Rapid discrimination of *Enterococcus faecium* strains using phenotypic analytical techniques

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1 **Rapid discrimination of *Enterococcus faecium* strains using phenotypic**
2 **analytical techniques and advanced chemometrics**

3

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13

14 **Keywords:**

15 *Enterococcus faecium*, classification, MALDI-TOF-MS, FT-IR, Raman, pulsed-field
16 gel electrophoresis, chemometrics.

17 ABSTRACT

18 Clinical isolates of glycopeptide resistant enterococci (GRE) were used to compare
19 three rapid phenotyping and analytical techniques. Fourier transform infrared (FT-
20 IR) spectroscopy, Raman spectroscopy and matrix-assisted laser
21 desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) were
22 used to classify 35 isolates of *Enterococcus faecium* representing 12 distinct pulsed-
23 field gel electrophoresis (PFGE) types. The results show that the three analytical
24 techniques provide clear discrimination among enterococci at both the strain and
25 isolate levels. FT-IR and Raman spectroscopic data produced very similar bacterial
26 discrimination, reflected in the Procrustes distance between the datasets (0.2125-
27 0.2411, $p<0.001$); however, FT-IR data provided superior prediction accuracy to
28 Raman data with correct classification rates (CCR) of 89% and 69% at the strain
29 level, respectively. MALDI-TOF-MS produced slightly different classification of
30 these enterococci strains also with high CCR (78%). Classification data from the
31 three analytical techniques were consistent with PFGE data especially in the case of
32 isolates identified as unique by PFGE. This study presents phenotypic techniques as
33 a complementary approach to current methods with a potential for high-throughput
34 point-of-care screening enabling rapid and reproducible classification of clinically
35 relevant enterococci.

36 INTRODUCTION

37 *Enterococcus* is a highly significant genus of bacteria, which causes important
38 clinical infections including urinary tract infections (UTIs), endocarditis, meningitis,
39 catheter-related infections, bacteremia, wound infections, pelvic and intra-abdominal
40 infections amongst others. Some of these Gram-positive cocci were originally
41 classified as *Streptococcus* spp. until genomic analysis by Schleifer and Kilpper-
42 Balz in 1984 demonstrated the requirement for a separate genus classification (1).
43 This well-known genus is part of the normal intestinal microflora of humans and
44 other animals (2). *Enterococcus* are also part of the lactic acid bacteria (LAB) group
45 present in foods, and whilst they are able to spoil fresh meats (3), they are important
46 in ripening and development of certain foods (i.e. dairy products), as well as being
47 used as probiotics in humans (4).

48 The majority of human clinical isolates of enterococci belong to two species,
49 *Enterococcus faecalis* and *Enterococcus faecium* (5). In addition to their prevalence
50 and pathogenicity, another very important factor associated with enterococcus is the
51 high level of antimicrobial resistance, particularly resistance to glycopeptide
52 antibiotics (such as vancomycin, teicoplanin and telavancin); resistant strains are
53 referred to as GRE (glycopeptide-resistant enterococci) (6, 7).

54 There is a constant requirement to develop analytical methods for the
55 discrimination of bacteria, which can be used in clinical diagnostics and food quality
56 control. These methods should ideally be rapid, reproducible, easy to use and
57 automated, in addition to having high resolution and sensitivity (8). Over a decade
58 ago, it was common to use methods, such as polymerase chain reaction (PCR) for
59 identification of specific DNA sequences and recognition by antibodies via enzyme-
60 linked immunosorbent assay (ELISA), to characterize bacteria. Although these

61 techniques are sensitive and specific, they are time-consuming and their use is
62 limited by the complexity of preparation procedures and the requirement for specific
63 primers and antibodies (9-12). Nowadays, modern analytical techniques, such as
64 matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
65 (MALDI-TOF-MS) (13-16), Fourier transform infrared (FT-IR) spectroscopy (17-
66 21) and Raman spectroscopy (22-24) are also used for the characterization of
67 bacteria. High dimensional and information rich datasets are produced from these
68 techniques, which has also directly led to the requirement of robust and reliable
69 chemometric methods to assist with data deconvolution and in-depth analysis (25).
70 This saw the introduction, acceptance and use of chemometrics, such as discriminant
71 function analysis (DFA) (22) and hierarchical cluster analyses (HCA) (26-28).

72 Previously, MALDI-TOF-MS has shown promising results for bacterial
73 characterization (13). FT-IR and Raman spectroscopy complement each other for
74 bacterial classification; both are robust metabolic fingerprinting techniques and need
75 little sample preparation (29, 30). FT-IR spectroscopy is used by many researchers
76 since it is not only rapid but also offers a high-throughput and non-destructive
77 method, allowing the analysis of intact bacteria and producing unique, reproducible
78 and distinct biochemical fingerprints (31). Raman spectroscopy shares similar
79 advantages to FT-IR spectroscopy and also has the additional advantage of water
80 being a very weak Raman scatter (32) so that samples do not need to be dried.

