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Comparative proteomics analyses of female *Culex pipiens* mosquito gut proteins after sugar and blood feeding using Nano LC-MS

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Abstract

Culex pipiens is a major vector transmitting a variety of arthropod-borne pathogens. *Cx pipiens* were collected then identified morphologically and by mitochondrial cytochrome oxidase I gene for accurate species confirmation. Mosquito's' gut plays an important role in infection susceptibility. A baseline proteomic dataset is needed to give insights into the physiology of blood feeding and to understand functional proteins in mosquito behavior following a blood meal ultimately leading to establish effective control strategies. As the changes in protein expression of *Cx. pipiens* gut after sugar and blood feeding have not been investigated, a proteomic analysis of gut tissue was carried out using Nano LC/mass spectrometry (LC-MS) and offline matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF)-MS. There were 56 functional putative proteins in the gut of sugar fed *Cx. pipiens* females and 37 proteins gut of blood fed *Cx. pipiens* females with molecular weight ranging from 800.4 to 2969.4 Da and from 818.3 to 3207.6 Da for sugar and blood fed gut females, respectively. 39 and 20 proteins were uniquely identified in sugar fed and blood fed, respectively, while 17 proteins were common by the two biological groups. Data are available via ProteomeXchange with identifier PXD030533.

Keywords: female, *Culex pipiens*, gut, blood feeding, sugar feeding, proteomics analysis.

1. Introduction

Mosquitoes are significant vectors with enormous economic and public health consequences. Worldwide, there are over 3,574 mosquito species **[1].** Hematophagous females are responsible for transmission of many pathogens that kill millions of people every year [**1, 2**]. Mosquitoes are vectors of several tropical infectious diseases to human such as lymphatic filariasis, malaria dengue fever, yellow fever and Chikungunya [**3, 4].** Mosquitoes in the *Cx. pipiens* complex have been identified as Rift Valley fever virus vectors **[5-7].** Since they require a blood meal for egg deposition and development to transmit these diseases, knowledge and understanding of the regulation of blood digestion may be important for future control [**8].** Many insects' transmitted pathogens interact with the vectors midgut to infect and, in some cases, develop **[9].** The act of feeding on blood is necessary for mosquito reproduction and the transmission of malaria parasites. Blood feeding, digestion, and subsequent physiological responses all have an impact on the gut microbial community [**10].**

Proteomics is a rapidly evolving study subject that has been used to profile and catalogue the proteomes of insects, along with mosquitoes at distinctive organelles, tissues and in distinctive physiological states [**11].** Their interactions with parasites, viruses and toxins have been additionally investigated [**11].** Mosquito proteomics studies have discovered the characteristics of midgut peritrophic matrix proteins, haemolymph proteins and mosquito-head proteins in the course of distinctive feeding

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(sugar or blood) [**12**]**.** The use of proteomics with LC-MS/MS (Liquid Chromatography-Mass Spectrometry) allowed recognition of host-virus interactions [**11].** Previous studies were performed to investigate differential expression of protein in mosquitoes with the intention to advantage higher information of protein regulation under numerous physiological conditions [**12-17].**

In mosquito proteomics research, most of the studies have emphasized the interrelation of proteome and physiological responses of vector against pathogen, characterizing proteins in mosquito tissue or organ at different cell state. In Malaysia, presented the first evidence of various protein bands found in different stages of *Aedes aegypti* and *Anopheles maculatus* development in Malaysian, hence demonstrated that midgut was the targeted organ for immunization study **[18].** Prevot *et al.* showed that 10 proteins that were absent in male mosquito but appeared in female mosquito during feeding were associated with blood digestion **[19]**.

Cx. pipiens is commonly distributed in all Egypt. However, there is no information regarding its gut proteome profiling. Thus, a proteomic analysis of *Cx. pipiens* gut is highly desirable and it is critical to investigate its various components. As a result, the current study was designed to assess the peptide variation in gut of *Cx. pipiens* after sugar and blood feeding using high performance liquid chromatography (HPLC) fractionation along with Nano LC/mass spectrometry (LC-MS) and offline matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF)-MS. This integrated approach provides a preliminary overview of *Cx. pipiens* gut protein constituents as well as information on potential bioactive peptide candidates for mosquito control.

2. Materials and Methods:-

1- Experimental insects:-

The Strain of *Cx. pipiens (L),* (*Diptera: Culicidae*) was used as an experimental insect in the present study. The culture was originated by collecting *Cx. pipiens* larvae from different locations at Al-Behera governorate, Egypt. During field surveillance activities, samples were collected using the dipping method and reared under controlled laboratory conditions **[20],** within the insectary of Zoology and Entomology Department, Faculty of Science, Al-Azhar University.

2- Laboratory maintenance of *Cx. pipiens* (L) and morphological identification:-

Mosquito larvae were reared in the insectary and fourth larval instars were used for morphological identification using taxonomic keys of **[21],** by the Public Organization for Institutes and Teaching Hospitals, Ministry of Health, Research Institute of Medical Entomology, Dokki, Giza, Egypt. Adult mosquitoes of known ages were kept under laboratory conditions (25 - 27 °C, 70-80% RH. and a photoperiod of 16 L : 8 D photoperiod) accordingly as described by **[20].** The collected larvae were reared to the third generation and larvae were ground with phosphate buffered saline (PBS) using a sterile mortar and pestle and the tissue homogenate was centrifuged for 10 minutes at 3000 rpm before being the supernatant fluid frozen at -70°C for further DNA extraction.

3- Molecular identification of Mosquito:-

a- DNA Extraction:

Extraction of DNA was performed at Animal Health Research Academy, Ministry of Agriculture, Dokki, Giza, Egypt using GeneJET Genomic DNA Purification Kit (Thermo Scientific, Catalog number K07210) as directed by the manufacturer.

b- Amplification of mitochondrial COI gene and sequencing:-

Amplification of mitochondrial COI gene was carried out according to **[22].** The PCR reaction mixture was adjusted to 25 µl and contained 12.5 µl Applied Biosystems™, AmpliTaq Gold® 360 Master Mix (Thermo Fisher Scientific, USA, Cat. no. 4398876), 1 µl of forward primer (LCO1490-F 5' - GGT CAA CAA ATC ATA AAG ATA TTG G- 3'), 1 µl of reverse primer (LCO1490-R 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA- 3'), 8 µl of extracted DNA and finally complete to 25 µl nuclease free water. PCR reaction conditions were adjusted as follows: an initial denaturation at 95°C for 5 minutes was followed by 35 cycles of denaturation at 94°C for 40 seconds, annealing at 51°C for 1 minute and extension at 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes **[23].**

A BIO-RAD® PCR system T100 thermocycler (BioRad, Hercules, California, USA) was used for DNA amplification. The amplified PCR products were visualized using Imager Gel Doc™ XR+ Imaging system (BIO-RAD) and Image lab™ software program for gel picture evaluation in contrast to the 100 bp DNA Ladder RTU (GeneDirex, cat. no. DM101-0100). PCR-product was purified using QIAquick® Gel Extraction Kit (QIAGEN, USA, Cat. no. 28704) and sequenced using BigDye® Terminator v3. and cycle sequencing kit (Applied Biosystems, USA), as directed by the manufacturer.

