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RESEARCH ARTICLE OPEN ACCESS

Comparative Analysis of Gastrointestinal Bacteria in *Ctenocephalides felis* and *Ctenocephalides canis*: Biochemical Traits, Antibiotic Resistance, and 16S rRNA Identification

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Abstract

Fleas of the genus *Ctenocephalides* that infest cats and dogs are known to harbor various pathogenic bacteria with potential zoonotic importance. This study aimed to isolate, characterize, and identify bacterial species from the gastrointestinal tracts of *Ctenocephalides felis* and *Ctenocephalides canis* using biochemical profiling, antibiotic susceptibility testing, and 16S rRNA gene sequencing. A total of ten bacterial isolates were obtained, showing different biochemical and antibiotic resistance profiles. Biochemical identification revealed the presence of *Staphylococcus, Pseudomonas, Francisella*, and *Sphingomonas* species, while molecular identification indicated variations at both the genus and species levels. Notably, isolates from *C. canis* exhibited higher resistance compared to those from *C. felis*, with Benzylpenicillin being the most resistant antibiotic. These findings highlight the species divergence and antibiotic resistance patterns of flea-associated bacteria, emphasizing their potential role in zoonotic transmission and the need for integrated monitoring of flea-borne pathogens.

Keywords: Pure Culture, Bacteria, *Ctenocephalides Felis, Ctenocephalides Canis*

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INTRODUCTION

Companion animals such as dogs and cats play an essential role in human society, offering emotional and social benefits but also serving as potential sources of zoonotic infections.[1,2] Their close association with humans increases the risk of exposure to ectoparasites, particularly fleas of the genus *Ctenocephalides*, which are among the most prevalent and medically significant parasites affecting domestic animals worldwide.[3] *Ctenocephalides felis* primarily infests cats, while *Ctenocephalides canis* is more frequently found on dogs.[4-6] Although these two species are morphologically similar, they differ in host specificity, ecological adaptation, and microbial associations.[7]

Fleas are well-documented vectors of various pathogens, including bacteria, viruses, and rickettsiae, that can cause diseases in animals and humans. Beyond their external role as vectors, the gastrointestinal tract of fleas constitutes a crucial internal niche for microbial colonization, survival, and transmission. Bacteria residing in this compartment may interact with both the flea host and ingested blood, influencing the flea's physiology and its competence as a disease vector.[8-11] Previous studies have identified several pathogenic bacterial taxa in fleas, such as *Bartonella henselae*, *Rickettsia felis*, and *Francisella tularensis*, highlighting their role in zoonotic cycles.[8,9] However, most of these studies have focused on metagenomic detection, with limited information available on the biochemical traits and antibiotic resistance patterns of individual bacterial isolates within flea species.

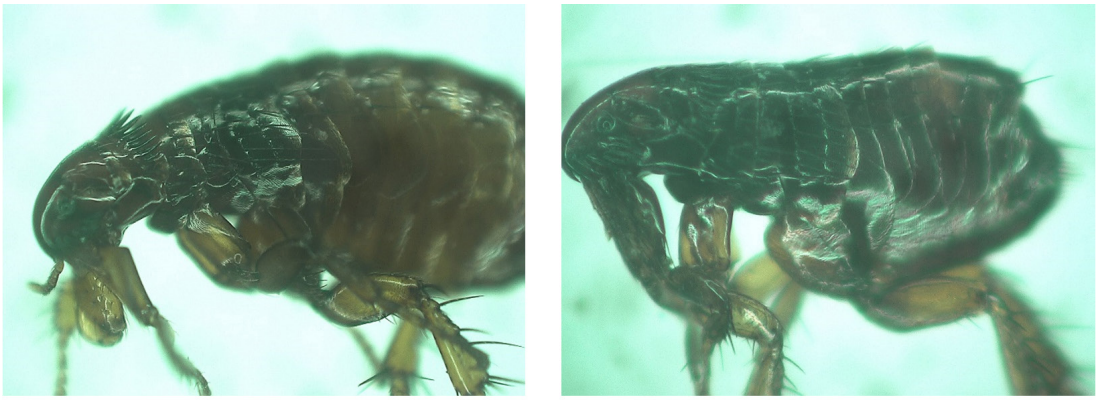
Understanding the biochemical and molecular characteristics of flea-associated bacteria is vital for evaluating their pathogenic potential and antimicrobial resistance, particularly in the context of the growing global concern over antibiotic-resistant zoonotic microorganisms. Comparative investigations between *C. felis* and *C. canis* may further reveal species-specific bacterial assemblages that reflect host preference, ecological behavior, and differing transmission capacities.[12-14]

