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Metagenomic analysis of *Fusarium oxysporum cv cubense*-infected soil in banana plantation, Sukabumi Indonesia

Yunus Effendi[1], A.Pambudi[1], A.Pancoro[2]

[1]Biology Department, Universitas Al Azhar Indonesia, Jakarta. Jalan Sisingamangaraja 12110 Jakarta - Indonesia

[2]School of Life Science and Technology, Bandung Institute of Technology - Indonesia

E-mail: effendiy@uai.ac.id

Abstract

Fusarium oxysporum (*Foc*) is a soil borne pathogen fungus that has been known as caused panama disease in many horticulture plants, especially banana. Currently, there is no effective method for solving panama disease in banana has been applied. Utilization of novel Rhizosphere microbes as bio-agent for preventing the dispersal of microbial pathogens is a method that recently has been developing in agriculture system of many countries. In this study, composition, richness and the abundance of soil microbes which is living in the rhizospheral area of banana plants were analyzed. Comparison of infected- and healthy soil with *Foc* was also performed for identifying key bacteria OUT which different in both soil condition. Using NGS of 16srRNA gene method, metagenomic analysis of soil microbes of those soils was performed. Data showed more than 9000 OTU of bacteria was identified. Of it, the *Foc*-infected soils showed a higher species abundance than healthy soil (ACE index 73,6 and 68,8 respectively). However, healthy soil has more taxa richness than infected-soil (Fisher index 447,7 and 343,4 respectively). It was confirmed with beta diversity analysis that showed infected-soils is has lower diversity in comparison with health soils. No statistically difference between both soil conditions. About 43 phyla have been identified and no significantly difference between both soil conditions. However, *Acidobacteria* (22%) and *Verrucomicrobia* (13%) were more abundance in the health soils in comparison in the infected soils 19% and 10% respectively, whereas *Proteobacteria* was found more abundance in the infected soil (11%) in comparison with the health soil (7%). High abundance of

Xantomonadaceae, member of Proteobacteria in the infected soils might contribute on Fusarium development in soils

Keywords: Rhizospere microbes, Metagenomic, Banana, *Fusarium oxysporum*

Introduction

Banana (*Musa* spp) is one of the most widely grown fruit along tropic and sub-tropic. Due to its high health benefit and nutrient contains, Banana is the most popular fruit which consumes many people and widely grown in the world. Its attractive flavor, high nutrition contains such as potassium, manganese, vitamin C, vitamin B, and high dietary fiber make banana one of most popular fruit in the world (Vazquezshy et al, 2012; Ellyn, 2011). Many countries especially Africa countries use banana as staple food instead of rice, maize, or wheat. The world banana production is in secondary rank of fruits production in the world after citrus (Ellyn, 2011). Indeed, banana has high economic value and currently its market in the world fruit trade is remaining growing especially in developing countries (Ellyn, 2011; FAOSTAT, 2014).

Banana is also a popular fruit and widely consumed in Indonesia. High demand on fruit market of Indonesia, making banana becomes as the most wide cultivated fruit in Indonesia. Mostly banana production is done by smalholders in almost area in Indonesia, although there are also present some Banana estates Sumatra and Java islands. Some area such as Lampung (Sumatra) and Sukabumi (East Java) has been known as center of banana production in Indonesia. About 74.751 hectare banana plantation has been reported in Indonesia and produce about 4.383.384 tons of bananas in 2003 (Anonimous, 2003). However, this number is remaining below target of national banana production. Pests and diseases contributed mainly in production decreasing and a limiting factor of banana production worldwide (FAO, 2015; Getha et al, 2002).

From many banana diseases have been identified worldwide, Fusarium wilt of banana which is known as Panama disease, is the most devastating banana disease in the world. This disease is caused by pathogenic soil-borne fungi, *Fusarium oxysporum*,

which is commonly colonizing in vascular tissues of Banana and prevent transportation of water and nutrient in the pseudosteam of infected banana. Thus, the plants are getting wilt which observed in yellowish leaves and later wilt totally on all leaves (Dita et al, 2010). *Fusarium oxysporum cv cubense* subgroup 'Tropical Race 4' (*Foc* TR4) is the most devastating race of *Foc* was recognized 1990s and has been identified as cause of serious losses of Cavendish banana in some areas of Southeast Asia (Ploetz and Churchill, 2011).

