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Microbial communities and key strains associated to banana spoilages through cultural plating and metagenomic analysis

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Abstract - This research investigated microbial communities associated to bananas spoilage by cultural plating and metagenomic analysis. Bananas were collected from three different sources including exported farm, domestic organic farm and marketed bananas. Bacteria and fungi on fresh through spoiled banana surfaces were determined. It was found that microbial communities observed from both methods were significantly different. Microbial communities on fresh and spoiled bananas observed from each method were relatively different. From cultural plating, bacteria observed on almost fresh bananas and spoilage, from export, domestic farms and wholesale fruits market, was *Enterobacter ludwigii*. Fungus, *Penicillium citrinum* was mainly observed from all fresh and spoiled bananas from marketed fruits. *Aspergillus inflatus* was mainly observed on spoiled bananas collected from export farm but was not found in domestic farm and marketed fruits. From metagenomics analysis, the most abundant bacteria on fresh and spoiled banana surface was *Enterobacteriacae* and *Pantoea*, respctively. *Moesziomyces* made up the largest division in both fresh and spoiled bananas. Information obtained also demonstrated that geographical factors had less impact on microbial flora associated to fruit spoilage. There was no specific microbial community pattern observed on banana collected from farms. Similar microbial profiles associated to the spoilage of banana collected form market were observed. Thus, data obtained could reinforce the importance of analytical methods for studies on the microbial ecology of banana and highlight the need for more detailed examination of this variable and its importance in post-harvest management.

Keywords: Cultural plating, banana spoilages, metagenomic analysis

1. Introduction

Bananas, major exported fruit of Thailand. From 2017 to 2021, Thailand's banana export value climbed by 1,331% and was worth up to 400 billion baht. (Office of Agricultural Economics, 2021). Microbial spoilage and pathogen in fruits cause significant economic loss throughout the fruit distribution chain. Therefore, the concern in fruit industry is to reduce such microorganism contaminations. Banana is a climacteric fruit and once ripening is initiated, it is irreversible and proceeds very rapidly, making the fruit highly perishable. The spoilage in this fruit is caused by bacteria, yeasts and molds and can be contaminated by these species at any stage after harvest (Yumbya *et al.*, 2021). Banana fruits may become contaminated by microbes as a result of mechanical damage or skin penetration. Fruits that have been spoiled by bacteria or fungi may be less appealing, have a lower market value, and when consumed, may have negative effects. There are some reports demonstrating bacteria; *Pediococcus* sp., *Propionibacterium* sp. and *Pseudomonas aeruginosa*), and fungal; *Botryodiplodia theobromae*, *Aspergillus niger* and *Rhizopus* sp.) were key microbes associated to banana spoilage (Okonkvvo *et al.*, 1990; Oyewole, 2012). Importantly, the study of microbial ecosystems on fruit surfaces could help better understand microbes and their roles associated to fruit spoilage. Methodology for the isolation and identification of microbes from fruit surfaces has followed conventional, cultural procedures, and inherent weaknesses in this approach have probably limited progress in understanding this ecology. Metagenomic analysis offers an alternative, culture-independent strategy to microbiological analysis (Forbes *et al.*, 2017), and is finding increasing application to food including agricultural products. In addition to detecting species normally found by cultural methods, it offers the prospect of detecting species that may be present in the habitat at viable but non-culturable states (Trevors, 2011).

This research aimed to investigate key factors such as geographical and seasonal influencing on microbial communities and key microbes associated to banana spoilage. Works included banana farm visits, collection of banana samples from the farms and marketed fruit samples. Microorganisms and important native flora associated to fresh through fruit spoilages were investigated by cultural plating and metagenomic analysis.

2. Materials and methods

2.1 Farm visit and fruit sample collection

Bananas (*Musa acuminata*, cultivar 'Hom Thong') were collected from the front and center of domestic organic farms located in Pathum Thani and export farms in Phetcha Buri. Marketed banana was bought from the wholesale market.

