Investigating the Immune Function and Proteomic Profiles of Plasmal Exosomes in Lactobacillus Plantarum-treated Immunosuppressive Broilers

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Research

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Abstract

Background: Exosomes are extracellular membranous nanovesicles that carry functional molecules, such as proteins, to mediate local and systemic cell-to-cell communication. Exosomes released by cells can present in the plasma and involved in the regulation of immunity. Probiotics play a beneficial role in improving the immune function of host through many mechanisms. However, whether probiotics can increase the immune function of broilers by regulating plasmal exosomal cargo is unclear.

Methods: Three hundred broilers were allocated to three treatments: control diet (CON group), control diet + dexamethasone (DEX) injection (DEX group), control diet containing $1 \times 10^8$ cfu/g *Lactobacillus plantarum* P8 + DEX injection (P8+DEX group). The immune function of broilers was detected by measuring the levels of inflammatory cytokines and immunoglobulins in plasma and jejunal mucosa. Exosomes were isolated from the plasma via EIQ3 isolation kits and characterized via transmission electron microscopy, nanoparticle tracking analysis, and Western blot. Then, exosomal protein profile was determined by proteomic. At last, correlation analysis was performed to figure out the potential role of exosomal proteins in regulating immune function of P8-treated broilers.

Results: P8+DEX treatment improved the immune function of DEX-induced immunosuppressive broiler through decreasing plasmal IL-1β, IL-6, TNF-α and jejunal IL-1β, as well as increasing plasmal IL-10 and jejunal IgM. The isolated extracellular vesicles had an average diameter of 125.8 nm, exhibited a cup-shaped morphology and expressed exosomal markers. A total of 784 proteins were identified in the exosomes. Among the 784 proteins, 126 differentially expressed proteins (DEPs) were found between DEX and CON groups, 102 DEPs were found between P8+DEX and DEX groups. Gene Ontology analysis indicated that DEPs between DEX and CON groups are mainly involved in metabolic process, cellular anatomical entity, cytoplasm, extracellular region and binding. DEPs between P8+DEX and DEX are mainly involved in multicellular organismal process, response to stimulus, cytoplasm, cell periphery, membrane, binding, protein binding and ion binding. Further, pathway analysis revealed that most of the DEPs between DEX and CON participated in the ECM-receptor interaction, focal adhesion, regulation of actin cytoskeleton, endocytosis and phagosome. Most of the DEPs between P8+DEX and DEX participated in the ErbB and PPAR signaling pathways. Moreover, many immunity-related DEPs were correlated with the altered immune parameters in plasma and jejunal in broilers fed with P8.

Conclusions: Our findings demonstrated that plasmal exosomes in immunosuppressive broilers fed with P8 carry proteins related to immune function, and may have immunomodulatory effects on the plasma and intestinal immunity.

Background

Stress-induced immunosuppression is a condition in which the immune system is affected by stress factors, and it can damage the immune organ cells and tissues, leading to abnormal immune function and the temporary or persistent dysfunction of the immune response [1, 2]. At present, stress-induced
Immunosuppression is a common condition in intensive breeding, especially in poultry breeding, resulting in serious threats to animal product food safety and public health [3, 4].

Exosomes are nanometer-sized (30–200 nm) membrane-enclosed extracellular vehicles (EVs) [5, 6] released from all cells that can enter into microenvironments and bloodstream [7]. Exosomes harbor a diverse of functional molecules (proteins, nuclear acids, and lipids) derived from their originating cells. This results in the formation of functionally diverse exosomes, capable of immune activation or immune suppression, respectively [8, 9]. For example, B-lymphocyte-derived exosomes display abundant MHC Class I and II molecules, co-stimulatory molecules CD80 and CD86, adhesion molecule ICAM-1 (CD54), also B cell marker CD20, and have the ability to activate CD4+ T cells in an antigen/MHC class II restricted way [10–12]. Tumor-derived exosomes cause time-dependent inhibition of the maturation of immature DCs via a dose-dependent, increased expression of IL-6 and phosphorylation of Stat 3 [13]. However, the role of exosomes in regulating broiler immunosuppression is little known.

Management of immunosuppression is complex. Under immunosuppression, the susceptibility of chickens to other bacterial and fungal infections enhanced [14]. Early usage of broad-spectrum antibiotics has achieved a significant reduction in the number of infections. However, there are problems resulting from the toxicity of these drugs and psychological decline [15]. Thus, finding safe immune-potentiating agents to improve the immune function in immunocompromised animals is important. Probiotics have been reported to improve the immune health status in immunocompromised animals or patients. For example, *Lactobacillus* strains protected mice from cyclophosphamide-caused myelosuppression and improved phagocytic cell recruitment to *C. albicans* infectious sites [15]. *Lactobacillus rhamnosus* GR-1 could lead to an increase in CD4 positive T cells and a decrease in febrile episodes in HIV patients [16]. Probiotics administration also reduced tumor incidence in Marek’s disease virus-infected chickens [17]. *Lactobacillus plantarum* P8 (P8) is a probiotic strain isolated from the natural fermented yogurt of the Inner Mongolian herder’s family. It is suggested that P8 could alleviate the hyperlipidemic of rat [18] and reduce the stress of adults [19]. Our previous study demonstrated that 1×10^8 cfu/g P8 inhibited oocyst shedding and elevated the growth performance as well as the intestinal health of broilers infected with *Eimeria* (data not shown). But whether P8 can improve the immune function and alter the exosomal composition of broilers under immunosuppression is unclear. Thus, in the present study, the levels of cytokines and immunoglobulins in the plasma and jejunal mucosa of dexamethasone sodium phosphate (DEX)-induced immunosuppressive broilers were measured, moreover, the quantitative proteomic analysis and potential biological functions of exosomal proteins were determined. In addition, correlation analysis was performed to figure out if the exosomal proteins play a potential role in regulating the immune function of immunosuppressive broilers.

**Methods**

**Materials**
The probiotic P8 was purchased from Beijing SciTop Biotechnology Co., Ltd. (Beijing, China). The DEX injection was obtained from Beian Feilong Animal Pharmaceutical Factory (Heilongjiang, China).

**Birds and diets**

Three hundred one-day-old male Cobb broilers with similar initial body weights were purchased from Henan Academy of Agricultural Sciences. The basal diet was obtained from Henan Academy of Agricultural Sciences. The composition and nutrient levels of the basal diet is listed in Additional file 1 Table S1.

**Purity and identification checks of bacteria**

The culture and preparation of P8 was prepared by the Department of Animal Nutrition, Qingdao Agricultural University, China. P8 was cultured on Man Rogosa Sharpe media, kept at 37 °C for 24 h. Pure bacterial cells were collected after centrifugation at 5000 × g for 10 min at 4 °C. Then, these cells were washed twice with sterile 0.85 % sodium chloride solution. Ultimately, the culture purity and identification were constantly checked by the spreading plate method [20].

**Experimental design**

A total of 300 broilers were equally divided into 4 treatments with 10 replicated cages of 10 birds each for a 21-day feeding period. The treatments were control diet (CON group), control diet + DEX intraperitoneal injection (DEX group), control diet containing 1 × 10^8 cfu/g P8 (P8 group), and control diet containing 1 × 10^8 cfu/g P8 + DEX intraperitoneal injection (P8+DEX group). At day 16, broilers in DEX and P8+DEX groups were injected with 3mg/Kg BW DEX, while broilers in the CON and P8 groups were injected with equal volume of saline. Fresh water and feed were provided ad libitum. The temperature of the room was set at 33-35 °C in the first week, and then decreased 2 °C every week until 24 °C.

**Sample collection**

At day 21, blood samples from 1 broiler of each replicate were randomly collected by cardiac puncture into vacuum tubes containing anticoagulant and centrifuged for 10 min (3000 × g) at 4 °C. Pure plasma samples were collected and stored in 1.5 mL Eppendorf tubes at -20 °C. The segments of jejunum from 1 broiler of each replicate were collected. Mucosa was scraped from 10 cm of the jejunum using a glass slide (5 cm proximal to the Meckel’s diverticulum). Jejunal mucosa samples were placed immediately in liquid nitrogen and then held at -80 °C.

