

# Prevalence and Relative Risk of *Cronobacter* spp., *Salmonella* spp., and *Listeria monocytogenes* Associated with the Body Surfaces and Guts of Individual Filth Flies

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Although flies are important vectors of food-borne pathogens, there is little information to accurately assess the food-related health risk of the presence of individual flies, especially in urban areas. This study quantifies the prevalence and the relative risk of food-borne pathogens associated with the body surfaces and guts of individual wild flies. One hundred flies were collected from the dumpsters of 10 randomly selected urban restaurants. Flies were identified using taxonomic keys before being individually dissected. *Cronobacter* spp., *Salmonella* spp., and *Listeria monocytogenes* were detected using the PCR-based BAX system Q7. Positive samples were confirmed by culture on specific media and through PCR amplification and sequencing or ribotyping. Among collected flies were the housefly, *Musca domestica* (47%), the blowflies, *Lucilia cuprina* (33%) and *Lucilia sericata* (14%), and others (6%). *Cronobacter* species were detected in 14% of flies, including *C. sakazakii*, *C. turicensis*, and *C. universalis*, leading to the proposal of flies as a natural reservoir of this food-borne pathogen. Six percent of flies carried *Salmonella enterica*, including the serovars Poona, Hadar, Schwarzengrund, Senftenberg, and Brackenridge. *L. monocytogenes* was detected in 3% of flies. Overall, the prevalence of food-borne pathogens was three times greater in the guts than on the body surfaces of the flies. The relative risk of flies carrying any of the three pathogens was associated with the type of pathogen, the body part of the fly, and the ambient temperature. These data enhance the ability to predict the microbiological risk associated with the presence of individual flies in food and food facilities.

Food-borne illnesses continue to be a serious public health problem. The Centers for Disease Control and Prevention (CDC) estimate that the consumption of contaminated food in the United States is the cause of illness in approximately one out of six people, leading to 128,000 hospitalizations and 3,000 deaths each year (13). These food-borne diseases are associated with 31 known pathogens (74) and with an assemblage of unspecified agents, including microbes, toxins, and other substances (73). The economic burden of these illnesses should not be underestimated. Recent assessments calculated the health care-related cost to be approximately \$51 billion per year in the United States, including productivity losses and mortality (75).

There are several mechanisms whereby food products can become contaminated. Some examples include improper agricultural practices, such as the use of nonpotable water for irrigation of crops, the use of improperly sanitized manufacturing equipment and utensils, inappropriate storage and transportation temperatures, poor food handling practices, and lack of rodent and/or insect control, to name a few (26, 88). Insects have been recognized as playing an important role in the spread of food-related diseases as they can deliver viable pathogens either directly, through contact with food, or indirectly, through contact with food contact surfaces and utensils (91). Among insects, flies have long been implicated in the transmission of pathogens, contaminating food and water and causing some of the most devastating diseases affecting humans, such as typhoid fever (1) and cholera (52). Their association with decaying matter, garbage, feces, and other similar sources and their endophilic (entering buildings) and synanthropic (associated with humans) behaviors make them vectors of pathogens that cause enteric diseases, such as *Salmonella* spp. (67), *Listeria monocytogenes* (39), *Campylobacter* spp.

(36), *Escherichia coli* O:157 (17), and the genus *Cronobacter* (formerly *Enterobacter sakazakii*) (60).

The indigenous bacterial community in the intestinal tracts of flies can be up to 10 times greater than the body surfaces (body, head, legs, and wings) (81). This indigenous microbiota has been shown to play an important role in the life cycle of flies and in their feeding, oviposition, and mating behaviors, possibly driving the evolution of new species (19, 79, 82). However, the role of food-borne pathogens on the body surfaces or in the intestinal tracts of flies is not totally understood. They can be opportunistic pathogens (89), but they can also establish symbiotic relationships with the host's commensal microbiota (56). Food-borne pathogens can remain in the intestines for a greater length of time than on the body surfaces (40, 71), where they are able to multiply (33) and colonize the fly's digestive tract (61), increasing the potential for dissemination.

Flies can be either mechanical or biological vectors of food-borne pathogens (22). As mechanical vectors, flies are vehicles of pathogens that do not develop or multiply in their body, contaminating food through contact with their body surfaces (57, 81). However, if the pathogen passes through the alimentary tract of the host and undergoes a stage of development or multiplication, the host is considered a biological vector (22, 64, 69). Since some

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food-borne pathogens have been shown to multiply in the intestinal tracts of the flies (33, 51, 61), flies can be considered biological vectors of these pathogens, contaminating food through regurgitation (vomit spots) and defecation (22, 64). Consequently, knowing if food-borne pathogens are located on the body surfaces or in the guts of an individual fly is important as this may have epidemiological implications. Unfortunately, most of the studies that have associated wild flies with food-borne pathogens have dealt with pooled groups of flies rather than individual specimens (27, 34, 39, 58, 67, 85), making food risk assessment on a per fly basis difficult. The few studies that have used single flies have macerated or homogenized the sample, making it impossible to differentiate if food-borne pathogens were on the body surfaces or in the guts of collected flies (43, 60).

From the 108 known families of flies, 29 are believed to be associated with the transmission of pathogens and even fewer with the transmission of food-borne pathogens (65). Four of these families, containing 12 fly species, were included in the list of pests that exhibit attributes contributing to the spread of food-borne pathogens. Such attributes include contacting potential sources of pathogens and endophilic, synanthropic, and communicative behaviors. Examples of pests exhibiting those attributes were published in the revised U.S. Food and Drug Administration (FDA) "filth strategy" (66) and have become known as the "Dirty 22." Evidence of the presence of any of these pests in food, food processing areas, or food storage facilities is considered an indication of insanitation. Although flies are well documented for being a public health threat, and they comprise the majority of pests included in the "Dirty 22," there is little information to accurately and quantitatively assess the food-related risk of the presence of an individual fly (65), especially in urban areas (42). Current food safety guidance lacks the scientific evidence required both to establish the prevalence of food-borne pathogens associated with these insects and to differentiate the species of flies that pose a factual threat for spreading food-borne pathogens (65).

The purpose of this study was to provide evidence for the prevalence of *Cronobacter* spp., *Salmonella* spp., and *L. monocytogenes* associated with the body surfaces and guts of individual flies collected from dumpsters outside restaurants in urban areas. Evidence for flies as a possible natural reservoir for *Cronobacter* spp. is also discussed. Additionally, the relative risk associated with a single fly transmitting these three food-borne pathogens was quantified. This information fills data gaps in the risk profile on the frequency of transmission of food-borne pathogens by different species of flies.

## MATERIALS AND METHODS

**Collection of flies.** A total of 100 flies were collected around the dumpsters of 10 randomly selected restaurants located in an 18-km radius from a centrally located point in the metropolitan area of Washington, DC. Each collection site was assigned a random number between 1 and 10. Flies were collected during the months of August and September 2011 between 10:00 a.m. and noon. The ambient temperature was recorded at the time of collection. Flies were collected individually using sterile entomological sweep nets. The nets were transferred to the lab in a cooler and placed at  $-20^{\circ}\text{C}$  for 2 to 5 min until the flies were immobilized. All procedures, from collection to analysis, were performed on individual flies.

**Identification and dissection of flies.** Using sterile forceps, each fly was transferred to 2-ml tubes containing 1 ml of buffered peptone water (BPW; Difco, Becton, Dickinson Company, Sparks, MD). Tubes were mixed gently by inversion for 2 min, allowing the whole body of the fly to

be in contact with the medium. Flies were then removed from the BPW medium, placed in clean 2-ml tubes, and surface disinfected as follows: they were submerged in 70% ethanol for 1 min and rinsed with sterile water, followed by submersion in 0.05% bleach for 1 min and then two rinses in sterile water (90). To verify the effectiveness of the disinfection process, 100- $\mu\text{l}$  aliquots of water from the last rinse were plated on Trypticase soy agar (TSA; Oxoid, Cambridge, United Kingdom) and incubated at  $37^{\circ}\text{C}$  for 24 h. Surface-disinfected flies were transferred to sterile petri dishes and identified to the species level using dichotomous keys for dipteran families (29, 32). The sex of the flies was determined through external morphology (21) before aseptic dissection of the guts. The guts of each fly were placed in 2-ml tubes containing 1 ml of BPW with 0.5-mm zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK) and vigorously shaken for 10 min in a Genie cell disruptor (Scientific Industries, Inc., Bohemia, NY). Vouchers of collected fly specimens were deposited at the Smithsonian National Museum of Natural History (USNM) (Diptera Unit, Alcohol Collection, lot no. 1205119) in Washington, DC.

**Pathogen detection.** The PCR-based BAX system Q7 (DuPont Qualicon, Wilmington, DE) was used to screen for *Salmonella* (standard assay), the *Listeria* genus (24E assay), and *E. sakazakii* (standard assay; used to detect *Cronobacter* spp.) as per the manufacturer's protocols. Primary enrichment was performed by transferring  $\sim 330\ \mu\text{l}$  of BPW-S (surface) and BPW-G (guts) mixtures to the following media: (i) for *Salmonella* spp., 1 ml of prewarmed ( $42^{\circ}\text{C}$ ) BPW with incubation in a recirculating water bath at  $42.5^{\circ}\text{C}$  for 22 to 24 h; (ii) for *Cronobacter* spp., 1 ml of prewarmed ( $37^{\circ}\text{C}$ ) BPW with Novobiocin (10 mg/liter) with incubation at  $37^{\circ}\text{C}$  for 22 to 26 h; and (iii) for *Listeria* spp., 1 ml of freshly prepared room temperature 24 *Listeria* enrichment broth (24 LEB) with selective supplement (Oxoid, Cambridge, United Kingdom) with incubation at  $37^{\circ}\text{C}$  for  $44 \pm 5$  h. Secondary enrichment was performed for the detection of *Salmonella* spp. and *Cronobacter* spp. For this, 100  $\mu\text{l}$  of enriched samples was transferred to 400  $\mu\text{l}$  of prewarmed ( $37^{\circ}\text{C}$ ) brain heart infusion (BHI) broth (Difco, Becton Dickinson Company, Sparks, MD) and the mixture was incubated at  $37^{\circ}\text{C}$  for 3 h. After enrichment was completed, all samples were prepared and processed according to the protocol described in the BAX system user's guide for each bacteria. To increase probability of detection, 20  $\mu\text{l}$  of enriched samples (instead of 5  $\mu\text{l}$ , as instructed by the manufacturer) were transferred to 200  $\mu\text{l}$  of lysis buffer. *Salmonella enterica* subsp. *arizonae*, *Cronobacter sakazakii*, and *L. monocytogenes* from FDA bacterial collections were included as positive controls, and sterile medium was used as a negative control. Tubes from primary and secondary enrichments were kept either in the refrigerator (*Salmonella* spp. and *Listeria* spp.) or at room temperature (*Cronobacter* spp.) for further confirmation analysis of BAX-positive samples.

