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RESEARCH ARTICLE

Genetic Characterization of Multidrug-Resistant Bacteria and Antibacterial Activity of Alpha-Mangostin and Clove Oil Nanostructured Lipid Carriers in Canine Periodontitis

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ABSTRACT

Canine periodontitis is a prevalent oral disease increasingly complicated by multidrug-resistant (MDR) bacteria, reducing the effectiveness of current antimicrobial therapies and creating treatment challenges. This study evaluated the antimicrobial efficacy of a nanostructured lipid carrier loaded with alpha-mangostin and clove oil (NLC-AMCO) against MDR oral bacteria. Forty-nine bacterial strains were isolated and characterized from thirty dogs diagnosed with clinical periodontitis. Antibacterial activity was assessed using standard phenotypic assays, including disc diffusion, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and total plate count (TPC), while whole genome sequencing (WGS) was applied to identify resistance gene profiles. NLC-AMCO demonstrated superior antimicrobial efficacy compared to alpha-mangostin and clove oil (AMCO) and commonly used antibiotics (amoxicillin, clindamycin, doxycycline, and metronidazole), exhibiting broader inhibition zones (up to 24-30 mm) and rapid bactericidal activity within 30 min. Time-dependent improvements in MIC and MBC values confirmed the sustained-release capability of the NLC-AMCO. At 48 h, MICs were reduced by half for key MDR isolates, including *Escherichia coli*_CU1 (31.25 to 15.62mg/mL), *Morganella morganii*_CU1 (15.62 to 7.8mg/mL), and *Acinetobacter baumannii* (3.9 to 1.95mg/mL), with comparable decreases in MBC values. WGS analysis revealed extensive resistomes, particularly in *Pseudomonas aeruginosa* and *E. coli*, carrying genes resistant to fluoroquinolones and last-resort agents such as colistin. These findings highlight the potential of NLC-AMCO as a plant-based nano-formulation for improving veterinary dental care in dogs with MDR infections and support its relevance for future antimicrobial-stewardship strategies in companion-animal medicine.

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INTRODUCTION

The oral microbiome is a complex ecosystem of microorganisms (Santibáñez *et al*., 2021) that play a crucial role in maintaining oral health by defending against pathogens, supporting digestion, and modulating the immune response (Davis and Weese, 2022). In healthy dogs, this balance supports stability. However, factors such as inadequate oral hygiene, poor diet, breed predispositions, and systemic illness can lead to microbial imbalance or dysbiosis (Bellows *et al.,* 2019; Cunha *et al.,* 2022). This disruption often results in periodontal disease, characterized by a shift from gram-positive aerobic bacteria to gram-negative anaerobes (Albuquerque *et al*., 2012; Silva *et al*., 2015).

Periodontitis has been reported in up to 90% of dogs over 2 years old (Niemiec, 2013), particularly in small breeds, and its severity increases with age in dogs (Yasuda *et al*., 2024). Periodontitis is a progressive inflammatory condition that affects the supporting tissues of the teeth. It is primarily caused by bacterial infections, including *Porphyromonas*, *Fusobacterium*, *Prevotella*, and *Treponema* species (Riggio *et al*., 2011; Santibáñez *et al*., 2021). These pathogens commonly form biofilms that protect them from host defenses and antimicrobial agents, contributing to treatment resistance and the emergence of antimicrobial resistance (AMR) (Pérez-Serrano *et al*., 2020; Kačírová *et al*., 2021). Some strains have evolved into multidrug-resistant (MDR) pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA). Beyond oral health, chronic periodontitis has been linked to systemic complications, including pneumonia, cardiovascular disease, systemic inflammation, and diabetes mellitus, particularly in geriatric or immunocompromised dogs (Glickman *et al*., 2009; Pereira Dos Santos *et al*., 2019).

The rising challenge of AMR in canine oral bacteria necessitates a reassessment of current treatment strategies. Although first-line antibiotics such as amoxicillin, doxycycline, clindamycin, and metronidazole remain commonly used (Bellows *et al*., 2019; Cunha *et al*., 2022; Albuquerque *et al*., 2012), their declining effectiveness due to resistance has led to the exploration of alternative therapies. Among these, nanostructured lipid carriers (NLCs) show great promise as advanced drug delivery systems (Yostawonkul *et al*., 2024; Yostawonkul *et al*., 2023). NLCs improve drug solubility and bioavailability, provide controlled release, and exhibit mucoadhesive properties that enhance drug retention at the site of infection (Kaewmalun *et al*., 2022; Kamble *et al*., 2024; Sawatphakdee *et al*., 2024). Importantly, NLCs can penetrate biofilms, making them suitable for treating persistent oral infections. Alpha-mangostin, derived from *Garcinia mangostana,* and clove oil from *Syzygium aromaticum*, possess strong antimicrobial, anti-inflammatory, and antibiofilm activities (Zhang *et al*., 2017; Nguyen *et al*., 2020; Tangsuksan *et al*., 2022). While traditional phenotypic methods, such as disc diffusion, remain the standard for assessing antibiotic susceptibility (Hudzicki, 2009), molecular techniques such as whole-genome sequencing (WGS) are becoming increasingly vital. WGS offers precise identification of bacterial species and resistance genes, supporting more targeted and effective treatment (Lewis *et al*., 2021).

The previous study demonstrated the efficacy of an NLC formulation loaded with alpha-mangostin and clove oil (NLC-AMCO) *in vitro*, including its stability, release kinetics, and antibacterial activity (Sawatphakdee *et al*., 2024). However, there is a lack of research specifically focused on the antibacterial activity against oral bacteria in dogs at different stages of periodontitis, as well as genomic investigations of MDR bacteria. Therefore, this study evaluates and compares the antimicrobial efficacy of NLC-AMCO and commonly used antibiotics against oral bacterial isolates from dogs with clinical periodontitis using disc diffusion, MIC, and MBC assays. In addition, whole-genome sequencing is applied to characterize resistance genes in MDR isolates and define their resistome profiles. The overall objective is to determine whether NLC-AMCO can serve as an alternative therapeutic option for MDR oral infections in dogs.

MATERIALS AND METHODS

Ethics approval: All animal procedures were approved by the Institutional Animal Care and Use Committee of Chulalongkorn University (Protocol No. 2331042), and the Institutional Biosafety Committee (IBC No. 2431043). Informed consent was obtained from dog owners in accordance with animal welfare guidelines.

Sample collection and bacterial isolation: Oral swabs were collected from 30 dogs diagnosed with various stages of periodontitis, classified using the AAHA Dental Care Guidelines (Bellows *et al*., 2019), ranging from gingivitis (stage 1) to severe periodontitis with >50% attachment loss (stage 4). A total of 49 bacterial isolates were obtained from the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University. Swabs from the gingival margin were transferred to Amies medium and streaked onto tryptic soy agar (TSA) followed by incubation at 37°C for 24 h under both aerobic and anaerobic conditions. Anaerobic gas packs (AnaeroPack™, Mitsubishi gas chemical company, Inc., Tokyo, Japan) were used in a 2500mL jar to achieve an atmosphere of 0% O_2, and 15% CO_2. Distinct colonies were sub-cultured for purity and identified using MALDI-TOF MS. Pure cultures were preserved in Cryoinstant® vials at –80°C. The general characteristics of the sampled dogs, including age, sex, breed, body condition score (BCS), diet, and stage of periodontitis (C1–C5), are summarized in Table 1.

