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 1×10^8 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-1 β , IL-6, TNF- α and jejunal IL-1 β , 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 now 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 paly 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 $5000 \times g$ 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 ($3000 \times g$) 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-1 β 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 μ L 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 jellylike status. The tubes were taped firmly to change the jellylike status into liquid status and then centrifuged at 10000 $\times g$ for 10 min at room temperature. The supernatant was transferred into a new 1.5 mL tube and then placed on ice. Thereafter, 50 μ L Reagent A was added in 200 μ L pre-treated plasma and mixed. The mixture was incubated at 4 °C for 30 min. After incubation, the supernatant was centrifuged at 3000 $\times g$ for 10 min at room temperature. The pellet at the bottom of the tube was resuspended completely with 200 μ L 1 $\times$ PBS and mixed well by vortexing until obtained a homogenous mixture. Fifty microlitre Reagent B was added into the re-suspension and mixed well by vortexing until obtained a homogenous mixture. The mixture was incubated at 4 °C for 30 min. After incubation, the mixture was centrifuged at 3000 $\times g$ for 10 min at room temperature. The exosomes pellet was obtained by removing the supernatant. The exosomes pellet completely in 50-120 μ L 1 $\times$ 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 $\times$ 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 μ L 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 ultrafiltration 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-1 β ($P < 0.01$), 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-1 β ($P < 0.01$), 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-1 β ($P < 0.01$), 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-1 β 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 ± 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 plasmal 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 plasmal 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-1 β 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 (ZYG) ($r = 0.818$, $P < 0.01$). 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-1 β 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 ($r = -0.803$, $P < 0.01$), 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) ($r = -0.857$, $P < 0.01$), A0A1L1RLL0 (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) ($r = -0.835$, $P < 0.01$), A0A1D5PXM8 (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 (NELF2) ($r = -0.840$, $P < 0.01$).

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- κ B signaling pathway by phosphorylating TAK1 and NF- κ B1 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- κ B 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-1 β , IL-6, TNF- α and jejunal IL-1 β , 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 play 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 highly 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 (PACSIN2), 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-1 β , 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-1 β , 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

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

^{a,b,c} 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	<i>P</i> -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 ^a	371.92 ^b	556.92 ^a	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 1×10^8 cfu/g P8; P8+DEX, control diet containing 1×10^8 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 (<i>P</i> -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

	domain-containing protein				
A0A3Q2U540	Ig-like domain-containing protein	-	2	4.55025	0.0134
Q5F442	Uncharacterized protein	RASA3	1	4.51919	0
F1NBW3	Serine incorporator 5	SERINC5	1	4.51106	0.0294
A0A3Q2U4V6	Ig-like domain-containing protein	-	1	4.41341	0.0005
P21760	Extracellular fatty acid-binding protein	LCN8; P20K; LCN15; EXFABP	2	4.01483	0.0015
Q27J90	Leukocyte ribonuclease A-2	RSFR	2	3.91679	0.0033
Q5ZKY7	Tetraspanin	CD82	3	3.85704	0.037
F1C6X7	MHC class II antigen alpha chain	B-LA; BLA; HLA-DRA	2	3.78031	0.0111
A0A3Q2UDP3	Uncharacterized protein OS=Gallus gallus OX=9031 GN=DOCK10 PE=3 SV=1	DOCK10	2	3.66783	0.0004
F1NV09	Epithelial cell adhesion molecule	EPCAM	4	3.66275	0.0001
A0A1D5PH32	Actin-depolymerizing factor	GSN	3	3.52032	0.0378
Q90593	Endoplasmic reticulum chaperone BiP	HSPA5	3	3.42985	0.0005
F1NW23	Clathrin heavy chain	CLTC	12	3.27741	0.0426
Q6IEC5	Putative ISG12(2) protein	ISG12-2; IFI6	5	3.19787	0.0203
E1C3Y3	Tetraspanin OS=Gallus gallus OX=9031 GN=TSPAN8 PE=3 SV=2	TSPAN8	1	3.05368	0
A0A3Q2U871	Uncharacterized protein	-	7	3.00759	0.049
P26007	Integrin alpha-6	ITGA6	6	2.87969	0.0326
Q2MJT5	Glycoprotein IIIb	CD36	2	2.80456	0.0445
R4GLT1	Cystatin domain-containing protein	CST3	1	2.7426	0
A0A1D5PT95	Uncharacterized protein	KRAS	1	2.68849	0.0117
A0A1D5P4G6	Integrin alpha-V	ITGAV	8	2.6846	0.0478

