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Characterization of protein composition of guinea fowl (*Numida meleagris*) egg white

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Summary

The guinea fowl is a poultry species whose breeding represents only a small portion of the poultry sector because of its low laying rate compared to the hen. The structure and interior of eggs show significant species differences, which may also contribute to differences in their biochemical composition. This may be particularly evident in the protein composition of the egg, which determines the specific profile of biological activity, including resistance to biotic and abiotic stresses. A detailed understanding of the egg proteome in various avian species, including guinea fowl, has both biological and technological relevance. Egg white proteins play essential roles in embryo protection, antimicrobial defence and determining the functional and nutritional properties of eggs as a food product. Therefore, identifying species-specific differences is important not only for comparative avian biology, but also for the development of novel applications in food processing, biotechnology, and breeding strategies aimed at improving the quality and storage stability of eggs. The vast majority of research has focused on determining the protein profiles of chicken egg components. Therefore, the present study focuses on the protein composition of the egg protein of another poultry species: the guinea fowl. Using two-dimensional electrophoresis (2-DE) combined with mass spectrometry (MALDI-TOF MS), we analyzed the protein composition of guinea fowl egg whites. We identified six key proteins, namely ovalbumin (OVAL), ovalbumin-related proteins X and Y (OVALX and OVALY), ovotransferrin (LTF), lysozyme C (LYSC) and GDP-mannose 4,6-dehydratase (GMDS). These proteins have primarily immune, reserve and embryonic development functions. They have previously been found in studies on eggs of other avian species. However, the presence of GMDS in avian eggs has not been confirmed before, except by one study on the protein profile of guinea fowl eggshells.

Keywords: guinea fowl, egg white, proteome, mass spectrometry

The guinea fowl (*Numida meleagris*) is a domesticated poultry species bred for meat and eggs. However, both the meat and egg production sectors of the poultry industry focus primarily on the domestic chicken (*Gallus gallus domesticus*). Guinea fowl breeding and raising make up a minor fraction of overall poultry production, largely because of the relatively low egg-laying capacity of these birds. The laying capacity of guinea fowl in intensive poultry farming is estimated at around 100 eggs per year (23), a notable contrast to domestic laying chickens, which, under intensive production conditions, are known to achieve an annual laying capacity of up to 300 eggs (37). The avian egg consists of the shell, subshell membranes, protein, vitelline membrane, and the yolk, which is attached to the subshell membranes through the chalazae. The unique structure of the egg ensures that the developing embryo is thoroughly nourished and effectively protected from unfavourable environmental conditions, including microorganisms, which are prevented from

entering the egg (2). Guinea fowl eggs are characterized by a thicker and more mechanically resistant shell, as well as a smaller number of pores in the shell. These attributes are believed to have a significant impact on the durability and overall quality characteristics of guinea fowl eggs (1, 2). Studies have shown that guinea fowl eggs maintain their high quality traits even after being stored at room temperature for 28 days (2). Additionally, there are reports indicating that the durability of these eggs at room temperature may extend up to 3 months (31). Differences in the biochemical composition of eggs, especially in their protein profile, may underlie the unique structural and mechanical properties of guinea fowl eggshells. The specific protein composition determines the characteristic profile of biological activity, including resistance to biotic and abiotic stress (2, 20). Prior investigations have predominantly concentrated on elucidating the protein composition of chicken eggs. Consequently, there is a valid rationale for studying the proteomes of eggs from other poultry species, given the substantial variations due to species differences. Examining the proteome composition of guinea fowl egg white protein has the potential to enhance our understanding of processes taking place within the egg. Furthermore, from a broader biological perspective, comparisons of the proteomes of eggs from different species may provide information on the evolution of reproductive strategies and the adaptation of bird species to different environmental conditions. From a technological point of view, egg proteins are key determinants of egg functionality in food processing. They affect foaming, emulsifying and gelling properties, as well as antibacterial activity. Understanding these properties of guinea fowl eggs can help optimize their potential use in food production, storage and processing, where their natural shelf life and protein profile may offer advantages over traditional chicken eggs. This includes evaluating how environmental factors influence the properties of eggs as a food resource, including various technological processes. Additionally, it can shed light on the developmental processes associated with embryonic development in this bird species. The objective of this study was therefore to elucidate the proteome composition of guinea fowl egg white protein.

