PRELIMINARY STUDY OF GUT BACTERIAL ABUNDANCE IN *Rhynchophorus ferrugineus* (COLEOPTERA: DRYOPHTHORIDAE) FED ON DIFFERENT DIETS

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ABSTRACT

Rhynchophorus ferrugineus or also known as RPW is a major pest in many parts of the world and has infested coconut plantations in Malaysia especially in Terengganu and Kelantan. To date, a combination of many methods such as trapping with pheromone, chemical pesticide, and biological pesticide (Integrated Pest Management) were applied to control RPW infestation. However, IPM often could not be used as a method for total eradication and in addition to that, IPM necessitates continuous laborious effort. Thus, a new target for RPW eradication method with high specificity must be identified. The purpose of this research is to study the effect of different diets towards gut bacterial abundance in RPW and thus widen the knowledge regarding their correlation. Parameter measured were abundance of gut bacteria following different diets treatment. 16S rRNA amplicon sequencing method was used in this research to identify all gut bacteria present in the RPW. Our analysis showed that there are six most abundant group of bacteria with different phylogenetic group rank present in RPW's gut. This include Enterobacteriaceae, *Leminorella grimontii*, Entomoplasmatales, *Erysipelothrix*, *Lactobacillus* and *Leuconostoc*. This groups of bacteria were known to have significant roles toward host's gut such as aiding in digestion, synthesizing hormone, and protecting from pathogenic bacteria growth. Comprehensive data obtained from this study on these microbes have potential to be used in exploring new dimension of RPW pest management.

Keywords: RPW, IPM, 16S rRNA amplicon sequencing

ABSTRAK

Rhynchophorus ferugineus atau lebih dikenali sebagai RPW ialah serangga perosak yang terkenal di serata dunia dan telah menyerang sebahagian besar tanaman pokok kelapa di Malaysia terutama di Terengganu dan Kelantan. Sehingga kini, gabungan beberapa kaedah seperti perangkap feromon, pestisid kimia dan pesitisid biologi (Pengurusan Perosak

Bersepadu) bagi pengawalan RPW telah dilakukan. Walaubagaimanapun, kaedah gabungan ini tidak dapat menghapuskan RPW secara menyeluruh. Tambahan lagi, ia memerlukan usaha dan pemantauan yang berterusan. Justeru itu, kaedah baru penghapusan RPW yang lebih spesifik dan berkesan perlu dibentuk. Kajian ini bertujuan untuk mengkaji kesan jenis diet terhadap kelimpahan bakteria usus RPW sekaligus menambah pengetahuan tentang korelasinya. Kaedah penjujukan amplikon 16S rRNA telah digunakan di dalam kajian ini bagi mengenalpasti bakteria usus yang terdapat di dalam RPW berbeza diet. Enam bakteria berbeza peringkat filogeni iaitu Enterobacteriaceae, *Leminorella grimontii*, Entomoplasmatales, *Erysipelothrix*, *Lactobacillus* and *Leuconostoc* telah dikenalpasti sebagai bakteria yang paling besar komunitinya di dalam sampel. Berdasarkan kajian lepas, bakteria-bakteria ini telah diketahui mempunyai beberapa kepentingan terhadap usus penghadaman hos seperti membantu sistem penghadaman, membantu penghasilan hormon dan merencat pertumbuhan bakteria patogenik di dalam usus perumah. Data komprehensif yang diperoleh daripada kajian ini berupaya untuk menerokai dimensi baru dalam pengurusan serangga perosak RPW.

