Isolation, Characterization and Antagonistic Activity of the External Microflora of the House fly, *Musca domestica* (Diptera: Muscidae)

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Abstract

Experiments were designed to isolate, characterize and study the interaction between external microbiota (bacteria and fungi) carried by adult *M. domestica* after dipping, then removal of the flies from distilled water, sugar solution and saline solution. *M. domestica* was collected from Sakaka city, Northwestern Saudi Arabia. Three groups of adult *M. domestica* were completely dipped and then removed from each of the above-mentioned solutions separately. Bacteria and fungi were isolated using corresponding media, characterized using macro and microscopic examinations, and then tested for antagonistic activity. Three bacterial species; *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa* and three fungi; *Candida albicans*, *Rhizopus stolonifer* and *Aspergillus niger* have been isolated, characterized and tested for antagonism. Biochemical tests of bacterial strains confirmed the ability to secrete economically important materials. Different efficiencies to ferment sugars and produce gases have been confirmed, too. Antagonistic tests between microorganisms have revealed that both *E. coli* and *P. aeruginosa* bacteria are antagonists to both *A. niger* and *C. albicans* fungi. However, *R. stolonifer* fungus is antagonist to both *E. coli* and *P. aeruginosa* bacteria. *B. subtilis* bacterium is antagonist to the 3 fungi and to the other 2 bacteria. The antagonistic activity of our bacterial strains could be attributed to the secretion of antimicrobial materials. Further study on the mechanism of antimicrobial activity of *B. subtilis* strain is recommended. It was concluded that this strain could be useful in controlling some bacterial and fungal infections.

Keywords: *M. domestica*, microbiota, bioactive materials, bacterial fungal antagonism.
INTRODUCTION

The house fly, Musca domestica, is one of the most common health pests worldwide. M. domestica possesses morphological and behavioral characteristics which make it not only annoying, but a mechanical vector of more than 100 pathogens. M. domestica is closely related to human activities and it breeds on decaying organic matter such as animal manure, human wastes, open toilets, garbage, foods, vegetables and plants. All of mentioned breeding media are full of diverse and active microbial communities. Many researchers have studied the microbes associated with the wings of some fly species. But only one article studied the effect of natural fall and dipping of M. domestica on microbial contamination of distilled water and milk.

The present study is based on interaction between the external microbiota (bacteria and fungi) carried by adult M. domestica after dipping, then removal of the flies from distilled water, sugar solution and saline solution. Consequently, the antagonism between the isolated strains was investigated.

MATERIALS AND METHODS

Collecting flies

The house fly, M. domestica, were collected from the Sakaka city, AlJouf, Northwestern Saudi Arabia. Collected flies were transported to the laboratory in sterile cups and then they were morphologically identified. M. domestica was reared and maintained in the insectary under controlled conditions (27±2 °C and 70±5% Relative humidity (RH) and 14/10 light/dark photoperiod cycle), according to. These flies were used as a stock for the experimental work.

Solutions used

The experimental solutions were chosen to represent the normal drinks and foods of the human beings. Distilled water represents the normal drinking water of human. The 10% sterile sugar solution represents juices and other sugary drinks consumed by human. The 10% sterile saline solution represents the balanced salting of all types of salads and cooked foods with sauces. All solutions were sterilized using bacterial filters and all tools were autoclaved.

Experimental design

Three groups of adult M. domestica (10 flies/group) were completely dipped in and then removed from each of the following solutions separately: 200 ml of sterilized distilled water (DW), 200 ml of 10% sterile sugar solution (SU), and 200 ml of 10% sterile saline solution (SA). Immediately after dipping and removal of flies, bacterial and fungal flora were cultured from the three solutions, separately (DW, SU and SA). One hour later after dipping and removal of flies, bacterial and fungal flora were cultured from the three solutions, separately (DW1, SU1 and SA1).

Bacterial isolation using differential media

A fixed volume (100 µl) of each of the solutions DW, DW1, SU, SU1, SA and SA1 was spread by sterilized scalpel on 20 cm diameter plates containing Nutrient agar (NA), Mannitol salt agar (MSA), MacConkey agar, Brilliant green agar (BGA) and Salmonella-Shigella agar (SSA) media, separately. Plates were sealed tightly with parafilm, placed upside down and incubated at 30 °C for 24-48 h. Plates were then investigated, bacteria were isolated, identified and stored until used in subsequent experiments. Procedure was carried out inside laminar air flow hood.

