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Micro-organisms associated with fruit fly infestation in mango fruits

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Abstract
One of the major setbacks with the mango production and export is the incidence tephritid fruit flies. As the female oviposits on the fruits, bacteria on the surface of the fruit are pushed into the fruit resulting in decay, allowing for secondary infestations by other microorganisms in addition to gut bacteria. Determination of the population of microorganisms associated with fruit flies in mangoes can give a clue about the possible health hazards associated with fruit fly infested fruits. Loops of pulp from feeding areas of infested mangoes were cultured on agar and growths were identified. The fruits were incubated and the flies reared out and identified. Four genera of fungi were identified, including *Aspergillus sp*. in 3 samples, *Phoma sp*. in 2 samples, *Penicillium sp*. in 8 samples, and *Trichoderma sp*. in 3 samples. Cross-infestations were recorded in some samples. *Bactrocera dorsalis* was the only fruit fly species reared from the mango samples collected.

Keywords: Mango, fruit fly, micro-organisms, health hazards, *Bactrocera dorsalis***,** fungi, bacteria

Introduction
Mango (*Mangifera indica* L.) is the third most important fruit crop in the tropics and is the most important of 41 species of the family Anacardiaceae [1]. It has an important socio-economic impact on households and the economies of West African countries. It is one of the most popular fruits consumed among millions of people worldwide, and gaining high demands in developed countries [2].

Production of mangoes however is faced with the challenge of pests and diseases worldwide with fruit flies as one of the major threats being battled against. Fruit flies (Diptera: Tephritidae) are among the most important and widespread insect pests of fruits and vegetables [3]. They cause great losses, from direct damage to fruits and vegetables to loss of market opportunities through imposition of strict quarantine regulations by importing countries (Europe and the Middle East) to prevent any entry and establishment of fruit flies in these countries [4,5]. In the European Union, US and Japan, both strict quarantine and Maximum Residue Levels are being implemented [6]. This is to ensure that as they insist on no importation of fruit flies, they also ensure that their commodities have less pesticide residues hence the need to adopt less insecticide control measures in the management of fruit flies.

Insects' guts harbor a wide range of microbes. These gut microbes contribute significantly to the development of their insect hosts, providing them with essential nutrients as well as protecting them against other harmful pathogens [7]. As female fruit flies puncture fruits to oviposit, micro-organisms on the fruit surface are pushed into fruit. This leads to fruit decay, which also serves as a conducive medium for the larvae to feed and mature. The points of puncture also serve as a medium for micro-organism introduction. As the larvae tunnel through the fruit and feed they introduce their gut microbes which cause the fruit to rot and fall [8, 9]. The potential of fruit flies as vectors of fruit-borne pathogens have not been extensively exploited, nevertheless the presence of micro-organisms in fruit fly infested fruits is well known. A number of symbiotic bacteria have been identified in various fruit fly species, including "*Candidatus* Stammerula trupanae", "*Candidatus* Erwinia dacicola" [10, 11]. The potential role of these flies in transmitting food-borne pathogens has not been taken seriously. A number of researches focused on isolation of the microorganisms from the fruit flies directly. This study focused on the micro-organisms in the infested fruits to establish a link between the micro-organisms from the fruits to micro-organisms reported in the fruit flies. There is the increasing concern of food borne diseases worldwide and it is estimated that one in forty Ghanaians suffer from serious food borne disease each year, posing an important drain

on the economy [12]. Some of the infested fruits are eaten either knowingly or unknowingly. In some cases, the infested area may be sliced off. This however does not imply that all the micro-organisms present in the fruit have been cut off. There could still be some residues of the micro-organisms which will be ingested. In some cases, newly deposited eggs might not have emerged but the host surface micro-organisms might already have been introduced into the fruit during oviposition. Besides, in the fruit processing industries too much care cannot be taken to select non-infested fruits. The various concerns and questions raised are the health implications of these ingested micro-organisms on humans when infested fruits are consumed. To address this concern, there is the need to first identify the micro-organisms which may be present in the infested fruits as well as their abundance and frequency. Their toxicity or otherwise can then be ascertained and their potential pathogenicity when consumed can then be assessed.

