Characterization of ESBL producing E. coli from chicken feces by phenotypic methods and MALDI-TOF MS

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Abstract

E.coli from broiler is a reservoir for ESBL (extended spectrum beta-lactamase) and presence of ESBL is a growing concern for antibiotic resistance. The aim of the study was to investigate and characterize ESBL and AmpC beta-lactamases in E. coli with traditional and new-generation methods. As well as biochemical analyses, the identification of isolates was performed with the MALDI-TOF MS. Within the scope of phyloproteomic analysis, all components of MALDI-TOF MS-based Principal Component Analysis (PCA) (dendrogram, scatter plotting, composit corelation index (CCI) and variance,) were applied. In the present study which is the first report for Duzce (Türkiye), 28.6% of 122 CFEC (chicken feces E. coli) isolates were identified as CFEC -ESBL. blaCTX-M, blaCTX-M-1, blaCTX-M-15, blaSHV, blaTEM, blaOXA-10, AmpC, blaCIT, blaMOX, blaSHV, blaCIT, and blaMOX genes were explored with PCR and blaCTX-M-1 gene was detected with the highest rate (68.5%). At least one of the resistance genes was detected in the phenotype screening tests, except one of the isolates (CFEC-ESBL-90). On the other hand CFEC-ESBL-38 contained only bla CTX-M-15 and the fact that this isolate was the only atypical ESBL strain with indole (-) and lac (-) characteristics among all isolates explains the highest variance (41%) and the most different from other PCA components. Also, this isolate had a high degree of similarity (87%; CCI) with the other isolate (CFEC-ESBL-90), which had low similarity to CFEC-ESBLs. As a result, phyloproteomic analyses with MALDI-TOF MS are considered to be beneficial in the characterization of phenotypic bacterial behavior.

Characterization of ESBL producing *E. coli* from chicken feces by phenotypic methods and MALDI-TOF MS

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Abbreviations:

CFEC: Chicken Feces *E. coli*	CHCA: Cyano-4-hydroxycinnamic acid
ESBL: Extended Spectrum Beta-Lactamase	ACN:Acetonitrile
PCA :Principal Component Analysis	TFA: Trifluoroacetic Acid
CCI: Composit Corelation Index	MSP: Micro Scout Plate (MSP
CFKO: Chicken Feces *Klebsiella oxytoca*	CFEBC: Chicken Feces *Enterobacter kobei*

Abstract

E.coli from broiler is a reservoir for ESBL (extended spectrum beta-lactamase) and presence of ESBL is a growing concern for antibiotic resistance. The aim of the study was to investigate and characterize ESBL and AmpC beta-lactamases in *E. coli* with traditional and new-generation methods.

As well as biochemical analyses, the identification of isolates was performed with the MALDI-TOF MS. Within the scope of phyloproteomic analysis, all components of MALDI-TOF MS-based Principal Component Analysis (PCA) (dendrogram, scatter plotting, composit corelation index (CCI) and variance,) were applied. In the present study which is the first report for Duzce (Türkiye), 28.6% of 122 CFEC (chicken feces*E. coli*) isolates were identified as CFEC -ESBL.*bla* $_{\text{CTX-M}}$, *bla* $_{\text{CTX-M-1}}$,*bla* $_{\text{CTX-M-15}}$, *bla* $_{\text{SHV}}$,*bla* $_{\text{TEM}}$, *bla* $_{\text{OXA-10}}$, AmpC,*bla* $_{\text{CIT}}$, *bla* $_{\text{MOX}}$,*bla* $_{\text{SHV}}$, *bla* $_{\text{CIT}}$, and*bla* $_{\text{MOX}}$ genes were explored with PCR and*bla* $_{\text{CTX-M-1}}$ gene was detected with the highest rate (68.5%). At least one of the resistance genes was detected in the phenotype screening tests, except one of the isolates (CFEC-ESBL-90). On the other hand CFEC-ESBL-38 contained only $bla_{\text{CTX-M-15}}$ and the fact that this isolate was the only atypical ESBL strain with indole (-) and lac (-) characteristics among all isolates explains the highest variance (41%) and the most different from other PCA components. Also, this isolate had a high degree of similarity (87%; CCI) with the other isolate (CFEC-ESBL-90), which had low similarity to CFEC-ESBLs.

As a result, phyloproteomic analyses with MALDI-TOF MS are considered to be beneficial in the characterization of phenotypic bacterial behavior.

Keywords: Antibiotic resistance, Extended-spectrum beta-lactamase, *E. coli* , MALDI-TOF MS, Principal component analysis

Introduction

Escherichia coli (*E. coli*) *is* a common member of the gut microbiota in humans and animals and it is characterized as an opportunistic pathogen. The bacterium is considered an important source of many antibiotic resistance genes in the ecosystem [1].

Antimicrobial resistance (AMR) is a very important growing crisis in the entire world. It is predicted by some experts that there will be 10 million deaths related to AMR every year in the world by 2050, and it is considered that the gene reservoir of *E. coli* is decisive for solution of the crisis [3]. Because *E. coli* is a zoonotic and spreads easily in food-environment-human interaction, which leads to a potential change in the microbiome at the global level. It was reported that *E. coli* plays an important role, especially in the spread of Extended-Spectrum Beta-Lactamases (ESBL), acquired AmpC beta-lactamases [4,5].

E. coli can hydrolyze almost all penicillin and cephalosporins via its ESBL encoding genes. The uninterrupted transmission and spread of these resistance genes, which can pass from one bacterium to another bacterium with their fast and easy mobility features, depend on hierarchically organized systems such as integron or it depends on their interactions with the network between ecologically related bacterial populations. In other words, the distribution of resistant bacteria is an ecological evolutionary process [5]. Understanding this can only be possible by examining the phylogenetic and phyloproteomic relationships between bacteria with various analytical methods. As well as the expensive and laborious sequence analyses (whole genome or multilocus sequencing etc.), the analysis of 16S ribosomal proteins, which are relatively inexpensive and effortless, has made a significant contribution to this field in recent years [6].

The use of the Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) method, which analyzes the proteins of bacteria with the help of validated databases created with reference spectra of standard strains, became widespread in the past 10 years. It allows sensitive and specific applications in food safety and many clinical studies and it is also a method approved by the FDA (Food and Drug Administration) for microbial identification [7-9]. In a comparative study was conducted in 2010, it was reported that MALDI-TOF MS had a very high rate of identifying the bacteria in cases (99.1%) compared to routinely used biochemical methods [10]. In another study, it was reported that Gram (-) bacteria were directly detected as a species in 99.2% of blood and urine samples [11]. MALDI-TOF MS identify after comparing highly conserved ribosomal proteins of microorganisms with reference proteomic profiles of standard strains that are abundantly available in the database. At the same time, it is possible to perform phyloproteomic analyzes in the case of working with multiple isolates. In this respect, it is possible to compare fingerprints with unique mass spectra created for each bacterium by MALDI-TOF MS, and then compare them with each other. Thus, with this series of analyses that can be performed with MALDI-TOF MS, besides microbial identification, contributes to an idea of similarities or differences in some processes operating in metabolism [9,12]. For example, Suen et al., (2019) identified pathogenic *Staphylococcus*species from indoor samples with MALDI-TOF MS and analyzed the multi-drug resistance profiles of isolates with MALDI biotyper software [13]. In another study, it was reported that *Mycobacterium*spp. identification takes 7-21 days after colony formation with tradinational biochemical methods, but it can be accurately identified in as little as 1 hour with MALDI-TOF MS [14]. Elbehiry et al. (2019) performed species discrimination in *Aeromonas* strains with MALDI-TOF MS-based Principle Component Analysis (PCA) and single peak analysis methods [15]. In another study, a new species of*Staphylococcus edaphicus* is identified with MALDI-TOF MS from sandy soil [16]. These studies show analytical identification potential of MALDI-TOF MS, along with the specificity of peptide and protein mass fingerprinting, and the identification of new species, with a constantly developed and updated database. Alharbi et al. [17] identified *S. aureus* and coagulase-negative staphylococci at 100% from 400 samples in their study comparing MALDI-TOF MS with conventional methods. There are other studies conducted on MALDI-TOF MS in which high rates of bacteria are identified from different sources (wastewater, rural areas, etc.) [18-20]. It is increasingly preferred for the identification of Gram (-) bacteria of fecal origin from food and farm animals [21,22].

The present study was designed as food safety research and broiler chickens were preferred. In general, there are microorganisms with very different characteristics in poultry, and many studies have shown that MALDI-TOF MS can reliably identify these bacteria [23]. Therefore this study was aim (i) to investigate presence of ESBL, AmpC beta-lactamase in *E. coli* obtained from the gut contents of broiler chickens and (ii) to characterize the isolates by phenotypic-proteomic analyses.

Materials and Methods

Chicken feces samples and chemicals

A total of 130 chicken feces samples were obtained fresh from 4 different slaughterhouses in the city centre of Duzce (Türkiye). Mac Conkey Agar (MAC) (Merck, Germany), Tryptic Soy Agar (TSA) (Merck, Germany) and Tryptic Soy Broth (TSB) (Condalab, Spain) were used for isolation and culture and ∝-Cyano-4-hydroxycinnamic acid (CHCA; Bruker, Germany) was used as a MALDI-TOF MS matrix. Acetonitrile (ACN, HPLC grade; Sigma-Aldrich, Missouri, USA), trifluoroacetic acid (TFA; Sigma-Aldrich, Missouri,

USA), 0.1 µm filtered ultrapure water (Sigma-Aldrich, Missouri, USA) free of DNAse and RNAse, and a Bruker Bacterial Test Solution (BTS) containing *E. coli* , RNAase and myoglobin protein profiles were also used.

