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Using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectroscopy (MALDI-TOF MS) for Determining Virulence of *Enterococcus faecalis* Isolates of Poultry

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ABSTRACT

Enterococcus faecalis (*E. faecalis*) is a ubiquitous microbe occurring in the environment and in the intestinal tract of poultry. *E. faecalis* is often isolated from the hatchery environment, where it may cause embryo mortality and rapidly spread amongst hatchlings. *E. faecalis* can also cause amyloid arthropathy in older laying chickens. Although some *E. faecalis* isolates cause disease, there is evidence indicating that not all *E. faecalis* isolates are virulent. Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) is a technology which allows a bacterium to be speciated accurately and rapidly following initial isolation. Using avirulent and highly virulent *E faecalis* isolates as determined by an embryo lethality assay, a MALDI-TOF MS biomarker model was developed to differentiate avirulent from highly virulent *E. faecalis* isolates. The biomarker model showed agreement with the ELA results in differentiating individual spectra of *E. faecalis* isolates as either virulent or avirulent. However, the biomarker model did not reliably classify intermediate virulence isolates as either avirulent or virulent on a consistent basis.

Key words: *Enterococcus faecalis*, Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS), Virulence determination.

INTRODUCTION

Enterococcus species are ubiquitous, occurring in soil and water and are a part of the intestinal microbiota of many animal species, including poultry (Devriese et al. 1991; García-Solache and Rice 2019; Ramos et al. 2020; Shah and Varahan 2024). *E. faecalis* often occurs in hatching eggs and the hatchery environment, where it may cause embryo mortality and can rapidly spread to hatchlings. *E. faecalis* is vertically transmitted either on or through the egg and may be at least partially responsible for egg infertility and/or decreased hatchability (Landman et al. 1999a; Fertner et al. 2011; Karunarathna et al. 2017; Reynolds and Loy 2020). *E. faecalis* can cause amyloid arthropathy in older laying chickens (Landman et al. 1998; Landman et al. 1999b; Blanco et al. 2016). *E. faecalis* has numerous virulence traits including antimicrobial resistance genes which can be transferred to other bacteria, formation of biofilms and an exotoxin that is often referred to in the literature as cytolysin or hemolysin (Coburn and Gilmore 2003; Fisher and Phillips 2009; Madsen et al. 2017; Fiore et al. 2019; Archambaud et al. 2024). However, there is evidence indicating that not all *E. faecalis* isolates are virulent (Blanco et al. 2016; Maasjost et al. 2019).

Virulence of bacteria has typically been assessed by an embryo lethality assay (ELA) by inoculating embryonating eggs via the chorioallantoic route and then assessing embryo mortality during a specified period of time (Goodpasture and Anderson 1937; Powell and Finkelstein 1966; Nolan et al. 1992; Borst et al. 2014; Blanco et al. 2017; Dolka et al. 2022). The use of ELAs may provide useful information; however, ELAs are time consuming and resource intensive and therefore are not ideal for (and may not be available as) a routine diagnostic assay. Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) is a technology which allows a bacterial isolate to be accurately identified within minutes following initial isolation and is now being used regularly in both medical and veterinary diagnostic laboratories (Hou et al. 2019; Ashfaq et al. 2022; Calderaro and Chezzi 2024).

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The use of MALDI-TOF MS to speciate *E. faecalis* in birds has been validated (Stępień-Pyśniak et al. 2017). MALDI-TOF MS has previously been shown to be capable of accurately classifying bacterial isolates into subtypes within a bacterial species based on genomic differences and/or whether the isolate represents a likely pathogen versus a commensal bacterial subtype (Loy and Clawson 2017; Hille et al. 2020; Hille et al. 2021; Olson et al. 2022). The objective of this study was to determine if MALDI-TOF MS could be used to differentiate virulent from avirulent *E. faecalis* isolates of poultry origin that were initially classified using ELA results.

MATERIALS AND METHODS

Ethical statement

This study was conducted at the University of Nebraska–Lincoln. The protocols for using 10-day-old chicken embryos do not require review and approval. The Institutional Animal Care and Use Committee has approved the specific pathogen-free (SPF) breeding flock used to produce the fertile eggs (Project ID 2599, approved May 20, 2024).

