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RESEARCH

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Immunogenicity of extracellular proteins of *Flavobacterium covae* in *Ictalurus punctatus*

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Abstract

Background Columnaris disease, instigated by Gram-negative *Flavobacterium covae* (*F. covae*), is a dreadful disease of many different genera of freshwater fish with alarmingly high mortality rates. *Ictalurus punctatus* (*I. punctatus*) is extremely prone to this disease. Despite the terrible implications of this disease, very little is known about how the host and the bacterium's virulence factor interact. Secreted extracellular proteins (SEPs) are key bacterial virulence agents that frequently provide immunogenicity. In a previous study, the major secreted extracellular proteins of *F. covae* were identified and their protective efficacy was verified in *I. punctatus*, and the results were published. In the current study, an endeavor has been made to examine the progressive shifts in *I. punctatus* immunity following immunization with *F. covae* SEPs in order to more thoroughly comprehend the host's intrinsic and acquired immune responses against *F. covae* infection. An enzyme-linked immunosorbent test (ELISA) is operated to analyze specific humoral immune responses against SEPs of *F. covae*. Moreover, the expressions of immune-associated genes that are related to inherent and acquired immunity were monitored in the anterior kidneys and spleens of the vaccinated *I. punctatus*.

Results Marked rise in immunoglobulin M (IgM) levels were detected in the serum of *I. punctatus* vaccinated with different SEP formulations, including crude SEPs, heat-deactivated SEPs, and SEPs combined with adjuvant, at 14 and 21 days post-vaccination. At the same time points, a marked increase in the expression levels of clusters of differentiation (CD4-1 and CD8-β) and major histocompatibility complex (MHC-II) genes was detected in the vaccinated fish's anterior kidneys and spleens, while the highest levels of interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α) up-regulations were detected in the immunized fish's anterior kidneys 14 days following vaccination.

Conclusions This work revealed that SEPs are an effective basis for vaccination against *F. covae* infection, eliciting humoral and inherent immune responses of *I. punctatus* after SEPs immunization. Improvements are needed to further enhance the *I. punctatus* immunological responses to SEPs of *F. covae* since these antigens remain a prospective candidate for future optimization and clinical trials in aquaculture settings.

Keywords Columnaris, *Flavobacterium covae*, *Ictalurus punctatus*, Immune responses

1 Background

F. covae, a bacterium that is Gram-negative, is the microbiological cause of columnaris disease, which affects numerous varieties of freshwater fish around the world [1]. *Ictalurus punctatus*, a channel catfish, is extremely prone to *F. covae* infection [2]. Disease often begins on the fish's exterior surfaces and may develop discolored abrasions along the back, which is known as saddleback disease [3]. Infected fish may have damaged or clumped gills, which can cause difficulty breathing

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and death [3, 4]. This disease frequently results in high mortality rates, which lead to significant economic losses in the fish farming sector [5].

F. covae pathogenesis is complicated, involving a number of cellular and extracellular proteins that aid in implantation as well as persistence inside the tissues of the host and provoke a variety of immunological responses. The significant loss of cutaneous, muscular, and gill tissues in infected host might be attributed in part to *F. covae*'s synthesis of numerous SEPs [6]. *Flavobacterium* species generate a variety of SEPs, such as proteases and chondroitin lysis enzymes, which are crucial determinants of the microbe's pathogenicity. Various reports have engrossed on the recognition of putative virulence factors in this bacterium [7, 8].

A sustained effort has been made over the past decades to create vaccine candidates against *F. covae*. For instance, the use of formalin killed microorganisms that elicited a particular immunoglobulin response in *Oreochromis niloticus* following intraperitoneal and intramuscular injection, but non-significant protection was detected after the experimental challenge [9]. Furthermore, *I. punctatus* vaccinated with the recombinant heat shock protein expressed by *dna*J gene or the recombinant protein encoded by *dna*K gene found in the extracellular portions of *F. columnare* showed highly specific antibody reactions [10, 11]. Likewise, five membrane proteins from *F. columnare* were highly immunogenic, causing a significant rise in IgM antibody serum levels and up-regulation in the activities of some immune-associated genes in grass carp [12].

Previously, we used mass spectrometry to identify some SEPs, comprising extracellular proteins such as hypothetical proteins, zinc metalloprotease, and chondroitin lyases of *F. covae* [13]. The separated SEPs provided significant protection to *I. punctatus* against *F. covae* infection [13]. As a result, it is suggested that SEPs could be employed as essential immunogenic proteins to elicit immune responses that are both innate and adaptive in *I. punctatus*.

In the present study, the potential immunological responses of *F. covae* SEPs were investigated in the *I. punctatus*, the commonly cultivated freshwater fish species that suffers greatly from columnaris disease in the US. An indirect (sandwich) ELISA was operated to assess the specific IgM serum levels. The immune response was additionally investigated at the genomic level in immunized fish within the anterior kidney and spleen, focusing on the expression of CD4-1, CD8-β, and MHC-II, in addition to other genes associated with innate immunity involving IL-8 and TNF-α.

