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GUT MICROBIOME OF HONEY BEE - AN INDUSTRIALLY RELEVANT POLLINATOR

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ABSTRACT

Metagenomics is an important tool to examine the gut microbiota of honey bee [Apis mellifera] – the producer of industrially relevant products such as honey, propolis, royal jelly and wax. Metagenomics approach is useful in analysis of various microbiota factors influencing apiculture: biofilm formation, carbohydrate metabolism, colony collapse disorder, disease progression, horizontal gene exchange, niche adaptation, nutrition.These factors are responsible for increase or decrease in the production of above said industrially relevant products. Causative agents of colony collapse disorder in honey bee namely bacteria, parasites, virus have been identified in the gut through metagenomics. Shotgun and high-throughput are the widely used methods for sequencing of honey bee gut metagenome. Bioinformatics tools, softwares and databases are used for sequence pre-filtering, assembly, identification of microbial diversity, data integration and data analysis of the honey bee gut metagenome. Honey bee gut metagenome serve as biologically relevant marker of colony health. Hygiene, disease resistance, antibiotic resistance, nutrient productions are the key areas governed by the gut microbiota of honey bee. Honey bee gut microbiota act as forerunner for studying gut of higher animals. Honey bee gut is the new paradigm to study the role of beneficial and pathogenic microorganisms.

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KEY WORDS

Antibiotic resistance; bioinformatics; colony collapse disorder; honey bee gut; metagenomics

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INTRODUCTION

When Beekeeping industry has gained importance because of two outcomes: direct products [honey, wax, propolis and royal jelly] of the industry and crop pollination of bees. The value of these direct products and pollination service industry is estimated to be around US$1.2 billion [1] and €22.8-57 billion respectively [2]. This industry is under threat due to Colony Collapse Disorder [CCD]- the sudden loss of worker bees attributed to pathogens [bacteria, parasites and virus], immunodeficiency [due to travel / poor diet], antibiotics, fungicides and pesticides [3, 4]. All these factors affect the normal flora of honeybee gut. In recent times, beekeeping industry has incurred losses worth billions of US$ worldwide due to CCD [4- 7]. Although, various physical and chemical methods [4, 6] have been put forth to analysis and contain CCD, it proved to be futile.

Metagenomics has emerged as a crucial method in assessment of gut microbiota of healthy as well as CCD honey bees [8, 9]. Shotgun [8] and high-throughput [9] are the widely used methods for sequencing of healthy and CCD honey bee gut metagenomes. Bioinformatics tools, softwares and databases are vital for genome [metagenome in particular] sequencing [10], pre-filtering [11], assembly [12] identification of microbial diversity [10, 11, 13-16], data integration and data analysis [12, 17- 20].

Metagenomics is useful in analysis of diverse microbiota factors influencing apiculture: biofilm formation [9], carbohydrate metabolism [9], colony collapse disorder [21], disease progression [22], horizontal gene exchange [23], niche adaptation [8, 9, 22, 23], nutrition [9, 22, 24]. Causative agents of CCD namely bacteria [25], parasites [21], virus [21] have been identified in the gut of the honey bee through metagenomics. Thus, metagenomics is a significant tool to study the gut of honey bee – the producer of industrially important products and crop pollinator.

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HONEY BEE GUT METAGENOME AND METATRANSCRIPTOME ISOLATION

Generally gut microbiota DNA [metagenomic DNA] is used as template for sequencing rather than RNA [metatranscriptome RNA] due to high abundance of rRNA's in the metatranscriptome [26] CTAB [DNA] and Trizol® [RNA] are the methods used for extractions for respective metagenome and metatranscriptome of honey bee gut [26]. These methods are time consuming and result in less purification factor. However, these methods require lesser monetary resources and the reagents used in these methods can be prepared in bulk for large number of metagenomic samples.

Apart from these conventional methods, a wide variety of kits are available in the market for gut metagenome / metatranscriptome isolation **[Table–1]**. Most of the honey bee gut metagenomic / metatranscriptome studies [9, 25, 26, 27, 28, 29, 30] utilize Qiagen [Hilden, Germany] manufactured kits [Gentra Puregene Cell Kit, Gentra Puregene Tissue Kit, DNeasy Blood & Tissue Kit, QIAamp DNA Mini Kit, RNeasy Mini Kit]. Other prominent kits utilised for gut metagenomic / metatranscriptome isolation are: UltraClean® Tissue & Cells RNA Isolation Kit, RNA PowerSoil® Total RNA Isolation Kit [31], NucliSENS® easyMAG®[32], RNAqueous®-Micro Total RNA Isolation Kit [33] and FastDNA® SPIN Kit [34] **[Table–1]**.