Therefore, the present study aims to isolate, characterize, and identify bacterial species from the gastrointestinal tracts of *Ctenocephalides felis* and *Ctenocephalides canis* through biochemical profiling, antibiotic susceptibility testing, and 16S rRNA gene-based molecular analysis (Figure 1). By integrating phenotypic and genotypic approaches, this work seeks to provide insights into the microbial diversity, antibiotic resistance, and potential zoonotic relevance of bacteria associated with cat and dog fleas.

MATERIALS AND METHODS

Samples

Adult fleas (*Ctenocephalides felis* and



(a) (b)

Figure 1. (a). *Ctenocephalides canis* (b). *Ctenocephalides felis*. Observed with a low range x250 lens, with a resolution of 0.8 mm; Hirox KH8700 3D Digital Stereo Microscope (Photo at the Laboratory of Bioactivity and Molecular Biology, FMIPA State University of Manado)

Ctenocephalides canis) were collected from domestic dogs and cats in Minahasa Regency (1°222 443 N, 124°332 523 E to 1°012 113 N, 125°042 213 E) and Tomohon City (1°152 N, 124°502 E), North Sulawesi, Indonesia. Thirty flea specimens were obtained from fifteen animals of each host species using sterile forceps. Live specimens were placed in sterile containers and transported immediately to the laboratory for bacterial isolation. For molecular analysis, representative samples were preserved in 95% ethanol and stored at -20 °C until DNA extraction.

Research Procedure

Bacterial isolation

Each flea was dissected aseptically on a sterile Petri dish under a stereomicroscope. The digestive tract was excised and streaked onto nutrient agar (NA) plates using a sterile inoculating loop. Plates were incubated at 37 °C for 48 hours. Colonies with distinct morphology were subcultured three times on nutrient agar slants to obtain pure cultures (Figure 2). Stable isolates were selected for further biochemical and molecular analyses.

Biochemical analysis with VITECT 2 compact

Biochemical characterization and identification were conducted using the VITEK 2 Compact automatic identification instrument (Figure 3). The analysis was conducted in a certified medical laboratory, the Laboratory of Dr. Kandouw Government General Hospital, Manado, North Sulawesi, Indonesia. VITEK 2 Compact is an automatic microorganism identification tool. The latest technology using VITEK 2 Compact consists of three stages of examination for biochemical identification and antibiotic sensitivity. Vitect2 Compact has been validated and interpreted according to the Clinical Laboratory Standard International (CLSI).[15,16] Before biochemical identification, Gram staining was performed on each isolate. The three stages are preparation or standardization of inoculum turbidity, data entry with a barcode system, and insertion of an identification card into the device.

Furthermore, the device will automatically carry out the entire process of inoculation, incubation, reading, validation, and interpretation of results. Furthermore, the completed examination will automatically produce a printout, while the system will automatically discard the ID/AST (Identification/Antimicrobial Sensitivity Test) card. The principle of automatic identification is to use an identification card; on the card, there is a well or a biochemical test

Table 1. VITEK 2 Compact Output Analysis Standard

Confidence Level	Choice	% Probability
Excellent	1	96 to 99
Very Good	1	93 to 95
Good	1	89 to 92
Acceptable	1	85 to 88

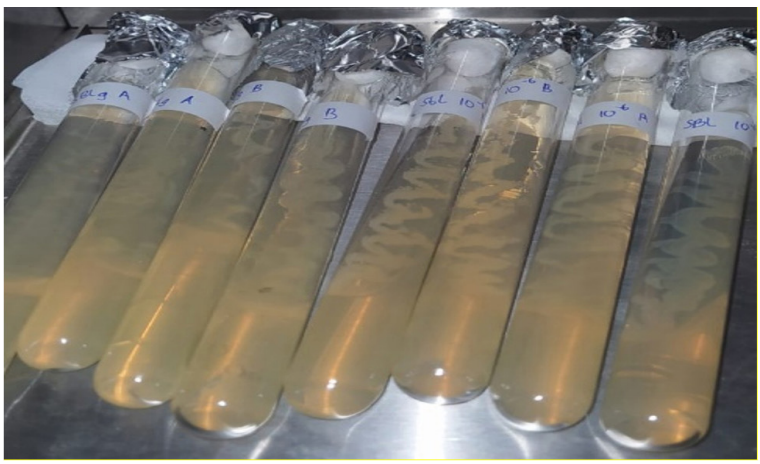


Figure 2. Pure culture of bacterial isolates from the digestive tract *Ctenocephalides felis* and *Ctenocephalides canis*

