Pathogens associated with houseflies from different areas within a New York State dairy

G. Gioia,1 J. Freeman,2 A. Sipka,3 C. Santisteban,1 M. Wieland,1 V. Alanis Gallardo,1,3 V. Monistero,4 J. G. Scott,2 and P. Moroni1,4*

Graphical Abstract

Summary

Houseflies in dairy farms can be a concern since they could play a crucial role in increased bacterial counts in milk and the potential occurrence of transmitted diseases that affect public and animal health. This study evaluated the bacterial communities associated with houseflies captured on a dairy farm in New York State. A total of 101 flies were collected at 3 farm sites: hospital pen, calf hutches, and milking parlor. Each housefly was tested by bacteriological analysis for microbial identification, and a total of 304 bacterial isolates were obtained. Twelve percent of the identified organisms are well known to affect dairy farms. We identified 26 bacterial species with implications for animal health since these are mastitis-causing pathogens; 5 bacteria are considered foodborne pathogens and, for this reason, represent a concern for human health, and last, but not least, we identified 5 milk spoilage bacteria species that affect the quality of dairy products.

Highlights

- We obtained 101 houseflies from 3 different sites of one commercial dairy farm.
- Bacteria culture was performed from external surfaces and internal parts of houseflies.
- We identified 26 mastitis-causing pathogens, 5 foodborne pathogens, and 5 milk-spoiling organisms.

1Quality Milk Production Services, Animal Health Diagnostic Center, Cornell University, Ithaca, NY 14850, 2Department of Entomology, College of Agriculture and Life Sciences, Cornell University, Ithaca, NY 14853, 3Departamento de Medicina Preventiva y Salud Pública, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Ciudad Universitaria, CDMX 04510, México, 4Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, Via dell'Università, 6, 26900 Lodi LO, Italy. *Corresponding author: pm389@cornell.edu. © 2022, The Authors. Published by Elsevier Inc. on behalf of the American Dairy Science Association®. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/). Received December 25, 2021. Accepted March 13, 2022.
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defies (Musca domestica) live in close association with
humans and domestic animals because both urban and rural
environments enable their development (Gopal et al., 2015; hu-
man excrement and garbage, as well as animal manure and bed-
ding, represent the main sources for their nutrition and oviposition
(West, 1951). This insect, having a dispersal range of 5 to 32 km,
can carry bacteria both on the surface of its exoskeleton and in
the alimentary canal, and disperse them through mechanical
translocation from the exoskeleton or by defecation and regurgita-
tion (Nazni et al., 2005). The presence of bristles and glandular
hairs on housefly legs enhances bacteria adhesion to their exterior
surface (Graczyk et al., 2001). Feces viscosity increases the ef-
ciency of flies’ bristles and hair in trapping bacteria suspended in
the manure (Graczyk et al., 1999). Bacteria can be stored in the
fly’s crop (Doud and Zurek, 2012) where they can multiply and
be regurgitated or pass through the gut, leading to the concept of
“bioenhanced transmission” (Onwugama et al., 2018). Flies can
contribute to the dissemination of bacteria of public health impor-
tance, such as Escherichia coli (Alves et al., 2018), Campylobacter
jejuni (Bahmendorff et al., 2014), Salmonella spp. (Holt et al., 2007),
and Staphylococcus spp. (Almeida et al., 2014), including strains
resistant to antibiotics (Macovei et al., 2008; Akhtar et al., 2009).
Flies are also discussed as reservoirs of mastitis pathogens such as
Staphylococcus aureus. Still, most of the data associated with the
dairy environment are related to horn flies (Haematobia irritans;
Anderson et al., 2012; Ryman et al., 2013). Otherwise, in other
studies conducted on dairies, the fly species are not mentioned
(Roberson et al., 1994). To the best of our knowledge, published
data of Staph. aureus isolated from houseflies are not specifically
associated with dairy environments (Nayduch et al., 2013).

Surveillance of pathogens in flies can identify persistent patho-
gens in farm environments and their potential association with
public and animal health. Houseflies can also act as vectors of
multidrug-resistant bacteria, contributing to the dissemination of
antimicrobial resistance between farm animals and humans (Zurek
and Ghosh, 2014; Usui et al., 2015). Despite the importance of
houseflies in disease transmission, little is known about the preva-
ience of pathogens on houseflies within different parts of a dairy,
which could affect udder health, human health, and food shelf life
(Geden et al., 2021). This study aimed to identify bacterial species
associated with houseflies captured in different locations on a dairy
farm structured by fly sex and bacterial presence in internal parts or
external surfaces of flies.

