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Review Article

Metabolomics applications in bee science

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ABSTRACT

Metabolomics is a powerfull approach to investigate metabolic networks. It combines acquisition of detailed and complete chemical information via chromatographic and nuclear magnetic resonance with multivariate statistics. We reviewed metabolomic studies in bee science: 1. Bee health: Gut microbiota, nutritional stress, virus. 2. Bee pollen: Chemical profiling, 3. Cerumen: Chemical profiling. 4. Honey: Authenticity, botanical and geographical origin, mature honey. 5. Pesticides: Impact of phytochemicals in bees exposed to pesticides. 6. Pot-honey: Entomological origin, spatial memory activity, storage changes of chemical profile. 7. Pot-Pollen: Chemical profiling. 8. Propolis: Antibacterial activity of bee collected resins, chemical profiling, review of honey bee and stingless bee propolis. 9. Royal jelly: Chemical profiling. Scientific research of this review on metabolomics was done with the following species of bees: *Apis mellifera*, *Austroplebeia australis*, *Bombus terrestris*, *Heterotrigona itama*, *Meliponula ferruginea*, *Tetragonula carbonaria* and *Tetragonula hockingsi*.

Keywords bee health, bee pollen, cerumen, GC-MS, honey, metabolomics, NMR, PCA, pesticides, propolis, OPLS-DA, PLS-DA

INTRODUCTION

Metabolomics is a powerfull approach to investigate metabolic networks in agriculture, environmental, food, pharmaceutical and veterinary platforms of bee science. It combines acquisition of more detailed and complete chemical information via chromatographic (LC-MS, GC-MS) and nuclear magnetic resonance (NMR) with multivariate statistics (HCA, PCA, OPLS-DA). The use of metabolomics provides insights into physiological, pathological, and biochemical changes as useful chemical fingerprints for traceability, quality control, processing and health applications and during cellular regulation (Shepherd et al., 2011). This combined chemical

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and multivariate approach allows discoveries. Bee science of such a biodiversity of bees, their health, the physiology of one bee and the behavior of the colony, their interactions with the environment and man, the products of the bee colony, their composition, origin, and bioactivity are a vast field of study that benefits from metabolomics approach.

Honey bees are globally important plant pollinators in agriculture and natural environments, but have suffered from colony declines in recent years. Therefore, bee health is important for fruit and seed supply in our planet. Social bees harbor a simple and conserved specialized microbiota that is spatially organized into different gut compartments for pathogen protection and nutritional function (Zheng et al., 2017). Individual communities contribute to the overall metabolomic of gut microbiota.

Propolis botanical origin is difficult to plan experimentally because resin foragers are relatively rare and often forage in unobservable far tree canopies (Wilson et al., 2013). These authors highlighted the benefits of metabolomics in bee science because such a small amount of resins from the corbiculae, collected by a single foraging bee was sufficient to identify the botanical source without prior knowledge of resin composition.

Honey, pollen, bee pollem, bee bread, royal jelly, bee venom, beeswax, so many compartments to observe. Qualitative or quantitative analysis are currently investigated –for all or some metabolites in bee science. They provide a wide range of metabolomic applications from discovery to targeted analysis. We reviewed metabolomic applications in bee science. A selected a group of published research and even one service are summarized, the chemical technology and the multivariate analysis use standard abbreviations that are informed before the references.

Bee health

Gut Microbiota

Guts of adult worker honey bees contain specialized bacteria. Experimental results show that gut bacteria increase weight gain in young adult bees, affect expression of genes governing insulin and vitellogenin levels, and increase sucrose sensitivity. They also shape the gut physicochemical microenvironment, lowering pH and oxygen levels. Peripheral resident bacteria consume oxygen, thus maintaining anoxia, as required for microbial activity. Additionally, gut bacteria produce short-chain fatty acids, with acetate and propionate as the major metabolites (Zheng et al., 2017). The honey bee gut is colonized by specialized bacteria that help digest components of the floral pollen diet and produce molecules that likely promote bee health. Contrary to human gut microbiota, the bee gut is composed of only a few bacterial species. The authors measured the repertoire of metaboloma from bee guts using untargeted metabolomics (MS-Q-TOF and OPLS-DA) to identifie contributions of bacterial species to bee digestion and various strategies bacteria deploy to co-exist in the animal gut. Several species of the genus *Lactobacillus* convert flavonoids. *Bifidobacterium asteroides* trigger bee hormones to modulate the immune system and behavior of host. The gut bacteria in bees are known for honey bee health, and may extend to colony health as a whole. (Kešnerová et al., 2017).

