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28 DISTINCTIVE BACTERIAL SPECIES WERE DETECTED USING 16S RIBOSOMAL RNA AT CATTLE MEAT RETAIL STORES

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ABSTRACT

The present work was carried out to detect and identify bacterial strains at cattle meat retail stores using the machinery of 16s ribosomal RNA. Meat samples were sampled from four different sources, specifically beef meat cutting boards, cutting tools (e.g. knives, saws, slicers,. etc), meat grinders, and meat refrigerators at four different retail stores located in Makkah, Saudi Arabia. 28 distinctive bacterial species were detected using 16s Ribosomal RNA at cattle meat retail stores. *Macrococcus caseolyticus* was ubiquitous where the species was found in all sources and locations. Species *Staphylococcus sciuri* has also occurred in all locations but not in all locations. DNA sequences length of the 49 isolates averaged 1106 pb, the shortest length was 1001 bp for *M. caseolyticus* sampled from meat boards at location A, where the longest length was 1280bp for *M. aseolyticus* sampled from meat tools at location B. The average content of bases was very similar to what was found in most of the four sources, that is T, A, C, and G were 22, 26, 23, and 29%. of A+T was 48 % much lower than that of C+G 52%. The percentage of GC ranged from 50% for *C. carnis* from meat grinders at location 4 up to 55% for *O.endophyticum* from cutting tools at location 2. Phylogenetic analysis of pooled sequences resulted in a rather complicated dendrogram. Only *M. aseolyticus* from cutting tools at location B had the longest brach length, which means this isolate is the most distantly related to the rest of isolates. However, *M. caseolyticus* from the rest of the locations and sources were mostly closely related. Pooled DNA sequence analysis resulted in 1349 sites of which 447 sites with alignment gaps or missing data. The number of monomorphic sites was 123 sites and 779 polymorphic sites. These polymorphic sites are divided into 77 singleton variable sites: and 702 parsimony informative sites. The total number of mutations was 1294. The number of conservative regions varied greatly among sequenced the four meat sources. Cutting boards had the lowest number of conserved regions 4 whereas both catting tools and meat grinders had the largest number 9. When all sequences were pooled together only 3 conserved regions were detected. The present work alarms serious sounds about the hygiene of tools used to cut, prepare, and cold store cattle meat in retail stores. As the number of detected bacterial strains reached 28 distinctive strains. These bacterial strains are genetically closely related. Some of these 28 strains are antibiotic-resistant Bactria might be opportunities-driven human pathogens and hence have earnest repercussions on the health of consumers.

INTRODUCTION

Cattle meat is one of the best and most important sources of dietary protein for humans. Cattle meat and its products are the most prevalent in addition to mutton and camel meat in Saudi Arabia (Al-Thubaiti *et al.*, 2021). Cattle meat has gained preference as it contains many essential amino acids and ease of metabolism, whose proteins decompose into many types of peptides important for good human health (Ebel *et al.*, 2004 and Alsayeqh, 2015). In addition, beef is a source of many micro and macro elements, which are included as enzyme accompaniments that are involved in the correct metabolism pathways, in addition to some vitamins and fatty acids necessary for the nervous system and the brain (Al-Mutairi & Dingwall, 2015 and WHO, 2017). Nowadays, food safety, including different types of meat, is one of the things that the consumer cares about, and this is because diseases that are transmitted through food and cause their spread are a great concern for humans (Biswas *et al.*, 2011 and Al-Mutairi & Dingwall, 2015). Cattle Meat may be contaminated at one of the

stages of its production and handling until it reaches the consumer. The meat of all kinds, including beef, is a rich food source suitable for the growth of many microbes that cause spoilage of meat and food poisoning (Ali *et al.*, 2010 and Kim & Yim, 2016)

Various microorganisms can grow on meat and meat products in an environment suitable for their growth. Many microorganisms including bacteria are responsible for meat spoilage, food poisoning, and carcass condemnation which negatively affects the economy of both farmers and sellers Ismail *et al.*, 2013 and Soepranianondo & Wardhana, 2019. Various infectious diseases may develop, the bacterial species that cause many diseases and spoilage of meat, such as *Aeromonas hydrophila, Yersinia enterocolitica, Listeria mono-cytogenese, Campylobacter jejuni, Salmonella spp, Clostridium perfringens, Escherichia coli, Staphylococcus aureus* and *Bacillus cereus* (Reid *et al.*, 2002, Da Silva *et al.*, 2014, Gogliettino *et al.*, 2020 and Marrone, 2021).