Isolation of Escherichia coli

The feces were inoculated directly into the medium with a sterile swab and incubated at 37°C for 24 hours and the identification was done by conventional methods (Gram stain, catalase, oxidase, oxidation/fermentation (OF), indole, methyl red (MR), voges Proskauer (VP), citrate, urease, triple sugar iron, and H_2S) [24]. Also, the isolates' ability to ferment simple sugars (inositol, lactose, xylose, and mannose) and hemolysis on an agar medium containing 5-10% defibrinated sheep blood were also recorded.

Phenotypic Determination of ESBL and AmpC

All isolates were passaged into MAC mediums containing 1 mg/L Cefotaxime, and growth was recorded [25]. Then, all isolates were subjected to double disk phenotype screening and confirmation test. The antibiotic susceptibility tests were performed according to the Kirby Bauer method recommended by the Clinical Laboratory Standards Institute [26]. Ceftazidime (30 µg), Cefotaxime (30 µg), Aztrenoam (30 µg), Ceftriaxone (30 µg), Cefpodoxime (10 µg), Ceftazidime-Clavulanic acid (40 µg), Cefotaxime-Clavulanic acid (40 µg), Cefoxitin (30 µg), Cefepime (30 µg) discs were used. The evaluation of zone diameters was made according to CLSI directives and the threshold values were determined according to CSLI 2018 and CTX: R [?]27, ATM: R [?]27, CPD: R [?]27, CAZ: R [?]22, CRO: R [?]25, FOX: R [?]14, FEP: R [?]18 were accepted. Also, Cefotaxime and Ceftazidime zone diameters progressing more than 5 mm in zone diameters with Clavulanic acid were evaluated as positive [26].

Investigation of Beta-Lactamase Genes

Genomic DNA was extracted by using boiling method (boiling in distilled water at 95^{o}C for 10 minutes). After boiling, it was centrifuged at 12000 rpm for 5 minutes and used as supernatant DNA. For beta-lactamase,*bla* $_{\text{CTX-M,}}$ *bla* $_{\text{CTX-M-1,}}$*bla* $_{\text{CTX-M-15,}}$*bla*$_{S\ \text{HV,}}$*bla* $_{\text{TEM,}}$*bla*$_{O\ \text{XA-10,}}$ with conventional PCR, and *bla* $_{\text{CIT,}}$ *bla* $_{\text{MOX genes}}$for AmpC beta-lactamase were investigated with multiplex PCR. The primers used in PCR reactions are given in Table 1. Each PCR reaction was run in 35 cycles with a total volume of 25 µl. For each gene, PCR mix (K0171 Thermo Scientific) 10 pmol reverse and forward primers and PCR water were used. In each reaction, pre-denaturation at 95^{o}C for 5 min, denaturation at 95^{o}C for 30 sec. *bla* $_{\text{CTX-M}}$at 57^{o}C, bla CTX- M-15 at 48^{o}C, *bla* $_{\text{CTX-M-1}}$ and*bla* $_{\text{OXA-10}}$ at 45^{o}C, *bla* $_{\text{TEM}}$at 44^{o}C, *bla* $_{\text{SHV}}$ primer bonding temperature at 42^{o}C, 45 sec synthesis at 72^{o}C and 7 min final synthesis at 72^{o}C. The primers for *bla* $_{\text{CIT}}$ and*bla* $_{\text{MOX}}$ were added in half and the same PCR cycle was run with an annealing temperature of 53^{o}C. *E. coli* NCTC 13461-NCTC 13462-NCTC 13463, *E. coli* ATCC 35218, *Klebsiella pneumoniae* ATCC 700603 strains were used as positive controls.

Identification of bacterial colonies from chicken feces by MALDI-TOF MS

For the identification of the peptide and protein spectra, the updated IVD database containing 10694 MSPs (Bruker Daltonics, respectively) was applied. For microbial biomass analysis using the MALDI-TOF MS method, a single colony was placed onto a special steel 96 micro scout plate (MSP) (Bruker Daltonics), which was spread onto the wells in the plate in the form of a thin film. After drying, 1 µL CHCA matrix solution (12.5 mg/mL CHCA in a 50% ACN and 2.5% TFA mixture) was added and allowed to dry completely at room temperature. The MALDI 96 MSP was placed in the MALDI-TOF MS Device, and the system was operated by using the optimized method for the identification of micro-organisms in linear positive ion mode at a 2.000-20.000 Dalton (Da) mass range. A 60 Hz Nitrogen laser was used at 337 nm as the ion source. The laser pulses consisting of 40 packets of 240 were applied in the measurement of each colony to obtain the spectra. Each sample was studied in triplicate, and the highest readings were included in the analysis. The internal quality control for MALDI-TOF MS in general bacteriology is in part achieved by using a Bruker BTS, consisting of an extract of *E. coli* proteins for mass calibration of the instrument [34]. Mass spectrum calibration was completed with seven peaks in the present study (m/z, 5095.39141 Da; 5381.28948

Da; 6265.88537 Da; 7254.94790 Da; 10289.99287 Da; 13692.32900 Da and 16962.67711 Da) assigned with a standard deviation of 58.52 ppm and maximum peak error of 78.19 ppm.

The Use of PCA in MALDI-TOF MS

The spectra were analyzed using Bruker Daltonics MALDI Biotyper Flex Analysis version 3.4 automation-controlled Biotyper Compass Explorer 1.4 software and the MALDI Biotyper 3.1 database. The identification score criteria used were applied following the recommendations of the manufacturer (Bruker). MALDI-TOF MS biotyping analysis elicits the characteristic mass and peak density distribution of ribosomal 16S proteins in the sample. Since this mass spectrum is species-specific for many microorganisms, it represents a "molecular fingerprint" [35]. The spectra were massed using the PCA method supported by external MATLAB software integrated into the MALDI Biotyper.

Based on the unique peptide and protein peaks within each spectrum, PCA helped to create clustered groups of spectra with similar variational properties and visualization of the differences among them. With phyloproteomic-PCA, the data were given on a three-dimensional (3D) coordinate system, and the dimensionality of the data set was reduced, preserving the original information. Optimized preliminary procedures (correction method: Savitski-Golay; subtraction method: multi-polygon; normalization method) were applied for each spectrum to increase the speed of the analysis and reduce the size of the data body [36]. The variance among the bacteria was automatically calculated with software support. In addition, virtual gel images (VGI) containing the projection of the peaks within the bacteria spectra were created. Vertical traces of VGI ranging from red to light blue corresponded to each peak within the spectrum and were given by a color scale ranging from low relative abundance (light green) to high relative abundance (red). For cluster analyses, PCA dendrograms and 3D or 2D scatter plots representing the relationship and closeness of each spectrum were created [37]. Finally, the similarity (proximity) and difference (distance) relationships of each bacteria to the others, whose composite correlation index (CCI) was calculated statistically using the software, were determined.

Results

3.1. The identification of bacteria from chicken feces samples by MALDI-TOF MS

A total of 124 isolates were obtained and then 122 *E. coli* were coded and numbered as CFEC (Chicken Feces *E.coli*), and these numbers were adhered to during the entire data evaluation process (Table 2). The other two isolates were chicken feces (CF), which were abbreviated with initials (*Klebsiella oxytoca:* CFKO; and*Enterobacter kobei:* CFEBC). CFKO and CFEBC isolates were not included in the phyloproteomic analysis.

3.2. The presence of ESBL and AmpC in CFEC bacteria

ESBL was detected in 28.6% (n=35) of 122 *E. coli.* Also, AmpC was not detected in any of the isolates (0 %). The genes responsible for ESBL and AmpC resistance were investigated with the PCR only in *E. coli* which are phenotypic ESBL-positive (Table 3). In this respect,*bla* $_{\text{SHV}}$ gene was not detected in any of the 35 isolates, and the *bla* $_{\text{CTX-M-1}}$ gene was detected at the highest rate (68.5%). It was found that 3 of the ESBL isolates contained 4 of the screened resistance genes. In another 4 isolates, 3 of the genes were screened and 2 different genes were detected in 21 isolates. While only one of the genes was detected in 6 isolates, none of the tested genes were detected in one isolate.

One or more of the other genes were detected in all isolates that contained the *bla* $_{\text{OXA-10}}$. Similarly, no isolates that contained only one *bla* $_{\text{TEM}}$ were detected, while all isolates containing the bla$_{\text{TEM}}$ gene as well as*the bla* $_{\text{TEM}}$ were detected as one or more of the CTX-Ms or *bla* $_{\text{OXA-10}}$ genes. Although phenotypic negative results, *bla* $_{\text{CIT}}$ and*bla* $_{\text{MOX}}$ genes were also investigated in ESBL-positive isolates and were not detected in any isolates. All results are presented in Table 4. The coding of ESBL detected*E.coli* is the same as the numbering in CFEC, and CFEC-ESBL is abbreviated by adding ESBL in front of this abbreviation.