Table 1: The general characteristics of the studied MDR dogs, including age, sex, BCS, breed, diet, and periodontitis stage of sampling dogs (C1-C5)

Dogs	Age	Sex	BCS (Score 1-5)	Breed	Diet	PD stage
C1	18	MC	3	Mixed	Commercial food (Wet food)	4
C2	10	FS	4	Poodle	Commercial food (Wet food)	4
C3	13	MC	3.5	Maltese	Commercial food (Wet food)	4
C4	10	MC	4	Chihuahuas	Commercial food (Wet food)	4
C5	14	MC	4.5	Pomeranians	Commercial food (Wet food)	4

Preparation of nanostructured lipid carrier loaded with alpha-mangostin and clove oil (NLC-AMCO) solution: A solution of alpha-mangostin (0.25mg) in 19.75mL of clove oil (carrier) was used as the alpha-mangostin-in-clove oil (AMCO) component. DMSO served as the diluent for AMCO dilutions and as the positive control. NLC-AMCO was prepared by forming separate oil and aqueous phases followed by high-speed homogenization (T25 digital ULTRA-TURRAX®, IKA, Staufen, Germany) at 6000 rpm for 5 min, according to the method described by Sawatphakdee *et al*. (2024). The emulsion was stored at 25°C for 24 h before use.

Disc diffusion assay: Antibacterial activity was evaluated using the Kirby–Bauer disc diffusion method (Hudzicki, 2009; Kamble *et al*., 2022). Sterile 6-mm paper discs were aseptically impregnated with 20µL of each test formulation: NLC-AMCO, AMCO, alpha-mangostin (AM), and clove oil (CO). The NLC-AMCO formulation

was applied in liquid form, as described by Sawatphakdee *et al*. (2024). AMCO was prepared by dissolving AM in CO at the same final concentration as in the NLC-AMCO formulation, while AM and CO were dissolved in DMSO prior to disc loading. Commercial antibiotic discs, amoxicillin (AML, 10µg), clindamycin (DA, 2µg), doxycycline (DO, 10µg), and metronidazole (MTZ, 5µg) (OXOID Ltd., UK), served as reference controls (Bellows *et al*., 2019; Cunha *et al*., 2022; Sawatphakdee *et al*., 2024). Plates were incubated at 37°C, after which inhibition zone diameters (IZDs) were measured.

Minimum Inhibitory and Bactericidal Concentrations (MIC and MBC): MICs were determined by broth microdilution using 96-well plates according to CLSI guidelines (Watts *et al*., 2018). MDR bacterial strains tested included *Escherichia coli* CU1, *Klebsiella pneumoniae* CU1, *Morganella morganii* CU1, *Pseudomonas aeruginosa* CU2, and *Acinetobacter baumannii*, all of which were clinical isolates obtained in the present study from the oral cavity of dogs with periodontal disease. These isolates were selected for MIC and MBC determination based on their multidrug-resistant profiles identified through preliminary Kirby–Bauer disk diffusion screening. NLC-AMCO and AMCO were serially diluted (500–0.97mg/mL). Bacterial suspensions (1.5×10^8 CFU/mL) were mixed with the test formulations and incubated for 24 and 48 h at 37°C. The MIC was defined as the lowest concentration that inhibited visible growth. For MBC determination, aliquots from wells without growth were plated, and the lowest concentration yielding no colonies was recorded.

Time-Kill Kinetics: To assess time-dependent killing, MDR bacteria were exposed to NLC-AMCO at a 1:1 ratio. Samples were collected at 0, 15, and 30 min, plated on plate count agar (PCA), and incubated at 37°C for 24 h. Colony-forming units (CFU/mL) were quantified using standard plate-count methods (ISO 4833-2:2013).

Whole Genome Sequencing (WGS): Genomic DNA from MDR bacterial isolates (Table 3) was extracted using the ZymoBIOMICS™ DNA Miniprep Kit following the manufacturer's protocol. Sequencing libraries were prepared and barcoded using the Oxford Nanopore Ligation Sequencing Kit (SQK-NBD 114.24) and sequenced on PromethION P2 Solo platform. Raw sequencing reads generated in POD5 format were basecalled and quality-filtered using Dorado (dna_r10.4.1_e8.2_400bps_sup, v5.0.0) with a minimum Q-score threshold of 15. Adapter trimming was performed using PoreChop, and high-quality reads were assembled de novo using Flye v2.9.4 (Kolmogorov *et al*., 2019). Assembly quality was evaluated using CheckM2 (Chklovski *et al*., 2023), and only assemblies classified as bacterial genomes with >90% completeness and <5% contamination were retained for downstream analysis. Taxonomic assignment of assembled contigs was performed using BLAST (ncbi-blast-2.15.0+). AMR genes were identified by querying assembled contigs against the Comprehensive Antibiotic Resistance Database (CARD v3.3.0) using the Resistance Gene Identifier (RGI v6.0.3). Only "strict" and "perfect" hit categories were considered, with an additional threshold of >80% gene coverage, in accordance with CARD-RGI recommended guidelines to minimize false-positive AMR predictions. WGS data have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1274155 (Alcock *et al*., 2023).

Statistical Analysis: All experiments were performed in triplicate, and the results are expressed as the mean ± SD. Categorical variables (e.g., food type proportions) were analyzed using Fisher's exact tests. Statistical significance was set at $p<0.05$. Heatmaps were generated for the disc diffusion data using RStudio (R Core Team, 2023).

RESULTS

Antimicrobial activity of NLC-AMCO against clinical oral isolates: Heatmap analysis (Fig. 1) highlighted the superior antimicrobial efficacy of NLC-AMCO compared with other treatments against both gram-negative and gram-positive oral bacteria. Of 49 isolates, 27 were gram-negative and 22 were gram-positive, including key periodontitis-associated pathogens such as *Bacteroides pyogenes* and *Porphyromonas gingivalis*.

NLC-AMCO exhibited the strongest antimicrobial effects among gram-negative strains, particularly against *E. coli* (CU1–CU6), *K. pneumoniae* (CU1–CU2), *Pasteurella multocida* (CU1–CU2), *Proteus mirabilis*, and *M. morganii* (CU1–CU2). In contrast, AMCO, AM, and CO exhibited weaker and inconsistent activities. Similarly, NLC-AMCO was effective against gram-positive strains such as *Streptococcus canis*, *Streptococcus anginosus*, *S. aureus*, and *Enterococcus faecalis*.

Notably, its efficacy matched or exceeded that of standard antibiotics, especially in strains resistant to clindamycin (CLI) and doxycycline (DOX). Metronidazole exhibited the highest resistance rate. Although DOX and CLI retained moderate effectiveness, resistance among certain strains reinforces the need for novel therapies.

Importantly, MDR was observed in *A. baumannii*, *E. coli*_CU1, *P. aeruginosa*_CU2, *K. pneumoniae*_CU1, and *M. morganii*_CU1. These findings underscore the potential of NLC-AMCO as a promising alternative for treating MDR infections associated with canine periodontitis.