Microbiological studies are still limited in results in the accurate identification of pathogenic microbes for humans, so the trend was toward studies at the molecular level (Jadidi A, 2012). Molecular methods based on polymerase chain reaction (PCR) offer a rapid and accurate alternative to conventional techniques. Through which the accurate identification of bacterial species under the same genus (Kairalla *et al.*, 2005 and Abd El-Razik *et al.*, 2019).

With the advent of polymerase chain reaction (PCR) techniques and the next generation of high-throughput sequencing (NGS) technologies, the 16S rRNA gene sequence has become more accurate and easier to identify microbes that cause spoilage of food and meat and cause many diseases in humans, especially Salmonella and Campylobacter, for which methods were used against them traditionally (Alsanie *et al.*, 2018 and BenAbdallah *et al.*, 2019).

However, reports regarding the molecular identification of bacterial communities present in beef and meat products using high-throughput sequencing techniques are still limited in Saudi Arabia. Therefore, the present work was carried out to detect and identify bacterial strains at cattle meat retail stores using the machinery of 16s ribosomal RNA.

MATERIALS & METHODS

The current work was carried out to isolate various bacterial strains from different sources within four retail stores located in Makkah, Saudi Arabia, with the purpose of identifying bacterial strains based on their 16S rRNA gene phylogeny. Meat samples were sampled from four different sources, specifically beef meat cutting boards, cutting tools (e.g. knives, saws, slicers,.. etc), meat grinders, and meat refrigerators. Nutrient agar (NA) plates were used to cultivate bacterial isolates, which were then incubated at 37 °C for 24 hours. NB was used to produce purified bacterial strains, which were then stored for further use in a 20% glycerol solution. The QIA- GEN Kit from Germany was used to extract bacterial genomic DNA in accordance with manufacturer's instructions. The universal primers 27F 5-AGAGTTTGATCCTGGCTCAG-3, and 1492R 5-AAGGAGGTGATCCAGCCGCA-3, were used to amplify the 16S rRNA gene. At Macrogen, South Korea, amplified products were purified before being Sanger sequenced. Following manual editing, the sequences were compared with the GenBank database of NCBI (http://www.ncbi.nlm.nih.gov) using the BLAST search in the SnapGene. Viewer program version 3.3.3. The MEGA program, which may be found on the NCBI website, was used to create phylogenetic trees.

Each sequence from various sources and locations was put through NCBI BLAST search tool http://blast.ncbi.nlm.nih.gov to investigate non-chance sequence comparability. BLAST search was limited to 16s ribosomal RNA sequences, models (XM/XP) as well as uncultured/environmental samples were also filtered off, therefore more trustworthy results would be extracted. Each sequence was exclusively *blastd*, where blast hit with the smallest expect-value (*which indicates the number of non-chance alignments*) was picked. To assure that Blast outputs were controlled by expected-value (aka e-value), Blast algorithm parameter was decreased such the expected threshold was set to a more stringent value of $1e^{-6}$. Alignment of the sequence was carried out Clustalx (Larkin *et al.*, 2007).

Exploratory data and phylogenetic analyses were carried out under R Project for Statistical Computing (R Core Team, 2020). Where Exploratory data analysis was done using Seqinr (Charif & Lobry, 2007) R package. Phylogenetic analysis was carried out by ape package (Paradis *et al.*, 2004). Reconstruction of the phylogenetic tree was done using the Neighbor joint method (Nei, 1987).

DnaSP (Librado & Rozas, 2009) software was used to analyze the hap- lotype diversity (H_d), the average number of nucleotide differences, the nucleotide diversity (n). The polymorphic site (S), the singleton variable sites (SP), and the parsimony informative sites (PIP) for each gene.

RESULTS AND DISCUSSION

To our surprise, this is the first work that uses 16s ribosomal RNA to detect cattle beef bacterial contamination in retail stores in Kingdom of Saudi Arabia. Table (1) shows species of different bacterial strains isolated from four sources as well as sequence length and the percentages of GC content.