**Analysis of biochemical indices**
The levels of immunoglobulin A (IgA), IgG, IgM, interleukin 6 (IL-6), IL-10, IL-1β and tumor necrosis factor α (TNF-α) in the plasma and jejunal mucosa were determined using ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd) according to manufacturer's protocol.

**Exosome Isolation**

Exosomes were isolated from plasmal samples by Exosome Isolation Q3 kit (EIQ3-02001, Wayen Biotechnologies, Shanghai, China). The frozen plasma samples were thawed in a 25 °C water bath and then placed on ice. Four microlitre Reagent C was added into 200 μL plasma and mixed well by vortexing until obtain a homogenous mixture. The mixture was incubated at 37 °C for 15 min. After incubation, the samples turned into jellylibe status. The tubes were taped firmly to change the jellylibe status into liquid status and then centrifuged at 10000 × g for 10 min at room temperature. The supernatant was transferred into a new 1.5 mL tube and then placed on ice. Thereafter, 50 μL Reagent A was added in 200 μL pre-treated plasma and mixed. The mixture was incubated at 4 °C for 30 min. After incubation, the supernatant was centrifuged at 3000 × g for 10 min at room temperature. The exosomes pellet was obtained by removing the supernatant. The exosomes pellet completely in 50-120 μL 1 × PBS and mixed well to obtain a homogenous mixture. Once the pellet was re-suspended, the exosomes re-suspension was aliquoted and stored at -80 °C till next experiments immediately.

**Exosomal protein extraction**

Exosomes samples were added the same value of protein lysis buffer (7 M Urea, 2 % SDS) containing 1 × protease inhibitor cocktail, followed by 1 min of sonication on ice using a ultrasonic processor (ultrasound on ice for 2 s, stop for 5 s), and rested on ice for 30 min. The lysate was centrifuged at 13000 rpm for 20 min at 4 °C, then the supernatant was transferred to a new 1.5 mL tube. Four times volume of 100 % acetone was added and the mixture was precipitated overnight at -20 °C. The sample solutions were centrifuged at the next day. The pellet at the bottom was collected and washed twice with 500 μL pre-cooling washing buffer (ethanol: acetone: acetic acid = 50: 50: 0.1). Finally, after centrifugation at 13000 rpm for 15 min at 4 °C, the precipitates were dissolved in buffer containing 6 M guanidine hydrochloride and 300 mM TEAB, and the protein concentration was quantified with BCA assay.

**Transmission Electron Microscope (TEM)**

Five microlitre exosome sample was deposited on Formvar-carbon-coated copper grids for 5 min at room temperature. The excess liquid was removed using Whatman filter paper. Add a drop of 2 % uranyl acetate and incubated for 1 min at room temperature. The excess liquid was removed using Whatman filter paper. After drying, samples were observed under a Tecnai G2 Spirit BioTwin TEM at 80 kV. The acquisitions were made with Gatan Orius SC200D camera.
Nanoparticle Tracking Analysis (NTA)

The frozen exosomal samples were thawed in a 25 °C water bath and then placed on ice. 1 × PBS was used to dilute exosomes for NTA. NTA was performed using a NanoSight instrument (PARTICLE METRIX Malvern Panalytical, Ltd., Malvern, United Kingdom) with a 488 nm laser and automated syringe pump as previously described [21]. The ZetaView 8.04.02 software was used to process the recorded movies.

Western Blot Analysis (WB)

Equal amounts of exosomal proteins from each group were subjected to SDS-PAGE, then proteins on the gel were transferred to nitrocellulose membrane. Membranes were blocked by 5 % skimmed milk and then incubated with the primary antibodies (anti-CD63, anti-TSG101, and anti-Calnexin) overnight at 4 °C. After washing with Tris Buffered Saline Tween, membranes were incubated with secondary antibody adjusted with Horseradish Peroxidase (Beyotime Biotechnology, China) [22].

Filter aided proteome preparation

Eighteen microgramme protein solution samples were taken from each sample, and the volume was determined to 100 μL with 25 mM ammonium bicarbonate. Then, 1 M DTT was added (terminal concentration 20 mM) and incubated at 57 °C for 1 h. Then, 10 μL 1 M iodoacetamide was added (terminal concentration 90 mM) and incubated for 40 min at room temperature under dark conditions. The sample solution was centrifuged on a 10 kDa ultraltration tube at 12,000 rpm, and dissolution buffer (ammonium bicarbonate) was added into the ultrafiltration tube to wash four times. The sample was digested with trypsin which was diluted with dissolution buffer at 37 °C overnight. Next day the peptides were collected by centrifugation, and dried by centrifugal concentration.

Desalination

The dried peptides were desalted on a Monospin desalting column for mass spectrometry analysis. Dissolution the dried mixed peptide using 0.1 % trifluoroacetic acid (TFA) solution. The 100 % acetonitrile was used to activate the desalting column. Then, the 0.1 % TFA solution was used to equilibrate the desalting column. The re-dissolved samples were added to the desalting column and centrifuged. Desalting column was cleaned using 0.1 % TFA solution. Thereafter, 50 % acetonitrile solution was added to collect the elution solution in a new tube. The elution solution was concentrated and dried by centrifugation to remove acetonitrile.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS)

The dried samples were re-dissolved in 0.1 % fluoroacetic acid (FA) solution and 1-2 μg sample was taken for mass spectrometry analysis. The on-line Nano-RPLC liquid chromatography was performed by Easy-nLC 1000 system (Thermo Scientific, USA). The trap column was home-made C18 (C18, 5 μm, 100 μm×2 cm) and the analytical column was C18 reversed-phase column (C18, 1.9 μm, 75 μm × 200 mm). The peptides results were subjected to nano electrospray ionization source followed by tandem mass
spectrometry in Orbitrap Fusion Lumos (Thermo Scientific, USA). The mass spectrometer was operated in the data-dependent mode. For MS scans, the scan ranged from 350 to 1,600 m/z. Intact peptides were detected at a resolution of 60,000 and peptides were then selected for MS/MS at a resolution of 15,000. Collision energy: 30% HCD [21].

Proteomic Analysis

The MS/MS data were analyzed with MaxQuant software (version 1.5.8.3, Max-Planck Institute for Biochemistry, Germany), and proteins were identified by comparing the peptide spectra against the Swissprot databases. The Trypsin was specified as the cleavage enzyme, and up to two missed cleavages were allowed. The mass tolerance value for the fragment ions was set to 0.05 Da. The FDR was set to < 1 %. Proteins were quantified using label-free quantification, and the relative protein abundances are presented as the mGC/HC ratios. The differential expression threshold was set to a 2-fold change. Data analysis was contract service offered by Wayen Biotechnologies (Shanghai), Inc. (Shanghai, China).

Statistical data analysis

One-way ANOVA was used for single factor analysis by SPSS 20.0 for windows (SPSS Inc. Chicago, IL). Spearman's correlation coefficient was calculated using SPSS Version 20.0 (SPSS Inc., Chicago, IL) and GraphPad Prism 8 (GraphPad Software, Inc.) software and used to assess bivariate relationships between variables. Results were expressed as means and the differences were considered significant at $P < 0.05$.

Results

Effects of P8 on the levels of cytokines in the plasma and jejunal mucosa in broilers

In the plasma, compared to the CON group, DEX significantly increased the level of IL-1β ($P < 0.01$), and significantly decreased the level of IL-10 ($P < 0.01$). Besides, compared to the DEX treatment, P8+DEX led to lower levels of IL-1β ($P < 0.01$), IL-6 ($P < 0.05$), TNF-α ($P < 0.01$), and higher level of IL-10 ($P < 0.01$) (Table 1).

In the jejunal mucosa, DEX significantly down-regulated the levels of IL-1β ($P < 0.01$), IL-6 ($P < 0.05$) and TNF-α ($P < 0.05$), and also up-regulated the level of IL-10 ($P < 0.01$) compared to the CON group. Additionally, broilers receiving P8+DEX had a decreased IL-1β level ($P < 0.01$) compared to the ones receiving DEX (Table 1).

