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ORIGINAL ARTICLE

Distribution of selected virulence genes and antibiotic resistance in *Enterococcus* species isolated from the South Nation River drainage basin, Ontario, Canada

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Abstract

Aims: Isolate and characterize water enterococci from the South Nation River drainage basin, an area dominated by agriculture.
Methods and Results: A total of 1558 enterococci were isolated from 204 water samples from the South Nation River obtained over a 3-year period. PCR was used to identify isolates to the species level and characterize them for carriage of 12 virulence determinants. Antibiotic resistance was evaluated phenotypically. *Enterococcus faecalis* (36·4%), *Enterococcus faecium* (9·3%) and *Enterococcus durans* (8·5%) were the major enterococci species isolated. Enterococci carrying more than two virulence determinants were more frequently detected in the summer (59·6%) than in other seasons (≤37·6%). Very few isolates (≤2·0%) were resistant to category I antibiotics ciprofloxacin and vancomycin.
Conclusions: Comparison of major water enterococci species with major faecal enterococci species obtained from various host groups (human, domesticated mammals and birds, wildlife) in this drainage basin suggest that water enterococci may have varied faecal origins. The low level of antibiotic resistance among enterococci suggests that dispersion of antibiotic resistance via waterborne enterococci in this watershed is not significant.
Significance and Impact of the Study: The data obtained in this study suggests that water enterococci in the SNR have a faecal origin and that their potential impact on public health regarding antibiotic resistance and virulence determinants is minimal.

Introduction

Enterococci are facultatively aerobic, Gram-positive bacteria. Being ubiquitous in the gastrointestinal tract of humans and animals, enterococci are useful as indicators of faecal contamination of water (Yost *et al.* in press). In clinical settings, *Enterococcus faecalis* and *Enterococcus faecium* are the most frequently encountered enterococci in nosocomial infections, the second most common cause of wound and urinary tract infections, and the third most common cause of bacteraemia (Fisher and Phillips 2009). Historically, the ratio of enterococci infection has been 10 : 1 for *Ent. faecalis*: *Ent. faecium*, but this ratio is slowly changing, with *Ent. faecium* infections increasing, while *Ent. faecalis* infections are decreasing (Sood *et al.* 2008). Enterococci are frequently resistant to antibiotics, a phenomenon thought to be occasioned by the use of antibiotics in animal husbandry and human medicine, and which complicates the treatment of enterococcal infections (Klare *et al.* 2003). A frequently cited example is the dissemination of glycopeptide-resistant *Ent. faecium* (GRE) strains through the food chain in Europe following the use of avoparcin in animal husbandry (Klare *et al.* 2003), which was then followed by a decrease in GRE after avoparcin use was banned (Hammerum *et al.* 2007). Likewise, vancomycin use in American hospitals was also

followed by an increase in enterococci resistant to this antibiotic (Klare *et al.* 2003).

In mixed activity watersheds, the magnitude, location and timing of faecal pollution events will vary according to watershed physical characteristics, land use and climate conditions, notably precipitation (Singer *et al.* 2006). Water can become contaminated through exposure to agricultural (livestock, poultry, biosolids use), urban (sewage, septage) or wildlife (mammalian, avian) faecal sources. Enterococci are commonly used as faecal indicators to monitor water quality because their presence correlates well with health risks in recreational and marine waters (Cabelli *et al.* 1982; U.S. Environmental Protection Agency 2003). Characterization of water enterococci isolates using techniques such as antibiotic resistance assay have also been used successfully to identify sources of faecal pollution in surface waters (Hagedorn *et al.* 1999; Harwood *et al.* 2003; Wiggins *et al.* 2003). The PCR detection of the *esp* gene variant of *Ent. faecium* (esp_{fm}) in surface water, a gene associated with urinary tract infection (Shankar *et al.* 2001), has also been described as a way to detect human faecal pollution in water (Scott *et al.* 2005), although mixed successes have been associated with the use of this technique (Byappanahalli *et al.* 2008; Layton *et al.* 2009).

Since 2004, we have been evaluating the distribution, densities and characteristics of faecal indicator bacteria and enteric pathogens in surface waters of the South Nation River watershed in Eastern Ontario, Canada (Lyautey *et al.* 2007, 2010; Ruecker *et al.* 2007; Lapen *et al.* 2008; Wilkes *et al.* 2009). Key objectives have been to identify predictive relationships between the densities of indicator bacteria and pathogenic micro-organisms and to identify key land use and climate conditions associated with pollution events. In the course of this work, we isolated large numbers of enterococci from surface water samples obtained from three sampling sites associated with various catchment size and land usage. The enterococci isolates were identified to the species level and characterized with respect to traits of public health significance, namely carriage of some virulence genes and resistance to selected antibiotics. Finally, the data were examined to identify seasonal variations in the frequency of traits of public health significance among water enterococci.

Materials and methods

Sampling sites and sampling strategy

The sampling sites were located within the South Nation River watershed, Ontario, Canada. The total area of the watershed is 3900 km^2, with a river length of approximately 175 km. A total of 24 sampling sites are covering an area of approximately 200 km^2 that has a generally flat topography, and where tile drainage and groundwater primarily contribute to flow. About 60% of this area is occupied by agricultural land, while about 40% of the area consists of urban developments and wildlife habitats. Agricultural land in the drainage basin is a mix of livestock and cash crop production. Manure is normally applied to the fields in the spring and in the fall. Water samples evaluated in this study were collected bi-weekly from three sites (MST-1, MST-5, MST-6) on the watershed representing a variety of catchment size and land usage (Table 1). More information about the drainage basin, land use in the area, specific details about the three sites used in the current study as well as ancillary and hydrological data can be found elsewhere (Lyautey *et al.* 2007, 2010; Ruecker *et al.* 2007; Wilkes *et al.* 2009).

Surface water samples were taken between March 2004 and December 2004 ($n = 68$), between January 2005 and December 2005 ($n = 71$), and between January 2006 and November 2006 ($n = 65$) from the three sites, for a total

Table 1 Land use and point source distribution for the South Nation River watershed

Site	Catchment Size (km^2)	Catchment area surveyed in 2005 (%)	Upstream distance from sampling site to upper margin of surveyed area (km)	Land surveyed (%)*			
				Dairy or cattle pasture	Crop†	Urban‡	Natural§
South Nation River, MST-1	2370·6	16	53·0	4	38	1	15
Little Castor River, MST-5	80·9	38	24·0	2	46	0	11
Payne River, MST-6	176·2	76	41·3	5	32	1	23

*Rounded to the nearest per cent.

†Crop land is land under corn, soybean, wheat or other production, excluding farmland devoted to forages (e.g. alfalfa, grass, clover).

‡Urban land excludes housing associated with a farming operation.

§Natural land includes wetland, exposed rock, shrub land or forest.

Source: Lyautey *et al.* (2007); Ruecker *et al.* (2007).

of 204 samples (Table 2). Water samples from sites MST-5 and MST-6 were collected within 0·5 m depth from the surface using a sampling pole to which a sterile 1-l PET jar was attached (Systems Plus, Baden, ON, Canada). Water samples from site MST-1 were taken from the intake of a municipal drinking water plant from a depth of about 6 m within the South Nation River and collected into PET jars. Containers were sealed immediately after collection, placed on ice and shipped overnight to the Agriculture and Agri-Food Canada laboratory in London, Ontario. Processing of the samples was carried out within 24 h of sample collection.

Isolation and phenotypic confirmation of enterococci

The water samples were processed according to the Method 9230 of the 21st edition of the Standard methods for the Examination of Water and Wastewater for faecal *Streptococcus* and *Enterococcus* groups (Eaton *et al.* 2005). For each sample, a volume of 100 μl, 1 ml, 10 ml, 50 ml and 100 ml was diluted in 100 ml of sterile sodium metaphosphate (2 g l^{-1}). Each of these dilutions was then filtered through a 47-mm 0·45-μm mixed cellulose ester filter with grid (Pall Corporation, Mississauga, ON, Canada). The filters were placed on Difco™ mEnterococcus agar (BD Biosciences, Mississauga, ON, Canada) plates and incubated for 48 h at 37°C. Because of varying numbers of colonies per sample, between 8 and 200 well-isolated colonies of presumptive *Enterococcus* spp. were picked from each sample of each site and inoculated into 100 μl of Bacto™ Brain Heart Infusion broth (BHI; BD Biosciences) in 96-well plates and incubated at 37°C for 24 h. Glycerol was then added to each well for a final concentration of 15%, and the plates were frozen at −80°C.

