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Proteomic Differences in the Milk Fat Globule Membrane (MGFM) Between High and Low Milk Yielding Sahiwal Cows

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ABSTRACT: In the present study we have studied the proteomic differences in the milk fat globule membranes of high versus low milk yielding Sahiwal cows (n=12 each). The proteins extracted from milk cream fat were subjected to trypsin digestion and peptides were labelled with Tandem Mass Tag (TMT) and identified quantitatively by LC- MS/MS. Identification using the Mascot search engine revealed 1716 proteins, out of which 439 proteins were differentially expressed (DEPs). Separated the down-regulated and up-regulated proteins based on the protein fold change data obtained from the LC- MS/MS protein analysis. We found 154 up-regulated Proteins and 285 down-regulated Proteins and further selected best 12 up-regulated proteins in low yielders sahiwal cattle and 12 down-regulated proteins from high milk producing sahiwal cattle. The DEPs were further subjected to gene ontology and pathway analysis using Cytoscape plug-in tool that showed involvement of proteins in catalytic activities, binding, enzyme regulatory activities, response to wounding and the acute inflammatory response, endopeptidase inhibitor activity and apoptosis. Proteome guided genomic selection gives accurate signature protein biomarkers for high versus low milk yielding Sahiwal cattle.

Keywords: Proteomics, Milk fat globule membrane, Sahiwal cattle, Differentially expressed proteins, LCMS.

INTRODUCTION

Cattle rearing has been a traditional livelihood in India and is closely linked to agricultural economy. The total number of Cattle in the country is 193.46 million in 2019 showing an increase of 1.3 % over previous Census. The Exotic/Crossbred and Indigenous/Non-descript Cattle population in the country is 51.36 million and 142.11 million respectively. Most of the indigenous cattle (about 80%) are non- descript and only 20% belong to Indigenous Breeds recognized by National Bureau of Genetic Resources. The Indigenous/Non-descript Female Cattle population has increased by 10% in 2019 as compared to previous census. The cattle genetic resource of India is represented by 37 well recognized Indigenous Breeds. Indigenous cattle, in India, are robust and resilient and are particularly suited to the climate and environment of their respective breeding tracts. They are endowed with qualities of heat tolerance, resistance to diseases and the ability to thrive under extreme climatic stress and less than optimal nutrition. The potential to enhance the productivity of the indigenous breeds of India through professional farm management and superior nutrition is immense. Therefore, it is essential to promote conservation and development of indigenous breeds. The Government of India launched Rashtriya Gokul Mission (RGM) is being implemented for development and conservation of indigenous bovine breeds since December 2014. The scheme is important in enhancing milk production and productivity of bovines to meet growing demand of milk and making dairying more remunerative to the rural farmers of the country. India's genetic cattle stock is represented by 46 well-known Indigenous Breeds. Indigenous cattle in India are hardy and adaptable, and they are well-suited to the climate and habitat of their breeding grounds. They have heat tolerance, disease resistance, and the ability to thrive in adverse climatic conditions and with less-than-ideal nutrition.

The number of secreting cells (MEC) and their activity determine the ability of the mammary gland to produce milk. As the MFGM proteome represents the whole protein content of the milk lipid fraction, we can obtain information regarding mammary epithelial cell health by analysing the protein composition of the milk fat globule membrane protein (MFGM). It's difficult to tell

whether an animal is a low or high yielder because milk yield is a multi-factor trait. Aside from the environment and management approaches, the genetic composition of the animal has a role in how the animal responds to the environment. The apical plasma membrane of mammary epithelial cells is believed to be the source of milk fat globule membrane protein (MFGM). The endoplasmic reticulum's precursor micro lipid droplets fuse together and proceed to the apical cytoplasm, where they are enclosed by the apical plasma membrane and secreted into the alveolar lumen. By investigating the nutritional profile of the MFGM, we can learn about the health of mammary epithelial cells. The proteome guided genomic selection research project has been designed with the following objectives to identifying and correlating the proteome and genetic signature of high and low milk producing Sahiwal cattle. Considering the importance of MGFM proteins, we hypothesised there are differences in the MGFM proteins in the milk of high and low milk yielders.

