African Journal of Biotechnology Vol. 7 (10), pp. 1432-1437, 16 May, 2008
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB08.078
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Aerobic and facultative anaerobic bacteria from gut of red palm weevil (*Rhynchophorus ferrugineus*)

Mohammad Khiyami* and Essam Alyamani

Natural Resources and Environment Research Institute, Biotechnology and Genetic Engineering Research Center, King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia.

Accepted 11 April, 2008

Red palm weevil (RPW), *Rhynchophorus ferrugineus* Oliver is one of the insects that attack date palm trees directly and cause its death. This infection poses serious economical consequences in Saudi Arabia and other gulf countries. RPW assimilates the components of palm tree tissues. Significant amount of microbiota in the gut of RPW may contribute to success of its pathogenesis. This study explored the nature of microbiota in RPW by culture-dependent and molecular-based techniques using PCR and sequencing analysis. Larvae of RPW were fed on an artificial diet and were fixed before opening its gut for microbial cultivation on enrichment media. Pure cultures were obtained after incubating the plates at different atmospheric conditions (aerobic, and strictly anaerobic). The majority of isolated microbiota observed were aerobes and facultative anaerobes (*Bacillus* sp., *Salmonella* sp., *Enterococcus* sp., and *Xanthomonas* sp.). These qualitative differences of bacteria, suggest the presence of a complex ecosystem in the gut of RPW. Subsequently, bacterial DNAs were extracted from pure cultures for definitive molecular identifications. Hot start-touchdown PCR was performed to amplify regions within 16S rDNA. Amplicons were cloned into the TOPO-TA vector for sequencing. The data reveal to some extent that aerobic and facultative anaerobic bacteria are more distributed in the gut of RPW.

Key words: 16S rDNA, microbiota, red palm weevil, date-palm.

INTRODUCTION

Date palm, *Phoenix dectyliferal*, is the most important fruit tree of arid, tropical and sub-tropical regions of the world including Saudi Arabia and many other Arab countries. Usually several insects attack date palms, but the red palm weevil (RPW), *Rhynchophorus ferrugineus* Oliver is the one that causes the most considerable damage. The RPW that invaded the Gulf region in the mid 1980s belongs to the order Coleoptera and family Curculionidae (Wattanapongsiri, 1966). The RPW is a concealed tissue borer and all of its life cycle stages are found inside the palm tree consuming the tender soft tissues. Damage symptoms of palm trees are indicated by the presence of tunnels in the trunk, oozing of thick yellow to brown fluid from the trees, the appearance of chewed plant tissues in and around the openings in the trunk, the presence of a fermented odor from the fluid inside infested tunnels in the trunk, and/or breaking of the trunk or toppling of the crown (Kaakeh, 2006). The attack by RPW would lead to death of palm tree within 6-8 months (Murphy and Briscoe, 1999). Some of the damage symptoms caused by the RPW may be also caused by other insects, such as termite. Studies during the last decade illustrate that symbiotic microorganisms in the intestinal termite gut play key physiological functions. These functions are: cellulose and Hemicellulose digestion, acetogenesis, hydrogenesis, methanogenesis, sulfate reduction, and nitrogen fixation in addition to its possibility of employing them as a biological control.

*Corresponding author. E-mail: Mkhiyami@kacst.edu.sa. Tel: +966-481-3772. Fax: +9661- 481-3611.

Table 1. Primers used for the amplification of the 16S rDNA gene.