2 Methods

2.1 Ethical approval

The *I. punctatus* tests were carried out in accordance with the specifications of an approved protocol by Institutional Animal Care and Use Committee (IACUC-19-388) at Mississippi State University, USA.

2.2 Fish immunization

Pathogen-free *I. punctatus* fingerlings ($n=300$) with a 35.45 g mean weight were placed at a density of 15 fish per tank in 20 circulating 40-L tanks with continual aeration. Following two weeks of acclimatization and three times-daily meals, the fish were arbitrarily allocated to four clusters, each consisting of five replicates. During the course of the experiments, the temperature of water was kept constant at 29 °C.

The fish were numbed with tricaine methanesulfonate (MS-222; Sigma®) before handling. Intraperitoneal injection (IP) was used for fish immunization in the current study to ensure complete absorption of the vaccine candidate formulations. The experimental fish were vaccinated with various formulations from previously generated SEPs as follows [13]: the group one was intraperitoneally inoculated with 150 µL of crude SEPs. The group two received 150 µL of heat-deactivated SEPs. The third group received 150 µL of SEPs blended with oil adjuvant (Freund's Complete Adjuvant, Seppic®, France) at a ratio of 30 proteins to 70 adjuvant. Group four received 150 µL of sterile phosphate buffer saline (PBS) as an imitation vaccination, representing the negative control.

2.3 Sample collection

After immunization, blood was drawn from the caudal veins of 25 fish per group (separate tanks) on days 14 and 21 post-vaccination, and serum was recovered with spinning at 3000 × g for 15 min for IgM measurement. The euthanized channel catfish was utilized to obtain anterior kidney and spleen tissues. Tissue specimens were promptly placed in sterilized RNase-free vials and soaked in RNA later (Ambion®, TX, USA) for a full day before beginning the process of extracting RNA for the immune genes analyses.

2.4 Serum antibody response

IgM, the most prevalent serum immunoglobulin that remains active for long periods in teleosts, was assessed using an indirect ELISA with modifications [14]. Overnight incubation at 4 °C for Immulon-2HB® 96-well micro-titer plates (Immuno-Chemistry Technologies®, Davis, CA, US) that were covered with purified SEPs, 150 µL/well. Following that, wells were cleaned and

occluded for 60 min at 25 °C with PBS dissolved 6% dry, skimmed milk (Bio-Rad®, California, US). PBS comprising 0.06% Tween-20 (PBS-T) was used to wash the wells a total of three times. Each well was filled with diluted serum (1:1000) (100 µL/well), kept for 60 min at 37 °C, and then rinsed with PBS-T. Monoclonal immunoglobulin 9E1 (anti-catfish Ig) in a 1:5 dilution was added to each well in an amount of 50 µL. Plates were rinsed with PBS-T after 60 min of incubation at 37 °C, and goat anti-mouse immunoglobulin (Thermo Fisher, Pittsburg, US) was introduced. Afterward, the plates were cleaned and kept at 25 °C for sixty minutes. Lastly, the plates were allowed to further incubate at 25 °C for a total of 60 min with 150 µL of p-nitrophenyl phosphate substrate (Sigma® US) diluted in 15% diethanolamine buffer. An ELISA Microplate Reader (DR-5000, Diatek, China) was used for measuring absorbance at 405 nm. Control wells in each plate contained PBS buffer instead of serum and were produced in the same manner as the blank.

2.5 Expression analyses of some immune genes following immunization

2.5.1 RNA abstraction

The whole RNA was extricated from tissue specimens exploiting Trizol (Sigma-Aldrich®, USA). To assess both the amount as well as the purity of the isolated RNA, a NanoDrop ND-1000 spectrophotometer (Marshall Scientific®, US) was utilized.

2.5.2 Complement DNA synthesis

The Maxima Strand cDNA Kit (Thermo Fisher®, TX, US) was utilized for transforming the entirety of RNA into complement, cDNA for RT-qPCR. cDNA reactions were carried out in 25-µL reaction sets according to the manufacturer's directions. Each tube had 4 µL of RNA, 8 µL of reaction mix, 1.5 µL of enzyme mix, and 11.5 µL of water that were used for molecular purposes. The reactions were allowed to remain for 6 min at 28 °C and 20 min at 55 °C. Lastly, the reactions were terminated at 88 °C for 5 min.