A subset of eight randomly selected isolates per water sample were used for confirmation and further analysis. Phenotypic confirmation of isolates was performed by plating onto Difco™ mEnterococcus agar plates (BD Biosciences), followed by BBL™ Enterococcosel Agar (BD Biosciences) and BHI agar (BD Biosciences). A single colony derived from each isolate was then inoculated in a well from a 96-well microplate containing 100 μl BHI broth per well and incubated at 37°C for 24 h. Cultures were then checked for salt tolerance and catalase activity (Lanthier *et al.* 2010). The purified and confirmed (salt tolerant and catalase negative) enterococci were inoculated into BHI broth and grown overnight at 37°C. The confirmed cultures were frozen at −80°C after the addition of glycerol to a final 15% concentration.

Molecular methods

DNA template for the PCR was prepared by proteinase K (Sigma-Aldrich, Oakville, ON, Canada) digestion

Table 2 Distribution of enterococci species per season in water samples from the South Nation River watershed

		Species identification with single mPCR (S1) panel only						Species identification with full mPCR (S1–S7) panel			
	No. of water samples	No. of isolates processed	*durans*	*faecalis*	*faecium*	No. of isolates identified	Unknown (S1)	No. of isolates processed	*asini*	*avium*	*casseliflavus*
Season	*n*	*n*	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Winter	24	181	16 (8·8)	42 (23·2)	37 (20·4)	95 (52·5)	86 (47·5)	25 (13·8)	0	2 (1·1)	0
Spring	60	462	62 (13·4)	88 (19·0)	62 (13·4)	212 (45·9)	250 (54·1)	42 (9·1)	1 (0·2)	3 (0·6)	1 (0·2)
Summer	64	495	23 (4·6)	285 (57·6)	15 (3·0)	323 (65·3)	172 (34·7)	7 (1·4)	0	0	0
Fall	56	420	32 (7·6)	152 (36·2)	31 (7·4)	215 (51·2)	205 (48·8)	40 (9·5)	0	0	6 (1·4)
Total	204	1558	133 (8·5)	567 (36·4)	145 (9·3)	845 (54·2)	713 (45·8)	114 (7·3)	1 (0·1)	5 (0·3)	7 (0·4)

	Species identification with full mPCR (S1–S7) panel									
	cecorum	*dispar*	*gallinarum*	*hirae*	*mundtii*	*raffinosus*	*seriolicida*	*solitarius*	No. of isolates identified	Unknown (S1–S7)
Season	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Winter	1 (0·6)	2 (1·1)	1 (0·6)	5 (2·8)	6 (3·3)	1 (0·6)	1 (0·6)	1 (0·6)	20 (80·0)	5 (20·0)
Spring	1 (0·2)	3 (0·6)	0	7 (1·5)	11 (2·4)	0	0	0	27 (64·3)	15 (35·7)
Summer	0	0	0	0	0	0	0	5 (1·0)	5 (71·4)	2 (28·6)
Fall	9 (2·1)	1 (0·2)	1 (0·2)	1 (0·2)	0	1 (0·2)	0	5 (1·2)	24 (60·0)	16 (40·0)
Total	11 (0·7)	6 (0·4)	2 (0·1)	13 (0·8)	17 (1·1)	2 (0·1)	1 (0·1)	11 (0·7)	76 (66·7)	38 (33·3)

(Lanthier *et al.* 2010). The enterococci DNA templates were diluted 10× with PCR-grade water and stored at −20°C.

Enterococci were identified by PCR as follows. Genus-specific primers E1 and E2 were used to target the 16S ribosomal RNA gene of all known *Enterococcus* spp. (Deasy *et al.* 2000). Species-specific primers were used to target the *sodA* gene (manganese-dependent superoxide dismutase) of 23 *Enterococcus* species (Jackson *et al.* 2004). Species-specific primers were grouped according to annealing temperatures and product size into seven multiplex PCR (mPCR) groups (S1–7) (Jackson *et al.* 2004). Primers used to target 12 enterococci pathogenicity genes were organized into four mPCR groups (V1–4) based on their annealing temperature and product size (Eaton and Gasson 2001; Bittencourt de Marques and Suzart 2004; Lanthier *et al.* 2010). Primers GM5F and 907R amplifying the 16S ribosomal gene of all Bacteria (Amann *et al.* 1992; Muyzer *et al.* 1993) were used as a positive control for groups S1–7 and V1–4. All primers were from Sigma-Aldrich.

The PCR mixture for the speciation mPCR with groups S1–7 was made of a volume of 2·5 μl of 10× diluted enterococci lysate as DNA template, 1× HF buffer (1·5 mmol l^{-1} $MgCl_2$ final; New England Biolab, Pickering, ON, Canada), 0·2 mmol l^{-1} each dNTP (Invitrogen, Burlington, ON, Canada), 0·3 U high fidelity Phusion *Taq* polymerase (New England Biolab) and 0·4 μmol l^{-1} of each primer (Jackson *et al.* 2004). Volume was completed to 25 μl with sterile Milli-Q water. The reaction mixture for the *Enterococcus* spp. genus identification or the virulence-determinant genes mPCR for groups V1–4 was made of a volume of 2 μl of 10× diluted enterococci lysate as DNA template, 1× *Taq* buffer with $(NH_4)_2SO_4$ [final concentrations of 75 mmol l^{-1} Tris–HCl (pH 8·8), 20 mmol l^{-1} $(NH_4)_2SO_4$, 0·01% (v/v) Tween 20; Fermentas, Burlington, ON, Canada], 2·5 mmol l^{-1} $MgCl_2$ (Fermentas), 0·2 mmol l^{-1} each dNTP (Invitrogen), 2·5 U recombinant *Taq* polymerase (Fermentas), and 0·2 μmol l^{-1} of each primer (Eaton and Gasson 2001). The volume was completed to 25 μl with sterile Milli-Q water. DNA extracted from reference strains of *Enterococcus* spp. was used as positive control for speciation (*Enterococcus avium* ATCC14025, ATCC49464; *Enterococcus casseliflavus* ATCC100327; *Enterococcus durans* ATCC6056, ATCC11576; *Ent. faecalis* ATCC19433, ATCC29212, ATCC49532; *Ent faecium* ATCC6569, ATCC27270, ATCC35667; *Enterococcus gallinarum* ATCC49573, ATCC700425; *Enterococcus hirae* ATCC8043, ATCC10544; *Enterococcus saccharolyticus* ATCC43076). *Enterococcus faecalis* ATCC19433 (*esp*$^+$), ATCC29212 (*gelE*$^+$, *EfaAfs*$^+$, *cpd*$^+$, *cob*$^+$, *cylA*$^+$, *cylM*$^+$, *cylB*$^+$, *ccf*$^+$, *eep*$^+$, *ccf*$^+$) and ATCC49532 (*enlA*$^+$, *agg*$^+$) were used as positive controls for virulence PCR. Autoclaved Milli-Q water replaced the DNA template in negative controls.

DNA amplification was performed with an MBS Satellite 2.0 (Thermo Fisher Scientific, Nepean, ON, Canada). The speciation PCR programme consisted of a denaturation step for 4 min at 95°C, followed by 30 cycles of amplification (denaturation for 30 s at 95°C, annealing for 1 min at a primer-dependent temperature, and elongation for 1 min at 72°C) (Jackson *et al.* 2004). Annealing temperatures of 55°C were used for groups S1, S2, S5 and S6, and 60°C for groups S3, S4 and S7. The amplification was followed by a final 7-min extension at 72°C. The virulence genes PCR program consisted of an initial denaturation step at 94°C for 10 min, followed by a 2-min annealing step at either 55°C or 57°C and a 2-min extension step at 72°C. This initial step was followed by 29 (V1, V3, V4) or 35 (V2) cycles of amplification (denaturation for 15 s at 92°C, annealing for 15 s at a primer-dependent temperature, and extension for 15 s at 72°C) and by a final extension step for 10 min at 72°C. Annealing temperatures of 55°C (V1, V2) and 57°C (V3, V4) were used.

PCR products were electrophoresed on 1·5–2% 1× Tris–acetate–EDTA (TAE) agarose gels. Gels were stained in an ethidium bromide solution before imaging with an Alpha Imager system (Cell Biosciences, Santa Clara, CA) equipped with DE500 Darkroom (Thermo Fisher Scientific).