Primer name	Direction	Primers sequence
8FPL	Forward	5'- GCG GAT CCG CGG CTG CAG AGT TTG ATC CTG GCT CAG-3'
806R	Reverse	5'-GCG GAT CCG CGG CCG CGG ACT ACC AGG GTA TCT AAT-3'

Significant counts of different aerobic, facultative anaerobic, microaerophilic and strictly anaerobic microbes were isolated from intestinal termite gut. Aerobic and facultative anaerobic bacteria isolated from termite's intestine including *Burkholderia* sp. and *Citrobacter* sp. were isolated under aerobic conditions as aromatics-degrading bacteria (Harazono et al., 2003). *Serratia marcescens*, *Enterobacter aerogens*, *Enterobacter cloacae*, and *Citrobacter farmeri* were isolated as facultative anaerobes (Adams and Boopathy, 2005). Additionally, under aerobic and facultative anaerobic conditions, several Gram-positive bacteria of the actinomycetes like *Cellulomonas*/*Oerskovia*, *Microbacterium*, *Kocuria* and bacterial genera including *Bacillus*, *Brevibacillu*s and *Paenibacillus* were isolated. Also, under the same conditions, Gram-negative bacteria related to the genera *Afipia*, A*grobacterium*, *Rhizobium*, *Brucella*/*Ochrobactrum*, *Pseudomonas* and *Sphingomonas*/*Zymomonas* and *Spirosoma* were isolated (Wenzel, et al., 2002). However, no information is available about the intestinal microbiota of red palm weevil guts. Very few studies have been conducted on the natural enemies of RPW such as *Beauveria bassiana, Pesudomonas aeruginosa, Bacillus sphaericus*, *B. megaterium* and *B. laterosporus* which were isolated from natural habitats and used as classical biological control of RPW (Hanounik et al., 2000; Murphy and Briscoe, 1999; Salama et al., 2004).

The objective of this study was to explore the nature of RPW gut microbiota and to determine whether the RPW gut can provide a distinctive environment for the isolation of several new aerobic and facultative anaerobic bacteria which might be exploited for biological control of RPW.

MATERIALS AND METHODS

Sample collection and microbiological techniques

Larvae of RPW were procured from Dr. Al-Ayedh at King Abdualziz City for Science and Technology, Saudi Arabia. They were fed on an artificial diet. The larvae were externally sterilized in 100% ethanol for about one minute and then allowed to air dry for one minute also. They were fixed on a chilled table before opening its gut using flame sterilized fine-tip forceps and placed in 10 ml of saline water. Serial dilution aliquots were used for the inoculation. Enrichment and isolation of bacteria were performed using two media. Medium one (M1) was minimal medium containing 8 g of $NH_2H_2PO_4$, 0.2 g of yeast extract, 2 g of K_2HPO_4, 0.5 g of $MgSO_2.7H_2O$, 0.5 g of Na_4SO_2, 0.5 g of NaCl, 10 mg of $ZnCl_2.2H_2O$, 8 mg of $MnSO_2.7H_2O$, 10 mg of $FeSO_4.7H_2O$, and 50 mg of $CaCl_2$ in 1 liter of distilled water, pH 7.0. Medium two (M2) was Tryptic Soy Broth (TSB). To prepare TSB (pH 7.0) an appropriate amount of propionic acid stock solution (1.0 M, pH 7.0) was added to 2x concentrated TSB media and pH was adjusted to pH 7.0 with 1.0 M HCl. For the preparation of anaerobic TSB, the aerobically prepared TSB was gassed with N_2, dispensed into 13 x 100 mm test tubes (4 mL/tube) under an N_2 stream and then sealed with butyl rubber stoppers, followed by autoclaving for 20 min (Kwon and Ricke, 1998). Sterilized aerobic and anaerobic tubes were inculcated and incubated for 3 days at 30°C. To isolate a single colony, 0.1 ml of each culture was spread on Tryptic Soy Agar (TSA) plates. The plates were incubated for 3 days at 30°C and isolates were transferred to fresh media approximately after every two weeks. The pure aerobic isolates were maintained in TSA slants at 4°C until needed for identification and characterization experiments. The pure anaerobic isolates were maintained on TSA, which were prepared aerobically. TSA plates were transferred into an anaerobic chamber (Bactron anaerobic chamers, model II, Sheldon manufacturing Inc. Cornelius, Oregon) having a mixed gas atmosphere (5% H_2, 10% CO_2, and 85% N_2). Finally, several media was used for isolation and characterization of specific microbial groups included MacConkey, thio-sulfate-citrate-bile-sucrose (Difco), and agar-blood media (I.P.A.) for the detection of *Enterobacteriaceae*, *Vibrionaceae*, and *Streptococcaceae*, respectively (Collins et al., 2004).

