RESEARCH PAPER

Functional Analysis of Lyophilized Human Breast Milk Used as a Biomaterial for Regeneration: In-silico Characterization

Rutuja Biradar[1], Ashmitha Kishan Shetty[2], Anbu Jayaraman[3], Deveswaran Rajamanickam[4], Asavari Desai[5]
[1,2]Department of Pediatric and Preventive Dentistry, Faculty of Dental Sciences, M S Ramaiah University of Applied Sciences, Bangalore 560054. Karnataka, India.
[3]Department of Pharmacology, Faculty of Pharmacy, M S Ramaiah University of Applied Sciences, Bangalore 560054. Karnataka, India.
[4]Department of Pharmaceutics, Faculty of Pharmacy, M S Ramaiah University of Applied Sciences, Bangalore -560054. Karnataka, India.
[5]Department of Orthodontics, Department of Orthodontics, Manipal College of Dental Sciences Mangalore, Manipal Academy of Higher Education, Manipal, Karnataka, India
Author for correspondence: ashmitha.pe.ds@msruas.ac.in

Abstract: Human breast milk (HBM) contains a rich repertoire of proteins that provide essential nutritional, immunological and developmental support to newborns. The protein composition varies significantly across lactation stages—colostrum, transitional and mature milk, with the highest protein concentration observed in colostrum. In the present in-silico study, HBM samples were collected from 10 healthy volunteer mothers within one week postpartum and lyophilized through controlled freezing and vacuum drying to obtain a powdered form. Proteins were identified using liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (LC/Q-TOF-MS). Bioinformatic analysis was conducted using the PANTHER (Protein Analysis Through Evolutionary Relationships) classification system to categorize the identified proteins by their Gene Ontology (GO) terms, molecular functions, cellular components, biological processes and signalling pathways. The analysis revealed that 42 HBM proteins were identified and 28 proteins were involved in biological regulation, immune response, cellular processes and hemostasis. These findings suggest that, beyond its nutritional value, HBM contains bioactive proteins with therapeutic and regenerative potential. The study enhances the understanding of the functional relevance of breast milk proteins and supports their application as natural biomaterials in fields such as tissue engineering, wound healing and neonatal medicine.

Keywords. Human Breast Milk, Proteomics, Lyophilization, Bioinformatics, Protein Classification, Immune Response, Tissue Regeneration

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1. Introduction

HBM has long been regarded as the optimal source of nutrition for infants offering a complex and dynamic mixture of nutrients with bioactive molecules and immunological components that support growth, development and disease resistance. HBM has been appreciated not only for its nutritional adequacy but also for its therapeutic potential, thus forming an integral part of traditional and natural medicine across cultures. In recent decades, advances in molecular biology and immunology have reinforced the perception of HBM as a biologically active substance with roles extending far beyond basic nutrition [1].

Composed of approximately 87–88% water and a balanced proportion of carbohydrates, fats and proteins, HBM provides an average of 65–70 kcal per 100 mL, with fats contributing around 50% of the energy and carbohydrates 40%. The solid fraction contains crucial macronutrients and micronutrients along with over 200 distinct proteins that serve structural, enzymatic and immunomodulatory functions. Key among these are caseins and whey proteins, including α-lactalbumin, lactoferrin, lysozyme and immunoglobulins such as secretory IgA. These proteins not only contribute to digestion and nutrient absorption but also play central roles in antimicrobial defense, immune regulation and tissue repair [2,3].

The immunological profile of breast milk is particularly remarkable. It contains a wide array of protective factors, including antibodies, leukocytes, defensins, cytokines (e.g., interleukins 1, 6, 8, and 10), transforming growth factor-beta (TGF-β), secretory leukocyte protease inhibitors (SLPI) and pattern-recognition receptors

such as Toll-like receptors (TLRs). Soluble TLR-2, CD14 and an 80kDa regulatory protein in breast milk help modulate microbial recognition in the gastrointestinal tract of the infant facilitating the establishment of beneficial microbiota while suppressing pathogenic responses [4,5]. The bifidus factor further promotes the growth of *Bifidobacterium* species, which are essential for intestinal health [2].

One of the most studied milk proteins is Lactoferrin which exhibits strong bacteriostatic activity particularly against *Escherichia coli* mainly by binding to free iron, an essential nutrient for microbial proliferation. Its iron-binding capacity in combination with its ability to modulate inflammation and immune cell recruitment positions it as a promising candidate for therapeutic applications [3,6]. Lysozymes and polymorphonuclear leukocytes present in HBM exert antimicrobial effects and contribute to non-specific immunity, while IgA provides mucosal protection and prevents pathogen adherence to epithelial surfaces [2].

