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Article

Exploration of Proteomics Analysis of Crop Milk in Pigeons (*Columba livia*) during the Lactation Period

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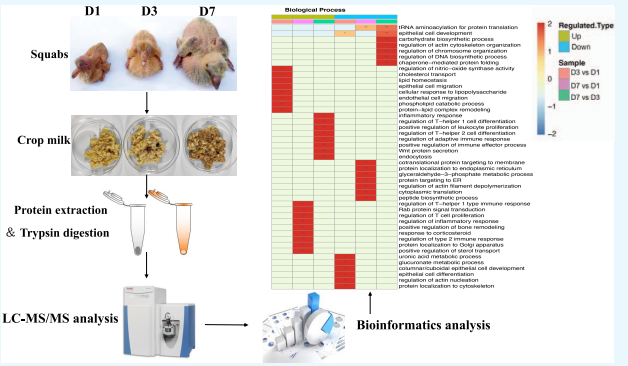
ABSTRACT: Pigeon milk is a curdlike substance separated from the mature crop epithelium of breeders, associated with the rapid growth and development of squabs. The aim of this study was to investigate in detail the variations in the content of several important ingredients in crop milk. In this study, we utilized proteomic techniques to investigate the composition and changing pattern of crop milk protein of squabs on days 1 (D1), 3 (D3), and 7 (D7). Our results indicated that the crude protein contents in crop milk decreased with age, and they were up to 50% during the first 3 days. The proteomic data showed that a total of 2558 proteins were identified in all samples from three stages, and the top 15% crop milk proteins were ribosomal protein, keratin, peroxiredoxin, annexin, heat shock protein, and eukaryotic translation protein based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis and normalized spectral abundance factors (NSAFs) calculation. Furthermore, the compositions of crop milk protein between D1 and D3 were quite similar [51 differentially expressed proteins (DEPs)], while great proteomic differences were observed between D1/D3 and D7 (more than 240 DEPs). Additionally, gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that up-regulated DEPs mainly participate in immune response, while down-regulated DEPs were involved in cell differentiation and development as well as tRNA aminoacylation biosynthesis. In conclusion, DEPs were mainly related to protein synthesis, immunity, and antioxidation, which provided effective information for the development of artificial squab milk products in the future.



INTRODUCTION

As altricial birds, domestic pigeon squabs (*Columba livia*) are fed by parents in a mouth-to-mouth manner for nearly 28 days. After birth, they live mainly on the crop milk to obtain nutrients up to 7 days of age.[1,2] Thereafter, the crop milk was mixed with the adult diet until the young squabs reached 28 days of age. The maturing rate of pigeon squabs is much faster than broilers, quails, or ostriches,[1] and the impressively rapid growth of squabs is likely attributable to their crop milk.[3] Like mammals, the process of crop milk production was regulated by the prolactin of pigeons during the lactation period.[4−6] However, unlike mammals, the histological analysis showed the pigeon crop tissue has no mammary alveolars, and pigeon milk is a curdlike substance separated from the mature crop epithelium of male and female breeders.[3,7,8] Transcriptome analysis showed that many genes related to crop cornification, including *β-keratin*, *S100-A9*, and *S100-A10*, are involved in pigeon milk production.[2,9] Besides, long noncoding RNA, microRNA, and endogenous RNA were demonstrated to be associated with milk production.[10,11] Although many studies investigated the mechanisms of pigeon milk production, the main components of curdlike pigeon milk are unclear at present. More importantly, the special feeding pattern makes it difficult for the pigeon industry to achieve intensive breeding.[12] Thus, it is essential to produce artificial pigeon milk by investigating the pigeon milk composition so as to improve the efficiency of the pigeon breeding industry.

There are several studies focused on the composition of mammalian milk, in which protein is an important nutritional component.[13] With the development of proteomics techniques, the protein compositions of human and ruminant milk have been extensively identified for the infant formula, including caseins, whey proteins, and milk fat globule membrane proteins.[14−16] Similar to mammalian milk, many studies have indicated that pigeon milk mainly consists of 60% protein, 30% fat, and a small amount of carbohydrate and minerals based on dry weight,[7,8] and the high levels of protein in crop milk may be an essential food source for rapidly growing squabs.[17] In functional studies, pigeon milk could cause precocial incisor eruption and eyelid opening in newborn

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mice and stimulate the growth of ovarian cells in hamsters, which were closely associated with an epidermal growth factor-like protein in pigeon milk.[18,19] Additionally, pigeon milk contains highly nutritious components such as immunoglobulins and cytokines that modulate the development of the immune system of chickens fed pigeon milk.[9] Therefore, it is very meaningful to determine the compositions and changing patterns of crop milk protein during the lactation period of pigeons by proteomics techniques so as to make clear the nutritional components of crop milk and develop artificial squab milk products.

RESULTS

Changes in the Protein Content of Pigeon Milk on Different Days. The crude protein contents in pigeon milk were all affected ($P < 0.05$, Figure 1) by age, and they

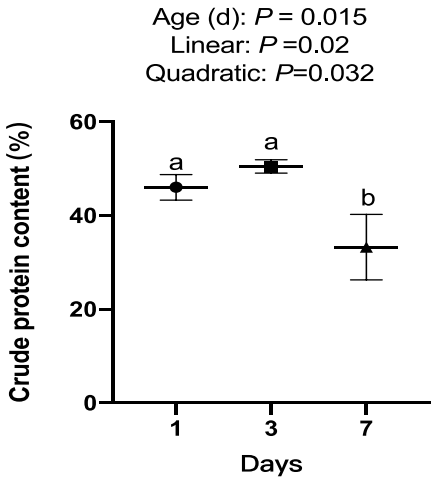

Figure 1. Content of crude protein in the crop milk of squabs on days 1, 3, and 7. All values are expressed as means ± standard error, $n = 6$. Lacking the same letters (a, b) means differences at $P < 0.05$.

decreased linearly and quadratically ($P < 0.05$) with age. The crude protein contents remained at a stabilized level from days 1 (D1) to 3 (D3) and then decreased on day 7 (D7).

Protein Expression Differences. There are 2558 identified proteins and 2116 quantifiable proteins of pigeon crop milk in all samples from the 3 ages. Among the 2558 identified proteins, the top 25% crop milk proteins were calculated by normalized spectral abundance factors (NSAFs) analysis, as shown in Table 1. We focused on the top 15% crop milk proteins, which were classified into ribosomal protein (5.61%), keratin (3.52%), peroxiredoxin (2.01%), annexin (1.71%), heat shock protein (1.26%), and eukaryotic translation (1.15%) in decreasing order.