DNA extraction and touchdown PCR

Bacterial DNA was isolated from bacterial samples by silica-based selective adsorption per manufacturer's instructions (QIAamp DNA Mini kit, Valencia, CA), followed by 1% agarose gel electrophoresis to evaluate the extraction. Extracted DNAs were quantified by using spectrophotometer (GeneQuant, Ge Healthcare). The yield was between 100-200 ng/µl with purity ratio of 1.8. Touchdown PCR was performed to minimize non-specific amplification. In touchdown PCR, the DNA polymerase was added to the reaction mixture at 80°C (after the denaturing step). The reaction was then ramped down to 65°C for primer annealing. Each subsequent annealing step decreased by 1°C until 55°C was reached (10 cycles), then the remaining cycles were performed at this annealing temperature. Touchdown PCR reactions included 10 x ExTaq buffer with $MgCl_2$ 2 mM /reaction, dNTP mix 2.5 mM each /reaction, ExTaq (Fisher Scientific, Pittsburgh, PA) DNA polymerase 1.25 U/reaction and 100 ng/µl of DNA, and total volume was brought up to 50 µl by ddH_2O. The PCR reactions were used to amplify regions within 16 S rDNA by using universal oligonucleotide primers targeting highly conserved regions (Table 1). The reaction tubes were placed in GenAmp 9700 thermal cycler (Applied Biosystems) 94ºC initial denaturation for 5 min, at 80ºC ExTaq with proofreading activity added, 94ºC for 30 s, 65ºC for 1 min. The annealing temperature declined 1ºC per cycle for 10 cycles until it reached 55, 72ºC for 3 min, 72ºC for 10 min for 35 cycles. All PCR products were gel-purified by QIAquick Gel extraction kit (Qiagen, Valencia, CA).

Cloning and sequencing 16S rDNA

To identify bacteria present in our samples, the regions of the 16S

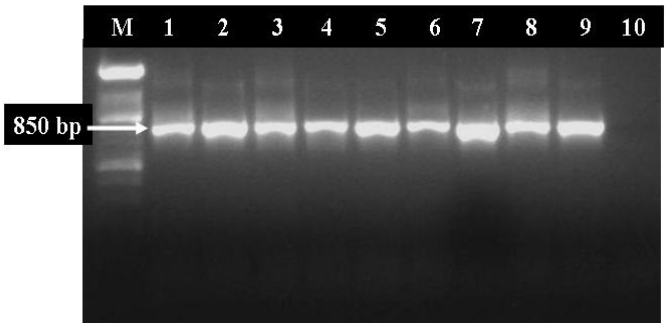

Figure 1. PCR amplicons of 16S rDNA from pure cultures of RPW gut microbiota. The expected size of amplicons was 850 bp from lane 1-9; lane 10 is negative control.

rDNA gene were PCR amplified. Amplicons were gel-purified using GFX PCR DNA and gel band purification kit (GE Healthcare) and sense and anti-sense strands of 16S rDNA were sequenced in an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA.) using ABI BigDye terminator cycle sequencing ready reaction kit chemistry according to manufacturer's recommendations. Following sequencing, each sequencing data were identified using basic local alignment search tool (BLAST). (www.ncbi.nlm.nih.gov/BLAST) and ribosomal databases of Michigan State University (http://rdp.cme.msu.edu/index.jsp). Sequencing data were aligned and the evolutionary relationship of the sequencing information was studied by phylogenetic analyses.