3.3. The use of PCA in MALDI-TOF MS for chicken feces E. coli

A general PCA analysis (I. Phyloproteomic study) of 122 CFEC isolates was performed, and a dendrogram profile and a 2D scattering profile was formed (Figure 1). Also, the variance values were automatically calculated and shown on the dendrogram in Figure 1A. With these three analyses (dendrogram, scattering profile, and variance), rough preliminary information was obtained according to the cluster formation of the most distant and the farthest and closest ones among 35 isolates among 122 CFECs and the scattering of these isolates. These two profiles and variance values were evaluated together. As seen in Figures 1A and 1B, seven CFEC isolates that are far from the main cluster as they have some differences are separated from other CFECs in the dendrogram profile and settled into separate clusters. For example, CFEC-38 (CFEC-ESBL-38), CFEC-90 (CFEC-ESBL-90), and CFEC-19 isolates with the highest variance (PC1) of 31% in this analysis were placed on the far right in a separate cluster. Also, three *E.coli* CFEC-108, CFEC-69, and CFEC-35 are separated by a separate line although they appear to be part of the larger cluster by the second variance value (PC2: 14%) (Fig. 1A and B). Consistent with the results in the dendrogram, the scattering profile in Figure 1C also shows that these seven isolates scatter far from the large cluster.

3.4. Inspection of biochemical results based on PCA results

In this part, the biochemical test results of some of the CFECs that were separated from the large cluster according to the cluster commonality, scattering profile, and variance values according to the MALDI-TOF MS-based PCA results were examined and it was seen that there were data compatible with the PCA analysis results. In this respect, for example, the indole test of two *E.coli* (CFEC-38, CFEC-90 far right) separated by 31% variance and CFEC-108 (PC2; 14% variance) were negative, and the remaining 119 *E. coli* were positive. On the other hand, one of the isolates separated from the large cluster with 14% variance (PC2) CFEC-108 was alpha hemolytic although there was no hemolysis in remaining strains CFEC-71, CFEC-69, and CFEC-35.

3.5. MALDI-TOF MS spectral analysis of CFEC- ESBL bacteria

Although general information was obtained firstly with PCA analyses of all 122 CFEC bacteria, the study focused on comparing the results of MALDI-TOF MS-based PCA analysis of 35 E. coli bacteria with ESBL detected with the results of other phenotypic methods. A representative mass spectrum (CFEC-ESBL-68) showing ESBL characteristics and gel images (virtual gel images; VGI) of 35 CFEC-ESBLs are given in Figure 2. In this respect, when the mass spectrum of the CFEC-ESBL-68 isolate in Figure 2A and the VGIs of all CFEC-ESBLs are examined, especially the high abundance (intensity) peptide (m/z; 5107 Da, 6270 Da, 7290 Da, 9085 Da) and 9762 Da) protein (m/z; 10489 Da) projections appear to be nearly identical for each. In this context, it is considered that there is no major difference when looked at roughly. However, when detailed analyzes of all components of PCA analyses were made (dendrogram, 2D/3D scatter plotting, variance, composite correlation index, etc.), it was determined that there were significant differences between these*E. coli* with ESBL characteristics.

3.6. Clusters, scatters, and composite correlation index analysis of ESBL-producing E. coli

At this step, with the support of the MATLAB program, the unique spectra, which are the fingerprints of each bacterium, were compared with each other and analyzed (II. Phyloproteomic study) and the data are given separately in Figure 3. First, the dendrogram (Fig. 3A) and 2D scattering profiles (Fig. 3B) generated for 35 CFEC-ESBLs together with a total of 10 variance values are shown (Fig. 3A).

As a result of the dendrogram analysis with of isolates showing only ESBL characteristics, only CFEC-ESBL-38 (CFEC-38) took its place on a separate line on the far right (Figure 3A), and it was located farthest (yellow dot) in the 2D scattering profile. It was found that this isolate differed from 34 CFEC-ESBLs with a variance of 41%. On the other hand, the second isolate, which is separated from the large cluster (n=33) with a 15% variance value, was also CFEC-ESBL-90.

Composite for calculating the distance and proximity indices of CFEC-ESBL CCI were also calculated automatically with the MATLAB program (data not shown). In the graphic given in Figure 3C, the total value of CCI % of 34 isolates with itself, calculated for 35 CFEC-ESBL and each isolate, is on the y-axis,

and each isolate itself is on the x-axis. As the closeness to the isolates within the group increases, the total %CCI value on the y-axis increases for each isolate. On the other hand, as the proximity value decreases, the total %CCI value also decreases. In this respect, when Figure 3C is examined, it is seen that the lowest total CCI percentage value belongs to CFEC-ESBL-38. Also, it was found that the total %CCI value of CFEC-ESBL-90 was lower than the other group members. It is a result that is highly compatible with dendrogram, variance, and 2D results. According to 33 isolates of CFEC-ESBL, CFEC-ESBL38 is very different and CFEC-ESBL-90 is different.

Another isolate that drew attention in 3C in the figure is CFEC-ESBL-93. It is seen that the total percent CCI value of this isolate is low. However, the variance value is 1% and is nested in the main cluster with 32 other CFEC-ESBLs in the dendrogram and scattering profile.

The CCI color matrix of all ESBL-CFEC isolates is presented in Figure 3D. In this color matrix, the dark red color represents the highest CCI% value (each isolate has 100% similarity to itself and this color is dark red), and the dark blue color corresponds to the lowest (0-1%) CCI value. If we take an overview of this, green tiles in the CCI color matrix correspond to 40% to 59%, and yellow to dark red tiles correspond to a CCI index of 60% to 100%. It indicates that the boxes from light blue to dark blue also have a CCI index from 39% to 0%.

The color matrices of two isolates with low affinity (CFEC-ESBL-38, CFEC-ESBL-90) and an isolate with the highest affinity (CFEC-ESBL-68) among 35 CFEC-ESBL are given in Figure 3E by matching their CCI% values. In this respect, it was found that the boxes corresponding to CFEC-ESBL-38 in the color matrix were mostly in blue tones and the %CCI values were low in proportion to this. In contrast, the box corresponding to CFEC-ESBL-90 appears to be light red (87% CCI). Also, the closeness of this isolate to CFEC-ESBL-41 and CFEC-ESBL-69 isolates, which are members of the large cluster, was determined to be 60% and 54%, respectively, in the dendrogram. On the other hand, it was reported to have the lowest affinity value (1%) against CFEC-ESBL-9, CFEC-ESBL-106, and CFEC-ESBL-110 isolates among other isolates (Figure 3 E). It is seen that CFEC-ESBL-38 has the lowest overall CCI % value since it has the lowest affinity values to other CFEC-ESBLs except for a few isolates.

It was found that the second different isolate, CFEC-ESBL-90, has the highest affinity (87%) against CFEC-ESBL-38 isolate. Similar to CFEC-ESBL-38, the affinity index to CFEC-ESBL-41 and CFEC-ESBL-69 isolates is 64% and 62%, respectively. Also, the affinity index values for some isolates (n=6) in the large cluster are over 50%, while the affinity indexes for 14 group members are between 30%-40%. This explains the relatively lower variance value compared to CFEC-ESBL-38 (Fig. 3 E).

As expected, the presence of mostly yellow to dark red colored boxes in the CCI color matrix of CFEC-ESBL-68, which has the highest percentage of closeness in total, draws attention. When we look at the color matrix in general, most of the CFEC-ESBL isolates that have a low variance value ([?]5%) in the large cluster in the dendrogram (Figure 3A) and are very close to each other in the scattering profile (Figure 3B) have plenty of colored boxes from yellow to red on the color scale (Figure 3D).

3.7. Compatibility of biochemical and gene analyses and PCA results of some CFEC-ESBL

These results from MALDI-TOF MS-based PCA analyses pointed to important clues that some of the 35 ESBL-CFEC isolates had significant differences. In this respect, the biochemical characteristics of the isolates with the highest affinity (CFEC-ESBL-68) and the lowest (CFEC-ESBL-38) and low affinity (CFEC-ESBL-90) to all isolates and findings for beta-lactam resistance genes were detected. The data is given in Figure 3F confirm all the PCA-based analysis results given above. Unlike the other 34 isolates, *bla* $_{\text{CTX-M-15}}$is one of the beta-lactam resistance genes in only CFEC-ESBL-38 isolates. It is considered that the presence of 41% variance causes the scattering profile to be located farthest in the scattering profile and with a different line in the dendrogram. Besides, in both (CFEC-ESBL-38 and CFEC-ESBL-90) isolates, *bla* $_{\text{CTX-M}}$,*bla* $_{\text{CTX-M-1}}$, *bla* $_{\text{TEM}}$,*bla* $_{\text{OXA-10}}$ The absence of their genes may be a reason for the 87% affinity between them, as well as leading to their separation from the main cluster (n=33).

Discussion

In the present study, 122 isolates were identified as *E. coli* by two methods, traditional (biochemical tests) and the new-generation method (MALDI-TOF MS). The identification scores obtained from MALDI-TOF MS are between [?]2.000, 16% were between 1.700-1.999 and there was no definition below the cut-off value ([?]1.699). Within the scope of phyloproteomic analysis, firstly, PCA analyses of all *E. coli* isolates were carried out and it was determined that there are isolates with different characteristics. Then, more detailed analyzes were carried out on a smaller group of *E. coli* with ESBL characteristics. With the support of all the analyzes performed, it was concluded that there were significant differences in the light of the data on variance, CCI index values, dendrogram, and scattering profile placements, even though hints on bacteria belonging to the same species are provided.