The proteomic data of the milk from the three groups of pigeon squabs are shown in Figure 2. The amount of differentially expressed proteins (DEPs) in pigeon milk increased with age, and the number of down-regulated proteins (385) was more than that of up-regulated proteins in the differentially expressed proteins (307) among all ages (Figure 2A,B). Besides, there are 51 (D3 vs D1), 393 (D7 vs D1), and 248 (D7 vs D3) differentially expressed proteins in pigeon milk, indicating that the number of differentially expressed proteins in pigeon milk between days 1 and 3 was less than that between days 1 and 7 or between days 3 and 7, and the number of differentially expressed proteins in pigeon milk

Table 1. Top 25% Crop Milk Proteins of Pigeon Squabs Identified Based on Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) analysis and NSAFs Calculations[a]

protein description	NSAFs (%)
guanine nucleotide-binding protein	0.329
proteasome	0.360
proteasome endopeptidase complement	0.463
glutathione *S*-transferase	0.466
NADH dehydrogenase	0.507
uncharacterized protein	0.526
aldehyde dehydrogenase	0.551
cystatin	0.566
actin-related protein	0.575
protein disulfide-isomerase	0.587
fatty acid-binding protein	0.618
chaperonin containing TCP1	0.618
tyrosine 3-monooxygenase	0.683
ATP synthase subunit	0.687
transglutaminase 4	0.718
cytochrome	0.762
RAB	0.965
eukaryotic translation	**1.153**
heat shock proteins	**1.263**
annexin	**1.706**
peroxiredoxin	**2.011**
keratin	**3.519**
ribosomal proteins	**5.613**
total	25.248

[a]NSAFs were used to rank the identified proteins, and their values should range from 0 to 100%, with values closer to 100% indicating higher protein levels. NSAFs were calculated as described as follows: spectral counts were divided by protein length, defining a spectral abundance factor (SAF), and SAF values were then normalized against the sum of all SAFs. The words in bold signify the top 15% proteins (NSAFs: normalized spectral abundance factors).

between days 1 and 7 was more than that between days 3 and 7. In addition, there were a total of 2140 common proteins in crop milk on days 1, 3, and 7, and there were 71, 28, and 24 unique proteins identified on days 1, 3, and 7, respectively (Figure 2C).

Gene Ontology (GO) Analysis. The GO analysis of all differentially expressed proteins is shown in Figure 3A−C. In the biological process (BP) category, most of the differentially expressed proteins between two random groups were mainly involved in cell process, metabolic process, biological regulation, response to stimulus, multicellular organismal process, cellular component (CC) organization or biogenesis, developmental process, and localization. Some differentially expressed proteins were assigned to the signaling, multi-organism process, reproduction, and immune system process. In the cellular component category, a large number of differentially expressed proteins were assigned to the cell, organelle, membrane, membrane-enclosed lumen, and macromolecular complex. However, there was a low proportion of differentially expressed proteins located in the extracellular region and cell junction. For the molecular function (MF) category, the GO terms including binding and catalytic activity were the predominant functions of differentially expressed proteins. Notably, a small number of differentially expressed proteins were associated with activities of transporter, structural molecule, and molecular transducer.

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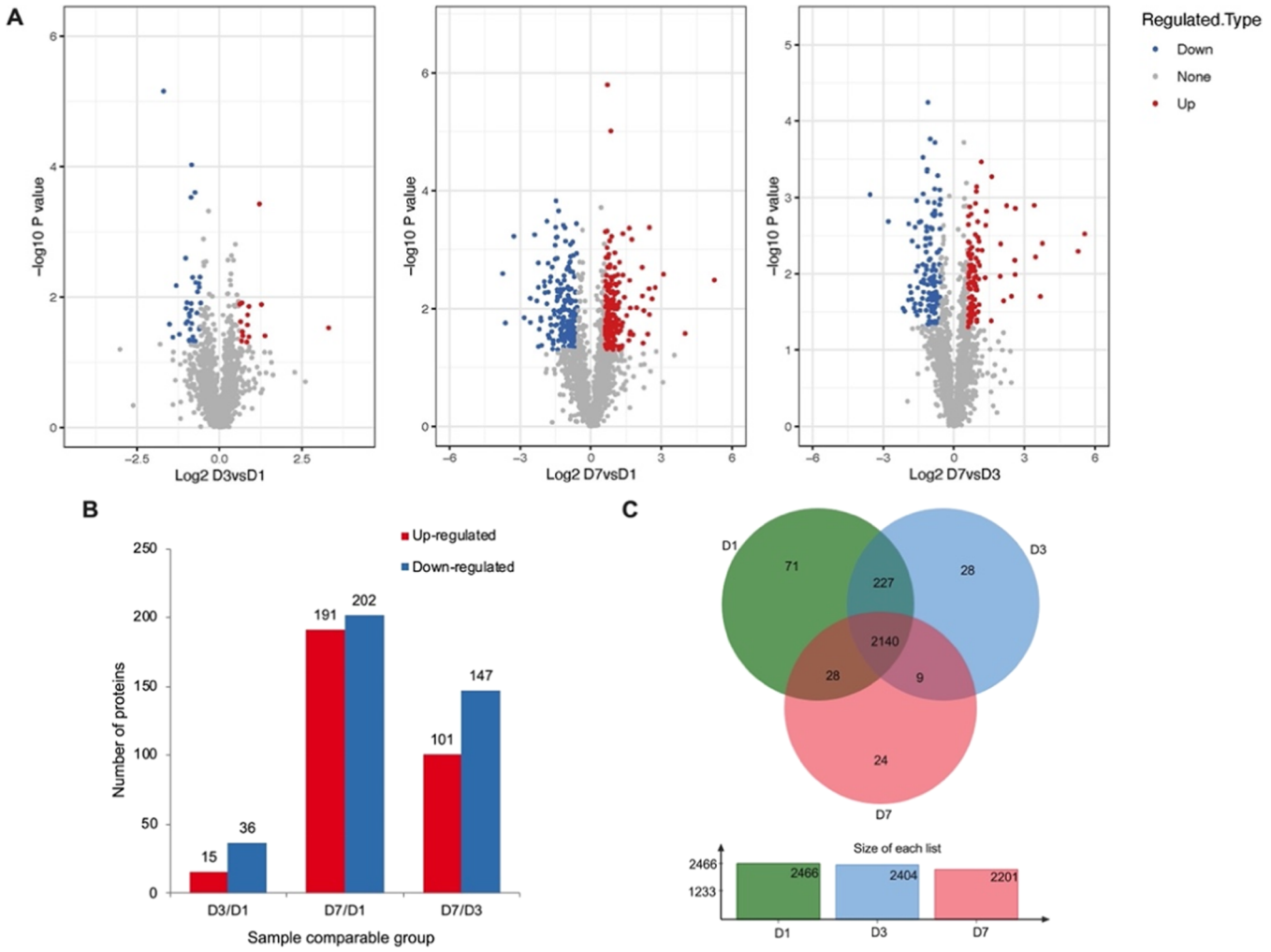