2.5.3 Quantitative real-time PCR

Specific primers to detect as well as evaluate the levels of transcription of CD4-1, CD8-β, MHC-II, IL-8 and TNF-α are listed in Table 1. Twist Bioscience® in California, US, provided them commercially. For qPCR, the FastStart Sybr Green Universal Master Kit (ROX®, Switzerland) was utilized. Each qPCR experiment was accomplished in a 50-µL reaction tube having primers (1 µL), Master Mix (25 µL), cDNA (4 µL), as well as water (19 µL). Quantitative PCR experiments were carried out using RT-qPCR from Applied Biosystems® US. The setting of the thermal cycler was set to 30 cycles, each consisting of initial denaturation (94 °C for 15 s), denaturation (94 °C for 45 s), annealing (56 °C for 30 s), and extension (74 °C for 45 s). Three duplicates of each sample were used. In order to normalize the results, a variance in cycle threshold (Ct) between the genes that were investigated and the gene of reference (18S rRNA) was computed [15, 16]. This difference was then used to calculate the fold changes for each gene. For the statistical evaluation, fold change estimates were employed to identify noteworthy variances between fish clusters that exemplify the sham-immunized and vaccine-treated fish.

Table 1 Genes, accession numbers from GenBank, and oligonucleotide sequences utilized in this work

Genes	Acces. Ns	Primers	References
18Sr RNA	AF021880	F-GAGAAACGGCTACCACATCC	[16]
		R-GATACGCTCATTCCGATTACAG	
CD4-1	DQ435305	F-GATGTCATCATTGTAGATCTCG	[15]
		R-GAGGTAGCTGGCATTTCACTCC	
CD8-β	HQ446240	F-CCATCAGGCCTGGAGAAAGCA	[15]
		R-TCACCACCAGGAGTAGGACA	
MHC-II	AF103002	F-GACACCAGGACATGGGAGGTG	[15]
		R-CGAGGAAGAAAGTTCCGGTAG	
IL-8	AY145142	F-CAATACTTTGTGAATTTCTGC	[15]
		R-TGTCCTTGGTTTCCTTCTGG	
TNF-α	AJ417565	F-GCACAACAAACCAGACGAGA	[15]
		R-TCGTTGTCCTCCAGTTTCAA	

2.6 Statistical assessment

Based on a one-way ANOVA test, the SAS program (Cary, North Carolina, USA) was exploited to determine the noteworthy differences between the investigated clusters. Comparisons with the sham-immunized group were done in order to assess IgM levels and immune gene up-regulation fold changes. The threshold for significance was set at $P \leq 0.05$.

3 Results

3.1 Serum immunoglobulin response

In *I. punctatus* serum that was drawn at 14 and 21 days after being immunized with various SEP formulations, the production of IgM to SEPs was assessed at 2 and 3 weeks' post-immunization, fish which had received vaccinations with crude SEPs, heat-deactivated SEPs, or SEPs joined with adjuvant had significantly greater antibody levels in their serum ($p \leq 0.05$) than the non-immunized control group (Fig. 1). Fish injected with crude SEPs and SEPs emulsified with oil adjuvant had higher levels of antibodies, according to ELISA, than fish immunized with heat-deactivated SEPs. At all levels,

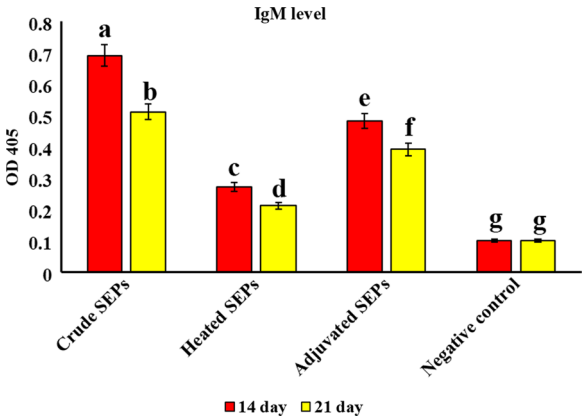

Fig. 1 *I. punctatus* serum antibody response at day 14 and day 21 after immunization. Fish groups immunized with raw SEPs are shown by crude SEPs. A fish group inoculated with heat-inactivated SEPs is indicated by heated SEPs. Adjuvated SEPs refer to a fish group that received a vaccination using SEPs emulsified with Freund's adjuvant as opposed to the negative control group, the sham-immunized group that received PBS injections. The average optical densities for 25 fish per group are measured at 405 nm. The standard errors (SE) of the mean are shown as vertical bars in each column. Different letters are used to signify differences at $P \leq 0.05$

the amounts generated from IgM at 14 days post-immunization were higher than those produced after 21 days post-immunization.