Antimicrobial activity of NLC-AMCO against MDR gram-negative bacteria: The antimicrobial efficacy of NLC-AMCO was assessed against MDR gram-negative bacteria using zone of inhibition analysis (Fig. 2A-F). NLC-AMCO consistently produced larger and more uniform inhibition zones than conventional antibiotics (AMX, CLI, DOX, MTZ) and other herbal-based treatments (AM, CO, AMCO). Notably, it was especially effective against highly resistant strains such as *A. baumannii*, *P. aeruginosa*_CU2, and *M. morganii*_CU1.

Average inhibition zone analysis (Fig. 2F) showed that both NLC-AMCO and DOX were the most effective; however, NLC-AMCO demonstrated a higher median inhibition zone with lower variability. In contrast, AMCO, AM, and CO exhibited limited and inconsistent activities. These findings emphasize the superior and broad-spectrum antimicrobial activity of NLC-AMCO, underscoring the advantages of nanoencapsulation in overcoming resistance in MDR gram-negative pathogens, where traditional therapies often fail.

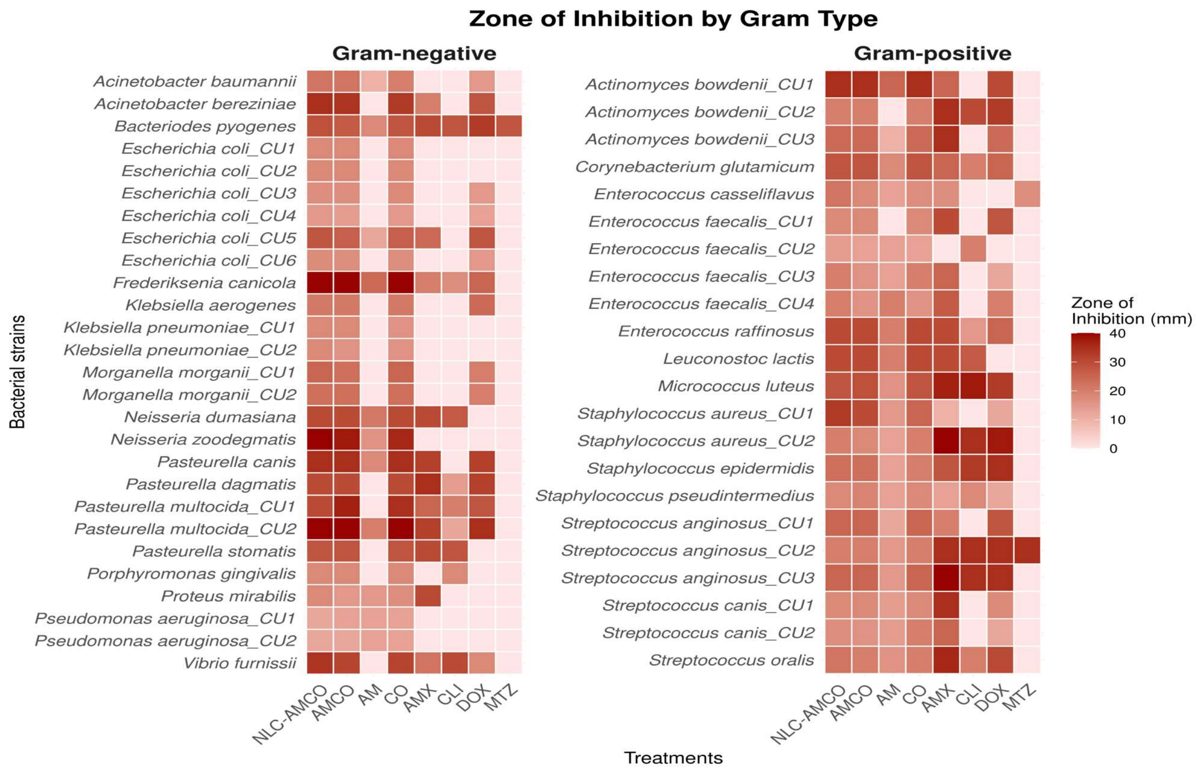

Fig. 1: Heatmap illustrating the comparative antimicrobial activity of eight treatments: nanostructured lipid carrier loaded with alpha-mangostin and clove oil (NLC-AMCO), alpha-mangostin and clove oil (AMCO), Alpha-Mangostin (AM), and clove oil (CO), amoxicillin (AMX), clindamycin (CLI), doxycycline (DOX), and metronidazole (MTZ). Color intensities in the heatmap indicate the degree of bacterial inhibition, categorized as weak, moderate, or strong.

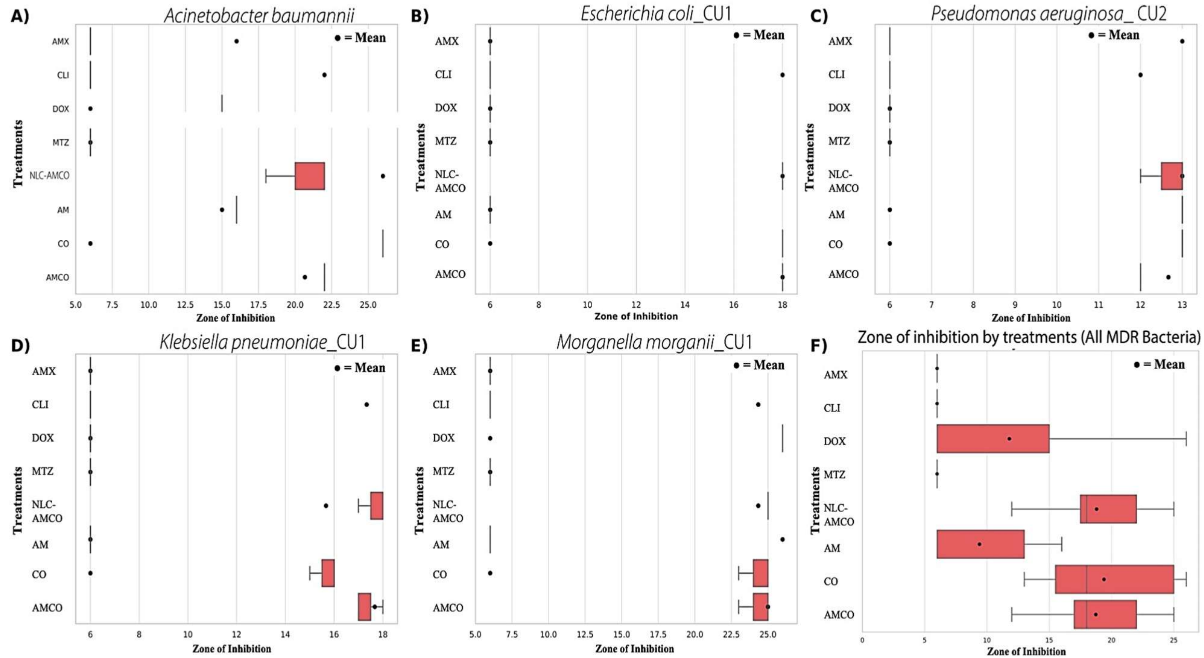

Fig. 2: Zones of inhibition (in mm) against multidrug-resistant (MDR) bacterial isolates recovered from the oral cavity of dogs. Individual panels represent: A) *Acinetobacter baumannii*, B) *Escherichia coli*_CU1, C) *Pseudomonas aeruginosa*_CU2, D) *Klebsiella pneumoniae*_CU1, and E) *Morganella morganii*_CU1. Panel F summarizes the comparative efficacy of each treatment across all MDR strains. Black dots indicate the mean inhibition zone for each treatment, while red box plots show the distribution and variability among replicates.