Figure 2. Distribution of differentially expressed proteins in the pigeon milk of squabs on days 1, 3, and 7. (A) Volcano plot of the differentially expressed proteins. The horizontal coordinate was the fold change (logarithmic transformation at the base of 2), and the vertical coordinate was the significant difference *P*-value (logarithmic transformation at the base of 10). Red dots represent the expression of up-regulated proteins, and blue dots represent the expression of down-regulated proteins; (B) up-regulated and down-regulated differentially expressed proteins (filtered with a threshold value of expression fold change, *P*-value < 0.05) (C) in different intervention groups; the Venn diagram of the common and differentially expressed proteins identified in the pigeon milk at different ages of pigeon squabs. D3/D1: the crop milk protein on day 3 versus day 1; D7/D1: the crop milk protein on day 7 versus day 1; D7/D3: crop milk protein on day 7 versus day 3.

GO Enrichment of Differentially Quantified Proteins. Following the GO classification with the biological process (BP), cellular component (CC), and molecular function (MF), the clustering analysis was carried out to compare the functional relevance of the identified proteins on different days of squabs.

For the biological process category (Figure 4A), compared with the crop milk on day 1, the up-regulated proteins of crop milk on day 3 were primarily enriched in cell migration and lipid metabolism process, including phospholipid catabolic process, cholesterol transport, and lipid homeostasis. The up-regulated proteins of crop milk on day 7 were enriched in immune response, including the regulation of type 2 immune response, regulation of inflammatory response, regulation of T-cell proliferation, and regulation of T-helper 1 type immune response. On the contrary, compared with the crop milk on day 1, the down-regulated proteins of crop milk on day 3 were enriched in cell differentiation and development as well as cytoskeleton formation. The down-regulated proteins of crop milk on day 7 were related to the peptide biosynthetic process, cotranslational protein targeting to membrane, tRNA amino-acylation for protein translation, and protein localization to the endoplasmic reticulum.

For the cellular component category (Figure 4B), compared with the crop milk on day 1, the up-regulated proteins of crop milk on day 3 were mainly originated from the cell surface and membrane, and the up-regulated proteins on day 7 mainly occurred in the extracellular space, mitochondria, and transport vesicle. However, the down-regulated proteins of crop milk on day 3 were mainly from the organellar ribosome, chromosome, and dynactin complex, and the down-regulated proteins on day 7 occurred in the cytosol ribosome, non-membrane-bounded organelle, and chaperone complex.

For the molecular function category (Figure 4C), compared with the crop milk on day 1, the up-regulated proteins of crop milk on day 7 resulted in enrichment in ribonucleoside binding, but the up-regulated proteins of 3-day-old crop milk had no related molecular functional clusters. In contrast, the down-regulated proteins of crop milk on day 3 were enriched in functional clusters associated with Notch binding,

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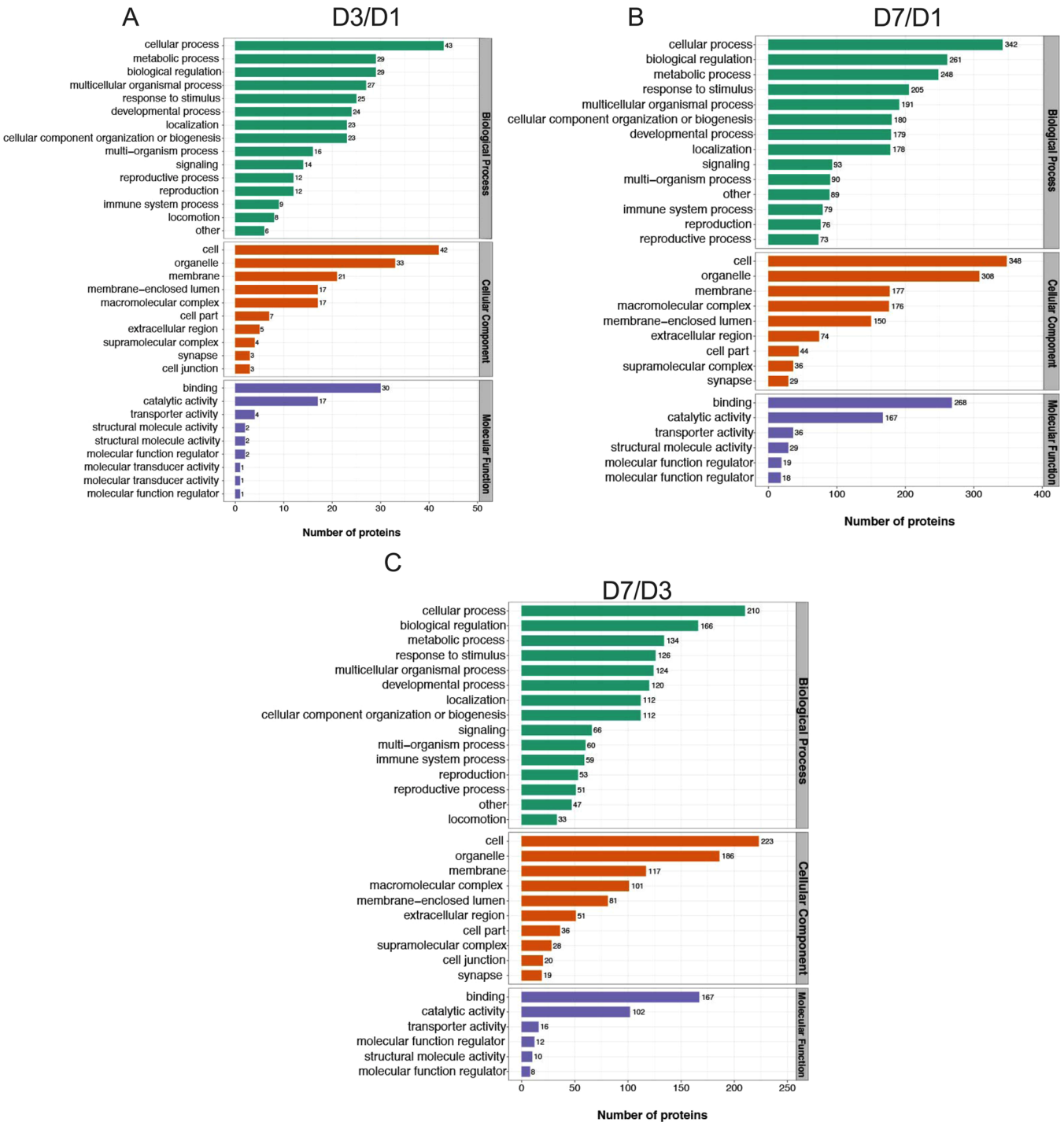