2.2 Screening for native microflora and key spoilage microbes on banana surface

2.2.1 Cultural plating

Fresh healthy bananas were collected, stored in sealed plastic bags and kept at room temperature. Microorganisms including bacteria and yeast mold were determined on storage banana surface every week until spoilage surfaces was observed. Microflora

on the surfaces of bananas was retrieved by using sterile cotton swabs soaked in a solution containing 0.15 M NaCl and 0.1% Tween 20 as described by Paulino *et al.* (2006) with modification. The head of 3 swabs was aseptically cut from the handle, placed into a microcentrifuge tube containing 500 µl of ST solution, centrifuged for 5 min, and then removed. Each 100 µl of this solution was spread on Nutrient agar (NA) and Dichloran-rose bengal chloramphenicol (DRBC) agar, respectively. (To *et al.*, 2021) The remaining volume was stored at -80 °C until DNA extraction.

Fresh healthy bananas were stored in the same condition until spoilage was observed (dark spot, pitting, juice leakage, etc) then surface microbes were retrieved in the same manner as fresh fruit. The swab solution was used for plate spreading and metagenomic DNA extraction. The Harrison disc method (Harrigan & McCance, 1976) was used to select the representative microbe on each plate. Colonies from each plate was selected for further identification, making it possible to calculate the percentage distribution of the various organisms present in the sample.

2.2.2 Strain idenfication

The colony isolates were identified by DNA sequencing analysis. DNA was extracted from pellets of bacterial cells using DNA extraction kit (Zymo Quick-DNA Fungal Bacterial Isolation Kits) The nucleic acid sequences were chosen from the conserved regions of the 16S rRNA/DNA V3 region and PCR was performed using primer set 338F/518R for bacteria and 26S D1/D2 rRNA/DNA with primer NL1 and LS2 for mold under the conditions. The PCR mixture volume was approximately 50 µl containing 1X Vi buffer, 0.1 mM dNTP, 0.1 mM each of primers, 2 U Taq DNA polymerase, 10-50 ng template DNA. The PCR for bacteria was accomplished using PCR machine with an initial step of 94°C for 2 min, followed by 35 cycles of 94, 55 and 72°C for 30 sec each step, and final step at 72°C for 7 min. The PCR for yeast mold was accomplished using PCR machine with an initial step of 95°C for 5 min, followed by 30 cycles of 95, 52 and 72°C for 1, 2 and 2 mins, and final step at 72°C for 7 min to ensure that the PCR product extension was completed.

PCR products were determined by running gel electrophoresis using agarose gel 1.5% under 100V for 30 minutes. The PCR amplicon was sent to commercial sequencing facility (Macrogen, Korea) after cleaning. The sequencing data was analyzed with nucleotide BLAST program of NCBI.

2.2.3 Metagenomics

Metagenomic DNA extraction were carried out using DNeasy PowerSoil Kit (Qiagen, Germany). 16S rRNA gene was amplified using 341F and 805R primers, targeting V3-V4 variable regions and using ITS primers, targeting ITS1-2 variable regions. Cluster generation and 250-bp paired-end read sequencing were performed on an Illumina MiSeq at Omics Sciences and Bioinformatics Center (Chulalongkorn University, Bangkok, Thailand).

2.3 Statistical analysis

Analysis of variance (ANOVA) and multiple comparisons by Turkey's test were performed using IBM-SPSS statistics package

version 22 (SPSS Inc., Chicago, IL, USA). A probability at $p < 0.05$ was considered statistically significant.

3. Results

3.1 Microorganism community and key microbes associated to banana surfaces and spoilage

3.1.1 Cultural plating

Bacteria and fungi associated to banana surfaces collected from different conditions were investigated. The data as shown in Table 1 and 2, it was found that diverse bacteria and fungi were observed as predominant native flora on fresh banana surfaces. The bacterial and fungi profiles as found on all fresh banana surfaces were relatively similar. Filamentous green mold as predominant was observed in all samples. Yellow bacteria were frequently observed on bananas. Bananas were spoiled by microorganisms ranging from 7 to 14 days. Spoilage of banana mainly began from the fruit stalk and migrated to a whole fruit observed as large dark spot on the skin with mushy flesh and musty smell. From this investigation, some key microbes associated to spoilage of bananas were observed and subjected to identification.