This study was conducted on a commercial dairy farm in
New York State that belongs to the client base of Quality Milk
Production Services, Cornell University, Ithaca, New York.
Approximately 1,300 lactating Holstein cows were housed in freestall
pens, bedded with manure solids, milked 3 times per day, and fed
a TMR consistent with National Research Council requirements
(NRC, 2001).

The farm was selected based on a client list of the daily milk
sample pick-up and 24-h result program of Quality Milk Produc-
CO2. After enrichment, each sample was inoculated using cotton balls
from the external surface, 2 mL of sterile 1X PBS (Hardy Diagnostics) was added to each vial and gently shaken by inversion
for 5 min at room temperature and cultured according to the
procedure outlined above for bacterial isolation of the external
surface.

For the isolation of bacteria from the internal part of flies, the
first step was to sterilize the external surface. For this purpose, the
remaining PBS was removed from each vial, and flies were washed
by inversion for 5 min with 70% isopropyl alcohol (VWR Interna-
tional). Subsequently, the alcoholic solution was removed, and flies
were exposed to UV light in a CLASS II biological safety cabinet
(The Baker Company) for 10 min. To verify the decontamination
efficiency, PBS was added to each vial containing the flies, and it
was streaked on medium (1) and incubated at 37°C for 48 h. Flies
were transferred into 2-mL sterile disruption tubes containing five
1.7-mm zirconium beads and 1 mL of PBS. Homogenates were
obtained with 2 cycles on a bead beater (HT24, OPS Diagnostics),
1 min each at 7,000 rpm. Homogenates were centrifuged at 2,000 ×
g for 5 min at room temperature and cultured according to the
procedure outlined above for bacterial isolation of the external
surface.

Bacteria were identified by MALDI-TOF using MALDI Bio-
typer Microflex LT (Bruker Daltonics). Adaption of the MALDI-
TOF library and sample preparation were performed as described
by Randall et al. (2015). For all bacteria except NAS, a result was
considered accurate if the score value was ≥1.7 for genus level
identification, and a score ≥2.0 was set for a match at the species
level. For NAS, a score value ≥1.7 was chosen (Cameron et al.,
2018) to be indicative of a valid result at the species level (no
scores <1.7 were obtained). Mycoplasma growth was detected by
visual inspection of culture plates under an illuminated stage ste-
reomicroscope. Mycoplasma colonies were confirmed by a colony
PCR specific for Mycoplasma and Acholeplasma followed by
Sanger sequencing for species identification (Gioia et al., 2016).

A total of 143 flies were caught from the 3 farm sites; 101 were
identified as houseflies, and the remaining 42 were stable flies.
Only houseflies were used for further investigation and from
those, 53 (52.5%) flies were female and 48 (47.5%) were male.
Out of the 101 flies, a total of 27 (26.7%) were found in the calf
area, 42 (41.6%) were collected in the sick pen, and 32 (31.7%)
were collected in the milking parlor. All colonies grown on plates
were subjected to MALDI. A total of 485 isolates were obtained;
484 were bacterial and 1 was fungal. Based on MALDI scores,
91 (18.8%) were identified at the genus level and 392 (80.8%) at
the species level. Multiple colonies of the same bacterial genus
and species, if isolated from the same fly body’s location, were
reported only once. Two isolates grew on Mycoplasma medium;
both were submitted for molecular confirmation and speciation.
The average (mean ± SD) number of isolates detected per fly was
4.8 ± 2.4 ranging between 1 and 11. Figure 1 illustrates the taxo-
nomic classification of 485 isolates (A) and their distribution di-
vided by farm sites where houseflies were collected (B), flies’ sex
(C), and flies’ body site from where the isolates were obtained (D).

