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Review

Bovine *Escherichia coli* Mastitis and Effects on Milk Microbiota

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A B S T R A C T

Escherichia coli is a microorganism that is found in the normal intestinal microbiota of humans and warm-blooded animals, causing intestinal or extra-intestinal infections. Many pathogenic *E. coli* strains can cause diarrhea, septicemia, neonatal meningitis, mastitis, urogenital system infections and various intra-abdominal, lung, soft tissue and skin infections in pets. Determining the genotypic and phenotypic characteristics of *E. coli* strains isolated from animals is very important for the prevention of infections caused by *E. coli*. In recent years, a new animal pathotype mammary pathogenic *E. coli* (MPEC), which causes mammary gland infections in animals has been included in the extraintestinal pathogenic *E. coli* group. The fact that approximately 25%-35% of the use of antimicrobials in the treatment of Gram-negative agents is unsuccessful indicates that the use of correct diagnostic tools should take place in routine before etiological diagnosis. Profiles in bovine milk with mastitis suggest that clinical mastitis is associated with dysbacteriosis and that the microbial community in an intact mammary gland helps prevent intramammary infection. In this review, the change in bacterial diversity of milk microbiota due to antimicrobial use in *E. coli*-induced mastitis cases is discussed together with current studies.

***Keywords**: Escherichia coli, mastitis, microbiota*

Sığır *Escherichia coli* Mastitisleri ve Süt Mikrobiyotası Üzerine Etkileri

Ö Z E T

Escherichia coli, insan ve sıcak-kanlı hayvanların normal barsak mikrobiyotasında yer alan ve aynı zamanda intestinal veya ekstra-intestinal infeksiyonlara yol açan bir mikroorganizmadır. Birçok patojenik *E. coli* suşları, evcil hayvanlarda diyare, septisemi, neonatal meningitis, mastitis, ürogenital sistem infeksiyonları ile çeşitli intra-abdominal, akciğer, yumuşak doku ve deri infeksiyonlarına neden olabilmektedirler. Hayvanlardan izole edilen *E. coli* suşlarının genotipik ve fenotipik özelliklerinin belirlenmesi, *E. coli*'nin neden olduğu infeksiyonların önlenmesi açısından oldukça önemlidir. Son yıllarda, ekstraintestinal patojenik *E. coli* grubuna yeni bir hayvansal patotip olan ve hayvanlarda meme bezi enfeksiyonlarına yol açan meme patojenik *E. coli* (MPEC) dahil edilmiştir. Gram negatif etkenlerin tedavisinde antimikrobiyallerin kullanımının yaklaşık %25-%35'inin olumsuz sonuçlanması doğru tanı araçlarının kullanımının etiyolojik tanıdan önce rutinde yerini alması gerektiğini göstermektedir. Mastitisli sığır sütündeki profiller, klinik mastitisin disbakteriyoz ile ilişkili olduğunu ve sağlam bir meme bezindeki mikrobiyal topluluğun meme içi enfeksiyonu önlemeye yardımcı olduğunu göstermektedir. Bu derlemede *E. coli* kaynaklı mastitis olgularında antimikrobiyal kullanımına bağlı olarak süt mikrobiyotasının bakteri çeşitliliğindeki değişim güncel çalışmalarla birlikte ele alınmıştır.

***Anahtar kelimeler**: Escherichia coli, mastitis, mikrobiyota*

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Introduction

Escherichia coli (E. coli) was isolated from infant feces in 1885 by the German microbiologist and pediatrician Theodor Escherich and named as *Bacterium coli commune* In 1895, with the revision of the Bacterium genus, it was reclassified by Migula as *Bacillus coli*. Finally, in 1919, it was revised as *Escherichia coli* in honor of Theodor Escherich (Blount, 2015). As a result of the increase in the amount of *E. coli* in the intestinal microbiota of humans and animals, various infections may ocur (İzgür et al., 2006). Pathogenic *E.coli* strains are zoonotic. It can be transmitted to humans through direct contact with the feces of animals or consumption of food and water contaminated with feces (Müştak et al., 2013). The factors that play a role in the emergence of *E. coli* infections can be listed as inadequate hygienic conditions, not giving colostrum to newborn puppies, not vaccinating, increase in the number of intestinal microbiota, nutritional disorders and stress (İzgür et al., 2006).

E. coli general characteristics, typing and pathotypes

E. coli is a Gram-negative, generally motile, cylindrical, non-spore-shaped, bacillus bacterium belonging to the *Enterobacteriaceae* family. Some strains of *E. coli* contain capsules and fimbriae (Gomes et al., 2016). Escherichia genus is located in the *Enterobacteriaceae* family in the *Gammaproteobacteria* class, which is the largest subclass of *Proteobacteria* (Brenner et al., 2005). *E. coli* replicates approximately every 20 minutes. It can grow rapidly in aerobic or anaerobic environment in a wide temperature range of 18 °C - 44 °C in liquid and general solid media (Jang et al., 2017). Colonies of *E. coli* strains formed on blood agar and nutrient agar are convex, shiny, smooth-edged and colorless. Due to lactose fermentation on MacConkey agar, it produces bright, pink colonies surrounded by a precipitate. On EMB agar, they form dark, green-black metallic green colonies (Prahlad et al., 2018). *E. coli* is an oxidase negative, catalase positive, nitrate reduction positive and mostly lactose fermenting bacteria (Al Humam, 2016). Commercial kits such as API 20E, which include customized tests for the determination of biochemical properties, can be used to distinguish *E. coli* from bacteria in the *Enterobacteriaceae* family and other *Escherichia* species (Liu et al., 2015).