3.2 Immune genes analyses

At 14 and 21 days after vaccination with various SEP formulations, the anterior kidneys and spleens of *I. punctatus* were examined for the transcription pattern of five immune-associated genes (CD4-1, CD8-β, MHC-II, IL-8, and TNF-α). When *I. punctatus* were vaccinated with raw SEPs, heat-deactivated SEPs, or SEPs mixed with adjuvant in comparison to the sham-vaccinated cluster, the transcription of prior genes was substantially ($p \leq 0.05$) up-regulated in the anterior kidney (Figs. 2, 3) and spleen (Figs. 4, 5) at 14 and 21 days post-immunization. At all levels, the anterior kidney's up-regulation levels were higher than those of the spleen. Fish immunized with heat-deactivated SEPs had the lowest up-regulated expressed level among all immunized groups, whereas fish immunized with crude SEPs had the greatest up-regulated expressed level. There was a non-significant alteration in the transcription of IL-8 in the anterior kidneys at 21 days post-vaccination among all immunized groups (Fig. 3). Additionally, there was a non-significant alteration in the transcription profiles of IL-8 and TNF-α in the spleen of the fish group that was immunized with heat-deactivated SEPs at both 14 and 21 days post-immunization (Fig. 5).

4 Discussion

The management of infectious diseases in animals, including fish, depends heavily on vaccinations. This study's purpose was to ascertain whether *I. punctatus* might generate immunogenic-reactive responses from isolated SEPs of *F. covae*. SEPs have a crucial role in the pathology of *Flavobacterium* species. The majorities of Gram-negative microorganisms release such substances to impact or injure the host's cells [17]. Extracellular substances and enzymes that are secreted during the pathogenesis of *F. covae* are imperative for the dissolution and attachment of gill, skin, and muscle tissues [6]. In our previous study, the SEPs from *F. covae* growth medium (FCGM) supernatant contained various proteins ranging in size from nearly 13 to 99 kDa [13]. Zinc-metalloprotease, chondroitin lyases, endonucleases and other putative proteins were recognized using mass spectrometry analysis [13]. These various proteins that are released may promote host tissue attachment or cellular disintegration as possible virulence factors [13]. The protein antigens of *Flavobacterium* species have been revealed through additional studies. For instance, different outer membrane proteins of *F. columnare* were identified, incorporating chondroitin lyases, collagenase, metalloprotease, oligopeptidase, and other hypothetical proteins [12]. Additionally, 15 immune-stimulating proteins from a virulent strain of *F. psychrophilum's* cellular and extracellular products, as well as 4 antigenic proteins from a non-pathogenic isolate, were recognized [18].

After receiving immunization with SEPs against various fish pathogens, these proteins might offer enough immunogenicity to provide protection against infections. Our reasoning for assessing SEPs relied on results from earlier studies that showed extracellular antigenic proteins could elicit an immune reaction and offer immune defense against piscine infectious agents like *Photobacterium damselae* [19], *Vibrio harveyi* [20], *Flavobacterium psychrophilum* [21], and *F. columnare* [12]. The ability of the immunogen(s) to induce immune responses that are both innate and adaptive is one of the most crucial factors considered during immunization efficacy studies. Following delivery of the SEPs to the host, the immunogenicity of the SEP fusion proteins often manifests as an IgM immunoglobulin reaction and transcription of immune-associated genes.

IgM serves as the majority serum immune globulin in fish, and it is important for both mucosal and systemic immune responses [22]. While IgZ mostly affects mucosal reactions also, it has an amino acid sequence that is extremely close to IgM, and it is a distinct teleost immunoglobulin that is equivalent to mammalian IgA [22, 23]. Furthermore, IgD's function in fish is not clearly understood [24]. Thus, in order to examine the immune