In addition to its internal effects, breast milk has demonstrated efficacy in treating a variety of external ailments such as conjunctivitis, nipple fissures, diaper rash and skin infections. Its regenerative properties, driven by components such as Epidermal Growth Factor (EGF), erythropoietin and anti-inflammatory cytokines, have led to increased interest in its topical and systemic applications [7,8]. Emerging evidence suggests that HBM can influence wound healing and oral tissue repair, including potential roles in pulpal regeneration and angiogenesis as key factors in dental tissue healing [13,14].

Despite this growing body of evidence, the molecular mechanisms underlying the functional diversity of HBM proteins remain only partially understood. In-silico methods provide a valuable complementary approach offering insights into the structural, functional and interactional characteristics of proteins through computational modeling and bioinformatics analyses. These tools enable the prediction of protein folding, active sites ligand interactions and post-translational modifications, helping to elucidate potential biological pathways and therapeutic applications.

The present study aims to perform an in-silico characterization of key proteins in HBM focusing on their physicochemical properties, structural domains, functional roles and potential therapeutic implications. Employing a range of bioinformatics tools and databases, seeking to enhance the molecular-level understanding of breast milk bioactive proteins and their roles in infant health and disease prevention. This work not only contributes to the scientific foundation of breastfeeding advocacy but also opens avenues for the development of novel therapeutic agents derived from naturally occurring human milk proteins.

2. METHODOLOGY

2.1 *Preparation of Lyophilized Human Breast Milk*

Institutional ethical clearance was obtained for collection of human milk samples from donor mothers. HBM samples were collected from healthy lactating mothers within one week postpartum. A pooled sample was prepared by mixing 100µL aliquots from 10 individual donors. The pooled HBM was stored at −80 °C for long-term preservation and −20 °C for short-term use prior to lyophilization (Figure 1).

2.2 Proteomic characterization: Protein identification was carried out using the UniProt Human Database (uniprot_human_2021, version 20211228). MS/MS Ion Search was performed with Trypsin as the digestion enzyme, allowing up to two missed cleavages. Carbamidomethylation of cysteine (C) was set as a fixed modification, while deamidation (NQ) and oxidation (M) were considered as variable modifications. Monoisotopic mass values were used, with peptide and fragment mass tolerances set at 10 ppm and 0.6 Da, respectively, while protein mass remained unrestricted. HBM samples were analyzed by Nano LC-MS/MS using an LC/Q-TOF-MS system and protein identification was performed using the PANTHER search engine against the UniProt-Swiss-Prot Human Database.

3. Results and discussion

3.1 *Qualitative Analysis*

The result was a dry, powdered form of both HBM suitable for proteomic analysis. Mass Spectrometry revealed about 42 proteins (Table1). Bioinformatics analysis using PANTHER software identified a total of 28 genes with various biological functions, molecular roles, cellular components and protein classes and pathways.

3.1a GO-Slim Biological Processes

The 28 representative genes were involved in 67 biological process hits Figure. 2 (https://pantherdb.org/servlet/GenomeFunctionChartServlet?chartType=2&listType=1&annotType=2&species=Homo%20sapiens) The most frequent processes included cellular process, developmental process response to stimulus, immune system process, growth and localization. This indicates that the proteins are not only structural and metabolic but also highly regulatory and immunological contributing to tissue repair, immune modulation and cellular homeostasis.

3.*1b GO-Slim Molecular Functions*

In the molecular function category Figure 3. (https://pantherdb.org/servlet/GenomeFunctionChartServlet?chartType=1&listType=1&annotType=1&species=Homo%20sapiens) 31 hits were found. The dominant molecular role was binding, especially protein and ion binding, followed by catalytic activity and transporter activity. Additional functions included molecular transducer and structural molecule activity. These functions are essential for signaling cascades and biochemical reactions involved in tissue regeneration and protection against pathogens.

3.1c GO-Slim Cellular Components

The cellular component analysis Figure. 4 (https://pantherdb.org/servlet/GenomeFunctionChartServlet?chartType=2&listType=1&annotType=4&species=Homo%20sapiens) showed 30 component hits. The majority of genes were localized to membrane-bound organelles and intracellular compartments, with a notable presence in extracellular regions. This suggests active roles in secretion, signaling and cell-cell communication especially important for maternal-neonatal immune interaction.