The *COI* sequence generated in this study was deposited in GenBank under the accession number MT199095.1 (http://www.ncbi.nlm.nih.gov). The BLAST similarity search (available at http://www.ncbi.nlm.nih. gov) was used to search the **BOLD** and GenBank databases for mosquito identification (National Center for Biotechnology Information, Rockville Pike, Bethesda, MD).

c- Phylogenetic analyses:-

A total of 20 COI sequences were used to construct the phylogenetic tree included 19 sequences downloaded from GenBank in addition to one sequences obtained in the current study. Maximum Likelihood based on Tamura-Nei model was used to build the tree **[24].** MEGAX was used to create the tree, which was inferred from 1000 bootstrap replicates **[25].**

1- Preparation of gut samples for analysis:-

A- *Cx. pipiens* gut dissection and extract preparation:

For proteomic analysis, guts were dissected from 500 *Cx. pipiens* females after 3~5 days of sugar feeding and other 500 guts from *Cx. pipiens* females were dissected after 6 h blood feeding as described by **[26]**. Phosphate buffered saline (0.150 M KH_2PO_4, 0.15 M Na_2HPO_4 and 0.9% NaCl) was used to dissect the guts.

B- Protein extraction and denaturation:

On each sample, 600 µl 8M Urea (500 mM Tris pH 8.5) with 60µl ultra-proteases (Roche, Mannheim) were added. The samples were homogenized, lively shaken and centrifuged at 10,000 RPM for 35 minutes then supernatants were taken to perform the total protein extraction, **[27].**

C- Protein quantification:

The extracted protein levels were determined by using the bicinchoninic acid assay (BCA assay) as follow, **[28].**

Sample	BSA (µl)	Sample (µl)	Sample Vehicle (µl)	MilliQ (µl)	BCA working solution (µl)
Blank	0	0	8	12	400
Standard (1.25 µg/ µl)	8	0	8	4	400
Sample	0	8	0	12	400

Sample	Conc. (ug/ µl)	Total protein needed for digestion (30 ug)	
		Sample (µl)	Urea Buffer (µl) (Mass up to 30 µl)
Mosquito A	9.257	3.240	26.759
Mosquito B	22.724	1.320	28.679

D- Protein digestion:

Directly digest the low samples mosquito A and mosquito B, with the above volume.

Each sample was reduced by adding 1.2 µl of 200 mM DTT. The samples were then incubated at room temperature for 45 minute. For alkylation, 1.2 µl of 1M iodoacetamide (IAA) was added to each sample and then incubated at room temperature for 45 h in far from light, followed by the addition 102 µl of 100mM Tris pH 8.4. For trypsinization, a modified procaine trypsin (Sigma, Germany) was added at a 1: 50 protease: protein mass ratio along with 1mM $CaCl_2$, and overnight incubated in a thermo-shaker at 900 rpm at 37 °C. The digested peptide solution was acidified with 6 µl 100 % formic acid to a final pH of 2-3, spin down for 30 minute at room temperature **[27, 29].**

E- Stage tip (MonoSpin Reversed Phase Columns) prod# 5010-21701:

The resultant peptide mixture was cleaned up using a stage tip (Pierce™ C 18 Spin Tips). In brief, 50 **µl** methanol was added for activation, 50 **µl** from solution B (0.2% formic acid (FA), 80% acetonitrile (CAN)) was added for initialization, 50 **µl** from solution A (0.2% FA) was added twice for re-equilibration, the Eppendorf tubes were changed, and all the samples were added for sample trapping. Then, each sample was washed with 15µl of solution A twice. For elution, 30µl of solution B was added 3 times, speed vacuuming was performed, and the residue was reconstituted in 20 µl of solution A. Centrifugation at 3000 RPM was performed between each step. Each sample was then injected into a mass spectrometer **(28).**

F- Peptide quantification:

The peptide quantification measured by using bicinchoninic acid assay (BCA assay) as follow:

Sample	BSA (µl)	Sample (µl)	Sample Vehicle (µl)	MilliQ (µl)	BCA working solution (µl)
Blank	0	0	10	15	25
Standard (1 µg/ µl)	10	0	10	5	25
Sample	0	10	0	15	25

Incubate each sample, standard and blank at 95 °C for 5 minutes, add 1000 µl of prepared BCA, and incubate at 60 °C for 30 minute, then cool down at room temperature for 20 minute before reading the samples at A562 against blank. After that, every sample was introduced into a mass spectrometer for analysis **[27, 30].**

2- Chromatography

A- Nano-LC MS/MS system:

Nano-LC MS/MS analysis was carried out using a TripleTOF™ 5600 + (AB Sciex, Canada) interfaced at the front end with an Eksigent nano LC 400 auto-sampler with an Ekspert nano LC425 pump. Evaluation was completed with an injection volume of 1 µg/10 µl. CHROMXP C18 CL, 5 µm (10 × 0.5 mm) (Sciex, Germany) was used to trap the peptides in trap and elute mode. Samples clean up after trapping the peptides in trap and elute mode at flow rate 10 µl/min for 3 min. using mobile phase A. (MilliQ containing 0.1% FA and acetonnitrile containing 0.1%FA).

The MS and MS/MS ranges were 400−1250 m/z (TOF mass range) and 170−1500 m/z (MS2 range, product ion), respectively. A linear gradient of 3–40% solution B (80% ACN, 0.2% formic acid) was applied for 55 minutes. Data-dependent acquisition (DDA) mode with a charge state of 2–5 was used to select the 40 strongest ions sequentially **[31]**. For each cycle, survey full scan MS/MS and MS spectra of the study were acquired at resolutions of 15,000 and 35,000, respectively. For high precision, external calibration was programmed and during the sample batches to correct for potential TOF deviation. The measurements were carried out in positive ion mode.

B- Data processing:

Using raw data, a file was generated in mascot general format (mgf), TripleTOF™ 5600+ MS file and analyzed by Protein pilot software (version 5.0.1.0, 4895), paragon algorithm (version 5.0.1.0, 4874). The organism *Culex pipiens* (2472 entries in the Swiss-Prot and TrEMBL databases) was used, as well as reversed decoy sequences. All complete and semi trypsin peptide candidates were included in the search space (up to 2 missed cleavages were lost at least 6 amino acids). The precursor mass and fragment masses had initial mass tolerances of 20 ppm and 10 ppm, respectively. Cysteine carbamidomethylation (mass 57.021460 amu) is classified as a static modification, wherease protein N-terminal methionine oxidation (mass 15.993 amu), pyrrolidone from carbamidomethylated C (-17.02 amu) and K acetylation (ass 42.01 amu) were classified as variable modifications. The false discovery rate (FDR) was kept at 1 % of the protein level to ensure high-quality results. By combining the sample replicates, the final protein list was created.

C- Bioinformatics research:

Search Gene Ontology annotation (GO)

using the UniProtKB database (www.uniprot.org) and Entrez PubMed database (www.ncbi.nih.gov) to

identify genes found in mosquito guts from known protein fragments yielded the molecular function and biological processe of the identified protein. The Kyoto Encyclopedia of Gene and Genome (KEGG) Orthology (KO) annotation for these proteins (https://www.genome.jp/kegg/) was used to perform KEGG Orthology annotation for these proteins. The proteomics data from mass spectrometry were submitted to the Proteome Xchange, (www.ebi.ac.uk/pride) Consortium via the PRIDE partner repository with the dataset identifier PXD030533.

According to the HUPO Proteomics Standards Initiatives, Minimum Information About a Proteomics Experiment (MIAPE) reporting guidelines for proteomics (http://www.psidev.info/index.php?q=node/91), our experiment is MIAPE compliant, and our results closely follow the Paris guideline (http://www.mcponline.org/site/misc/ParisReportFinal.xhtml).

The two lists of proteins designated for the gut strain used in this study are summarized as follows: gut of sugar fed *Cx. pipiens* females (Mosquito A) and gut of blood fed *Cx. pipiens* females (Mosquito B). Venny 2.1.0 - BioinfoGP – Csic was used to identify the proteins that were unique to sugar and blood fed *Cx. pipiens* guts, (http://bioinfogp.cnb.csic.es/tools/venny/**).**

3. Results

Mosquito identification

The partial COI DNA gene sequence isolated from larvae of *Cx. pipiens* resulted in 678 bp amplicon (**Figure 1**). The average frequencies of T, C, A, and G were 39.9, 14.5, 28.7, and 16.9% respectively. The A + T contents (68.6 %) were higher than the C + G contents (31.4 %). The sequence was blasted to *Cx. pipiens* sequences in GenBank with an overall nucleotide identity of 98.9%–100%. The resulted sequence was deposited in Genbank under the accession numbers MT199095.1. Sequence comparison with other *Cx. pipiens* mitochondrial DNA sequences on Genbak showed no difference in Egyptian isolates from field. The species studied in the current study thus could be specified based on their COI gene, resulting in 100 % compatibility between molecular and taxonomic identification.