In the present study, the population (diversity and abundance) of micro-organism in mango fruits collected from some selected mango growing areas in Ghana was determined, as well as their associations with fruit fly infestations and their potential health hazards to consumers.

Materials and Methods

Study and sample site

Samples were collected from Somanya in the Eastern Region of Ghana, Dodowa and the University of Ghana, Legon both in the Greater Accra Region of Ghana. Study was conducted from August, 2011 to June, 2012. Dodowa and Somanya are renowned mango growing communities in these regions. Mango varieties cultivated in these areas include Keitt, Kent and Palmer. Keitt is the major variety cultivated by the farmers. They are mainly cultivated for exports and for juice producing companies in the country. Kent, Keitt and the local varieties were included in the study.

To obtain flies for the laboratory infestation of mangoes, a colony of fruit flies was established. The colony was kept running throughout the study.

Field and Laboratory infested samples

For field-infested samples, fruit fly-infested mangoes were collected from the different study sites, transported to the laboratory and subjected to microbiological analysis.

For laboratory-infested samples, fruits were selected and thoroughly examined to ensure there were no oviposition marks. To determine if micro-organisms were from the fruit surface or from the flies, two set ups were established. In one set up, fruits were washed with 70% ethanol, 2ml of 2% sodium hypochlorite and distilled water [13] (hereafter referred to as WF fruits) and presented to the flies in the 'infestation cage'. In the other set, unwashed fruits were presented to flies (hereafter referred to as UF). The fruits were removed from the cages after 72 hours and kept in a separate cage for 7 days for rotting to begin and subjected to microbiological analysis.

Un-infested mango samples

To determine if micro-organisms were from fruit surfaces or the environment and not flies, there were three set ups. One set up involved washed and punctured fruits (hereafter referred to as WP fruits), the second involved unwashed and punctured fruits (hereafter referred to as UP fruits). The fruits were punctured using entomological pins, which were heat-sterilised after each fruit, with 25 punctures on each fruit surface. The fruits were kept for 7 days and subjected to micro-organism tests.

The third set up involved non-punctured fruits (hereafter referred to as NP fruits). Mango pulp samples were cut out of whole mangoes and sterilised with 1% sodium hypochlorite and distilled water. The samples were then subjected to microbiological analysis. These were carried out for each mango variety.

Matured ripening mangoes were sampled for the laboratory-infested and un-infested set ups.

Determination of micro-organisms

Isolation of micro-organisms was done following the procedure outlined by Collins and Lyne [14] with some modifications. Petri dishes were sterilized in a dry oven at 120 °C for 90 minutes and allowed to cool. Water agar was prepared in the ratio of 15 gram agar to 1000 ml distilled water and sterilized at 121 ℃ for 15 minutes and allowed to cool. Ten millilitres of water agar was dispensed in each petri dish in a sterile inoculation chamber and allowed to solidify. Loops of pulp from larval feeding areas in fruit fly infested mangoes were placed on the water agar using sterilised inoculation pins and incubated at 25 °C for 7 days. Cotton-like growths observed around the inoculation were sub-cultured onto Potato dextrose agar (PDA) medium in a sterile inoculation chamber. The PDA was prepared in the ratio of 39 grams of powdered PDA to 1000 ml of distilled water and autoclaved at 121 °C for 15 minutes. Ten millilitres of the PDA medium was dispensed into each petri dish and allowed to solidify. These were incubated at 25 °C and observed daily for about 7 days. Slides of fungal growths were prepared and examined under a Leica DMZ 50 light microscope. The organisms were identified with identification aid from Samson and van Reenen-Hoekstra [15] and Barnett and Barry [16]. Bacterial growth observed on the PDA media were further sub-cultured on nutrient agar media in a sterile inoculation chamber. The nutrient agar was prepared in the ratio of 35 grams of powdered nutrient agar to 1000ml of distilled water. Ten millilitres of the media was dispensed into each petri dish and allowed to solidify. Strips of the bacteria sample were produced on the media and allowed to grow for less than 36 hours. Bacterial staining and identification was done using the procedure outlined by Tuite [17]. Fruits from some set ups were cultured on some selective media including Sabouraud dextrose agar (SDA) which selects yeasts and moulds, Eosin Methylene Blue agar (LEVINE) for some intestinal bacteria, and Manitol salt agar and the Baird Parker Agar Base for selection of *Staphylococcus* species. Six fruit samples were incubated on these media, including field infested Local and Keitt varieties, unwashed whole mangoes (un-infested and not punctured) of Keitt, Local and Kent varieties, and unwashed laboratory punctured Keitt mango.

