African Journal of Microbiology Research

Volume 11 Number 29 7 August, 2017

ISSN 1996-0808

Academic Journals

ABOUT AJMR

The African Journal of Microbiology Research (AJMR) is published weekly (one volume per year) by Academic Journals.

The African Journal of Microbiology Research (AJMR) provides rapid publication (weekly) of articles in all areas of Microbiology such as: Environmental Microbiology, Clinical Microbiology, Immunology, Virology, Bacteriology, Phycology, Mycology and Parasitology, Protozoology, Microbial Ecology, Probiotics and Prebiotics, Molecular Microbiology, Biotechnology, Food Microbiology, Industrial Microbiology, Cell Physiology, Environmental Biotechnology, Genetics, Enzymology, Molecular and Cellular Biology, Plant Pathology, Entomology, Biomedical Sciences, Botany and Plant Sciences, Soil and Environmental Sciences, Zoology, Endocrinology, Toxicology. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles are peer-reviewed.

Contact Us

Editorial Office: ajmr@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJMR

Submit manuscript online http://ms.academicjournals.me/

Editorial Board Members

Editorial Board Members

Editorial Board Members

Editorial Board Members

African Journal of Microbiology

Table of Contents: Volume 11 Number 29 7 August, 2017

ARTICLES

academicJournals

Vol. 11(29), pp. 1150-1154, 7 August, 2017
DOI: 10.5897/AJMR2017.8581
Article Number: B30595965421
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Immunological evaluation of sequential poliovirus vaccination among Saudi and non-Saudi children living in Jeddah

Sana G. Alattas[1, 2]

[1]Department of Biological Sciences, Faculty of Science, King Abdulaziz University (KAU) P. O. Box 4702, Jeddah 21412, Saudi Arabia.
[2]Center for Clean Water and Clean Energy, Department of Civil and Environmental Engineering, Massachusetts Institute of Technology-MIT, United States of America.

Received 6 May, 2017; Accepted 14 July, 2017

Poliomyelitis is a life-threatening acute paralytic disease caused by Poliovirus (PV). In the present study, the immunostatus of polio-vaccinated children and young adults (1 to 21 years old) living in Jeddah, Saudi Arabia was investigated to ascertain their extent of protection against the virus. Children were categorized in three groups: (A) Immunocompetent: Group I: Vaccinated by IPV at first and, Group II- Vaccinated by OPV at first; (B) Immunocompromised: Tuberculosis (T.B), diabetes, AIDS, congenital immunodeficiency; and (C) Control group: healthy children vaccinated at a private hospital in Jeddah-IMC hospital. Blood samples (692) were collected from the children admitted to Hospital children wards of King Abdulaziz University Hospital-KAUH (Government), and International Medical Center Hospital-IMC (Private) in Jeddah City, for routine medical examination checkup, during a 24-month period, from January 2015 to December 2016. A total of 228 (32.95%) were Saudis and 464 (67.05%) were non-Saudi individuals. The number of samples found to be negative for polio immunoglobulin G (IgG) and were considered as non-immune children was 72 (10.4%) while the overall immune responders were 584 (84.4%). 36 (5.2%) were low positive and their immunity against polio infection was doubtful. Non-Saudi seronegative subjects varied from 28 (6.03%) Yemani, 24 (5.17%) Somalian, 8 (1.72%) Afghani, 4 (0.86%) Indians, 3 (0.65%) Chadian, 2 (0.43%) Pakistanis; to 2 (0.43%) Nigerians. Based on the present data, we recommend higher vaccination coverage and sensitive surveillance investigation in polio-free countries. Evaluation of vaccination programmes should be carried out for the early detection of immune negative and disease-susceptible individuals.

Key words: Poliomyelitis, poliovirus vaccination, seronegative, immunization, immune response.

INTRODUCTION

Poliomyelitis is a life-threatening acute paralytic disease caused by poliovirus (PV). Coxsackie A7, however, causes a non-Poliovirus flaccid paralysis (Bodian et al., 1949; Bodian, 1972; Nathanson and Martin, 1979; Brack, 1987; Moriniere et al., 1993; Hovi et al., 2005; Thompson et al., 2006; Vancelik et al., 2007; Patel and Orenstein, 2016).

It is one of the major four contagious diseases in the world, with low mortality, but high morbidity rates (Bodian et al., 1949; Thompson et al., 2006; Dhole et al., 2009;

Mugisha et al., 2010). Poliovirus belongs to the Enterovirus genera, Picornaviridae family with three distinctive serotypes (Type 1, 2, and 3) (Bodian et al., 1949; Thompson et al., 2006; Dhole et al., 2009; Mugisha et al., 2010). Poliovirus can be transmitted primarily through the fecal-oral route and also the respiratory system.

In the 1970s, the World Health Organization (WHO) recommended and introduced an expanded immunization programme in which a Poliovirus vaccine dose was given to each child. Still, this never reached a complete coverage with adequate high levels; hence at the beginning of the millennium, the Wild Polio Virus was re-emanated in considerable number of supposed polio-free countries, which confirms the ultimate fragile herd immunity in those countries (Hovi et al., 2011).

In humans, the virus replicates at the intestinal tract and it is released with the stool usually for 2 to 4 weeks after infection. The virus spread is related to poor hygiene, and sewage-treatment services. Faeces serve as a contamination source of water, milk, and food. Hence, young children are probably the most important transmitters of Enteroviruses.

The accreditation of inactivated poliovirus vaccine (IPV) in 1955 and Oral Polio Vaccine (OPV) in 1962 encouraged the worldwide beginning of vaccination programmes (Nathanson and Martin, 1979; Cheuk, 2007). The IPV is prepared by inoculating the monkey kidney tissue culture (vero cell line) with the poliovirus (CDC, 2001a, b).

The vaccine contains the three poliovirus serotypes (CDC, 2001a, b), which induces effective circulation of antibodies in blood, thereby preventing any polio virus that finds its way to the intestine from entering and replicating in the central nervous system (Vancelik et al., 2007).

On the other hand, the live-attenuated vaccine is a trivalent vaccine, containing the three serotypes of poliovirus in a ratio of 10:1:6 (CDC, 2001a, b; Kew et al., 2004). These weakened PV strains replicate in the human intestine and induce mucosal immunity that prevents the viral replication at the gastrointestinal tract (CDC, 2001a, b; Kew et al., 2004; Cheuk, 2007). The OPV yields lifelong mucosal immunity by encouraging production of IgA antibody in the intestinal tract and furthermore serum antibodies in the circulating blood (Pelczar et al., 1993; Cheuk, 2007).

Polio national immunization schedule in Saudi Arabia includes a vaccination with IPV at 2 months of age, followed by OPV in 4,6,12,18 months, and an OPV booster dose at the primary school entry (MOH). In the meantime, starting from April 2016, all nations using OPV have converted to bivalent OPV (bOPV) as part of the last steps for universal elimination of all-cause poliomyelitis. bOPV retains safety against type 1 and 3 polioviruses, but leaves young children susceptible to infection by type 2 vaccine-derived polioviruses (Bandyopadhyay et al., 2015; Patel and Orenstein, 2016).

To support the population immunity and confirm that all children are safe against type 2 polioviruses in nations that are polio-endemic, or at great danger of the virus importation, the WHO Strategic Advisory Group of Experts (SAGE) recommends at least one dose of IPV, given with the third dose of bOPV at 14 weeks of age or older, to decrease the interloping from maternally-derived antibodies (WHO, 2013).

In nations with 90 to 95% immunization report of low importation threat, IPV-OPV sequential schedules can be used to reduce the risk of vaccine-associated paralytic polio (VAPP) (Lopez-Medina et al., 2017; WHO, 2004).

Moreover, studies that approved a sequential schedule of immunization from other nations that administered multiple IPV doses followed by various OPV doses received by infants, have furthermore established that VAPP was eradicated. Nevertheless, the risk of VAPP was not explicitly estimated in nations that embraced the recently recommended universal polio immunization schedule which is: 3 doses of OPV plus a singular dose of IPV at 14 weeks of age (Progress toward Interruption of Wild Poliovirus Transmission-Worldwide, 2006).

A major difference exists between private and government hospitals (MOH) in Jeddah, Saudi Arabia, in terms of quality and standards. Aside the low standard of some Health centres or hospitals, there are many other factors that also influence the efficacy of the administered vaccines such as: storage, transportation, and availability of qualified health providers. Since most pilgrims with unidentified vaccination status constitute a group at high risk in the situation of wild polioviruses importation into the Saudi Arabian Kingdom, it is thus essential to continue seroepidemiological monitoring.

And to effectively evaluate the influence of vaccination schedules on the people's immune status as well as to improve immunization programmes, virological and immunological studies are, no doubt, required (Patriarca et al., 1991; Moriniere et al., 1993; Pelczar et al., 1993; Fine and Carneiro, 1990; The Annual Statistics Book of Health, 2004; Progress toward Interruption of Wild Poliovirus Transmission-Worldwide, 2006; Certification of Poliomyelitis Eradication- European Region, 2002; Tafuri et al., 2008; Dhole et al., 2009; Platt et al., 2014).

In the present study, therefore, the immunostatus of polio-vaccinated children and young adults (1-21 years old) living in Jeddah was investigated to ascertain their extent of protection against the virus, via estimation of circulating immunoglobulin G (IgG).

E-mail: sangtas@mit.edu; sgalattas@kau.edu.sa.

Table 1. All Polio-ELISA results among Saudi and non-Saudi cases.

Nationality	Polio ELISA				Total
	Positive	Low Positive	Equivocal	Negative	
Saudi	192	12	16	8	228
Non Saudi	392	24	24	24	464
Total	584	36	40	32	692

MATERIALS AND METHODS

Sampling and experimental design

The present study was carried out within a 24-month period between January 2015 and December 2016. A total of 692 blood specimens were gathered from children admitted to the children wards of King Abdulaziz University Hospital-KAUH (Government) and International Medical Center Hospital-IMC (Private) in Jeddah City, Saudi Arabia for routine medical examination checkup. Each child's data was collected on a precoded inquiry form recording name, age, sex, nationality, and hospital entry reasons. It is worth mentioning that the consents of participants (the children) were acquired from the cases or their custodians earlier in the study and were revised and accepted by the Ethics committee of Clinical Microbiology Research Center, King Abdulaziz University. Of the 692 children and adults enrolled in the study, 228 children and young adults were Saudi residents. The remaining 464 screened individuals were children and young adults from Chad, Yeman, Pakistan, Somalia or other nationalities. The participants were categorized into the following groups based on their health or hospital records regarding anti-poliovirus vaccination:

(A) Immunocompetent:

Group I: Vaccinated by IPV at first
Group II: Vaccinated by OPV at first

(B) Immunocompromised:

Tuberculosis (T.B.), diabetic, AIDS, and/ or congenital immunodeficiency patients.

(C) Control group:

50 healthy children who have completed all vaccination doses from a private hospital in Jeddah- IMC hospital were used as controls.

The inclusion criteria for children used in the study were that, they were children between 1 to 21 years, and have completed the anti-poliovirus vaccination program (3 doses+1booster dose). Exclusion measures were; new administration of immunoglobulin, blood products or immunosuppressive treatment.

Specimen collection and handling

5 ml of venous blood samples was collected from each participant in plain tubes under complete aseptic conditions, following standard precautions. The samples were left to coagulate for some minutes and thereafter centrifuged at 3000 g for 5 min. The supernatant (serum) was later collected and stored at -20°C till use. Prior to use for analysis, the sera were diluted 1:101 with ready to-use sample diluent (e.g. 5 µl serum sample diluents).

Detection of IgG in serum

Specific Polio immunoglobulin G antibody (IgG Ab) was quantified by using ELISA kit (IMMUNOLAB GmbH, Otto-Hahn-Str. 16, D-34123 Kassel) for semiquantitative detection of IgG anti-Poliovirus in children's serum samples. The assay results were collated based on the instructions on the manufacturer's assay protocol.

Statistical analysis

The results of the anti-poliovirus data for children from Saudi and non-Saudi were analyzed using the Student's t-test. Where $p>0.05$, the compared means were considered as non-significantly different. Data computation was done using SPSS (version 20) for windows.

RESULTS AND DISCUSSION

A total of 692 serum specimens were collected and tested from January 2015 to December 2016. 72 (10.4%) of these specimens were found to be seronegative for polio IgG and were regarded as non-immune children. Overall immune responders were 584 (84.4%) out of which 36 (5.2%) were low positive and hence their immunity against polio infection is doubtful. A total of 228 (32.95%) were Saudis, and 464 (67.05%) were non-Saudi individuals (Table 1). Non-Saudi (N.S.) seronegative subjects varied from 28 (6.03%) Yemani, 24 (5.17%) Somalian, 8 (1.72%) Afghani, 4 (0.86%) Indians, 3 (0.65%) Chadian, 2 (0.43%) Pakistanis; and 2 (0.43%) Nigerians (Table 1).

Vaccination

The schedule for Polio vaccination in the government hospital in Jeddah was to give IPV in 2 months age, followed by OPV in 4, 6, 12, 18 months, and an OPV booster dose at the primary school entry, while, in the private hospital was as follows: IPV in 2, 4, 6 months age, followed by OPV in 12, 18 months, and an OPV booster dose at the primary school entry.

However, at the end of the study all vaccinations schedules were standardized among governmental and private hospitals in Jeddah, to give IPV in 2,4,6 with a drop of OPV in 6 months age, followed by OPV drop in 12, 18 months, and an OPV booster dose at the primary school entry.

The principal sequence of IPOL vaccine comprises of three 0.5 ml doses intramuscularly or subcutaneously administered, and it is advisable to be eight or more weeks separately and typically at ages 2, 4, and 6 to 18 months (Progress toward Interruption of Wild poliovirus Transmission-Worldwide, 2006). The vaccine should be

given more often than four weeks separately under no circumstances. The first immunization dose could be given at primary as six weeks of age. For this sequence, a booster dose of IPOL vaccine is given at 4 to 6 years of age (Progress toward Interruption of Wild poliovirus Transmission-Worldwide, 2006).

In recent United States studies, a combination of IPV and OPV was utilized which efficiently generated high neutralization titers (Ertem et al., 2000; Saleem et al., 2014).

Conclusion

In this study, we found that a number of children were still seronegative for circulating IgG [72 (10.4%)], and 36 (5.2%) were low seropositive. Seronegative children are at high risk and vulnerable to Poliovirus; it is a strong indicator for failure of vaccination.

The study results also showed that 5.2% of the participants were weak responders to the vaccine with low seroconversion. Low seroconversion rate might be due to a number of reasons such as incomplete vaccination, simultaneous enteroviral infections, interloping between serotypes of OPV and deprived sanitization in water supply and seawage treatment (Faden et al., 1990; Vancelik et al., 2007; Tao et al., 2013, 2016). Accidental occupation and great inhabitance expansion level may be additional reasons for low seroconversion rates, in addition to illiteracy of parents.

On the other hand, poor maintenance in cold chain and, suboptimal habits of vaccine processing could result in low seropositivity (CDC, 2006). From the observed variations in responders status, it could be inferred that cultural differences may play a role in formation of attitudes and behaviours towards vaccination, as we found immune non-responders among non-Saudi varied from 28 (6.03%) among Yemani, 24 (5.17%) among Somalian, 8 (1.72%) among Afghani, 4 (0.86%) among Indians, 3 (0.65%) among Chadian, 2 (0.43%) among Pakistanis; to 2 (0.43%) among Nigerians (Table 1), even though all of them were from the government hospital in Jeddah.

Ultimately, the study data showed that Polio vaccination programme failed for 10.4% of studied children, and was insufficient for 5.2% of studied children. As Jeddah city remains a risk subject of the poliovirus importation from endemic regions, it is important to reach high immunization proportions to sustain current situation. If any country or its neighbors have a WPV importation recognition, then all health specialists need to quickly introduce additional immunization actions to limit the spread of WPV and achieve outbreaks interruption.

Based upon the present data, we recommend higher vaccination coverage and precise monitoring systems in polio-free countries. Evaluation of vaccination programmes should be implemented for early detection of immuno negative disease-susceptible individuals.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

ACKNOWLEDGMENTS

Authors would gratefully acknowledge the unconditional support of Prof. Maha Kawashti, and also to thank the participating children, the administrative staff of the health care centers who collaborated in obtaining clinical samples and the KFGH -Immunology laboratory senior Mrs. Hanan Al Wajeeh for her technical help and cooperation. The effort of Mr. Salah Bernawe is also highly appreciated for his help with the statistical analysis.

REFERENCES

Bandyopadhyay AS, Garon J, Seib K, Orenstein WA (2015). Polio vaccination: past, present and future. Future Microbiol. 10:791-808.

Bodian D (1972). Poliomyelitis. In. Pathology of the Nervous System, Vol 3. Minckler (ed.). New York: McGraw-Hill, pp. 2323-2344.

Bodian D, Morgan I M, Howe HA (1949). Differentiation of types of poliomyelitis viruses. III. The grouping of fourteen strains into three basic immunological types. Am. J. Hyg. 49:234-245.

Brack M (1987). Agents Transmissible from Simians to Man. Berlin: Springer-Verlag.

Centers for disease Control and prevention (CDC) (2006). Resurgence of wild poliovirus type 1 transmission and consequences of importation-21 countries, 2002–2005. MMWR 55:145-150.

Centers for disease Control and prevention (CDC) (2001a). Circulation of a type 2 vaccine-derived poliovirus-Egypt, 1982–1993. Morbid. Mortal. Weekly Rep. 50:41-51.

Centers for disease Control and prevention (CDC) (2001b). Progress toward global eradication of poliomyelitis. Morbid. Mortal. Weekly Rep. 2002a. 51:253-256.

Certification of poliomyelitis eradication- European Region (2002). MMWR -Morb. Mortal. Weekly. Rep. 51:572-574.

Cheuk DKL (2007). New Childhood Immunisation Programme in Hong Kong. The Hong Kong Medical Diary 12(7):22-24.

Dhole TN, Ayyagari A, Chowdhary R, Shakya AK , Shrivastav N, Datta T, Prakash V (2009). Non-Polio Enteroviruses in acute flaccid paralysis children of India: Vital assessment before Polio eradication. J. Paediatr. Child Health 45:409-413.

Ertem M, Sarac A, Tumay S (2000). Poliomyelitis eradication programme: acute flaccid paralysis surveillance in Mardin and five other provinces around Mardin, Turkey 1998. Public Health 114:286-290.

Faden H, Modlin JF, Thoms ML, McBean AM, Ferdon MB, Ogra PL (1990). Comparative evaluation of immunization with live attenuated and enhanced-potency inactivated trivalent poliovirus vaccines in childhood: systemic and local immune responses. J. Infect. Dis. 162(6):1291-1297.

Fine EMP, Carneiro AM (1990). Transmissibility and persistence of oral polio vaccine viruses: Implications for the global poliomyelitis eradication initiative. Am. J. Epidemiol. 150:1011-1012.

Hovi T, Blomqvist S, Nasr E, Burns CC, Sarjakoski T, Ahmed N, Savolainen C, Roivainen M, Stenvik M, Laine P, Barakat I, Wahdan MH, Kamel FA, Asghar H, Pallansch MA, Kew OM, Gary HE Jr, de Gourville EM, El Bassioni L(2005). Environmental surveillance of wild poliovirus circulation in Egypt-balancing between Mugisha et al. Oral polio vaccination in captive chimpanzees detection sensitivity and workload. J. Virol. Methods 126:127-134.

Hovi T, Shulman LM, Vander Avoort H, Deshpande J, Ro Iva Inen M, De Gourville EM (2011). REVIEW ARTICLE Role of environmental poliovirus surveillance in global polio eradication and beyond. Epidemiol. Infect. Page 1 of 13. f Cambridge University Press and World Health Organization.

Kew OM, Wright PF, Agol VI, Delpeyroux F, Shimizu H, Nathanson N, Pallansch M (2004) . Circulating vaccinederived polioviruses: current state of knowledge. Bull. World Health Organ. 82:16-32.

Lopez-Medina E, Melgar M, Gaensbauer JT, Bandyopadhyay AS, Borate BR, Weldon WC, Rüttimann R, Ward J, Clemens R, Asturias EJ (2017). Inactivated polio vaccines from three different manufacturers have equivalent safety and immunogenicity when given as 1 or 2 additional doses after bivalent OPV: Results from a randomized controlled trial in Latin America. Vaccine 35(28):3591-3597.

Moriniere BJ, van Loon FP, Rhodes PH, Klein-Zabban ML, Frank-Senat B, Herrington JE, Patriarca PA (1993). Immunogenicity of a supplemental dose of oral versus inactivated poliovirus vaccine. Lancet 341:1545-1550.

Mugisha L, Pauli G, Opuda-Asibo J, Joseph OO, Leendertz FH, Diedrich S (2010). Evaluation of poliovirus antibody titers in orally vaccinated semi-captive chimpanzees in Uganda. J. Med. Primatol. 39:123-128.

Nathanson N, Martin JR (1979). The epidemiology of poliomyelitis: enigmas surrounding its appearance, epidemiology, and disappearance. Am. J. Epidemiol. 110:672-691.

Patel M, Orenstein WA (2016). A World free of polio – the final steps. N. Engl. J. Med. 374:501-503.

Patriarca PA, Wright PF, John TJ (1991). Factors affecting immunogenicity of oral polio vaccine in developing countries: A review. Rev. Infect. Dis. 13:926-939.

Pelczar MJ Jr, Chan ECS, Krieg NR (1993). Microbiology: Concepts and Applications. McGraw-Hill, Inc., New York. 698.

Platt LR, Estívariz CF, Sutter RW (2014). Vaccine-Associated Paralytic Poliomyelitis: A Review of the Epidemiology and Estimation of the Global Burden. Epidemiology and Global Burden of VAPP. J. Infect. Dis. 210(suppl_1):S380- S389.

Progress toward interruption of wild poliovirus transmission--Worldwide January 2005-March 2006 (2006). MMWR Morb. Mortal Wkly. Rep. 55(16):458-462.

Saleem AF, Mach O, Yousafzai MT, Khan A, Weldon WC, Oberste MS, Sutter RW, Zaidi AKM (2014). Needle adapters for intradermal administration of fractional dose of inactivated poliovirus vaccine: Evaluation of immunogenicity and programmatic feasibility in Pakistan. Vaccine 35(24):3209-3214.

Tafuri S, Prato R, Martinelli D, Calvario A, Bozzi A , Labianca M , Patti A, Lopalco PL, Germinario C (2008). Serological survey on immunity status against polioviruses in children and adolescents living in a border region, Apulia (Southern Italy). BMC Infect. Dis. 8(1):150.

Tao Z, Zhang Y , Liu Y, Xu A, Lin X, Yoshida H, Xiong P, Zhu S, Wang S, Yan D, Song L,Wang H, Cui N, Xu W(2013). Isolation and Characterization of a Type 2 Vaccine-Derived Poliovirus from Environmental Surveillance in China, 2012. PloS One 8(12):e83975.

Tao Z, Wang Z, Lin X, Wang S, Wang H, Yoshida H, Xu A, Song Y (2016). One-year Survey of human enteroviruses from sewage and the factors affecting virus adsorption to the suspended solids. Sci. Rep. 6:31474.