**Confirmation of *Cronobacter* spp.** To confirm the presence of *Cronobacter* spp. in BAX-positive samples, a 3-mm loopful (10  $\mu\text{l}$ ) of BHI broth from the secondary enrichment was streaked on violet red bile glucose agar (VRBG; Difco, Becton, Dickinson Company, Sparks, MD) and on Brilliance *Cronobacter* agar, also known as the Druggan-Forsythe-Iversen formulation (DFI; Oxoid, Cambridge, United Kingdom), for isolation of single colonies. All plates were incubated at  $36^{\circ}\text{C}$  for 22 to 24 h. Five presumptive *Cronobacter* colonies from the plates described above were subcultured on DFI and incubated at  $36^{\circ}\text{C}$  for 22 to 24 h. One blue-pigmented colony on DFI was randomly selected and further streaked on TSA plates and onto two chromogenic media, DFI and ChromID (bio-Mérieux, S.A., Marcy l'Etoile, France). Plates were incubated at  $25^{\circ}\text{C}$  for 48 to 72 h (TSA) and at  $36^{\circ}\text{C}$  for 22 to 24 h (ChromID and DFI). The color of bacterial colonies on each medium was recorded. Additionally, presumptive identification of the selected colony was performed using the commercial Rapid API-20E biochemical identification system (bio-Mérieux, S.A., Marcy l'Etoile, France), according to the manufacturer's instructions. Because several other microorganisms exhibited blue-pigmented colonies on DFI agar and were shown to be non-*Enterobacter* spp. in the Rapid API-20E system, DNA from presumptive target bacteria was extracted for molecular identification.

**Molecular identification of *Cronobacter* spp.** Bacterial DNA was extracted from TSA cultures using the PrepMan Ultra reagent (Applied Biosystems) as indicated by the manufacturer. Ribosomal 16S rRNA and 1,6- $\alpha$ -glucosidase genes were amplified from 1  $\mu$ l of 2- to 10-fold-diluted DNA using the primer pairs P0-P6 (46) and EsAgf-EsAgr (55), respectively, at the recommended concentrations. The PCR mixtures were set up in a total volume of 50  $\mu$ l using the EmeraldAmp GT-PCR master mix (TaKaRa Bio, Inc.). The PCR cycling conditions for each gene were the same as those described by the authors. PCR products were purified and sequenced by Retrogen, Inc. (San Diego, CA), using the respective primer pairs. Sequence files were imported into Sequencher 5.0 (GeneCodes, Ann Arbor, MI). The primers were trimmed off using default parameters, and bidirectional sequences were assembled into contigs with default settings. Sequences were compared against those in the NCBI nucleotide BLAST (nr/nt) database (<http://blast.ncbi.nlm.nih.gov>) using the megablast algorithm. Sequence similarities of  $\geq 99\%$  from presumptive *Cronobacter* isolates were considered reliable identifications for this organism.

*E. sakazakii* has been reclassified into the new genus *Cronobacter* (47). This genus contains seven species: *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, *C. condimenti*, and *C. universalis* (formerly *Cronobacter* genomospecies 1) (49). Full-length 16S rRNA sequences of 64 *Cronobacter* strains, representative of five species, along with two sequences from *Pantoea* species and one sequence from *Enterobacter helveticus* (included as an outgroup) were retrieved from GenBank (46, 47, 49). Retrieved sequences and sequences obtained in this study were aligned using the ClustalX software (Lasergene, Madison, WI). To clarify the taxonomic position of *Cronobacter* strains obtained in this study, phylogenetic analyses were performed using the neighbor-joining (NJ) method, combined with the Hasegawa, Kishino and Yano (HKY) nucleotide substitution model, found in Geneious Pro software (version 5.6). A consensus tree was created using the resample tree option and 1,000 bootstrap replicates.

**Confirmation of *Salmonella* spp.** To confirm the presence of *Salmonella*, two 100- $\mu$ l aliquots of BHI broth were removed from the secondary enrichment. The first aliquot was added to 10 ml of Rappaport-Vassiliadis (RV) medium (Oxoid, Cambridge, United Kingdom). The second one was added to 1 ml of tetrathionate (TT) broth (Difco, Becton, Dickinson Company, Sparks, MD). Both media were incubated at 42.5°C for 22 to 24 h. After incubation, samples were plated as recommended by the FDA *Bacteriological Analytical Manual* (BAM) protocol for *Salmonella* (2). Presumptive *Salmonella* colonies were identified using the commercial Rapid API-20E system according to the manufacturer's instructions. Ribotyping was performed on all presumptive *Salmonella* strains identified by the commercial Rapid API-20E system using the RiboPrinter microbial characterization system (DuPont Qualicon, Wilmington, DE) with the PvuII protocol, following the manufacturer's instructions. Identified *Salmonella* serovars were tested again using the BAX system with the *Salmonella*-2 standard assay.

**Confirmation of the *Listeria* genus and *L. monocytogenes* strains.** BAX samples that were positive for the genus *Listeria* 24E assay were run using the *L. monocytogenes* BAX standard assay, according to the manufacturer's instructions. Ten microliters of primary enrichment medium from 24E *Listeria* genus BAX-positive samples was streaked on two Brilliance *Listeria* agar (BLA) plates (Oxoid, Cambridge, United Kingdom; formerly Oxoid chromogenic *Listeria* agar [OCLA]). The plates were incubated at 37°C for 22 to 26 h and then examined for both the presence of blue-green colonies and the presence of an opaque white halo indicating activity of lecithinase, an enzyme associated with virulence in *Listeria* spp. Two BAX-negative samples were randomly selected and cultured on BLA plates to corroborate the accuracy of the BAX assay. Presumptive *Listeria* species were confirmed by ribotyping as described before using the EcoRI protocol according to the manufacturer's instructions. Stocks of overnight cultures of all confirmed bacterial strains were placed in 1 ml of 30% glycerol-BPW at -20°C for long-term storage. Vouchers of bacterial strains isolated from flies in this study were also deposited at the Food and

Drug Administration, Center for Food Safety and Applied Nutrition, Office of Regulatory Science, Division of Microbiology, College Park, MD (FDA/CFSAN/ORS/DM; collection no. SAL3538 to SAL3544, LIS0149 to LIS0156, and ENTB0350 to ENTB0368).

**Data analysis.** To estimate the proportion of food-borne pathogens carried by a population of flies, a random sample size of 96 flies was the minimum sample size required to be 95% confident that the sample percentage was in error by no more than 10% (84). Hence, a sample of 100 flies was selected for this study. Statistical analyses of the presence of food-borne pathogens on the body surfaces and in the guts of collected flies were performed using the  $\chi^2$  test (PROC FREQ; SAS v9.3, 2005; SAS Institute, Inc.). Overall associations between the presence of food-borne pathogens, the fly's body part, fly species, and collection sites were analyzed using the Fisher's exact probability test (PROC FREQ) (72). If significant overall associations were detected, pairwise comparisons among the levels of the significant variables were performed. The Fisher's exact test was used instead of the  $\chi^2$  test, since  $\chi^2$  analysis does not accurately calculate expected frequencies that are below 5. A two-tailed *P* value of  $< 0.05$  indicated statistical significance.

The predicted probability of a fly carrying food-borne pathogens on the body surfaces or in the guts was analyzed using the SAS logistic regression procedure (PROC LOGIT, 2005; SAS Institute, Inc.). Pathogen presence was the categorical dichotomous response variable (positive or negative; *n* = 600). The relationship between the response variable and the predictor variables, along with their interactions, was analyzed with the full logistic probability model described in equation 1

$$\text{Logit}(P) = \alpha + \beta_1 \times \text{pathogen} + \beta_2 \times \text{body part} + \beta_3 \times \text{fly species} + \beta_4 \times \text{fly sex} + \beta_5 \times \text{ambient temperature} \quad (1)$$

where  $\text{logit}(P) = \ln [P/(1 - P)]$ ,  $\ln$  is the natural log, *P* is the probability of the presence of bacterial food-borne pathogens,  $\alpha$  is the *P* intercept,  $\beta_1$  to  $\beta_5$  are regression coefficients, and pathogen (*Cronobacter* spp., *Salmonella* spp., or *L. monocytogenes*), body part (surface and guts), fly species, fly sex (male or female), and the ambient temperature (°C) at the time of collection are the predictor variables. The stepwise variable selection method with analysis of maximum likelihood estimates based on a Wald  $\chi^2$  *P* value of  $< 0.05$  was used to specify the best-fit, reduced model. The receiver operating characteristics (ROC) curve was used as a measurement of the goodness-of-fit of the model. The ROC curve quantifies the power of the predicted values using the area under the ROC curve (AUC). AUC values of  $> 0.7$  are considered to show acceptable discrimination, those of  $> 0.8$  are considered to show excellent discrimination, and those of  $> 0.9$  are considered to show outstanding discrimination (44).

**Nucleotide sequence accession numbers.** Full-length sequences of 16S rRNA ( $> 1,300$  bp) and sequences from the 1,6- $\alpha$ -glucosidase gene of *Cronobacter* strains obtained from this study have been deposited in the GenBank database (<http://www.ncbi.nlm.nih.gov/GenBank/index.html>) under accession no. JQ963896 to JQ963914 and JX315535 to JX315553, respectively.