Kata kunci: RPW, Pengurusan Perosak Bersepadu, Penjujukan amplikon 16S rRNA

INTRODUCTION

Red Palm Weevil consists of two species that are *Rynchophorus ferrugineus* and *R. vulneratus*. However, *R. ferrugineus* is more prevalent of the two species due to its prominency as palm tree pest around the world. Earliest reported case came from United Arab Emirates country in the year 1985 where hundreds thousands of date palm trees has been infected by this species, causing a great loss in date fruit production (El-Juhany 2010; Gush 1997). Despite its smaller size as compared to *R. vulneratus*, this RPW has a wider range of target and more ferocious in many parts of Malaysia especially in east coast. In Terengganu alone, 55 000 coconut trees have been infected with RPW damaging coconut market in the state (Azmi et al. 2013). Besides the fact that coconut trees in Malaysia are in great danger of being eradicated by this pest, there is a far greater concern lies ahead. There is high probability of RPW to start infesting the most valuable and economical plantation in Malaysia, the oil palm tree (Azmi et al. 2013). Thus, new RPW eradication method with high specificity targets needs to be explored, in order to have alternative to the current conventional Integrated Pest Management (IPM) method. Rapid and specific technique is required due to the fast breeding nature of RPW itself. RPW breeds faster than we can eradicate them, and can sum up to a staggering amount of 531 eggs per female RPW in its lifetime (Ince et al. 2011).

Diet has been known to affect the community of gut bacteria and vice versa. For example, according to one study, crickets without hindgut bacteria performed worst on diets with low nutritional value as compared to crickets with normal hindgut bacteria (Ulrich et al. 1981). This study proves that gut bacteria plays an important role in ensuring insect host survivability even in time of stress. In another study, cockroaches with eliminated hindgut symbionts and mycetomes develop smaller hindguts and have higher concentration of uric acid (Cochran 1985). These effects correlated with the role of gut bacteria in digestion and nitrogen fixation mechanism. Certain gut bacteria with nitrogen fixing ability tend to utilize the host's nitrogen contents by altering it to a consumable and harmless form. Most insects lack this ability and have to depend entirely on its symbionts in order to prevent wasting precious nitrogen that are needed in building essential amino acids (Douglas 2009). This symbiotic relationship shows that insect's gut bacteria will not only affect the diet intake and

digestion process but also partake in host's regulation process. Besides that, gut microbes such as *Eserichia coli* and other Enterobacteriaceae bacterial family has been known to produce digestive hormone which is serotonin inside various organisms' guts including insects (Brenner&Farmer 1984; French et al. 2014). In a recent study by Harris et al. (2016), immunohistochemistry technique has proven the presence of 5-HT receptor on plasma membrane, basement membrane and cytoplasm of *Rhynchophorus ferrugineus*'s whole gut cells. This is another evidence indicating the importance of serotonin in digestion process specifically in Red Palm Weevil and the possibility of its production by gut microbes.

In a more recent study, four scarabids beetle species of different eating habits are studied and their gut microbial communities were identified. Surprisingly, one of the predatory beetle species has a much similar gut microbial community to another predatory beetle species despite having a closer phylogenetic relationship to the omnivorous beetle species (Colman et al. 2012). This strong evidence indicate the impact of diet on structuring the microbial community in insect. The impact of diet is further characterized in termites and beetles which shared xylophagous diet. Study showed that both do not have the same gut microbial community. However, this might be due to different preferences on types of lignocellulose tissue. The beetle preferred living lignocellulose tissue while termites choose the opposite (Colman et al. 2012). The types of diet influence different gut bacterial growth in host as each bacterium has its own preferences to nutrition. Nucleic acid probes targeting 16S-like ribosomals RNAs is used in a previous study and it was understood that soil-feeding termites has higher relative abundance of microorganism when compared to wood-feeding termites (Brauman et al. 2001).

Although research regarding the effects of diet on gut bacterial community in insects is scarce and not profound, this studies has been done in depth in human and mice. For example, one study has been done on the effect of transferring gut bacteria between different mice. In this study, gut bacteria from obese mice was transferred to lean mice. Surprisingly, this pool of gut bacteria have positively caused the lean mice to gain extra weight as well as succumbed to diabetic issue similar to the obese mice (Nicholson et al. 2012). This case should not be any different to other organisms especially in simpler organism such as insect. Other than that, the same bacteria could either be beneficial or parasitic towards its host depending on the type of diet intake. *Erwinia* sp. bacteria in thrips become beneficial when the host is fed with cucumber leaves (De Vries et al. 2004). Shorter maturation time and higher oviposition rate were observed in *Erwinia* infected thrips compared to aposymbiont thrips. Meanwhile, the same bacteria became parasitic towards its host when fed with cucumber leaves as well as pollen when aposymbiont thrips develops faster and lay more eggs compared to infected thrips (De Vries et al. 2004).