Currently, no effective methods have been applied for avoiding Foc spreading. Few methods have been reported applied in some areas, but mostly has less effective impact not only economically but also less environmentally safe (Lin et al, 2016). Timely eradication of Foc-infected banana and avoiding Foc-contaminated soil are a less methods which are applied in industrial banana plantation, although economically the methods are not applicable. A Foc-infected soil is difficult to be eradicated since Foc able to produce thick-cell wall chlamydospores, making it resistant to fungicides and fumigates (Shi et al, 1991). The spores of Foc can survive for long period under unsuitable soil condition for more than 30 years (Ploetz, 2006. Ghag et al, 2015) and beginning actively infect banana root during banana plantation (O'Donnell et al, 1998).

During its life, plant develops important processes that necessary for its life. One of important processes is interaction with rhizospere microbiome nearby plant root areas include bacteria, fungi, nematodes, protozoa, algae, and microarthrops (Raaijmakers et al, 2001). These microbiomes play important roles in ecological fitness of plants which interact with the microbiomes (Kent and Triplett, 2002). However, the microbiomes contribute not only a positive interaction with plants but also negative interaction such as causing disease. Plant-microbe interactions may thus be considered beneficial, neutral, or harmful to the plant, depending on the specific microorganisms and plants involved and on the prevailing environmental conditions (Bais et al., 2006). In general, diversity of microbiomes in the soil is an important factor that has significant impacts on plant growth and development. However, to what level specificity of microbiomes especially bacteria, will contribute to plant – microbes interactions is remain unclear. Most of bacteria in soils are unculturable (Nihorimbere et al, 2011), thus knowing their diversity

and their functional role in rhizosphere areas of plants is important information for understanding the role of bacteria and other microbes for plant growth.

In this study, analysis of soil bacterial diversity from banana plantation in Sukabumi, West Java – Indonesia was performed. Metagenomic analysis was performed by sequencing 16srRNA gene using NGS illumina platform. Comparison of diversity, richness and abundance of soil bacteria between Foc-infected soils and Foc-uninfected soils were done.

Materials and Methods

Sampling

Soil samples were collected from Banana plantation of PTPN VIII in Parakan Salak Sukabumi – West Java Indonesia. Four soils samples were collected from 2 different sites. Two samples were collected from Foc-uninfected soil, whereas two others samples were collected from Foc-infected soils. Each sample was consisted of 500 gr soil samples which collected from 3 different sites nearby rhizosphere areas of infected or uninfected banana plants. The soil mixes were then homogenized and took 100 gr. The soil samples were keep in -4^{o}C until used.

DNA extraction and molecular works

Whole genomic DNA was extracted using PowerSoil DNA kit (MoBio). DNA extraction was done following manual procedure of the kit. A 0.25 gr soil of each samples were used as source of the genomic DNA. All procedures were performed aseptically for avoiding contamination. The DNA results were quantified using NanoDrop and checked its quality using gel electrophoresis. The DNA was used as template for PCR. A 2µL of DNA was added to 10µL PCR mix (GoTaq ® Green Master mix - PROMEGA) and 1 nmol of each forward and reverse 16srRNA primer. Reaction was performed 35 cycles which consisted of 30 sec at 94^{o}C and continued with 57^{o}C of annealing for 20 sec, followed with 2 min 72^{o}C for elongation.

A region V4 of 16srRNA gene was amplified with primer F515 (5'-GTGCCAGCMGCCGCGGTAA-'3) and 907R (5'-CCGTCAATTCMTTTRAGTTT-'3) (Lane et al, 1991). The PCR products were purified and subjected for automated Illumina Miseq platform (1st BASE – Malaysia) after the PCR products were normalized in equimolar amounts.

Sequences analysis

Raw sequence data generated from Illumina Miseq platform were processed in QIIME Ver 6.0 (Caporaso et al., 2010). All sequences are shorter than 150 bp or longer than 600 bp are removed from downstream processing. Read were then aligned with 16srRNA SILVA database (www.arb-silva.de) and GRD database (metasystems.riken.jp/grd/), then followed inspected for chimeric errors. "Species-level" of OUT was used in analysis, thus reads then were clustered at 97% similarity into OTUs. In this step, rare OTUs with only 1 (singleton) or 2 reads (doubleton) are deleted from downstream processing. Taxonomic assignment was carried out with the RDP Classifier (Wang et al., 2007).

