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Comparison of biochemical and genotypic speciation methods for vancomycin-resistant enterococci isolated from urban wastewater treatment plants

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ABSTRACT

Enterococci species in wastewater including *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus casseliflavus* and *Enterococcus gallinarum* isolates (n = 308) with low or high level vancomycin resistance were determined and compared using a phenotypic method (RapID™ STR system), 16S rRNA sequencing, and multi-locus (*atp*A, *gro*ESL, and *phe*S) sequence analysis (MLSA). Error rates for the RapID™ STR system were *E. faecalis* (15.9%), *E. faecium* (21.5%), and *E. casseliflavus/E. gallinarum* (56.9%) when referenced to the consensus of all methods tested. Comparison of single nucleotide polymorphism (SNP) distances and phylogenetic trees suggested that the *gro*ESL locus delineated species more effectively than other loci. The *gro*ESL locus was the most reliable loci for the correct identification of *Enterococcus spp.*, including *E. casseliflavus* and *E. gallinarum*, with high congruence compared to the consensus (Adjusted Rand Index = 0.954; Adjusted Wallace Co-efficient = 0.941). All of the methods were compared to whole genome sequencing, which acted as a gold standard, for the isolates from this study and those downloaded from NCBI.

1. Introduction

Enterococci are ubiquitous in the environment and given their resilience to environmental stresses and extreme conditions, they have been used as an indicator for fecal contamination in water and food products (Manero and Blanch, 1999). Enterococci are Gram positive cocci, catalase negative, facultative anaerobic bacteria that can be cultivated in broth containing 6.5% NaCl, 40% bile salts, and 0.1% methylene blue. Depending on species and strain, enterococci can grow over a range of temperatures (10–45 °C) and at pHs as high as 9.6, and remain viable even after 30 min at 60 °C (Devriese et al., 1987; Devriese et al., 1993; Domig et al., 2003; Hudson et al., 2003). Enterococci were formally classified with fecal streptococci and were seen as opportunistic pathogens that were not particularly virulent. However, *Enterococcus* spp. have the ability to acquire multiple antimicrobial resistance (AMR) determinants and can spread these determinants to more virulent pathogens (Moellering Jr, 1998). Two species, *E. faecalis* and *E. faecium*, are the most common etiological agents of nosocomial infections. However, other *Enterococcus* species including *E. durans*, *E. hirae*, *E. gallinarum* and *E. casseliflavus* have been increasingly associated with opportunistic infections (Kirschner et al., 2001). *E. faecalis* and *E. faecium* are also the most common *Enterococcus* spp. isolated from urban wastewater (Kirschner et al., 2001).

Vancomycin-resistant enterococci (VRE) are high priority targets for

Abbreviations: SDI, Simpson's Diversity Index; ARI, Adjusted Rand Index; AWC, Adjusted Wallace Coefficient; MLSA, multi-locus sequence analysis; SNP, single nucleotide polymorphism; AMR, antimicrobial resistance; VRE, vancomycin-resistant enterococci; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; THB, Todd-Hewitt broth; TSA, Tryptic Soy Agar; FAC, ferric ammonium citrate

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the development of new antimicrobial agents due to their prominence as nosocomial pathogens, lack of treatment options, and the potential for rapid dissemination within both the clinic and the environment (Ramotar et al., 2000; Ahmad et al., 2014). There are both acquired and intrinsic mechanisms of vancomycin resistance in enterococci. Three genotypes (vanA-type, vanB-type, vanC-type) are common in VRE isolated from wastewater. VanA and vanB-type VRE have high level vancomycin resistance and vanA-type VRE may also be teicoplanin resistant. Both vanA and vanB-type VRE are usually either *E. faecalis* or *E. faecium*. VanC-type VRE possesses intrinsic low-level resistance to vancomycin, but not to teicoplanin, a phenotype most often found in *E. casseliflavus* and *E. gallinarum*. Although VRE have potential as an indicator of environmental AMR, vanC-type VRE do not necessarily have the same health risks as vanA- or vanB-type VRE (Ramotar et al., 2000). Consequently, differentiation between vanC-type VRE and other VRE through speciation is important for accurate environmental risk assessments.

In clinical and public health laboratories, enterococci species are primarily identified through biochemical characterization or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Wieser et al., 2012). Phenotypic profiles can include Gram stain, colony morphology, growth requirements, typical carbohydrate utilization and enzyme activities specific to each *Enterococcus spp* (Domig et al., 2003; Petti et al., 2005). These characteristics are not static and can change with stress or evolution (Devriese et al., 1993; Petti et al., 2005). Differences in the phenotypic profiles among *Enterococcus spp* may misrepresent the phylogenetic relationship among species, further complicating classification (Domig et al., 2003). The formation of certain metabolites in defined media can also be used to differentiate and identify *Enterococcus spp.* (Domig et al., 2003; Tan et al., 2017). In this study, the commercial RapID™ STR system was evaluated for its ability to identify enterococci isolated from wastewater. It uses chromogenic substrates to rapidly identify medically relevant streptococci and related organisms isolated from human clinical specimens, including enterococci.

While there are a variety of techniques used to identify *Enterococcus* species, the phylogenetic relationship of species within this genus has been determined primarily by comparative sequencing of the 16S rRNA gene. However, limited differences in the sequence of the 16S rRNA among species impedes the ability of this method to differentiate those that are closely related (Devriese et al., 1993; Naser et al., 2005a; Naser et al., 2005b). There have been a variety of different loci proposed for the speciation of closely related enterococci including the loci for alpha subunits of ATP synthases (*atp*A) (Naser et al., 2005a), chaperonins (*gro*ESL) (Zaheer et al., 2012), and phenylalanyl-tRNA synthase alpha-subunits (*phe*S) (Naser et al., 2005b; Ahmad et al., 2014). In this study, the ability of the RapID™ STR system to identify enterococci species with low and high level vancomycin resistance isolated from urban wastewater was compared to 16S rRNA sequencing and the sequence analysis of *atp*A, *phe*S, and *gro*ESL loci. The ability of four loci (16S rRNA, *atp*A, *phe*S, and *gro*ESL) to discriminate between *E. casseliflavus* and *E. gallinarum* was determined and compared to that of whole genome sequencing.

2. Material and methods

2.1. Selection of isolates

A total of 308 presumptive VRE isolates from treated and untreated urban wastewater were characterized in this study. All isolates were confirmed as enterococci using 23S rRNA sequencing (Table 1). Isolates were initially obtained from wastewater samples upon growth on Slanetz and Bartley agar (Oxoid, Ontario, Canada) and were then grown on tryptic soy agar (TSA; Sigma-Aldrich Canada, Ontario, Canada) with 1 g/L esculin (Sigma Aldrich Canada, Ontario, Canada) and 0.6 g/L ferric ammonium citrate (FAC, Ward's Science, New York, USA) and a single colony was then transferred to 5 mL of THB and grown overnight. An aliquot (1 μL) of the overnight culture was transferred to each well of a 96-well plate containing THB or THB supplemented with vancomycin (4 or 32 mg/L; Bio Basic Inc., Ontario, Canada), esculin (1 g/L) and FAC (0.6 g/L). Isolates were recovered following growth overnight in the presence of vancomycin and confirmed by the visualization of a black pigment (Lindell and Quinn, 1975). Isolates were then stored at −20 °C in THB with 7.5% dimethyl sulfoxide (DMSO; Sigma Aldrich Canada, Ontario, Canada).