Metadata analysis of periodontitis-affected dogs: Table 2 presents the distribution of periodontitis cases diagnosed at the Small Animal Teaching Hospital in Bangkok, Thailand, from January to December 2024. Extra-small breeds such as Pomeranians, Chihuahuas, Maltese, and Yorkshire Terriers accounted for the majority of cases, followed by small breeds (6.5–9kg), including Poodles and Dachshunds. Only one medium-sized dog (a Beagle) and eight mixed-breed

medium- to large-sized dogs were recorded. This suggests a higher prevalence of periodontitis among smaller-breeds, consistent with the weight distribution shown in Fig. 3B.

Table 2: Number and percentage of dogs in each of the four weight categories diagnosed with periodontitis and sampled between January to December, 2024 at the Small Animal Teaching Hospital, Chulalongkorn University, Bangkok, Thailand

Weight Size Category	Number of Dogs	Percentage of Dogs
Extra-small (<6.5kg)	28	57.14%
Small (6.5–9kg)	12	24.49%
Medium-small (9–15kg)	1	2.04%
Medium-large (15–<30kg)	8	16.33%

Figs. 3A and 3C further indicate that periodontitis severity was greater in male dogs and in those infected with gram-negative bacteria. Diet was categorized into dry food (pellets), wet commercial food, and homemade food. A statistically significant association was found between diet type and periodontitis stage using Fisher's Exact Test (p=0.002), highlighting the potential role of diet in disease progression.

MIC and MBC of NLC-AMCO against MDR gram-negative bacteria: Table 3 summarizes the MIC and MBC values of AMCO and NLC-AMCO against five MDR gram-negative bacteria isolated from dogs with periodontitis, assessed at 24 and 48 h. At 24 h, AMCO exhibited stronger immediate antibacterial activity, with MICs ranging from 0.98-15.62mg/mL. In contrast, NLC-AMCO exhibited higher MICs (31.25mg/mL), indicating lower initial efficacy. However, by 48 h, NLC-AMCO demonstrated time-dependent enhancement in activity. MIC values decreased for *E. coli*_CU1 (31.25 to 15.62mg/mL), *M. morganii*_CU1 (15.62 to 7.8mg/mL), and *A. baumannii* (3.9 to 1.95mg/mL), while *K. pneumoniae*_CU1 and *P. aeruginosa*_CU2 remained unchanged.

Table 3: MIC (mg/mL) and MBC (mg/mL) values of NLC-AMCO and AMCO Against MDR bacteria isolated from dogs with periodontitis

Sample	MIC (mg/mL)				MBC (mg/mL)			
	Time (24 h)		Time (48 h)		Time (24 h)		Time (48 h)	
Bacteria species (MDR)	AMCO	NLC-AMCO	AMCO	NLC-AMCO	AMCO	NLC-AMCO	AMCO	NLC-AMCO
*Klebsiella pneumoniae*_CU1	1.95	31.25	1.95	31.25	1.95	62.5	1.95	62.5
*Pseudomonas aeruginosa*_CU2	15.62	31.25	15.62	31.25	15.62	31.25	15.62	31.25
*Escherichia coli*_CU1	0.98	31.25	0.98	15.62*	1.95	62.5	1.95	31.25*
*Morganella morganii*_CU1	0.98	15.62	0.98	7.8*	1.95	31.25	1.95	15.6*
Acinetobacter baumanii	0.98	3.9	0.98	1.95*	0.98	7.8	0.98	3.9*

The asterisk (*) denote changes in MIC or MBC values observed after extended contact time.

The MBC results reflected a similar pattern. The most notable reduction was observed in *A. baumannii* (7.8 mg/mL to 3.9mg/mL), followed by *E. coli*_CU1 (62.5 to 31.25mg/mL) and *M. morganii*_CU1 (31.25 to 15.6mg/mL). *K. pneumoniae*_CU1 showed no change, while *P. aeruginosa*_CU2 had identical MIC and MBC values (31.25mg/mL), indicating bactericidal action. Overall, AMCO showed superior immediate efficacy, whereas NLC-AMCO exhibited enhanced and sustained antibacterial activity over time, particularly against selected MDR strains, highlighting its potential as a controlled-release therapeutic agent.

Bacterial survival rate: Total plate count analysis demonstrated a clear relationship between bacterial viability and time following exposure to NLC-AMCO. Most bacterial populations declined rapidly, with no observable growth as early as 15 min post-treatment. However, *E. coli*_CU1 exhibited a more gradual reduction beginning at 15 min, followed by a continuous decrease in colony numbers. After 30 min, no bacterial growth was detected on plate count agar (Fig. 4).

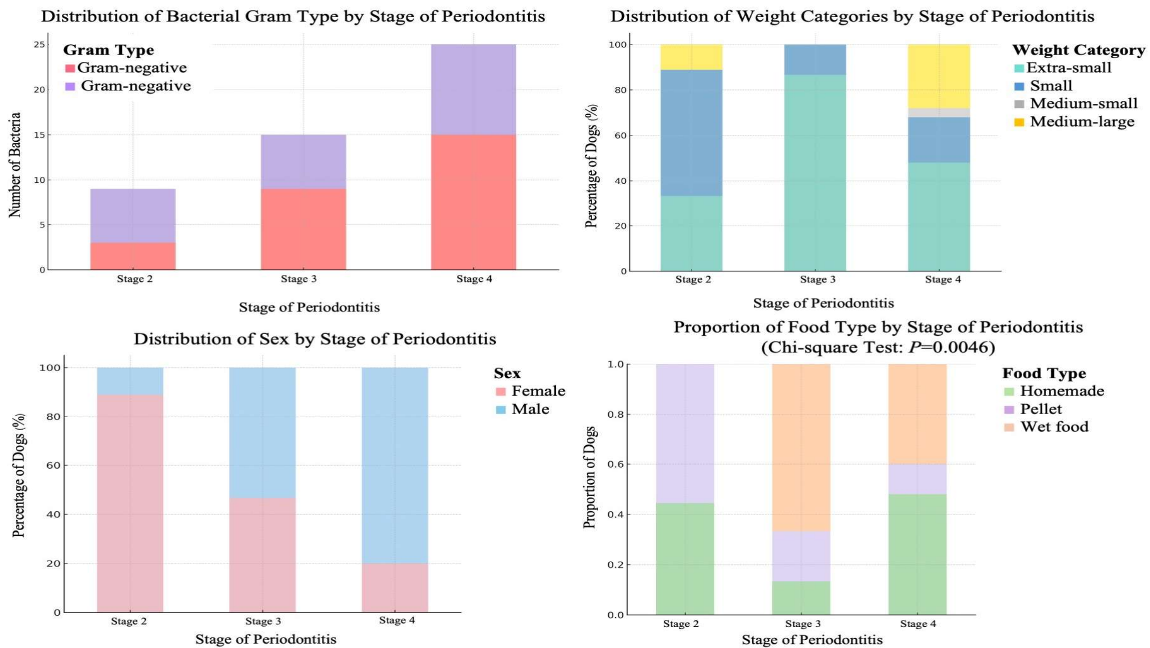

Fig. 3: Stacked bar graphs depicting the distribution of selected metadata variables across periodontitis stages. A) the number of gram-positive and gram-negative bacteria, B) distribution by weight categories, C) distribution by sex, and D) the proportion by type of food. A statistically significant association was found between food type and periodontitis stage (Fisher's exact test, P=0.002).