Out of the 101 flies, a total of 27 (26.7%) were found in the calf
area, 42 (41.6%) were collected in the sick pen, and 32 (31.7%)
were gram negative, and 1 isolate (0.4%) was identified as a fun-
gus. From the 2 isolates growing on Mycoplasma agar, one
was identified as Mycoplasma arginini and one as Acholeplasma
laidlawii. Both cases represent bacterial species classified as moli-
lices and for the present study were included in the gram-positive
cluster according to Razin et al. (1998). The isolate classified as
a fungus was identified by MALDI as Candida krusei and it was
obtained from the external surface of a male fly collected in the
sick pen. Based on the site of collection (Figure 1B), a total of 211
(43.5%) isolates were obtained from 42 flies captured in the sick
pen. Out of those, 126 (59.7%) were gram positive, 84 (39.8%)
were gram negative, and 1 isolate (0.4%) was identified as a fun-
gus. We obtained a total of 148 (30.5%) isolates from 27 flies of
the calf hutch area; 70 (47.3%) were gram positive and 78 (52.7%)
were gram negative. From 32 flies collected in the milking parlor,
a total of 126 (25.9%) isolates were obtained; 82 (65%) were gram
positive, and 44 (34.9%) were gram negative. The number of bac-
teria isolated from male or female flies was similar (Figure 1C). A
total of 252 (51.9%) isolates were from 48 male flies, 150 (59.5%)
were gram positive, 101 (40%) were gram negative, and 1 (0.4%)
was a fungus. In 53 female flies we found 233 (48%) isolates, of
which 128 (54.9%) were gram positive and 105 (45%) were gram
negative. The majority, 349 (71.9%), of isolates were retrieved
from internal parts, of which 170 (48.7%) were gram positive and
179 (51.3%) were gram negative. Only 136 (28%) isolates were
obtained from the external part of the flies, of which 108 (79.4%)
were gram positive, 27 (19.8%) were gram negative, and 1 (0.7%)
was a fungus (Figure 1D).

Infestations of houseflies on a dairy farm represent a concern
due to possible microorganisms they could carry. Some of those

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milk-spoiling organisms. We divided the isolates into 3 groups based on their potential impact on milk quality and health of both humans and animals. We detected a total of 17 species of pathogens that, to our knowledge, have not been previously reported from houseflies at dairies (Table 1); most of these are mastitis pathogens and some are milk-spoiling organisms. Some of the bacterial species found in the present study have been previously classified as milk-spoiling organisms (Scheldeman et al., 2004; Scatamburlo et al., 2015; Awasti et al., 2019), and these included *B. licheniformis* and *Lysinibacillus sphaericus*, which were found in flies collected in the milking parlor and the sick pen, *Pseudomonas aeruginosa* found in the calf hatch and the sick pen, and *Paenibacillus lactis* isolated from 1 fly of the milking parlor. Before this study, none of these 3 organisms have been isolated from houseflies collected in dairies. In most previous publications, these organisms were found in water, soil, or milking equipment.

We detected a total of 17 species of pathogens that, to our knowledge, have not been previously reported from houseflies at dairies (Table 1); most of these are mastitis pathogens and some are milk-spoiling organisms. Species of pathogens that we and others have detected in houseflies from dairies include *Enterococcus faecalis, E. coli*, and *Salmonella spp.* Other foodborne pathogens were *B. cereus, B. subtilis*, and *E. coli* carried by flies collected from all 3 farm locations. Other foodborne pathogens were *Salmonella spp.* found in the milking parlor, and *Staph. aureus* found in the calf hutch and the milking parlor. In our study, we found only 1 *Salmonella* isolate compared with Almeida et al. (2014) where *Salmonella* was found in 9.5% of muscoids.

1. In the group of organisms that can invade the teat canal and cause mastitis, we found NAS, *Enterococcus* spp., *Klebsiella* spp., and *E. coli* present in flies from all 3 farm locations. *Citrobacter* spp., *Enterobacter cloacae*, and *Pseudomonas aeruginosa* were only found in flies from calf hutches and the sick pen. *Lactococcus* spp. and *Staph. aureus* were found in flies from calf hutches and milking parlor. Only flies found in the sick pen carried *Corynebacterium stationis* and *Mycoplasma arginini*. *Streptococcus uberis* was only found in flies from the milking parlor. Except for *Enterococcus* spp., *Klebsiella pneumoniae*, *E. coli*, *Enterobacter cloacae*, *Staph. aureus*, and most of the NAS species, all other bacteria here classified as mastitis pathogens have not been reported to be isolated from houseflies in dairy farms before this study to our knowledge. Both *Staph. aureus* and *Mycoplasma* spp. are considered contagious mastitis pathogens and require timely management to prevent new infections within a dairy herd. It is well known that flies may be carriers of *Staph. aureus*, but no previous reports exist on the possibility to isolate *Mycoplasma* spp. from flies. *Mycoplasma arginini* is not one of the most pathogenic species, but it can persist for a long time under different environmental conditions (Nagatomo et al., 2001).

