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RESEARCH ARTICLE

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16S ribosomal RNA (rRNA) sequences for a phylogenetic analysis of milk contaminants

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ABSTRACT: Milk is considered a highly nutritious food as it contains essential macronutrients necessary for human growth and overall well-being. Previous research indicates that raw milk serves as an ideal medium for bacterial growth, which makes it a potential source of contamination. Pathogenic bacteria in milk pose a major public health risk, contributing to approximately 90% of dairy-related illnesses. This study examined five selected bacterial genera, four of which belong to the ESKAPE group, while the fifth is a globally recognized foodborne pathogen. The study focused on analyzing their evolutionary and phylogenetic relationships using 16S rRNA sequences. The phylogenetic relationships shown in the phylogenetic tree illuminate both the common and unique evolutionary paths that culminate in the formidable virulence of ESKAPE pathogens. Whether through shared inheritance or the dynamic exchange of genetic material, these relationships help explain why these bacteria are so adept at causing life-threatening infections and resisting antibiotics.

KEYWORDS: 16s rRNA, ESKAPE, foodborne pathogen, milk

INTRODUCTION

Milk is considered a complete food due to its essential macronutrients necessary for human growth and well-being [1]. Kubala and Stanuch [2] highlighted that milk is a rich source of vitamins, minerals, and proteins. In the Philippines, total dairy consumption reached approximately 3.27 million metric tons in 2022, while local production only meets 1% of the country's annual dairy requirement, with the remaining 99% being imported [3]. Raw milk, however, provides a conducive environment for pathogenic bacteria, which causes contamination making it a potential source of contamination. Pathogenic bacteria in milk are a significant public health concern, accounting for about 90% of dairy-related illnesses [4]. The genera *Campylobacter*, *Acinetobacter*, *Enterobacter*, *Enterococcus*, and *Pseudomonas* are frequently identified as contaminants in raw milk [1-7]. With this group are the ESKAPE pathogens, (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) that are known for their ability to resist multiple antibiotics, biofilm production and enzymatic degradation of drugs, and cause serious infections [1, 2, 7]. On the other hand, *Campylobacter* is excluded from the ESKAPE group since it is less commonly associated with multidrug resistance [8, 9]. These bacteria utilize distinct virulence factors, such as motile flagella and cytolethal distending toxins, to colonize and damage the intestinal lining. Although not part of the ESKAPE pathogens, *Campylobacter* remains a critical public health issue due to its high prevalence and increasing resistance trends [10-12]. Meanwhile, *Enterobacter* species can contaminate milk and can survive pasteurization and persist in dairy environments, which can lead to off-flavors and safety issues in milk [5, 13]. On the other hand, *Acinetobacter* species can survive in milk under refrigeration and may contribute to spoilage and contamination, posing risks to milk safety [14]. In addition, *Enterococci* are particularly concerning in dairy due to their persistence in harsh conditions and ability to survive pasteurization, leading to potential milk contamination and reduced product safety [15]. Moreover, *Pseudomonas aeruginosa*, can grow and produce heat-stable enzymes that break down milk proteins, fats, and carbohydrates. This enzymatic activity results in off-flavors, changes in texture, and spoilage, which shorten the shelf life of dairy products [16]. Contamination with *Pseudomonas* often arises from poor hygiene practices, or insufficient cleaning of milking equipment, highlighting the importance of implementing strict control measures to

maintain milk quality and safety [17]. Meanwhile, the ability to form biofilms and adapt to low-temperature storage conditions of *Campylobacter* causes contamination of milk and dairy products [9, 10, 18, 19]. Contaminated milk poses a public health risk, especially when proper hygiene and pasteurization practices are not consistently applied [1, 2, 20].

Phylogenetic analysis of ESKAPE pathogens in milk contaminants is crucial for several scientific and practical reasons. This can uncover how ESKAPE pathogens adapt to survive in diverse environments, including milk and dairy products. This is vital for improving sanitation practices during production and processing. This could also provide critical insights into the evolution, resistance mechanisms, and ecological adaptations of ESKAPE bacteria in milk.