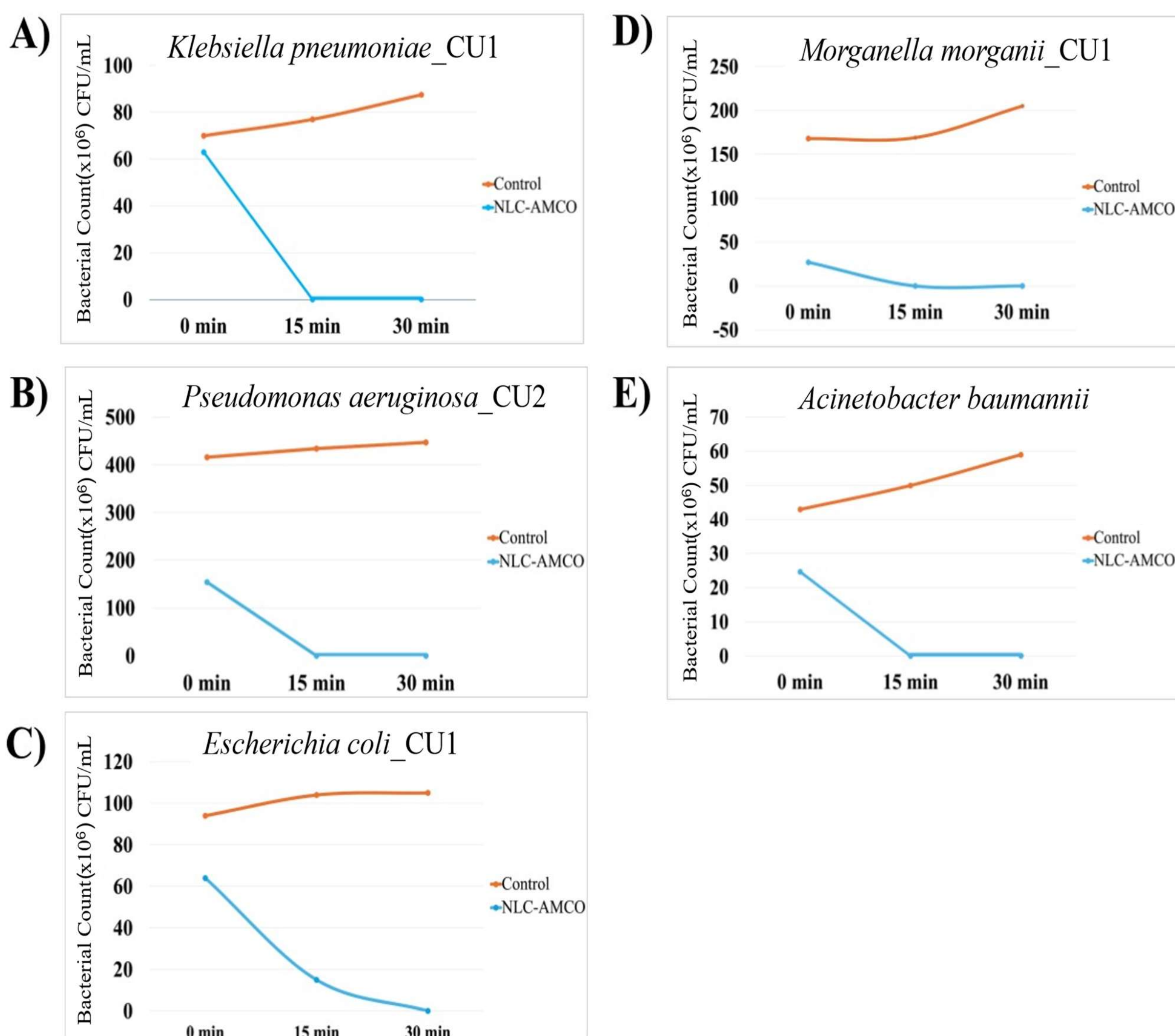

Fig. 4: Total plate count (TPC) showing the number of viable bacterial colonies after treatment with NLC-AMCO compared to the control. Colony-forming units (CFU) were quantified to evaluate the antimicrobial efficacy of each treatment against MDR bacteria isolated from dogs with periodontitis.

Whole-genome sequencing and antimicrobial resistance profiles: Whole-genome sequencing identified extensive AMR determinants distributed across multiple drug classes, with the largest resistomes observed in *P. aeruginosa* and *E. coli* (Fig. 5). Fluoroquinolone-associated genes were the most frequently detected across MDR isolates, followed by resistance determinants linked to β-lactam subclasses (cephalosporins and penams). *P. aeruginosa* encoded the broadest repertoire, including multiple hits associated with ciprofloxacin, nalidixic acid, and norfloxacin, as well as peptide-class resistance affecting last-line polymyxins (colistin A and colistin B).

A heat map (Fig. 5) summarized the number of unique resistance genes ("hits"), as annotated by CARD/RGI, for 45 antibiotics stratified into functional classes (e.g., fluoroquinolones, cephalosporins, aminoglycosides) across the assembled genomes of *P. aeruginosa*, *M. morganii* subsp. *morganii*, *K. pneumoniae* subsp. *pneumoniae*, *E. coli*, and *A. baumannii*. *P. aeruginosa* possessed the most extensive resistome. Notably, high copy numbers were observed within the peptide and fluoroquinolone classes, including hits for the last-line polymyxins (colistin A and colistin B), ciprofloxacin (seven genes), whereas three to four hits were detected for the carbapenems imipenem and meropenem. *E. coli* also encoded a broad panel, encompassing six genes for ciprofloxacin and determinants against multiple aminoglycosides (amikacin, gentamicin C) and cephalosporins.

Fig. 6 further categorized the resistance genes into 12 families, clustered into three major mechanisms: (i) Efflux systems, especially resistance-nodulation–cell division (RND) pumps, which were highly expanded in *P. aeruginosa* and moderately present in *E. coli*, along with ATP-binding cassette (ABC-type) and major facilitator superfamily (MFS) pumps found at lower levels in *M. morganii* and *E. coli*; (ii) Target-site modifications, including resistant *gyrA*, *gyrB* and *parC* alleles associated with fluroquinolone resistance, and elongation-factor-Tu-variants linked to elfamycin resistance in *K. pneumoniae* and *E. coli*; and (iii) Cell-envelope adaptations, such as porin mutations reducing β-lactam uptake and modified penicillin-binding-protein (PBPs).

