each gene are indicated with an asterisk (*). Error bars represent the standard error (n = 6). TMED7, Transmembrane emp24 domain-containing protein 7; CAPZA2, F-actin-capping protein subunit alpha-2; VAMP3, Vesicle-associated membrane protein 3; ZCH10, zinc finger CCHC domain-containing protein 10; GABARAP, gamma-aminobutyric acid receptor-associated protein; DESI, desumoylating isopeptidase 2; IscU, iron-sulfur cluster assembly scaffold protein; PPIL, peptidyl-prolyl cis-trans isomerase; MTAP, S-methyl-5'-thioadenosine phosphorylase.

Another interesting signal transduction molecule we identified was 14-3-3-like protein. Numerous parasites, including *Plasmodium* sp., *Toxoplasma* sp., *Neospora* sp., *Eimeria* sp., *Schistosoma* sp., and *Echinococcus* sp., have been found to contain this highly conserved protein (Siles-Lucas and Gottstein, 2003). In many of these parasites, the protein is suggested to play significant roles during their life cycle. For example, in *Eimeria tenella*, this protein plays an important role in the regulation of mannitol cycle metabolic pathway, which is an important energy source for their sporulation process (Liberator et al., 1998). These proteins modulate protein kinase C (PKC) activity and translocation in *Schistosoma japonicum* and *Schistosoma mansoni*, during their life cycles (Zhang et al., 2000; Siles-Lucas and Gottstein, 2003). Similar to these parasites, 14-3-3 protein might have important roles in sporogony and translocation during the development of *T. bryosalmonae* in its host. Considering its important role, 14-3-3 protein is suggested as a potential vaccine candidate against parasitic infections such as *Schistosoma* sp. and *Echinococcus* sp. (Siles-Lucas and Gottstein, 2003; Siles-Lucas et al., 2008).

Genes involved in actin cytoskeleton organization

Genes associated with actin cytoskeleton organization that were identified in the present work included actin (cytoplasmic 1 and 2), tubulin (alpha-1 and beta-1), thymosin beta-4, F-actin-capping protein subunit alpha-2 (CAPZA2), and cell division control protein 42 (CDC42). The organization of the actin cytoskeleton is a process performed at the cellular level that results in the assembly, placement, or degradation of the cytoskeleton structure containing actin filaments and other related proteins. Actin is important in a multitude of functions such as the maintenance of shape, structural integrity of the cell, cytokinesis, cellular endocytosis and the transport of membrane vesicles in eukaryotic cells (Pollard and Cooper, 2009; Suarez and Kovar, 2016). In endoparasites like *T. bryosalmonae*, the genes involved in actin cytoskeleton organization might be highly dynamic in their function, particularly when the parasite changes its morphology, shifts within host (one organ to another) and transmits from one host to complete its life cycle. In *M. cerebralis*, a transcript similar to actin related protein 3 homologue (ARP3) was found with a putative function of sporoplasm's movement regulation during host detection and penetration (Eszterbauer et al., 2009). Furthermore, the genes involved in the cytoskeleton organization (b-actin, Talin and RhoA), and cytoskeletal-extracellular matrix interaction (Integrin-b) were observed to be significantly upregulated in the virulent genotypes of *C. shasta* (Alama-Bermejo et al., 2019; Alama-Bermejo et al., 2020). We also identified an actin protein of *T. bryosalmonae* in the infected kidney of brown trout by anti-parasite antibody-based protein purification followed by mass spectrometry analysis (Kumar et al., 2015). Another study

confirmed the important role of actin in cellular motility, which supports the parasite in the evasion from the host immune system (Hartigan et al., 2016). Based on these findings, we speculate that actin cytoskeleton related genes of *T. bryosalmonae* contribute to invasion, host-parasite interaction, growth, migration, and establishment of disease in salmonids.

Small GTPases belonging to the Rho family are important modulators of the actin cytoskeleton. We found Rho family genes of *T. bryosalmonae* such as rho-related GTP-binding protein RhoC, and rho GTPase-activating protein 44-like by immunoscreening analysis. Additionally, CDC42, which belongs to the Rho family of actin regulators, was also revealed in our study. This protein is known to regulate many cell signaling pathways of eukaryotes (Watson et al., 2017). The F-actin-capping protein subunit alpha-2 is another antigenic protein identified by adsorbed sera of infected fish in the present experiment and is known for its role in actin cytoskeleton organization. Capping protein is an alpha/beta heterodimer and it is an important protein in eukaryotes for actin cytoskeleton development (Hart et al., 1997). The role of F-actin has been confirmed in motility and spore formation in *C. shasta* (Brekhman et al., 2021). In light of these reports, and our present study, Rho family genes in *T. bryosalmonae* could play important roles and interact with antibodies of infected fish during the development of the disease. These findings open a new research area on cytoskeleton associated proteins in *T. bryosalmonae*, with the aim of understanding their precise molecular function.

Genes involved in cellular transportation

Cellular transportation is essential for the normal functioning of a cell tomaintain homeostasis. Inthe current study, we identified zinc transporters such as zinc transporter 1 and zinc transporter 7 as antigenic genes of *T. bryosalmonae*. Zinc acts asa cofactor for various biological systems including cell signaling (Hara et al., 2017), and its deficiency or excess is detrimental to cells (Sugarman, 1983; Wessells et al., 2012). Cells are endowed with zinc transporter proteins to maintain the optimum level of zinc import, intracellular storage and efflux (Dufner-Beattie et al., 2003). In many parasitic organisms, zinc transporters are involved in parasite development and are known virulence factors in *T. gondii* and *L. infantum* (Carvalho et al., 2015; Chasen et al., 2019). In line with these reports, we assume that zinc transporters in *T. bryosalmonae* transport zinc into the cytoplasm and localize to vesicles. However, how zinc transporters contribute in the invasion process of *T. bryosalmonae* needs to be investigated.

Vesicle-associated membrane protein 3-like (VAMP3) of *T. bryosalmonae* was found to be antigenic and upregulated in the infected brown trout (Figure 5). VAMP3 is a membrane protein involved in the movement of materials across the cell membrane and it is also necessary for the homeostasis and other physiological processes of the cell (Feldmann et al., 2011). Silencing of VAMP3 affects the migration of cells and cell-mediated adhesion by integrin (Luftman et al., 2009). Furthermore, in the human parasite *Leishmania amazonensis*, VAMP3 has an important function in the formation of parasitophorous vacuoles (Séguin et al., 2022). In the case of malaria parasite, *Plasmodium falciparum* vesicle-mediated trafficking transports parasite proteins into the infected host cytosol and cell membrane (Taraschi et al., 2001). Similarly, transmembrane emp24 domain-containing protein 7 (TMED7) was identified; this protein is involved in cellular transport and is important for the cellular homeostasis (Aber et al., 2019). Currently, there is not much information on membrane proteins of myxozoan parasites, suggesting the need for further studies to understand their molecular and cellular functions.

Genes involved in protein folding

Heat shock proteins (HSPs) are molecular chaperones that play an essential role in development of parasite and host-parasite interactions (Polla, 1991). HSPs were found to be abundant in the mature spore of *C. shasta* by proteome analysis (Brekhman et al., 2021). HSP60, 70 and 90 were found to be enriched in *T. bryosalmonae* by transcriptome analysis (Faber et al., 2021). By immunoscreening, we found IVI genes associated with HSPs such as HSP12.2, 70, and 90, DNJA1 protein, activator of 90 kDa heat shock protein ATPase homolog 1, and molecular chaperone ABC1 in *T. bryosalmonae*. HSPs have been identified as major antigens of parasitic nematodes and flukes (Arizono et al., 2011; Chung et al., 2017). These findings suggest that HSPs of *T. bryosalmonae* may be involved in the induction of host immunity. We propose that HSPs could be a potential interest for vaccine development or therapeutic target against *T. bryosalmonae*.

While we determined peptidyl-prolyl cis-trans isomerase-like (PPIL) to be antigenic using IVIAT, in qRT-PCR analysis, it was only slightly upregulated (1.30 fold). PPIL molecules are known to have a functional role in host-parasite interaction and anti-parasitic drug action (Bell et al., 2006). Moreover, PPIL plays an important role in the protein folding mechanism, a process making proteins biologically functional. The cis-trans isomerization of peptide bonds N-terminal to proline residues in polypeptide chains is catalysed by peptidyl-prolyl isomerases. This protein folding machinery has been targeted for antagonizing drug resistance in the malaria causing parasite *P. falciparum* (Wilkinson, 2020). Despite the statistically insignificant upregulation of PPIL in our study, in light of its important role and its demonstrated vaccine potential in other parasites, this gene should be further investigated for its functional role and prospective use as a vaccine candidate against *T. bryosalmonae*.

Genes involved in cellular metabolic processes

Many of the genes identified in this experiment, such as ceramide synthase 1-like (CERS1), COP9 signalosome, hydroxyacyl-coenzyme A dehydrogenase (HADH), and enolase (ENO) have already been shown to have a role in cellular metabolic processes of other parasitic organisms and have been regarded as potential vaccine candidates or drug targets. The CERS1 gene has a crucial role in the sphingolipid biosynthetic pathway and encodes for the catalytic enzyme ceramide synthase that aids in the synthesis of ceramide. In fungal pathogens, ceramide synthase is identified to play an important role in the virulence (Munshi et al., 2018). Ceramide synthase activity is observed in infectious parasites such as *Leishmania* sp. and *T. cruzi* that manipulates host lipidome to favour the establishment of the parasite within the host (Rub et al., 2013). In parasitic (Figueiredo et al., 2012) and fungal pathogens (McEvoy et al., 2020), ceramide synthase is identified as a novel drug target that could interfere with the sphingolipid biosynthetic pathway.

Several parasites such as *Entamoeba histolytica*, *Toxoplasma* sp., *Trypanosoma* sp., and *Leishmania* sp. are known to encode for COP9 signalosome. This gene regulates the ubiquitin-proteasomal system (UPS) protein degradation pathway of *E. histolytica* (Ghosh et al., 2020). Additionally, zinc-ditiocarb complex inhibited *E. histolytica* development by targeting the COP9 signalosome. HADH is an important enzyme involved in the b-oxidation of fatty acids that helps to metabolize lipids as the source of energy. In canines, recombinant HADH protein of *Echinococcus granulosus* offered protection against cystic echinococcosis infection (Xian et al., 2021). Thus, *T. bryosalmonae* might depend on HADH mediated b-oxidation of fatty acids for its energy metabolism. If so, it would be interesting to explore the potential of a recombinant HADH protein of *T. bryosalmonae* as a vaccine candidate to develop prophylactic measures against PKD in salmonids.

The interconversion of 2-phosphoglycerate into phosphoenolpyruvate during glycolysis is mediated by a metalloenzyme ENO (Didiasova et al., 2019). ENO is known to play a key role in enhancing the virulence of trypanosomatid parasites such as *Trypanosoma* sp. and *Leishmania* sp. (Avilán et al., 2011). ENO is highly conserved across species with similar catalytic residues and is identified as a candidate target for antiparasitic drugs against trypanosomatid parasites (Avilán et al., 2011). Hence, experiments are needed to identify potential candidate drugs targeting ENO of *T. bryosalmonae* to treat PKD in salmonids.

Small ubiquitin-like modifier (SUMO) plays an important role in protein modification and regulates a diverse range of cellular processes. Desumoylating isopeptidases are enzymes that reverse protein SUMOylation (Shin et al., 2012). In the present work, desumoylating isopeptidase 2 is identified as an antigenic gene of *T. bryosalmonae* in brown trout. SUMOylation could be potential drug target for treating parasitic diseases (Sumam de Oliveira et al., 2021).