Table 2. Biochemical parameters in VITEK 2 Compact

No.	Symbol	Chemical nomenclature	No	Symbol	Chemical nomenclature
1	H_2S	H_2S Production	25	ProA	L-Proline Arylamidase
2	BGLU	Beta-Glucose	26	GGAA	Glu-Gly-Arg-Arylamidase
3	BGURr	Beta-Glucuronidase	27	PLE	Palatinose
4	PyrA	L-Pyrrolydonyl-Arylamidase	28	AGLTp	Glutamyl Arylamidase Pna
5	SAC	Saccharose/Sucrose	29	SUCT	Succinate alkalinization
6	dTRE	D-Trehalose	30	ELLM	Ellman
7	dMAN	D-Mannitol	31	BGAL	Beta-Galactosidase
8	APPA	Ala-Phe-Pro Arylamidase	32	OFF	Fermentation Glucose
9	ILATk	L-Lactate alkalinization	33	LDC	Lysine Decarboxylase
10	GlyA	Glycine Arylamidase	34	IMTLa	L-Malate assimilation
11	O129r	O/129 Resistance	35	IARL	L-Arabitol
12	dMAL	D-Maltose	36	NAGA	Beta-N-Acetyl
13	LIP	Lipase	37	IHISa	Histidine assimilation
14	dTAG	D-Tagatosa	38	BAlap	Beta-Alanine Arylamidase
15	AGLU	Alpha-Glucosidase	39	dSOR	D-Sorbitol
16	ODC	Ornithine Decarboxylase	40	5KG	5-Keto-D-Gluconate
17	dGLU	D-Glucose	41	PHOS	Phosphatase
18	dMNE	D-Mannose	42	ADO	Adonitol
19	TyrA	Tyrosine Arylamidase	43	BNAG	Beta-N-Acetyl-Glucosaminidase
20	CIT	Citrate/Sodium	44	ILATa	L-Lactate assimilation
21	dCEL	D-Cellobiose	45	MNT	Malonate
22	GGT	Gamma-Glutamyl-Transferase	46	AGAL	Alpha-Galactosidase
23	BXYL	B-Xylose	47	CMT	Coumarate
24	URE	Urease			

medium modified for rapid bacterial identification. The testing procedure with the VITEK 2 Compact device starts from the gram test, card selection, and making bacterial suspensions according to the McFarland standard and identification using the device until the identification results sheet is issued. Based on the theory that the results obtained in identification with the VITEK 2 Compact are expressed in percentages for the correctness of the identified organisms (Table 1).[16,17]

A total of 47 biochemical test parameters were used for each bacterial isolate. The biochemical test result indicators are positive (+) and/or negative (-) (Table 2).

Antibiotic resistance test

Antimicrobial susceptibility testing (AST) was conducted concurrently with biochemical identification using the VITEK 2 Compact system. A panel of 21 antibiotics representing different classes (β-lactams, aminoglycosides, macrolides, tetracyclines, glycopeptides, and fluoroquinolones) was employed. Minimum inhibitory concentrations (MICs) were automatically calculated by the instrument. Resistance and susceptibility interpretations were determined according to CLSI (2021) breakpoints. Antibiotic resistance was analyzed using variance and Tukey's test.[6,17]

Molecular identification of pure cultures of bacteria

DNA extraction

Genomic DNA from pure bacterial cultures was extracted using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, USA) following the manufacturer's protocol. Briefly, 50-100 mg of bacterial cells were lysed in BashingBead™ buffer and processed in a bead beater at maximum speed for 5 min. The lysate was centrifuged at 10,000 × g for 1 min, and the supernatant was purified through a Zymo-Spin™ column. DNA was eluted in 100 µL of elution buffer and stored at -20 °C for PCR amplification.[18,19]

Table 3. Results of biochemical tests of bacterial isolates from *C. felis* and *C. canis*