Results

Population of micro-organisms isolated from mangoes (Tables 1, 2, 3)

A total of 180 mango samples were examined with 4 replicates for each, all of which had some micro-organisms. A total of 357 isolates were recovered, of which 113 (31.65%) were found to be fungi and 244 (68.35%) were bacteria. Forty-six fruit samples (25.56%) had both fungi and bacteria, 52 fruit samples (28.89%) had only fungi, while 82 (45.56%) had only bacteria. Five identified and one unidentified genera of fungi were isolated. The identified genera included *Penicillium*, *Aspergillus*, *Trichoderma*, *Rhizopus* and *Phoma*. *Aspergillus* was isolated from 54 samples (30.00%) representing 15.13% of total micro-organisms recorded.

Almost all Keitt mango varieties used as control had *Aspergillus* (Table 1). *Trichoderma* was isolated from 24 samples (13.33%), representing 6.72% of the total micro-organisms recorded and this was present in all field-infested samples and some laboratory infested samples, but absent in the un-infested samples. *Penicillium* was isolated from 20 samples (11.11%) representing 5.60% of the total micro-organisms recorded. *Penicillium* was more from field infested mangoes than from laboratory samples.

Rhizopus and Phoma were isolated from 3 samples each (1.67%), representing 0.84% of the total micro-organisms. Rhizopus was absent in the local varieties and from both laboratory- and field-infested mangoes while *Phoma* was present only in the field-infested Keitt from Somanya. The unidentified fungi were isolated from 9 samples (5.00%), representing 2.52% of the total micro-organisms recorded. This was not recorded in any of the field infested samples.

Bacteria could not be identified to genus level; identification was based on gram type. Four bacteria types were recorded. These included gram-positive cocci, gram-positive rods, gram-negative rods and gram-negative cocci. Gram-positive cocci were isolated from 106 samples (58.89%), representing 29.69% of the total micro-organisms. They were the highest number of micro-organisms recorded, and it was present in all set ups studied, except in the Keitt control, where no bacteria were isolated (Table 1). Gram-positive rods were isolated from 92 samples (51.11%), representing 25.77% of the total micro-organisms. This bacteria type was also isolated from all set ups, except the control for Keitt. Gram-negative rods were isolated from 41 samples (22.78%), representing 11.48% of the total micro-organisms recorded. They were absent from unwashed and punctured (UP) mango samples of all the varieties. Gram-negative cocci were isolated from 6 samples (3.33%), representing 1.68% of the total micro-organisms. Most of the cultures had mixed cultures of bacteria. About 128 samples had bacteria of which 38 had single bacteria, either gram-positive rods, cocci, or gram-negative rods, the remaining 90 were combinations of different types.

Population of micro-organisms isolated from field infested mangoes (Tables 1, 2)

Keitt and Local mango varieties were the field infested samples examined. In Dodowa, three genera of fungi and three types of bacteria were isolated from Keitt mangoes (Table 1). The fungi included *Penicillium*, *Aspergillus* and *Trichoderma*. *Penicillium* species was isolated from 8 samples, *Aspergillus* species from 4 mango samples and *Trichoderma* species from 1 sample. The bacteria types isolated included gram-negative rods from 6 samples, gram-positive rods from 7 samples and gram-positive cocci from 8 samples. In Somanya, four genera of fungi and three types of bacteria were isolated from Keitt mangoes (Table 1). The fungi included *Penicillium*, *Aspergillus*, *Phoma* and *Trichoderma*. They were recorded from 5, 4, 3 and 2 samples respectively. The types of bacteria included gram-positive cocci from 6 samples and gram-negative rods from 3 samples, gram-positive rods from 5 samples. The Local mangoes sampled from the University of Ghana campus, recorded three genera of fungi and three types of bacteria (Table 2). *Penicillium*, *Aspergillus* and *Trichoderma* were recorded from 3 samples each. The bacteria included gram-negative rods from 3 samples, gram-positive rods from 8 samples and gram-positive cocci from 9 samples. All three localities had common micro-organisms present except for *Phoma* species which was present only in some samples of Keitt from Somanya. There were however differences in the number of the micro-organisms from each category.