Effects of P8 on the levels of immunoglobulins in the plasma and jejunal mucosa in broilers

In the plasma, the levels of IgM, IgG and IgA were not altered significantly by different treatments. However, in the jejunal mucosa, DEX treatment led to a lower level of IgM ($P < 0.01$), which was reversed
by the treatment of P8+DEX ($P < 0.01$). But there were no significant differences of the IgG and IgA secretions among groups (Table 2).

**Characterization of exosomes**

The characterization of exosomes was performed by TEM, NTA and WB. TEM analysis demonstrated cup-shaped vesicles with a size range from 100-150 nm in diameter (Fig. 1A). NTA showed that the mean size of purified exosomes was $125.8 \pm 3.6$ nm, and the primary peak size was $129.3$ nm (Fig. 1B). Moreover, WB analysis revealed that exosomal marker proteins (TSG101 and CD63) were obviously expressed in the exosome samples. However, calnexin, which generally represents contamination by intracellular proteins, was absent (Fig. 1C).

**Proteomic analysis of exosomes**

A total of 784 proteins were identified in plasma exosomes by label-free quantitative proteomic analysis, indicating that the exosomes contained abundant exosomal proteins (Additional file 1 Table S2). Through exploration, we found that 126 differentially expressed proteins (DEPs) ($P < 0.05$) between DEX group and CON group were screened from the results based on the differential expression threshold (fold change > 2 times) (Fig. 2). Among the 126 DEPs, 58 proteins were up-regulated (Table 3), while 68 proteins were down-regulated (Table 4) in plasma exosomes isolated from broilers injected with DEX relative to those isolated from the control ones. Moreover, 102 DEPs were screened between the exosomes from the P8+DEX group and DEX group (Fig. 2). Among the 102 DEPs, 40 proteins were up-regulated (Table 5), while 62 proteins were down-regulated (Table 6) in plasma exosomes isolated from broilers receiving P8+DEX relative to those isolated from broilers receiving DEX.

**Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of DEPs**

GO and KEGG analysis were conducted to understand the functional significance of DEPs. The results of GO enrichment analysis were classified into three sections: cellular component (CC), molecular function (MF), and biological process (BP). Compared to the CON group, DEPs in exosomes from the DEX group mainly participate in organic substance metabolic process (BP), nitrogen compound metabolic process (BP), macromolecule metabolic process (BP), cellular anatomical entity (CC), cytoplasm (CC), extracellular region (CC), binding (MF), protein binding (MF) and ion binding (MF) ($P < 0.05$) (Fig. 3). Furthermore, compared with the DEX group, DEPs in exosomes from the P8+DEX group mainly participate in multicellular organismal process (BP), response to stimulus (BP), cytoplasm (CC), cell periphery (CC), membrane (CC), binding (BP), protein binding (BP), ion binding (BP) ($P < 0.05$) (Fig. 3).

KEGG pathway analysis showed that the enriched pathways within the DEPs between DEX and CON groups were mainly involved in ECM-receptor interaction (TNC/CD47), focal adhesion (PAK3/TNC/RAP1A/ZYX), regulation of actin cytoskeleton (PAK3/WASF2/RDX/ITGB2/BAIAP2), endocytosis (RABEP1/VPS37C/HSPA2/CHMP5/BF2/CHMP1A) and phagosome (ITGB2/BF2) ($P < 0.05$). In addition, the DEPs between P8+DEX and DEX groups were mainly involved in ErbB signaling.
(PAK3/KRAS/CAMK2D), PPAR signaling pathway (ILK/FABP6) and proteasome (PSMA5/PSMA3) ($P<0.05$) (Fig. 4, Additional file 1 Table S3 and S4).

**Correlation analysis between immune indices and proteomic of exosomes**

The correlation between immune indices and proteomic of exosomes was illustrated in Fig. 5 and Additional file 2. Results with the correlation coefficient ($r$) larger than 0.8 or less than -0.8 and the $P$ value less than 0.01 were selected. As for the plasmal indices, the IL-1β was negatively correlated with the protein under accession number A0A3Q2U3V9 ($r = -0.833, P < 0.01$) and positively correlated with the protein under accession number E1C007 (PACSIN2) ($r = 0.817, P < 0.01$). IL-6 was positively correlated with the protein under accession number O93410 (CALM) ($r = 0.800, P < 0.01$), F1NCZ2 (GDI1) ($r = 0.831, P < 0.01$) and Q04584 (ZYX) ($r = 0.818, P < 0.01$). TNF-α was positively correlated with protein under accession number O93410 (CALM) ($r = 0.817, P < 0.01$). IgA was negatively correlated with the protein under accession number A0A3Q2U775 ($r = -0.840, P < 0.01$) and F1NL81 (PI16) ($r = -0.803, P < 0.01$).

As for the jejunal mucosal indices, the IL-1β was negatively correlated with the protein under accession number F1NWN4 (FBLN2) ($r = -0.837, P < 0.01$), A0A3Q2U324 ($r = -0.800, P < 0.01$), F1P201 (VCAM1) ($r = -0.967, P < 0.01$), A0A3Q2U775 ($r = -0.857, P < 0.01$), R4GKL8 (C1QTNF3) ($r = -0.867, P < 0.01$), F1NPN5 (SPIA3) ($r = -0.837, P < 0.01$), Q90WD0 (ACTR3) ($r = -0.857, P < 0.01$), Q9DER4 (ZP1) ($r = -0.836, P < 0.01$), E1BUA6 (VNN1) ($r = -0.826, P < 0.01$), A0A3Q3AJD3 ($r = -0.900, P < 0.01$), and positively correlated with the protein under accession number A0A1L1RMF4 ($r = -0.803, P < 0.01$) and Q9BD54 (CD74) ($r = -0.840, P < 0.01$) and A0A3Q2TUM9 (C5AR1) ($r = -0.810, P < 0.01$). IL-10 was positively correlated with the protein under accession number A0A1D5P6B0 ($r = -0.857, P < 0.01$), A0A2H4C5L1 (BF) ($r = -0.857, P < 0.01$), A0A1L1RL0 (RPL24) ($r = -0.865, P < 0.01$), F1NPS5 (CHMP1A) ($r = -0.884, P < 0.01$) and Q7T2X3 (LDLR) ($r = -0.898, P < 0.01$), and negatively correlated with the protein under accession number Q7T190 (TIMP3) ($r = -0.924, P < 0.01$). IgM was negatively correlated with the protein under accession number A0A3Q2U540 ($r = -0.921, P < 0.01$), Q5W9C5 (BF1) ($r = -0.829, P < 0.01$), P35062 (HIST1H2A3) ($r = -0.829, P < 0.01$), E1C007 (PACSIN2) ($r = -0.979, P < 0.01$), A0A1L1RMF4 ($r = -0.824, P < 0.01$), R4GLT1 (CST3) ($r = -0.835, P < 0.01$), A0A1D5PX8M (VPS37C) ($r = -0.824, P < 0.01$), Q6PW00 (CD3D) ($r = -0.866, P < 0.01$), E1C3Y3 (TSPAN8) ($r = -0.834, P < 0.01$), F1NLE7 (AIMP1) ($r = -0.800, P < 0.01$), A0A1D5PMA3 (NELL2) ($r = -0.840, P < 0.01$).

**Discussion**

In recent decades, more and more reports have proved the effective roles of exosomes involved in immunomodulation [13, 23, 24]. However, most of the research were done in human or murine models, little is known about the biofunction of exosomes in chickens. Limited reports on chicken exosomes have suggested that chicken biliary exosomes possess the capacity to influence the immune responses of lymphocytes and inhibit avian leukosis virus subgroup J [25]. Polyinosinic-polycytidylic acid-stimulated exosomes from chicken macrophage cell line HD11 induced the NF-κB signaling pathway by phosphorylating TAK1 and NF-κB1 in HD11 and chicken T-cell line transformed by reticuloendotheliosis
virus type T (REV-T) CU91 [26]. Exosomes of lipopolysaccharide-stimulated chicken macrophages modulated immune response through the MyD88/NF-κB signaling pathway [27]. Hence, the regulation of exosomes may be useful for improving the immune function of chickens.

