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Characterization of Virulence Genes of Food Borne Bacteria from Beef Meat Isolated from Traditional and Modern Markets in Jakarta

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Foodborne illness is a disease caused by the presence or contamination of pathogenic microbes in food. Meat and its processed product are very potential to cause foodborne illness cases, especially if the meat is cooked at an inappropriate temperature. This study aims to identify bacteria in meat and determine their potential pathogenicity by identifying pathogenic genes. The meat samples were obtained from traditional and modern markets in the Jakarta area. Bacterial identification was carried out using 16S rRNA gene, while bacterial pathogenicity profiles were identified using PCR amplification of pathogenicity-causing genes namely *STM4497, SefA, Prot6E, MecA, MeCI, Hly,* and *Stx1*. Two pathogenic bacteria namely *Proteus mirabilis* and *Enterobacter sp* were found dominantly in meat samples from traditional market. Whereas in modern market, more pathogenic various bacteria were found namely *Enterobacter hormaechei, Escherichia coli, Lactococcus garvieaea, Morganella morganii, Proteus Sp, Proteus terrae,* and *Salmonella enterica*. Moreover, *STM4497* and *Prot6E* were common pathogenic genes identified in most of bacteria.

Keywords: Gene, Pathogenic Bacteria, Foodborne Illness, Polymerase Chain Reaction, 16S sRNA.

Foodborne illness is a disease caused by the presence or contamination of pathogenic microbes in food. Microbes that cause disease may come from food products such as meat. Meat is a perishable food, due to the high free water content in meat, and the many nutrients that can be accessed by microbes. Therefore, the handling of perishable products is highly dependent on temperature regulation along the supply chain system (Haji *et al.* 2020).

Beef and its processed products are considered a favourable environment for microorganisms, one of which is bacteria. These bacteria are *Escherichia coli, Staphylococcus aureus, Streptococcus spp, Shigella spp, Salmonella spp,* and *Clostridium perfringens* (Al Hilu and Al-Shujairi 2020). These bacteria can cause a range of gastrointestinal symptoms, from mild discomfort to severe illness, depending on the specific strain and individual susceptibility due to their ability to produce various toxin. Molecular analysis from several studies revealed that there are some common virulence genes responsible for the production of those toxins. It is clear that enterohemorrhagic *E. coli* infections are related to the expression of certain virulence genes such as *stx1, stx2, eae,* and *hly* (Kholdi *et al.*, 2021). Additionally, enterotoxin production by Staphylococcus is mostly caused by the expression of virulence genes such as *SefA, MecA, Sed, eta, tsst, Prot6e,* and *STM4497* (Dorjgochoo *et al.*, 2023; Nurjayadi *et al.*, 2021; Hu *et al.*, 2019). Thus, it is important to study the expression levels of those genes in food microbes that contaminate food, particularly meat. In this study, some common virulence genes (*STM4497, SefA, Prot6E, MecA, MeCI, Hly,* and S*tx*) are the subject of analysis to

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determine their presence in contaminated microbes. Microbial contamination of meat can reflect the sanitary conditions of meat processing, which if the conditions are poor, can damage the content of the meat. Meat and processed products can contain pathogens. Pathogenic bacteria are the cause of diseases often associated with the field of food sanitation, such as diarrhea, vomiting, typhoid, and gastrointestinal infections due to high levels of microorganism contamination (Rudin *et al.* 2019).

Microbiological testing is needed to determine the quality of the product and the feasibility of the product for consumption. Culture-based methods are often regarded as the standard for microbiological analysis of food, particularly in detecting viable pathogens. However, this approach is generally not rapid, as it tends to be laborious, time-consuming, and slow in yielding results (Bhunia, 2014; Zhao et al, 2014). Over the last two decades, nucleic acid–based methods have operated by detecting specific DNA or RNA sequences of the target pathogenic organism using Polymerase Chain Reaction (PCR), which has been commonly employed. Rapid, specific, and sensitive characteristics are among the advantages of standard PCR-based detection methods compared to culture-based methods (Foddai and Grant, 2020; Priyanka *et al.* 2016).

