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Diversity, distribution and antibiotic resistance of *Enterococcus* spp. recovered from tomatoes, leaves, water and soil on U.S. Mid-Atlantic farms

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ABSTRACT

Antibiotic-resistant enterococci are important opportunistic pathogens and have been recovered from retail tomatoes. However, it is unclear where and how tomatoes are contaminated along the farm-to-fork continuum. Specifically, the degree of pre-harvest contamination with enterococci is unknown. We evaluated the prevalence, diversity and antimicrobial susceptibilities of enterococci collected from tomato farms in the Mid-Atlantic United States. Tomatoes, leaves, groundwater, pond water, irrigation ditch water, and soil were sampled and tested for enterococci using standard methods. Antimicrobial susceptibility testing was performed using the Sensititre microbroth dilution system. *Enterococcus faecalis* isolates were characterized using amplified fragment length polymorphism to assess dispersal potential. Enterococci ($n = 307$) occurred in all habitats and colonization of tomatoes was common. Seven species were identified: *Enterococcus casseliflavus*, *E. faecalis*, *Enterococcus gallinarum*, *Enterococcus faecium*, *Enterococcus avis*, *Enterococcus hirae* and *Enterococcus raffinosus*. *E. casseliflavus* predominated in soil and on tomatoes and leaves, and *E. faecalis* predominated in pond water. On plants, distance from the ground influenced presence of enterococci. *E. faecalis* from samples within a farm were more closely related than those from samples between farms. Resistance to rifampicin, quinupristin/dalfopristin, ciprofloxacin and levofloxacin was prevalent. Consumption of raw tomatoes as a potential exposure risk for antibiotic-resistant *Enterococcus* spp. deserves further attention.

1. Introduction

Enterococci are enteric, commensal bacteria that colonize the digestive tracts of a wide range of vertebrate hosts, and are therefore, widespread in the environment and in agricultural settings (Fisher and Phillips, 2009). Some species, including *Enterococcus faecalis* and *Enterococcus faecium*, are among the most important hospital-acquired, multidrug-resistant microorganisms, causing severe, life-threatening infections of the bloodstream, urinary tract, skin and soft-tissue (Arias and Murray, 2012). Enterococci are also accepted as suitable indicators of fecal contamination for recreational waters (USEPA, 2002), and have been used as indicators of microbiological quality of fresh produce (Ailes et al., 2008; Johnston et al., 2006).

The pathogenicity of antibiotic-resistant enterococci in hospital settings and the possibility of community-acquired infections emphasize the potential importance of these microorganisms with regard to food safety (Arias and Murray, 2012; Franz et al., 2003; Giraffa, 2002). Although hospital-acquired infections are more prevalent than community-acquired infections, cases of community-acquired urinary tract infections and other illnesses associated with multidrug-resistant enterococci have been reported, with higher risks of infection being associated with antibiotic therapy (Aznar et al., 2004; Tang et al., 2007; Fazal et al., 1995; Kwan and Onyett, 2008; Raja et al., 2005). Foodborne antibiotic-

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resistant enterococci may colonize human digestive tracts, and could become dominant gastrointestinal tract inhabitants in hospitalized patients being administered antibiotics, potentially serving as a source of hospital-acquired infections (Arias and Murray, 2012). In spite of this potential threat to public health, the prevalence of multidrug-resistant enterococci in the environment and the community and the possibility of foodborne routes of exposure remain under-researched.

Nevertheless, *Enterococcus* spp. have been isolated from various vegetables, leafy greens and fruits obtained from retail markets (Johnston and Jaykus, 2004; Johnston et al., 2006; Ronconi et al., 2002) and specifically from tomatoes (Abriouel et al., 2008; McGowan et al., 2006; McGowan-Spicer et al., 2008). McGowan et al. (2006) found that 9 of 27 (33.3%) tomato samples harbored *Enterococcus* spp., the most predominant being *Enterococcus casseliflavus*, an organism that rarely causes human illness (Gascon et al., 2003; Iaria et al., 2005; Pappas et al., 2004). Since the few studies that have evaluated *Enterococcus* on tomatoes have tested retail tomatoes, it remains unclear whether colonization occurs mainly pre-harvest during the production stage versus post-harvest during packing, handling and transport. Furthermore, information regarding the antimicrobial susceptibilities of field-derived enterococci is scarce.

This study aimed to evaluate the distribution, diversity and antimicrobial susceptibilities of *Enterococcus* spp. recovered from tomato farms in the Mid-Atlantic region of the U.S. The relatedness among environmental and tomato-associated *E. faecalis* was also compared by amplified fragment length polymorphism (AFLP) as a means to evaluate relationships among isolates, and therefore, dispersal potential from one habitat to another within a farm setting. In addition, pre-harvest tomatoes and leaves were divided into top-, middle- and bottom-portions of the plant to assess whether tomato location on the vine is a risk factor for bacterial contamination.