Enterococcus faecalis isolates

The Nebraska Veterinary Diagnostic Center (NVDC) has a repository of *E. faecalis* field isolates from various avian species including chickens (broilers and layers), turkeys, and game birds. A total of 43 isolates were evaluated including 33 from chickens, 8 from turkeys and 2 from pheasants.

Culture conditions

Frozen stocks were plated on tryptic soy agar with 5% sheep blood (Remel, Lenexa, KS) and incubated in 5% CO_2 at 37°C for 24 hours. The isolates were then passaged onto another tryptic soy blood agar plate which was incubated for 24 hours prior to ELA assessment and/or generation of MALDI-TOF spectra.

Embryo lethality assay (ELA)

A chicken embryo lethality assay was developed to determine virulence of *E. faecalis* isolates. Briefly, the ELA used 10-day-old specific pathogen free chicken embryos that were inoculated with approximately 100 colony forming units of *E. faecalis*, contained in 0.1mLs of phosphate buffered saline (PBS), by the chorioallantoic route and then the eggs were placed in a 37.8ºC incubator. Twenty embryos per *E. faecalis* isolate, along with another group of 20 embryos (as a control group, inoculated with 0.1mLs of sterile PBS) were used. The embryos were assessed for livability/mortality by candling at 7 days post-inoculation. The student t-test was employed to compare the livability/mortality of the controls to the livability/mortality of the *E. faecalis* isolate inoculated groups. The parameters for the ELA were developed such that the control group needed to have a livability of at least 80% (i.e., 20% mortality=4 embryo deaths). Individual experiments that did not meet this threshold were repeated. Using simulations, we established the criteria for a virulent *E. faecalis* isolate would have embryo livability of less than 20% when compared to the sterile PBS inoculated control group ($P<0.05$). In order to more easily compare virulence between different *E. faecalis* isolates, we established a virulence index by using the following formula: Virulence index (VI)=Percent livability of PBS control embryos / percent livability *E. faecalis* inoculated embryos. Example: PBS inoculated control embryos=19/20 live, (95% livability), *E. faecalis* inoculated embryos=3/20 live (15% livability), virulence index=95/15=6.33. The VI parameters were as follows: less than 1.25=avirulent, greater than 1.25 but less than or equal to 2.0=low virulence, greater than 2.0 but less than 4.0=moderate virulence and greater than 4.0=high virulence.

MALDI-TOF MS

Spectra was obtained using the manufacturers recommended methods that have previously been described (Khot et al. 2012). To summarize, 2-3 colonies were dissolved in 300µL HPLC water, and 900µL of ethanol was then added to the mixture. The sample was centrifuged at 16,000 x g for 2min and the supernatant was discarded. The sample was allowed to air dry completely. The resulting pellet was then dissolved in 25µL acetonitrile and 25µL 70% formic acid before another centrifugation at 16,000 x g for 2min. Then, 1µL of the fluid mixture was placed on a single well of the MALDI-TOF target plate and allowed to air dry completely. Finally, 1µL of α-cyano-4-hydroxycinnamic acid matrix solution (Bruker Daltonik, Billerica, MA) was added to each sample well on the target plate. Once completely air dried, the target plate was analyzed using a MALDI Biotyper system (Bruker Daltonik) set to a positive linear mode with a mass range of 2-20 *m/z* and a laser frequency of 60 Hz. Samples were plated onto three separate target wells and each well was analyzed three times, resulting in nine technical replicates of spectra for each isolate. Any null spectrum observed was removed from further analysis.

Spectra comparison and biomarker model development

A main spectrum profile (MSP) was made which incorporated spectra from all MALDI-TOF MS technical replicates from each isolate using MBT Compass Explorer software (Bruker Daltonik). Dendrograms were developed using the resulting MSPs and the "View MSP dendrogram" function within MBT Compass Explorer. Biomarker model generation utilized ClinProTools 3.0 software (Bruker Daltonik) and the quick classifier (QC) classification algorithm. To generate the QC biomarker model, eight avirulent isolates (VI range 0.8 – 1.18) and three highly virulent isolates (VI range of 10 - 17) were used to train the model. Recognition capability and cross validation accuracies were calculated automatically by ClinProTools 3.0.

