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Proteomic profile of epidermal mucus from *Labeo rohita* reveals differentially abundant proteins after *Aeromonas hydrophila* infection

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ABSTRACT

We report the proteomic profile of Epidermal Mucus (EM) from *Labeo rohita* and identified the differentially abundant proteins (DAPs) against *Aeromonas hydrophila* infection through label-free liquid chromatography-mass spectrometry (LC-MS/MS). Using discovery-based proteomics, a total of 2039 proteins were quantified in non-treated group and 1,328 proteins in the treated group, of which 114 were identified as DAPs in both the groups. Of the 114 DAPs, 68 proteins were upregulated and 46 proteins were downregulated in the treated group compared to nontreated group. Functional annotations of these DAPs shows their association with metabolism, cellular process, molecular process, cytoskeletal, stress, and particularly immune system. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis and Fisher's exact test between the two groups shows that most of the proteins were immune-related, which were significantly associated with the proteasome, phagosome, and *Salmonella* infection pathways. Overall, this study shows a basic and primary way for further functional research of the involvement of vitellogenin 2, alpha-2-macroglobulin-like protein, toll-like receptors (TLR-13), calpain, keratin-like proteins, and heat shock proteins against bacterial infection. Nonetheless, this first-ever comprehensive report of a proteomic sketch of EM from *L. rohita* after *A. hydrophila* infection provides systematic protein information to broadly understand the biological role of fish EM against bacterial infection.

Introduction

Bacterial diseases are one of the important cause of mortality in freshwater fish that result in a huge economic loss [27,64]. A number of bacteria that cause disease in almost all fish and every culture condition, has been reported and need to be focused [5,70]. *Aeromonas hydrophila* is one of the most harmful bacteria in almost all animal taxa, and causes septicemia and ulceration in freshwater fish [11,33]. *A. hydrophila* enters fish body through the skin and gills [13], stimulating macrophage apoptosis and destroying the mucus layer [59]. This bacterium also affects various internal organs such as the liver, kidneys, and spleen [35]. Contrarly, fish is equipped with many immune factors that confer protection against *A. hydrophila* [74,76]. Particulalry, fish epidermal mucus (EM) is proven to protect the host body against bacterial infection [21,49].

The fish EM mucus is multifunctional with a role in respirations, osmeoregulation and homeostasis. The composition of fish EM vary across the body surface and species to species [3,57]. It contain immune relevant components with a significant role in both innate and adaptive immunity [71]. The structural part of the fish EM is mainly composed of glycoprotein (Mucins) [14,18,47,48] lipid, ions, enzymes, other immune-related components such as lysozyme [28,44], immunoglobulin [7,53,71], lectins [47], complement [1], antimicrobial peptides [26,73], proteases [8,66], and heat shock proteins [46]. Though studies of fish mucosal immunity at protein level have been conducted [50,68] yet study of host immune factors in fish has been further recommended [15,16].

In Pakistan, freshwater fish are widely cultivated, though the fish

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industry of this country is not well developed. In addition, pathogenic bacteria have been reported in fish [60,69]. Previously, we have confirmed several pathogenic bacteria from diseased fish inhabiting in different farms [2]. Consequently, we found that skin mucus from both infected and healthy fish, from the aquatic environment of Pakistan, exhibit strong bactericidal activity against different kinds of bacteria [2, 4]. However, studies regarding fish immune system around this country are still very rare. Therefore, we have established the proteomic profile of EM isolated from *L. rohita* treated with *A. hydrophila*. The DAPs we discovered from EM are known for their immune functions against bacteria. This basic report will pave new ways for further studies and will add important understandings to the local and global field of molecular immunology of fish.

Materials and methods

Fish collection

Fish with an average weight of 799 ± 0.577 g was collected from the local fish farm of district Kohat and brought to the Fisheries and Aquaculture Laboratory, Department of Zoology, KUST, Kohat. Before bacterial infections, 36 fish were distributed in 6 rectangular glass aquaria (each with 6 fish) with 200-liter capacity and fed with commercial pelleted feed. Three aquaria were considered as control whereas three aquaria were considered as treated group. Fish were acclimatized for two weeks, with water temperature ranged from 26–28 °C, and feces were removed daily and 30 % of tank water was renewed on each alternate day. All these experimental procedures were permitted by the Ethical Research Committee of KUST wide letter number "KUST/Ethical Committee/1447".

Collection of EM and A. hydrophila challenge

The median lethal dose (LD50) of *A.hydrophila* (ON920871) (that was isolated and identified from naturally infected fish [2] was determined as described earlier [37], cultured in Tryptic Soy Broth (TSB) and incubated overnight at 37 °C. For the treated group, each of fish was treated with *A.hydrophila* at amount of 100 μL of around 1.5×10^9 CFU/mL intra-peritoneally, whereas each of nontreated fish (control group) were injected with an equal volume of phosphate buffer saline (PBS). Skin lesions were examined in the bacterial-treated group of fish after 48 hours while these symptoms were not found in the nontreated group. Fish were kept starved for one day after 15- days of acclimatization to maximize the secretion of mucus and prevent defecation during the process of mucus collection. Fish were anesthetized with tricaine Methane sulfonate (MS-222) then placed on a sterile tray, cleaned with phosphate buffer saline solution, and gradually scraped with a sterile slide from lateral sides. Mucus was gently collected without creating any dent on fish surface and to prevent fecal contamination. Mucus was collected from both control (three aquaria each with 6 fish) and treated (three aquaria each with 6 fish) groups and labeled as NT-LR 1-3 and T-LR 1-3 respectively. The collected mucus was put into falcon tubes (15 mL) and then were sent to Malaysia Genome and Vaccine Institute (MGVI) Jalan Bangi, 43000 Kajang, Selangor, Malaysia for lyophilization and further process.

Protein extraction and quantifications

Proteins were extracted from preserved and lypholized mucus samples by following the protocol of [42,67,72] with little changes. Epidermal mucus was melted on dry ice and diluted with 1000 μL PBS. Samples were sonicated (2 × 5 s) with an ultrasonic device (SONICS Vibracell VCX750, USA) and centrifuged at 4 °C for 30 min at 15,000 g and then the supernatant was collected. A 10 % w/v mixture of trichloroacetic acid (TCA) and 0.1 % DL-Dithiothreitol, Sigma, USA (DTT) was added to the collected supernatant and incubated at -20 °C for 120 min. The TCA and DTT mixture containing mucus samples were centrifuged at 10000 g for thirty min at 4 °C. The pellet was redissolved in chilled acetone containing 0.1 % DTT and incubated at -20 °C for 60 min. The mucus sample was again centrifuged at 10,000 g for 30 min, at 4 °C, and the obtained pellet was air-dried for 2–3 min and suspended in rehydration buffer (9.8 M urea, 20 mM DTT). The same protein extraction procedure was used for both treated and nontreated groups. After extraction, protein content was calculated with standard Bovine Serum Albumin (BSA), and a protein curve was made by using a colorimetric Bradford assay. SDS-PAGE was performed and each protein was extracted by the method of [32] with little modifications. The protein samples were put on a 5 % stacking gel at 75V and a 12 % resolving gel at 125V. The gel was stained by using Coomassie Brilliant Blue (CBB) staining protocol and heated for 1 minute in the microwave oven. Then, the gel was kept at room temperature on a shaker for 15 min before being destained with a destaining solution (10 % Acetic acid, 20 % Methanol, 70 % distal water). For visualization of gel, a Bio-Rad GS800 calibrated densitometer (Bio-Rad Laboratories, Hercules, CA, USA) with a 32-bit pixel depth and 600-dpi resolution were used (Fig. 1).