In this current study, we would like to understand the effect of different diets towards gut bacterial diversity in RPW as well as to identify its potential as the new target for RPW eradication. In addition, the targeted bacteria must confer to the rules that it causes harm to RPW but not to humans and other animals. In order to screen for suitable bacteria candidate, gut microbes in RPW with different diets were compared to each other and their respective control. Wild strain RPW act as control in this study where captured wild strain were processed immediately after sampling unlike the other two treated samples which were reared in lab for more than two weeks.

MATERIALS AND METHODS

RPW Sampling: Adult RPW were sampled from February until August 2016. Sampling location was situated at Seberang Takir, Terengganu. The GPS coordinates of all eight locations are as follows, 5°22'11.8"N 103°06'41.4"E, 5°21'47.4"N 103°06'57.9"E, 5°21'18.2"N 103°07'05.1"E, 5°20'47.7"N 103°07'01.8"E, 5°20'30.3"N 103°07'02.7"E, 5°20'08.6"N 103°06'26.2"E, 5°20'02.3"N 103°06'33.6"E and 5°20'01.7"N 103°06'32.6"E. Trapping method was conducted by using perforated bucket containing pheromone P028 Ferrolure+700 mg product code cta-7239, mass trapping lure for *Rhynchophorus ferrugineus*'s male adults. Sackcloth was wrapped around each bucket to ease the movement of RPW into the trap. Traps were put near infested coconut trees. The distance between each trap was set according to the previous study which is one hectare from each other (El- Shafie et al. 2011). The duration of trapping was between two to four weeks and traps were visited twice a month in order to replace the food, pheromone and collected the trapped samples.

RPW Rearing and Treatment: RPW adults were collected and reared inside transparent cage for minimal of two weeks. The RPW were separated into four groups as Table 1 below. Each group has equal amount of male and female RPW in order to ensure its survivability. The diets were replaced every four days for coconut's crown and every seven days for sugarcane. The weight of each diet was fixed that was approximately at 10 g for each 1 g of RPW's weight. The difference in diet replacing time was due to the difference in decaying duration of each diet. The diets were given for consecutive of two weeks and above. Group 1 and 2 were hatched from egg and reared in laboratory with the constant condition of 27°c, wet, and aerated. Meanwhile, group 3 was taken and processed straight away after sampling. This group is considered wild and acts as a positive control in the experiment.

The rearing procedure of RPW requires the utmost care in every life stages. Eggs obtained from adult were kept in dark, aerated box with humid environment until it hatched into larva. The larva were given individual sugarcane stick measuring to approximately 20 cm in length and 2.5 cm in width. These sugarcanes were changed once in a two weeks or when it becomes dry. After two months, larva will turned into cocoon and kept inside dark, aerated box. Humidity must be maintained at 40% and above. The adult RPW hatched from the cocoon after two weeks were used for Group 1 and 2 treatments.

TABLE 1. RPW's treatment for each group is shown in the table below. All groups are kept at constant condition which are 27°c, wet, and aerated.

Group	Diet	Treatement	Role in Experiment
1	Sugarcane	2 weeks; Lab-rear and food replaces every week	Lab-reared RPW
2	Coconut's crown	2 weeks; Lab-rear and food replaces every four days	Lab-reared RPW
3	Natural coconut plant	Processed directly from sampling	Wild RPW (+ve control)

Sterile Dissection and DNA Extraction: Dissecting kits were autoclaved before usage and phosphate buffer saline were made from sterile distilled water. This was to ensure no bacteria from environment will contaminate the samples. The guts obtained from RPW were separated into three parts which are foregut, midgut, and hindgut. This separation was made by referring to previous studies relating to Red Palm Weevil gut structure (Harris et al. 2015). Two RPW samples were used in each extraction to ensure high DNA concentration. DNA extraction was carried out by using Analytic Jena Kit brand.