81 Here, the aim was to use these three distinct phenotypic approaches (namely
82 MALDI-TOF-MS, FT-IR and Raman spectroscopies) in combination with rigorous
83 chemometric analysis of the resultant datasets to classify 35 clinically relevant
84 isolates of enterococci, which had been previously typed by pulsed-field gel
85 electrophoresis (PFGE). This was carried out in order to compare the results from,

86 and determine the efficiency of, these analytical techniques for the rapid
87 differentiation of *E. faecium* strains. In future, this may allow clinical diagnostic
88 laboratories to analyze multiple bacterial samples rapidly for infection control
89 purposes in point-of-care setting within hospitals, clinics, or GP surgeries which
90 would significantly accelerate diagnosis, and potentially ensure that the correct
91 antimicrobial therapies were used if required, and eliminate the delay associated with
92 sending strains to reference laboratories when analyzing patient samples.

93

94

95 **MATERIALS AND METHODS**

96 **General chemicals.** Trifluoroacetic acid (TFA), HPLC grade water, acetonitrile,
97 sinapinic acid (SA), α -cyano-4-hydroxycinnamic acid (CHCA), and ferulic acid
98 (FA) were purchased from Sigma-Aldrich (Dorset, UK).

99 **Enterococci strains.** Isolates were from faecal samples from patients in a surgical
100 ward in a hospital in Belfast, UK and were collected following an increase in
101 enterococcal infections on the ward. They were identified as *E. faecium* by a
102 VITEK® system (bioMérieux) and their identity confirmed by MALDI-ToF analysis
103 using a Bruker microflex instrument. The 35 isolates were typed using pulsed-field
104 gel electrophoresis (PFGE) of *Sma*I-digested genomic DNA by Public Health
105 England's National Reference Laboratory as described previously (33). Table S1
106 summarizes information on the 35 clinical isolates, which were classified into 12
107 groups (12 PFGE-defined types) named: EC04, EC09, EC10, EC13, EC14, EC15,
108 EC19, EC20, UNI 156, UNI 178, UNI 191 and UNI 214, where 'UNI' types
109 describe isolates that were unique within the set.

110 **Bacterial isolates.**

111 The samples analyzed by the three techniques (*viz.* MALDI-TOF-MS, FT-IR and
112 Raman) were collected from the same flask to avoid any variations between different
113 preparations that may affect results obtained using the different analytical platforms.
114 First, enterococci were cultured on nutrient agar (NA) plates for 24 h at 37°C. A
115 single colony from the agar culture was used to inoculate 50 mL of Lysogeny broth
116 (LB) in a 250 mL flask which was incubated overnight at 37°C with shaking at 200
117 rpm. This was followed by measuring the optical density (OD) at 600 nm using a
118 Biomate 5 spectrophotometer (Thermo, Hemel Hempstead, UK) for each isolate.
119 The volume of analyzed bacterial suspension was then normalized to account for
120 variation in cell biomass in the different replicate cultures (4 biological replicates
121 were prepared for each isolate) and used to inoculate a fresh flask of broth, which
122 was incubated at 37°C for 11 h (when the bacteria reached the stationary phase).
123 Then, 10 mL from each flask was collected and centrifuged at 4800 g for 10 min and
124 the pellet washed three times with sterile deionized water. Figure S1 illustrates the
125 preparation process.

126 For vibrational spectroscopic analysis, the collected pellets were suspended in
127 suitable volumes of saline (0.9% (w/v) NaCl) depending on the OD (all isolates had
128 approximately the same cell density). Then, 15 µL was spotted onto a silicon plate
129 (Bruker Ltd., Coventry, UK) and was allowed to dry at 40°C for 45 min before
130 analysis with FT-IR spectroscopy. For Raman spectroscopy, 4 µL of each sample
131 was spotted onto a stainless steel plate and then allowed to dry at 40°C for 45 min.

132 For MALDI-TOF-MS, three different matrices were tested to find the most
133 compatible matrix with enterococci; these matrices were: FA, SA and CHCA. In
134 addition, 3 different deposition methods (sample-matrix) were tested as described
135 previously (16) to find the best method for depositing the samples: mix, overlay and

underlay (data not shown). SA matrix and the mix deposition method were found to be the optimal combination for MALDI-TOF-MS analysis for these samples. On the day of analysis of the samples, the biomass was suspended in 1000 μL of 2% TFA then vortexed for 3 min. An equal volume of 10 μL of bacterial suspension and matrix were vortexed for 2 s and 2 μL of this mixture spotted onto a MALDI stainless steel plate and allowed to dry at ambient temperature.

Fourier transform infrared (FT-IR) spectroscopy. FT-IR spectroscopy plate (Bruker Ltd., Coventry, UK) which contained 96 locations/spots was washed using 5% sodium dodecyl sulfate (SDS) solution. This was followed by washing the plate using deionized water and allowing it to dry at room temperature (34). High-throughput screening (HTS) was carried out using a Bruker Equinox 55 FT-IR spectrometer. The HTXTM module described by Winder *et al.* (35) was used with this instrument. Transmission mode was used to analyze the dried biomass to produce FT-IR spectra. The parameters used for FT-IR analysis included the following: spectra were collected in the wavenumber range between 4000 and 600 cm^{-1} , resolution was 4 cm^{-1} and each spectrum was the average of 64 co-adds. Spectral acquisition and subtracting the background were achieved using Opus software (Bruker Ltd.). Four biological replicates, each in four analytical replicates, were analyzed and analysis was performed in three machine runs, resulting in 1680 FT-IR spectra.