2.2. RapID™ STR system

The RapID™ STR system (Thermo Fisher Scientific, Ontario, Canada) was used for biochemical identification of enterococci species following the manufacturer's instructions. Briefly, isolates were initially cultured on TSA for 18–24 h at 37 °C, and confirmed as Gram positive and oxidase positive (Oxoid Canada, Ontario, Canada). Colonies were picked using a sterile inoculation loop, and transferred to RapID™ inoculation fluid (Thermo Fisher Scientific, Ontario, Canada) to achieve a #2 McFarland turbidity. The test panels were then inoculated with the entirety of the RapID™ inoculation fluid. Inoculated panels were subsequently incubated at 37 °C without CO_2 for 4 h. The panels were then scored based on biochemical reactions to known substrates (colour) and the scores were used to identify the isolates using the Electronic RapID™ Compendium (ERIC), a comprehensive database that supports the RapID™ STR system. *E. casseliflavus* H88S1, *E. faecalis* ATCC 51299, *E. faecium* ATCC 27270, and *E. faecalis* ATCC 700221 were used as reference strains.

2.3. Single and multi-locus sequence analysis for species identification

Sequence analysis of four loci including 16S rRNA, ATP synthase subunit alpha (*atp*A) (Naser et al., 2005a), phenylalanine–tRNA ligase alpha subunit (*phe*S) (Naser et al., 2005b; Ahmad et al., 2014), and chaperonins (*gro*ESL) (Zaheer et al., 2012) was assessed as means of speciating isolates. Isolates were grown overnight on TSA with esculin (1 g/L) and FAC (0.6 g/L). An aliquot of each overnight culture was transferred to 5 mL of TE buffer to achieve a standard turbidity of OD_{600} of 2 (1×10^9 CFU/mL). This suspension (1 mL) was transferred to a micro-centrifuge tube (Eppendorf Canada Ltd., Ontario, Canada) and centrifuged for 2 min at 14000 x*g*. The supernatant was discarded and the pellet was re-suspended in 1 mL of TE buffer. The suspension was boiled at 99 °C for 10 min to lyse the cells and lysates were then used as the template for PCR reactions. Reactions were performed using an Eppendorf Mastercycler Pro S (Eppendorf Canada Ltd., Ontario, Canada). The same primers were used for both amplification and sequencing of amplicons using the PCR conditions shown in Table 1. Each PCR reaction consisted of 50 μL using the HotStar Taq Plus Mastermix Kit (Qiagen Inc., Ontario, Canada), 5 μL of DNA template and final primer concentrations of 500 nM for all of the targets, except *atp*A, which had a final concentration of 1000 nM. PCR products were separated using a 1–2% agarose gel containing 0.2 μg/mL ethidium bromide (BioRad Canada, Quebec, Canada). The gel was visualized and analyzed using an Alpha Innotech AlphaImager Gel Imaging System (Sacramento, California, USA). *E. faecium* ATCC 700221, *E. faecalis* ATCC 51299, *E. casseliflavus* H88S1, *E. gallinarum* K4H1 and *Staphylococcus aureus* ATCC 25923 were used as control strains for both PCR and sequencing.

The PCR products were purified using MinElute 96 UF PCR Purification Kit (Qiagen Inc., Ontario, Canada) and sequenced by Genome Quebec (Quebec, Canada) for the *gro*ESL locus and Eurofins MWG Operon Corporation Canada (Ontario, Canada) for the 16S rRNA, *atp*A, and *phe*S loci. Bi-directional sequencing was performed for all loci, except *gro*ESL. Assembled sequences underwent BLASTn searches using Geneious 10.2.3 (Biomatters Ltd., New Zealand) in order to identify isolates. Consensus identification was the species identified

Table 1
Summary of primers and PCR parameters.

Primer name	Primer sequence (5′ - > 3′)	Amplicon size (bp)	Annealing temperature (°C)	Number of cycles	Extension time (s)	Reference
23S-F	GAGAAATTCCAAACGAACTTG	90–91	60	40	60	[9]
23S-R	CAGTGCTCTACCTCCATCATT					
16S_27F_Dgen	AGAGTTTGATCMTGGCTCAG	~1400	58	35	90	This study
16S_1492R_Dgen	TACGGYTACCTTGTTACGACTT					
atpA_all_F	GGDYTWGAAAAYGCVATGAGTG	1070	49	40	60	[3]
atpA_all_R	CCRAAYTGNGTRAADGCTTC					
atpA_E.cass_F	ACGGTTCTTATGGGATGGCA	945	58	35	60	[3]
atpA_E.cass_R	TCTGTGCAGAACCACCAACC					
Ent-ES-211-233-F	GHACAGAAGTRAAATAYGAAGG	185–226	51	45	20	[40]
Ent-EL-74-95-R	GGNCCTAABGTHACTTTNACTG					
pheS_spec_F	CGDACVATGGAAAAACATG	337	51	35	40	[3]
pheS_spec_R	CWGCNCCTAARATYTCRATC					
pheS_E.cass_F	GAAGTGCTGATTCGGACCCA	415	58	35	40	[3]
pheS_E.cass_R	CGATCCCTGACATTTCTAAGACG					

through the majority of methods (at least 3 of 4 methods) for each isolate. Concatenation of sequences, SNP analysis and phylogenetic trees for the single and concatenated sequences were generated using Geneious 10.2.3. Concatenated sequences were generated by linking the sequences of each loci together in a particular order and consolidating them into one sequence. To generate the phylogenetic trees, all of the sequences for each locus were *de novo* assembled to create a multiple sequence alignment. This enabled representation of deletions and/or insertions in the sequences compared to others in the alignment. Heatmaps depicting the raw SNP distances between isolates and their relationships to species were generated using the heatmaply package version 0.14.1 (Galili et al., 2017) in R version 3.4.3 (R Core Team, 2017). Hierarchical clustering was performed in R using complete linkage with the hclust function. The hierarchical clustering trees of each SNP distance matrix were cut at the height that resulted in 7 groups/clusters using the cutree function. Concordance between the clusters of each method and the reference assignment was evaluated with the Comparing Partitions online tool (http://www.comparingpartitions.info/). Comparing Partitions was used to calculate the Simpsons Index of Diversity (Hunter and Gaston, 1988) for diversity estimation, the Adjusted R and Coefficient (Hubert and Arabie, 1985), the Adjusted Wallace Coefficient (Pinto et al., 2007; Pinto et al., 2008), and to determine the congruence between typing methods (Carriço et al., 2006).