Nutritional stress

Pollinators are stressed by environments with limited offer of nutrients. Metabolomics-based diagnostic biomarkers in hemolymph of *Bombus terrestris* fed with low-carbohydrate diet (UHPLC-Q-Orbitrap-HRMS and PCA, OPLS-DA) using machine learning algorithms on two selected biomarker sets showed that gluconeogenesis contributed significantly to sugar stability in stressed bumblebees (Wang et al. 2019).

Virus

Identifying dangerous bee viral diseases is critical to safeguard bees (Wang et al., 2021) designed a predictive model for standardized bumble bees, with non-infected bees metabolically differentiated from infected bees with Israeli acute paralysis virus (IAPV) and slow bee paralysis virus (SBPV) infections. Biomarkers for the metabolomic (UHPLC-Q-Orbitrap-HRMS and CV-ANOVA, OPLS-DA, WEKA machines learning classification algorithms) changes in the *Bombus terrestris* hemolymph following viral infection in the model successfully discriminated virus infection versus non-virus infection in wild bumble bees.

Bee Pollen

Chemical profiling

The metabolome of canola pollen is affected by irrigation showing differences in lipids and non-polar secondary metabolites. Metabolome of beehive pollen by plant source showed differences in pentose sugars, myo-inositol and furanose (Arathi et al., 2018). Honey bee colony decline world-wide may be caused by malnutrition. Optimal nutrition is a defense against multiple stressors such as parasites/pathogens and pesticides. Pollen is the primary source of protein for bees and is critical for brood rearing and colony growth. Targeted sterol analysis and untargeted metabolomics (LC-APCI-MRM, UPLC-Q-TOF-MS and ANOVA, PCA, SAMplot, dendrograms, correlation matrix) on five commercially available

crop pollens and three bee-collected crop pollens. Key phytosterols and metabolites are present in bee diets, including some of the major bee-pollinated crop pollens. The highest concentrations were observed for 24-methylenecholesterol (Chakrabarti et al., 2019).

Cerumen

Chemical profiling

Cerumen is an admixture of resins an stingless bees (Meliponini) wax used to build and protect rhe nest (Roubik, 1989). Two cerumen of *Meliponula ferruginea* from Tanzania contained substantial amounts of carbohydrates (one with major arabitol, mannitol and minor monosaccharides, one with major monosaccharides and minor sugar alcohols) and similar triterpene fingerprint possibly from a common resin source (Popova et al., 2021).

Honey

Authenticity

Control of honey frauds is needed in Ecuador to protect bee keepers and consumers because simple syrups and new syrups with eucalyptus are sold as genuine honeys. Authenticity was confirmed with ^{1}H NMR and PLS-DA. Spectra of honey dilutions in deuterated water with an enhanced amino acid region with signals for proline, phenylalanine and tyrosine were present in genuine honey. Classic quality indicators were also tested with this method (sugars, HMF), indicators of fermentation (ethanol, acetic acid), and residues of citric acid used in the syrup manufacture. Sucrose and HMF were higher in fake honey, out of honey standards. Sensory analysis was the final test to recognize the honey produced in combs by *Apis mellifera*, fake honey, and honey produced in cerumen pots by stingless bees. (Schievano et al., 2015).

Botanical origin

Honey traceability and quality analysis was done with non-targeted metabolomics-based (UPLC-Q-TOF-MSE and ANOVA, PCA, PLS-DA, VIP multivariate, Chemspider and HMDB databases) after solid-phase HLB cartridge extraction pretreatment of eight (acacia, jujube, vitex, linden, buckwheat, manuka, wolfberry, motherwort) botanical types if honey by Shen et al. (2021). Thirty-two metabolites were identified (18 flavonoids, 7 phenolic acids, 6 phenyl and terpenoid glycosides, 1 steroid). Various flavonoids were common in buckwheat and manuka honeys. Manuka honey had highest gnaphaliin and galangin 3-methyl ether. Linden honey was characterized by phenyl glycosides. Vitex honey was rich in quinic acid derivatives in addition to the flavonoids of vitexin. Ponasteroside A was a marker of jujube honey. Acacia had distinctive kaempferol derivative and 6-C-fucosyl luteolin.

Geographical origin

Geographical indication (GI) of honey by metabolomic studies for certified regions, through maps produced by VOSviewer software. Metabolomic approaches can relate specific honey attributes to the region's terroir and know-how. The evidence of GIs and metabolomics, is viewed as potential tool for marketing purposes (e.g., to assist communication of positive aspects and quality), and legal protection. This review provides a taxonomic categorization useful to government agencies to improve GIs registration systems and promotion strategies (Cassago et al., 2021).