No.	Isolate	Positive Biochemical Test Results	Number of Biochemical Parameters tested	Species/Gram Staining	% Probability
Ctenocephalides felis					
1	CF1	AMY, dRIB, dXYL, BGURr, NAG, dMAL, PHOS, BGUR dan BACi	43	*Staphylococcus sciuri*/ +	97
2	CF2	AMY, dRIB, OPTO, NC6.5, dXYL, dMANE, SAL, NAG, dMNE, SAC, PLYB, dMAL, MBdG, dTRE, BACi	47	*Staphylococcus lentus*/ +	96
3	CF3	ProA, PyrA	47	*Francisella tularensis*/ -	93
4	CF4	dRIB, NOVO, OPTO, dXYL, LAC, dMAN, URE, SAC, dMAL, dTRE, BGUR	43	*Staphylococcus arlettae*/ +	94
Ctenocephalides canis					
1	CC1	AMY, LeuA, dRIB, dXYL, BGURr, NAG, dMAL, PHOS, BGUR dan BACi, ADH2s	47	*Staphylococcus aureus*/ +	96
2	CC2	AMY, AlaA dRIB, OPTO, PIPLC, NC6.5, dXYL, dMANE, SAL, NAG, dMNE, SAC, PLYB, dMAL, MBdG, dTRE, BACi	43	*Staphylococcus aureus*/ +	95
3	CC3	APPA, ProA, PyrA, dGLU, BGAL, BGUR	47	*Staphylococcus haemolyticus*/ +	96
4	CC4	AMY, APPA, dRIB, NOVO, OPTO, dXYL, LAC, dMAN, URE, SAC, dMAL, dTRE, BGUR, BACl	43	*Pseudomonas fluorescens*/ -	90
5	CC5	AMY, APPA, dRIB, NOVO, OPTO, dXYL, LAC, dMAN, URE, NAG, SAC, dMAL, dTRE, BGUR, BACl	47	*Pseduomonas stutzeri*/ -	90
6	CC6	LeuA, dRIB, NOVO, OPTO, dXYL, LAC, dMAN, URE, SAC, dMAL, dTRE, BGUR, BACl	43	*Sphingomonas paucimobilis*/ -	94

16S rRNA amplification and sequencing

16S rRNA gene amplification was performed using primers: 16sA (5'CGC CTG TTT AAC AAA AAC AT 3') (Forward), 16sB2 (5'TTT AAT CCA ACA TCG AGG 3') (Reverse). PCR amplification was performed with 2x PCR components MyTaq HS Red Mis Bioline 25 µl; 10 pmol primers consisting of 1 µl forward primer, 1 µl reverse primer, 2 µl DNA template, and 21 µl ddH_2O. At the same time, the PCR conditions used a 35x cycle with Denaturation of 94 °C for 70 seconds, annealing of 55 °C for 35 seconds, extension of 70 °C for 40 seconds, and a final extension of 70 °C for 40 seconds. Amplicon visualization used the 0.8% agarose gel electrophoresis method. The PCR products were then sent to the sequencing service company 1st BaseTM in Singapore for sequencing using the Sanger method.[18]

Analysis of sequencing results

Raw sequences were trimmed and assembled using MEGA version 12. Consensus sequences were aligned using BLASTn (National Center for Biotechnology Information, NCBI) to determine taxonomic identity based on sequence similarity. Phylogenetic trees were constructed

in MEGA 12 using the Neighbor-Joining and Minimum Evolution methods with 1,000 bootstrap replications. Sequences showing ≥97% identity were considered to belong to the same bacterial species.[18,19]

RESULTS AND DISCUSSION

Bacterial Isolation

A total of ten pure bacterial isolates were successfully recovered from the gastrointestinal tracts of *Ctenocephalides felis* (four isolates) and *Ctenocephalides canis* (six isolates). All isolates exhibited distinct colony morphologies and stable growth following three successive subcultures. The higher bacterial diversity observed in *C. canis* may reflect its broader host interactions and greater environmental exposure, consistent with previous reports suggesting that *C. canis* exhibits higher microbial carriage variability compared to *C. felis*.