Protein analysis and trypsin digestion

The aggregated protein bands were cut from the stacking gel after running 15 min of SDS-PAGE and the sliced gel bands of size 1mm × 1mm, were kept in a clean microcentrifuge tube as described earlier [19]. For destaining, 100 μL of 50 % acetonitrile (ACN) in 50 mM ammonium bicarbonate (ABC) was transferred into the tube with excised gel and thus the Coomassie staining was washed off. The tube was vortex and shaken for 15 min and this step was repeated 3 times until the gel became destained. After destaining, proteins were reduced with 300 μL of 10 mM DTT in 100 mM ABC into the tube with excised gel and incubated for 30 min at 60 °C. After reduction, proteins were alkylated with 300 μL of 55 mM indole acetic acid (IAA) in 100 mM ABC into the tube with excised gel and incubated for 20 min at room temperature in the dark. The reduced and alkylated gel was washed with 1000 μL of 50 % ACN in 100 mM ABC for 20 min and 100 μL of 100 % ACN was added and vortex for 15 min. Without disposing of the excess solution, the gel was dehydrated for 60 min at room temperature in a SpeedVac. The gel was rehydrated with 50 μL of 6 ng/μL trypsin in 50 mM ABC along with 50 μL of 50 mM ABC. The trypsin digested samples were vortexed after overnight incubation at 37 °C and then 100 μL of 100 % ACN was added. The liquid was collected in a separate clean tube,

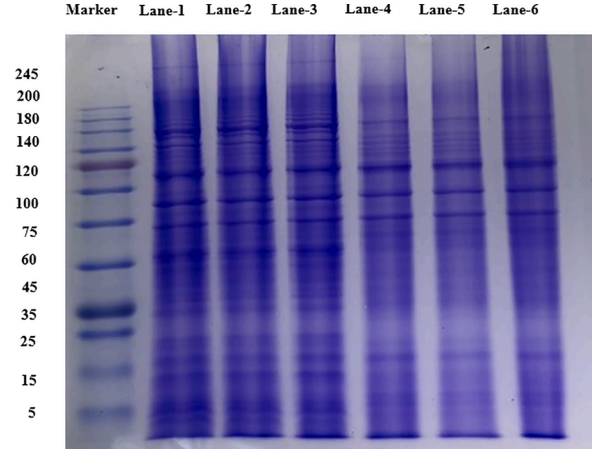

Fig. 1. SDS-PAGE showed the separation of proteins extracted from all samples of EM of *L. rohita* of nontreated NT-LR 1-3 EM and treated T-LR 1-3 with *A. hydrophila*: Lane 1 (N-T1), Lane 2 (N-T2), Lane 3 (N-T3), Lane 4 (T1), Lane-5 (T2), Lane-6 (T6). Smobio Enhanced 3-colour High Range Protein Marker (5-245 kDa) was used as a standard. Approximately 10 μg protein was loaded/lane.

add 100 μL of 50 % ACN, stirred for 15 min. The digested samples were dried completely under SpeedVac and then stored at -20 °C for further analysis.

LC-MS/MS analysis

A microgram of the sample peptide was subjected after quantifications into the column and thus LC-MS/MS was run with a LC gradient of 120 min from all the samples. Data were attained through the Orbitrap fusion tribrid Mass Spectrometer that was linked with an Easy-nLC Nano Liquid Chromatographer (system 1200). Then the samplas subjected to and EASY-spray column Acclaim pep map TM C18 100 A^0, 2 μm particle size, 50 μm id x 15 cm at a flow rate of 5 μL/minute. Peptides were dissolved in an Acclaim spray column (50 μm × 15 cm, 2 μm particles, pore size of 100 Angstrom (Thermo Fisher Scientific), with a flow rate of 250 nL/minute for 120 min and meanwhile the gradient was used as solvent B (Acetonitrile with 0.1 percent Formic acid). Spectrometric acquisition was done through the Orbitrap Mass analyzer (Data Dependent Acquisition mode) with complete scan ranged (310–1800 *m/z*) where the Mass Resolution was 60000. The dynamic exclusion time was set at 20 s and the mas window was set at 10 ppm. Collision-Induced Dissociation (CID) and High-Energy Collision Dissociation (HCD) methods were used for fragmentation at the MS1 and MS2 levels, with AGC targets set at 400000 and 10000, respectively, to acquire all MS/MS spectra. Meanwhile, for positive internal calibration, a lock mass of 445.12003 *m/z* was used.

Label free quantifications and identification of proteins (Proteome Discoverer Version 2.1)

Integrated search engine in NCBI and Thermo Scientific TM Proteome Discoverer Version 2.1 was used to analyse the raw MS data against the *L. rohita* protein database (taxonomy ID - 84645, retrieved on 09/27/2022). In Proteome Discoverer, the mode of Orbitrap fusion was chosen as the tool where trypsin was selected as the protease. Overall, 2-missed cleavages were permitted as oxidation on methionine selected as the variable modification and carbamidomethylation on cysteine as the inert alteration. All peptides were validated by using the percolator® algorithm, based on a q-value smaller than 1 % False Discovery Rate (FDR). The reverse mode was elected as the decoy mode, and proteins were detected based exclusively on their unique peptide.

Statistical analysis

Data obtained with Proteome Discoverer Software were subsequently analyzed with the Perseus framework, accessed on 15-10-2022. Utilizing the Proteome Discoverer output files, statistical analysis was performed by Perseus software (version 2.0.7.0) for the identification of DAPs between the two groups. Statistical assessment was executed in R programming language to differentiate the DAPs between treated and nontreated EM samples. The logarithmic values and missing data were overwritten by imputing missing values following the normal distribution. Then, the rows were filtered for valid values that contained at least 3 values in one group. All missing values were replaced by a normal distribution using the imputation function. To obtain an estimate of the variabilities among the biological replicates of the nontreated and treated, DE analysis (RStudio) was applied.