RESULTS AND DISCUSSION

Several studies have been performed about the function of the intestinal microbiota. The digestive system and the absorption of the nutrients are highly dependent on a balanced intestinal microbiota. Further, intestinal microbiota produces useful compounds such as vitamins and enzymes. This study focused on the distribution of aerobic and facultative anaerobic bacteria in RPW larvae. Limited number of bacteria was isolated and identified from the gut of larvae grew on artificial diet. PCR amplicons were obtained from pure culture of RPW gut microbiota with the expected size of 850 bp (Figure 1). Analysis of 16S rDNA sequences revealed that bacterial isolates represented four genus and six species; *Bacillus* sp, *Salmonella* sp, *Enterococcus* sp, and *Xanthomonas* sp. Neither enrichment-selective media nor conditions were adequate for RPW gut microflora. Normally, insect gut microbiota is considered as a complex ecosystem containing over a hundred of bacterial species including anaerobes and facultative anaerobes (Varma, 1994; Brauman, 2000). The role of facultative anaerobes in the termite gut is to scavenge oxygen, which has permeated the exoskeleton into the gut (Madigan et al., 2002). Hence, the presence of large quantities of oxygen can be deadly to strictly anaerobic organisms, such as the protozoa and spirochete found in the termite gut. The oxygen that permeates from the exoskeleton to the gut is effectively scavenged by the facultative organisms and thus protecting the strict anaerobes in the center of the gut, which are essential for cellulose digestion and termite survival (Adams and Boopathy, 2005). Isolating aerobic and facultative anaerobic from larva gut demonstrated a potentially deep penetration of oxygen into the gut and an essential role of oxygen in the mineralization of aromatic compounds. Further, bacteria gut might play several physiological functions. For example, *Enterococcus* strain RfL6, representing the most abundant physiotype among the carbohydrate-utilizing gut bacteria, was not purely fermentative, but consumed oxygen during growth on glucose, accompanied by a complete shift in the product spectrum from lactate to acetate, and was able to oxidize lactate to acetate when oxygen was present (Tholen el al., 1997).

Based on the results of this study, we assume that the majority of the intestinal RPW bacteria are fastidious and/or extremely anaerobic. Several studies have emphasized that the older methods of anaerobic culture, in particular the anaerobic jar and liquid media containing reducing agents, are inadequate for the recovery of extreme anaerobes. Sensitive anaerobes will die if exposed to room air for more than 5 min. The results of this study should have considerable importance for future work on RPW intestinal microbiota. The samples of intestinal larvae should be removed and placed on selective media under anaerobic conditions; anaerobic chamber may be an advantage for the isolation process. On the other hand, growing the larvae on an artificial diet might affect or change the intestinal microbiota (Kane and Breznak, 1991). Hence, the gut of insect acts as continuous culture systems whereby microbes that cannot degrade diet compounds are washed out whereas those that can degrade diet compounds are retained and made to multiply in the gut (Hayashi, et al., 2007). Therefore, it is important to use RPW larvae grown on natural diet as a control to study the inhabiting bacteria of RPW intestine.

One of the most effective and applied approach to study gut microbiota is to use molecular techniques. Recently, the development and application of molecular techniques have facilitated and revolutionized the analysis of mixed bacterial populations in different species. The 16S rDNA based phylogenetic approach has been used to study human and animal intestinal microbial ecology and specific groups of bacteria (Felske et al., 1998). Regions within 16S rDNA evolved at different and slow rates, resulting in alternating conserved and variable regions (Kolbert and Persing, 1999). The presence of conserved regions of 16S rDNA allows the selection of universal oligonucleotide primers for PCR amplification of most prokaryotic organisms. The most significant advantage of using bacterial 16S rDNA is the ability to establish