Raman Spectroscopy. This was carried out using a confocal Raman system (inVia, Renishaw plc., Wotton-Under-Edge, UK) coupled with a 785 nm wavelength laser. A power intensity of ~ 30 mW was applied on the samples at an exposure time of 20 s. Four biological replicates and seven different locations within each sample spot were analyzed, resulting in a total of 980 Raman spectra.

161 **MALDI-TOF-MS.** The enterococci isolates were analyzed using an AXIMA-
162 Confidence MALDI-TOF-MS (Shimadzu Biotech, Manchester, UK), equipped with
163 a nitrogen pulsed UV laser with a wavelength of 337 nm. The parameters of this
164 device were set as follows: 90 mV laser power, 91 acquired profiles with each
165 profile containing 20 shots, linear TOF, positive ionization mode, and mass-to-
166 charge (m/z) range of 1,000-18,000. The spectra were collected using a circular
167 raster pattern. The MALDI-TOF-MS device was calibrated using a protein mixture:
168 insulin (5,735 Da), cytochrome c (12,362 Da), and apomyoglobin (16,952 Da)
169 (Sigma-Aldrich). Each of 4 biological replicates from the 35 isolates was analyzed in
170 four technical replicates on four different days; this led to the generation of a total of
171 560 MALDI-TOF-MS spectra (35 isolates $\times$ 4 biological replicates $\times$ 4 analytical
172 replicates).

173 **Data analysis**

174 **Data pre-processing.** Opus software was used to export FT-IR data into ASCII
175 format; the data were then transferred into MATLAB 2012a (The Mathworks Inc.,
176 MA, US). All FT-IR spectra were baseline corrected using standard normal variate
177 (SNV) to remove any light scattering effect. The three analytical replicates were then
178 averaged to reduce the number of redundant samples. Due to the large number of
179 samples, 8 separate (96 spot silicon) sampling plates were used; therefore, it was
180 necessary to correct for the subtle differences in signals from different silicon plates.
181 This was achieved by using a piece-wise direct standardization (PDS) model (36).
182 The PDS model was built on two different 'reference' isolates which were spotted on
183 every plate. The pre-processed FT-IR spectra were then subjected to multivariate
184 analysis (MVA, see below). Raman spectra were also normalized using standard
185 normal variate (SNV) and then subjected to MVA.

186 MALDI-TOF-MS data were pre-processed as follows: (i) the baseline was
187 corrected using asymmetric least squares (AsLS) (37), and (ii) spectra were
188 normalized by dividing each individual baseline corrected spectrum by the square
189 root of the sum of squares of the spectrum (38). The pre-processed MALDI-TOF-
190 MS data were subjected to the same data analysis flow as Raman and FT-IR spectral
191 data.

192 **Multivariate data analysis.** A flowchart of multivariate data analysis is provided in
193 Figure 1. For all three datasets, two types of classification were performed: one at
194 the strain level (i.e. 12 classes) defined by PFGE, and the other at the isolate level
195 (i.e. 35 classes, one for each isolate).

196 For cluster analyses, principal components-discriminant function analysis (PC-
197 DFA) (39-41) was applied to reduce the dimensionality of the data and discriminate
198 samples from the designated classes. The PC-DFA scores of each class were then
199 averaged and subjected to hierarchical cluster analysis (HCA) (42). Dendrograms
200 from each analysis were generated to illustrate the relative relatedness of these
201 bacteria.

202 Partial least squares-discriminant analysis (PLS-DA) (43), with 1,000
203 bootstrapping validations (44), was also applied to obtain a validated supervised
204 classification model for discriminating different strains or isolates. In each
205 bootstrapping process, the data were randomly split into two different sets: a
206 training set and a test set. A PLS-DA model was trained on the training set and then
207 applied to the test set to predict the class membership of the samples in the test set.
208 This process was repeated 1,000 times and the results were recorded and averaged to
209 produce a $c \times c$ confusion matrix (c is the number of designated classes, either 12
210 (strains) or 35 (isolates)), in which the element at the i^{th} row, j^{th} column is the

percentage of samples in class i being predicted as class j on average. In order to assess the statistical significance of the predictive performance of the PLS-DA models, a corresponding permutation test within each bootstrapping resampling was also performed. This means that in addition to building the PLS-DA model using the known class membership, another model (called the ‘null’ model) was also built using a randomly permuted class membership. The results of the null model were also recorded and from this the null distribution was obtained. An empirical p -value was calculated by counting the number of cases where the null model had obtained better predictive accuracy than the real model and dividing the obtained number by the total number of bootstrapping resampling (i.e. 1,000 in this study).