MATERIALS AND METHODS

Data mining

Seventy species of pathogenic bacteria with about 1,450-bp 16S rRNA gene sequences from NCBI database were selected of the selected species of bacteria belonging the genera of ESKAPE pathogens but are part of the ESKAPE such as *Pseudomonas*, *Enterococcus*, *Enterobacter*, *Acinetobacter*, *Klebsiella* and *Staphylococcus* were selected. *Campylobacter* was also included to the ingroup while *Escherichia coli*, *Mycobacterium tuberculosis*, and *Bacillus subtilis* served as the outgroups. Presented in Table 1 are the selected taxa with their corresponding class and accession number.

Multiple sequence alignment

Multiple sequence alignment was performed using the ClustalW in MEGA with 15.00 gap opening penalty and 6.66 gap extension penalty. Further, realignment was done using clustal Omega v1.2.4 [21], using fasttree v2.1.8 default and clustalo default [22].

Phylogenetic analysis

The phylogenetic trees were constructed using MEGA X. The aligned sequences were trimmed to eliminate biases. Phylogenetic trees were constructed using maximum-likelihood methods evaluated by a bootstrap analysis with 1000 replicates. Evolutionary relationships of the sampled species were inferred using the bootstrap consensus trees. The best maximum-likelihood model was determined and applied for the construction of each phylogenetic tree. Phylogenetic analysis was carried out through interpretation of the phylogenetic tree based on the relationship of the sample.

Table 1. Classification of bacteria used in the study.

Class	Species	Accession Number
Gammaproteobacteria	*Pseudomonas azotoformans*	D84009.1
Gammaproteobacteria	*Pseudomonas synxantha*	D84025.1
Gammaproteobacteria	*Pseudomonas taetrolens*	D84027.1
Gammaproteobacteria	*Pseudomonas putida*	AB109013.1
Gammaproteobacteria	*Pseudomonas fragi*	D84014.1
Gammaproteobacteria	*Pseudomonas aeruginosa*	AB126582.1
Gammaproteobacteria	*Enterobacter chengduensis*	NR_179167.1
Gammaproteobacteria	*Enterobacter mori*	ON600471.1
Gammaproteobacteria	*Enterobacter sp*	JF939050.1
Gammaproteobacteria	*Enterobacter mori*	ON600470.1
Gammaproteobacteria	*Enterobacterquasiroggenkampii*	NR_179166.1
Gammaproteobacteria	*Enterobacter cloacae*	MF953264.1
Gammaproteobacteria	*Enterobacter kobei*	MF953259.1
Gammaproteobacteria	*Enterobacter cloacae*	ON384772.1
Gammaproteobacteria	*Enterobacter cancerogenus*	MH024380.1
Gammaproteobacteria	*Acinetobacter baumannii*	*U10874.1*
Gammaproteobacteria	*Acinetobacter junii*	*AB101444.1*
Gammaproteobacteria	*Acinetobacter pullicarnis*	*NR_169498.1*
Gammaproteobacteria	*Acinetobacter sp.*	*KX955254.1*
Gammaproteobacteria	*Acinetobacter wuhoensis*	*KY853661.1*
Gammaproteobacteria	*Acinetobacter defluvii*	*NR_156989.1*
Gammaproteobacteria	*Acinetobacter chinensis*	*NR_165666.1*
Gammaproteobacteria	*Acinetobacter johnsonii*	*AB099655.1*
Gammaproteobacteria	*Acinetobactervariabilis*	*NR_134685.1*
Psilonproteobacteria	*Campylobacter iguanorium*	*KF425533.1*
Epsilonproteobacteria	*Campylobacterfetus subsp. Veneralis*	*M65011.1*
Epsilonproteobacteria	*Campylobacter fetus*	*AF482990.1*
Epsilonproteobacteria	*Campylobacter sputorum*	*AF022768.1*