3.1d Protein Class Categorization

Protein class analysis Figure 5. (https://pantherdb.org/servlet/GenomeFunctionChartServlet?chartType=2&listType=1&annotType=5&species=Homo%20sapiens) revealed 28 hits. Transport/carrier proteins were the most abundant, followed by nucleic acid binding proteins, enzyme modulators and cytoskeletal proteins. These classes of proteins reflect the dynamic metabolic and regulatory landscape of HBM aiding not only in nutrient delivery but also in wound healing and immune response.

3.1e Pathway analysis

Several signaling pathways were identified (https://pantherdb.org/servlet/GenomeFunctionChartServlet?chartType=1&listType=1&annotType=3&species=Homo%20sapiens), including the Arachidonoylglycerol pathway, CCKR signaling pathway, FGF signaling pathway and cytoskeleton regulation by Rho GTPase. These pathways are known to regulate cellular proliferation, migration, morphogenesis and inflammatory responses essential for epithelial regeneration, angiogenesis and host defense.

The proteomic and bioinformatics profiling of HBM in this study provides compelling insights into its multifaceted role in neonatal development, immune defense and potential wound-healing applications. The identification of 42 proteins and the subsequent in-silico characterization of 28 key genes demonstrates the rich complexity of HBM.

The wide variety of biological processes such as development immune modulation and signaling reflected in the GO-Slim analysis, supports existing literature that HBM is a bioactive fluid with therapeutic potential [15]. Proteins involved in epithelial cell proliferation and matrix remodeling, such as those in the FGF and Rho GTPase signaling pathways, offer promising regenerative cues for tissue engineering and oral wound care.

The elevated presence of immune-related proteins such as lactoferrin, immunoglobulins, and lysozyme C, especially in colostrum, affirms HBM role in passive immunity. The discovery of TNF and TGFB1 proteins involved in inflammatory regulation suggests that HBM plays an active role in modulating the neonatal immune system and inflammatory response, potentially accelerating recovery from tissue injury [16,17].

The cellular localization of these proteins in secretory vesicles, membranes and extracellular spaces aligns with their immunological and reparative functions [18]. The abundance of transport and enzyme modulator proteins highlights the role of HBM in delivering regulatory molecules and catalyzing healing processes at the cellular level [19].

The CCKR signaling pathway (https://pantherdb.org/pathway/pathwayDiagram.jsp?catAccession=P06959) is an important regulator of cellular homeostasis acting through classical CCK-B receptors, their isoforms and alternative receptor subtypes. Activation by gastrin and cholecystokinin peptides initiates multiple intracellular cascades that modulate the expression of genes associated with cell survival, angiogenesis and invasion. These signaling events are strongly implicated in processes of tissue remodeling and disease progression [20,21].

The Arachidonoylglycerol (2-AG) pathway (https://pantherdb.org/pathway/pathwayDiagram.jsp?c

atAccession=P05726), represents a major endocannabinoid signaling route, exerting its effects primarily via CB_1 receptors in the central nervous system and CB_2 receptors in the immune system. 2-AG has been shown to modulate neuroinflammation, enhance neuronal survival and contribute to tissue regeneration and wound repair thereby maintaining physiological balance under both normal and stress conditions [22]. These pathways highlight the significance of receptor-mediated signaling in cellular adaptation, repair and remodelling and may provide valuable therapeutic insights in contexts where enhanced regeneration or controlled remodelling is desired [23,24].

This study also adds weight to the therapeutic use of HBM in traditional medicine for treating infections, conjunctivitis and nipple wounds and extends its potential to clinical applications such as pulpal healing and tissue regeneration. Given the presence of growth factors, antimicrobial agents and signaling molecules, HBM may serve as a natural and biocompatible wound-healing agent.

4. Conclusion

This in-silico study provides a comprehensive proteomic and functional overview of lyophilized human breast milk. The 42 identified proteins encompass a wide spectrum of molecular functions, cellular components and biological pathways critical for immune protection, cellular regeneration and growth. Key findings demonstrate that HBM contains immune-modulatory, growth-promoting and anti-inflammatory proteins that could potentially accelerate wound healing and tissue repair. This supports the concept of utilizing HBM as a natural bio-therapeutic agent particularly in pediatric, dermatological and dental applications. Future studies should aim to validate these findings in-vivo and explore clinical formulations of HBM-derived proteins for therapeutic use in regenerative medicine

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Electronic supplementary material

Supplementary material pertaining to this article is available on the *Bulletin of Materials Science* website (www.ias.ac.in/matersci).