The high risk posed by pathogenic bacteria makes it necessary to conduct research to detect the presence or absence of pathogenic bacterial contamination in meat sold in traditional markets and modern markets in the Jakarta area. Information on the presence of pathogenic bacteria in meat products sold in traditional and modern markets in the Jakarta area will increase the awareness of the people of Jakarta in buying and consuming meat sold in markets in Jakarta. The purpose of this study is to identify the types of bacteria in meat from traditional and modern markets in the Jakarta area and their potential pathogenicity by identifying virulence genes in these bacteria and comparing the types of bacteria in meat from traditional and modern markets in the Jakarta area. The benefit of this research is to provide information to the public about pathogenic bacterial contamination in meat products and minimize the number of microbial contaminations in meat so that its safety is guaranteed.

MATERIAL AND METHODS

Sampling. The samples used in this analysis were beef from one of the traditional and modern markets in Jakarta. Sampling was done by putting the meat samples obtained into sterile plastic, then stored in a coolbox and taken to the laboratory. Meat samples of 100 grams each were taken for analysis.

Microbial isolation. In the first stage, the commonly used pre-enrichment step was carried out by taking 25 grams of meat and putting it into 225 ml of TSB (Tryptone Soya Broth) Incubation was carried out for 24 hours at 37ºCelcius and 120 rpm. After that, dilution was done with 0.9 % NaCl solution. The results of the dilution were then inoculated by the scatter method onto the surface of TSA (Tryptone Soya Agar) media and incubated at 32ºC for 24 hours. The number of microbes that show different colony characteristics will be separated and cultured in fresh media. Isolation of microbial cultures was carried out by the scratch method using tilted TSA media and then incubated at 32ºC for 24 hours. After that, pure isolate culture was carried out with LB (Luria Bertani) media. The isolates consisted of 10 bacterial isolates from traditional market beef samples and 10 bacterial isolates from modern market beef samples. Then the 20 isolates were continued DNA extraction stage.

DNA Extraction. The extraction method used refers to the research of Aris *et al.* (2013). The stages are bacteria that have been cultured taken 1.5 ml and put into the tube and then centrifuged for 5 minutes at 6000 rpm. After that, the supernatant was discarded and the pellet was added with 1.5 ml of bacterial cells and then centrifuged for 5 minutes at 6000 rpm and repeated 3 times. Then the resuspended pellet was added with 1ml Tris-EDTA 1x and centrifuged for 2 minutes at 6000 rpm. Then the supernatant was discarded and the remaining pellet was added 50 µl TE 1x, 100 µl SDS 10%, 10 µl proteinase K, 100 µl 5M NaCl, and 100 µl warm CTAB. After that, it was incubated for 20 minutes at 65°C. After that, the solution was added with 500 µl PCI and vortexed. Then, it was centrifuged again for 10 minutes at

10,000 rpm. The resulting liquid will become 2 phases which are separated and added 1:1 isopropanol and then incubated at -20°C for 20-24 hours. After incubation, the solution was centrifuged for 10 minutes at a maximum speed of 17,000 g. The supernatant was discarded and the supernatant was removed. Then the supernatant was discarded and the pellet obtained was added 1 ml of 70% ethanol and centrifuged again for 5 minutes at a maximum speed of 17,000 g. After that, the supernatant was discarded and 100 µl of 1x TE was added and the DNA was stored to proceed to the PCR process.

Polymerase Chain Reaction (PCR). The universal primers used in this study were 27 F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492 R (5'-GGTTACCTTGTTACGACTT- 3') (Richard and Farooq, 2001). PCR was also performed with several primers to profile pathogenic genes (Table 1). The PCR technique was performed using a 0.5 ml microtube. The total volume of the reaction mixture was 25 µl containing 10pmol of each primer, 2 µl template DNA, 12.5 µl 2x My TAQ Polymerase, and 8.5 µl ddH2O. PCR amplification was performed under conditions of predenaturation at 95°C for 1 minute, denaturation at 95°C for 15 seconds, annealing for 15 seconds with various annealing temperatures (56°C for *Stx1, Stx2, SefA*; 55°C for *STM4467, Pro6E, 16S rRNA*; 51°C for *MecI*, 52°C for *MecA*; 70°C for *Hly*), and extension at 72°C for 15 minutes with a final extension at 72°C for 5 minutes for a total of 30 cycles. The PCR products were visualized using 2% agarose containing RedSafe dye and running in 1X TAE buffer with 100V for 40 min, before gel were documented using UV Transllluminator.