Finally, similarities between the three different datasets (FT-IR spectroscopy, Raman spectroscopy and MALDI-TOF-MS data) were measured using Procrustes analysis (45). Procrustes analysis is an excellent approach for assessing the differences and similarities between different ordination space from cluster analyses and has been used previously for the assessment of different analytical techniques (46). The distances were calculated based on the averaged PC-DFA scores for the biological replicates.

RESULTS AND DISCUSSION

Table S1 shows all 35 isolates belonging to 12 strains (PFGE-defined 12 types) including: EC04, EC09, EC10, EC13, EC14, EC15, EC19, EC20 UNI 156, UNI 178, UNI 191 and UNI 214. These strains were previously confirmed to belong to *E. faecium* using a VITEK[®] system and by MALDI-ToF analysis using a Bruker Microflex system (data not shown). The PFGE results (Fig. S2) were compared to

235 results obtained in this study using FT-IR spectroscopy (17, 30, 46-49), Raman
236 spectroscopy (25, 30, 50, 51) and MALDI-TOF-MS (13, 14, 16, 52-54). We believe
237 that these analytical techniques in combination with chemometrics offer an
238 improvement in the classification of bacteria due to their higher biochemical
239 resolution.

240 **Classification using FT-IR spectroscopy.**

241 In this study, four biological replicates of bacterial isolates were analyzed in four
242 analytical replicates and analysis was performed in three machine runs, resulting in a
243 total of 1680 FT-IR spectra. The three machine replicate measurements were
244 performed in order to evaluate the reproducibility of the FT-IR technique. Typical
245 spectra based on four biological replicates of representatives of 12 strains from
246 enterococcus (EC04, EC09, EC10, EC13, EC14, EC15, EC19, EC20, UNI 156, UNI
247 178, UNI 191 and UNI 214) are provided in Figure S3A. The infrared spectra
248 contain different distinct regions that can be used to characterize bacterial samples.
249 These have been well documented previously and include: wavenumbers around
250 3400-2850 cm^{-1} corresponding to fatty acids, at 1705-1454 cm^{-1} related to amide I
251 and II regions attributed to peptides and proteins, and around 1085-1052 cm^{-1}
252 corresponding to polysaccharides (19, 55, 56).

253 Discrimination between the strains based on visual inspection of the spectra was
254 difficult (17) because these strains are very similar phenotypically. Therefore, in
255 order to develop a classification model to distinguish between bacterial samples
256 based on similarities in the spectral data, multivariate analysis was used to reduce the
257 high dimensionality of the data. First, PC-DFA was applied using 40 principal
258 components (PC) to the 12 strains (i.e. 12 classes) and 35 isolates (i.e. 35 classes)
259 using the pre-processed FT-IR spectra (Fig. 2A and 3A, respectively). Figure 2A

shows a clear separation between the 12 strains, displaying 4 main clusters; Cluster 1 is a single-member cluster (SMC) containing only (EC10), Cluster 2 includes (EC20 and UNI 156), Cluster 3 (UNI 191, EC04 and EC15) and Cluster 4 formed a large group and is a combination of (EC13, EC19, EC14, EC09, UNI 214 and UNI 178). Each cluster is represented by a different color in the figure. As described above, HCA was undertaken using spectral data in order to simplify the DFA plot and to illustrate the related strains. Cluster analysis was based on averaged DFA scores (12 classes/strains), using Ward's linkage as shown in Figure 2B. Clusters seen in Figure 2A are reflected in the HCA dendrogram plot (Fig. 2B).

PC-DFA was subsequently performed for all the 35 isolates and the results are provided in Figure 3. Clear separation between all 35 isolates was observed despite the fact that there were a much higher number of classes to be separated than the number of strains. For example, clear separation was observed between the two representatives of EC10 (139 and 151). Furthermore, results generated using PFGE correlated well with FT-IR spectroscopic data. For example, the UNI 156 and UNI 178 were seen as unique by both techniques. In addition, the three EC20 isolates (192, 198 and 204) and EC19 isolates (173, 174 and 175) clustered together and were not differentiated using FT-IR spectroscopy, which was also observed in the PFGE results, where the bands were quite similar (Fig. 3B). This implies that the isolates within each of these groups are highly similar to each other phenotypically and genetically. Finally, two more clusters were observed, with one cluster containing all the EC04, EC15 and UNI 191 strains and the remainder of the isolates forming another cluster.

The PLS-DA classification using FT-IR spectral data achieved an average correct classification rate (CCR) of 89.4% at the strain level and 54.3% at the isolate level,

285 both with an empirical p -value of <0.001 , i.e. not a single case where the null model
286 obtained better results, indicating that the predictive accuracies were highly
287 significant. The null distributions are provided in Figure S4A and B at the two
288 levels.

289 The confusion matrices of strains and isolates classification are presented in
290 Table 1 and Table S3, respectively. Most of the 12 strains showed high prediction
291 accuracies, for example EC04, EC10, EC13 and EC20 had accuracies of 89.9%,
292 99.7%, 99.8% and 99.2%, respectively. However, EC14 and UNI 214 had lower
293 prediction accuracies of 47.3% and 58.9%, respectively. The confusion matrix
294 showed that there was a certain level of overlap between (EC14 and EC09) and (UNI
295 214 and EC19).