Data analysis

Alpha and Beta diversity analysis were performed using Explicet ver 2.10.5 software (Robetson et al, 2013). Statistical comparison of alpha diversity between samples was carried out with Excel (Microsoft) whereas statistically different (*P-value*) was calculated with T-test. Sample-specific OTUs (showing significantly different relative abundances between samples) were assessed by T-test. Heat map which showed relative abundances between samples in certain taxa level was generated with Explicet ver 2.10.5 software. Venn diagrams were made to visualize which OTUs were shared between infected and health soils using Explicet ver 2.10.5 software.

Result and discussion

Structure and diversity of soil bacteria

A total of 37,909,152 reads was obtained using Illumina Miseq sequencing of 16srRNA gene. Of it total of 35,149,668 reads (87.5%) passed filter. After primer removal and

length- and quality filtering, followed with removal of singleton as well as doubletons, about 20.000 reads for each samples were obtained. These reads have passed quality filtering control (mean of read length and GC%). “Species-level” of OUT was used in analysis, thus reads were clustered at 97% similarity into OTUs. About 9.000 – 11.000 OTUs were identified for each sample (Table 1).

Bacterial community analyses showed that the infected soils had relative higher species richness and had more species abundance than the health soils. Nevertheless, there was no significant different in species richness between the infected soils and the health soils. Alpha diversity analysis summarized that species diversity (richness) of health soils was higher than infected soils which showed on higher Simpson diversity index (7.5-7.7) and Fisher alpha indexes (379.4-516.1). The richness index of the Chao1 estimator (Chao1) (Chao, 1984) and the abundance-based Coverage estimator (ACE) (Eckburg et al., 2005) was calculated to estimate the number of observed OTUs that were present in the sampling assemblage. The diversity within each individual sample was estimated using the nonparametric Shannon diversity index (Washington, 1984)

Table 1. Number of sequences and OTUs after filtering

Group	**Screen < 150 bp and > 600 bp**	**Chimera, Singleton and doubletons removal**	**Number of OTUs**
Health soil1	244147	118229	11364
Health soil2	213990	105205	9630
Infected soil1	205317	91449	10575
Infected soil2	247097	109523	11880

The ACE estimator indicated that species abundance was observed relative higher in the infected soils than in the health soils, even Chao1 index indicated only a slight difference of species abundance between these soils (Tabel 3).

Tabel 3. Diversity and richness indexes

Group	Chao1	ACE	Shannon	Simpson	Fisher
Infected soils1	9956	79.33211	48.0574	7.798442	504.8816
Infected soils2	8278	67.98454	42.94576	7.239521	181.9179
Health soils1	9300	63.20111	43.50184	7.773869	516.0863
Health soils2	9469	74.49042	46.99294	7.540323	379.3965

Metagenomic analysis showed that bacteria dominate the diversity of microbiome in the soil samples (99%) of both soil conditions. Archaea has only 0.04% and 0.012% in health soil and infected soil respectively. A total 73 phyla were identified in the soil samples, however the abundance of these phyla are statistically not different (Table 2).

Tabel 2. Abundance of taxonomic phyla groups health and infected soils

Phylum	**Health soil**	**Infected soil**	***p-value***
Archaea			
Crenarchaeota	0.00033±0.00006	0.00017±0.00004	0.27
Euryarchaeota	0.00003±0.00002	0.00026±0.00020	0.07
Parvarchaeota.	0.00005±0.00005	0.00000±0.00000	0.26
Bacteria			
AD3	0.05517±0.02247	0.08320±0.01472	0.41
Acidobacteria	0.20938±0.01099	0.17915±0.00467	0.06
Actinobacteria	0.02296±0.00359	0.02922±0.00246	0.31
Armatimonadetes	0.00410±0.00054	0.00356±0.00041	0.42
BHI80.139	0.00010±0.00003	0.00009±0.00002	0.17
BRC1	0.00025±0.00025	0.00020±0.00020	0.56
Bacteroidetes	0.00789±0.00019	0.00232±0.00192	0.07