Gentra Puregene Cell and Gentra Puregene Tissue Kits utilize salting-out precipitation technology to isolate metagenomic DNA from honey bee gut [35, 36]. DNeasy Blood & Tissue kit, QIAamp DNA Mini Kit and RNeasy Mini Kit utilizes Silica technology [37]. Compared to CTAB [DNA] and Trizol® [RNA] methods, these kits provide high quality metagenomic DNA / metatranscriptome in terms of recovery, purification factor and less processing time [35- 37].

BIOINFORMATICS TOOLS, SOFTWARES AND DATABASES – ESSENTIAL FOR HONEY BEE METAGENOMICS

After the isolation, purification and estimation of metagenomic DNA from honey bee gut, the next step is the sequencing. Bioinformatics softwares, tools, on-line programs / resources and databases are highly essential for metagenome sequencing, pre-filtering, assembly, classification of microbial diversity, data incorporation and its analysis **[Table–2]**. Metagenome sequencing requires robust platforms to complete the task with high accuracy, low cost and greater sequencing reads output. IGA IIx [Illumina Genome Analyzer IIx] and R 454 TS [Roche 454 Titanium Sequencer, Roche Applied Science, Indianapolis, IN] are extensively used sequencing platforms for honeybee gut metagenome sequencing projects [8, 9, 21, 23, 24]. IGA IIX generates reads of length between 35-100 bp. It creates an output of 100 billion bp. [38]. R 454 TS creates ~1,000,000 shotgun Reads per Run and generates reads of length up to 1,000 bp and [8, 9, 22- 24]. It has the utmost consensus precision of about 99.97 % [39]. These two robust sequencing platforms have insured success of honeybee gut metagenome projects.

After the sequencing is completed, pre-filtering of the sequences is essential to sustain the quality of read pairs [9]. Further, the sequences are subjected to a gap-closing examination. RDP-II [40] is the most accepted database for sequence pre-filtering and gap-closing [41]. RDP-II has many applications namely: alignment, annotation, examination and phylogenetically consistent taxonomic support for metagenomic data [40]. Other pipeline includes Pyrotagger [42]. Pyrotagger provides following metagenomic services: filtering of quality reads, trimming of the read span, high proportion of clustering [using pyroclust algorithm], sorting and cataloguing of data [42]. It processes reads of up to 100000 quantities [42].

Assembly of the sequences is the next step. RDP-II [30, 40], Velvet v1.2.10 [43] are used for the assembly of honeybee gut metagenome [9]. Velvet v1.2.10 is a de novo metagenome assembler [44]. It works on the principle of de Bruijn graphs and able to assemble short and longer reads [43, 44].

Annotation of the genes is the key step in the identification of microbial diversity [taxonomic profiling] in the honey bee gut metagenome. MetaPhyler [45], Integrated Microbial Genomes /Metagenome Expert Review [IMG/MER] [46] are used for annotation of assembled honeybee gut metagenome with the availability of metagenome datasets. MetaPhyler [45] uses phylogenetically linked marker genes for taxonomic cataloguing of metagenomic reads. It works on the principle of BLAST. MetaPhyler is considered to be superior compared to its predecessors [PhymmBL, MEGAN] [47]. IMG/MER [46] has numerous tools for analysis [IMG/M UI Map] that provides the

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users [with private password] for thorough assessment and modification of the honey bee gut metagenome sample[s]' annotations [48].

Geneious Pro v5.6 [49] is used for honeybee gut metagenome data integration. It has various applications: metagenome browsing, phylogenetic tree construction, metagenome grouping visualization [50]. It can handle data from wide variety of sequencing platforms: Sanger, NGS, barcoded. It works on almost in all computer platforms: Windows, Mac and Linux [50].

IMG/MER [46], MEGAN [47], MOTHUR [30, 51] are used for honeybee gut metagenome data analysis. MEGAN performs taxonomic study, efficient examination using following classifications: COG/NOG, KOG, KEGG, SEED [52]. It is used for relative visualization, rarefaction analysis, principal coordinate analysis and clustering of metagenome data [52]. It also provides various charts and plots: space-filling radial trees, wordclouds, bubble charts, co-occurrence plot [52]. Metadata specifying and viewing of metagenome samples as rared/shared/core/total biomes is possible with the use of MEGAN [53]. MOTHUR [30, 51] analyzes honey bee gut metagenome data generated by wide range of sequencing platforms namely: Illumina [HiSeq/MiSeq], IonTorrent, Sanger and 454 [54].

NCBI, ARB-SILVA, Greengenes and RDP databases [55] serve as vital repository for honey bee gut metagenomes. In NCBI, there are at present 3 BioProjects related to honey be gut metagenomes [1, 56]. SILVA is a database of ARB software package [57]. ARB has a graphic, integrated environment of software tools for receiving and analysis of honey bee gut metagenome sequence information [57]. Greengenes is used for browsing, exporting, comparing, searching, aligning, trimming of honey bee gut metagenome data [58].