Another interesting protein that we identified was calmodulin, a highly conserved calcium binding protein. Additionally, the result of qRT-PCR confirmed the upregulation of calmodulin in infected kidney during PKD development. Calmodulin regulates various cellular activities such as cell motility, and it has been identified as a part of the invasion motor in *T. gondii* (Nebl et al., 2011). Interestingly, immunogenicity and pathogenicity of calmodulin have been confirmed in *T. gondii* by IVIAT and gene silencing (Tao et al., 2014). In accordance with the above mentioned findings, calmodulin may play an important role as a calcium-binding protein in *T. bryosalmonae* for invasion and motility and could be a possible vaccine candidate against *T. bryosalmonae* infection.

Genes with unknown function

Among the 136 *in vivo* induced genes of *T. bryosalmonae* identified in the present experiment, we found ten hypothetical proteins. These genes are unique and their functional roles are not known. Further studies are needed to characterize these genes and to understand their role in the pathogenesis of *T. bryosalmonae* in salmonids.

Conclusion

In this study, we identified *in vivo* induced antigens of *T. bryosalmonae*, a salmonid parasite of economic and ecological significance. This is the first investigation that provides a piece of comprehensive information about the antigenic genes of the parasite during host-pathogen interactions. The identified IVI genes may play critical roles during parasite development and pathogenesis in salmonids. Taken together, the results enhance our understanding of *T. bryosalmonae* virulence mechanism in salmonids. Moreover, this study provides an extensive list of *in vivo* induced genes that could be further evaluated for their potential as drug targets and vaccine candidates against PKD in salmonids.

Data availability statement

The data presented in the study are deposited in the FigShare repository, accession number 10.6084/m9.figshare.20775592 https://figshare.com/s/dac3158e101e02659af7.

Ethics statement

The animal study was reviewed and approved by the institutional ethics committee of the University of Veterinary Medicine Vienna and the national authority, according to §26 of the Austrian Law for Animal Experiments, Tierversuchsgesetz 2012 under approval number BMWFW-68.205/0181-WF/V/3b/2017. All methods of this study are reported in accordance to the ARRIVE guidelines for animal research.

Author contributions

GK conceived and designed the experiments, performed the experiments and wrote the manuscript. AS and SS performed the experiment and reviewed the manuscript. FN, JB, and ME-M designed the experiments and reviewed the manuscript. All the authors approved the final draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1032347/full#supplementary-material

SUPPLEMENTARY FIGURE 1
PCR analysis of identified recombinant clones. Gel image showing different sized cDNA inserts from *in vivo* induced positive clones obtained by PCR with vector specific primers. L: 100 bp ladder (Qiagen), Lanes 1–38 PCR-amplified IVI clones and NTC: negative template control.

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3.3 Differentially expressed transcripts of *Tetracapsuloides bryosalmonae* (Cnidaria) between carrier and dead-end hosts involved in key biological processes: novel insights from a coupled approach of FACS and RNA sequencing

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Differentially expressed transcripts of *Tetracapsuloides bryosalmonae* (Cnidaria) between carrier and dead-end hosts involved in key biological processes: novel insights from a coupled approach of FACS and RNA sequencing

Saloni Shivam[1,2], Reinhard Ertl[3], Veronika Sexl[4], Mansour El-Matbouli[1,5] and Gokhlesh Kumar[1*]

Abstract

Tetracapsuloides bryosalmonae is a malacosporean endoparasite that infects a wide range of salmonids and causes proliferative kidney disease (PKD). Brown trout serves as a carrier host whereas rainbow trout represents a dead-end host. We thus asked if the parasite adapts to the different hosts by changing molecular mechanisms. We used fluorescent activated cell sorting (FACS) to isolate parasites from the kidney of brown trout and rainbow trout following experimental infection with *T. bryosalmonae*. The sorted parasite cells were then subjected to RNA sequencing. By this approach, we identified 1120 parasite transcripts that were expressed differentially in parasites derived from brown trout and rainbow trout. We found elevated levels of transcripts related to cytoskeleton organisation, cell polarity, peptidyl-serine phosphorylation in parasites sorted from brown trout. In contrast, transcripts related to translation, ribonucleoprotein complex biogenesis and subunit organisation, non-membrane bounded organelle assembly, regulation of protein catabolic process and protein refolding were upregulated in rainbow trout-derived parasites. These findings show distinct molecular adaptations of parasites, which may underlie their distinct outcomes in the two hosts. Moreover, the identification of these differentially expressed transcripts may enable the identification of novel drug targets that may be exploited as treatment against *T. bryosalmonae*. We here also describe for the first time how FACS based isolation of *T. bryosalmonae* cells from infected kidney of fish fosters research and allows to define differentially expressed parasite transcripts in carrier and dead-end fish hosts.

Keywords Proliferative kidney disease, malacosporean endoparasite, differential transcript expression, brown trout, rainbow trout

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Introduction

Tetracapsuloides bryosalmonae, the causative agent of proliferative kidney disease (PKD) is a malacosporean endoparasite of the phylum Cnidaria [1, 2]. It is a major parasite affecting freshwater salmonids in Europe and North America [3]. Similar to other myxozoans, *T. bryosalmonae* has a life cycle involving two hosts (vertebrate and invertebrate) [4]. The bryozoans serve as its primary host and act as reservoirs of *T. bryosalmonae* spores infective to their secondary hosts, the salmonid fish [4, 5]. The parasite undergoes a series of developmental alterations within both primary [6] and secondary hosts [7]. Spores released from infected bryozoan colonies infect salmonid fish mainly through their gills [8]; thereafter traverse through the blood vascular system to reach the main target organ kidney [9]. Within the kidney of fish host, the parasite transitions through extrasporogonic and sporogonic stages [7]. Subsequently, an intratubular development leads to the formation of spores [10], which leave the fish host via urine and are infective for the bryozoans [4, 8].

Although, numerous salmonids are susceptible to *T. bryosalmonae* presenting similar clinical signs and symptoms, currently only brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*) are known to be carriers of the parasite [8]. Carrier fish are capable of releasing viable parasite spores, which are infective for the bryozoans. Rainbow trout, a dead-end host, upon infection exhibit similar clinical manifestations of the disease but do not shed spores infective for bryozoans, thereby terminating the parasite life cycle [8]. Interestingly, under similar environmental conditions, the appearance of clinical symptoms in both the fish host and also parasite development follows similar path. However, in contrast to brown trout, European strain of *T. bryosalmonae* never develops intra-luminal sporogonic stages in rainbow trout [7, 8].

Factors contributing to the different fate of *T. bryosalmonae* in rainbow trout and brown trout remain elusive. Apparently, the carrier state of brown trout suggests towards the existence of certain immune evasion strategies or manipulation of host pathways by the parasite. Likewise, the dead-end status of rainbow trout for this parasite might echo a successful and robust host response or the inefficient parasite survival tactic. Given the differential outcome of the parasite, compara- tive transcriptional profiling of *T. bryosalmonae* from rainbow trout and brown trout can be useful in gaining insights on the differential behavior of this parasite and elucidating important genes necessary for their survival and pathogenesis in the host. Knowledge of protective host immune responses and parasite biology could pave the way for developing successful PKD management measures including vaccines. Rational design of vaccines against any parasite including *T. bryosalmonae* could be based on either eliciting the host immune responses or targeting the parasite biology and/or virulence factors.

Transcriptomics has emerged as a powerful tool in advancing our understanding of host, parasite, and their interactions [11–14]. However, dominance of host transcripts has been highlighted as a major issue during dual RNA sequencing of parasite infected host samples, sometimes the parasite transcripts making a mere 0.1% [11]. Besides, a high probability of contamination from host exists. A possible solution would be to purify parasites from infected host tissues prior to RNA sequencing using techniques such as fluorescence-activated cell sorting (FACS). It is a high-throughput technique for selectively separating a desired cell type from a mixture of heterogeneous cell suspension with a high degree of purity [15]. Though this technique has emerged as a method of choice for isolating specific cell populations, it is also being used for the isolation and purification of parasites from a mixture of cell types [16].

In this study, we isolated *T. bryosalmonae* from the main target organ kidney of infected brown trout and rainbow trout using FACS and performed RNA sequencing of the sorted parasite cells to determine its differentially expressed transcripts (DETs) during the clinical phase of infection between the two fish hosts.

Materials and methods

Brown trout and rainbow trout sampling

Specific pathogen-free brown trout and rainbow trout (mean length 12 ± 2 cm) were purchased from a certified local hatchery and transported to our laboratory. The trout were acclimatized for a month in 1000 L tanks with continuous aeration and water flow. The temperature in tanks was maintained at 15 ± 1 °C. Fish were fed to satiation with commercial trout feed. Ten fish from each species were randomly sampled and their health status was ascertained by our routine diagnostic procedures. The examined fish were found to be negative for *T. bryosalmonae*, and other parasites and also for bacterial and viral pathogens.

The experimental exposure of fish was carried out with parasite spores released from infected bryozo- ans, according to Kumar et al. [17]. Briefly, prior to parasite exposure, the fish were kept in an aquarium with 100 L of water. To this, a spore suspension from 30 mature parasite sacs was added and mixed properly to aquaria. The water flow in the aquaria was stopped for 6 h with vigorous aeration and then re-started very slowly overnight. Following this, both brown trout and rainbow trout (n = 69 per fish species) were randomly divided into 3 groups as three replicates (n = 23 in each

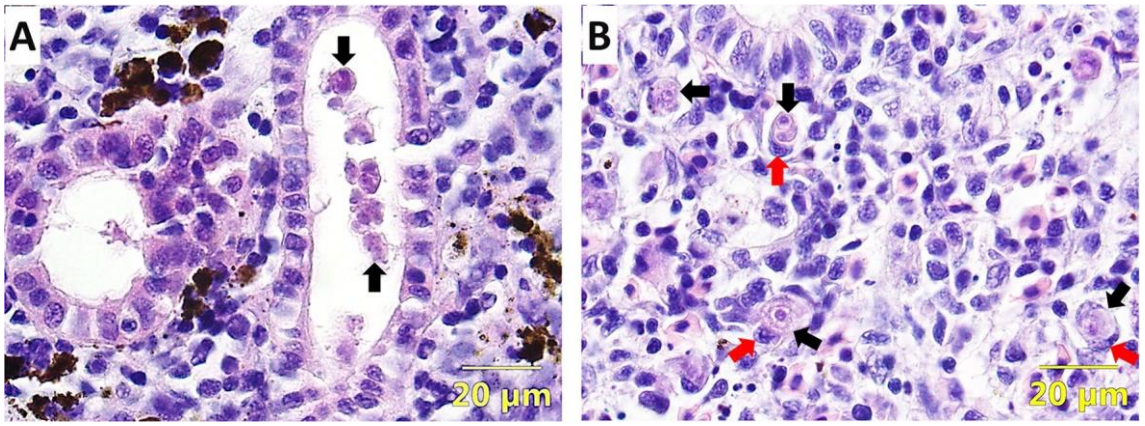

Figure 1 Histological sections of infected kidneys. A Brown trout kidney section shows intra-luminal sporogonic stages of *Tetracapsuloides bryosalmonae* (arrows), **B** rainbow trout kidney section shows interstitial pre-sporogonic stages (black arrows) of *T. bryosalmonae* associated with macrophages (red arrows) (H&E staining).

aquarium). Similarly, three replicates for each fish species (n = 69 per fish species and n = 23 for each replicate group) were maintained as uninfected controls without adding parasite spores. Fish (n = 9) were randomly sampled at 2-, 4-, 6-, 8-, 10-, 12-, and 17-weeks post-exposure (wpe) from both infected and unin- fected control groups. The presence of parasite was confirmed in the kidneys using histology and immunohistochemistry. At 10 wpe, numerous intra-luminal sporogonic and pre-sporogonic stages (Figures 1A and 2A) in brown trout and interstitial pre-sporogenic stages of parasite in rainbow trout (Figures 1B and 2B) were observed. A description of experimental method for FACS and RNA-seq is presented in a flowchart (Figure 3).