Mature honey

During the production of honey by the honey bee, the nectar is transformed from a vegetal matrix, to a mixed vegetal-animal product. The bee transports the nectar to the hive in a stomach known as proventriculus, it is deposited in the comb and transformed with substances added by the bees and dehydration. When the honey reaches < 20% water content, the bees operculate the comb with a thin layer of beeswax. The operculated honey is mature, safely stored for the bee colony, and also ready for commercial use. Stomach honey (SH), immature honey (IMH), and mature honey (MH) were analyzed by an UPLC-QTOF-MS-based metabolomics approach with multivatiate PCA, OPLS-DA and VIP during a rapeseed flower season. MH and IMH had different metabolic profile, 3 metabolites –including decenedioic acid, were accumulated in MH. Decenedioic acid is a bee-originated fatty acid (FA) and was further confirmed in acacia, and jujube honeys by GC-MS, making it a potential marker to discriminate IMH and MH (Sun et al., 2021).

Pesticides

Impact of phytochemicals in bees exposed to pesticides

Honey bee colony losses are a negative side effect caused by pesticides used in agriculture. (Ardalani et al., 2021) investigated the effect of phytochemicals on the metabolism of pesticides with untargeted metabolomics (phytochemical composition of the diets were profiled with an UHPLC-Q Exactive-MS/MS and exposure residual pesticide concentrations were measured by LC-QTRAP-MS/MS). Honey bees were feed for two days with diets based on pollen or nectar (*Reseda odorata*, *Borago officinalis*, *Phacelia tanacetifolia*, *Trifolium repens*) and later pesticide exposure of oral 10 ng/bee imidacloprid, or contact 0.9 µg/bee tau-fluvalinate or 0.5 µg/bee tebuconazole. After 1 h oral or 24 h contact dietary pollen reduced residual imidacloprid (*Reseda*, *Phacelia*) and tebuconazole (*Reseda*) in honey bees.

Pot-Honey

Entomological origin

Here a reference from a service by PREMIER Biosoft (since 1994) on Metabolomics as last endpoint of the omics cascade, the comprehensive study of metabolites within a biological system. Due to its recent advances, metabolomics has emerged as an important tool for entomological studies. Metabolite profiling of pot-honey extracted from stingless honey bees serves to authenticate their origin. Moreover, the potential antioxidant properties of stingless bee products are used to treat diseases associated with oxidative stress, microbial infections, and inflammatory disorders.

Honeys are produced by *Apis mellifera* and stingless bees (Meliponini) in Ecuador. We studied honey produced in beeswax combs by *Apis mellifera*, and honey produced in cerumen pots by *Geotrigona* and *Scaptotrigona* bees. Signals of ^{1}H NMR spectra were integrated and used as inputs for PCA, PLS-DA analysis, and labelled sets of classes were successfully identified, enhancing the separation between the three groups of honey according to the entomological origin: *A. mellifera*, *Geotrigona* sp. and *Scaptotrigona* sp. This procedure is therefore recommended for authenticity test of honey in Ecuador (Vit et al., 2015).

Spatial Memory Activity in Mice

Heterotrigona itama honey from Malaysia was tested on Swiss albino mice during 7 and 35 days 2000 mg/kg daily oral intake (equivalent to equivalent to a human dose of 162 mg/kg). RT-PCR for gene expression of mice striatum, and NMR for metabolomics analysis of the honey A Morris water maze (MWM) behaviour analysis. Spatial working memory and spatial reference memory of mice improved in the honey-treated groups compared to the control group. Gene expression demonstrated significant upregulation of BDNF and Itpr1 genes involved in synaptic function. Phenylalanine, an essential precursor for tyrosine that plays a role at the BDNF receptor, was estimated with NMR. Spatial working memory improved with 7-day honey intake, and prolonged intake up to 35 days increased spatial reference memory in the mice model. The phenylalanine present in honey may have triggered the upregulation of BDNF genes in honey-treated mice and improved their spatial memory performance (Mustafa et al., 2019).

Storage changes of chemical profile.

The difference between *Meliponula ferruginea* and *Apis mellifera* honey kept at environmental temperature during 18 months was compared by PCA and hierarchical cluster analysis based on the NMR metabolomics (Popova et al., 2021). Acetic acid (3.05 g/100 g) and lactic acid (2.38 g/100 g), ethanol, the amino acid pyroglutamic acid, the nucleobase trigonelline and uridine were higher in *M. ferruginea* pot-honey than *Apis mellifera*. The stored pot-honey increased fructose possibly after degradation of some fructose containing di- and trisaccharides. Pyroglutamic acid increased probably due to microbiological activity or Maillard reactions. HMF concentration increased with time.