## RESULTS

Four families were identified from collected flies: Calliphoridae (49%), Muscidae (48%), Sarcophagidae (2%), and Anthomyiidae (1%). All specimens but one were identified to the species level using taxonomic keys. Six different species of flies were found. The most abundant fly species was the housefly, *Musca domestica* (47%), followed by the blowflies, *Lucilia cuprina* (33%) and *Lucilia sericata* (14%). Other species included the secondary screw-worm, *Cochliomyia macellaria* (2%), the red-tailed flesh fly, *Sarcophaga haemorrhoidalis* (2%), and the black garbage fly, *Ophyra leucostoma* (1%). One specimen (1%) was identified to the family level as Diptera: Anthomyiidae (Norman E. Woodley, Systematic Entomology Laboratory, Agricultural Research Service, U.S. Department of Agriculture). Sixty-nine flies were males, and 31 were

females. The ambient temperature at the time of collection ranged between 16 and 36°C.

**Detection and confirmation of *Cronobacter* spp.** Forty-five samples were positive for the *E. sakazakii* (*Cronobacter*) BAX standard assay. BAX-positive samples required several subculturing steps to obtain well isolated presumptive *Cronobacter* colonies on specific media. Nineteen out of the 45 BAX-positive samples exhibited typical pigmentation of the colonies on all three media: yellow, pale yellow, or cream-white pigmentation on TSA, blue-green pigmentation on DFI, and blue-black or blue-gray pigmentation on ChromID. However, the Rapid API-20E system identified 26/45 samples belonging to the genus *Enterobacter*: 24 samples were identified as *E. cloacae*, with percent identities ranging from 58.5 to 99.9%, and two samples were identified as *E. aerogenes*, with percent identities of 83.7 and 96% (see Table S1 in the supplemental material). The Rapid API-20E system also identified some typical *Cronobacter* pigmented colonies on chromogenic media as non-*Enterobacter* but belonging to some neighboring genera, such as *Citrobacter freundii*, *Providencia stuartii*, *Proteus vulgaris*, *Pantoea* spp., and *Klebsiella pneumoniae*. Therefore, the pigmentation of presumptive *Cronobacter* colonies on TSA or either of the two chromogenic media used, along with the identification of the Rapid API-20E system, was not a definitive criterion to confirm *Cronobacter* spp. on all BAX-positive samples from collected flies. DNA was subsequently obtained from individual colonies of all 45 *E. sakazakii* (*Cronobacter*) BAX-positive samples and PCR amplified using the 16S rRNA and 1,6- $\alpha$ -glucosidase (EsAg) primers. All 45 samples gave a PCR product of the expected size (>1,300 bp) when the 16S rRNA primers were used. However, just 19 samples amplified a specific fragment of approximately 1,500 bp when the EsAg primers were used (see Table S1).

Edited sequences from all amplified fragments were compared against those in the NCBI nucleotide database. Sixteen out of the 24 samples that were identified as *E. cloacae* using the Rapid API-20E system were identified as *C. sakazakii* after BLAST searches of the 16S rRNA sequences against those in the nucleotide database. One more sample was identified as *C. turicensis* and another as *C. malonaticus* (see Table S1 in the supplemental material). The remaining six samples were identified as *Enterobacter* spp. (4 samples), *Enterobacter cancerogenus* (1 sample), and *E. cloacae* (1 sample). Additionally, 16S rRNA sequences from the two bacterial strains identified as *E. aerogenes* with the Rapid API-20E system were identified as *C. turicensis* and *Enterobacter* spp. (see Table S1).

Overall, 16S rRNA sequences from 19 bacterial strains were identified as *Cronobacter* spp., with percent identities of  $\geq 99\%$ . Results were consistent as these 19 bacterial strains were the only ones that amplified the 1,6- $\alpha$ -glucosidase gene and were identified as *C. sakazakii* (17 strains) and *C. turicensis* (2 strains) using BLAST. The same 19 *Cronobacter* strains were the only ones showing typical pigmentation of the colonies on all three media used (see Table S1 in the supplemental material). Consequently, only these 19 samples were considered positive for *Cronobacter* spp. and included for further analysis.

A 16S rRNA NJ phylogenetic tree was constructed to clarify the taxonomic position of *Cronobacter* species obtained in this study (Fig. 1). Five well-defined clusters corresponding to five *Cronobacter* biogroups were delineated on the phylogenetic tree. Seventeen *Cronobacter* sequences from collected flies clustered within the *C. sakazakii* group. One sequence, isolated from the surface of

*S. haemorrhoidalis*, clustered within the *C. turicensis* group, and another sequence, isolated from the surface of *M. domestica*, clustered within the *C. universalis* group (Fig. 1).

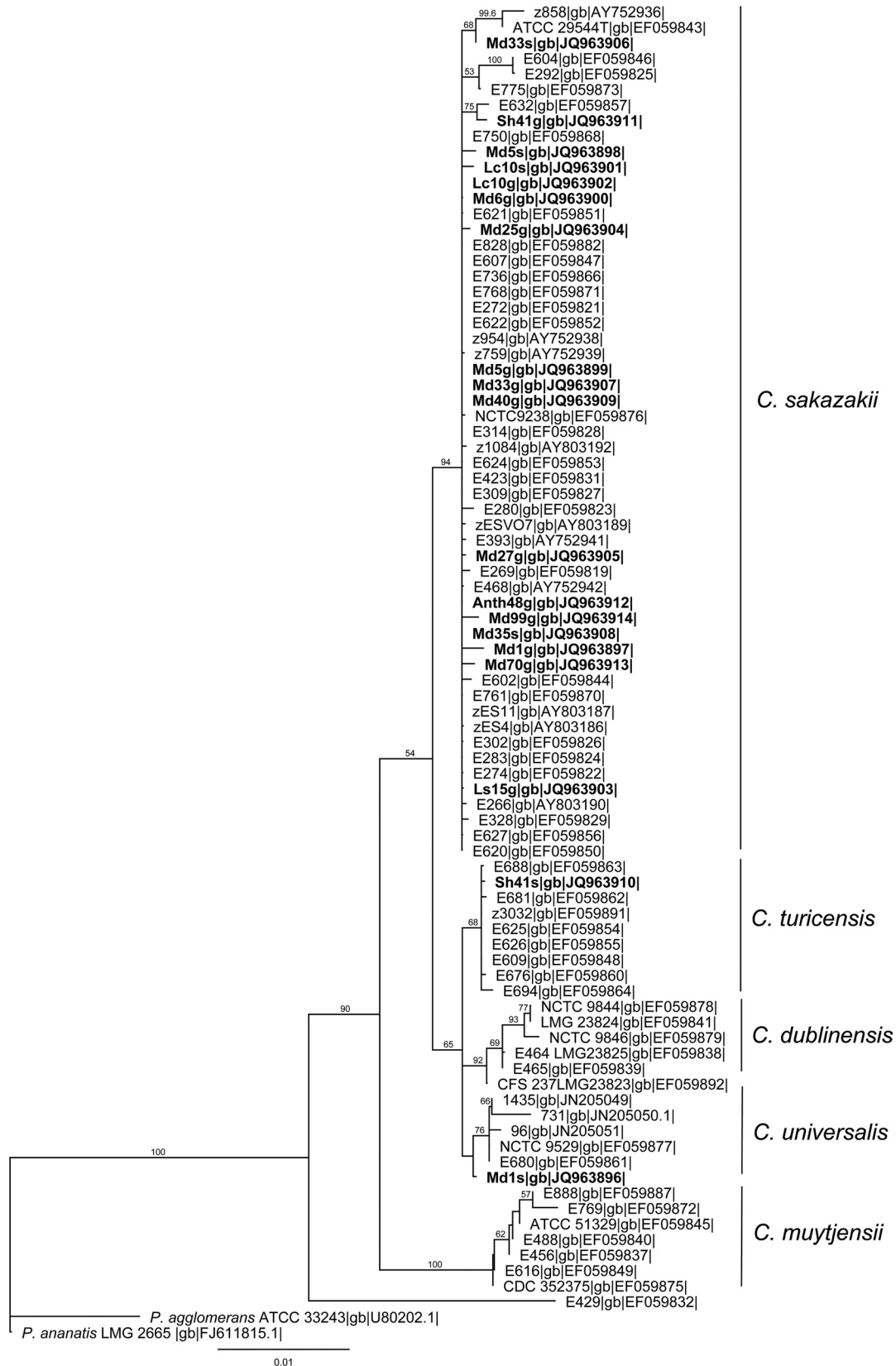
**Detection and confirmation of *Salmonella* spp.** Seven samples were positive for the *Salmonella* BAX assay. Typical *Salmonella* colonies with black centers were observed on bismuth sulfite (BS) agar, xylose lysine desoxycholate (XLD) agar, and Hektoen enteric (HE) agar. From each sample, one to three typical and well-isolated *Salmonella* colonies were randomly selected for presumptive generic identification of *Salmonella* with the commercial Rapid API-20E system. All colonies were identified as *Salmonella* spp., with percent identities ranging from 67.5 to 99.5%. One colony from each sample was selected for ribotyping analysis using the PvuII restriction enzyme protocol.

Five ribotype patterns, corresponding to five *Salmonella* serovars, were obtained from colonies recovered from the seven *Salmonella* BAX-positive samples (Fig. 2a). Two *Salmonella enterica* serovar Senftenberg (DuPont ID pattern DUP-PUVII-1153) strains were isolated from the guts of two flies (*M. domestica* and *L. cuprina*), both collected from site 10, with identity similarities of 0.97 and 0.87, respectively. One *S. enterica* serovar Hadar strain (DUP-PUVII-3173), with identity similarity of 0.98, was isolated from the guts of one *M. domestica* fly collected from site 7. Three other distinctive *Salmonella* serovars were recovered from three flies collected from site 6: *S. enterica* serovar Poona (DUP-PUVII-3216) was recovered from both the body surfaces and guts of *L. cuprina*, with identity similarities of 0.94 and 0.96, respectively; *S. enterica* serovar Schwarzengrund/Bredeney (DUP-PUVII-1148) was recovered from the guts of *L. cuprina*, with 0.97 identity similarity; and *S. enterica* serovar Brackenridge (DUP-PUVII-3161) was recovered from the guts of *L. sericata*, with 0.96 identity similarity. All bacterial strains were also positive for the *Salmonella*-2 BAX assay.