Concreting the variation of species found in all four sources and locations, a total of 28 distinctive species were detected out of the 49 samples. *Macrococcus*

caseolyticus was ubiquitous where the species was found in all sources and locations. Species *Staphylococcus sciuri* was also occurred in all locations but not in all locations (1). The number and variation of bacterial species found in the present study outnumbered the species reported by Bughti *et al.* (2017) on cattle beef in Pakistan, as well as Kang *et al.* (2019) in extortion slaughterhouses of Australia. However, the lower number of bacterial strains detected by Kang *et al.* (2019) maybe attributed to sampling area.

The whole 49 sequences from the four sources were pooled together to introduce a comprehensive picture of all isolates. The sequence length of all 49 DNA sequences averaged 1106 pb, the shortest length was 1001 bp for *M. caseolyticus* sampled from meat boards at location A, where the longest length was 1280bp for *M. aseolyticus* sampled from meat tools at location B. For percent of GC content. The average content of bases was very similar to what was found in most of the four sources, that is T, A, C, and G was 22, 26, 23, and 29%. of A+T was 48 % much lower than that of C+G 52%. The percentage of GC ranged from 50% for *C. carnis* from meat grinders at location 4 up to 55% for *O. endophyticum* from cutting tools at location 2.

The distance matrix of all 49 sequences is presented graphically in Figure (1). Only *M. caseolyticus* from cutting tools at location B, was found to be distantly related to the rest of the isolated strains, (as the darker shades of gray mean a larger distance). Another 3 strains were distantly related to the other strains but those found in meat fridges. These three strains are namely, *P. sanguinis* and *P. piechaudii* from meat grinders at locations A and C, as well as *P. sanguinis* from meat fridges at location C.

Phylogenetic analysis of pooled sequences resulted in a rather complicated dendrogram is shown in (Figure 2). One good way to elaborate phylogenetic relationships among these strains is to consider branch lengths. Only *M. aseolyticus* from cutting tools at location B had the longest brach length, which means this isolate is the most distantly related to the rest of isolates. However, *M. caseolyticus* from the rest of the locations and sources were mostly closely related. *C. carnis* from the meat grinder at C location had the second longest branch length. *P. sanguinis* and *P. piechaudii* from meat grinders locations A and C as well as *P. sanguinis* from meat fridges location C also have long branch lengths and clustered together.

DNA Sequences Analysis

Polymorphic Sites

DNA sequence analysis introduces a coherent tool for good comprehending of evolutionary forces that shaped nucleotide variations as well as introduce consciousness of the significance of particular genomic regions (Hutter *et al.*, 2006).

Analyses of polymorphic sites for the four sources as well as the pooled analysis are shown in table (2). The total number of aligned sites ranged from 1171 for cutting tools to 1324 for cutting boards. The number of sites without alignment gaps or missing data ranged from 189 for cutting tools to 337 for cutting boards. The number of monomorphic sites ranged from 179 for meat grinders to 698 sites for cutting tools. The cutting tools have the lowest number of polymorphic sites (284 sites) whereas cutting boards have the largest (775 sites). For the number of mutations, again cutting tools have the lowest number 362 whereas meat grinders have the largest number of mutations 1126.

Pooled Sequence analysis comprised 49 sequences that resulted in 1349 sites of which 447 sites with alignment gaps or missing data. The number of monomorphic sites was 123 sites and 779 polymorphic sites. These polymorphic sites are divided into 77 singleton variable sites: and 702 parsimony informative sites. The total number of mutations was 1294.

Haplotype & nucleotide analyses

Haplotype diversity (aka gene diversity) is the probability that two arbitrary sampled alleles are different (Nie, 1987). The number of haplotypes, haplotype diversity, and Standard deviation (SD) of haplotype diversity are shown in table (3). The number of haplotypes ranged from 10 for cutting boards and meat fridges to 13 for meat grinders. When all sequences pooled together haplotype number was 34. Haplotype diversity (H) of the four meat sources, as well as the pooled sequences, almost reached 1, which indicated great divergences among haplotypes of the isolated species, either for the four meat sources as well as the pooled sequences. Moreover, the values standard deviation of haplotypes diversity was rather small ranged 0.02 to 0.05.