2.4. Whole genome sequencing of selected isolates

Thirty-nine (n = 39) enterococci isolates, chosen based on species and AMR phenotypic profile from the 308 isolates, underwent whole genome sequencing and sixty-two (n = 62) additional genomes from NCBI (Appendix A) were used to compare the four loci *in silico* to identification based on whole genome analysis using Geneious 10.2.3 and BLASTn searches. A small number of *E. casseliflavus* (n = 14) and *E. gallinarum* (n = 6) genomes, including new genomic sequences of *E. casseliflavus* (n = 2) and *E. gallinarum* (n = 2) isolated from wastewater in this study were also compared. Briefly, isolates were grown overnight on TSA with esculin (1 g/L) and FAC (0.6 g/L), suspended in TE buffer and DNA was extracted using a modified protocol based on the DNeasy Blood & Tissue Method (Qiagen Inc., Ontario, Canada). The modification involved the addition of an enzymatic lysis step where bacterial cells were incubated at 37 °C with shaking (150 rpm) in a lysis buffer consisting of 20 mM Tris-Cl (pH 8.0), 2 mM sodium EDTA, 1.2% Triton X-100 and 40 mg/mL lysozyme (Sigma Aldrich Canada, Ontario, Canada), prior to the addition of the proteinase K (35 µL) and 5 µL of 100 mg/mL RNase A (Qiagen Inc., Ontario, Canada). The mixture was then incubated at room temperature for 10 min. Genomic DNA was sequenced using an Illumina MiSeq platform (Illumina Canada, British Columbia, Canada). Sequence data were assembled using SPAdes genome assembler version 3.6.0 software (Bankevich et al., 2012) and annotated using Prokka version 1.12 (Seemann, 2014) using the Galaxy instance (Giardine et al., 2005) of the National Microbiology Laboratory (Public Health Agency of Canada). Comparative genomic analysis was undertaken using the Gview Server with alignment and percent identity cut-offs of 80% (Petkau et al., 2010). Species identity was determined *in silico* based on the four loci and then compared to the identity indicated by the BLAST atlases generated using the genomes and searching them with BLAST against a reference genome for each species (*E. casseliflavus* EC20, *E. gallinarum* F1212F, *E. faecalis* ATCC 29212, *E. faecium* DO).

3. Results

The methods of identification were compared in three ways: 1) among the 308 isolates, we used the identifications from each method to compare the extent to which the methods exhibited consensus in species identification; 2) evaluation of the distances of single nucleotide polymorphisms (SNPs) in the four loci; and 3) *in silico* analysis of the four loci and BLAST atlases using whole genomes (n = 101). When each

Table 2
Summary of error rates for each identification method compared to the consensus identification[a] derived from all of the five methods.

Species	Biochemical method	Genotypic methods			
	RapID STR (%)[a]	*gro*ESL (%)	16S rRNA (%)	*atp*A (%)	*phe*S (%)
E. faecalis; n = 151	15.9(22)	0	0.7 (1)	0	0
E. faecium; n = 94	21.5 (20)	2.2[b](1)	1.1 (1)	0	0
E.casseliflavus/ E.gallinarum; n = 58	56.9 (21)	5.2 (3)	5.2(3)	6.9 (4)	13.8(8)
Other[c]; n = 5	40 (2)	0	0	0	0

[a] Consensus identification was defined as the species identified by the majority of methods (at least 3 of 4) used in this study. Percent and number of misidentified isolates in brackets that failed to meet the criteria for consensus identification.
[b] These sequences clustered with the correct species during phylogenetic analysis.
[c] Other refers to *Enterococcus spp*. other than *E. faecalis*, *E. faecium*, *E. casseliflavus* or *E. gallinarum*.

method was compared to the consensus identification of all the methods (Table 2), the RapID™ STR system was the least reliable.

The error rates for the RapID™ STR system were 15.9%, 21.5%, and 56.9% for *E. faecalis*, *E. faecium* and *E. casseliflavus/E. gallinarum*, respectively. It correctly identified all of the *E. hirae* ($n = 3$) isolates but misidentified *E. mundtii* ($n = 1$) and *E. saccharolyticus* (n = 1). For *E. casseliflavus* and *E. gallinarum*, the majority of the misidentified isolates ($n = 21$) were identified as *E. avium* ($n = 16$). Others were identified as *E. faecium* ($n = 4$), *E. malodortus* (n = 1) or were incorrectly identified ($n = 7$) among *E. casseliflavus* and *E. gallinarum*. The remaining *E. casseliflavus/ E. gallinarum* isolates were either identified as *Streptococcus spp* ($n = 3$) or could not be identified (n = 2). For *E. faecium*, all of the misidentified isolates ($n = 20$) were identified as *E. casseliflavus/ E. gallinarum* ($n = 13$), *E. faecalis* (n = 4) and *E. hirae/ E. durans* (n = 3). For *E. faecalis*, the majority of misidentified isolates ($n = 22$) were identified as *E. casseliflavus/ E. gallinarum* (n = 20) as well as *E. faecium* ($n = 1$) and *E. avium* ($n = 1$), while other isolates were identified as *Aerococcus spp* (n = 1) and *Streptococcus spp* (n = 1).

In this study, error rates from 16S rRNA gene analysis were lower than that derived from biochemical testing, but higher than using MLSA, at 0.7% for *E. faecalis*, 1.1% for *E. faecium*, and 5.2% for *E. casseliflavus/E. gallinarum* (Table 2). The misidentified *E. faecalis* isolate (n = 1) was identified as *E. faecium*, the misidentified *E. faecium* (n = 1) as *E. faecalis* and the misidentified *E. casseliflavus* isolates ($n = 3$) as *E. gallinarum*. The error rate for MLSA was lower than for 16S rRNA locus and the RapID™ STR system. The *atp*A and *phe*S loci correctly identified all *E. faecium* and *E. faecalis* isolates, but did not align in 6.9% and 13.8% of the *E. casseliflavus* and *E. gallinarum* isolates, respectively (Table 2). In both cases, the misidentified isolates were still identified as either *E. casseliflavus* or *E. gallinarum*. The *gro*ESL loci only failed to identify one *E. faecium* isolate ($n = 1$, 2.2%), which was identified as *E. faecalis* by the other loci. Furthermore, this isolate did cluster with *E. faecalis* during phylogenetic analysis. Some additional disagreements were noted between *E. casseliflavus/E. gallinarum* (5.2%) when identified using *gro*ESL sequences.

The *gro*ESL phylogenetic tree more clearly clustered based on species as compared to the other 3 loci which clustered into two clades as opposed to distinct species (Fig. 1; Appendix B). Using the Simpson's Diversity Index (SDI), the atpA (SDI = 0.796) locus exhibited the highest diversity, while the *gro*ESL (SDI = 0.608) exhibited the lowest (Table 3). The SDI of the consensus identifications was 0.614, which was most similar to the diversity indices of the *gro*ESL locus. Both the adjusted R and index (ARI) and the adjusted Wallace coefficient (AWC) were used to determine the agreement between the consensus identification and each of the loci and concatenated loci (Tables 4 and 5). Both measures indicated that the *gro*ESL locus was most similar to the consensus identification with an ARI of 0.954 and an AWC of 0.941. The second most similar identifier was the *atp*A locus with an ARI of 0.523 and AWC of 0.904. The other two loci, 16S rRNA and *phe*S, had similar ARI ($p = .069$) and AWC ($p = .051$). All of the concatenated loci were more dissimilar to the consensus identification than any of the individual loci. The *gro*ESL locus had the greatest discriminatory power as compared to the other loci, even if the loci sequences were concatenated (Fig. 1). Consequently, *gro*ESL was the preferred locus for identifying enterococci at the species level, while *atp*A and *phe*S were capable of differentiating between *E. faecium* and *E. faecalis*.

In addition to the amplicon sequencing, thirty-nine (39) isolates from the collection were sequenced and additional genomes ($n = 62$) from NCBI (Appendix A) were identified using *in silico* analysis of the 4 loci and the BLAST atlases (Fig. 2). The concatenated sequences were not included because they did not improve species identification in the larger dataset. Identification using the four loci was in agreement for all of the *E. faecalis* ($n = 41$) and *E. faecium* ($n = 43$) isolates examined. Identification of the *E. casseliflavus* and *E. gallinarum* genomes ($n = 17$) was incorrect for 23.53% (n = 4) of the 16S rRNA sequences, 11.76% ($n = 2$) for *atp*A and *gro*ESL sequences, and 5.88% (n = 1) for *phe*S sequences (Appendix E). The errors in identification using the 16S rRNA and *atp*A locus and one of the errors for the *gro*ESL locus was attributed to the inability of these methodologies to identify the genomes to the species level. The additional errors (for *gro*ESL and *phe*S) were cases where *E. casseliflavus* was misidentified as *E. gallinarum*.