Sample preparation

During preliminary studies, the sample preparation protocol for the FACS analysis was optimized. Uninfected and infected kidney samples were used for the optimiza- tion of staining protocol. Different concentration of pri- mary antibody, secondary antibody, incubation time, and temperature were tested. Additionally, during optimization a primary antibody alone control, secondary anti- body alone control, unstained and stained (using both primary and secondary antibodies) uninfected control and infected kidney samples were tested before carry- ing out the following protocol. We used a combination of Dulbecco's phosphate buffered saline (DPBS, Sigma) and 2% newborn calf serum (NCS) as blocking buffer. NCS was used to promote cell viability while also acting as a

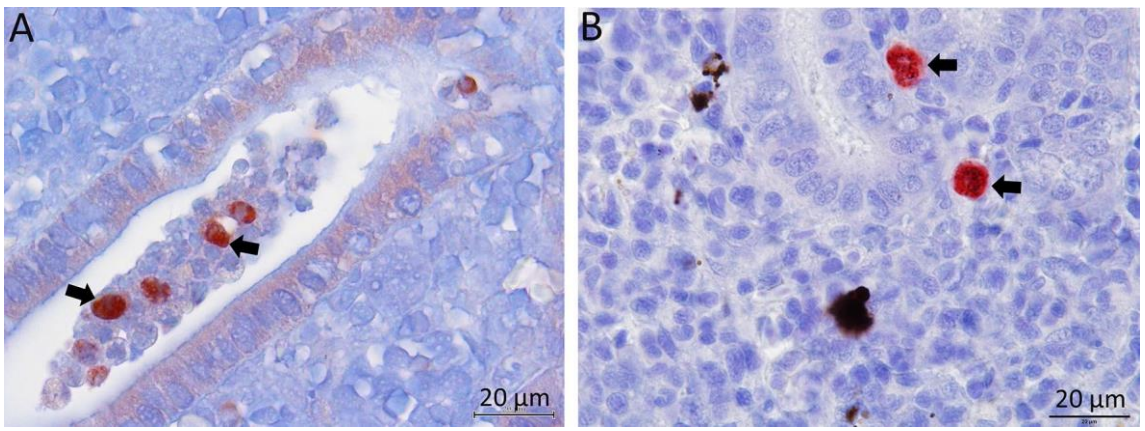

Figure 2 Immunohistochemistry of infected kidneys. A Brown trout kidney section shows intra-luminal sporogonic stages of *Tetracapsuloides bryosalmonae* (arrows), **B** rainbow trout kidney section shows interstitial pre-sporogonic stages of *T. bryosalmonae*. Parasite stages were visualized by immunohistochemistry using monoclonal antibody against *T. bryosalmonae* and counterstained with haematoxylin.

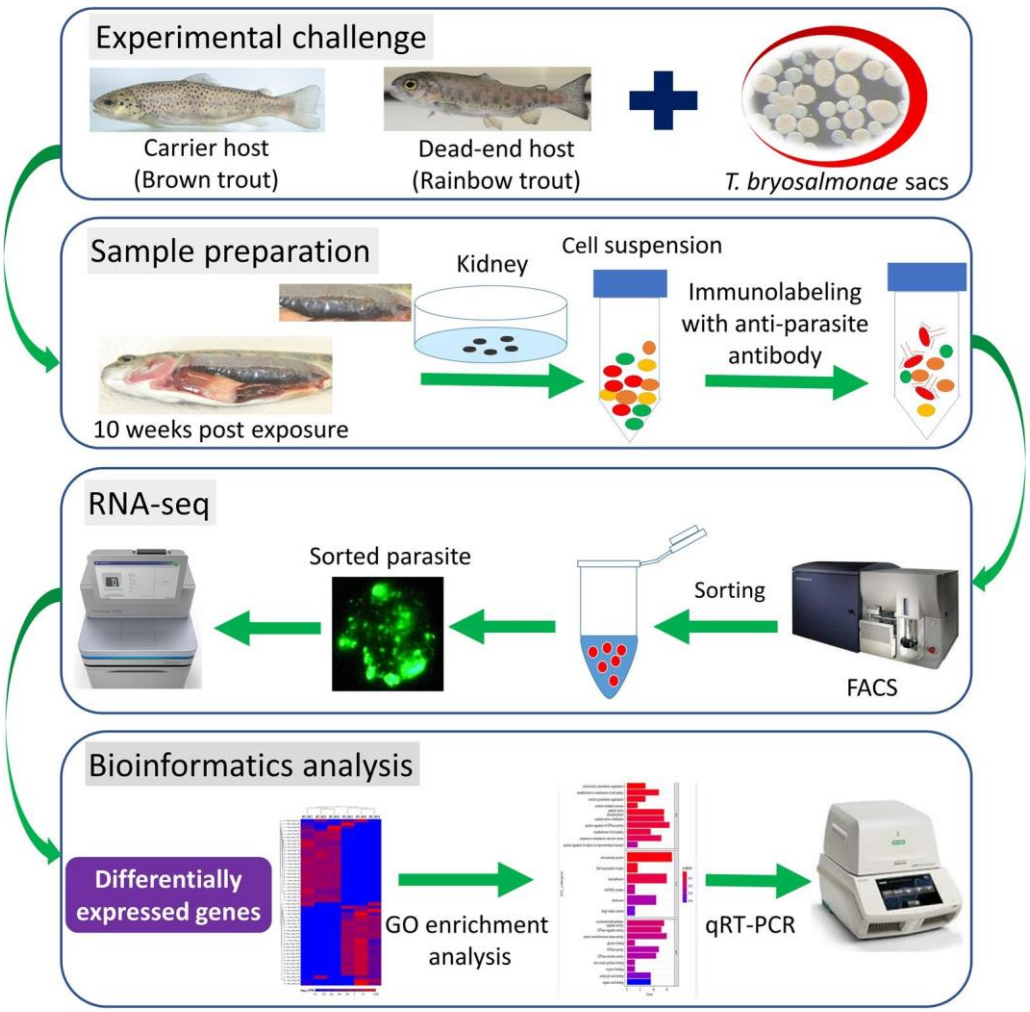

Figure 3 Flowchart of the experimental setup used to sort *Tetracapsuloides bryosalmonae* from infected kidney of fish and RNA sequencing. Brown trout and rainbow trout were experimentally challenged with laboratory cultured *T. bryosalmonae*. Ten weeks post-exposure kidney samples were collected and cell suspension was made from them. Cell suspension from kidney was stained with anti-*Tetracapsuloides bryosalmonae* antibody and anti-mouse antibody Alexa 488 and then subjected to sorting by FACS Aria III cell sorter. Samples were then used for RNA extraction and cDNA library preparation followed by sequencing. Subsequently, data was analyzed using bioinformatics approaches to identify differentially expressed genes of *T. bryosalmonae* between the two fish species.

blocking agent. A portion of posterior kidney of sampled uninfected control and infected groups at 10 wpe were excised and placed in a Petri dish filled with pre-chilled DPBS. Each kidney sample was washed three time with cold DPBS to remove fat and blood clumps. Kidney sam- ple was cut into small pieces (2–4 mm) with the scalpel and was washed 3 times with PBS containing 2% new- born calf serum (NCS) and kept at 4 °C until further processing. Each kidney tissue was disrupted manually with gentle pressure using cell strainer (70 µm) and a syringe barrel and the volume was made up to 8 mL with cold DPBS containing 2% NCS. The cell suspension was centrifuged at 2000 rpm for 10 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 1 mL of DPBS containing 2% NCS solution. The cells were counted with hemocytometer. Afterwards, cell suspension (4×10^6 cells) was distributed to 1.5 mL Eppendorf tube and then centrifuged at 2000 rpm for 10 min at 4 °C. The supernatant was discarded, and the cell pellet was resuspended in 150 µL of the commercially available anti-*T. bryosalmonae* monoclonal antibody (1:10 diluted with cold DPBS containing 2% NCS) (Aquatic diagnostics ltd., Stirling, Scotland, UK) and incubated for 60 min at 4 °C. After incubation, cell suspension was washed two times with 800 µL of cold DPBS containing 2% NCS and centrifuged at 2000 rpm for 10 min at 4 °C. Subsequently, cell pellet was incubated with 100 µL of anti-mouse antibody Alexa 488 and incubated for 30 min and then washed two

times with cold DPBS containing 2% NCS. Finally, cell pellet was resuspended in 400 µL of DPBS containing 2% NCS and then transferred in a FACS tube through tube filter and kept on ice for until use.

Parasite cell sorting from infected kidney

A FACS Aria III cell sorter (Becton Dickinson, San Jose, USA) equipped with a 488 nm blue solid laser was used for analysis. Forward-scatter characteristics (FSC) resulted from the small-angle scatter, while side-scatter characteristics (SSC) were recorded as orthogonal scatter of the 488 nm laser. Fluorescence (eYFP/Venus) was detected by a 502-nm long-pass and a 530/30 nm band-pass filter set. The FACS software DIVA 7.0 was used for the recording. Thresholding on the FSC/SSC was applied for all measurements and removed for the sorting proce- dure. Initially, to enable gate settings uninfected kidney cell suspension were analysed. Cell sorting was achieved by pre-gating the cells twice. FSC and SSC characteristics were used to set the first gate to remove non-cell parti- cles. The next gate was set around the FSC height and fluorescence area signal to select stained population of cells. From these pre-gating settings, the cells were sorted with the four-way purity mask and a threshold rate of 8000–25 000 events/s with 70 µm nozzle. Cells were sorted directly into 1.5 mL Eppendorf tubes contain- ing cold lysis buffer with beta-mercaptoethanol (Qiagen, Germany) and the sorted cells were stored immediately at −80 °C. The parasite viability was examined visually under the fluorescence microscope before and after cell sorting.

RNA extraction, library preparation and sequencing

Total RNA was extracted from FACS-sorted *T. bryosalmonae* from brown trout and rainbow trout using RNe- asy UCP Micro Kit (Qiagen) along with an on-column DNase digestion step following the manufacturer's proto- col. RNA integrity was assessed on the 4200 TapeStation with the High Sensitivity RNA ScreenTape Kit (Agilent, Santa Clara, CA, USA). Only samples with RNA integrity numbers > 8.0 were used for further analysis.

Library preparation was done with 60 ng total RNA input using the Poly(A) RNA Selection Kit V1.5 and the CORALL mRNA-Seq Library Prep Kit (Lexogen, Vienna, Austria) according to the manufacturers pro- tocol. Six cDNA libraries (3 each for sorted parasite samples from brown trout and rainbow trout) were prepared. Library quality control was done with the High Sensitivity D1000 ScreenTape Kit on the 4200 TapeStation (Agilent). Libraries were sequenced on a NovaSeq 6000 system (Illumina, San Diego, CA, USA) implementing 150-bp paired-end reads. Sequencing was done by the NGS unit of the Vienna Biocenter Core Facilities (VBCF, Vienna, Austria).

Analysis of RNA-seq data

Data were analyzed with CLC Genomics Workbench software v22 (Qiagen, Aarhus, Denmark). The raw reads were subjected to quality filtering: low quality bases (Phred score ≤ 30), reads shorter than 50 nucleotides, adapter and unique molecular identifier sequences from library prep were removed. To remove host sequences, the filtered reads were mapped to either brown trout (NCBI accession: GCA_901001165.2) or rainbow trout genome (GCA_013265735.3) with the CLC mapping tool using the default mapping parameters (mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, similarity fraction = 0.8). Three publicly available transcriptome assemblies from *T. bryosalmo- nae* [18–20] were combined into one file and redundant sequences were removed using the clustering software cd-hit-est with a sequence identity threshold of 0.95 [21]. The remaining unmapped reads were mapped to the available myxozoan genomes and the combined, non-redundant *T. bryosalmonae* transcriptome assem- bly. The details of datasets used for mapping are pro- vided in Table 1.