Nucleotide diversity (π) is an efficient measure of the extent of DNA polymorphism. Nucleotide diversity is the average number of either nucleotide differences or substitutions per site for a set of DNA sequences randomly sampled (Nie, 1987, chap. 10) chap. 10).

Nucleotide diversity, Average number of nucleotide differences (k), the total variance of nucleotide differences (free mutations) sequences isolated from cutting boards, cutting tools, meat grinders, meat fridges and pooled sequences are shown in table (4) The nucleotide diversity (n) was quite small, in general, ranged from 0.11 for cutting tools to 28 for meat grinders. For average number of nucleotide differences (k), cutting tools isolates had the lowest k estimate (107), while cutting boards isolates had the largest k estimate (264). For pooled sequences, the k estimate was moderate 194.

The length of conserved sequences in isolates of the four meat sources as well as the pooled sequences is shown

graphically in figure (3). The number of conservative regions varied greatly among sequenced the four meat sources. Cutting boards had the lowest number of conserved regions 4 whereas both catting tools and meat grinders had the largest number 9. When all sequences were pooled together only 3 conserved regions were detected. The length of conserved regions also vary greatly varied within and among meat sources. The length of cutting boards has the widest range of conserved regions, which varied from 62 to 244, for cutting tools the length ranged from 74 to 204. The length of 9 conserved regions of meat grinders ranged from 38 to 170. The narrowest range was for the 5 conserved regions of meat fridges ranged from 86 to 142, while the range of the conserved regions of the pooled sequences ranged from 57 to 198.

The present work alarms serious sounds about the hygiene of tools used to cut, prepare, and cold store cattle meat in retail stores. As the number of detected bacterial strains reached 28 distinctive strains. These bacterial strains are genetically closely related. Some of these 28 strains are antibiotic- resistant Bactria might be opportunities-driven human pathogens and hence have earnest repercussions on the health of consumers.

Table 1: Description of bacteria Samples collected from different sources and location along with DNA sequence length and percentage GC content.

Source	location	Strain	Sequence length	GC%
Cutting Boards	A	*Macrococcus caseolyticus*	1110	52
		Klebsiella pneumonia	1080	55
	B	*Staphylococcus saprophyticus*	1133	51
		Ochrobactrum endophyticum	1078	55
		Macrococcus caseolyticus	1280	51
		Staphylococcus sciuri	1174	52
	C	*Bacillus sfensis*	1158	53
		Macrococcus caseolyticus	1099	52
		Hafnia paralvei	1120	53
	D	*Macrococcus caseolyticus*	1071	52
		Enterobacter hormaechei	1100	55
Cutting Tools	A	*Staphylococcus epidermidis*	1049	51
		Macrococcus caseolyticus	1001	52
		Staphylococcus sciuri	1049	51
	B	*Lysinibacillus boronitolerans*	1100	53
		Macrococcus caseolyticus	1088	52
		Lactococcus garvieae	1158	53
	C	*Citrobacter freundii*	1117	54
		Macrococcus caseolyticus	1030	52
		Staphylococcus sciuri	1050	51
	D	*Macrococcus caseolyticus*	1099	52
		Enterobacter hormaechei	1119	55
Meat Grinders	A	*Macrococcus epidermidis*	1085	52
		Lactococcus garvieae	1119	52
		Macrococcus caseolyticus	1059	53
		Psychrobacter sanguinis	1090	50
	B	*Lactococcus garvieae*	1158	53
		Raoultella ornithinolytica	1144	53
		Macrococcus caseolyticus	1202	52
		Hafnia alvei	1146	53
	C	*Macrococcus caseolyticus*	1098	52
		Psychrobacter piechaudii	1063	50
	D	*Macrococcus caseolyticus*	1100	52
		Staphylococcus sciuri	1096	51
		Brevibacillus borstelensis	1120	55
		Bacillus piscis	1085	55
		Acinetobacter baumannii	1121	52
Meat Fridges	A	*Macrococcus caseolyticus*	1059	52
	B	*Escherichia marmotae*	1144	54
		Acinetobacter lactucae	1166	52
		Acinetobacter nosocomialis	1166	52