Numerous reports demonstrated that probiotics can enhance the immune function of hosts through non immune mechanisms (stabilization of the gut mucosal barrier, competition for adhesion, secretion of antimicrobial substances, etc.) and the modulation of the mucosal and systemic immune responses [28]. A recent study also reported that the serum exosomes isolated from *Lactobacillus plantarum* No.14-treated mice reduced in vitro cytokine production [29]. Thus, we hypothesized that probiotics may elevate the immune function of broilers through the circulating exosomes with functional biomolecules, such as proteins, lipids and nucleic acids.

In the present study, we used DEX to induce the immunosuppression of broilers [30] and we found that P8 could improve the immune function of DEX-treated broilers, reflected by the decreased plasmal IL-1β, IL-6, TNF-α and jejunal IL-1β, as well as the increased plasmal IL-10 and jejunal IgM. Then, we isolated exosomes from plasma samples by using EIQ3 exosome isolation kit. The isolated plasmal exosomes were identified by morphological observation and biochemical analysis. We observed that the ultrastructure and size of plasma exosomes complied with the typical morphology of exosomes [31, 32]. The surface markers of exosomes mainly included CD9, CD63, CD81, CD82, HSP27, HSP90, TSG101 and ALIX [33]. Here, the presence of exosomes was confirmed with the detection of CD63 and TSG101, and the purity of exosomes was confirmed by the absence of Calnexin.

In the past decades, the proteomic cargo of exosomes under immunosuppression have been investigated. Osteosarcoma exosomes contained immunosuppressive proteins including TGF-β, α fetoprotein and heat shock proteins [34]. Collagen type V alpha 2 chain (COL5A2) and lipoprotein lipase (LPL) were significant higher in ovarian cancer cells derived exosomes than ovarian surface epithelial cells [35]. In the present study, proteomic analysis uncovered that a total of 784 proteins were present in plasmal exosomes. Out of the total 784 proteins, DEX induced 126 DEPs compared to the CON group, while P8 + DEX induced 102 DEPs compared to the DEX group. Unfortunately, no other studies using DEX or probiotics have reported data on exosomal proteomic to serve for comparison with our results. Further, we explored the general trends in functional changes of exosomal proteins identified in the present study via GO analysis. Most of the DEPs between DEX and CON groups were in the organic substance metabolic process, nitrogen compound metabolic process, cellular anatomical entity, binding, protein binding and ion binding. Besides, most of the DEPs between P8 + DEX and DEX were in the multicellular organismal process and response to stimulus, cytoplasm and binding, indicating their critical roles in the metabolism, stimulation and cell differentiation, yet their verifications merit further evaluating.

Furthermore, the proteins were analysed using KEGG database. DEPs between DEX and CON groups were mainly included in endocytosis (RABEP1/VPS37C/HSPA2/CHMP5/BF2/CHMP1A), phagosome (ITGB2/BF2) signaling pathway and so on, which might be involved in inflammation [36]. Besides, DEPs between P8 + DEX and DEX groups were mainly involved in ErbB signaling (PAK3/KRAS/CAMK2D), PPAR (ILK/FABP6) signaling pathway and so on. The ErbB signaling pathway is related to the development of
cancer [37]. PAK3, KRAS and CAMK2D are genes involved in the ErbB signaling pathway. The decreased abundances of KRAS and CAMK2D in P8 + DEX group implied the attenuation of immunosuppression [38, 39]. PAKs are important regulators of the inflammatory response. As reported by Taglieri et al. [40], only PAK1 and PAK2, but not PAK3, have been thus far associated with inflammation, immunity, and infective disease. Thus, the increased PAK3 expression in the present study may paly other biological roles rather than regulating the immunosuppression. Moreover, PPAR signaling pathway has anti-inflammatory effects [41]. ILK and FABP6 are genes involved in the PPAR signaling pathway. The activation of PPAR upregulates ILK gene expression [42]. The elevated ILK abundance might indicate the decreased inflammation. In addition, FABP6 was high expressed in patients with cancer [43, 44]. Hence, the decreased FABP6 level implied the alleviation of immunosuppression. This investigation offers insight into a potential role for circulating exosomes in regulation and function during immunosuppression.

To further confirm the effect of exosomal proteins on the immune function of broilers, the correlation analysis was performed between exosomal proteomic and immune parameters in plasma and jejunal mucosa. Among the DEPs that correlated with the immune parameters, we found that the expressions of protein E1C007 (PACSIN2), P35062 (HIST1H2A3), A0A1D5PMA3 (NELL2), A0A1L1RMF4, A0A3Q2U540, Q5W9C5 (BF1), R4GLT1 (CST3), E1C3Y3 (TSPAN8) and F1NLE7 (AIMP1) in the DEX group was higher than those of the CON and were lower than those of P8 + DEX group. Moreover, the expressions of protein R4GKL8 (C1QTNF3), Q9DER4 (ZP1), Q90WD0 (ACTR3) and A0A3Q2U3V9 in the DEX group were lower than those of the CON group and were higher than those of the P8 + DEX group. Reports have suggested that some of the aforementioned proteins, including E1C007 (PASCSIN2), A0A1D5PMA3 (NELL2), Q5W9C5 (BF1), R4GLT1 (CST3), E1C3Y3 (TSPAN8), were associated with the impairment of immune function, leading to immunosuppression [45–49], whereas, C1QTNF3 and ACTR3 were crucial for the normal immune function [50, 51]. Results of the correlation analysis revealed that E1C007 was positively correlated with plasmal IL-1β, and E1C007, A0A1D5PMA3, Q5W9C5, R4GLT1, E1C3Y3 as well as F1NLE7 were negatively correlated with jejunal IgM, besides, R4GKL8 and Q90WD0 was negatively correlated with the jejunal IL-1β, indicating that the P8-induced plasmal exosomal proteins play an important role in improving the immune function of broilers.

**Conclusion**

In summary, we demonstrated that P8 effectively improved the immune function of DEX-induced immunosuppressive broilers. Moreover, a remarkable number of proteins involved in various biological processes, including ErbB and PPAR signalings are packed with plasmal exosomes from P8-treated immunosuppressive broilers. Correlation analysis indicated that the exosomal cargo of immunosuppressive broilers fed with P8 were involved in the improvement of immune function. These findings shed some light on the beneficial role of probiotic in regulating immune function of broilers through plasmal exosomal proteins.
Abbreviations

P8: *Lactobacillus plantarum* P8; CON: control diet; DEX: dexamethasone; IL: interleukin; TNF-α: tumor necrosis factor; Ig: immunoglobulin; DEP: differentially expressed protein; WB: western blot; NTA: nanoparticle tracking analysis; TEM: transmission electron microscope; GO: gene ontology; KEGG: kyoto encyclopedia of genes and genomes.

Declarations

Acknowledgements

Not applicable.

Authors’ contributions

HWL and YW designed the study. FZ and KZ performed the research; YW analyzed data and wrote the paper. JSZ and HWL contributed to revision of the manuscript. The authors read and approved the final manuscript.

Consent for publication

Not applicable.

Competing interests

The authors declare that there is no conflict of interest.

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Availability of data and materials

All the protein data are available via ProteomeXchange with identifier PXD026588.

Ethics approval

The animal experiment was approved and performed in accordance with the guidelines of Ethics and Animal Welfare Committee of Qingdao Agricultural University.