Phenotypic characterization

Phenotypic characterization of all isolates studied were performed and compared to phenotypic data of known organisms described in the Bergey's Manual of systematic Bacteriology as well as Gram's staining according to the standard gram staining protocol.

Antagonistic activity between bacterial isolates

Antagonistic activity was tested according to. Briefly, 0.5 ml of a bacterial suspension was spread on the surface of solidified nutrient agar and paper-disc diffusion method was used for the other bacterial strains. Clear inhibition zones were measured and compared to positive and negative controls. Each experiment was repeated thrice.

Fungal isolation

A fixed volume (100 µl) of the solutions DW, DW1, SU, SU1, SA and SA1 was spread onto 20 cm diameter plates containing Czapek-Dox's agar medium and Potato Dextrose Agar (PDA) medium, separately. Chloramphenicol (25.0 mg/L) or Chlortetracycline (40.0 mg/L) was added to the media to inhibit bacterial growth. Plates were
sealed tightly with parafilm, placed upside down and incubated at 28 °C for 7-15 days.

Identification of fungal isolates

Purification of the colonies was carried out by transferring each single colony to a sterile PDA plate and incubating plates at 28 °C for 7-15 days. The propagated colonies were mounted on slides and stained with lactophenol cotton blue to be examined under light microscope. Macroscopic morphology of mycelium and conidia was observed and used for fungal identification.

Antagonism between fungi and associated bacteria

Antagonistic activity was tested according to (26). Briefly, one ml of each fungus was spread onto the surface of solidified Czapek-Dox’s agar media. A paper-disc diffusion method was used as described above. Three replicates were incubated at 30 °C for 15 days, and inhibition zones were measured and compared to a reference chart.

RESULTS

Characterization of bacterial strains

A total of 18 bacterial isolates were identified during this study from all samples. These isolates were isolated from DW, DW1, SU, SU1, SA and SA1. Isolates were definitely characterized as three species; Escherichia coli, Bacillus subtilis and Pseudomonas aeruginosa (Table 1).

### Table 1. Isolation of bacterial species from different solutions after dipping and removal of M. domestica immediately and one hour later

| Solution | DW | SU | SA | DW1 | SU1 | SA1 |
|----------|----|----|----|-----|-----|-----|
| Bacterial Species |    |    |    |     |     |     |
| E. coli | ✓  | ✓  | ✓  | ✓   | ✓   | ✓   |
| P. aeruginosa | ✓  | ✓  | ✓  | ✓   | ✓   | ✓   |
| B. subtilis | ✓  | ✓  | ✓  | ✓   | ✓   | ✓   |

Morphological characterization of bacterial colonies

Shapes, sizes, elevation, opacity and margins of the bacterial colonies are summarized in Table (2). All colonies were elevated and opaque except the translucent colony of E. coli. Circular colonies of E. coli and P. aeruginosa and irregular B. subtilis colony were observed, too. In addition, small-sized with entire margin colonies of E. coli, medium-sized with undulate margin colonies of P. aeruginosa and large-sized with lobate margin colonies of B. subtilis were noticed (Table 2).

Gram characteristics of the bacterial species

Table (3) summarizes Gram’s staining and cell morphology of the bacterial species. All bacterial cells were Gram-negative except B. subtilis which was Gram-positive. Meanwhile, all cells were rod-shaped except P. aeruginosa which were coccrobacilli.

Biochemical characterization of bacterial species

Specific biochemical assays were carried out to evaluate economic and commercial values of the species. All bacterial species secrete catalase, B. subtilis and P. aeruginosa secrete oxidase and only B. subtilis secretes urease (Table 4). These enzymes can be commercially harnessed and marketed.

IMViC tests indicated that only E. coli secretes tryptophanase enzyme and indole. Additionally, E. coli is glucose-acidic-fermenter.