		Psychrobacter cibarius	1054	52
	C	*Chryseobacterium carnis*	1119	50
		Raoultella ornithinolytica	1118	53
		Macrococcus caseolyticus	1029	52
		Psychrobacter sanguinis	1070	51
	D	*Staphylococcus sciuri*	1099	51
		Macrococcus caseolyticus	1101	52
		Acinetobacter variabilis	1120	53

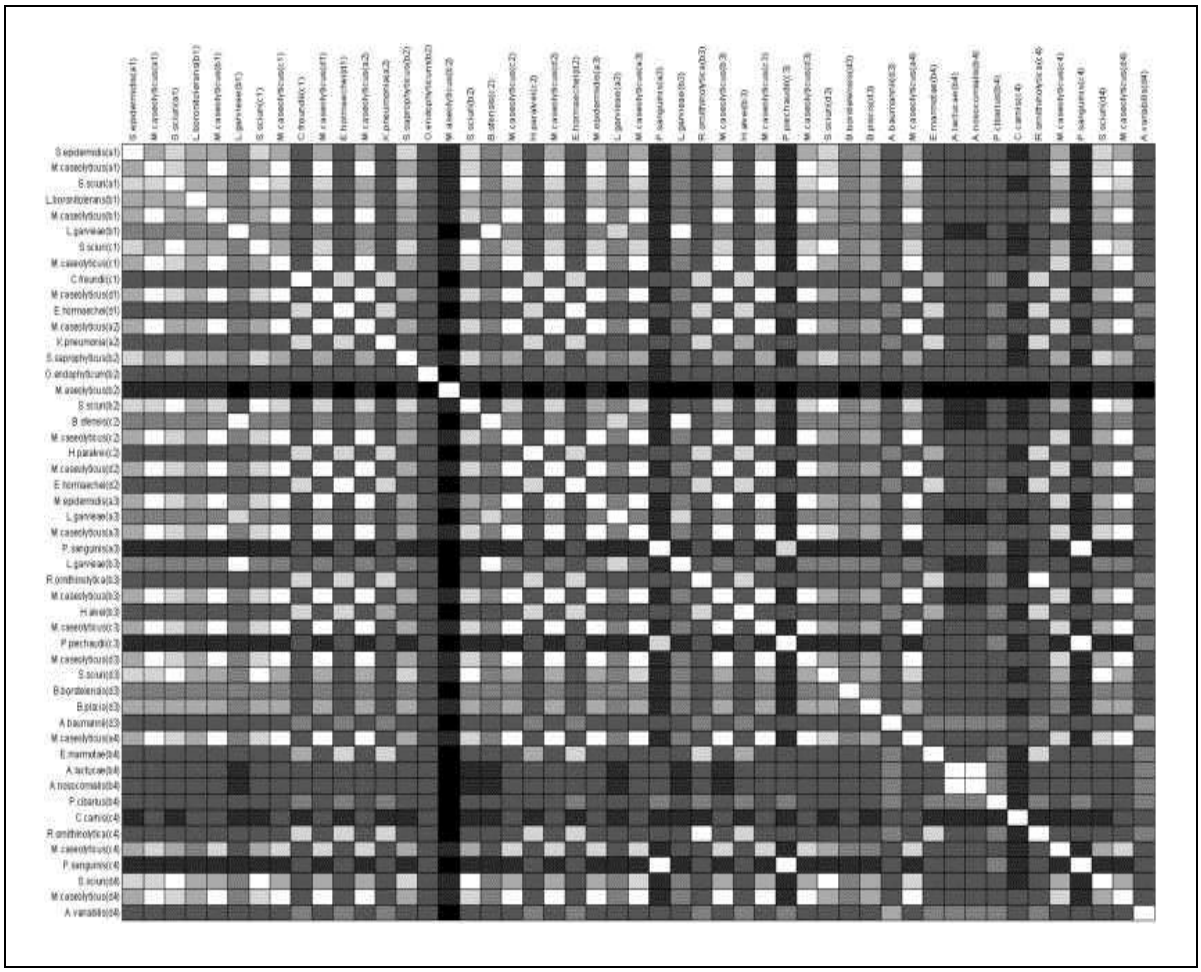

Figure 1: Distance matrix of pooled isolates, (*letter in brackets is an indicative of the location, number in the bracket refer to source, 1= cutting boards, 2= cutting tools, 3=meat grinders, and 4 = meat fridges *) darker shades of gray mean a larger distance.