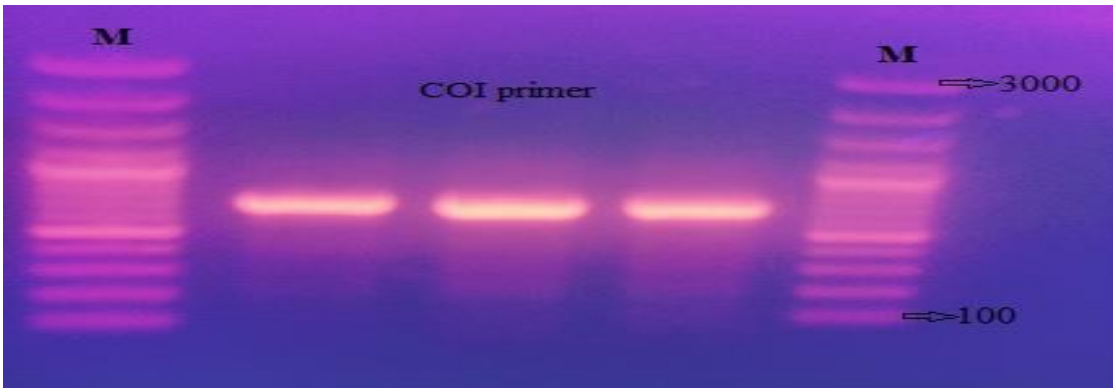

Figure 1: Amplification of *COI* gene produces 678 bp of PCR products from mosquito species. Lane M: 100 bp DNA ladder, 14 bands ranged from 100 to 3000 bp.; Lanes: DNA of field mosquito, *Cx. pipiens*, collected from Al-Behera governorate, Cairo, Egypt.

Proteomic profiling of the gut of *Cx. pipiens*:

The proteomic profile for gut of sugar fed female *Cx. pipiens* after 3-5 days and blood fed after 6 hrs was obtained using Nano-LC MS/MS analysis. Over peak list files were merged and used to identify proteins by X Tandam, which was incorporated into the Peptide shaker pipeline (version 1.16.261). These analyses, combined with pamphlet examination of the specified proteins, yielded datasets of high-confidence identified proteins. There were 56 functional putative proteins in the gut of sugar fed *Cx. pipiens* females (Mosquito A) and 37 proteins in the gut of blood fed *Cx. pipiens* females were identified (Mosquito B), (**Figure 2, tables 1-3**).

The current study identified 93 proteins in both type of feeding (**Figure 3**). As shown in Fig. 3, 39 and 20 proteins were uniquely identified in sugar fed (Mosq. A) and blood fed (Mosq. B), respectively, while 17 proteins were common by the two biological groups. Unique proteins were abundant in both the sugar fed and blood fed producing groups, according

to the findings. Based on Gene Ontology annotation, over of the specified ideal proteins were assigned to a specific function under the categories of molecular functions and biological process.

Among identified total 63 significant putative proteins using in solution trypsin digestion in *Cx. pipiens* after sugar fed, Myosin (tr|Q45FA2) was identified with highest score and highest peptide matches (30 peptides), while in blood feeding of *Cx. pipiens* with peptide matches (29 peptides).

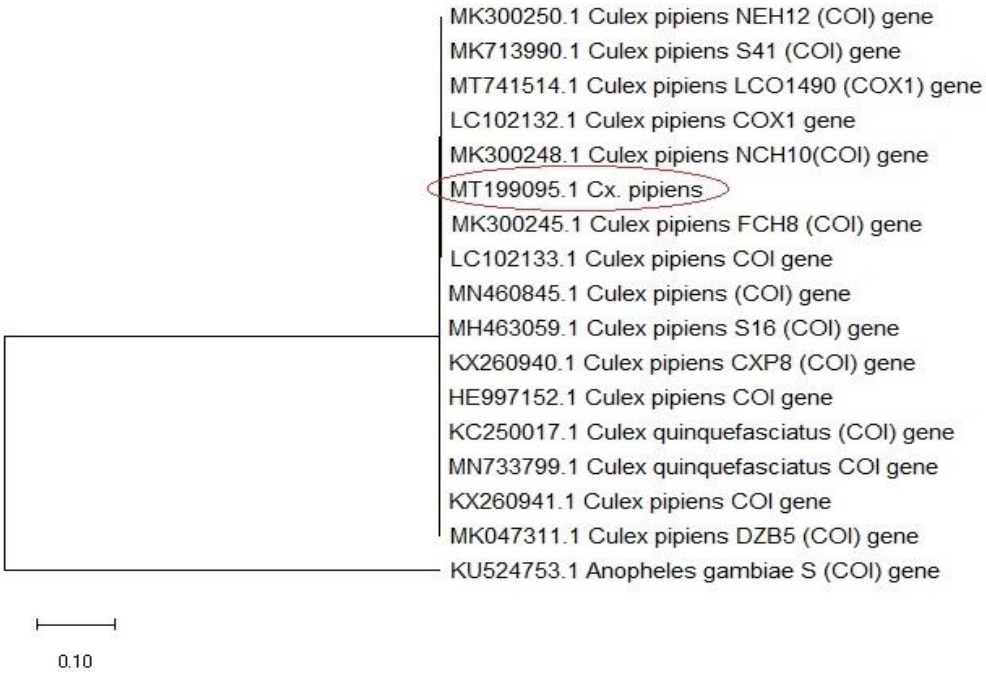

Figure 2: The Maximum Composite Likelihood method on MEGA X software was used to draw a phylogenetic tree of *Cx. pipiens* mosquitoes in Egypt. Next to the branches is the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). As an outgroup, the *Anopheles gambiae* S (COI) sequence was used.

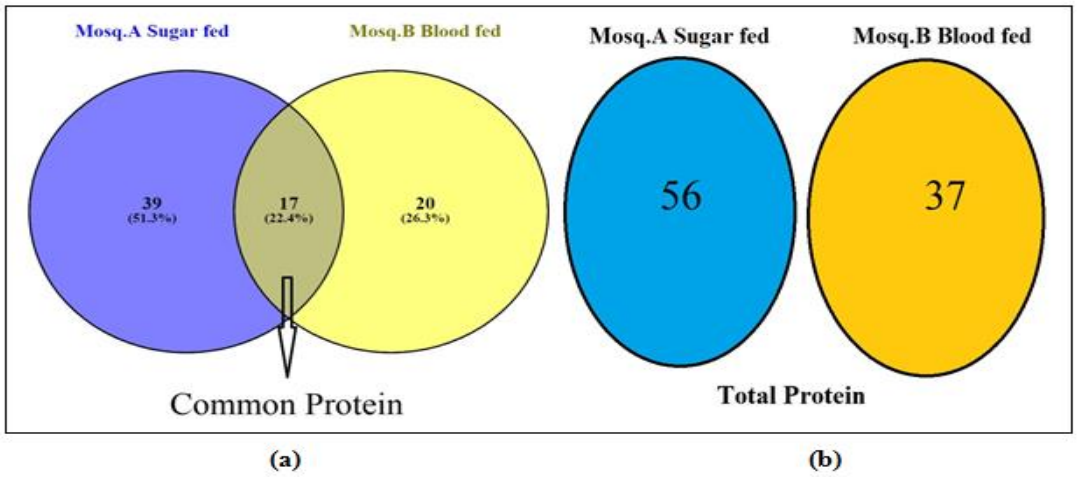

Figure 3: Venn diagram integration of identified proteins commonly and exclusively in gut of sugar fed *Cx. pipiens* females (Mosquito A) and gut of blood fed *Cx. pipiens* females (Mosquito B) based on the gene name (a). Total proteins were represented in both samples (b).