Venn diagram was constructed using the online tool InteractiVenn [22] to exhibit the number of shared and unique transcripts of *T. bryosalmonae* in brown trout and rainbow trout. The parasite transcripts (average RPKM > 1.0) from brown trout and rainbow trout were used to construct the Venn diagram. For differential expression, brown trout samples (n = 3) were compared to rainbow trout samples (n = 3). Transcripts showing absolute fold changes > 2.0 and FDR-corrected p-val- ues < 0.01 were considered as differentially expressed. The calculation of p-values implements the "exact nega- tive binomial test" proposed by Robinson and Smyth [23]. Heatmaps of differentially expressed transcripts were generated with the single linkage method based on Euclidean distance matrix. Heatmap and volcano plot were generated with the SRplot online tool [accessed on 21 March 2023]. GO categories (biological process, molecular function, and cellular components) enriched in DETs were identified using the clusterProfiler pack- age enrichGO in R (version 4.2.1) [24]. For each com- parison, upregulated and downregulated transcript sets were input separately into enrichGO. A p-value cut-off of 0.05 was used. All quality control steps followed in this study right from sample preparation to RNA-seq data analysis are provided in Additional file 1.

Table 1 Summary of mapping data of FACS sorted *Tetracapsuloides bryosalmonae*

Samples	Number of raw reads	Number of quality-filtered reads	Number of reads mapping to brown trout or rainbow trout	Remaining: number of unmapped reads	Mapping of unmapped reads: number of mapping reads to different references						
					E. leei mitochondrial genome (5 genes) GCA_001455295.2	*K. iwatai* mitochondrial genome (10 genes) GCA_001407235.2	*M. squamalis* (5710 genes) GCA_010108815.1	*H. salminicola* (8187 genes) GCA_009887335.1	*S. zaharoni* (number annotated genes) GCA_001455285.1	*T. kitauei* (15 020 genes) GCA_000827895.1	Combined *T. bryosalmonae* transcriptome (31 464 contigs, Ahmad et al. [20]; Faber et al. [19]; Kumar et al. [18]
BT_SC1	71 360 140	68 363 654	43 666 912	24 696 742	2 308 164	3 837 812	2 121 654	2 121 654	2 383 909	3 297 048	13 551 795
BT_SC2	76 160 696	73 576 346	44 245 459	29 330 887	2 936 500	4 862 586	2 656 946	2 656 946	3 017 066	4 109 527	16 276 440
BT_SC3	80 297 000	76 804 534	48 592 048	28 212 486	2 495 135	4 166 910	2 332 754	2 332 754	2 576 958	3 586 536	15 870 805
RT_SC1	59 057 686	56 787 460	37 180 648	19 606 812	1 991 590	3 036 937	1 659 712	1 659 712	1 859 904	2 641 455	8 123 191
RT_SC2	87 380 018	83 725 584	51 212 973	32 512 611	3 551 579	5 591 392	2 989 800	2 989 800	3 505 367	4 802 022	14 838 808
RT_SC3	72 073 626	68 923 366	48 272 618	20 650 748	1 801 703	2 812 857	1 467 204	1 467 204	1 770 479	2 411 495	7 777 966

The filtered clean reads of sorted *T. bryosalmonae* obtained from infected kidney of brown trout and rainbow trout were mapped to different genome and transcriptome references (fish hosts and myxozoan parasites). The remaining unmapped reads were mapped against the genomes of different myxozoan parasites and the combined transcriptome of *T. bryosalmonae* from brown trout, rainbow trout and bryozoan hosts. RNA-seq analysis was performed using CLC Genomics Workbench software version 22. *T. bryosalmonae* samples from brown trout: BT_SC1, BT_SC2, BT_SC3; *T. bryosalmonae* samples from rainbow trout: RT_SC1, RT_SC2, RT_SC3.

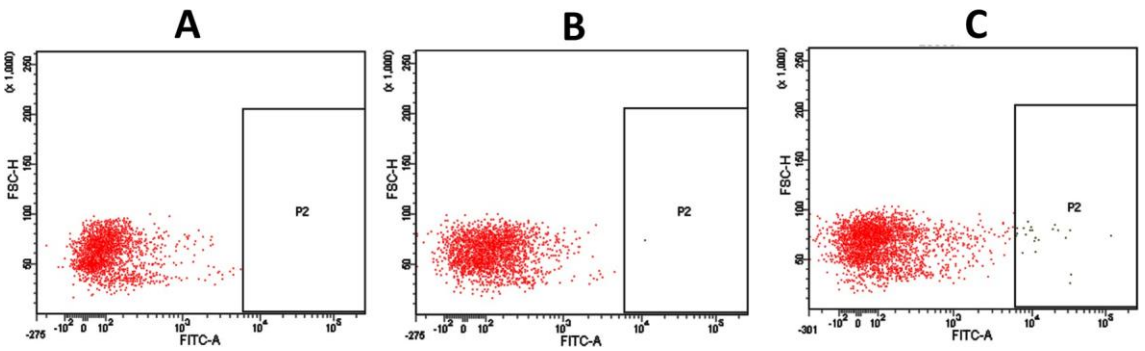

Figure 4 Isolation of *Tetracapsuloides bryosalmonae* by FACS Aria III cell sorter. Figures represent a two-dimensional scatterplot which corresponds to Fluorescein isothiocyanate area (FITC-A) versus forward scatter height (FSC-H). **A** Unstained infected kidney cells as control; **B** stained infected kidney cells with anti-mouse antibody Alexa 488 as a secondary antibody alone control; **C** stained infected kidney cells with anti-*T. bryosalmonae* monoclonal antibody and anti-mouse antibody Alexa 488.

Validation of differentially expressed transcripts by quantitative real time PCR

To test the validity of RNA-seq results, we performed a quantitative analysis of six randomly selected differentially expressed transcripts (Additional file 2) by quantitative real-time PCR (qRT-PCR) on both RNA extracted from sorted cells as well as from infected kidney samples. The analysed transcripts included ATP-binding cassette subfamily G member 4-like, gag-pol fusion protein, leukocyte surface antigen CD53-like, predicted protein, CD63 antigen, NHP2-like protein 1. Transcript-specific primers were designed using the online tool NCBI Primer-BLAST. Gradient PCR was used to evaluate optimum annealing temperature of designed primers, and serial dilutions were used to test each transcript's primer efficiency. To further ensure their specificity and sensitiv- ity, each primer set's distinctive amplicon was sequenced and subjected to a BLAST analysis. Sixty ng of total RNA from each sorted sample and 1 µg of RNA from infected kidney samples was used to synthesize cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, USA). The cDNA samples (n = 3) of *T. bryosalmonae* from brown trout and rainbow trout were subjected to qRT-PCR with two technical replicates using the optimized primers. qRT-PCR was performed in a final volume of 10 µL, which contained 3 µL parasite sorted cells and infected kidney diluted cDNA samples, 0.5 µM of each primer, 1× SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad) and DEPC-treated sterile distilled water. After 5 min of cDNA denaturation at 95 °C, 37 cycles were performed at 95 °C for 30 s, annealing at 58–60 °C for 30 s and 72 °C for 30 s in a CFX96 Touch Real-Time PCR detection system (Bio-Rad). At the end of all gene expression cycling protocols, melting curve analysis was performed to validate amplification specificity under the following conditions: 58–60 °C for 30 s to 95 °C with an increment of 0.5 °C for 10 s. CWF19-like protein 2 and RPL-18 [19] were used as reference genes to normalize the test samples. The $2^{-\Delta\Delta Ct}$ method was calculated to determine the relative gene expression of *T. bryosalmonae* from the brown trout group relative to the rainbow trout group. The statistical difference between groups was determined using unpaired Student's t-test. Linear regression analysis was performed on corresponding $\log_2$ fold change values of RNA-seq and qRT-PCR to evalu- ate the relationship between them. For all statistical tests, P-value of < 0.05 was regarded as significant.

Results

Isolation of *T. bryosalmonae* from infected kidney

To isolate parasites from the infected kidney cell suspension of brown trout and rainbow trout, the *T. bryosalmonae* populations were characterized using the representative two-dimensional scatterplots. The scatterplots in Figures 4A–C represent forward scatter height (FSC-H) as a function of the fluorescein isothiocyanate area (FITC-A). Unstained infected kidney cells and secondary antibody alone stained infected cells respectively, allowed to detect and eliminate autofluorescence of different cell types (Figure 4A) and non-specific binding (Figure 4B). Figure 4C shows the distribution of para- sites within the rectangular gate from stained infected cells. Each dot inside this gate is considered as one event defined as a parasite cell. For infected kidney samples from each fish host, parasites were always sorted from the rectangular gate. The parasite cell after sorting is demonstrated in Figure 5.

RNA-seq analysis

Sequencing of the six libraries yielded 446.32 mil- lion raw reads. The number of raw reads from each library ranged from 59.05 to 87.38 million (Table 1).

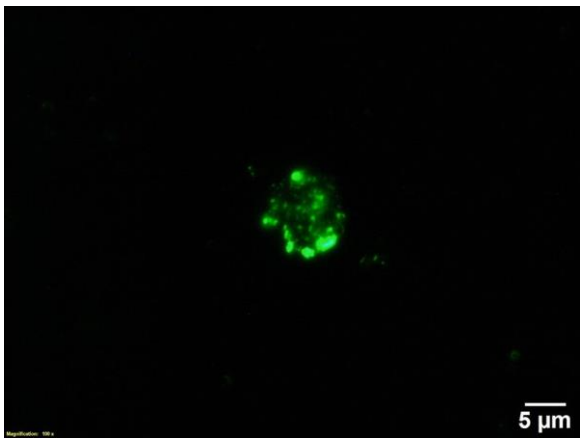

Figure 5 The sorted cell of *Tetracapsuloides bryosalmonae*. Cell suspension from infected kidney was stained with anti-*T. bryosalmonae* monoclonal antibody and anti-mouse antibody Alexa 488 and then subjected to sorting by FACS Aria III cell sorter.

Figure 6 Venn diagram of total identified transcripts of *Tetracapsuloides bryosalmonae*.. Venn diagram representing number of unique and shared genes in *T. bryosalmonae*. The number of common genes of *T. bryosalmonae* between the brown trout and rainbow trout was 3784, whereas 255 and 4253 genes were found to be unique to rainbow trout and brown trout, respectively.

After quality filtering, 56.78–83.72 million reads were retained. Following mapping to brown trout and rainbow trout genomes, 82.24 (37.59%) and 72.77 million reads (34.74%) remained, respectively. After mapping to myxozoan genomes and the *T. bryosalmonae* transcriptome the highest mapping percentage was obtained with the combined *T. bryosalmonae* transcriptome assembly. Therefore, we used the combined *T. bryosalmonae* transcriptome assembly as a reference for mapping of RNA-seq data. 15.2 million and 10.2 million average parasite reads mapped from brown trout and rainbow trout respectively (Table 1). These reads were assembled into 4039 and 8037 contigs of *T. bryosalmonae* (transcripts with average RPKM > 1.0) from rainbow trout and brown trout, respectively. The expression values (read counts and RPKM) for all parasite transcripts is provided in Additional file 3.

The Venn diagram showed 3784 shared parasite con- tigs between the two fish hosts, whereas 255 and 4253 transcripts were found to be unique in rainbow trout and brown trout, respectively (Figure 6).

Differential transcript analysis

Comparative transcript expression analysis between *T. bryosalmonae* from brown trout and rainbow trout revealed 1120 DETs of *T. bryosalmonae* (fold change > 2 or < −2, FDR *p*-value < 0.01). This accounts for 3.6% of the total contigs available in the reference *T. bryosalmonae* transcriptome. DETs were visualized in a heatmap and a volcano plots (Figures 7 and 8). Out of 1120 DETs, 548 transcripts (48.93%) were upregulated, and 572 transcripts (51.07%) were downregulated (Additional file 4).

Gene ontology

Out of the identified 1120 DETs, 371 transcripts (33.12%) were assigned to gene ontology terms. The important biological processes with which upregulated transcripts were associated included cortical actin cytoskeleton organization, establishment or maintenance of cell polarity, and peptidyl-serine phosphorylation and modification. The upregulated transcripts were part of cellular components such as cell substrate junction, focal adhesion, and golgi medial cisterna. The upregulated transcripts were involved in molecular functions such as integrin binding, glucose binding, protein serine kinase activity, GTPase binding, and peptidyl-proline dioxygenase activity (Figure 9).