Table 2: Number of sites, monomorphic, polymorphic sites, and number of mutations of cutting bards, cutting tools, meat grinders, meat Fridges and pooled sequence analysis.

Source	Number of sites	Monomorphic Sites	Polymorphic sites		No. mutations
			Singleton	Parsimony informative	
Cutting Board	1324	212	482	293	1108
Cutting Tools	1171	698	63	221	362
Meat Grinder	1239	179	181	592	1126
Meat Fridges	1237	223	436	312	1012
Pooled Sequences	1394	123	77	702	1294

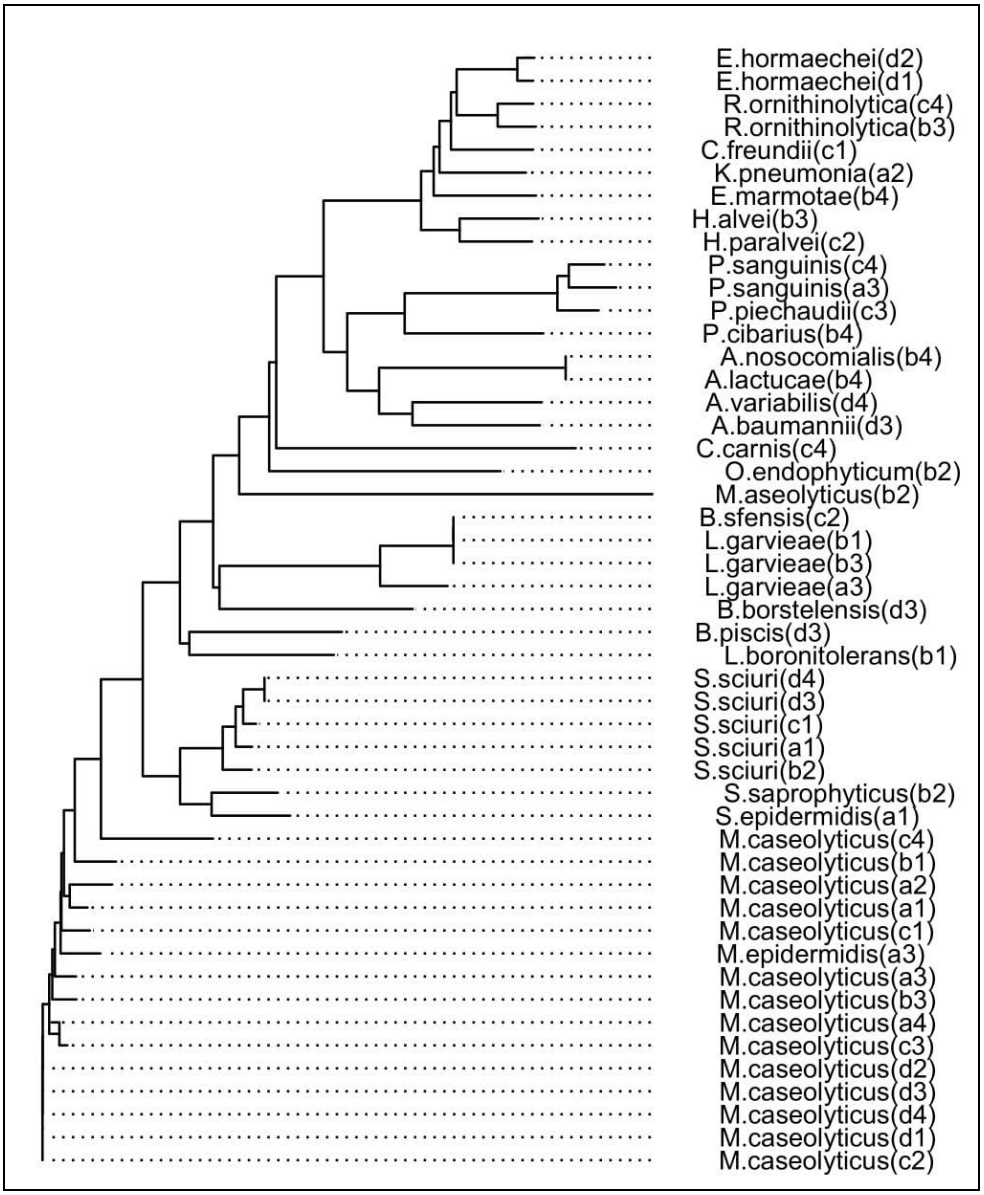

Figure 2: Neighbor-joining phylogenetic tree of 49 isolates from pooled sequences, *(letter in brackets is an indicative of the location, number in the bracket refer to source, 1= cutting boards, 2= cutting tools, 3=meat grinders, and 4 = meat fridges*).

Table 3: Number of haplotypes , haplotypes diversity and Standard deviation (SD) of haplotypes diversity for sequences isolated from cutting boards, cutting tools, meat grinders, meat fridges and pooled sequences.