Figure 3. Gene ontology (GO) categories assigned to the differentially expressed proteins in the pigeon milk of squabs on days 1, 3, and 7. (A) Differentially expressed proteins between days 3 and 1 were classified into biological process, cellular component, and molecular function. (B) Differentially expressed proteins between days 7 and 1 were classified into biological process, cellular component, and molecular function. (C) Differentially expressed proteins between days 7 and 3 were classified into biological process, cellular component, and molecular function. D3/D1: the crop milk on day 3 versus day 1; D7/D1: the crop milk on day 7 versus day 1; D7/D3: crop milk on day 7 versus day 3.

cytoskeletal protein binding, and uridine diphosphate (UDP)-glycosyltransferase activity, and the down-regulated proteins of crop milk on day 7 were related to protein binding.

Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis of Differentially Expressed Proteins. The KEGG pathway enrichment analysis of the differentially expressed proteins is shown in Figure 5 and Table S1. A total of 12, 28, and 20 KEGG pathways were significantly enriched in D3/D1, D7/D1, and D7/D3 groups, respectively. Importantly, the cholesterol metabolism pathway was significantly up-regulated in both D3/D1 and D7/D1 groups (Figure 5A1,A2), and the pathogenic *Escherichia coli* infection was the common up-regulated pathway (Figure 5A2,A3) in both D7/D1 and D7/D3 groups. On the contrary, the drug metabolism pathway was significantly down-regulated in the D3/D1 group (Figure 5B1), and aminoacyl-tRNA biosynthesis, pentose

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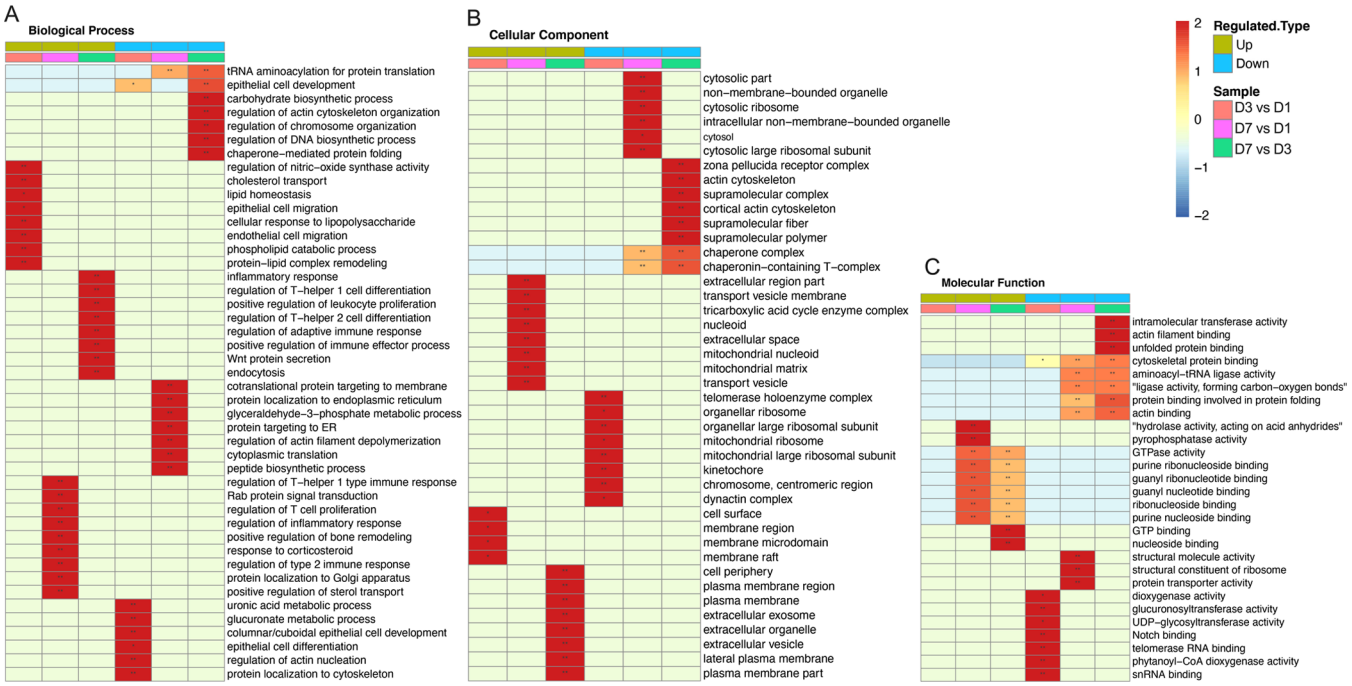

Figure 4. Cluster analysis heat map based on GO enrichment. (A) Biological process. (B) Cellular component. (C) Molecular function. The differential proteins in the different comparison groups were subjected to GO enrichment. The horizontal direction represents the enrichment test results of the different groups, and the vertical direction is the enrichment-related functions. Different color blocks in a row indicate the degree of enrichment. The red color indicates a strong degree of enrichment (the deeper the red, the stronger the enrichment), and the blue color indicates a weaker enrichment (the lighter the blue, the weaker the enrichment). *$P < 0.05$; **$P < 0.01$. D3/D1: the crop milk protein on day 3 versus day 1; D7/D1: the crop milk protein on day 7 versus day 1; D7/D3: the crop milk protein on day 7 versus day 3.

phosphate pathway, and glycolysis/gluconeogenesis were the common down-regulated pathways in both D7/D1 and D7/D3 groups (Figure 5B2,B3). Noticeably, we found that a large number of differentially expressed proteins related to the ribosome pathway were down-regulated in the D7/D1 group.