Fig. 5: Integrated heat-map visualization of predicted antimicrobial-resistance determinants across five multidrug-resistant (MDR) bacterial genomes. The heat-map displays the number of unique AMR gene hits annotated by the Comprehensive Antibiotic Resistance Database (CARD) using RGI (strict mode, ≥80 % coverage threshold) across 45 antimicrobial compounds grouped by functional class (e.g., fluoroquinolones, cephalosporins, aminoglycosides, carbapenems, penams, macrolides, peptide antibiotics, disinfectants/antiseptics, and others) in *Pseudomonas aeruginosa*, *Morganella morganii* subsp. *morganii*, *Klebsiella pneumoniae* subsp. *pneumoniae*, *Escherichia coli*, and *Acinetobacter baumannii*. Each row represents a bacterial species and each column represents an antibiotic agent, with color intensity indicating the abundance of predicted resistance determinants associated with that compound.

Fig. 6: Distribution of antimicrobial-resistance gene families in five multidrug-resistant (MDR) bacterial species isolated from dogs with periodontitis. The heatmap displays the number of hits (Hit) for each antimicrobial resistance gene. The color intensity represents the number of hits per gene family in each species, with higher values shown in yellow and lower in purple.

DISCUSSION

Periodontitis is one of the most common oral diseases in dogs and is widely recognized as a multifactorial condition (Wallis *et al.,* 2021; Gonçalves-Anjo *et al.,* 2022; O'Flynn *et al.,* 2025). Its progression is influenced by age, diet, breed, and oral hygiene (Watson, 1994; Gorrel, 1998; Gonçalves-Anjo *et al*., 2022). Poor oral care accelerates disease onset, leading to significant periodontal destruction and eventual tooth loss (Cunha *et al*., 2022). Mechanistically, the disease

begins with bacterial biofilm formation on gingival surfaces, triggering an immune response marked by neutrophil infiltration and the release of proinflammatory cytokines such as *IL-1β* and *TNF-α*, which ultimately drives the degradation of periodontal ligaments and alveolar bone (Wallis *et al*., 2025). Advanced cases typically require dental scaling, antibiotics, and anti-inflammatory medications (Bellows *et al*., 2019).

In this study, 49 bacterial isolates were obtained from dogs with clinical signs of periodontitis, some of which harbored multiple strains. Dogs with advanced periodontitis had higher proportions of gram-negative bacteria, consistent with their known virulence mechanisms such as lipopolysaccharide production and immune evasion (Cekici *et al*., 2014; Silva *et al*., 2015; Wallis *et al*., 2015). Small breed dogs, including Pomeranians, Chihuahuas, Maltese, and Dachshunds, were overrepresented, similar to trends observed in other countries (Wallis *et al*., 2021). These breeds are commonly kept as household pets, increasing the likelihood of early recognition of symptoms such as halitosis, drooling, and reduced appetite, which may contribute to higher diagnosis rates.

Dietary patterns emerged as a significant risk factor. Dogs fed soft or wet diets had a higher prevalence of severe periodontitis. These diets adhere to tooth surfaces, encouraging plaque formation, whereas dry kibble provides mild abrasive action that helps reduce plaque buildup. Fisher's exact test confirmed a significant relationship between diet type and periodontitis severity ($p = 0.002$), reinforcing prior findings that soft diets may exacerbate disease progression (Wallis *et al*., 2021; O'Flynn *et al*., 2025). Dogs with stage 2 disease often continued eating dry pellets or homemade diets because of milder symptoms. In contrast, those with advanced stages exhibited symptoms such as oral pain and excessive salivation, prompting a shift to softer diets. However, in the absence of oral hygiene, these diets can worsen plaque accumulation and accelerate disease progression.

Five of the bacterial strains isolated were identified as MDR: *A. baumannii*, *E. coli*_CU1, *P. aeruginosa*_CU2, *K. pneumoniae*_CU1, and *M. morganii*_CU1. These organisms were found resistant to tested antibiotics (penicillin, cephalosporins, fluoroquinolones, and tetracyclines), which are commonly used in veterinary medicine. This aligns with global trends in AMR in companion animals (Basak *et al*., 2016; Soonthornsit *et al*., 2022; Authority *et al*., 2025). Fluoroquinolone such as enrofloxacin, marbofloxacin, and pradofloxacin, are typically used to treat respiratory and urinary tract infections in dogs (Grabowski *et al*., 2022); however resistance to these drugs was observed in all oral isolates, mirroring previous findings (Vingopoulou *et al*., 2018).

Penams and penems, which inhibit bacterial cell wall synthesis, are generally reserved for serious infections due to concerns regarding rising AMR (Basak *et al*., 2016). In canine periodontal disease, penems are used only when first-line agents fail, particularly in cases of osteomyelitis (Conrads *et al*., 2021). Tetracyclines resistance was notably high in *P. aeruginosa*_CU2, *E. coli*_CU1, and *K. pneumoniae*_CU1, consistent with genotypic findings and phenotypic disc diffusion testing.

Both phenotypic and genotypic analyses confirmed resistance in multiple isolates. These findings underscore the urgent need for antimicrobial stewardship and the development of alternative therapeutic strategies. The presence of MDR bacteria in companion animals also poses a zoonotic threat with broader public health implications (McEwen and Collignon, 2018).

Resistance is not solely chromosomal; plasmid-mediated resistance is a major contributor to the spread of MDR. Plasmids carrying multiple resistance genes can be transferred horizontally, facilitating the dissemination of AMR traits. For example, *E. coli* strains isolated from dogs have been shown to carry plasmid-borne *mcr-1* genes, which confer resistance to colistin, a last-resort antibiotic (Wu *et al*., 2024). Supporting this, a Thai veterinary teaching hospital reported high resistance rates of MDR *E. coli* isolates to ampicillin (96.1%), enrofloxacin (80.4%), and tetracycline (78.4%). In Malaysia, 72% of *E. coli* isolates from dogs were MDR, with notable resistance to cephalexin and amoxicillin/clavulanic acid (Haulisah *et al*., 2022; Soonthornsit *et al*., 2022).

Whole-genome analysis of *P. aeruginosa* and *E. coli* isolates revealed resistance genes to colistin, ciprofloxacin, and carbapenems (Poirel *et al*., 2018; Shoaib *et al*., 2025; Wallis *et al*., 2025). Mechanisms such as efflux pumps (e.g., AcrAB-TolC), porin mutations, and target-site alterations further reduce antibiotic efficacy (Shoaib *et al*., 2025) and enhance persistence in the oral environment (Elfadadny *et al*., 2023; Wallis *et al*., 2025).