**Detection and confirmation of the *Listeria* genus and *L. monocytogenes*.** Eight samples tested positive for the 24E *Listeria* genus BAX kit. Four of these were positive for the *L. monocytogenes* BAX standard assay. All *Listeria* BAX-positive samples were confirmed by cultures showing typical blue-green *Listeria* colonies on chromogenic BLA plates. However, none of the strains showed an opaque white halo. No bacterial growth was observed from *Listeria* genus BAX-negative samples.

The four samples that were BAX positive for both the *Listeria* genus and *L. monocytogenes* were confirmed as *L. monocytogenes* by the DuPont ID pattern DUP-1042 of the RiboPrinter database, with identity similarities ranging from 0.92 to 0.95 (Fig. 2b). The four samples that were BAX positive for the *Listeria* genus but BAX negative for the *L. monocytogenes* assay were confirmed as *Listeria innocua* and presented three different DuPont ID patterns—DUP-1007, DUP-1009, and DUP-1010—with identity similarities ranging from 0.89 to 0.98.

The four samples that were positive for *L. innocua* were from the guts of four different flies (two *M. domestica* flies, one *L. cuprina* fly, and one *L. sericata* fly) collected from four different sites (sites 1, 3, 5, and 7). The four samples that were positive for *L. monocytogenes* were isolated from three flies (two *L. cuprina* flies and one *L. sericata* fly) collected from site 6, with one of the *L. cuprina* flies carrying *L. monocytogenes* on both the body surfaces and the guts (Fig. 2b). Because *L. innocua* is not considered a human food-borne pathogen, only the four strains that were con-



**FIG 1** Phylogenetic tree of 16S rRNA full-length sequences of *Cronobacter* strains. The neighbor-joining method combined with the model of Hasegawa, Kishino, and Yano (HKY) and 1,000 bootstrap replicates were used. *Cronobacter* strains obtained in this study and their GenBank accession numbers are in boldface. The bar indicates 1% estimated sequence divergence.

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Site #	Fly		Body Part	Sample ID/ Strain	DuPont Label	DuPont ID	ID Similarity	Riboprinter Pattern	BAX-Salmonella-2 Assay		
	Family	Species							BAX-24E <i>Listeria</i> genus	BAX-24E <i>L. monocytogenes</i>	
-	-	-	-	Reference	<i>S. Senftenberg</i>	DUP_PVUII-1153	-		-	-	
10	6	Muscidae	<i>Musca domestica</i>	Guts	Md6g	<i>S. Senftenberg</i>	DUP_PVUII-1153	0.97		Positive	Positive
	10	Calliphoridae	<i>Lucilia cuprina</i>	Guts	Lc10g	<i>S. Senftenberg</i>	DUP_PVUII-1153	0.87		Positive	Positive
7	11	Muscidae	<i>Musca domestica</i>	Guts	Md11g	<i>S. Hadar</i>	DUP_PVUII-3173	0.98		Positive	Positive
6	81	Calliphoridae	<i>Lucilia cuprina</i>	Surface	Lc81s	<i>S. Poona</i>	DUP_PVUII-3216	0.94		Positive	Positive
				Guts	Lc81g	<i>S. Poona</i>	DUP_PVUII-3216	0.96		Positive	Positive
	82	Calliphoridae	<i>Lucilia cuprina</i>	Guts	Lc82g	<i>S. Schwarzenrund/Bredeney</i>	DUP_PVUII-1148	0.97		Positive	Positive
	90	Calliphoridae	<i>Lucilia sericata</i>	Guts	Ls90g	<i>S. Brackenridge</i>	DUP_PVUII-3161	0.96		Positive	Positive

Site #	Fly		Body Part	Sample ID/ Strain	DuPont Label	DuPont ID	ID Similarity	Riboprinter Pattern	BAX-24E <i>Listeria</i> genus		
	Family	Species							<i>Listeria</i> genus	<i>L. monocytogenes</i>	
6	81	Calliphoridae	<i>Lucilia cuprina</i>	Surface	Lc81s	<i>Listeria monocytogenes</i>	DUP-1042	0.92		Positive	Positive
				Guts	Lc81g	<i>Listeria monocytogenes</i>	DUP-1042	0.93		Positive	Positive
	82	Calliphoridae	<i>Lucilia cuprina</i>	Guts	Lc82g	<i>Listeria monocytogenes</i>	DUP-1042	0.93		Positive	Positive
	90	Calliphoridae	<i>Lucilia sericata</i>	Guts	Ls90g	<i>Listeria monocytogenes</i>	DUP-1042	0.95		Positive	Positive
7	12	Calliphoridae	<i>Lucilia sericata</i>	Guts	Ls12g	<i>Listeria innocua</i>	DUP-1009	0.98		Positive	Negative
1	31	Muscidae	<i>Musca domestica</i>	Guts	Md31g	<i>Listeria innocua</i>	DUP-1010	0.94		Positive	Negative
3	63	Muscidae	<i>Musca domestica</i>	Guts	Md63g	<i>Listeria innocua</i>	DUP-1007	0.92		Positive	Negative
5	75	Calliphoridae	<i>Lucilia cuprina</i>	Guts	Lc75g	<i>Listeria innocua</i>	DUP-1010	0.89		Positive	Negative

FIG 2 Ribotype patterns of *Salmonella* (a) and *Listeria* (b) strains isolated from flies collected from dumpsters outside restaurants in urban areas.

firmed as *L. monocytogenes* were included for further statistical analysis.

**Prevalence of food-borne pathogens.** The percentages of flies that were found positive for the presence of *Cronobacter* spp., *Salmonella* spp., and *L. monocytogenes*, either in the guts or on the surface, were 14%, 6%, and 3%, respectively. The species of flies that were positive for *Cronobacter* spp. were *M. domestica* (10%), *L. cuprina* (1%), *L. sericata* (1%), and *S. haemorrhoidalis* (1%), along with the fly in the family Anthomyiidae (1%). The flies that tested positive for *Salmonella* spp. were *M. domestica* (2%), *L. cuprina* (3%), and *L. sericata* (1%), while the flies positive for *L. monocytogenes* were *L. cuprina* (2%) and *L. sericata* (1%). Thirty samples were positive for the presence of any of the three food-borne pathogens on either the body surfaces or the guts of collected flies. Aliquots of water from the last disinfection rinse of individual flies showed no bacterial growth on TSA plates, allowing us to conclude that there was no cross-contamination between the fly's body surfaces and the guts. The frequency of food-borne pathogens was statistically higher in the guts (22 positives), than on the body surfaces (8 positives;  $\chi^2 = 6.8772$ ,  $df = 1$ ,  $P = 0.0087$ ) (Fig. 3a). A significant overall association was observed among bacteria detected in the flies' guts (Fisher's exact test,  $P = 0.0304$ ) but not on the body surfaces (Fisher's exact test,  $P = 0.0549$ ). Pairwise comparison among bacterial pathogens in the guts showed that the presence of *Cronobacter* spp. was statistically higher (13%) than that of *L. monocytogenes* (3%) (Fisher's exact test,  $P = 0.0165$ ). However, no statistical differences were found between the presence of *Cronobacter* spp. and *Salmonella* spp. or between *Salmonella* spp. and *L. monocytogenes* (Fisher's exact test,  $P = 0.1464$  and  $P = 0.4977$ , respectively) (Fig. 3a).

The overall association between the presence of pathogens and the species of flies was not statistically different (Fisher's exact test,  $P = 0.3275$ ). Figure 3b shows the percentage of flies per species that were positive for pathogens found on the body surfaces and in the guts. The greatest prevalence of *Cronobacter* spp. was mostly

found in the guts (9%) and on the body surfaces (4%) of *M. domestica*, whereas *Salmonella* spp. (3%) and *L. monocytogenes* (2%) were more abundant in the guts of *L. cuprina*. No bacterial pathogens were detected on *C. macellaria* and *O. leucostoma*.

A significant overall association was observed among the presence of pathogens and collection sites (Fisher's exact test,  $P = 0.0003$ ). Multiple pairwise comparisons showed that sites 3 and 9, each containing one sample positive for *Cronobacter* spp. (Fisher's exact test,  $P = 0.0166$  each), were significantly lower than site 10, where seven samples were positive for *Cronobacter* spp. and two samples were positive for *Salmonella* spp., and site 6, where four samples were positive for both *L. monocytogenes* and *Salmonella* spp. (Fisher's exact test,  $P = 0.0322$  each).

**Relative risk for the presence of food-borne pathogens.** Stepwise selection of the full logistic regression model gave rise to the final model shown in equation 2:

$$\text{Logit}(P) = \alpha + \beta_1 \times \text{pathogen} + \beta_2 \times \text{body part} + \beta_3 \times \text{ambient temperature} \quad (2)$$

The estimates indicate that the probability of a single fly being positive for the presence of any of the three food-borne pathogens evaluated in this study was associated with the type of food-borne pathogen, the body part of the fly, and the ambient temperature. The reduced model showed good performance, as indicated by the AUC value of 0.73 (acceptable discrimination) with 95% Wald confidence limits of 0.6306 and 0.8296 (Table 1).