Table (1): List of identified proteins in gut of *Cx. pipiens* common (C) in both sugar and blood fed.

No.	Identified Protein	Protein Accession No.	No. of Protein	
			Mosq.A	Mosq. B
C 1	Actin OS=Culex pipiens pallens	tr|A0A173GY68	283	141
C 2	Putative myosin light chain 2 OS=Culex pipiens pallens	tr|Q45FA2	43	15
C 3	Actin OS=Culex pipiens pipiens; Actin OS=Culex pipiens pipiens	tr|Q4JQ54 tr|Q2LEY8	396	134
C 4	Triosephosphate isomerase (Fragment) OS=Culex pipiens	sp|P91919	38	3
C 5	Mitochondrial F-type H+-transporting ATPase subunit b OS=Culex pipiens pallens	tr|A0A1Z3GD36	26	7
C 6	Arginine--tRNA ligase OS= Culex pipiens (strain wPip); Arginine--tRNA ligase OS= Culex pipiens (strain wPip); Arginine--tRNA ligase OS= Culex pipiens (strain wPip)	sp|B3CNF3 tr|B3CN49 sp|B3CNF3	2	17
C 7	14-3-3 zeta OS=Culex pipiens	tr|E3T3S3	15	5
C 8	Glucose-6-phosphate isomerase OS=Culex pipiens pallens	tr|A0A514K1U5	13	1
C 9	Superoxide dismutase (Fragment) OS=Culex pipiens	tr|F8RUB7	11	1
C 10	ATP synthase subunit OS=Culex pipiens pallens ATP synthase subunit alpha OS= Culex pipiens ATP synthase subunit beta OS= Culex pipiens	tr|A0A1Z3GD37 SP|B3CN53 SP|B3CN17	40	4
C 11	Phosphotransferase OS=Culex pipiens pallens	tr|A0A514K1U6	11	2
C 12	Cytochrome c oxidase subunit 2 OS=Culex pipiens pipiens; Cytochrome c oxidase subunit 2 OS=Culex pipiens pallens; Cytochrome c oxidase subunit 2 OS=Culex pipiens; Cytochrome c oxidase subunit 2 OS=Culex pipiens; Cytochrome c oxidase subunit 2 OS=Culex pipiens; Cytochrome c oxidase subunit 2 OS=Culex pipiens	tr|E9NVN2; tr|A0A1B0XB79; tr|A0A0N7AL38; tr|A0A0N7AFM9; tr|A0A0N6XW16; tr|A0A0N6W8Y0	10	7
C 13	Beta-actin OS=Culex pipiens pipiens	tr|Q27S91	145	69
C 14	Ankyrin repeat domain protein OS= subsp. Culex pipiens; Ankyrin repeat domain protein OS= subsp. Culex pipiens; Ankyrin repeat domain protein OS= subsp. Culex pipiens; Ankyrin repeat domain protein OS= subsp. Culex pipiens; Ankyrin repeat domain protein OS= subsp. Culex pipiens	tr|B3CPL2 tr|B3CNN3 tr|B3CLM0 tr|B3CP71 tr|B3CP79	7	4
C 15	Heat shock protein 70 OS=Culex pipiens; Heat shock protein 70 B2 OS=Culex pipiens complex sp.; Heat shock protein 70 B2 OS=Culex pipiens complex sp.; Heat shock protein 70 B2 OS=Culex pipiens complex sp.; Heat shock protein 70 B2 OS=Culex pipiens complex sp.; Heat shock protein 70 B2 OS=Culex pipiens complex sp.; Heat shock protein 70 B2 OS=Culex pipiens complex sp.; Heat shock protein 70 B2 OS=Culex pipiens complex sp.; Heat shock protein 70 B2 OS=Culex pipiens complex sp.; Heat shock protein 70 B2 OS=Culex pipiens complex sp.; Heat shock protein 70 B2 OS=Culex pipiens complex sp.; Heat shock protein 70 B2 OS=Culex pipiens complex sp.; Heat shock protein 70 B2 OS=Culex pipiens complex sp.; Heat shock protein 70 B2 OS=Culex pipiens complex sp.	tr|Q52QQ9 tr|E9P4J9; tr|E9P4I3; tr|E9P4I2; tr|E9P4F9; tr|E9P4F7; tr|E9P4F2 tr|E9P4K3; tr|E9P4J9T; tr|E9P4I3; tr|E9P4I2; tr|E9P4F9; tr|E9P4F7; tr|E9P4F2	8	12
C 16	Tubulin beta chain OS=Culex pipiens pipiens Tubulin beta chain (Fragment) OS=Culex pipiens molestus	tr|Q15G69 tr|A0A516AFJ1	32	8

C 17	**Carboxylic ester hydrolase OS=Culex pipiens pipiens; Carboxylic ester hydrolase OS=Culex pipiens; Carboxylic ester hydrolase OS=Culex pipiens complex sp. Carboxylic ester hydrolase OS=Culex pipiens pipiens; Carboxylic ester hydrolase OS=Culex pipiens pipiens; Carboxylic ester hydrolase OS=Culex pipiens; Carboxylic ester hydrolase OS=Culex pipiens; Carboxylic ester hydrolase OS=Culex pipiens; Carboxylic ester hydrolase OS=Culex pipiens; Carboxylic ester hydrolase OS=Culex pipiens complex sp. Carboxylic ester hydrolase OS=Culex pipiens complex sp. Carboxylic ester hydrolase OS=Culex pipiens complex sp. Carboxylic ester hydrolase OS=Culex pipiens pipiens; Carboxylic ester hydrolase OS=Culex pipiens pipiens; Carboxylic ester hydrolase OS=Culex pipiens pipiens; Carboxylic ester hydrolase OS=Culex pipiens;**	**tr|R9RHA5; tr|Q6QDR2; tr|V9IQI6; tr|R9RHA0; tr|R9RFM0; tr|Q8WQ89; tr|P91597I; tr|P91596I; tr|B6HY21; tr|V9IQQ3; tr|V9IQZ3; tr|V9IQQ3; tr|R9RIG8; tr|R9RIF8; tr|R9RHA5; tr|Q6QDR2; sp|P16854**	**80**	**6**

Table (2): List of identified proteins in gut of sugar fed (S) females *Cx. pipiens.*