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Tables

Table 1  Effects of P8 on the levels of cytokines in the plasma and jejunal mucosa of broilers

| Item       | CON   | DEX   | P8+DEX | SEM   | P-value |
|------------|-------|-------|--------|-------|---------|
| Plasma     |       |       |        |       |         |
| IL-1β (ng/L) | 89.67b | 108.12a | 85.00b | 6.23  | 0.004   |
| IL-6 (ng/L)  | 38.76ab| 40.10a | 30.73c | 3.55  | 0.032   |
| TNF-α (ng/L) | 55.94ab| 62.00a | 46.30c | 3.81  | 0.001   |
| IL-10 (ng/L) | 39.81ab| 29.16c | 35.45b | 2.56  | 0.000   |
| Jejunal mucosa |       |       |        |       |         |
| IL-1β (ng/mg) | 141.51b| 154.71a| 138.58b| 4.06  | 0.000   |
| IL-6 (ng/mg)  | 31.78b | 40.47a | 35.63ab| 3.71  | 0.040   |
| TNF-α (ng/mg) | 62.50b | 69.98a | 63.78ab| 3.37  | 0.012   |
| IL-10 (ng/mg) | 55.66a | 39.71b | 44.34b | 2.65  | 0.000   |

Mean value within a role with no common superscript differ significantly (P < 0.05). CON, control diet; DEX, control diet + DEX injection; P8, control diet containing 1 × 10⁸ cfu/g P8; P8+DEX, control diet containing 1 × 10⁸ cfu/g P8 + DEX injection

Table 2  Effects of P8 on the levels of immunoglobulins in the plasma and jejunal mucosa of broilers
| Item                      | CON   | DEX   | P8+DEX | SEM  | P-value |
|--------------------------|-------|-------|--------|------|---------|
| **Plasma**               |       |       |        |      |         |
| IgM (ng/mL)              | 290.25| 283.16| 239.00 | 32.29| 0.406   |
| IgG (μg/mL)              | 3.46  | 4.54  | 3.78   | 0.61 | 0.352   |
| IgA (ng/mL)              | 727.25| 827.25| 716.63 | 73.79| 0.441   |
| **Jejunal mucosa**       |       |       |        |      |         |
| IgM (ng/mg)              | 584.42| 371.92| 556.92 | 57.34| 0.001   |
| IgG (ng/mg)              | 6.24  | 5.92  | 4.30   | 0.74 | 0.058   |
| IgA (ng/mg)              | 914.75| 809.75| 901.63 | 68.88| 0.341   |

a,b Mean value within a role with no common superscript differ significantly ($P < 0.05$). CON, control diet; DEX, control diet + DEX injection; P8, control diet containing $1 \times 10^8$ cfu/g P8; P8+DEX, control diet containing $1 \times 10^8$ cfu/g P8 + DEX injection

**Table 3** The up-regulated DEPs between DEX and CON groups
| Accession | Description | Gene symbol | Unique peptides | DEX vs CON (ratio) | DEX vs CON (P-value) |
|-----------|-------------|-------------|----------------|-------------------|----------------------|
| P35062    | Histone H2A-III | HIST1H2A3; LOC427881; HIST1H2A4L3; LOC417955 | 2              | 64.54129          | 0                    |
| F1NIW7    | Cubilin | CUBN | 4              | 20.68636          | 0                    |
| A0A3Q2UFJ3 | Calcium/calmodulin-dependent protein kinase | CAMK2D | 3              | 19.65484          | 0                    |
| F1NBT0    | Serine/threonine-protein kinase 10 | STK10 | 5              | 13.74323          | 0.0323               |
| Q7T190    | Tissue inhibitor of metalloproteinase 3 (Fragment) | TIMP3 | 1              | 13.6144           | 0.0238               |
| A0A3Q2TX84 | Ig-like domain-containing protein | - | 1              | 11.68156          | 0.0183               |
| A0A3Q2TUE5 | Ig-like domain-containing protein | - | 2              | 11.43221          | 0.0362               |
| F1NUJ7    | FABP domain-containing protein | FABP6 | 2              | 9.59988           | 0                    |
| A0A3Q2UIJ5 | Uncharacterized protein | LOC769729 | 1              | 9.57246           | 0                    |
| A0A1D5PMA3 | Uncharacterized protein | NELL2 | 1              | 8.64332           | 0.0119               |
| A0A1D5PY04 | Uncharacterized protein protein | CLIP2 | 2              | 8.4481            | 0.0354               |
| A0A1D5NTE7 | Fibrinogen C-terminal domain-containing protein | - | 2              | 7.76389           | 0.0476               |
| A0A3Q2TXS3 | Ig-like domain-containing protein | - | 1              | 7.54106           | 0                    |
| A0A3Q2TXN1 | Uncharacterized protein | - | 4              | 6.04833           | 0.0079               |
| A0A3Q3APB3 | Uncharacterized protein | LOC101748084 | 1              | 5.86691           | 0                    |
| A0A1D5PGD5 | Collagen alpha-3 (VI) chain | COL6A3 | 10             | 5.72437           | 0.0207               |
| A0A1D5PKY9 | Septin | SEPT6 | 2              | 5.21007           | 0.0151               |
| F1NEB3    | Uncharacterized protein | HABP2 | 5              | 5.04958           | 0.0013               |
| Q5F3I1    | Fibrinogen C-terminal | FGL2 | 3              | 4.76188           | 0.0006               |
| Accession | Description                                      | Gene ID | Fold Change | p-Value |
|-----------|--------------------------------------------------|---------|-------------|---------|
| A0A3Q2U540 | Ig-like domain-containing protein                |         | 4.55025     | 0.0134  |
| Q5F442    | Uncharacterized protein                          | RASA3   | 4.51919     | 0       |
| F1NBW3    | Serine incorporator 5                            | SERINC5 | 4.51106     | 0.0294  |
| A0A3Q2U4V6 | Ig-like domain-containing protein                |         | 4.41341     | 0.0005  |
| P21760    | Extracellular fatty acid-binding protein         | LCN8; P20K; LCN15; EXFABP | 4.01483 | 0.0015  |
| Q27J90    | Leukocyte ribonuclease A-2                       | RSFR    | 3.91679     | 0.0033  |
| Q5ZKY7    | Tetraspanin                                      | CD82    | 3.85704     | 0.037   |
| F1C6X7    | MHC class II antigen alpha chain                 | B-LA; BLA; HLA-DRA | 3.78031 | 0.0111  |
| A0A3Q2UDP3 | Uncharacterized protein OS=Gallus gallus OX=9031 GN=DOCK10 PE=3 SV=1 | DOCK10 | 3.66783     | 0.0004  |
| F1NV09    | Epithelial cell adhesion molecule                | EPCAM   | 3.66275     | 0.0001  |
| A0A1D5PH32 | Actin-depolymerizing factor                     | GSN     | 3.52032     | 0.0378  |
| Q90593    | Endoplasmic reticulum chaperone BiP              | HSPA5   | 3.42985     | 0.0005  |
| F1NW23    | Clathrin heavy chain                             | CLTC    | 3.27741     | 0.0426  |
| Q6IEC5    | Putative ISG12(2) protein                        | ISG12-2; IFI6 | 3.19787 | 0.0203  |
| E1C3Y3    | Tetraspanin OS=Gallus gallus OX=9031 GN=TSPAN8 PE=3 SV=2 | TSPAN8 | 3.05368     | 0       |
| A0A3Q2U871 | Uncharacterized protein                          |         | 3.00759     | 0.049   |
| P26007    | Integrin alpha-6                                 | ITGA6   | 2.87969     | 0.0326  |
| Q2MJT5    | Glycoprotein IIIb                                | CD36    | 2.80456     | 0.0445  |
| R4GLT1    | Cystatin domain-containing protein               | CST3    | 2.7426      | 0       |
| A0A1D5PT95 | Uncharacterized protein                          | KRAS    | 2.68849     | 0.0117  |
| A0A1D5P4G6 | Integrin alpha-V                                 | ITGAV   | 2.6846      | 0.0478  |
| Protein ID | Description                                                      | Drug | Log2 Fold Change | P Value |
|-----------|------------------------------------------------------------------|------|-----------------|---------|
| Q5W9C5   | MHC class I antigen                                               | BF1; HFE | 1               | 2.67921 | 0    |
| Q5F3U5   | Uncharacterized protein                                           | RAP2C | 2               | 2.57332 | 0.0043 |
| F1P4U3   | Secreted phosphoprotein 2                                         | SPP2  | 1               | 2.44087 | 0.0006 |
| F1NDH2   | Angiotensin 1-10                                                  | AGT   | 5               | 2.39932 | 0.0412 |
| E1C007   | Protein kinase C and casein kinase substrate in neurons protein 2 | PACSIN2 | 3               | 2.37931 | 0.0221 |
| E1C0F3   | Uncharacterized protein                                           | RAB7A | 1               | 2.2642  | 0.0003 |
| A0A3Q2TZ57 | Ig-like domain-containing protein                               | -     | 1               | 2.10007 | 0.0482 |
| A0A3Q2U9L1 | Uncharacterized protein                                         | -     | 2               | 1.97347 | 0.0005 |
| A0A1L1RMF4 | Uncharacterized protein                                      | -     | 1               | 1.91233 | 0.0064 |
| Q5ZLI2   | Proteasome subunit alpha type                                     | PSMA3 | 1               | 1.86183 | 0    |
| Q6Q1Q8   | Mannan-binding lectin associated serine protease 3               | MASP1 | 1               | 1.8393  | 0    |
| A0A3Q2U7Z1 | Uncharacterized protein                                        | LPXN  | 2               | 1.72145 | 0.0416 |
| E1C1Q3   | Uncharacterized protein                                           | SLC29A1 | 1              | 1.64852 | 0    |
| Q9YHD2   | Nuclear calmodulin-binding protein (Fragment)                    | URP; HNRNPUL2 | 1              | 1.63727 | 0.0447 |
| A0A3Q2TUM9 | C5a anaphylatoxin chemotactic receptor                            | C5AR1 | 1               | 1.57463 | 0    |
| P68139   | Actin, alpha skeletal muscle                                      | ACTA1 | 1               | 1.44516 | 0    |
| E1C857   | Tetratraspanin                                                   | TSPAN6 | 1              | 1.43924 | 0.0315 |
| F1NLE7   | tRNA-binding domain-containing protein                            | AIMP1 | 1               | 1.28265 | 0    |