RESULTS AND DISCUSSION

Chicken embryo lethality assays have previously been used to assess virulence amongst various bacterial pathogens (Goodpasture and Anderson 1937; Finkelstein and Ransom 1960; Powell and Finkelstein 1966; Goodpasture 1983; Dolka et al. 2022). Typically, 9–10-day-old embryos are inoculated with bacteria by the allantoic / chorioallantoic route and assessed for mortality at some predetermined time interval following inoculation. The ELA method has been used for a number

of poultry pathogens including *Escherichia coli* (*E. coli*) (Nolan et al. 1992; Wooley et al. 2000), *Salmonella* species (Zhang et al. 2020), *Enterococcus cecorum* (Borst et al. 2014; Dolka et al. 2022) and *E. faecalis* (Blanco et al. 2017; Maasjost et al. 2019). In a study whereby an LD_{50} was established for a strain of *E. faecalis*, Blanco et al. (2017) utilized 3443 chicken egg embryos. This study illustrates the resource intensiveness of the ELA method. Moreover, virulence and ELA results have been reported to correlate for *E. cecorum* (Borst et al. 2014), but others have reported variability when using ELAs to assess other bacteria including *E. coli* and *E. faecalis* (Powell and Finkelstein 1966; Nolan et al. 1992; Maasjost et al. 2019).

The results of the ELA for *E. faecalis* isolates in this study were as follows: avirulent (VI ranged from, 0.80 to 1.18)=8 isolates, low virulence (VI ranged from 1.25 to 1.82)=21 isolates, moderate virulence (VI ranged from 2.22 to 3.00)=7 isolates and high virulence (VI ranged from 4.50 to 17.0)=7 isolates. The percentage of *E. faecalis* isolates evaluated by the ELA with their associated virulence is indicated in Fig. 1. Note that more than half (67.4%) were found to be either avirulent or low virulent isolates and 32.6% of the isolates were either moderate or highly virulent isolates. Avirulent isolates made up 18.6% (8/43) while 16.3% (7/43) were highly virulent.

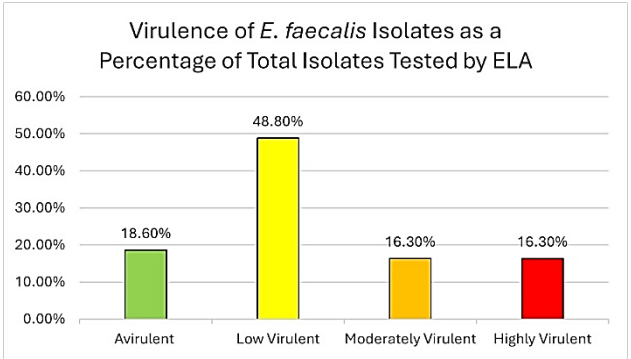

Fig. 1: Virulence of *E. faecalis* isolates.

The *E. faecalis* isolates that were used to build the MALDI-TOF QC biomarker model are listed in Table 1 along with their species of origin and their respective ELA VI. The resulting QC biomarker model incorporated four total peaks with m/z of: 3375, 6752, 10578 and 13053. The accuracy for internal recognition capability was 93.06% for avirulent strains, and 100% for the highly virulent strains, resulting in a 96.53% overall recognition capability. The cross-validation results were 91.89% for avirulent strains, and 98.08% for highly virulent strains, with an overall accuracy of 94.98%. Fig. 2 displays a scatter plot of individual spectra used to develop the model using the magnitude of peaks with m/z of 3375 and 6752, which are the two peaks assigned the most weight in the biomarker model algorithm. The plot shows general clustering of the two levels of virulence. Among the spectra generated from avirulent isolates, there were nine spectra that were outside the otherwise well grouped spectra data points that represent all other avirulent isolates. The nine spectra that are present as outliers are all from a single isolate used in the avirulent training set and indicates there is variability among this group of isolates at these specific peaks.

