Supplementary Information (SI) for Analyst.

1. EXPERIMENTAL METHODS

1.1 BACTERIAL INCUBATION AND VANCOMYCIN SUSCEPTIBILITY OF *ENTEROCOCCUS* ISOLATES

The isolates used in this study were categorised as susceptible or resistant via antimicrobial susceptibility testing for vancomycin using the clinical breakpoints determined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The clinical isolates used in this study are listed in Table 2. To construct the predictive model, three vancomycin-susceptible *E. faecium* (VSE) isolates and three vancomycin-resistant *E. faecium* isolates (VRE) were used (Table 1), with three independent biological replicates of each. Blind testing was performed using four *Enterococcus* isolates (two *E. faecium* and two *E. faecalis*) with unknown vancomycin susceptibility. All isolates used in this study were sourced from The Alfred Hospital, Melbourne, Victoria, Australia, with one isolate from the American Type Culture Collection.

Table 1. Clinical *E. faecium* isolates used in this study

Name	Vancomycin susceptibility	Genotype
D045	Susceptible	N/A
J060	Susceptible	N/A
J034	Susceptible	N/A
C003	Resistant	Van A/B
B030	Resistant	Van A/B
B025	Resistant	Van A/B

The bacterial isolates were cultured overnight in sterile Brain Heart Infusion (BHI) broth at 37°C with constant shaking. The overnight cultures were used to inoculate 50 mL of BHI broth in 200 mL flasks to a bacterial cell density of 0.2, measured by a cell densitometer set to $OD_{600\,nm}$. Vancomycin was added to a final concentration of 4 mg/mL to the antibiotic treatment flasks, while the control flasks received an equal volume of Milli-Q water. Cultures were then incubated at 37 ºC with aeration for 120 min and 240 min. At each time point, the cell density was measured again to check for growth and cells were collected from approximately 25 mL of culture by centrifugation at 600 *g* for 5 min at room temperature. The bacterial isolates were washed with MilliQ water, spun down via benchtop centrifuge at 600 *g*, and then resuspended in 1 mL MilliQ water six times to remove any residual BHI media. After the sixth wash, the final supernatant was removed, and the resulting pellet was measured.

1.2 DATA COLLECTION

ATR-FTIR spectra were recorded using a Bruker Alpha FT-IR spectrometer with an attenuated total reflection (ATR) sampling device. The instrument was equipped with a single bounce diamond internal reflection element, a globar source, a KBr beam splitter, and a deuterated triglycine sulfate detector. All the spectra were collected in the range of 4000 – 900 cm^{-1} with a spectral resolution of 6 cm^{-1}, with 64 scans for sample measurement and a background scan rate of 128 scans. The background spectra were collected before the first sample measurement, and recollected every three experimental spectra (approx. every three minutes) to ensure that no significant humidity changes occurred. For each sample, 0.5 µL of bacterial pellet was placed directly on the ATR crystal and air-dried for approx. 4 min. The sample was determined to be dried once a significant reduction in the shape and intensity of the O-H band at 3200 cm^{-1}, and the spectral intensity of the amide I band remained unchanged for approx. 1 min. For each biological replicate of the sample, three technical replicates were collected ($n_{isolate}$ = 6, $n_{total\_spectra}$ = 108, n_{VSE} = 54, n_{VRE} = 54). To ensure against bias and the influence of background data, the collection of experimental spectra was randomised on a technical replicate level.

1.3 DATA ANALYSIS USING CHEMOMETRIC ANALYSIS

Principal component analysis was performed using MATLAB R2024a (MathWorks, Natick, USA) with PLS toolbox v8.2 (Eigenvector Research, Manson, USA). All the ATR-FTIR spectra for each isolate were divided into two new datasets, VSE or VRE, depending on the isolates' susceptibility to vancomycin. Each dataset was subjected to the same preprocessing protocol; the spectral region was cut to 1800 – 900 cm^{-1}, pre-processed to the 2nd derivative

(Savitzky-Golay algorithm, 11 smoothing points), and normalised using the Standard Normal Variate (SNV) algorithm before applying Principal Component Analysis (PCA). The data was mean-centred, and the number of components used was decided via a set threshold of 80% of the variance explained. The percentage cumulative variance captured by the model's first two principal components at 120 min was 83.9% for VSE and 81.56% for VRE. After the preliminary model was loaded, extreme score outliers were removed to ensure the best pattern visualisation, as the data was mean-centred. As such, score plots were employed to visualise data clustering, with corresponding loading plots used to determine which spectral bands were contributing to the separation.

Partial least squares discriminant analysis (PLS-DA) was used to generate the predictive antibacterial resistance diagnostic model. To do so, a difference spectrum was created by subtracting the spectral information of the isolates exposed to 4 mg/L vancomycin from the spectral information of the control isolates. The differences spectra were calculated post-processing. This results in a single spectrum for each isolate, representing the spectral alterations induced by drug exposure. A positive drug response was indicative of susceptibility in VSE, whereas the absence of such a response suggested resistance in VRE. These difference spectra were then calculated for VSE and VRE datasets, and used for the construction of the predictive model using PLS-DA.