4. Discussion

The identification of enterococci to the species level is crucial for proper clinical interventions and for epidemiological and infection control purposes (Haritsa et al., 2014). Conventional culture and biochemical tests have been used in most clinical microbiology laboratories for the identification of *Enterococcus spp.* (Kirschner et al., 2001; Fang et al., 2012). This is usually in the form of commercially available kits (Ramotar et al., 2000) which are often less expensive, have standardized methods, and are adaptable to high throughput systems (Hudson et al., 2003). A number of biochemical tests (Devriese et al., 1987; Devriese et al., 1993; Domig et al., 2003) have been packaged into commercial test kits. Previous studies have found these kits to be unreliable for the speciation of enterococci (Ramotar et al., 2000; Kirschner et al., 2001; Hudson et al., 2003;Fang et al., 2012), particularly for environmental isolates.

Misidentification of environmental *Enterococcus* spp. can result in an over- or underestimation of the human health risk, given that the prevalence of antimicrobial resistance and the ability to cause clinical infections varies among species. There are a variety of factors that can result in misidentification of enterococci, regardless of the method used. The handling of strains including user error, culture and subculture methods (Devriese et al., 1993), number of passages of an isolate, use of mixed cultures and contamination can also result in discrepancies in genus and species identification (Hudson et al., 2003).

In the clinic, enterococci are usually isolated from blood, faeces or urine and frequently form biofilms in catheters. Whereas, non-clinical enterococci have adapted to survive in the broader environment and possess genes that enable them to absorb and metabolize additional nutrients and produce metabolites that enable them to overcome other selective pressures (Hudson et al., 2003). The variation in the biochemical profile of environmental isolates can result in increased error rates for speciation methods that rely on biochemical tests. For example, the error rate for BBL Crystal ID kit was reported to be 44% for non-clinical isolates (Hudson et al., 2003).

Studies which have evaluated commonly used commercial identification kits for enterococci reported error rates of 2–21% for *E. faecalis*, 5–9% for *E. faecium* and 14–79% for other entercoccal species, including *E. casseliflavus* and *E. gallinarum* (Willey et al., 1999; Kirschner et al., 2001). A comparison of the Vitek system and API 20S for identification of enterococcal species produced misidentification in 2.4–20.8%, 4.7–8.8% and 14–79.3% of the isolates for *E. faecalis*, *E. faecium* and *E. casseliflavus/E. gallinarum*, respectively (Sadar et al., 1995).

Biochemical identification has multiple drawbacks that can, in part, account for the disagreements with the other methods used in this study. Some of the general limitations include the need to ensure that the isolate is an *Enterococcus* spp. for the commercial kit before it is used, given the close biochemical relatedness of enterococci and other genera (Devriese et al., 1993; Hudson et al., 2003). Additionally, some species and strains within a genera do not share the same biochemical traits (Manero and Blanch, 1999). For example, strains from clinical, agricultural and environmental origins can differ in metabolic profile, including the ability to utilize different carbohydrates and other substrates, hindering biochemical identification (Devriese et al., 1987). The ability of a biochemical commercial kit to correctly speciate an isolate is limited by the database used to create the system and grading scheme (Devriese et al., 1993; Petti et al., 2005). Identification of rare enterococcal species and strains with unusual phenotypic profiles can also be limited (Kirschner et al., 2001; Hudson et al., 2003; Fang et al.,