**Table 4** The down-regulated DEPs between DEX and CON groups
| Accession | Description                          | Gene symbol | Unique peptides | DEX vs CON (ratio) | DEX vs CON (P-value) |
|-----------|--------------------------------------|-------------|----------------|--------------------|----------------------|
| E1C3D2    | Septin                               | SEPT2       | 1              | 0.75954            | 0                    |
| E1BWW2    | Uncharacterized protein              | TSG101; UEVLD | 1              | 0.75747            | 0                    |
| Q5ZKS2    | Uncharacterized protein              | RHOF        | 1              | 0.73229            | 0.0152               |
| Q9DF58    | Integrin-linked protein kinase       | ILK         | 1              | 0.6349             | 0.0156               |
| A5HUM6    | Tenascin X B                         | TNXB; TN; TNX | 8              | 0.6265            | 0.0001               |
| A0A1D5PMN6| Uncharacterized protein              | KIF1B       | 1              | 0.62134            | 0.0186               |
| F1NSM7    | Ovocleidin-116                       | MEPE        | 3              | 0.62087            | 0.0448               |
| F1N851    | Uncharacterized protein              | ENTPD1      | 1              | 0.60771            | 0.0026               |
| P00565    | Creatine kinase M-type               | CKM         | 3              | 0.58617            | 0.0003               |
| P46157    | Gallinacin-1 alpha                   | GAL1; AvBD1 | 2              | 0.57304            | 0.0231               |
| Q90631    | Kinectin                             | KTN1        | 4              | 0.55989            | 0.0284               |
| H9L0D7    | Wiskott-Aldrich syndrome protein family member | WASF2 | 1 | 0.55391 | 0.0061 |
| Q7SX63    | Heat shock protein 70                | HSPA2       | 2              | 0.5128             | 0.0328               |
| Q5ZHM4    | CN hydrolase domain-containing protein | VNN1     | 2              | 0.50947            | 0.0447               |
| E1BTI7    | SMB domain-containing protein        | TINAG       | 13             | 0.50845            | 0.0299               |
| Q90WD0    | Actin-related protein 3              | ACTR3       | 2              | 0.50512            | 0                    |
| Q5G8Y9    | Apolipoprotein D                     | APOD        | 1              | 0.49876            | 0                    |
| E1BZN8    | Uncharacterized protein              | F12         | 4              | 0.49585            | 0.0122               |
| P09244    | Tubulin beta-7 chain                 | TUBB        | 1              | 0.49186            | 0.0115               |
| F1C6U4    | MHC class II antigen beta chain      | LOC101747454; BLB2 | 1 | 0.48491 | 0.0001 |
| A0A1D5P9U9| Aquaporin-5                          | AQP5        | 2              | 0.47223            | 0.0008               |
| Q90XB2    | Surfactant protein A                 | SFTPA1; SFTPA; SFTPA2 | 1 | 0.4565 | 0 |
| R4GM71    | Phosphatidylcholine-sterol acyltransferase | LCAT   | 6              | 0.45467            | 0.0035               |
| Accession | Description                                           | LOC/Reference          | Log2FC | FDR   |
|-----------|-------------------------------------------------------|------------------------|--------|-------|
| R4GJX3    | Uncharacterized protein                               | LOC770612; IFITM3      | 2      | 0.44714 0.0071 |
| R4GKL8    | C1q domain-containing protein                         | C1QTNF3                | 2      | 0.44141 0.0011 |
| F1NLW7    | Suppressor of tumorigenicity 14 protein homolog       | ST14                   | 3      | 0.43546 0   |
| F1NSA8    | Uncharacterized protein                               | RAP1A                  | 1      | 0.41077 0.0028 |
| P01994    | Hemoglobin subunit alpha-A                            | LOC100858011; HBAA; HBA1| 6      | 0.40104 0.0401 |
| A0A3Q2U3V9| Uncharacterized protein                               | LOC100858647           | 6      | 0.39477 0.0235 |
| A0A1L1RUW5| Uncharacterized protein                               | NMI                    | 1      | 0.36985 0   |
| A0A1D5PIF2| LIM domain-containing protein                          | LIMS1                  | 1      | 0.36686 0.0169 |
| Q9DER4    | Zona pellucida protein 1                              | ZP1                    | 2      | 0.36395 0   |
| Q04584    | Zyxin                                                 | ZYX                    | 1      | 0.35897 0.0001 |
| A0A1D5NXR0| VWFD domain-containing protein                         | -                      | 3      | 0.3521 0.0086 |
| E1BY44    | TGc domain-containing protein                          | TGM2                   | 7      | 0.32088 0.0003 |
| Q8UWG7    | 60S ribosomal protein L6                              | RPL6                   | 1      | 0.31974 0   |
| A0A3Q2U0U0| SH3 domain-containing protein                          | EPS8                   | 3      | 0.31449 0   |
| A0A0K0PUH6| Chemerin                                              | RARRES2                | 3      | 0.29516 0.0032 |
| A0A1D5PGI9| BUD13 homolog                                         | BUD13                  | 1      | 0.29403 0.0457 |
| A0A3Q2TSW8| Uncharacterized protein                               | NID2                   | 4      | 0.27915 0   |
| R4GIW4    | Uncharacterized protein                               | XPNPEP2                | 2      | 0.27031 0.0021 |
| A0A3Q2UIT0| Tetraspanin                                           | UPK1B                  | 1      | 0.27014 0.0001 |
| A0A1D5NUZ0| Uncharacterized protein                               | NAPA                   | 1      | 0.26364 0.0001 |
| F1NVY4    | Uncharacterized protein                               | GGT1                   | 3      | 0.26308 0   |
| Q5ZJX9    | Proteasome subunit alpha type                         | PSMA5                  | 2      | 0.23715 0.0003 |
| A0A1D5PNT8| VPS10 domain-containing protein                        | SORT1                  | 1      | 0.20383 0   |
| F1P2W2    | Uncharacterized protein                               | ATRN                   | 5      | 0.19855 0.0023 |
| F1P3P3    | Uncharacterized protein                               | ARHGDI A               | 3      | 0.18905 0.0004 |
| Protein ID | Description                                      | p-value | q-value |
|-----------|--------------------------------------------------|---------|---------|
| F1NNF9    | Ankyrin repeat and kinase domain containing 1    | 0.18577 | 0       |
| F1P386    | Uncharacterized protein                          | 0.18534 | 0       |
| Q90944    | Epiphycan                                        | 0.1505  | 0       |
| A0A2H4C5L1| MHC class I antigen                              | 0.14362 | 0       |
| F1NWP1    | Christmas factor                                 | 0.14221 | 0       |
| E1BSI4    | Uncharacterized protein                          | 0.1414  | 0       |
| A0A1D5PGT3| Brain-specific angiogenesis inhibitor 1-associated protein 2 | 0.12844 | 0       |
| Q9W6V5    | Receptor-type tyrosine-protein phosphatase eta    | 0.1273  | 0       |
| B5BSS3    | MHC class I alpha chain 2                        | 0.09117 | 0       |
| P87362    | Bleomycin hydrolase                              | 0.07845 | 0.0004  |
| Q7T2X3    | Low-density lipoprotein receptor                 | 0.07034 | 0.0002  |
| F1NPS5    | Charged multivesicular body protein 1a            | 0.0702  | 0       |
| A0A3Q2U504| Vitamin K-dependent protein S                     | 0.0574  | 0       |
| Q5ZL65    | Integrin-associated protein                       | 0.05374 | 0       |
| A0A3Q2U3X0| LRRCT domain-containing protein                   | 0.03605 | 0       |
| A0A1L1RLL0| TRASH domain-containing protein                   | 0.02835 | 0       |
| A0A1D5P6B0| Procollagen C-endopeptidase enhancer             | 0.02774 | 0       |
| A0A3Q2TZU8| Protein tweety homolog                           | 0.00996 | 0.0075  |
| A0A3Q2UHT9| Uncharacterized protein                          | 0.00933 | 0       |
| A0A1L1RZV0| Ubiquitin-conjugating enzyme E2 variant 2         | 0.00282 | 0       |