From Fig. 4a to c, inferences can be made about the predicted probability of a fly carrying one of the three different pathogens at a given temperature. For example, at 27°C the probabilities of a fly carrying *Cronobacter* spp., *Salmonella* spp., or *L. monocytogenes* in the guts are 12.0%, 4.2%, or 3.0%, respectively. At the same ambient temperature, the probability of a fly carrying any of the three pathogens, on the body surfaces or in the guts is 2.8% or 4.4%, respectively (Fig. 4d). The ability to make these predictions is also confirmed by the positive coefficients associated with the predic-

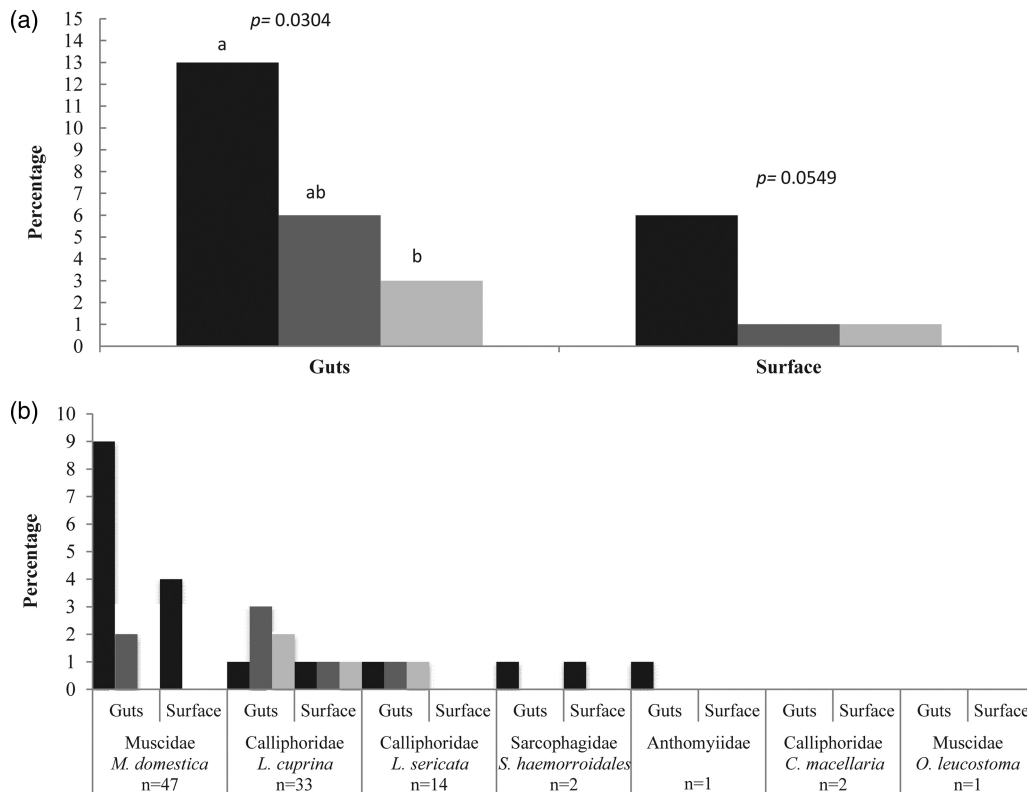


FIG 3 Presence of *Cronobacter* spp. (black bars), *Salmonella* spp. (dark gray bars), and *L. monocytogenes* (light gray bars) on flies collected from dumpsters outside restaurants in urban areas. (a) Overall presence of pathogens on the body surfaces and in the guts of collected flies. (b) Presence of pathogens by fly species and body part. Means with the same letter are not statistically different from each other (Fisher's exact test,  $P < 0.05$ ).

tor parameters, *Cronobacter* spp. (0.9208), *Salmonella* spp. (−0.1694), fly guts (0.5472), and temperature (0.0743) (Table 1).

Our results also indicate that it is three times more likely to find any of the three bacterial pathogens in the guts than on the body surfaces of the flies (Table 2). Likewise, the presence of *Cronobacter* spp. in an individual fly was five times greater than that of *L. monocytogenes* and three times greater than that of *Salmonella* spp., while the presence of *Salmonella* spp. was two times greater than that of *L. monocytogenes*. These results are illustrated as changes in the probability of the presence of these pathogens on both the body surfaces and in the guts of a fly as a function of ambient temperature (Fig. 4a to c).

## DISCUSSION

Food-borne illness due to *Cronobacter*, a ubiquitous and opportunistic genus of bacteria, has historically been found among

TABLE 1 Analysis of maximum likelihood estimates of the logistic regression model<sup>a</sup>

Parameter	$\beta$ (SE)	Wald $\chi^2$	$P$
Intercept	−5.3400 (1.0547)	25.6335	<0.0001
<i>Cronobacter</i> spp.	0.9208 (0.2689)	11.7231	0.0006
<i>Salmonella</i> spp.	−0.1694 (0.3196)	0.2810	0.5960
Guts	0.5472 (0.2137)	6.5598	0.0104
Temp	0.0743 (0.0357)	4.3239	0.0376

<sup>a</sup> For each parameter shown,  $df = 1$ .  $R^2 = 0.04$ , maximum-rescaled  $R^2 = 0.122$ , Kendall's  $\tau$ - $\alpha = 0.044$ , Goodman-Kruskal  $\gamma = 0.47$ , Somers's  $D_{xy} = 0.46$ , and AUC = 0.73. Test values are as follows, with  $df = 4$  for each: likelihood ratio,  $\chi^2 = 24.4707$ ,  $P < 0.0001$ ; score,  $\chi^2 = 24.3809$ ,  $P < 0.0001$ ; and Wald,  $\chi^2 = 21.4047$ ,  $P = 0.0003$ .

high-risk groups, especially neonates, making it a public health concern. It has been confirmed as the cause of severe systematic neonatal infection and mortality in premature newborns with underlying medical conditions (86) and also in apparently healthy full-term infants (7). *Cronobacter* spp. rarely affect adults, causing less severe infections (18, 38). Recorded cases and outbreaks in infants have been associated with the ingestion of milk-based powdered infant formula (20, 62, 80), but in a number of other cases involving infants and adults, the vehicle of transmission was not confirmed.

Species of *Cronobacter* have been isolated from a variety of foods, beverages (28, 48), and many other environmental sources, such as soil (50), air (63), household vacuum dust (48), and food preparation utensils, such as blenders (8). *Cronobacter* spp. have also been isolated from *M. domestica*, collected from dumpsters outside restaurants in Gainesville, FL (10), from urban areas in Maharashtra, India (35), and also from the guts of the stable fly, *Stomoxys calcitrans* (37, 60, 61), and the Mexican fruit fly, *Anastrepha ludens* (53). Despite the many sources from which *Cronobacter* spp. have been isolated, *Cronobacter*'s natural habitat and primary reservoir still remain unknown.

Here we demonstrated that wild flies carry *Cronobacter* spp. both externally and internally, possibly serving as their natural reservoir. To be a natural reservoir of a particular microorganism, certain criteria need to be met (69). The host species must be capable of maintaining the pathogen in wild populations, without being negatively affected by the pathogen. Additionally, the pathogen must remain within the host species for a sufficient

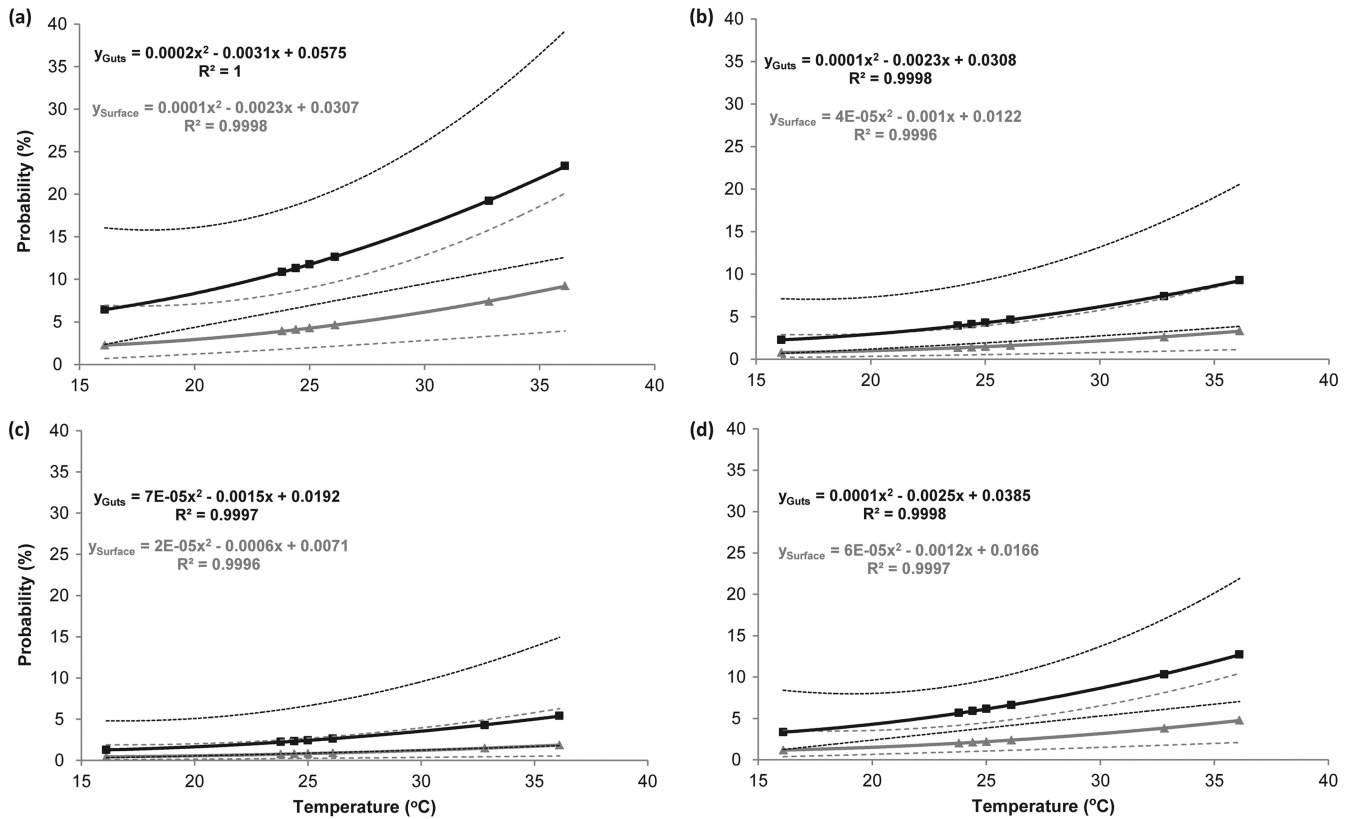


FIG 4 Relative risk of the presence of *Cronobacter* spp. (a), *Salmonella* spp. (b), *L. monocytogenes* (c), and any of the three pathogens (d) on body surfaces (gray triangles with dotted lines) and in guts (black squares with dotted lines) of wild flies according to ambient temperature (°C). Dotted lines represent the 95% upper and lower confidence intervals.

amount of time to be able to transmit it to the affected organisms (69). Our study showed that *Cronobacter* spp. were present either in the guts or on the body surfaces of 14% of the wild flies collected from dumpsters outside urban restaurants (see Table S1 in the supplemental material). This prevalence is greater than that reported by Mramba et al. (60) from wild stable flies collected in rural sites from Kansas and Florida, where only 2/928 (0.2%) flies were positive for *Cronobacter* spp. Occurrences of *Cronobacter* spp. greater than our findings have only been found in foods of plant origin, such as herbs and spices, seeds, organic breakfast cereals, and on animal feed or grain (41, 45, 48, 59), thus suggesting plants as another possible reservoir of this pathogen (76).

