Significance and Survival of Enterococci During the House Fly Development

ANURADHA GHOSH,1 MASTURA AKHTAR,2,3 CHRIS HOLDERMAN,2,4 AND LUDEK ZUREK1,2,5


ABSTRACT House flies are among the most important nonbiting insect pests of medical and veterinary importance. Larvae develop in decaying organic substrates and their survival strictly depends on an active microbial community. House flies have been implicated in the ecology and transmission of enterococci, including multi-antibiotic-resistant and virulent strains of Enterococcus faecalis. In this study, eight American Type Culture Collection type strains of enterococci including Enterococcus avium, Enterococcus casseliflavus, Enterococcus durans, Enterococcus hirae, Enterococcus mundtii, Enterococcus gallinarum, Enterococcus faecalis, and Enterococcus faecium were evaluated for their significance in the development of house flies from eggs to adults in bacterial feeding assays. Furthermore, the bacterial colonization of the gut of tenerial flies as well as the importance of several virulence traits of E. faecalis in larval mortality was assessed. Overall survival of house flies (egg to adult) was significantly higher when grown with typically nonpathogenic enterococcal species such as E. hirae (76.0% survival), E. durans (64.0%), and E. avium (64.0%) compared with that with clinically important species E. faecalis (24.0%) and E. faecium (36.0%). However, no significant differences in survival of house fly larvae were detected when grown with E. faecalis strains carrying various virulence traits, including isogenic mutants of the human clinical isolate E. faecalis V583 with in-frame deletions of gelatinase, serine protease, and capsular polysaccharide serotype C. Enterococci were commonly detected in fly puparia (range: 75–100%; concentration: 10^3–10^5 CFU/puparium); however, the prevalence of enterococci in teneral flies varied greatly: from 25.0 (E. casseliflavus) to 89.5% (E. hirae). In conclusion, depending on the species, enterococci variably support house fly larval development and colonize the gut of teneral adults. The human pathogenic species, E. faecalis and E. faecium, poorly support larval development and are likely acquired in nature by adult flies during feeding. House fly larvae do not appear to be a suitable model organism for assessment of enterococcal virulence traits.

KEY WORDS house fly, Enterococcus spp., larval development, gut colonization
Furthermore, some insects (e.g., Manduca sexta and Galleria mellonella) have been used as animal models for studying bacterial virulence factors (Gaspar et al. 2009, Mason et al. 2011). Due to rapid development, nonexpensive rearing, absence of adaptive immunity, and the gut microbial community that commonly comprises enterococci, house flies could be a suitable model system for assessing enterococcal virulence traits.

Our study aimed to: 1) assess the significance of different enterococcal species in the development of house fly larvae, 2) determine the transtidial survival of enterococci from larva to adult, and 3) test whether house fly larvae can serve as a model to assess virulence traits of enterococci.

Materials and Methods

Enterococcal Strains. Eight enterococcal species type strains (from American Type Culture Collection, ATCC) were used in our experiments: Enterococcus avium ATCC 14025, E. casseliflavus ATCC 25788, Enterococcus durans ATCC 19432, E. hirae ATCC 8043, Enterococcus mundtii ATCC 43186, E. gallinarum ATCC 49573, E. faecalis ATCC 19433, and E. faecium ATCC 19434. In addition, eight E. faecalis strains and isogenic deletion mutants of E. faecalis ATCC 19434 were tested (Table 1).

Table 1. Description of strains and mutants of E. faecalis used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>V583</td>
<td>Clinical strain (ATCC 700602), serotype C, vancomycin-resistant, gelatinase-, and serine protease-positive</td>
<td>Sahm et al. 1989</td>
</tr>
<tr>
<td>V583Δeg/hΔsprE</td>
<td>Isogenic deletion mutant of V583, gelatinase-, and serine protease-defective, tetracycline- and spectinomycin-resistant</td>
<td>Hancock and Perego 2004</td>
</tr>
<tr>
<td>V583Δeg/C</td>
<td>Isogenic deletion mutant of V583, capsule serotype C-deficient</td>
<td>Thurlow et al. 2009</td>
</tr>
<tr>
<td>MMHE94</td>
<td>Epidemic clinical strain, serotype C, hemolytic, high-level gentamicin-resistant</td>
<td>Hueyke et al. 1991</td>
</tr>
<tr>
<td>OG1X</td>
<td>Clinical strain, streptomycin-resistant, aggregation substance-, cytolysin-, and gelatinase-defective</td>
<td>Ike et al. 1983</td>
</tr>
<tr>
<td>OG1RF</td>
<td>A derivative of clinical strain OG1, serotype B laboratory strain (ATCC 47077), rifampicin- and fusidic acid-resistant, gelatinase-positive</td>
<td>Dunny et al. 1978</td>
</tr>
<tr>
<td>JH2-2</td>
<td>A derivative of clinical strain JH2, plasmid-free, aggregation substance-, cytolysin-, and gelatinase-defective</td>
<td>Yagi and Clewell 1980</td>
</tr>
<tr>
<td>FA2-2(pAM714)</td>
<td>Laboratory strain, serotype C, cytolysin-positive, gelatinase-, and serine protease-defective</td>
<td>Ike and Clewell 1984</td>
</tr>
</tbody>
</table>