Biochemical identification and characterization

Biochemical profiling using the VITEK 2 Compact system revealed clear interspecific differences between bacterial isolates from *C. felis* and *C. canis*. Isolates from *C. felis* predominantly belonged to the genus *Staphylococcus* (e.g., *S. sciuri*, *S. lentus*), whereas isolates from *C. canis* included *Staphylococcus aureus*, *Pseudomonas fluorescens*, and *Sphingomonas paucimobilis* (Table 3). The presence of *Staphylococcus* spp. in both flea species is noteworthy, as these bacteria are known for their adaptability and frequent association with skin and mucosal surfaces of mammals. However, the occurrence of *Francisella tularensis* like isolates in *C. felis* and *Pseudomonas* spp. in *C. canis* indicates species-specific microbial assemblages. This variation could be influenced by physiological differences between the flea hosts, their feeding patterns, or the microbial composition of their respective mammalian hosts. These findings reinforce the role of the flea gastrointestinal tract as an ecological reservoir supporting taxonomically diverse bacteria. Such diversity may contribute to the fleas' competence as potential vectors of opportunistic and pathogenic microorganisms.

Antibiotic resistance test

The results of antibiotic resistance tests of four *Ctenocephalides felis* bacterial isolates and six *Ctenocephalides canis* bacterial isolates showed different resistance and susceptibility responses. A total of 21 types of antibiotics were used to test the resistance of bacterial isolates. Bacterial isolates CF1, CF2, CF3 only showed resistance to Benzylpenicillin (MIC: $\leq$0.03; $\leq$0.5; $\leq$0.03; $\leq$0.03). Isolate CF4 showed resistance to Benzylpenicillin and Vancomycin (MIC: $\leq$0.03 and $\leq$0.05) (Appendix 1).

Antibiotic resistance test on *C. canis* isolates using 20 types of antibiotics. Isolate CC1 showed resistance to Benzylpenicillin (MIC: $\geq$0.5), Oxacillin (MIC: $\geq$4), and Trimethoprim/Sulfamethoxazole (MIC: $\geq$320). Isolate CC2 showed resistance to Cefazolin (MIC: $\geq$64) and Aztreonam (MIC: 32). Isolate CC3 showed resistance to Benzylpenicillin (MIC: $\geq$0.5), Oxacillin (MIC: $\geq$4) and Trimethoprim/Sulfamethoxazole (MIC: 80). Isolate CC4 did not show resistance to the antibiotics tested. Isolate CC5 showed resistance to Cefazolin (MIC: $\leq$4) and Aztreonam (MIC: $\geq$64). Isolate CC6 showed resistance to Cefazolin (MIC: $\geq$64) and Aztreonam (MIC: 32) (Appendix 2).

Analysis of antibiotic susceptibility revealed distinct resistance profiles among bacterial isolates from *Ctenocephalides felis* and *C. canis* (Table 4). Overall, isolates from both flea species exhibited high resistance to Benzylpenicillin, with all tested isolates classified as resistant (score = 3), indicating no interspecific variation for this antibiotic. In contrast, a pronounced difference was observed for Oxacillin resistance. ANOVA indicated a statistically significant variation between the two flea species ($F = \infty$, $p < 0.001$), with isolates from *C. canis* showing complete resistance (score = 3) whereas those from *C. felis* were fully susceptible (score = 1). This suggests species-specific bacterial communities or differential exposure to β-lactam antibiotics in their respective hosts. For Vancomycin, no significant difference was detected between *C. felis* and *C. canis* ($p = 0.356$), though *C. felis* isolates displayed slightly higher mean resistance scores (1.75 ± 0.50) compared to *C. canis* (1.33 ±

Table 4. Analysis of variance of antibiotic resistance

Antibiotic	Mean ± SD (*C. felis*)	Mean ± SD (*C. canis*)	F-statistic	p-value	Significance
Benzylpenicillin	3.00 ± 0.00	3.00 ± 0.00	–	–	ns
Oxacillin	1.00 ± 0.00	3.00 ± 0.00	∞	0.0000	*
Vancomycin	1.75 ± 0.50	1.33 ± 0.52	1.00	0.356	ns

Description: S = 1, I = 2, R = 3; * = significant ($p < 0.05$); ns = no significant

Table 5. Differences in resistance levels

Antibiotic	F-statistic	P-value	Interpretation
Benzylpenicillin	–	–	Cannot be calculated (all values are the same = R, no variation).
Oxacillin	∞	0.0000	The differences are very significant between *C. felis* (all S) and *C. canis* (all R).
Vancomycin	1.00	0.356	Not significant; resistance was similar between the two species.

0.52). These findings indicate that while both flea species harbor bacteria with similar glycopeptide sensitivity, β-lactam resistance profiles vary markedly between hosts.