The flies collected in this study carried *Cronobacter* species from three distinctive groups (Fig. 1). Strains from two of these groups, *C. sakazakii* and *C. turicensis*, have been isolated from various food sources (41, 59). The third group, *C. universalis*, has

been defined by five strains: its representative strain, NCTC 9529, was collected from a United Kingdom water sample in 1954, strain 96 was isolated from onion powder purchased in the United Kingdom, strain 1435 was isolated from rye flour purchased in Turkey, strain 731 was recovered from a leg infection of a 9-year-old boy (49), and strain E680 was recovered from an unknown source (46).

Seventeen of the strains isolated from collected flies belonged to the *C. sakazakii* group. These strains were isolated from *M. domestica*, *S. haemorrhoidalis*, *L. cuprina*, *L. sericata*, and Anthomyiidae. Strain Sh41s (JQ963910), isolated from the surface of *S. haemorrhoidalis*, belonged to the *C. turicensis* group, whereas strain Md1s (JQ963896), isolated from the surface of *M. domestica*, clustered with the five strains belonging to the *C. universalis* group. A BLAST search of the 16S rRNA nucleotide sequence of the Md1s strain, gave 99% identity to the *C. universalis* strain E680 (EF059861) (see Table S1 in the supplemental material). However, we did not perform further biochemical tests on the newly obtained Md1s strain, as indicated by Iversen et al. (47) and Joseph et al. (49).

There is evidence that flies are not negatively affected by harboring *Cronobacter* spp. in their guts. Despite the low prevalence of *Cronobacter* spp. in wild stable flies (60), it was demonstrated that this bacterial pathogen not only can persist in the guts of laboratory-reared stable flies for at least 20 days, but the bacteria can also support larval development in the absence of other microbes and can colonize the guts of newly emerged flies (61).

TABLE 2 Odds ratio estimates of the presence of food-borne pathogens from collected flies

Effect	Point estimate	95% Wald confidence limits
<i>Cronobacter</i> spp. vs <i>Salmonella</i> spp.	2.975	1.209, 7.322
<i>Cronobacter</i> spp. vs <i>L. monocytogenes</i>	5.325	1.762, 16.090
<i>Salmonella</i> spp. vs <i>L. monocytogenes</i>	1.790	0.512, 6.250
Guts vs body surfaces	2.988	1.293, 6.900
Temp	1.077	1.004, 1.155



Therefore, some food-borne pathogens such as *Cronobacter* spp. may be harbored in the flies' digestive tracts as normal gut flora that can be passed on to the next generation, enhancing their vector potential.

*Cronobacter* species are both thermo- and osmotolerant (9, 70, 78). These physiological characteristics suggest that they are probably able to survive on the body surfaces of the fly for longer periods of time than other food-borne pathogens, thus increasing the likelihood of mechanical transmission by flies. The environmental tolerance of *Cronobacter* spp. also makes them capable of withstanding food processing (3, 9, 68, 78), allowing flies to potentially contaminate food products, including those of plant origin, before or after processing.

Not only are the physiological characteristics of the bacteria conducive to survival on and inside the fly, but the flies themselves also exhibit the characteristics necessary for spreading food-borne pathogens, such as endophily, synanthropy, and communicative behaviors (65). Flies demonstrating the same characteristics that allow for the transmission of food-borne pathogens were shown to carry *Salmonella* spp. and *L. monocytogenes*. Both *Salmonella* (nontyphoidal) and *L. monocytogenes* are among the top five pathogens causing food-borne mortality in the United States (13), with nontyphoidal *Salmonella* being the leading cause of food-related hospitalizations (74).

*Salmonella* is commonly found in the environment and the gastrointestinal tracts of wild and farmed animals and humans. While it can be disseminated through a wide variety of routes, the consumption of food contaminated with animal feces is the most common route of dissemination. Outbreaks of *Salmonella* have implicated both animal- and produce-based products (25). *Salmonella* has also been associated with wild flies collected from animal farms (5, 32, 34, 39, 58, 67, 87) and, less frequently, with wild flies collected from urban areas (6, 16, 85). However, the reported frequencies of *Salmonella* spp. associated with flies vary greatly among studies. Some examples include 0% (27), 13.3% (16), 26.4% (87), 61.7% (85), and 100% (6). This great variability is explained mainly by the lack of systematic methods used and the use of either individual flies or pooled samples of flies, which varied from 10 to 50 flies per pool. In addition, only a handful of studies have identified *Salmonella* to the serovar level (16, 32, 67, 87).

In our study, *Salmonella* spp. were present in 6% of flies collected from urban areas, and a total of five serovars were identified: Seftenberg, Hadar, Poona, Brackenridge, and Schwarzengrund/Bredeney (Fig. 2a). *S. enterica* serovar Hadar was also reported from the internal contents and the surfaces of pooled samples of *M. domestica* collected from an animal facility in Malaysia (16), whereas *S. enterica* serovar Schwarzengrund and *S. enterica* serovar Senftenberg were recovered from *M. domestica* flies collected from swine farms in Taiwan (87). With the exception of *S. enterica* serovar Brackenridge, all *Salmonella* serovars isolated from the flies collected in this study have caused food-borne illness in the United States (14).

*L. monocytogenes* is the third leading cause of death from food-borne illness in the United States (74). A recent multistate outbreak of *L. monocytogenes* in the United States, linked to whole cantaloupes, led to the death of 30 people (15). *L. monocytogenes* is ubiquitous in agricultural settings (soil, decaying vegetation, plants, and water) and human and animal feces (24), and it can also persist in food manufacturing environments. Although *L. monocytogenes* has been found in many environments, 99% of

human listeriosis cases appear to originate from food consumption (31), affecting mainly older adults, the immunocompromised, neonates, and pregnant women.

Even though flies have been implicated as vectors of *L. monocytogenes*, up to now, there is no scientific evidence associating this food-borne pathogen with wild flies collected from urban areas. *L. monocytogenes* was not found on *M. domestica* flies collected from an artisan cheese factory in Campinas, Brazil (11), but *Listeria* spp. were found on 5 out of 180 total *M. domestica* flies collected from 12 animal farms in Nuevo León, Mexico (39). However, the species of *Listeria* was not identified. Additionally, insects were shown to be the source for *L. monocytogenes* in an outbreak associated with Quargel cheese produced in Austria (D. Schoder and M. Wagner, presented at the IAFP European Symposium on Food Safety, Ede, The Netherlands, 19 May 2011). However, the specific type of insect that carried the pathogen was never identified.

To the best of our knowledge, this is the first evidence of flies acting as vectors of *L. monocytogenes*: 3% of collected flies were positive for this pathogen. Positive flies belong to the family Calliphoridae (*L. cuprina* and *L. sericata*) (Fig. 2b). *L. innocua* was also recovered from 4% of collected flies. It is possible to have false-negative results for the presence of *L. monocytogenes* when *L. innocua* is present. However, bacterial strains may behave differently, particularly when the concentrations of these two species in the sample differ (12). No further experiments were performed on strains isolated from collected flies to determine the possibility of inhibition among *Listeria* species and/or strains.

In our study, none of the flies was positive for all three pathogens evaluated. However, one *L. cuprina* fly (fly 81) carried *Salmonella* spp. and *L. monocytogenes* in the guts and on the body surfaces, and two more flies, *L. cuprina* (fly 82) and *L. sericata* (fly 90), had the same two pathogens in the guts. These flies were all collected from a single site (site 6), demonstrating that flies pick up bacterial pathogens from the surrounding environment (Fig. 2a and b). The same pattern was observed in site 10, where one *M. domestica* fly (fly 6) and one *L. cuprina* fly (fly 10) were found to carry both *S. Seftenberg* and *C. sakazakii* in their guts (Fig. 2a; see Table S1 in the supplemental material).

Studies on the interactions of the complex bacterial community that populates the fly's gut have mainly focused on nonpathogenic bacteria (19). We have demonstrated a statistically significant association between collection sites and the presence of food-borne pathogens, and it is assumed that microbiota in the fly's gut can be derived from their surrounding environment (18). The fact that some of the sites were "hot spots" for particular bacteria does not negatively affect the ability to assess the risk of pathogens associated with flies, because our sites were chosen at random. To more accurately assess how these "hot spots" may affect the ability of flies to transmit disease, more research on the persistence of ingested pathogenic bacterial strains by flies is needed. Preliminary studies have demonstrated that adult houseflies that ingested food with different concentrations of *Salmonella* and *L. monocytogenes* transmit these pathogens to their progeny (unpublished data).

Other bacteria identified from collected flies in this study, including *Proteus* spp., *Proteus vulgaris*, *Proteus hauseri*, *Proteus penneri*, *Klebsiella pneumoniae*, *Providencia alcalifaciens*, *Providencia stuartii*, *Enterobacter* spp., *Enterobacter cloacae*, *Enterobacter cancerogenus*, and *Citrobacter freundii* (see Table S1 in the supplemental material), have also been isolated from flies in other stud-

ies (10, 19, 35, 54, 83). These microorganisms are considered indigenous microbiota in flies as they can establish symbiotic relationships with the host, providing nutrients or other defensive compounds (56).

No false positives were detected when the BAX standard assays were used for *Salmonella*, the *Listeria* genus, and *L. monocytogenes*, and confirmation of viable pathogens on agar plates was in agreement with BAX-positive results. However, the *E. sakazakii* (*Cronobacter*) BAX standard assay showed 56% (25/45) false-positive presumptive *Cronobacter* spp., and cross-reactivity with other bacteria, such as *C. freundii*, was confirmed (data not shown). Hence, the BAX system alone is not sufficient to confirm the presence and the identity of *Cronobacter* spp. from individual flies. Additional steps, including determination of the phenotypic characteristics of the colonies in several specific media and 16S rRNA sequence analysis, should be included to confirm this microorganism.