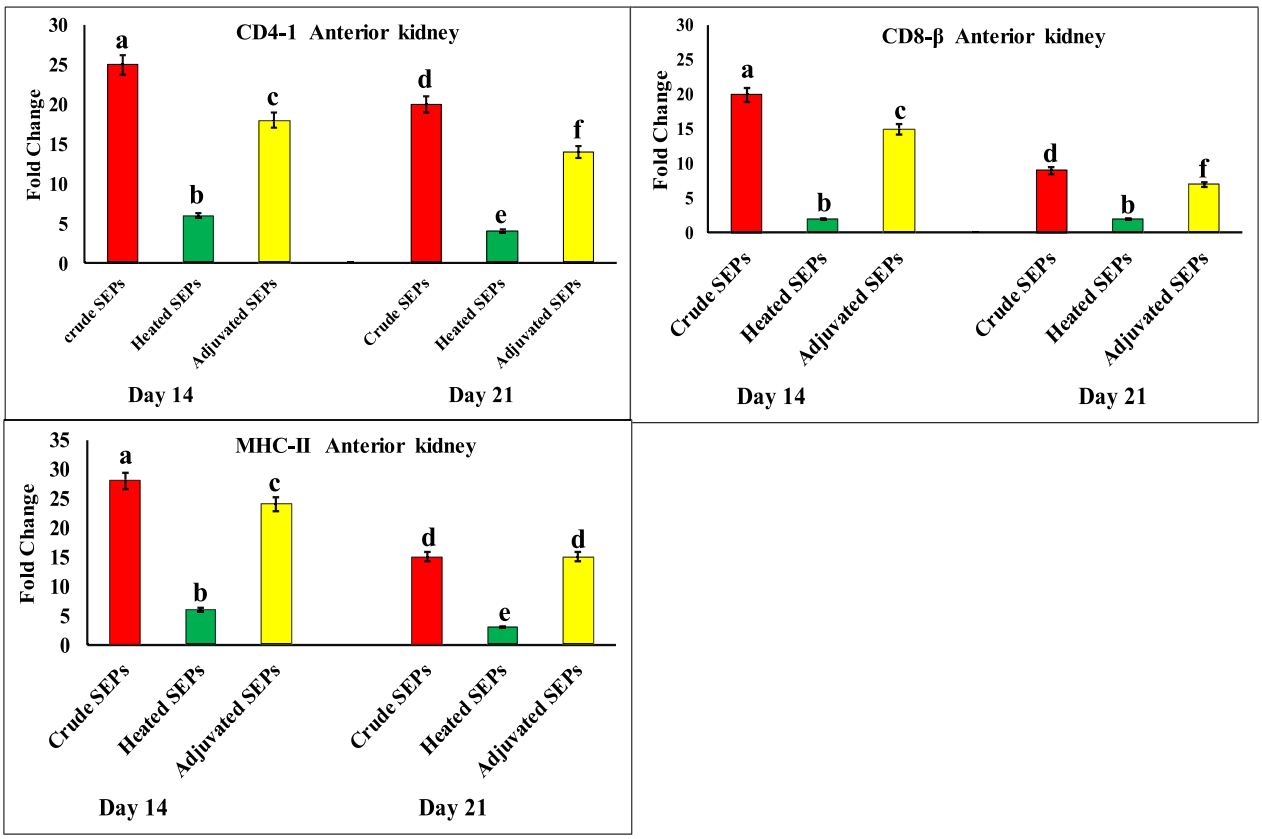

Fig. 2 Alterations in the expressions of CD4-1, CD8-β, and MHC-II genes in the anterior kidneys of *I. punctatus* after immunization. Fish groups immunized with raw SEPs are shown by crude SEPs. A fish group inoculated with heat-inactivated SEPs is indicated by heated SEPs. Adjuvated SEPs refer to a fish group that received a vaccination using SEPs emulsified with Freund's adjuvant. Data are donated as fold differences in gene transcription compared to assessments presented by the negative control group, the sham-immunized group that received PBS injections. Specimens were examined in quadruplicate from a pull of organs from 25 fish per group and offered as average ± SD. Dissimilar letters are used to signify differences at $P \leq 0.05$

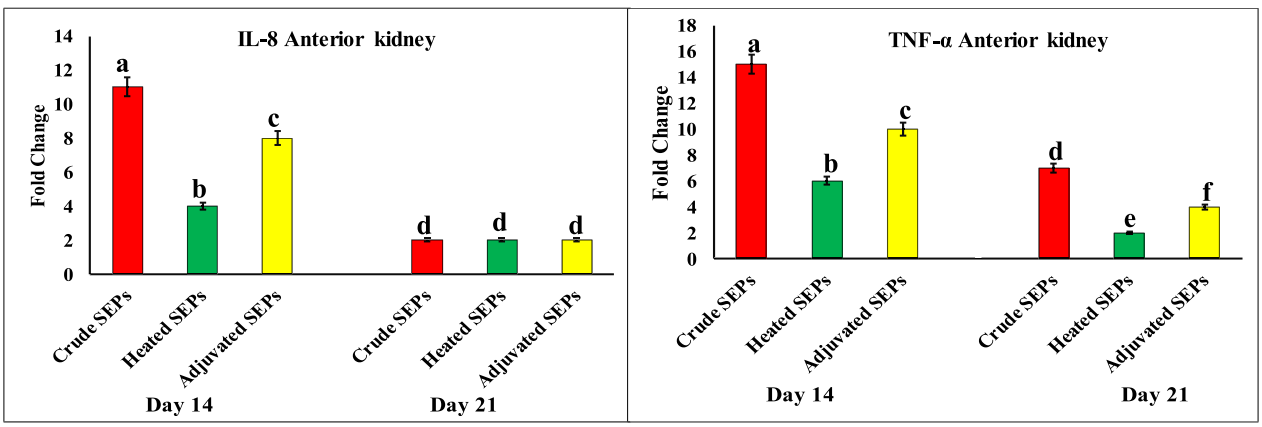

Fig. 3 Alterations in the expressions of IL-8, and TNF-α genes in the anterior kidneys of *I. punctatus* after immunization. Fish groups immunized with raw SEPs are shown by crude SEPs. A fish group inoculated with heat-deactivated SEPs is indicated by heated SEPs. Adjuvated SEPs refer to a fish group that received a vaccination using SEPs emulsified with Freund's adjuvant. Data are donated as fold differences in gene transcription compared to assessments presented by the negative control group, the sham-immunized group that received PBS injections. Specimens were examined in quadruplicate from a pull of organs from 25 fish per group and offered as average ± SD. Dissimilar letters are used to signify differences at $P \leq 0.05$