296
297 Furthermore, in-depth analysis of the confusion matrix (Fig. 4) showed that
298 classification of unique strains was generally in line with PFGE results. In Figure 4,
299 high percentage class membership assignments are represented by warm colors (e.g.
300 red), indicating agreement between predicted classes and known classes. It is also
301 interesting to see that representatives from EC19 and EC20 formed two “squares” of
302 “tiles” on the diagonal line, in which the colors were similar to each other. Results
303 from Figure 4 suggest that the PLS-DA model was not able to differentiate the
304 isolates within EC19 and EC20, yet another observation that is consistent with PFGE
305 results. On the other hand, all representatives of EC04 and EC09 (160 and 133) were
306 unique in the FT-IR spectroscopy profile using the PLS-DA model but had visually
307 similar PFGE profiles. This is most likely due to PFGE providing genetic
308 information (57, 58) while FT-IR spectroscopy describes phenotypes (27, 59). This
309 implies that isolates from EC19 and EC20 are highly conserved phenotypically,

310 whereas those from EC04 and EC09 are not, and such subtle differences in
311 phenotypes were detected by FT-IR spectroscopy. Our observations showed that FT-
312 IR spectroscopy appears to be a very promising analytical approach for
313 discrimination of enterococci at different levels. In line with the results presented in
314 this study, work carried out by Guibet *et al.* showed that clear discrimination and
315 classification of enterococci strains can be achieved using FT-IR spectroscopy (60,
316 61).

317 **Classification using Raman spectroscopy.** In addition to the FT-IR spectroscopy
318 technique used in this study, Raman spectroscopy was used as a complementary
319 technique (17, 61-63). As expected, the two techniques generated different spectra.
320 These two approaches are complementary due to the selection rules, whereby
321 infrared causes a change in the net dipole moment in a particular functional group,
322 induced by molecular vibrations, whereas Raman causes a change in the polarization
323 of bonds within a molecule. Therefore, bonds within a molecule are generally
324 infrared or Raman active with the result being that the two techniques can provide
325 complementary (bio) chemical information (29, 64).

326 Raman spectra of the 12 *E. faecium* strains are shown in Figure S3B. Raman
327 spectra for these types appeared almost indistinguishable and no differences were
328 detected on visual inspection. Moreover, some specific peaks which were identified
329 in these spectra included: peaks at around 722 cm⁻¹, 783 cm⁻¹, 854 cm⁻¹, 1004 cm⁻¹,
330 1098 cm⁻¹, 1334 cm⁻¹, 1451 cm⁻¹ and 1664 cm⁻¹, which correspond to adenine,
331 cytosine/uracil, tyrosine, phenylalanine, phosphate, guanine, protein and amide I,
332 respectively (65-67).

333 PC-DFA scores plot of pre-processed Raman spectra for the 12 PFGE-defined
334 types is shown in Figure S5A. The figure shows classification results similar to those

335 seen with FT-IR spectroscopy data. There was an obvious overlap between the two
336 spectroscopic techniques, especially with representatives of EC10. However, EC20
337 overlapped with UNI 156 in FT-IR spectroscopy data, whereas EC20 was closer to
338 UNI 178 based on Raman spectroscopy data. These observations can be seen in the
339 HCA dendrogram based on Raman data (Fig. S5B), which was quite similar to the
340 HCA results generated from FT-IR data. Looking back at the dendrogram in
341 Figure S2 based on PFGE data, visual inspection showed that there were some
342 similarities between results generated via spectroscopic techniques and those based
343 on PFGE; for example, EC04 and EC15 were shown to overlap in both sets of results
344 (Fig. S2).

345 As with FT-IR data, Raman spectroscopy data on the 35 isolates were also
346 analyzed using to PC-DFA and HCA (Fig. S6A and B, respectively). The results
347 suggested that Raman spectroscopy was also successful in discriminating the two
348 representatives of EC10 (139 and 151), which was also the case using FT-IR
349 analysis (Fig. 3). Furthermore, in order to ensure the classification is robust, the data
350 were analyzed using a heat map based on PLS-DA (Fig. S6C). The results suggested
351 that all the isolates indicated as unique (UNI) by PFGE were also unique in the PLS-
352 DA model generated using Raman spectroscopy data.

353 In addition, chemometric-based identification was carried out using PLS-DA at
354 both the strain and isolate levels and the predictive accuracies were calculated based
355 on 1,000 bootstrapping resampling using Raman spectral data. The null distribution
356 was obtained (Fig. S4C and D) at both the strain (12 classes) and isolate levels (35
357 classes) resulting in average CCR of 69.3% ($p<0.001$) and 21.1% ($p<0.001$),
358 respectively. The CCR from FT-IR data was higher at both levels compared to
359 Raman data possibly due to the higher reproducibility of FT-IR data. Confusion

matrices were also generated at both the strain level (Table S2A) and the isolate level (data not shown); these results suggested that Raman spectroscopy can also be used as a robust technique for bacterial discrimination. In-depth analysis showed that Raman spectroscopy generated around 70% prediction accuracy at the strain level which is lower than that of FT-IR spectroscopy (nearly 90%). This is most likely due to the low concentration of cells used for analysis: the infrared interrogation beam used was *ca.* 1 mm and passes completely through the dried bacterial film; while the Raman microscope delivers a highly focussed laser beam with an interrogation volume of ~ 1 pL and therefore measures very few bacteria. To overcome this limitation with Raman, bacteria can be analyzed directly from the agar plates or surface-enhanced Raman spectroscopy (SERS) as an alternative technique (68-70), but this is an area for future study.