To further understand protein−protein interactions (PPIs) among the differentially expressed proteins in the crop milk of squabs on different days, the databases of IntAct Uniprot and molecular interaction (MINT) were queried to determine the interaction between target proteins and other proteins directly acting with them. Cytoscape software was used to generate the interaction network. The concentrated nets were obtained by protein−protein interaction (PPI) analysis (Figure 6). These proteins were enriched in protein synthesis and folding, which were consistent with the results of GO enrichment and KEGG pathway enrichment. There were five differentially expressed proteins with a high degree of connectivity with ribosomes, including tyrosine−tRNA ligase (YARS, A0A2I0R7VUG7), phenylalanyl-tRNA synthetase (FARSB, A0A2I0MBE8), tryptophanyl-tRNA synthetase (WARS, A0A2I0MPR9), T-complex 1 (TCP1, A0A2I0MU42), and eukaryotic translation initiation factor 2 (EIF2S3, A0A2I0MLE0); most of them were involved in aminoacyl-tRNA biosynthesis. Besides, there were 29 differentially expressed proteins that belong to ribosomes, including 22 ribosomal proteins and seven proteins related to protein translation, fold, and processing.

Verification of Differentially Expressed Proteins by Parallel Reaction Monitoring (PRM) Analysis. To confirm the label-free mass spectrometry results, a PRM analysis was performed to detect the expression of 14 differentially expressed proteins (Table 2). Among the down-regulated differentially expressed proteins, we focused on those having predicted annotations related to epithelial cell development, protein translation (tRNA aminoacylation for protein translation), folding (chaperone-mediated protein folding), and localization (protein localization to endoplasmic reticulum). Besides, the proteins involved in KEGG pathways, including aminoacyl-tRNA biosynthesis, pentose phosphate pathway, and glycolysis/gluconeogenesis, were also focused on. From the down-regulated proteins in D3/D1, D7/D1, and/or D7/D3 groups, nine proteins including WD repeat domain 1 (WDR1), cytoplasmic FMR1-interacting protein (CYFIP1), phenylalanyl-tRNA synthetase (FARSB), cysteinyl-tRNA synthetase (CARS), tyrosine−tRNA ligase (YARS), tryptophanyl-tRNA synthetase (WARS), T-complex protein 1 subunit eta (CCT7), 6-phosphogluconate dehydrogenase (PGD), and fructose-bisphosphate aldolase (ALDOB) were selected to confirm the label-free mass spectrometry results by PRM. The PRM analysis results showed that the expression levels of all of these nine down-regulated proteins had a consistent trend with the mass spectrometry results. In contrast, the proteins related to lipid homeostasis, cholesterol metabolism, immune response, and pathogenic *E. coli* infection were up-regulated in D3/D1, D7/D1, and/or D7/D3 groups. From the up-regulated proteins, we selected five proteins, including lipocalin (LCNL1), prostaglandin E synthase 3 (PTGES3), lipase, endothelial (LIPG), sodium/potassium-transporting ATPase subunit α (ATP1A1), and transferrin receptor (TFRC) for validation by PRM. However, the PRM analysis results showed that the LCNL1 was the only confirmed protein, as being consistent with the label-free mass spectrometry results.

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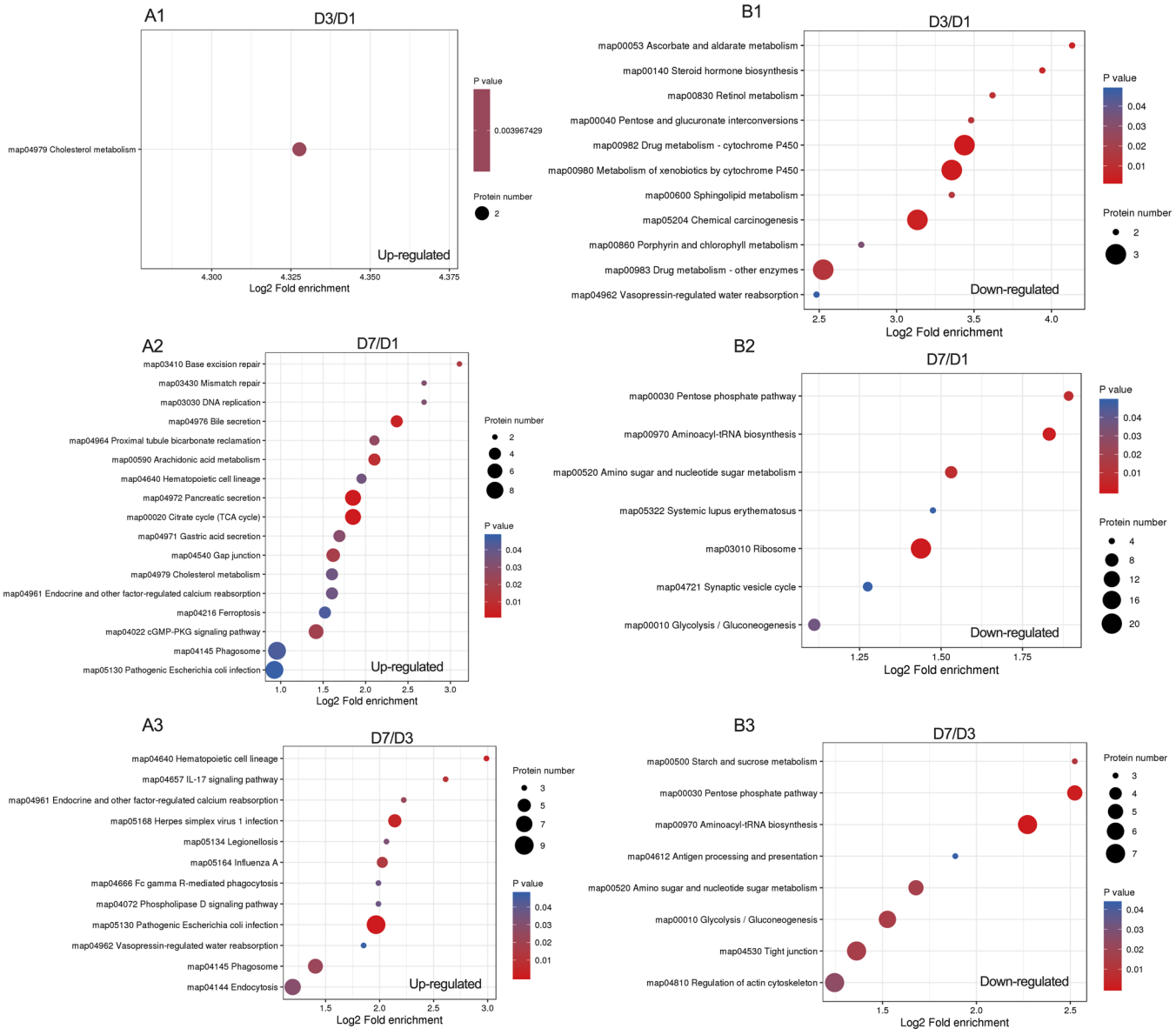