Chlamydiae	0.00094±0.00047	0.00102±0.00053	0.33
Chlorobi	0.00012±0.00001	0.00056±0.00044	0.11
Chloroflexi	0.21542±0.02283	0.20454±0.01260	0.46
Cyanobacteria	0.00129±0.00004	0.00194±0.00054	0.11
Elusimicrobia	0.00197±0.00028	0.00144±0.00011	0.25
FBP	0.00000±0.00000	0.00001±0.00001	0.21
FCPU426	0.00282±0.00067	0.00212±0.00015	0.51
Fibrobacteres	0.00014±0.00004	0.00042±0.00031	0.23
Firmicutes	0.00054±0.00032	0.00054±0.00012	0.24
GAL15	0.00716±0.00309	0.01918±0.00502	0.09
GN02	0.00001±0.00001	0.00001±0.00001	0.22
Gemmatimonadetes	0.00942±0.00192	0.00976±0.00069	0.31
Kazan.3B.28	0.00004±0.00004	0.00000 0.00000	0.34
NC10	0.00000±0.00000	0.00003±0.00003	0.44
NKB19	0.00124 ±0.00123	0.00058 ±0.00058	0.43
Nitrospirae	0.01218±0.00175	0.01494±0.00217	0.12
OD1	0.00004±0.00004	0.00000±0.00000	0.24
OP11	0.00000±0.00000	0.00003±0.00003	0.32
OP3	0.00124±0.00123	0.00058±0.00058	0.09
Planctomycetes	0.01218±0.00175	0.01494±0.00217	0.33
Proteobacteria	0.02411±0.00601	0.02245±0.00378	0.15
Synergistetes	0.00000±0.00000	0.00002±0.00002	0.23
TM6	0.00118±0.00092	0.00044±0.00025	0.15
TM7	0.16714±0.01058	0.00820±0.00639	0.37
Verrucomicrobia	0.06428±0.00510	0.10006±0.02639	0.31
WPS.2	0.00004±0.00004	0.00012±0.00009	0.25
WS3	0.00292±0.00269	0.00007±0.00002	0.24
Thermi	0.00294±0.00049	0.00820±0.00639	0.16

Analysis of taxonomic abundance of species between the health soils and the infected soils showed varied diversity within samples. The abundance of major bacterial phylum was observed less different (Figure 1). From 11 major bacteria phylum which was compared, there was no significant difference between health soils and infected soils. However, some phyla were found present more abundance in one of the samples.

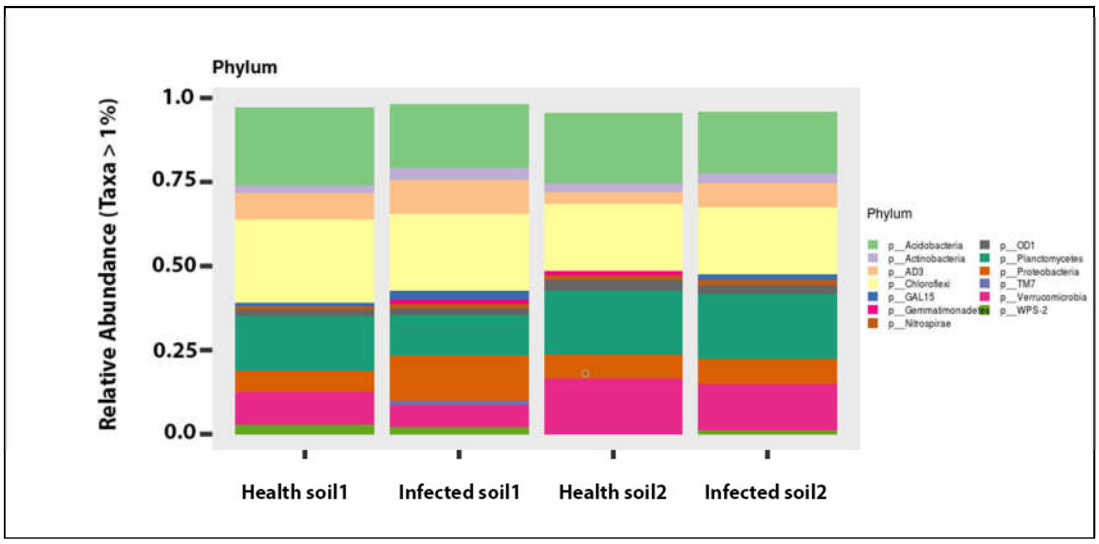

Figure 1. Relative abundance of phylum taxa.