Epsilonproteobacteria	*Campylobacter lanienae*	*OQ561783.1*
Epsilonproteobacteria	*Campylobacter sp*	*PP989493.1*
Epsilonproteobacteria	*Campylobacter lari*	*L04316.1*
Bacilli	*Enterococcus faecalis*	*AB036835.1*
Bacilli	*Enterococcus sanguinicola*	*FJ378700.1*
Bacilli	*Enterococcus durans*	*EU333895.1*
Bacilli	*Enterococcus faecium*	*FJ378666.2*
Gammaproteobacteria	*Pseudomonas syringae*	*NR_043716.1*
Gammaproteobacteria	*Enterobacter hormaechei*	*NR_042154.1*
Gammaproteobacteria	*Enterobacter asburiae*	*NR_145647.1*
Gammaproteobacteria	*Enterobacter soli*	*NR_117547.1*
Gammaproteobacteria	*Enterobacter bugandensis*	*NR_148649.1*
Gammaproteobacteria	*Acinetobacter pittii*	*NR_116774.1*
Gammaproteobacteria	*Acinetobacter lwoffii*	*NR_026209.1*
Gammaproteobacteria	*Acinetobacter calcoaceticus*	*NR_042387.1*
Gammaproteobacteria	*Acinetobacter nosocomialis*	*NR_117931.1*
Gammaproteobacteria	*Acinetobacter indicus*	*NR_117784.1*
Epsilonproteobacteria	*Campylobacter jejuni*	*NR_041834.1*
Epsilonproteobacteria	*Campylobacter helveticus*	*NR_025948.1*
Epsilonproteobacteria	*Campylobacter concisus*	*NR_043604.1*
Epsilonproteobacteria	*Campylobacter upsaliensis*	*NR_043602.1*
Bacilli	*Enterococcus cecorum*	*NR_024905.1*
Bacilli	*Enterococcus hirae*	*NR_037082.1*
Bacilli	*Enterococcus mundtii*	*NR_024906.1*
Bacilli	*Enterococcus casseliflavus*	*NR_104560.1*
Bacilli	*Enterococcus gallinarum*	*NR_113924.1*
Bacilli	*Staphylococcus aureus*	*NR_118997.2*
Bacilli	*Staphylococcus epidermidis*	*NR_036904.1*
Bacilli	*Staphylococcus warneri*	*NR_025922.1*
Bacilli	*Staphylococcus saprophyticus*	*NR_074999.2*
Gammaproteobacteria	*Klebsiella pneumoniae*	*NR_036794.1*
Gammaproteobacteria	*Klebsiella aerogenes*	*NR_102493.2*
Gammaproteobacteria	*Klebsiella variicola*	*NR_025635.1*
Gammaproteobacteria	*Klebsiella oxytoca*	*NR_041749.1*
Gammaproteobacteria- outgroup	*Escherichia coli*	*NR_024570.1*
Bacilli- outgroup	*Bacillus subtili*	*NR_112116.2*
Actinobacteria- outgroup	*Mycobacterium tuberculosis*	*NR_102810.2*

RESULTS AND DISCUSSION

In the phylogenetic tree of ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter*) and related bacteria, distant species serve as outgroups (Figure 1). The *M. tuberculosis, E.coli* and *Bacillus subtilis* are included to provide a reference point for rooting the tree and understanding the evolutionary relationships. *M. tuberculosis* is known for its unique cell wall structure and slow growth, which differentiate it from the faster-growing ESKAPE pathogens while *B. subtilis* belongs to the phylum Firmicutes, making it more closely related to the Gram-positive ESKAPE pathogens (*Enterococcus faecium* and *Staphylococcus aureus*) [23, 24]. However, it is not associated with antibiotic resistance as ESKAPE pathogens. *E. coli*, on the other hand is part of the same phylum (*Proteobacteria*) and shares functional similarities [24].