MATERIALS AND METHODS

The present study was conducted in different farms of Punjab and Haryana including Kamdhenu Goushala, Divya Jyoti Jagrati Sansthan (DJJS), Nurmahal, Ludiana and Livestock Research Centre (LRC), ICAR-National Dairy Research Institute , Karnal. The study duly followed the regulations of institutional animal ethics committee, National Dairy Research Institute, Karnal.

A. Selection and classification of Sahiwal Cows

Apparently healthy Sahiwal cows that were between third and fourth parity, lactation days between 70 and 90 days were utilized in the study. The animals yielding >18 litres were considered as high yielders while those yielding <8 litres were considered as low yielding Sahiwal cows. The milk samples were subjected to somatic cell count, electrical conductivity and pH analysis to rule out both acute and chronic mastitis. The animals which had the SSC count of <100000 cells/mL of milk, electrical conductivity of <5mS/cm and the pH between 6 and 6.5 were considered for further analysis.

B. Milk collection and isolation of milk sat globule membrane protein (MGFM)

Milk collected during morning hours were used for the study, the MGFM was isolated as per the method described by Leonardo Murgiano *et al.* (2013). Briefly, milk (500mL) was centrifuged at 2000g for 30 minutes at 4°C, the cream obtained was washed five times with phosphate buffered saline (Ph 7.4) at 2000g for 10 minutes, the cream was stored at -80°C till further use. The milk fat cream was mixed 1:3 with a solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% Triton X-100, 20 mMTris, 1% DTT and incubated on ice for 60 minutes with periodic vortexing and centrifuged at 10000g for one hour. The supernatant was mixed with precipitation solution (methanol: chloroform: water :: 4:3:3) by vortexing and centrifuged at 10000g for 60 minutes. The aqueous phase was discarded and organic phase was mixed with three volumes of methanol and centrifuged at 9000g for 20 min. supernatant was discarded and pellet was airdried followed by lyophilisation using vacuum. The final pellet was resuspended in 1X PBS solution.

C. Mass Spectrometric Analysis of Peptide Mixtures

Mass Spectroscopy was performed using EASY-nLC 1000 system (Thermo Fisher Scientific) coupled to Thermo Fisher-Q Exactive equipped with nanoelectrospray ion source. 1.0 µg of the peptide mixture was resolved using 25 cm PicoFrit column (360µm outer diameter, 75µm inner diameter, 10µm tip) filled with 1.9 µm of C18-resin (Dr Maeisch, Germany). The peptides were loaded with buffer A and eluted with a 0–40% gradient of buffer B (95% acetonitrile, 0.1% formic acid) at a flow rate of 300 nl/min for 100 min. MS data was acquired using a data-dependent top10 method dynamically choosing the most abundant precursor ions from the survey scan.

D. Data Processing

All samples were processed and RAW files generated were analyzed with Proteome Discoverer (v2.2) against the Uniprot BOVINE reference proteome database. For Sequest search, the precursor and fragment mass tolerances were set at 10 ppm and 0.5 Da, respectively. The protease used to generate peptides, i.e. enzyme specificity was set for trypsin/P (cleavage at the C terminus of “K/R: unless followed by “P”) along with maximum missed cleavages value of two. Carbamidomethyl on cysteine as fixed modification and oxidation of methionine and N-terminal acetylation were considered as variable modifications for database search. Both peptide spectrum match and protein false discovery rate were set to 0.01 FDR.

Resultant protein was analysed using PANTHER (Protein Analysis Through Evolutionary Relationships) classification system available at http://www.pantherdb.org/. The energy pathways and metabolism categories were combined into a single category of Biological Process and functions of classified proteins were categorized in Molecular function. Proteins were also classified according to cellular component and protein class. Pathway analysis was performed using Pathway Studio 8.0 software (Ariadne Genomics, Rockville, MD, USA). We obtained genes involved in different pathways and interrelationship between genes, proteins, cell processes, and diseases.

RESULTS AND DISCUSSION

We have analyzed the major MFGM proteins present in milk fat globule by SDS-PAGE (Fig. 1-3) revels major MFGM proteins found in MFGM are Mucin 1, Xanthene dehydrogenase/Xanthene oxidase, PAS III, CD 36, Butytophillin, ADRP, Lactadherin and GTP-Binding proteins and Compare the proteomic differences observed in the milk fat globule membrane proteins between high versus low yielding Sahiwal cattle using two dimensional electrophoresis (2DE) (Fig. 4) and Table 1and Panther system.