Antibiotic resistance analysis

The resistance of *Enterococcus* spp. isolates to antibiotics was determined phenotypically as follows. First, 96-well microplates containing 100 μl of Difco™ Mueller–Hinton Broth (MHB; VWR International, Ville Mont-Royal, QC, Canada) per well were inoculated with the original frozen enterococci isolates. Eight microlitres of an overnight MHB culture plate grown at 37°C was transferred into a 96-well microplate containing 200 μl of 0·02% Tween 20 (Sigma-Aldrich) per well to stabilize emulsions and suspensions. Five microlitres from each well was taken up using a 96-pin VP Floating Pin Replicator (V&P Scientific, San Diego, CA, USA) and used to inoculate 245 mm^2 square plates containing BD Bacto™ Mueller–Hinton Agar (MHA; VWR International) supplemented with various antibiotics. Filter-sterilized aqueous solutions of antibiotic (Sigma-Aldrich) solutions were added to the MHA before the plates were poured. Final antibiotic concentrations were adjusted to half, equal and twice the breakpoint concentrations used in the 2004 Canadian Integrated Program for Antibiotic Resistance Surveillance (CIPARS) report (Health Canada 2006). Antibiotic concentrations used were (in μg ml^{-1}) bacitracin (64, 128, 256), chloramphenicol (16, 32, 64), ciprofloxacin (2, 4, 8), erythromycin (4, 8, 16), gentamicin (250, 500, 1000), kanamycin (1024, 2048, 4096), lincomycin (4, 8, 16), penicillin (8, 16, 32), streptomycin (500, 1000, 2000),

tetracycline (8, 16, 32), tylosin (4, 8, 16) and vancomycin (16, 32, 64). The MHA plates were incubated at 37°C for 24 h following which the growth of each isolate was determined by eye and referenced to an antibiotic-free control plate. Presence of small, visible colonies was considered positive growth.

Statistical analyses

Pearson χ^2 test of contingency tables (2 × 4) with Yates' correction for continuity was used to evaluate if the distribution (%) of each enterococci species, each virulence determinant and each antibiotic resistance was different between seasons. For these analyses, data from all sites and years were combined and then grouped per season, and a *P*-value ≤0·05 was considered significant. However, the Pearson χ^2 test will only identify whether the distribution is different between the four seasons, but cannot identify which season differs from the others. To clarify this, Fisher's exact test of contingency tables (2 × 2) was used to evaluate whether the distribution was different for each season (winter *vs* spring, winter *vs* summer, winter *vs* fall, spring *vs* summer, spring *vs* fall, summer *vs* fall) when the Pearson χ^2 test identified different distribution between seasons. A *P*-value ≤0·05 was also considered significant for this test. All statistical tests were performed using Analyse-It for Microsoft Excel ver. 2.21 [Analyse-It Software, Ltd, Leeds, UK (http://www.analyse-it.com/)] and Microsoft Excel 2002.

Results

Confirmation and identification of enterococci isolates as *Enterococcus* spp.

A total of 1558 presumptive enterococcal isolates from 204 water samples were obtained by membrane filtration of water samples taken from the watershed (Table 2). All of the 1558 isolates were confirmed as *Enterococcus* spp. by PCR using the genus-specific primers E1 and E2 (Deasy *et al.* 2000).

A mPCR protocol distinguishing 23 *Enterococcus* species was used for the identification to the species level of the water enterococci (Jackson *et al.* 2004). The group 1 primer mix (S1) was used on all of the 1558 isolates to identify *Ent. durans*, *Ent. faecalis*, *Ent. faecium* and *Enterococcus malodoratus* (Table 2). *Enterococcus faecalis* was the most frequently identified *Enterococcus* species (36·4% of the collection), and this species was most frequently ($P \leq 0{\cdot}05$) identified among isolates recovered in the summer (57·6%), followed by fall (36·2%), while no difference ($P > 0{\cdot}05$) was observed between isolates recovered in winter and spring (19·0–23·2%) for this species. *Enterococcus faecium* was the second most frequently identified species among the collection (9·3%), and it was most frequently ($P \leq 0{\cdot}05$) identified among isolates recovered in winter (20·4%), followed by spring (13·4%), fall (7·4%) and summer (3·0%). *Enterococcus durans* was the third most frequently identified ($P \leq 0{\cdot}05$) enterococci species (8·5%) among the collection. The frequency of identification of this species was higher only ($P \leq 0{\cdot}05$) in spring (13·4%) compared to summer and fall (4·6–7·6%), and no difference ($P > 0{\cdot}05$) was observed between the frequency of identification of this species between winter (8·8%) and the other seasons (Table S1). No isolate could be identified as *Ent. malodoratus*. Overall, 845 (54·2%) of the isolates were assigned to an *Enterococcus* species using the S1 primer mix.

Further characterization with the remaining sets of mPCR primers (S2–S7) of 114 of the isolates that could not be identified with the S1 primer set was performed, but only allowed identification of 10 minor (≤1·1%) species (Table 2). Out of these minor species, *Enterococcus mundtii* was the most abundant (1·1%) among the collection and was only identified among isolates recovered in winter (3·3%) and spring (2·4%), but no difference ($P > 0{\cdot}05$) was observed between the frequency of identification of this species between these two seasons. There were significant ($P \leq 0{\cdot}05$) differences between seasons in the identification of minor enterococci species for *Ent. avium*, *Ent. casseliflavus*, *Enterococcus cecorum* and *Ent. hirae*, but because of the small number of isolates obtained for each of these species, statistical analyses could not clearly identify the differences between the frequency of identification of these species per season (Table S1). The proportion of isolates that could be assigned a species following the full panel species identification mPCR was 66·7% ($n = 76$).

Frequency of virulence determinants among *Enterococcus* spp. isolates

The distribution of 12 virulence determinants within the collection of 1558 *Enterococcus* spp. isolates was determined (Table 3). The virulence determinants most frequently detected were *ccf* (55·9%), followed by *eep* (29·7%), *cpd* (29·5%), *EfaAfs* (28·5%), *gelE* (18·2%) and *cob* (17·8%). All of the other virulence determinants were found in <10% of the isolates. Only seven isolates (0·4%) harboured *cylABM*.

A total of eight virulence determinants were more frequently found ($P \leq 0{\cdot}05$) among isolates from the collection obtained in the summer compared to the other three seasons: *agg* (22·0% of summer isolates), *ccf* (71·9%), *cob* (34·7%), *cpd* (49·5%), *eep* (50·9%), *EfaAfs* (48·9%), *enlA* (13·7%) and *gelE* (37·8%). Isolates obtained

Table 3 Distribution of selected virulence genes per season in *Enterococcus* spp. isolated from the South Nation River watershed

	No. of isolates processed	Virulence genes						
		agg	*ccf*	*cob*	*cpd*	*cylA*	*cylB*	*cylM*
Season	*n*	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Winter	181	14 (7·7)	88 (48·6)	7 (3·9)	30 (16·6)	11 (6·1)	3 (1·7)	8 (4·4)
Spring	462	10 (2·2)	176 (38·1)	25 (5·4)	56 (12·1)	55 (11·9)	9 (1·9)	32 (6·9)
Summer	495	109 (22·0)	356 (71·9)	172 (34·7)	245 (49·5)	59 (11·9)	7 (1·4)	42 (8·5)
Fall	420	15 (3·6)	251 (59·8)	74 (17·6)	128 (30·5)	15 (3·6)	8 (1·9)	12 (2·9)
Total	1558	148 (9·5)	871 (55·9)	278 (17·8)	459 (29·5)	140 (9·0)	27 (1·7)	94 (6·0)

	Virulence genes					
	eep	*EfaAfs*	*enlA*	*esp*	*gelE*	*cylABM*
Season	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Winter	33 (18·2)	28 (15·5)	0	0	9 (5·0)	2 (1·1)
Spring	47 (10·2)	57 (12·3)	16 (3·5)	3 (0·6)	17 (3·7)	2 (0·4)
Summer	252 (50·9)	242 (48·9)	68 (13·7)	13 (2·6)	187 (37·8)	2 (0·4)
Fall	131 (31·2)	117 (27·9)	20 (4·8)	9 (2·1)	71 (16·9)	1 (0·2)
Total	463 (29·7)	444 (28·5)	104 (6·7)	25 (1·6)	284 (18·2)	7 (0·4)

in the fall were found to harbour the second highest ($P \leq 0{\cdot}05$) frequency for six of those determinants, namely *ccf* (59·8% of fall isolates), *cob* (17·6%), *cpd* (30·5%), *eep* (31·2%), *EfaAfs* (27·9%) and *gelE* (16·9%). The *cylA* gene was also present at a higher ($P \leq 0{\cdot}05$) frequency (11·9% for both season) among both spring and summer isolates, compared to the other two seasons. The frequency of distribution was different between all seasons among isolates from the collection for only two virulence determinants, namely *ccf* and *eep*. In the case of *ccf*, the frequency of this gene was highest ($P \leq 0{\cdot}05$) among summer isolates (71·9%), followed by fall (59·8%), winter (48·6%) and spring (38·1%). In the case of *eep*, this gene was also more frequent ($P \leq 0{\cdot}05$) among summer isolates (50·9%), followed by fall (31·2%), winter (18·2%) and spring isolates (10·2%) (Tables S2, S3). Overall, selected virulence genes were detected more frequently in isolates obtained in the summer and fall period than in the winter or spring among the collection.