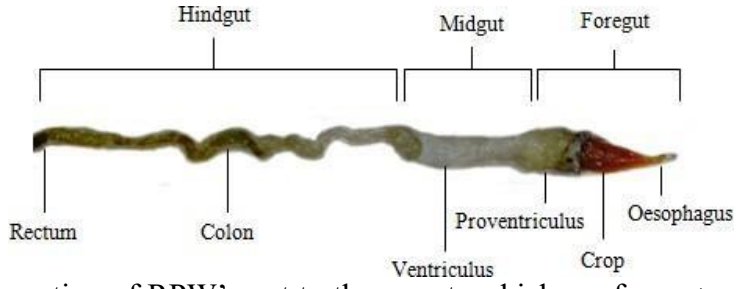

Figure 1. The separation of RPW's gut to three parts which are foregut, midgut, and hindgut. Other parts of gut are labeled as above based on previous study (Harris et al. 2015).

Polymerase Chain Reaction of Bacterial 16S rRNA genes: PCR amplification of the V1-V2 region of the 16S rRNA gene was performed by using the following primers, which contain linker sequences (TC or CA) and eight base, sample specific bar-coded sequences (designated X): 8F (5'-X-TC- AGAGTTTGATCCTGGCTCAG-3') and 338R (5'-X-CA-TGCTGCCTCCCGTAGGAGT-3'). The conditions for PCR are as follows: initial denaturation at 94°C (2 mins), 30 cycles of denaturation at 94°C (1 min), annealing at 60°C (30 secs), extension at 72°C, and final extension step of 10 mins at 72°C. Bright main strip between 400-450 bp were chosen and purified by using Qiagen Gel Extraction Kit (Qiagen, Germany).

PCR Products Purification and HiSeq Sequencing: PCR products obtained were sent to First Base Asia Sdn Bhd for purification, quality control check, library preparation and HiSeq sequencing. Purified PCR products were evaluated on a Agilent 2100 Bioanalyzer to ensure sample used is of high DNA quality. Library preparation chosen for this DNA samples is 16S microbial amplicon library and were later analyzed with Illumina HiSeq 2500 platform sequencing technology. 250 bp paired-end reads were generated from this process.

16S rRNA Data Analysis via QIIME Pipeline: Generated paired-end reads from the three samples undergone four process in order to obtain clean effective tags for operational taxonomic unit (OTU) annotation step later on. Data splitting is the first process in which paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. In the second process, the sequences were assembled by using analysis tool FLASH (V1.2.7, http://ccb.jhu.edu /software/ FLASH/). In the third process, the raw tags obtained were filtered under specific conditions to obtain high-quality clean tags by using Qiime (V1.7.0, http://qiime.org /scripts/split_libraries_fastq.html). Lastly, the tags were compared with the reference database (Gold database, http://drive5.com/ uchime/uchime download.html) using UCHIME algorithm (UCHIME Algorithm http://www.drive5.com/ usearch/manual/ uchime_algo.html) to detect chimera sequences. The chimera sequences were then removed. Next, effective tags for each

samples were obtained. These effective tags were then used in OTU clustering and species annotation step in which the types of each bacteria present in samples were finally identified. In OTU production step, sequences analysis were performed by Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/) Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. Lastly in species annotation step, the GreenGene Database (http://greengenes.lbl.gov/ cgi-bin/nph-index.cgi) was used based on RDP classifier (Version 2.2, http://sourceforge.net/projects/rdp- classifier/) algorithm to annotate taxonomic information of each representative sequence. The types of bacteria present and its abundance are finally identified for each samples.

RESULTS AND DISCUSSION

Table 2 presents a comparison of gut bacterial community between different diets and different parts of gut. Initial F, M and H in each sample's label represent foregut, midgut, and hindgut while initial 1, 2 and 3 represents the group number as in Table 1. Each OTU represents identified bacteria that have been sequenced and matched onto database in order to identify its phylogenetic rank. These are the top OTUs identified in all samples and the highest OTU consists of the family Enterobacteriaceae. The OTU 1 sequence tag amounted to 58 060 out of 68 526 total tags sequenced in sample H1. This shows that 84.73% of bacteria in sample H1 consist of Enterobacteriaceae bacteria.