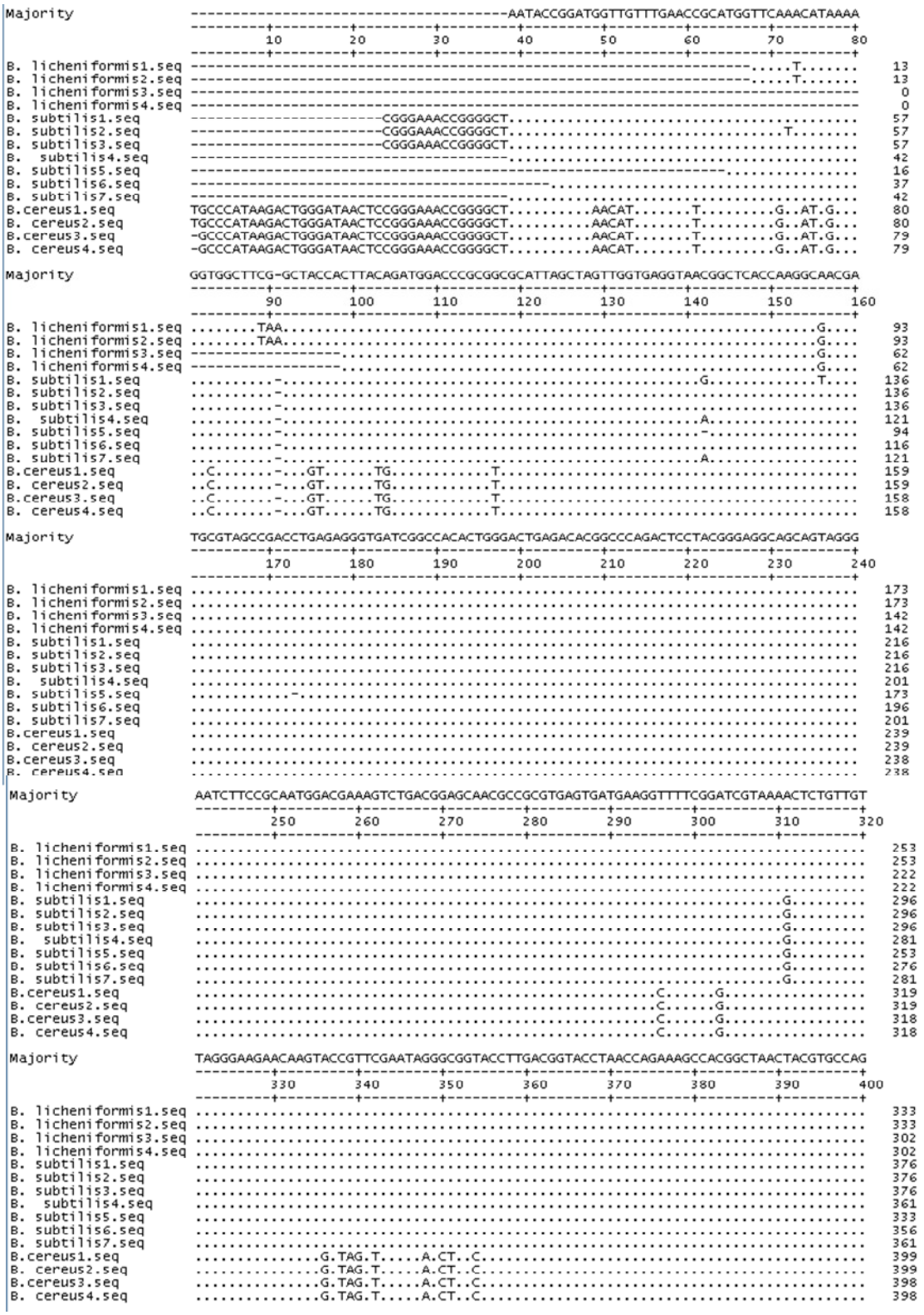

Figure 2. Multiple sequence alignments of 16S rDNA of identified bacterial species was executed by DNAstar LaserGene software version 7, highlighting the similarities and differences among the identified bacteria from pure cultures of RPW gut microbiota. Dot lines indicate consensus sequences.