Relationship between soil conditions and microbial abundances

Acidobacteria (22%) (*P<0.06*) and *Verrucomicrobia* (13%) (*P<0.31*) were more abundance in the health soils in comparison in the infected soils 19% and 10% respectively, whereas *Proteobacteria* was found more in the infected soil (11%) (*P<15*) in comparison with the health soil (7%) (Figure 2).

Nevertheless, there were no significant differences in the relative abundances of these dominant species in both soil conditions. Rarefaction curve confirmed this data that number of OTUs is varied abundance in range from 9360 – 11880 OTUs in the soil samples (Figure 3). *Proteobacteria* was found higher in infected soil than in health soils. The more abundance of *Proteobacteria* in infected soils in this study was suggested as one of important factors which composed a suitable environment for pathogenic microbes growing. Sanguin et al (2009) reported *Proteobacteria* abundance was negatively correlated with disease suppression.

The relative high abundance of *Acidobacteria* in both soils in our study might correspond with acid soil condition (pH 4.9 – 5.1). In addition, the data showed that the *Acidobacteria* was found relative higher (22%) in the health soils than in the infected soil (19%). Whether it contributed to incidence of higher number of Foc in the soils or not, it was a still a hypothesis. However, several study of application of a bio-organic fertilizer (BIO) to various orchard with serious Fusarium wilt disease have been reported effectively enhancing suppression of Fusarium wilt disease by ameliorating structure of the microbial community (Kavino et al, 2010; Shen et al, 2013; Cotxarrera et al, 2002; Zhao et al, 2011). Shen et al (2014) showed that BIO-treated soil has higher abundances of *Gemmatimonadetes* and *Acidobacteria*, while *Bacteroidetes* were found in lower abundance.

In addition, out of top 10 phyla taxa in this study (Figure 2), *Bacteroidetes* phylum showed a slightly difference in abundance between in health soils and infected soils (*P-value* > 0.07) (Tabel 2). The *Bacteoroidetes* phylum has been reported that its abundance is associated with Fusarium wilt incidence (Shen et al., 2014). In a high abundance, the *Bacteroidetes* has positive corresponding to initial and disease stage of Fusarium wilt disease incidence whereas decreasing of the Fusarium disease incidence was significantly shown when suppressiveness of this phyla was reached (Shen et al., 2014; Kyselková et al., 2009).

The composition of the soil microbial community and induced changes caused by its amendment, provide useful information on soil health and quality (Poulsen et al, 2013). Maintaining biodiversity of soil microbes is crucial to soil health because a decrease in soil microbial diversity is responsible for the development of soil-borne diseases (Mazzola, 2004). Determining the responses of soil bacterial communities to different organic amendments is particularly important because the bacterial community is one of the main components that determine soil health and is believed to be one of the main drivers in disease suppression (Garbeva et al., 2004)