ESBL was detected in almost one of the 3 isolates. It is a very high rate especially according to the scales of industrial production facilities. Because *E. coli* is a very important strain in terms of food pathogens and antibiotic resistance profile. Unfortunately, similar or even higher rates of ESBL were detected in some studies. For example, Yang et al. [38] similarly reported that 22.9% of the bacteria which were ESBL- *E. coli.* In other studies, Badr et al. [39] determined the rate of ESBL producing *E. coli* isolated from chickens is 46.7%, while Gazal et al. [40] detected 66% and Fournier et al. [41] detected ESBL 84%. Cormier et al. [1] reported that this rate reached up to 90%. According to the findings of the present study, predominantly *bla* $_{\text{CTX-M-1}}$resistance genes were detected. In previous studies, the most frequently reported resistance genes were CTX-Ms and their derivatives [42]. These genes, which are reported to be common on a global scale, may be an indication that bacteria are in contact with each other [2,39].

CFEC-ESBL-38 is a strain that came to the fore with its difference in the study as the only atypical ESBL strain that shows only indole (-) and lac (-) among 122 isolates.*E. coli* is lac (+) commonly and lactose permease enzyme (LacY protein) is a very important protein that enables the use of lactose in*E.coli.* However, in some bacteria, lac (-) variants occur due to deficiencies in the level of this enzyme encoded by this LacY gene [43,44]. Stepień-Pyśniak et al. [8] showed that*Enterecoccus faecalis* and *E. mundtii isolates* were separated in the dendrogram with phyloproteomic analysis by using the spectral profiles of the isolates. In the same study, it was also noted that there was clustering of very similar strains in terms of phenotype and genotype according to galactosidase and mellobiose characteristics, and it was stated in the study that a single gelatinase negative isolate gave a different peak. Unlike the other 34 CFEC-ESBL isolates, it is thought that the presence of *bla* $_{\text{CTX-M-15}}$ only in CFEC-ESBL-38 causes it to settle furthest in the scattering profile and with a different line in the dendogram with a variance of 41%. The expression of a peptide/protein that is directly or indirectly related to phenotypic resistance might be cause the difference of this strain [45-47].

In the I. Phyloproteomic study, the CFEC-ESBL-90 was completely excluded from the large cluster with 14% variance in the full dendrogram (n=122, for CFEC) profile while it was located on a separate line in the dendrogram connected to the large cluster with 15% variance in the II. Phyloproteomic study (n=35, for CFEC-ESBL). Additionally, it was determined that the closeness ratios (CCI) to large cluster members, except for a few, were at a low level. Biochemically, this strain had weak catalase ability. The presence of catalase enzyme is very characteristic of *E. coli.* In general, *E. coli* harbors two different catalase genes: katG encodes hydroperoxidase I (HPI) and katE encodes HPII. The activity of both catalases increases when both are present together and the expression level of genes increases [48]. In the dendrogram profile, the difference in common with the members of the cluster including CFEC-ESBL-90 (CFEC-19 and CFEC-ESBL-38) is that it does not have hemolysis activity. The presence of the hemolysis enzyme and the observed hemolysis ability are mostly variable within the species in *E.coli* [49,52]. On the other hand, another possible feature makes this strain different from others is that the indole test is negative. Because the indole test is an indicator of the tryptophanase and tryptophan permease enzymes of the tryptophanase operon (TNA Operon), and the indole test of *E. coli* (90-95%) is mostly positive [51]. Deficiency of these proteins was also determined as a possibile affecting all PCA analysis results. Torres-Corral and Santos [52] pointed out that *Lactococcus garvieae* isolates gave characteristic 3 peaks and as the reason for grouping of isolates, enzymes such as epimerase, methyltransferases, and acetylphophatases possessed by the isolates. There are

some studies suggesting that changes in enzyme structures may be affect results because protein analyses of biological structures are performed with MALDI-TOF MS [53,54]. On the other hand, according to the present study, catalase, tryptophanase, and tryptophan permease enzymes of*E . coli* may be the reasons for the differences, but it must be supported by further studies. Because bacteria produce many specific and non-specific proteins [55].

Besides, other genes (which were not tested in the study) are considered to responsible for ESBL in CFEC-ESBL-90. The majority of genes responsible for ESBL appear as CTX-M, SHV, and TEM variants. However, there are also other genes responsible for resistance [39, 56-58]. In the study of Laudy et al. [59], similar to the present study, although ESBL-producing *Pseudomonas aeruginosa* strains obtained from phenotypic test results, they could not detect all the genes it screened at the same rate. They also reported new 3 different ESBL-producing genes with their further studies. Because many genes responsible for beta-lactamases were reported and continue to be reported [2,39,59]. Further studies can be carried out to detect ESBL genes in CFEC-ESBL-90.

In the present study, as well as the detection of differences between*E.coli* and ESBL -*E.coli,* it was observed that similar ones were numerically higher. For example, the greatest affinity was detected in the CFEC-ESBL-68 isolate. Although the ratio of CFEC-ESBL-68 to 13 cluster members was 49-69%, the closeness ratio to the remaining 21 cluster members was 70-99%. This might be because CFEC-ESBL-68 has typical biochemical features for *E.coli* like most isolates in the study (strong catalase property, alpha hemolysis ability, indole and lactose positive, etc.). However, performing further tests (other simple sugar fermentation, gelatinase, nitrate, arginine, biofilm, multidrug resistance profile, etc.) are needed to understand.

The present study was conducted to determine the differences and similarities between *E. coli* isolates with all PCA analysis (Dendrogram, scatter plotting, variance, and CCI). It was also found that the ESBL group generally differed from susceptible strains and there were some heterogeneities and homogeneities in the isolates. Alharbi et al. [17] showed that MSSA and MRSA can be separated in dendrogram cluster analysis. Similarly, in another study, it was shown that MSSA and MRSA were distinguished by peaks of different masses, and it was emphasized that MALDI TOF MS saves time according to molecular studies [60]. All these data suggest that ESBL producing *E. coli* are phylogenetically separated and may differ greatly in natural ecosystems. Previous studies have shown the feasibility of MALDI-TOF-MS for the clonal identification of bacteria. Our study is also an example for that.

In conclusion, phyloproteomic analyses with MALDI-TOF MS may be useful for characterization of phenotypic behaviors. Unfortunately, the high cost of analysis of multiple samples with high-cost methods such as Whole Genome Analysis is a major challenge for analytical studies. Thus, the most important result was found in the present study is that performing advanced analyzes as well as identification with the less costly MALDI-TOF MS contributes significantly to the validation of traditional analysis results. Also, this study represents a first in terms of ESBL screening and characterization in broiler chickens for the region (Duzce, Türkiye). There is no previous study that was conducted or published in the region. In recent years, epidemiological studies have focused on the spread of resistant strains which are extremely important in terms of clinically and food safety. In this sense, it is anticipated that this study will contribute to the monitoring of the data in the region. Although important clues were obtained, further analyzes are planned to make sense of the effect of biochemical characteristics on variance values.

References

[1] Cormier, A., Zhang, P. L., Chalmers, G., Weese, J. S., Deckert, A., Mulvey, M., McAllister T., Boerlin, P. (2019). Diversity of CTX-M-positive *Escherichia coli* recovered from animals in Canada. *Vet Microbiol* , 231, 71-75. https://doi.org/10.1016/j.vetmic.2019.02.031

[2] Carvalho, I., Carvalho, J. A., Martínez-Álvarez, S., Sadi, M., Capita, R., Alonso-Calleja, C., Rabbi F, Dapkevicius MdLNE, Igrejas G, Torres C, Poeta, P. (2021). Characterization of ESBL-producing*Escherichia coli* and *Klebsiella pneumoniae* isolated from clinical samples in a northern Portuguese hospital: predominance of CTX-M-15 and high genetic diversity. *Microorganisms* , 9(9), 1914. htt-

ps://doi.org/10.3390/microorganisms9091914

[3] Tian, M., He, X., Feng, Y., Wang, W., Chen, H., Gong, M., Liu, D., Clarke, J.L., van Eerde, A. (2021). Pollution by antibiotics and antimicrobial resistance in livestock and poultry manure in China, and Countermeasures. *Antibiotics,* 10, 539. https://doi.org/10.3390/antibiotics10050539

[4] Zhang, P.L., Shen, X., Chalmers, G., Reid-Smith, R.J., Slavic, D., Dick, H., Boerlin, P. (2018). Prevalence and mechanisms of extended spectrum cephalosporin resistance in clinical and fecal Enterobacteriaceae isolates from dogs in Ontario, Canada. *Vet Microbiol,* 213, 82–88. https://doi.org/10.1016/j.vetmic.2017.11.020

[5] Hernando-Amado, S., Coque, T. M., Baquero, F., Martínez, J. L. (2019). Defining and combating antibiotic resistance from One Health and Global Health perspectives. *Nat. Microbiol,* 4(9), 1432-1442. https://doi.org/10.1038/s41564-019-0503-9

[6] Taban, B. M., Numanoglu Cevik, Y. (2021). The efficiency of MALDI-TOF MS method in detecting *Staphylococcus aureus* isolated from raw milk and artisanal dairy foods. *CyTA-J Food* , 19(1), 739-750. https://doi.org/10.1080/19476337.2021.1977392

[7] Cheng, K., Chui, H., Domish, L., Hernandez, D., Wang, G. (2016). Recent development of mass spectrometry and proteomics applications in identification and typing of bacteria. *Proteomics–Clin Appl.*10(4), 346-357. https://doi.org/10.1002/prca.201500086

[8] Stepień-Pyśniak, D., Hauschild, T., Dec, M., Marek, A., Urban-Chmiel, R., & Kosikowska, U., (2021). Phenotypic and genotypic characterization of *Enterococcus* spp. from yolk sac infections in broiler chicks with a focus on virulence factors. Poult Sci, 100(4), 100985. https://doi.org/10.1016/j.psj.2021.01.008