The annual statistics book of health (2004). Ankara, Turkey: The Republic of Turkey Ministry of Health. P 68.

Thompson KM, Duintjer Tebbens RJ, Pallansch MA (2006). Evaluation of response scenarios to potential polio outbreaks using mathematical models. Risk Anal. 26:1541-1556.

Vancelik S, Guraksin A, Ayyildiz A, and Beyhun N E (2007). Seroepidemiology of poliovirus antibody among the children in Eastern Turkey. Indian J. Med. Res. 126:528-533.

World Health Organization (WHO) (2004). Polio eradication initiative. Office of the Director-General and Department of Immunization, Vaccines and Biologicals, Family and Community Health. The Global Polio Eradication Initiative Strategic Plan 2004-2008. WHO: Geneva.

World Health Organization (WHO) (2013). Global Polio Eradication Initiative. Polio Eradication & Endgame Strategic Plan 2013-2018. Geneva, Switzerland: World Health Organization (2013). <http://polioeradication.org/wpcontent/uploads/2016/07/PEESP_EN_A4.pdf>[accessed 23.01.17].

academicJournals

Vol. 11(29), pp. 1155-1161, 7 August, 2017
DOI: 10.5897/AJMR2017.8567
Article Number: F6DB6AD65426
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

In vitro evaluation of compost extracts efficiency as biocontrol agent of date palm *Fusarium* wilt

El kinany Said[1,2,3*], Achbani El hassan[2], Haggoud Abdellatif[3], Ibijbijen Jamal[4], Belmalha Saadia[1], Rachidi Fouad[1], Echchgadda Ghizlane[1] and Bouamri Rachid[1*]

[1]Department of Plant and Environment Protection, National School of Agriculture, Meknes, Morocco.
[2]Laboratory of Plant Protection URPP, National Institute for Agricultural Research, Meknes, Morocco.
[3]Laboratory of Microbial Biotechnology, Faculty of Sciences and Technology, Sidi Mohamed Ben Abdellah University, Fez, Morocco.
[4]Department of Biology, Faculty of Sciences, Moulay Ismail University, Meknes, Morocco.

Received 20 April, 2017; Accepted 14 June, 2017

Bayoud, vascular wilt of date palm caused by *Fusarium oxysporum* f. sp. *albedinis* (*Foa*), is widely distributed in all date palm growing regions of Morocco. It is the most serious disease of the date palm. Compost is recognized for their ability to improve soil characteristics and to protect the crops against biotic and abiotic stress. In this experiment, *in vitro* effects of different concentrations of sterilized and unsterilized compost extract on the growth of *F. oxysporum* f. sp. *albedinis* were evaluated. All concentration of unsterilized compost extract decreased radial growth of *Foa*. In fact, fungal radial growth inhibition ranged from 20 to 97%. Higher antifungal activity was noted in 30 and 40% concentration (more than 93%). Nevertheless, sterile compost extract inhibited mycelia growth only for the 40% concentration with 18% fungal growth inhibition, while lower concentrations were not effective.

Key words: Date palm, *Fusarium oxysporum* f. sp. *albedinis*, compost extract, mycelium growth, inhibition rate.

INTRODUCTION

The date palm, *Phoenix dactylifera* L. is one of the most important species in the palm family (*Palmaceae*) which includes 200 genera and more than 2500 species (El Hadrami and Al-Khayri, 2012; Hadrami and Hadrami, 2009). The genus *Phoenix* consists of fourteen species distributed in the tropical and sub-tropical regions (Al-balqa, 2016). *P. dactylifera* L. is claimed to encompass over 5000 cultivars, some of which have been more or less characterized in detail (El-Hadrami and Al-Khayri, 2012). Date palm is of great economic importance to oasis agriculture where abiotic factors are extreme. Otherwise it creates favorable conditions for improving growth of secondary crops like olive tree, wheat and others leguminous plants. In the world, 100 million trees were estimated with an average of production of 7.62 million tons (FAO, 2010). Moroccan palm groves alone cover 50 000 ha corresponding to 5 million tree and 100.000 tons/year (Sedra, 2012). In Morocco, 223 cultivars have been absolutely characterized since 1992 (Saaidi, 1992; http://www.agriculture.gov.ma).

*Corresponding author: E-mail: rbouamri@enameknes.ac.ma or said.elkinany@usmba.ac.ma.

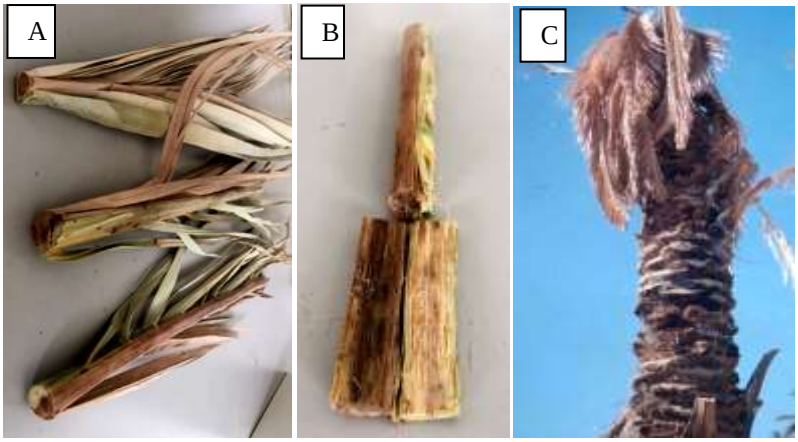

Figure 1. Bayoud symptoms: **A,** unilateral wilting of rachis; **B,** Fusarium wilt within rachis; **C,** late stage of infection (plant death).

Economically, the date palm fruit and by-product are precious for their nutritional and dietetic properties and income generating for oasis' populations (Al-shahib and Marshall, 2003; Chao and Krueger, 2007; Saafi et al., 2008). "Bayoud" disease (Figure 1) is the most destructive fungal disease of date palm. Its causal agent is *Fusarium oxysporum* f. sp. *albedinis* (*Foa*). The impact of the disease is very serious in North Africa, particularly in Morocco where 2/3 of palm tree were destroyed so far (Chakroune et al., 2008; Pereau-Leroy, 1958). In addition, *Fusarium* wilt has killed more than 10 million palm trees during the last 100 years (Dihazi et al., 2012; Saaidi, 1992). The control of the disease using chemicals products is not effective and implies negative effects on environmental and human health (Bernal-Vicente et al., 2008; Boulter et al., 2000; Brimner and Boland, 2003). Prophylactic methods are not of interest due to the contamination of several date palm groves and to their non-durable impact (Jaiti et al., 2007; Saaidi, 1992). Planting resistant cultivars constitutes the only efficient and economic method to control *Fusarium* wilt despite the fact the available cultivars have produced poor date (Saaidi, 1992). In Morocco, cultivars that are sensitive are economically important (Mejhoul and Boufegous).

Another alternative method to control phytopathogenic fungi consists of applications of compost and/or its extract (Alberto et al., 2016; El-Masry et al., 2002; Markakis et al., 2016; Pane et al., 2013, 2012; Sghir et al., 2015). The compost extract is an organic product obtained after fermentation of compost in liquid phase for a few days to up two weeks or just for few hours of mixing with or without aeration (short preparation) (Ingham, 2003; Lanthier, 2007). A number of factors which are involved in the compost extraction process, such as temperature, aeration, organic matter and microbiological properties, are responsible for their efficiency in plant disease suppression (Pane et al., 2012). The use of compost extract as a biological control agent (BCA) against soil borne diseases has increased in the last years. Several researchers showed that the compost extract can control several pathogenic fungi like *Botrytis cinerea, Alternaria alternata, Pyrenochaeta lycopersici* (Palou et al., 2013; Pane et al., 2012), *Pythium debaryanum, Sclerotium bataticola* and *Fusarium oxysporum* f. sp. *lycopersici* (El-Masry et al., 2002). Nine compost extracts based on animal manures (cattle manure, sheep manure, chicken manure and horse manure) were used *in vitro* against numerous pathogenic fungi causing different plant diseases (*Fusarium oxysporum* f. sp. *radici-lycopercisi*, *Fusarium solani*, *Fusarium graminerum, Fusicoccum amugdalis, Alternaria sp*., *Colletotrichum coccodes, Botrytis cinerea, Sclerotinia sclerotiorumn Aspergilus niger, Rhizoctonia solani, Rhizoctonia bataticola, Phytium* sp. and *Verticilium dahliae*) and revealed important results (Kerkeni and Khedher, 2007). The suppressiveness of compost extract is mostly due to its microbial community (Koné et al., 2010; Lin et al., 2014; Pane et al., 2012; Powell and Barry, 2017; Suárez-Estrella et al., 2013; Ventorino et al., 2016). These microbes exert their antagonism by microbiostasis, antibiosis and hyperparasitism and/or stimulate systemic resistance in host plants (Le Page and Bousquet, 2007). However, other researchers reported that compost actions are due to physical, chemical, biochemical and microbiological dynamic interactions with plant-pathogen system (Le Page and Bousquet, 2007). Indeed, several organic chemicals present in compost or released by compost-inhabiting microorganisms have been identified as providing disease suppressive effects, including phenolic compounds, volatile fatty acids and salicylic acid (Le Page and Bousquet, 2007). It is also demonstrated that compost or its extract induce systemic resistance against pathogenic fungi in numerous plants (Kavroulakis et al., 2005; Sang et al., 2010).

Therefore, the main objective of this study was to investigate the suppressive capacity of unsterilized (UCE) and sterilized (SCE) compost extract against *Foa* growth,

Table 1. Rates of raw materials used in composting process.

Raw matter	CM (%)	OMWW (%)	DOMW (%)	OMW (%)	VS (%)
Rate (%)	47	5	12	17	19

CM: Chicken manure, OMWW: olive mill waste water, DOMW: dry olive mill waste, OMW: two phases olive mill waste, VS: vine shoot.

Table 2. Physical and chemical properties of different composting raw materials.

Parameter	CM	OMWW	DOMW	OMW	VS
Moisture (%)	63.5	77.7	19.8	54.2	39.2
pH	7.9	4.9	8.5	6.3	8.7
CE (mS. Cm^{-1})	12.0	29.9	6.6	2.4	3.3
C/N	7.4	27.0	44.8	29	41
Organic carbon (%)	13.2	6.7	76.1	20.3	29.2
Total nitrogen (%)	1.8	0.3	1.7	0.7	0.7
P2O5 (%)	1.3	0.3	0.3	1.0	0.2
K2O (%)	1.1	3.0	1.0	0.4	0.5

CM: Chicken manure, OMWW: olive mill waste water, DOMW: dry olive mill waste, OMW: two phases olive mill waste, VS: vine shoot.

focusing on the abiotic and biotic factors involved in this suppressive mechanism.

MATERIALS AND METHODS

Fungal isolate

In the Tafilalet date palm grove, the leaf samples were collected from symptomatic and non-symptomatic Mejhoul cultivar trees and used for *Fusarium* species isolation and identification. Twenty fragments of date palm leaf of each sample were surface-sterilized for 5 min with a 4% sodium hypochlorite solution, rinsed twice in sterile distilled water and dried in a laminar flow cabinet. The growth medium potato dextrose agar (PDA) was used for fungal isolation. The plates were incubated at 28°C in the dark for 7 days. All *Fusarium* isolates were subcultured on PDA medium and incubated for purification and spores production for 7 days in the dark and two weeks of light. After that, cultural characters were assessed by microscopic examination. The morphology of macroconidia, microconidia and the chlamydospores was assessed and the identification was made using the criteria of Sedra (2012).

Compost source

The compost used in this experiment was produced in a private composting unit in Meknes, Morocco using a mixture of agricultural waste (chicken manure and vine shoot) and agro-industrial waste (olive mill waste and olive mill waste water) (Table 1). The windrow composting system was used in which mixtures were subjected to interval turning over every two weeks. Different raw material and compost were analyzed in a private laboratory to determine their physical and chemical characteristics (Table 2).

Compost extract preparation

Compost extract were prepared following the method of El Masry et al. (2002). The mature compost was suspended in phosphate buffer containing K_2HPO_4 (8 g/l) and NaH_2PO_4 (0.34 g/l) at a ratio of 1:2 (w/v). Then, it was well shacked (150 rpm) for 72 h under room temperature in natural photoperiod (24/11°C day/night). The mixture was splitted in sterile centrifuge tubes (50 ml) and centrifuged at 500 g (gravity) for 10 min, to remove large particles, then, at 1000 *g* for 10 min to obtain the active supernatant (compost extract). A portion of compost extract was autoclaved (120°C for 20 min) to obtain a sterile compost extract (SCE).

In vitro suppressive effect of compost extract

The suppressive effect of the compost extract against *F. oxysporum* f. sp. *albedinis* (*Foa*) was examined using well-cut diffusion technique (Pane et al., 2013). Both sterilized and unsterilized composts extracts were used in five different concentrations: 10, 15, 20, 30 and 40% (v/v). PDA medium (before cooling step) was used for preparation of compost extract concentrations, and mixtures were cooled into Petri dishes (90 mm of diameter). For control plates, the PDA medium was supplemented with phosphate buffer PBS sterile. An active mycelia disk (5 mm in diameter) of pathogen was placed at the center of the Petri plates. All Petri plates were then incubated at 28°C and evaluated for pathogen growth monitoring during 8 days of incubation. Five replicate were used per elementary concentration and experiment was repeated twice.

To determine the inhibition rate (IR) of the pathogen by applying each of the tested compost extract, the radial fungal growth of *Foa* was monitored by measuring the colony diameters for the control and treated plates at 0, 2, 4, 6 and 8 days. The inhibition rate was calculated according to the formula used by Hibar et al. (2006):

IR (%) = (1–(Average diameter of the treated / Average diameter of the control)) x 100

Five repetitions were carried out for each UCE or SCE concentrations and controls. All plates were incubated at 26°C until

Table 3. Physical and chemical properties of mature compost.

Parameter	Value
Moisture (%)	44.83
pH	6.69
Organic matter (%)	32.48
Total Kjeldhal nitrogen (NTK) (%)	1.29
Phosphorus (P_2O_5) (%)	1.74
Potassium (K_2O) (%)	1.22
Organic carbon (%)	16.24
C/N	12.59
Electric conductivity ($ms.cm^{-1}$)	24.69

control plates were fully covered by mycelium (8 days). After incubation, linear reduction of the radius-growth of *Foa* was measured.

Experimental design and statistical analysis

Petri plates were distributed in a completely randomized design with five Petri plates per elementary treatment and the whole experiment was repeated two times. Data were analyzed using SPSS statistical program version 12.0 and subjected to the analysis of regression relation between unsterilized extract concentration and rate inhibition of *Foa*.

RESULTS AND DISCUSSION

Organic matter, C/N ratio, total nitrogen, phosphorus, potassium and pH of raw material and final compost are presented in Table 3. The compost produced and used showed pH with suitable value of 6.69 and the electrical conductivity of about 24 $ms.cm^{-1}$. Compost had more than 32% organic matter content. The total nitrogen, the phosphorus and the potassium were greater than 1%. The C/N ration is suitable for sustainable agriculture, because ratio of 27 in OMSW (Table 1) in raw materials was previously confirmed for increasing pore space and allowing bulk oxygenation (Barje et al., 2016). In addition, the same compost composition was used to promote date palm (cv. Mejhoul) at 1:3 ratio (v/v). It has increased significantly all growth parameters as well as nutrient and chlorophyll content without any toxicity (data not shown).

After 8 days of incubation at 28°C, results shown in Figures 2, 3 and 4 revealed that unsterilized compost extract caused an inhibiting effect on mycelium development of *Foa* when compared with untreated control (*Foa* alone). However, the mycelia growth of *Foa* measured in the different Petri plates during incubation varied with the compost extract concentration (Figure 4). The results of the regression analysis showed a significant relationship, at 5% levels, between suppressive effect and concentration of unsterilized compost extract (Table 4). The regression coefficient was 0.966 (Table 5).

The most effective concentrations were 30 and 40% where the pathogen development was completely inhibited (more than 97%). For the remaining concentrations (10, 15 and 20%), mycelium growth decrease ranged from 20 to 44% as compared to the control (Figures 1, 2 and Table 6). After the period of incubation, some compost-inhabiting microorganisms (bacteria and fungi species) were observed to develop in the Petri dishes and had antagonistic effect against *Foa* (Figure 2). On the other hand, no suppressive effect towards *Foa* was observed in the sterilized compost extract except for high concentration (40%) which showed an inhibitory effect of about 18% (Figure 3). This reduction of mycelium growth in the 40% concentration of sterilized compost extract may be due to some chemical compounds elaborated during composting process and remained after the sterilization step or some thermostable extracellular metabolites. These results confirm the findings of Kerkeni and Khedher (2007) and El-Massry et al. (2002) who showed that compost extract prepared from various animal manures had high inhibitory effects of *F. oxysporum* f. sp. *radices-lycopersici* which can be attributed to active microorganisms inhabiting the unsterilized compost extract. Indeed, as confirmed by other research, compost extract might have contained antagonistic mycoparasites, such as *Trichoderma* sp., *Penicillium* sp. and *Petriella* sp. (Danon et al., 2007; Zmora-Nahum et al., 2008) or plant growth promoting bacteria (PGPB) like genera of *Azotobacter, Pseudomonas, Stenotrophomonas, Bacillus, Flavobacterium, Streptomyces* or Actinomyces group found in the chestnut compost which has colonized mycelia and inhibited sclerotia germination of *Sclerotium rolfsii* (Danon et al., 2007; Ventorino et al., 2016; Zmora-nahum et al., 2008). In addition, it could be other antagonistic microorganisms inhabiting compost such as *Nocardiopsis* sp., *Streptomyces violaceorubidus* and *Streptomyces* sp. which were identified and screened for their antifungal activities by bio-active substance production (peptides) (Su et al., 2014). These peptides were characterized for their antibiosis mechanism (surfactins) and tested for growth inhibition of tomato pathogens such as *Alternaria solani* and *Botrytis cinerea* (On et al., 2015). It was also reported that the sterilization of compost destroyed its active microorganisms and consequently nullified their antagonistic effect (El Khaldi et al., 2016; Hoitink et al., 1997; Raviv, 2014; Zhang et al., 1998) and the growth inhibition was related to pH value and ammonium (NH_4^+) concentration in the culture medium (Danon et al., 2007; Zmora-Nahum et al., 2008). Fungal colonies grown in plates containing weak concentration of UCE showed that fungal species inhabiting compost were inhibited by high compost extract (Figure 2). Hence, microbial activities have particularly reduced growth radius of *Foa* pathogen. For sterilized compost extract, no limited growth was detected in weak percentage because only chemical

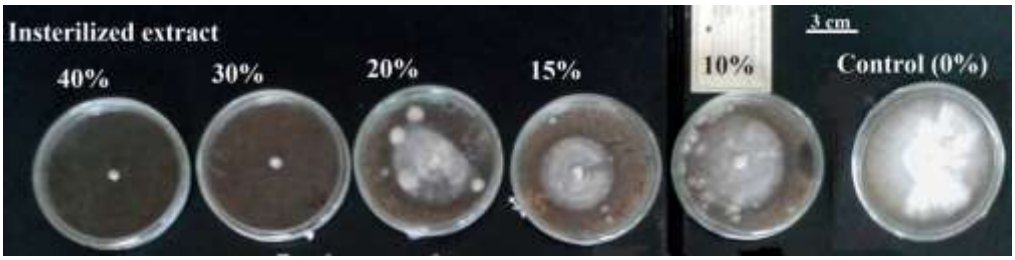

Figure 2. Mycelium growth of *F. oxysporum* f.sp albedinis in response to different unsterilized compost extract concentrations after 8 days of incubation in PDA medium. Control plate contains Foa pathogen alone.

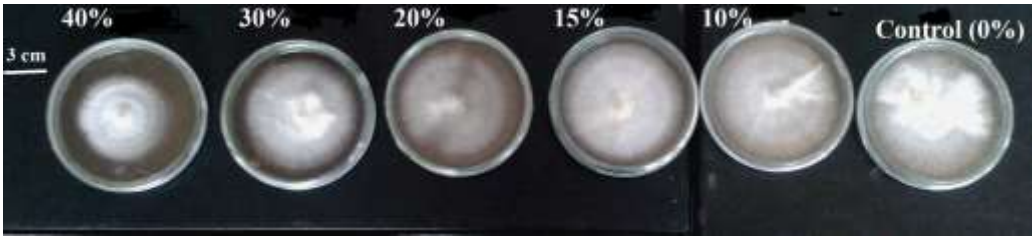

Figure 3. Mycelium growth of *F. oxysporum* f.sp albedinis in response to different sterilized compost extract concentrations after 8 days of incubation in PDA. Control plate contains *Foa* pathogen alone.

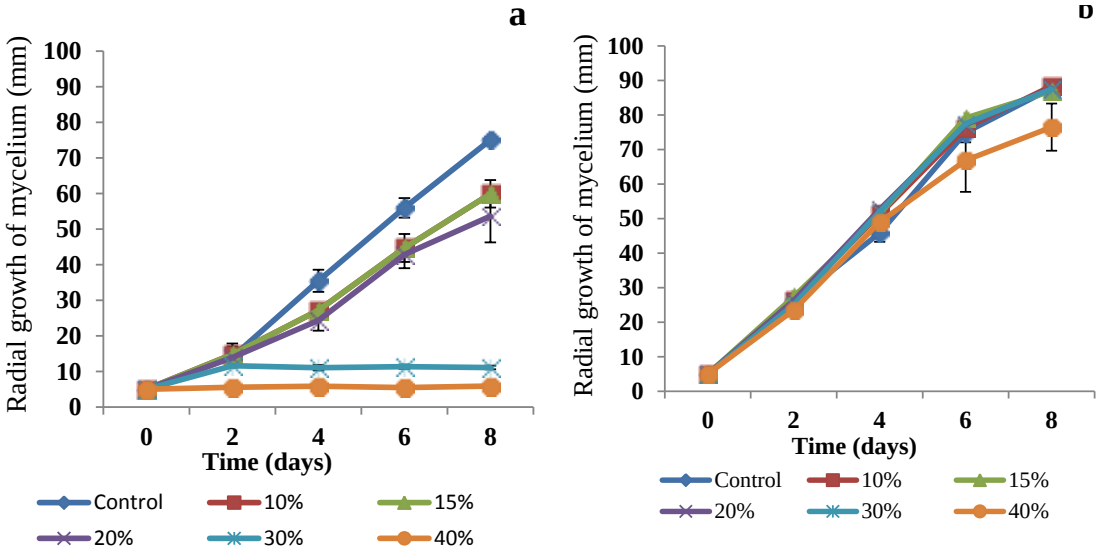

Figure 4. Evolution of mycelia growth of *F. oxysporum* f.sp *albedinis* with unsterilized compost extract (a) and sterilized compost extract (b).

Table 4. Significant regressions of compost extract and rate inhibition of radius growth of *Foa*

Model		Sum of squares	ddl	Average of squares	D	Sig.
1	**Regression**	**4532.931**	**1**	**4532.931**	**41.623**	**0.008**
	Residue	326.717	3	108.906		
	Total	4859.648	4			

Table 5. Model of linear regression.