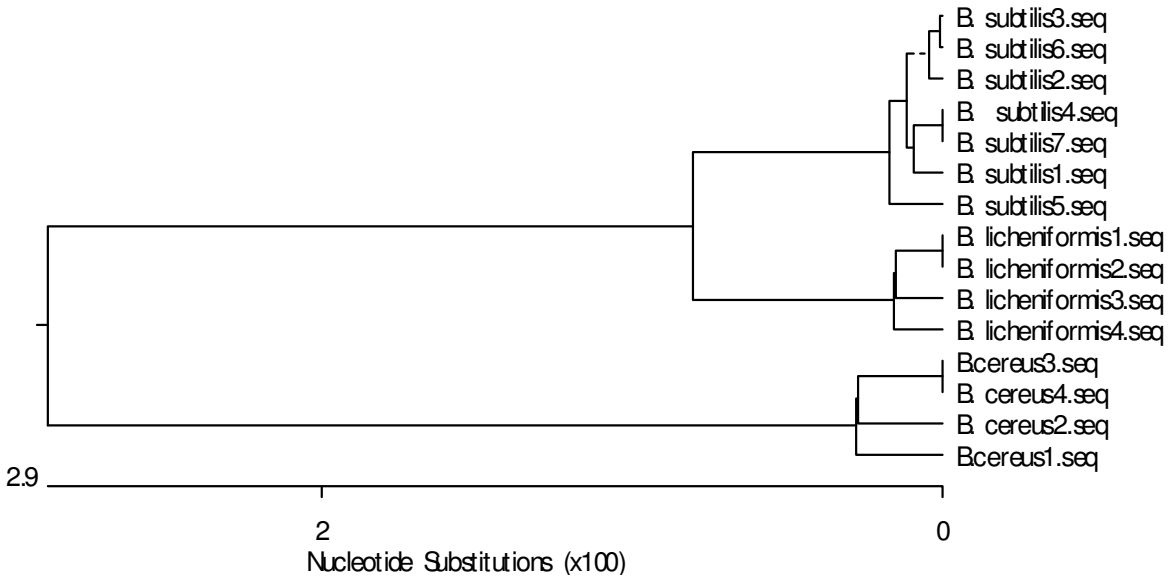

Figure 3. Phylogenetic analysis showing the evolutionary relationship of the bacteria from pure cultures of RPW gut microbiota based on 16S rDNA sequence information.

phylogeny by identification of polymorphisms (Persing, 1993). The 16S rDNA sequences are also used as a marker for genetic bacterial activity (Woese, 1987). Highly variable portions of 16S rDNA sequence provide useful information about the bacteria and the relationships among different genera and species. The 16S rDNA Amplicons may be used directly for sequencing analysis to provide definitive identification of mixed bacterial population and give an indication of representative members in the natural microbial community. The identification is based on BLAST alignment of query sequence against Gene bank sequence database (Figure 2), which was used to construct the evolutionary relationship analysis (Figure 3).

The diversity of intestinal microbiota signifies the need for special requirements for cultivation. Therefore, the use of molecular methods to analyze the intestinal microbiota directly permits global analysis of mixed bacterial population and is a proven tool for rapid assessment of the biodiversity in gut ecosystem. Culture-independent studies by PCR amplification of 16S rDNA followed by sequence determination, and comparative sequence analysis have surpassed the limitation of microbiological identification by conventional culturing techniques.

ACKNOWLEDGMENTS

The authors acknowledge Dr. Hassan Al-Ayedh at KACST for providing the larvae of Red Palm Weevil. This project was supported by a grant from King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia.

REFERENCES

Adams L, Boopathy R (2005). Isolation and characterization of enteric bacteria from the hindgut of Formosan termite. Bioresour. Technol. 96: 1592-1598.