[9] Ha, S., M., Kim, C., K., Roh, J., Byun, J. H., Yang, S. J., Choi, S. B., Chun, J., Yong, D. (2019). Application of the whole genome-based bacterial identification system, TrueBac ID, using clinical isolates that were not identified with three matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) systems. *Ann Lab Med,* 39(6), 530-536. https://doi.org/10.3343/alm.2019.39.6.530

[10] Cherkaoui, A., Hibbs, J., Emonet, S., Tangomo, M., Girard, M., Francois, P., & Schrenzel, J. (2010). Comparison of two matrix-assisted laser desorption ionization-time of flight mass spectrometry methods with conventional phenotypic identification for routine identification of bacteria to the species level. *J. Clin. Microbiol,* 48(4), 1169-1175. https://doi.org/10.1128/JCM.01881-09

[11] Faron, M.L., Buchan, B.W., Hyke, J., Madisen, N., Lillie, J.L., Granato, P.A, et al. (2015). Multicenter evaluation of the Bruker MALDI biotyper CA system for the identification of clinical aerobic gram-negative bacterial isolates. *PLoS One* 10, e0141350. https://doi.org/10.1371/journal.pone.0141350

[12] Clark, C. M., Costa, M. S., Sanchez, L. M., & Murphy, B. T. (2018). Coupling MALDI-TOF mass spectrometry protein and specialized metabolite analyses to rapidly discriminate bacterial function.*Proc. Natl. Acad. Sci,* 115(19), 4981-4986. https://doi.org/10.1073/pnas.1801247115

[13] Suen, L., Siu, G., Guo, Y.P., Yeung, S., Lo, K., O'Donoghue, M. (2019). The public washroom - friend or foe? An observational study of washroom cleanliness combined with microbiological investigation of hand hygiene facilities. *Antimicrob. Resist. Infect. Control* 8, 47. https://doi.org/10.1186/s13756-019-0500-z

[14] Hou, T. Y., Chiang-Ni, C., Teng, S. H. (2019). Current status of MALDI-TOF mass spectrometry in clinical microbiology. *J Food Drug Anal.* 27(2), 404-414. https://doi.org/10.1016/j.jfda.2019.01.001

[15] Elbehiry, A., Marzouk, E., Abdeen, E., Al-Dubaib, M., Alsayeqh, A., Ibrahem, M., Hamada, M., Alenzi, A., Moussa, I., Hemeg, H. A. (2019). Proteomic characterization and discrimination of*Aeromonas* species recovered from meat and water samples with a spotlight on the antimicrobial resistance of Aeromonas hydrophila.*Microbiologyopen* , 8(11), e782. https://doi.org/10.1002/mbo3.782

[16] Pantucek, R., Sedlacek, I., Indrakova, A., Vrbovska, V., Maslanova, I., Kovarovic, V., Svec, P., Kralova, S., Kristofova, L., Keklakova, J., Petras, P., Doskar, J. (2018). *Staphylococcus edaphicus* sp. nov., iso-

lated in Antarctica, harbours mecC gene and genomic islands with suspected role in adaptation to extreme environment. *Appl. Environ. Microbiol.* 84 https://doi.org/10.1128/AEM.01746-17. AEM.01746-17

[17] Alharbi, A., Al-Dubaib, M., Elhassan, M. A., Elbehiry, A. (2021). Comparison of MALDI-TOF mass spectrometry with phenotypic methods for identification and characterization of *Staphylococcus aureus* causing mastitis. *Trop. Biomed* , 38(2), 9-24. https://doi.org/10.47665/tb.38.2.032

[18] Suzuki, Y., Niina, K., Matsuwaki, T., Nukazawa, K., & Iguchi, A. (2018). Bacterial flora analysis of coliforms in sewage, river water, and ground water using MALDI-TOF mass spectrometry. *J. Environ. Sci. Health A.* 53(2), 160-173. https://doi.org/10.1080/10934529.2017.1383128

[19] Jančová, P., Pachlová, V., Čechová, E., Cedidlová, K., Šerá, J., Pištěková, H., Buňka, F., Buňková, L. (2020). Occurrence of biogenic amines producers in the wastewater of the dairy industry.*Molecules.* 25, 5143. https://doi.org/10.3390/molecules25215143

[20] Ogutcu, H., Kantar, F., Alaylar, B., Numanoglu Cevik, Y., Gulluce, M. (2022). Isolation and characterization of hydrocarbon and petroleum degrading bacteria from polluted soil with petroleum and derivatives by MALDI-TOF MS method. *Geomicrobiol J.* 1-10. https://doi.org/10.1080/01490451.2022.2074575

[21] Araújo, T. M. C., Pereira, R. D. C. L., Freitag, I. G. R., Rusak, L. A., Botelho, L. A. B., Hofer, E., Hofer C. B., Vallim, D. C. (2020). Evaluation of MALDI–TOF MS as a tool for detection of Listeria spp. directly from selective enrichment broth from food and stool samples. *J. Microbiol. Methods* , 173, 105936. https://doi.org/10.1016/j.mimet.2020.105936

[22] Nees, M., Hess, M., Hess, C. (2022). Discrimination and characterization of *Escherichia coli* originating from clinical cases of femoral head necrosis in broilers by maldi-tof mass spectrometry confirms great heterogeneity of isolates.*Microorganisms.* 10, 1472. https://doi.org/10.3390/microorganisms10071472

[23] Dandachi, I., Sokhn, E. S., Dahdouh, E. A., Azar, E., El-Bazzal, B., Rolain, J. M., Daoud, Z. (2018). Prevalence and characterization of multi-drug-resistant gram-negative bacilli isolated from lebanese poultry: A nationwide study. *Front. Microbiol.* 9, 550. https://doi.org/10.3389/fmicb.2018.00550

[24] Krieg, N., Holt, J. (2005). Facultatively anaerobic gram-negative rods. Family I. Enterobacteriaceae. Bergey's Manual of Systematic Bacteriology. Baltimore. Williams & Wilkins 1, 408–420.

[25] Wilson, G., McCabe, D. (2007). The use of antibiotic-containing agars for the isolation of extended-spectrum β-lactamase-producing organisms in intensive care units. *Clin. Microbiol. Infect,*13(4), 451-453. https://doi.org/10.1111/j.1469-0691.2006.01667.x

[26] Clinical and Laboratory Standards Institute (CLSI), 2018. M100S 28th Edition. Wayne, PA:Quinn PJ, Carter ME, Markey B, Carter GR. Clinical Veterinary Microbiology. London: Wolfe Publishing; 1994. p. 42-126.

[27] Saladin M., Cao V.T., Lambert T., Donay J.L., Herrmann J.L., Ould-Hocine Z. Verdet, C., Françoise Delisle, F., Philippon, A., Arlet G. (2002). Diversity of CTX-M β-lactamases and their promoter regions from Enterobacteriaceae isolated in three Parisian hospitals. *FEMS Microbiol Lett,* 209(2), 161-8. https://doi.org/10.1111/j.1574-6968.2002.tb11126.x

[28] Woodford, N., Fagan, E.J., Ellington, M.J. (2006). Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum β-lactamases. *J Antimicrob Chemother,* 57(1), 154- 5. https://doi.org/10.1093/jac/dki412

[29] Dhanji, H., Doumith, M., Clermont, O., Denamur, E., Hope, R., Livermore, D. M., Woodford, N. (2010). Real-time PCR for detection of the O25b-ST131 clone of Escherichia coli and its CTX-M-15-like extended-spectrum β-lactamases. *Int. J. Antimicrob. Agents,* 6(4), 355-358. https://doi.org/10.1016/j.ijantimicag.2010.06.007

[30] Chanawong A., M'Zali F.H., Heritage J., Lulitanond A., Hawkey P.M. (2000). Characterisation of extended-spectrum β-lactamases of the SHV family using a combination of PCR-single strand conformational polymorphism (PCR-SSCP) and PCR-restriction fragment length polymorphism (PCR-RFLP). *FEMS Microbiol Lett,* 184(1), 85-9. https://doi.org/10.1111/j.1574-6968.2000.tb08995.x

[31] Vahaboglu H., Ozturk R., Akbal H., Saribas S., Tansel O., Coskunkan F. (1998). Practical approach for detection and identification of OXA-10-derived ceftazidime-hydrolyzing extended-spectrum β-lactamases. *J Clin Microbiol,* 36(3), 827-9. https://doi.org/10.1128/JCM.36.3.827-829.1998

[32] Arlet G., Rouvea M., Casin I., Bouvet P.J., Lagrange P.H., Philippon A. (1994). Molecular epidemiology of Klebsiella pneumoniae strains that produce SHV-4 β-lactamase and which were isolated in 14 French hospitals. *J Clin Microbiol,* 32, 2553-8. https://doi.org/10.1128/jcm.32.10.2553-2558.1994

[33] Pérez-Pérez F.J., Hanson N.D. (2002). Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol.* 40(6), 2153-62. https://doi.org/10.1128/JCM.40.6.2153-2162.2002

[34] Szabados, F., Tix, H., Anders, A., Kaase, M., Gatermann, S. G., Geis, G. (2012). Evaluation of species-specific score cutoff values of routinely isolated clinically relevant bacteria using a direct smear preparation for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry-based bacterial identification. Eur. J. Clin. Microbiol. Infect. Dis. 31, 1109-1119. https://doi.org/10.1007/s10096-011-1415-7