From identification results based on DNA sequencing analysis as shown in Table 2. Normal flora found on bananas was relatively similar and also predominant species associated with the spoilage of bananas were relatively similar. Bacteria observed on almost fresh bananas and spoilage, from export, domestic farms and wholesale fruits market, was *Enterobacter ludwigii*. Fungus, *Penicillium citrinum* was mainly observed from all fresh and spoiled bananas from marketed fruits. *Aspergillus inflatus* was mainly observed on spoiled bananas collected from export farm but was not found in domestic farm and marketed fruits.

Based on microbial profiles found from cultural plating with statistically collected as summarized in Table 2 could be the key microbes associated to banana spoilage. To intensively investigate the role of spoilage associated strains, metagenomic analysis of total DNA directly extracted from fruit surfaces was conducted to compare to the results obtained from these cultural plating methods.

Table 1. Spoilage time and characteristics of bananas from 3 different sources

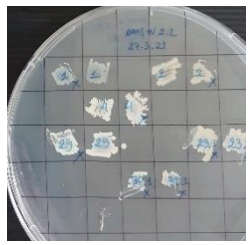
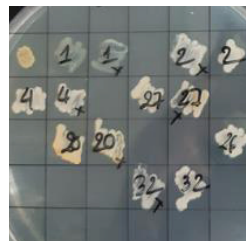
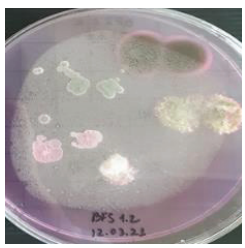
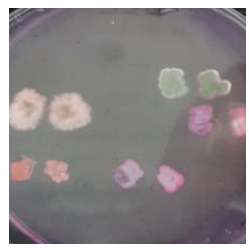
Location		Domestic organic farms in Pathum Thani	Export farms in Phetcha Buri	Wholesale fruits market
Spoilage time		**2 weeks**	**2 weeks**	**1 week**
Spoilage characteristic		Mold on bunch, dark spot on skin, mushy flesh, musty smell	Dark spot on skin, mushy flesh, musty smell	Mold on bunch, dark spot on skin, mushy flesh, musty smell
				
Main spoilage microbes	Bacteria			
	Yeast & Mold			

Table 2. Microorganisms isolated of fresh and spoiled banana from 3 different sources

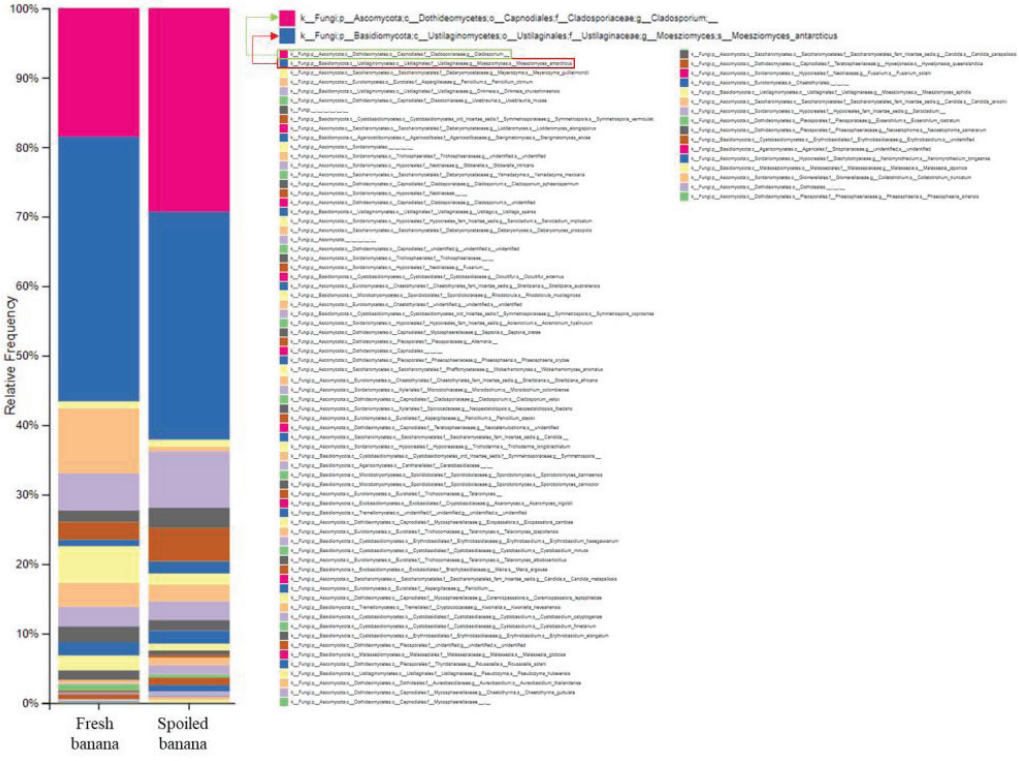
Source of Bananas	* Microorganisms isolated from fresh sample		** Microorganisms isolated from spoilage (%)	
	Bacteria	Yeast & Mold	Bacteria	Yeast & Mold
Domestic organic farms in Pathum Thani	*Sphingobacterium* sp., *Microbacterium* sp., *Enterobacter ludwigii* and *Staphylococcus cohnii*,	*Meyerozyma caribbica*, *Aspergillus oryzae*, *A. novoparasiticus*, *Penicillium citrinum* and *Fusarium solani*	*Enterobacter ludwigii* (30%), unknow2 (30%) and *Sphingobacterium* sp. (30%)	*Penicillium citrinum* (61.7%), *Aspergillus oryzae* (35%) and *A. novoparasiticus* (15.6%)
Export farms in Phetcha Buri			*Staphylococcus cohnii* (77.55%), unknow26 (9.5%) and *Microbacterium* sp. (7.4%)	*Penicillium citrinum* (84.6%) and *Aspergillus inflatus* (15.4%)
Wholesale market			*Enterobacter ludwigii* (50%), unknow2 (45%) and unknow20 (1.16%)	*Aspergillus oryzae* (91.7%), *Penicillium citrinum* (3.3%) and *Meyerozyma caribbica* (5%)