Table 1: *E. faecalis* isolates used to build MALDI-TOF MS biomarker model. Green shaded=avirulent isolates. Red shaded=highly virulent isolates

Isolate#	Isolate Origin	Virulence Index
2	Chicken-Broiler	0.8
6	Chicken-Broiler	0.9
4	Chicken-Broiler	0.95
5	Turkey	0.95
3	Pheasant	1.06
7	Chicken-Broiler	1.11
1	Chicken-Broiler	1.14
8	Chicken-Broiler	1.18
43	Chicken-Broiler	10
38	Turkey	17
40	Turkey	17

Detecting variability among spectra within the virulence categories is supported by the fact the model did not reliably classify isolates with a VI greater than 1.18 or less than 10. Additionally, when different biomarker models were developed using isolates with these more intermediate levels of VI, the software reported recognition capability and cross validation results that were substantially less than the reported model. The resulting accuracies of these models were unreliable in classifying isolates that were not present in the training set. When comparing the overall spectrum differences using the MSP of the isolates, there is a trend toward grouping based on virulence level among isolates used to develop the model (Fig. 3a). But, when all isolates are included in the dendrogram comparison, the trend toward clustering based on virulence class is less noticeable (Fig. 3b). This is in agreement with the interpretation that there are potential differences in isolates in the avirulent categories, but the isolates with an intermediate VI display substantial variability and do not show a tendency to group with isolates on either end of the virulence spectrum which is problematic for the biomarker model.

Other techniques have been used previously for evaluating the virulence of *E. faecalis* isolates including using microarray assays, polymerase chain reactions (PCRs) for detecting virulence genes (Poeta et al. 2006; Champagne et al. 2011; Hwang et al. 2011; Silva et al. 2011; Olsen et al. 2012; Stępień-Pyśniak et al. 2021), Multi Locus sequence typing (Blanco et al. 2016; Blanco et al. 2018) and ERIC-PCR (Blanco et al. 2018). Similar to the findings in this study, none of the aforementioned techniques have demonstrated a definitive correlation between a biomarker and *E. faecalis* virulence.

Conclusion

The results of this study indicate there are potential reliable differences in MALDI-TOF MS spectra obtained from *E. faecalis* isolates that exhibit no virulence versus those with very high virulence, as determined by ELA. However, isolates that fall outside of these groups and are more intermediate in terms of virulence have enough spectra variability among them to inhibit reliable and repeatable MALDI-TOF MS virulence classification using the methods described here. Despite the unreliable accuracy of the model in terms of classifying isolates with more intermediate virulence, the biomarker model