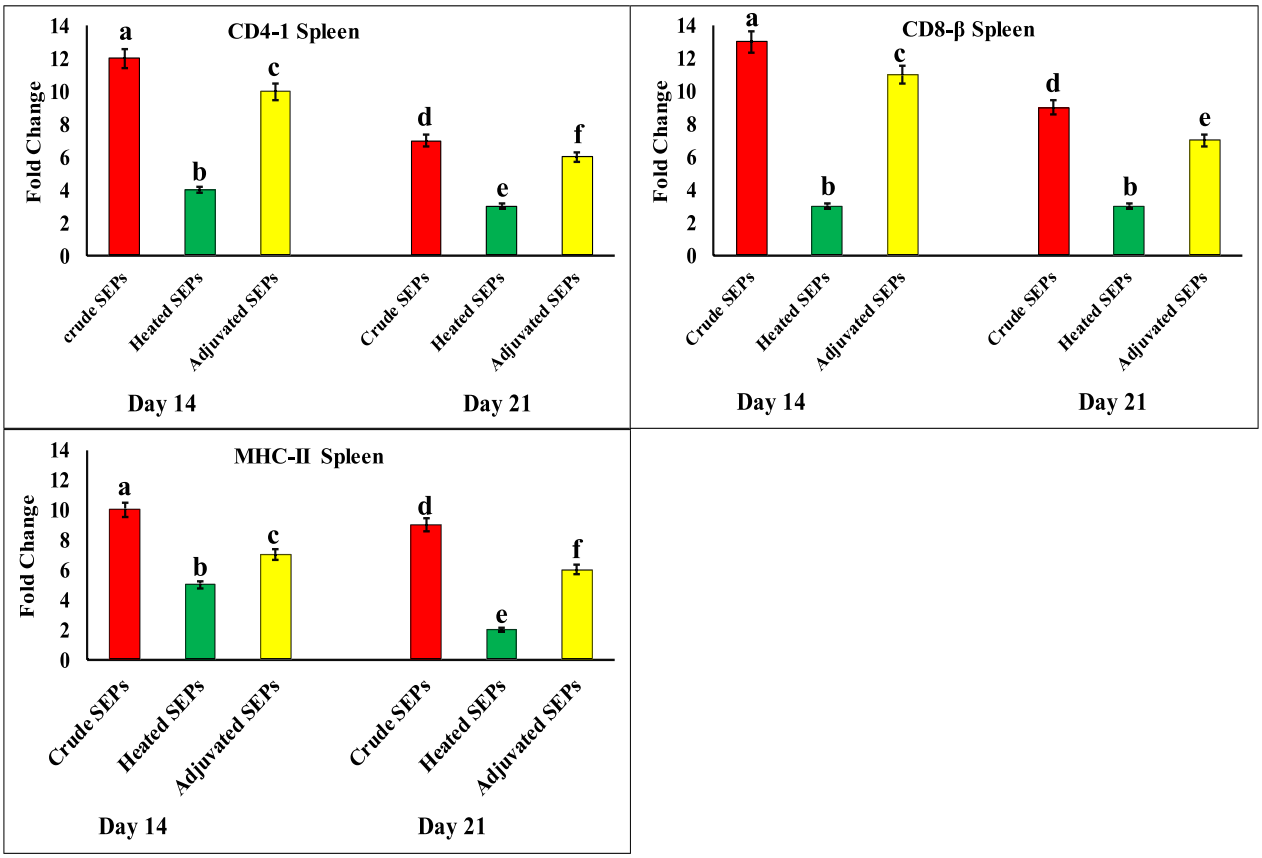

Fig. 4 Alterations in the transcriptions of CD4-1, CD8-β and MHC-II genes in the spleens of *I. punctatus* after immunization. Fish groups immunized with raw SEPs are shown by crude SEPs. A fish group inoculated with heat-inactivated SEPs is indicated by heated SEPs. Adjuvated SEPs refer to a fish group that received a vaccination using SEPs emulsified with Freund's adjuvant. Data are donated as fold differences in gene transcription compared to assessments presented by the negative control group, the sham-immunized group that received PBS injections. Specimens were examined in quadruplicate from a pull of organs from 25 fish per group and offered as average ± SD. Dissimilar letters are used to signify differences at $P \leq 0.05$