No.	Identified Protein	Protein Accession No.	No. of Protein	
			Mosq.A	Mosq .B
S 1	**1,4-alpha-glucan branching enzyme OS=Culex pipiens pallens**	**tr|Q4F8A2**	**19**	**--**
S 2	**Putative acyl-CoA dehydrogenase OS=Culex pipiens pipiens**	**tr|C7E0P7**	**8**	**--**
S 3	**40S ribosomal protein S4 OS=Culex pipiens pallens**	**tr|Q152V2**	**7**	**--**
S 4	**Putative mitochondrial malate dehydrogenase OS=Culex pipiens pipiens**	**tr|Q15G76**	**3**	**--**
S 5	**14-3-3 epsilon (Fragment) OS=Culex pipiens**	**tr|C3V8W1**	**14**	**--**
S 6	**Ribosomal protein L22 OS=Culex pipiens pallens**	**tr|A7LBG7**	**8**	**--**
S 7	**Ribose-phosphate diphosphokinase OS=Culex pipiens**	**tr|H9D1K2**	**6**	**--**
S 8	**Catalase (Fragment) OS=Culex pipiens**	**tr|F8RUB4**	**3**	**--**
S 9	**60S ribosomal protein L18 OS=Culex pipiens pipiens**	**tr|Q15G74**	**3**	**--**
S 10	**Fatty acid synthase (Fragment) OS=Culex pipiens**	**tr|Q56GM1**	**2**	**--**
S 11	**Putative fatty acid synthase S-acetyltransferase OS=Culex pipiens pipiens**	**tr|C7E0P0**	**6**	**--**
S 12	**Translation initiation factor IF-2 OS= subsp. Culex pipiens**	**tr|B3CPD4**	**3**	**--**
S 13	**Uncharacterized protein OS= subsp. Culex pipiens**	**tr|B3CLW0**	**1**	**--**
S 14	**Uncharacterized protein OS= subsp. Culex pipiens**	**tr|B3CN19**	**2**	**--**
S 15	**Chaperone protein ClpB OS= subsp. Culex pipiens**	**tr|B3CPG5**	**1**	**--**
S 16	**Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex OS=Wolbachia pipientis subsp. Culex pipiens (strain wPip)**	**tr|B3CMX8**	**3**	**--**

S 17	**Putative 3-hydroxyacyl-CoA dehyrogenase OS=Culex pipiens**	**tr|C7E0Q3**	**5**	**--**
S 18	**Putative beta-ketoacyl-CoA thiolase OS=Culex pipiens**	**tr|C7E0Q9**	**1**	**--**
S 19	**Putative 3,2-trans-enoyl-CoA isomerase OS=Culex pipiens**	**tr|C7E0Q5**	**2**	**--**
S 20	**Putative fatty acid synthase S-acetyltransferase OS=Culex pipiens pipiens**	**tr|C7E0P1**	**2**	**--**
S 21	**Glycogen [starch] synthase OS=Culex pipiens pipiens**	**tr|A0A0E3X6T0**	**1**	**--**
S 22	**Phosphoglycerate kinase OS= subsp. Culex pipiens**	**SP|B3CM25**	**2**	**--**
S 23	**Complex III subunit 3 (Fragment) OS=Culex pipiens pipiens;** **Complex III subunit 3 (Fragment) OS=Culex pipiens pipiens;** **Complex III subunit 3 OS=Culex pipiens pipiens x Culex pipiens molestus;** **Complex III subunit 3 OS=Culex pipiens pipiens x Culex pipiens molestus;** **Complex III subunit 3 OS=Culex pipiens molestus;** **Complex III subunit 3 OS=Culex pipiens pipiens x Culex pipiens molestus;** **Complex III subunit 3 OS=Culex pipiens pipiens x Culex pipiens molestus;** **Complex III subunit 3 OS=Culex pipiens molestus;** **Complex III subunit 3 OS=Culex pipiens molestus;** **Complex III subunit 3 OS=Culex pipiens pipiens;** **Complex III subunit 3 OS=Culex pipiens molestus;** **Complex III subunit 3 (Fragment) OS=Culex pipiens pipiens;** **Complex III subunit 3 (Fragment) OS=Culex pipiens pipiens;** **Complex III subunit 3 OS=Culex pipiens pipiens**	**tr|F8T4N5;** **tr|F8T4N3;** **tr|A0A221HK11;** **tr|A0A221HK04;** **tr|A0A221HK01;** **tr|A0A221HK00;** **tr|A0A221HJZ8;** **tr|A0A221HJZ7;** **tr|A0A221HJZ5;** **tr|A0A221HJY8;** **tr|A0A221HJY7;** **tr|A0A221HJY5;** **tr|A0A221HJY0;** **tr|A0A221HJX8;**	**1**	**--**
S 24	**Putative acyl-CoA dehydrogenase OS=Culex pipiens pipiens**	**tr|C7E0P9**	**1**	**--**
S 25	**Cytochrome b OS=Culex pipiens pallens;** **Cytochrome b OS=Culex pipiens pipiens;** **Cytochrome b OS=Culex pipiens pipiens;**	**tr|A0A1B0XB39;** **tr|E9NVR7;** **tr|E9NVP1**	**5**	
S 26	**30S ribosomal protein S15 OS= subsp. Culex pipiens**	**SP|B3CM20**	**1**	**--**
S 27	**Putative fatty acid binding protein OS=Culex pipiens pipiens**	**tr|C7E0R0**	**1**	**--**
S 28	**CidA IV(Epsilon/1) protein OS= Culex pipiens;** **CidA III(Gamma/8) protein OS= Culex pipiens ;** **CidA III(Gamma/5) protein OS= Culex pipiens;** **CidA III(Gamma/6) protein OS= Culex pipiens ;** **CidA_IV delta/1 protein OS= Culex pipiens;** **CidA_IV alpha/1 protein OS= Culex pipiens;** **CidA_IV beta/1 protein OS= Culex pipiens;** **CidA_I alpha/1 protein OS= Culex pipiens;** **CidA III(Beta/8) protein OS= Culex pipiens;** **CidA_III beta/2 protein OS= Culex pipiens;** **CidA_III beta/1 protein OS= Culex pipiens**	**tr|A0A6M2YSV0;** **tr|A0A5B8WRL3;** **tr|A0A5B8WMH2** **tr|A0A5B8WM71;** **tr|A0A2K9VS11;** **tr|A0A2K9VS06;** **tr|A0A2K9VS04;** **tr|A0A2K9VRZ6;** **tr|A0A2K9VRZ1;** **tr|A0A2K9VRZ0;** **tr|A0A2K9VRY9**	**2**	**--**

S 29	Peptide deformylase OS= Culex pipiens (strain wPip)	SP|B3CMB1	1	--
S 30	Uncharacterized protein OS= Culex pipiens	tr|B3CPS2	1	--
S 31	Uncharacterized protein OS= Culex pipiens	tr|B3CP14	1	--
S 32	Putative ribosomal protein L19 OS=Culex pipiens pipiens	tr|B9V0F4	1	--
S 33	BolA-like protein OS= Culex pipiens (strain wPip)	tr|B3CND3	1	--
S 34	Puative minor tail protein z OS= Culex pipiens	tr|B3CP29	1	--
S 35	Putative exported protein OS= Culex pipiens	tr|B3CLI7	6	--
S 36	Type I secretion system ATPase OS= Culex pipiens	tr|B3CLX9	1	--
S 37	Putative phage protein OS= Culex pipiens (strain wPip)	tr|B3CNU6	1	--
S 38	TrbL/VirB6 plasmid conjugal transfer family protein OS=Wolbachia pipientis subsp. Culex pipiens (strain wPip)	tr|B3CLE1	1	--
S 39	Proteasome subunit beta OS=Culex pipiens pallens	tr|G9L9K7	1	--

Table (3): List of identified proteins in gut of blood fed (B) females *Cx. pipiens.*