Guanin sitozin percentage (G+C) content of *E. coli* strains is between 48 and 59. The average DNA relationship calculated based on DNA-DNA hybridization (DDH) varies between 29% and 94% within *Escherichia* species, and the DNAs of different *E. coli* strains are closely related with an average of 84% (Scheutz and Strockbine, 2015).

E. coli strains are subtyping using the combination of the three main surface antigens, somatic O, flagellar H and capsular K antigens (O:K:H), and serotyping based on O and H antigens is considered the gold standard, since the possibility of K antigen typing is limited (DebRoy et al., 2011).

Phage typing reveals the pathogenic roles of serologically unidentified *E. coli* strains. Due to the production difficulties of antisera, phage typing of *E. coli* K1, K3, K5, K7, K12, K13 and K95 strains is very useful for some antigens (Scheutz and Strockbine, 2015).

Clermont et al. (2000) in 2000, they defined the triplex PCR method called Clermont Typing by providing a simple and rapid detection of *E. coli* phylogroups based on the detection of *E. coli* haem-utilization (chuA) gene, uncharacterized protein yjaA gene and tail - specific protease (TSPE4.C2). Later, in the light of new genomic data, the arpA gene was added to the protocol as an additional gene region, and the triplex PCR method was revised as the quadruplex PCR method (Clermont et al., 2013). In recent years, with the evaluation of whole genome sequence data, some *E. coli* strains have been included in a new phylogroup defined as phylogroup G between phylogroup B2 and phylogroup F (Clermont et al., 2019).

Virulence genes are not the only feature used to distinguish pathogenic *E. coli* strains from commensal strains, but phenotypic determination of virulence genes is also an important factor. *E. coli* strains are divided into two as those that cause intestinal (intestinal) and extra-intestinal (extraintestinal) infections (Omerovic et al., 2017). *E. coli* strains isolated from intestinal diseases were divided into different main categories according to phenotypic features, epidemiological features, clinical features of the diseases they cause, and specific virulence factors. Intestinal *E. coli* pathotypes; It consists of Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Diffuz Aderent *E. coli* (DAEC) and Vero- or Shiga-toxin producing *E. coli* (VTEC or STEC) (Scheutz and Strockbine, 2015). Extraintestinal pathogenic *E. coli* (ExPEC) strains are facultative pathogens and are commensal in the intestinal microbiota of healthy animals. Pathotypes in the ExPEC group; It includes Septicemic Pathogenic *E. coli* (SEPEC), Neonatal Meningitis *E. coli* (NMEC), Uropathogenic *E. coli* (UPEC), Avian Pathogenic *E. coli* (APEC) and other potential *E. coli* pathotypes. In recent years, mammary pathogenic *E. coli* (MPEC), which causes mammary gland infections in animals, and endometrial pathogenic *E. coli* (EnPEC), which causes uterine infections, have been added to other potential *E. coli* pathotypes (Omerovic et al., 2017).

Mammary pathogenic E. coli (MPEC)

Apart from the typical factors involved in *E. coli* virulence in general, the specific pathogenic mechanisms specific to MPEC strains are not yet clearly known. It is stated that mastitis occurs after local immune response triggered after detection of various *E. coli* compounds by mammary gland epithelium and immune cells. The most known of these compounds is lipopolysaccharide, which is considered to be the major virulence factor in mastitis caused by *E. coli*. In studies conducted on *E. coli* mastitis, MPEC strains were determined to cause acute (VL2874 and P4 strains) or chronic (VL2732 strain) mas-

Table 1. Characteristics of MPEC strains (Blum et al., 2015).

Strain	Source	Serotype	Phylogenetic group	Development in milk/ Phagocytosis resistance	Mammary pathogenic Mouse/Cow
VL2874	Acute masititis	O141:H4	A	Fast/High	Yes/Yes
VL2732	Chronic mastitis	O8:H30	A	Fast/High	Yes/Yes
P4	Acute masititis	O32:H37	A	Fast/High	Yes/Yes
K71	Barn	O58:H40	B1	Slow/Low	No/No

titis and shown in Table 1 (Blum et al., 2015).

E. coli VL2874 strain has the RTX toxin cluster containing the hemolysin genes hlyA, hylC, and hylD. VL2874 is the only strain that shows hemolytic character in sheep blood agar among other mastitis agent MPEC strains. hylA has been found to increase pathogenicity in ExPEC strains. In addition, TosA, which is very similar to the RTX toxin, has been associated with pathogenicity in UPEC strains (Vigil et al., 2011). In the search for the *E. coli* plasmids database in NCBI, it was determined that Col (RNAI) identified in the genome of the VL2874 strain, is a plasmid type that is very closely related to the ColE-1-like plasmid p302S (AY333433) found in *Salmonella enterica subsp. enterica*. VL2732 strains contain the toxin mRNA interferase YgiU or MqsR, which transforms into permanent cells under biofilm formation and stress, and the yersiniabactin siderophore, which is also associated with biofilm formation in iron-limited environments such as milk. Yersiniabactin, which is also found in APEC strains, plays an important role in the formation of chronic mastitis (Blum et al., 2015).

Effects of E. coli on microbiota in clinical mastitis

A 100-year review of the Journal of Dairy Science states that mastitis, defined as inflammation or inflammation of the mammary gland, remains the most common bacterial disease in dairy farms. In addition, it is stated that studies continue on the treatment of mastitis or preventing the use of frequently used antibacterials. In clinical mastitis cases, a treatment protocol is established depending on the results of aerobic culture, and the treatment of Gram-positive mastitis agents is generally successful. Studies on pathogen-based protocols later showed that the efficacy of intramammary antimicrobial applications against Gram-negative mastitis agents may be insufficient (Vasquez et al., 2019). The fact that approximately 25%-35% of the use of antimicrobials in the treatment of Gram-negative agents is unsuccessful indicates that the use of correct diagnostic tools should take place in routine before etiological diagnosis (Oliveira and Ruegg, 2014). Sequencing and analysis of highly variable regions in the 16S rRNA gene can assess bacterial diversity in the milk of cows with mastitis, as well as reveal which organisms contribute to culture-negative and other etiological diseases (Oikonomou et al., 2014).