Model	R	R^2	R^2 ajusted	standard error of the estimation
1	0.966	0.933	0.910	10.43578

Table 6. Rate inhibition of *F. oxysporum* f.sp *albedinis* at different concentration of sterilized and unsterilized compost extracts.

Concentration	10%	15%	20%	30%	40%
Unsterilized compost extract	20.1±0.5	37.3±2.7	44.5 ± 3.2	93.0 ± 5.1	97.9±1.7
Sterilized compost extract	4.8±0.5	6.6±0.7	5.6±0.1	5.9±0.53	18.4±2.6

factors have influenced mycelium growth in elevated SCE concentration.

Conclusion and perspective

This study revealed that unsterilized compost extracts were efficient for *in vitro* suppression of *Foa*. They could constitute an alternative for biological control of “Bayoud”. Further studies are required to isolate and select effective microorganisms inhabiting this compost. Also, it is necessary to evaluate the antifungal activity of this compost *in vivo* and in naturally infected soils in date palm grove.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Al-balqa MAA (2016). Economic importance of date palm Phoenix dactylifera L. (Liliopsida : Arecales : Arecaceae) pests in Jordan Valley. Braz. J. Biol. Sci. 2(3):101-109

Alberto C, Gava T, Pinto JM (2016). Biocontrol of melon wilt caused by Fusarium oxysporum Schlect f. sp. melonis using seed treatment with Trichoderma spp. and liquid compost. Biol. Control 97:13-20

Al-shahib W, Marshall RJ (2003). The fruit of the date palm : its possible use as the best food for the future ? Int. J. Food Sci. Nutr. 54(4):247-259.

Barje F, Meddich A, El Hajjouji H, El Asli A, Ait Baddi G, El Faiz A, Hafidi M (2016). Growth of date palm (*Phoenix dactylifera* L.) in composts of olive oil mill waste with organic household refuse. Compost Sci. Util. 24:273-280.

Bernal-Vicente A, Ros M, Tittarelli F, Intrigliolo F, Pascual JA (2008). Citrus compost and its water extract for cultivation of melon plants in greenhouse nurseries. Evaluation of nutriactive and biocontrol effects. Bioresour. Technol. 99:8722-8728.

Boulter JI, Boland GJ, Trevors JT (2000). Compost: A study of the development process and end-product potential for suppression of turfgrass disease. World J. Microbiol. Biotechnol. 16:115-134.

Brimner TA, Boland GJ (2003). A review of the non-target effects of fungi used to biologically control plant diseases. Agric. Ecosyst. Environ. 100:3-16.

Chao CT, Krueger RR (2007). The date palm (Phoenix dactylifera L.): Overview of biology, uses, and cultivation. HortScience 42:1077-1082.

Chakroune K, Bouakka M, Lahlali R, Hakkou A (2008). Suppressive effect of mature compost of date palm by-product on *Fusarium oxysporum* f.sp. *albedinis*. Plant Pathol. J. 7(2):148 -154

Danon M, Zmora-Nahum S, Chen Y, Hadar Y (2007). Prolonged compost curing reduces suppression of Sclerotium rolfsii. Soil Biol. Biochem. 39:1936-1946.

Dihazi A, Jaiti F, Jaoua S, Driouich A, Baaziz M, Daayf F, Serghini MA (2012). Use of two bacteria for biological control of bayoud disease caused by Fusarium oxysporum in date palm (Phoenix dactylifera L) seedlings. Plant Physiol. Biochem. 55:7-15.

El Hadrami A, Al-Khayri JM (2012). Socioeconomic and traditional importance of date palm. Emir. J. Food Agric. 24:371-385.

El Khaldi R, Daami-Remadi M, Cherif M (2016). Biological Control of Stem Canker and Black Scurf on Potato by Date Palm Compost and its Associated Fungi. J. Phytopathol. 164:40-51.

El-Masry MH, Khalil AI, Hassouna MS, Ibrahim HAH (2002). In situ and in vitro suppressive effect of agricultural composts and their water extracts on some phytopathogenic fungi. World J. Microbiol. Biotechnol. 18:551-558.

FAO (2010). Food and Agriculture Organization of United State, http://www.fao.org/home/en/.

Hadrami I El, Hadrami A El (2009). Breeding date palm. Breeding Plantation Tree Crops: Tropical Species. pp.191-216.

Hibar K, Daami-Remadi M, Jabnoun-Khiareddine H, Znaïdi IE, El Mahjoub M (2006). Effet des extraits de compost sur la croissance mycélienne et l’agressivité du Fusarium oxysporum f. sp. radicis-lycopersici. Biotechnol. Agron. Soc. Environ. 10:101-108.

Hoitink HAJ, Stone AG, Han DY (1997). Suppression of plant diseases by composts. HortScience 32(2):184-187.

Ingham E (2003). Compost tea. Acres USA 33:1-6.

Jaiti F, Meddich A, El Hadrami I (2007). Effectiveness of arbuscular mycorrhizal fungi in the protection of date palm (Phoenix dactylifera L.) against bayoud disease. Physiol. Mol. Plant Pathol. 71:166-173.

Kavroulakis N, Ehaliotis C, Ntougias S, Zervakis GI, Papadopoulou KK (2005). Local and systemic resistance against fungal pathogens of tomato plants elicited by a compost derived from agricultural residues. Physiol. Mol. Plant Pathol. 66:163-174.

Kerkeni A, Khedher MB (2014). *In vitro* bioassay of the antagonistic activity of some bacteria isolated from compost extracts. Sci. Agric. 2:2-7

Koné SB, Dionne A, Tweddell RJ, Antoun H, Avis TJ (2010). Suppressive effect of non-aerated compost teas on foliar fungal pathogens of tomato. Biol. Control 52:167-173.

Lanthier M (2007). Compost Tea and Its impact On Plant Diseases. BC Organic Grower 10(2):8-11.

Le Page C, Bousquet F (2007). Sustainable development and biodiversity – 2, serie of Composing for Sustainable Agriculture (volume 3). M.L. Sukhadia University Botany Department, Udaipur, Rajasthan, India. Dinesh K. Maheshwari Editor.

Lin Y, Du D, Si C, Zhao Q, Li Z, Li P (2014). Potential biocontrol Bacillus sp. strains isolated by an improved method from vinegar waste compost exhibit antibiosis against fungal pathogens and promote growth of cucumbers. Biol. Control 71:7-15.

Markakis EA, Fountoulakis MS, Daskalakis GC, Kokkinis M, Ligoxigakis EK (2016). The suppressive effect of compost amendments on Fusarium oxysporum f.sp. radicis-cucumerinum in cucumber and Verticillium dahliae in eggplant. Crop Prot. 79:70-79.

On A, Wong F, Ko Q, Tweddell RJ, Antoun H, Avis TJ (2015). Antifungal effects of compost tea microorganisms on tomato pathogens. Biol. Control 80:63-69.

Palou L, Taberner V, Guardado A, Del Río MÁ, Montesinos-Herero C (2013). Incidence and etiology of postharvest fungal diseases of pomegranate (Punica granatum cv. Mollar de Elche) in Spain. Phytopathol. Mediterr. 52:478-489.

Pane C, Celano G, Villecco D, Zaccardelli M (2012). Control of Botrytis cinerea, Alternaria alternata and Pyrenochaeta lycopersici on tomato with whey compost-tea applications. Crop Prot. 38:80-86.

Pane C, Piccolo A, Spaccini R, Celano G, Villecco D, Zaccardelli M (2013). Agricultural waste-based composts exhibiting suppressivity to diseases caused by the phytopathogenic soil-borne fungi Rhizoctonia solani and Sclerotinia minor. Appl. Soil Ecol. 65:43-51.

Pereau-Leroy P (1958). Le palmier dattier au Maroc. Ministère de l'Agriculture, Instit Franc Rech Outremer, Paris. 142 p.

Powell WKMSM, Barry KJEKM (2017). Diverse microbial communities in non-aerated compost teas suppress bacterial wilt. World J. Microbiol. Biotechnol. 33(3):49.

Raviv M (2014). Compost as a Tool to Suppress Plant Diseases : Established and Putative Mechanisms. In. III International Symposium on Organic Matter Management and Compost Use in Horticulture 1146. pp. 11-24.

Saafi EB, Trigui M, Thabet R, Hammami M, Achour L (2008). Common date palm in Tunisia: Chemical composition of pulp and pits. Int. J. Food Sci. Technol. 43:2033-2037.

Saaidi M (1992). Comportement au champ de 32 cultivars de palmier dattier vis-à-vis du bayoud : 25 années d'observations. Agronomie 12:359-370.

Sang MK, Kim J-G, Kim KD (2010). Biocontrol Activity and Induction of Systemic Resistance in Pepper by Compost Water Extracts Against Phytophthora capsici. Phytopathology 100:774-783.

Sedra H (2012). Guide du phoeniciculteur. Mise en place et conduite des vergers phoénicicoles INRA- Edition 2012, ISBN: 978-9954-8602-8

Sghir F, Touati J, Chliyeh M, Mouria B, Touhami O, Filali-maltouf A, Modafar CEl, Moukhli A, Benkirane R, Douira A (2015). Effect of Trichoderma harzianum and endomycorrhizae on the suppression of Fusarium wilt in plants of two date palm varieties: Majhoul and Boufeggous. Int. J. Adv. Pharm. Biol. Chem. 4(2):2277-4688.

Su A, Ting Y, Hermanto A, Lin K (2014). Biocatalysis and Agricultural Biotechnology Indigenous actinomycetes from empty fruit bunch compost of oil palm : Evaluation on enzymatic and antagonistic properties. Biocatal. Agric. Biotechnol. 3(4):310-315.

Suárez-Estrella F, Jurado MM, Vargas-García MC, López MJ, Moreno J (2013). Isolation of bio-protective microbial agents from eco-composts. Biol. Control 67:66-74.

Ventorino V, Parillo R, Testa A, Viscardi S, Espresso F, Pepe O (2016). Chestnut green waste composting for sustainable forest management : Microbiota dynamics and impact on plant disease control. J. Environ. Manage. 166:168-177.

Zhang W, Han DY, Dick WA, Davis KR, Hoitink HAJ (1998). Compost and compost water extract-induced systemic acquired resistance in cucumber and Arabidopsis. Phytopathology 88:450-455.

Zmora-Nahum S, Danon M, Hadar Y, Chen Y (2008). Chemical properties of compost extracts inhibitory to germination of Sclerotium rolfsii. Soil Biol. Biochem. 40(10):2523-2529.

academicJournals

Vol. 11(29), pp. 1162-1170, 7 August, 2017
DOI: 10.5897/AJMR2017.8607
Article Number: DD9246D65428
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Effects of antifungal activity of essential oils, salts and antioxidants acids on pathogenic fungi and their application methods for controlling postharvest diseases in banana fruits

Zoeir H. A.[1], El Zahaby H. M.[1], Ziedan E. H.[2]* and Maswada H. F.[1]

[1]Botany Department, Faculty of Agriculture, Tanta University, Tanta, Egypt.
[2]Plant Pathology Department, National Research Centre, Dokki, Cairo, Egypt.

Received 29 May, 2017; Accepted 28 June, 2017

In vitro **clove essential oil (1.0%), sodium carbonate (2.0%) and sorbic, salycilic and propinic acids (0.5%) completely (100%) inhibited mycelial linear growth of *Colletotrichum musae* and *Fusarium moniliforme,* which is the cause of postharvest diseases in banana fruits. *In vivo* different application methods, that is, spray, soaking and dusting were tested on crown rot, neck rot, finger rot and flower end rot under artificial infestation with causal pathogens. Soaking method significantly reduced major postharvest diseases incidence in banana fruits than spray and dusting treatments. Soaking banana fruits in clove suspension (2%) and sodium carbonate (4.0%) was the best treatment that completely inhibited (100%) crown rot and flower end rot diseases and significantly reduced finger rot and neck rot diseases. So, clove oil and sodium carbonate were the most promising agents for controlling major postharvest diseases of banana fruits as eco-friendly and alternative synthetic fungicides.**

Key words: Banana, antioxidants, fungi, diseases, essential oil, salts.

INTRODUCTION

Banana (*Musa* species) is an economic and important fruit crop grown worldwide in more than 120 countries throughout tropical and subtropical regions. It is a popular worldwide staple food for more than 400 million people (Zhang et al., 2005). Postharvest diseases destroy 10 to 30% of the total yield of crops during handling, transportation, storage and marketing (Agrios, 2005). *Colletotrichum musae* and *Fusarium* species, that is, *Fusarium solani*, *Fusarium semitectum*, *Fusarium moniliforme* and *Fusarium musae* are the major fungi causing postharvest complex diseases (fruit rot, crown rot, finger rot, cigar end rot) of banana fruits (Bhattacharyya and Chakraborty, 2007; Diedhiou et al., 2014; Khleekorn et al., 2015; Marin et al., 1996; Abd-Alla et al., 2014; Abdullah et al., 2016; Triesta et al., 2016). Application of cultural, physical and biological methods

*Corresponding author: E-mail: ziedanehe@yahoo.com.

is an alternative to synthetic fungicides for controlling postharvest diseases of banana fruits (Dionisio and Natsuaki, 2007; Lassois et al., 2008). Recently, essential oils, antioxidants, organic and inorganic salts were used as fungicidal alternatives, that is, basil oil (*Ocimum basilicum*), cinnamon oil (*Cinnamomum zeylanicum*) and clove oil (*Cymbopogon nardus*). Low concentrations of essential oils were used against banana crown rot disease and their fungal pathogens; they were also used to maintain quality during storage (Ranasinghe et al., 2002; Anthony et al., 2003, 2004; Maqbool et al., 2010, 2011; Abd-Alla et al., 2014). Salts, that is, potassium sorbate, calcium propionate, sodium bicarbonate, sodium carbonate, and ammonium bicarbonate reduced crown rot incidence of banana fruits (Dionisio and Kobayashi, 2004). Antioxidants, that is, salicylic, citric, benzoic, ascorbic acids and hydroquinone have been used as protective and therapeutic treatments for plant diseases caused by a wide range of viral, fungal and bacterial pathogens (Mandal et al., 2009). Ascorbic acid, benzoic acid and butylated hydroxyl anisole (BHA) reduced mycelial growth of *C. musae,* the cause of anthracnose disease of banana fruits (Khan et al., 2001). Citric extract at 4% significantly reduced fruit rot incidence of banana, plus propionic acid (Ranasinghe et al., 2002; Cruz et al., 2013). The objectives of this study were to screen some essential oils, salts, and antioxidants against causal pathogens and their control of postharvest diseases of banana fruits.

MATERIALS AND METHODS

Causal pathogenic fungi

Highly aggressive isolates causing major postharvest diseases of banana fruit, that is, *C. musae* and *F. moniliforme* isolates were provided from Botany Department, Faculty of Agriculture, Tanta University, Egypt.

Agents tested

The agents tested included commercial essential oils of cinnamon oil (*C. zeylanicum* Blume), thyme oil (*Thymus vulgaris* L.), clove oil (*Syzygium aromaticum*), lemongrass oil (*Cymbopogon citratus* Stapf) and Black seed (*Nigella sativa* L.), antioxidants, that is, benzoic, sorbic, malic, salicylic and propionic acids and salts, that is, sodium benzoate, sodium carbonate, sodium carbonate sodium chloride, and sodium hypochlorite. All agents were provided from Chemical Industrial Development Company (CID), Egypt.

Antifungal against causal organisms

Antifungal activity against mycelia linear growth of *C. musae* and *F. moniliforme* on potato dextrose agar (PDA) of essential oils, that is, cinnamon, clove, lemon grass, and black seed were tested at 0.0, 0.1, 0.5, and 1.0% concentrations. Antioxidants, that is, benzoic, sorbic, malic, salicylic and propionic acids concentrations (0.1, 0.5, and 1.0%) and salts, that is, sodium chloride, sodium hypochlorite, sodium benzoate, and sodium carbonate at 1.0, 2.0, and 4.0% were tested at different concentrations by dissolving the requisite amounts in 0.5 ml of 0.1% Tween 80. Then, they were mixed completely in 100 ml of PDA medium before pouring in Petri dishes (9 cm-diameter). The control sets were prepared similarly using equal amounts of tween 80 on sterilized distilled water. Each plate was inoculated with 5 mm central disk for each fungus. Five plates were used as replicates and five plates free of each agent tested served as a control. The plates were incubated at 27±2°C for 6 days. Percentage of mycelial linear growth inhibition was calculated by the following formula (Skidmore and Dickinson, 1976) as follows:

Inhibition of fungal growth % = C - T /C×100

where C = the radial mycelia growth in control and T = the radial mycelia growth in the treatment

Application methods on postharvest diseases incidence of banana fruits

Different treatment methods used for banana fruits, that is, spray, soaking and dusting were the most effective agents used for mycelial linear growth. Suspension of clove oil (1.0%) and propionic acid (0.5%) were used as spray and soaking for 5 min. Meanwhile, sodium carbonate (4.0%) and salicylic acid (0.5%) were used as spray, soaking and dust treatments on banana fruits before artificial infestation with pathogens.

Fruit samples

Banana fruits Cv. Balady were purchased from private orchard in El-Gharbeia Governorate Egypt at maturity stage. They were disinfected by double immersion in 2 of 70% ethanol for 5 min and allowed to dry at room temperature under sterile conditions. Fruits were separated in polyethylene bags previously disinfected with 70% ethanol and exposed to UV light for 20 min. They were infested with mixture spore (1:1). Suspension (1×10^6/ml) of either *C. musae* or *F. moniliforme* was used for inoculation of banana fruits freshly prepared from 7 days old (PDA) cultures.

Determination of postharvest disease incidence

Postharvest disease incidence was calculated as the number of infected fruits showing symptom of crown rot, neck rot, finger rot, and flower end rot as follows:

Disease % of diseased fruits = Number of diseased banana fruits/Total number of banana fruits × 100

Disease severity was ranked by observing percentage of rotten symptom based on linear scale (0-4) as follows:

Disease severity (%) = Σ (n × r) × 100/N

where n= Number of fruits in each numerical disease grade; r = Number of the disease grade and N= Total number of inoculated fruits multiplied by the maximum numerical disease grade as follows:

0 = healthy fruit free rotten and discoloration;
1= 1-25% rotten and discoloration area;
2 = 26-50% rotten and discoloration area;
3= 51-75% rotten and discoloration area;
4 = 76-100% rotten and discoloration area.

Statistical analysis

Data were analyzed with analysis of variance (ANOVA).

Table 1. Effect of essential oil on mycelia growth *C. musae* and *F. moniliforme*.

Treatment		Mycelium fungal growth(cm)			
		C. musae		*F. moniliforme*	
Essential oil	**Conc. (%)**	**L. growth**	**Reduction (%)**	**L. growth**	**Reduction (%)**
Control	0.0	8.37[a]	0.00	7.87[a]	0.00
Cinnamon	0.1	7.00[bc]	16.41	6.66[b]	15.35
	0.5	7.00[bc]	16.41	6.00[bc]	23.80
	1.00	5.33[e]	36.32	6.00[bc]	23.80
Clove	0.1	6.00[d]	28.35	6.00[bc]	23.80
	0.5	3.00[f]	64.17	5.00[d]	36.50
	1.0	0.00[h]	100.00	0.00[e]	100.00
Lemongrass	0.1	7.50[b]	10.44	6.83[b]	13.23
	0.5	7.50[b]	10.44	6.66[b]	15.35
	1.0	7.50[b]	10.44	6.66[b]	15.35
Black seed	0.1	6.00[d]	28.35	6. 00[bc]	23.80
	0.5	6.50[cd]	22.38	5.33[cd]	32.27
	1.0	2.50g	70.149	5.33[cd]	32.27

Values in each column followed by the same letter are not significantly different at *P≤ 0.05* according to Duncan's multiple range.

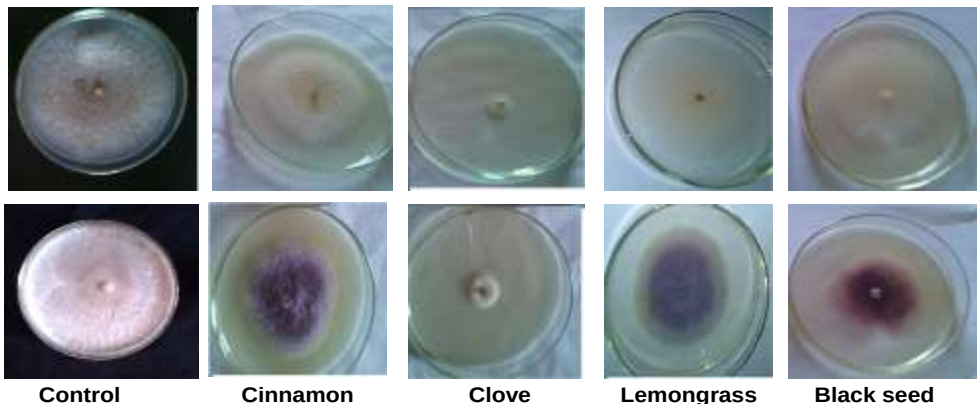

Figure 1. Effect of cinnamon, clove, lemongrass and black seed oils (1.0%) on mycelia growth of *C. musea* (above) and *F. moniliforme* (below).

Comparisons among means were made using Duncan's multiple range test (Snedecor and Cocharn, 1980) at *P* = 0.05.

RESULTS

Laboratory study

Antifungal activity of essential oils against causal organisms

Data presented in Table 1 and Figure 1 shows that all the different concentrations of essential oils tested significantly reduced mycelia linear growth of both tested fungi, that is, *C. musae* and *F. moniliformae* than the control. Data in Table 1 showed that increased essential oil concentrations led to the reduction of mycelial linear growth of two fungi tested. Clove oil (1.0%) was the best essential oil that completely inhibited (100%) linear growth of *C. musae* and *F. moniliforme* followed by black seed oil and then cinnamon oil. Clove essential oil included phenylpropanoids such as carvacrol, thymol, eugenol and cinnamaldehyde; its antimicrobial, antifungal, and antiviral activity (Chaieb et al., 2007). On

Table 2. Effect salts on mycelia growth *C. musae* and *F. moniliforme*.