The number of virulence determinant per isolates was also evaluated among isolates from the collection (Table 4). A large proportion of isolates did not carry any of the virulence determinant selected for the current study (39·9%), and these isolates were less frequently ($P \leq 0{\cdot}05$) obtained in the summer (24·6%) compared to other seasons (39·3–53·9%). Similarly, the frequency of carriage of two or more virulence determinants was higher ($P \leq 0{\cdot}05$) among isolates obtained in the summer (59·6%), compared to other seasons (27·5–37·6%) (Table S4).

Antibiotic resistance of the *Enterococcus* spp. isolates

At breakpoint concentrations, the most common resistance was against lincomycin (28·5%), followed by bacitracin (23·4%), and resistance to all of the other antibiotics was equal or below 2·0% (Table 5) among isolates from the collection. Regarding category I antibiotics, no isolate was found to be resistant to breakpoint concentrations of vancomycin, and resistance to ciprofloxacin was low (2·0%). At breakpoint concentrations, significant ($P \leq 0{\cdot}05$) differences in the frequency of resistance between at least two seasons were found for seven antibiotics (bacitracin, chloramphenicol, ciprofloxacin, kanamycin, lincomycin, streptomycin and tetracycline) among isolates from the collection (Tables S5, S6).

Similarly, at twice the breakpoint concentration, the most common resistance among isolates from the collection was also against lincomycin (56·0%), followed by bacitracin (8·1%) and tetracycline (7·8%), while resistance to all other antibiotics was equal or below 3·0%. Regarding category I antibiotics, no isolate was found to be resistant to twice the breakpoint concentrations of vancomycin, while resistance to ciprofloxacin was low (0·8%). At twice the breakpoint concentration, significant ($P \leq 0{\cdot}05$) differences in the frequency of resistance between at least two seasons were found for eight antibiotics (bacitracin, ciprofloxacin, erythromycin, gentamicin, lincomycin, streptomycin, tetracycline and tylosin) (Tables S5, S6).

The number of antibiotic resistance per isolate was also evaluated per season (Table 6) among isolates from the

Table 4 Number of selected virulence genes per season in *Enterococcus* spp. isolated from the South Nation River watershed

	No. of isolates processed	No. of virulence gene per isolate												
		0	1	2	3	4	5	6	7	8	9	10	11	2 or more
Season	*n*	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Winter	181	85 (47·0)	43 (23·8)	26 (14·4)	6 (3·3)	8 (4·4)	1 (0·6)	4 (2·2)	7 (3·9)	1 (0·6)	0	0	0	53 (29·3)
Spring	462	249 (53·9)	86 (18·6)	65 (14·1)	19 (4·1)	20 (4·3)	7 (1·5)	6 (1·3)	6 (1·3)	2 (0·4)	0	1 (0·2)	1 (0·2)	127 (27·5)
Summer	495	122 (24·6)	78 (15·8)	39 (7·9)	14 (2·8)	44 (8·9)	25 (5·1)	40 (8·1)	76 (15·4)	39 (7·9)	12 (2·4)	5 (1·0)	1 (0·2)	295 (59·6)
Fall	420	165 (39·3)	97 (23·1)	32 (7·6)	18 (4·3)	21 (5·0)	20 (4·8)	33 (7·9)	22 (5·2)	10 (2·4)	0	2 (0·5)	0	158 (37·6)
Total	1558	621 (39·9)	304 (19·5)	162 (10·4)	57 (3·7)	93 (6·0)	53 (3·4)	83 (5·3)	111 (7·1)	52 (3·3)	12 (0·8)	8 (0·5)	2 (0·1)	633 (40·6)

collection. At breakpoint concentration, the number of isolates sensitive to all antibiotics was 50·1%, with a higher ($P \leq 0{\cdot}05$) proportion of isolates from the collection not carrying any resistances found in fall (55·1%) compared to winter (44·6%) and spring (46·2%), but not summer (52·0%; $P > 0{\cdot}05$). Resistance to two or more antibiotics at breakpoint concentration was more frequent ($P \leq 0{\cdot}05$) in isolates from the collection obtained in the winter (16·4%) compared to other seasons (5·8–8·1%). At twice the breakpoint concentration, the frequency of isolates not resistant to any antibiotics among the collection was lower ($P \leq 0{\cdot}05$) among summer isolates (28·7%), compared to isolates obtained in the other three seasons (39·5–49·8%). The frequency of carriage of multiple resistances against twice the breakpoint concentration of antibiotics was higher ($P \leq 0{\cdot}05$) among isolates obtained in both winter and spring (15·8–20·3%), followed by fall (8·4%) and summer (4·7%) (Table S7).

The most common resistance profiles at breakpoint concentrations were also determined per species among isolates from the collection. When combining results from all seasons, the most common profile was lincomycin resistance only for frequently identified species among the collection, such as *Ent. durans* (52·1%; $n = 63$), *Ent. faecalis* (66·1%; $n = 363$) and *Ent. faecium* (33·3%; $n = 48$). Lincomycin resistance only was the most common antibiotic resistance profiles for isolates which could not be identified to the species level using the S1 mPCR panel (48·6%; $n = 245$), and also with the full S1–S7 panel (40·0%; $n = 14$). The most common profile was also lincomycin resistance only for 6 of the 11 rarely identified species among isolates from the collection.

Discussion

Seasonal distribution of *Enterococcus* species in the South Nation River watershed

Faecal contamination of surface water can favour the dispersion of various enterococci species in the environment. To evaluate the identity of enterococci species in the South Nation River, enterococci were isolated from surface water samples and were identified to the species level by mPCR. It was revealed that the dominant enterococci species isolated from the South Nation River was *Ent. faecalis*, but *Ent. faecium* and *Ent. durans* were also commonly isolated. These results are similar to what has been described elsewhere with *Ent. faecalis* and *Ent. faecium* usually the dominant species of enterococci isolated from surface water (Kuhn *et al.* 2003; Kuntz *et al.* 2003; Sapkota *et al.* 2007; Pangallo *et al.* 2008; Lata *et al.* 2009). *Enterococcus durans* has likewise been detected in surface waters, although at lower frequencies than that found in our study (Sapkota *et al.* 2007; Pangallo *et al.* 2008).

The distribution of each *Enterococcus* species was also compared between seasons and revealed that *Ent. faecalis* was most often recovered in the summer, while *Ent. faecium* was most often recovered in the winter and *Ent. durans* most often recovered in spring. Multiple factors can explain the seasonal variation of these three *Enterococcus* species. In this watershed, *Ent. faecalis* is more abundant in faeces of livestock, poultry and wildlife than in sewage effluent and septic sludge (Lanthier *et al.* 2010). Dominance of this species during the summer suggests that pollution from non-human sources may be dominant at the sampling sites during this time of the year. On the other hand, a higher frequency of *Ent. faecium* suggests that contamination from human sources may be of more importance during the winter, because *Ent. faecium* is more abundant in human wastewater than in faeces from livestock, poultry or wildlife (Lanthier *et al.* 2010). This is consistent with the absence of water flow and manure application during winter freeze up. *Enterococcus durans* in the South Nation River was found to have a higher frequency in the spring, but compared to summer and fall only. This species has been shown to be a minor resident of the intestinal tract of humans, domesticated mammals and birds, and wildlife (Poeta *et al.* 2007; Lanthier *et al.* 2010; Yost *et al.* in

Table 5 Distribution of maximum antibiotic resistance levels per season in *Enterococcus* spp. isolated from the South Nation River watershed*