References

[1] Witkowska-Zimny M, Kaminska-El-Hassan E. Nutritional and therapeutic properties of human breast milk: a review. *Nutrients*. 2017;9(9):doi:10.3390/nu9090997.
[2] Kasrae H, Rahnama S, Amini H, et al. Antimicrobial factors of human breast milk and their role in infant immune development. *J Matern Fetal Neonatal Med*. 2015;28(12):1435–1440.
[3] Lönnerdal B. Bioactive proteins in human milk: mechanisms of action. *J Pediatr*. 2017;177(Suppl):S7–S9.
[4] Labéta MO, et al. Soluble CD14 and Toll-like receptor 2 (TLR2) in human milk and their role in neonatal immune regulation. *J Immunol*. 2000;165(7):4243–4249.
[5] Andreas NJ, Kampmann B, Mehring Le-Doare K. Human breast milk: a review on its composition and bioactivity. *Early Hum Dev*. 2015;91(11):629–635.
[6] Bullen JJ, Rogers HJ, Spalding PB, Ward CG. Lactoferrin: iron-binding and antibacterial activity. *J Med Microbiol*. 2017;66(10):1347–1354.
[7] Barel M, Bosse R, Royer A. Pasteurization processes and impact on bovine milk nutritional properties. *Dairy Sci Technol*. 2020;100(3):413–428.
[8] Witkowska-Zimny M, Kaminska-El-Hassan E. The role of human milk in wound healing and regenerative medicine. *Adv Wound Care*. 2019;8(7):345–352.
[9] Sánchez L, et al. Therapeutic uses of human breast milk in traditional medicine: a review. *Complement Ther Clin Pract*. 2021;43:101343.
[10] Perrine CG, et al. Vitamin D supplementation for breastfeeding infants: recommendations and evidence. *Pediatrics*. 2010;126(6):e1506–e1511.
[11] Flint DJ, et al. Vitamin and mineral content of human milk and nutritional considerations for infants. *Clin Nutr*. 2012;31(6):929–934.
[12] Decsi T, et al. Mineral composition of human milk during lactation: implications for infant nutrition. *J Pediatr Gastroenterol Nutr*. 2003;37(1):105–109.
[13] Shah R, et al. Biological mechanisms of pulpal healing and regeneration. *J Endod*. 2020;46(3):287–293.
[14] Islam MA, et al. Molecular mechanisms in dental pulp regeneration: roles of growth factors and stem cells. *J Oral Biosci*. 2023;65(1):1–10.
[15] El-Hatmi H, Girardet JM, Gaillard JL, Yahyaoui MH, Attia H. Characterisation of whey proteins of camel milk: a proteomic approach. *J Dairy Res*. 2015;82(3):270–278. doi:10.1017/S0022029915000276.
[16] D'Alessandro A, Scaloni A, Zolla L. Human milk proteins: an interactomics and updated functional overview. *J Proteome Res*. 2010;9(7):3339–3373. doi:10.1021/pr100168u.

[17] Lopez Alvarez M, Lopez Morato E, Rodriguez Palmero M, Castell M, Rivero M, Franch A. Immunomodulatory and anti-inflammatory effects of human milk in vitro. *Clin Exp Immunol*. 2007;147(3):480–488. doi:10.1111/j.1365-2249.2007.03300.x.

[18] Kierson JA, Dimatteo DM, Locke RG, MacKley AB, Spear ML. Ghrelin and cholecystokinin in term and preterm human breast milk. Acta Paediatrica. 2006 Aug;95(8):991-5.

[19] Gaitán AV, Wood JT, Liu Y, Ji L, Nikas SP, Makriyannis A, Lammi-Keefe CJ. Maternal dietary fatty acids and their relationship to derived endocannabinoids in human milk. Journal of Human Lactation. 2021 Nov;37(4):813-20.

[20] Yang Q, Chen D, Liu X, Li W, Zheng H, Cai X, Li R. Identification of nanoparticle infiltration in human breast milk: Chemical profiles and trajectory pathways. Proceedings of the National Academy of Sciences. 2025 May 20;122(20):e2500552122.