No.	Identified Protein	Protein Accession No.	No. of Protein	
			Mosq.A	Mosq .B
B 1	Esterase B1 OS=Culex pipiens	sp|P16854	--	5
B 2	Putative chymotrypsin-like protein OS=Culex pipiens pallens	tr|Q8T9R6	--	10
B 3	Putative transposase OS= Culex pipiens (strain wPip)	tr|B3CP43	--	31
B 4	Trypsin 5G1 (Fragment) OS=Culex pipiens complex sp. Trypsin 5G1 (Fragment) OS=Culex pipiens complex sp. Trypsin 5G1 (Fragment) OS=Culex pipiens complex sp. Trypsin 5G1 (Fragment) OS=Culex pipiens complex sp. Trypsin 5G1 (Fragment) OS=Culex pipiens complex sp. Trypsin 5G1 (Fragment) OS=Culex pipiens complex sp. Trypsin 5G1 (Fragment) OS=Culex pipiens complex sp. Trypsin 5G1 (Fragment) OS=Culex pipiens complex sp. Trypsin 5G1 (Fragment) OS=Culex pipiens complex sp. Trypsin 5G1 (Fragment) OS=Culex pipiens complex sp. Trypsin 5G1 (Fragment) OS=Culex pipiens complex sp. Trypsin 5G1 (Fragment) OS=Culex pipiens complex sp. Trypsin 5G1 (Fragment) OS=Culex pipiens complex sp. Trypsin 5G1 (Fragment) OS=Culex pipiens complex sp.	tr|E9P4Z4; tr|E9P4Z3; tr|E9P4Z0; tr|E9P4Y9; tr|E9P4Y8; tr|E9P4Y7; tr|E9P4Y5; tr|E9P4Y4; tr|E9P4Y2; tr|E9P4Y0; tr|E9P4X9; tr|E9P4X8; tr|E9P4X7; tr|E9P4W6	--	1
B 5	Vacuolar ATP synthase subunit B, OS=Culex pipiens complex	tr|E9P430	--	1
B 6	VP1 (Fragment) OS=Culex pipiens densovirus; VP1 (Fragment) OS=Culex pipiens densovirus; VP1 (Fragment) OS=Culex pipiens densovirus; VP1 (Fragment) OS=Culex pipiens densovirus; VP1 (Fragment) OS=Culex pipiens densovirus; VP1 (Fragment) OS=Culex pipiens densovirus; VP1 (Fragment) OS=Culex pipiens densovirus; VP1 (Fragment) OS=Culex pipiens densovirus;	tr|A0A6B9LUW1; tr|A0A6B9LSB6; tr|A0A6B9LLF6; tr|A0A6B9LLD6; tr|A0A6B9LKF9; tr|A0A6B9LJV8; tr|A0A6B9LJC3; tr|A0A6B9LJ96;	--	30

	VP1 (Fragment) OS=Culex pipiens densovirus	**tr|A0A6B9LJ84**		
B 7	**Efflux transporter, RND family, MFP subunit OS=Wolbachia pipientis subsp. Culex pipiens (strain wPip)**	**tr|B3CND0**	**--**	**1**
B 8	**Putative juvenile hormone resistant protein OS=Culex pipiens; Methoprene-tolerant protein OS=Culex pipiens pipiens**	**tr|Q5EFD7; tr|Q4ZH01**	**--**	**6**
B 9	**Putative membrane protein OS= Culex pipiens**	**tr|B3CLV5**	**--**	**1**
B 10	**ORF3 OS=Culex pipiens associated Tunisia virus**	**tr|A0A345GPS1**	**--**	**2**
B 11	**30S ribosomal protein S20 OS= Culex pipiens; 30S ribosomal protein S2 OS= Culex pipiens**	**sp|B3CMH0 sp|B3CNG9**	**--**	**3**
B 12	**Chymotrypsin-like OS=Culex pipiens**	**tr|Q56GM2**	**--**	**1**
B 13	**Pdz protein (Fragment) OS=Culex pipiens pipiens**	**tr|A0A0E3T0Z1**	**--**	**1**
B 14	**Outer membrane protein assembly factor BamD OS=Wolbachia pipientis subsp. Culex pipiens (strain wPip)**	**tr|B3CND9**	**--**	**1**
B 15	**Chaperone protein HtpG OS= Culex pipiens**	**tr|B3CLW2**	**--**	**10**
B 16	**GTP-binding protein, typA OS= Culex pipiens**	**tr|B3CNK4**	**--**	**2**
B 17	**Structural protein VP1 OS=Culex pipiens densovirus**	**tr|C4P0S3**	**--**	**3**
B 18	**Cytochrome P450 OS=Culex pipiens pallens; Cytochrome P450 OS=Culex pipiens pallens**	**tr|Q6DLW0; tr|Q58I35**	**--**	**1**
B 19	**Single-stranded-DNA-specific exonuclease RecJ OS=Culex pipiens (strain wPip)**	**tr|B3CLH0**	**--**	**2**
B 20	**Putative phage related protein OS= Culex pipiens; Putative phage related protein OS= Culex pipiens; Putative phage related protein OS= Culex pipiens**	**tr|B3CPN8 tr|B3CPB5; tr|B3CNV7**	**--**	**20**

LC/MS instrument with a TripleTOF^{TM} 5600 + (AB Sciex, Canada) an online was used to analyse quantified samples of the gut to study the total number of peptide profiles produced in the sugar and blood feeding of *Cx. pipiens* female mosquitoes. The total ion chromatography (TIC) the LC/MS spectra of the extracted gut from *Cx. pipiens* females at sugar and blood feeding show the remarkable complexity of peptides found in this species (**Figure 4**). The LC/MS analysis revealed molecular weight of proteins ranging from 800.4 to 2969.4 Da in sugar fed and from 818.3 to 3207.6 Da in blood fed *Cx. pipiens*. The box blot chart depicts the molecular mass delivery of the components in *Cx. pipiens* gut blood and sugar feeding (**Figure 5**).

Figure (6), Tow way heat map cluster showing 17 common protein between females of *Cx. pipiens* at 3-5 days of sugar and after 6 hrs. blood feeding mosquito in addition to specific protein presence only in blood feeding mosquito and sugar feeding mosquito, revealing that the blood feeding mosquito characterized by the presence of 20 proteins while the sugar feeding of mosquito were characterized by the presence of 39 proteins.

RA ordination graph observed the distance correlation between gut of *Cx. pipiens* females revealing the specific protein content for sugar and blood feeding mosquitoes and proving the direct effect of changing the feeding diet and it relation to the protein creation and activation within the gut of each mosquito, which means that the direct change to diet of mosquitoes can have a direct effect on protein creation and digestion system modification according to diet changing. Thus the food type has ignition to cell genomic protein creation for investigated mosquito this action could be globally to animal kingdom. There are for 56 exclusive proteins in sugar fed mosquito and 37 exclusive proteins in blood fed mosquito these shows in **Figure (7).**

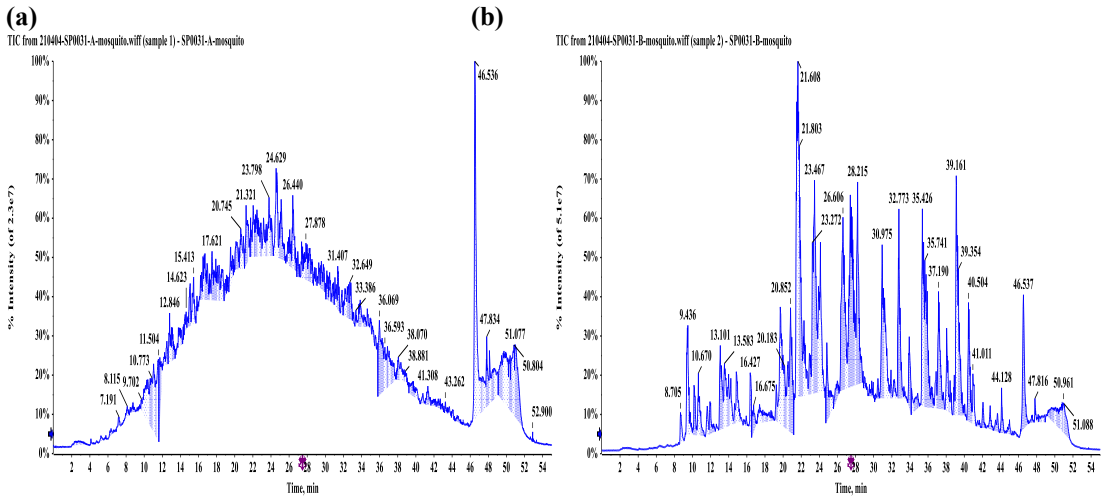

Figure 4: Total ion chromatography (TIC), shows LC/MS chromatograms of (a) Gut of *Cx. pipiens* after 3-5 days of sugar fed, (b) Gut of *Cx. pipiens* after 6 hrs of blood fed.