			Antibiotic resistance																	
	Total isolates	No. of isolates processed	Bacitracin			Chloramphenicol			Ciprofloxacin			Erythromycin			Gentamicin			Kanamycin		
			64	128	256	16	32	64	2	4	8	4	8	16	250	500	1000	1024	2048	4096
Season	*n*	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Winter	181	177 (97·8)	68 (38·4)	39 (22·0)	30 (16·9)	1 (0·6)	0	0	19 (10·7)	10 (5·6)	1 (0·6)	5 (2·8)	1 (0·6)	0	0	0	1 (0·6)	0	9 (5·1)	5 (2·8)
Spring	462	450 (97·4)	157 (34·9)	101 (22·4)	40 (8·9)	6 (1·3)	0	0	9 (2·0)	15 (3·3)	1 (0·2)	6 (1·3)	4 (0·9)	4 (0·9)	0	0	0	1 (0·2)	0	6 (1·3)
Summer	495	429 (86·7)	172 (40·1)	127 (29·6)	14 (3·3)	0	0	0	13 (3·0)	1 (0·2)	0	1 (0·2)	0	3 (0·7)	0	0	0	0	0	2 (0·5)
Fall	420	370 (88·1)	152 (41·1)	66 (17·8)	32 (8·6)	3 (0·8)	6 (1·6)	0	8 (2·2)	3 (0·8)	9 (2·4)	8 (2·2)	1 (0·3)	10 (2·7)	0	1 (0·3)	7 (1·9)	7 (1·9)	0	9 (2·4)
Total	1558	1426 (91·5)	549 (38·5)	333 (23·4)	116 (8·1)	10 (0·7)	6 (0·4)	0	49 (3·4)	29 (2·0)	11 (0·8)	20 (1·4)	6 (0·4)	17 (1·2)	0	1 (0·1)	8 (0·6)	8 (0·6)	9 (0·6)	22 (1·5)

	Antibiotic resistance																	
	Lincomycin			Penicillin			Streptomycin			Tetracycline			Tylosin			Vancomycin		
	4	8	16	8	16	32	500	1000	2000	8	16	32	4	8	16	16	32	64
Season	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Winter	15 (8·5)	64 (36·2)	91 (51·4)	0	0	1 (0·6)	1 (0·6)	1 (0·6)	5 (2·8)	9 (5·1)	6 (3·4)	20 (11·3)	2 (1·1)	1 (0·6)	10 (5·6)	50 (28·2)	0	0
Spring	77 (17·1)	144 (32·0)	197 (43·8)	0	1 (0·2)	0	5 (1·1)	4 (0·9)	5 (1·1)	12 (2·7)	10 (2·2)	63 (14·0)	22 (4·9)	4 (0·9)	12 (2·7)	85 (18·9)	0	0
Summer	23 (5·4)	93 (21·7)	302 (70·4)	1 (0·2)	0	1 (0·2)	2 (0·5)	0	1 (0·2)	3 (0·7)	6 (1·4)	9 (2·1)	10 (2·3)	5 (1·2)	6 (1·4)	89 (20·7)	0	0
Fall	44 (11·9)	106 (28·6)	209 (56·5)	0	1 (0·3)	0	1 (0·3)	7 (1·9)	3 (0·8)	12 (3·2)	0	19 (5·1)	8 (2·2)	5 (1·4)	15 (4·1)	48 (13·0)	0	0
Total	159 (11·2)	407 (28·5)	799 (56·0)	1 (0·1)	2 (0·1)	2 (0·1)	9 (0·6)	12 (0·8)	14 (1·0)	36 (2·5)	22 (1·5)	111 (7·8)	42 (2·9)	15 (1·1)	43 (3·0)	272 (19·1)	0	0

*All concentrations are in μg ml^{-1}.

Table 6 Distribution of maximum antibiotic resistance levels per season in various enterococci species isolated from the South Nation River watershed

			No. of antibiotic resistance per isolate														
	Total isolates	No. of isolates processed	0			1			2			3			4		
			1/2 BP	BP	2xBP	1/2 BP	BP	2xBP	1/2 BP	BP	2xBP	1/2 BP	BP	2xBP	1/2 BP	BP	2xBP
Season	*n*	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Winter	181	177 (97·8)	59 (33·3)	79 (44·6)	72 (40·7)	77 (43·5)	69 (39·0)	69 (39·0)	32 (18·1)	25 (14·1)	17 (9·6)	7 (4·0)	4 (2·3)	16 (9·0)	2 (1·1)	0	2 (1·1)
Spring	462	450 (97·4)	164 (36·4)	208 (46·2)	224 (49·8)	208 (46·2)	207 (46·0)	155 (34·4)	62 (13·8)	29 (6·4)	51 (11·3)	16 (3·6)	6 (1·3)	13 (2·9)	0	0	4 (0·9)
Summer	495	429 (86·7)	185 (43·1)	223 (52·0)	123 (28·7)	184 (42·9)	181 (42·2)	286 (66·7)	51 (11·9)	24 (5·6)	15 (3·5)	8 (1·9)	1 (0·2)	2 (0·5)	1 (0·2)	0	1 (0·2)
Fall	420	370 (88·1)	136 (36·8)	204 (55·1)	146 (39·5)	188 (50·8)	136 (36·8)	193 (52·2)	37 (10·0)	30 (8·1)	15 (4·1)	7 (1·9)	0	5 (1·4)	2 (0·5)	0	1 (0·3)
Total	1558	1426 (91·5)	544 (38·1)	714 (50·1)	565 (39·6)	657 (46·1)	593 (41·6)	703 (49·3)	182 (12·8)	108 (7·6)	98 (6·9)	38 (2·7)	11 (0·8)	36 (2·5)	5 (0·4)	0	8 (0·6)

	No. of antibiotic resistance per isolate														
	5			6			7			8			2 or more		
	1/2 BP	BP	2xBP	1/2 BP	BP	2xBP	1/2 BP	BP	2xBP	1/2 BP	BP	2xBP	1/2 BP	BP	2xBP
Season	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Winter	0	0	1 (0·6)	0	0	0	0	0	0	0	0	0	41 (23·2)	29 (16·4)	36 (20·3)
Spring	0	0	2 (0·4)	0	0	1 (0·2)	0	0	0	0	0	0	78 (17·3)	35 (7·8)	71 (15·8)
Summer	0	0	1 (0·2)	0	0	0	0	0	1 (0·2)	0	0	0	60 (14·0)	25 (5·8)	20 (4·7)
Fall	0	0	1 (0·3)	0	0	3 (0·8)	0	0	0	0	0	6 (1·6)	46 (12·4)	30 (8·1)	31 (8·4)
Total	0	0	5 (0·4)	0	0	4 (0·3)	0	0	1 (0·1)	0	0	6 (0·4)	225 (15·8)	119 (8·3)	158 (11·1)

BP, breakpoint concentration.

press), making it difficult to identify a single source being responsible for its presence in the watershed. However, in a survey of faecal enterococci in the South Nation River area (Lanthier *et al.* 2010), it was shown that this species was found in a higher proportion among wildlife hosts, but only compared to human wastewaters and not to domesticated mammals and birds. This may suggests that the higher frequency of *Ent. durans* in the spring could be related to the release of faeces of wildlife animals that accumulated in the winter or from spring manure application.

Some of the *Enterococcus* water isolates obtained in this study could have an environmental origin instead of a faecal origin. An Australian study showed that <5% of surface water enterococci isolates could not be associated with a known faecal source and were suspected to be of environmental origin (Ahmed and Katouli 2008). Possible environmental sources of enterococci in the South Nation River may include sediments (Badgley *et al.* 2010a; Balzer *et al.* 2010), biofilms (Balzer *et al.* 2010), plants (Muller *et al.* 2001) and submerged aquatic vegetation (Badgley *et al.* 2010a,b).

Seasonal distribution of virulence genes among *Enterococcus* isolates from the South Nation River watershed

For the purposes of the study, sex pheromone genes (*ccf*, *cob*, *cpd*) and sex pheromone-related genes (*agg*, *eep*) were considered virulence determinants (Valenzuela *et al.* 2008). While these genes are responsible for the conjugative transfer of sex pheromones plasmids, they can also be involved in the pathogenicity process as well. For example, there is evidence that the sex pheromone genes are involved in the inflammation process and that both sex pheromones genes and *agg* are involved in host colonization (Klare *et al.* 2001; Kayaoglu and Orstavik 2004). Furthermore, sex pheromone may favour dissemination of virulence determinants and antibiotic resistances (Valenzuela *et al.* 2008).

Among the sex pheromone genes, *ccf* was the gene who had the highest frequency among the *Enterococcus* water isolates, followed by *cpd* and *cob*. Previous studies reported frequent detection of these three sex pheromone genes in *Ent. faecalis* strains of various origins (Eaton and Gasson 2001; Abriouel *et al.* 2008; McGowan-Spicer *et al.* 2008; Valenzuela *et al.* 2008; Ozmen Togay *et al.* 2010). Results were mixed about the distribution of these genes in *Ent. faecium*, with studies showing their presence (Abriouel *et al.* 2008; Ozmen Togay *et al.* 2010) and absence in this species (Eaton and Gasson 2001; Valenzuela *et al.* 2008) in various environments. All three genes were found to be present in a high proportion among the enterococci obtained in the present study, suggesting that these genes have a large distribution among surface water enterococci.