Material and methods

Materials and protein sample preparation. The research material used in the current study consisted of egg white protein derived from three freshly laid guinea fowl eggs. Upon cracking the eggs, the egg whites were mechanically separated from the yolks. To liquefy the protein, it was homogenized for 30 minutes by gentle mixing on a magnetic stirrer. Subsequently, the protein was subjected to a 12-hour lyophilization process (ALPHA 1-2 LDplus lyophilizer, Christ). The protein lyophilizates were mixed with a buffer containing 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl pH 7.8, 1 mM EDTA and a cocktail of protease inhibitors. The mixtures were homogenized (30 minutes, 21 Hz, Tissue Lyser, Qiagen) with stainless steel beads of 5 mm diameter. Subsequently, the samples were centrifuged to eliminate protein precipitates. The resulting supernatant was mixed with chilled acetone at a ratio of 1 : 4 and placed at a temperature of –20°C for 2 hours. After centrifugation at 21,000 g and 4°C for 30 minutes, the supernatant was carefully decanted. The resultant protein pellets were then dried using a vacuum concentrator (Eppendorf 5301 Vacumfuge Concentrator) and reconstituted in a lysis buffer containing 5 M urea (w/v), 2 M thiourea (w/v), 2% CHAPS (w/v), and 0.5% Tris (w/v).

Two-dimensional electrophoresis (2-DE). The total protein concentration in samples prepared in that manner was assessed with a Protein Assay Kit (Bio-Rad) in accordance with the manufacturer's instructions. The protein concentration was determined by extrapolating from a 5-point standard curve of bovine serum albumin. Absorbance readings for the samples were acquired at a wavelength of 595 nm using a microplate spectrophotometer (PowerWave XS, Bio-Tek Instruments).

After dilution in a lysis buffer to obtain a protein concentration of 150 µg in 125 µl of solution, DTT (0.1% w/v) and ampholyte (0.2% v/v, BioLyte® 3/10, Bio-Rad) were added. Subsequently, the samples were analysed by two-dimensional gel electrophoresis (2-DE). Isoelectric focusing (IEF) was conducted on 7 cm strips with immobilized pH gradient (IPG Strips, pH 3-10, nonlinear, Bio-Rad). The initial step involved rehydration, which was divided into passive and active stages, to subsequently conduct isoelectric focusing (Protean i12 IEF Cell, Bio-Rad). After the separation of proteins based on their isoelectric points, the IPG strips were equilibrated in two steps in a buffer containing 0.5 M Tris pH 6.8, 6 M urea, 10% SDS solution and glycerol. The first step consisted of equilibration in a buffer with DTT (1%) for 15 minutes and was followed by equilibration in a buffer containing iodoacetamide (2.5%) for 20 minutes. The equilibrated IPG strips were placed onto 12% polyacrylamide gels, and SDS-PAGE electrophoresis was conducted.

Gel staining and image acquisition. After completing the electrophoretic separation, protein visualization was achieved by staining the gels with Coomassie G-250 according to the methodology previously described by Lepczyński et al. (10). Briefly, the staining procedure initially involved fixing proteins on the gel in a fixing buffer (50% ethanol, 5% phosphoric acid) for 3 hours. Subsequently, the gels were stained in a 20-fold diluted concentrate of Bradford's solution (Bio-Rad Protein Assay) for 12 hours. In the final step of staining, the gel was incubated in deionized water for 3 hours. Finally, stained 2-D gels were archived using a calibrated optical densitometer GS-800 (Bio-Rad).

Protein identification – MALDI-ToF mass spectrometry. After scanning, protein spots were excised from the gels and placed in Eppendorf tubes. Initially, the samples were destained with two series of washes in a buffer composed of 25 mM ammonium bicarbonate and 50% acetonitrile, using incubation in an ultrasonic bath (10 min). The subsequent step involved dehydrating the protein spots. For

this purpose, 100% acetonitrile was added to the destained spots, which were incubated for 15 minutes in an ultrasonic bath. Finally, the samples were dried with a vacuum concentrator (Eppendorf). In the subsequent step, the protein spots were subjected to trypsin digestion to obtain peptides characteristic of these proteins („protein fingerprint" pattern). The entire mixture was then placed in an incubator for a period of 12 hours at a temperature of 37°C. The peptide mixture was then placed on AnchorChip 600/96 plates (Bruker Daltonics) and mixed 1:1 with a CHCA (α-cyano-4-hydroxycinnamic acid) matrix solution by the dry droplet method. The samples were further analysed with a MALDI-TOF (matrix-assisted laser desorption/ionization time of flight) mass spectrometer (Microflex, Bruker). The MALDI-TOF parameters were as follows: ion mass range – 400-3400 m/z, laser shots per sample – 50, ion source voltage – 16.5 V and 19 V, and lens voltage – 9.50 V. Mass spectra were acquired with the Flex Control software, while spectrum processing and annotation were performed with the Flex Analysis software. Subsequently, the peptide spectra were compared with protein sequence databases using the MASCOT software and data available in the NCBI and UniProt databases.