Source	No. of Haplotypes(H)	Haplotype(gene) diversity, (Hd)	SD Haplotype diversity
Cutting boards	10	0.98	0.05
Cutting tools	11	1.00	0.04
Meat grinders	13	0.97	0.04
Meat Fridges	10	0.97	0.04
Pooled Sequences	34	0.96	0.02

Table 4: Nucleotide diversity, Average number of nucleotide differences (k) , total variance of nucleotide differences (free mutations) sequences isolated from cutting boards, cutting tools, meat grinders, meat fridges and pooled sequences.

Source	Nucleotide diversity (π)	Average no. of nucleotide differences (K)	Total variance of k (free recombination), V(k)
Cutting boards	0.27	264	106
Cutting tools	0.11	107	42
Meat grinders	0.28	262	100
Meat Fridges	0.25	245	96
Pooled Sequences	0.22	194	67

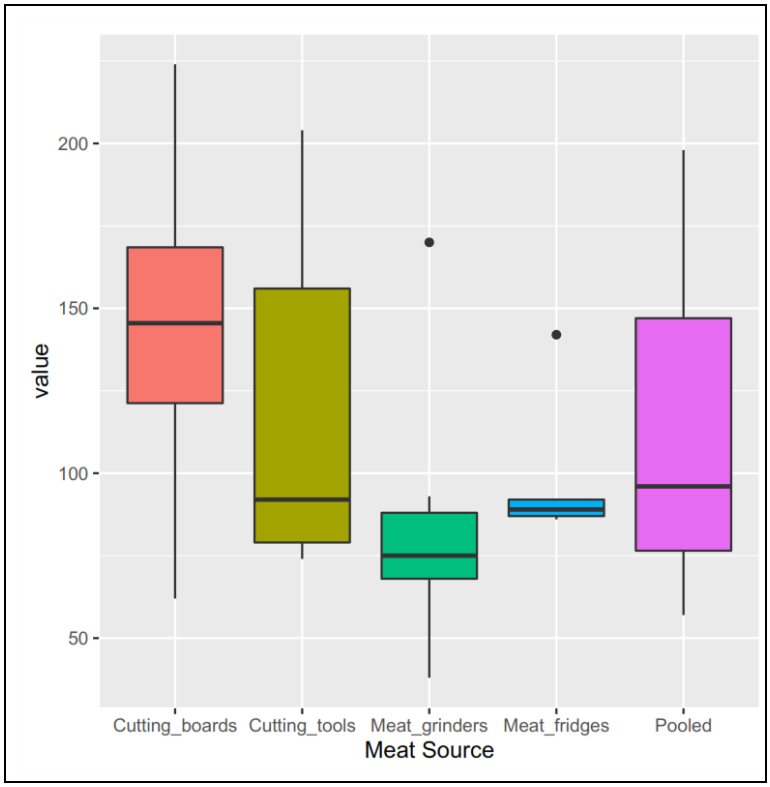

Figure 3: Boxplot of length of conservation regions for isolates collected from meat boards, meat tools, meat grinders and meat fridges.

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