RESULT

Sequencing Results of 16S rRNA Primers. Sequencing results from 16S rRNA primers produced diverse bacteria (Table 1). Among 20 isolation samples, 18 samples were amplified with 16S rRNA primers and were identified on NCBI Genebank database. These results generated both pathogenic and non-pathogenic bacteria. Dominantly, the isolates from traditional market beef meat were identified as common pathogenic bacteria such *as Kurthia gibsonii, Proteus mirabilis, Proteus sp, Enterobacter sp* and *Kurthia sp.* While the isolates from modern market showed a more diverse species of pathogenic bacteria such as *Enterobacter hormaechei, Escherichia coli, Lactococcus garvieaea, Morganella morganii, Proteus sp, Proteus terrae, and Salmonella enterica.*

Pathogen Gene Profiling in Beef Meat Samples from Traditional and Modern Markets. The results of pathogenic gene profiling on isolated bacteria can be seen from table 3 where all samples are positive with primer *STM4497* (Fig 1) and most of them also showed positive for the present of *Prot6E* gene (Fig 1). Then other gene that dominate was *Stx1* (Table 3). *SefA, MecA,* and *Hly* genes were only found in three bacteria, *Salmonela enterica* (M9)*, E. coli (M8)* and *Morganella morganii (M6)* respectively, which those were isolated from samples modern market. Interestingly, 6 isolates of 10 isolates from samples of traditional market, showed positively present of 3 genes (*STM4497, Prot6E* and *Stx1*) namely *Proteus mirabilis (T3), Uncultured bacterium (T4), Proteus sp (T6), Kurthia gibsonii (T7), Enterobacter sp (T8),* dan *Fictibacillus rigui (T9)*. However, from sample of modern market, only two isolates have those three genes (Isolat 5 (M5) and *Salmonela enterica (M9)*.

DISCUSSION

The DNA sequencing results obtained from beef meat samples using 16S rRNA primers revealed the presence of pathogenic bacteria in each of the samples (Table 2). In the case of samples collected from the traditional market, several pathogenic bacteria were identified. Specifically, these included *Proteus mirabilis* (T3), *Proteus sp* (T6), and *Enterobacter sp* (T8). *Proteus mirabilis* belongs to the group of gram-negative bacteria and falls within the enterobactericeae family. This bacterium is pathogenic to humans due to its capability to induce urinary tract infections (Meyby, 2014). It is imperative to address the contamination of meat by *Proteus mirabilis*, as it can lead to various health issues among consumers, including diarrhea, nausea, and gastritis (Prozesky, 1968). While *Kurthia gibsonii* bacteria pose a potential pathogenic threat in poultry,

Table 1 Primer List of Pathogenicity Test

No	Primer	Sequence (5'→3')	Tm (°C)	Species identification	Gene Detection	Reference
1	Stx1-F	TCTCAGTGGGCGTTCTTATG	56	*E. coli* O157 H7 A	Shiga Toxin Protein	Allotaibi & Khan, 2023
2	Stx1-R	TACCCCCTCAACTGCTAATA	56			
3	MecA-F	TCACCAGGTTCAACCCAAAA	52	*Staphylococcus aureus*	High level resistance to antibiotics	Cuny *et al.*, 2011
4	MecA-R	CCTGAATCTGCTAATAATATTC	52			
5	MecC1-F	GACACGTGAAGGCTATGATATAT	51	*Staphylococcus aureus*	Repressor protein for MRSA	García-Álvarez *et al.*, 2011
6	MecC1-R	ATTCTTCAATATCATCTTCGGAC	51			
7	STM4497-F	AACAACGGCTCCGGTAATGA	55	*S. typhimurium*	Hypothetical protein code	Kim *et al.*, 2006
8	STM4497-R	TGACAAACTCTTGATTCTGA	55			
9	Prot6E-F	ATATCGTCGTTGCTGCTTCC	55	*S. enterica*	Surface fimbriae specific in plasmid	Malorny *et al.*, 2007
10	Prot6E-R	CATTGTTCCACCGTCACTTG	55			
11	Hly-F	GCTGCAAGTCCTAAGACGCC	70	*L. monocytogenes*	The listeriolysin O	Seethalakshmi *et al.*, 2008
12	Hly-R	GGGCGGCGGAGGACAGGGG	70			
13	SefA-F	GATACGTTGAAAACTGAGGA	56	*E. enteritidis*	A fimbrial operon gene	Doran *et al.*, 1996
14	SefA-R	CGGGAATACCGCATAGTAGC	56			

it is noteworthy that the *Kurthia gibsonii* strain NICMB 9758 is not pathogenic (Kusumaningsih and Mustika, 2020). Additionally, the bacteria *Bacillus amyloliguefaciens* (T2) and *Fictibacillus rigui* (T9) are non-pathogenic gram-positive bacteria (Glaeser *et al.*, 2013). Isolate T5, on the other hand, could not be identified during the sequencing process, indicating that it might be an uncultured bacterium—a type of bacteria that has not been cultured.