Gene Ontology (GO) analysis. To categorize the proteins based on their participation in the biological processes described, a functional protein association analysis was conducted using the STRING v11.5 software, available on-line https://www.string-db.org/ (33). Functional protein-protein interaction (PPI) enrichment was carried out using *Numida meleagris* and *Gallus gallus domesticus* as the reference genomes. Interaction search parameters included an interaction score of 0.400 (indicating medium confidence), and significant results were identified using the Benjamini-Hochberg False Discovery Rate, set at $p < 0.05$. Functional associations were determined based on the enrichment results obtained from STRING clusters.

Results and discussion

In the present study, we employed 2-DE in combination with mass spectrometry (MALDI-TOF MS) to characterise the protein composition of guinea fowl (*Numida meleagris*) egg white protein. Figure 1A shows a 2-D pattern of guinea fowl egg white proteins, which contains 43 distinct protein spots in the pH range of 3-10 and of a molecular mass between 10 and 250 kDa. All protein spots (43) were excised from the gels and subjected to peptide mass fingerprinting using MALDI TOF MS. In total, 11 spots were successfully identified, corresponding to 6 different gene products: ovalbumin (OVAL), ovalbumin-related protein X (OVALX), ovalbumin-related protein Y (OVALY), ovotransferrin (LTF), lysozyme C (LYSC), and GDP-mannose 4,6-dehydratase (GMDS). Detailed information concerning identification parameters are listed in Table 1. The GO enrichment analyses revealed a statistically significant association between all 6 gene products (AVAL, OVALX, OVALY, LTF, LYZC and GMDS), with a protein-protein interaction (PPI) p-value of 2.5×10^{-10} for *Numida meleagris* and 7.17×10^{-9} for