Bioassays. EYTSA was inoculated with fresh cultures of the individual bacterial strains and incubated overnight at 37°C. The first-instar larvae were transferred aseptically with a sterile brush to EYTSA. Each bioassay was conducted with five larvae per plate per enterococcal strain in five replicates. We used 10 larvae per plate per strain in three replicates for the bioassays performed with E. faecalis strains. Un-inoculated EYTSA with first-instar larvae was used as negative control. All plates were incubated at 25°C and examined daily for larval mortality and pupation. The pupae were weighed, surface sterilized, and transferred on sterile filter paper in sterile petri dishes for incubation at 25°C until adult emergence. Pupation, weight of pupae, adult emergence, and survival rates (egg to adult) were recorded.

Determination of Enterococcal Concentration in Teneral Adults and Puparia After Adult Emergence. Each emerged adult fly was immediately surface sterilized as described earlier for eggs. Surface-sterilized adults and empty puparia were homogenized in 1.0 ml phosphate buffered saline (pH 7.2; MP Biomedicals, Solon, OH) and diluted plated on m-Enterococcus agar (BBL, BD Diagnostic Systems, Sparks, MD). Enterococcal colonies were confirmed phenotypically and by the esculin hydrolysis test as described previously (Macovei and Zurek 2007). Concentration of enterococci was calculated as CFU/puparium or CFU/fly.

Statistical Analysis. Data on pupation, fly emergence, fly survival (egg to adult), and enumeration of enterococci in adults and puparia were checked for normal distribution by Shapiro–Francia test (Royston 1993), then transformed with arcsine square root (arc-sine sqrt [percent/100]) to stabilize error variance (Gomez and Gomez 1984), and analyzed using analysis of variance. Means were compared by the least-squares means protocol (P = 0.05) of the general linear model (SAS Institute 2003). Although all tests of significance (with exception of pupal weight) were based on the transformed data, the untransformed percent values are reported. Percent pupation and survival of flies reared on various strains and mutants of E. faecalis were analyzed using analysis of variance (P < 0.05) and the post hoc Tukey test.

Results

Significance of Enterococci in Larval Development and Gut Colonization of Teneral Adults. Bioassays using EYTSA confirmed that house fly larvae fail to
develop beyond the first instar in sterile media and bacterial strains were required to complete larval development. Overall, the highest proportion (80.0%) of larvae reached the pupa stage when grown with *E. hirae* and this was statistically significant compared with larvae reared on *E. mundtii* (*P* = 0.0014), *E. faecalis* (*P* = 0.0004), and *E. faecium* (*P* = 0.0176) (Table 2). A significantly lower proportion of fly pupation was observed with the potentially human pathogenic species *E. faecalis* (48.0%) compared with that of all other strains, except *E. faecium* (*P* = 0.1461) and *E. mundtii* (*P* = 0.6266) (Table 2).

The mean pupal weight ranged from 0.018 to 0.020 g and did not differ significantly among *E. avium*, *E. gallinarum*, *E. durans*, *E. hirae*, *E. mundtii*, *E. faecalis*, and *E. faecium* (Table 2). Only fly larvae grown with *E. casseliflavus* had a significantly greater pupal weight compared with those fed on *E. durans* (*P* = 0.0117) (Table 2).

Regardless of the strain, adult flies started to emerge in 4–5 d after pupation. The proportion (%) of adult emergence was significantly higher with *E. hirae* (95.0%, *P* = 0.0003, 0.0013, 0.0002), *E. avium* (94.1%, *P* = 0.0003, 0.0013, 0.0002), *E. mundtii* (92.3%, *P* = 0.0007, 0.0026, 0.0003), and *E. durans* (84.2%, *P* = 0.0052, 0.0173, 0.0025) compared with that with *E. faecalis* (50.0%), *E. faecium* (60.0%), and *E. casseliflavus* (50.0%). The adult emergence on *E. hirae* did not differ significantly from that with *E. avium* (94.1%, *P* = 1.0), *E. mundtii* (92.3%, *P* = 0.8), and *E. durans* (84.2%, *P* = 0.3228) (Table 2).