Comparison of micro-organisms from field- and laboratory-infested fruits

A total of five fungal genera and four bacteria types were isolated from both field- and laboratory-infested fruit samples. *Penicillium*, *Aspergillus* and *Trichoderma* were common in both field and laboratory infested fruits. *Phoma* was isolated from field infested fruits and an unidentified fungus from the laboratory infested fruits. Gram-negative rods, gram-positive rods and gram-positive cocci were common to both field- and lab-infested fruits while gram-negative cocci were isolated from only one sample of lab-infested fruits. With the washed Keitt mangoes 2 fungi genera and 3 types of bacteria were isolated. The fungi included *Aspergillus* from 2 samples and an unidentified genus from 4 samples. The bacteria included gram-negative rods from 3 samples, gram-positive rods from 2 samples and gram-positive cocci from 3 samples. For the unwashed Keitt mangoes, 3 genera of fungi and 3 types of bacteria were isolated. The fungi included *Penicillium* (from one sample), *Aspergillus* (4 samples) and *Trichoderma* (2 samples). The bacteria included gram-negative cocci (one sample), gram-positive rods (6 samples) and gram-positive cocci (6 samples). From washed Local mangoes, *Aspergillus* (1 sample) and an unidentified species (2 samples) were isolated. Gram-negative rods were isolated from 3 samples, gram-positive rods from 6 samples and gram-positive cocci from 6 samples. From unwashed Local mangoes, *Aspergillus* was the only fungus isolated from 4 mango samples, while Gram-negative rods were isolated from 1 sample, gram-positive rods from 4 samples and gram-positive cocci from 7 samples.

Population of micro-organisms from un-infested fruits

Five genera of fungi and 4 types of bacteria were isolated from the clean un-infested fruit samples. The fungi included *Aspergillus*, *Penicillium*, *Trichoderma*, *Rhizopus* and the unidentified fungi. Two genera of fungi and 2 types of bacteria were isolated from the washed Keitt mango. *Trichoderma* was isolated from 1 sample and the unidentified fungi from 2 samples. The bacteria included gram-positive rods from 5 samples and gram-positive cocci from 8 mango samples. From the unwashed Keitt mango samples, 2 genera of fungi and 3 types of bacteria were isolated. *Aspergillus* was isolated from 6 samples and *Trichoderma* from 1 sample. Gram-negative cocci were isolated from 1 sample, gram-positive rods from 4 samples and gram-positive cocci from 4 samples. With the non-punctured mango samples of the Keitt variety all samples had fungi and there were no bacteria isolated. Three genera of fungi were recorded, *Penicillium* (2 samples), *Aspergillus* (9 samples) and *Rhizopus* (1 sample). Three types of bacteria were isolated from the washed Local mango samples, but there were no fungi isolated. Gram-negative rods were isolated from 6 samples, gram-positive rods from 8 samples and gram-positive cocci from 8 samples. With the unwashed samples, *Aspergillus* (from 2 samples) and *Trichoderma* (7 samples) and gram-positive rods (7 samples) and gram-positive cocci (6 samples) were also isolated. More diverse micro-organisms were recorded in the Kent mango samples (Table 3). From the washed samples 3 genera of fungi and 3 types of bacteria were isolated. The fungi included *Aspergillus*, *Trichoderma* and *Rhizopus*. Bacteria included Gram-negative rods (from 4 samples), gram-positive rods (8 samples) and gram-positive cocci (9

samples). The unwashed samples recorded 4 genera of fungi and 2 types of bacteria. Fungi comprised *Penicillium* (1 sample), *Aspergillus* (7 samples), *Rhizopus* (1 sample) and the unidentified fungus (1 sample).