The downregulated transcripts were associated with the biological processes such as cytoplasmic translation, ribonucleoprotein complex biogenesis, subunit organisa- tion and assembly, translational elongation, regulation of translation, non-membrane bounded organelle assembly, and regulation of protein cellular catabolic process. The cellular component analysis of these transcripts indicated that they belonged to the following GO categories: focal adhesion, cell-substrate junction, ribosomal subunit, ficolin-1-rich granule, secretory granule lumen and cytosolic ribosome. In addition, the downregulated transcripts have molecular functions such as GTPase activity, GTP binding, cadherin binding, guanyl nucleotide binding, structural constituent of ribosomes and cytoskeleton and translation regulator activity (Figure 10).

Validation of selected transcripts

Expression profiles of the randomly selected transcripts by qRT-PCR were consistent with the patterns of expression revealed by the RNA-seq (Figure 11). The parasite transcripts that were upregulated and downregulated by RNA-seq and qRT-PCR of sorted cells showed similar

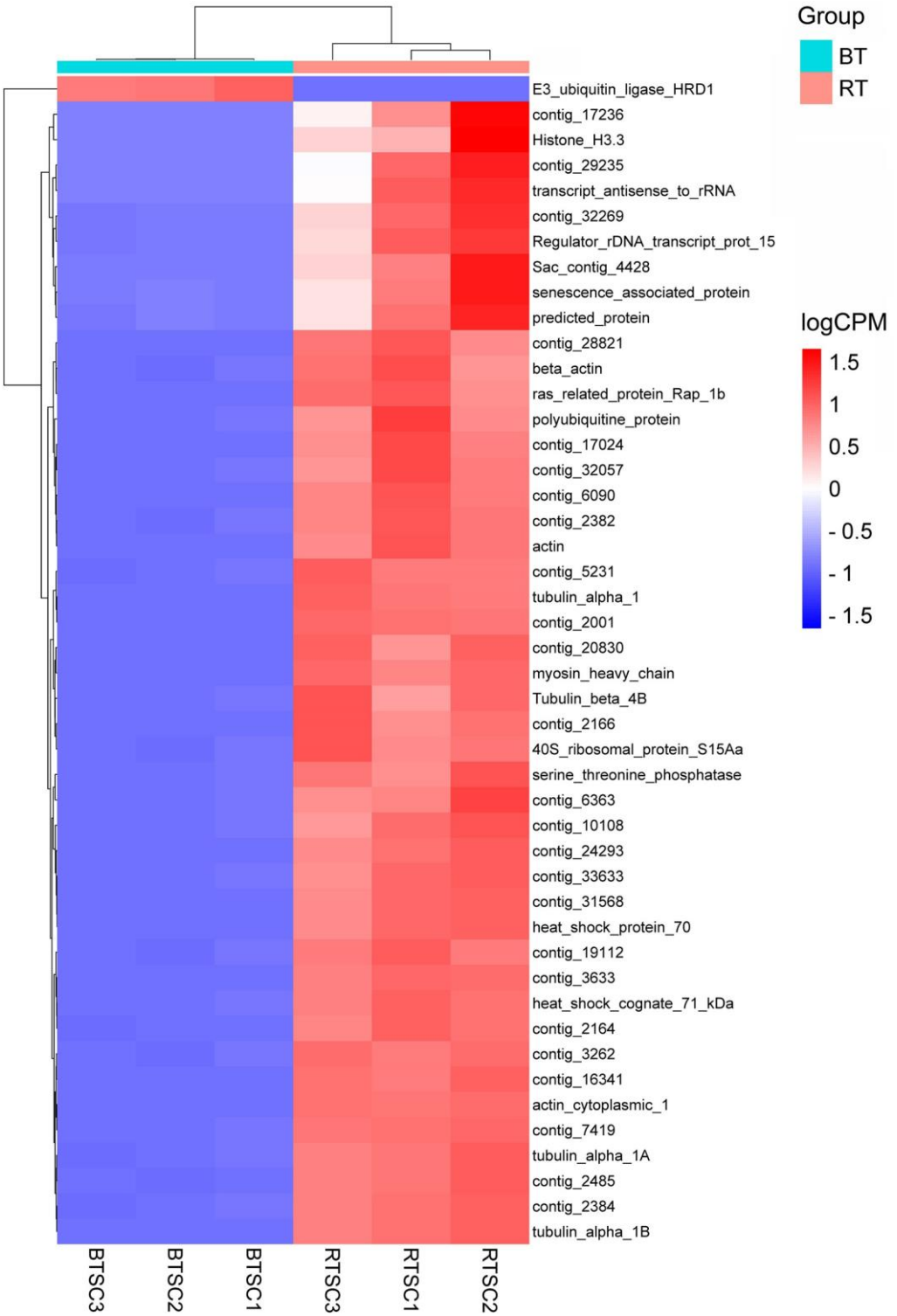

Figure 7 Heatmap visualization and hierarchical clustering of top 50 differentially expressed transcripts. The heatmap shows 50 differentially expressed transcripts of *Tetracapsuloides bryosalmonae* between brown trout and rainbow trout selected based on FDR adjusted *P*-value. Hierarchical clustering was performed using the single linkage method based on Euclidean distance matrix. The columns represent *T. bryosalmonae* samples and rows represent selected genes.

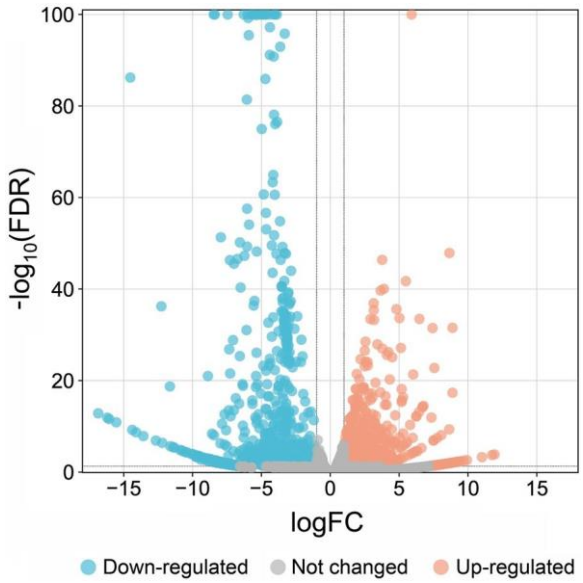

Figure 8 Volcano plot of *Tetracapsuloides bryosalmonae* transcripts in brown trout compared to rainbow trout. The x-axis shows $\log_2$ transformed fold change, and the y-axis shows $\log_{10}$ transformed adjusted significance. Each dot represents an individual gene that is significantly downregulated (blue), significantly upregulated (red) or unchanged (grey) in brown trout compared to rainbow trout. The dashed horizontal line represents the 0.01 cut-off for adjusted *P*-value, and the dashed vertical lines represent the fold change cut-off of two.

expression pattern in the fish kidney tissue, thereby confirming the reliability of our approach.

Discussion

The behavior and outcome of *T. bryosalmonae* within hosts can be profoundly influenced by a myriad of host-associated factors, highlighting their indispensable role in shaping the dynamics and ultimate outcomes of the biological system. However, the differential behavior of parasite between both the hosts can also be a response of the parasite itself and the focus of this study was to look into such parasite associated factors. We conducted this study to identify differentially expressed transcripts of *T. bryosalmonae* between brown trout (carrier host) and rainbow trout (dead-end host). We used FACS to obtain pure parasites in large numbers from infected kidney of both the fish species. To our knowledge, this is the first study reporting the isolation of *T. bryosalmonae* from fish host by FACS. The commercially available monoclo- nal antibody against *T. bryosalmonae* that was used for staining cells has been found to identify both pre-sporogonic stages and sporogonic stages of the parasite [25]. Subsequent to FACS isolation, sorted parasite cells were subjected to RNA-seq. In recent years, RNA-seq is being widely used for generating huge amount of transcriptome data for many myxozoan parasites, the analysis of which is leading to impactful information [14]. Concerning *T. bryosalmonae*, some of the previous studies utilized dual RNA-seq to study its transcriptome from infected kid- ney of brown trout [20], and the overlap transcriptome between infected kidney of rainbow trout and bryozoan hosts [19]. However, the comparative transcriptome of this parasite between infected kidney of rainbow trout and brown trout has not been attempted earlier. This is remarkable considering the disparity in the fate of the parasite in these hosts; successful completion of life cycle in brown trout whereas clearance or degradation in rainbow trout [26]. Therefore, in our work, we applied RNA-seq to analyze the comparative transcriptome of FACS purified *T. bryosalmonae* from brown trout and rainbow trout. From our approach, we obtained more than twice host-filtered reads as reported by Faber et al. [19] through dual RNA-seq of infected kidney of rainbow trout. Similarly, we obtained greater than 10 times host-filtered reads as reported by Ahmad et al. [20] from infected kidney of brown trout. This is despite the fact that the total number of reads obtained by Faber et al. [19] and Ahmad et al. [20] was 1.6 and 2 times higher than what we found. This suggests towards the enrich- ment of parasite reads in our study by using FACS. Nev- ertheless, we too had 62–65% host reads. However, this might be related to the close association of *T. bryosal- monae* with host macrophages [7], also shown in the his- tology figure from our study (Figure 1B). Additionally, a cursory glance at the host mapped reads also showed that many genes are macrophage associated (such as interleu- kin, nitric oxide synthase, Zinc Finger CCCH-Type Con- taining 12 A, Nuclear Factor Kappa B Subunit 2, Retinal Dehydrogenase 2), which could be a possible explanation for the presence of host reads. Our study revealed many differentially expressed transcripts, likely to be involved in the fundamental processes required for its survival in the fish host. Understanding these differences in tran- script expression of *T. bryosalmonae* in brown trout, and rainbow trout can be instrumental in future development of control measures.

In comparison to the dead-end host, *T. bryosalmonae* seemingly reflect an adaptive plastic strategy in the car- rier host. Parasites are detectable in blood initially during the pre-clinical [9, 27], and later in the post-clinical phase of infection in brown trout [28]. Additionally, probably the parasites are maintained throughout their lives in this host [28, 29]. On the contrary, in rainbow trout the parasites are suggested to be cleared in the fish surviving the infection [30]. From the host perspective, B cell and Th1-like cytokines mediated immune responses probably confer greater tolerance and lower resistance to brown trout in comparison to rainbow trout [26]. However,