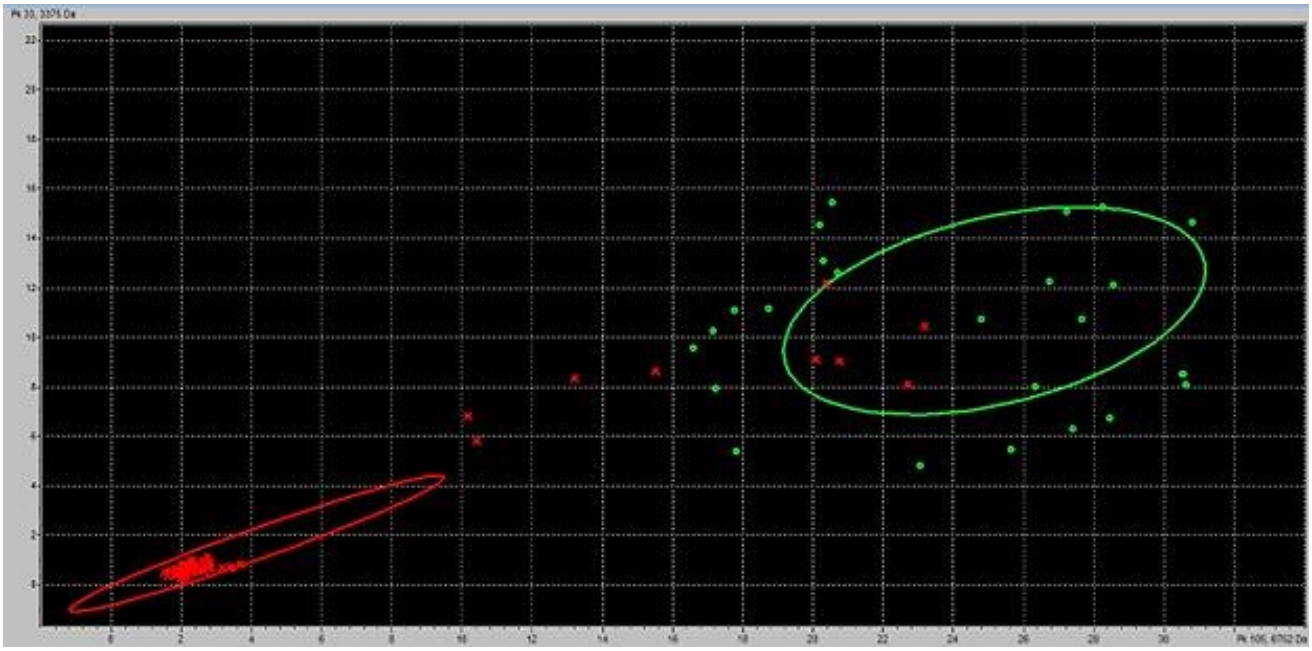

Fig. 2: Scatter plot of spectra data from 8 avirulent (red x) vs 3 highly virulent (green circle) *E. faecalis* isolates. Peaks used to generate the plot included those with m/z of 3375 (Y axis) and 6752 (X axis). Units are arbitrary units of magnitude.

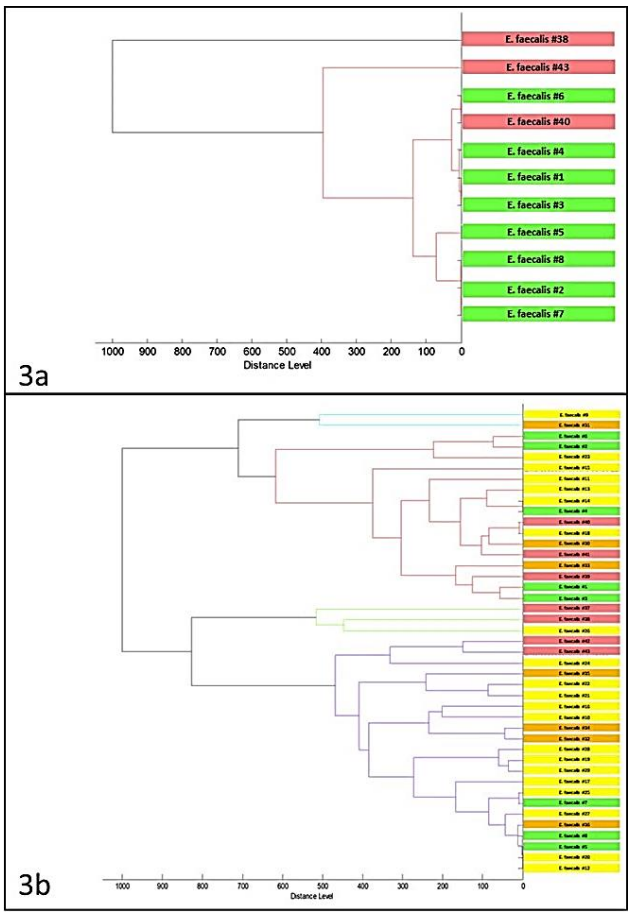

Fig. 3: Main spectrum profile (MSP) dendrogram comparison of isolates used to develop the biomarker model (3a) and all isolates used in the study (3b). Color shade indicates virulence category. Green: avirulent, yellow: low virulent, orange: moderate virulent, red: high virulent.

developed here may have utility for the poultry industry and veterinary diagnosticians. If the biomarker model can accurately classify isolates that are avirulent or have very low virulence potential, this would help in determining if a particular isolate warrants further investigation from a management standpoint. The model reported here was developed using a limited number of isolates based on availability of isolates and the fact that the differences between spectra were only detectable in isolates from the extreme ends of the virulence spectrum. In the future, using the reported model to classify more known avirulent and highly virulent isolates would be beneficial to confirm the accuracy of the model and/or develop a model that is even more robust in its training set, which may result in a model that could be more accurate.

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Conflict of Interest Statement: The authors declare no conflict of interest.

Author's Contributions: Conceptualization, DLR and MMH; Methodology, DLR, MMH, EMH and EBS; Software, MMH and EMH; Validation, MMH and EMH; Formal Analysis, MMH and EMH; Investigation, DLR and MMH.; Resources, DLR and MMH; Data Curation, EMH and EBS; Writing – Original Draft Preparation, DLR; Writing – MMH; Supervision, DLR and MMH; Project Administration, DLR; Funding Acquisition, DLR and MMH. All authors have read and agreed to the published version of the manuscript.

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