It was clearly shown that the modern markets exhibit a higher prevalence of pathogenic bacteria compared to beef samples from traditional markets. Specifically, the identified pathogenic bacteria in modern market samples include *Enterobacter hormaechei, Escherichia coli, Lactococcus garvieae, Morganella morganii, Proteus species, Proteus terrae,* and *Salmonella enterica*. Isolates M1, and M5 represent uncultured bacteria. In the case of M9 and M10 isolates, they correspond to *Salmonella enterica*, which are pathogenic microorganisms responsible for causing foodborne diseases, particularly a condition known as Salmonellosis. It is worth noting that Salmonella cannot be easily distinguished from *E. coli* under microscopic examination or by growing them on nutrient-rich media (Sulistiani and Hafiludin, 2022). In instances where food becomes contaminated with *E. coli* bacteria and is subsequently consumed, it can result in diarrhea (Bahri *et al.*, 2019).

In the field of food sanitation, two bacteria, namely *Enterobacter hormaechei* and *Morganella morganii*, have connections to other domains. *Enterobacter hormaechei* is generally regarded as pathogens caused nosocomial infections and typically do not induce diseases in animals (Wang *et al.*, 2020).

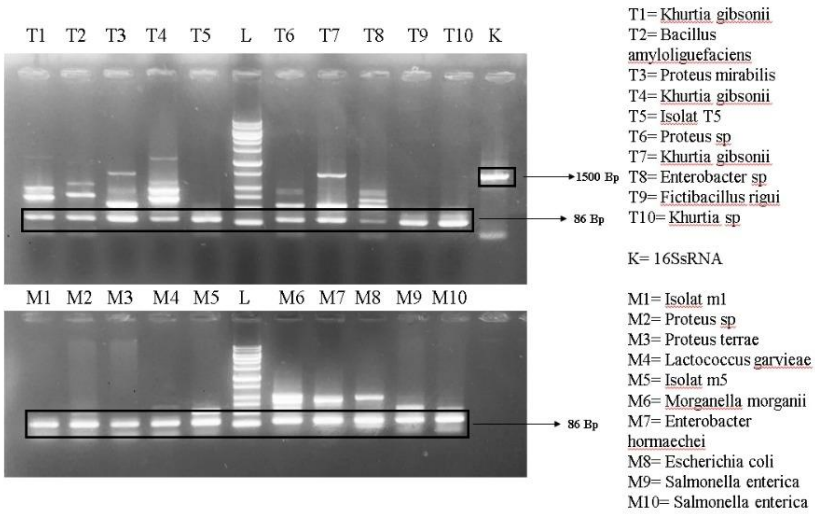

Figure 1 Results with Primer *STM4497* with Beef Meat Bacteria Samples from Traditional (T) and Modern (M) Market.

On the other hand, *Morganella morganii* represent clinically atypical pathogens and frequently suggested as the reason of nosocomial infections in adults, particularly within the urinary tract infections or wound-related complications.

In the case of Proteus species and *Proteus terrae*, they belong to the category of gram-negative bacteria and typically exist as part of the normal flora within the human gastrointestinal tract. However, if these bacteria able to penetrate into the urinary tract, open wounds, or the respiratory system, they can become pathogenic. Shifting the focus to *Lactococcus garvieaea*, this gram-positive bacteria is recognized for triggering outbreaks of infection in fish residing in warm waters. The primary source of contamination often stems from the consumption of raw seafood. Nevertheless, as indicated by prior research, other animal-derived food sources like unpasteurized dairy products can also be potential contributors. These sources have been associated with gastrointestinal disorders that disrupt the mucosal layer (Sari *et al.*, 2019).