Meanwhile, sample M has a slightly higher number of Enterobacteriaceae which is 92.17%. In overall, sample F2, F3, M1, M2, H1, H2, and H3 have high number of Enterobacteriaceae family ranging from 58.76% to 92.71%. Even though sample M2 only shows a total of 33.24% Enterobacteriaceae family, 60.49% of OTU 3 was found to consist of bacteria *Leminorella grimontti* that also originated from Enterobacteriaceae family. Sample M3 however, was observed to have only 2% of Enterobacteriaceae family. Enterobacteriaceae is a very common gut bacterium in a wide range of organisms including insects. It has been shown to aid in host's digestion processes and synthesizes essential hormones for host such as serotonin (Brenner & Farmer 1984). The fact that this bacterial family was barely found in sample M3 in contrary to the sample M1 and M2, shows that the types of diet given can actually effect the present of bacteria in RPW's gut. The lab-reared food given to the RPW might contained more bacteria than natural coconut tree due to its staling state. Thus, wild strain RPW (group 3) act as control that eliminate the presence of transient bacteria which comes and go with the food intake. In both lab-rear and wild strain RPW, it is shown that there are high abundance of bacteria in hindgut ranging above a staggering amount of 78.34%. This might be caused by the long physical length of hindgut compared to midgut and foregut as shown in Figure 1 which enabled it to harbor more bacterial community.

Moreover, hindgut carries an important function of converting toxic ammonium in insect's hindgut into uric acid for excretion. Most insect such as termite depends entirely on gut bacteria to help convert the ammonium into uric acid as well as recycling the nitrogen molecule for amino acid building in host (Potrikus & Breznak 1981). The high percentage of bacteria especially Enterobacteriaceae in the hindgut part of RPW regardless of the diet indicates its significance in this process. Other than that, foregut also shows high percentage of Enterobacteriaceae which might be due to the presence of mycetomes attached to the foregut. This organ carries and grow beneficial symbionts for insects' function (Musgrave

1964; Vega & Dowd 2005).

Aside from OTU percentage, the bacteria ecological niche and known role in gut are also stated in Table 2 based on previous reported studies. Besides that, CAZY enzymes or carbohydrate active enzymes from CAZY bacterial database is also stated in the table. Some of these bacteria have the ability to produce CAZY enzymes that function in degrading polysaccharides in gut. If the bacteria has the ability to produce this enzyme, it is highly likely that the bacteria will play an important role in RPW's digestion.

Using clustering threshold of 97% and normalization cut-off at 34, 778 tag sequences has been chosen to be further analyze using alpha diversity index data as in Table 3. Observed species shows the number of OTU for each samples. Shannon index (H) was used to characterize OTU diversity in a bacterial community and measure its level of diversity. Species diversity is measured based on two factors; 'species richness' which is the number of OTU present in a sample and 'species evenness' which is the evenness of bacterial species present in all samples. of OTU distribution in each samples. Higher H index indicates higher diversity of that particular sample. Based on Table 3, sample F3 has the highest H index followed closely by sample F1. This indicate that these two samples has the highest bacterial diversity compared to other samples. Sample F2 also shows high bacterial diversity ranking after sample F3. This prove that bacterial diversity regardless of diet are heavily concentrated in the foregut part of RPW.

TABLE 2. Table below shows 6 highest abundance bacteria and its percentage in each samples. Previous studies regarding ecology niche, role in gut, and CAZY enzymes from database of each bacteria are discussed as well in the table.

Name	OTU Percentage %									Related CAZY	Ecology Niche	Known Role in Gut	References
	F1	M1	H1	F2	M2	H2	F3	M3	H3				
Enterobacteriaceae	31.93	92.02	84.98	71.79	33.24	78.34	58.76	2	79.63	Too wide	Host gut	Digestion, synthesize hormone	Brenner&Farmer 1984
Entomoplasmatales	34.15	4.11	0.04	2.36	0.94	1.63	10.43	97.09	0.95	Too wide	Ant's gut	Unknown	Funaro et al. 2011
Leminorella grimontii	0.02	0.63	1.19	0.05	60.49	3.66	-	-	-	Unknown	Host gut	Unknown	Brenner& Farmer 1984; Lombard et al. 2014
Leuconostoc	10.88	0.19	0.01	1.75	0.07	0.06	0.75	0.14	0.06	GH1,GH13, GH43, GH36, GT28, etc	Fermented food, host gut, Meat	Protect from pathogenic bacteria	Bernborn et al. 2006; Lombard et al. 2014
Erysipelothrix	-	0.06	0.09	0.01	2.64	11.27	-	-	-	GH1,GH13, GH18, GT35, etc	Soil, insect and pig's gut	Unknown	Borchardt et al. 1977; Bang et al. 2015; Lombard et al. 2014
Lactobacillus	1.37	0.01	0.01	0.85	0.04	0.02	8.15	0.05	0.05	GH1, GH2, GT2, etc	Dairy product, host gut	Protect from pathogenic bacteria	Cai et al. 2007; Sandine 1979; Lombard et al. 2014

TABLE 3. Alpha Indices Table.