Figure 5. KEGG functional pathway analysis of the differentially expressed proteins in the pigeon milk of squabs on different days based on the bubble chart. (A1−A3) Up-regulated pathway of differentially expressed proteins in D3/D1, D7/D1, and D7/D3 groups, respectively. (B1−B3) Down-regulated pathway of differentially expressed proteins in D3/D1, D7/D1, and D7/D3 groups, respectively. The pathway with a corrected *P*-value <0.05 was significant. The horizontal coordinate in each graph represents the fold change (logarithmic transformation at the base of 2), and the vertical coordinate represents the enriched pathways. The color gradient represents the size of the *P*-value. The color gradient changes from blue to red, and the closer the red, the higher the significance level of enrichment. The larger the circle, the more proteins enriched. D3/D1: the crop milk protein on day 3 versus day 1; D7/D1: the crop milk protein on day 7 versus day 1; D7/D3: the crop milk protein on day 7 versus day 3.

DISCUSSION

Here, we show the changes of crop milk proteins during lactation from hatching to the end of the 1st week. Consistent with previous studies, our study demonstrated that the crude protein contents in crop milk decreased with age, suggesting that the protein contents are affected by the lactation periods.[13,18] Moreover, we found that the content of crop milk protein during the first 3 days was nearly up to 50%, and previous studies demonstrated that the crop milk protein accounted for approximately 55−60% of the total milk composition by dry weight.[8,17] By contrast, mammary milk contains only 25% protein on a dry matter basis.[20] The high level of protein in pigeon milk might have essential functions for the rapid growth and development of squabs. Pigeon milk has been shown to contain a number of bioactive proteins, including IgA, growth factor, and transferrin,[19−21] which could promote chicken growth and induce the innate immune response.[9] Currently, it is difficult to produce industrialized artificial pigeon milk because of many unidentified protein components in pigeon milk.[12] Thereby, this is the first proteome study to investigate the composition and function of pigeon milk. Proteomic data showed that the protein composition of crop milk was relatively stable during the first 3 days after hatching, while the number of differentially expressed proteins increased significantly on the 7th day, and the number of down-regulated proteins was more than that of up-regulated proteins. These results suggested that the changing pattern of protein composition was consistent with

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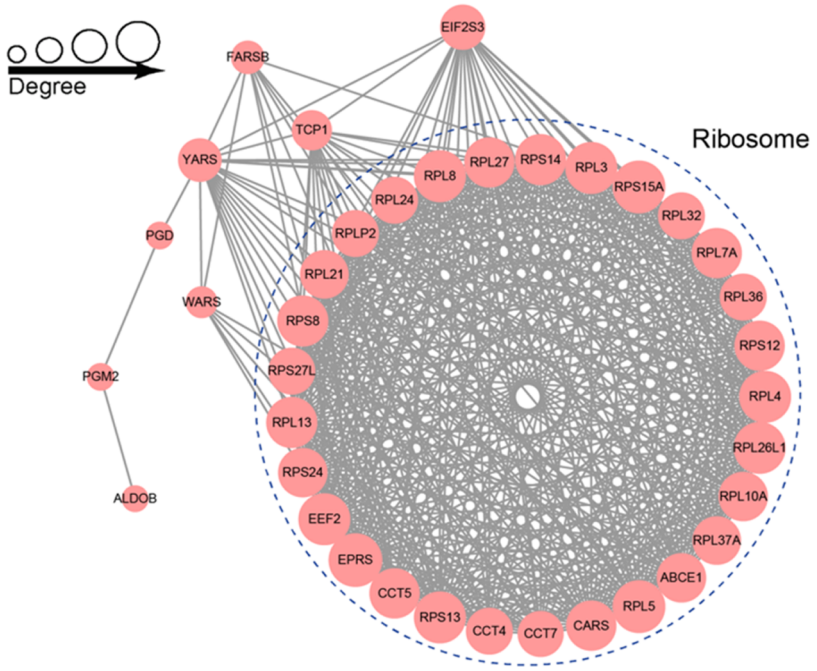

Figure 6. Concentrated net was obtained by protein−protein interaction (PPI) analysis. The gray lines represent direct interactions between two proteins. The circles stand for differentially expressed proteins. The circle size correlates with the number of interacting proteins with differentially expressed proteins. The larger the circle, the more proteins it interacts with, indicating that the protein is more important in the network.

Table 2. Comparison between the Label-Free Mass Spectrometry and Parallel Reaction Monitoring (PRM) Results[a]

protein accession	protein names	label-free mass spectrometry (D3/D1)	PRM (D3/D1)	label-free mass spectrometry (D7/D1)	PRM (D7/D1)	label-free mass spectrometry (D7/D3)	PRM (D7/D3)
A0A2I0MMS6	WD repeat domain 1 (WDR1)	**0.66**	**0.64**	**0.35**	**0.22**	**0.53**	**0.34**
A0A2I0MLK0	cytoplasmic FMR1-interacting protein (CYFIP1)	**0.53**	**0.72**	**0.17**	**0.30**	**0.31**	**0.42**
A0A2I0MBE8	phenylalanyl-tRNA synthetase (FARSB)	0.96	0.91	**0.54**	**0.33**	**0.56**	**0.36**
A0A2I0MHX5	cysteinyl-tRNA synthetase (CARS)	0.73	0.75	**0.21**	**0.15**	**0.29**	**0.19**
R7VUG7	tyrosine−tRNA ligase (YARS)	1.18	1.58	**0.52**	**0.41**	**0.44**	**0.26**
A0A2I0MPR9	tryptophanyl-tRNA synthetase (WARS)	1.02	**0.75**	**0.33**	**0.15**	**0.32**	**0.20**
A0A2I0MME0	T-complex protein 1 subunit eta (CCT7)	1.13	0.99	**0.63**	**0.31**	**0.55**	**0.31**
A0A2I0M3D0	6-phosphogluconate dehydrogenase (PGD)	1.03	0.77	**0.55**	**0.01**	**0.54**	**0.02**
A0A2I0LUV9	fructose-bisphosphate aldolase (ALDOB)	0.93	0.91	**0.44**	**0.32**	**0.47**	**0.35**
A0A2I0LIE4	lipocalin (LCNL1)	0.53	0.52	**5.52**	**3.99**	**10.47**	**7.71**
A0A2I0LJ55	prostaglandin E synthase 3 (cytosolic) (PTGES3)	**1.43**	1.22	**1.97**	0.94	**1.38**	0.77
A0A2I0M0K5	lipase, endothelial (LIPG)	**1.58**	1.38	**1.68**	0.84	1.06	0.61
A0A2I0MTD0	sodium/potassium-transporting ATPase subunit α (ATP1A1)	1.14	0.96	**1.75**	1.01	**1.54**	1.05
A0A2I0LU15	transferrin receptor (TFRC)	1.02	1.13	**1.55**	0.88	**1.52**	0.78