Treatments		Mycelum fungal growth (cm)			
		C .musae		*F. moniliforme*	
Salts	**Conc. (%)**	**L. growth**	**Reduction (%)**	**L. growth**	**Reduction (%)**
Control	0.0	8.100[a]	00.00	8.200[a]	00.00
Sodium chloride	1.0	6.83[b]	15.64	6.00[de]	26.82
	2.0	6.33[bc]	21.8	6.00[de]	26.82
	4.0	6.500[bc]	19.75	6.50[cd]	20.73
Sodium hypochlorite	1.0	6.00[c]	25.92	5.33[efg]	34.96
	2.0	3.83[e]	52.76	5.16[fg]	37.00
	4.0	0.00[g]	100.00	0.00[i]	100.00
Sodium bicarbonate	1.0	6.00[c]	25.92	7.50[ab]	8.53
	2.0	4.16[e]	48.65	6.50[cd]	20.73
	4.0	2.66[f]	67.8	5.66[ef]	30.90
Sodium carbonate	1.0	2.66[f]	67.8	6.66[cd]	18.70
	2.0	0.00[g]	100.00	4.33[h]	47.15
	4.0	0.00[g]	100.00	0.00[i]	100.00
Sodium benzoate	1.0	6.33[bc]	21.81	7.16[bc]	12.60
	2.0	5.00[d]	38.27	5.33[efg]	34.96
	4.0	3.00[f]	62.86	4.83[gh]	41.06

Values in each column followed by the same letter are not significantly different at *P≤0.05* according to Duncan's multiple range.

the other hand, lemon grass oil was the least oil that reduced mycelial linear growth of two fungi tested. These results are in agreement with the results obtained *in vitro*, on fungi causing crown rot in banana fruits, that is, *Lasiodiplodia theobromae, Fusarium proliferatum* and *C. musae*. Ranasinghe et al. (2002) found that cinnamaldehyde (66.2%) is the major constituent of cinnamon bark oil, its fungistatic and fungicidal at 0.64 and 1.00 mg/ml of *C. nardus* and 0.2 to 0.6% (v/v) of *O. basilicum.* Anthony et al. (2004) and Maqbool et al. (2010) found that cinnamon oil suppressed mycelial growth and inhibited conidial germination (83.2%) of *C. musae*. Maqbool et al. (2011) reported that lemon grass at 0.05 and 0.4% had fungicidal activity against *C. musae* and *Colletotrichum gloeosporioides,* the causal organisms of banana and papaya anthracnose diseases, respectively. Abd-Alla et al. (2014) found that cinnamon, thyme oils completely inhibited 100% mycelium growth and conidial germination of *F. semitectum,* the cause of crown rot of banana fruits. In addition, Idris et al. (2015) reported that basil, cinnamon and rosemary oil (0.1%) completely inhibited mycelia growth of *C. musae*.

Screening of salts against causal organisms

Data in Table 2 and Figure 2 showed that all salts tested significantly reduced linear growth of both fungi tested more than the control. In general, increased essential oils concentration increased their reduction of mycelial growth of the two fungi tested. Sodium carbonate was the best salt that completely (100%) inhibited the linear growth of *C. musae* at 2% and *F. moniliforme* at 4% followed by sodium hypochlorite at 4%. Data in Table 2 indicated that sodium bicarbonate and sodium benzoate significantly reduced linear growth of *C. musae* by 67.8 and 62.8, respectively. Data in Table 2 and Figure 2 showed that sodium carbonate and sodium hypochlorite completely (100) reduced mycelial linear growth of *F. moniliforme* at 4%, but sodium benzoate had moderate effect on mycelial growth of *F. moniliforme* followed by sodium bicarbonate. On the other hand, sodium chloride had the least effect on mycelial growth of two fungi. These results are in agreement with Dionisio and Koabyashi (2004) who found that organic and inorganic salts, that is, Na_2CO_3 (4 g/L), NaClO (5 g/L), $NaHCO_3$, $CaCl_2$, and NaCl (6 g/L) completely inhibited spore germination of fungi causing crown rot diseases in banana, that is, *L. theobromae*, *Thielaviopsis paradoxa, C. musae, C. gloeosporioides, Fusarium verticillioides,* and *Fusarium oxysporum.* Turkkan and Erper (2014) found that sodium metabisulfite completely inhibited mycelial growth of *F. oxysporum.*f.sp.*capae,* the cause of onion basal rot.

Effect of organic and antioxidant acids against causal organisms

Data in Table 3, Figures 3 and 4 shows that all organic

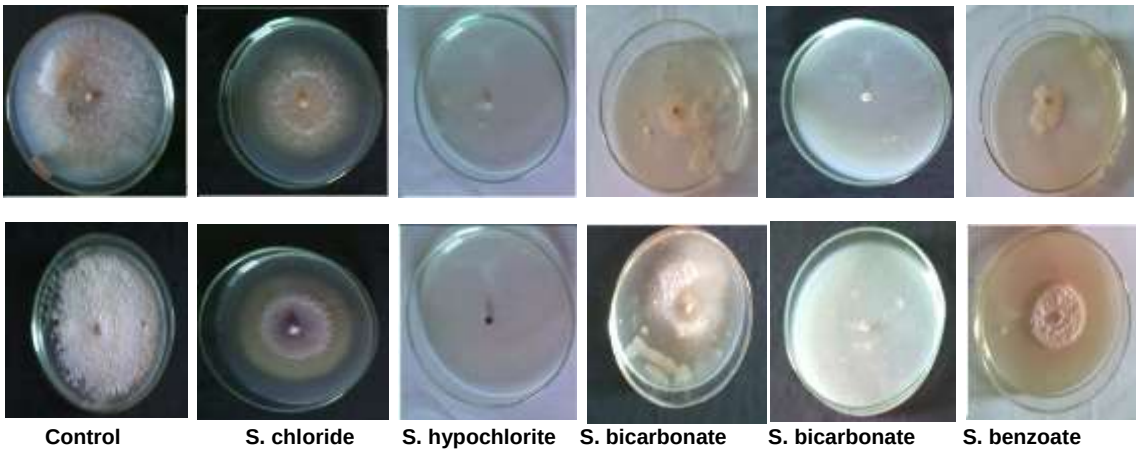

Figure 2. Effect of sodium chloride, sodium hypochlorite, sodium bicarbonate, sodium bicarbonate, and sodium benzoate salt on mycelia growth of *C. musea* (above) and *F. moniliforme* (below).

Table 3. Effect antioxidants on mycelial growth of *C. musae* and *F. moniliforme*.

Treatments		Mycelium fungal growth(cm)			
		C. musae		*F. moniliforme*	
Antioxidants	**Conc. (%)**	**L. growth**	**Reduction (%)**	**L. growth**	**Reduction (%)**
Control	0.0	8.58^{a}	0.00	8.00^{a}	0.00
	0.1	6.33^{e}	26.21	7.00^{b}	12.50
Benzoic acid	0.5	7.00^{d}	18.44	6.83^{b}	14.58
	1.0	1.50^{h}	82.52	6.00^{c}	25.00
	0.1	7.50^{c}	12.61	2.83^{f}	64.58
Propionic acid	0.5	0.00^{i}	100.00	0.00^{i}	100.00
	1.0	0.00^{i}	100.00	0.00^{i}	100.00
	0.1	1.33^{h}	84.46	2.00^{gh}	75.00
Sorbic acid	0.5	0.00^{i}	100.00	0.00^{i}	100.00
	1.0	0.00^{i}	100.00	0.00^{i}	100.00
	0.1	2.33^{g}	72.81	3.33^{e}	58.337
Citric acid	0.5	0.00^{i}	100.00	2.33^{g}	70.837
	1.0	0.00^{i}	100.00	1.66^{h}	79.175
	0.1	2.83^{f}	66.99	3.66^{e}	54.175
Malic acid	0.5	0.00^{i}	100.00	2.16^{g}	72.925
	1.0	0.00^{i}	100.00	0.00^{i}	100.00
	0.1	8.00^{b}	6.79	5.00^{d}	37.500
Salicylic acid	0.5	0.00^{i}	100.00	0.00^{i}	100.00
	1.0	0.00^{i}	100.00	0.00^{i}	100.00

Values in each column followed by the same letter are not significantly different at $P \leq 0.05$ according to Duncan's multiple range.

and antioxidants acids tested significantly reduced linear growth of *C. musae* and *F. moniliforme* more than the control (untreated). In general, increased organic and antioxidants acids concentrations increased their reduction of mycelial growth of two fungi tested. Data in Table 3 clearly shows that propionic, salicylic and sorbic, and malic acids at 0.05 and 100% inhibited mycelial linear growth of fungi tested. Data in Table 3 shows that

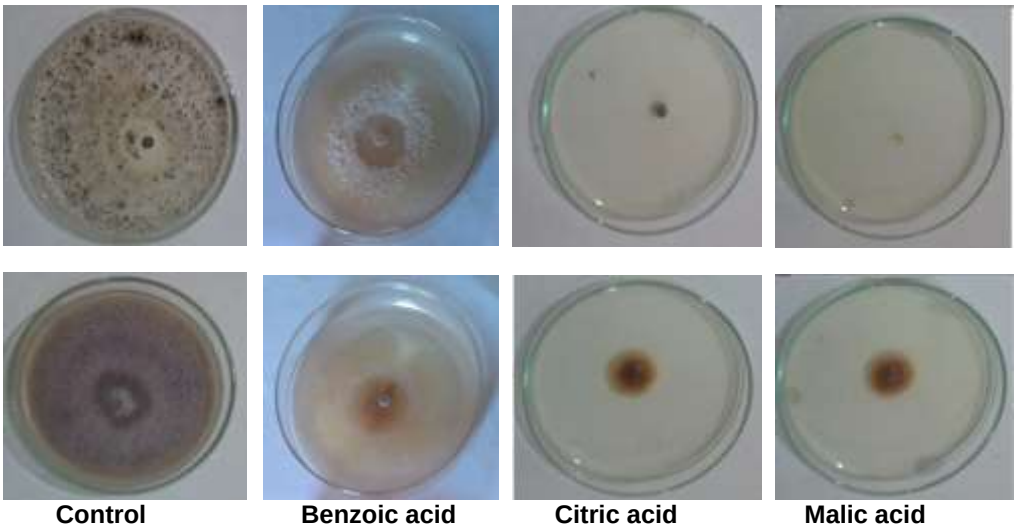

Figure 3. Effect of benzoic, citric, malic acids at (0.5%) on mycelia growth *C. musae* (above) and *F. moniliforme* (below).

citric acid (1.0%) completely (100%) inhibited mycelial growth of *C. musae* and significantly reduced linear growth of *F. moniliforme* by 79.1%.

On the other hand, benzoic acid was the least agent that reduced linear growth of both fungi. Antioxidants safe natural phenolic compound chemicals, that is, salicylic, citric, benzoic, hydroquinone and ascorbic acids had been used as alternative pesticides to protective and therapeutic treatments of plant diseases caused by a wide range of viral, fungal and bacterial pathogens. Mandal et al. (2009) and Khan et al. (2001) found the greatest antifungal activity of BHA and benzoic acid against *C. musae,* the cause of banana fruit anthracnose disease.

Effect of treatment methods on postharvest diseases of banana fruit Cv. Balady

Different treatment methods, that is, spraying, soaking and dusting were tested against postharvest diseases of banana, such as, crown rot, neck rot, finger rot, and flower end rot. Data in Table 4 showed that clove oil (1.0%) and propionic acid (0.5%) were used as spray and soaking treatments for banana fruits. Meanwhile, spraying, soaking and dusting treatment were used with sodium carbonate (4.0%) and salicylic acid (0.5%). Data in Table 4 indicated that all the different treatments with clove oil, propionic acid, sodium carbonate and salicylic acid significantly reduced crown rot incidence of banana fruits more than the control. Soaking treatment significantly reduced crown rot incidence and disease severity with clove oil, propionic acid, sodium carbonate and salicylic acid compared to spray and dusting treatments. Data in Table 4 clear indicated that they were no significant differences between all the treatment methods (spray, soaking and dusting) on neck rot diseases incidence of banana fruit, except soaking treatment with sodium carbonate. Table 4 indicated that soaking treatment in clove oil and sodium carbonate significantly reduced finger rot of banana fruits more than propionic acid, salicylic acid as spray and dusting treatments.

Furthermore, data in Table 4 shows that there are no significant differences in percentage of flower end rot disease and disease severity between treatments tested and control, except soaking treatment. Clove oil was the best and significantly treatment that reduced flower end rot incidence at zero level on banana fruit. In general, soaking treatment with clove and sodium carbonate were the best treatments that reduced crown rot and neck rot. These results are in agreement with Anyhony et al. (2003) and Abd-Alla et al. (2014).

Effect of different treatments on the management of postharvest diseases of banana fruits

Healthy ripe banana fruits Cv. Balady were soaked for 5 min, in each suspension of clove oil (2.0%), sodium carbonate (6.0%), sorbic acid (2.0%), propionic acid (1.0%) and salicylic acid (1.0%) before artificial infestation with causal pathogens (Figure 5). Data in Table 5 showed that banana fruits soaked for 5 min in clove oil suspension (2.0%), sodium carbonate (6.0%), sorbic acid (2.0%), propionic acid (1.0%) and salicylic acid (1.0%) significantly reduced postharvest diseases of banana fruits, that is, crown rot, neck rot, finger rot and flower end rot more than the control. Soaking banana fruit in clove suspension was the best and significantly treatment that

Table 4. Effect of treatment methods on postharvest diseases of banana fruits.

Treatment			Postharvest diseases incidence on banana fruits Cv. Balady							
			Crown rot		Neck rot		Finger rot		Flower end rot	
Agents	Conc. (%)	Method	%	D.S	%	D.S	%	D.S	%	D.S
Control	-	-	50.0^{a}	2.0^{a}	30.0^{b}	2.0^{a}	40.0^{b}	2.0^{a}	30.0^{a}	2.0^{a}
Clove	1.0	Spray	30.0^{c}	2.0^{a}	30.0^{b}	2.0^{a}	30.0^{c}	2.0^{a}	30.0^{a}	2.0^{a}
		Soaking	20.0^{d}	1.0^{b}	30.0b	2.0^{a}	20.0^{d}	1.0^{b}	0.0^{c}	0.0^{c}
Propionic acid	0.5	Spray	30.0^{c}	2.0^{a}	40.0^{a}	2.0^{a}	30.0^{c}	2.0^{a}	20.0^{b}	1.0^{b}
		Soaking	20.0^{d}	1.0^{b}	40.0^{a}	2.0^{a}	40.0^{b}	2.0^{a}	30.0^{a}	2.0^{a}
Sodium carbonate	4.0	Spray	30.0^{c}	2.0^{a}	30.0^{b}	2.0^{a}	20.0^{d}	1.0^{b}	30.0^{a}	2.0^{a}
		Soaking	10.0^{e}	1.0^{b}	10.0^{d}	1.0^{b}	20.0^{d}	1.0^{b}	30.0^{a}	2.0^{a}
		Dusting	40.0^{b}	2.0^{a}	30.0^{b}	2.0^{a}	50.0^{a}	2.0^{a}	30.0^{a}	2.0^{a}
Salicylic acid	0.5	Spray	20.0^{d}	1.0^{b}	20.0^{c}	1.0^{b}	30.0^{c}	2.0^{a}	30.0^{a}	2.0^{a}
		Soaking	30.0^{c}	2.0^{a}	20.0^{c}	10.0^{b}	30.0^{c}	2.0^{a}	20.0^{b}	1.0^{b}
		Dusting	40.0^{b}	2.0^{a}	30.0^{b}	2.0^{a}	40.0^{b}	2.0^{a}	20.0^{b}	1.0^{b}

Values in each column followed by the same letter are not significantly different at *P≤ 0.05* according to Duncan's multiple range.

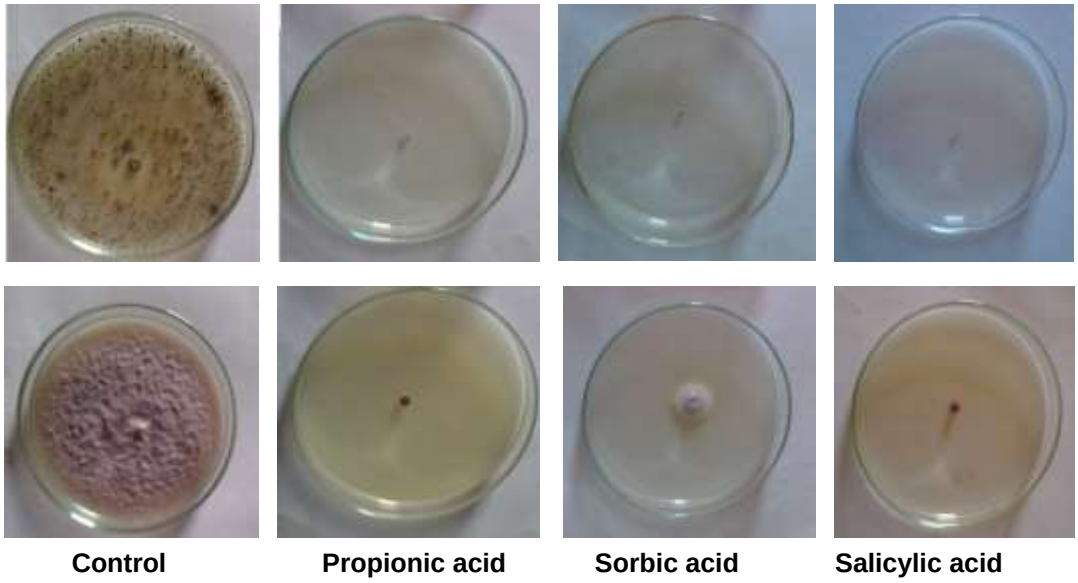

Figure 4. Effect of propionic, sorbic and salicylic acids at (0.5%) on mycelia growth of *C. musae* (above) and *F. moniliforme* (below).

completely suppressed (100%) crown rot and flower end rot incidence of banana fruits and significantly reduced finger rot. On the other hand, salicylic acid followed by propionic acid was the lowest treatments that reduced crown, neck and finger rots. These results are in line with that of Ranasinghe et al. (2002) study who found that, spraying embul banana with emulsions of cinnamom oil prior storage controlled crown rot stored up to 14 days at ambient temperature (28±20°C) and 21 days at 40°C in modified atmosphere. Also, banana fruits treated with emulsions of cinnamon oil combined with modified atmosphere packaging extended the storage life of Embul banana up to 21 days in a cold room and 14 days at 28 ± 2°C without affecting the organoleptic and

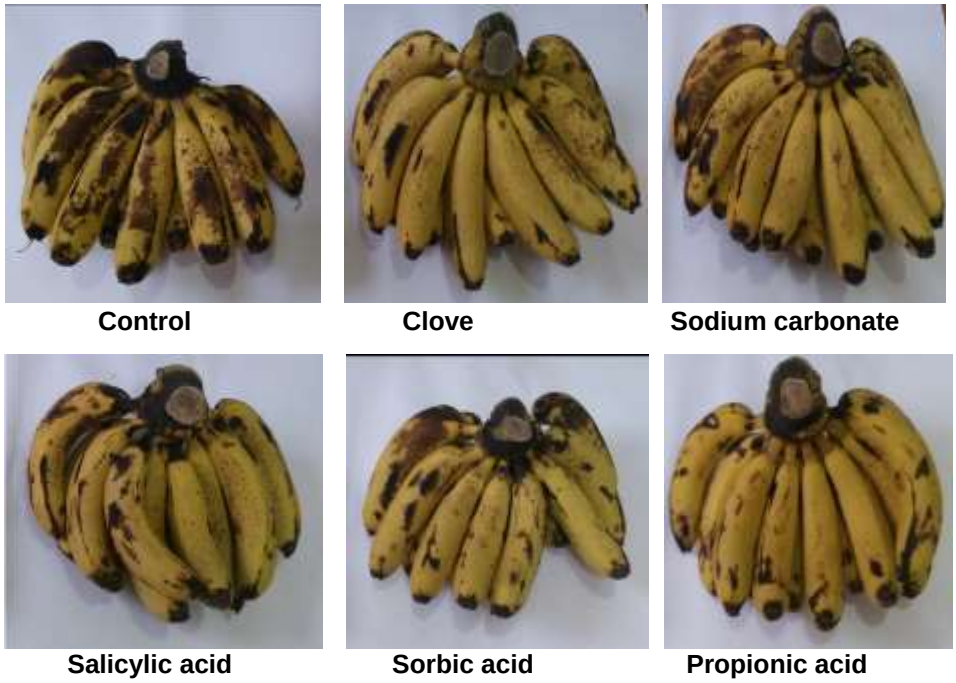

Figure 5. Effect of some essential oil clove (0.2%), sodium carbonate (6.0%), propionic and salycilic acids (1.0%) and sorbic acid (2.0%) for management of postharvest diseases banana fruit. After 15 days after artificial infestation by causal organisms.

Table 5. Management of post-harvest diseases after storage 15 days at 25 2°C.

Treatment		Postharvest diseases incidence on banana fruits							
Material	Conc. (%)	Crown rot		Neck rot		Finger rot		Flower end rot	
		%	D.S	%	D.S	%	D.S	%	D.S
Control	0.0	100.0^{a}	4.0^{a}	60.0^{a}	3.0^{a}	90.0^{a}	4.0^{a}	80.0^{a}	4.0^{a}
Clove	2.0	0.0^{f}	0.0^{e}	20.0^{d}	1.0^{c}	30.0^{d}	2.0^{c}	0.0^{d}	0.0^{c}
Sodium carbonate	6.0	20.0^{e}	1.0^{d}	20.0^{d}	1.0^{c}	40.0^{c}	2.0^{c}	20.0^{b}	1.0^{b}
Sorbic acid	2.0	30.0^{d}	2.0^{c}	40.0^{b}	2.0^{b}	40.0^{c}	2.0^{c}	20.0^{b}	1.0^{b}
Propionic acid	1.0	80.0^{b}	4.0^{a}	30.0^{c}	2.0^{b}	60.0^{b}	3.0^{b}	0.0^{d}	0.0^{c}
Salicylic acid	1.0	60.0^{c}	3.0^{b}	20.0^{d}	1.0^{c}	40.0^{c}	2.0^{c}	10.0^{c}	1.0^{b}

Values in each column followed by the same letter are not significantly different at *P≤ 0.05* according to Duncan's multiple range.

physico-chemical properties (Ranasinghe et al., 2005; Maqbool et al., 2010; Abd-Alla et al., 2014). Idris et al. (2015) found that treatment of banana fruits Cvs. Cavendish and Williams treated with essential oil (0.20%) of basil, cinnamon and rosemary essential oils reduced anthracnose of banana fruits after 19 days of storage. Singh and Tripathi (2015) showed that, *C. zeylanicum* oil treated banana fruits showed enhancement storage life up to 4 days. Dionisio and Kobayashi (2004) reported that dipping banana fruits in NaClO or $NaHCO_3$ for 10 to 15 min reduced the incidence of crown rot 17 days after harvest. Kazemi et al. (2013) reported that dipping of pomegranates fruits for 4 min in 4% calcium chloride solution combined with sodium hypochlorite (10%) was the best treatment to enhance postharvest factors of pomegranates. Cruz et al. (2013) reported that citric extract at 4% significantly reduced fruit rot incidence of banana by 19.44% more than 90.16% in the control.

Conclusion

Clove essential oil, sodium carbonate and sorbic acid were the most promising eco-friendly, antifungal and alternative synthetic fungicides against pathogenic fungi and their ability to deteriorate banana fruits during

storage, marketing and transportation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Abd-Alla MA, El-Gamal NG, El-Mougy NS, Abdel-Kader MM (2014). Post-harvest treatments for controlling crown rot disease of Williams banana fruits (*Musa acuminata* L.) in Egypt. Plant Pathol. Q. 4(1):1-12.

Abdullah Q, Mahmoud A, Al-Harethi A (2016). Isolation and identification of fungal postharvest rot of some fruits in Yemen. PSM Microbiol. 1(1):36-44.