2. Materials and methods

2.1. Sampling sites

Eight tomato farms in the Mid-Atlantic Region of the U.S. were sampled during the 2009 tomato-harvesting season, from July to October. Six were large-scale industrial productions – coded TFL3, TFL9, TFL19, TFL25, TFL32 and TFL37 – that used plasticulture and chemical fertilization. On these large-scale farms, a single field was randomly selected and sampled in both July and October. The two other farms included in the study were small-scale, family-owned operations – coded TFS1 and TFS2. On TFS1, two fields were sampled, TFS1-PC and TFS1-CC, where plasticulture or zero tillage with a cover crop were used, respectively. On TFS2, one field was sampled where plasticulture was used. The fields on the small-scale farms were sampled in August and September. Small-scale operations occasionally used composted poultry litter for fertilization. All fields on both large-scale and small-scale farms had tomatoes planted on raised beds in rows that were drip-irrigated using sand-filtered pond water.

2.2. Sample collection

Irrigation pond water, groundwater (water located underground) and water pooled in irrigation ditches between tomato rows were collected with gloved hands by filling sterile 1 L polyethylene Nalgene Wide Mouth Environmental Sample Bottles (Nalgene, Lima, OH, U.S.) as previously described (Micallef et al., 2012). Tomato and leaf samples as well as soil samples (200 g) from irrigation ditches, were collected from three randomly selected locations in each sampled field with gloved hands using sterile 798 ml whirl-pak bags (Nasco, Fort Atkinson, WI, U.S.) as described previously (Micallef et al., 2012). Tomatoes and leaves were sampled in triplicate (nine samples total) in a tiered fashion along the vine: bottom (<30 cm from the ground), middle (30–60 cm from the ground) and top (>60 cm from the ground) plant portions as previously described (Micallef et al., 2012). All samples were transported to the lab on ice and stored at 4 °C. Water samples were analyzed within 24 h of collection.

2.3. Sample analysis

Standard membrane filtration was used to recover *Enterococcus* spp. from water samples (EPA, 2002). Briefly, ten-fold serial dilutions in the range of 100–10^{-1} ml for pond water, 100–1 ml for groundwater and 10–10^{-2} ml for irrigation ditch water were filtered through 0.45 μm, 47 mm mixed cellulose ester filters (Millipore, Billerica, MA, U.S.). Filters were placed on mEI agar (EMD Chemicals, Gibbstown, NJ, U.S.) and incubated at 42 °C for 24 h. Blue colonies typical of enterococci on mEI were counted and the number of colony forming units in CFU/100 ml of water was determined. One to six colonies were picked and purified on Brain Heart Infusion Agar (BD Diagnostic Systems, Franklin Lakes, NJ, U.S.). Confirmed catalase-negative isolates were tested for pyrrolidonyl peptidase (pyr) activity (Remel, Lenexa, KS, U.S.) before archiving in Brucella Broth (BD Diagnostic) with 15% glycerol at −80 °C.

For isolation of *Enterococcus* spp. from tomatoes, 100 ml of Buffered Peptone Water (HiMedia Laboratories, Mumbai, India) was added to each bag and tomatoes were washed by hand rubbing the bag for 2 min. A 5 ml aliquot of the rinsate was transferred to 15 ml Enterococcosel Broth (EB), (BD Diagnostic Systems) for a 48 h enrichment at 42 °C, and a 10 μl loopful of the enrichment was streaked onto Enterococcosel Agar (EA) (BD Diagnostic Systems) and incubated at 42 °C for 24 h. From each EA plate, up to six colonies were purified, confirmed and archived as noted above.

For isolation of *Enterococcus* spp. from leaves, 100 ml of phosphate buffered saline (PBS) was added to each bag and hand rubbed for 30 s, vortexed for 2 min and the process repeated before transferring a 2 ml aliquot to 18 ml of EB in a culture tube and incubating and processing as described above. For isolation of *Enterococcus* spp. from soil, 100 g of soil were re-suspended in 100 ml of EB for a 48 h enrichment at 42 °C, and a 10 μl loopful of the enrichment was streaked on EA and incubated at 42 °C for 24 h. From each EA plate, up to 6 black colonies were purified on BHI agar plates, tested for catalase and pyr activity and archived as described above.

2.4. Enterococcus spp. identification

Identification of presumptive *Enterococcus* spp. was performed on the Vitek 2.0 Compact 2.0 System (Biomeriuex, Marcy l'Etoile, France) using 24 h cultures grown on trypticase soy agar with 5% sheep's blood (BD Diagnostic Systems) and GP cards with suspensions made up according to the manufacturer's recommendations. For confirmation, a multiplex PCR assay described by Kariyama et al. (2000) was modified as described below. Genomic DNA from *Enterococcus* was extracted by heat lysis as previously described (Micallef et al., 2012). Three microliters of this product were used directly in a PCR reaction targeting the D-alanine:D-alanine ligase (*ddl*) genes of *E. faecalis* and *E. faecium*, the vancomycin resistance-encoding *van*C1 and *van*C2/3 genes of *Enterococcus gallinarum* and *E. casseliflavus*, respectively, and an internal control targeting a 350 base pair portion of the 16S rRNA genes using primers as described in Micallef et al. (2012). Primers for *Enterococcus avium* and *Enterococcus raffinosus* targeted the