Functional annotation

Selected differentially abundant proteins (DAPs) from the Perseus data were used for functional annotation and biological pathway analysis. The DAPs identified from Perseus and the NCBI database were integrated into Ubuntu to obtain the sequence for each accession and then submitted to Euro-galaxy to blast the sequences. Subsequently, the blastp.xml files and FASTA files retrieved from Ubuntu were used for functional annotations by using Blast2GO and OMICSBOX. Enrichment analysis (Fisher's exact test) was performed for all DAPs (upregulated v downregulated proteins) ($P \leq 0.05$).

Results

A. hydrophila infection alters proteomic profile of L. rohita EM

Discovery-based proteomics data by high-resolution MS identified and quantified the proteins in the EM. The significantly unique peptides ($P \leq 0.05$) were used to identify the group of proteins that differentiate the treated and nontreated groups, where comparison was performed using nontreated group as a reference group. A sum of 2039 in non-treated (NT-LR-1 (2932), NT-LR-2 (1516), NT-LR-3 (1669), and 1,328 proteins were found in the treated groups T-LR-1 (1365), T-LR-2 (1102), T-LR-3 (1519). Furthermore, a total of 114 proteins were found as DAPs after Perseus analysis between the two groups (NT-LR v T-LR). Of these 114 DAPs, 46 were downregulated and 68 were upregulated in the treated group compared to nontreated group. The plots of all the measured upregulated and downregulated proteins along with the fold changes are shown in Table 1.

Function annotations of DAPs

We have compared DAPs between treated and nontreated which showed an enrichment of specific functions using functional analysis (BLAS2GO). Based on the functions they annotated to, the DAPs were further divided into 3 categories: A) biological processes, B) molecular functions, and C) cellular components. Due to the importance of EM in the processes of immunity, inflammation, stress, skin regeneration, and antibacterial activity, we found the proteins sequence mainly associated with immune relevant, stress, antibacterial and structural proteins. The number of proteins in most of the GO terms in the treated and nontreated groups was annotated to developmental process (GO: 0032502); regulation of biological process(GO: 0048856); metabolic process (GO: 0008152); response to stimulus (GO: 0050896); (GO:0005575); Intracellular anatomical entity (GO:0110165); Protein containing complex (GO: 0032991); membranes (GO: 00432246): **C):** (GO: 0003674); Structural molecular activity (GO: 0005515): binding (GO: 0003824) and catalytic activity (GO: 0003824). The 5 GO terms annotated with the highest number of proteins were the developmental process, biological process, metabolic process, response to stimulus, internal anatomy, and molecular functions (Fig. 2).

Functional annotation of EM proteins belonged to specific pathways and has an important role in stress, immune, and cytoskeleton and are significantly upregulated after treated with *A. hydrophila.* The notable proteins were Ubiquitin-conjugating enzyme (E3 ubiquitin- ligase TRIM32) and 26S proteasome non-ATPase regulatory subunit 7 and etcetera (Table 1). Many biological processes depend on ubiquitination, including regulation of transcription, protein transport, progression of the cell cycle, immune response, signal transduction, and pathogenesis. Bacterial infections can get the benefit of the fish ubiquitin system to use the responses of the immune system for their purpose. Proteins as transferrin variant C, type I cytoskeletal 13-like protein type I cytoskeletal 18, type I cytoskeletal 19-like protein, 14-3-3 protein zeta/delta, apolipo A-I, Galectin-4, which are significantly upregulated in the EM of treated group. Galectin is a lectin binding protein that has increased significantly after exposure to *A. hydrophila.* Intelectin has an important function in clumping bacteria and has significant role in the nonspecific immune system (Table 1).

Upregulated and Downregulated proteins of L. rohita EM

Comparison between the treated and nontreated groups discovered DAPs against *A. hydrophila* infection. Functional annotations showed that most of the downregulated DAPs involved in developmental

Table 1
Differentially abundant proteins (DAPs) found between treated and nontreated Epidermal mucus of *L. rohita* samples