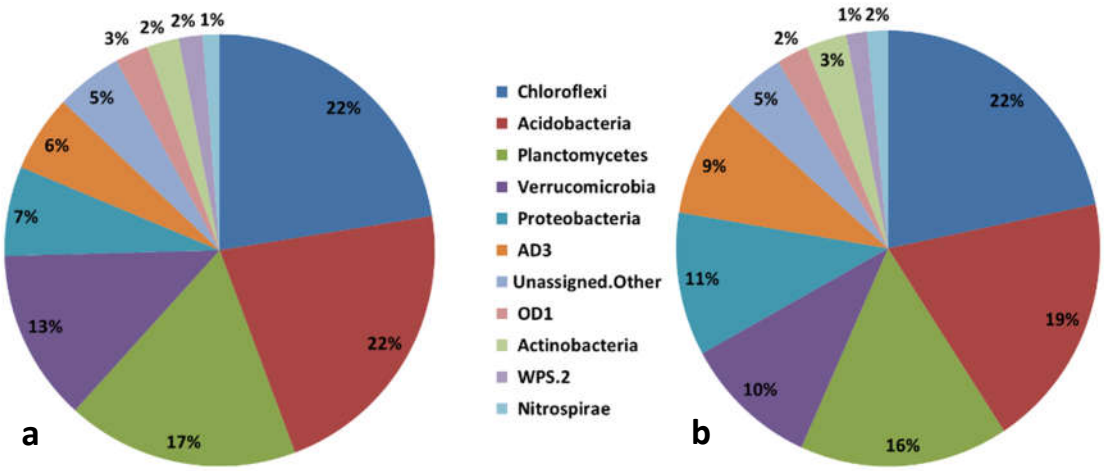

Figure 2. Relative abundance of major phyla taxa of both soil conditions. a) Relative abundance of phyla in health soils indicated that *Acidobacteria* and *Verrucomicrobia* were higher than in infected soil, b) Relative abundance of phyla in infected soils, *Proteobacteria* has more abundance in comparison to health soil.

Naturally, suppressive soil condition on certain pathogenic microbes relies on at least two important factors, first a general mechanism of competition for nutrients caused by the whole soil microflora and the second a specific competition between pathogenic and non-pathogenic microbes strains. Composition and diversity of microflora at the end will determine whether certain pathogenic microbes dominate the soil or not.

The abundance of Proteobacteria members on healthiness banana plants has been reported in several studies (Köberl et al., 2017; Shen et al., 201; Shen 2014). Comparative microbiome analyses between healthy and diseased Gros Michel plants on Fusarium Wilt-infested farms in Nicaragua and Costa Rica revealed significant shifts in the gammaproteobacterial microbiome (Köberl et al., 2017). The Author found that Gammaproteobacterial diversity and community members were identified as potential health indicators. Indeed, increasing of plant-beneficial *Pseudomonas* and *Stenotrophomonas* correlated positively with healthy plants (Köberl et al., 2017).

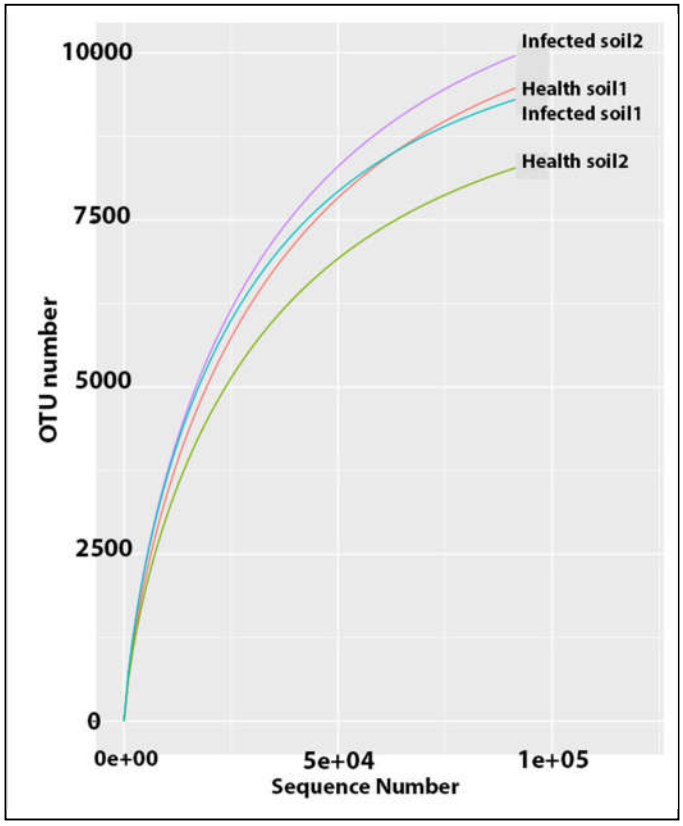

Figure 3. Rarefaction curve of all soil samples. Data showed number of OTUs abundance of each soil condition.