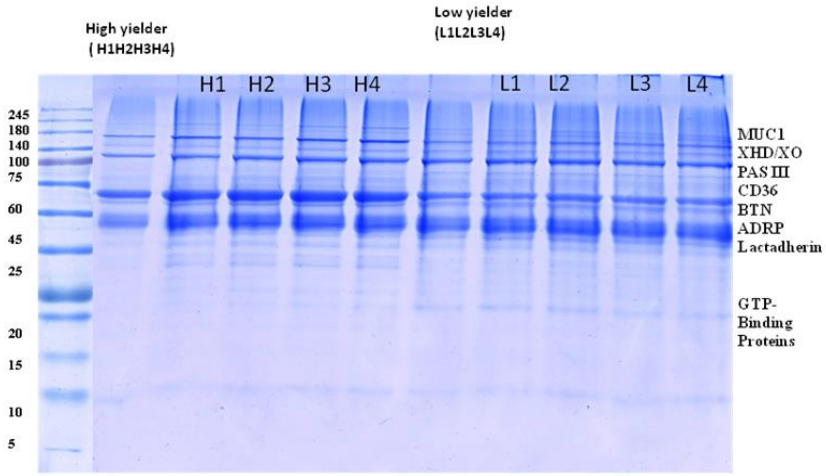
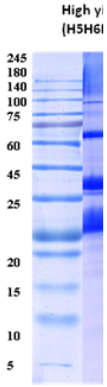


Fig. 1. SDS-PAGE of major MFGM proteins.



Proteins	Molecular Weight (Da)
Mucin I (MUC 1)	160,000-200,000
Xanthine Oxidase	150,000
PAS III	95,000-100,000
CD 36 or PAS IV	76,000-78,000
Butyrophilin	67,000
Adipophilin (ADPH)	52,000
PAS 6/7	48,000-54,000
Fatty acid binding protein (FABP)	13,000
BRCA 1	210,000

Molecular Weights of Major MFGM Proteins

Fig. 2. SDS-PAGE of major MFGM proteins.

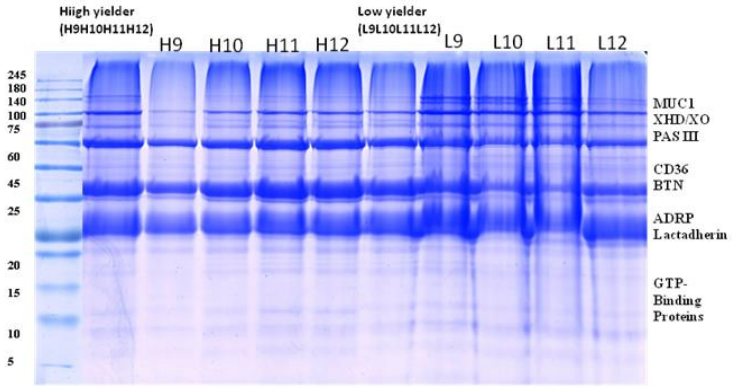


Fig. 3. SDS-PAGE of major MFGM proteins.

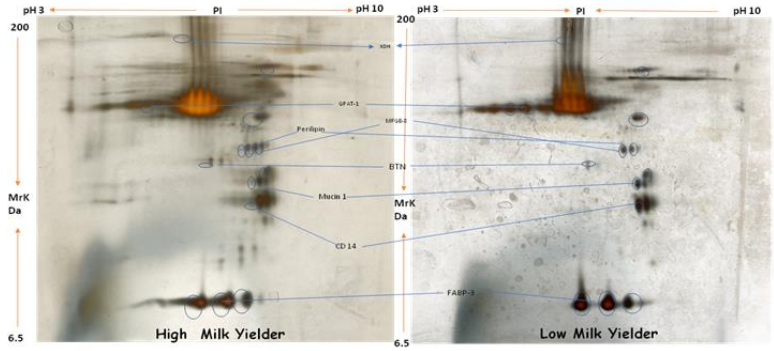


Fig. 4. Two-dimensional gel electrophoresis (12% polyacrylamide gel–silver staining technique) of milk fat globule membrane proteins.

Table 1: Major MFGM proteins identified in 2D images.