The members of the family Enterococcaceae, Staphylococcaceae, Oraxellaceae, Pseudomonadaceae and Enterobacteriaceae have diverse evolutionary linkage provide ESKAPE pathogens wide range or resistance mechanisms and ecological niches [25, 26]. Based from the selected taxa of bacteria in the study, three major clades were formed namely the Bacilli clade (*Staphylococcus* and *Enterococcus*), Gammaproteobacteria (*Acinetobacter*, *Enterobacter*, and *Pseudomonas*), and the Epsilonproteobacteria (*Campylobacter*). Gammaproteobacteria cluster tightly, indicating a strong evolutionary relationship, especially within the *Enterobacter* and *Klebsiella* species. Bacilli clade encompasses *S. aureus*, *S. epidermidis*, and related species. This group is distinct from the Gammaproteobacteria, representing an older Gram-positive lineage, distinct from the predominantly Gram-negative Gammaproteobacteria [26,

27]. Meanwhile, Epsilonproteobacteria clade further diverges, suggesting early separation from the other major bacterial classes. The Gammaproteobacteria and Epsilonproteobacteria families differ in ecological functions and metabolic strategies [27]. Gammaproteobacteria, including species like *E. coli* and *P. aeruginosa*, display high metabolic diversity and are found in a wide range of environments such as soil, water, and host-associated niches, often using aerobic or facultative anaerobic metabolism [23]. Among the ESKAPE pathogens, the oldest group in evolutionary terms is the Gram-positive bacteria, which include *E. faecium* and *S.aureus*. These bacteria belong to the phylum *Firmicutes*, which is considered evolutionarily older compared to the Gram-negative bacteria in the ESKAPE group, such as *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species.

It is also inferred in the phylogenetic tree that *Campylobacter* is evolutionarily distinct from *Enterobacter* and *Acinetobacter*, as they belong to different classes and phyla. It represents a lineage characterized by its helical shape and microaerophilic growth and are primarily associated with animal hosts and are significant pathogens causing gastroenteritis in humans. Accordingly, they have evolved genomic traits, such as a smaller genome size and specialized metabolic pathways for survival in microaerophilic conditions (low oxygen environments) [28]. The divergence of these groups is driven by different selective pressures, such as host specialization (*Campylobacter*), environmental adaptability (*Enterobacter*), and antibiotic resistance (*Acinetobacter*) [29, 30, 31]. These factors collectively explain the evolutionary distance and distinct characteristics of *Campylobacter*, *Enterobacter*, and *Acinetobacter*.

Separate phylogenetic trees per genera were also analyzed. Figure 2, presents the phylogenetic tree for *Pseudomonas* bacteria. The tree reveals the formation of several distinct clades among the genus *Pseudomonas* and related species. This include Clade I of *P. synxantha* and *P. azotoformans* with bootstrap value of 99, which depicts the close evolutionary ties. The second clade is comprised of *P. fluorescens, P. taetrolens,* and *P. syringae* of bootstrap values of 98 and 83 indicating evolutionary adaptations within this subgroup. And lastly, Clade III where ESKAPE bacteria, *P. auroginosa* belong together with *P. viridiflava, P.fragi,* and *P. putida*. Focusing on Clade III, *P. aeruginosa* stands out due to its pathogenic potential, resistance mechanisms, and ability to thrive in diverse environments, making it a significant concern in healthcare.

P.aeruginosa has several advantages compared to *P. viridiflava*, *P.fragi*, and *P. putida*, primarily due to its pathogenicity, adaptability, and resistance mechanisms.

It produces a wide array of virulence factors, such as exotoxins, elastases, and proteases, which damage host tissues and evade immune responses. These are tightly regulated by quorum sensing, a sophisticated communication system. *P. aeruginosa* can colonize a variety of niches, including the human respiratory tract, urinary system, and wounds. In contrast, *P. viridiflava* is more plant-associated, *P. fragi* is linked to food spoilage, and *P. putida* is primarily environmental. Also, according to Peix [32], *Pseudomonas fragi* and *Pseudomonas aeruginosa* may cluster together with a high bootstrap value in certain phylogenetic analyses, but this grouping typically represents broader evolutionary ties rather than a close genetic relationship. In addition, since *Pseudomonas* was first described in 1897, numerous new species have been discovered and identified in recent years, leading to the reclassification of certain species [6, 24, 33, 34].