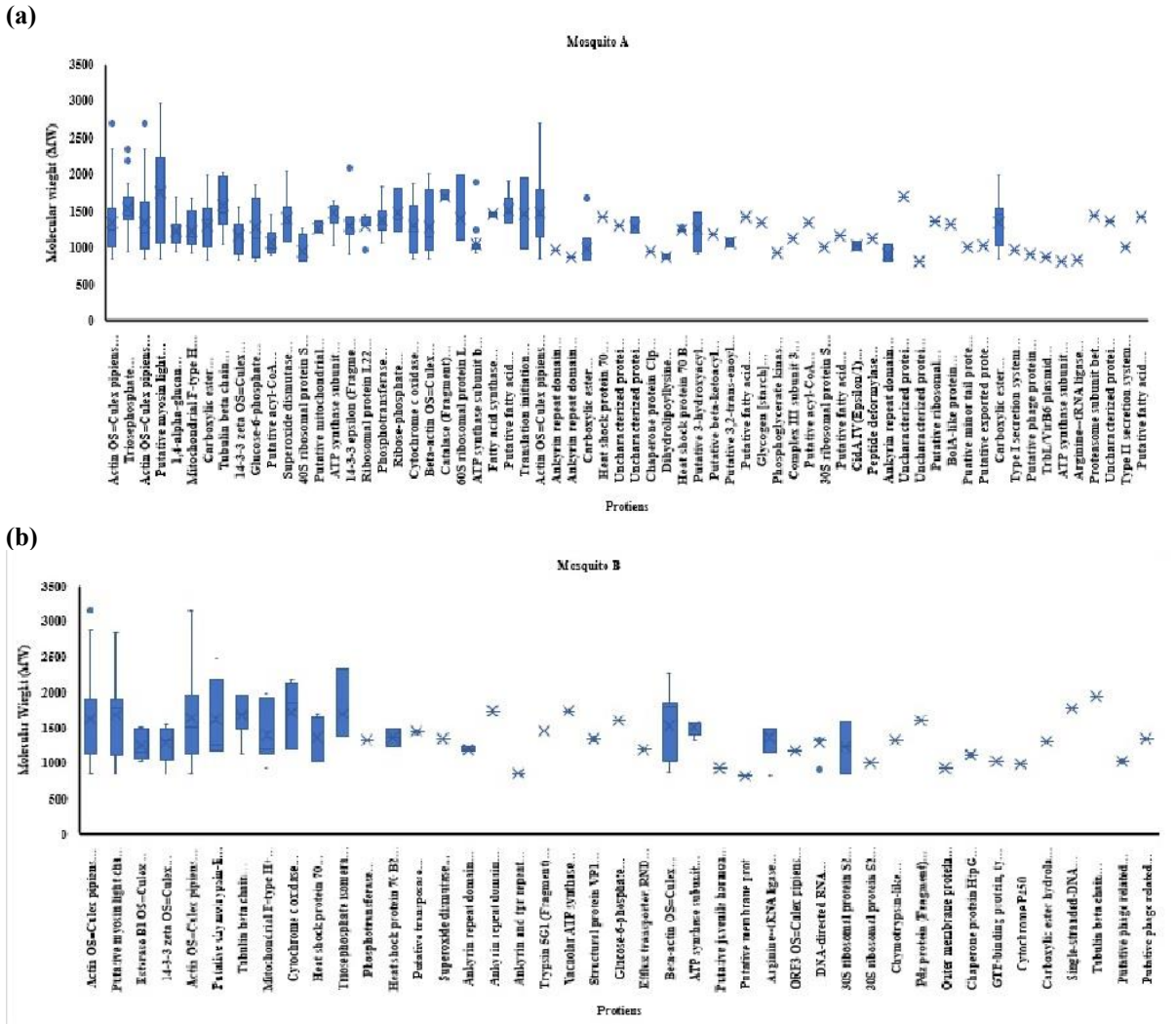

Figure 5: Box blot chart revealing the molecular weight distribution of protein for gut of *Cx. pipiens* females at sugar feeding (a) and blood feeding (b).

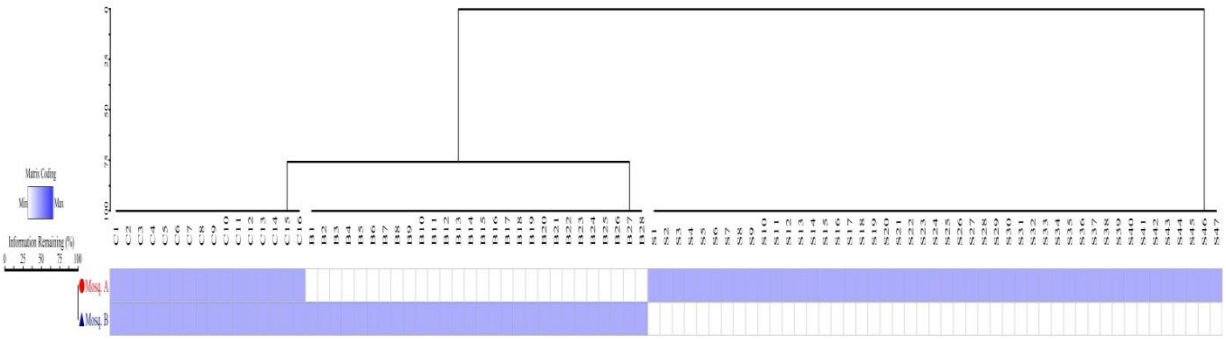

Figure 6: Clustering Heatmap analysis showing 17 common protein, 20 exclusive proteins in gut of blood fed *Cx. pipiens* females (Mosq. B) and 39 proteins in gut of sugar fed (Mosq. A), C 1-17 represents common protein, S 1-39 represents sugar fed mosquito (Mosq. A) and B 1-20 represents blood fed (Mosq. B).

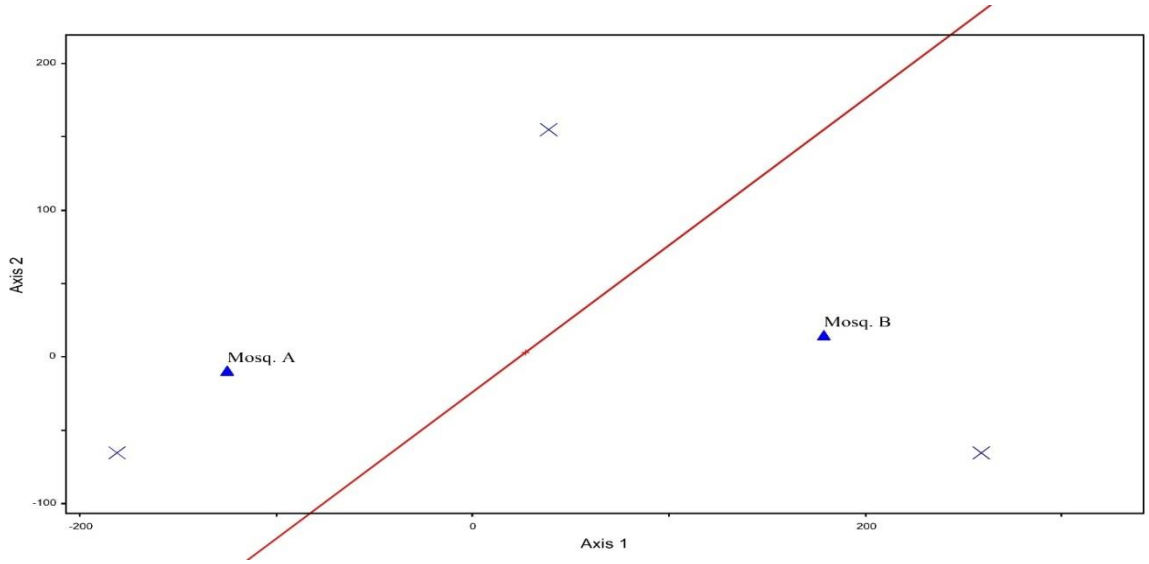

Figure 7: Principal components of RA ordination chart revealed the distance correlation between gut of female *Cx. pipiens* after 3-5 days of sugar fed (Mosq. A) and after 6 hrs from blood fed.