Porcellato et al. (2020) described that milk in healthy cattle has a complex microbiota, mainly composed of *Corynebacteriaceae* and *Staphylococcaceae* families. They identified these two families in all cows and therefore identified them as part of the core microbiota with potential implications for udder health. Profiles formed in bovine milk with mastitis suggest that clinical mastitis is associated with dysbacteriosis and that the microbial community in an intact mammary gland helps prevent intramammary infection (Fernández et al., 2013; Oikonomou et al., 2014).

Ganda et al. (2017) reported high microbial diversity in the microbiota before the experimental infusion of *E. coli* into the breast (72 hours before). It was determined that there was no difference between the microbial profile in the 4 groups formed before the study. In the samples of all groups before the study, the most abundant families were found as, *Ruminococcaceae* (16.8%±10.1%), *Lachnospiraceae* (7.0%±5.1%), *Aerococcaceae* (6.8%±8.2%), *Enterobacteriaceae* (6.3%±13.5%), *Planococcaceae* (5.7%±9.5%), *Bacteroidaceae* (5.4%±3.3%), *Corynebacteriaceae* (5.1%±7.3%), *Clostridiaceae* (4.2%±3.1%), *Bacillaceae* (3.5%±3.7%) ve *Staphylococcaceae* (2.8%±4.9%) in order from the most dense to the least dense. It is seen that the microbial diversity is much higher in the unchallenged groups than in those with experimental mastitis infection with *E. coli* (Figure 1a). It was determined that there was no significant change in the mean densities of the 25 families, which were determined to be the most intense before the study, in the group in which intramammary ceftiofur was administered without any experimental infection (Figure 1b). In the challenged untreated group after the challenge application, it remained above the 30% average intensity between 12 and 150 hours and reached a peak level of 64.7% at the 12th hour (Figure 1c). In the challenged treated group after the challenge application, it remained above the 30% average intensity between 12 and 600 hours and reached a peak level of 77.9%

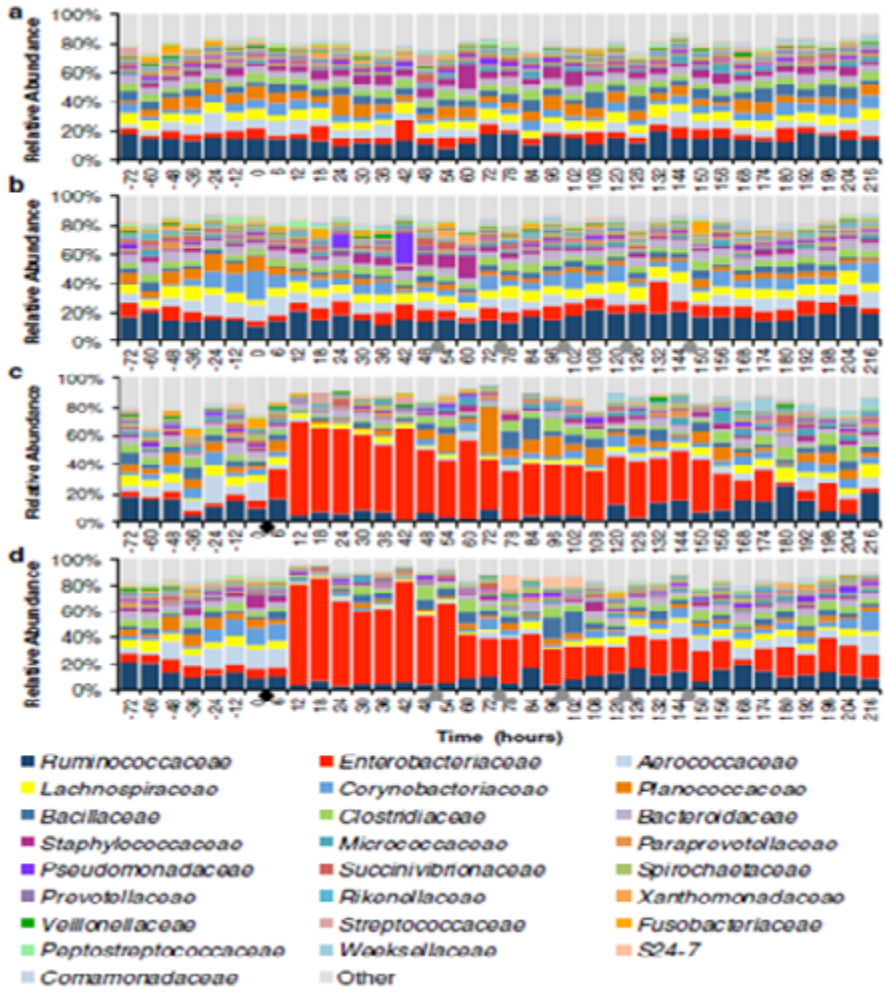

Figure 1. The effect of ceftiofur treatment and *E. coli* challenge application on the average densities of 25 families in milk microbiota in cattle (Ganda et al., 2017).
a. Unchallenged and untreated group
b. Unchallenged and treated group,
c. Challenged and untreated group,
d. Challenged and treated group.
(Black backpoints represent experimental infection with 100 CFU of *E. coli* and grey backpoiints represent intramammary treatment with ceftiofur)

at the 18th hour (Figure 1d). It was observed that the microbial profile of the milk changed significantly in the groups challenged with *E. coli* (Figure 1c, 1d).