To address this issue, a nano-herbal formulation, NLC-AMCO, was developed using alpha-mangostin and clove oil, both of which possess known antimicrobial properties. NLCs improve solubility and bioavailability and provide sustained drug release (Sawatphakdee *et al*., 2024). Mechanistically, these compounds disrupt bacterial membranes (Zhang *et al*., 2017; Sivaranjani *et al*., 2019; Deng *et al*., 2023), inhibit ATP synthesis, and interfere with quorum sensing (Krishnan *et al*., 2012). Nano-formulations also penetrate biofilms more effectively (Nguyen *et al*., 2020) and maintain longer drug activity (Jamil *et al*., 2016). Antimicrobial tests showed that NLC-AMCO produced broader inhibition zones and improved heatmap profiles compared with unformulated extracts and conventional antibiotics. Although MIC values were initially higher, they declined over time, suggesting that NLC-AMCO operates through a sustained-release mechanism (Sawatphakdee *et al*., 2024), thereby enhancing antimicrobial activity.

Total plate count assays demonstrated rapid bactericidal activity. Most bacterial strains were significantly reduced within 15 min, with complete inhibition by 30 min. Even *E. coli*_CU1, which showed slower decline, was fully eliminated within the same period. Certain host characteristics were also associated with the presence of MDR strains. Affected dogs were often older, neutered males from small breeds, fed soft diets, and had higher body condition scores. These observations mirror those in human studies, where diet and metabolic status influence periodontitis severity and oral microbiota (Saito and Shimazaki, 2007; Kania *et al*., 2025).

Although no dogs had received antibiotics in the month prior to sampling, many had a history of systemic illness and antibiotic exposure, suggesting that cumulative treatment history and host immune status may drive resistance development. One limitation of this study was

the underrepresentation of obligate anaerobic bacteria owing to culture constraints. Future research should consider molecular and anaerobic approaches, such as broth microdilution or metagenomics, to better characterize the canine oral microbiome (Benning and Mathers, 1999; Geaman *et al*., 2024).

Conclusions: This study demonstrated the potent antimicrobial efficacy of the novel NLC-AMCO formulation, which combines alpha-mangostin and clove oil, against MDR bacterial isolates recovered from dogs with periodontitis. NLC-AMCO consistently outperformed its individual components and several conventional antibiotics *in vitro*, including colistin- and ciprofloxacin-resistant strains. WGS analysis further revealed species-specific resistome complexity, with *P. aeruginosa* exhibiting the broadest profile, particularly enriched for fluoroquinolones resistance and peptide-antibiotic determinants, followed by *E. coli*. The distribution of resistance genes suggested dominant efflux-mediated mechanisms, including RND pumps in *P. aeruginosa* and moderate representation of ABC-type and MFS transporters in *E. coli*. Together, these genomic insights complement the phenotypic findings and support NLC-AMCO as a promising, sustainable veterinary alternative that may contribute to improved antimicrobial stewardship in companion-animal dentistry.

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REFERENCES

Albuquerque C, Morinha F, Requicha J, *et al*., 2012. Canine periodontitis: the dog as an important model for periodontal studies. The Veterinary Journal 191:299-305.

Alcock BP, Huynh W, Chalil R, *et al*., 2023. CARD 2023: Expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. Nucleic Acids Research 51:D690-D699.

Authority EFS, Prevention ECfD and Control, 2025. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2022–2023. EFSA Journal 23:e9237.

Basak S, Singh P and Rajurkar M, 2016. Multidrug resistant and extensively drug resistant bacteria: A study. Journal of Pathogens 2016:4065603.

Bellows J, Berg ML, Dennis S, *et al*., 2019. 2019 AAHA dental care guidelines for dogs and cats. Journal of the American Animal Hospital Association 55:49-69.

Benning VR and Mathers JJ, 1999. Comparison of agar dilution and broth microdilution methods of anaerobic antimicrobial susceptibility testing using several veterinary antibiotics against *Clostridium perfringens* strains originating from porcine and avian sources. Anaerobe 5:561-569.

Cekici A, Kantarci A, Hasturk H, *et al*., 2014. Inflammatory and immune pathways in the pathogenesis of periodontal disease. Periodontology 2000 64:57-80.

Chklovski A, Parks DH, Woodcroft BJ, *et al*., 2023. CheckM2: a rapid, scalable and accurate tool for assessing microbial genome quality using machine learning. Nature Methods 20:1203-1212.

Conrads G, Klomp T, Deng D, *et al*., 2021. The antimicrobial susceptibility of *Porphyromonas gingivalis*: Genetic repertoire, global phenotype, and review of the literature. Antibiotics 10:1438.

Cunha E, Tavares L and Oliveira M, 2022. Revisiting periodontal disease in dogs: How to manage this new old problem? Antibiotics 11:1729.

Davis EM and Weese JS, 2022. Oral microbiome in dogs and cats: Dysbiosis and the utility of antimicrobial therapy in the treatment of periodontal disease. Veterinary Clinics: Small Animal Practice 52:107-119.

Deng X, Xu H, Li D, *et al*., 2023. Mechanisms of rapid bactericidal and anti-biofilm alpha-mangostin in vitro activity against *Staphylococcus aureus*. Polish Journal of Microbiology 72:199-208.

Elfadadny A, Uchiyama J, Goto K, *et al*., 2023. Antimicrobial resistance and genotyping of *Pseudomonas aeruginosa* isolated from the ear canals of dogs in Japan. Frontiers in Veterinary Science 10:1074127.

Geaman W, Choi BI, Kaindl J, *et al*., 2024. Microbroth dilution method for antibiotic susceptibility testing of fastidious and anaerobic bacteria of the urinary microbiome. Microbiology Spectrum 12:e0031424.

Glickman LT, Glickman NW, Moore GE, *et al*., 2009. Evaluation of the risk of endocarditis and other cardiovascular events on the basis of the severity of periodontal disease in dogs. Journal of the American Veterinary Medical Association 234: 486-94.

Gonçalves-Anjo N, Requicha J, Teixeira A, *et al*., 2022. Genomic medicine in periodontal disease: old issue, new insights. Journal of Veterinary Dentistry 39:314-322.

Gorrel C, 1998. Periodontal disease and diet in domestic pets. The Journal of Nutrition 128: S2712-S2714.

Grabowski Ł, Gaffke L, Pierzynowska K, *et al*., 2022. Enrofloxacin—the ruthless killer of eukaryotic cells or the last hope in the fight against bacterial infections? International Journal of Molecular Sciences 23:3648.

Haulisah NA, Hassan L, Jajere SM, *et al*., 2022. High prevalence of antimicrobial resistance and multidrug resistance among bacterial isolates from diseased pets: retrospective laboratory data (2015–2017). PLoS One 17:e0277664.

Hudzicki J, 2009. Kirby-Bauer disk diffusion susceptibility test protocol. American Society for Microbiology 15:55-63.

Jamil B, Abbasi R, Abbasi S, *et al*., 2016. Encapsulation of cardamom essential oil in chitosan nano-composites: in-vitro efficacy on antibiotic-resistant bacterial pathogens and cytotoxicity studies. Frontiers in Microbiology 7:1580.

Kačírová J, Maďari A, Mucha R, *et al*., 2021. Study of microbiocenosis of canine dental biofilms. Scientific Reports 11:19776.

Kaewmalun S, Yata T, Kitiyodom S, *et al*., 2022. Clove oil-nanostructured lipid carriers: a platform of herbal anesthetics in Whiteleg shrimp (*Penaeus vannamei*). Foods 11:3162.