Accession	Description	Sum PEP Score	Coverage [%]	# Peptides	# PSMs	# Unique Peptides	# Protein Groups	# AAs	MW [kDa]	calc. pI
P80457	Xanthine dehydrogenase/oxidase OS=Bos taurus GN=XDH PE=1 SV=4	519.134	63	59	940	1	1	1332	146.7	7.74
F1N647	Fatty acid synthase OS=Bos taurus GN=FASN PE=4 SV=2	348.421	30	51	201	51	1	2512	274.1	6.46
Q71SP7	Fatty acid synthase OS=Bos taurus GN=FASN PE=2 SV=1	286.519	24	43	166	43	1	2513	274.4	6.7
B2D1N9	ATP-binding cassette sub-family G member 2 OS=Bos taurus GN=ABCG2 PE=2 SV=1	272.952	50	28	222	28	1	658	73.1	8.73
Q4GZT4	ATP-binding cassette sub-family G member 2 OS=Bos taurus GN=ABCG2 PE=2 SV=2	272.952	51	28	222	28	1	655	72.7	8.66
P18892	Butyrophilin subfamily 1 member A1 OS=Bos taurus GN=BTN1A1 PE=1 SV=2	271.891	59	23	1807	13	1	526	59.2	5.2
F1MXX6	Lactadherin OS=Bos taurus GN=MFGE8 PE=2 SV=1	247.165	64	32	1267	32	1	431	47.8	7.15
F1N1N6	Perilipin-2 OS=Bos taurus GN=PLIN2 PE=2 SV=1	229.728	68	23	516	23	1	450	49.3	8.56
E1BGW1	Mucin-15 OS=Bos taurus GN=MUC15 PE=2 SV=2	67.848	22	6	47	6	1	330	35.7	5.02
P02662	Alpha-S1-casein OS=Bos taurus GN=CSN1S1 PE=1 SV=2	53.201	29	5	72	5	1	214	24.5	5.02
P02754	Beta-lactoglobulin OS=Bos taurus GN=LGB PE=1 SV=3	52.632	55	8	113	8	1	178	19.9	5.02
Q5GJ77	Glycerol-3-phosphate acyltransferase 1, mitochondrial OS=Bos taurus GN=GPAM PE=2 SV=1	24.454	12	6	7	6	1	825	93.1	7.93
A6QNL0	Monocyte differentiation antigen CD14 OS=Bos taurus GN=CD14 PE=2 SV=1	6.361	4	1	2	1	1	375	39.9	5.77

The gene ontology analysis revealed that Most of them were involved in cellular process (27%) and others include in response to stimuli (11%), signalling (8%), developmental process (10%), cellular component organization (6%), immune system process (4%), and developmental process (10%). Our study reveals the present finding suggests that proteins confined to metabolic pathways were differentially expressing during different stages of lactation leading to variation in milk yield during different stages of lactation (Fig. 4). Significant enrichment of functions like antiviral, anticarcinogenic, and antimicrobial properties (Singh, 2006) as well as important synergies with probiotics (Brisson *et al.,* 2010) and preventing infection of enteric pathogens, promoting immune and neurological functions, as well as the development of new-borns (Lee *et al.,* 2018) and also proteins associated with these biological processes are essential for maintaining the structure and function of mammary epithelial cells which intern maintains the mammary gland function.

Molecular function (Fig. 5-7) more than one-half of identified DEPs proteins accounted for binding activity (54%) and catalytical activity (23%) which is essential for cell to cell interaction and metabolic activity. Cell-matrix interaction plays role in growth, development and remodelling of mammary gland throughout the stages of lactation. Rest other proteins were found to be involved in structural molecule activity (7%), molecular function (8%), ATP dependent activity (4%) and majority of the differentially expressed MFGM proteins of cellular components Fig. 2 were mainly enriched in the processes of response to wounding and the acute inflammatory response, in the cellular components of the extracellular region.