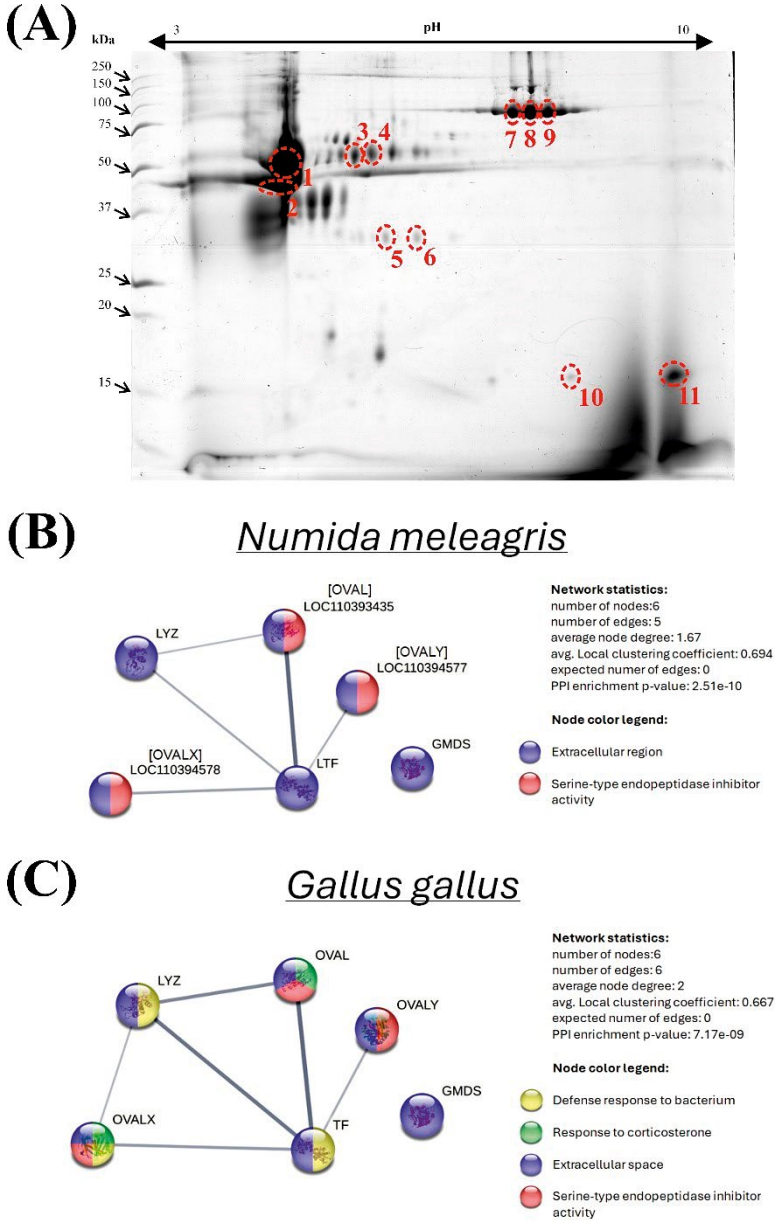

Fig. 1. (A) A representative 2-D protein profile of guinea fowl egg white protein with labelled protein spots. The spot numbers correspond to those presented in Table 1. (B) STRING analysis of gene expression for *Numida meleagris*. (C) STRING analysis of gene expression for *Gallus gallus*

Explanations: Both images illustrate protein-protein interactions, with nodes representing gene products and edges indicating interactions identified within the egg white. Varied edge thicknesses signify the strength and nature of protein interactions. Node colours correspond to selected gene ontology (GO) terms, such as biological processes and molecular functions. Network statistics show the number of nodes and edges, the average node degree, the average local clustering coefficient, the expected number of edges and the enrichment p-value of protein-protein interactions (PPI). Gene name abbreviations: LYZ – lysozyme C, LTF/TF – ovotransferrin, OVAL – ovalbumin, OVALY – ovalbumin-related protein Y, OVALX – ovalbumin-related protein X, GMDS – GDP-mannose 4,6-dehydratase.

Tab. 1. Parameters of guinea fowl egg white protein determined by MALDI-TOF mass spectrometry

Spot No.	Protein name	Gene name	Accession number	Peptides matched	Sequence coverage (%)/ MASCOT score	Theoretical pI/ MW	Species
1	Ovalbumin	OVAL	XP_021241976.1	7	31/105	5,13/43,140	*Numida meleagris*
2	Ovalbumin	OVAL	XP_021241976.1	5	25/59	5,13/43,140	*Numida meleagris*
3	Ovalbumin-related protein Y	OVALY	BAM13279.1	8	25/62	5,27/44,042	*Gallus gallus*
4	Ovalbumin-related protein Y	OVALY	BAM13279.1	9	30/95	5,27/44,042	*Gallus gallus*
5	Ovalbumin-related protein X	OVALX	XP_021244206.1	8	28/83	5,91/44,496	*Numida meleagris*
6	Ovalbumin-related protein X	OVALX	XP_021244206.1	9	35/84	5,91/44,496	*Numida meleagris*
7	Ovotransferrin	LTF	XP_021249482.1	10	21/87	7,57/112,166	*Numida meleagris*
8	Ovotransferrin	LTF	XP_021249482.1	16	24/187	7,57/112,166	*Numida meleagris*
9	Ovotransferrin	LTF	XP_021249482.1	9	18/93	7,57/112,166	*Numida meleagris*
10	GDP-mannose 4,6-dehydratase	GMDS	XP_005481742.1	10	38/88	6,09/42,878	*Zonotrichia albicollis*
11	Lysozyme C	LYSC	P00698.1	8	59/132	9,37/16,741	*Gallus gallus*

and *Gallus gallus domesticus*. The GO enrichment analyses were performed using two different genomes as a background. It was determined by the limited enrichment results for the Helmeted Guineafowl. The enrichment results obtained for this species showed that all gene products identified are extracellular region proteins (P = 0.0011), and three of the gene products (OVAL, OVALX, OVALY) exert serine-type endopeptidase inhibitor activity (p = 0.0051). The GO enrichment performed for *Gallus gallus domesticus* made it possible to categorize the gene products into known biological processes: response to corticosterone (OVALX, OVALY; p = 0.0436) and defence response to bacterium (LYZ, TFE, OVALX; p = 0.0436). The OVAL, OVALX and OVALY were categorized as proteins with serine-type endopeptidase inhibitor activity (p = 0.009). Similar to enrichment performed for the *Numida meleagris* genome, the GO analysis using chicken genome as a background revealed that all gene products identified are extracellular space proteins (9.14×10^{-5}). The GO analyses for *Numida meleagris* and *Gallus gallus domesticus* are presented in Figure 1 sections B and C, respectively.