This research also provides information on the relative risk of urban flies carrying *Cronobacter* spp., *Salmonella* spp., and *L. monocytogenes*. The predicted probability of the presence of these pathogens on either the body surfaces or in the guts of collected flies was positively correlated with ambient temperature (Fig. 4a to d). Higher ambient temperatures have been shown to increase the replication cycles of both food-borne pathogens (23, 77) and populations of flies (30), combined factors that could help to explain the reported increase of food-borne illnesses during the summer months (4).

Even though the relative risk of an individual fly carrying *Cronobacter* spp., *Salmonella* spp., and *L. monocytogenes*, externally or internally, has been correlated with ambient temperature, the question of whether a single fly could potentially deliver infectious or lethal doses of these food-borne pathogenic bacteria is still dependent on other factors. The behavioral pattern of the flies is one factor to consider, particularly because we have demonstrated that flies carry up to three times more bacterial food-borne pathogens in the guts than on the body surfaces. Thus, if a fly has sufficient time to feed, regurgitate, and defecate on the food, the chance of delivering those pathogens increases. Other factors include the physiological characteristics of the bacterial strain, the survival and/or growth of the pathogen on food, the conditions under which potentially contaminated food is maintained, and the susceptibility of the consumer population. Overall, this research provides quantitative data that contribute to assessment of the risk of the presence of flies in food or food facilities. Appropriate control of these insects may decrease the spread of food-borne pathogens and the risk to public health.

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## REFERENCES

- Anderson JF. 1909. The differentiation of outbreaks of typhoid fever due to water, milk, flies and contact. *Am. J. Public Hyg.* 19:251–259.
- Andrews WH, Jacobson A, Hammack TS. 2011. *Salmonella*. Bacteriological analytical manual. US Food and Drug Administration, Washington, DC.
- Arku B, Mullane N, Fox E, Fanning SA, Jordan K. 2008. *Enterobacter sakazakii* survives spray drying. *Int. J. Dairy Technol.* 61:102–108.
- Arthur A, Gournis E, McKeown D, Yaffe B. 2009. Toronto public health: foodborne illness in Toronto. Toronto Public Health, Toronto, Ontario, Canada. [www.toronto.ca/health](http://www.toronto.ca/health).
- Barber DA, Bahnson PB, Isaacson R, Jones CJ, Weigel RM. 2002. Distribution of *Salmonella* in swine production ecosystems. *J. Food Prot.* 65:1861–1868.
- Barro N, Aly S, Tidiane OCA, Sababenedjo TA. 2006. Carriage of bacteria by proboscises, legs, and feces of two species of flies in street food vending sites in Ouagadougou, Burkina Faso. *J. Food Prot.* 69:2007–2010.
- Biering G, et al. 1989. Three cases of neonatal meningitis caused by *Enterobacter sakazakii* in powdered milk. *J. Clin. Microbiol.* 27:2054–2056.
- Block C, et al. 2002. Cluster of neonatal infections in Jerusalem due to unusual biochemical variant of *Enterobacter sakazakii*. *Eur. J. Clin. Microbiol. Infect. Dis.* 21:613–616.
- Breeuwer P, Lardeau A, Peterz M, Joosten HM. 2003. Desiccation and heat tolerance of *Enterobacter sakazakii*. *J. Appl. Microbiol.* 95:967–973.
- Butler JF, Garcia-Maruniak A, Meek F, Maruniak JE. 2010. Wild Florida house flies (*Musca domestica*) as carriers of pathogenic bacteria. *Fla. Entomol.* 93:218–223.
- Cardozo GMBQ, et al. 2009. *Musca domestica* L. as a vector of pathogenic microorganisms in ultra-filtered fresh Minas cheese. *Braz. J. Food Technol.* 12:85–91.
- Carvalho A, Eusebio C, Silva J, Gibbs P, Teixeira P. 2010. Influence of *Listeria innocua* on the growth of *Listeria monocytogenes*. *Food Control* 21:1492–1496.
- CDC. 7 February 2012, posting date. CDC estimates of foodborne illness in the United States. CDC 2011 estimates: findings. Centers for Disease Control and Prevention, Atlanta, GA. <http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html>.
- CDC. 16 January 2012, posting date. Foodborne Outbreak Online Database (FOOD). Centers for Disease Control and Prevention, Atlanta, GA. <http://www.cdc.gov/foodborneoutbreaks/Default.aspx>.
- CDC. 7 February 2012, posting date. Investigation update: multistate outbreak of listeriosis linked to whole cantaloupes from Jensen Farms, Colorado. Centers for Disease Control and Prevention, Atlanta, GA. <http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/101211/index.html>.
- Choo LC, Saleha AA, Wai SS, Fauziah N. 2011. Isolation of *Campylobacter* and *Salmonella* from houseflies (*Musca domestica*) in a university campus and a poultry farm in Selangor, Malaysia. *Trop. Biomed.* 28:16–20.
- De Jesus AJ, Olsen AR, Bryce JR, Whiting RC. 2004. Quantitative contamination and transfer of *Escherichia coli* from foods by houseflies, *Musca domestica* L. (Diptera: Muscidae). *Int. J. Food Microbiol.* 93:259–262.
- Dennison SK, Morris J. 2002. Multiresistant *Enterobacter sakazakii* wound infection in an adult. *Infect. Med.* 19:533–535.
- Dillon RJ, Dillon VM. 2004. The gut bacteria of insects: nonpathogenic interactions. *Annu. Rev. Entomol.* 49:71–92.
- Drudy D, Mullane NR, Quinn T, Wall PG, Fanning S. 2006. *Enterobacter sakazakii*: an emerging pathogen in powdered infant formula. *Clin. Infect. Dis.* 42:996–1002.
- Dübendorfer A, Hediger M, Burghardt G, Bopp D. 2002. *Musca domestica*, a window on the evolution of sex-determining mechanisms in insects. *Int. J. Dev. Biol.* 46:75–79.
- Ekdahl K, Normann B, Andersson Y. 2005. Could flies explain the elusive epidemiology of campylobacteriosis? *BMC Infect. Dis.* 5:11.
- FAO. 2008. Climate change: implications for food safety, vol 1196. Food and Agriculture Organization of the United Nations, Rome, Italy.
- Farber JM, Losos JZ. 1988. *Listeria monocytogenes*, a foodborne pathogen. *Can. Med. Assoc. J.* 138:413–418.