Agrios GN (2005). Plant Pathology, 5th ed Academic Press, USA. 922p.

Anthony S, Abeywickrama K, Wijeratnam SW (2003).The effect of spraying essential oils of *cymbopogon nardus, cymbopogan flexuosus* and *Ocimum basilicum* on postharvest diseases and storage life of Embul banana. J. Hortic. Sci. Biotechnol. 78:780-785.

Anthony S, Abeywickrama K, Dayananda R, Wijeratnam SW, Arambewela L (2004). Fungal pathogens associated with banana fruit in Sri Lanka, and their treatment with essential oils. Mycopathologia 157:91-97.

Bhattacharyya A, Chakraborty S (2007). Effect of inorganic salts on *Colletotrichum musae* and *Fusarium solani* - causal organisms of crown rot disease of banana. Assam Agricultural University, AICRP on Fruits, Jorhat, India, 290 p.

Chaieb K, Hajlaoui H, Zmantar T, Kahla-Nakbi AB, Rouabhia M, Mahdouani K, Bakhrouf A (2007).The chemical composition and biological activity of clove essential oil, *Eugenia caryophyllata* (*Syzigium aromaticum* L. Myrtaceae): a short review. Phytother. Res. 21(6):501-506.

Cruz M, Estrada KRF, Clemente E, Itako AT, Stangarlin JR, Cruz M (2013). Plant extracts for controlling the post-harvest anthracnose of banana fruit. Rev. Bras. Plant. Med. 15(4):727-733.

Diedhiou MP, Zakari HA, Mbaye N, Rokhaya F, Samb LP (2014). Control methods for postharvest diseases of banana (*Musa sinensis*) produced in Senegal. Int. J. Sci. Environ. Technol. 3:1648-1656.

Dionisio G, Kobayashi T (2004). Inhibitory influence of inorganic salts on banana postharvest pathogens and preliminary application to control crown rot. J. Gen Plant Pathol. 70:61-65.

Dionisio GA, Natsuaki KT (2007). Control of crown rot-causing fungal pathogens of banana by inorganic salts and a surfactant. Crop Prot. 26(11):1667-1673.

Idris MF, Ibrahim MA, Forsido FS (2015). Essential oils to control *Colletotrichum musae in vitro* and *in vivo* on banana fruits. Am-Eur. J. Agric. Environ. Sci. 15(3):291-302.

Kazemi F, Jafararpoor M, Golparvar A (2013). Effects of sodium and calcium treatments on the shelf life and quality of pomegranate. Intl. J. Farm Allied Sci. 2(S2):1375-1378.

Khan SHJ, Aked J, Magan N (2001). Control of the anthracnose pathogen of banana (*Colletotrichum musae*) using antioxidants alone and in combination with thiabendazole or imazalil. Plant Pathol. 50(5):601:608.

Khleekorn S, McGovern J, Wongrueng S (2015). Control of the banana anthracnose pathogen using antagonistic microorganisms. J. Agric. Technol. 11(4):965-973.

Lassois L, Bellaire L, Jijakli MH (2008). Biological control of crown rot of bananas with *Pichia anomala* strain K and *Candida oleophila* strain. Biol. Control 45:410-418.

Mandal S, Mallick N, Mitra A (2009). Salicylic acid induced resistance to *Fusarium oxysporum* f.sp.*lycopersici* in tomato. Plant Physiol. Biochem. 47:642-649.

Maqbool M, Ali A, Peter G, Alderson PG (2010). Effect of cinnamon oil on incidence of anthracnose disease and postharvest quality of bananas during storage. Int. J. Agric. Biol. 12:516-520.

Maqbool M, Ali A, Peter G, Aldersona PG, Mohamed MTM, Siddiqui Y, Zahida N (2011). Postharvest application of gum Arabic and essential oils for controlling anthracnose and quality of banana and papaya during cold storage. Postharvest Biol. Technol. 62:71-76.

Marin H, Sutton B, Blankenship M, Swallow H (1996). Pathogenicity of fungi associated with crown rot of banana in Latin America on grande naine and disease resistant hybrid banana. Plant Dis. 80(5):525-528.

Ranasinghe L, Jayawardena B, Abeywickrama K (2005). An integrated strategy to control post- harvest decay of Embul banana by combining essential oils with modified atmosphere packaging. Intl. J. Food Sci. Technol. 40:97-103.

Ranasinghe L, Jayawardena B, Abeywickrama K (2002). Fungicidal activity of essential oils of *Cinnamomum zeylanicum* (L.) and *Syzygium aromaticum* (L.) Merr et L M Perry against crown rot and anthracnose pathogens isolated from banana. Lett. Appl. Microbiol. 35:208-211.

Singh R, Tripathi P (2015). *Cinnamomum zeylanicum* essential oil in the management of anthracnose of banana fruits. J. Innov. Pharm. Biol. Sci. 2(3):290-299.

Skidmore AM, Dickinson CH (1976). Colony interactions and hyphal interference between *Septoria nodorum* and phylloplane fungi. Trans. Br. Mycol. Soc. 66:57-64.

Snedecor GW, Cochran WG (1980). Statistical Methods. 7th Ed. Iowa State Univ. Press, Ames. pp. 476-480.

Triesta D, Pierard D, De Cremer K, Hendrickx M (2016). *Fusarium musae* infected banana fruits as potential source of human Fusariosis : may occur more frequently than we might think and hypotheses about infection. Commun. Integr. Biol. 9(2):e1162934.

Turkkan M, Erper I (2014). Evaluation of antifungal activity of sodium salts against onion basal rot caused by *Fusarium oxysporum* f. sp. cepae. Plant Prot. Sci. 50(1):19-25.

Zhang P, Whistler RL, BeMiller JN, Hamaker BR (2005). Banana starch: Production, physicochemical properties, and digestibility-a review. Carbohyd. Polym . 59:443-458.

academicJournals

Vol. 11(29), pp. 1171-1183, 7 August, 2017
DOI: 10.5897/AJMR2016.8256
Article Number: 6648E3F65431
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Midgut bacterial diversity analysis of laboratory reared and wild *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes in Kenya

Erastus Mwaringa Mwadondo[1], Amanuel Ghilamicael[2], Amos Ematiti Alakonya[2,3] and Remmy Wekesa Kasili[2]*

[1]Department of Zoology, Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000-00200, Nairobi, Kenya.
[2]Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology P. O. Box 62000-00200, Nairobi, Kenya.
[3]International Institute of Tropical Agriculture, Headquarters, PMB, 5320, Ibadan Oyo State, Nigeria.

Received 5 August, 2016; Accepted 21 June, 2017

Midgut symbiotic bacteria are known to play fundamental roles in the biology of mosquitoes, however knowledge of midgut bacterial communities associated with mosquitoes is scanty due to limitation of the isolation techniques based on culturing. In this study, the composition and diversity of midgut bacteria in field collected and lab reared adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes was explored using the Illumina sequencing. Deoxyribonucleic acid was isolated from the pooled midgut extracts and their 16S rRNA gene sequenced using Illumina sequencing platform. Operational taxonomic units (OTUs) were analyzed using QIIME 1.8.0; taxonomy was assigned using BLASTn against SILVA 119 and hierarchical clustering was done using R program software. Out of the total number of sequence reads obtained, 145 OTUs were realized at 3% genetic distance. The 145 OTUs spanned 12 phyla; *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, *Euryarchaeota*, *Gemmatimonadetes*, *Spirochaetae*, *Archeabacteria* *Verrucomicrobia*, *Chloroflexi*, *Bacteriodetes*, *Acidobacteria* and *Actinobacteria*. Microbial community composition based on OTUs showed significant difference between field collected and lab reared mosquitoes (χ^2 = 45.0799, p = 3.2 × 10^{-5}). Similarly, there was a significant difference in community composition at OTU level between *Anopheles gambiae* and *Culex quinquefasciatus* (χ^2 = 31.2257, p = 7.7 × 10^{-4}). The bacterial composition and diversity appeared to be influenced by the environment and the species of the mosquitoes.

Key words: *Anopheles gambiae*, *Culex quinquefasciatus,* midgut, DNA, diversity.

INTRODUCTION

Mosquitoes transmit diseases like malaria, dengue, lymphatic filariasis, yellow fever among others. Among these diseases, malaria is the most important mosquito borne disease with an estimated 214 million new cases of malaria worldwide (World Health Organization (WHO), 2015). The African region accounted for most of the global cases of malaria (88%), followed by South-East Asia region (10%) and Eastern Mediterranean region (2%) (WHO, 2015). In Kenya, there were an estimated 6.7 million new clinical cases and 4,000 deaths each year

and those living in Western Kenya have an especially high risk of malaria (Centers for Disease Control and Prevention (CDC), 2015). Most of the deaths are caused by the parasite *Plasmodium falciparum* whose major vector in Africa is the mosquito species *Anopheles gambiae* that is widely distributed throughout the Afro-tropical belt (Boissière et al., 2012).

Another mosquito species *Culex quinquefasciatus* is the principal vector for *Wuchereria bancrofti*, the filarial worm that causes filariasis and Japanese encephalitis (Agrawal and Sashindran, 2006). Lymphatic filariasis is a major public health problem worldwide. It is estimated that 1.3 billion people from 83 countries are living with the disease or are at risk of infection (Agrawal and Sashindran, 2006). Lymphatic filariasis is present on the East African coast especially in Kenya (Njenga et al., 2011).

Current mosquito vector control strategies include insecticide treatment delivered through spraying houses and insecticide-impregnated mosquito nets. While these methods are effective at decreasing mosquito vector numbers, they have also contributed to the rise in insecticide resistant mosquitoes (Bando et al., 2013).

Various alternative approaches are being tried to reduce malaria cases in the world, and one such approach is paratransgenesis. Paratransgenesis is a method where by a symbiotic bacteria is used to express effector molecules inside a targeted vector. The symbiotic bacteria are genetically modified to produce effector molecules and then reintroduced into the mosquito to produce the required effect (Chavshin et al., 2012). Understanding the microbial community structure of the mosquito midgut is therefore necessary in order to identify possible bacterial candidates for paratransgenesis. The mosquito midgut plays a critical role to the survival and development of the parasites and is therefore, the most attractive site to target malaria parasites (Whitten et al., 2006). The midgut microbiota of mosquitoes is still not well investigated and a few studies have been carried out on microflora of wild caught malaria vectors (Wang et al., 2011). The available conventional culture techniques limit the scope in determination of the microbial diversity since it sometimes misses out on non-culturable microbes (Pidiyar et al., 2004).

In laboratory-raised mosquitoes, the midgut bacteria can be acquired through contaminated sugar solutions, blood meals and transmitted transstadially. However, in wild mosquitoes, the origin of the midgut bacteria, is still unknown (Riehle and Jacobs-Lorena, 2005). In the current study the bacterial composition and diversity in the midgut of lab reared and field collected *A. gambiae* and *C. quinquefasciatus* mosquitoes were determined using the illumina sequencing.

MATERIALS AND METHODS

Study site

The study area for field collected mosquitoes was Ahero, Kenya, which is a malaria endemic region. It is located at latitude 0º 11'S and longitude 34º 55'E and is approximately 1153 m above sea level. The area has a tropical climate with significant rainfall throughout the year and with an average temperature of 23.0°C.

Collection of field *A. gambiae* and *C. quinquefasciatus* mosquitoes

Adult *A. gambiae* and *C. quinquefasciatus* mosquitoes were captured from pit shelters by use of Centre for Disease Control and Prevention (CDC) light traps. The CDC light traps were hung at least one meter above the ground on a tree or pole between 6:00 and 7:00 pm in the evening and left overnight. The collection bags containing the mosquitoes were picked between 6.00 and 6.30 am in the morning. The mosquitoes were then put into vial/jars from the collection bags using mouth aspirators and stored at 4°C. One hundred and thirty eight adult female *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes were identified to species level using a standard morphological key according to Gillies and De Meillon, (1968). The specimens were then transferred to the laboratory at the Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology (JKUAT).

Acquisition of laboratory-reared *A. gambiae* and *C. quinquefasciatus*

One hundred and thirty eight laboratory reared adult female *A. gambiae* and *C. quinquefasciatus* mosquitoes were acquired from the International Centre of Insect Physiology and Ecology (ICIPE) Kasarani, Nairobi. They were transferred live to the laboratory at the Institute for Biotechnology Research, (JKUAT) and maintained in a mosquitarium at 28°C and 70 to 80% humidity until dissection. The mosquitoes were offered resins and 1% glucose solution as a source of energy and were not fed on blood.

Dissection of mosquitoes and isolation of DNA

Dissection of mosquitoes was done according to Rani et al. (2009). Before dissecting, the mosquitoes were chilled to death and sterilized with 70% ethanol then transferred into sterile distilled water in a sterile hood. The mosquitoes were dissected individually under sterile conditions. The midguts were mashed and suspended in 100 µl of sterile phosphate buffered solution (PBS). The mashed midguts were ground to homogeneity. Each midgut extract consisted of 20 pooled midguts of adult female mosquitoes. Field collected and lab reared mosquitoes had seven pooled midgut extracts each. The midgut extracts were stored at -80ºC until further analysis.

The total microbial genomic DNA was extracted separately from each group of mosquito midgut extracts using purelink genomic DNA mini kit (Invitrogen), following the manufacturer's

*Corresponding author: E-mail: rkasili@gmail.com. Tel: +254713800972.

instructions (CAT number, K1820-02 Life technologies, California, USA). Genomic DNA concentration was quantified using a nano drop spectrophotometer and the DNA stored at -20°C until further analysis.

Polymerase chain reaction amplification

Polymerase chain reaction (PCR) amplification of the 16S rRNA gene V4 variable region was carried out on the extracted DNA using primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) that had a barcode (Caporaso et al., 2010). PCR amplification was carried out in 30 cycles using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 min of intitial heating, followed by 30 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, followed by a final elongation step at 72°C for 5 min. PCR products were visualized on 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together in equal proportions based on their DNA concentrations from the gel images. Pooled samples were purified using calibrated Ampure XP beads (Agencort Bioscience Corporation, MA, USA).

Amplicon library preparation

The pooled and purified PCR products were used to prepare DNA library by following Illumina TruSeq DNA library protocol (Yu and Zhang, 2012). Sequencing was performed at Molecular Research DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq platform following the manufacturer's guidelines. The resulting raw sequences were submitted to NCBI (Sequence Read Archive) with the following study accession numbers; sequences for field collected *A. gambiae* SAMN04386463; field collected *C. quinquefasciatus* SAMN04386464; lab reared *A. gambiae* SAMN04386465 and lab reared *C. quinquefasciatus* SAMN04386466.

Sequence analysis and taxonomic classification

Sequences obtained from the Illumina sequencing platform were depleted of barcodes and primers using a proprietary pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX) developed at the service provider's laboratory. Short sequences < 200 bp, sequences with ambiguous base calls, and those with homopolymer runs exceeding 6 bp were removed. The sequences were denoised, chimeras and singleton sequences removed (Capone et al., 2011; Dowd et al., 2011; Eren et al., 2011). De novo OTU clustering was done with standard UCLUST method using the default settings as implemented in QIIME Version 1.8.0 at 97% similarity level (Caporaso et al., 2010). Taxonomy was assigned to each OTU using BLASTn against SILVA SSU Reference 119 database at default e-value threshold of 0.001 in QIIME (Quast et al., 2013).

Diversity indices

Diversity indices (Shannon, Inverse Simpson, Evenness), rarefaction, Venn diagram (to compare the shared OTUs between the samples of mosquitoes) and hierarchical clustering were computed, using Vegan package version 1.16 to 32 in R software (R development Core Team, (2012). Kruskal-Wallis rank sum test was used to compare the relative abundance of gut microflora among *A. gambiae* and *C. quinquefasciatus* from lab reared and field collected samples using R programming language (R development Core Team (2012). Significance was tested at 95% confidence interval (p = 0.05). To support OTU-based analysis, taxonomic groups were derived from the number of reads assigned to each taxon at all ranks from domain to species using the taxa_summary.txt output from QIIME pipeline Version 1.8.0.

RESULTS

Assemblage and diversity of the microbial communities

After removing chimeras, denoising and demutiplexing, a total of 24,025 sequence reads greater than 200 bp were attained from the 16S rRNA data. Total OTU richness at 3% distance amounted to 145. The OTUs per data set ranged between 26 and 102. OTUs comprised 87% bacteria, 0.7% Archaea, 2% Fungi, 1.4% Eukarya and 8% no blast hit (sequences reads that were not assigned). Rarefaction curve was plotted in order to evaluate if all the diversity within the samples had been exhaustively recovered (Figure 1).

The slopes of the curves flatten out in cases where full diversity has been detected. This indicates that even if more sequences were obtained, the number of OTUs detected in the samples would not increase. However, more sequences would be required to exhaust the full diversity within the samples if the slopes did not flatten out (Chao et al., 2014). The sequencing depth as shown by the rarefaction curve was exhaustive enough to ensure the inclusion of the entire diversity of the microbes in the midgut of the two species of mosquitoes collected from field and lab reared.

The distribution of shared OTUs across the two species of mosquitoes and the sample source (lab reared and field collected) is shown in (Figure 2). Seven OTUs were common in all the samples, fifty four (54) OTUs were only found in field collected *A. gambiae* while 18 OTUs were detected only from the field collected *C. quinquefasciatus* samples. Lab reared *C. quinquefasciatus* and *A. gambiae* samples had one and 10 unshared OTUs, respectively.

A diversity index is a quantitative measure that reflects how many different types of species there are in a community and simultaneously takes into account how evenly the individuals are distributed among them.The estimated Shannon diversity index varied between (3.54) for field collected *A. gambiae* and (1.93) for lab reared *C. quinquefasciatus* (Table 1). The Shannon diversity index for field collected *C. quinquefasciatus* (2.73) was higher than lab reared *A. gambiae* (2.52) and lab reared *C. quinquefasciatus* (1.93). The Shannon index is a representation of species abundance and evenness, when either of these two factors increases, the diversity index also increases. Evenness index was used to estimate how well the species are evenly distributed in a community. The highest evenness was recorded from field collected *A. gambiae* (0.767) indicating that OTUs were evenly distributed as compared to other samples. The lowest evenness was recorded from lab reared *C.*

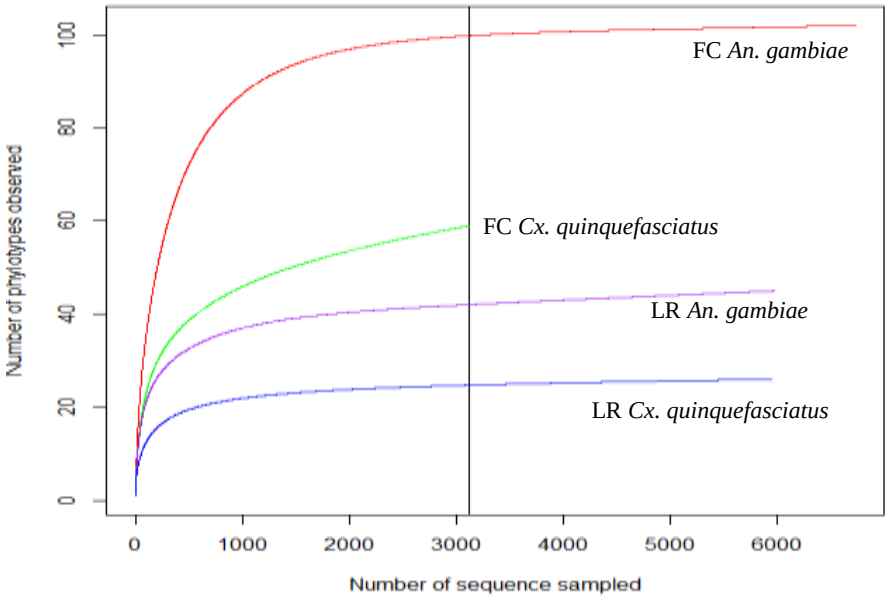

Figure 1. Rarefaction curve analysis in field collected (FC) *Cx.* (*Culex*) and lab reared (LR) *An.* (*Anopheles*) samples.

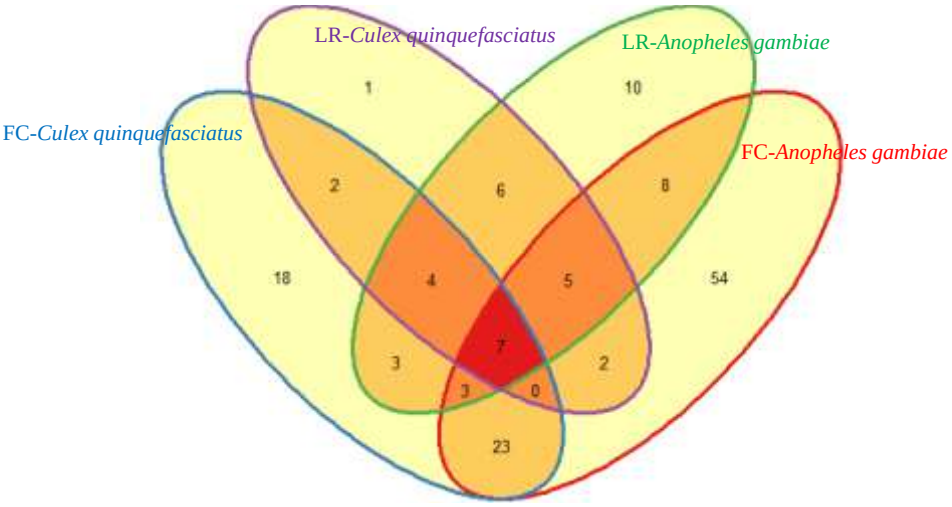

Figure 2. Venn diagram showing the distribution of shared OTUs across the 4 samples. Numbers indicate OTUs enumerated in field collected (FC) and lab reared (LR) samples.

Table 1. Diversity indices computed at OTU-based bacterial taxonomic units obtained from samples of the Field collected (FC) and Lab reared (LR) mosquitoes.

Sample	No. of sequences after filtering	Richness (OTUs)	Shannon (H)	Inverse Simpson	Evenness (J)	Effective no. of sp.
FC *Anopheles gambiae*	7516	102	3.54	19.98	0.767	34.47
FC *Culex quinquefasciatus*	3465	59	2.73	8.72	0.67	15.33
LR *Anopheles gambiae*	6669	45	2.52	5.98	0.661	12.43
LR *Culex quinquefasciatus*	6375	26	1.93	4.65	0.593	6.89
Total	**24,505**	**145**				

The microbial community composition, based on Kruskal-Wallis test, at OTU level showed significant difference between field collected and lab reared mosquitoes (χ^2= 45.0799, p = 3.2 × 10^{-5}). Similarly, there was significant difference in microbial community composition at OTU level between *Anopheles gambiae* and *Culex quinquefasciatus* (χ^2= 31.2257, p = 7.7 × 10^{-4}).