Sample name	observed_species	shannon	simpson	goods_coverage
F1	76	3.003	0.813	1
M1	69	0.628	0.149	1
H1	51	1.074	0.279	1
F2	78	1.983	0.529	1
M2	104	1.648	0.556	1
H2	55	1.443	0.417	1
F3	115	3.073	0.803	1
M3	68	0.322	0.061	1
H3	107	1.603	0.376	1

The reasons behind this might relate to the gut's function in which foregut focused more on churning and mechanically digesting the food which allows many transient bacteria to remain in it. Meanwhile, other parts of gut which focused on chemical digestion and secretes enzyme produced an environment that might not be suitable for some bacteria to live and grow (Chapman 1998). Furthermore, the symbiotic bacteria that colonize midgut and hindgut might secrete chemicals that inhibit the growth of other bacteria considered as pathogenic (Dillon et al. 2005). Sample M3 has the lowest H index which indicates the presence of dominant bacteria with possible significant towards RPW. This bacteria is known as Entomoplasmatales with OTU percentage of 97.09%. Simpson Index (D) also shows the level of diversity via the two factors which are 'species richness' and 'species evenness'. This index ranging from 0 to 1 in which 1 indicates highest diversity. This index also shows almost similar results as Shannon index where sample F3 and F1 has the highest diversity while sample M3 has the lowest diversity.

As stated before, foregut regardless of diets has the highest gut bacterial diversity compared to other two parts of gut as can be seen in Table 2. This is followed by hindgut and middle gut. However, this is only true for sample from RPW with sugarcane diet and positive control. Middle gut of RPW with coconut's crown as diet has a higher gut bacterial diversity compared to its hindgut and has even greater differences with the middle gut of sugarcane diet RPW and positive control RPW. This shows the potential effects of different diets to bacterial abundance and its localization in different parts of gut.