Pot-Pollen

Chemical profiling

Australian stingless bees (Meliponini) process pollen in cerumen pots. Pot-pollen was harvested from *Tetragonula carbonaria* (Smith), *Tetragonula hockingsi* (Cockerell) and *Austroplebeia australis* (Friese) foraging in the same geographic area and season. Ethanolic extracts of pot-pollen were analyzed for volatile organic compounds (VOCs) and secondary metabolites from. Metabolomic approach (HS-SPME-GC-MS and LC-ESI-HRMS/MS, and statistical variability assessed by two-way ANOVA and PCA). VOCs contain similar mono-sesqui-terpenes across pot-pollens. Acetic acid was associated with *T. hockingsi*, p-anisaldehyde with *A. australis* and a methanone derivative with *T. carbonaria*. Ethanol extracts of the pot-pollens contained glycosyl-flavonoids and phenolics, including traces of secondary metabolites typical of stingless bee propolis (Massaro et al., 2018) possibly originated from the cerumen pot.

Propolis

Antibacterial activity of bee collected resins

The deposition of antibacterial plant resins in honey bee *Apis mellifera* nests has important physiological benefits. Wilson et al. (2013) observed bees from the experimental apiary foraging resin from eastern cottonwood (*Populus deltoides*) and balsam poplar (*Populus balsamifera*) among many available resinous plants. Resins from the bee corbiculae were diluted in acetonitrile and analyzed (HPLC, MS-Q-Exactive-TOF-MS) Antibacterial analysis revealed that resins varied in inhibition of the bee pathogen *Paenibacillus larvae*. These authors concluded that honey bees foraged a single source during a trip and the differential inhibition of *P. larvae* by *Populus* spp., suggests that resins from closely related plant species many have different benefits to bees.

Anticancer activity

See below in Chemical Profiling the propolis groups from Mato Grosso do Sul state, Midwest Brazil (Costa et al., 2020). Propolis from group II exhibited antitumor potential against prostate and breast carcinoma cells, as did propolis in groups III and IV against the btrast cell line. The propolis in

group I, despite the highest amount of total phenolic compounds and its unique DPPH scavenging activity, was not the most cytotoxic against the cell lines tested (Costa et al., 2020).

Natural products combined with standard chemotherapeutic agents are a strategy to reduce the negative side effects of chemotherapy. Doxorubicin (DOX) is a breast cancer drug with several side effects and drug resistance. Synergistic interactions between Australian propolis and DOX in the MCF7 breast adenocarcinoma cells were quantified using different synergy quantitation models. Biochemometric and metabolomics-driven analysis was performed to identify the potential anticancer metabolites. Shotgun proteomics identified 21 significantly dysregulated proteins in the synergistic combination-treated cells versus the mono treatments. The overexpression of UPF2 in the synergistic combination treatment, could assist in overcoming doxorubicin resistance. The significant synergy and key molecular pathways in the interaction between AP-1 and DOX in the MCF7 cells together with the AP-1 anticancer metabolites were identified (Alsherbiny et al., 2020).

Chemical profiling

Chemical profiling of *Apis mellifera* propolis from Malaysia and New Zealand was done with LC-HRMS and NMR processed with MZmine 2.10 and the Dictionary of Natural Products 2016 database, and a multivariate analysis by OPLS-DA. After chemical profiling and isolation work, triterpenoid and flavonoid derivatives revealed anticancer properties on lung, breast and ovary cancer cell lines (Yusnaini, 2018).

Propolis samples collected from Mato Grosso do Sul state, Midwest Brazil, were investigated for metabolomic profiles (HPLC-DAD-MS/MS, ^{1}H NMR, ^{13}C NMR and PCA, HCA multivariate statistics). Based on phytogeographical origin and chemical composition, 20 potential markers were identified and five groups were proposed: (I) Cerrado/Central, (II) Atlantic Forest/South, (III) Cerrado–Pantanal transition area/Northwest, (IV) Cerrado/North, and (V) Pantanal/West. A total of 47 compounds were identified, including prenylated phenylpropanoids, flavonoids, isoflavonoids, and di- and triterpenoids, among other constituents. Isoflavonoids, typically found in red propolis from Northeast Brazil, are being reported for the first time in a propolis sample from the Midwest. A new prenylated aromatic compound, (E)-3-[4-hydroxy-3-(2-hydroxy-3-methylbut-3-en-1-yl)-5-(3-methylbut-2-en-1-yl) phenyl] propenoic acid, was obtained (Costa et al., 2020).