[35] Oviaño, M., Sparbier, K., Barba, M. J., Kostrzewa, M., Bou, G. (2016). Universal protocol for the rapid automated detection of carbapenem-resistant Gram-negative bacilli directly from blood cultures by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS). *Int. J. Antimicrob. Agents,* 48(6), 655-660. https://doi.org/10.1016/j.ijantimicag.2016.08.024

[36] Samad, R. A., Al Disi, Z., Ashfaq, M. Y. M., Wahib, S. M., & Zouari, N. (2020). The use of principle component analysis and MALDI-TOF MS for the differentiation of mineral forming *Virgibacillus* and*Bacillus* species isolated from sabkhas. *RSC Advances,* 10(25), 14606- https://doi.org/14616.10.1039/D0RA01229G

[37] Zhang, H., Yamamoto, E., Murphy, J., & Locas, A. (2020). Microbiological safety of ready-to-eat fresh-cut fruits and vegetables sold on the Canadian retail market. *Int. J. Food Microbiol,* 335, 108855. https://doi.org/10.1016/j.ijfoodmicro.2020.108855

[38] Yang, F., Zhang, Sd., Shang, X., Wang, X., Wang, L., Yan, Z., Li, H. (2018). Prevalence and characteristics of extended spectrum β-lactamase-producing *Escherichia coli* from bovine mastitis cases in China. *J. Integr. Agric,* 17(6), 1246-1251. https://doi.org/10.1016/S2095-3119(17)61830-6

[39] Badr, H., Reda, R. M., Hagag, N. M., Kamel, E., Elnomrosy, S. M., Mansour, A. I., Shahein, M.A., Ali S. F., Ali, H. R. (2022). Multidrug-resistant and genetic characterization of extended-spectrum beta-lactamase-producing *E. coli* recovered from chickens and humans in Egypt. *Animals* , 12(3), 346. https://doi.org/10.3390/ani12030346

[40] Gazal, L. E. D. S., Medeiros, L. P., Dibo, M., Nishio, E. K., Koga, V. L., Gonçalves, B. C., Grassotti T.T., Camargo T.C.L., Pinherio J. J., Vespero, Brito K. C. T., Brito B. G., Nakazato, G., Kobayashi, R. K. T. (2021). Detection of ESBL/AmpC-producing and fosfomycin-resistant*Escherichia coli* from different sources in poultry production in Southern Brazil. *Front in Microbiol,* 11, 604544. https://doi.org/10.3389/fmicb.2020.604544

[41] Fournier, C., Aires-de-Sousa, M., Nordmann, P., Poirel, L., 2020. Occurrence of CTX-M-15-and MCR-1-producing Enterobacterales in pigs in Portugal: Evidence of direct links with antibiotic selective pressure. *Int. J. Antimicrob. Agents,* 55(2), 105802. https://doi.org/10.1016/j.ijantimicag.2019.09.006

[42] Higgins, O., Chueiri, A., O'Connor, L., Lahiff, S., Burke, L., Morris, D., Pfeifer N.M., Smith, T. J. (2022). Portable differential detection of CTX-M ESBL gene variants, *bla* $_{\text{ctx-m-1}}$and *bla* $_{\text{ctx-m-15}}$, from *Escherichia*

*coli*isolates and animal fecal samples using loop-primer endonuclease cleavage loop-mediated isothermal amplification. *Microbiol Spectr,* e03316-22. https://doi.org/10.1128/spectrum.03316-22

[43] Gajdács, M., Ábrók, M., Lázár, A., Burián, K. (2020). Differential epidemiology and antibiotic resistance of lactose-fermenting and non-fermenting Escherichia coli: Is it just a matter of taste?. *Biol Futura.* 71, 175-182. https://doi.org/10.1007/s42977-020-00016-6

[44] Sun, H. (2022). Equilibrium properties of *E. coli*lactose permease symport—A random-walk model approach. *PloS One* , 17(2), e0263286. https://doi.org/10.1371/journal.pone.0263286

[45] Ank, N., Sydenham, T.V., Iversen, L.H., Justesen, U.S., Wang, M. (2015). International journal of antimicrobial agents characterisation of a multidrug-resistant Bacteroides fragilis isolate recovered from blood of a patient in Denmark using whole-genome sequencing. *Int. J. Antimicrob. Agents,* 46, 117–120. https://doi.org/10.1016/j. ijantimicag.2015.02.024.

[46] Vrioni, G., Tsiamis, C., Oikonomidis, G., Theodoridou, K., Kapsimali, V., Tsakris, A., 2018. MALDI-TOF mass spectrometry technology for detecting biomarkers of antimicrobial resistance: current achievements and future perspectives. *Ann. Transl. Med,* 6, 240. https://doi.org/10.21037/atm.2018.06.28

[47] Flores-Trevino, S., Garza-Gonzalez, E., Mendoza-Olazar´ an, S., Morfín-Otero, R., Camacho-Ortiz, A., Rodríguez-Noriega, E., Bocanegra-Ibarias, P. (2019). Screening of biomarkers of drug resistance or virulence in ESCAPE pathogens by MALDI-TOF mass spectrometry.*Sci. Rep,* 9, 1–10. https://doi.org/10.1038/s41598-019-55430-1

[48] Doukyu, N., Taguchi, K. (2021). Involvement of catalase and superoxide dismutase in hydrophobic organic solvent tolerance of*Escherichia coli. AMB Express* , 11(1), 1-11. https://doi.org/10.1186/s13568-021-01258-w

[49] Schwidder, M., Heinisch, L., Schmidt, H. (2019). Genetics, toxicity, and distribution of enterohemorrhagic *Escherichia coli*hemolysin. *Toxins,* 11(9), 502. https://doi.org/10.3390/toxins11090502

[50] Ndegwa, E., Alahmde, A., Kim, C., Kaseloo, P., O'Brien, D. (2020). Age related differences in phylogenetic diversity, prevalence of Shiga toxins, Intimin, Hemolysin genes and select serogroups of*Escherichia coli* from pastured meat goats detected in a longitudinal cohort study. *BMC Vet Res,* 16(1), 1-15. https://doi.org/10.1186/s12917-020-02479-0

[51] Rezwan, F., Lan, R., & Reeves, P. R. (2004). Molecular basis of the indole-negative reaction in Shigella strains: extensive damages to the tna operon by insertion sequences. *J. Bacteriol.* 186(21), 7460-7465. https://doi.org/10.1128/JB.186.21.7460-7465.2004

[52] Torres-Corral, Y., Santos, Y. (2022). Predicting antimicrobial resistance of Lactococcus garvieae: PCR detection of resistance genes versus MALDI-TOF protein profiling. *Aquaculture,* 553, 738098. https://doi.org/10.1016/j.aquaculture.2022.738098

[53] Ashfaq, M. Y., Da'na, D. A., & Al-Ghouti, M. A. (2022). Application of MALDI-TOF MS for identification of environmental bacteria: A review. *J. Environ. Manage* , 305, 114359. https://doi.org/10.1016/j.jenvman.2021.114359

[54] Ribeiro, D.G., Carmo, L., Santos, I.R., Almeida, R.F., Silva, L.P., Oliveira-Neto, O.B., Scherwinski-Pereira, J.E., Mehta, A. (2020). MALDI TOF MS-profiling: applications for bacterial and plant sample differentiation and biological variability assessment. *J. Proteonomics,* 213, 103619. https://doi.org/10.1016/j.jprot.2019.103619.

[55] Macek, B., Forchhammer, K., Hardouin, J., Weber-Ban, E., Grangeasse, C., Mijakovic, I. (2019). Protein post-translational modifications in bacteria. *Nat Rev Microbiol,* 17(11), 651-664. https://doi.org/10.1038/s41579-019-0243-0

[56] Ranjbar, R., Farahani, A. (2019). Study of genetic diversity, biofilm formation, and detection of Carbapenemase, MBL, ESBL, and tetracycline resistance genes in multidrug-resistant Acinetobacter baumannii isolated from burn wound infections in Iran. *Antimicrobial Resistance & Infection Control* , *8* , 1-11. https://doi.org/10.1186/s13756-019-0612-5

[57] Silago, V., Kovacs, D., Samson, H., Seni, J., Matthews, L., Oravcová, K., Lupindu, A.M., Hoza, A.S., Mshana, S.E. (2021). Existence of multiple ESBL genes among phenotypically confirmed ESBL producing*Klebsiella pneumoniae* and *Escherichia coli* concurrently isolated from clinical, colonization and contamination samples from Neonatal Units at Bugando Medical Center, Mwanza, Tanzania.*Antibiotics,* 10, 476. https://doi.org/10.3390/antibiotics10050476

[58] Wibisono, F. J., Sumiarto, B., Untari, T., Effendi, M. H., Permatasari, D. A., Witaningrum, A. M. (2020). CTX Gene of extended spectrum beta-lactamase (ESBL) producing *Escherichia coli* on broilers in Blitar, *Indonesia. Sys Rev Pharm,* 11(7).