Before applying the PLS-DA model to the new difference datasets (both VSE and VRE), the data were cut to an 1800 – 900 cm^{-1} wavenumber range, pre-processed to the 2^{nd} derivative (Savitzky-Golay algorithm, 11 smoothing points), and normalised using Standard Normal Variate (SNV). The differences spectra were calculated post-processing. To generate the model, the new dataset was divided into a calibration and validation set using random sampling, with approx. 66% of the data was assigned for model calibration, and 34% assigned for validation. The data was mean-centred and the model constructed using three loading vectors (LV), with the percentage cumulative variance captured by the model of 80.69%. Scores plots were employed to visualise the predicted data classification and the probability of classification of the two classes (VSE or VRE).

1.4 BLIND TESTING

For blind testing, four independent isolates were grown and prepared using the same protocol as described above. Cells were collected at 0 and 120 minutes for ATR-FTIR measurements. To ensure outcomes were independent, the overnight cultures were performed by a researcher who was different from the researcher who undertook the ATR-FTIR measurements. The collected spectra underwent the data processing procedure stated earlier, followed by the extraction of the spectra differences between vancomycin exposure and internal control of the same isolate at 120 min. Subsequently, resistance profiles were predicted using the PLS-DA models for the 1800 – 900 cm^{-1} spectral range using the same protocol mentioned above. The predicted resistance profile was then compared with the actual resistance profile, determined via minimum inhibitory concentration (MIC) testing using vancomycin.

2. ADDITIONAL DATA

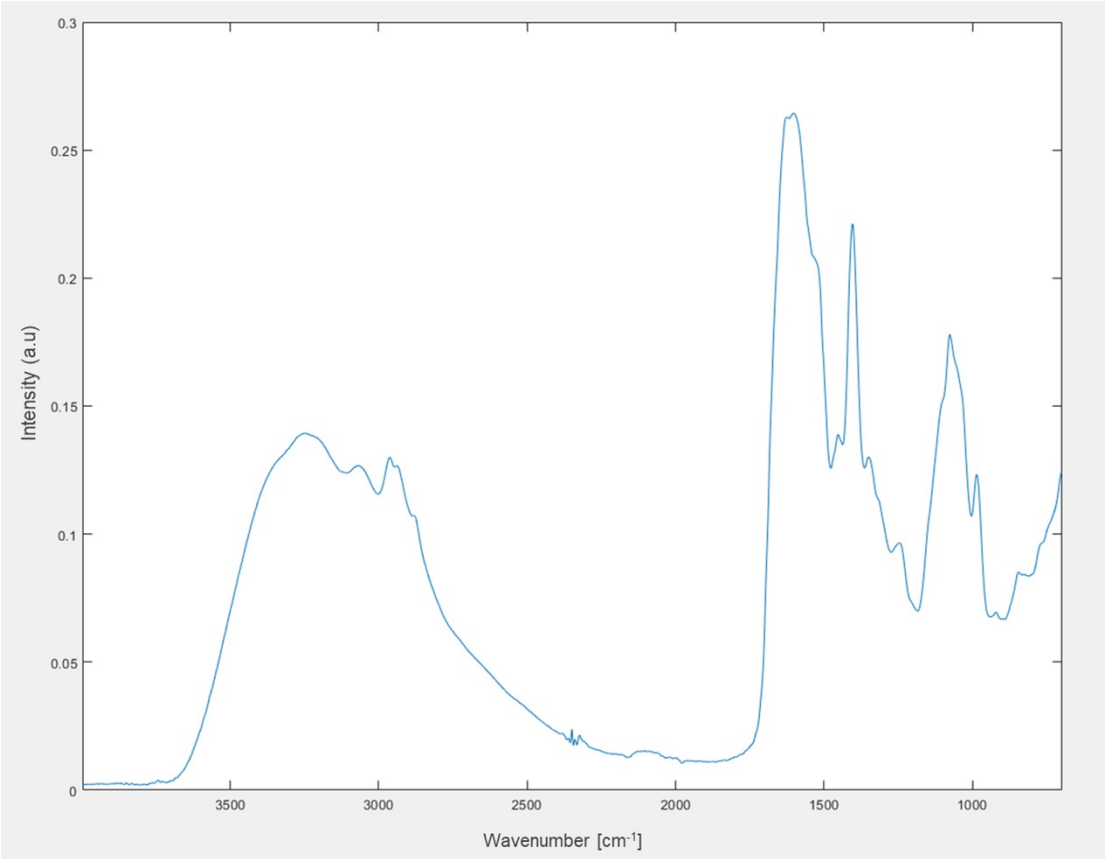

Figure S1. Raw ATR-FTIR spectrum of sterile Heart Brain Infusion broth used to culture bacteria.