The propolis of *Apis mellifera* from Bulgaria and *Meliponula ferruginea* from Tanzania were studied by GC-MS. *Pinus* species and a second unknown plant were the propolis source (Popova et al., 2021). Alkylphenols/resorcinols, anacardic acids, and mangiferolic acid, most probably originating from *Mangifera indica* fruit bark. They have been detected in propolis of stingless bee species in Asia and South America (Gallardo et al., 2020).

Pharmacological activity

Chemical composition, anti-proliferative, antibacterial, antifungal, anti-inflammatory and antioxidant properties of Australian propolis compared with Brazilian and Chinese propolis extracts. The Australian propolis extract was more anti-proliferative against the MCF7 and the MDA-MB-231 metastatic breast adenocarcinoma cell lines compared to Brazilian and Chinese propolis. Similarly the antibacterial (*Escherichia coli* and *Staphylococcus aureus*), anti-inflammatory (lipopolysaccharide-induced RAW264.7 macrophages) and antioxidant assays (ABTS, DPPH, CUPRAC). The metabolomic approach (UPLC-Q-TOF-MS and chemometrics unsupervised PCA and supervised OPLS-DA) analyses of propolis from Australia, China and Brazil revealed 67 key discriminatory metabolites from seven main chemical classes including flavonoids, triterpenes, acid derivatives, stilbenes, steroid derivatives, diterpenes and miscellaneous compounds. Seven common phenolic compounds were quantified (Bhuyan et al., 2021).

Review of honey bee and stingless bee propolis

Metabolomic studies of propolis were mostly done by (HPLC, GC-MS, LC-MS) but recently also (^{1}H NMR, ^{13}C NMR and multivariate analysis). In a comprehensive review on propolis, Tran et al. (2020) dissects publications on propolis composition: In the early 2000s, most studies were on Brazilian stingless bee propolis but later Southeast Asia and Australia increased their studies. A total of 100 compounds have been identified from stingless bee propolis (2000-2019), 24 of them were previously identified in honey bee propolis. A number of propolis compounds have been isolated and identified in each continent America (352), Asia (166), Africa (100), Europe (72), and Oceania (68), and country Brazil (158), Mexico (69), Nepal (37), Australia (36), and Greece (35). These authors considered the chemical structural diversity of propolis according to fingerprint-based diversity and to scaffold-based diversity (chemoinformatic analysis), and provide a unique supplementary material with this information for all the propolis compounds, 502 for honey bees and 100 for stingless bees. They noticed that bees in different regions harvested similar compounds from different plant families,

such as chrysin, pinocembrin, mangiferonic acid and isocupressic acid. And also observed that stingless bees and honey bees are attracted by similar flavanone and cycloartane-type triterpenes. Bees selectively choose resins with antimicrobial activity to protect themselves, but the mechanism of this choice is still unknown.

Royal jelly

Chemical profiling

The NMR-based metabolomics precisely and significantly authenticated the RJ products, by reliably tracing both their geographical and botanical origin, as well as their production period. In particular, Chinese RJ products exhibited a larger amount of citrate and lysine, accompanied by a lower content of 10-HDA. Our results showed that NMR spectroscopy can recognize low-quality fraudulent products and become an useful analytical tool to certify and trace the RJ molecular composition. The application of NMR spectroscopy promises to enable the efficient protection of both the producers and consumers of high-quality royal jelly. In addition, the proposed method may be used in combination with the conventional melissopalynological methods for recognizing frauds (Mazzei et al., 2020).

Chemical profiling of Indonesian royal jelly compositions by untargeted metabolomics approach (UPLC-Q-TOF-MS and LC–MS-MS with PCA) identified >30 compounds. Oligosaccharides, fatty acids, and adenosine monophosphate derivatives were major components. Sucrose and planteose were highest in the samples from Banjarnegara and Kediri, dimethyloctanoic acid was unique in Banjarnegara royal jelly. It was also found that royal jelly from Demak and Tuban contained more organic fatty acids and oligosaccharides than the others. The PCA scores plot separated the four geographical origins of royal jelly. The high content of fatty acids and oligosaccharides in the sample from Tuban makes it the most suitable for use in cosmetic, skin care, and bio-supplement industries (Sari et al., 2021).

Conclusions

The metabolomics applied in bee science is not a new approach, but the instruments and bioinformatics have modernized into a new dimension for understanding bee processes, and related topics such as those retrieved in the current review. Databases are growing fast and demand more discipline of scientists to manage them. Some are interested in marketing, others in control, bee protection or exploitation, or just mining new metabolites and fix them into a scheme of progress. Misuses of the word metabolomics were not included here.

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