* Microorganism isolated from fresh samples were relatively similar and equally distributed
** Percentage of each morphology in total microflora presents in each sample

3.1.2 Metagenomics

Metagenomics is the analysis of total microbial genetic material directly recovered from environmental samples, which enables the analysis of genomes of at least most abundant microbial species without the need to isolate and cultivate individual microbial species (Handelsman *et al.*, 1998). In this study, the DNA extracted from mixed microflora on banana surfaces were subjected to metagenomic analysis to see the whole picture of microbial community might associated to banana spoilage. The results as shown in Figure 1a and Figure 1b. The microbial profiles on each type of fruits predicted from metagenomic analysis were more diverse with no specific pattern and relatively different from cultural plating. The most abundant bacteria on fresh and spoiled banana surface was *Enterobacteriacae* and *Pantoea*, respctively. *Moesziomyces* made up the largest division in both fresh and spoiled bananas.

(a)



(b)

Figure 1. Bacterial profile (a) and fungi profile (b) of fresh and spoiled banana

3.2 Comparison of microbial profile on cultural plating and metagenomic analysis

Microbial community profiles observed from cultural plating and metagenomics were significantly different as shown in Table 3. By using the cultural plating approach, the microbes in a fresh banana were discovered to be evenly distributed, while the metagenomics method revealed different microbial abundances. In addition, the main microbes associated to fresh bananas were also different. *Sphingobacterium* sp., *Microbacterium* sp., *Staphylococcus cohnii* and *Enterobacter ludwigii* were discovered through cultural plating while *Enterobacteriaceae*, *Pantoea*, and *Klebsiella* were observed through metagenomics. The microbiological profiles of spoiled bananas clearly showed the difference between these 2 analysis methods. Cultural plating revealed that *Staphylococcus cohnii* and *Penicillium citrinum* mostly detected whereas metagenomics displayed that *Pantoea* and *Moesziomyces* were major microbes in spoiled banana.