Kamble MT, Rudtanatip T, Soowannayan C, *et al*., 2022. Depolymerized fractions of sulfated galactans extracted from *Gracilaria fisheri* and their antibacterial activity against *Vibrio parahaemolyticus* and *Vibrio harveyi*. Marine Drugs 20:469.

Kamble MT, Yostawonkul J, Medhe SV, *et al*., 2024. Innovative feed additives for sustainable aquaculture: phytobiotics encapsulated in organic nanoparticles. In: Sustainable Feed Ingredients and Additives for Aquaculture Farming: Perspectives from Africa and Asia (Gabriel N, Abasubong K, Erasmus V, Kamble M, eds): Springer Nature, Singapore, pp:501–520.

Kania M, Drab Z, Dąbrowska M, *et al*., 2025. Impact of diet and nutritional status on gingival crevicular fluid metabolome and microbiome in people with type 1 diabetes: a cross-sectional study. Scientific Reports 15:32781.

Kolmogorov M, Yuan J, Lin Y, *et al*., 2019. Assembly of long, error-prone reads using repeat graphs. Nature Biotechnology 37:540-546.

Krishnan T, Yin W-F, Chan K-G, 2012. Inhibition of quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa* PAO1 by ayurveda spice clove (*Syzygium aromaticum*) bud extract. Sensors 12:4016-4030.

Lewis S, Nash A, Li Q, *et al*., 2021. Comparison of 16S and whole genome dog microbiomes using machine learning. BioData Mining 14:41.

Mcewen SA, Collignon PJ, 2018. Antimicrobial resistance: a One Health perspective. Microbiology Spectrum 6:26.

Nguyen PT, Nguyen MT, Quach LT, *et al*., 2020. Antibiofilm activity of alpha-mangostin loaded nanoparticles against *Streptococcus mutans*. Asian Pacific Journal of Tropical Biomedicine 10:325.

Niemiec BA, 2013. Veterinary Periodontology, Wiley- Blackwell, Hoboken, NJ, USA.

O'Flynn C, Wright H, Williams T, *et al*., 2025. Veterinary assessment of periodontitis disease risk in dogs: a multi-country survey of clinical decision-making. Preventive Veterinary Medicine 247:106735.

Pereira dos Santos JD, Cunha E, Nunes T, *et al*., 2019. Relation between periodontal disease and systemic diseases in dogs. Research in Veterinary Science 125:136-140.

Pérez-Serrano RM, Domínguez-Pérez RA, Ayala-Herrera JL, *et al*., 2020. Dental plaque microbiota of pet owners and their dogs as a shared source and reservoir of antimicrobial resistance genes. Journal of Global Antimicrobial Resistance 21:285-290.

Poirel L, Madec JY, Lupo A, *et al*., 2018. Antimicrobial resistance in *Escherichia coli*. Microbiology Spectrum 6:e0026-2017.

Riggio MP, Lennon A, Taylor DJ, *et al*., 2011. Molecular identification of bacteria associated with canine periodontal disease. Veterinary Microbiology 150:394-400.

Saito T, Shimazaki Y, 2007. Metabolic disorders related to obesity and periodontal disease. Periodontology 2000 43:254-266.

Santibáñez R, Rodríguez-Salas C, Flores-Yáñez C, *et al*., 2021. Assessment of changes in the oral microbiome that occur in dogs with periodontal disease. Veterinary Sciences 8:118.

Sawatphakdee G, Yostawonkul J, Oontawee S, *et al*., 2024. Feasibility of nanostructured lipid carrier loaded with alpha-mangostin and clove oil for canine periodontal therapy. Animals 14:2084.

Shoaib M, Gul S, Majeed S, *et al*., 2025. Pathogenomic characterization of multidrug-resistant *Escherichia coli* strains carrying wide efflux-associated and virulence genes from the dairy farm environment in Xinjiang, China. Antibiotics 14(5):511.

Silva N, Abusleme L, Bravo D, *et al*., 2015. Host response mechanisms in periodontal diseases. Journal of Applied Oral Science 23:329-355.

Sivaranjani M, Leskinen K, Aravindraja C, *et al*., 2019. Deciphering the antibacterial mode of action of alpha-mangostin on *Staphylococcus epidermidis* RP62A through an integrated transcriptomic and proteomic approach. Frontiers in Microbiology 10:150.

Soonthornsit J, Apiratwarrasakul S, Phumthanakorn N, 2022. Clinical characteristics, antimicrobial resistance and treatment outcomes of multidrug-resistant *Escherichia coli* infection in dogs and cats at a veterinary teaching hospital in Thailand. The Thai Journal of Veterinary Medicine 52:207-212.

Tangsuksan P, Srichana T, Kettratad M and Nittayananta W, 2022. Antimicrobial and anti-inflammatory effects of α-mangostin soluble film. Journal of International Society of Preventive and Community Dentistry 12:189-198.

Vingopoulou EI, Delis GA, Batzias GC, *et al*., 2018. Prevalence and mechanisms of resistance to fluoroquinolones in *Pseudomonas aeruginosa* and *Escherichia coli* isolates recovered from dogs suffering from otitis in Greece. Veterinary Microbiology 213:102-107.

Wallis C, Colyer A and Holcombe LJ, 2025. Bacterial associations with periodontal disease in Yorkshire terriers. BMC Veterinary Research 21:296.

Wallis C, Marshall M, Colyer A, *et al*., 2015. A longitudinal assessment of changes in bacterial community composition associated with the development of periodontal disease in dogs. Veterinary Microbiology 181:271-282.

Wallis C, Saito EK, Salt C, *et al*., 2021. Association of periodontal disease with breed size, breed, weight, and age in pure-bred client-owned dogs in the United States. The Veterinary Journal 275:105717.

Watts JL, Sweeney MT and Lubbers BV, 2018. Antimicrobial susceptibility testing of bacteria of veterinary origin. Microbiology Spectrum 6:16.

Wu Y, Wang CH, LI X, *et al*., 2024. Characteristics of the plasmid-mediate colistin-resistance gene mcr-1 in *Escherichia coli* isolated from pig farm in Jiangxi. Pakistan Veterinary Journal 44(4):1303-1307.

Yasuda J, Narita T, Shimizu T, *et al*., 2024. Investigation of periodontal disease development and *Porphyromonas gulae* FimA genotype distribution in small dogs. Scientific Reports 14:5360.

Yostawonkul J, Kitiyodom S, Kamble MT, *et al*., 2024. Immersion of nanostructured lipid carriers loaded with 17-alpha methyltestosterone for masculinization of red tilapia (*Oreochromis* sp.). Aquaculture 586:740780.

Yostawonkul J, Kitiyodom S, Supchukun K, *et al*., 2023. Masculinization of red tilapia (*Oreochromis* spp.) using 17α-methyltestosterone-loaded alkyl polyglucosides integrated into nanostructured lipid carriers. Animals 13:1364.

Zhang Y, Wang Y, Zhu X, *et al*., 2017. Antibacterial and antibiofilm activities of eugenol from essential oil of *Syzygium aromaticum* (L.) Merr. & L. M. Perry (clove) leaf against periodontal pathogen *Porphyromonas gingivalis*. Microbial Pathogenesis 113:396-402.