Both sex pheromone-related genes *agg* (coding for the aggregation substance) and *eep* (coding for a protein enhancing the expression of pheromones) were detected in *Enterococcus* isolates. Previous studies do not agree on the frequency of *agg* among enterococci. Some studies have shown the presence of *agg* among *Ent. faecalis* but not *Ent. faecium* isolates from food and clinical origin (Eaton and Gasson 2001; Franz *et al.* 2001; Valenzuela *et al.* 2008), while others have shown its presence in both *Ent. faecalis* and *Ent. faecium* from food, clinical and environmental origins (Semedo *et al.* 2003; Abriouel *et al.* 2008; Ozmen Togay *et al.* 2010) and in other *Enterococcus* species (Semedo *et al.* 2003). In our study, the *agg* gene was found in *Ent. durans*, *Ent. faecalis*, *Ent. faecium* and unknown (S1) isolates (Table S3). To our knowledge, only one study examined the frequency of *eep* among enterococci, and the study focused on *Ent. faecalis*, revealing that *eep* was present in more than half of the clinical isolates (Bittencourt de Marques and Suzart 2004). Currently, the knowledge related to the contribution of this virulence determinant is limited, but it is suspected that it may have a role in cow mastitis (Denham *et al.* 2008).

The frequency of cytolysin genes *cylA*, *cylB* and *cylM* was low among the water *Enterococcus* isolates obtained in the current study. This contrasts with the perception that β-haemolysis activity is widespread in enterococci (Semedo *et al.* 2003). The relationship between the presence of *cyl* genes or β-haemolysis activity and pathogenicity is unclear, as demonstrated by the varying frequency of *cyl* genes among clinical isolates (Bittencourt de Marques and Suzart 2004; Abriouel *et al.* 2008). Furthermore, the presence of *cyl* genes in isolates is not always correlated with β-haemolysis activity, because a complete *cylL*$_L$*L*$_S$*ABM* operon is needed for expression of active cytolysin (Poeta *et al.* 2008). In the current study, only 7 of 1558 isolates (0·4% of the total collection) carried *cylABM*, suggesting that the risk of waterborne infection by β-haemolytic enterococci is very low.

The *EfaAfs* gene was found frequently among the water enterococci isolates obtained in the current study. This gene has been associated with endocarditis and is suspected to be involved in the biotic and abiotic surface adhesion mechanism in enterococci, and in immune system evasion (Lowe *et al.* 1995; Singh *et al.* 1998; Abriouel *et al.* 2008). This gene has been frequently found in clinical *Ent. faecalis* isolates (Eaton and Gasson 2001; Bittencourt de Marques and Suzart 2004; Abriouel *et al.* 2008), but also in non-clinical isolates such as *Ent. faecalis* vegetable and food isolates, suggesting that its role in adhesion is also important outside a human host (Eaton and Gasson 2001; Abriouel *et al.* 2008). The *gelE* gene,

coding for an extracellular gelatinase, was also found to occur among water enterococci in the current study. This gene has been found previously among clinical, but also in food isolates (Eaton and Gasson 2001; Semedo *et al.* 2003; Bittencourt de Marques and Suzart 2004; Creti *et al.* 2004; McGowan-Spicer *et al.* 2008). The *enlA* and *esp* genes have been both associated with urinary track infections (Shankar *et al.* 2001; Bittencourt de Marques and Suzart 2004). The *esp* gene has also been shown to occur with varying frequency in enterococci from other sources such as clinical, food and the environment (Eaton and Gasson 2001; Semedo *et al.* 2003; Abriouel *et al.* 2008; McGowan-Spicer *et al.* 2008). Detection of *EfaAfs*, *gelE*, *enlA* and *esp* among water isolates, as well as from a variety of environments in other studies, suggests that these may have other uses than pathogenicity and that they may prove useful for survival in environments other than clinical.

Overall, our results suggests that enterococci pose a higher risk to public health in the summer season because (i) 8 of the 12 virulence determinants (*agg*, *ccf*, *cob*, *cpd*, *eep*, *EfaAfs*, *enlA* and *gelE*) were more frequently found among summer isolates, while *cylA* was found more frequently among both spring and summer isolates, and (ii) isolates obtained in the summer were more likely to harbour two or more virulence determinants than isolates recovered in other seasons. Comparison of the frequency of these virulence determinants among water enterococci with their frequency among faecal enterococci from the South Nation River watershed suggest that the higher frequency of *cpd*, *EfaAfs* and *cylA* among summer isolates could have an agricultural origin (Lanthier *et al.* 2010). However, other contamination sources are also plausible because, with the exception of *enlA*, the carriage of each of these nine virulence determinants among faecal enterococci was high in all three host groups (humans, domesticated mammals and birds, wildlife) in the South Nation River basin (Lanthier *et al.* 2010).

Using a primer set that targeted the *esp* gene of both *Ent. faecalis* and *Ent. faecium* [i.e. $esp_{fs/fm}$ (Eaton and Gasson 2001)], *esp* was only infrequently detected, although more often in the summer. Some reports have described that the *Ent. faecium* variant of the gene (esp_{fm}) was abundant among human sewage, wastewater and septic samples and absent into animal faeces and have related detection of this gene in water to human faecal pollution (Scott *et al.* 2005; Ahmed *et al.* 2008). However, a survey of faecal enterococci isolated from the South Nation River basin showed that no significant difference was found between the frequency of the *esp* gene in human wastewaters, domesticated mammals and birds and wildlife faeces, suggesting that this gene is not useful to track human source pollution, and these results were similar to those obtained by other studies (Whitman *et al.* 2007; Byappanahalli *et al.* 2008; Layton *et al.* 2009).

Seasonal distribution of antibiotic resistances among *Enterococcus* isolates from the South Nation River watershed

To evaluate the pool of resistances among South Nation River enterococci, the collection of water enterococci isolates was evaluated for resistance against 12 antibiotics based on clinical breakpoints. Overall, results revealed that at breakpoint and twice the breakpoint concentrations, South Nation River water enterococci were not frequently (≤3·0%) resistant to any of the antibiotics tested (including category I antibiotics ciprofloxacin and vancomycin), with the exception of lincomycin, bacitracin and tetracycline. At breakpoint and twice the breakpoint concentration, lincomycin resistance was the most common antibiotic resistance encountered among South Nation River enterococci isolates. These results are consistent with enterococci being intrinsically resistant to this antibiotic (Klare *et al.* 2003), and similar results were obtained previously showing a high frequency of resistance to this antibiotic among freshwater enterococci (Meinersmann *et al.* 2008). The second most frequent antibiotic resistance at breakpoint and twice the breakpoint concentration among enterococci isolates obtained in this study was against bacitracin. Again, a similar frequency of resistance to bacitracin was observed among freshwater enterococci isolated (Meinersmann *et al.* 2008). Variable levels of tetracycline resistance were observed before in surface water enterococci, which may be attributed to environmental variations between studies (Meinersmann *et al.* 2008; Lata *et al.* 2009; Servais and Passerat 2009). Overall, these results show that the frequency of antibiotic resistance among water enterococci in the South Nation River is generally low, suggesting that the public health risk regarding transmission of antibiotic-resistant waterborne enterococci to humans is low in the study area.

We also observed differences in the carriage of antibiotic resistance against bacitracin, lincomycin and tetracycline among water enterococci between seasons. In the case of lincomycin, a lower incidence of resistance to breakpoint concentration was found among summer isolates compared to other seasons, while a higher frequency of resistance was found among summer isolates when examining resistance against twice the breakpoint concentration. While lincomycin resistance among enterococci is intrinsic (Klare *et al.* 2003), the higher frequency of resistance to twice the breakpoint concentration of this antibiotics among summer isolates suggests an agricultural or human source, because the use of this antibiotic in animal feed or human medicine may increase resistance to this antibiotic. In fact, a higher frequency of resistance to lincomycin was observed among domesticated mammals

and bird isolates compared to human and wildlife isolates in a previous survey of faecal enterococci in the South Nation River drainage basin (Lanthier *et al.* 2010).

A higher frequency of resistance to bacitracin at breakpoint concentration was observed among wildlife faecal isolates in the South Nation River area compared to human and agricultural sources in a previous survey (Lanthier *et al.* 2010). However, no difference in the resistance to bacitracin at twice the breakpoint concentration was observed between host groups in that survey, making the origin of this higher frequency of bacitracin resistance in the winter unclear.