Oxacillin showed a significant difference in resistance ($p < 0.001$) between *C. felis* and *C. canis*. Isolates from *C. canis* had higher resistance to Oxacillin than *C. felis*. Benzylpenicillin could not be analyzed because all isolates showed the same resistance pattern (R), so the variance = 0. Vancomycin showed no significant difference ($p > 0.05$) between the two tick species (Table 5).

Oxacillin menunjukkan perbedaan resistansi yang signifikan ($p < 0.001$) antara *C. felis* dan *C. canis*. Isolat dari *C. canis* memiliki resistansi yang lebih tinggi terhadap Oxacillin dibanding *C. felis*. Benzylpenicillin tidak dapat dianalisis karena semua isolat menunjukkan pola resistansi yang sama (*R*), sehingga varians = 0. Vancomycin menunjukkan tidak ada perbedaan signifikan ($p > 0.05$) antara kedua spesies kutu. Bars represent the mean (±SD) resistance scores for each antibiotic, where S = 1, I = 2, and R = 3. The two flea species exhibited distinct resistance profiles, with *C. canis* showing significantly higher resistance to Oxacillin ($p < 0.001$), as indicated by the red asterisk. No significant differences were observed for Benzylpenicillin or Vancomycin. These results highlight host-specific variations in bacterial resistance patterns associated with different flea species (Figure 3).

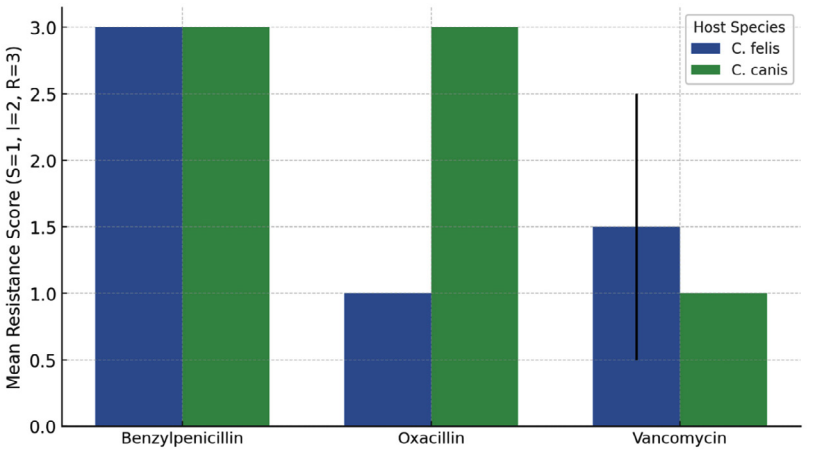


Figure 3. Comparison of mean antibiotic resistance scores between *C. felis* and *C. canis*

Table 6. Identical species of 16S rRNA gene sequences of bacterial isolates *C. felis* and *C. canis* using the NCBI BLAST method

No.	Isolate	Species termirip hasil BLAST NCBI	Perc. Ident	Accession Number	Data Source
Ctenocephalides felis					
1	CF1	*Pedobacter suwonensis*	88.33%	CP031708.1	https://blast.ncbi.nlm.nih.gov/Blast.cgi
2	CF2	*Achromobacter insolitus*	85.96%	CP038034.1	
3	CF3	*Pseudochrobactrum* sp. XF203	96.28%	CP084392.1	
4	CF4	*Sphingobacterium faecium*	98,76%	CP123861.1	
Ctenocephalides canis					
1	CC1	*Bacillus* sp. (in: *firmicutes*)	100%	PQ097125.1	
2	CC2	*Gamma proteobacterium*	97.35%	ON406394.1	
3	CC3	*Enterobacter cloacae*	100%	MT613361.1	
4	CC4	*Bacillus paralicheniformis*	94.45%	CP043501.1	
5	CC5	*Sphingobacterium* sp.	99.10%	CP079104.1	
6	CC6	*Pseudochrobactrum* sp.	97.53%	CP084392.1	

Molecular identification of bacterial isolates

The results of molecular identification using the 16S rRNA gene showed the similarity of each species of bacterial isolates obtained from *C. felis* and *C. canis*, which differed from the results of biochemical tests. Each 16S rRNA gene sequence of bacterial isolates from *C. felis* and *C. canis* was aligned with BLAST on the NCBI site. Based on the percent identity obtained, CF2 showed similarity to *Achromobacter insolitus* with a percent identity of 85.96%, while CF4 showed similarity to *Sphingobacterium faecium* with the highest percent identity of 98.76%. On the other hand, isolate CC1 showed similarity to *Bacillus* sp. (in: firmicutes) with the highest percent identity of 100%. In comparison, isolate CC4 showed similarity to *Bacillus paralicheniformis,* with the lowest percent identity being 94.45% (Table 6).