MICROBIOTA OF HONEY BEE IDENTIFIED BY METAGENOMICS

After performing metagenome sequencing, pre-filtering, assembly, identification of microbial diversity, "data integration and its analysis" is the key to unlock the potential of the microbiota of honey bee gut. Gilliamella and Snodgrassella **[Table–3]** are found to be predominantly present [59] in all the honey bee gut metagenomes: Gilliamella apicola [9, 8, 29], Gilliamella sp. [9], G. apicola wkB1T [23], Snodgrassella alvi [8,9], Snodgrassella sp. [9], S. alvi wkB2T [23]. Both, G. apicola and S. alvi are present only in pH neutral hive niches [60]. G. apicola and S. alvi showed high percentage of 16S rRNA similarity and multiple strains are present in the same gut sample [59] because of their frequent recombination. Both, phylogenies of G. apicola and S. alvi show major similarity with the phylogenies of their hosts [29]. G. apicola is present generally in the midgut and hindgut regions [60]. Moreover, significant numbers of recombination events occur in Gilliamella sp when compared to S. alvi [61, 62]. One phylotype within Betaproteobacteria ["Candidatus S. alvi"], and one within Gammaproteobacteria ["Candidatus G. apicola"] are present in each and every one of the gut metagenomes suggesting the importance of Snodgrassella and Gilliamella [61].

Actinobacteria, γ-Proteobacteria [Frischella perrara] and Bacilli, the core group of bacteria responsible for breakdown of polysaccharides, fermentation and production of honey [24, 63] have been identified by metagenomics. A key family namely Acetobacteraceae responsible for microbial persistence in larval stage is present in first and second larval instars[22]. γ-proteobacterial species [having genes encoding pectin-degrading enzymes] responsible for breakdown of pollen walls and honey formation [9] have been identified by metagenomics. Bifidobacterium sp. [31, 34], Lactobacillus kunkeei [60], Acetobacteraceae and Lactobacillus sp., the probiotics species are present in honey bee gut [9]. Bifidobacterium sp. provides defensive mechanism to honey bee to ward off potential pathogens [31]. L. kunkeei controls the larval gut and beebread, the key niches of honey bee [22]. These species are responsible for maintaining the probiotic nature of the gut.

CCD as described earlier is responsible for loss of billions of US$ to the beekeeping industries in the continents of Americas [4, 6], Europe [5], and Africa [7]. Indiscriminate use of pesticides, antibiotics in beekeeping industries has led to development of pesticide and antibiotic resistance in the worker honeybees [3]. All these factors affect the normal flora of honeybee gut leading to the large scale collapse of honeybee colonies [2, 21]. Metagenomics unlike erstwhile physical and chemical methods has able to recognize and differentiate the normal gut microbiota and causative agents [bacteria, parasites and virus] of CCD. Metagenomics analysis has prevented further loss of worker bees due to CCD.

Pathogenic bacteria, fungi, parasite, virus to blame for loss of honey bee colonies have been identified by metagenomics. Principal pathogens include Burkholderia [25], Wolbachia, Mucor hiemalis, Nosema apis, N.

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ceranae [64], Crithidia [65], Lake Sinai virus [LSV] [27, 33], black queen cell virus [BQCV], acute bee paralysis virus [ABPV], deformed wing virus [DWV] [28], Israeli acute paralysis virus [IAPV] [27], Kashmir bee virus [KBV] and sacbrood virus [SV] [21]. Wolbachia is an persistent honey bee associate. Among these pathogens Israeli acute paralysis virus is found to be highly contagious [21]. Burkholderia [25] is found in lone individual bees. M. hiemalis kills honey bees under various conditions [21]. Nosema species [N. apis, N. ceranae] is very much prevalent [21] in honey bees [100% in case of CCD]. N. ceranae works in synergistic with the wide variety of virus: BQCV, ABPV, DWV, KBV [33] to cause CCD.

Through gut metagenomics, an 18S rRNA gene of about ~700 nucleotide section is identified to be of the parasite Crithidia [21]. Crithidia is controlled in gut by the presence of Gilliamella [65]. New strains of LSV [27,33] have emerged in recent times in both USA and Europe. ABPV and KBV are closely related virus responsible for CCD [33] and KBV in particular is prevelant in CCD colonies in major parts of USA [33]. DWV is responsible for loss of colonies during fall season in USA [5]. Recently, a new variant of IAPV has been identified [27]. Through metagenomics approach, it is proved that Iridovirus does not cause CCD [32].