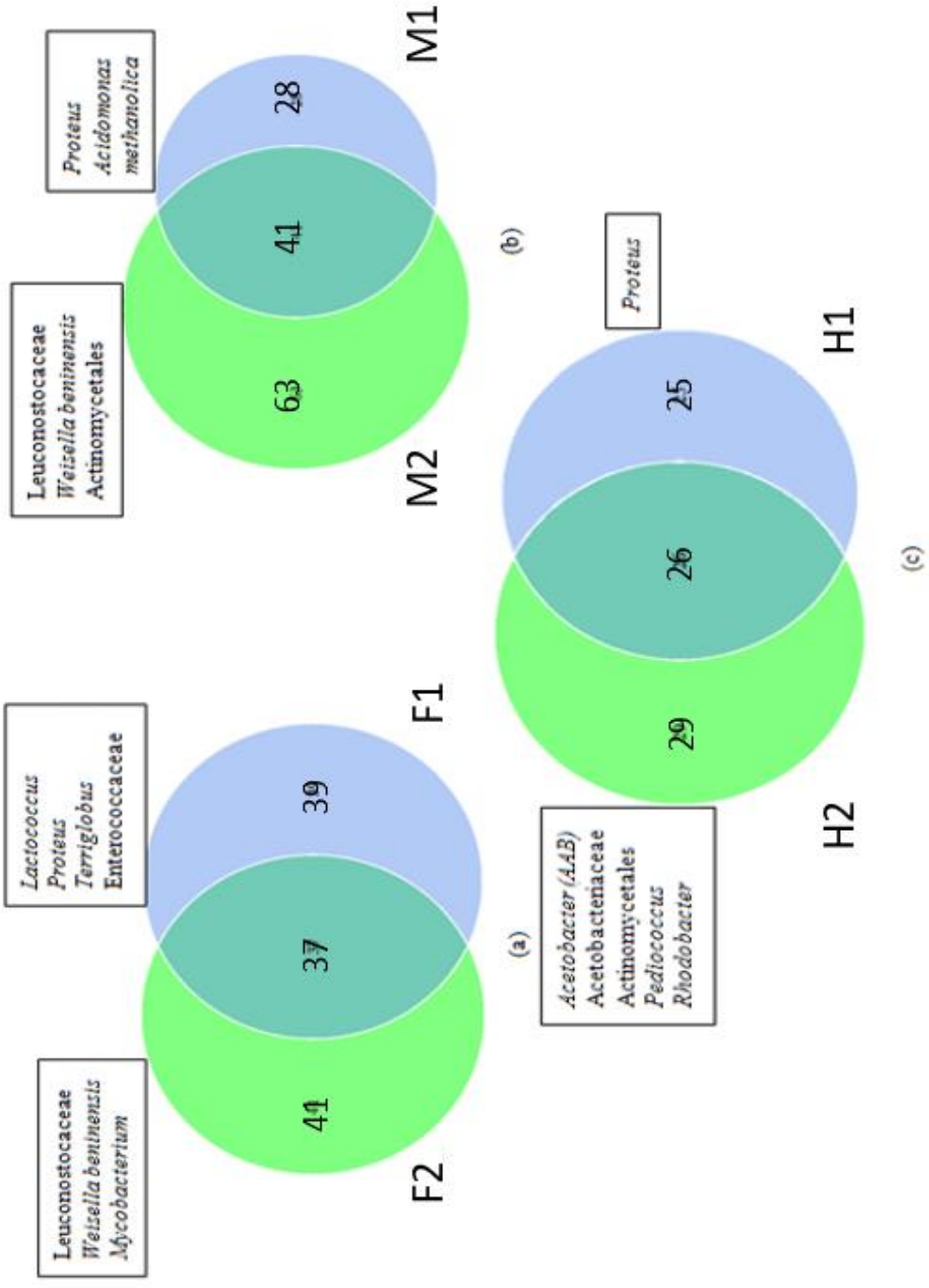

FIGURE 2 Venn Diagram showing both the unique and shared gut bacterial abundance between RPW fed on sugarcane and coconut's crown diet.

Coconut's crown has more nutritional value preferred by various gut bacterial species and its moist condition allows optimal growth of bacteria unlike the drier sugarcane. However, this factors of diet should be further clarified via analytical quantifications of water and nutrition level contents in future experiments. Middle gut gives the most distinct comparison in which RPW eith coconut's crown diet has far greater unique OTU number compared to RPW with sugarcane diet. This evidence might point to the fact that coconut's crown diet builds a more diverse gut bacterial community.

CONCLUSION

This study identifies 6 bacteria with different phylogenetic group ranks as the most abundant bacteria in RPW's gut namely Enterobacteriaceae, *Leminorella grimontii*, Entomoplasmatales, *Erysipelothrix*, *Lactobacillus* and *Leuconostoc* that have the potential to be developed as biocontrol towards RPW. However, further studies and experiments need to be done in order to use one of this strains as target in RPW eradication in the near future. Comprehensive data obtained from this study is hoped to widen the knowledge and potential of RPW pest management such as in biological control or biotechnology area.

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REFERENCES

Azmi, W.A., Chik, Z., & Abdul Rahman, A. 2013. A new invasive coconut pest in Malaysia: the red palm weevil (Curculionidae: *Rhynchophorus ferrugineus*), *Planter* 89(1043): 97-110.

Brauman, A., Doré, J., Eggleton, P., Bignell, D., Breznak, J.A, & Kane, M.D. 2001. Molecular phylogenetic profiling of prokaryotic communities in guts of termites with different feeding habits, *FEMS Microbiology Ecology* 35(1): 27-36.

Cai, H., Rodriguez, B. T., Zhang, W., Broadbent, J. R. & Steele, J. L. 2007. Genotypic and phenotypic characterization of Lactobacillus casei strains isolated from different ecological niches suggests frequent recombination and niche specificity, *Microbiology* 153(8): 2655-2665.

Chapman, R.F. 1998. *The insects: structure and function* Ed., Cambridge University Press.

Cochran, D.G. 1985. Nitrogen excretion in cockroaches, *Annual Review of Entomology* 30(1): 29-49.