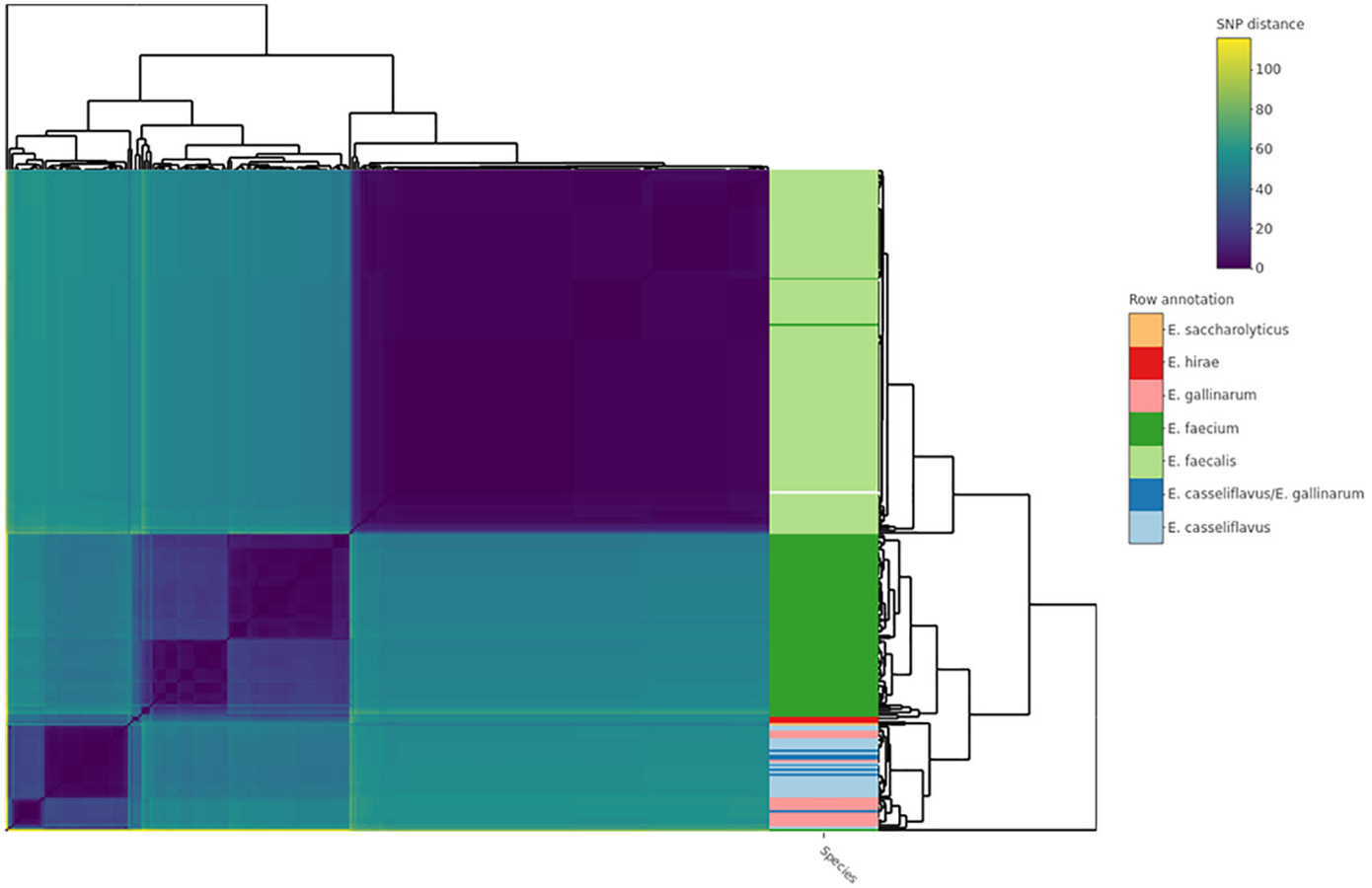

Fig. 1. SNP distances between isolates and the clustering of species within the phylogenetic tree generated using the *gro*ESL (A), *atp*A (B), *phe*S(C), 16S rRNA loci (D), *atp*A*phe*S (E), 16S*atp*A*phe*S (F), 16S*atp*A*phe*S*gro*ESL (G) loci (B-G are in Appendix B). The SNP distances are depicted in the heatmap with yellow indicating a long distance and blue indicating a short distance. The species of each isolate is depicted by colored bar with *E. saccharolyticus* (orange), *E. hirae* (red), *E. gallinarum* (pink), *E. faecium* (dark green), *E. faecalis* (light green) and *E. casseliflavus* (light blue) included. Isolates that were either *E. casseliflavus* or *E. gallinarum,* but could not be speciated are indicated in dark blue. Note that the *gro*ESL loci sequences cluster by species, while the other loci cluster into two clades, then by species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2012). A higher speciation error rate for biochemical-based commercial identification kits has also been suggested for clinically important VRE (Ramotar et al., 2000; Kirschner et al., 2001; Winston et al., 2004).

The use of 16S rRNA gene sequencing in combination and/or as an alternative method to biochemical testing is considered to be a more accurate and objective method for identification as it reduces interpretative bias and provides more meaningful results than biochemical testing alone (Domig et al., 2003; Petti et al., 2005). The 16S rRNA locus is still used as a universal marker for basic evolutionary analysis of bacteria and fulfills most of the requirements of a good phylogenetic marker including: ubiquity, an indispensable function that is linked to evolutionary conservation, and a wide spectrum of sequence variation (Glaeser and Kämpfer, 2015). In addition, there are generally accepted recommendations for sequence length, alignment and tree building.

Table 3
Simpson's Indices of Diversity for each locus and concatenated loci with *p*-values between each loci, concatenated sequences, and the consensus identification[a].

Loci	Simpson's ID[b]	CI (95%)[c]	CINA (95%)[d]	Jackknife pseudo-value CI (95%)[e]	p-value (*vs.* consensus ID)[f]
16S rRNA	0.622	0.573–0.671	0.572–0.671	0.572–0.672	0.795
16S*atp*A*phe*S	0.763	0.748–0.778	0.748–0.779	0.748–0.779	< 0.001
16S*atp*A*phe*S*gro*ESL	0.787	0.770–0.804	0.769–0.804	0.770–0.804	< 0.001
*atp*A	0.796	0.777–0.816	0.777–0.816	0.777–0.816	< 0.001
*atp*A*phe*S	0.778	0.766–0.791	0.765–0.791	0.766–0.791	< 0.001
*gro*ESL	0.608	0.563–0.653	0.563–0.653	0.562–0.653	0.120
*phe*S	0.635	0.587–0.683	0.586–0.683	0.586–0.684	0.470
Consensus ID[g]	0.614	0.569–0.659	0.569–0.659	0.569–0.660	

[a] Seven partitions were used in the analysis to represent the seven *Enterococcus* spp. of interest.
[b] Simpson's Index of Diversity is indicative of the probability of two randomly sampled strains from a population belonging to each *Enterococcus* species.
[c] Confidence Intervals: interval estimate.
[d] Non-Approximated Confidence Interval.
[e] Jackknife pseudo-value confidence interval (95%) which is an indication of the variability in estimated values relative to those of the true population.
[f] *P*-values denote the difference in the diversity within partitions between each typing method and the consensus identification (ID).
[g] Consensus Identification (ID): the species that was identified as by the majority of methods compared (at least 3 or more of the 4 methods).

Table 4
Adjusted R and[a] and Jackknife Pseudo-values CI (95%)[b] for each locus pairing.

Loci	16S rRNA	16S*atp*A*phe*S	16S*atp*A*phe*S*gro*ESL	*atp*A	*atp*A*phe*S	*gro*ESL	*phe*S	Final ID
16S rRNA								
16S*atp*A*phe*S	0.434 (0.415–0.454)							
16S*atp*A*phe*S*gro*ESL	0.468 (0.442–0.494)	0.865 (0.820–0.911)						
*atp*A	0.106 (0.068–0.144)	0.300 (0.270–0.331)	0.362 (0.329–0.395)					
*atp*A*phe*S	0.024 (0.072–0.172)	0.300 (0.277–0.322)	0.435 (0.367–0.504)	0.435 (0.367–0.504)				
*gro*ESL	0.189 (0.129–0.250)	0.010 (0.000–0.028)	0.069 (0.043–0.094)	0.510 (0.483–0.538)	0.122 (0.072–0.172)			
*phe*S	0.042 (0.000–0.093)	0.000 (0.000–0.005)	0.008 (0.000–0.030)	0.150 (0.105–0.195)	0.354 (0.319–0.391)	0.269 (0.196–0.343)		
Consensus ID[c]	0.185 (0.128–0.241)	0.013 (0.000–0.030)	0.069 (0.044–0.094)	0.523 (0.509–0.538)	0.125 (0.075–0.175)	0.954 (0.915–0.993)	0.268 (0.194–0.343)	

[a] Adjusted R and: overall concordance of two methods taking into account that the agreement between partitions could arise by chance alone.
[b] Jackknife Pseudo-Values Confidence Intervals (95%): variability in the estimated values relative to those of the true population.
[c] Consensus Identification: the species that the isolate was identified as by the majority of methods compared (at least 3 or more of the 4 methods).

Large databases with 16S rRNA sequences from a large variety of known taxa are available. However, there is limited resolution at the species level which has been demonstrated both in this study and by others (Glaeser and Kämpfer, 2015). Consequently, the 16S rRNA locus can be limited in its ability to differentiate closely-related *Enterococcus spp.* (Naser et al., 2005ab). The presence of multiple copy numbers of the 16S rRNA gene and sequence variation between copies within the same genome further complicate identification and quantification of isolates using this loci (Větrovský and Baldrian, 2013).

Besides 16S rRNA genes, a variety of other loci may be used for the identification of enterococci. Partial sequences of genes coding for proteins with housekeeping functions have been used to deduce phylogeny, generate phylogenetic trees, and delineate species through multi-locus sequence analysis (MLSA). In this study, three genes, *atp*A (Naser et al., 2005a), *phe*S (Naser et al., 2005b) and *gro*ESL (Zaheer et al., 2012) previously used for MLSA were compared to the phenotypic testing and 16S rRNA sequencing. The utility of the multi-loci sequence analysis and 16S rRNA sequencing for species identification was further assessed by comparing single nucleotide polymorphism (SNP) distances between isolates of the same species and among species. SNPs are inherited single nucleotide substitutions between individuals within a species and the SNP distances can be used to estimate the phylogenetic distances between individual isolates (Mooney et al., 2010).