Klebsiella and *Enterobacter*, both belong to the Enterobacteriaceae family within the Gammaproteobacteria class (Figure 4). The tree shows distinct clades for *Klebsiella* species and *Enterobacter* species. These clades are connected by a common node, reflecting their genetic and evolutionary proximity. Within the *Klebsiella* clade, *K. pneumoniae* and *K. oxytoca* are more closely related to each other than to *Enterobacter* species. Similarly, *E.cloacae* and *E. asburiae* form a tight sub-clade within the *Enterobacter* group. The branches connecting *Klebsiella* and *Enterobacter* species have high bootstrap values indicating strong confidence in their evolutionary relationship. The genus *Klebsiella* was first described in the late 19th century, with *Klebsiella pneumoniae* being one of the earliest identified species. It is considered one of the older genera within the Enterobacteriaceae family, with its evolutionary traits like capsule formation and carbohydrate fermentation being well-established. *Enterobacter* diverged later, adapting to different ecological niches and acquiring distinct traits [36, 37].

The Enterobacter species *E. chengduensis, E. cancerogenus, E.*mori, *Enterobacter* sp., and *E. cloacae* are closely related due to their shared phylogenetic lineage within the Enterobacteriaceae family. They are characterized by metabolic versatility, commonly inhabiting environments such as soil, water, and host-associated settings [15, 26, 31]. However, while their evolutionary connections are strong, differences in their specific strain characteristics and ecological adaptations contribute to variations in their roles and functions [7, 27, 34].

Figure 1. Phylogenetic tree of the selected bacteria based on the phylogenetic analysis using 16s rRNA gene sequences.

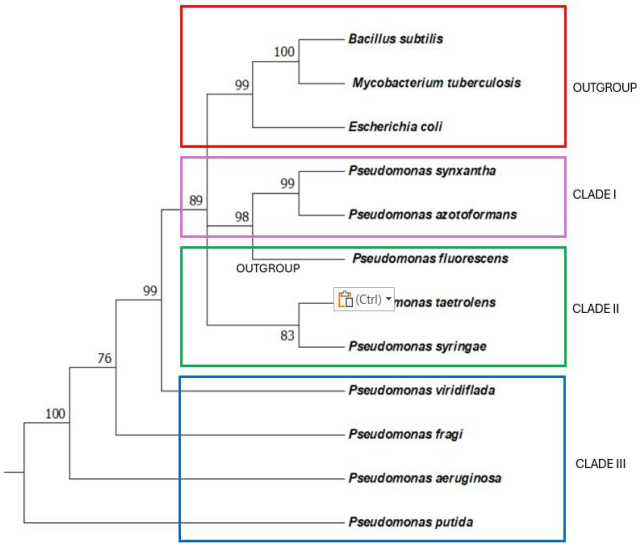

Figure 2. Phylogenetic tree of the selected species of *Pseudomonas*

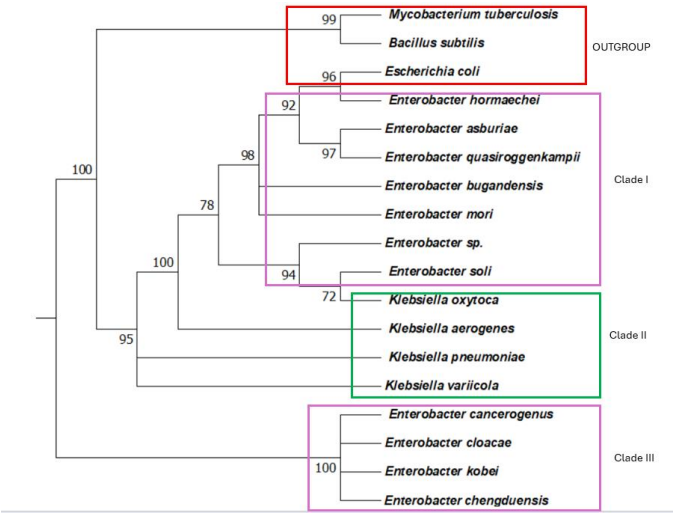

Figure 3. Phylogenetic tree of the selected species of *Enterobacter and Klebsiella*