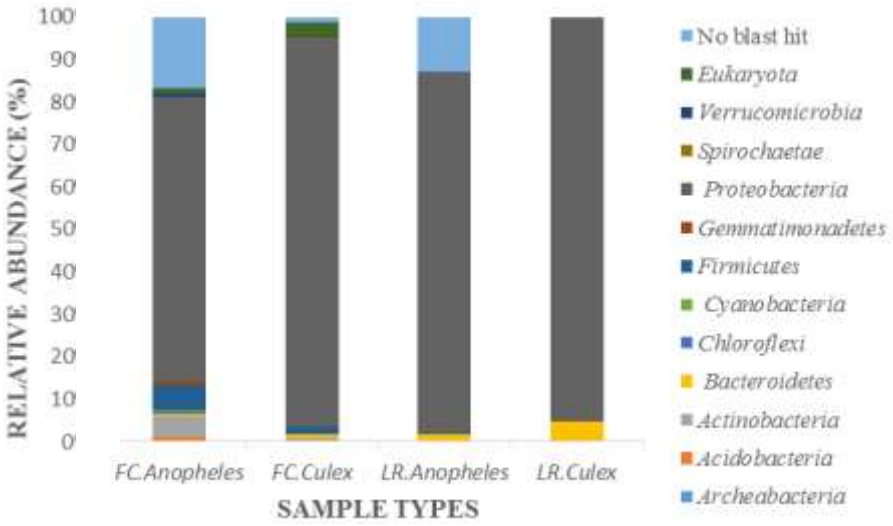

Figure 3. Relative abundance at phylum level from the field collected (FC) and lab reared (LR) samples.

quinquefasciatus (0.593) indicating that bacterial species are less evenly distributed and some species are more dominant than the others. The value of Inverse Simpson index ranged from 4.65 for lab reared *C. quinquefasciatus* to 19.98 for field collected *A. gambiae*. The value of Inverse Simpson index was observed to increase with increase in diversity**.**

Microbial taxonomic community composition

The SILVA SSU Reference 119 database (Quast et al., 2013) was used to assign reads to appropriate taxonomic ranks. The OTUs spanned 12 phyla (Figure 3); *Proteobacteria* (62.04-95.11 %)*, Firmicutes* (0.00-6.13 %), *Bacteriodetes* (0.42-4.89 %), *Actinobacteria* (0.00-4.97%), *Eukaryota* (0.00-3.46%), *Gemmatimonadetes* (0.00-0.86%), *Spirochaetae* (0.00-0.21%), *Verrucomicrobia* (0.00-0.76%), *Chloroflexi* (0.00-0.80%), *Acidobacteria* (0.00-0.68%), *Archeabacteria* (0.00-0.39%) and *Cyanobacteria* (0.00-0.10%). The no blast hits had relative abundance ranging from 0.00 to 16.58%.

OTUs belonging to the Phylum *Proteobacteria* were the most abundant and were represented by the most genera as shown in Figure 4. In lab reared *C. quinquefasciatus* sample the OTUs were affiliated to following genera; *Aeromonas*, *Asaia*, *Elizabethkingia*, *Enterobacter*, *Pseudomonas*, *Rahnella*, *Serratia* and *Wolbachia*. *Serratia marcescens* was the most abundant species in this sample with a relative abundance of 64.29%. Other species present in higher abundance were *Rahnella* uncultured bacterium 18.13%, *Serratia* uncultured bacterium 5.08%, *Wolbachia Embioptera* sp. 4.37% and *Elizabethkingia meningoseptica* 4.88% (Figure 4). However, in field collected *Culex quinquefasciatus* sample genera represented were, *Wolbachia*, *Sphingomonas*, *Streptococcus*, *Serratia*, *Rhizobium*, *Rahnella*, *Pseudomonas*, *Methylobacterium*, *Ixodes*, *Helicobacter*, *Gamma proteobacterium*, *Enterobacter*, *Corynebacterium*, *Bartonella*, *Bacteroidetes*, *Bacillus*, *Asaia*, *Arcobacter*, *Akkermansia*, *Agrobacterium*, and *Aeromonas*. The most abundant species in field collected *C. quinquefasciatus* sample were *Arcobacter* uncultured bacterium with relative abundance of 34.83%, while *Bartonella grahamii* as4aup had 24.45% (Figure 4). *Arcobacter* uncultured bacterium, *Bacteroidetes* uncultured bacterium, *B. grahamii* as4up, *Gamma Proteobacteria* uncultured bacterium, *Helicobacter* sp. B52Seymour and *Ixodes scapularis* were unique species in the field collected *C. quinquefasciatus* sample.

In lab reared *A. gambiae* sample, *Asaia* uncultured bacterium was the most abundant species with 39.30% relative abundance. Other taxa represented in the sample include *Aeromonas* sp. DMA1, *Rahnella* uncultured bacterium and *Serratia marcescens* each scoring a relative abundance of 10%. The genera found in lab reared *Anopheles gambiae* sample include; *Aeromonas, Serratia, Bacillus*, *Asaia, Chryseobacterium, Gluconacetobacter, Delftia, Pseudomonas, Rahnella, Thorsellia, Enterobacter* and *Stenotrophomonas*. *Thorsellia anophelis* was unique to lab reared *A. gambiae* sample (Figure 5). The field collected *A. gambiae* sample was found to harbor a higher diversity of bacterial species. The most abundant species were *Agrobacterium* sp. 12.63% and *Methylobacterium* uncultured bacterium at 11.14% relative abundance. The most predominant genera found in field collected include; *Serratia, Bacillus*, *Agrobacterium Stenotrophomonas, Gluconacetobacter, Methylobacterium*. *Rahnella* (Figure 5). The unique species in field collected *A. gambiae* sample include

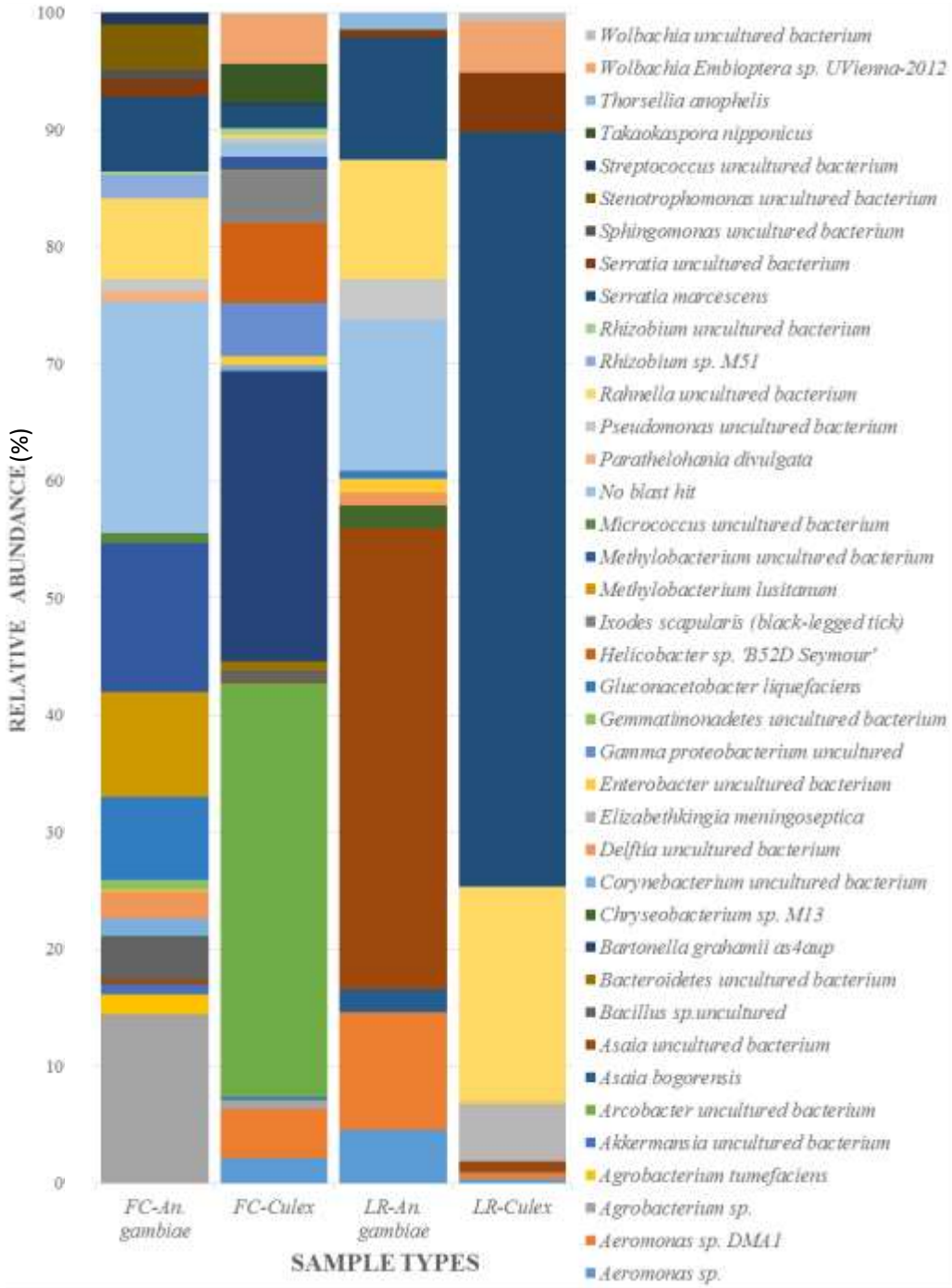

Figure 4. Relative abundance of the most predominant bacterial species from the field collected (FC) and lab reared (LR) samples.

Agrobacterium tumefaciens, *Gemmatimonadetes* uncultured bacterium, *Micrococcus* uncultured bacterium and *Rhizobium* sp. M51 (Figure 5).

Bacterial species which were recovered from all the four samples include, *Serratia marcescens*, *Asaia* uncultured bacterium, *Enterobacter* uncultured bacterium, *Pseudomonas* uncultured bacterium and *Rahnella* uncultured bacterium. *Parathelohania divulgata* and *Takaokaspora nipponicus* are fungal species recovered from the field collected *A. gambiae* and *C.quinquefasciatus* respectively (Figure 5). Detailed information on all the bacterial species recovered in this study is given in additional file 1 Table S1.

Hierarchical clustering, based on Bray-Curtis

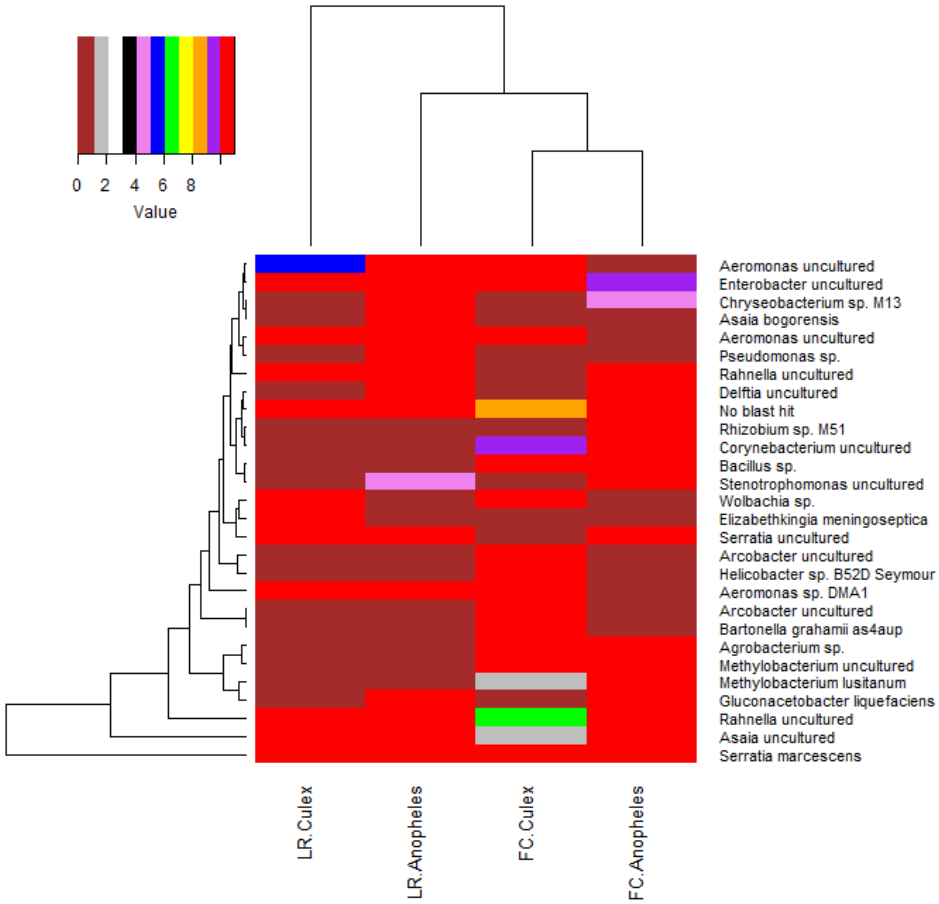

Figure 5. Hierarchical clustering of most abundant midgut bacterial species of the field collected (FC) and lab reared (LR) mosquitoes. Species level was chosen to be used in hierarchical clustering to assess the relationship between sample and taxa.

dissimilarity, showed two clusters (Figure 5). The dendogram shown on the top signify the relationship between the four samples. The bacteria composition of lab reared *A.gambiae*, field collected *A. gambiae* and field collected *C. quinquefasciatus* samples were clustered together. Within this cluster the field collected *A. gambiae* and field collected *C. quinquefasciatus* samples were more closely related to each other. The bacterial community recovered from the lab reared *C. quinquefasciatus* samples was observed to form a distinct cluster.

DISCUSSION

The microbes inhabiting mosquito midgut have drawn special attention in the recent past due to their interactions with both the mosquito host as well as disease causing parasites. The present study sought to investigate the composition and diversity of microbes in midguts of lab reared and field collected *A. gambiae* and *C. quinquefasciatus* mosquitoes. The field collected mosquitoes showed more midgut bacterial composition and diversity than the lab reared mosquitoes. A similar observation was reported by Rani et al. (2009) in their study involving the analysis of bacterial diversity in larvae and adult midgut microflora in lab reared and field collected *Anopheles stephensi* mosquito vectors. The higher bacterial diversity in field collected mosquitoes could probably be due to the fact that wild mosquitoes are exposed to the natural environment where they feed on various natural foods that could be the source of the diverse microbes, whereas the lab reared mosquitoes are fed on artificial diet of glucose and resins. Furthermore, adult female mosquitoes require a blood meal for their egg development and the blood acquired in the field could also be a source of various bacterial flora. On the other hand, the blood given to the adult female lab reared mosquitoes is from infection-free rabbits/mice (Rani et al., 2009). In the present study, the highest number of bacterial species was detected from field collected *A. gambiae* mosquitoes followed by field collected *C. quinquefasciatus* and lab reared *A. gambiae*. The least number of bacterial species were detected from lab

reared *C. quinquefasciatus*.

Diversity indices analysis at OTU level from field collected and laboratory reared mosquitoes revealed a significant difference in microbial community composition. Field collected *A. gambiae* had the highest value of Inverse Simpson index and while the lowest was lab reared *C. quinquefasciatus* samples. The value of Inverse Simpson increases with diversity (Chandel et al., 2013). The Shannon index is another widely used index for comparing diversity between various habitats (Chandel et al., 2013). The Shannon index is a representation of both species abundance and evenness, when either of these two factors increase, the diversity index increases. Evenness was used for the estimating how well the species are evenly distributed among the samples. The lowest evenness was recorded from laboratory reared *C. quinquefasciatus* sample indicating that the bacterial species in this sample are less evenly distributed, that is, some species are more dominant than others. Comparative diversity was visualized using heatmap based on Bray-Curtis dissimilarities at species level. The microbial composition of the field collected samples at species level, were more similar compared to the laboratory reared. However, the laboratory reared samples the bacterial composition seemed to differ between *A. gambiae* and *C. quinquefasciatus*.

Members of the phylum Proteobacteria, were predominantly recovered from both the field collected and lab reared samples than those of phylum *Firmicutes*, *Actinobacteria* and *Bacteriodetes*. *Proteobacteria* were also shown to be dominant in a previous study conducted in Kenya on *A. gambiae* mosquitoes (Wang et al., 2011). Proteobacteria was the largest phylum represented by 43 species belonging to 26 genera. Some of the groups of bacteria recovered in the present study are similar to those recovered from previous culture dependent and culture-independent studies (Rani et al., 2009). Phylum *Firmicutes* consisted of ten species which were affiliated to nine genera. *Actinobacteria* represented fifteen species belonging to thirteen genera whereas *Bacteriodetes* consisted of five species belonging to five genera. Phylum *Cyanobacteria*, *Gemmatimonadetes*, *Spirochaetae*, *Verrucomicrobia*, *Chloroflexi*, *Archeabacteria* and *Acidobacteria* represented only a small portion of the mosquito midgut communities.

The dominant genera recovered in the present study belong to *Serratia, Asaia*, *Arcobacter*, *Rahnella*, *Bartonella*, *Aeromonas*, *Agrobacterium*, *Methylobacterium* and *Wolbachia*. The results of the study are consistent with earlier reports which have shown that that above genera are dominant (Pidiyar et al., 2004; Demaio et al., 1996; Favia et al., 2007; Dong et al., 2009). This suggests that at least a fraction of the mosquito midgut inhabitants could be common for different mosquito species inhabiting similar environments and may represent evolutionary conservation of association between bacteria and mosquito gut. Members of the genera *Acinetobacter*, *Aeromonas*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Serratia*, *Asaia*, *Rahnella*, and *Stenotrophomonas* have been frequently reported in mosquito gut in previous studies (Pidiyar et al., 2004; Boissière et al., 2012; Chandel et al., 2013). Sequences belonging to genera *Asaia*, *Enterobacter*, *Pseudomonas*, *Rahnella* and *Serratia* were recovered from all the samples and comprise a major part of microbiota of *A. gambiae* and *C. quinquefasciatus* mosquitoes in the present study.

Serratia marcescens appeared to be the core species (23.6%) in the present study, as it was observed in both lab reared and field collected samples, suggesting that it possesses a competitive advantage over other bacterial species. *S. marcescens* is abundant in nature, and especially in the artificial foods given to the lab reared mosquitoes. Similar results were reported in five generations of lab reared *A. gambiae* (Dong et al., 2009).

Asaia uncultured bacterium species was recovered at 39.30% was more abundant in lab reared *A.gambiae*. *Asaia* has been associated with *Anopheles* species, in particular field collected *Anopheles funestus*, *Anopheles Maculipennis, Anopheles gambiae* and *Anopheles coustani*, and *Anopheles stephensi* in which *Asaia* bacteria was dominant and stably associated (Favia et al., 2007). The presence of *Asaia* species in *Anopheles* mosquito could be a target for malaria control it produces antiparasite molecules in mosquitoes that could be exploited in paratransgenic control of malaria (Damiani et al., 2010; Favia et al., 2007).

Elizabethkingia meningoseptica and *Wolbachia* sp. have repeatedly been detected in both lab reared and wild caught mosquitoes (Pumpuni et al., 1996) indicating their prevalent symbiotic association with mosquitoes. In the present study, *Wolbacha* was detected in both field collected and lab reared *C. quinquefasciatus*, previously it has been reported in several other mosquito vectors including, *Aedes*, *Coquillettida* and *Masonia* (Ricci et al., 2012).

Bacillus sp., *Stenotrophomonas*, *Micrococcus Acinetobacter*, and *Rhizobium* frequently isolated from soil and environmental samples were recovered at significantly greater numbers from the field collected mosquitoes. This suggests that the local soil and water environment plays an important role in colonization of the mosquito midgut at breeding sites or during nectar/blood feeding (Chandel et al., 2013). *Parathelohania divulgata*, *Parathelohania obesa* and *Takaokaspora nipponicus* fungal species were recovered from the field collected *A. gambiae* and *C. quinquefasciatus* but were absent in lab reared mosquitoes.

From the foregoing, the mosquito midgut has a rich diversity of symbiotic bacteria. The parasite mosquito relationship is believed to have been in existence for many years and it is likely that the acquired microflora permit the maintenance of pathogenic parasites in mosquitoes. The microbes could be benefiting the

mosquitoes by protecting them against harmful bacteria and the mosquitoes could be benefiting the parasites by lowering the mosquito immunity against the parasites.

Conclusion

The results obtained present an analysis of the composition and bacterial diversity of lab reared and wild mosquitoes using Illumina sequencing technology. The bacterial flora of adult female *A. gambiae* and *C. quinquefansciatus* midgut is diverse and is dominated by bacterial species *S. marcescens*. In future, understanding the tripartite mosquito-microbes-parasite interaction will enable us gain more insight that may be useful in the development of novel approaches for the control of malaria and other mosquito borne diseases like filariasis, dengue, Zika and Chikungunya.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Agrawal V, Sashindran V (2006). Lymphatic Filariasis in India : Problems, Challenges and New Initiatives. Med. J. Armed Forces India 62:359-362.

Bando H, Okado K, Guelbeogo WM, Badolo A, Aonuma H, Nelson B, Fukumoto S, Xuan X, Sagnon NF, Kanuka H (2013). Intra-specific diversity of *Serratia marcescens* in *Anopheles* mosquito midgut defines Plasmodium transmission capacity. Sci. Rep. 3:1641.

Boissière A, Tchioffo MT, Bachar D, Abate L, Marie A, Nsango SE, Shahbazkia HR, Awono-Ambene PH, Levashina EA, Christen R, Morlais I (2012). Midgut Microbiota of the Malaria Mosquito Vector *Anopheles gambiae* and Interactions with *Plasmodium falciparum* Infection. PLoS Pathog. 8(5):e1002742.

Capone KA, Dowd SE, Stamatas GN, Nikolovski J (2011). Diversity of the Human Skin Microbiome Early in Life. J. Invest. Dermatol. 131:2026-2032.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010). QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7:335-336.

Centres for Disease Control and Prevention, CDC (2015). Malaria - Malaria Worldwide - CDC–Activities in Kenya. Available at: http://www.cdc.gov/malaria/malaria_worldwide/cdc_activities/kenya.html.

Chandel K, Mendki MJ, Parikh RY, Kulkarni G, Tikar SN, Sukumaran D, Prakash S, Parashar BD, Shouche YS, Veer V (2013). Midgut Microbial Community of *Culex quinquefasciatus* Mosquito Populations from India. PLoS One 8(11):e80453.

Chao A, Gotelli NJ, Hsieh TC, Sander EL, Ma KH, Colwell RK, Ellison AM (2014). Rarefaction and extrapolation with Hill numbers: A framework for sampling and estimation in species diversity studies. Ecol. Monogr. 84:45-67.

Chavshin AR, Oshaghi MA, Vatandoost H, Pourmand MR, Raeisi A, Enayati AA, Mardani N, Ghoorchian S (2012). Identification of bacterial microflora in the midgut of the larvae and adult of wild caught *Anopheles stephensi*: a step toward finding suitable paratransgenesis candidates. Acta Trop. 121:129-134.