In the present study, the discovery rate of protein spots representing the Guinea Fowl egg white protein profile was assessed at 25.58%. The proteins identified, except for GMDS, are proteins most abundant in egg white. To increase the number of separated gene expression products and substantially increase the number of identified proteins, the enrichment of the mid- and low-abundant proteins should be performed using, for example, hexapeptide libraries or immune depletion-based tools. However, our initial attempts to perform such enrichment with hexapeptide libraries-based tools were insufficient, because of the physical properties of egg white (data not published). On the other hand, the limited identification rate may reflect the completeness of the protein databases for *Numida meleagris*. In the UniProt and NCBI databases, only limited data are available regarding the protein products of gene expression characteristic of the guinea fowl. Therefore, due to the above and relatively poor description of the guinea fowl egg white proteins in the literature, the discussion of the results obtained was based primarily on data and literature related to the most extensively studied poultry species, such as the domestic chicken. It should be emphasised that albumin fractions were the most abundant protein spots among all proteins identified. OVAL, a phosphoglycoprotein with a molecular weight of 45 kDa, belongs to the serpin superfamily and is known for its ability to inhibit the activity of serine proteases (11, 21). However, OVAL is an exception, as it does not exhibit typical properties of a proteolytic enzyme inhibitor. Instead, it has been reported to exert an effective antioxidant capacity due to its functional groups that perform metal ion binding (7, 30). It is also hypothesised that OVAL may primarily serve as a biological reserve material for the developing embryo (6). Evidence from previous studies also points to a role for OVAL in embryo development, where this protein migrates from egg white to the embryo. This process involves a conformational change in its structure, rendering it more amenable to transport and interaction with other proteins, including LYSC, in fertilized chicken eggs (27, 32). Thus, the role of OVAL seems to be more complex and is not yet fully understood. Based on the analysis of the protein profile obtained, it can be unequivocally stated that OVAL constitutes the principal component of egg protein, similarly as in other poultry species (13, 15, 16, 21, 22, 27). In addition to being abundantly accumulated in the egg white proteins of diverse poultry species, this protein is ubiquitously found in many components of chicken eggs, including the shell (17), yolk (18) and vitelline membrane (14). OVAL has been identified as a constituent of the proteome in the shells of guinea fowl eggs, suggesting its possible role in regulating calcium precipitation within the shell. This function aligns it with ovocleidin-17, ovocleidin-116 and lysozyme C (8). With no available reports on the

role of OVAL in guinea fowl egg white protein and considering the suggested function in chicken egg white protein, it is reasonable to assume a similar role for OVAL in this bird species as well. However, given the interspecies differences in the structure of avian ovoglobulins, such as the higher molecular weight of OVAL in ostriches (21), future research may unveil entirely new additional functions for OVAL in guinea fowl egg white protein.

Further two proteins identified in this study are OVALX and OVALY, which, like OVAL, belong to the serpin superfamily (28). Both of these proteins are homologs of OVAL (34). They have been identified in the eggshell, egg white, yolk and vitelline membrane of chicken eggs (13, 15-19, 25), and OVALY has also been found in the shell of guinea fowl eggs (8). In contrast to OVAL, OVALY is not a phosphoglycoprotein, but a glycoprotein. The quantitative ratio of OVALY to OVAL in chicken egg protein is 13 : 100 (25). A previous study demonstrated that the expression of OVALY increases approximately 10-fold in the white protein of freshly fertilized chicken eggs, suggesting its significant role in embryonic development processes (26). OVALX is also a glycoprotein, whose estimated expression in chicken egg protein is about one-hundredth of that for OVAL (3). This glycoprotein is attributed mainly protective functions. Through its heparin-binding domain, OVALX shields the embryo from pathogens, thus playing a predominantly protective and defensive role. The effectiveness of its bacteriostatic and bactericidal properties has been confirmed for at least two pathogens: *L. monocytogenes* and *S. enterica enteritidis* (28). However, there is a lack of similar reports on the functions of OVALY and OVALX in guinea fowl eggs. It can be hypothesized that OVALY and OVALX in the shell of guinea fowl eggs may be involved in the shell mineralization process. This assumption is based on the fact that the expression of OVALY and OVALX significantly decreases during the mineralization process of chicken eggshells (8, 19). Although OVALX has been identified in the shell of chicken eggs, and not guinea fowl eggs, its isoform has been identified in the latter, allowing us to suggest that OVALX is also expressed in this component of guinea fowl eggs (8).