[59] Laudy, A. E., Róg, P., Smolińska-Król, K., Ćmiel, M., Słoczyńska, A., Patzer, J., Dzierżanowska, D., Wolinowska, R., Starościak, B., Tyski, S. (2017). Prevalence of ESBL-producing*Pseudomonas aeruginosa* isolates in Warsaw, Poland, detected by various phenotypic and genotypic methods. *PloS one,* 12(6), e0180121. https://doi.org/10.1371/journal.pone.0180121

[60] Tang, W., Ranganathan, N., Shahrezaei, V., & Larrouy-Maumus, G. (2019). MALDI-TOF mass spectrometry on intact bacteria combined with a refined analysis framework allows accurate classification of MSSA and MRSA. *PloS one,* 14(6), e0218951. https://doi.org/10.1371/journal.pone.0218951

Table 1. Oligonucleotides used in the study for PCR

Gene	5' — 3'	References
$bla_{\text{CTX-M}}$	F-SCSATGTGCAGYACCAGTAA R-CCGCRATATGRTTGGTGGTG	[27]
$bla_{\text{CTX-M-1}}$	F-AAAAATCACTGCGCCAGTTC R-AGCTTATTCATCGCCACGTT	[28]
$bla_{\text{CTX-M-15}}$	F-TGG GGG ATA AAA CCG GCA G R-GCG ATA TCG TTG GTG GTG C	[29]
bla_{SHV}	F-CTTTACTCGCTTTATCG R-TCCCGCAGATAAATCACCA	[30]
$bla_{OXA\text{-}10}$	F-GTCTTTCGAGTACGGCATTA R-ATTTTCTTAGCGGCAACTTAC	[31]
bla_{TEM}	F-ATGAGTATTCAACATTTCCG R-CCAATGCTTAATCAGTGAGC	[32]
bla_{CIT}	F-TGGCCAGAACTGACAGGCAAA R-TTTCTCCTGAACGTGGCTGGC	[33]

Gene	5' — 3'	References
bla_{MOX}	F-GCTGCTCAAGGAGCACAGGAT R-CACATTGACATAGGTGTGGTGC	[33]

Table 2. Identified bacteria from chicken feces by MALDI-TOF MS

Sample code	MALDI score	Sample code	MALDI score	Sample code	MALDI score	Sample code	M
CFEC-1	2.197	CFEC-32	2.405	CFEC-63	2.271	CFEC-94	2
CFEC-2	2.271	CFEC-33	2.437	CFEC-64	2.186	CFEC-95	2
CFEC-3	2.464	CFEC-34	2.071	CFEC-65	2.320	CFEC-96	1
CFEC-4	2.399	CFEC-35	2.032	CFEC-66	2.218	CFEC-97	2
CFEC-5	2.213	CFEC-36	2.131	CFEC-67	2.359	CFEC-98	2
CFEC-6	2.299	CFEC-37	2.238	CFEC-68	2.230	CFEC-99	2
CFEC-7	2.367	CFEC-38	2.133	CFEC-69	2.174	CFEC-100	2
CFEC-8	2.334	CFEC-39	1.974	CFEC-70	2.302	CFEC-101	2
CFEC-9	2.016	CFEC-40	2.184	CFEC-71	2.069	CFEC-102	2
CFEC-10	2.016	CFEC-41	2.068	CFEC-72	2.296	CFEC-103	2
CFEC-11	2.148	CFEC-42	2.472	CFEC-73	2.237	CFEC-104	2
CFEC-12	1.939	CFEC-43	2.085	CFEC-74	2.186	CFEC-105	2
CFEC-13	2.322	CFEC-44	2.438	CFEC-75	1.987	CFEC-106	2
CFEC-14	2.111	CFEC-45	2.060	CFEC-76	1.872	CFEC-107	1
CFEC-15	2.272	CFEC-46	2.186	CFEC-77	2.514	CFEC-108	2
CFEC-16	2.149	CFEC-47	2.230	CFEC-78	2.276	CFEC-109	2
CFEC-17	2.524	CFEC-48	1.948	CFEC-79	1.876	CFEC-110	1
CFEC-18	2.250	CFEC-49	2.274	CFEC-80	1.997	CFEC-111	2
CFEC-19	2.151	CFEC-50	2.515	CFEC-81	2.146	CFEC-112	2
CFEC-20	2.214	CFEC-51	2.234	CFEC-82	1.954	CFEC113	2
CFEC-21	2.262	CFEC-52	2.304	CFEC-83	1.809	CFEC-114	2
CFEC-22	1.957	CFEC-53	2.415	CFEC-84	2.042	CFEC-115	2
CFEC-23	2.434	CFEC-54	2.374	CFEC-85	2.367	CFEC-116	2
CFEC-24	2.110	CFEC-55	2.176	CFEC-86	2.011	CFEC-117	2
CFEC-25	2.380	CFEC-56	2.038	CFEC-87	2.068	CFEC-118	1
CFEC-26	2.277	CFEC-57	2.422	CFEC-88	2.246	CFEC-119	2
CFEC-27	2.214	CFEC-58	2.422	CFEC-89	2.466	CFEC-120	1
CFEC-28	2.357	CFEC-59	2.424	CFEC-90	1.756	CFEC-121	2
CFEC-29	1.855	CFEC-60	2.441	CFEC-91	1.919	CFEC-122	2
CFEC-30	2.216	CFEC-61	1.775	CFEC-92	2.119	CFKO -123	2
CFEC-31	2.417	CFEC-62	2.069	CFEC-93	2.253	CFEBC -124	1

CFEC: Chicken feces *E. coli* , CFKO: Chicken feces *Klebsiella oxytoca,* CFEBC: Chicken feces *Enterobacter cobei*

Table 3. Rate of resistance genes for ESBL *E. coli*

ESBL n=35

$bla_{\mathbf{CTX\text{-}M}}$	$bla_{\mathbf{CTX\text{-}M\text{-}1}}$	$bla_{\mathbf{CTX\text{-}M\text{-}15}}$	$bla_{\mathbf{SHV}}$	$bla_{\mathbf{TEM}}$	$bla_{\mathbf{OXA\text{-}10}}$

23 (65.7%) AmpC n=35 (%) *bla*$_{\mathbf{CIT}}$ 0	24 (68.5%) AmpC n=35 (%) *bla*$_{\mathbf{CIT}}$ 0	1 (2.8%) AmpC n=35 (%) *bla*$_{\mathbf{CIT}}$ 0	0 AmpC n=35 (%) *bla*$_{\mathbf{MOX}}$ 0	9 (25.7%) AmpC n=35 (%) *bla*$_{\mathbf{MOX}}$ 0	14 (40%) AmpC n=35 (%) *bla*$_{\mathbf{MOX}}$ 0

Table 4. Biochemical and genetic features of ESBL-*E.coli* isolates

Sample code for ESBL- *E.coli*	**Biochemical characteristics**	**Biochemical characteristics**	**Biochemical cha**
	Indole	**Lactose**	**Catalase**
CFEC-ESBL-1	+	+	W
CFEC-ESBL-7	+	+	W
CFEC-ESBL-9	+	+	S
CFEC-ESB-14	+	+	S
CFEC-ESBL-16	+	+	W
CFEC-ESBL-18	+	+	W
CFEC-ESBL-22	+	+	S
CFEC-ESBL-27	+	+	S
CFEC-ESBL-28	+	+	W
CFEC-ESBL-38	-	-	St
CFEC-ESBL-40	+	+	St
CFEC-ESBL-41	+	+	St
CFEC-ESBL-43	+	+	S
CFEC-ESBL-45	+	+	S
CFEC-ESBL-47	+	+	S
CFEC-ESBL-51	+	+	S
CFEC-ESBL-55	+	+	St
CFEC-ESBL-62	+	+	St
CFEC-ESBL-67	+	+	St
CFEC-ESBL-68	+	+	S
CFEC-ESBL-69	-	+	W
CFEC-ESBL-70	+	+	S
CFEC-ESBL-73	+	+	S
CFEC-ESBL-77	+	+	S
CFEC-ESBL-79	+	+	W
CFEC-ESBL-80	+	+	S
CFEC-ESBL-85	+	+	S
CFEC-ESBL-89	+	+	S
CFEC-ESBL-90	-	+	W
CFEC-ESBL-93	+	+	W
CFEC-ESBL-105	+	+	St
CFEC-ESBL-106	+	+	St
CFEC-ESBL-107	+	+	St
CFEC-ESBL-108	+	-	S
CFEC-ESBL-110	+	+	S

Other biochemical features (such as urease, methyl red, H_2S, etc.) were not presented in the table because they are the same for each bacterium.

Also, since bla_{SHV}, bla_{CIT}, and bla_{MOX} were not detected in any isolate, they were not given in the table.

**Bacterium has shown variable hemolysis. W: Weak; S: Strong*

Figure Captures

Figure 1 . Dendrogram Profile of 122 CFEC isolates (A and B) and 2D scattering profile (C).

Figure 2. (A) Representative mass spectrometry of CFEC-ESBL-68 and (B) Virtual gel profile of 35 CFEC-ESBL isolates.

Figure 3. The Principal Component Analysis of the total 35 CFEC-ESBL isolates and comparison of both genetic and biochemical analysis results of them. (A) Dendrogram and variance analysis (B) 2D Scatter plotting (C) The total CCI % value corresponds to each isolate (The -axis is the total CCI% value which includes itself and is calculated for each isolate and has a projection of each isolate of those values on the x-axis) (D) The color matrix of the total CFEC-ESBL isolates, (E) The color matrix and CCI % value of three isolates (CFEC-ESBL-38, CFEC-ESBL-68, and CFEC-ESBL-90 (F) All characteristic data of three isolates (CFEC-ESBL-38, CFEC-ESBL-68, and CFEC-ESBL-90).