Pathway (String bioinformatics search engine) Studio analysis was performed to anlayse the pathways involved in the differentially expressed proteins of MFGM. The results revealed that Humoral immune response-prevent autoimmune diseases (URLY), TLR2 and TLR4 pathway by inhibiting inflammation-oxidative activity, MFGM with BFB and LAB-Immune response, Apoptotic signalling pathway, mitogen-activated protein kinase (MAPK) signalling pathway, PI3K-Akt signalling pathway, TCA Cycle, Oxidative phosphorylation, Insulin signalling pathway, Pathways in cancer, fatty acid metabolism, Mitochondrial membrane transport-ATP synthase. Network pathway analysis of the up-regulated proteins in later stage connect to the NF-kB and Jak-MAPK pathways causing decline in milk production Ruiz *et al*. (2021). Akt, PI3K and p38/MAPK signalling pathways are associated with high milk production mediated through insulin hormone signalling (Janjanam *et al*., 2014) whereas the extent of a triglyceride catabolic process, which occurs via acid lipolysis in the lysosome and neutral lipolysis in the cytoplasm, can regulate lipid droplet size (El-Tarabany *et al.,* 2008). It was confirmed that the controlling lipid metabolism in the MFGM proteins of the goat was superior to that of the bovine MFGM proteins by the identification of LPL and APOE as the key enzymes involved in the triglyceride catabolic process. The crucial catabolic and transport process known as the phagosome pathway has been observed in mature milk from the Guanzhong goat and Holstein cow (Sun *et al*., 2020). According to Yang *et al*. (2020), the phagosome pathway is a convoluted organic process of apoptotic cell uptake and pathogen elimination that supports tissue homeostasis, host defence, and inflammation. The MFGM proteins may therefore be essential for developing animals' immune systems. Additionally, the string pathway analysis revealed that the various MFGM proteins in cattle greatly influenced glycometabolism through the pathway of galactose metabolism. According to (Sun *et al.,* 2019), the

majority of goat MFGM proteins were connected to metabolic processes, such as lipid and glucose metabolism. In mammary epithelial cells, fatty acid synthases are found on the endoplasmic reticulum surface and on cytoplasmic lipid droplets. A number of membrane enzymes, including palmitoyl-CoA and glycerol 3-phosphate, can generate triglycerides from activated fatty acids in the endoplasmic reticulum. Fatty acid synthases were directly linked to palmitoyl-CoA, a significant by-product of fatty acid synthases, in the fatty acid synthesis pathway (Moriya *et al*., 2011). Additionally, glucose and fructose encourage the build-up of triglycerides in milk, which helps turn sugar into fat, as well as the allosteric inhibition of fatty acid oxidation by increasing the availability of triose phosphate precursors, acetyl-CoA, and metabolites like malonyl-CoA for the formation of fatty acids through glycerol-3-phosphate biosynthesis and de novo lipogenesis (Haile *et al.,* 2016). Hepatic steatosis and lipid build-up could be prevented by controlling fatty acid biosynthesis.

The differential expression of proteins in low and high milk yielding MEC samples, investigated in the present study has focused on important target proteins related to lactation.

Table 2: Up-regulated proteins in low yielders.

Uniprot ID	Uniprot ACC	protein description	protein length	percent coverage	percent share of spectrum ids	Average 114|117 (A)	Average 115|116 (B)	Fold change A/B
A3FPG8	GPAT4_BOVIN tr	Glycerol-3-phosphate acyltransferase 4	456	35.1	0.124	3.675	1.09375	3.360
Q2KJE5	G3PT_BOVIN tr	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	395	9.6	0.006	4.01875	1.137668919	3.532
P62998	RAC1_BOVIN tr	Ras-related C3 botulinum toxin substrate 1	192	42.2	0.024	4.46875	0.994087838	4.495
L8IXH1	L8IXH1_9CETA	Ras-related protein Rab-2A	199	56.8	0.093	4.05625	1.239864865	3.272
Q58CW5	SERC2_BOVIN tr	Serine incorporator 2	452	13.1	0.026	2.64375	0.793918919	3.330
Q8WML4	MUC1_BOVIN	Mucin-1	580	20.9	0.388	5.525	0.815878378	6.772
E1BG26	E1BG26_BOVIN	Cation-transporting ATPase	1226	13.5	0.054	2.83125	0.538006757	5.262
A4IFP2	A4IFP2_BOVIN	KRT4 protein	549	18.8	0.061	3.16875	0.554898649	5.711
L8IAU7	L8IAU7_9CETA	Nogo-B receptor	196	13.3	0.005	4.7875	1.14527027	4.180
Q3ZBD0	PSMD7_BOVIN tr	26S proteasome non-ATPase regulatory subunit 7	322	3.8	0.003	5.31875	0.66722973	7.971
L8HUH4	L8HUH4_9CETA	Putative serine protease 47	342	7.6	0.005	2.71875	0.685810811	3.964
A5D7D1	ACTN4_BOVIN	Alpha-actinin-4	911	18.4	0.036	8.55625	0.237331081	36.052
P26201	CD36_BOVIN tr	Platelet glycoprotein 4	472	51.1	0.843	2.81875	1.288851351	3.187

Table 3: Down-regulated proteins in high yielders.