[21] Davenport C, Yan J, Taesuwan S, Shields K, West AA, Jiang X, Perry CA, Malysheva OV, Stabler SP, Allen RH, Caudill MA. Choline intakes exceeding recommendations during human lactation improve breast milk choline content by increasing PEMT pathway metabolites. The Journal of Nutritional Biochemistry. 2015 Sep 1;26(9):903-11.

[22] Baranyi J, Pacza T, Martins ML, Thakkar SK, Samuel TM. Modelling the temporal trajectories of human milk components. BMC Pregnancy and Childbirth. 2024 Nov 11;24(1):739.

[23] Slater CN, Juntereal NA, Kral TV, Spatz DL, Chao AM. Comparison of the effect of direct breastfeeding, expressed human milk, and infant formula feeding on infant weight trajectories: A systematic review. Breastfeeding Medicine. 2024 Apr 1;19(4):235-47.

[24] Christensen SH, Rom AL, Greve T, Lewis JI, Frøkiær H, Allen LH, Mølgaard C, Renault KM, Michaelsen KF. Maternal inflammatory, lipid and metabolic markers and associations with birth and breastfeeding outcomes. Frontiers in nutrition. 2023 Sep 4;10:1223753.

Figures

Figure 1. Image of Lyophilized Human Breast Milk

Table1: List of proteins identified by mass spectrometry

1.	P02788	Lactotransferrin
2.	P05814	Beta-casein
3.	P00709	Alpha-lactalbumin
4.	P01833	Polymeric immunoglobulin receptor
5.	P01876	Immunoglobulin heavy constant alpha 1
6.	P04264	Keratin, type II cytoskeletal 1
7.	P19835	Bile salt-activated lipase
8.	A0A0G2JMB2	Immunoglobulin heavy constant alpha 2 (A2m marker)
9.	P02768	Albumin
10.	P47710	Alpha-S1-casein
11.	P47989	Xanthine dehydrogenase/oxidase
12.	P07498	Kappa-casein
13.	P01011	Alpha-1-antichymotrypsin
14.	A0A0B4J231	Immunoglobulin lambda like polypeptide 5
15.	H0YKB5	Milk fat globule EGF and factor V/VIII domain
16.	P35527	Keratin, type I cytoskeletal 9
17.	Q13410	Butyrophilin subfamily 1 member A1
18.	P10909	Clusterin
19.	A0A1B0GVI3	Keratin, type I cytoskeletal 10
20.	P01834	Immunoglobulin kappa constant
21.	P04433	Immunoglobulin kappa variable 3-11
22.	P01619	Immunoglobulin kappa variable 3-20
23.	Q86XA6	B4GALT1 protein

24.	Q9P0W8	Spermatogenesis-associated protein 7
25.	P02750	Leucine-rich alpha-2-glycoprotein
26.	A0A075B6Z2	T cell receptor alpha joining 56
27.	P35908	Keratin, type II cytoskeletal 2 epidermal
28.	D6RD17	Joining chain of multimeric IgA and IgM
29.	F5GXS0	Complement C4B (Chido blood group)
30.	S4R3A2	Fatty acid-binding protein, heart
31.	I3L4N8	Actin gamma 1
32.	A0A024R6N5	Serpin family A member 1
33.	E9PC84	Tenascin C
34.	Q99698	Lysosomal-trafficking regulator
35.	Q3LGB0	Osteopontin
36.	P12273	Prolactin-inducible protein
37.	P01700	Immunoglobulin lambda variable 1-47
38.	P61626	Lysozyme C
39.	P23280	Carbonic anhydrase 6
40.	P06858	Lipoprotein lipase
41.	Q9954	Perilipin-2
42.	P01871	Immunoglobulin heavy constant mu

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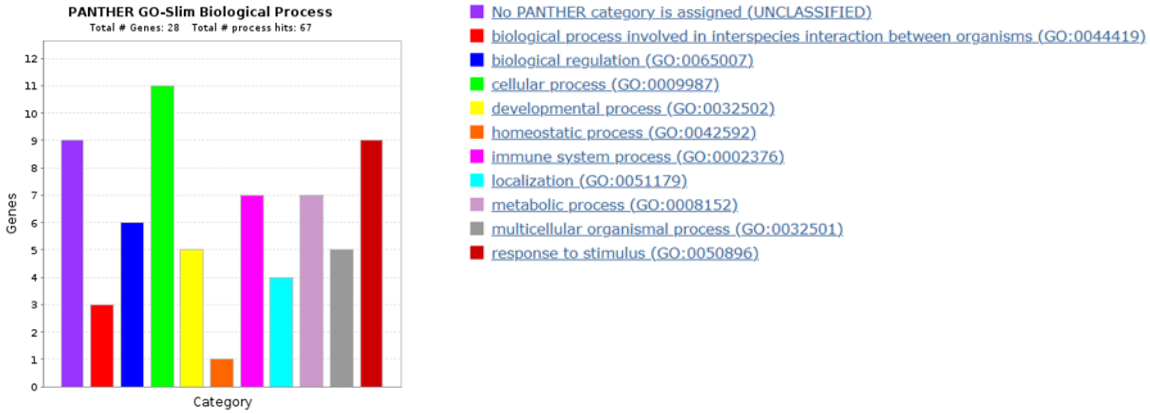