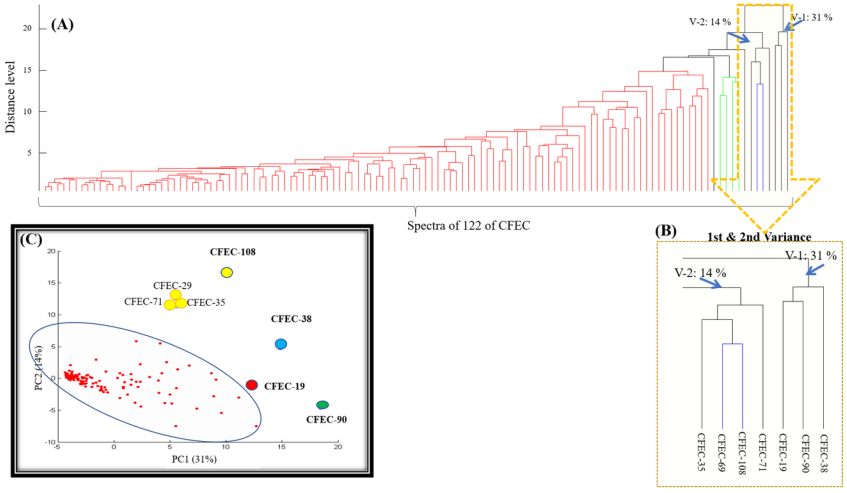
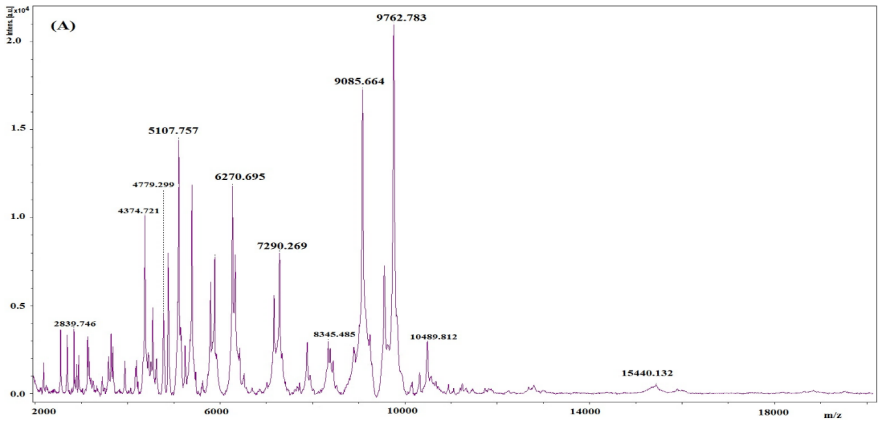


Figure 1 . Dendrogram Profile of 122 CFEC isolates (A and B) and 2D scattering profile (C)



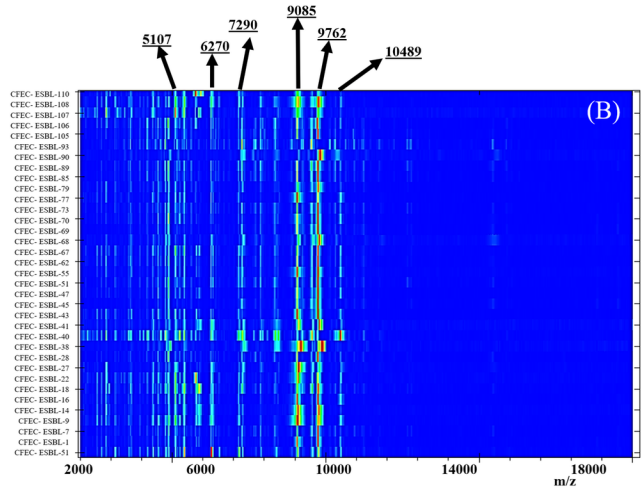
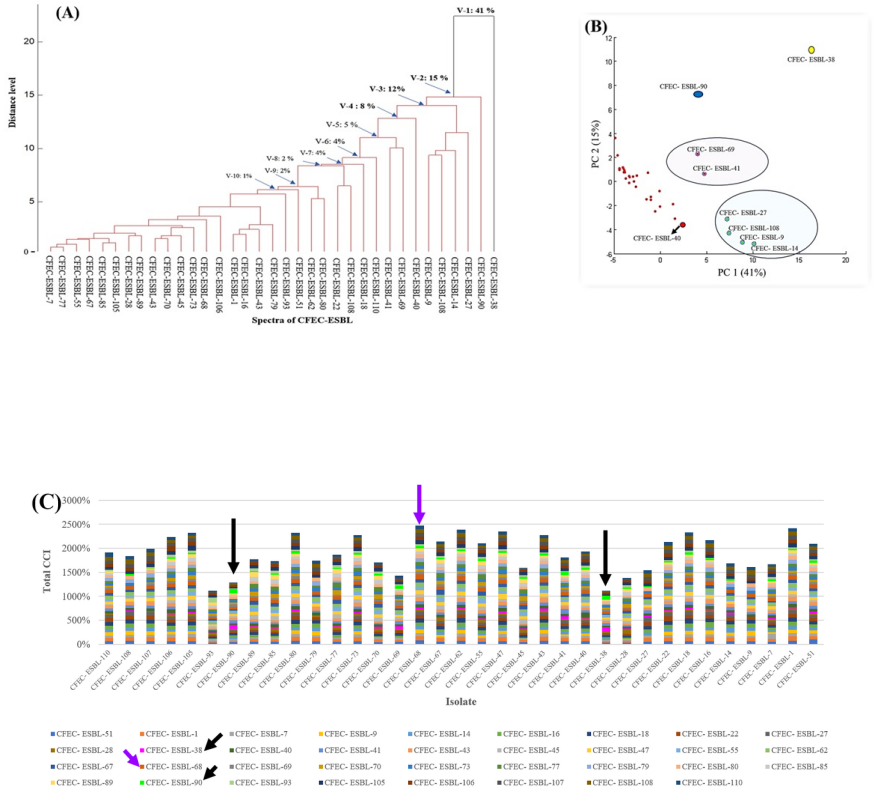


Figure 2. **(A)** Representative mass spectrometry of CFEC-ESBL-68 and (B) Virtual gel profile of 35 CFEC-ESBL isolates



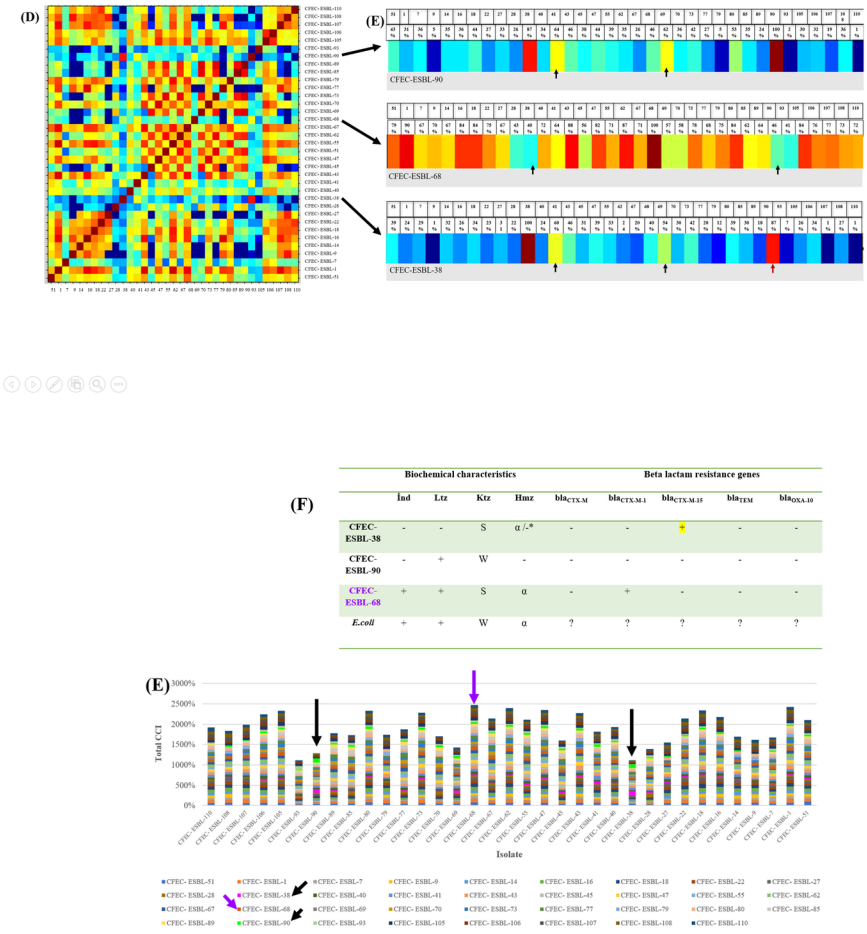


Figure 3. The Principal Component Analysis of the total 35 CFEC-ESBL isolates and comparison of both genetic and biochemical analysis results of them. (A) Dendrogram and variance analysis (B) 2D Scatter plotting (C) The total CCI % value corresponds to each isolate (The -axis is the total CCI% value which includes itself and is calculated for each isolate and has a projection of each isolate of those values on the x-axis) (D) The color matrix of the total CFEC-ESBL isolates, (E) The color matrix and CCI % value of three isolates (CFEC-ESBL-38, CFEC-ESBL-68, and CFEC-ESBL-90 (F) All characteristic data of three isolates (CFEC-ESBL-38, CFEC-ESBL-68, and CFEC-ESBL-90)