### Table 2. Colony characteristics of the isolated bacterial species

| Colony Characteristic | Shape | Size  | Elevation | Opacity  | Margin |
|-----------------------|-------|-------|-----------|----------|--------|
| Bacterial Species     |       |       |           |          |        |
| E. coli               | Circular | Small | Raised   | Translucent | Entire |
| P. aeruginosa         | Circular | Medium | Raised   | Opaque   | Undulate |
| B. subtilis           | Irregular | Large  | Raised   | Opaque   | Lobate  |
Table 3. Gram’s characteristics and cell morphology of the isolated bacterial species

| Bacterial species | Cell Gram Character | Cell Morphology |
|-------------------|---------------------|-----------------|
| *E. coli*         | -ve                 | Rod shaped      |
| *P. aeruginosa*   | -ve                 | Coccobacilli    |
| *B. subtilis*     | +ve                 | Rod shaped      |

Table 4. Biochemical characteristics of the isolated bacterial species

| Biochemical test | *E. coli* | *P. aeruginosa* | *B. subtilis* |
|------------------|-----------|-----------------|---------------|
| Catalase         | +ve       | +ve             | +ve           |
| Oxidase          | -ve       | +ve             | +ve           |
| Urease           | -ve       | -ve             | +ve           |
| Tryptophanase    | +ve       | -ve             | -ve           |
| Indole           | +ve       | -ve             | -ve           |
| Glucose fermentation | +ve Acidic | +ve Alkaline | +ve Alkaline |
| Sucrose fermentation | -ve     | -ve             | +ve Alkaline |
| Lactose fermentation | +ve Acidic | -ve           | -ve           |
| TSI- test        | +ve Acidic | -ve           | +ve Acidic   |
| CO₂ production   | +ve       | -ve             | -ve           |
| H₂S production   | +ve       | -ve             | -ve           |

However, both *B. subtilis* and *P. aeruginosa* are glucose-alkaline-fermenters. Sugar fermentation tests revealed that *E. coli* and *P. aeruginosa* are non-sucrose-fermenters. Both *B. subtilis* and *P. aeruginosa* are non-lactose-fermenters (Table 4). In addition, TSI and H₂S tests revealed that *B. subtilis* is trisugar-acidic-fermenter lacking both CO₂ and H₂S gas production. *E. coli* is trisugar-acidic-fermenter producing CO₂ and lacking H₂S gas production. Whilst, *P. aeruginosa* is non-trisugar-fermenter (Table 4).

Table 5. Colony characterization by using differential media

| Media      | Bacteria         | Color                          |
|------------|------------------|-------------------------------|
| MacConkey agar | Tow growths;     | Pink colonies.                |
|            | *E. coli*        |                               |
|            | *P. aeruginosa*  | Colorless colonies with dark centers. |
| MSA        | *E. coli*        | Pink colonies.                |
| SSA        | *E. coli*        | Pink colonies.                |
| BGA        | *E. coli*        | Greenish colonies.            |
| NA         | Tow growths;     | Creamy or brown color colonies. |
|            | *B. subtilis*    |                               |
|            | *P. aeruginosa*  | Greenish color colonies.      |

Characterization by differential media

In order to differentiate between the obtained bacterial species, 5 differential media were employed. Bacterial growth and characteristic colors of bacterial colonies were summarized in Table (5). Three growths with two characteristic colors were observed with MacConkey agar, two growths with two characteristic colors with NA media, only one growth with a characteristic color was observed with SSA, BGA and MSA media (Table 5). Insufficient characterization has been observed when using differential media.
Antagonistic activity between bacterial species

Growth of two or more microorganisms in a single culture medium may indicate synergistic activity. However, growth of a single species on the medium may indicate antagonistic activity of the growing species. Our results revealed that *B. subtilis* is antagonistic to both *E. coli* and *P. aeruginosa* (Table 6).

Table 6. Antagonistic activity of the isolated bacterial species

| Bacterial combination                  | Antagonism | Growth                                |
|--------------------------------------|------------|---------------------------------------|
| E. coli + P. aeruginosa              | -ve        | Two growths and no inhibition         |
| E. coli + B. subtilis                | +ve        | Growth of *B. subtilis* only          |
| P. aeruginosa + B. subtilis          | +ve        | Growth of *B. subtilis* only          |
| E. coli + P. aeruginosa + B. subtilis| +ve        | Growth of *B. subtilis* only          |

Fungal isolation

A total of ten fungal isolates were isolated during the current work. Only one isolate from DW and DW1, two isolates from SU and SU1, two isolates from SA and SA1 were isolated. Fungal isolates were identified as *Candida albicans*, *Rhizopus stolonifer* and *Aspergillus niger* (Table 7). *C. albicans* was persistent in all solutions, *R. stolonifer* appeared in sugar solutions and *A. niger* grew in salt solutions (Table 7).