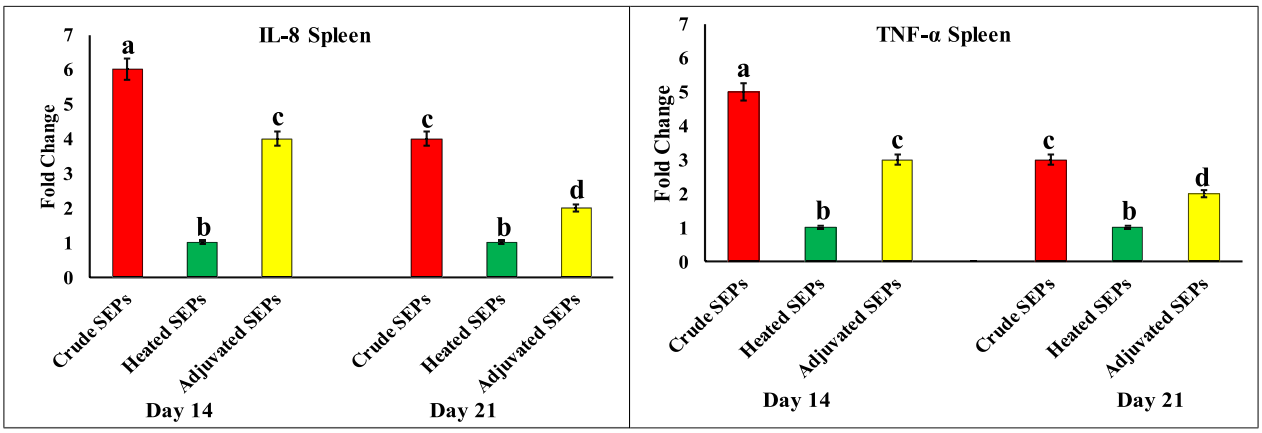

Fig. 5 Alterations in the transcriptions of IL-8, and TNF-α genes in the spleens of *I. punctatus* after immunization. Fish groups immunized with raw SEPs are shown by crude SEPs. A fish group inoculated with heat-deactivated SEPs is indicated by heated SEPs. Adjuvated SEPs refer to a fish group that received a vaccination using SEPs emulsified with Freund's adjuvant. Data are donated as fold differences in gene transcription compared to assessments presented by the negative control group, the sham-immunized group that received PBS injections. Specimens were examined in quadruplicate from a pull of organs from 25 fish per group and offered as average ± SD. Dissimilar letters are used to signify differences at $P \leq 0.05$

system's adaptive responses in *I. punctatus*, IgM antibodies produced at various times were assessed using a SEP-specific ELISA. In the current investigation, we found that all levels of the immunized fish showed an antibody reaction at 14 and 21 days after vaccination, which was considerably stronger at day 14 than day 21 after immunization (Fig. 1). Higher significant antibody titers were generated by both crude SEPs and SEPs blended with oil adjuvant in *I. punctatus* at day 14 after vaccination than by heat-deactivated SEPs, and the same antibody pattern was maintained at day 21 after immunization. Similarly, grass carp, *Ctenopharyngodon idella*, vaccinated intraperitoneally with the recombinant fusion protein of the antigenic domains from various membrane proteins of *F. columnare* involving collagenase, prolyl oligopeptidase, zinc metalloprotease, thermolysin, and chondroitin AC lyase showed substantial rises in IgM antibody levels [12]. In a different investigation, the extracellular portion of *F. columnare* contains the chaperone protein DnaK, which is encoded by the *Dna*K gene, was used for immunization of channel catfish [11]. The mucosal IgM immunoglobulin was found in considerable amounts in the cutaneous tissues of channel catfish group's bath vaccinated with recombinant DnaK protein at 4 and 6 weeks after vaccination [11]. The production of IgM to start protective adaptive immune responses may be induced by an increase in the activities of phagocytic and microbicidal B-cells and the development of IgM Memory B-cells during the immunization process, which typically serve as professional antigen-presenting cells [15]. The magnitude of antibody reactions to specific proteins may be influenced by the quantity of antigen(s), the length of contact, and the degree of intricacy of the antigen [25]. The lower serum level of IgM in the fish group that vaccinated with the heat-deactivated SEPs in comparison with the IgM level of the fish groups that immunized with the crude SEPs and SEPs mixed with oil adjuvant may be attributed to the denaturation in the antigenic structure caused by heat [26]. The generated IgM may help explain why SEPs can deliver meaningful vaccination effectiveness [13]. The humoral immune response of vertebrates includes immunoglobulin and serum total proteins, both of which are vital components [27]. In our investigation, the IgM was substantially higher in the immunized fish than in the non-immunized fish, implying that the immunization promotes the synthesis of particular antigen-specific immunoglobulin and subsequently favorably influences the concentration of circulating total IgM, like previously recorded in rainbow trout vaccinated against *Lactococcus garvieae* [28, 29]. In contrast to our finding, pike perch's serum total protein did not significantly increase after immunization against *Aeromonas salmonicida* [30]. These variations might be attributed to the immunization methods and the vaccine's ultimate efficacy, as it has been generally accepted that vaccine efficacy is represented by the ability of the vaccine to elicit an adequate antibody response in vivo, and this efficacy can be dramatically affected by the vaccination strategy used, including the method of administration (IP, IM, or oral), the antigenic nature of the vaccine preparations, the number of administered doses, and other vaccine modulators as adjuvants.