Table 5 The up-regulated DEPs between P8+DEX and DEX groups
| Accession   | Description                                      | Gene symbol | Unique peptides | P8+DEX vs DEX (ratio) | P8+DEX vs DEX (P-value) |
|-------------|--------------------------------------------------|-------------|----------------|------------------------|-------------------------|
| A0A3Q3AJD3  | WH1 domain-containing protein                    | -           | 1              | 828.76722              | 0                       |
| A0A3Q2UHW3  | Guanine nucleotide-binding protein subunit gamma | -           | 1              | 56.42243               | 0                       |
| A0A3Q2TSK8  | Rho family-interacting cell polarization regulator | FAM65B      | 1              | 14.49547               | 0                       |
| A0M8U0      | F-actin-capping protein subunit alpha            | CAPZA2      | 1              | 14.25679               | 0                       |
| A0A3Q2TZA4  | A2M_recep domain-containing protein              | -           | 2              | 13.59721               | 0.0242                  |
| F1P201      | Uncharacterized protein                          | VCAM1       | 5              | 6.71103                | 0.0029                  |
| F1NL81      | SCP domain-containing protein                    | PI16        | 1              | 5.31015                | 0.0118                  |
| A0A3Q2U775  | Ig-like domain-containing protein                | -           | 1              | 5.30131                | 0                       |
| F1P291      | Osteonectin                                      | LOC415258   | 4              | 4.66856                | 0.0243                  |
| Q91017      | Gizzard PTB-associated splicing factor (Fragment) | SFPQ        | 2              | 4.03185                | 0                       |
| F1P2W2      | Uncharacterized protein                          | ATRN        | 5              | 3.83935                | 0.0119                  |
| F1N8W8      | Serine/threonine-protein kinase PAK 3            | PAK3        | 1              | 3.76494                | 0                       |
| E1BUA6      | CN hydrolase domain-containing protein            | VNN1        | 2              | 3.7602                 | 0                       |
| F1W2N4      | Uncharacterized protein                          | FBLN2       | 6              | 3.64213                | 0.0429                  |
| Q9DER4      | Zona pellucida protein 1                         | ZP1         | 2              | 3.49814                | 0                       |
| A0A173G7D2  | Mannose-binding lectin                           | MBL2; MBL   | 1              | 3.43387                | 0.0011                  |
| R4GL8       | C1q domain-containing protein                    | C1QTNF3     | 2              | 3.32231                | 0                       |
| F1NWP1      | Christmas factor                                 | F9          | 6              | 3.27458                | 0.0037                  |
| Q5ZJX7      | Multivesicular body subunit 12A                  | FAM125A; MVB12A | 1  | 3.05845                | 0.0001                  |
| A0A3Q2U324  | A2M domain-containing protein                    | -           | 4              | 2.96109                | 0.0087                  |
| ID       | Description                                           | Symbol   | Fold Change | p-value |
|----------|-------------------------------------------------------|----------|-------------|---------|
| Q90933  | Neuron-glia cell adhesion molecule (Ng-CAM)           | L1CAM    | 14          | 2.88677 | 0.0443  |
| A0A1D5PW36 | Uncharacterized protein                          | BPIL3    | 1           | 2.82844 | 0.0007  |
| A0A1D5PCD2 | A2M_recep domain-containing protein            | -        | 11          | 2.70052 | 0.0007  |
| A0A3Q2U0U0 | SH3 domain-containing protein                     | EPS8     | 3           | 2.69614 | 0       |
| F1N851  | Uncharacterized protein                            | ENTPD1   | 1           | 2.55443 | 0       |
| A0A3Q2U504 | Vitamin K-dependent protein S                   | PROS1    | 2           | 2.4876  | 0       |
| Q90WD0  | Actin-related protein 3                            | ACTR3    | 2           | 2.36804 | 0       |
| A0A3Q2U3V9 | Uncharacterized protein                         | LOC100858647 | 6         | 2.3079  | 0.0422  |
| F1NPN5  | SERPIN domain-containing protein                   | SPIA3    | 3           | 2.29543 | 0.0116  |
| A0A1D5P7Y2 | Uncharacterized protein                         | TSPAN13  | 1           | 2.24296 | 0       |
| P23498  | Osteopontin                                        | SPP1     | 1           | 2.14947 | 0.0193  |
| Q5ZJX9  | Proteasome subunit alpha type                     | PSMA5    | 2           | 2.0583  | 0.0478  |
| A0A3Q2UBB3 | Microfibril associated protein 2                  | -        | 1           | 1.93253 | 0.0368  |
| Q90974  | Anti-Muellerian hormone                           | AMH      | 1           | 1.85467 | 0       |
| Q9DF58  | Integrin-linked protein kinase                    | ILK      | 1           | 1.82115 | 0.0031  |
| Q2IAL7  | Cathelicidin-2                                    | CAMP; CATH2 | 1         | 1.74816 | 0.0134  |
| A0A1D5PYR9 | VWFA domain-containing protein                | ITGAD    | 3           | 1.73532 | 0.0068  |
| A0A1D5P5T7 | GP-PDE domain-containing protein               | GDPD2    | 1           | 1.69604 | 0.0059  |
| F1NIM0  | Transmembrane channel-like protein                | TMC7     | 1           | 1.49182 | 0.0347  |
| P00565  | Creatine kinase M-type                            | CKM      | 3           | 1.29087 | 0.0194  |