The error rates for the identification of *E. casseliflavus* and *E. gallinarum* were higher for all of the tested methods compared to error rates for *E. faecium* and *E. faecalis*. For biochemical testing, the profiles of *E. casseliflavus* and *E. gallinarum* are very similar and differentiating the two species is limited to the identification of the inconsistent formation of a yellow pigment by *E. casseliflavus* (Cartwright et al., 1995). The biochemical test used in this study was also designed to primarily identify clinical isolates, which are mostly *E. faecium* and *E. faecalis*, and not *E. casseliflavus* and *E. gallinarum*. The extent to which the RapID™ STR kit has been tested for the identification of environmental isolates is unknown. Additionally, compared to *E. faecium* and *E. faecalis*, there are relatively few publically available genomes of *E. casseliflavus* and *E. gallinarum*, limiting the number of sequences available for the identification of these species.

Given the difficulty in correctly identifying *E. casseliflavus* and *E. gallinarum* regardless of the method, the genomes of both species were sequenced and compared to the reference genomes of, *E. casseliflavus* EC20 (human clinical) and *E. gallinarum* F1212F (bovine), using BLAST atlases (Fig. 2ab) (Grant et al., 2012). Interestingly, *E. gallinarum* FDAARGOS 163 (human commensal) and *E. gallinarum* FDAARGOS 375 (human commensal) genomes were more similar to *E. casseliflavus* EC20 than the other *E. gallinarum* isolates from cattle and wastewater

Table 5
Adjusted Wallace Coefficient[a] and Jackknife Pseudo-Values CI (95%)[b] for each locus and concatenated sequence pairing.

Loci	16S rRNA	16S*atp*A*phe*S	16S*atp*A*phe*S*gro*ESL	*atp*A	*atp*A*phe*S	*gro*ESL	*phe*S	Final ID
16S rRNA		0.328 (0.320–0.336)	0.338 (0.314–0.362)	0.075 (0.044–0.105)	0.017 (0.000–0.035)	0.195 (0.112–0.274)	0.041 (0.000–0.091)	0.188 (0.111–0.261)
16S*atp*A*phe*S	0.643 (0.547–0.737)		0.810 (0.740–0.880)	0.274 (0.235–0.312)	0.287 (0.262–0.313)	0.016 (0.000–0.043)	0.000 (0.000–0.008)	0.020 (0.000–0.046)
16S*atp*A*phe*S*gro*ESL	0.759 (0.674–0.844)	0.928 (0.881–0.975)		0.352 (0.301–0.402)	0.322 (0.291–0.352)	0.116 (0.065–0.166)	0.013 (0.000–0.047)	0.114 (0.065–0.162)
*atp*A	0.179 (0.131–0.228)	0.332 (0.300–0.364)	0.373 (0.346–0.400)		0.460 (0.393–0.527)	0.899 (0.840–0.957)	0.243 (0.180–0.307)	0.904 (0.850–0.959)
*atp*A*phe*S	0.037 (0.001–0.073)	0.313 (0.281–0.344)	0.306 (0.278–0.335)	0.413 (0.336–0.489)		0.199 (0.112–0.286)	0.535 (0.436–0.632)	0.200 (0.114–0.285)
*gro*ESL	0.184 (0.137–0.232)	0.008 (0.000–0.020)	0.049 (0.032–0.066)	0.356 (0.332–0.380)	0.088 (0.052–0.124)		0.254 (0.190–0.319)	0.941 (0.891–0.992
*phe*S	0.043 (0.000–0.095)	0.000 (0.000–0.004)	0.006 (0.000–0.022)	0.108 (0.071–0.144)	0.265 (0.233–0.296)	0.285 (0.186–0.381)		0.280 (0.179–0.378)
Consensus ID[c]	0.182 (0.137–0.228)	0.010 (0.000–0.022)	0.049 (0.032–0.066)	0.368 (0.352–0.384)	0.091 (0.054–0.127)	0.967 (0.935–1.000)	0.257 (0.193–0.321)	

[a] Adjusted Wallace Co-efficient: directly indicates the agreement between partitions.
[b] Jackknife Pseudo-Values Confidence Intervals (95%): variability in the estimated values relatively to those of the true population.
[c] Consensus Identification (ID): the species that the isolate was identified by the majority of the compared methods (at least 3 or more of the 4 methods).