In addition to the presence of pathogenic bacteria, there are also identified gram-positive bacteria. According to research conducted by Feito *et al.* (2022) regarding this specific bacterium, it has the potential for virulence and zoonosis. Therefore, both in vitro and in vivo research are necessary to better understand and characterize its pathogenicity regulation pathways. Further in vitro and in vivo studies are thus required to comprehensively comprehend the pathogenicity regulation pathways of *L. garvieae*. *Morganella morganii* is a gram-negative nosocomial pathogenic bacterium that is also identified as one of the causes for the decarboxylation of the free amino acid histidine into histamine, as researched by Setiaji and Widantari (2015). The histamine produced has the potential to induce poisoning in humans. Since meat contains the free amino acid histidine, when the enzyme histidine decarboxylase from *Morganella morganii* breaks down histidine, histamine formation occurs, leading to the occurrence of crusting. This histamine-induced crusting can result in poisoning for humans.

The STM4497 gene has been identifies in all bacteria isolates, with an amplification length of 86 base pairs (bp). However, multiple bands in electrophoresis were identified. It may cause by several factor related with PCR process, like suboptimal annealing temperature (Nurjayadi *et al.*, 2021). Identification the presence of the *SefA* gene revealed its existence solely in bacteria from modern markets, specifically *Salmonella enterica.* Although *SefA gene* (fimbrin gene) exhibits low sequence variability, individual base pairs hold potential for distinguishing *Salmonella enteritidis, Salmonella dublin,* and *Salmonella gallinarum* strains (Doran *et al.*, 1996).

Table 2 Sequencing Results of 16S rRNA sequence

Sample Code	Scientific Name	Query Cover	E-Value	Identity %	Accession
T1	*Kurthia gibsonii*	97%	6,00E-151	96.94%	MT305989.1
T2	*Bacillus amyloliquefaciens*	97%	9,00E-165	95.21%	MH921988.1
T3	*Proteus mirabilis*	99%	6,00E-146	97.44%	OQ414204.1
T4	*Kurthia gibsonii*	94%	1,00E-105	82.71%	OP457917.1
T5	*NA – uncultured organism*	13%	2,00E-04	100%	JN499939.1
T6	*Proteus sp.*	73%	1,00E-40	78.91%	MF327051.1
T7	*Kurthia gibsonii*	93%	e-90	97.54%	EU253503.1
T8	*Enterobacter sp.*	61%	1,00E-10	80.43%	KC833510.1
T9	*Fictibacillus rigui*	36%	2,00E-20	90.59%	KP236176.1
T10	*Kurthia sp.*	93%	7,00E-100	99.03%	CP126689.1
M1	*NA – uncultured bacterium*	46%	1,00E-05	82.50%	GQ932772.1
M2	*Proteus sp.*	95%	1,00E-59	100%	EU530206.1
M3	*Proteus terrae*	98%	1,00E-13	83.52%	OP778634.1
M4	*Lactococcus garvieae*	91%	4,00E-79	97.73%	OL823047.1
M5	*NA – uncultured bacterium*	28%	3,00E-06	91.30%	KX821736.1
M6	*Morganella morganii*	68%	2,00E-52	94.20%	CP043955.1
M7	*Enterobacter hormaechei*	79%	1,00E-132	95.38%	MT471013.1
M8	*Escherichia coli*	94%	4,00E-83	95.59%	MF511712.1
M9	*Salmonella enterica*	94%	1,00E-32	94.53%	JQ267507.1
M10	*Salmonella enterica*	54%	5,00E-65	98%	MH548471.1

Amplification using the Prot6E primer yields a 135 bp product. From traditional market samples, it was identified mostly of the bacterial isolates contain *Prot6E* gene, except for *Kurthia gibsonii* and *Khurtia sp*. In modern market samples, the absent of *Prot6E* genes were found in isolate M1*, Lactococcus garvieae*, *Escherichia coli,* and *Salmonella enterica*. Hu et al (2019) reported that *Prot6E* gene is common found in in Brazilian egg and chicken samples, along with a *SefA* in *Salmonella ser. Enteritidis*-infected US egg samples, reflecting genetic diversity among isolates from different regions (Hu *et al.*, 2019).