Before the challenge was applied, *Ruminococcaceae* was determined as the most dense family. The mean densities of the *Ruminococcaceae* family were found to be 14.3% and 13.3% respectively in the challenged treated and challenged untreated groups. A significant increase in the densities of the *Enterobacteriaceae* family was detected in the challenge applied groups (Figure 2). Intramammary administration with ceftiofur hydrochloride did not show a significant effect on the average density ratio of the *Enterobacteriaceae* family, and it also did not provide a significant decrease in any time zone after the challenge test when compared to the untreated group (Figure 2).

They stated that Shannon diversity index values were high and similar in all groups before the challenge application. Diversity values decreased sharply after *E. coli* challenge application and the lowest diversity values were observed 30 and 78 hours after challenge application. They found that challenged treated animals had significantly different indices of diversity than challenged untreated animals at 78, 102 and 180 hours. They did not detect any significant difference between the diversity levels of the unchallenged treated and unchallenged untreated groups (Figure 3).

Ganda et al. (2017) found that 3 of 12 cows that were challenged in the study were not infected. Schukken et al. (2009) and Burvenich et al. (2003) stated that the high number of somatic cells in the time period before the challenge was stated as the best explanation for this. Animals infected after challenge administration showed a sharp increase in linear score 18 hours after administration. They found that linear scores were higher in animals that were challenged but not infected, compared to those that were infected before the challenge (Figure 4).

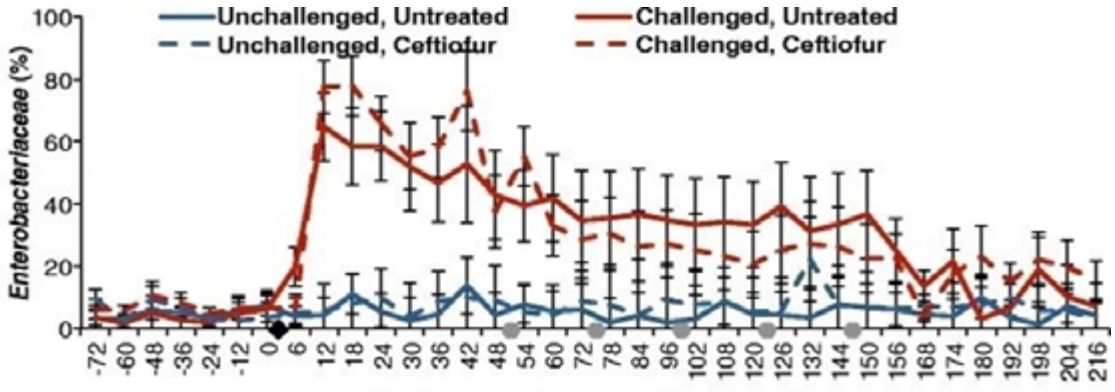

Figure 2. Effects of experimental infection with *E. coli* and treatment with ceftiofur on average densities of the *Enterobacteriaceae* family (Ganda et al., 2017).
(Black backpoints represent experimental infection with 100 CFU of *E. coli* and grey backpoints represent intramammary treatment with ceftiofur)

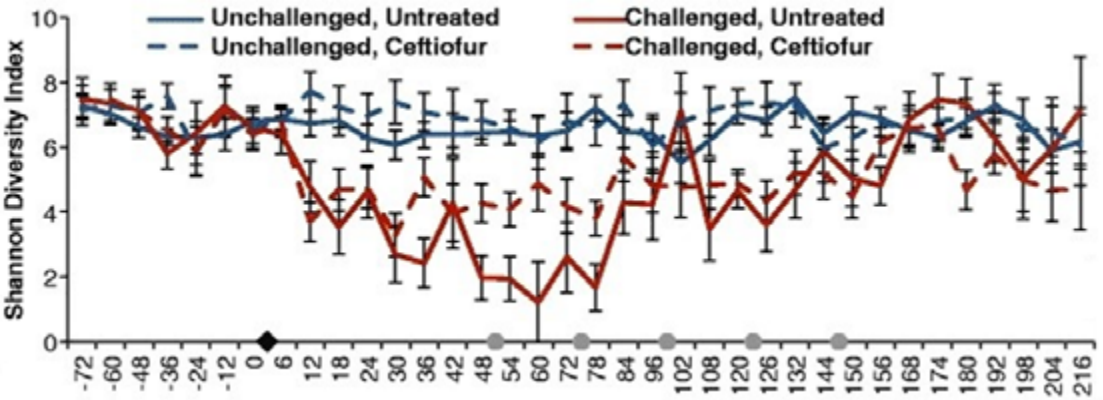

Figure 3. Shannon diversity index graph of microbiota as a result of experimental infection with *E. coli* and treatment with ceftiofur (Ganda et al., 2017).
(Black backpoint represent experimental infection with 100 CFU of *E. coli* and grey backpoints represent intramammary treatment with ceftiofur)