In terms of the teleost immune system, the spleen and head kidney are thought to be the mainly significant immune-associated organs in teleost because they are responsible for producing all blood components [31]. When taken as a whole, immunity generated by cells in fish is the most significant component of T-lymphocyte-expressed adaptive immune reactions [32]. The receptors CD4-1, CD8-β, and MHC-II are glycoproteins in nature and present on T lymphocytes, which are T-cytotoxic and T-helper cells. These receptors are in charge of trapping and destroying foreign antigenic proteins and other intracellular infected host cells [33, 34]. It would be interesting to track the expression of MHC-II once the antigens are presented in teleost fish because the presentation of MHC-II antigens in fish has not gotten much study attention. In the current study, marked rises in the transcription of the CD4-1, CD8-β, and MHC-II genes were recorded at 14 and 21 days post-immunization in the head kidneys (Fig. 2) and spleens (Fig. 4) of immunized *I. punctatus* in comparison to sham-immunized fish. Similar to our findings, noteworthy transcriptions of MHC-Iα and MHC-IIβ were recorded in the grass carp, *Ctenopharyngodon idella,* vaccinated intraperitoneally with the recombinant fusion membrane proteins of *F. columnare* [12]. Also, a marked surge in the transcription profile of the MHC-Iα was recorded in *Paralichthys olivaceus,* immunized with recombinant Eta2, a protein belonging to the family of outer membrane protein chaperones of *Edwardsiella tarda* [35]. The capacity of SEPs of *F. covae* to sensitize the adaptive immunity in *I. punctatus* may be supported by rises in the regulation of transcription of CD4-1, CD8-β, and MHC-II genes, which have roles in antigen digestion and presentation, jointly accompanied by elevated serum IgM levels.

Regarding the inherent immunity, our findings illustrated that, uppermost up-regulation transcription level of IL-8 and TNF-α was obtained in the anterior kidneys (Fig. 3) and spleens (Fig. 5) of the immunized fish with raw SEPs and SEPs blended with oil adjuvant at 14 days post-immunization in comparison to the sham-immunized group. Although the transcription level of IL-8 and TNF-α was recorded as a marked reduction at 21 days post-vaccination in the fish group vaccinated with heat-deactivated SEPs, the fish groups that immunized with raw SEPs and SEPs blended with

oil adjuvant maintained the high transcription levels of both IL-8 and TNF-α, particularly in the anterior kidneys, at 21 days post-vaccination. Similarly, high expression levels of IL-10, IL-1β, IL-8, and interferon II, I were observed in the grass carp, *Ctenopharyngodon idella* subjected to immunization with recombinant fusion outer membrane proteins of *F. columnare* at 21 and 28 days post-vaccination [12]. As IL-8 and TNF-α are initial immune genes that are expressed at the beginning of infection and/or immunization process in fish and play a critical role in controlling inflammatory responses, pro-inflammatory cytokines may be a possible explanation for the increased transcription levels of these genes [36]. The CXC-chemokine subfamily member IL-8 has strong neutrophil chemotactic properties [37]. Along with increasing the phagocytic activity of fish leucocytes, TNF-α also causes the expression of a variety of immunological genes linked to inflammation, such as IL-1β [38, 39]. The increased expression of the IL-8 and TNF-α genes found in the current study may indicate that *I. punctatus* had a stronger innate immune system after receiving the SEPs vaccine formulations particularly the crude SEPs and adjuvated SEPs.

5 Conclusion

In conclusion, the current study revealed that giving SEPs obtained from virulent *F. covae* to *I. punctatus* fingerlings significantly stirred both inherent and acquired immune responses, suggesting that the SEPs may serve as protective antigens in vaccination against columnaris disease in channel catfish. By igniting the innate immune system through immunomodulation, the knowledge gained from our work is helpful for better understanding the interaction between *I. punctatus* host and *F. covae*. Furthermore, it will be valuable for creating innovative strategies to increase the host resistance to *F. covae* infection.

Abbreviations

SEPs	Secreted extracellular proteins
OMPs	Outer membrane proteins
IgM	Immunoglobulin M
CD4-1	Clusters of differentiation 4-1
CD8-β	Clusters of differentiation 8-beta
MHC-II	Major histocompatibility complex II
IL-8	Interleukin-8
TNF-α	Tumor necrosis factor alpha
cDNA	Complement DNA

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Author contributions
MS theorized the study, designed the methodology, and carried out the experiments. RO performed the statistical analysis. MS and RO prepared and wrote the original draft, edited the draft, and reviewed and approved the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data are accessible with the corresponding author on demand.

Declarations

Ethics approval and consent to participate
The channel catfish tests were carried out in accordance with the specifications of an approved protocol by the Institutional Animal Care and Use Committee (IACUC-19-388) at Mississippi State University, USA.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no conflict of interest.

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