Stx1 gene which produces shiga-like toxin, was common identified from *Escherichia coli.* Suardana *et al.* (2010) reported that cow feces, chicken feces, beef, and human feces contain *E.coli* 0157:H7 which produced shiga like toxin. This is attributed to the highly pathogenic nature of toxin types Stx1 and Stx2 found in *Escherichia coli* O157:H7, with the primary clinical symptom being bloody diarrhea and Stx2 toxin will have the opportunity to suffer from HUS (hemolytic uremic syndrome) (Wirathi *et al.* 2020). Interestingly, our data showed that *Stx1* gene was identified not in E. coli (M8), but in others bacterial isolates. The traditional market samples showed the presence of the *Stx1* toxin gene in several bacteria, including *Proteus mirabilis*, uncultured bacterium, *Proteus sp, Kurthia gibsonii, Enterococcus sp,* and *Fictibacillus rigui*. Conversely, samples from modern markets—namely, isolate M1, *Proteus sp*, isolate M5, and *Salmonella enterica*—also exhibit this gene. The MecA primer identification process did not yield bacteria with the gene in traditional market samples. However, modern market samples, specifically *Escherichia coli* contained this gene. The *MecA* gene-associated *Staphylococcus aureus* has the potential to induce a wide range of diseases, spanning from mild skin infections to systemic conditions like pneumonia and meningitis (Ramandinianto *et al.*, 2020). Notably, *S. aureus* can be transmitted to humans through the contamination of milk, untreated milk, and dairy products.

Table 3 Pathogen Gene Profiling In Beef Meat Samples From Traditional And Modern Market

Kode	Nama	*STM4497*	*Sef*A	*Prot*6E	*Stx*1	*Mec*A	*Me*CI	*Hly*
T1	Kurthia gibsonii	+	-	+	-	-	-	-
T2	Bacillus amyloliguefaciens	+	-	+	-	-	-	-
T3	Proteus mirabilis	+	-	+	+	-	-	-
T4	Kurthia gibsonii	+	-	-	-	-	-	-
T5	Uncultured bacterium	+	-	+	+	-	-	-
T6	Proteus sp	+	-	+	+	-	-	-
T7	Kurthia gibsonii	+	-	+	+	-	-	-
T8	Enterobacter sp	+	-	+	+	-	-	-
T9	Fictibacillus rigui	+	-	+	+	-	-	-
T10	Kurthia sp	+	-	+	-	-	-	-
M1	Isolat m1	+	-	-	+	-	-	-
M2	Proteus sp	+	-	+	+	-	-	-
M3	Proteus terrae	+	-	+	-	-	-	-
M4	Lactococcus garvieae	+	-	-	-	-	-	-
M5	Isolat m5	+	-	+	+	-	-	-
M6	Morganella morganii	+	-	+	-	-	-	+
M7	Enterobacter hormaechei	+	-	+	-	-	-	-
M8	Escherichia coli	+	-	-	-	+	-	-
M9	Salmonella enterica	+	+	-	+	-	-	-
M10	Salmonella enterica	+	-	-	-	-	-	-

T = Sample from Traditional Market M = Sample from Modern Market

The *Hly* gene was not detected within samples collected from the bacterial isolates of traditional market samples. However, it was identified within *Morganella morganii*. The *Hly* gene encodes *L. monocytogenes* hemolysin listeriolysin O (LLO), a key element contributing to *L. monocytogenes* virulence, extensively recognized for its pivotal role in interactions with the host during pathogenicity. The presence of the *Hly* gene within *L. monocytogenes* is indicative of its potential to induce foodborne illness, specifically listeriosis (Price *et al.*, 2018).

CONCLUSION

Based on the findings of this research, it can be deduced that distinctions exist in the characteristics of pathogenic genes prevalent among bacteria within beef samples from both traditional and modern markets. The traditional markets displayed the presence of pathogenic bacteria, particularly Proteus and Enterobacter strains. In traditional market samples, the dominant distribution of pathogenic genes comprises *STM4497, Prot6E,* and *Stx1* genes. These genes span across various bacterial types identified within beef samples sourced from traditional markets. On the other hand, the array of pathogenic bacteria types observed in modern markets is more diverse. These encompass *Enterobacter hormaechei, Escherichia coli, Lactococcus garvieae, Morganella morganii, Proteus species, Proteus terrae,* and *Salmonella enterica*. Furthermore, the distribution of pathogenic genes in modern markets is primarily governed by *STM4497, Prot6E,* and *Stx1* genes, with several other genes, such as *MecA* and *Hly*, identified in specific bacterial isolates.

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