Genetic diversity can be inferred from the phylogenetic tree of *Entercoccus*, forming a single clade signifies that they evolved from a shared ancestral bacterium. Within this clade, species that branch off closely together are likely to have similar genetic makeups, which can translate into comparable physiological traits and perhaps similar responses to environmental pressures, including the use of antibiotics. High bootstrap values suggest the relationships are well supported by the genetic data. Clinically significant species like *E. faecium* and *E. faecalis* may occupy branches that not only highlight their close relation but also their adaptations such as the evolution of particular antibiotic resistance mechanisms. However, other species in the group represent more environmental or less virulent forms. Among which, only *E. hirae* is closely related to the ESKAPE *E. faecium* and it is more distantly related to other *Enterococcus* species like *E. faecalis* and *E. casseliflavus.*

For the phylogenetic analysis of *Acinetobacter* (Figure 5), high bootstrap values reinforce that these species are closely related. This also suggests that these strains have evolved under similar selective pressures. In the context of hospitals and antibiotic-rich environments, these closely knit clades may be hotspots for horizontal gene transfer. Mobile genetic elements (such as plasmids and integrons) can move resistance genes not just vertically (from parent to offspring) but also laterally across these species [39].

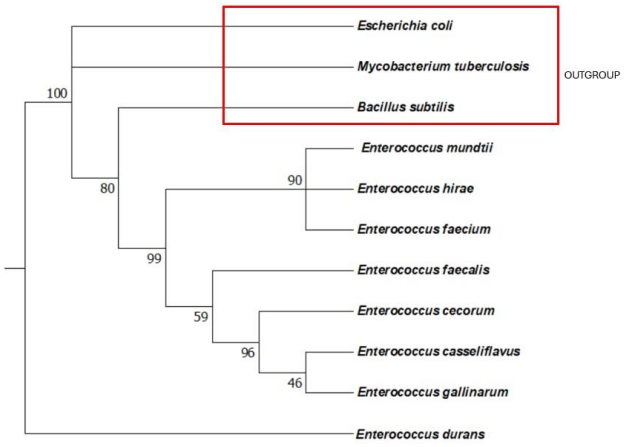

Figure 4. Phylogenetic tree of the selected species of *Enterococcus*

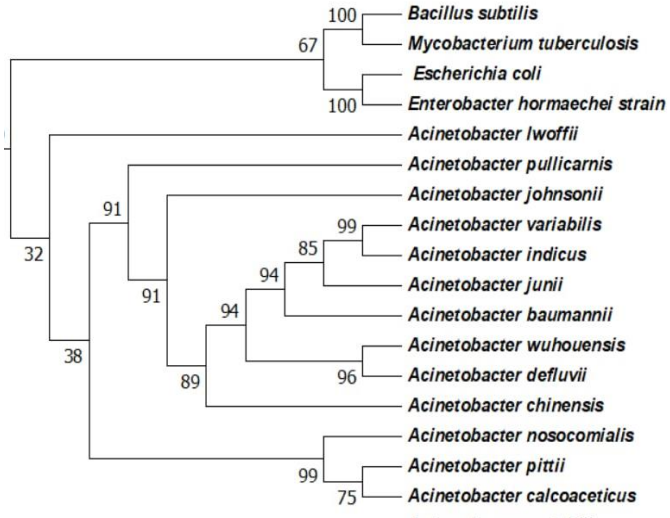

Figure 5. Phylogenetic tree of the selected species of *Acinetobacter*

Table 2. Summary of characteristics of ESKAPE bacteria [40-46]