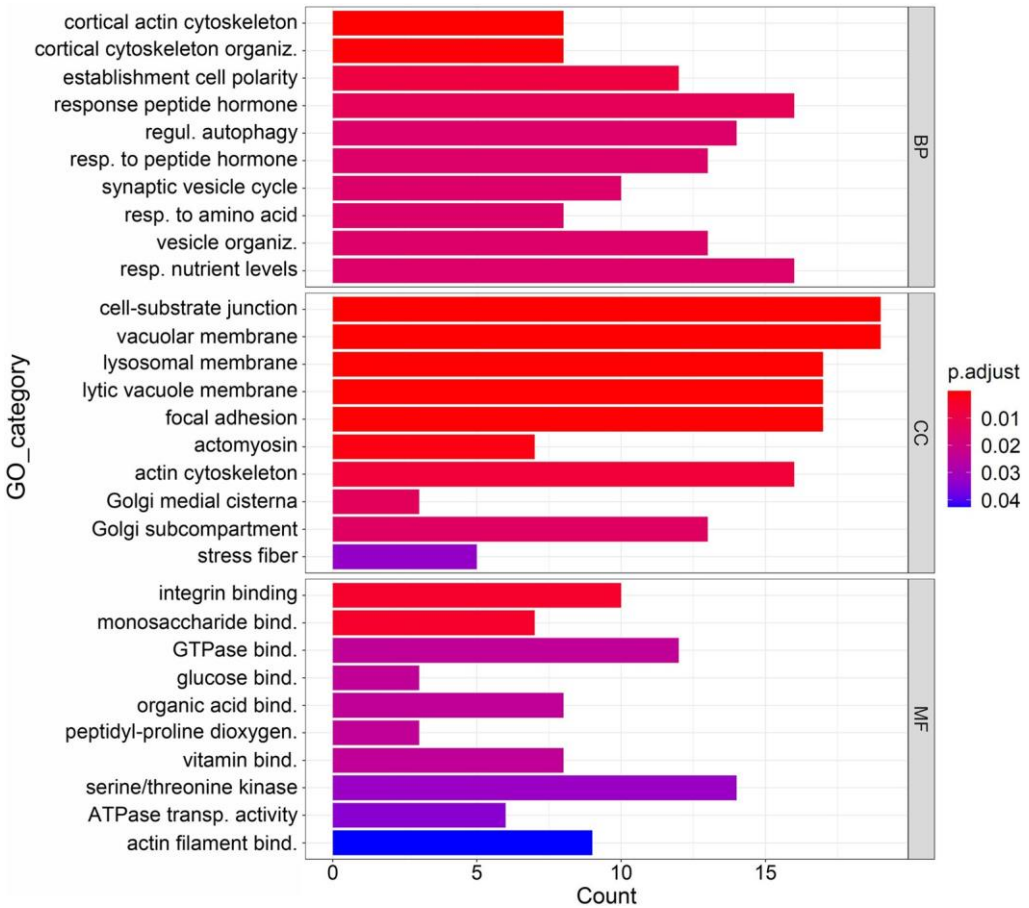

Figure 9 Functional enrichment analysis of upregulated DETs shown by bar plot. Three sub ontologies of GO enrichment analysis: the biological process (BP), cellular component (CC) and molecular function (MF). GO terms are represented on y-axis and number of genes (count) in each GO term are represented on x-axis.

information on the response of the parasite at the molecular level in both fish hosts are obscure. Nevertheless, there is widespread recognition that transcriptional modulation is critical for the survival of parasites in order to adapt to host immune responses and physiological changes [31]. Accordingly, it is plausible that the parasite is able to adapt to the host immune response in brown trout by varying its gene expression, whereas it cannot do so adequately or appropriately in rainbow trout [26]. In the present work, we experimentally infected both brown trout and rainbow trout with the same *T. bryosalmonae* lineage, indicating that the parasite had to adapt its gene expression levels in each of the fish to overcome immune defences and differences in the physiological conditions in the host. Changes in the *T. bryosalmonae* transcriptome can hence, be directly linked to the different host environments and immune responses, which is supported by the biological function of differentially expressed transcripts. Table 2 summarises some of the important physiological functions and the differentially expressed parasite transcripts possibly associated with them. Here, we attempt to highlight and discuss some of the enriched biological processes of DETs, which might be crucial for parasite development in the fish host and require closer examination.

Evidence of different gene regulatory mechanisms in *T. bryosalmonae*

Like all organisms, the survival of parasites depends on stringent spatiotemporal regulation of gene expres- sion finally leading to the synthesis of diverse proteins that are the principal functional units. The translation of mRNAs into proteins is dependent on many other RNAs, ribosomal proteins and protein-RNA complexes. In our study, the GO analysis of downregulated DETs of *T. bryosalmonae* (from brown trout compared to rainbow trout) revealed that the top ten GO terms were mainly processes associated with translation and proteins. The cells can respond to environmental cues much faster through translational control rather than transcriptional

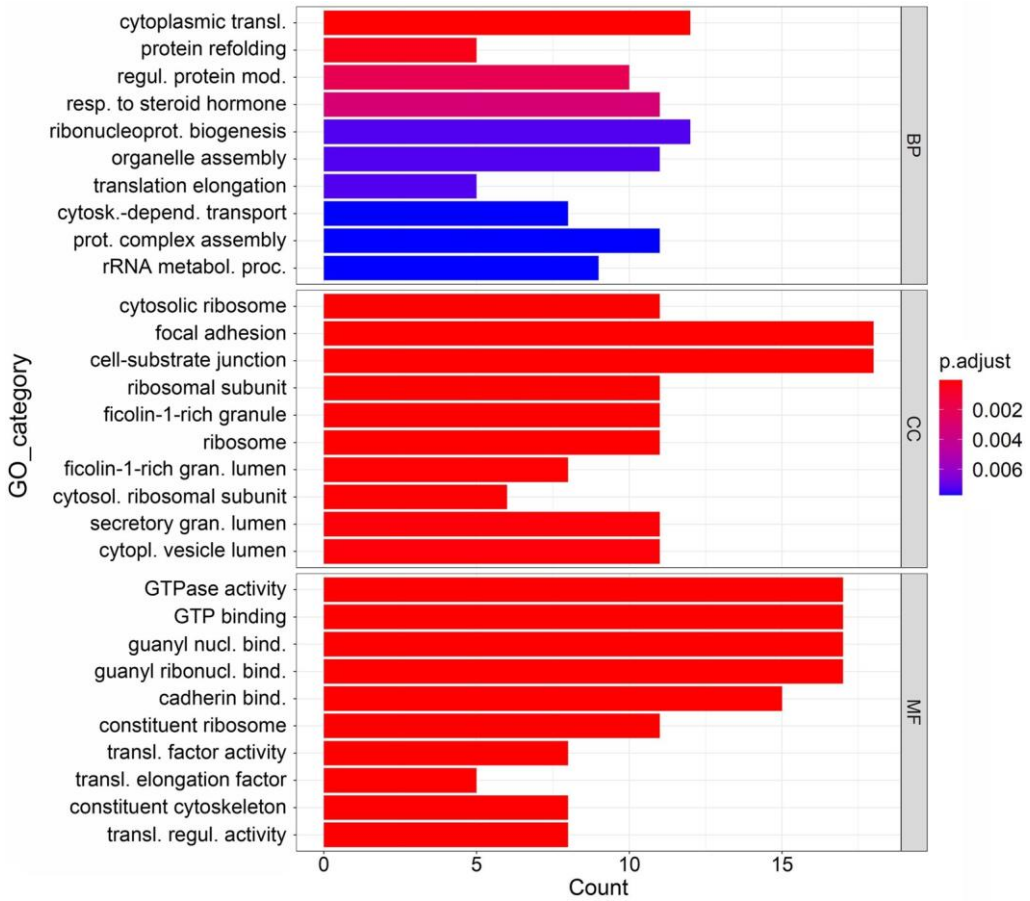

Figure 10 Functional enrichment analysis of downregulated DETs shown by bar plot. Three sub ontologies of GO enrichment analysis: the biological process (BP), cellular component (CC) and molecular function (MF). GO terms are represented on y-axis and number of genes (count) in each GO term are represented on x-axis.

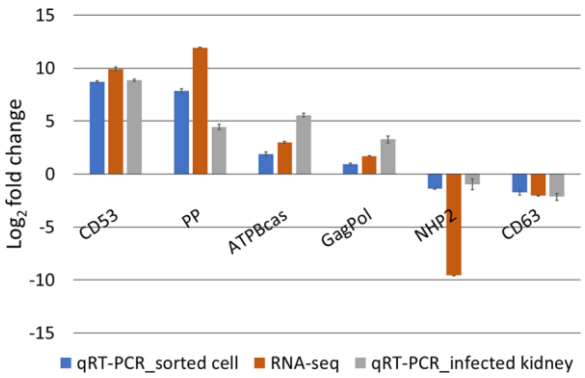

Figure 11 Validation of RNA-seq analysis by qRT-PCR on selected genes. The gene expression values are represented as relative log_2-fold change (mean ± SEM) of *Tetracapsuloides bryosalmonae*-brown trout group compared to the rainbow trout group ($n = 3$). *CD53:* leukocyte surface antigen CD53-like, *PP* predicted protein: *ATPBcas:* ATP-binding cassette sub-family G member 4-like, *GagPol:* gag-pol fusion protein, *NHP2:* NHP2-like protein 1, and *CD63:* CD63 antigen. *qRT-PCR:* Quantitative Real time Polymerase chain reaction, *RNA-seq:* RNA sequencing.

regulation [32]. This is because the easiest strategy to regulate the protein function over time is to inhibit or promote its synthesis. This might be significant for *T. bryosalmonae*, which has a complex life cycle undergoing different developmental stage transitions in its fish hosts during which gene expression regulation might occur essentially at the post-transcriptional level.

The occurrence of post-transcriptional regulation is supported by the presence of many of the genes among the DETs. For instance, argonaute genes, the component of RNA interference (RNAi) machinery [33], as well as protein phosphatase 1 catalytic subunit that play key roles in post-transcriptional gene regulation [34]. Among the different mechanisms, RNA degrada- tion is an important method of post-transcriptional regulation [35]. Exosome complex (EXOSC) genes are important mediators of RNA degradation pathway that encode a multi-protein intracellular complex [36]. We found downregulation of EXOSC7 and its association

Table 2 Some major physiological functions of parasites and associated differentially expressed genes found in this study

Function	Gene	Log_2 fold change (p value < 0.001) *T. bryosalmonae*(brown trout vs. rainbow trout)
Adherens junctions linking proteins	Catenin beta-like	−3.879
Actin polymerization	ras-like GTP-binding protein Rho1 isoform X2	−3.985
	ras-related GTP-binding protein A	2.796
	ras-related protein Rac1	−14.379
	T-cell activation Rho GTPase-activating protein	−1.711
	rho GTPase-activating protein 5 isoform X1	1.361
	rho GTPase-activating protein 39-like	1.948
	rho-associated protein kinase 1 isoform X1	1.980
	Afadin isoform X5	5.159
Adhesion molecules	Putative alpha P integrin isoform X1	−3.688
	Neural cell adhesion molecule 2	5.028
Tight junction proteins	Tetraspanin-9	1.769
	Tetraspanin-4	2.245
Extracellular matrix	fibronectin type-III domain-containing protein 3 A isoform X1	1.295
	collagen alpha-1 (XII) chain	3.639
	matrix metalloproteinase-25-like	3.271
Integrin-cytoskeleton links	talin-1 isoform X1	1.55
	alpha-actinin-1 isoform X3	2.113
	alpha-actinin, sarcomeric-like isoform X2	−5.290
	AChain A, X-ray structure of the human alpha-actinin isoform 3	−3.039
Nucleotide transporter	adenosine 3'-phospho 5'-phosphosulfate transporter 1	4.3565
Amino acid transporters	ATP-binding cassette sub-family G member 4-like	1.801
	ATP-binding cassette sub-family A member 12	1.537
	high affinity cationic amino acid transporter 1-like	3.088
	cationic amino acid transporter 2-like	3.310
Ion transporters (channel/Pore type transporters)	solute carrier organic anion transporter family member 4A1 isoform X2	−1.594
	vesicular glutamate transporter 2-like	2.149
	voltage-dependent calcium channel type A subunit alpha-1-like	4.013
	voltage-dependent N-type calcium channel subunit alpha-1B-like isoform X1	5.495
Ion transporters (ATP driven Na + dependent or cotransporters	Kanadaptin	1.478
	putative glycerol-3-phosphate transporter 5	1.407
Cell signalling	EGF-like domain-containing protein	6.451
	CREB-binding protein	−5.874

The table describes some of the key physiological functions of differentially expressed transcripts identified in this study. The log_2 fold change of the parasite transcripts between brown trout and rainbow trout at a p value < 0.001 are presented.

with the GO category of regulation of translation. The mature spores of another myxozoan parasite, *C. shasta* were found to contain EXOS1 and EXOS2 [37]. These findings hint at important roles of EXOSC genes in the maturation of myxozoan spores. Beside these genes, the presence of CPEB [38], RACK1 [39], and CNOT1 [40], probably emphasise major roles of post-transcriptional as well as post-translational regulation during the parasite development in the fish host.