Classification using MALDI-TOF-MS. As described in the Materials and Methods section, four biological replicates were analyzed in four analytical replicates for each bacterial strain, resulting in 560 MALDI-TOF-MS spectra; both the biological and technical replicates clustered closely together ensuring good bioanalytical reproducibility (data not shown). The spectra for the 35 enterococci isolates were pre-processed before data analysis. The typical pre-processed positive ion mode MALDI-TOF-MS spectra for all 12 *Enterococcus* strains (EC04, EC09, EC10, EC13, EC14, EC15, EC19, EC20, UNI 156, UNI 178, UNI 191 and UNI 214) are provided in Figure S3C. In general, the MALDI-TOF-MS spectra were of high quality with high signal-to-noise ratios in the acquisition m/z range 1,000-18,000 and a high number of peaks for each studied strain were detected. There are many factors that can affect MALDI-TOF-MS results and some of these can differ from lab to another, such as the type of medium used (71), sample handling, type of matrix (72),

385 sample deposition method (73), solvents, instrument settings (74, 75) and the type of
386 data analysis chosen (41, 76). These can inadvertently affect MALDI-TOF-MS
387 results and subsequent PC-DFA and HCA.

388 MALDI-TOF-MS spectra are not readily interpretable from the 35 isolates as
389 they are similar phenotypically and MALDI-TOF-MS spectra show only two
390 dimensions ($m/z \times$ intensity). Therefore, as is the case for the vibrational
391 spectroscopy techniques, robust multivariate analysis methods were employed for
392 this purpose. The results of PC-DFA using 12 classes (12 strains) in a three-
393 dimensional plot of DF1 vs DF2 vs DF3 and a two-dimensional plot of DF2 vs DF3
394 are shown in Figure S7A and B, respectively. Four main clusters were observed in
395 the PC-DFA plots; SMC (Cluster) 1 contains only UNI 178; Cluster 2 contains
396 EC20; Cluster 3 consists of EC04, EC10, EC15 and UNI 191; and Cluster 4 formed
397 a large group of (EC13, EC19, EC14, EC09, UNI 214 and UNI 156). Results from
398 the HCA dendrogram (Fig. S7C) confirmed the separation between the 12 classes
399 (i.e. 12 strains). This indicated that UNI 178 is phenotypically very different from
400 the other strains based on MALDI-TOF-MS data.

401 PC-DFA was also applied to data from the 35 isolates; the results showed
402 that isolates number 160 and 219 (both from EC09) were very different from the
403 other isolates. Therefore, another PC-DFA was carried out with these two outliers
404 removed and the HCA results are shown in Figure S8D. It appears that all
405 representatives of EC20 (204, 198 and 192) overlap with each other, which was also
406 observed in FT-IR and Raman spectroscopy data, with the exception that isolate 192
407 slightly differed from the other two representatives (204 and 198) in the HCA
408 dendrogram when using Raman data (Fig. S6B). However, analysis by PFGE

409 showed that isolates 192 and 198 clustered more closely with each other than with
410 isolate 204.

411 Furthermore, PLS-DA model applied to MALDI-TOF-MS data achieved an
412 average CCR of 78.2% ($p<0.001$) and 35.7% ($p<0.001$) for the 12 (strains) and 35
413 (isolates) classes, respectively. When PLS-DA was undertaken with 33 isolates (with
414 isolates 160 and 219 removed), the average CCR for the isolates increased to
415 53.95% ($p<0.001$). The prediction accuracies for the 12 classes are shown in Table
416 S2B and those for the 35 classes (isolates) are shown in Table S4. Table S2B shows
417 that discrimination between most of the strains (12 classes) using MALDI-TOF-MS
418 data achieved high correct classification rates, except for EC14 and UNI 191, which
419 had rather low classification rates. Confusion matrices for the 35 classes and the 33
420 classes (160 and 219 isolates removed) are shown in Figure S8A and C, respectively.
421 From these matrices, it can be seen that all the isolates identified by the reference
422 laboratory as unique (UNI), which included isolates 156, 178, 191 and 214, were
423 also classified as unique based on MALDI-TOF-MS data. Moreover, EC20 and
424 EC19 were assigned the same classification in PFGE typing, and this was in
425 agreement with MALDI-TOF-MS, FT-IR spectroscopy and Raman spectroscopy
426 data. In addition, based on MALDI-TOF-MS data (Fig.S8A and C), representatives
427 of EC13 (152, 154 and 155) belonged to the same cluster, and isolates 177 from
428 EC13 was significantly different from the remaining EC13 strains; this was also
429 observed in FT-IR and PFGE data. Looking back at Figure S8C, it can be seen that
430 all the strains from EC04 were unique in MALDI-TOF-MS and FT-IR profiles when
431 using PLS-DA modelling.