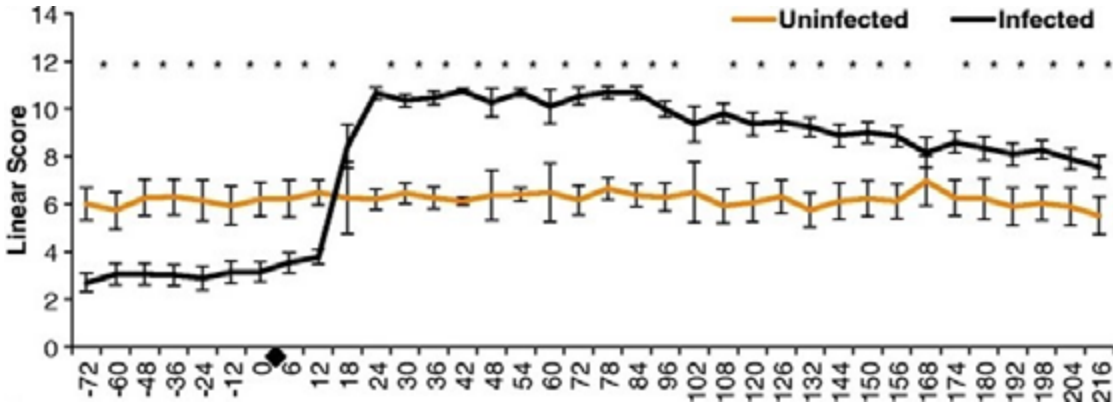

Figure 4. Effects of experimental infection with *E. coli* on linear score values in infected and uninfected cows (Ganda et al., 2017). (Black backpoint represent experimental infection with 100 CFU of *E. coli*)

As a result of the study, Ganda et al. (2017) determined that the application of ceftiofur treatment in *E. coli*-induced mastitis did not cause a significant change in the milk microbiome.

Lima et al. (2018) determined that *Firmicutes* (57.7%±7.6%) and *Proteobacteria* (26.0%±7.6%) phyla predominate as a result of sequence analysis performed on healthy milk samples. When milk sample fractions (whole milk, fat, oil+pellet, pellet) and extraction kits (PowerFood, PowerSoil) were compared, they found that there was no significant difference in the mean values of these two phyla. They reported that the *Proteobacteria* phylum in *E. coli* and *Klebsiella spp.* mastitis samples comprised approximately 98% of the 16S rRNA sequences detected independently of milk fractions and DNA extraction kits. In milk samples with mastitis caused by *Streptococcus spp.* most of the sequences were

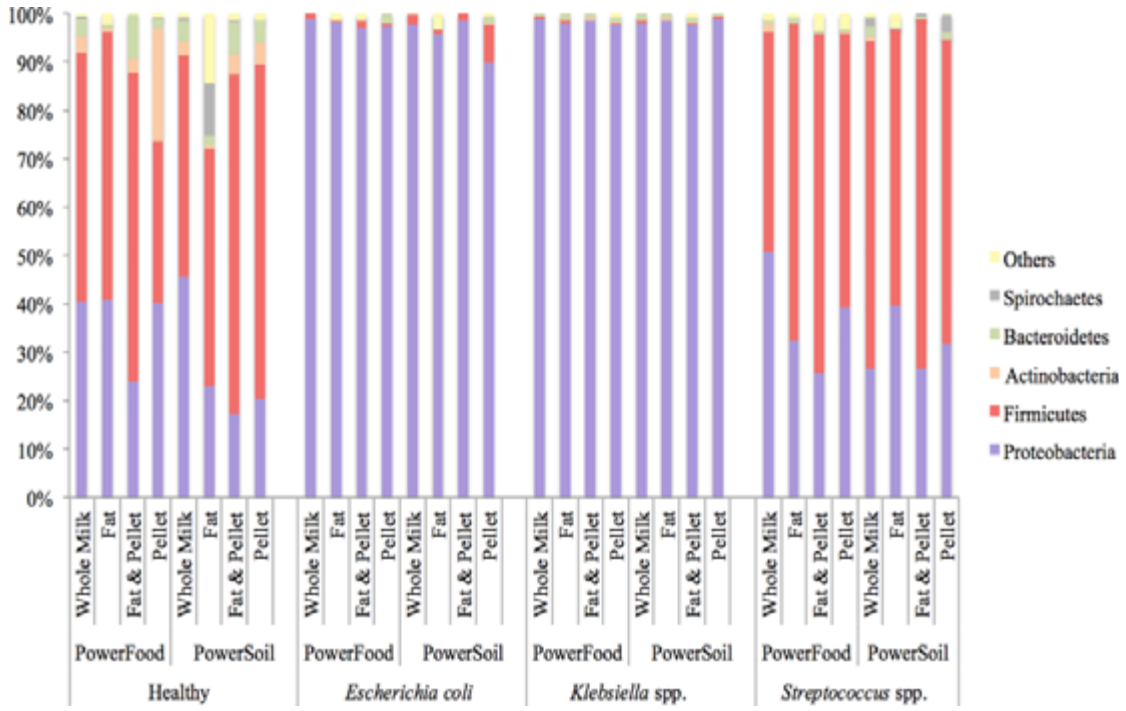

Figure 5. Mean relative abundance of the most prevalent bacterial phyla identified in healthy milk samples and milk samples from cows diagnosed with clinical mastitis due to Escherichia coli, Klebsiella spp. and Streptococcus spp. infection according to four milk sample fractions (whole milk, fat, fat + pellet, and pellet) and two different DNA extraction kits (PowerFood and PowerSoil) (Lima et al., 2018).

found to be associated with *Firmicutes* (69.6%±9.5%) and *Proteobacteria* (30.1%±9.4%) (Figure 5).

They stated that they detected 62 families in common in two DNA extraction kits in healthy milk samples on the Venn diagram (Figure 6a). It has been determined that these 62 families constitute 95.65% of the average density in healthy milk samples. *Ruminococcaceae*, *Enterobacteriaceae*, *Staphylococcaceae*, *Bacillaceae*, *Streptococcaceae* and *Pseudomonadaceae* were the most common bacterial families detected in all fractions of healthy milk samples (Figure 6b). On the venn diagram created according to the sample fractions, it was stated that 4 families within 28 common families constitute the main microbiota (87.8%±2.4%) defined among all milk sample fractions (Figure 6c). They determined that the four families that make up this main microbiota consist of *Ruminococcaceae*, *Enterobacteriaceae*, *Bacillaceae* and *Pseudomonadaceae*.