Accession	Protein Name	Coverage[a]	Sequence score[b]	Molecular weight[c]	P-value[d]	Fold change logFC[e]	Peptides[f]
UPREGULATED PROTEINS							
KAI2654466.1	14-3-3 protein zeta/delta	32.24489796	356.0382429	27.83	1.22E-19	22.91038174	7
RXN25852.1	transferrin variant C	6.433824	118.9949	60.348	0.002643	21.8081482	3
RXN12930.1	WD repeat-containing 34	3.225806452	51.80760956	82.672	0.008918	21.78980799	2
RXN05447.1	toll-like receptor 13	3.067484663	10.5339762	74.829	0.004373	21.6107732	2
RXN03646.1	type I cytoskeletal 19-like protein	7.136715391	147.1744276	127.673	0.008918	21.47489227	5
RXN39545.1	apolipo A-I	11.95652174	102.0174513	31.868	4.35E-20	21.43188226	3
RXN02394.1	type I cytoskeletal 18	4.147465438	19.3421793	48.967	0.004373	21.17485778	2
RXN13896.1	GTP-binding nuclear Ran	7.726269316	55.95985115	50.788	0.038607	20.95849278	3
RXN32036.1	annexin A1-like protein	10.52631579	141.2285335	37.693	0.007678	20.8807011	3
RXN34607.1	type I cytoskeletal 13-like protein	14.25178147	143.731734	46.866	0.028555	20.85871854	6
RXN28728.1	calpain small subunit 1-like protein	12.03703704	9.394081831	24.41	0.009598	20.80147232	2
RXN28728.1	calpain small subunit 1-like protein	12.03703704	9.394081831	24.41	0.009598	20.80147232	2
RXN24735.1	coactosin	31.69014085	85.82882822	16.084	0.028555	20.79489129	3
KAI2649895.1	Keratin, type II cytoskeletal 8	12.09540034	424.1445801	64.975	0.014761	20.43275733	7
RXN29259.1	tubulin beta-4B chain	21.7847769	496.9043911	84.759	0.007678	20.42212607	14
RXN22594.1	AP-2 complex subunit beta isoform X2	4.124860647	43.48040962	99.817	0.038607	20.35167945	3
KAI2659941.1	14-3-3 protein gamma	21.95121951	172.9431078	27.915	0.028555	20.21073983	5
RXN10255.1	14 kDa apolipo	16.25441696	133.5789763	31.576	0.000432	20.11850056	3
KAI2657905.1	Phosphoglycerate mutase 1	18.11023622	60.80386984	28.908	0.004373	20.08165743	4
RXN34193.1	heterogeneous nuclear ribonucleo A B-like isoform X2	6.707317073	62.40562987	36.374	0.004373	20.06275069	2
KAI2664700.1	Upstream stimulatory factor 1	4.081632653	36.81055641	54.029	0.000432	19.92284092	2
KAI2648171.1	L-lactate dehydrogenase A chain	17.22222222	282.8044053	39.534	0.028555	19.91698552	5
RXN24522.1	proliferating cell nuclear antigen	12.30769231	6.560997844	28.622	0.011435	19.58838676	2
KAI2647796.1	Epidermal differentiation-specific protein	14.53488372	56.28749657	19.287	0.011435	19.58555809	2
RXN12433.1	profilin-2-like isoform X2	26.61870504	195.6835451	15.092	0.024958	19.51813189	4
RXN18447.1	F-actin-capping subunit alpha-1-like protein	15.01831502	36.7423625	31.309	3.89E-22	19.51565971	2
RXN05295.1	iron-responsive element-binding 2	1.565074135	45.93264389	133.37	0.007678	19.36186956	2
RXN31617.1	calcium calmodulin-dependent kinase type II delta 1 chain isoform X4	9.241706161	55.17066252	48.085	0.004373	19.34781695	3
ACJ53925.1	Annexin A4	14.64174455	40.63251173	35.56	0.007678	19.3164431	3
RXN07412.1	ADP ATP translocase 2 protein heart-	2.258355917	26.85654998	123.753	0.000425	19.26803822	2
KAI2659359.1	Actin-related protein 2/3 complex subunit 4	13.21585903	42.43894362	26.366	0.011435	19.25705307	3
KAI2660878.1	Keratin, type I cytoskeletal 18	2.11673614	0.066	549	6.75E-23	19.2502566	1.91925991
RXN20411.1	ras-related Rab-11B	15.59633028	33.24337554	24.524	0.007678	19.23104757	3
RXN22617.1	gelsolin-like isoform X2	8.923512748	102.4767544	77.409	0.002643	19.16384178	3
KAG7252158.1	hypothetical protein CRUP_019583	10.45454545	62.98877025	25.631	0.002643	19.06175059	2
RXN23302.1	F-actin-capping subunit	29.28870293	122.9185866	26.827	0.007678	18.93273531	5
KAI2652355.1	Heat shock protein HSP 90-alpha 1	14.69387755	307.9437141	168.849	0.025211	18.89629376	10
RXN09298.1	lysozyme g-like protein	11.73469388	59.77187526	21.751	0.008215	18.79699104	2
RXN23886.1	tubulin alpha chain	33.33333333	773.8345332	65.228	0.004373	18.7601335	9
RXN03073.1	40S ribosomal S12	18.18181818	14.07801127	14.457	0.008918	18.74137322	2
KAI2668313.1	NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial	10.95238095	11.1543324	23.722	1.42E-27	18.64521205	2
RXN34167.1	Peptide-N4-N-acetyl-beta-D-glucosaminylasparagine amidase F precursor	1.968911917	37.69668472	109.2	0.007678	18.55714769	2
RXN33499.1	carboxylesterase notum2-like protein	2.812148481	38.19678736	99.267	0.038607	18.50430993	2
RXN15989.1	annexin A4	13.75892287	0.445	35.662	1.26E-29	18.49957111	4
KAI2664763.1	Annexin A1	4.517704518	25.98113525	89.231	0.001942	18.43592754	3
RXN33734.1	E3 ubiquitin- ligase TRIM32	5.461768	0.166	1007	0.002643	18.43159015	89.88571
RXN25902.1	complement C3-like protein	2.873563	107.5233	175.633	0.007678	18.26903485	4
RXN12299.1	40S ribosomal S3a	16.10486891	75.73952174	30.196	0.009598	18.24984963	4
RXN23916.1	alpha-2-macroglobulin-like protein	5.094112	205.2019	645.484	0.004373	18.19080816	7
RXN23912.1	alpha-2-macroglobulin-like protein	9.89011	0.432	1274	1.01E-27	18.17669055	572.9518
RXN31849.1	26S proteasome non-ATPase regulatory subunit 7	7.317073	52.61989	37.211	0.008918	17.83203548	4
RXN19027.1	actin-related 2-like protein	7.868020305	84.86260021	44.307	7.67E-37	17.72308091	2
AUG68890.1	RAB7	17.87439614	114.1709089	23.538	0.008918	17.68621479	3
KAI2657013.1	Macrophage migration inhibitory factor	26.95652174	16.91188049	12.371	0.008918	17.58408073	2
APB93353.1	vitellogenin 2	4.679802956	125.8058792	178.33	0.011435	17.57444871	7
RXN07670.1	calpain small subunit 1-like protein	10.56910569	94.73768556	41.925	0.014761	17.51999958	3
RXN23913.1	alpha-2-macroglobulin-like protein	8.767577	99.81071	135.223	0.007678	17.41664103	6
KAI2646821.1	Histone H4	25.29833	868.5344	46.687	0.004373	4.552064	13
AVP32215.1	14-3-3 protein beta/alpha-A	42.21311	420.4139	27.616	0.007678	3.797519	7
KAI2653804.1	Galectin-4	24.80276	430.8551	225.596	0.008918	3.676603	7
KAI2655256.1	14-3-3 protein beta/alpha-A	6.811146	109.3536	36.085	0.011435	3.235041	2
RXN10339.1	60S ribosomal L12 isoform X1	23.39181	159.9536	18.361	0.004373	3.222491	3
RXN05230.1	type I cytoskeletal 13-like protein	22.34568	1008.685	89.213	0.007678	2.973874	13
RXN24768.1	epithelial cell adhesion molecule-like protein	5.096661	82.86241	64.769	0.028555	2.967842	2
KAI2666578.1	40S ribosomal protein S2	7.865169	78.21855	48.399	0.038607	2.962757	3
KAI2655262.1	40S ribosomal protein S19	13.42282	64.83232	16.334	0.008918	2.871513	2
RXN02566.1	cytolysin Src-1-like protein	9.803922	109.0125	22.992	0.002643	2.737077	2
DOWNREGULATED PROTEINS							
RXN14569.1	type I cytoskeletal 47 kDa-like isoform X1	7.129094	71.91799	54.307	0.011435	-4.549691	4

(*continued on next page*)

Table 1 (*continued*)