Uniprot ID	Uniprot ACC	protein description	protein length	percent coverage	percent share of spectrum ids	Average 114|117 (A)	Average 115|116 (B)	Fold change A/B
P30932	CD9_BOVIN tr	CD9 antigen	226	26.5	0.039	0.29375	2.578547	0.114
P02722	ADT1_BOVIN tr	ADP/ATP translocase 1	298	10.1	0.018	0.51875	1.5	0.346
P04896	GNAS2_BOVIN	Guanine nucleotide-binding protein G(s) subunit alpha isoforms short	394	49.7	0.177	0.65625	1.978885	0.332
P81125	SNAA_BOVIN tr	Alpha-soluble NSF attachment protein	295	52.5	0.083	0.35625	2.853041	0.125
L8J084	L8J084_9CETA tr	Ras-related protein Rab-9A	203	12.3	0.006	0.6625	1.331926	0.497
F1MD43	F1MD43_BOVIN tr	Mothers against decapentaplegic homolog	465	7.2	0.009	0.45625	2.630912	0.173
F1N647	F1N647_BOVIN	Fatty acid synthase	2512	65.6	1.052	0.6875	1.983108	0.347
L8I5S0	L8I5S0_9CETA	Alpha-S1-casein	214	68.7	0.781	0.65	1.857264	0.350
L8J2E9	L8J2E9_9CETA	Long-chain-fatty-acid--CoA ligase 3	720	55.8	0.224	0.8875	1.849662	0.480
A0A0B7G4D8	A0A0B7G4D8_THACB	Magnesium transporter NIPA2	743	1.6	0.003	0.85625	1.867399	0.459
L8I9P7	L8I9P7_9CETA	Monocyte differentiation antigen CD14	381	35.2	0.053	0.55625	1.810811	0.307
Q2TJL4	Q2TJL4_BOSIN	Butyrophilin	130	87.7	0.619	1.64375	1.713682	0.959
P80025	PERL_BOVIN tr	Lactoperoxidase	712	25.6	0.059	1.4625	1.589527	0.920
L8HR34	L8HR34_9CETA	Lactadherin OS=Bos mutus GN=M91_06780 PE=4 SV=1	431	94	3.068	1.225	1.585304	0.77272243

Depending on the protein fold change obtained from the mass spectroscopic analysis of MFGM proteins selected 12 up-regulated proteins in high yielders (Table 3) and 12 down-regulated proteins in high yielders (Table 4) were selected and Further, the validation of these proteins at both the levels of transcripts and the protein provides us the insights about the mechanisms involved in the lactation and the expression differences that results in high milk yield in Sahiwal cows.