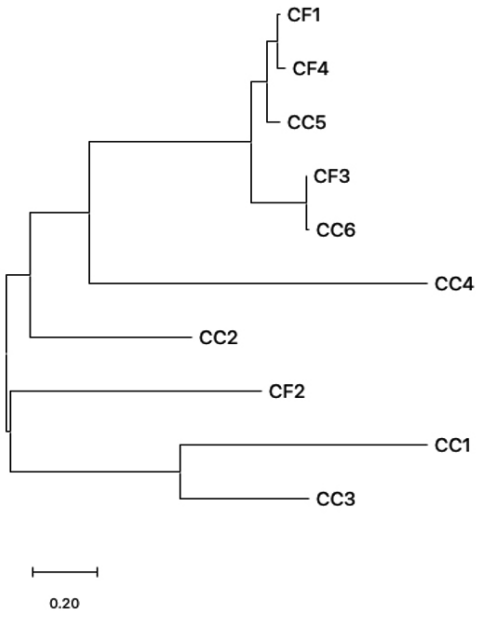


Figure 4. Phylogeny Relationship of Bacterial Isolates from *Ctenocephalides felis* (CF) and *Ctenocephalides canis* (CC) based on 16S rRNA gene. Phylogenetic tree was constructed using Neighbor Joining model (Bootstrap 1000 x)

To obtain phylogenetic relationships, the 16S rRNA gene sequences of bacterial isolates from *C. felis* and *C. canis* were used to construct a phylogenetic tree. The phylogenetic construction produced two main monophyletic groups (Figure 4). Based on the phylogenetic tree formed, three bacterial isolates were in the same monophyletic group, namely CC2, CC1, and CC3. Thus, the three isolates have a close relationship. At the same time, the CC2 isolate is in its monophyletic group. CC4 is in the same group as CF1, CF4, CF3, and CC6, but not at the same node. This means that CC2 and CC4 have genetic variations compared to CF3, CC6, CC5, CF4 and CF1 (Figure 4). The results of this study indicate that bacterial isolates in both *C. canis* and *C. felis* have high species divergence.

DISCUSSION

The combined biochemical, antimicrobial, and molecular data from this study reveal

distinct and ecologically meaningful differences in the bacterial assemblages associated with *Ctenocephalides felis* and *C. canis*. Phenotypically, isolates from both species exhibited broad carbohydrate metabolism and enzymatic versatility, including the ability to utilize D-amygdalin, D-ribose, D-galactose, D-xylose, and N-acetylglucosamine.[20] However, specific enzymatic activities—such as leucine arylamidase and phosphatidylinositol phospholipase C in *C. canis* isolates, and L-proline and alanine arylamidases in *C. felis* suggest niche-driven adaptations to differing gut environments or host-derived nutritional substrates.[21] Such host-specific biochemical patterns align with previous metagenomic reports showing that flea microbiota composition varies according to host species, geography, and blood meal origin.[22-25]

Molecular identification based on 16S rRNA gene sequencing revealed that the bacterial isolates belonged to multiple genera, including *Staphylococcus*, *Pseudomonas*, *Sphingomonas*, and *Francisella*, thereby extending the known microbial diversity of flea-associated bacteria.[26] Several discrepancies between biochemical and molecular identifications were observed, for instance where isolates biochemically classified as *Staphylococcus* were genetically aligned with *Achromobacter* or *Sphingobacterium*.[27-29] These inconsistencies highlight the limitations of phenotypic methods such as VITEK 2 for environmental or host-associated isolates, due to restricted clinical reference databases and phenotypic plasticity within bacterial taxa.[30-33] Such findings underscore the necessity of a polyphasic taxonomic approach integrating phenotypic, genotypic, and phylogenetic analyses to achieve accurate identification, as has been emphasized in comparative microbiome studies of arthropod vectors.[34-36]