Accession	Protein Name	Coverage[a]	Sequence score[b]	Molecular weight[c]	P-value[d]	Fold change logFC[e]	Peptides[f]
KAI2645748.1	Caspase-6	5.333333	0.122	300	0.004373	-17.24014683	9.682151
KAI2664457.1	Vitamin D-binding protein	2.591793	0.155	463	0.004373	-17.42566514	16.42327
KAI2665451.1	Cullin-associated NEDD8-dissociated protein 1	1.572739	40.56822	169.257	3.27E-34	-17.49760959	2
KAI2662642.1	Cysteine–tRNA ligase, cytoplasmic	18.98	6.166388	93.666	0.007678	-17.50674528	2
KAI2668644.1	Phosphoglucomutase-2	4.746318	0.059	611	0.011435	-17.54393459	24.30084
RXN18438.1	kelch 12	15	471.1007	111.734	0.008918	-17.89792701	6
RXN27388.1	calpain-2 catalytic subunit-like protein	3.555556	0.062	675	0.004373	-17.91502237	13.33443
RXN14036.1	cytochrome b-c1 complex subunit mitochondrial-like protein	3.043478	0.28	460	0.007678	-18.03000664	43.02423
AFY09726.1	ceruloplasmin, partial	2.299908	0.041	1087	0.007678	-18.05918035	9.313047
KAI2651062.1	Fructose-bisphosphate aldolase C	25.89532	0.551	363	0.008918	-18.20300257	64.69082
KAI2665055.1	Cystatin-B	15	0.292	100	0.008918	-18.23523105	34.78967
RXN13828.1	complement C3-like protein	3.011803	45.17195	274.04	4.49E-27	-18.23523105	2
RXN13544.1	guanine nucleotide-binding G(I) G(S) G(T) subunit beta-1	1.84667	64.88922	199.371	1.14E-26	-18.27436767	4
RXN18980.1	alpha-2-macroglobulin-like protein	4.251208	53.82425	115.324	0.038607	-18.31396833	3
RXN04861.1	serine threonine- phosphatase 2A catalytic subunit alpha isoform	18.08118	0.668	271	3.33E-26	-18.32181989	87.60896
KAI2664321.1	Heterogeneous nuclear ribonucleoprotein U-like protein 1	3.272727	0.099	825	0.007678	-18.32781097	50.25338
KAI2664776.1	AP-1 complex subunit beta-1	2.495379	18.65408	119.297	0.011435	-18.34401964	2
KAI2661976.1	Voltage-dependent anion-selective channel protein 2	7.389163	0.105	406	0.038607	-18.3863855	12.7973
RXN20681.1	GC-rich sequence DNA-binding factor 2-like protein	3.49345	207.1441	104.327	0.007678	-18.45002047	2
KAI2651791.1	hypothetical protein H4Q32_014542	8.520179	0.369	446	0.038607	-18.46458762	138.7432
KAI2663457.1	Envoplakin	0.73107	0.017	1915	0.028555	-18.47001487	28.23277
RXN09211.1	vinculin	2.439024	51.58951	116.273	0.011435	-18.52705845	3
RXN12839.1	ATP-citrate synthase	11.34884	87.34674	117.863	0.008918	-18.74670881	5
RXN26255.1	programmed cell death 6-interacting -like isoform X2	0.785714	21.48167	155.173	0.004373	-18.87969881	3
KAI2664610.1	Beta-hexosaminidase subunit beta	4.32526	37.01671	66.088	0.008918	-18.93798233	4
KAI2647972.1	Tubulin beta-4B chain	28.15315	170.167	49.585	0.011435	-18.94326785	10
RXN26318.1	catenin beta-1	2.5	55.20581	87.574	2.26E-22	-18.96900941	2
RXN06610.1	fibrinogen gamma chain-like protein	8.788599	103.2149	48.172	2.51E-22	-18.9854998	2
KAI2657647.1	Annexin A11	4.562044	42.94753	57.791	0.028555	-19.02265132	2
RXN27779.1	glutamate mitochondrial-like protein	15.38462	0.719	520	0.008918	-19.04012907	210.2785
RXN24956.1	stress-70 mitochondrial	6.811594	96.2167	74.697	0.008918	-19.16444136	7
RXN23518.1	elongation factor 2	11.30742	196.7013	94.419	0.028555	-19.1948385	8
RXN09288.1	fructose-bisphosphate aldolase B	20.32967	0.823	364	0.008918	-19.34806304	115.8411
RXN03876.1	dihydrolipoyl mitochondrial	10.43307	36.79569	53.493	0.002643	-19.3620008	2
RXN06894.1	clathrin heavy chain 1	6.416465	212.0428	188.344	0.007678	-19.8346728	8
RXN02662.1	ubiquitin-like modifier-activating enzyme 1	5.253104	273.5401	116.745	0.008918	-19.87320864	5
KAI2667771.1	Coatomer subunit beta'	3.584906	69.11857	119.633	0.004373	-19.93365687	4
RXN26576.1	leukocyte elastase inhibitor-like isoform X1	8.702409	292.963	148.004	2.12E-20	-20.05134713	5
RXN26308.1	prolyl endopeptidase-like protein	1.849218	167.7424	79.763	0.028555	-20.08363065	5
KAI2647051.1	Class I histocompatibility antigen, F10 alpha chain	5.112474	96.79037	54.663	0.008918	-20.09890655	2
RXN12794.1	heat shock 70	17.88491	446.8307	70.451	0.004373	-20.31155099	5
RXN26579.1	GDP-mannose 4,6 dehydratase	18.88889	114.4027	29.937	4.70E-20	-20.50182008	3
AAY43356.2	intelectin	16.03774	100.6411	35.241	0.007678	-20.66881279	3
RXN23766.1	plastin-3	20.25518	377.2351	70.129	4.53E-20	-20.80099656	8
RXN04579.1	calpain-2 catalytic subunit-like protein	1.525659	34.32399	80.19	0.028555	-21.63974299	2
RXN07826.1	gelsolin-like protein	7.494145	277.8087	94.005	0.004373	-22.49026236	7

[a] Coverage shows the protein sequence
[b] Sequence Score shows identification of protein score
[c] Molecular weight indicates the proteins size
[d] Proteins values considered significant ($P \leq 0.05$)
[e] Fold change are measured as ≥ 1 and ≤ -1
[f] Peptide labels peptide number sequences matched a protein.
[g]The proteins numbers are calculated: 46 (Downregulated); 68 (Upregulated) proteins.

processes, metabolism process, gene regulation processes, immune related proteins, and oxidoreductase activity. While upregulated DAPs were mainly mapped to antibacterial proteins, immune related proteins, developmental and metabolic processes, cellular metabolism, cytoplasmic components, molecular components, carboxylic acid metabolism, and binding proteins (Fig. 3).

As shown the list of DAPs, the immune response against *A. hydrophila* upregulates and abound the immune-related protein, viz., annexin A1-like protein, apolipo A-I, Galectin-4, epithelial cell adhesion molecule-like protein, calpain, keratin like proteins, hypothetical protein CRUP_019583, Histone H4, heat shock HSPs 90, Complement C3 like protein and TLR-13. Whereas, unlikely heat shock protein 70, Class I histocompatibility antigen, F10 alpha chain, hypothetical protein H4Q32_014542 were downregulated (Table 1).