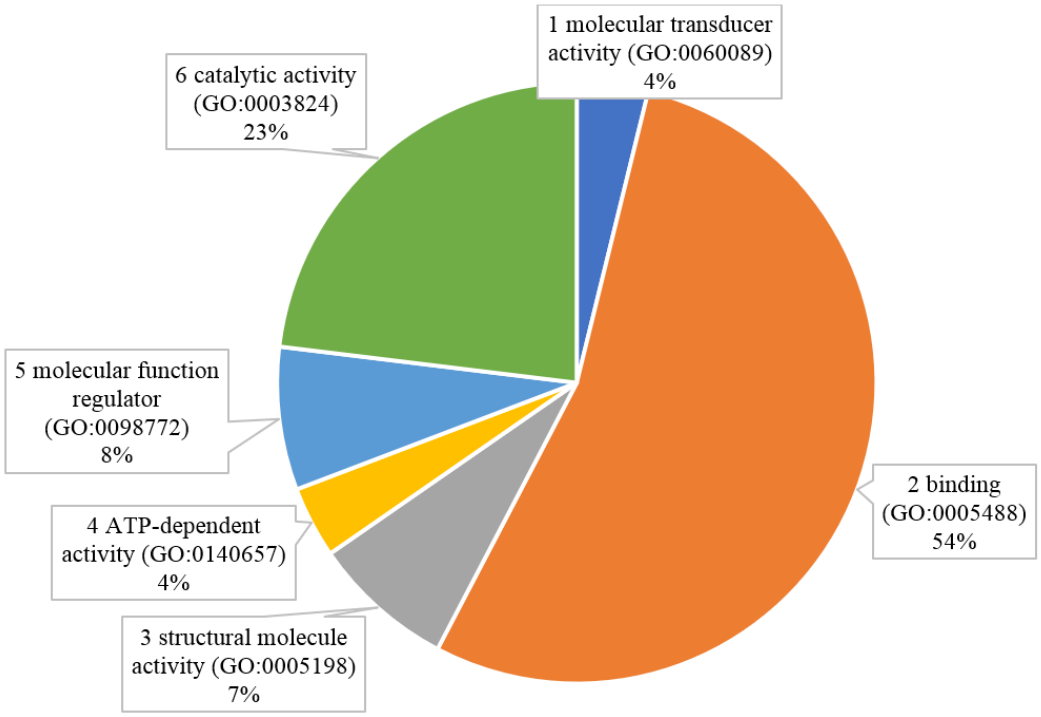


Fig. 5. Molecular Function.

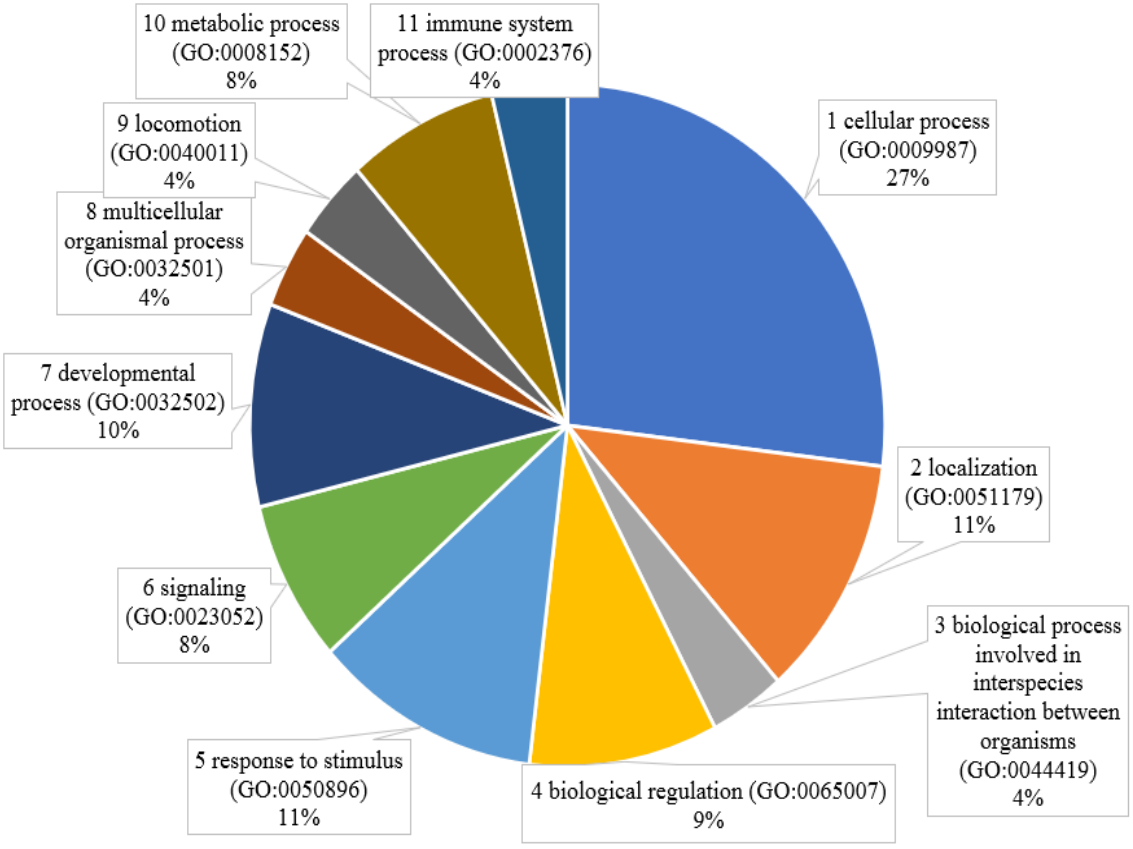


Fig. 6. Biological Process.