Lima et al. (2018) identified 4 families as common by two extraction kits among 30 families detected on the venn diagram in milk with mastitis originating from *E. coli* (Figure 7).

It has been revealed that the determination of the *Enterobactericeae* family in the isolations made in the two DNA isolation kits was done correctly and there was no significant difference between the average densities determined in the protocols. It was determined that only in the pellet sample fraction, the PowerFood extraction kit found the average density of the *Enterobacteriaceae* family 10% higher than that of PowerSoil (Figure 8).

As a result of the study by Lima et al. (2018) the total number of families determined as a result of sequencing in *E. coli* and *Klebsiella spp.* mastitis samples were 30 and 28, respectively; It was determined as 64 in mastitis samples originating from *Streptococcus spp.* The results of this study show that the milk microbiome changes very significantly in cases of mastitis originating from the *Enterobacteriaceae* family, especially *E. coli* and *Klebsiella spp*.

Ganda et al. (2016) in metagonomic analyzes of milk microbiota, showed that the mean density of *Proteobacteria* phylum was higher in milk with mastitis caused by *E. coli*, *Klebisella spp.* and *Pseudomonas spp.* compared to healthy milk. In the comparison made with the causative agent, the rise of the phylum it belongs to during the infection was mostly seen in the milk with mastitis caused by *E. coli* (Figure 9).

Ganda et al. (2016) applied ceftiofur to animals with *E. coli* mastitis for 5 days in their study. They stated that the general bacterial load decreased on the 3rd day in the treated group compared to the untreated group, but no effect was observed at the end of the 8th day. As a result of the study, the control group (infected and non-infected), treated and untreated groups were thoroughly examined. At the end of the 14th day, they determined that the bacterial diversity was higher in the milk samples of the treated and untreated milk samples of those who did not show clinical signs. The mean relative abundance of bacteria from the phylum *Proteobacteria* was greater in the milk from mastitic quarters infected by *E. coli* and *Pseudomonas spp.* compared

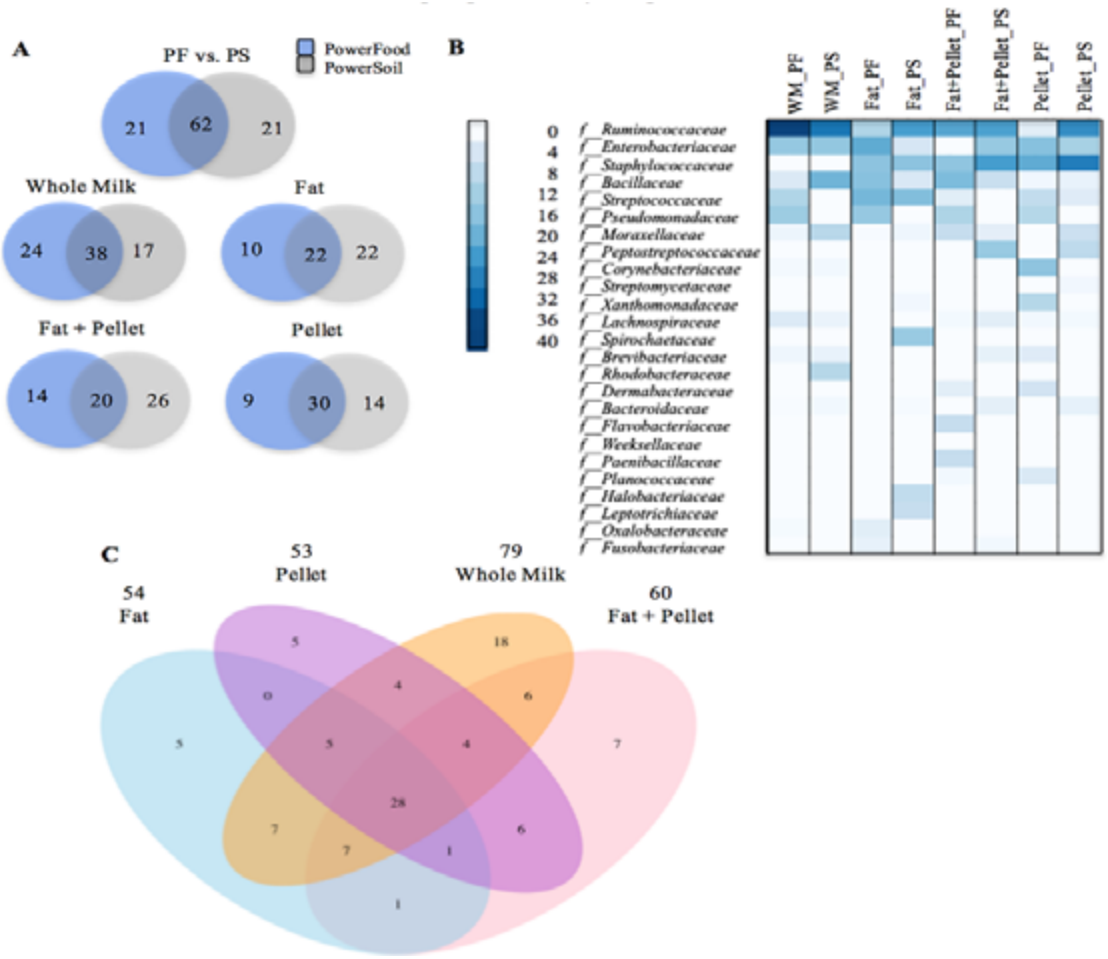

Figure 6. The most common bacterial families detected by Venn diagram showing the degree of overlap of bacterial families between two DNA extraction kits in 4 sample fractions in healthy milk samples (Lima et al., 2018).
a. Venn diagrams showing the numbers of unique and shared bacterial families for healthy milk samples,
b. Heatmap illustrating the 25 most common bacterial families ranking by relative abundance identified in healthy milk samples according to milk sample fractions: fat, fat + pellet, pellet, and whole milk and DNA extraction kit,
c. Venn diagram showing the numbers of unique and shared bacterial operational taxonomic unit according to milk sample fractions. Numbers at the top of each milk sample type are the total number of families detected in samples processed by that protocol.