Determining if micro-organism populations are directly due to presence of fruit fly

Larvae in fruits

Diversity of micro-organisms isolated from the infested fruits showed no difference from those of the un-infested fruits. They were different in abundance. For the fungi, all genera isolated from the infested fruits were present in the un-infested fruits, except for *Phoma* species which was isolated only from the field-infested Keitt mangoes from Somanya. Fruit samples cultured on the specific media gave some clue to the distinctions in the bacteria isolated from the samples. *Staphylococcus* species was isolated from the field infested Keitt mango sample, while the un-infested samples showed only the presence of moulds. Some intestinal bacterium (Enterobacteriaceae) was also isolated from the field infested Local mango samples but it was absent in the un-infested mango samples. No bacteria were isolated from the un-infested cultured samples of the Kent variety.

For all the 3 study sites, *Bactrocera dorsalis* was the only fruit fly species isolated from the reared fruits, indicating the displacement of the other tephritid fruit fly species by this invasive species.

Table 1: Diversity and abundance of micro-organisms isolated from the Keitt variety of mango fruits sampled

Micro-organisms isolated		Number of sample with micro-organisms						
		Field-infested fruits		Laboratory infested fruits		Uninfested fruits		
		Dodowa	Somanya	W.F	U.F	W.P	U.P	N.P
Fungi	*Penicillium* species	8	5	-	1	-	-	2
	Aspergillus species	4	4	2	4	-	6	9
	Trichoderma species	1	2	-	2	1	1	-
	Rhizopus species	-	-	-	-	-	-	1
	Phoma species	-	3	-	-	-	-	-
	Unidentified	-	-	4	-	2	-	-
Bacteria	Gram –ve rods	6	3	3	-	-	-	-
	Gram –ve cocci	-	-	-	1	-	1	-
	Gram +ve rods	7	5	2	6	5	4	-
	Gram +ve cocci	8	6	3	6	8	4	-

W.F=Washed and infested, U.F=Unwashed and infested, W.P=Washed and punctured, U.P=Unwashed and Punctured, and N.P=Not punctured

Table 2: Diversity and abundance of micro-organisms isolated from the local variety of mango fruits sampled

Micro-organisms isolated		Number of sample with micro-organisms					
		Field infested	Laboratory infested fruits			Uninfested fruits	
		Legon	W.F	U.F	W.P	U.P	N.P
Fungi	*Penicillium* species	3	-	-	-	-	-
	Aspergillus species	3	1	4	-	2	-
	Trichoderma species.	3	-	-	-	7	-
	Unidentified	-	2	-	-	-	-
Bacteria	Gram –ve rods	3	3	1	6	-	5
	Gram –ve cocci	-	-	-	-	-	3
	Gram +ve rods	8	6	4	8	7	10
	Gram +ve cocci	9	6	7	8	6	8

W.F=Washed and infested, U.F=Unwashed and infested, W.P=Washed and punctured, U.P=Unwashed and Punctured, and N.P=Not punctured

Table 3: Diversity and abundance of micro-organisms isolated from the Kent variety of mango fruits sampled

Micro-organisms isolated		Number of sample with micro-organisms				
		Laboratory infested fruits			Uninfested fruits	
		W.F	U.F	W.P	U.P	N.P
Fungi	*Penicillium* species.	-	-	-	1	-
	Aspergillus species.	3	4	1	7	-
	Trichoderma species.	1	2	4	-	-
	Rhizopus species.	-	-	1	1	-
	Unidentified	-	-	-	1	-
Bacteria	Gram -ve rods	-	4	4	-	3
	Gram -ve cocci	1	-	-	-	-
	Gram +ve rods	5	1	8	2	4
	Gram +ve cocci	4	3	9	1	9

W.F=Washed and infested, U.F=Unwashed and infested, W.P=Washed and punctured, U.P=Unwashed and Punctured, and N.P=Not punctured