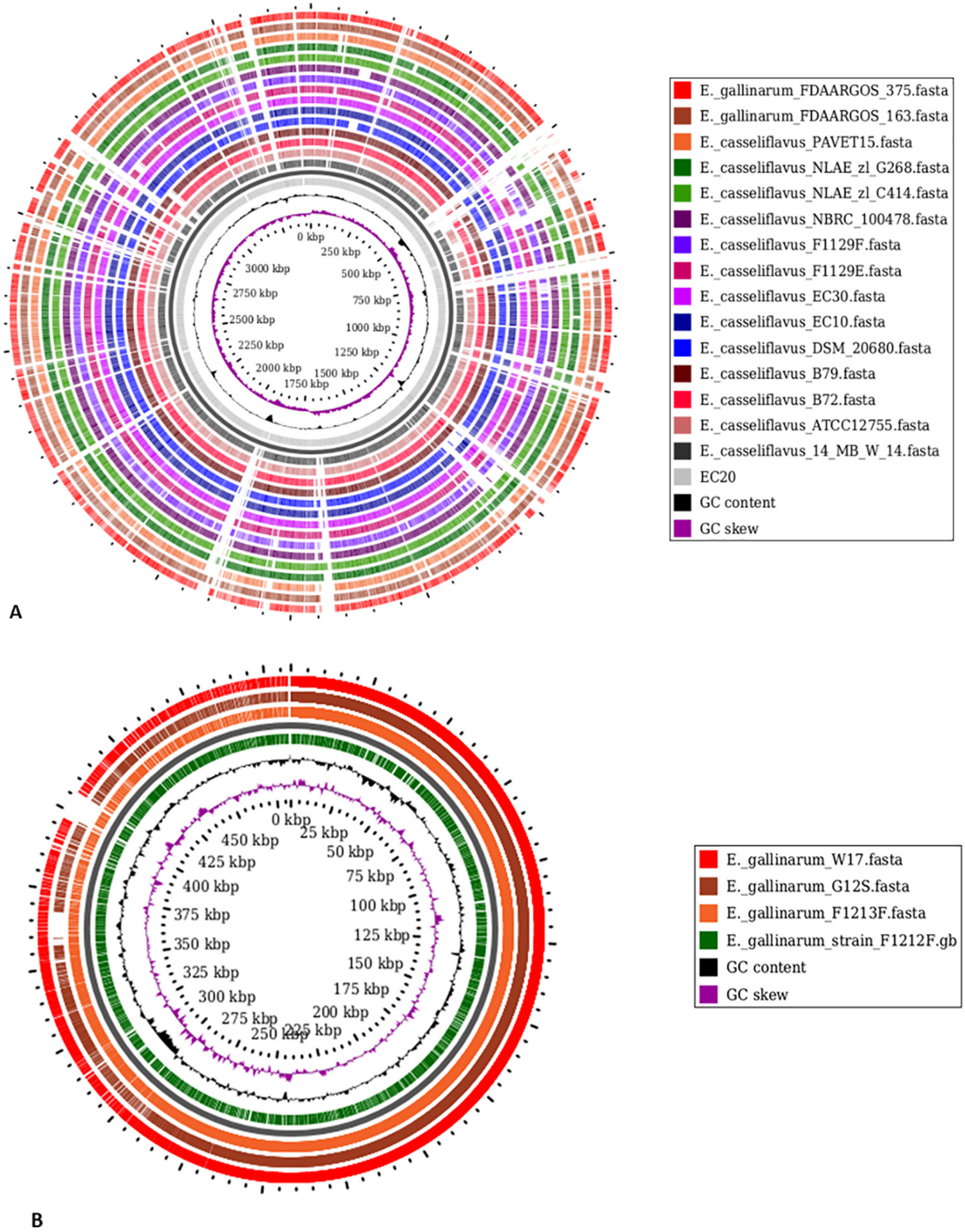

Fig. 2. *E. casseliflavus* and *E. gallinarum* BLAST atlases illustrating the sequence diversity and similarity among isolates of the same species and other species by mapping the genomes against a reference strain A) *E. casseliflavus* BLAST atlas using *E. casseliflavus* EC20 as the reference genome. B) *E. gallinarum* BLAST atlas using *E. gallinarum* F1212F as the reference genome. C) Combined *E. casseliflavus* and *E. gallinarum* BLAST atlas using *E. casseliflavus* EC20 as the reference genome, illustrating the similarity among FDAARGOS genomes and the *E. casseliflavus* genomes. Note that the two genomes of *E. gallinarum* FDAARGOS shared more genes with *E. casseliflavus* genomes than *E. gallinarum* genomes, suggesting that these genomes are misidentified in NCBI.

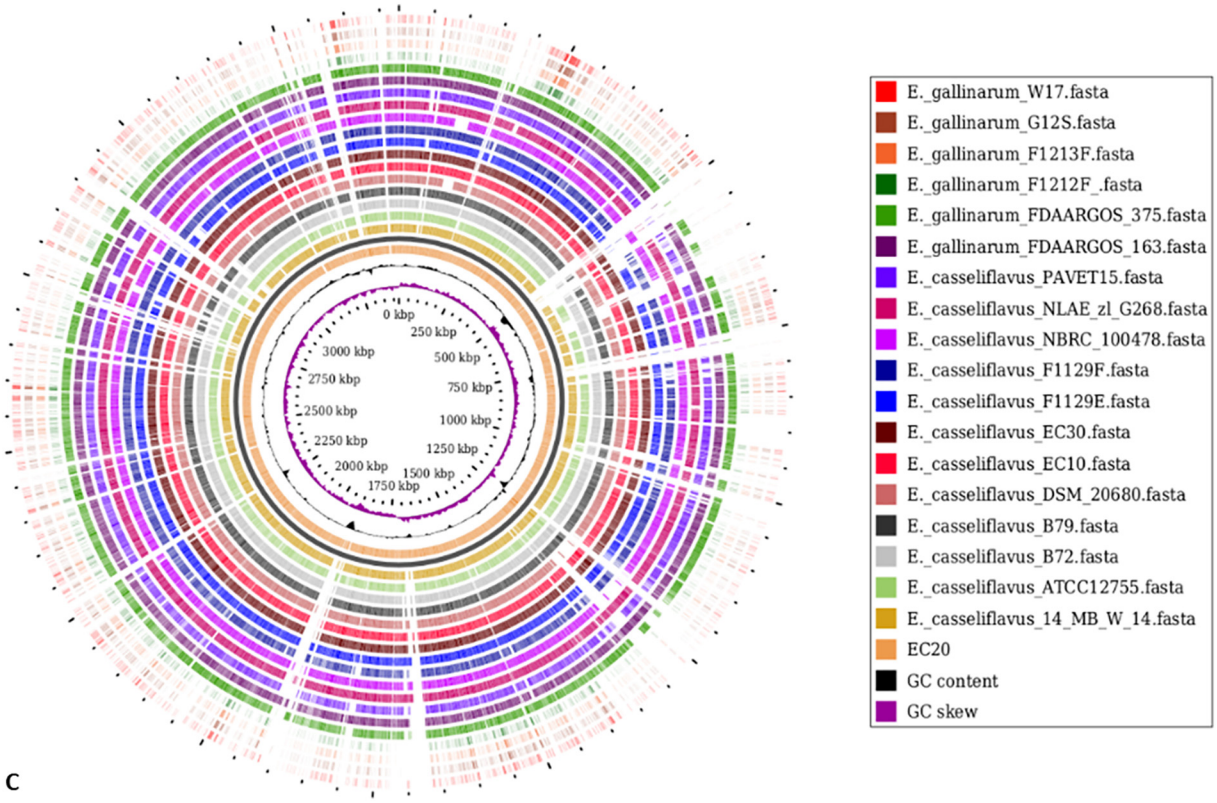

Fig. 2. (*continued*)

(Fig. 2c). The FDAARGOS genomes were assessed *in silico* using the 16S rRNA, *atp*A, *phe*S and *gro*ESL loci. The *atp*A, *phe*S and *gro*ESL loci identified both FDAARGOS genomes as *E. casseliflavus* while the 16S rRNA locus was unable to speciate these isolates. Subsequently, the two *E. gallinarum* FDAARGOS genomes were determined to have been misidentified and the designation for *E. gallinarum* FDAARGOS 375 has since been changed in the NCBI database to *Enterococcus* sp. FDAARGOS 375. However, the designation for *E. gallinarum* FDAARGOS 163 remains the same. All of the other *E. gallinarum* and *E. casseliflavus* genomes were identified correctly when compared to the whole genome.

The *groESL* locus was more reliable as compared to the other methods tested. This locus encodes the 60 kDa molecular chaperonin protein which is conserved in both bacteria and eukaryotes and has been used as a target for the detection, identification, and quantification of both bacteria, eukaryotes, and protozoa (Links et al., 2012). It is known as a preferred barcode for bacteria and as an indicator of microbiome diversity and provides more precise resolution than 16S rRNA of closely related taxa at the species and subspecies level (Links et al., 2012). This work verifies that the assertion that the *gro*ESL loci is favourable for inter-species and intra-species identification of bacterial isolates. For instance, this locus can also help to identify unique variants and differentiate isolates based on sequence alignment and SNP distances (Appendix C and D).

5. Conclusions

Commercial biochemistry-based identification methods, such as the RapID™ STR system, are shown to be less reliable in identifying enterococci species, including VRE, as compared to using the sequences of the four loci used in this study. The *gro*ESL locus was the most discriminatory while 16S rRNA was the least. The error rates for the identification of *E. casseliflavus* and *E. gallinarum* were higher for all identification methods when compared to more clinically-relevant *E. faecium* and *E. faecalis*. The difficulty in identifying and delineating these two species was also illustrated when these methods were compared to whole genome analysis. Laboratories looking for a quick, accurate method for identification of enterococci, and low-level VRE, without the use of specialized equipment such as MALDI-TOF should consider sequencing the *gro*ESL locus, a conclusion supported by our comparison to WGS.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.04.019.