Table 7. Isolation of fungal species from different solutions after dipping and removal of *M. domestica* immediately and one hour later

| Solution | DW | SU | SA | DW1 | SU1 | SA1 |
|----------|----|----|----|-----|-----|-----|
| Fungal Species |    |    |    |     |     |     |
| *C. albicans* | √  | √  | √  | √   | √   | √   |
| *R. stolonifer* | —  | √  | —  | —   | √   | —   |
| *A. niger* | —  | —  | √  | —   | —   | √   |

branching globular structures with pseudohyphae. *R. stolonifer* appeared as dense, cottony structures which fill culture plate. Branched aerial mycelia with filamentous non-septate hyphae were observed. Sporangia with many spores are carried by sporangiophores. *A. niger* was reported as dichotomous branched mycelia with septate hyphae. Numerous black spores are carried by long, smooth and hyaline conidiophores (Table 8).

Antagonistic activity

*E. coli* and *P. aeruginosa* bacteria prohibited growths of both *A. niger* and *C. albicans*, whatever bacteria have applied individually or in combination. However, *R. stolonifer* prohibited growths of *E. coli* and *P. aeruginosa* whatever applied to the fungus individually or mixed with each other. Interestingly, *B. subtilis* bacteria prohibited the growths of all fungi whatever it has applied individually or in combination with other bacteria (Table 9).

DISCUSSION

The current study presents 3 bacterial and 3 fungal colonies with distinct morphological characters were identified. Two Gram negative Proteobacteria; *E. coli* (Enterobacteriales, Enterobacteriaceae) and *P. aeruginosa* (Pseudomonadales, Pseudomonadaceae) and one Gram positive Firmicutes bacteria; *B. subtilis* (Bacillales, Bacillaceae) were isolated. In addition, 2...
Ascomycotic fungi; *C. albicans* (Saccharomycetales, Saccharomycetaeaceae), *A. niger* (Eurotiales, Trichocomaceae) and one Zygomycotic fungus; *R. stolonifer* (Mucorales, Mucoraceae). Bacterial association with flies is attracting subject to authors from 1912 up till now. Due its accessibility to humane living, special attention to house fly was markedly noticeable. Several authors have isolated more than 32 bacterial genera including our species from the house fly; *M. demestica*. The

Table 8. Macroscopic and microscopic characterization of the isolated fungi

| Fungi       | *A. niger* | *R. stolonifer* | *C. albicans* |
|-------------|------------|-----------------|---------------|
| On agar plate | Powdery structures with numerous black dots. | Dense, cottony, aerial mycelia fill the plate. It appears white then became grey. | White colony. |
| Branching    | Dichotomous branching. | Branched. | Non-branching. |
| Hyphae       | Septate and hyaline. | Non-septate. Stolons connecting fungal bodies. | Pseudohyphae. |
| Conidiophores | Conidiophores are long, smooth, hyaline and darker at the apex. | Noticeable sporangiophores. | Absent. |
| Spores       | Numerous and black. | Globose sporangia with many spores, and flattened base. | Reproduction by budding. |

Table 9. Antagonistic activity between fungi and bacteria

| Bacteria      | Fungi       | Antagonism | Growth |
|---------------|-------------|------------|--------|
| *E. coli*     | *A. niger*  | +ve        | Growth of *E. coli* |
| *P. aeruginosa* | +ve        | Growth of *P. aeruginosa* |
| *B. subtilis* | +ve        | Growth of *B. subtilis* |
| *E. coli* + *P. aeruginosa* | +ve | Growth of *E. coli* + *P. aeruginosa* |
| *E. coli* + *B. subtilis* | +ve | Growth of *B. subtilis* |
| *P. aeruginosa* + *B. subtilis* | +ve | Growth of *B. subtilis* |
| *E. coli* + *P. aeruginosa* + *B. subtilis* | +ve | Growth of *B. subtilis* |
| *E. coli*     | *R. stolonifer* | +ve | Growth of *R. stolonifer* |
| *P. aeruginosa* | +ve | Growth of *R. stolonifer* |
| *B. subtilis* | +ve | Growth of *B. subtilis* |
| *E. coli* + *P. aeruginosa* | +ve | Growth of *R. stolonifer* |
| *E. coli* + *B. subtilis* | +ve | Growth of *B. subtilis* |
| *P. aeruginosa* + *B. subtilis* | +ve | Growth of *B. subtilis* |
| *E. coli* + *P. aeruginosa* + *B. subtilis* | +ve | Growth of *B. subtilis* |
| *E. coli* | *C. albicans* | +ve | Growth of *E. coli* |
| *P. aeruginosa* | +ve | Growth of *P. aeruginosa* |
| *B. subtilis* | +ve | Growth of *B. subtilis* |
| *E. coli* + *P. aeruginosa* | +ve | Growth of *E. coli* + *P. aeruginosa* |
| *E. coli* + *B. subtilis* | +ve | Growth of *B. subtilis* |
| *P. aeruginosa* + *B. subtilis* | +ve | Growth of *B. subtilis* |
| *E. coli* + *P. aeruginosa* + *B. subtilis* | +ve | Growth of *B. subtilis* |
reported 32 genera belong to 3 phyla, 12 orders and 21 families within bacterial kingdom (e.g. 10, 27-42). In parallel, more than 21 fungal genera including our species have been isolated from the house fly; M. domestica. The reported 21 genera belong to 4 phyla, 13 orders and 12 families within fungal kingdom (e.g. 33, 43-49). More than 100 species of parasites and microorganisms have been isolated from the house fly61,36,37. Authors have paid attention to the bacterial communities of other flies50-52.