Regarding tetracycline resistance, clear distinctions between seasons could only be made for resistance against twice the breakpoint concentration of this antibiotic, and it was shown that resistance was higher among both winter and spring isolates. These results suggest an agricultural or human origin, because our survey of South Nation River faecal enterococci revealed that tetracycline resistance to twice the breakpoint concentration was higher among domesticated mammals and birds isolates compared to wildlife isolates (Lanthier *et al.* 2010). This observation is also consistent with tetracycline resistance being acquired in enterococci (Klare *et al.* 2003).

Finally, the frequency of carriage of two or more antibiotic resistance at breakpoint concentration was higher among winter isolates, while resistance to twice the breakpoint concentration was higher among both winter and spring isolates. Isolates resistant to two or more antibiotics at twice the breakpoint could have an agricultural origin, because a higher proportion of carrier of two or more antibiotic resistances was observed among domesticated mammals and birds at twice the breakpoint concentration in our survey of faecal enterococci in the South Nation River area (Lanthier *et al.* 2010). However, at breakpoint concentration, no difference could be observed between hosts regarding carriage of two or more antibiotic resistance, making the origin of these isolates unclear in the South Nation River.

In conclusion, *Ent. faecalis*, *Ent. faecium* and *Ent. durans* were the three major enterococci species isolated from the South Nation River in summer, winter and spring, respectively. It was hypothesized that they may have a non-human, human and unknown origin, respectively, based on the comparison with dominant enterococci species found in various hosts in the South Nation River basin (Lanthier *et al.* 2010). The frequency of carriage of multiple virulence determinants was higher in the summer compared to other seasons, but the risk to public health is probably tempered by the lower enterococci concentration found in spring and summer in the South Nation River compared to other seasons (Wilkes *et al.* 2009). Carriage of antibiotic resistance was generally low among water enterococci, and very few isolates were found to be resistant to category I antibiotics such as ciprofloxacin and vancomycin, showing that dispersion of antibiotic resistance through surface water by enterococci does not pose a high risk to public health.

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References

Abriouel, H., Omar, N.B., Molinos, A.C., Lopez, R.L., Grande, M.A., Martinez-Viedma, P., Ortega, E., Canamero, M.M. *et al.* (2008) Comparative analysis of genetic diversity and incidence of virulence factors and antibiotic resistance among enterococcal populations from raw fruit and vegetable foods, water and soil, and clinical samples. *Int J Food Microbiol* **123**, 38–49.

Ahmed, W. and Katouli, M. (2008) Phenotypic variations of enterococci in surface waters: analysis of biochemical fingerprinting data from multi-catchments. *J Appl Microbiol* **105**, 452–458.

Ahmed, W., Stewart, J., Powell, D. and Gardner, T. (2008) Evaluation of the host-specificity and prevalence of enterococci surface protein (*esp*) marker in sewage and its application for sourcing human fecal pollution. *J Environ Qual* **37**, 1583–1588.

Amann, R.I., Stromley, J., Devereux, R., Key, R. and Stahl, D.A. (1992) Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. *Appl Environ Microbiol* **58**, 614–623.

Badgley, B.D., Nayak, B.S. and Harwood, V.J. (2010a) The importance of sediment and submerged aquatic vegetation as potential habitats for persistent strains of enterococci in a subtropical watershed. *Water Res* , in press. doi: 10.1016/j.watres.2010.07.005.

Badgley, B.D., Thomas, F.I. and Harwood, V.J. (2010b) The effects of submerged aquatic vegetation on the persistence of environmental populations of *Enterococcus* spp. *Environ Microbiol* **12**, 1271–1281.

Balzer, M., Witt, N., Flemming, H.C. and Wingender, J. (2010) Faecal indicator bacteria in river biofilms. *Water Sci Tech* **61**, 1105–1111.

Bittencourt de Marques, E. and Suzart, S. (2004) Occurrence of virulence-associated genes in clinical *Enterococcus*

faecalis strains isolated in Londrina, Brazil. *J Med Microbiol* **53**, 1069–1073.

Byappanahalli, M.N., Przybyla-Kelly, K., Shively, D.A. and Whitman, R.L. (2008) Environmental occurrence of the enterococcal surface protein (*esp*) gene is an unreliable indicator of human fecal contamination. *Environ Sci Technol* **42**, 8014–8020.

Cabelli, V.J., Dufour, A.P., McCabe, L.J. and Levin, M.A. (1982) Swimming-associated gastroenteritis and water quality. *Am J Epidemiol* **115**, 606–616.

Creti, R., Imperi, M., Bertuccini, L., Fabretti, F., Orefici, G., Di Rosa, R. and Baldassarri, L. (2004) Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. *J Med Microbiol* **53**, 13–20.

Deasy, B.M., Rea, M.C., Fitzgerald, G.F., Cogan, T.M. and Beresford, T.P. (2000) A rapid PCR based method to distinguish between *Lactococcus* and *Enterococcus*. *Syst Appl Microbiol* **23**, 510–522.

Denham, E.L., Ward, P.N. and Leigh, J.A. (2008) Lipoprotein signal peptides are processed by Lsp and Eep of *Streptococcus uberis*. *J Bacteriol* **190**, 4641–4647.

Eaton, T.J. and Gasson, M.J. (2001) Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Appl Environ Microbiol* **67**, 1628–1635.

Eaton, A.D., Clesceri, L.S., Rice, E.W. and Greenberg, A.E. (2005) Fecal *Streptococcus* and *Enterococcus* groups. In *Standard Methods for the Examination of Water and Wastewater* ed. Franson, M.A.H. pp. 9-86–9-90. Baltimore: APHA/AWWA/WEF.

Fisher, K. and Phillips, C. (2009) The ecology, epidemiology and virulence of *Enterococcus*. *Microbiology* **155**, 1749–1757.

Franz, C.M., Muscholl-Silberhorn, A.B., Yousif, N.M., Vancanneyt, M., Swings, J. and Holzapfel, W.H. (2001) Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. *Appl Environ Microbiol* **67**, 4385–4389.

Hagedorn, C., Robinson, S.L., Filtz, J.R., Grubbs, S.M., Angier, T.A. and Reneau, R.B. Jr (1999) Determining sources of fecal pollution in a rural Virginia watershed with antibiotic resistance patterns in fecal streptococci. *Appl Environ Microbiol* **65**, 5522–5531.

Hammerum, A.M., Heuer, O.E., Lester, C.H., Agerso, Y., Seyfarth, A.M., Emborg, H.D., Frimodt-Moller, N. and Monnet, D.L. (2007) Comment on: withdrawal of growth-promoting antibiotics in Europe and its effects in relation to human health. *Int J Antimicrob Agents* **30**, 466–468.

Harwood, V.J., Wiggins, B., Hagedorn, C., Ellender, R.D., Gooch, J., Kern, J., Samadpour, M., Chapman, A.C. *et al.* (2003) Phenotypic library-based microbial source tracking methods: efficacy in the California collaborative study. *J Water Health* **1**, 153–166.

Health Canada (2006) *Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS)*, 2004 edn. p. 151. Guelph: Public Health Agency.

Jackson, C.R., Fedorka-Cray, P.J. and Barrett, J.B. (2004) Use of a genus- and species-specific multiplex PCR for identification of enterococci. *J Clin Microbiol* **42**, 3558–3565.

Kayaoglu, G. and Orstavik, D. (2004) Virulence factors of *Enterococcus faecalis*: relationship to endodontic disease. *Crit Rev Oral Biol Med* **15**, 308–320.

Klare, I., Werner, G. and Witte, W. (2001) Enterococci. Habitats, infections, virulence factors, resistances to antibiotics, transfer of resistance determinants. *Contrib Microbiol* **8**, 108–122.

Klare, I., Konstabel, C., Badstubner, D., Werner, G. and Witte, W. (2003) Occurrence and spread of antibiotic resistances in *Enterococcus faecium*. *Int J Food Microbiol* **88**, 269–290.

Kuhn, I., Iversen, A., Burman, L.G., Olsson-Liljequist, B., Franklin, A., Finn, M., Aarestrup, F., Seyfarth, A.M. *et al.* (2003) Comparison of enterococcal populations in animals, humans, and the environment – a European study. *Int J Food Microbiol* **88**, 133–145.

Kuntz, R.L., Hartel, P.G., Godfrey, D.G., McDonald, J.L., Gates, K.W. and Segars, W.I. (2003) Targeted sampling protocol as prelude to bacterial source tracking with *Enterococcus faecalis*. *J Environ Qual* **32**, 2311–2318.

Lanthier, M., Scott, A., Lapen, D., Zhang, Y. and Topp, E. (2010) Frequency of virulence genes and antibiotic resistances in *Enterococcus* spp. isolates from wastewater and feces of domesticated mammals and birds, and wildlife. *Can J Microbiol* **56**, 715–729.