The overall survival rate of house fly larvae (egg to adult) was highest from EYTS with *E. hirae* (76.0%), followed by *E. avium* (64.0%) and *E. durans* (64.0%). A significantly lower survival to the adult stage was recorded with *E. faecalis* (24.0%) compared with that on *E. hirae* (*P* < 0.0001), *E. avium* (*P* < 0.0001), *E. mundtii* (*P* = 0.0013), *E. durans* (*P* < 0.0001), and *E. casseliflavus* (*P* = 0.0013) (Table 2).

Transstadi al Survival of Enterococci From Larva to Adult. Prevalence of enterococci in the gut of teneral adults ranged from 25.0 to 89.5% (Table 2). The most frequent gut colonization was recorded from flies with *E. hirae* (89.5%) followed by *E. durans* (87.5%) and that was significantly higher (*P* < 0.0001 and *P* < 0.01, respectively) compared with that of all other enterococcal species. The lowest colonization rate was observed from flies with *E. casseliflavus* (25.0%). The overall enterococcal concentration ranged from 10^2 to 10^5 CFU/ fly and varied widely among individual flies. *Enterococcus gallinarum* survived in the fly gut throughout the development with the highest concentration of 6.6 ± 5.7 × 10^4 CFU/ fly (Table 2).

Empty puparia were also examined for the presence of enterococci. The majority of puparia (75–100%) were positive for enterococci. All puparia were positive for *E. hirae*, *E. faecalis*, *E. gallinarum*, and *E. mundtii* on EYTS (Table 2). Across different enterococcal species, the mean bacterial concentration per puparium ranged from 10^2 to 10^5 CFU (Table 2).

**Table 2. Significance and survival of enterococci in the gastrointestinal tract during the house fly development (egg to adult) (*n* = 25 per enterococcal species)**

<table>
<thead>
<tr>
<th>Enterococcus species</th>
<th>Pupation (%)</th>
<th>Pupa wt (g) mean ± SD</th>
<th>Adult emergence (%)</th>
<th>Survival to adult (%)</th>
<th>Teneral adults with enterococci (%)</th>
<th>Puparia with enterococci (%)</th>
<th>Enterococci (CFU/ml) mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. avium</td>
<td>68.0^ab</td>
<td>0.019 ± 0.003^ab</td>
<td>94.1^*</td>
<td>64.0^*</td>
<td>37.5^*</td>
<td>85.0^*</td>
<td>5.9 ± 8.2 by 10^2 1.2 ± 2.6 by 10^5</td>
</tr>
<tr>
<td>E. casseliflavus</td>
<td>68.0^ab</td>
<td>0.021 ± 0.002^a</td>
<td>70.6^b</td>
<td>48.0^b</td>
<td>25.0^*</td>
<td>85.7^cd</td>
<td>7.7 ± 9.4 by 10^2 1.4 ± 1.6 by 10^2</td>
</tr>
<tr>
<td>E. durans</td>
<td>76.0^ab</td>
<td>0.018 ± 0.006^b</td>
<td>84.2^ab</td>
<td>64.0^*</td>
<td>87.5^*</td>
<td>85.9^*</td>
<td>0.7 ± 2.6 by 10^3 2.7 ± 3.8 by 10^3</td>
</tr>
<tr>
<td>E. hirae</td>
<td>50.0^*</td>
<td>0.020 ± 0.004^ab</td>
<td>95.0^*</td>
<td>76.0^*</td>
<td>89.5^*</td>
<td>100^*</td>
<td>0.6 ± 2.4 by 10^3 1.8 ± 3.6 by 10^3</td>
</tr>
<tr>
<td>E. mundtii</td>
<td>52.0^ab</td>
<td>0.019 ± 0.003^ab</td>
<td>92.3^ab</td>
<td>52.0^b</td>
<td>30.8^*</td>
<td>100^*</td>
<td>3.0 ± 3.2 by 10^3 5.3 ± 5.7 by 10^3</td>
</tr>
<tr>
<td>E. gallinarum</td>
<td>72.0^ab</td>
<td>0.019 ± 0.036^a</td>
<td>50.0^d</td>
<td>36.0^b</td>
<td>33.3^a</td>
<td>100^*</td>
<td>6.6 ± 5.7 by 10^3 4.7 ± 2.6 by 10^3</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>48.0^d</td>
<td>0.020 ± 0.002^ab</td>
<td>50.0^d</td>
<td>24.0^*</td>
<td>50.0^*</td>
<td>100^bc</td>
<td>0.6 ± 1.0 by 10^4 1.1 ± 0.8 by 10^3</td>
</tr>
<tr>
<td>E. faecium</td>
<td>60.0^cd</td>
<td>0.019 ± 0.002^ab</td>
<td>60.0^cd</td>
<td>36.0^bc</td>
<td>33.3^a</td>
<td>75.0^*</td>
<td>1.1 ± 1.3 by 10^2 1.6 ± 3.7 by 10^3</td>
</tr>
</tbody>
</table>

Values within the same column followed by the same letter are not significantly different (*P* > 0.05).