Another protein identified in our study is LTF. This protein has previously been reported as a protein constituent of eggs across various bird species (13, 16, 21). It has been found in different components of chicken eggs, including the shell (14, 19), yolk (18) and vitelline membrane (14). LTF, a glycoprotein with a typical molecular weight of approximately 70 kDa in most bird species (21), stands out as one of the most highly expressed proteins in egg white protein, accounting for 12-13% of the total protein concentration in chicken eggs (9, 22). The primary function of LTF is to bind iron ions, thereby conferring bacteriostatic properties. By forming a complex of ovotransferrin-iron, it inhibits microbial development by reducing the bioavailability of this element. Bacterial species most sensitive to the iron-depriving effect of LTF include *Pseudomonas* spp., *Escherichia coli* and *Streptococcus mutans*, while *Proteus* spp. and *Klebsiella* spp. are among the most resistant (9, 35). In addition to its bacteriostatic properties, LTF plays a significant role in embryo development. In fertilized eggs, this protein migrates from the egg white to the embryo, ensuring the embryo's access to iron during its rapid growth period. This hypothesis is supported by the decrease in LTF concentration observed during embryonic development (13). LTF has also been identified in the shells of guinea fowl eggs, where, similarly to OVALX and OVALY, it is probably involved in the process of shell mineralization, particularly in the early stages, influencing the formation of calcium carbonate crystals (8, 19).

LYSC, belonging to the defensin family, is an antimicrobial protein with a molecular weight of 14 kDa (5, 12). It is among proteins found in a relatively high concentration in chicken egg white, contributing approximately 3.4% to the total protein pool in chicken eggs (15, 22). LYSC has been identified in all components of the chicken eggs: the shell, egg white, yolk and vitelline membrane (14, 16-19, 21, 26). Moreover, it has been detected in the shells of guinea fowl eggs, where, like LTF, it participates in the formation of calcium carbonate crystals during shell mineralization (8, 19). In the egg white protein, LYSC exhibits antimicrobial properties by breaking down β-glycosidic bonds in polysaccharides that constitute the cell walls of Gram-positive bacteria. It demonstrates bactericidal activity, particularly against bacteria of the *Bacillus*, *Micrococcus* and *Streptococcus* genera. Its bacteriostatic properties have also been demonstrated against bacteria belonging to the *Pseudomonas*, *Escherichia*, *Moraxella*, *Campylobacter*, *Salmonella* and *Shigella* genera (4, 36).

The last protein identified in our study is GMDS, an enzyme belonging to the lyase class. GMDS catalyzes the breakdown of GDP-mannose into GDP-4-dehydro-6-deoxy-D-mannose and water. This enzyme initiates *de novo* synthesis of GDP-fucose, a crucial step for the transfer of fucose, a component of many signalling and immunogenic oligosaccharides. Consequently, this biocatalyst is indirectly involved in immune processes and intercellular signalling (24, 29). Previous studies aimed at characterising the protein profiles of both chicken egg white components and the shells of guinea fowl did not include an examination of GMDS. As a result, the specific function of GMDS in eggs remains elusive.

In summary, this study has identified six proteins that constitute the protein profile of guinea fowl egg white, namely OVAL, OVALY, OVALX, LTF, LYSC and GMDS. These proteins play pivotal roles in im-

mune processes through their antimicrobial properties, act as reserve entities, exhibit catalytic functions and contribute to embryo development. Most of these gene products have previously been found in the egg white protein and other components of eggs from diverse bird species. The presence of GMDS in bird eggs has not been confirmed until now. Notably, except for a single study aimed at characterizing the protein profile of guinea fowl eggshells, the protein composition of both the yolk and vitelline membrane of this avian species remains unexplored. The present findings appear to represent the initial effort in characterizing the proteome composition of this egg component. We hypothesize that the proteins identified in this study mirror the functions of their counterparts in chicken eggs. Therefore, these proteins may undergo concentration changes during egg aging and guinea fowl development. The ongoing challenge lies in characterizing alterations in the proteome of guinea fowl egg white protein due to technological and physiological processes.

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