Lapen, D.R., Topp, E., Edwards, M., Sabourin, L., Curnoe, W., Gottschall, N., Bolton, P., Rahman, S. *et al.* (2008) Effect of liquid municipal biosolid application method on tile and ground water quality. *J Environ Qual* **37**, 925–936.

Lata, P., Ram, S., Agrawal, M. and Shanker, R. (2009) Enterococci in river Ganga surface waters: propensity of species distribution, dissemination of antimicrobial-resistance and virulence-markers among species along landscape. *BMC Microbiol* **9**, 140.

Layton, B.A., Walters, S.P. and Boehm, A.B. (2009) Distribution and diversity of the enterococcal surface protein (*esp*) gene in animal hosts and the Pacific coast environment. *J Appl Microbiol* **106**, 1521–1531.

Lowe, A.M., Lambert, P.A. and Smith, A.W. (1995) Cloning of an *Enterococcus faecalis* endocarditis antigen: homology with adhesins from some oral streptococci. *Infect Immun* **63**, 703–706.

Lyautey, E., Lapen, D.R., Wilkes, G., McCleary, K., Pagotto, F., Tyler, K., Hartmann, A., Piveteau, P. *et al.* (2007) Distribution and characteristics of *Listeria monocytogenes* isolates from surface waters of the South Nation River watershed, Ontario, Canada. *Appl Environ Microbiol* **73**, 5401–5410.

Lyautey, E., Lu, Z., Lapen, D.R., Wilkes, G., Scott, A., Berkers, T., Edge, T.A. and Topp, E. (2010) Distribution and diversity of *Escherichia coli* populations in the South Nation River drainage basin, eastern Ontario, Canada. *Appl Environ Microbiol* **76**, 1486–1496.

McGowan-Spicer, L.L., Fedorka-Cray, P.J., Frye, J.G., Meinersmann, R.J., Barrett, J.B. and Jackson, C.R. (2008) Antimicrobial resistance and virulence of *Enterococcus faecalis* isolated from retail food. *J Food Prot* **71**, 760–769.

Meinersmann, R.J., Berrang, M.E., Jackson, C.R., Fedorka-Cray, P., Ladely, S., Little, E., Frye, J.G. and Mattsson, B. (2008) *Salmonella*, *Campylobacter* and *Enterococcus* spp.: their antimicrobial resistance profiles and their spatial relationships in a synoptic study of the Upper Oconee River basin. *Microb Ecol* **55**, 444–452.

Muller, T., Ulrich, A., Ott, E.M. and Muller, M. (2001) Identification of plant-associated enterococci. *J Appl Microbiol* **91**, 268–278.

Muyzer, G., de Waal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**, 695–700.

Ozmen Togay, S., Celebi Keskin, A., Acik, L. and Temiz, A. (2010) Virulence genes, antibiotic resistance and plasmid profiles of *Enterococcus faecalis* and *Enterococcus faecium* from naturally fermented Turkish foods. *J Appl Microbiol* **190**, 1084–1092.

Pangallo, D., Drahovska, H., Harichova, J., Karelova, E., Chovanova, K., Aradska, J., Ferianc, P., Turna, J. *et al.* (2008) Evaluation of different PCR-based approaches for the identification and typing of environmental enterococci. *Antonie Leeuwenhoek* **93**, 193–203.

Poeta, P., Costa, D., Rojo-Bezares, B., Zarazaga, M., Klibi, N., Rodrigues, J. and Torres, C. (2007) Detection of antimicrobial activities and bacteriocin structural genes in faecal enterococci of wild animals. *Microbiol Res* **162**, 257–263.

Poeta, P., Igrejas, G., Costa, D., Sargo, R., Rodrigues, J. and Torres, C. (2008) Virulence factors and bacteriocins in faecal enterococci of wild boars. *J Basic Microbiol* **48**, 385–392.

Ruecker, N.J., Braithwaite, S.L., Topp, E., Edge, T., Lapen, D.R., Wilkes, G., Robertson, W., Medeiros, D. *et al.* (2007) Tracking host sources of *Cryptosporidium* spp. in raw water for improved health risk assessment. *Appl Environ Microbiol* **73**, 3945–3957.

Sapkota, A.R., Curriero, F.C., Gibson, K.E. and Schwab, K.J. (2007) Antibiotic-resistant enterococci and fecal indicators in surface water and groundwater impacted by a concentrated swine feeding operation. *Environ Health Perspect* **115**, 1040–1045.

Scott, T.M., Jenkins, T.M., Lukasik, J. and Rose, J.B. (2005) Potential use of a host associated molecular marker in *Enterococcus faecium* as an index of human fecal pollution. *Environ Sci Technol* **39**, 283–287.

Semedo, T., Santos, M.A., Lopes, M.F., Figueiredo Marques, J.J., Barreto Crespo, M.T. and Tenreiro, R. (2003) Virulence factors in food, clinical and reference Enterococci: a common trait in the genus? *Syst Appl Microbiol* **26**, 13–22.

Servais, P. and Passerat, J. (2009) Antimicrobial resistance of fecal bacteria in waters of the Seine river watershed (France). *Sci Total Environ* **408**, 365–372.

Shankar, N., Lockatell, C.V., Baghdayan, A.S., Drachenberg, C., Gilmore, M.S. and Johnson, D.E. (2001) Role of *Enterococcus faecalis* surface protein Esp in the pathogenesis of ascending urinary tract infection. *Infect Immun* **69**, 4366–4372.

Singer, R.S., Ward, M.P. and Maldonado, G. (2006) Can landscape ecology untangle the complexity of antibiotic resistance? *Nat Rev Microbiol* **4**, 943–952.

Singh, K.V., Coque, T.M., Weinstock, G.M. and Murray, B.E. (1998) In vivo testing of an *Enterococcus faecalis efaA* mutant and use of *efaA* homologs for species identification. *FEMS Immunol Med Microbiol* **21**, 323–331.

Sood, S., Malhotra, M., Das, B.K. and Kapil, A. (2008) Enterococcal infections & antimicrobial resistance. *Indian J Med Res* **128**, 111–121.

U.S. Environmental Protection Agency (2003) *Bacterial Water Quality Standards for Recreational Waters (Freshwater and Marine Waters)*No. EPA-823-R-03-008 . Washington, D.C.: U.S. Environmental Protection Agency.

Valenzuela, A.S., Omar, N.B., Abriouel, H., Lopez, R.L., Ortega, E., Canamero, M.M. and Galvez, A. (2008) Risk factors in enterococci isolated from foods in Morocco: determination of antimicrobial resistance and incidence of virulence traits. *Food Chem Toxicol* **46**, 2648–2652.

Whitman, R.L., Przybyla-Kelly, K., Shively, D.A. and Byappanahalli, M.N. (2007) Incidence of the enterococcal surface protein (*esp*) gene in human and animal fecal sources. *Environ Sci Technol* **41**, 6090–6095.

Wiggins, B.A., Cash, P.W., Creamer, W.S., Dart, S.E., Garcia, P.P., Gerecke, T.M., Han, J., Henry, B.L. *et al.* (2003) Use of antibiotic resistance analysis for representativeness testing of multiwatershed libraries. *Appl Environ Microbiol* **69**, 3399–3405.

Wilkes, G., Edge, T., Gannon, V., Jokinen, C., Lyautey, E., Medeiros, D., Neumann, N., Ruecker, N. *et al.* (2009) Seasonal relationships among indicator bacteria, pathogenic bacteria, *Cryptosporidium* oocysts, *Giardia* cysts, and hydrological indices for surface waters within an agricultural landscape. *Water Res* **43**, 2209–2223.

Yost, C.K., Diarra, M. and Topp, E. (in press) Animals and humans as sources of fecal indicator bacteria. In *The Fecal Bacteria* ed. Sadowsky, M.J. and Whitman, R.L. Washington DC: American Society for Microbiology.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Distribution of enterococci species per season in water samples from the South Nation River watershed

Table S2 Distribution of selected virulence genes per season in *Enterococcus* spp. isolated from the South Nation River watershed

Table S3 Distribution of selected virulence genes in *Enterococcus* spp. isolated from the South Nation River watershed

Table S4 Number of selected virulence genes per season in *Enterococcus* spp. isolated from the South Nation River watershed

Table S5 Distribution of maximum antibiotic resistance levels per season in *Enterococcus* spp. isolated from the South Nation River watershed

Table S6 Distribution of maximum antibiotic resistance levels in *Enterococcus* spp. isolated from the South Nation River watershed

Table S7 Distribution of maximum antibiotic resistance levels per season in various enterococci species isolated from the South Nation River watershed

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