In contrast with study, the abundance *Xantomonadaceae* one of *Proteobacteria* phyla member was found relative higher in infected soils than in health soils (Figure 4). The family of *Xantomonadaceae* has been known well as one of the pathogenic family which caused Banana Xanthomonas Wilt (BXW) (Biruma et al, 2007). However, Köberl et al., 2017 reported that *Xantomonadaceae* presented in higher number in healthy plants. In other study by Sanguin et al (2009) *Proteobacteria* abundance was negatively correlated with *Fusarium* disease suppression thus confirming that the outbreak stage of wheat take-all disease is mainly attributed to the prevalence of Proteobacteria.

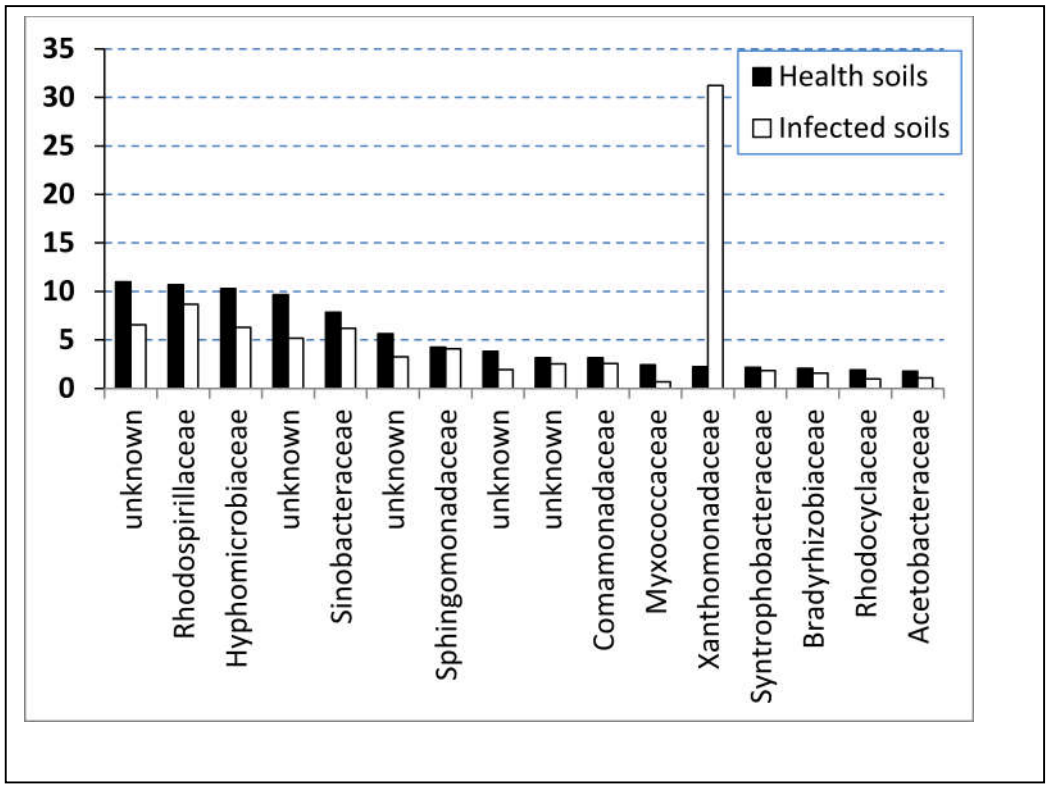

Figure 4. Top 16 families belong Proteobacteria which identified in healths and infected soils. Xanthomonadaceae showed more abundance in infected soils than in health soils (*P-value* > 0.07)

It seems that Proteobacteria might present as positive or negative factors on the development of pathogenic bacterial, depend on which specific bacterial taxa dominate (Köberl et al., 2017; Biruma et al, 2007; Shen et al, 2015).

In conclusion, the result from the present study demonstrated that composition, diversity and richness of microbiome in rhizosperal areas of banana plants in banana plantation Sukabumi might correspond with the incidence of Fusarium development in the rhizosfere soils. The more abundances of bacteria belongs of *Acidobacteria* and *Verrucomicrobia* phyla might associated with the healthiness of the soils, whereas higher abundances of *Proteobacteria*, particularly *Xanthomonadaceae* family might contributed positively to Fusarium development in the soils.

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