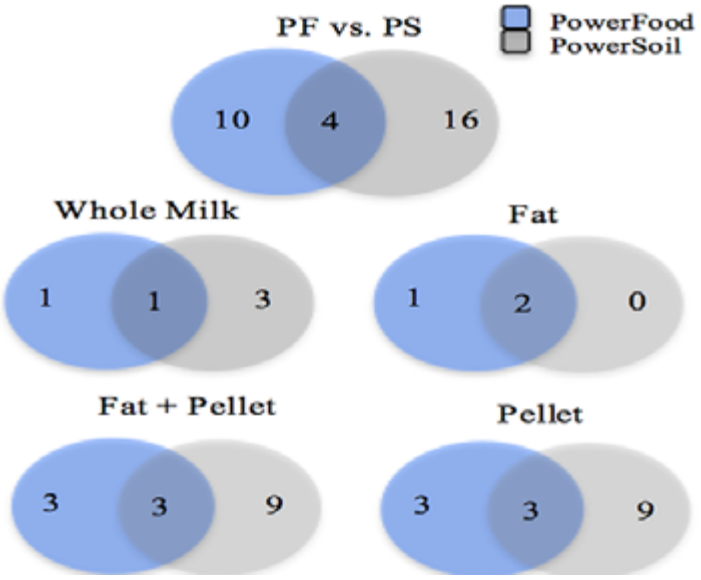

Figure 7. Venn diagrams showing the degree of overlap of bacterial families between two DNA extraction kits relative to sample fractions of milk with E. coli-induced mastitis (Lima et al., 2018).

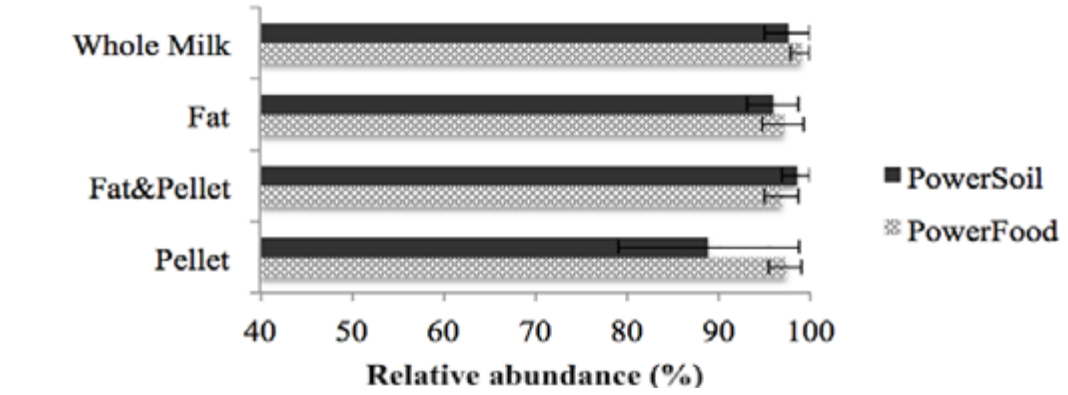

Figure 8. Average densities of the Enterobacteriaceae family detected in each milk sample fraction (Lima et al., 2018).