Brauman A (2000). Effect of gut transit and mound deposit on soil organic matter transformations in the soil feeding termite. A review Eur. J. Soil Biol. 36:117–125.

Collins CH, Lyne PM, Grange JM, Falkinham III JO (Eds). (2004). Microbiological methods. Eight edition. London: Arnold, UK.

Felske A, Wolterink A, Van Lis R, Akkermans A (1998). Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils. (The Netherlands) Appl. Environ. Microbiol. 64(3): 871-879.

Hanounik SB, Saleh MME, Abuzuairah RA, Alheji M, Aldhahir H, Aljarash Z (2000). Efficacy of entomopathogenic nematodes with antidesiccants in controlling the red palm weevil *Rhynchophorus ferrugineus* on date palm trees. Int. J. Nematol. 10: 131-134.

Harazono K, Yamashita N, Shinzato N, Watanabe Y, Fukatsu T, Kurane R (2003). Isolation and characterization of aromatics-degrading microorganisms from the gut of the lower termite Coptotermes formosanus. Biosci. Biotechnol. Biochem. 67: 889-929.

Hayashi A, Aoyagi H, Yoshimura T, Tanaka H (2007). Development of novel method for screening microorganisms using symbiotic association between insect (Coptotermes formosanus shiraki) and intestinal microorganisms. J. Biosci. Bioeng. 103: 358-367.

Kaakeh W (2006). Toxicity of imidacloprid to developmental stages of *Rhynchophorus ferrugineus* (Curculionidae: Coleoptera).Lab. Field Tests Crop Prot. 25: 432-439.

Kane MD, Breznak JA (1991). Effect of Host Diet on Production of Organic Acids and Methane by Cockroach Gut Bacteria. Appl. Environ. Microbiol., 2628-2634.

Kolbert CP, Persing DH (1999). Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. Curr. Opin. Microbiol. 2: 299-305.

Kwon YM, Ricke SC (1998). Survival of a Salmonella typhimurium Poultry Isolate in the Presence of PropionicAcid underAerobic and Anaerobic Conditions. Anaerobe 4: 251-256.

Madigan MT, Martinko JM, Parker J (2002). Brock biology of microorganisms. prentice Hall, Upper Saddle River NJ, pp. 477-485.

Murphy ST, Briscoe BR (1999). The red weevil as an alien invasive: biology and prospects of biological control as component of IPM. Biocontrol News Inform. 20:35-46

Persing DH (1993).Diagnostic molecular microbiology. Current challenges and future directions. Diagn. Microbiol. Infect. Dis, 16: 159-163.

Salama HS, Fouda MS, El-Bendary MA, Abdel-Razek A (2004). Infection of red palm weevil *Rhynchophorus ferrugineus,* by spore-forming bacilli indigenous to its natural habitat in Egypt. J. Pest Sci. 77: 27-31.

Tholen A, Schink AB, Brune A (1997). The gut microflora of *Reticulitermes flavipes*, its relation to oxygen, and evidence for oxygen-dependent acetogenesis by the most abundant *Enterococcus* sp. FEMS Microb. Ecol. 24: 137-149.

Varma A, Kolli KB, Pau J, Saxena S, Konig, H (1994). Lignocellulose degradation by microorganisms from termite hills and termite guts: A survey on the present state of art. FEMS Microbiol. Rev., 15: 9-28.

Wattanapongsiri A (1966). Rivision of the Genera *Rhynchophorus* and *Dynamis* (Coleoptera: Curculionidae). Department of agriculture science Bulletin 1, Department of agriculture Bangkok.

Wenzel M, Schonig I, Berchtold M, Kampfer P, Konig H (2002). Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite *Zootermopsis angusticollis*. J. Appl. Microbiol. 92: 32-40.

Woese CR (1987). Bacterial evolution. Microbiol Rev. 51: 221-71.