Table 3. Comparison of microbial profile on 2 analytical methods

Analytical methods	**Microorganisms isolated from fresh sample		**Microorganisms isolated from spoilage	
	Bacteria	Yeast & Mold	Bacteria	Yeast & Mold
Cultural plating (Phetcha Buri) *(%)	*Sphingobacterium* sp., *Microbacterium* sp., *Staphylococcus cohnii* and *Enterobacter ludwigii*	*Meyerozyma caribbica*, *Aspergillus oryzae*, *A. novoparasiticus*, *Penicillium citrinum* and *Fusarium solani*	*Staphylococcus cohnii* (77.55%), and *Microbacterium* sp. (7.4%)	*Penicillium citrinum* (84.6%) and *Aspergillus inflatus* (15.4%)
Metagenomics ***(%)	*Enterobacteriaceae* (21.95%), *Pantoea* (16.4%) and *Klebsiella* (7.66%)	*Moesziomyces* (32.87%), *Cladosporium* (30.85%) and *Dirkmeia* (8.11%)	*Pantoea* (27.55%), *Corynebacterium* (12.72%) and *Curtobacterium* (8.58%)	*Moesziomyces* (38.08%), *Cladosporium* (20.78%) and *Penicillium* (10.13%)

* P ercentage of each morphology in total microflora present in each sample
** Only a few illustrative numbers, information was shown to briefly give an idea how data are analyzed. Microorganism isolated from fresh sample were relatively similar and equally distributed.
*** Percentage of abundance according to taxonomic classifications

4. Discussion and conclusion

Different microorganism profiles were identified using two different analytical techniques. Using the cultural plating method, it was discovered that fresh bananas included bacteria; *Sphingobacterium* sp., *Microbacterium* sp., *Enterobacter ludwigii*, *Staphylococcus cohnii*, *Meyerozyma caribbica*, fungal; *Aspergillus oryzae*, *A. novoparasiticus*, *Penicillium citrinum* and *Fusarium solani* while spoiled bananas had bacteria; *Staphylococcus cohnii*, *Microbacterium* sp., fungal; *Penicillium citrinum* and *Aspergillus inflatus*. On the other hand, the metagenomics method

indicated that bacteria; *Enterobacteriaceae*, *Pantoea*, *Klebsiella, Moesziomyces*, fungal; *Cladosporium* and *Dirkmeia* were the most common microorganisms in fresh bananas while bacteria; *Pantoea*, *Corynebacterium*, *Curtobacterium*, *Moesziomyces*, fungal; *Cladosporium* and *Penicillium* were found in spoiled bananas. The microbial profiles from both analyses were also different from the previous reports of Okonkvvo *et al.* (1990) and Oyewole (2012). The role of these microorganisms associated to fruit spoilage has been already well described.

Penicillium was reported as fungal genera frequently occurring during post-harvest stages of fruits and vegetables. *Penicillium* rots in fruits mainly included green mold and blue mold spoilage. They produced many hydrolytic enzymes to pierce into host tissue, directly penetrated fruits through the wound or injuries and present on the fruit surface and cause cross-contamination. Upon invasion, these fungi utilized host nutrients for spore germination, growth, and metabolism (Dukare *et al.*, 2019). *Aspergillus* was demonstrated to grow on the outer layer of damaged fruits, causing black mold, heart rot development on certain fruits and vegetables and producing mycotoxins. The mold grew optimally at pH 3.0. Citric and malic acids, at concentration present in the unripe fruits could enhance its growth (Palejwala *et al.*, 1987). *Aspergillus* was reported as main fungi found on mangoes, oranges, apples, guava, grapes, watermelons and papaya.

For bacteria, *Bacillus*, some species such as *B. pumilus* has been reported to cause disease in a variety of plants. It was reported to cause bacterial fruit rot on muskmelon and ficus lacor (Song *et al.*, 2017; Hakim *et al.* 2015). *Bacillus subtilis*, *Bacillus coagulans*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus polymyxa*, and *Bacillus macerans* might responsible for spoilage in less acid fruit pulps such as tomato, pear, peach, mango, mandarin, and orange with pH between 3.7 and 4.5. *Sphingobacterium* sp., Gram-stain-negative, strictly aerobic, rod-shaped, non-motile, non-spore-forming, originates from soil, plants, water, food. Information regarding to fruit spoilage caused by this genera has not been found. *Enterobacter* was pathogenic potential, causing many outbreaks relating to consumption of fresh produce. It was reported that normal flora of many fruits and vegetables contained high counts of this group. *Enterobacter cloacae* was reported to be the main pathogenic agents of yellowish to brownish soft and watery symptoms on infected stem and fruit in dragon fruit (Masyahit *et al.*, 2009). *Microbacterium*, Gram-positive motile short rod, facultatively aerobic was reported as bacteria that dominated at the early stage of fruit development. Information regarding to fruit spoilage caused by this genera has not been found.