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	Tetraspanin	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 (<i>P</i> -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

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

F1NNF9	Ankyrin repeat and kinase domain containing 1	-	1	0.18577	0
F1P386	Uncharacterized protein	CR1L	1	0.18534	0
Q90944	Epiphycan	EPYC	4	0.1505	0
A0A2H4C5L1	MHC class I antigen	BF	1	0.14362	0
F1NWP1	Christmas factor	F9	6	0.14221	0
E1BSI4	Uncharacterized protein	CHMP5	1	0.1414	0
A0A1D5PGT3	Brain-specific angiogenesis inhibitor 1-associated protein 2	BAIAP2	2	0.12844	0
Q9W6V5	Receptor-type tyrosine-protein phosphatase eta	PTPRJ	1	0.1273	0
B5BSS3	MHC class I alpha chain 2	BF2	1	0.09117	0
P87362	Bleomycin hydrolase	BLMH	1	0.07845	0.0004
Q7T2X3	Low-density lipoprotein receptor	LDLR	1	0.07034	0.0002
F1NPS5	Charged multivesicular body protein 1a	CHMP1A	1	0.0702	0
A0A3Q2U504	Vitamin K-dependent protein S	PROS1	2	0.0574	0
Q5ZL65	Integrin-associated protein	CD47	1	0.05374	0
A0A3Q2U3X0	LRRCT domain-containing protein	LOC101750889	4	0.03605	0
A0A1L1RLL0	TRASH domain-containing protein	RPL24	1	0.02835	0
A0A1D5P6B0	Procollagen C-endopeptidase enhancer	-	2	0.02774	0
A0A3Q2TZU8	Protein tweety homolog	TTYH3	2	0.00996	0.0075
A0A3Q2UHT9	Uncharacterized protein	EHD4	3	0.00933	0
A0A1L1RZV0	Ubiquitin-conjugating enzyme E2 variant 2	UBE2V2	1	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 (<i>P</i> -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 2	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
F1NWN4	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
R4GKL8	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

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 (<i>P</i> -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

A0A3Q2TUM9	C5a anaphylatoxin chemotactic receptor	C5AR1	1	0.26213	0
F1NV09	Epithelial cell adhesion molecule	EPCAM	4	0.25783	0.0001
A0A1D5PXM8	VPS37 C-terminal domain-containing protein	VPS37C	1	0.25558	0.0005
A0A1D5PNT8	VPS10 domain-containing protein	SORT1	1	0.25327	0.0005
F1NSM7	Ovocleidin-116	MEPE	3	0.25238	0.0212
A0A3Q2U540	Ig-like domain-containing protein	-	2	0.23225	0.0143
A0A1L1RMF4	Uncharacterized protein	-	1	0.22209	0.0005
Q5F442	Uncharacterized protein	RASA3	1	0.22128	0
Q5F3U5	Uncharacterized protein	RAP2C	2	0.22044	0.0013
F1C6X7	MHC class II antigen alpha chain	B-LA; BLA; HLA-DRA	2	0.21122	0.0081
A0A1D5PPP9	Uncharacterized protein	PTMA	2	0.17915	0.0427
R4GLT1	Cystatin domain-containing protein	CST3	1	0.15362	0
A0A3Q3APB3	Uncharacterized protein	LOC101748084	1	0.11231	0
A0A3Q2UIJ5	Uncharacterized protein	LOC769729	1	0.10447	0
F1NIW7	Cubilin	CUBN	4	0.10149	0
Q5F3I1	Fibrinogen C-terminal domain-containing protein	FGL2	3	0.0853	0.0003
A0A3Q2UFJ3	Calcium/calmodulin-dependent protein kinase	CAMK2D	3	0.05437	0
F1NUJ7	FABP domain-containing protein	FABP6	2	0.0372	0
P35062	Histone H2A-III	HIST1H2A3; LOC427881; HIST1H2A4L3; LOC417955	2	0.01549	0

Figures

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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