Damiani C, Ricci I, Crotti E, Rossi P, Rizzi A, Scuppa P, Capone A, Ulissi U, Epis S, Genchi M, Sagnon N, Faye I, Kang A, Chouaia B, Whitehorn C, Moussa GW, Mandrioli M, Esposito F, Sacchi L, Bandi C, Daffonchio D, Favia G (2010). Mosquito-bacteria symbiosis: the case of *Anopheles gambiae* and *Asaia*. Microb. Ecol. 60:644-654.

Demaio J, Pumpuni CB, Kent M, Beier JC (1996). The midgut bacterial flora of wild *Aedes triseriatus*, *Culex pipiens*, and *Psorophora columbiae* mosquitoes. Am. J. Trop. Med. Hyg. 54:219-223.

Dong Y, Manfredini F, Dimopoulos G (2009). Implication of the Mosquito Midgut Microbiota in the Defense against Malaria Parasites. PLoS Pathog. 5:e1000423.

Dowd SE, Delton Hanson J, Rees E, Wolcott RD, Zischau AM, Sun Y, White J, Smith DM, Kennedy J, Jones CE (2011). Survey of fungi and yeast in polymicrobial infections in chronic wounds. J. Wound Care 20:40-47.

Eren AM, Zozaya M, Taylor CM, Dowd SE, Martin DH, Ferris MJ (2011). Exploring the Diversity of *Gardnerella vaginalis* in the Genitourinary Tract Microbiota of Monogamous Couples Through Subtle Nucleotide Variation. PLoS One 6(10):e26732.

Favia G, Ricci I, Damiani C, Raddadi N, Crotti E, Marzorati M, Rizzi A, Urso R, Brusetti L, Borin S, Mora D, Scuppa P, Pasqualini L, Clementi E, Genchi M, Corona S, Negri I, Grandi G, Alma A, Kramer L, Esposito F, Bandi C, Sacchi L, Daffonchio D (2007). Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an *Asian* malarial mosquito vector. Proc. Natl. Acad. Sci. U. S. A. 104:9047-9051.

Gillies MT, DE Meillon B (1968). The Anophelinae of Africa south of the Sahara (Ethiopian Zoogeographical Region). 343 p.

Njenga SM, Mwandawiro CS, Wamae CN, Mukoko DA, Omar AA, Shimada M, Bockarie MJ, Molyneux DH (2011). Sustained reduction in prevalence of lymphatic filariasis infection in spite of missed rounds of mass drug administration in an area under mosquito nets for malaria control. Parasit. Vectors 4(1):90.

Pidiyar VJ, Jangid K, Patole MS, Shouche YS (2004). Studies on Cultured and Uncultured Microbiota of Wild *Culex quinquefasciatus* Mosquito Midgut Based on 16s Ribosomal Rna Gene Analysis. Am. J. Trop. Med. Hyg. 70:597-603.

Pumpuni CB, Demaio J, Kent M, Davis JR, Beier JC (1996). Bacterial population dynamics in three anopheline species: the impact on *Plasmodium* sporogonic development. Am. J. Trop. Med. Hyg. 54:214-218.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner F O (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41:D590-D596.

R development Core Team (2012). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing: Vienna, Austria.

Rani A, Sharma A, Rajagopal R, Adak T, Bhatnagar RK (2009). Bacterial diversity analysis of larvae and adult midgut microflora using culture-dependent and culture-independent methods in lab-reared and field-collected *Anopheles stephensi*-an Asian malarial vector. BMC Microbiol. 9(1):96.

Ricci I, Damiani C, Capone A, DeFreece C, Rossi P, Favia G (2012). Mosquito/microbiota interactions: from complex relationships to biotechnological perspectives. Curr. Opin. Microbiol. 15:278-284.

Riehle MA, Jacobs-Lorena M (2005). Using bacteria to express and display anti-parasite molecules in mosquitoes: current and future strategies. Insect Biochem. Mol. Biol. 35:699-707.

Wang Y, Gilbreath TM, Kukutla P, Yan G, Xu J (2011). Dynamic Gut Microbiome across Life History of the Malaria Mosquito *Anopheles gambiae* in Kenya. PLoS One 6:e24767.

Whitten MMA, Shiao SH, Levashina EA (2006). Mosquito midguts and malaria: cell biology, compartmentalization and immunology. Parasite Immunol. 28:121-130.

WHO- World Health Organisation (2015). World malaria report. http://www.who.int/malaria/publications/world-malaria-report-2015/report/en/.

Yu K, Zhang T (2012). Metagenomic and Metatranscriptomic Analysis of Microbial Community Structure and Gene Expression of Activated Sludge. PLoS One 7(5):e38183.

Additional file 1

Table S1. Midgut bacterial composition at species level and their abundance.

Species level	FC.*Anopheles*	FC.*Culex*	LR.*Anopheles*	LR.*Culex*	Total
Phylum *Archeabacteria*					
Archaea, Euryarchaeota, Methanobacteria, Methanobacteriales, Methanobacteriaceae, *Methanobrevibacter*, uncultured archaeon	24	0	0	0	
Phylum *Acidobacteria*					
Bacteria, Acidobacteria, Acidobacteria, Subgroup 4, Unknown Family, Blastocatella, uncultured *Acidobacteria* bacterium	10	0	0	0	
Bacteria, Acidobacteria, Acidobacteria, Subgroup 6, uncultured *Acidobacteria* bacterium	32	0	0	0	
Phylum *Actinobacteria*					
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Corynebacteriaceae, *Corynebacterium* uncultured bacterium	72	10	0	0	
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Corynebacteriaceae, Corynebacterium, uncultured *Corynebacterium* sp.	6	6	0	0	
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Corynebacteriaceae, *Corynebacterium* unidentified marine bacterioplankton	10	3	0	0	
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Corynebacteriaceae, uncultured, uncultured bacterium	31	1	0	0	
Bacteria, Actinobacteria, Actinobacteria, Corynebacteriales, Dietziaceae, *Dietzia* uncultured bacterium	8	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Frankiales, Geodermatophilaceae, *Blastococcus* uncultured bacterium	7	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Cellulomonadaceae, *Actinotalea* uncultured bacterium	17	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Intrasporangiaceae, *Terrabacter* uncultured bacterium	38	1	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Microbacteriaceae, *Curtobacterium* uncultured bacterium	24	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae, Arthrobacter, *Arthrobacter* sp. TSBY-23	7	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae, Enteractinococcus, *Yaniella* sp. YUAB-SO-24	7	1	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae, Kocuria, *Kocuria* sp. oral clone AW006	11	2	0	0	
Bacteria, Actinobacteria, Actinobacteria, Micrococcales, Micrococcaceae, *Micrococcus* uncultured bacterium	43	0	0	0	
Bacteria, Actinobacteria, Actinobacteria, Propionibacteriales, Nocardioidaceae, *Nocardioides* uncultured bacterium	4	2	0	0	
Bacteria, Actinobacteria, Actinobacteria, Streptomycetales, Streptomycetaceae, Streptomyces, *Streptomyces ferralitis*	12	0	0	0	
Bacteria, Actinobacteria, Thermoleophilia, Gaiellales, uncultured, uncultured bacterium	12	0	0	0	
Phylum *Bacteroidetes*					
Bacteria, Bacteroidetes, Bacteroidia, Bacteroidales, Rikenellaceae, RC9 gut group, uncultured bacterium	6	0	0	0	
Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Cryomorphaceae, *Fluviicola* uncultured bacterium	14	0	0	0	
Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Chryseobacterium, *Chryseobacterium* sp. M13	5	0	113	0	
Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, Elizabethkingia, *Elizabethkingia meningoseptica*	1	0	0	291	
Bacteria, Bacteroidetes, Flavobacteriia, Flavobacteriales, Flavobacteriaceae, uncultured, uncultured Bacteroidetes bacterium	0	21	0	0	
Phylum *Chloroflexi*					
Bacteria, Chloroflexi, Thermomicrobia, JG30-KF-CM45, uncultured soil bacterium	31	0	0	0	
Bacteria, Chloroflexi, Thermomicrobia, Sphaerobacterales, Sphaerobacteraceae, Nitrolancea, uncultured *Chloroflexi* bacterium	19	0	0	0	
Phylum *Cyanobacteria*					
Bacteria, Cyanobacteria, *Chloroplast* uncultured bacterium	6	0	0	0	
Phylum *Firmicutes*					
Bacteria, Firmicutes, Bacilli, Bacillales, Bacillaceae, *Anoxybacillus* uncultured bacterium	0	10	1	0	
Bacteria, Firmicutes, Bacilli, Bacillales, Bacillaceae, Bacillus, uncultured *Bacillus* sp.	183	31	1	0	

Table S1. Contd.

Bacteria, Firmicutes, Bacilli, Bacillales, Bacillaceae, *Bacillus* uncultured bacterium	5	1	0	0
Bacteria, Firmicutes, Bacilli, Bacillales, Planococcaceae, *Lysinibacillus* uncultured bacterium	22	5	0	0
Bacteria, Firmicutes, Bacilli, Bacillales, Staphylococcaceae, *Salinicoccus* uncultured bacterium	39	0	0	0
Bacteria, Firmicutes, Bacilli, Lactobacillales, Carnobacteriaceae, *Atopostipes* uncultured bacterium	32	1	0	0
Bacteria, Firmicutes, Bacilli, Lactobacillales, Carnobacteriaceae, uncultured, uncultured bacterium	6	0	0	0
Bacteria, Firmicutes, Bacilli, Lactobacillales, Streptococcaceae, *Streptococcus* uncultured bacterium	53	4	0	0
Bacteria, Firmicutes, Clostridia, Clostridiales, Peptostreptococcaceae, *Incertae Sedis* uncultured bacterium	26	4	1	0
Bacteria, Firmicutes, Clostridia, Clostridiales, Ruminococcaceae, uncultured, uncultured bacterium	15	0	0	0
Phylum *Gemmatimonadetes*				
Bacteria, Gemmatimonadetes, Gemmatimonadetes, AT425-EubC11 terrestrial group, uncultured bacterium	42	0	0	0
Phylum *Proteobacteria*				
Bacteria, Proteobacteria, Alphaproteobacteria, Caulobacterales, Caulobacteraceae, *Brevundimonas* uncultured bacterium	12	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Bartonellaceae, Bartonella, *Bartonella grahamii* as4aup	0	737	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Bradyrhizobiaceae, Bosea, uncultured *Bosea* sp.	37	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Methylobacteriaceae, Methylobacterium, *Methylobacterium lusitanum*	468	2	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Methylobacteriaceae, *Methylobacterium* uncultured bacterium	665	32	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, *Agrobacterium tumefaciens*	87	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, *Rhizobium* sp. JC140	32	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, *Rhizobium* sp. M51	106	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, uncultured *Agrobacterium* sp.	754	21	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, *Rhizobium* uncultured bacterium	10	18	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhizobiales, Rhizobiaceae, Rhizobium, uncultured *Paracoccus* sp.	19	1	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodobacterales, Rhodobacteraceae, *Paracoccus* uncultured bacterium	9	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, Acetobacter, uncultured *Acetobacter* sp.	0	0	8	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, Asaia, *Asaia bogorensis*	0	0	116	1
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, *Asaia* uncultured bacterium	16	2	2223	55
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, *Asaia* uncultured bacterium	0	0	47	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, *Asaia* uncultured bacterium	5	0	39	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rhodospirillales, Acetobacteraceae, Gluconacetobacter, *Gluconacetobacter liquefaciens*	370	0	37	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, Anaplasmataceae, *Wolbachia* Embioptera sp. UVienna-2012	0	125	0	261
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, Anaplasmataceae, *Wolbachia* uncultured bacterium	0	5	0	43
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, mitochondria, *Triticum aestivum*	17	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, Rickettsiaceae, Rickettsia, uncultured Rickettsia sp.	19	0	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, *Sphingomona*s uncultured bacterium	21	2	0	0
Bacteria, Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, *Sphingomonas*, uncultured *Firmicutes* bacterium	24	0	0	0
Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae, *Delftia* uncultured bacterium	74	0	59	0
Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae, Delftia, uncultured *Delftia* sp.	46	0	7	0
Bacteria, Proteobacteria, Betaproteobacteria, Burkholderiales, Oxalobacteraceae, Oxalicibacterium, *Oxalicibacterium flavum*	30	0	0	0
Bacteria, Proteobacteria, Betaproteobacteria, Neisseriales, Neisseriaceae, uncultured, uncultured bacterium	7	1	0	0

Table S1. Contd.

Bacteria, Proteobacteria, Epsilonproteobacteria, Campylobacterales, Campylobacteraceae, *Arcobacter* uncultured bacterium	0	1050	0	0
Bacteria, Proteobacteria, Epsilonproteobacteria, Campylobacterales, Helicobacteraceae, Helicobacter, *Helicobacter* sp. 'B52D Seymour'	0	204	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Aeromonadales, Aeromonadaceae, Aeromonas, *Aeromonas* sp. DMA1	0	125	588	37
Bacteria, Proteobacteria, Gammaproteobacteria, Aeromonadales, Aeromonadaceae, Aeromonas, uncultured *Aeromonas* sp.	0	0	12	2
Bacteria, Proteobacteria, Gammaproteobacteria, Aeromonadales, Aeromonadaceae, *Aeromonas* uncultured bacterium	0	64	254	18
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Enterobacter* uncultured bacterium	10	23	71	13
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Escherichia-Shigella, *Serratia marcescens*	0	0	1	4
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Rahnella* uncultured bacterium	364	7	399	1050
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Rahnella* uncultured bacterium	207	0	210	31
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Rahnella* uncultured bacterium	12	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	168	48	365	2146
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	157	14	232	1236
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	0	0	15	98
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	11	3	69	399
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia marcescens*	1	0	7	23
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Serratia, *Serratia* sp. DR.Y5	0	0	1	13
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	4	0	4	18
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	28	0	0	1
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	42	0	12	59
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	0	0	11	91
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, *Serratia* uncultured bacterium	19	0	10	53
Bacteria, Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Thorsellia, *Thorsellia anophelis*	0	0	81	0
Bacteria, Proteobacteria, Gammaproteobacteria, Orbales, Orbaceae, Gilliamella, uncultured *gamma proteobacterium*	0	69	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Orbales, Orbaceae, Gilliamella, uncultured *gamma proteobacterium*	0	65	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, Acinetobacter, *Acinetobacter* sp. B7_2TCO2	9	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, *Acinetobacter* uncultured bacterium	6	3	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, *Acinetobacter* uncultured bacterium	6	1	1	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, *Enhydrobacter* uncultured proteobacterium	13	4	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, *Psychrobacter* uncultured bacterium	11	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas* uncultured bacterium	21	14	26	6
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas* uncultured bacterium	28	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, *Pseudomonas* uncultured Pseudomonas sp.	1	0	177	0
Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, *Stenotrophomonas* uncultured bacterium	196	0	5	0
Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, *Stenotrophomonas*, uncultured bacterium	15	0	0	0
Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadales Incertae Sedis, *Steroidobacter* uncultured bacterium	16	0	0	0
Phylum *Spirochaetae*				
Bacteria,Spirochaetae,Spirochaetes,Spirochaetales,Spirochaetaceae,uncultured,*Spironema culicis*	0	6	0	0
Phylum *Verrucomicrobia*				
Bacteria,Verrucomicrobia,Verrucomicrobiae,Verrucomicrobiales,Verrucomicrobiaceae,*Akkermansia*uncultured bacterium	47	0	0	0

Table S1. Contd.

Bacteria,Verrucomicrobia,Verrucomicrobiae,Verrucomicrobiales,Verrucomicrobiaceae,*Akkermansia*uncultured bacterium	0	7	0	0	
Phylum *Eukaryota*					
Eukaryota, Opisthokonta, Nucletmycea, Fungi, Microsporidia, Incertae Sedis, Amblyosporidae, Parathelohania, *Parathelohania divulgata*	51	0	0	0	
Eukaryota, Opisthokonta, Nucletmycea, Fungi, Microsporidia, Incertae Sedis, Amblyosporidae, *Parathelohania Parathelohania obesa*	33	0	0	0	
Eukaryota, Opisthokonta, Nucletmycea, Fungi, Microsporidia, Incertae Sedis, Amblyosporidae, *Takaokaspora Takaokaspora* nipponicus	0	100	0	0	
No blast hit	1030	34	757	0	1721
Total abundance	6214	2890	5960	5949	21666

academicJournals

Vol. 11(29), pp. 1184-1190, 7 August, 2017
DOI: 10.5897/AJMR2016.8086
Article Number: D2DA87C65432
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Identification of intestinal microbiota and microbiological quality of the king weakfish (*Macrodon ancylodon*)

Rafaela Cristina Barata Alves[1], Hamilton Mendes Figueredo[2], Rafaela Santos Oliveira da Silva[3] and Lúcia de Fátima Henriques Lourenço[1]*

[1]Food Science and Technology, Graduate Program in Food Science and Technology, Federal University of Pará (Universidade Federal do Pará), UFPA, Belém, PA, Brazil
[2]Prof. Dr., School of Food Engineering, Institute of Technology, UFPA, Belém, PA, Brazil
[3]School of Food Engineering, Institute of Technology, UFPA, Belém, PA, Brazil.

Received 30 April, 2016; Accepted 30 June, 2016

The king weakfish (*Macrodon ancylodon*) has a wide geographic distribution in South America and provides a positive economic outlook for the fishing industry in Brazil. The present study aimed to identify the intestinal microbiota and bacterial strains that can increase during storage of the king weakfish. Characterization of the microbiological quality was performed on samples to identify the microflora through isolation on culture media and identification using BD BBL kits for Gram-negative and Gram-positive bacteria. The growth of isolated strains was tested at conventional storage temperatures of 7 and 3°C used to limit the expansion of pathogenic bacteria populations. All king weakfish samples were within the limits established by Brazilian law for microbiological standards. Twenty strains that were isolated from the intestinal microbiota of *M. ancylodon* were identified; five stood out and may play a role in the safety and/or shelf-life of this fish species: *Staphylococcus cohnii* subsp. *urealyticum*, *Burkholderia cepacia*, *Pseudomonas stutzeri*, *Pseudomonas fluorescens* and *Corynebacterium* sp. However, only the first two strains showed significant growth at 3°C after three days of storage, mainly *B. cepacia* which is also considered an opportunistic pathogen.

Key words: King weakfish, intestinal microbiota, opportunistic pathogen, shelf-life.

INTRODUCTION

The king weakfish (*Macrodon ancylodon*) is an important species nationally and along the northern coast of Brazil, in terms of both the volume captured and its consumption by the local population. The species *M. ancylodon* belongs to the family Sciaenidae and has a wide geographic distribution in South America, ranging from the tropical waters of Venezuela to the subtropical waters of Argentina. It can grow to an average of 40 cm long,

*Corresponding author. E-mail: luciahl@ufpa.br.

weighing less than 1 kg, by feeding on shrimp, fish and squid (Santos et al., 2003).

Fish can harbor a large number of microorganisms that are pathogenic to humans and are acquired from environmental pollution as well as improper handling between capture and final consumption. Due to its high perishability, fish should be stored at low temperatures and handled under adequate sanitary conditions throughout the production chain in order to deliver a safe product to consumers (Mol and Tosun, 2011). Importantly, most pathogenic bacteria do not grow at temperatures below 4.4°C (Jay, 2005).

Improper handling results in significant post-capture losses of wild-caught and cultured fish, mainly due to microorganisms. Because they are extremely perishable, fish lose their initial freshness due to enzymatic and chemical reactions, which is closely followed by a complete loss in quality due to microbial activity that occurs primarily on the skin, gills and intestines (Gram and Dalgaard, 2002). During fish processing, microorganisms that are naturally found in the intestine and on the skin may contaminate the muscle, utensils and equipment, leading to cross-contamination and the transmission of food-borne illnesses due to improper handling and inadequate storage (Jay, 2005). It is important to identify the microbial species that contribute to spoilage and are involved in quality loss in fish such that appropriate quality control measures can be implemented.

The total number of microorganisms in the gastrointestinal tract of fish is small compared to that of warm-blooded animals and varies with the age, food source and environmental conditions of the fish (Nayak, 2010). The primary microbial groups that colonize the intestinal tracts of temperate water fish vary based on the fish species but overall are predominantly psychrotrophic, aerobic, anaerobic or facultative Gram-negative bacteria, including *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Shewanella*, *Flavobacterium*, *Cytophaga*, *Vibrio*, *Corynebacterium*, *Photobacterium* and *Alteromonas* (Gram and Huss, 1996; International Commission on Microbiological Specifications for Foods (ICMSF), 2005). Some Gram-positive bacteria, including lactic acid-producing bacteria, *Bacillus sp.* and *Staphylococcus sp.*, are also present (Lalitha and Surendran, 2006). However, only a few members of the fish microbiota, known as "spoilage-specific organisms" or SSOs, are responsible for spoilage (Gram and Dalgaard, 2002).

There are no current data available in the literature on the intestinal microbiota and there are few studies on the microbiological quality of the king weakfish (*Macrodon ancylodon*). Therefore, the aim of the present study was to identify the bacterial populations in the microflora, microbiological characterization this species and study the growth temperature characteristics of the micro-organisms during storage.

MATERIALS AND METHODS

Samples

King weakfish were captured at sea, 70 km from the municipality of Salinópolis, located in the state of Pará, Brazil, and whole fish samples were collected directly from commercial fishing vessels after docking. Four fish were collected at two different periods (beginning and end) of the months of May and June, 2014, which are the months of highest annual production of the specie. The samples were placed in polyethylene bags and transported in coolers on ice to the Food Microbiology Laboratory, Federal University of Pará where the analyses were performed.

Microbiological quality of the samples

Twenty-five grams of each fish sample (ventral part of the fillet) were aseptically removed for each collection period. Samples were transferred to sterile pouches, and 225 ml of sterile saline peptone water was added. Samples were then homogenized at 2,300 rpm for 30 s (Stomacher 400 Circulator SEWARD). The total counts of heterotrophic aerobic mesophilic and aerobic psychrotrophic bacteria counts were determined using standard count in plates of plate count agar followed by incubation at 36°C for 48 h and 7°C for 10 days. Analyses of coliforms at 45°C, *Salmonella sp*. and coagulase-positive Staphylococci were also performed. All analyses were conducted according to the methods described in Compendium of Methods for the Microbiological Examination of Foods (Downes and Ito, 2001).

Intestinal microbiota

Bacterial isolation

Sample material was extracted from fecal matter and the internal intestinal walls of the king weakfish using a sterile loop. All extracted material was then homogenized in 10 ml of sterile saline peptone water. The entire procedure was performed using aseptic techniques. For identification of intestinal microbiota of the king weakfish the homogenates were inoculated in aliquots of 0.1ml on the surface using two types of agar media, violet red bile glucose (VRBG) agar for enterobacteria strains and Baird-Parker agar for gram-positive bacteria, and both plates were incubated at 36°C for 48 h. Next, one plate was selected for each medium and five to eight colonies per plate were randomly chosen and the selected colonies were striated to obtain a pure culture isolation on the same medium (VRBG or Baird-Parker Agar). After incubation at 36°C for 48 h, the colonies were transferred to tubes containing brain heart infusion broth (BHI) supplemented with 10% glycerol and stored frozen for subsequent identification.