Enrichment analysis of DAPs and pathways

KEGG enrichment pathways analysis of the 114 annotated DAPs was involved in the regulation of actin cytoskeleton, calcium signalling pathway, NOD like receptor signalling pathways, metabolic pathways, phagosome, complement pathways, biosynthesis of amino acids, Proteasome, and *salmonella* infection. Moreover, *salmonella* infections, metabolic pathways, phagosome, and Proteasome pathways were the significant identified pathways in the treated EM of *L. rohita* after KEGG analysis (Fig. 4).

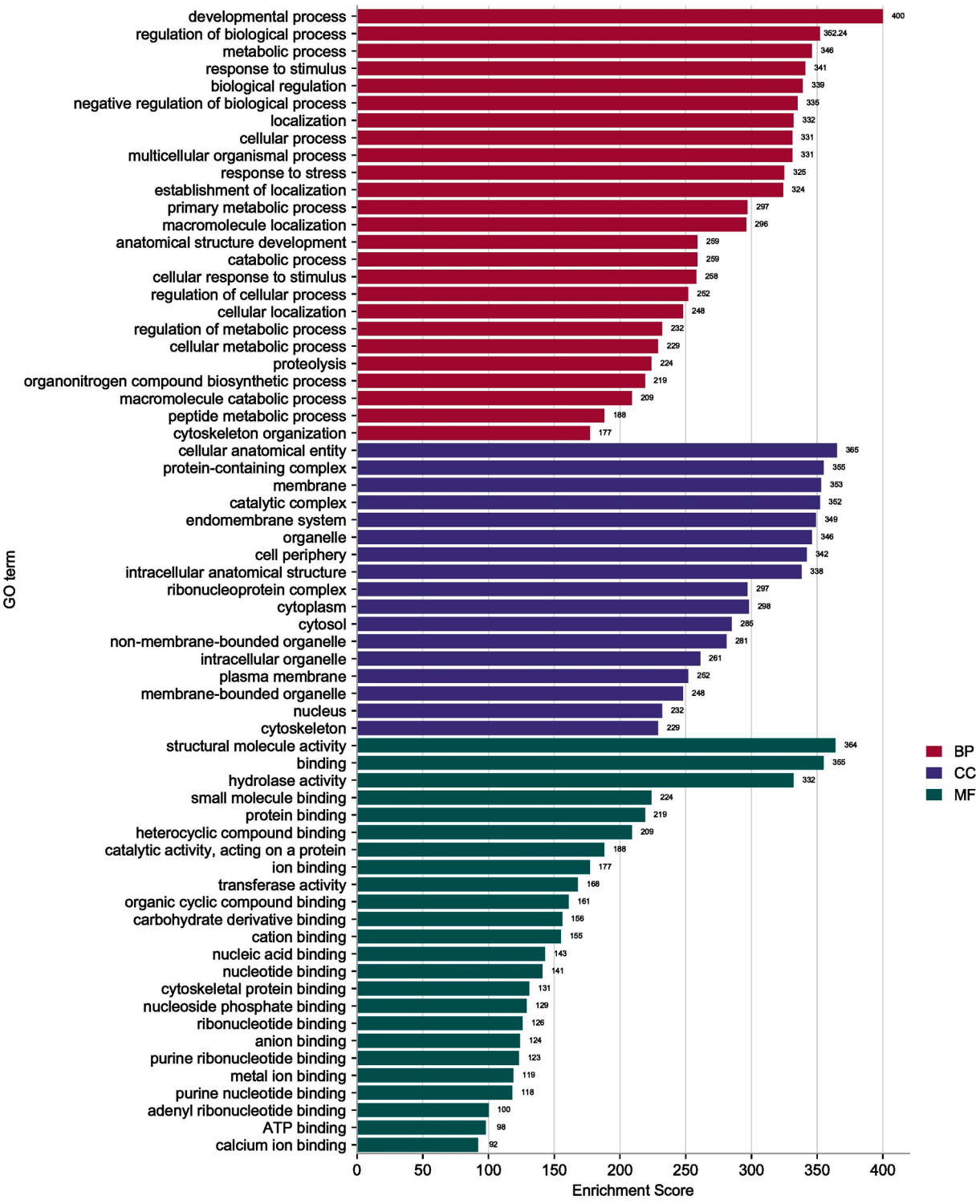

Fig. 2. Gene Ontology annotations of the DAPs of both NT-LR and T-LR groups based on the BLAST2GO ($P \leq 0.05$). Bar graph is the identified proteins enriched with gene ontology (GO) after classification into 3-categories on the basis of enrichment score. GO terms: A): Biological Process (GO:0065007); developmental process (GO: 0032502); regulation of biological process (GO: 0048856); metabolic process (GO: 0008152); response to stimulus (GO: 0050896); B): Cellular Component (GO:0005575); Intracellular anatomical entity (GO:0110165); Protein containing complex (GO: 0032991); membranes (GO: 00432246): C): Molecular Function (GO: 0003674); Structural molecular activity (GO: 0005515): binding (GO: 0003824).

A. Hydrophila abound the cytoskeleton, lysosomal and apoptotic proteins in L. rohita EM

The cytoskeletal proteins including Actin-related protein 2/3 complex subunit 4, Keratin, tubulin alpha chain, tubulin beta-4B chain, type I cytoskeletal 13-like protein, type I cytoskeletal 18, type I cytoskeletal 19-like protein, was found to be upregulated. ATP-binding component of the Arp2/3 (Actin-related protein 2/3 complex subunit 4) complex maintains the cytoskeletal homeostasis without being treated with bacterial infections that aid cell motility by facilitating the production of

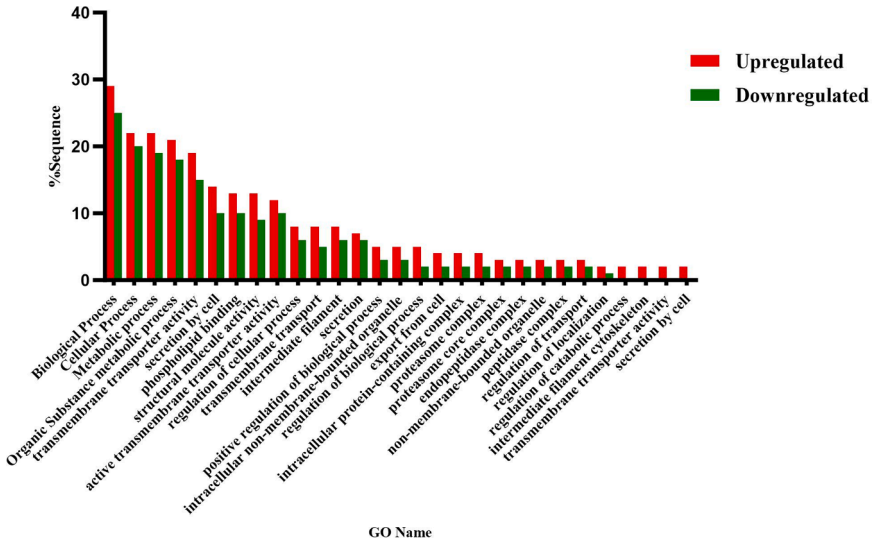

Fig. 3. Fisher Exact Test (Enrichment analysis): Fisher exact test was performed for the comparison of GO terms based on sequence percentage (Biological process, Cellular components, and Molecular function) of the DAPs (Upregulated v downregulated).