2. In the group of organisms previously classified as potential foodborne pathogens, we found *B. cereus, B. subtilis*, and *E. coli* carried by flies collected from all 3 farm locations. Other foodborne pathogens were *Salmonella* spp. found in the milking parlor, and *Staph. aureus* found in the calf hutch and the milking parlor. In our study, we found only 1 *Salmonella* isolate compared with Almeida et al. (2014) where *Salmonella* was found in 9.5% of muscoids.

3. Some of the bacterial species found in the present study have been previously classified as milk-spoiling organisms (Scheldeman et al., 2004; Scatamburlo et al., 2015; Awasti et al., 2019), and these included *B. licheniformis* and *Lysinibacillus sphaericus*, which were found in flies collected in the milking parlor and the sick pen, *Pseudomonas aeruginosa* found in the calf hatch and the sick pen, and *Paenibacillus lactis* isolated from 1 fly of the milking parlor. Before this study, none of these 3 organisms have been isolated from houseflies collected in dairies. In most previous publications, these organisms were found in water, soil, or milking equipment.

**Figure 1.** Bacterial isolate counts found in 101 houseflies obtained from 3 sites of 1 dairy farm. (A) Taxonomic classification of the isolates. Bacteria are classified as gram positive, gram negative, and fungus. (B) Bacteria count distribution between farm locations where flies were collected. (C) Bacteria count distribution between female and male flies. (D) Bacteria count distribution based on the flies’ body site.

Microorganisms are harmless, but others could cause an increase of infectious diseases in cows or negatively affect the milk quality. Mastitis is one of the most common diseases of dairy cattle with high economic impact (Halasa et al., 2007). It has been demonstrated that different species of flies, including head flies (*Hydrotaea irritans*) and horn flies, could act as a possible vector for mastitis pathogens, particularly summer mastitis in heifers (McDougall et al., 2009). Implications on milk quality could be associated with foodborne pathogens that represent a hazard for human health or spoilage microorganisms that compromise milk’s quality, flavor, and texture. From the total 392 organisms identified at the species level, 304 were selected based on their importance and possible implications in a dairy farm. Table 1 depicts the frequency distribution of 304 bacteria species isolated from external surfaces and internal parts of flies collected at different farm sites and their potential impact on milk quality and health of both humans and animals. We divided the isolates into 3 groups based on (1) mastitis-causing pathogens, (2) foodborne pathogens, and (3) milk-spoiling organisms.
healthy animals, humans, and even insects. In a dairy farm, these organisms are typically found in feces, manure, and bedding; all are places where houseflies habitually go for feeding. Bacteria present in the organic matter stick on flies’ body surfaces and can be carried around. The constant movement of houseflies back and forth from feces to food and drinking water places humans and animals at risk of infection. Enterococcus faecalis, E. coli, Klebsiella pneumoniae, and Enterobacter cloacae can be opportunistic invaders of the mammary glands and are considered environmental mastitis-causing pathogens. Mastitis caused by E. coli and Klebsiella spp. can be severe and occasionally fatal. The predominance of a single strain of E. coli or Klebsiella spp. in a dairy farm could indicate contagious transmission or exposure of multiple cows to an environmental point source (Muñoz et al., 2007). For the aim of the present study, all isolates were identified at the genus level; none of them were further tested by strain typing. For future studies, it would be interesting to see if there are matches between strains found in flies and strains of the same organism found in other sources such as milk samples from cows with clinical mastitis. The identification at the strain level could also be relevant to point out the presence of critical foodborne pathogens such as Shiga toxin-producing E. coli; this is important mainly for dairy farms that sell unpasteurized milk (Murinda et al., 2019). To our knowledge, this is the first study describing the distribution of the bacterial communities carried by flies in different areas of the same farm. Our results showed that the pathogens found differ by the farm location where flies were collected. Although 9 of the pathogens were detected in all 3 collection sites, 13 pathogens were found in only one of the locations within the facility. Considering that flies travel 5 to 32 km, our data give a snapshot of what these flies carried in the given location at sampling time. For future research, it would be interesting to collect flies on specific sites over time to see if there are circadian or seasonal changes or if there is a concentration of certain organisms on one site despite the flight radius of the fly. Indeed, the observations of this study need to be specifically associated with the farm selected where flies were collected. Because bacterial communities might be highly variable from farm to farm, it would be necessary for future studies to conduct the same kind of investigation in more than one farm, even located in different areas.