The antagonistic activity of our bacterial strains could be interpreted by the ability of bacteria to secrete enzymes and other economic materials as shown in biochemical characterization. Antagonistic tests between microorganisms have revealed that both E. coli and P. aeruginosa bacteria are antagonists to A. niger and C. albicans fungi. Agreeable results have been presented by61 who revealed that E. coli secretes a fungicide that kills C. albicans. Also P. aeruginosa was reported as antagonist to A. niger53. Other studies have reported that P. aeruginosa is antagonist to Aspergillus fumigatus in planktonic growth54 and in bio lm, too55-58. Contrary to our results, no antagonism between E. coli and C. albicans has been found56. Interestingly, P. aeruginosa and A. fumigatus have been reported to possess mutual antagonism at different stages of bio lm development59. Recently, the complexity beyond the simple antagonistic interaction between P. aeruginosa and C. albicans has been intensively reviewed60. E. coli, Pseudomonas sp. and Bacillus sp. have been reported as antagonists to A. niger and could be used in biocontrol of the fungus61. E. coli has exhibited antagonistic activity to pathogenic Aspergillus spp.62. However, R. stolonifer fungus is antagonist to E. coli and P. aeruginosa bacteria. A previous study has presented that R. stolonifer fungus showed antagonistic effect to A. niger and C. albicans fungi and to P. aeruginosa and E. coli bacteria. This activity was attributed to toxic secondary metabolites secreted by the fungus63. B. subtilis bacterium is antagonist to the 3 fungi and to the other 2 bacteria. In antagonistic study, B. subtilis has proved to produce a biosurfactant that prohibited the growth of Salmonella, Shigella and Staphylococcus bacteria64. Antifungal activity of Bacillus isolates against phytopathogenic fungi may be attributed to the cyclic lipopeptide; fungycin which plays important role in this process65-68. Recently, the antimicrobial compounds of B. subtilis have been intensively reviewed69. No microbial competition between bacteria and fungi was recorded in the present study. However, microbial competition after natural falling and dipping of house fly in water and milk has been reported15. The total number of microbes has decreased within one hour after dipping in the case of water. Meanwhile, immediate decrease in total number of microbes in the case of milk has been reported15. Further research on the effect of falling and dipping of M. domestica using electron microscopy and molecular techniques is recommended.

Overall, the current work presents isolation, characterization and antagonistic activity of six microorganisms isolated from external surface of the house fly; M. domestica after dipping in DW, SU and SA solutions. Our results revealed that our bacterial strains secrete many economically important materials which could be harnessed and marketed. Different efficiencies of sugar fermentation and gas production have been observed, too. In addition the antagonistic activity, especially the ability of B. subtilis bacterium to prohibit growth of all bacterial and fungal strains could be interpreted in the light of its production of bioactive materials. Further study on the mechanism of antimicrobial activity of B. subtilis strain is recommended. We concluded that this strain could be useful in controlling some bacterial and fungal infections.

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CONFLICTS OF INTEREST

The authors declares that there is no conflict of interest.

AUTHORS’ CONTRIBUTION

Conceived and designed the experiments: FHG, AMS. Performed the experiments: FHG, AMS, TES. Analyzed the data: FHG, AMS. Wrote the paper: AMS, FHG. All authors have approved the final manuscript.
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DATA AVAILABILITY
All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT
Not applicable.

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