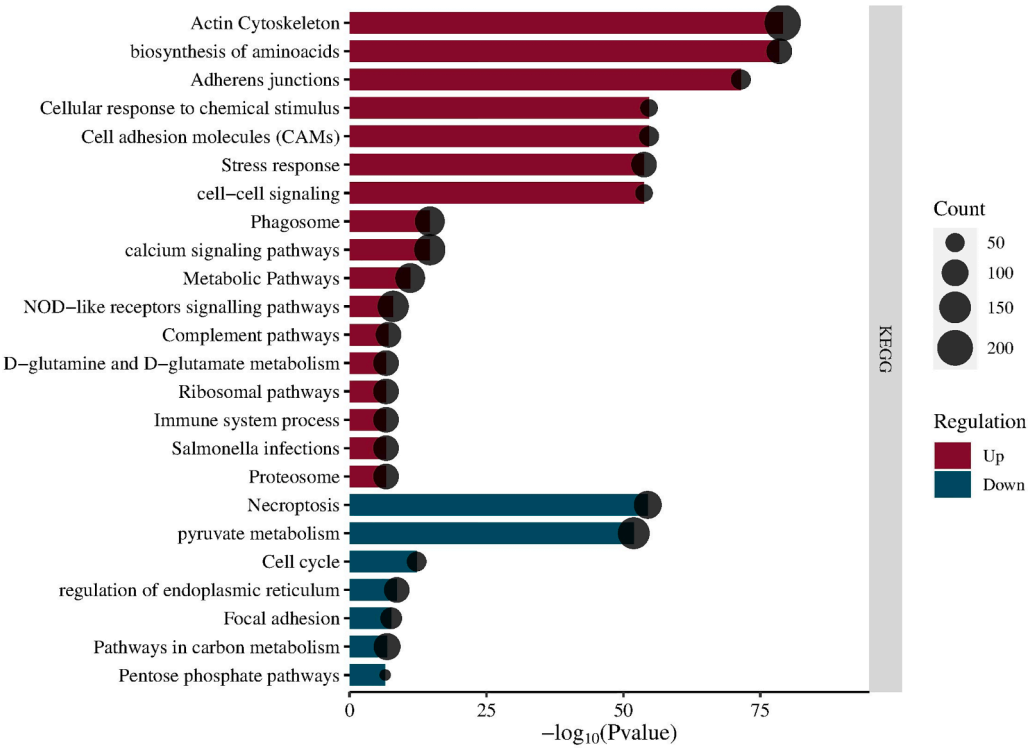

Fig. 4. KEGG pathways enrichment analysis for the identified DAPs. Each row denotes an enriched function, and the count denotes the enriched ratio which is reflected as the "input proteins number". The colour of the bar differentiates Up and downregulated pathways.

branching actin systems in the cytoplasm. The Arp2/3 complex protein is essential for lamellipodium protrusion, vesicle transferring, and entry of bacteria in the fish cell during exposure.

Another significant protein, TLR-13 is the recognizer of pathogens, found to be upregulated in the EM of treated group (Table 1). TLRs recognize molecular pattern pathogen-associated derived from bacteria, perform an important role in the maturation and activation of macrophages, and C-type lectins are also recognized to boost specific immune responses.

Discussion

This first-ever comprehensive proteomic profile of EM of nontreated and bacterial-treated *L. rohita* reveals unique DAPs and pathways. Pathway enrichment analysis and Fisher exact test discovered the contribution of these proteins in biological processes, cellular components, and molecular functions. Notably, Toll-like receptor-13, Histone H4, Galectin-4, complement C3-like proteins, Heat shock proteins HSP90, calpain-like small subunit, keratin-like proteins, hypothetical protein CRUP_019583 were mapped upregulated proteins. Whereas proteins like Class I histocompatibility antigen, F10 alpha chain,

hypothetical protein H4Q32_014542, were mapped as downregulated proteins.

In our study, TLR-13 was upregulated in the EM of treated fish (Table 1), unlikely it was downregulated in the liver of *L. rohita* treated with *A.hydrophila* [39]. TLRs are the important epidermal proteins of innate immune system that acts as a receptor and help in the recognition of pathogen-associated molecular patterns (PAMPs) [40]. TLRs can initiate the TIR-domain comprising adapter-inducing interferon-dependent signaling pathway (TRIF) developed by tumor necrosis receptor-associated factor, which ultimately activates MAP kinase (MEK1/2), NF-κB, or activator protein (AP) [69]. TLR-13 is generally identified as an endosomal receptor, which is a cell surface receptor capable of TRIF-mediated signal transduction in *L. rohita* [56]. TLR-13 was the 1st identified antiviral protein that has been described to distinguish dsRNA of several RNA viruses and initiate the TIRF pathway, which develops type I interferon (IFN) and proinflammatory cytokines [29]. Furthermore, TLR-13 has also been reported in channel catfish, zebrafish, mice, and yellow croaker to work against bacteria and TLR-2 microbial ligand peptidoglycan in immature dendritic cells [29]. Upregulation of TLR-13 in treated Zebrafish with *Edwardsiella tarda* has been reported at transcriptomic level [34]. TLR-13 was found to be downregulated in kidney and spleen of treated blue catfish [6] and rainbow trout with *Edwardsiella ictulari* and *Yersinia ruckeri* [51]. Taking together, this altered expression of TLR-13 may be due to the nature of stimulus and may be organs and species-specific. In fact, during *A. hydrophila* infection, the infected cell of fish would have tried to remove the bacterium by antimicrobial processes, thus interfered with signal transduction through the TLR [9].

Calpain small subunit 1-like protein was found to be upregulated in the EM of the treated group, whereas calpain-catalytic subunit 2 was downregulated in non-treated group (Table 1). Calpain is a regulatory component that acts as a calcium-dependent cysteine protease and is abundant in mammalian cells [65]. Calpain-mediated proteolysis is lacking and mostly alters substrate proteins rather than complete degradation, suggesting that calpain has a specific role in suppressing the physiological response [54]. In addition, inflammatory cytokines such as TNF-α and interleukins can suppress calpain levels in cells and tissues [23]. Therefore, the inflammatory cytokines induced during exposure of cod fish to Vibrio anguillarum may suppress calpain levels [48]. In addition, epidermal lesions caused by bacteria disrupt the amount of calcium and displace the cell membrane, which may affect the stimulation of calpain. In our study, no epidermal lesion was found because calpain protein was upregulated in the treated group. However, mucosal calpains, which are found in higher vertebrates, are thought to play an imperative role in mucosal defense [55].