432 **Procrustes distance test of the three analytical techniques.** Analytical techniques
433 such as FT-IR spectroscopy, Raman spectroscopy and MALDI-TOF-MS are

434 currently used in clinical research studies worldwide and many reports have been
435 published showing advantages of using such techniques (24, 54, 77, 78). Kirschner
436 *et al.* (61) demonstrated accurate identification and classification of 18 strains from 6
437 different species belonging to enterococci using vibrational spectroscopic techniques
438 in combination with chemometrics. This study suggested that FT-IR and Raman
439 spectroscopies can offer potential alternatives to the conventional typing tests due to
440 their speed and ease of use. Oliveira *et al.* (51) showed that Raman spectroscopy, in
441 combination with a chemometric algorithm, can be used to discriminate between
442 seven different colonies of Gram-positive and Gram-negative bacteria. In another
443 previous study, it was also shown that 59 clinical bacterial strains associated with
444 urinary tract infections (UTIs) could be identified using FT-IR and Raman
445 spectroscopy (17). As an alternative to vibrational spectroscopic techniques,
446 MALDI-TOF-MS is a relatively new technique which has shown very promising
447 results for identification in agreement with methodologies carried out in
448 microbiological laboratories, and therefore has been used for the identification and
449 classification of bacterial species (15, 79, 80) and is appearing in many clinical
450 microbiology testing laboratories (54, 81, 82).

451 Previous studies have generally focussed on the application of just one or two
452 analytical techniques for the classification of *Enterococcus* spp. However, to
453 generate complementary data and more comprehensive analysis, this study combines
454 three different analytical techniques – FT-IR spectroscopy, Raman spectroscopy and
455 MALDI-TOF-MS – to analyze whole bacterial cells. Successful classification was
456 demonstrated at the strain (i.e. 12 classes) and isolate (i.e. 35 classes) levels based on
457 data generated by the three analytical platforms. In order to assess the overall
458 information content in the spectra that has been revealed by the cluster analysis from

the scores plots, Procrustes analysis was employed to assess the overall similarity between the patterns detected by these three platforms. The results are presented in terms of Procrustes distance (Table 2A and B), where the Procrustes distance varies from 0 to 1; the lower the distance, the higher the similarity between the results. The comparisons were made using averaged PC-DFA scores. For each dataset, there were two sets of PC-DFA scores, one at the strain level (12 classes) and another for isolates classification (33 classes). For each set of PC-DFA scores, the scores were then averaged according to their strain label and isolate label to give two sets of *averaged* PC-DFA scores.

The findings in Table 2 can be summarized as follows:

- (i) The patterns in the PC-DFA scores at strain and isolate levels were highly similar to each other for all the three analytical platforms. The Procrustes distances varied from 0.0681 to 0.1812. This suggested that the variation originating from different bacteria is the main factor in PC-DFA, i.e. the differences between different bacterial genotypes were significantly higher than those between different isolates.
- (ii) The two vibrational spectroscopic techniques (FT-IR and Raman) generated highly similar results both at the strain and isolate classification levels, with the corresponding Procrustes distances varying from 0.2112 to 0.3187.
- (iii) However, the results generated by MALDI-TOF-MS were significantly different from those generated by the two spectroscopic techniques, and the corresponding Procrustes distances were all above 0.8. Such differences can be mainly attributed to data on isolate UNI 178, which appeared to be very different to other strains in the MALDI-TOF-MS dataset.

483 Table S5 shows a summative comparison of the 4 main clusters identified based on
484 the three analytical techniques using PC-DFA plots of the 12 *E. faecium* strains (12
485 classes). It can be seen from this table that despite the large Procrustes distances
486 between data generated by MALDI-TOF-MS and those generated by the other two
487 techniques, the main identified clusters patterns observed in all three datasets were
488 still largely consistent.

489

490

491 **CONCLUSIONS**

492 The results obtained from the two vibrational spectroscopic techniques
493 demonstrated that good discrimination can be achieved at both the strain and isolate
494 levels and the detected patterns from the two techniques were highly similar. In
495 addition, bacterial classification results from MALDI-TOF-MS were generally
496 consistent with these vibrational spectroscopic techniques. However, UNI 178 was
497 detected to be very different in MALDI-TOF-MS data, which differed from the other
498 two analytical techniques employed in this study.

499 The results obtained using these spectroscopic phenotyping approaches were
500 mostly consistent with previous results obtained from experiments carried out using
501 the genotypic classification method, PFGE. Some of the results differed when
502 directly comparing our analytical approach with results from the molecular approach
503 and these differences may be due to comparing phenotypic differences from whole-
504 organism fingerprinting with genotypic differences using PFGE.

505 In conclusion, we have assessed multiple analytical phenotypic as
506 complementary approaches to current molecular methods. All methods provided
507 excellent clustering which was in general agreement with genotypic baseline

508 methods, as well as allowing excellent discrimination to the strain level and good
509 resolution at the sub-strain level. We believe that these three different
510 physicochemical techniques have excellent potential as high-throughput point-of-
511 care screening tools, and for the rapid and reproducible classification of clinically
512 relevant bacteria, such as *E. faecium*.