[a]Numbers <1 in bold indicate that the proteins were significantly down-regulated with age; numbers >1 in bold indicate that the proteins were significantly up-regulated with ages. Numbers not in bold indicate that the proteins were not affected by different ages.

that of the protein content. Similar to the colostrum of mammals, the pigeon milk during the first 3 days contains numerous nutrients.[19−21] After that, the pigeon milk secretion decreased and was mixed with a large amount of the adult diet on day 7.[12] Therefore, the content and diversity of crop milk proteins declined after 3 days. The GO term annotation was conductive to characterize the physiological and functional changes associated with the protein expression in tissues.[22] The KEGG pathway analysis was widely used for a systematic understanding of the gene functions in organisms.[23] In the present study, GO enrichment results showed that the up-regulated proteins on the 7th day mainly participate in the immune response, including the regulation of T-cell proliferation and differentiation and inflammatory response. The further analysis of the KEGG pathway suggested that the pathogenic *E. coli* infection was also up-regulated with age, indicating that the pigeon milk protein may play an important role in resistance to external pathogen infection by activating

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the immune function of pigeons.[9] Mammalian milk can deliver immune molecules such as immunoglobulins and cytokines to modulate the development of the immune system of newborn mammals.[24,25] Moreover, we observed an IgA-associated protein in pigeon milk (joining chain of multimeric IgA and IgM, A0A2I0LR47), but it is not in the top 30% identified proteins, constituting only 0.089%, which might be lost in the experimental processes including protein extraction, separation, and digestion. On the contrary, the down-regulated proteins on the 3rd day participated in cell differentiation and development in pigeon milk, and the related proteins, including WDR1 and CYFIP1, had a high expression level on the 1st day during lactation and then decreased. It has been demonstrated that the lactating crop epithelium begins to proliferate and subsequently differentiates into keratinocytes that eventually form pigeon milk.[7] A previous study found that the relative weight and thickness of crops are at a maximum on the 3rd day of lactation, and the 1st week of lactation is the high-yield period of crop milk.[8] Besides, the KEGG enrichment pathway showed that tRNA aminoacylation biosynthesis was down-regulated with age, and the levels of related proteins including FARSB, CARS, YARS, and WARS in pigeon milk decreased using a PRM analysis. The protein synthesis was indicated by aminoacyl-tRNA biosynthesis in the skeletal muscle.[26] It has been reported that aminoacyl-tRNA synthetases and glycyl-tRNA synthetase might be involved in the regulation of the cell signal transduction pathway for milk protein synthesis.[27] Therefore, the down-regulated tRNA aminoacylation biosynthesis in pigeon milk indicated that the protein synthesis of crop milk was reduced with lactating pigeon age, leading to a decrease of the crude protein contents in crop milk with age.

Although crop milk is similar to mammalian milk in its function, the composition of these products is obviously different. The whey protein and casein protein were the main proteins in mammalian milk, including human and ruminant milk.[15,16] However, our PPI network analyses showed that ribosomal proteins were abundant in pigeon milk, especially the milk on the first day during lactation. In accordance with our results, Sun et al.[28] found that ribosome was abundant in goat colostrum and mature milk. It is well known that ribosomes consisting of a small 40S and a large 60S subunit are the cellular organelles responsible for protein synthesis in cells, which function to translate messenger RNAs into proteins.[29] In addition, our results indicated that eukaryotic translation initiation factor 2 and tRNA synthetase have a high degree of connectivity of ribosome. Cellular protein synthesis consists of three distinct stages, initiation, elongation, and termination, and all stages depend on translation factors.[30] Herein, we speculate that ribosomal proteins in pigeon milk might be from the exfoliated crop epithelium and mainly participated in the synthesis of other pigeon milk proteins with biological functions. In addition to ribosomes, we also found the keratin, peroxiredoxin, annexin, and heat shock proteins were the main pigeon milk proteins. Keratin and annexin were the cornification-associated proteins in the crop milk, and Gillespie et al.[2] found that several cornification-associated genes, including α and β keratin and annexin *cp35*, were present in lactating pigeon crop cells using transcriptome analysis. Peroxiredoxin is a major cytosolic antioxidant protein involved in cell redox homeostasis,[7] and heat shock proteins are the markers of multiple types of cellular stress, including inflammation and hypoxia.[31] It is possible that a hyperplastic response in the pigeon crop epithelium during lactation leads to localized cellular stress and the expression of peroxiredoxin and heat shock proteins.[7] These antioxidant proteins in pigeon milk may directly protect the developing squab as well as the parental crop tissue from external stress.

CONCLUSIONS

In summary, we have used proteomics and PRM approaches to determine the compositions, functions, and changing patterns of pigeon milk at different ages. From the 2558 proteins identified in all samples from 3 ages, the top 15% crop milk proteins were classified into ribosomal protein, keratin, peroxiredoxin, annexin, heat shock protein, and eukaryotic translation protein. Also, we found that the content and diversity of crop milk proteins declined after 3 days. According to GO functional annotation and KEGG pathway analysis, we observed that up-regulated differentially expressed proteins were mainly related with immune and antioxidation, while down-regulated differentially expressed proteins are involved in protein synthesis. All of these findings provided effective information for the accurate preparation of the staged artificial pigeon milk.

MATERIALS AND METHODS

Animals and Treatment Procedure. The study was carried out in accordance with the guidelines set by the Animal Care and Use Committee (permit number: SYXK-2017-0005) of the Institute of Animal Husbandry and Veterinary Medicine, Beijing Academy of Agriculture and Forestry Sciences (IAHVM-BAAFS), Beijing, China. The protocols were approved by the Animal Care and Use Committee of IAHVM-BAAFS.

Birds, Diets, and Treatments. A total of six pairs of healthy White King breeding pigeons were obtained from a commercial pigeon farm (Miyun District, Beijing, China). Two squabs were raised by a pair of pigeons. According to the farming methods used by the pigeon industry, the parent pigeons were fed a formulated diet consisting of maize, peas, soybean meal, and wheat. The feed, sand, and water were provided ad libitum. Birds were housed in a room under a lighting cycle of 16 h light and 8 h darkness. The mean daily temperature was 22 ± 6 °C.