References

Ahmad, A., Dada, A.C., Usup, G., 2014. Application of multilocus sequence analysis for molecular characterization of enterococci with virulence factors recovered from a

tropical recreational beach. Southeast Asian J. Trop. Med. Public Health 45, 700.
Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A., 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19, 455–477.
Carriço, J.A., Silva-Costa, C., Melo-Cristino, J., Pinto, F.R., de Lencastre, H., Almeida, J.S., Ramirez, M., 2006. Illustration of a common framework for relating multiple typing methods by application to macrolide-resistant *Streptococcus pyogenes*. J. Clin. Microbiol. 44, 2524–2532.
Cartwright, C.P., Stock, F., Fahle, G.A., Gill, V.J., 1995. Comparison of pigment production and motility tests with PCR for reliable identification of intrinsically vancomycin-resistant enterococci. J. Clin. Microbiol. 33, 1931–1933.
Devriese, L.A., Van de Kerckhove, A., Kilpper-Bälz, R., Schleifer, K.H., 1987. Characterization and identification of *Enterococcus* species isolated from the intestines of animals. Int. J. Syst. Evol. Microbiol. 37, 257–259.
Devriese, L.A., Pot, B., Collins, M.D., 1993. Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. J. Appl. Microbiol. 75, 399–408.
Domig, K.J., Mayer, H.K., Kneifel, W., 2003. Methods used for the isolation, enumeration, characterisation and identification of *Enterococcus spp.*: 2. Pheno-and genotypic criteria. Int. J. Food Microbiol. 88, 165–188.
Fang, H., Ohlsson, A.K., Ullberg, M., Özenci, V., 2012. Evaluation of species-specific PCR, Bruker MS, VITEK MS and the VITEK 2 system for the identification of clinical *Enterococcus* isolates. Eur. J. Clin. Microbiol. Infect. Dis. 31, 3073–3077.
Galili, T., O'Callaghan, A., Sidi, J., Sievert, C., 2017. Heatmaply: an R package for creating interactive cluster heatmaps for online publishing. Bioinformatics 1, 3.
Giardine, B., Riemer, C., Hardison, R.C., Burhans, R., Elnitski, L., Shah, P., Zhang, Y., Blankenberg, D., Albert, I., Taylor, J., Miller, W., Kent, W.J., Nekrutenko, A., 2005. Galaxy: a platform for interactive large-scale genome analysis. Genome Res. 15, 1451–1455.
Glaeser, S.P., Kämpfer, P., 2015. Multilocus sequence analysis (MLSA) in prokaryotic taxonomy. Syst. Appl. Microbiol. 38, 237–245.
Grant, J.R., Arantes, A.S., Stothard, P., 2012. Comparing thousands of circular genomes using the CGView comparison tool. BMC Genomics 13, 202.
Haritsa, K.B., Shashikala, N., Chavan, S.K.D., Sangeetha, S., 2014. Isolation, identification and speciation of enterococci and their antimicrobial susceptibility in a tertiary care hospital. J. Evol. Med. Dent. Sci. 3, 13893–13900.
Hubert, L., Arabie, P., 1985. Comparing partitions. J. Classif. 2, 193–218.
Hudson, C.R., Fedorka-Cray, P.J., Jackson-Hall, M.C., Hiott, L.M., 2003. Anomalies in species identification of enterococci from veterinary sources using a commercial biochemical identification system. Lett. Appl. Microbiol. 36, 245–250.
Hunter, P.R., Gaston, M.A., 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. 26, 2465–2466.
Kirschner, C., Maquelin, K., Pina, P., Thi, N.N., Choo-Smith, L.P., Sockalingum, G.D., Sandt, C., Ami, D., Orsini, F., Doglia, S.M., Allouch, P., Mainfait, M., Puppels, G.J., Naumann, D., 2001. Classification and identification of enterococci: a comparative phenotypic, genotypic, and vibrational spectroscopic study. J. Clin. Microbiol. 39, 1763–1770.
Lindell, S.S., Quinn, P., 1975. Use of bile-esculin agar for rapid differentiation of Enterobacteriaceae. J. Clin. Microbiol. 1, 440–443.
Links, M.G., Dumonceaux, T.J., Hemmingsen, S.M., Hill, J.E., 2012. The chaperonin-60 universal target is a barcode for bacteria that enables de novo assembly of metagenomic sequence data. PLoS ONE 7 (11), e49755.
Manero, A., Blanch, A.R., 1999. Identification of *Enterococcus spp*. with a biochemical key. Appl. Environ. Microbiol. 65, 4425–4430.
Moellering Jr., R.C., 1998. Vancomycin-resistant enterococci. Rev. Infect. Dis. 26, 1196–1199.
Mooney, S.D., Krishnan, V.G., Evani, U.S., 2010. Bioinformatic tools for identifying disease gene and SNP candidates. In: Genetic Variation. Humana Press, Totowa, NJ, pp. 307–319.
Naser, S., Thompson, F.L., Hoste, B., Gevers, D., Vandemeulebroecke, K., Cleenwerck, I., Thompson, C.C., Vancanneyt, M., Swings, J., 2005a. Phylogeny and identification of enterococci by atpA gene sequence analysis. J. Clin. Microbiol. 43, 2224–2230.
Naser, S.M., Thompson, F.L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M., Swings, J., 2005b. Application of multilocus sequence analysis (MLSA) for rapid identification of enterococcus species based on rpoA and pheS genes. Microbiology 151, 2141–2150.
Petkau, A., Stuart-Edwards, M., Stothard, P., Van Domselaar, G., 2010. Interactive microbial genome visualization with GView. Bioinformatics 26, 3125–3126.
Petti, C.A., Polage, C.R., Schreckenberger, P., 2005. The role of 16S rRNA gene sequencing in identification of microorganisms misidentified by conventional methods. J. Clin. Microbiol. 43, 6123–6125.
Pinto, F.R., Carriço, J.A., Ramirez, M., Almeida, J.S., 2007. Ranked adjusted Rand: integrating distance and partition information in a measure of clustering agreement. BMC Bioinformatics 8, 44.
Pinto, F.R., Melo-Cristino, J., Ramirez, M., 2008. A confidence interval for the Wallace coefficient of concordance and its application to microbial typing methods. PLoS ONE 3, e3696. https://doi.org/10.1371/journal.pone.0003696.
R Core Team, 2017. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria online. https://www.R-project.org/>.
Ramotar, K., Woods, W., Larocque, L., Toyea, B., 2000. Comparison of phenotypic methods to identify enterococci intrinsically resistant to Vancomycin (VanC VRE)☆. Diagn. Microbiol. Infect. Dis. 36, 119–124.
Sadar, H.S., Biedenbach, D., Jones, R.N., 1995. Evaluation of Vitek and API 20S for species identification of enterococci. Diagn. Microbiol. Infect. Dis. 22, 315–319.
Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30, 2068–2069.
Tan, T.Y., Jiang, B., Ng, L.S.Y., 2017. Faster and economical screening for vancomycin-resistant enterococci by sequential use of chromogenic agar and real-time polymerase chain reaction. J. Microbiol. Immunol. Infect. 50, 448–453.
Větrovský, T., Baldrian, P., 2013. The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. PLoS ONE 8 (2), e57923.
Wieser, A., Schneider, L., Jung, J., Schubert, S., 2012. MALDI-TOF MS in microbiological diagnostics—identification of microorganisms and beyond (mini review). Appl. Microbiol. Biotechnol. 93 (3), 965–974.
Willey, B.M., Jones, R.N., McGeer, A., Witte, W., French, G., Roberts, R.B., Jenkins, S.G., Nadler, H., Low, D.E., 1999. Practical approach to the identification of clinically relevant *Enterococcus* species. Diagn. Microbiol. Infect. Dis. 34, 165–171.
Winston, L.G., Pang, S., Haller, B.L., Wong, M., Chambers, H.F., Perdreau-Remington, F., 2004. API 20 strep identification system may incorrectly speciate enterococci with low level resistance to vancomycin. Diagn. Microbiol. Infect. Dis. 48, 287–288.
Zaheer, R., Yanke, L.J., Church, D., Topp, E., Read, R.R., McAllister, T.A., 2012. High-throughput species identification of enterococci using pyrosequencing. J. Microbiol. Methods 89, 174–178.