4. Discussion:-

Exact identification of mosquito species is climacteric in vector control programmes because only a few mosquito species play an important role in disease transportation **[32].**

A mass spectrometry based proteomic analysis was performed in this study to identify protein changes after blood feeding in the guts of *Cx pipiens* which may provide better understanding the interactions between vectors and their hosts subsequently, developing effective control strategies as well as facilitating further reverse genetic analyses (RNAi mediated knockdown). According to our findings, the majority of the annotated proteins are predicted to be involved in carbohydrate metabolism, energy metabolism, biosynthesis, protein synthesis, cytoskeleton nuclear function associated, transport, protein folding, signal transduction, unassigned, cell redox haemostasis, protease, anti-haemostatic, stress response, development,calcium haemostasis, diverse functions, cell adhesion, detoxification, immune iron haemostasis and metabolic process.

Common proteins were Actin, Beta-actin, Tubulin beta chain, Putative myosin light chain, Triosephosphate isomerase, carboxylic ester hydrolase, 30S ribosomal protein, Mitochondrial -transporting ATPase subunit b,,Arginine--tRNA ligase 3-3-14 zeta, Glucose-6-phosphate isomerase, Superoxide dismutase, ATP synthase subunit alpha and beta, Phosphotransferase, Cytochrome c oxidase subunit, Ankyrin repeat domain protein and Heat shock protein.

In sugar fed, the proteins were 1,4 -alpha-glucan branching enzyme, Putative acyl-CoA dehydrogenase, 3-hydroxyacyl-CoA dehyrogenase, Putative beta-ketoacyl-CoA thiolase, Putative 3,2-trans-enoyl-CoA isomerase, 40 and 60 S ribosomal protein, Putative mitochondrial malate dehydrogenase Epsilon, Ribose-phosphate diphosphokinase, cabalase, Fatty acid synthase, Putative fatty acid synthase S-acetyltransferase, Translation initiation factor, Chaperone protein ClpB, Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase, Glycogen synthase, Phosphoglycerate kinase, Cytochrome b, Putative fatty acid binding protein, CidA IV(alpha, beta, delta, Epsilon), CidA III(Gamma/5,6,8) CidA_I alpha, CidA III(Beta/1,2,8), Peptide deformylase, BolA-like protein, Puative minor tail protein, Putative phage protein and TrbL/VirB6 plasmid conjugal transfer family.

In blood fed, the identified proteins were Esterase, Putative chymotrypsin-like protein, Putative transposase, Trypsin 5G1, Vacuolar ATP synthase subunit B, Structural protein VP1, Efflux transporter RND family MFP subunit, Putative juvenile hormone resistant protein, Methoprene-tolerant protein, Putative membrane protein, ORF3 associated Tunisia virus, Chymotrypsin-like, Pdz protein, Outer membrane protein assembly, Chaperone protein HtpG, GTP-binding protein, Cytochrome P450, Single-stranded-DNA-specific exonuclease and Putative phage related protein. As a result, our findings suggest that the protein identified in the midgut of blood fed mosquitoes may act as a survival factor against unwanted microorganisms or pathogens, as well as external stress that is exacerbated during blood feeding. Autophagy, as previously stated, has been linked to insect epithelium protection against blood digestion products **[33].**

Mosquito midgut is responsible for maintaining ion transmit, lipid, amino acid and sugar absorption and is made up of a monocular layer of columnar epithelial cells resting on a continuous basal lamina or basement membrane **[34].** The midgut is important in physiological processes because it is the first place for blood digestion and nutrient absorption **[35].** Adult female mosquitoes expend two to three times their normal body weight when they take a blood meal **[36].** The release of a significant quantity of free heme into the midgut cavity due to the digestion of the blood meal **[37, 38].** The many digestive enzymes that digest the protein-rich blood meal in the midgut into free amino acids that are used for vitellogenin, after a blood meal (egg yolk protein) biosynthesis and egg development are trypsin and chymotrypsin. **[39-41],** indicated that the blood meal stimulates the gut epithelial cells to synthesize and secrete trypsin-like proteins into the lumen. Within an hour of a female mosquito consuming a blood meal the gut surrounds the blood meal with a perithrophic membrane **[42].** The membrane acts like a sieve and retains molecules that are larger than 25 KD including trypsin inhibitors that are part of the blood meal. Trypsin is secreted from the epithelial cells into a discrete compartment between the perithrophic membrane and the gut epithelial cells called the ectoperithrophic space. Trypsin with a Mr of less than 25 KD penetrates the perithrophic membrane and starts to digest the blood clot around its periphery, reducing exposure to trypsin-retrained subfractions. *Ae. aegypti* females midguts undergo two stages of trypsin synthesis after ingesting a blood meal. The first stage, which lasts 4–6 hrs. after a blood meal, is distinguished by the presence of small amounts of early trypsin. The second stage, which occurs between 8 and 36 hrs. after blood feeding, is distinguished by the presence of large amounts of late trypsin **[43].**

After a blood meal, enzymes involved in power metabolism, like glucose dehydrogenase and aldehyde dehydrogenase, were found to be over expressed. The enzyme aldehyde dehydrogenase has been linked to the control of juvenile hormone (JH) synthesis in blood fed mosquitoes **[44].** JH regulates reproductive maturation in adult female mosquitoes **[45]** and induces the vitellogenesis **[46].** Other over, the antennal carrier protein TOL-2 (JH binding domain), takeout protein (JH binding domain) and odorant-binding proteins (OBP39) were expressed proteins involved in blood feeding method regulation **[47, 48].**

Mosquitoes face significant oxidative stress, which must be prevented by antioxidant defenses or heme detoxification mechanisms due to blooding feeding **[37 and 49]. [50 and 51],** used LC-MS/MS analysisfor protein profiling of both permethrin-resistant and susceptible strain of *Aedes aegypti* the most significant related proteins that functions to

protect and defence for the survival against pyrethroids insecticides are alkaline phosphatase, apolipophorin II, E3 ubiquiton-protein ligase, heat shock protein HSP70, serine protease IV, serine threonine related protein, DNAJ chaperone and cytochrome c oxidase subunit. While, the remaining proteins expressed in pyrethroid resistant *Ae*. *aegypti* are proteins that functions for transportation, storage, midgut proteins and regulation of tissue and organ.

Proteins were involved in carbohydrate metabolism including malate dehydrogenase, dehydrogenase was reported in sugar-fed. Additionally, proteins involved in energy metabolism; ATP synthase subunit proteins are also recorded in sugar fed. ATP synthase subunit α are involved in ATP metabolic process and are the major source of energy utilized for secretions **[52 and 53].**

Conclusions:-

In this study, mitochondrial COI was used to confirm morphological identification and discrimination of *Cx. pipiens* mosquitoes. Identifying protein differences between sugar and blood feeding of *Cx. pipiens* guts provides effective and novel targets for molecular-based vector borne disease control.

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Author contributions:

RE and SM designed, performed experiments, analyzed the data and wrote the original draft. AB and WE reviewed and edited the final version. All authors read and approved the final manuscript.

Declarations

Data availability:

The datasets generated and analyzed during the current study are available in the manuscript.

Funding:

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Consent for publication: Not applicable.

Conflict of interest: The authors declare that they have no conflict of interest.

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