Discussion

A number of micro-organisms, especially bacteria, have been isolated from fruit flies associated with various fruits, and some of these micro-organisms may be pathogenic or opportunistic pathogens to humans and animals [18-20]. Many raw fruits and vegetables carry gram-negative bacteria which

are essentially of environmental origin and express resistance to antibiotics [21]. It was observed that the gram-negative bacteria isolated in this study were generally not exclusive to fruit fly infested fruits. However, with the specific culture, only the infested local mango variety had some Enterobacteriaceae, a gram-negative bacteria present. This shows a direct link between fruit fly infestation and the presence of the bacteria. Most of the bacteria isolated could be from the environment, especially the gram-positive bacteria which were present in all fruit samples which had bacteria. Pankaj and Amit [22] isolated eleven different types of bacteria from the fruit fly *Bactrocera tau*. These included 6 gram-negative bacteria; *Pseudomonas putida* (rods or coccobacilli), *Cedacea davisae* (Enterobacteriaceae), *Pantoa agglomerans* (Enterobacteriaceae), *Stenotrophomonas* (*Xanthomonas*) *maltophilia* (rods), *Acinetobacter* species (coccobacilli), *Actinobacillus* species, and 5 gram-positive rods; *Arthrobacter* species, *Coryneform* species, *Bacillus subtilis*, *B. sphaericus* and *B. brevis*. Some of these bacteria are known to be attaining high pathogenicity in humans [23]. Though isolated bacteria from this study were not identified to the genus level, it is possible that some of these bacteria could be those reported by Pankaj and Amit [22]. Further identification of the bacteria type will give a clearer picture of the different species present.

The results obtained show that micro-organisms isolated from the field samples, especially the fungi, may not necessarily be restricted to any of the localities or the mango varieties. Any mango variety from different localities may be prone to any of these fungi. *Penicillium* and *Aspergillus* for example are known to be ubiquitous [24, 25], hence any mechanical damage to the fruit can be a source of secondary infection by any micro-organisms in the environment. The Fungi isolated were found to be present in both infested and un-infested samples, hence the presence of fungi may not be directly linked to fruit fly presence. This study confirmed some findings by other authors about the association of micro-organisms with fruit flies. Enterobacteriacae and Staphylococcus were isolated from only the infested samples and these were isolated from fruit flies [10, 11, 22, 26]. This indicates a direct link between fruit fly infestation and the presence of these bacteria and the health implications of these bacteria cannot be overemphasized.

The presence of *Staphylococcus* species and Enterobacteriaceae exclusively in the infested fruit samples confirms the associations of these bacteria with fruit flies [26]. The un-infested fruit samples cultured on the selective media were not washed, so the absence of *Staphylococcus* species and the Enterobacteriaceae is an indication that these bacteria are directly associated with the presence of the fruit flies and not surface bacteria. "*Candidatus* Stammerula trupanae", and "*Candidatus* Erwinia dacicola" isolated from fruit flies by Viale *et al*. [10] and Ben-Yosef *et al*. [11] are enterobacteria. Enterobacteriaceae are primarily known to cause intestinal upset and are responsible for a variety of human illnesses including urinary tract infections, wound infections, gastroenteritis, meningitis, septicaemia and pneumonia [27, 28]. *Staphylococcus* species is also a pathogenic bacterium, with *Staphylococcus aureus* of increasing medical importance.

Some of the other bacteria isolated may however be surface bacteria on the fruits and others could be secondary bacteria from environment as observed on the washed un-infested fruit samples. Infestation of fruits by fungi and bacteria may occur during the growing period, at harvest time, during handling, storage, transport and marketing, or even after purchase by consumers [29, 30]. This confirms the report by Bateman [31] that yeasts, fungi, and gram-positive bacteria have been isolated from a number of fruit fly species, but are usually shown to be simply "associated organisms" rather than symbionts. There is the need to ascertain the status of the gram-negative bacteria isolated whether they are also "associated organisms" or symbionts.

Conclusion

The findings from this work show that whether fruit flies are present or absent in a fruit, there are micro-organisms associated with fruits, some of which could be plant pathogenic, opportunistic pathogens or human pathogens. Almost all the fungi, especially the most abundant genera (*Aspergillus*, *Trichoderma* and *Penicillium*) were isolated from both infested and un-infested fruit samples. But there are some bacteria that a are only present with the presence of fruit flies in the fruit. *Staphylococcus* species and Enterobacteriaceae isolated were mainly associated with fruit fly infestation.

It is important to identify the specific bacteria isolated from these fruit samples to better ascertain the status, whether they are phytopathogens or are potential opportunistic or human pathogens.

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