**Table 6** The down-regulated DEPs between P8+DEX and DEX groups
| Accession | Description | Gene symbol | Unique peptides | P8+DEX vs DEX (ratio) | P8+DEX vs DEX (P value) |
|-----------|-------------|-------------|----------------|-----------------------|------------------------|
| O42351    | Rabaptin-5  | RABEP1      | 1              | 0.75201               | 0                      |
| Q90593    | Endoplasmic reticulum chaperone BiP | HSPA5 | 3              | 0.73616               | 0.0473                 |
| E1BWW2    | Uncharacterized protein | TSG101; UEVLD | 1              | 0.72709               | 0                      |
| F1P386    | Uncharacterized protein | CR1L | 1              | 0.71003               | 0                      |
| P68139    | Actin, alpha skeletal muscle | ACTA1 | 1              | 0.69197               | 0                      |
| F1P4U3    | Secreted phosphoprotein 2 | SPP2 | 1              | 0.68375               | 0.012                  |
| A0A3Q2TXS3 | Ig-like domain-containing protein | - | 1              | 0.67825               | 0.0036                 |
| P21760    | Extracellular fatty acid-binding protein | LCN8; P20K; LCN15; EXFABP | 2              | 0.66739               | 0.0496                 |
| A0A1L1RUW5 | Uncharacterized protein | NMI | 1              | 0.65965               | 0.0275                 |
| A0A3Q2U7A2 | Ig-like domain-containing protein | - | 1              | 0.62714               | 0.0159                 |
| A0A3Q2UGD4 | Ig-like domain-containing protein | - | 1              | 0.62172               | 0.0396                 |
| E1C1Q3    | Uncharacterized protein | SLC29A1 | 1              | 0.6066               | 0                      |
| Q5ZLI2    | Proteasome subunit alpha type | PSMA3 | 1              | 0.55381               | 0                      |
| F1NLE7    | tRNA-binding domain-containing protein | AIMP1 | 1              | 0.55353               | 0                      |
| Q6Q1Q8    | Mannan-binding lectin associated serine protease 3 | MASP1 | 1              | 0.54368               | 0                      |
| F1NNF9    | Ankyrin repeat and kinase domain containing 1 | - | 1              | 0.53991               | 0                      |
| E1C0F3    | Uncharacterized protein | RAB7A | 1              | 0.53376               | 0.0008                 |
| Q9BD54    | MHC class II-associated invariant chain (Fragment) | CD74 | 1              | 0.51598               | 0                      |
| A0A1D5PT95 | Uncharacterized protein | KRAS | 1              | 0.51464               | 0.0327                 |
| P01875 | Ig mu chain C region | - | 16 | 0.51124 | 0.0242 |
|---|---|---|---|---|---|
| F1NCZ2 | Rab GDP dissociation inhibitor | GDI1 | 3 | 0.50966 | 0.0131 |
| A0A3Q2U9L1 | Uncharacterized protein | - | 2 | 0.50672 | 0.0005 |
| Q6PW00 | T-cell receptor T3 delta chain | CD3D | 1 | 0.49022 | 0.0071 |
| E1C857 | Tetraspanin | TSPAN6 | 1 | 0.47527 | 0.003 |
| F1NEB3 | Uncharacterized protein | HABP2 | 5 | 0.44691 | 0.0081 |
| Q8UWG7 | 60S ribosomal protein L6 | RPL6 | 1 | 0.40712 | 0 |
| F1NQD9 | Radixin | RDX | 1 | 0.39935 | 0.0006 |
| A0A3Q2UDP3 | Uncharacterized protein | DOCK10 | 2 | 0.39916 | 0.0011 |
| F1NDH2 | Angiotensin 1-10 | AGT | 5 | 0.39399 | 0.036 |
| Q5W9C5 | MHC class I antigen | BF1; HFE | 1 | 0.37324 | 0 |
| E1C007 | Protein kinase C and casein kinase substrate in neurons protein 2 | PACSIN2 | 3 | 0.37321 | 0.0161 |
| A5HUM6 | Tenascin X B | TNXB; TN; TNX | 8 | 0.36364 | 0 |
| P07630 | Carbonic anhydrase 2 | CA2 | 4 | 0.36017 | 0.0256 |
| R4GM71 | Phosphatidylcholine-sterol acyltransferase | LCAT | 6 | 0.3509 | 0.0451 |
| A0A1D5PMA3 | Uncharacterized protein | NELL2 | 1 | 0.34434 | 0.0386 |
| E1C7T9 | Uncharacterized protein | BAIAP2L1 | 4 | 0.34145 | 0.032 |
| A0A3Q2U0N4 | Ig-like domain-containing protein | - | 1 | 0.33343 | 0.0045 |
| Q5ZL35 | Arginine and glutamate-rich protein 1 | ARGLU1 | 1 | 0.33275 | 0.0193 |
| E1C3Y3 | Tetraspanin | TSPAN8 | 1 | 0.32747 | 0 |
| P00337 | L-lactate dehydrogenase B chain | LDHB | 2 | 0.29927 | 0.0471 |
| O93410 | Calmodulin | CALM; CALM2 | 8 | 0.27975 | 0.0262 |
| E1C7C1 | Complement component 8 subunit beta | C8B | 8 | 0.27619 | 0.0044 |
| F1NSD3 | Ig-like domain-containing protein | - | 1 | 0.27103 | 0.0491 |
| Accession | Description                                           | Symbol | ID    | q-value | p-value |
|-----------|-------------------------------------------------------|--------|-------|---------|---------|
| A0A3Q2TUM9 | C5a anaphylatoxin chemotactic receptor                | C5AR1  | 0.26213 | 0       |
| F1NV09    | Epithelial cell adhesion molecule                     | EPCAM  | 0.25783 | 0.0001  |
| A0A1D5PX8 | VPS37 C-terminal domain-containing protein            | VPS37C | 0.25558 | 0.0005  |
| A0A1D5PNT8 | VPS10 domain-containing protein                       | SORT1  | 0.25327 | 0.0005  |
| F1NSM7    | Ovocleidin-116                                       | MEPE   | 0.25238 | 0.0212  |
| A0A3Q2U540 | Ig-like domain-containing protein                     | -      | 0.23225 | 0.0143  |
| A0A1L1RMF4 | Uncharacterized protein                               | -      | 0.22209 | 0.0005  |
| Q5F442    | Uncharacterized protein                               | RASA3  | 0.22128 | 0       |
| Q5F3U5    | Uncharacterized protein                               | RAP2C  | 0.22044 | 0.0013  |
| F1C6X7    | MHC class II antigen alpha chain                     | B-LA; BLA; HLA-DRA | 0.21122 | 0.0081  |
| A0A1D5PPP9 | Uncharacterized protein                               | PTMA   | 0.17915 | 0.0427  |
| R4GLT1    | Cystatin domain-containing protein                    | CST3   | 0.15362 | 0       |
| A0A3Q3APB3 | Uncharacterized protein                               | LOC101748084 | 0.11231 | 0       |
| A0A3Q2UIJ5 | Uncharacterized protein                               | LOC769729 | 0.10447 | 0       |
| F1NIW7    | Cubilin                                              | CUBN   | 0.10149 | 0       |
| Q5F3I1    | Fibrinogen C-terminal domain-containing protein       | FGL2   | 0.0853  | 0.0003  |
| A0A3Q2UFJ3 | Calcium/calmodulin-independent protein kinase         | CAMK2D | 0.05437 | 0       |
| F1NUJ7    | FABP domain-containing protein                        | FABP6  | 0.0372  | 0       |
| P35062    | Histone H2A-III                                      | HIST1H2A3; LOC427881; HIST1H2A4L3; LOC417955 | 0.01549 | 0       |
Figure 1

Identification of plasmal exosomes of broilers. (A) The observation of exosomes by TEM, bar = 200 nm, (B) NTA of exosomes, (C) Identification of CD63 and TSG101 in plasmal exosomes by WB, Calnexin is a negative control protein were used to validate the quality of our isolation technique.
Figure 2

Differentially expressed proteins in plasmal exosomes. (A) Volcano plot, (B) Hierarchical clustering of heatmap.
Figure 3

GO analysis of proteins in plasmal exosomes. BP, biological process; CC, cellular components; MF, molecular function. Pie diagrams show the top 10 enriched GO terms.

Figure 4

KEGG pathway analysis of proteins in plasmal exosomes.
Figure 5

Spearman correlation analyses of exosomal proteins and immune parameters.

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