Table 1. Selected bacterial species isolated from 101 houseflies in which bacterial selection was based on effects on udder health, human health, and milk quality

| Genus          | Species                  | Effect | Farm site | Location | n (%) |
|----------------|--------------------------|--------|-----------|----------|-------|
| Citrobacter    | freundii                 | M      | H, C      | E, I     | 4 (1.0) |
| Enterobacter   | cloacae                  | M      | H, C, P   | E, I     | 25 (6.4) |
| Escherichia    | coli                     | M, F   | H, C, P   | E, I     | 59 (15.1) |
| Klebsiella     | pneumoniae               | M      | H, C, P   | E, I     | 26 (6.6) |
|                 | varicola                 | M      | H, P      | E, I     | 7 (1.8)  |
|                 | oxytocis                 | M      | C, P      | E, I     | 4 (1.0)  |
| Salmonella     | spp.                     | F      | P         | I        | 1 (0.3)  |
| Pseudomonas    | aeruginosa               | M, S   | H, C      | E, I     | 7 (1.8)  |
| Enterococcus   | faecalis                 | M      | H, C, P   | E, I     | 1 (0.3)  |
|                 | hirae                    | M      | C         | I        | 1 (0.3)  |
|                 | faecium                  | M      | H, C, P   | E, I     | 18 (4.6) |
|                 | gallinarum               | M      | H         | I        | 2 (0.5)  |
| Lactococcus    | garvieae                 | M      | C, P      | E, I     | 6 (1.5)  |
|                 | lactis                   | M      | C         | E        | 2 (0.5)  |
| Streptococcus  | uberis                   | M      | P         | I        | 1 (0.3)  |
| Staphylococcus | aureus                   | M, F   | C, P      | I        | 2 (0.5)  |
|                 | capitis                  | M      | P         | E        | 1 (0.3)  |
|                 | chromogenes              | M      | H, P      | I        | 1 (0.3)  |
|                 | haemolyticus             | M      | H, C, P   | E, I     | 10 (2.6) |
|                 | lugdunensis              | M      | H         | E, I     | 2 (0.5)  |
|                 | saprophyticus            | M      | H, C, P   | E, I     | 10 (2.6) |
|                 | scini                    | M      | H, C, P   | E        | 2 (0.5)  |
|                 | succinarius              | M      | H, P      | E        | 10 (2.6) |
|                 | xylosus                  | M      | C         | E        | 1 (0.3)  |
| Corynebacterium| stationis                | M      | H         | E        | 1 (0.3)  |
| Bacillus       | cereus                   | F, S   | H, C, P   | E, I     | 16 (4.1) |
|                 | subtilis                 | F      | H         | E        | 1 (0.3)  |
|                 | licheniformis            | S      | H, P      | E        | 7 (1.8)  |
| Lysinibacillus | sphaericus               | S      | H, P      | I        | 3 (0.8)  |
| Paenibacillus  | lactis                   | S      | P         | E        | 1 (0.3)  |
| Mycoplasma     | arginini                 | M      | H         | I        | 1 (0.3)  |

1Effect on udder health (M), human health (F), and milk quality (S).
2Farm site (sick pen, H; calf area, C; and milking parlor, P).
3Location (external surface, E; internal parts, I).
4Number and percentage (calculated from all isolates).
5Species has not been previously identified from houseflies at dairies.
faecalis, and E. coli. However, contrary to our study, Bahrndorff et al. (2017) used a culture-independent amplicon sequencing approach to characterize the bacterial communities of houseflies on dairy farms.

The large number of pathogens detected in this study, several reported for the first time, illustrate that surveys of pathogens on houseflies can help to understand and ultimately reduce disease spread.

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