Antibiotic resistance poses severe threat for survival of honey bee colonies. Tetracyclin resistance is a leading cause for loss of colonies [66]. Through metagenomics approach, 8 tetracycline resistance loci namely: genes coding for efflux pump and genes coding for ribosome protection are identified from microbiota associated with tetracycline exposed bee colonies [66].

GUT MICROBIOTA FACTORS INFLUENCING APICULTURE: IDENTIFIED BY METAGENOMICS

Microbiota factors are responsible for the increase in the yield of apiculture products [67]. Various metagenomics sequencing methods have been employed to study these factors **[Table–3]**. Biofilm formation by the gut microbiota is responsible for pathogen defense, thereby preventing the loss of worker bees against protozoan parasite [9]. Carbohydrate metabolism by gut microbiota results in efficient nutrient utilization leading to increase in honey production [9, 22, 24]. Disease progression and resistance is influenced by gut microbiota, thereby maintaining the general health honeybee colony [22]. Horizontal gene exchange among gut symbionts is responsible for the host specificity [23].

Niche adaptation is a critical factor for diversification of gut microbiota among different species of honey bee. This factor is responsible for breakdown of pollen walls for nutrition, energy metabolism, microbial succession [8, 9, 22, 23].

FUTURE DIRECTIONS OF HONEY BEE GUT METAGENOMICS

Future research in honey bee gut metagenomics depends upon the information generated through the sequencing projects. Further, a fully dedicated database would serve as a repository of data generated and further relevant information would be obtained. Using this information, new drug targets can be developed to counter the menace of CCD. This would result in enhancement of life span of honey bees resulting increased pollination and production of honey, propolis, royal jelly and wax.

CONCLUSION

Honey bee gut metagenome serve as indicator of its health [34] and a marked change in the metagenome composition can be used as biological marker of the colony health [25, 27, 33]. Broad-spectrum hygiene, disease prevention, synthesis of nutrients in the honey bee colonies is due to the core beneficial microbial community present in the gut metagenome [24, 31, 60]. Well-organized evolutionary aspects of honey bee gut microbiota serve as model to study gut microbiota of higher animals [8]. CCD and antibiotic resistance is comprehensively studied using metagenomics [3, 32, 66]. Metagenomics has revealed that vertical transmission of gut microbiota from mother to daughter honey bees and also role of worker bees [29, 63]. Thus, honey bee emerged as front runner in the study of gut metagenomics in particular to that of beneficial bacteria [9, 23, 30, 55].

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Table: 1. Honey bee gut metagenome DNA/RNA isolation Kits

Kit	Manufacturers	References
FastDNA® SPIN Kit	MP Biomedicals [India] Pvt Ltd, Mumbai, India	34
RNAqueous®-Micro Total RNA Isolation Kit	Invitrogen BioServices India Pvt. Ltd, Bangalore, India	33
Gentra Puregene Cell Kit	Qiagen, Hilden, Germany	9
DNeasy Blood & Tissue Kit	Qiagen, Hilden, Germany	26
QIAamp DNA Mini Kit	Qiagen, Hilden, Germany	27
RNeasy Mini Kit	Qiagen, Hilden, Germany	28
Gentra Puregene Tissue Kit	Qiagen, Hilden, Germany	25
UltraClean® Tissue & Cells RNA Isolation Kit	MO BIO Laboratories, Inc, Carlsbad, USA	31
NucliSENS® easyMAG®	bioMerieux, Inc, Durham, USA	32

Table: 2. Bioinformatics tools, softwares and databases used in honey bee gut metagenomics

Databases, Platforms, Softwares, Tools	References
Pre-filtering and gap-closing	
RDP-II	40
Pyrotagger	42
Assembly of the sequences	
RDP-II	40
Velvet v1.2.10	43
Annotation of the genes	
MetaPhyler	45
IMG/M ER	46
Data integration	
Geneious Pro v5.6	49
Data analysis	
IMG/M ER	46

Table: 3. Factors influencing apiculture identified by etagenomics

Factors	Microbiome	Sequencer
Biofilm formation	G. apicola S. alvi	IGA IIx*
Carbohydrate metabolism	Gilliamella sp. Snodgrassella sp.	IGA IIx*
	γ-Proteobacteria, Bacilli	IGA IIx*
Horizontal gene exchange	G. apicola wkB1T S. alvi wkB2T	IGA IIx*
Niche adaptation	Gilliamella sp. Snodgrassella sp.	IGA IIx*
	G. apicola S. alvi	IGA IIx*
	G. apicola wkB1T S. alvi wkB2T	IGA IIx*

IGA IIx* - Illumina Genome Analyzer IIx;

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CONFLICT OF INTEREST

There is no conflict of interest.

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