**Fig. 1.** Pupation and survival of house flies on different strains of *Enterococcus faecalis* (*n* = 30 per strain).
Discussion

House flies have been implicated as mechanical or bioenhanced vectors for several human pathogenic bacteria such as *Salmonella* spp., *Campylobacter* spp., *Pseudomonas aeruginosa*, *Listeria* spp., *Vibrio* spp., and *Escherichia coli* O157:H7 (reviewed by Graczyk et al. 2001, Zurek and Gorham 2008). Previous studies have shown that house flies also commonly carry antibiotic-resistant and potentially virulent enterococci (Macovei and Zurek 2006, Graham et al. 2009, Ahmad et al. 2011). Furthermore, the house fly digestive tract provides a suitable habitat for enterococcal growth (Doud and Zurek 2012) and horizontal transfer of antibiotic resistance genes (Akhtar et al. 2009). Previously, we have also demonstrated that house flies have a great potential to contaminate human food with enterococci in a short period (Macovei et al. 2008, Doud and Zurek 2012). Consequently, this insect may represent a link between the agricultural and urban environment for antibiotic resistance traits. However, the significance of enterococci in house fly larval development and the gut colonization of teneral adult flies by enterococci were unknown.

Our bioassays confirmed the data from previous studies (Schmidtmann and Martin 1992, Zurek et al. 2000) showing that live bacteria are required for the successful house fly development to the adult stage. The overall fly survival rate from eggs to adults varied greatly depending on the enterococcal species and this likely reflects differences in metabolic properties (e.g., utilization and fermentation of carbohydrates, hydrolysis of amino acids) among individual enterococcal species (Farrow and Collins 1985, De Vaux et al. 1998, Vancanneyt et al. 2001). The highest percentage of house fly pupation and survival to the adult stage was observed with *E. hirae* and this species also commonly colonized the gut of teneral adults. This indicates that *E. hirae* is well adapted to the house fly gut environment and fly developmental processes from larvae to adults. *Enterococcus hirae* is also the most common enterococcal species detected in manure of pigs (Ahmad et al. 2011), pastured cattle and bison (Anderson et al. 2008), and feedlot cattle (L. Z., unpublished). In contrast, *E. hirae* was not detected in wild house fly adults, including those from fast food restaurants (Macovei and Zurek 2006) and poultry farms (Graham et al. 2009), and it was found only in very low prevalence in house flies from swine farms (Ahmad et al. 2011), feedlot and pastured cattle (L. Z., unpublished), and waste water treatment plants (Doud et al. 2014). It is possible that the gut microbiome of adult house flies changes over time depending on their food sources and *E. hirae* in adult house flies is digested and replaced by other enterococcal species, primarily by *E. faecalis*. This is corroborated indirectly by the fact that although *E. faecalis* supported the larval development of house flies to the least extent, it was the most commonly detected enterococcal species in the digestive tract of adult house flies collected from various environments (Macovei and Zurek 2006, Graham et al. 2009, Ahmad et al. 2011, Doud et al. 2014). In addition, our recent study (Doud and Zurek 2012) reported the colonization and proliferation of *E. faecalis* in the crop and midgut of adult house flies, demonstrating that this insect is a bioenhanced vector for *E. faecalis*. Future studies focusing on the analysis of the bacterial community in the digestive tract of wild teneral adults are needed to better understand the transstadial bacterial survival and how this affects the vector capacity of flies for animal and zoonotic pathogens.

We were also interested in assessing house fly larvae as a novel model organism for testing putative virulence traits including gelatinase, serine protease, aggregation substance, capsular polysaccharide, and cytolsin that are associated with pathogenic strains of *E. faecalis* (Gilmore 2002). Although, on the species level, *E. faecalis* supported house fly development poorly (24.0% survival), there was no significant difference in the fly development among *E. faecalis* strains with or without putative virulence factors. This includes the clinical strain *E. faecalis* V583 and its isogenic mutants without gelatinase, serine protease, and capsular polysaccharide serotype C. Therefore, based on our bacterial feeding assays, larvae of *Musca domestica* were not found to be suitable as a model organism for testing enterococcal infection and virulence.

In conclusion, enterococci, depending on the species, support house fly larval development and colonize the gut of teneral adults to various degrees. The human pathogenic species, *E. faecalis* and *E. faecium*, do not support larval development to great extent and are likely acquired in nature by adult flies during feeding and eventually outcompete other enterococcal species in the fly digestive tract. House fly larvae do not appear to be a suitable model organism for assessment of enterococcal virulence traits.

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