Feature	*E.faecium*	*S. aureus*	*K. pneumoniae*	*A. baumannii*	*P.aeruginosa*	*Enterobacter spp.*
Taxonomic Group	Firmicutes, Bacilli	Firmicutes, Bacilli	Proteobacteria,	Proteobacteria,	Proteobacteria,	Proteobacteria,
Gram Staining	Gram-positive	Gram-positive	Gram-negative	Gram-negative	Gram-negative	Gram-negative
Shape	Cocci	Cocci	Rod-shaped	Rod-shaped	Rod-shaped	Rod-shaped
Spore Formation	Absent	Absent	Absent	Absent	Absent	Absent
Pathogenicity	bloodstream infections, endocarditis	skin infections, pneumonia, sepsis	pneumonia, UTIs, sepsis	pneumonia, wound infections	pneumonia, UTIs, sepsis	Causes pneumonia, UTIs, sepsis
Antibiotic Resistance	Vancomycin-resistant (VRE)	Methicillin-resistant (MRSA)	Carbapenem-resistant (CRE)	Carbapenem-resistant (CRAB)	Multidrug-resistant (MDR)	Carbapenem-resistant (CRE)
Biofilm Formation	Moderate	High	High	High	High	High
Metabolic Traits	Facultatively anaerobic; lactic acid fermentation	Facultatively anaerobic; capable of fermentation and respiration	Facultatively anaerobic; nitrogen fixation in some strains	Obligate aerobe; highly versatile metabolism	Obligate aerobe; capable of anaerobic respiration under certain conditions	Facultatively anaerobic; capable of fermentation and respiration
Aerobic /Anaerobic	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe	Obligate aerobe	Obligate aerobe	Facultative anaerobe
Key Metabolic Pathways	Fermentation of carbohydrates	Fermentation of glucose; aerobic respiration	Fermentation of lactose; nitrogen metabolism	Utilization of diverse carbon sources	Aerobic respiration; denitrification	Fermentation of lactose; aerobic respiration
Habitat	Human gut microbiota	Skin, mucous membrane	Gastrointestinal tract	Soil and hospital environments	Soil, water, and hospital environments	Gastrointestinal tract

In summary, the different charcaterics of the ESKAPE bacteria are presented in Table 2. As shown in the inferred phylogenetic trees, both the common and unique evolutionary paths that culminate in the formidable virulence of ESKAPE pathogens. Whether through shared inheritance or the dynamic exchange of genetic material, these relationships help explain why these bacteria are so adept at causing life-threatening infections and resisting antibiotics. This evolutionary perspective is essential in guiding both current clinical practices and future research aimed at mitigating the impact of these dangerous pathogens. Moreover, diversity suggests that virulence and the mechanisms allowing them to overcome antibiotic challenges—has evolved along different trajectories even when the end result is a highly successful pathogen. Understanding where these species sit in the tree helps in distinguishing whether common virulence traits result from a shared ancestral strategy or from convergent evolution driven by similar environmental pressures. Species that cluster closely together in the tree tend to share more of their genomic heritage like the virulence factors such as adhesins, toxins, and biofilm-forming capabilities [41, 42].

CONCLUSION

This study highlights the different evolutionary relationships of the milk contaminant bacteria that are either food borne pathogens or belong to the ESKAPE group. While these organisms are clinically united by their multidrug resistance and virulence, the tree reveals that they have distinct evolutionary origins. This close genetic relationship suggests that traits such as biofilm formation, toxin production, or specific resistance enzymes might be shared among these pathogens, facilitating their survival in antibiotic-rich environments. Thus, this study can provide framework for predicting resistance patterns and guiding effective intervention strategies.

DECLARATION

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Authorship Contributions

Concept and Writing: Ms. Marinette Rose M. Carpio, Dr. Mary Jhane G. Valentino; Critic and final revision: Ms. Marinette Rose M. Carpio & Dr. Mary Jhane G. Valentino

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Consent for publication

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Competing interests

The authors declared that there is no conflict of interest.

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