Colman, D. R., Toolson, E. C. & Takacs- Vesbach, C. 2012. Do diet and taxonomy influence insect gut bacterial communities? *Molecular Ecology* 21(20): 5124-5137.

De Vries, J., Jacobs, G., Sabelis, M.W., Menken, S.B. & Breeuwer, J.A. 2004. Diet dependent effects of gut bacteria on their insect host: the symbiosis of *Erwinia sp*. and western flower thrips, *Proceedings of the Royal Society of (B: Biological Sciences, London*) 2171- 2178.

Dillon, R., Vennard, C., Buckling, A. & Charnley, A. 2005. Diversity of locust gut bacteria protects against pathogen invasion. *Ecology Letters* 8(12): 1291-1298.

Douglas, A.E. 2009. The microbial dimension in insect nutritional ecology. *Functional Ecology* 23(1): 38-47.

El-Juhany, L.I. 2010. Degradation of date palm trees and date production in Arab countries: causes and potential rehabilitation. *Australian Journal of Basic and Applied Sciences* 4(8): 3998-4010.

El-Shafie, H., Faleiro, J., Al-Abbad, A., Stoltman, L. & Mafra-Neto, A. 2011. Bait-free attract and kill technology (Hook™ RPW) to suppress red palm weevil, Rhynchophorus ferrugineus (Coleoptera: Curculionidae) in date palm. *Florida Entomologist* 94(4): 774-778.

Funaro, C. F., Kronauer, D. J., Moreau, C. S., Goldman- Huertas, B., Pierce, N. E. & Russell, J. A. 2011. Army ants harbor a host-specific clade of Entomoplasmatales bacteria. *Applied and Environmental Microbiology* 77(1): 346-350.

French, A. S., Simcock, K. L., Rolke, D., Gartside, S. E., Blenau, W. & Wright, G. A. 2014. The role of serotonin in feeding and gut contractions in the honeybee. *Journal of Insect Physiology,* 61: 8-15

Gush H., 1997. Date with disaster, *The Gulf Today*.

Harris, M.N., Norzainih, J. & Wahida, O. N. 2015. Morphology and histology of the digestive System of the red palm weevil larva, *Rhynchophorus Ferrugineus,* Olivier (Coleoptera: Dryophthoridae*). Proceedings of the rd International Conference on Chemical, Agricultural and Medical Sciences (Singapore)* 32-37.

Harris, M.N, Norefrina Shafinaz, M.N., Salmah, Y. & Nurul Wahida, O. 2016. Distribution of serotonin (5-HT) and dopamine (DA) on digestive tract of red palm weevil larva, *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae). *Serangga* 21(1): 39-50.

Ince, S., Porcelli, F. & Al-Jboory, I. 2011. Egg laying and egg laying behavior of red palm weevil Rhynchophorus ferrugineus (Olivier) 1790 (Coleoptera: Curculionidae), *Agriculture and Biology Journal of North* 2: 1368-1374.

Murphy, S. & Briscoe, B. 1999. The red palm weevil as an alien invasive: biology and the prospects for biological control as a component of IPM, *Biocontrol news and information* 20: 35N-46N.

Musgrave, A. 1964. Insect mycetomes. *The Canadian Entomologist* 96(1-2): 377-389.

Nicholson, J.K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W. & Pettersson S. 2012. Host-gut microbiota metabolic interactions. *Science* 336(6086): 1262-1267.

Potrikus, C.J. & Breznak, J.A. 1981. Gut bacteria recycle uric acid nitrogen in termites: a strategy for nutrient conservation, *Proceedings of the National Academy of Sciences* 78(7): 4601-4605.

Sandine, W.E. 1979. Roles of Lactobacillus in the intestinal tract. *Journal of Food Protection* 42(3): 259-262.

Ulrich, R., Buthala, D. & Klug, M. 1981. Microbiota associated with the gastrointestinal tract of the common house cricket, *Acheta domestica*. *Applied and Environmental Microbiology* 41(1): 246-254.

Vega, F.E. & Dowd, P. F. 2005. *The role of yeasts as insect endosymbionts, Insect-Fungal Associations: Ecology and Evolution.* Oxford University Press, New York 211- 243.