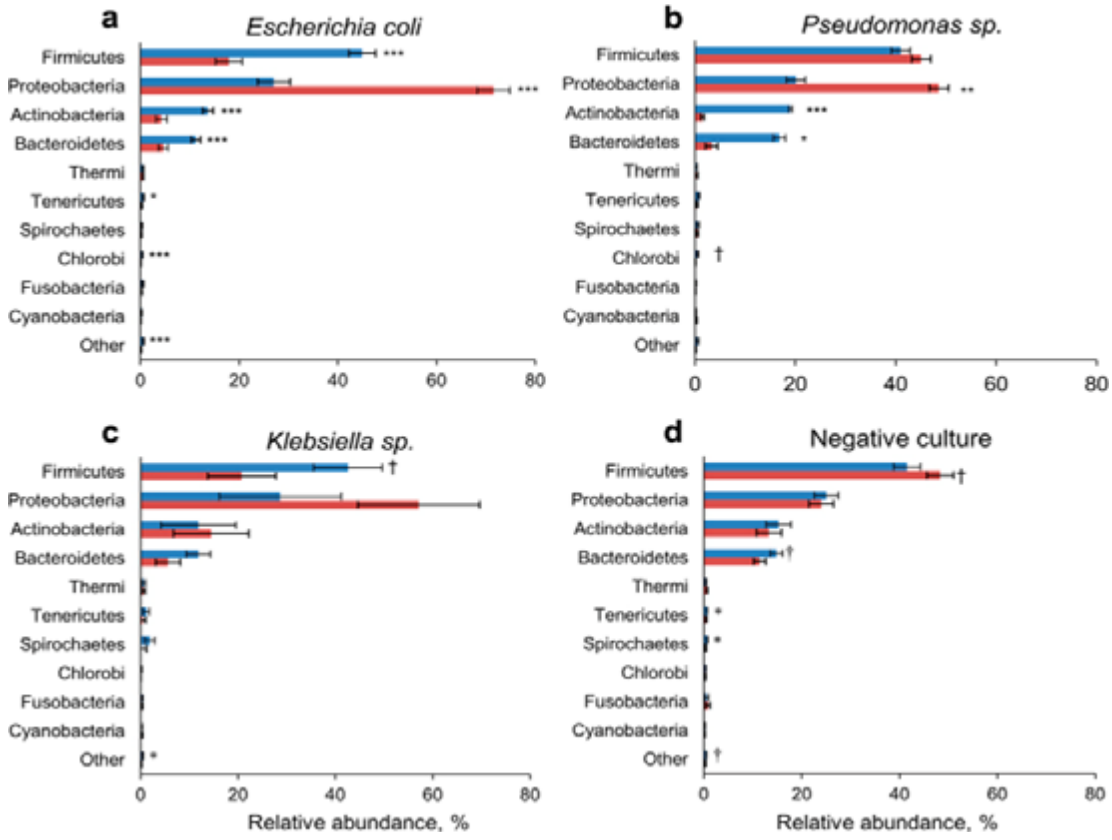

Figure 9. Average phylum densities in milk with clinical mastitis of different origin (red bars) and healthy milk (blue bars) (Ganda et al., 2016).

with that of healthy quarters. This was driven mostly by greater abundances of *Enterobacteriaceae* (Figure 10).

Conclusion

In dairy farming the inability to make a correct diagnosis against mastitis infections, the use of wrong antibiotics, the increase in antibiotic resistant bacterial populations caused by antibiotic use, and the decrease in bacterial diversity in milk microbiota are stated as the most basic problems today. The use of antimicrobials in the food industry is considered as one of the potential factors that may affect human health apart from its effective use. Ceftiofur is the only third generation cephalosporin FDA approved for use in food production animals, especially against gram-negative agents and *Enterobacteriaceae* family infections such as *E. coli*. However, it has been classified as one of the critically important antimicrobials for humans by the World Health Organization. Studies on milk microbiota have also shown that the efficacy of ceftiofur treatment is not sufficient in cases of culture-negative, *E. coli* and other causative mastitis. Studies have shown that bacterial diversity in milk microbiota decreases more significantly in *Enterobacteriaceae* and especially in *E. coli*-induced mastitis cases compared to other causative mastitis cases. It is necessary to characterize dysbacteriosis cases on the basis of the agent in mastitis cases, and to consider the bacterial diversity in the microbiota, treatment protocols, linear score and changes in milk yield after infection. In addition to these factors, it has been concluded that long-

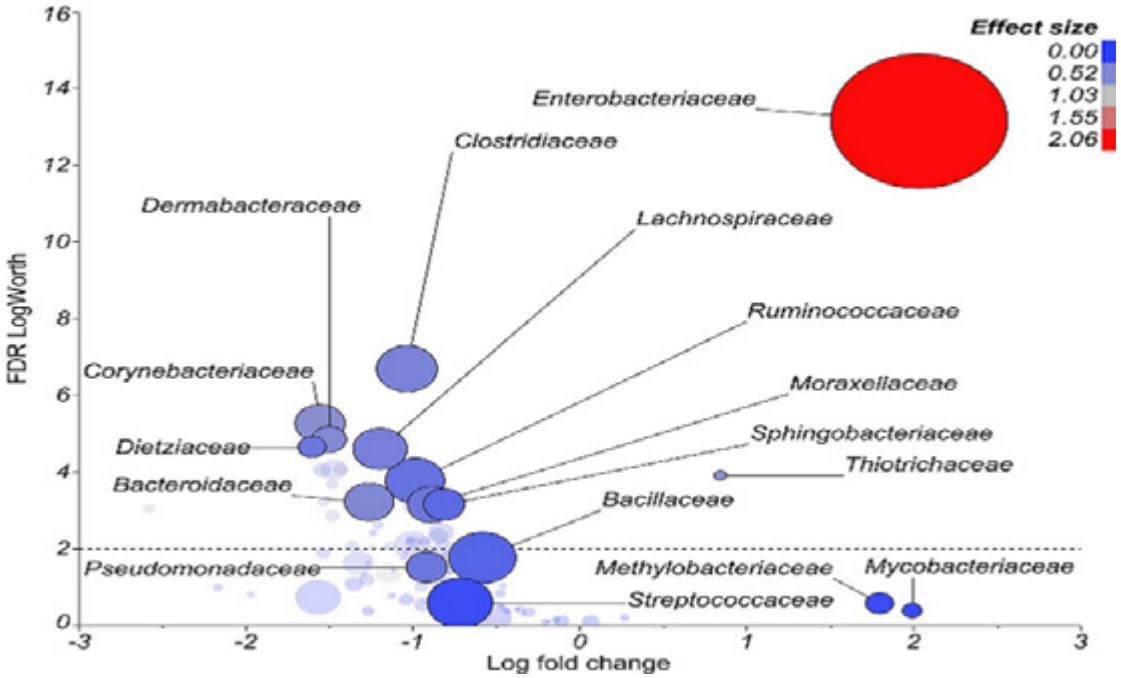

Figure 10. Comparison of the microbiome from quarters with clinical mastitis associated with E. coli and healthy quarters (Ganda et al., 2016).

term and detailed evaluations are required in scientific studies on the milk microbiome, taking into account the clinical condition of the animals.

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Conflict of Interest

The authors declare that they have no conflict of interest in this study.

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