Figure 2. Graphical representation of biological process of the genes composed in lyophilised HBM by PANTHER software

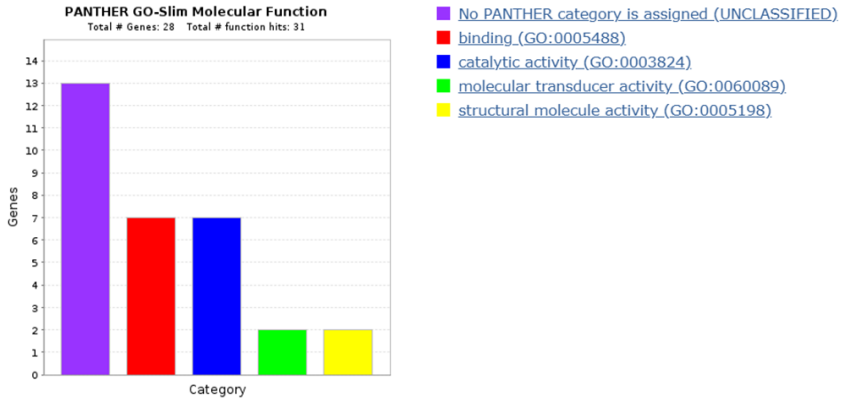

Figure 3. Graphical representation of molecular function of the genes composed in lyophilised HBM by PANTHER software

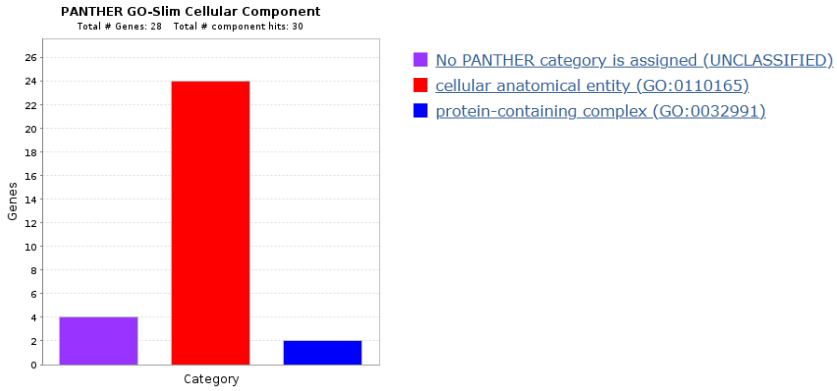

Figure 4. Graphical representation of cellular component of the genes composed in lyophilised HBM by PANTHER software

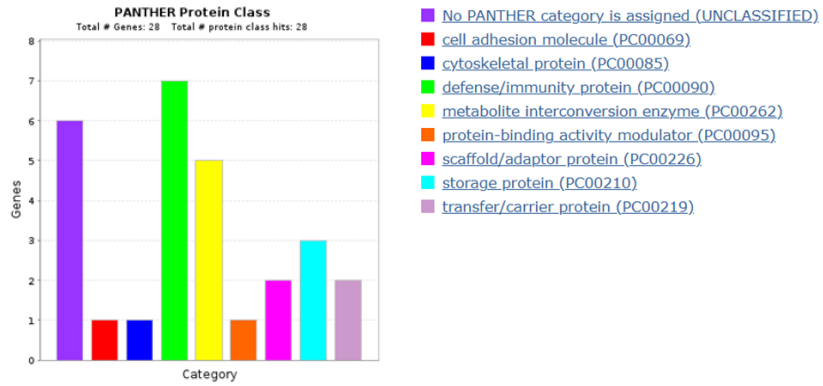

Figure 5. Graphical representation of protein class of the genes composed in lyophilised HBM