Role of ribonucleoprotein complex (RNPs) in parasite gene regulation

Interestingly, our data revealed that many downregulated DETs were associated with the ribonucleoprotein complex biogenesis and ribonucleoprotein complex subu- nit organization categories. Ribonucleoprotein complex (RNPs) play important role in determining the fate of RNA in eukaryotes. RNPs consist of mRNA bound to RNA-binding proteins (RBPs). In addition to providing

protection to the RNA molecule, RNPs are involved in several processes during its transport from the site of transcription to the site of translation in the cytoplasm. Ribosomal proteins are involved in regulation of gene-specific transcription and translation processes [41]. Additionally, alternative ribosome variants help eukaryotic cells to adapt to changing conditions [42]. Currently information on gene regulatory mechanisms in myxozoan parasites is scarce and needs to be investigated.

Downregulated biological process of cytoplasmic translation

The biological process of cytoplasmic translation was the top process related to the downregulated DETs. A possible explanation for this could be that downregulation of genes related to translation in *T. bryosalmonae* from brown trout might be a probable parasite strategy similar to other parasites such as *Plasmodium.* These parasites employ translational delay wherein protein expression is actively suspended for expressed mRNA transcripts to quickly adapt to new environments and undergo developmental switching facilitating their survival. Storing transcripts required for such adaptations allows for rapid changes in gene expression by bypassing the time needed for transcription [43]. Additionally, many coccidian protozoan organisms are known to regulate the transcription of ribosome biosynthesis in order to adapt to changes that accompany stage transitions during their developmental life cycles [44, 45]. Previous studies have reported the presence of *T. bryosalmonae* sporogonic stages in the kidney lumen of brown trout but not in rainbow trout [25]. These findings are suggestive of employment of translational delay by *T. bryosalmonae* as an immune evasion mechanism and a strategy for their development from extrasporogonic to sporogonic stages in brown trout when compared to rainbow trout. Nevertheless, functional studies would be required to validate this notion.

Expression of transcripts related to formation of processing bodies (P bodies)

An interesting enriched biological process was non-membrane-bounded organelle assembly, which refers to the aggregation and arrangement of a variety of biomolecules to form a non-membrane-bounded organelle. These cellular compartments tend to assemble and disassemble rapidly. Some important organelles belonging to this category include nucleoli [46], P-granules [46], Cajal bodies [47], stress granules [48] and signalling complexes on the cytosolic face of membranes [49]. Each of these organelles have a unique molecular composition despite sharing some similarities. Processing bodies (P bodies) are primarily composed of mRNAs in complex with proteins associated with translation repression and 5′-to-3′ mRNA decay (e.g., deadenylation complex Ccr4-Not, decapping activators DDX6). Recently, myosin (MYO1C, MYO1D, MYO6 and MYH10) have been identified as P-body proteins [50]. In our study, the presence of genes (e.g., CNOT1, MEF2A and MYH6), linked to the biological process of non-membrane-bounded organelle assembly might indicate the presence of P bodies. Different functions have been proposed for P bodies such as storage, decay and release of mRNA upon stress removal [51]. The formation of these organelles increases in response to diverse stress stimuli and disappear during mitosis. In view of the proposition of coevolution of *T. bryosalmonae* with brown trout and not with rainbow trout, the downregulation of DETs related to the tentative P bodies is expected in the former host. Nevertheless, there is no information on the formation of RNP granules in *T. bryosalmonae*, as well as in any of the myxozoans in general.

T. bryosalmonae heat shock proteins (HSPs)

In the present study, the GO term protein refolding was one of the significant biological processes in downregulated DETs. Genes linked with this process included HSP70, HSP90 and HSP60 family members. Parasites express HSPs in response to diverse stimuli such as heat and oxidative stress that confer them resistance to these harsh conditions and are therefore critical to their survival [52]. Additionally, HSPs play important roles in several other processes such as protein homeostasis. They are known to bind to non-native forms of proteins to facilitate their folding to native conformations [53]. Both brown trout and rainbow trout are known to mount robust immune responses against *T. bryosalmonae* [26]. In turn, *T. bryosalmonae* need to overcome these challenges, which might be adversely affecting many of their physiological processes including protein structure and function. Thus, the upregulation of these parasite HSPs might be suggestive that they are the main strategy used by *T. bryosalmonae* for maintaining protein homeostasis in rainbow trout, whereas some other strategy might be employed solely or in combination with HSPs in case of brown trout. Additionally, HSPs in several parasites such as *T. cruzi* [54] and Schistosomes [55] have been found to be immunogenic in their hosts. In our recent study, we found the HSP70 and HSP90 of *T. bryosalmonae* to be immunogenic using in vivo induced antigen technology [56].

Cytoskeleton organisation and cell polarity crucial for parasite development

GO analysis of upregulated differentially expressed transcripts of *T. bryosalmonae* (brown trout vs. rainbow trout) showed significant enrichment in the biological

processes of cytoskeleton organisation and cell polarity. The actin cytoskeleton is a dynamic network playing important roles in many cellular mechanisms such as division, motility and shape maintenance in addition to generating mechanical forces within the cell [57]. In our study, the genes associated to the cytoskeleton organisation included talin 1 (TLN1), and erythrocyte membrane protein band 4.1 like protein 5 (EPB41L5). TLN1 is responsible for connecting integrins to actin and the regulation of integrin adhesion complexes [58]. Reportedly, this gene is involved in the motility of highly virulent *Ceratomyxa shasta* strains but not in avirulent strains [59]. Protein 4.1 [encoded by erythrocyte mem- brane protein band 41 (EPB41) gene] is a membrane-cytoskeleton protein cross-linker and adaptor that connects cytoplasmic spectrin-actin filament complexes and a wide variety of transmembrane proteins [60]. The erythrocyte membrane protein of *Plasmodium falci- parum* is shown to promote adherence of infected host erythrocytes to microvascular epithelium [61] in a pro- cess called as sequestration which allows the parasite to escape the host immune response [62]. Besides, *P. falci- parum* EMP1 has been demonstrated to adhere to brain, intestinal and kidney endothelial cells [63]. Earlier stud- ies have reported the attachment of *T. bryosalmonae* to the vascular endothelium of host [64, 65]. Though unex- plored in *T. bryosalmonae* immune evasion does occur as evident from persistence of the parasites in brown trout. Together, these findings suggest that EMPs might be an important player in the attachment of parasites to vascu- lar endothelium and also to kidney cells, thereby in some way aiding in their evasion from host defenses. Therefore, the role of *T. bryosalmonae* EMPs needs to be further explored.

Cell polarity involves polarized organization of cell membrane-associated proteins and the asymmetric organization of organelles and cytoskeleton [66]. It governs diverse cellular processes as differentiation, localized membrane growth, directional cell migration, and vectorial transport of molecules across cell layers [67]. Under this GO term, we found many members of serine-threonine kinase family. Additionally, integrin linked kinase (ILK) was also associated to this process, which is known to play an important role in collective cell migration and establishment of cell polarity [68].

Extrasporogonic stages of *T. bryosalmonae* present in kidney interstitium migrate to kidney lumen by amoeboid movement for further development to form sporogonic stages [7]. While extrasporogonic stages of *T. bryosalmonae* have been reported in both brown trout and rainbow trout, sporogonic stages in renal lumen are reported only in the carrier host (Figure 1A). In myxozoan parasites, many studies have pointed towards the fundamental importance of motility during invasion, en route target tissue and during development in their hosts [69, 70]. Besides, the involvement of actin cytoskeleton in motility of this group of parasites is also demonstrated [59, 71, 72]. Consistent with these findings, we postulate that higher expression of *T. bryosalmonae* genes related to cytoskeleton organization and cell polarity in carriers as compared to dead-end hosts may be playing an important role in the development and differentiation of the extrasporogonic stages of the parasite to sporogonic stages in brown trout.

Post-translational modifications (PTMs) of serine phosphorylation

Two interesting processes containing upregulated DETs were peptidyl-serine phosphorylation and peptidyl-serine modification. In eukaryotes, PTMs govern various essential functions including cell signaling, protein trafficking, epigenetic control of gene expression, cell-cell interactions, and cell proliferation and differentiation [73]. One of the most studied and important PTMs is phosphorylation [74]. Phosphorylation occurs at many amino acid residues including serine [75]. In the present study, many members of serine-threonine kinase family were found to be involved in the processes of peptidyl-serine phosphorylation and modification (Additional file 5).

An interesting serine-threonine kinase present was doublecortin like kinase 3 (DCLK3). DCLK3 is a DCX-domain (doublecortin) containing protein with a kinase domain attached to them. In the Apicomplexan parasites such as *Toxoplasma*, DCX loss causes impaired host-cell invasion and slow growth [76]. As with other myxozoan parasites, the role of post-transcriptional and post-translational modifications during the parasite life-cycle remains unexplored in *T. bryosalmonae*. However, the findings in this study suggest towards their potential role in regulating parasite life-cycle.

Possible role of cysteine in mitigating oxidative stress

We determined cysteine metabolic process to be amongst the upregulated processes other than the top ten biologi- cal processes. Under this process the transcripts of CDO, glutamate-cysteine ligase catalytic subunit (GCLC) and GGT1 (65-folds, 29-folds and 3.0-foldss, respectively) were included. By involving in numerous biological pathways, these genes have been found to be important mediators of cysteine based antioxidative response [77, 78]. The upregulation of these genes in the carrier host probably indicate that cysteine metabolism may be a major oxidative stress management strategy employed by *T. bryosalmonae* in brown trout as compared to rainbow trout.

Comparison of RNA sequencing and qRT-PCR data

Validation of RNA sequencing data by qRT-PCR is a routinely used approach. In our study, in general, a high correlation was obtained between the expression level obtained by RNA-seq data and qRT-PCR for the randomly selected transcripts. However, for one of the parasite transcripts NHP2, although both techniques revealed its downregulation in brown trout relative to rainbow trout; the difference was relatively very high. This could be merely due to the fact that both are different techniques (utilizing different normalization methods, have different dynamic range of expression detection etc.), or due to the biological complexity of the investigation. Additionally, the gene or transcript under investigation could be the potential reason [79]. This has also been observed for genes which exhibit very high or very low expression levels [80].

Overall, our study suggests that *T. bryosalmonae* regulates its gene expression depending on the host. Gene ontology enrichment analysis enabled us to identify many biological processes to which the DETs were associated such as the post-transcriptional and post-translational regulatory mechanisms, cytoskeleton organisation, stress response mediated by HSPs and cysteine metabolism, which could be important determinants of *T. bryosalmonae* fate in these two hosts. Considering the multitude of important functions that may be executed by the DETs, the proteins encoded by them should be further evaluated by functional studies as molecular targets for developing therapeutics against *T. bryosalmonae*. Additionally, functional studies will be required to determine the precise roles of identified hypothetical proteins and genes with unknown function.

Limitations and future prospects

Though we successfully employed FACS to isolate *T. bryosalmonae* from the fish host for the first time, we acknowledge the limitations associated with our study. While we visually examined cell viability pre and post sorting and ensured the inclusion of living cells based on the forward and side scatter characteristics provided by FACS, we did not assess it using other techniques such as live and dead cell staining or the incorporation of fluorescent markers. In future, incorporating live and dead cell staining or other fluorescent markers in the FACS technique would enhance the suitability of the technique for conducting further studies as these complementary methods would enable a more precise assessment of cell viability. This technique could be pivotal in aiding the in vitro culture of this parasite.

Abbreviations

T. bryosalmonae	*Tetracapsuloides bryosalmonae*
PKD	proliferative kidney disease
DPBS	Dulbecco's phosphate buffered saline
NCS	newborn calf serum
FACS	fluorescent activated cell sorting
RNA-seq	RNA sequencing
DETs	differentially expressed transcripts
qRT-PCR	quantitative real-time PCR

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13567-023-01185-7.

Additional file 1. Quality control pipeline applied in the analysis of RNA-seq data.

Additional file 2. List of quantitative qRT-PCR primers used in this study.

Additional file 3. List of expression values for all *T. bryosalmonae* transcripts.

Additional file 4. List of differentially expressed transcripts of *T. bryosalmonae*.

Additional file 5. List of top ten GO biological process categories of upregulated and downregulated *T. bryosalmonae* transcripts.