Pigeon Milk Collection. Pigeon milk samples were collected from 12 squabs at 1, 3, and 7 days after hatching (D1, D3, and D7). As soon as squabs were fed by the parent pigeon, the pigeon milk was forced out from the squab crop to mouth immediately and then collected into 5 mL sterile tubes. Equal weight crop milk from two squabs in each pair pigeons was pooled into one sample and a total of six biological samples were stored at −80 °C for further analyses. Among them, three biological replicates for each age were used for label-free proteomics and parallel reaction monitoring (PRM) analysis, and six biological replicates for each age were used for crude protein determination.

Crude Protein Determination. The crude protein content in pigeon milk was analyzed with the Kjeldahl method using an automatic instrument (K9860, Hanon, China).[32] The content of crude protein was determined based on the air-dry weight of pigeon milk.

Protein Extraction. Frozen pigeon milk samples were ground to powder by liquid nitrogen, and then transferred into ice-cold lysis buffer [8 M urea and 1 mM tetraethylammonium

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bromide (TEAB)] supplemented with 1% protease inhibitor cocktail, followed by sonication for 3 min on ice using a high-intensity ultrasonic processor (Scientz-5T, Scientz, China). The mixture was centrifuged at 12 000*g* for 10 min at 4 °C, and the supernatant was collected for total protein determination using a BCA Protein Assay kit (Thermo Scientific Pierce) according to manufacturer instructions.

Trypsin Digestion and Desalination. For digestion, the same amount of protein from different samples was used for this experiment. Lysis buffer was used to make up the volume of each sample to a certain volume, and then 1 volume of precooled acetone was added into the protein solution. After shortly mixing, 4 volumes of precooled acetone were added into the protein solution. The mixture was incubated at −20 °C for 2 h and centrifuged at 8000*g* for 5 min at 4 °C. The supernatant was discarded, and the precipitate was washed three times with precooled acetone. After drying the pellet, 200 mM TEAB was added to resuspend the protein pellet, and trypsin was added at 1:50 trypsin-to-protein mass ratio for the digestion overnight at 37 °C. The digests were incubated at 37 °C for 30 min with the final concentration of 5 mM dithiothreitol (DTT) and then incubated at room temperature for 15 min in the dark with the final concentration of 11 mM iodoacetamide (IAM).

The digested protein sample was acidified to pH 2−3 with 10% trifluoroacetic acid (TFA) and centrifuged at 12 000*g* for 10 min at room temperature. The supernatant was transferred to a new tube and desalination was performed. Briefly, the SPE column was activated by 100% methanol and stabilized by 0.1% TFA. Then, the acidified sample was loaded into the SPE column. Then, the trapped peptide sample was washed with 0.1% TFA. Finally, the sample was eluted with 80% acetonitrile. The sample is quantified by the BCA kit, and the same amount of peptide was taken using a vacuum dryer.

LC-MS/MS Analysis. The tryptic peptides were dissolved in solvent A (0.1% formic acid, 2% acetonitrile in water) and directly loaded onto a homemade reversed-phase analytical column (25 cm length, 100 μm i.d.). Peptides were separated with a gradient from 6 to 24% solvent B (0.1% formic acid in acetonitrile) over 70 min, 24 to 35% in 14 min and increasing to 80% in 3 min and then holding at 80% for the last 3 min, all at a constant flow rate of 450 nL/min on a nanoElute UHPLC system (Bruker Daltonics).

The peptides were subjected to the capillary source, followed by the timsTOF Pro (Bruker Daltonics) mass spectrometry. The electrospray voltage applied was 1.75 kV. Precursors and fragments were analyzed at the time-of-flight (TOF) detector, with an MS/MS scan ranging from 100 to 1700 m/z. The timsTOF Pro was operated in the parallel accumulation serial fragmentation (PASEF) mode. Precursors with charge states 0−5 were selected for fragmentation, and 10 PASEF-MS/MS scans were acquired per cycle. The dynamic exclusion was set to 30 s.

Database Search and Bioinformatics Analysis. The resulting MS/MS data were processed using the MaxQuant search engine (v.1.6.6.0). Tandem mass spectra were searched against the *C. livia* proteome database (17 309 entries; the last update date was 20200521) concatenated with reverse decoy database. Trypsin/P was specified as the cleavage enzyme allowing up to two missing cleavages. The mass tolerance for precursor ions was set as 20 ppm in the First search and 20 ppm in the Main search, and the mass tolerance for fragment ions was set as 20 ppm. Carbamidomethyl on cysteine residues was specified as the fixed modification, and acetylation on protein N-terminal and oxidation on methionine residues were specified as variable modifications. False-discovery rate (FDR) was adjusted to <1%. The proteins with a fold change of 1.50 or 0.67 and *P*-value <0.05 were considered as differentially expressed proteins.

For GO annotation, proteins were classified by GO annotation into three categories: biological process, cellular compartment, and molecular function. For protein pathway, the Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to identify enriched pathways. Both GO annotation and protein pathway were analyzed by a two-tailed Fisher's exact test with a corrected *P*-value <0.05 considered as significant, which was employed to test the enrichment of the differentially expressed protein against all identified proteins. Correction for multiple-hypothesis testing was performed using standard FDR control methods.

Normalized spectral abundance factors (NSAFs) were used to rank the protein identifications and calculated as described as follows: spectral counts were divided by protein length, defining a spectral abundance factor (SAF), and SAF values were then normalized against the sum of all SAFs.[33]

PRM Validation. To determine the reliability of label-free quantitation results, a PRM assay was performed using the original protein samples (i.e., from D3/D1, D7/D1, D7/D3). According to the results from the primary assessment, a total of 11 peptides were selected and added to the inclusion list. The experimental method for PRM validation was performed as described by Jia et al.[34] The PRM data were processed using Skyline (v.3.6) and statistically analyzed using Student's *t*-test with a *P*-value <0.05 considered as significant.

Statistical Analysis. The experiment of pigeon milk proteomics and PRM validation was performed in three biological replicates, and the experiment of pigeon milk crude determination was performed in six biological replicates. The data are presented as the mean ± standard error. Charts were made by GraphPad Prism 7.0. Statistical analysis was conducted using SPSS 19 software, and one-way analysis of variance (ANOVA) methods were used for testing differences and $P < 0.05$ was considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c02977.

KEGG pathway enrichment by up-/down-regulated proteins in the crop milk of pigeon squabs at different ages (Table S1) (PDF)

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Notes

The authors declare no competing financial interest.

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