25. Fatica MK, Schneider KR. 2011. *Salmonella* and produce survival in the plant environment and implications in food safety. *Virulence* 2:573–579.
26. FDA. 2012. Bad bug book: foodborne pathogenic microorganisms and natural toxins handbook, 2nd ed. US Food and Drug Administration, Washington, DC.
27. Förster M, Sievert K, Messler S, Klimpel S, Feffer K. 2009. Comprehensive study on the occurrence and distribution of pathogenic microorganisms carried by synanthropic flies caught at different rural locations in Germany. *J. Med. Entomol.* 46:1164–1166.
28. Friedemann M. 2007. *Enterobacter sakazakii* in food and beverages (other than infant formula and milk powder). *Int. J. Food Microbiol.* 116:1–10.
29. Gagné R. 1991. Flies (Diptera), p 269–296. In Gorham JR (ed), *Insect and mite pests in food, an illustrated key*, vol 1. US Department of Agriculture and US Department of Health and Human Services, Washington, DC.
30. Goulson D, Derwent LC, Hanley ME, Dunn DW, Abolins SR. 2005. Predicting calyptate fly populations from the weather, and probable consequences of climate change. *J. Appl. Ecol.* 42:795–804.
31. Gray MJ, et al. 2004. *Listeria monocytogenes* isolates from foods and humans form distinct but overlapping populations. *Appl. Environ. Microbiol.* 70:5833–5841.
32. Greenberg B. 1971. Flies and diseases, vol I. Ecology and biotic associations. Princeton University Press, Princeton, NJ.
33. Greenberg B, Kowalski JA, Klowden MJ. 1970. Factors affecting the transmission of *Salmonella* by flies: natural resistance to colonization and bacterial interference. *Infect. Immun.* 2:800–809.
34. Greenberg B, Varela G, Bornstein A, Hernandez H. 1963. *Salmonellae* from flies in a Mexican slaughterhouse. *Am. J. Hyg.* 77:177–183.
35. Gupta AK, et al. 2012. Phylogenetic characterization of bacteria in the gut of house flies (*Musca domestica* L.). *FEMS Microbiol. Ecol.* 79:581–593.
36. Hald B, Skovgård H, Pedersen K, Bunkenborg H. 2008. Influxed insects as vectors for *Campylobacter jejuni* and *Campylobacter coli* in Danish broiler houses. *Poult. Sci.* 87:1428–1434.
37. Hamilton JV, Lehane MJ, Braig HR. 2003. Isolation of *Enterobacter sakazakii* from midgut of *Stomoxys calcitrans*. *Emerg. Infect. Dis.* 9:1355–1356.
38. Hawkins RE, Lissner CR, Sanford JP. 1991. *Enterobacter sakazakii* bacteremia in an adult. *South. Med. J.* 84:793–795.
39. Hernández-Escareño JJ, et al. 2012. Presence of *Enterobacteriaceae*, *Listeria* spp., *Vibrio* spp. and *Staphylococcus* spp. in house fly (*Musca domestica* L.), collected and macerated from different sites in contact with a few animal species. *Rev. Cient. Fac. Cienc. Vet.* 22:128–134.
40. Hewitt CG. 1912. Houseflies and how they spread disease. Cambridge University Press, Cambridge, United Kingdom.
41. Hochel I, Růžičková H, Krásný L, Demnerová K. 2012. Occurrence of *Cronobacter* spp. in retail foods. *J. Appl. Microbiol.* 112:1257–1265.
42. Hogsette JR, Amendt J. 2008. Flies, p 209–237. In Bonnefoy X, Kampen H, Sweeney K (ed), *Public health significance of urban pests*. World Health Organization, Geneva, Switzerland.
43. Holt PS, Geden CJ, Moore RW, Gast RK. 2007. Isolation of *Salmonella enterica* serovar Enteritidis from houseflies (*Musca domestica*) found in rooms containing *Salmonella* serovar Enteritidis-challenged hens. *Appl. Environ. Microbiol.* 73:6030–6035.
44. Hosmer DW, Lemeshow S. 2000. Applied logistic regression, 2nd ed. John Wiley and Sons, New York, NY.
45. Iversen C, Forsythe S. 2004. Isolation of *Enterobacter sakazakii* and other *Enterobacteriaceae* from powdered infant formula milk and related products. *Food Microbiol.* 21:771–777.
46. Iversen C, et al. 2007. The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov., *Cronobacter sakazakii* subsp. *sakazakii* comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter* genomospecies I. *BMC Evol. Biol.* 7:1–11.
47. Iversen C, et al. 2008. *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactaridi* subsp. nov. *Int. J. Syst. Evol. Microbiol.* 58:1442–1447.
48. Jaradat ZW, Ababneh QO, Saadoun IM, Samara NA, Rashdan AM. 2009. Isolation of *Cronobacter* spp. (formerly *Enterobacter sakazakii*) from infant food, herbs and environmental samples and the subsequent identification and confirmation of the isolates using biochemical, chromogenic assays, PCR and 16S rRNA sequencing. *BMC Microbiol.* 9:225. doi: 10.1186/1471-2180-9-225.
49. Joseph S, Cetinkaya E, Drahovska H, Levican A. 2012. *Cronobacter condimenti* sp. nov., isolated from spiced meat and *Cronobacter universalis* sp. nov., a novel species designation for *Cronobacter* sp. genomospecies 1, recovered from a leg infection, water, and food ingredients. *Int. J. Syst. Evol. Microbiol.* 62:1277–1283.
50. Khan AA, Jones RA, Cerniglia CE. 1998. Rapid method for the detection of genetically engineered microorganisms by polymerase chain reaction from soil and sediments. *J. Ind. Microbiol. Biotechnol.* 20:90–94.
51. Kobayashi M, et al. 1999. Houseflies: not simple mechanical vectors of enterohemorrhagic *Escherichia coli* O157: H7. *Am. J. Trop. Med. Hyg.* 61:625–629.
52. Kotenok YF, Chicherin YV. 1977. Houseflies (*M. domestica* L.) as transmitters of the agent of cholera. *Zh. Mikrobiol. Epidemiol.* December:23–27. (In Russian.)
53. Kuzina LV, Peloquin JJ, Vacek DC, Miller TA. 2001. Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens* (Diptera: Tephritidae). *Curr. Microbiol.* 42:290–294.
54. Lam K, et al. 2007. Proliferating bacterial symbionts on house fly eggs affect oviposition behaviour of adult flies. *Anim. Behav.* 74:81–92.
55. Lehner A, et al. 2006. Comparison of two chromogenic media and evaluation of two molecular based identification systems for *Enterobacter sakazakii* detection. *BMC Microbiol.* 6:15. doi:10.1186/1471-2180-6-15.
56. Leroy PD, et al. 2011. The semiochemically mediated interactions between bacteria and insects. *Chemoecology* 21:113–122.
57. Levine OS, Levine MM. 1991. Houseflies (*Musca domestica*) as mechanical vectors of shigellosis. *Rev. Infect. Dis.* 13:688–696.
58. Mian LS, Maag H, Tacal JV. 2002. Isolation of *Salmonella* from muscoid flies at commercial animal establishments in San Bernardino County, California. *J. Vector Ecol.* 27:82–85.
59. Molloy C, et al. 2009. Surveillance and characterisation by pulsed field gel electrophoresis of *Cronobacter* spp. in farming and domestic environments, food production animals and retail foods. *Int. J. Food Microbiol.* 136:198–203.
60. Mramba F, Broce A, Zurek L. 2006. Isolation of *Enterobacter sakazakii* from stable flies, *Stomoxys calcitrans* L. (Diptera: Muscidae). *J. Food Prot.* 69:671–673.
61. Mramba F, Broce AB, Zurek L. 2007. Vector competence of stable flies, *Stomoxys calcitrans* L. (Diptera: Muscidae), for *Enterobacter sakazakii*. *J. Vector Ecol.* 32:134–139.
62. Mullane NR, et al. 2006. *Enterobacter sakazakii*: biological properties and significance in dried infant milk formula (IMF) powder. *Int. J. Dairy Technol.* 59:102–111.
63. Mullane NR, et al. 2008. Dissemination of *Cronobacter* spp. (*Enterobacter sakazakii*) in a powdered milk protein manufacturing facility. *Appl. Environ. Microbiol.* 74:5913–5917.
64. Naydich D, Noblet GP, Stutzenberger FJ. 2002. Vector potential of houseflies for the bacterium *Aeromonas caviae*. *Med. Vet. Entomol.* 16: 193–198.
65. Olsen AR. 1998. Regulatory action criteria for filth and other extraneous materials. III. Review of flies and foodborne enteric disease. *Regul. Toxicol. Pharmacol.* 28:199–211.
66. Olsen AR, Gecan JS, Ziobro GC, Bryce JR. 2001. Regulatory action criteria for filth and other extraneous materials. V. Strategy for evaluating hazardous and nonhazardous filth. *Regul. Toxicol. Pharmacol.* 33:363–392.
67. Olsen AR, Hammack TS. 2000. Isolation of *Salmonella* spp. from the housefly, *Musca domestica* L., and the dump fly, *Hydrotaea aeneascens* (Wiedemann) (Diptera: Muscidae), at caged-layer houses. *J. Food Prot.* 63:958–960.
68. Osaili T, et al. 2008. Effect of environmental stresses on the sensitivity of *Enterobacter sakazakii* in powdered infant milk formula to gamma radiation. *Lett. Appl. Microbiol.* 47:79–84.
69. Putt SNH, Shaw APM, Woods AJ, Tyler L, James AD. 1988. Veterinary epidemiology and economics in Africa: a manual for use in the design and appraisal of livestock health policy. *Epidemiology: some basic concepts and definitions*. Veterinary Epidemiology and Economics Research Unit, Department of Agriculture, University of Reading, Reading, Berkshire, England.

70. Riedel K, Lehner A. 2007. Identification of proteins involved in osmotic stress response in *Enterobacter sakazakii* by proteomics. *Proteomics* 7:1217–1231.
71. Rochon K, Lysyk TJ, Selinger LB. 2005. Retention of *Escherichia coli* by house fly and stable fly (Diptera: Muscidae) during pupal metamorphosis and eclosion. *J. Med. Entomol.* 42:397–403.
72. SAS Institute. 2005. SAS 9.1.3 help and documentation. SAS Institute, Inc., Cary, NC.
73. Scallan E, Griffin PM, Angulo FJ, Tauxe RV, Hoekstra RM. 2011. Foodborne illness acquired in the United States, unspecified agents. *Emerg. Infect. Dis.* 17:16–22.
74. Scallan E, et al. 2011. Foodborne illness acquired in the United States, major pathogens. *Emerg. Infect. Dis.* 17:7–15.
75. Scharff RL. 2012. Economic burden from health losses due to foodborne illness in the United States. *J. Food Prot.* 75:123–131.
76. Schmid M, et al. 2009. Evidence for a plant-associated natural habitat for *Cronobacter* spp. *Res. Microbiol.* 160:608–614.
77. Semenza JC, Suk JE, Estevez V, Ebi KL, Lindgren E. 2012. Mapping climate change vulnerabilities to infectious diseases in Europe. *Environ. Health Perspect.* 120:385–392.
78. Shaker RR, Osaili TM, Abu Al-Hasan AS, Ayyash MM, Forsythe SJ. 2008. Effect of desiccation, starvation, heat, and cold stresses on the thermal resistance of *Enterobacter sakazakii* in rehydrated infant milk formula. *J. Food Sci.* 73:M354–M359.
79. Sharon G, et al. 2010. Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* 107:20051–20056.
80. Simmons BP, Gelfand MS, Haas M, Metts L, Ferguson J. 1989. *Enterobacter sakazakii* infections in neonates associated with intrinsic contamination of a powdered infant formula. *Infect. Control Hosp. Epidemiol.* 10:398–401.
81. Steinhaus EA. 1967. Insect microbiology. Facsimile of 1947 edition. Hafner Publishing Co., Ltd., New York, NY.
82. Su ZJ, et al. 2010. Comparison of bacterial diversity in wheat bran and in the gut of larvae and newly emerged adult of *Musca domestica* (Diptera: Muscidae) by use of ethidium monoazide reveals bacterial colonization. *J. Econ. Entomol.* 103:1832–1841.
83. Sulaiman S, Othman MZ, Aziz AH. 2000. Isolations of enteric pathogens from synanthropic flies trapped in downtown Kuala Lumpur. *J. Vector Ecol.* 25:90–93.
84. Triola M. 2010. Elementary statistics using Excel, 4th ed, p 340–403. Pearson, Boston, MA.
85. Ugboogu OC, Nwachukwu NC, Ogbuagu UN. 2006. Isolation of *Salmonella* and *Shigella* species from house flies (*Musca domestica* L.) in Uturu, Nigeria. *Afr. J. Biotechnol.* 5:1090–1091.
86. van Acker J, et al. 2001. Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. *J. Clin. Microbiol.* 39:293–297.
87. Wang YC, et al. 2011. Transmission of *Salmonella* between swine farms by the housefly (*Musca domestica*). *J. Food Prot.* 74:1012–1016.
88. WHO. 2012. Food production to consumption. World Health Organization, Geneva, Switzerland. [http://www.who.int/foodsafety/fs\\_management/en/](http://www.who.int/foodsafety/fs_management/en/).
89. Yano T, et al. 2008. Autophagic control of *Listeria* through intracellular innate immune recognition in *Drosophila*. *Nat. Immunol.* 9:908–916.
90. Zurek L, Denning SS, Schal C, Watson DW. 2001. Vector competence of *Musca domestica* (Diptera: Muscidae) for *Yersinia pseudotuberculosis*. *J. Med. Entomol.* 38:333–335.
91. Zurek L, Gorham JR. 2008. Insects as vectors of foodborne pathogens, p 1–16. In Voeller JG (ed), *Wiley handbook of science and technology for homeland security*. Wiley Inc., Hoboken, NJ.