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Authors' contributions

GK and MEM conceptualized and supervised this study. SS performed the experiment. RE, SS and GK analyzed RNA-seq data and performed bioinformatics. SS drafted the manuscript. GK, RE, VS and MEM reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated for this study are available in the NCBI Short Read Archive (SRA) portal under NCBI Bioproject ID PRJNA837187.

Declarations

Ethics approval and consent to participate

The animal study was reviewed and approved by the institutional ethics committee of the University of Veterinary Medicine Vienna and the national authority approved this study under the approval number BMWFW-68.205/0181-WF/V/3b/2017, in accordance with § 26 of the Austrian Law for Animal Experiments, Tierversuchsgesetz 2012. The methods employed in this work are reported in accordance with the ARRIVE guidelines for animal research.

Competing interests

The authors declare that they have no competing interests.

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4. Discussion

PKD caused by *T. bryosalmonae* in salmonids remains a pressing concern for salmonid populations throughout Europe and North America, with its significance poised to escalate in response to rising temperature (Sterud et al., 2007; Bettge et al., 2009). Therefore, with the broader goal of facilitating vaccine development against *T. bryosalmonae*, initially a comprehensive literature review was conducted to explore potential avenues. Literature survey enabled us to identify certain critical knowledge gaps pertaining to the host-parasite interactions and the parasite biology. Specifically, there was a lack of information regarding the parasite-specific antibody response in the host fish as well as potential vaccine candidates. Additionally, in the previous work at our lab, some B-cell-associated genes linked to haematopoiesis and B-cell receptor signaling exhibited variable expression during PKD pathogenesis in brown trout. Therefore, we studied the systematic serum antibody response using indirect ELISA (enzyme linked immunosorbent assay) and systematic B-cell-associated gene expression in kidney and spleen of *T. bryosalmonae*-infected brown trout at different time points. In our next work, we examined the *in vivo* induced parasite antigens in brown trout using *in vivo* induced antigen technology (IVIAT).

Another important knowledge gap was the lack of information on the differential behavior of *T. bryosalmonae* between its carrier (brown trout) and dead-end host (rainbow trout). In addition to host-associated factors playing a key role in influencing the behavior and outcomes of *T. bryosalmonae* within its hosts, the notvaried behavior of this parasite between hosts might also stem from its intrinsic attributes. Consequently, our subsequent study specifically delved into these parasite-associated factors. By focusing on this comparison, we aimed to unravel the parasite-specific contributors to its differential behavior and provide insights into the underlying mechanisms.

Herein, the important findings of this work are discussed.

4.1. Host-parasite interaction

An outcome of the host-parasite interaction is the generation of immune response by the host which is aimed at limiting parasite burden (resistance) and/or reducing adverse health

impact due to the parasite (tolerance) (Råberg et al., 2008). Brown trout act as a carrier host to the European strain of *T. bryosalmonae* whereas rainbow trout act as a dead-end host. As *T. bryosalmonae* develops chronic infections in brown trout, it is considered to have greater tolerance and lower resistance towards this parasite. Rainbow trout on the other hand is not found to carry the parasite after a period of time, and is considered to have greater resistance (Bailey et al., 2019). Among the extensively studied host immune responses, a key mechanism involves, vertebrate hosts producing antibodies against invading pathogens including parasites. Antibodies are effector molecules of humoral immunity in vertebrates. In fish, the significance of antibody-mediated immune response has been demonstrated during various parasitic infections either to protect, clear parasite or to confer resistance to the host (Alvarez-Pellitero 2008). In the dead-end host (rainbow trout) sera, anti-*T. bryosalmonae* antibodies were detected at six weeks post-exposure (wpe) through indirect immunofluorescence (Hedrick et al., 1993). However, antibody response had not been studied in the carrier host, brown trout. Therefore, we conducted an experiment to quantify anti-parasite antibody levels in the serum of *T. bryosalmonae*-exposed brown trout using indirect ELISA and to investigate systematic B-cell-associated gene expression in kidney and spleen at different time points. In this study, we assessed anti-*T. bryosalmonae* antibody levels in serum from exposed brown trout from 2 to 17 wpe. Initially, antibody levels increased, then declined in the later infection phase. We observed the highest antibody titer at 8 wpe. Additionally, antibodies were detected even at 17 wpe, suggesting a sustained immune stimulation by the parasite. Notably, antiparasitic antibodies correlated positively with parasite intensity in the kidney and spleen. This suggests potential applications in profiling PKD-associated clinical manifestations, vaccine development, and assessing humoral response efficacy. Three antibody isotypes (IgM, IgD, and IgT) exist in fish and five isotypes (IgM, IgG, IgD, IgT, and IgA) are known in mammals (Pettinello and Dooley 2014). Similar to mammals, fish antibody isotypes have specific roles, but their functions remain mostly unexplored. Identifying the immunoglobulin type present at this time point could provide insights into the generated immune response.

From our study on the gene expression patterns of B cell associated genes (CD79A, CD74, CD34, BCL7, BLNK, and CD22) in both the kidney and spleen of *T. bryosalmonae* infected

fish, it can be inferred that B-cell activity is modulated and involved in the immune responses of both organs throughout the study period.

The upregulation of CD79A, CD74, BLNK, and CD22 in both the studied organs at various time points suggests the activation and participation of B cells in the immune response against the parasite. These genes are associated with different aspects of B-cell function, including B-cell receptor signaling, antigen presentation, and immune regulation (Lund et al., 2008; Myers 1991). The downregulation of CD34 and BCL7 expression in both organs also indicates the dynamic nature of B-cell responses during the infection. CD34 is known to be involved in stem cell regulation and hematopoiesis (Salati et al., 2008), while BCL7 is linked to immune system regulation (Uehara et al., 2015). These changes could reflect shifts in the types or states of B cells present, potentially indicating the progression of the immune response or regulatory mechanisms.

The expression pattern of some of the genes studied here have also been investigated during *Cryptocaryon irritans* infection in the grouper (*Epinepheles coides*). CD79A upregulation in fish kidney at early stages and in spleen at later stage is reported during *C. irritans* infection, possibly indicating the B-cell differentiation in the kidney and maturation in the spleen (Mo et al., 2016). The differential regulation of BLNK gene in different organs (head kidney, spleen, skin and gill) of grouper following *C. irritans* infection, suggested proliferation and differentiation of B cells and macrophages in the primary haematopoietic organ, followed by migration to infected sites (Mo et al., 2018). Additionally, upregulation of CD22 has been demonstrated in skin during *C. irritans* infection in grouper (Hu et al., 2017). It would be interesting to study the B-cell mediated immune response during other parasitic infection of fish especially the myxozoan parasites. We also examined the correlation between the parasite intensity and the expression of the above-discussed B-cell-associated genes in kidney and spleen. Overall, parasite intensity in kidney significantly correlated only with CD74 expression. Likewise, the expressions of CD79A and BCL7 showed a positive association with parasite intensity in spleen.

Taken together, the collective expression patterns of these genes suggest that B cells are actively engaged in responding to the *T. bryosalmonae* infection in both the kidney and spleen.

The differential expression levels at different time points highlight the complexity of the immune response and the different roles that B cells play at different stages of the infection.

In the succeeding study, we identified 136 *in vivo* induced genes of *T. bryosalmonae* in brown trout using IVIAT. These genes were involved in a variety of functions critical for the parasite including signal transduction, actin cytoskeleton organization, transport, metal ion binding, transcription, mitochondrial organization, translation, protein folding, metabolic process, cell division, and DNA repair.

4.2. Comparative transcriptome of *T. bryosalmonae*

Our subsequent project dealt with the identification of differentially expressed transcripts of *T. bryosalmonae* between carrier and dead-end host. In this study, we used FACS to obtain pure parasites in large numbers from infected kidneys of both the fish species. To our knowledge, this study marks the first report of *T. bryosalmonae* isolation from a fish host using FACS. Following this isolation, the sorted parasite cells were used for RNA-seq analysis. Through this approach, we identified 1120 parasite transcripts showing differential expression patterns between those derived from brown trout and rainbow trout. Parasites obtained from brown trout exhibited higher transcript levels associated with cytoskeleton organization, cell polarity, and peptidyl-serine phosphorylation. In contrast, rainbow trout-derived parasites displayed upregulation in transcripts linked to translation, ribonucleoprotein complex biogenesis and organization, assembly of non-membrane bounded organelles, regulation of protein catabolic processes, and protein refolding. These revelations illuminate distinct molecular adaptations in the parasites, potentially underpinning the divergent outcomes within the two hosts. Additionally, many of the DETs were found to be *in vivo* induced in our previous study. Therefore, these differentially expressed transcripts hold promise for their further evaluation as novel drug targets for *T. bryosalmonae* management.

4.3. Limitations and future prospects

Our study documents the systematic antibody response of brown trout against *T. bryosalmonae*, with certain aspects requiring thoughtful consideration. We utilized antigens from parasite sacs developed in invertebrate bryozoans, which infect fish. *T. bryosalmonae*

stages vary between primary (bryozoan) and secondary (salmonid) hosts. For instance, spores differ in structure between hosts. In fish, developmental stages include presporogonic and sporogonic phases. Antibody responses against these stages from fish might differ due to distinct antigenic characteristics compared to bryozoan-derived stages. Thus, the observed antibody levels could be similar to those against kidney-derived parasite antigens in infected trout. Prior research showed antigenicity of parasite antigens from infected kidneys but did not examine systematic antibody responses (Saulnier and de Kinkelin, 1996). Assessing sera against highly infected fish tissues could have addressed this. Further investigation is needed to explore antigenic differences between bryozoan and kidney-derived parasites.

In our subsequent project wherein, we employed IVIAT technique, it is important to note that this technique does not guarantee the antigenicity of identified genes. While it can help identify genes that are expressed *in vivo* during infection, further studies using different tools such as RNA interference are required to determine if these genes are truly antigenic and play a role in the immune response. Antigenicity depends on factors such as the interaction between the pathogen and the host's immune system, and additional validation is typically needed to establish the antigenic properties of the identified genes.

Isolation of *T. bryosalmonae* by FACS holds tremendous potential as an indispensable tool in establishing the *in vitro* culture of this parasite, representing a critical milestone in advancing research in this field. The precision and versatility of FACS, especially when combined with live and dead cell staining or fluorescent markers, can significantly enhance our ability to isolate and manipulate viable parasite cells. This precision is pivotal for maintaining the integrity of the culture, enabling more controlled experiments, and ultimately deepening our understanding of *T. bryosalmonae* and its interactions. As such, FACS emerges as an essential technique that can catalyze breakthroughs in *T. bryosalmonae* research and contribute to the development of targeted treatments and preventive measures.

The salient findings of this study are:

1. Brown trout mounts a parasite specific antibody response during the course of *T. bryosalmonae* infection.
2. 136 *in vivo* induced genes of *T. bryosalmonae* involved in critical biological functions were identified in brown trout using *in vivo* induced antigen technology.
3. Fluorescence-activated cell sorting could be a feasible approach to isolate *T. bryosalmonae* from infected tissues. Parasites purified in this way are suitable for transcriptomic analysis.
4. Differentially expressed transcripts of *T. bryosalmonae* between carrier and dead-end host are involved in key biological functions of the parasite.

5. Conclusion

Our study employed a dual approach to unravel the intricate dynamics of the host-*T. bryosalmonae* interaction. By investigating the antibody response through indirect ELISA, we gained valuable insights into the immune reaction of the host to the presence of parasite. This allowed us to gauge the ability of the host to mount an effective defense against *T. bryosalmonae*. Subsequently, we identified 136 *in vivo* induced genes of *T. bryosalmonae* using IVIAT in the sera of infected brown trout with potentially important roles for the parasite. In parallel, our in-depth exploration of parasite biology via RNA sequencing offered a unique vantage point into the molecular intricacies of the strategies of this parasite within the host environment. We identified key gene expression patterns that shed light on various aspects, including survival mechanisms, potential drug targets, and interaction with the host's immune system.

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