Identification of bacterial strains

The isolated colonies were characterized using Gram stains for morphology (microscopy) as well as the oxidase and indole reactions. The strains were then identified using kit recognized worldwide of the BD BBL Crystal including the Enteric/Nonfermenter ID System for Gram-negative bacteria and the Gram-positive ID System for Gram-positive bacteria. The assays were performed according to the manufacturer's protocol.

Temperature limits for growth of the microflora

The methodology employed here was adapted from the method of

Table 1. Results for mesophiles, psychrotrophs and coliforms at 45°C of the king weakfish by collection (periods).

Analyses	Collections			
	1st	2nd	3rd	4th
Mesophiles (Log CFU/g)	3.58^{cd}	3.53^{bd}	3.42^{bc}	3.83^{a}
Psychrotrophs (Log CFU/g)	4.55^{c}	4.79^{b}	5.22^{a}	5.4^{a}
Coliforms at 45°C (MPN/g)	75^{b}	3.6^{c}	3.6^{c}	150^{a}

*Different letters in each line means difference at 95% level of significance.

Table 2. Identification of bacteria isolated from intestinal microbiota of the king weakfish and their frequency (%).

Bacteria isolated	Strains (%)	ID (%)
Staphylococcus cohnii spp. *urealyticum*	6.67	99.99
Burkholderia cepacia	26.67	97.22
Pseudomonas fluorescens	46.67	91.24
Pseudomonas stutzeri	13.33	62.66
Corynebacterium sp.	6.67	91.33

% ID indicates the similarity profile of the isolates according to the manufacturer's internal standards for the kits.

Damasceno et al. (2015), where the optical density was used to identify strains that could grow at the different storage temperatures. The bacterial strains previously identified and frozen in BHI broth were first reactivated in proportion 1:10 ml in the medium for 24 h at 36°C. The 0.6 ml from isolates identified were then transferred to tubes containing 9 ml of BHI medium and incubated at 7°C (mean temperature for conventional refrigeration) and 3°C (below the temperature limits for the growth of pathogens) for 56 and 80 h, respectively. The spectrophotometric readings were taken at four-hour intervals using a UV spectrophotometer (Modelo Nova 2000) at different wavelengths determined based on the highest absorbance for each strain identified after scanning the entire spectrum in the UV-visible (200 to 800 nm).

Statistical analysis

Analysis of variance (ANOVA) and Tukey's test at a 5% significance level were used to determine the significance of the microbiological quality between samples of periods and the temperature limits for growth of the identified microorganisms. The program *Statistica* 8.0 was used for these analyses.

RESULTS AND DISCUSSION

Characterization of the microbiological quality

All samples were within the microbiological standards established by the Brazilian legislation for fresh fish, demonstrating the good quality of the samples collected at the four time periods in May and June. This standard was based on the absence of *Salmonella* spp. and less than 10^3 coagulase-positive *Staphylococci* in 25-gram samples of king weakfish (Brazil, 2001). The results of the additional analyses used to characterize the microbiological quality of the samples are presented in Table 1.

Total and thermotolerant coliforms are used as quality indicators because fish do not usually carry those organisms, particularly *E. coli* and faecal coliform, their presence reflect the degree of microbial contamination to which food is exposed (ICMSF, 1986). The results of the analysis of coliforms at 45°C indicated low levels of contamination for the king weakfish samples over the collections periods. In the standard plate count analysis for aerobic mesophiles and psychrotrophs, all samples were below the total counts established by the International Commission on Microbiological Specifications for Foods (ICMSF, 2005), which recommends a maximum of 7 Log CFU/g of refrigerated fish. Counts of psychrotrophic bacteria were higher than those reported by Thong et al. (2013) for the species *Pangasius hypophthalmus* at 4.3 Log CFU/g. In addition, the total counts for these microorganisms were higher than the counts for mesophiles, indicating that the storage of king weakfish until 4°C favors the growth of microorganisms spoilage (Jay, 2005).

Identification of the intestinal microbiota

Table 2 lists the bacteria identified from the intestinal microbiota of king weakfish. Twenty isolates were evaluated using bacterial identification kits; 90% (18

strains) were Gram-negative and 10% (two strains) were Gram-positive. However, five isolates could not be identified using the kit standards or the manufacturer's identification software. There is ample evidence that the gastrointestinal microbiota of fish is highly variable, largely reflecting the aquatic environment and food source of each fish (Al-Harbi and Uddin, 2004). The microbiota of most fish is dominated by psychrotrophic Gram-negative species; however, the bacterial load in tropical fish often contains more Gram-positive and enteric bacteria than temperate fish species (Gram and Huss, 1996). Gram-positive strains such as *Staphylococcus* spp., *Micrococcus* spp. and *Bacillus* spp. have been isolated from tropical marine fish, although reports on the identity or specific sources of these bacteria are limited (Al-Bulushi et al., 2010).

Most bacteria identified by the technique used have a very high similarity profile (over 90%), demonstrating security in the identification of the microorganism in microflora. In general, the identified strains from the king weakfish intestine are considered spoilage bacteria and may directly play a role in the safety and/or shelf-life of this fish species. *Staphylococci* are not part of the normal fish microbiota (Van den Broek et al., 1984), given that coagulase-negative *Staphylococci* are normally found in the mucous membranes and skin of samples of human origin (Piette and Verschraegen, 2009). The *Staphylococcus cohnii* subsp. *Urealyticum* isolated from king weakfish intestines was classified as a coagulase-negative *Staphylococcus* and is able to form large colonies and aerobically produce acid byproducts when metabolizing lactose (Tammy and Bannerman, 1996). Because it is not part of the normal fish microbiota, these bacteria only accounted for 6.67% of the strains identified in king weakfish. Most *Staphylococcal* species are harmless, although some species of this genus cause various diseases by producing enzymes and toxins, such as *S. cohnii,* which encodes a gene for enterotoxin production (Zell et al., 2008).

Studies characterizing the microbiota of foods *in natura* and food products are important in assessing the safety, spoilage and the influence of these microorganisms on sensory characteristics. Studies on salted and dried salted cod identified Gram-positive bacteria such as *S. cohnii* and Gram-negative bacteria such as *Pseudomonas fluorescens*, a bacterium that is associated with food spoilage due to its ablity to produce H_2S and/or biogenic amines, especially in foods stored at low temperatures (Rodrigues et al., 2003). In the present study, 46.67% of the isolates from the king weakfish intestinal microbiota were identified as *P. fluorescens* (Table 2). *P. fluorescens*, an aquaculture pathogen that also infects many other fish species, produces extracellular enzymes that contribute to skin diseases, primarily in fish that are injured during improper handling and transport (Zhang et al., 2009). However, these and other virulence mechanisms of *P. fluorescens* remain poorly understood (Zhang et al., 2009).

Over the past decade, some non-fermentative Gram-negative bacteria have emerged as important fish and human pathogens (Enoch et al., 2007). In addition to the Gram-negative bacterium, this study identified two other strains *Burkholderia cepacia* and *Pseudomonas stutzeri* accounted for 26.67 and 13.33%, respectively, of the intestinal microbiota in king weakfish. *B. cepacia* is a well-known pathogen opportunistic associated with hospital-acquired infections (Enoch et al., 2007) due to its resistance to many antimicrobial agents (Spencer, 1995).

Miranda and Zelmeman (2002) reported antibiotic resistance in Gram-negative bacteria, which they attributed to the use of antibiotics to control bacterial pathogens in salmon farming. The authors evaluated strains isolated from water, food and minnows, where the most prevalent species was *P. fluorescens.* Other Gram-negative bacteria were also resistant, including *Pseudomonas aeruginosa*, *S. maltophilia, B. cepacia* and *Acinetobacter*. This factor has been extensively explained about the low permeability of the outer-membrane of these strains, which excludes a wide range of antimicrobial compounds (antibiotics, dyes and organic solvents) (Hancock, 1998).

Pseudomonas stutzeri is also known for its metabolic diversity; it is widely distributed in natural environments and frequently isolated from contaminated soil and wastewater (Lalucat et al., 2006). In a study that included more than 100 bacterial isolates from the intestinal tract of fish in Pakistan, the marine bacterium *P. stutzeri* isolated from the intestinal tract of ribbonfish (*Desmodema spp*.) exhibited antimicrobial activity against various bacterial species, including antibiotic resistant for *Staphylococcus aureus* (Lalucat et al., 2006). However, besides the ability to act as a producer of the antimicrobials, some microorganisms isolated from fish may be responsible for foodborne diseases. Al-Harbi and Uddin (2004) reported that the bacterial microbiota in hybrid tilapia intestines varied seasonally, and the species *Aeromonas hydrophila, Shewanella putrefaciens*, *Corynebacterium urealyticum*, *Escherichia coli* and *Vibrio cholerae* were the predominant bacterial isolates. Also isolated strains of opportunistic bacteria including *Flavobacterium* sp. *Pseudomonas fluorescens, Salmonella* sp., *Staphylococcus* sp. and *Streptococcus* sp., which have high importance among microorganism presents in intestinal microflora of fish, because under stress conditions, these bacteria may be food poisoning food agents (Al-Harbi and Uddin, 2004).

In addition to the seasonal factors that influence the bacterial microbiota, some storage conditions also alter bacterial colonization in fish. Lalitha and Surendran (2006) characterized the microorganisms in refrigerated shrimp, and during the first two weeks of storage, the microbiota was largely composed of the Gram-negative bacterial genera *Aeromonas, Shewanella, Moraxella* and *Pseudomonas* and the Gram-positive genera

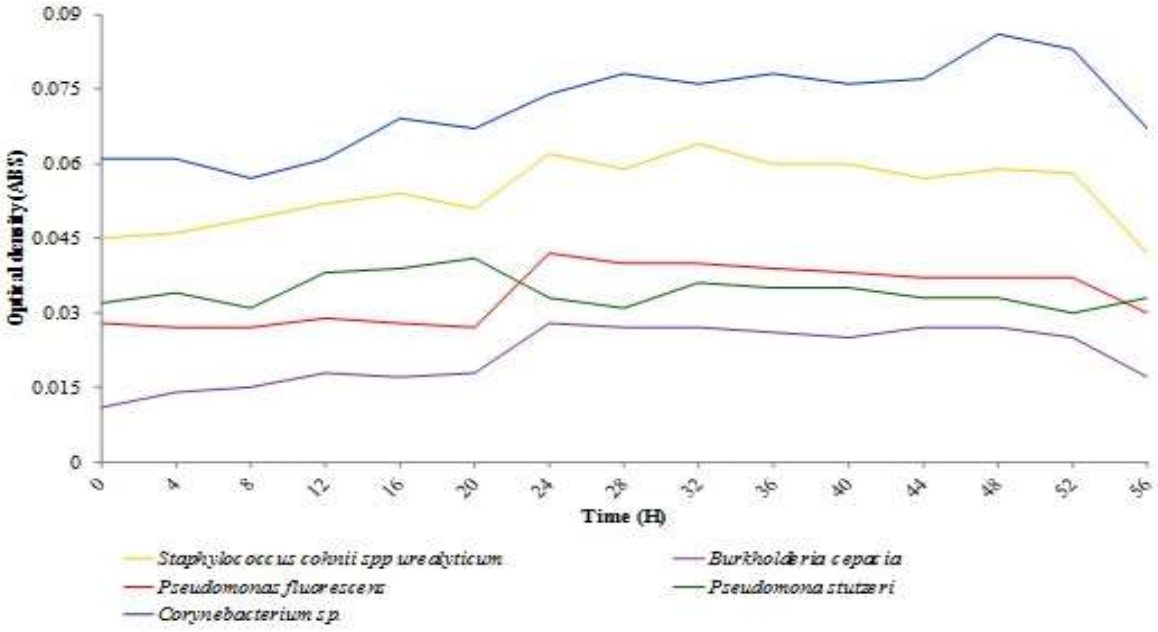

Figure 1. Growth of the intestinal microbiota of king weakfish at 7°C for 56 hours. *Specific wavelengths for each bacteria: *Staphylococcus cohnii* spp. *urealyticum* (618 nm), *Burkholderia cepacia* (583 nm), *Pseudomonas fluorescens* (640 nm), *Pseudomonas stutzeri* (580 nm), *Corynebacterium* sp. (593 nm).

Enterococcus, *Micrococcus* and *Corynebacterium*. At the end of storage, the predominant organisms were *Aeromonas, S*. *putrefaciens* and *Pseudomonas*. Oku and Amakoromo (2013) also isolated 12 bacterial strains in freshwater fish in Nigeria, and the predominant strains were identified as *B. subtilis, Corynebacterium, Lactobacillus, Pseudomonas* and *S. aureus.* The identification of spoilage or pathogenic bacteria in the microbiota of fish species during their shelf life and an understanding of how they cause spoilage is extremely important in preserving fish quality under different storage conditions (temperature) and maintaining commercial stability.

Temperature growth limits of the microbial isolates

Monitoring the microbial content in the internal organs of fish helps estimate how their food-related qualities will be affected when stored under appropriate conditions during their storage. Therefore, the growth of bacterial isolates from the king weakfish microflora was assessed at 7 and 3°C, which correspond to conventional storage temperatures in household refrigerators and are growth-limiting conditions for most pathogenic bacteria. Figure 1 illustrates the growth of king weakfish intestinal microbiota isolates at 7°C for 56 h. None of the five strains showed significantly different ($P > 0.05$) growth rates over time. These data indicate that over a storage period of more than two days at a temperature of 7°C, neither Gram-negative or Gram-positive bacteria isolated from the intestine showed a significant increase in growth.

At a temperature of 3°C (Figure 2), the spoilage bacteria *S. cohnii* subsp. *urealyticum* and the opportunistic pathogenic *B. cepacia*, showed significant differences between their growth over time ($P < 0.05$). The growth of these microorganisms can be attributed to their ability to better adapt to the storage in low temperatures in experiment cultivation medium.

Damasceno et al. (2015) assessed the growth of bacterial isolates at 10 and 15°C for six hours and found that all 16 of the strains identified that composed the microbiota of the muscle of the butterfly peacock bass (*Cichla ocellaris*) and the piraiba catfish (*Brachyplatystoma vaillantii*) could not grow under the studied conditions. The authors further noted a correlation between high-temperature habitats (fish from tropical regions) and microbiological quality, decreases pathogens ability and limits the spoilage processes when these fish are stored at temperatures below 10°C. This behavior was also observed for king weakfish, as the identified isolates showed slow growth because of characteristics: good microbiological quality of samples fish and difficult in growth growth of some bacterial present in the intestinal microflora.

Conclusion

The acceptable microbiological quality and according to the identification of intestinal microbiota, the king

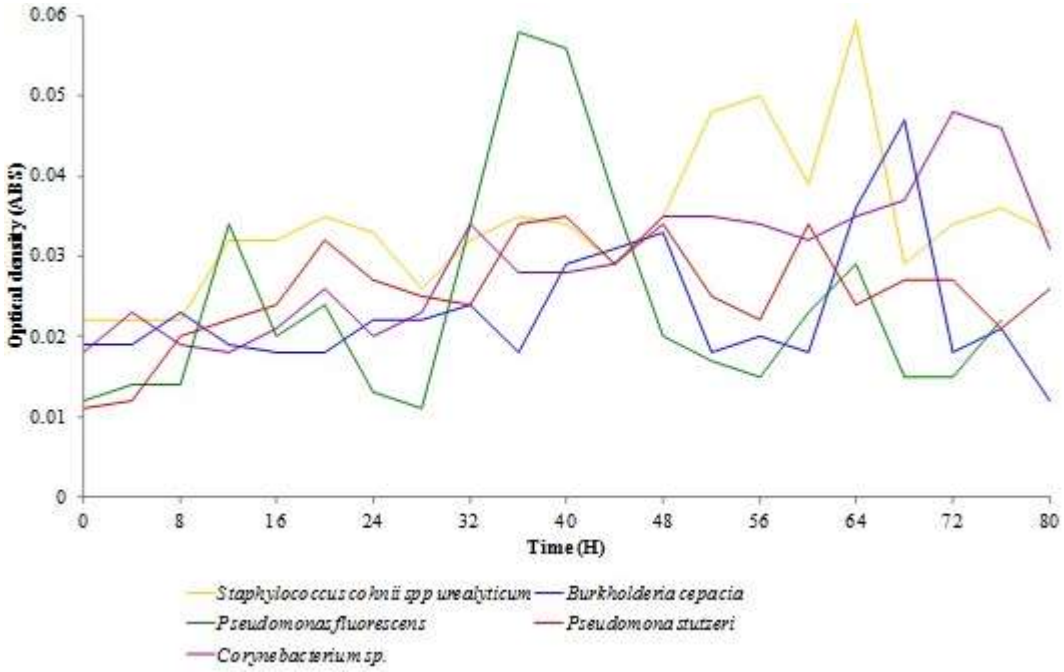

Figure 2. Growth of the intestinal microbiota of king weakfish at 3°C for 80 h. *Specific wavelengths for each bacteria: *Staphylococcus cohnii* spp. *urealyticum* (618 nm), *Burkholderia cepacia* (583 nm), *Pseudomonas fluorescens* (640 nm), *Pseudomonas stutzeri* (580 nm), *Corynebacterium* sp. (593 nm).

weakfish can extend its shelf-life when stored at low temperatures. This affirmative is based on low counts of mesophilic, psychrotrophic bacteria and thermotolerant coliforms and the five species identified in microflora can cause deterioration and/or may play a role of opportunistic pathogen, but that did not grow significantly at 7°C after 56 h. However, at 3°C, the bacteria *S. cohnii* subsp. *urealyticum* and opportunistic pathogens *B. cepacia* showed significant growth, implying in the food safety after three days if stored under these conditions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

To the National Council for Scientific and Technological Development (CNPq) and to PROPESP (Pró-reitoria de Pós-graduação da UFPA).

REFERENCES

Al-Bulushi IM, Poole SE, Barlow R, Deeth HC, Dykes GA (2010). Speciation of Gram-positive bacteria in fresh and ambient-stored sub-tropical marine fish. Int. J. Food Microbiol. 138:32-38.

Al-Harbi AH, Naim UM (2004). Seasonal variation in the intestinal bacterial flora of hybrid tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) cultured in earthen ponds in Saudi Arabia. Aquaculture 229:37-44.

Brazil (2001). Ministry of Health. Resolution RDC nº12, of january 2nd, 2001. Aprove the Technical Regulation on Microbiological Standards for Foods. Official Gazette of the Federative Republic of Brazil. Brasilia – DF.

Damasceno EIT, Pantoja LNG, Figueiredo HM, Silva LHM, Rodrigues AMC (2015). Microbiota of two species of commercially important fish in the Amazon region (Belém-Pará-Brazil): Butterflypeacock bass (*Cichia ocelarris*) and piramutaba (*Brachyplatystoma vailantii*). Afr. J. Microbiol. Res. 9(9):572-580.

Downes FP, Ito K (2001). *Compendium of methods for the microbiological*. Examinations of Foods. 4ª ed. Washington (DC): APHA.

Enoch DA, Birkett CI, Ludlam HA (2007). Non-fermentative gram-negative bacteria. Int. J. Antimicrob. Agents 3:S33-S41.

Gram L, Dalgaard P (2002). Fish spoilage bacteria- problems and solutions. Curr. Opin. Biotechnol. 13:262-266.

Gram L, Huss HH (1996). Microbiological spoilage of fish and fish products. Int. J. Food Microbiol. 33:121-137.

Hancock REW (1998). Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative Gram negative bacteria. Clin. Infect. Dis. 27(1):S93-S99.

ICMSF (International Comission on Microbiological Specifications for Foods) (1986). Microorganisms in Food. Sampling for microbiological analysis: Principles and specific applications. 2 ed. Londres: Blackwell Scientific Publications.

ICMSF (International Commission on Microbiological Specifications for Foods) (2005). Microorganisms in Foods 6: Microbial Ecology of Food Commodities, 2nd edition. Chapter 3. New York: Kluwer Academic/Plenum Publishers.

Jay JM (2005). Food of Microbiology. Brazil: Artmed. 6º ed. 712 p.

Lalitha KV, Surendran PK (2006). Microbiological changes in farm reared freshwater prawn (*Macrobrachium rosenbergii*) in ice. Food Control 17:802-807.

Lalucat J, Bennasar A, Bosch R, Garcia-Valdes E, Palleroni NJ (2006). Biology of *Pseudomonas stutzeri*. Microbiol. Mol. Biol. Rev. 70:510-547.

Miranda CD, Zemelman R (2002). Bacterial resistance to oxytetracycline in Chilean salmon farming. Aquaculture 212:31-47.

Mol S, Tosun Y (2011). The quality of fish from retail markets in Istanbul, Turkey. J. Fish. Sci. 5(1):16-25.
Nayak SK (2010). Role of gastrointestinal microbiota in fish. A Review. Aquac. Res. 41:1553-1573.
Oku I, Amakoromo ER (2013). Microflora of fresh and smoke-dried fish in Yenagoa metropolis, Nigeria. Afr. J. Microbiol. Res. 7(35):4451-4456.
Piette A, Verschraegen G (2009). Role of coagulase-negative staphylococci in human disease. Vet. Microbiol. 134:45-54.
Rodrigues MJ, Ho P, López-Caballero ME, Vaz-Pires P, Nunes ML (2003). Characterization and identification of microflora from soaked cod and respective salted raw materials. Food Microbiol. 20:471-481.
Santos S, Schneider H, Sampaio I (2003). Genetic differentiation of *Macrodon ancylodon* (Sciaenidae, Perciformes) populations in Atlantic coastal waters of South America as revealed by mtDNA analysis. Genet. Mol. Biol. 26(2):151-161.
Spencer RC (1995). The emergence of epidemic, multiple-antibiotic-resistant *Stenotrophomonas* (*Xanthomonas*) *maltophilia* and *Burkholderia* (*Pseudomonas*) *cepacia*. J. Hosp. Infect. 30:453-464.
Tammy L, Bannerman TL (1996). New *Staphlococcus* Species. Clin. Microbiol. Newsl. 18(10):73-76.
Thong TAN, Noseda B, Samapundo S, Nguyen BL, Broekaert K, Rasschaert G, Heyndrickx M (2013). Microbial ecology of Vietnamese Tra fish (*Pangasius hypophthalmus*). Int. J. Food Microbiol. 167:144-152.
Van den Broek MJM, Mossel DAA, Mol H (1984). Microbiological quality of retail fresh fish fillets in The Netherlands. Int. J. Food Microbiol. 1:53-61.
Zell C, Resch M, Rosenstein R, Albrecht T, Hertel C, Gotz F (2008). Characterization of toxin production of coagulase-negative staphylococci isolated from food and starter cultures. Int. J. Food Microbiol. 127:246-251.
Zhang W, Hu Y, Wang H, Sun L (2009). Identification and characterization of a virulence-associated form a pathogenic *Pseudomonas fluorescens* strain. Vet. Microbiol. 139:183-188.

African Journal of Microbiology Research

Related Journals Published by Academic Journals

- *African Journal of Biotechnology*
- *African Journal of Biochemistry Research*
- *Journal of Bacteriology Research*
- *Journal of Evolutionary Biology Research*
- *Journal of Yeast and Fungal Research*
- *Journal of Brewing and Distilling*

academicJournals