The antibiotic susceptibility data demonstrated marked interspecific variation. All isolates were resistant to Benzylpenicillin, confirming the ubiquity of β-lactam resistance, while ANOVA revealed a significant difference in Oxacillin resistance ($p < 0.001$) between the two flea species.[37,38] Isolates from *C. canis* were consistently resistant, whereas those from *C. felis* were fully susceptible. This divergence likely reflects differential exposure to antimicrobial residues through host blood or environmental contact, leading to distinct microbial selection pressures. Such results are consistent with reports that coagulase-negative *Staphylococcus* (CoNS) species, including *S. sciuri* and *S. lentus*, can act as reservoirs of β-lactamase and mecA resistance determinants transferable to more virulent species like *S. aureus*.[39,40] The presence of *S. arlettae* and *S. haemolyticus* both recognized for their genomic plasticity and multidrug-resistance further reinforces the fleas' potential role as carriers of resistance genes in domestic ecosystems.

Among Gram-negative isolates, *Pseudomonas fluorescens*, *P. stutzeri*, and *Sphingomonas paucimobilis* were recovered predominantly from *C. canis*. Although typically environmental, these bacteria have been associated with opportunistic and nosocomial infections, particularly in immunocompromised hosts.[38] Their presence within flea gastrointestinal tracts may therefore represent an indirect but significant link between environmental microbial pools and companion animals, suggesting that fleas could serve as intermediate reservoirs facilitating the persistence and potential transfer of antimicrobial-resistant bacteria across ecological boundaries. The detection of *Francisella tularensis* related sequences in *C. felis* isolates also warrants attention, as it implies the coexistence of potentially pathogenic taxa within the flea microbiome that might interact with symbiotic or commensal populations.[30-33]

The integrated interpretation of biochemical, molecular, and resistance data provides a more holistic understanding of the microbial ecology of *C. felis* and *C. canis*. The metabolic flexibility observed among isolates likely confers survival advantages within the nutrient-variable flea gut, while differences in resistance profiles suggest that the microbiota of *C. canis* is under greater selective pressure, possibly due to repeated environmental exposure or differences in host pharmacological backgrounds. Furthermore, the observed discrepancies between phenotypic and genotypic identifications support the hypothesis that environmental bacterial isolates within fleas are evolving under unique selective regimes, potentially facilitating horizontal gene transfer events that contribute to antimicrobial

resistance dissemination. This interpretation is consistent with the broader One Health perspective, which posits that arthropods, particularly ectoparasites, can function as micro-reservoirs within the interconnected network of human, animal, and environmental health.

Collectively, these findings advance our understanding of the complex microbial ecology of cat and dog fleas. They reveal that flea-associated bacteria are metabolically diverse, taxonomically heterogeneous, and variably resistant to clinically relevant antibiotics. Importantly, they suggest that fleas may represent overlooked but ecologically significant reservoirs and vectors of antimicrobial-resistant bacteria. Future research should employ whole-genome sequencing and resistome analysis to elucidate the genetic mechanisms underlying these resistance patterns, trace the mobility of resistance genes across taxa, and quantify the potential contribution of fleas to AMR transmission within domestic and public health contexts.

CONCLUSION

This study provides integrative evidence that the gastrointestinal bacterial communities of *Ctenocephalides felis* and *C. canis* differ substantially in their biochemical characteristics, molecular composition, and antimicrobial resistance profiles. The bacterial isolates displayed host-specific metabolic adaptations and variable antibiotic resistance, particularly toward β-lactam antibiotics, with *C. canis* associated isolates exhibiting broader resistance patterns. Molecular identification through 16S rRNA sequencing confirmed a diverse microbiota encompassing both opportunistic and environmental taxa, including *Staphylococcus*, *Pseudomonas*, *Sphingomonas*, and *Francisella* species. The coexistence of metabolically versatile and multidrug-resistant bacteria within these flea species suggests that fleas may act as ecological reservoirs and potential vectors for antimicrobial resistance within domestic and peri-domestic environments. These findings underscore the importance of including ectoparasites in One Health surveillance frameworks and highlight the need for genomic-based approaches to characterize the resistome and mobile genetic elements contributing to bacterial persistence and resistance dissemination across host species.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

DVR, MYS, and OAW conceptualized the study. DVR and JSBT applied methodology. DVR, OAW, and MYS investigated the study. MYS and JSBT performed data and formal analysis. DVR and JSBT collected the resources. DVR and MYS wrote the original draft. MYS JSBT and OAW wrote, reviewed, and edited the manuscript, and HHA performed supervision. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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