Form the reports about yeasts, *Meyerozyma* yeast species could be isolated from a wide number of environmental sources. They were predominant in spoiled blueberry juice. *Meyerozyma caribbica* was one of spoilage isolates on pineapple cubes. However, some species were found associated with fruits or plant surfaces and showed antagonistic activities against fungal pathogens (Salas *et al.*, 2017). *Candida* which are pathogenic causing different forms of candidiasis in human and frequently found associated with fruits or plant surfaces. They were dominant pathogen cause spoilage in date fruit (Aljasass *et al.*, 2016).

From cultural plating, Bacteria observed on almost fresh bananas and spoilage, from export, domestic farms and wholesale fruits market, was *Enterobacter ludwigii*. Fungus, *Penicillium citrinum* was mainly observed from all fresh and spoiled bananas from marketed fruits. *Aspergillus inflatus* was mainly observed on spoiled bananas collected from export farm but was not found in domestic farm and marketed fruits. From metagenomics analysis, the most abundant bacteria on fresh and spoiled banana surface was *Enterobacteriacae* and *Pantoea*, respctively. *Moesziomyces* made up the largest division in both fresh and spoiled bananas.

Form this observation, key spoilage fungi found on bananas was *Penicillium citrinum* using culture method while the key microbe predicted by metagenomic data were *Moesziomyces*. From non-culture method (metagenomic analysis), *Enterobacteriaceace* was abundant in banana. For key bacteria in culture method, the bacteria found was also diverse with no specific pattern.

The different result from 2 methods could be due to the fact that many microbes could not be cultured with generally used media. The key microbes found by culture approach could be the key spoilage microbes because they could be cultured and express activity on fruit surface. Metagenomic method could not confirm if the most abundant microbes cause spoilage since they were directly extracted from DNA of both viable and dead cells. They could be dead cell and could not exert any activity. Thus, the viable strains well develop on fruit surfaces during spoilage development as observed by cultural plating could be the key microbes associated to fruit spoilage. Based on microbial profiles found from the cultural plating with statistically collected and selected to be representative as summarized in Table 3. could be the key microbes to be selected for further post-harvest management for control these strains. However, microbial profiles as observed from metagenomic analysis could be also important to further study their key roles in terms of antagonistic effect and/or their metabolic activity associated to banana spoilage, since properties of some abundant microbes observed have not been characterized and reported before.

As previously mentioned, microbial spoilage causes significant economic loss throughout the banana distribution chain. Therefore, the concerns in food industry is to reduce such microorganism contaminations. Fruits from tropical areas are more susceptible to different microorganisms than those grown in subtropical or temperate climates. In addition, most tropical and subtropical fruits are injured by low temperatures and therefore spoilage control cannot be assisted by refrigeration. Information observed in this study could help better understand the step of spoilage, spoilage characteristic, and particularly, key microbes associated to those spoilages. Possible roles and sources of these microbes were also explained. Thus, our data could reinforce the importance of analytical methods for studies on the microbial ecology of fruits and highlight the need for more detailed examination of this variable and its importance in post-harvest management including selection of decontamination means and chemical and/or even bio-control agents used.

Generally, data previously demonstrated give only simple qualitative

descriptions of the microbes isolated and make little attempt to ask why certain species are predominant and what factors affect their occurrence. Answers to these questions are important in managing the indigenous flora of the fruits and optimizing their potential impact on the fruit quality to improve microbiological safety and to extend the shelf-life of the fruits. From this study, information obtained also demonstrated that geographical factors had less impact on microbial flora associated to fruit spoilage. There was no specific microbial community pattern observed on banana collected from farms. Interestingly, similar microbial profiles associated to the spoilage of banana collected from market were observed. This reflected the key impact of post-contamination and/or handing process on microbial quality of the fruits. This information is key for managing the manufacturing process more efficiently and producing fresh bananas with excellent quality.

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