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518

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759 **FIGURE LEGENDS**

760 **FIG 1.** Workflow of data analysis undertaken for FT-IR spectroscopy, Raman spectroscopy
761 and MALDI-TOF-MS. The data were first pre-processed then multivariate analysis MVA
762 was applied using PC-DFA at both the (ST) strain (12 classes) and (IS) isolate (35 classes)
763 levels. This was followed by PLS-DA.

764 **FIG 2.** (A) Discriminant function analysis (DFA) scores plot from FT-IR data after pre-
765 processing, illustrating the relationship between the 12 enterococci. (B) Cluster analysis on
766 averaged PC-DFA scores (12 classes/strains) using Ward's linkage.

767 **FIG 3.** (A) PC-DFA plot from FT-IR data after pre-processing which illustrates the
768 relationship between the 35 enterococcus isolates. (B) Hierarchical cluster analysis on
769 averaged PC-DFA scores (35 classes/isolates) using Ward's linkage (right) and PFGE
770 results (left). Each isolate is represented by the same color in both the boxes around the
771 PFGE images and the FT-IR dendrogram.

772 **FIG 4.** PLS-DA trained on 35 classes (i.e. 35 isolates) from FT-IR spectral data. High
773 percentage class membership assignments are represented by warm colors (e.g. red) whilst
774 the cold colors (e.g. blue) represent low percentage class membership assignments. The
775 diagonal "tiles" are much warmer than off-diagonal "tiles", which indicates agreement
776 between predicted classes and known classes.

777

778

Figure 1

Figure 2

A

PFGE

B

Figure 3

785

786

Figure 4

787 **Table 1.** The prediction accuracies of the 12 enterococci strains using FT-IR spectroscopy
788 data

Class Known/Predicted	EC04	EC09	EC10	EC13	EC14	EC15	EC19	EC20	UNI 156	UNI 178	UNI 191	UNI 214
EC04	89.9%	0.5%	0.0%	0.0%	0.4%	8.3%	0.1%	0.0%	0.0%	0.0%	0.7%	0.1%
EC09	0.1%	90.3%	0.0%	1.3%	4.8%	0.0%	3.5%	0.0%	0.0%	0.0%	0.0%	0.0%
EC10	0.0%	0.1%	99.7%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.1%
EC13	0.0%	0.0%	0.0%	99.8%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%
EC14	0.1%	48.9%	0.0%	1.1%	47.3%	1.0%	1.4%	0.1%	0.0%	0.0%	0.1%	0.0%
EC15	6.8%	1.4%	0.0%	0.0%	0.5%	91.1%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%
EC19	1.6%	9.3%	0.0%	0.2%	3.6%	0.0%	83.5%	0.0%	0.0%	0.0%	0.0%	1.8%
EC20	0.0%	0.1%	0.0%	0.0%	0.0%	0.7%	0.0%	99.2%	0.0%	0.0%	0.0%	0.0%
UNI 156	0.4%	0.0%	0.0%	0.5%	0.0%	0.0%	0.1%	0.9%	98.1%	0.0%	0.0%	0.0%
UNI 178	0.0%	5.3%	0.0%	0.1%	0.0%	0.0%	0.4%	0.0%	0.0%	93.9%	0.2%	0.0%
UNI 191	6.5%	0.9%	0.0%	25.2%	0.0%	1.3%	0.0%	0.0%	0.0%	0.0%	66.1%	0.1%
UNI 214	1.9%	13.4%	0.0%	1.0%	0.1%	0.0%	20.4%	0.0%	0.0%	0.0%	4.2%	58.9%

789

790 **Table 2.** The similarity between three different datasets using Procrustes distance

791 (A) PC-DFA at the strain level

Averaging on ST level	FT-IR (IS)	FT-IR (ST)	Raman (IS)	Raman (ST)	MALDI (IS)	MALDI (ST)
FT-IR (IS)	-					
FT-IR (ST)	0.0858	-				
Raman (IS)	0.2125	0.2933	-			
Raman (ST)	0.2314	0.3187	0.1502	-		
MALDI (IS)	0.8602	0.889	0.899	0.8202	-	
MALDI (ST)	0.9125	0.8846	0.9149	0.8988	0.1812	-

792

793 (B) PC-DFA at the isolate level

Averaging on IS level	FT-IR (IS)	FT-IR (ST)	Raman (IS)	Raman (ST)	MALDI (IS)	MALDI (ST)
FT-IR (IS)	-					
FT-IR (ST)	0.1085	-				
Raman (IS)	0.2112	0.2446	-			
Raman (ST)	0.2411	0.3168	0.1132	-		
MALDI (IS)	0.8593	0.8719	0.8196	0.8001	-	
MALDI (ST)	0.8975	0.8608	0.8841	0.8703	0.0681	-

794 (ST) and (IS) indicate the PC-DFA was calculated at the strain (12 classes, PFGE-
795 defined 12 types) and isolate (33 classes) levels, respectively.