Keratin, type I cytoskeletal-13, 18, and 19 were upregulated in treated *L. rohita* EM (Table 1). Keratin is a cell structural protein whose main role is to defend cells from physical and mechanical injury [55]. Several studies have suggested that keratin present in fish epidermal mucus has a bactericidal activity due to its pore-forming characteristics [36]. The amount of Keratin depends on the ubiquitin-proteasome pathway, and its expression levels can be changed upon cell damage [52]. Keratin II was observed to be downregulated in treated Atlantic cod mucus with *Vibrio* bacteria [48].

Annexins are an important protein of fish EM and have anti-inflammatory and antibacterial properties [20,75]. Six different types of annexins proteins were reported upregulated in the gills of treated channel catfish (i.e., A-1, A-2, A-4, A-5, A-6, and A11) with *Edwardsiella ictaluri* [30,75]. Besides, annexin A-13 can persuade a defensive response against the parasite fluke *Microcotyle sebastis* infected the fish gills [12]. Annexin A-2 protein was significantly increased in the rainbow trout gills by uncovered to X rays [62]. Annexin max 3 found in medaka (Japanese rice fish) are similar to annexin A-2 found in gills of rainbow trout, which showed that expression of annexin max 3 protein was upregulated in gills by exposure to direct radiation [63]. In this study, the annexin A1 and A4 like protein was upregulated in treated *L. rohita*, which may be indication of an active immune response in epidermal mucus against bacteria.

Histones play an imperative role in the chromatin structure and transcriptional regulation and also participate actively in the fish immune system [38,58]. Histone proteins such as H2A, H2B, H3, and H4 were upregulated in shrimp (*Litopenaeus vannamei*) in response to *Micrococcus luteus* [41]. Histone 4 has bactericidal role in oyster (*Crassostrea virginica*) treated with *E. coli, Staphylococcus aureus, V. anguillarum*, and *M. luteus* [10,17]. Histone 4 protein was also found to be overexpressed in the treated zebrafish gills with *A. hydrophila*, while linker histone like protein H1M was upregulated in EM of zebrafish exposed to *Citrobacter freundii* [31]. In our study, Histone 4 was upregulated in the EM of treated group signifying that histones proteins act as a possible bactericidal agent for the prevention and treatment of fish bacterial infection [38].

Beta-tubulin was upregulated in the EM of *Gadus morhua* treated with *V. anguillarum* suggesting its role in phagocytic activities [48]. Cytoskeleton proteins such as actin and tubulin were identified in the gills of zebrafish treated with *A. hydrophila* possibly has immunological role to activate the phagosome pathways. In mammals, myosins have an important role in muscle contraction and relaxation processes. Tropomyosin, along with the troponin complex, was related to actin in muscle fibers and regulates muscle contraction by regulating the myosin binding [22]. The upregulation of all these proteins found in our study suggests that these proteins play an important role in the EM of *L. rohita* against *A. hydrophila* infection.

Heat shock proteins (HSPs) are conserved and have role in the stress response, counting heat, tissue damage, heavy metal contact, and bacterial infection. They are characterized into families based on their molecular weight: HSPs (15–30 kDa), HSP60s, HSP70s (66–79 kDa), HSP90s (83–90 kDa) and HSP100s. HSPs proteins help in the recovery of fish cells in stress conditions and promote cyto-protection [43,61]. HSP60 is an inducible stress chaperone that is primarily found in the eukaryotic cells' mitochondria and can initiate a strong pro-inflammatory response in cells of the fish's innate immune system [61]. The rapid response of HSP70s to environmental stresses in the presence of infections has led to its observation as a biomarker [25]. Heat shock proteins (HSP90) works also has an important role in several activities such as protein degradation, cell signaling, and intracellular transport [77]. Heat shock proteins (HSP) has been found in EM of *Dicentrarchus labrax* [77], *Sparus aurata* [26], *Cyclopterus lumpus* [43], and *Salmo salar* [46]. In this study, HSP 90-alpha 1 was upregulated in EM of treated group and HSP-70 was downregulated (Table 1). This altered abundance may indicate their role against *A. hydrophila* infection and immune regulation in *L. rohita*.

In our study, the complement protein C3-like protein was upregulated in the EM of treated group of *L. rohita*, while downregulated in the nontreated group, suggesting that it may intermediate the recruitment of immune cells (chemoattractant), opsonin (stimulation of phagocytosis), and agglutinin (coagulation of pathogens) found in the skin mucus of Sea bass [14]. Apolipo-A1 and 14 kDa Apolipo protein were upregulated in the EM of treated group by 21 and 20-fold respectively in the present study. Apolipo proteins were upregulated in the EM of Atlantic cod and salmon treated with sea lice and bacterial infection, respectively [18,48]. Furthermore, Apolipo-1 in Striped bass has bactericidal activity *in vitro* [24] and lytic activity in the skin mucus of Channel catfish [45]. Functional diversity of EM is linked to the complex components, and identification of DAPs against *A. hydrophila* might be helpful to understand the possible mechanism of EM of *L. rohita* against bacterial infection. The upregulation of EM proteins might be due to due to excess proliferation of goblet cell. These results together indicate that *A. hydrophila* infection possibly triggers goblet cells to secret more immune components against bacterial infection. Thus, more studies are required to determine the function of each described proteins, as well as their accurate role and mechanism in the immune response against bacterial infection.

Conclusions

The study presents the comparative proteomic sketches of EM from bacterial-treated and nontreated *L. rohita*. Functional annotations of the DAPs show that *A. hydrophila* treatment may alter the abundance of protein in the EM of fish. Most of the DAPs found in this study were immune-related, stress-related, bactericidal, and cytoskeletal proteins. The significant signalling pathways in which the DAPs were involved, were related to *Salmonella* infection, proteasome, phagosome, and metabolism. This study will act as a base for further research underpinning the molecular immune response of fish EM against bacterial infection.

CRediT authorship contribution statement

Shandana Ali: Conceptualization, Data curation, Formal analysis, Investigation, Methodology. **Waheed Ullah:** Conceptualization, Supervision, Writing – review & editing. **Ahmad Faris Seman Kamarulzaman:** Data curation, Formal analysis. **Maizom Hassan:** Formal analysis. **Muhammad Rauf:** Investigation, Methodology. **Muhammad Nasir Khan Khattak:** Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing. **Farman Ullah Dawar:** Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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