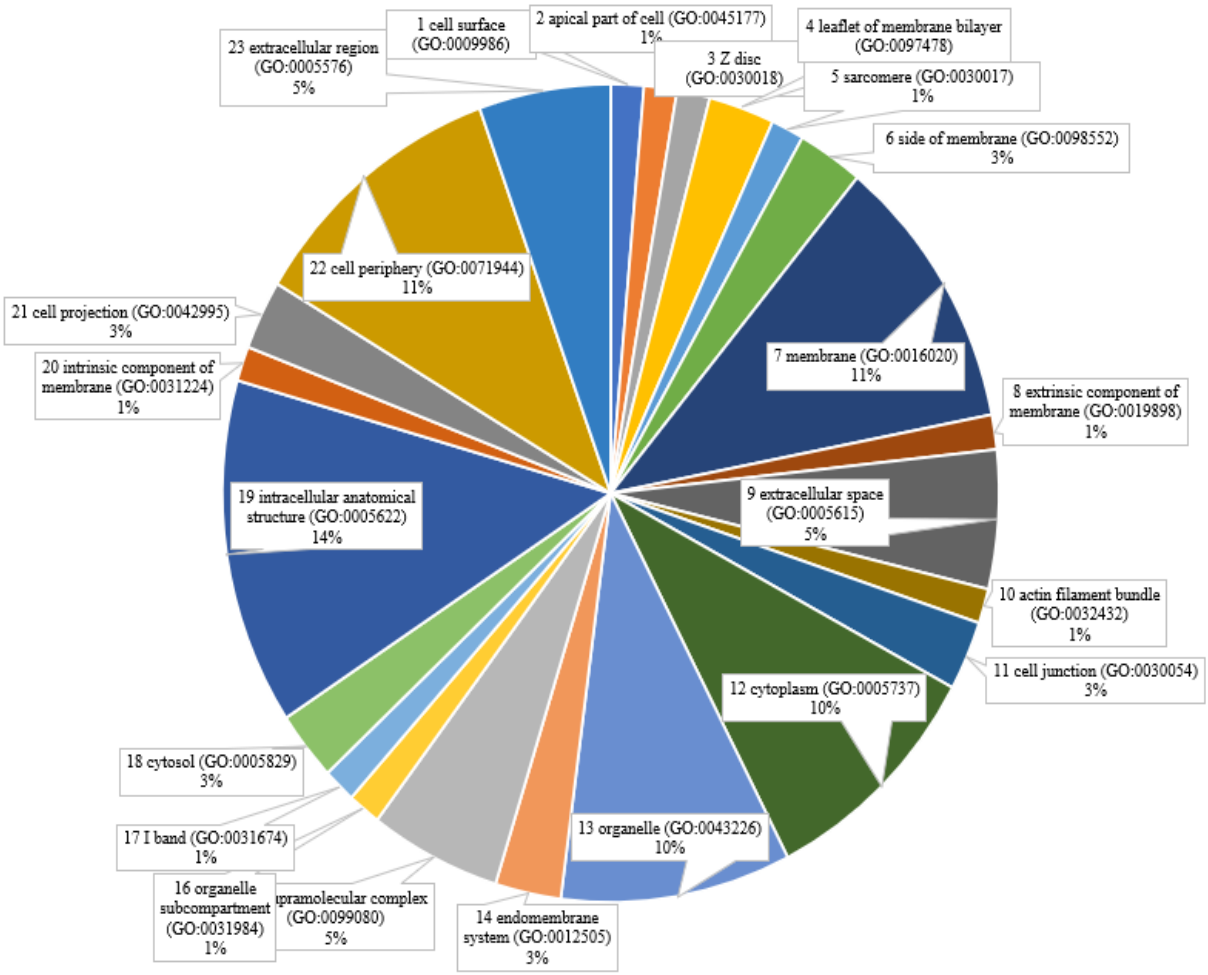


Fig. 7. Cellular Components.

CONCLUSIONS

In present study we show those 12 up-regulated proteins in high yielders and 12 down-regulated proteins in high yielders of Sahiwal cattle. Therefore, these differentially expressed proteins in high versus low milk yielding Sahiwal cattle may be considered as protein biomarkers for differentiating between high versus low milk yielding Sahiwal cattle.

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Conflict of Interest. None.

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