

RESEARCH ARTICLE

Selection and Phenotypic Characterization of Potential Age-Associated Probiotic Candidates from Canine Fecal Microbiota

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Article ID: KVFD-2026-37251**Received:** 08.06.2026**Accepted:** 23.10.2026**Published Online:** 26.10.2026**Abstract**

The term “microbiota,” formerly known as “microflora,” generally refers to bacterial communities found on mucosal surfaces or other parts of the body, such as the skin. The canine gut microbiota plays a critical role in host health and undergoes dynamic changes across life stages. This study aimed to isolate, screen, and presumptively characterize potential probiotic candidate bacteria from canine fecal samples obtained from puppy, young, and adult dogs. Fecal samples were collected from three healthy dogs in each of three age groups (nine dogs): puppies (<6 months), young dogs (6-18 months), and adults (>18 months). From an initial pool of approximately 1000 bacterial isolates, 90 Gram-positive, catalase-negative, and oxidase-negative isolates were selected after a multi-stage *in vitro* screening for tolerance to pH 2.0, resistance to 3% porcine bile, blood agar hemolysis, and growth under combined stress (pH 4.5 and 2.5% sodium cholate). Following functional and safety screening, 30 representative candidate strains (10 from each age group, selected via balanced randomization among eligible non-redundant isolates) were biochemically characterized using the VITEK® 2 GP system. Presumptively identified taxa belonged predominantly to lactic acid bacteria, including *Lactiplantibacillus* (*Lactobacillus*) *plantarum*, *Enterococcus faecium*, *Pediococcus acidilactici*, *Limosilactobacillus* (*Lactobacillus*) *reuteri*, *Enterococcus faecalis*, and *Pediococcus pentosaceus*, which were detected across all three age cohorts, as well as stage-associated taxa such as *Lactobacillus acidophilus* and *Weissella confusa*. Isolates exhibited distinct substrate utilization profiles, yielding 17, 16, and 10 positive reactions across variable markers in puppy, young, and adult groups, respectively. These findings establish a well-characterized strain collection and demonstrate that canine fecal microbiota harbors viable, stress-tolerant lactic acid bacteria with age-associated phenotypic profiles, providing candidate strains for future molecular confirmation and functional evaluation.

Keywords: Animal probiotics, Canine intestinal microbiota, Lactic acid bacteria, Phenotypic characterization, Presumptive identification

INTRODUCTION

The gastrointestinal (GI) tract of dogs harbors a highly diverse and dynamic microbial ecosystem that plays essential roles in host metabolism, immune modulation, nutrient absorption, and maintenance of intestinal barrier integrity. The gut microbiota is now recognized as a key determinant of overall health in companion animals, and disturbances in microbial composition (dysbiosis) have been associated with a range of pathological conditions including inflammatory bowel disease, acute diarrhea, metabolic disorders, and immune-mediated diseases [1,2].

In healthy dogs, the intestinal microbiota is predominantly composed of bacterial phyla such as *Firmicutes*, *Bacteroidetes*,

Fusobacteria, *Proteobacteria*, and *Actinobacteria*. The balance among these microbial communities is critical for maintaining gut homeostasis, and alterations in their relative abundance have been linked to gastrointestinal and systemic diseases [1]. Consequently, modulation of gut microbiota has emerged as a central strategy in veterinary medicine to improve animal health and performance.

Probiotics, defined as live microorganisms that confer health benefits to the host when administered in adequate amounts, have gained increasing attention as functional feed additives in companion animals [2,3]. Numerous studies have demonstrated that probiotic supplementation in dogs can enhance intestinal microbial balance,



improve immune responses, and reduce the incidence and severity of gastrointestinal disorders ^[4,5]. In addition, certain probiotic strains have shown anti-inflammatory properties and the ability to strengthen intestinal barrier function by modulating tight junction proteins and cytokine expression ^[5].

Host specificity has been identified as a critical factor influencing probiotic efficacy. Early studies demonstrated that lactic acid bacteria (LAB) isolated from canine gastrointestinal tracts exhibit superior adaptation and functional performance compared to non-host-derived strains ^[6]. Subsequent research has further supported the importance of selecting probiotic candidates directly from the target host to ensure better colonization, survival, and biological activity within the gastrointestinal environment ^[7].

Importantly, the canine gut microbiota is not static but undergoes significant changes throughout the life cycle. During early life, puppies exhibit an immature and unstable microbiota characterized by higher variability and increased susceptibility to dysbiosis. As dogs mature, the microbial community becomes more stable and diverse, resembling the adult microbiota typically after several months of age ^[8]. In contrast, aging is associated with shifts in microbial composition, reduced production of beneficial metabolites such as short-chain fatty acids, and increased prevalence of potentially pathogenic taxa ^[9,10].

These age-related changes in gut microbiota have important physiological and clinical implications. Variations in microbial composition have been linked not only to intestinal health but also to systemic functions including metabolism, immune competence, and even cognitive performance in dogs ^[10]. Furthermore, recent studies indicate that the response to probiotic supplementation may also be age-dependent, with older dogs often showing more pronounced improvements in microbial balance and health-related outcomes following probiotic intervention ^[4].

Despite growing evidence highlighting the importance of microbiota, most commercially available probiotic products for dogs are formulated with generic, single-formula compositions that do not account for the life-stage-dependent characteristics of the host. This conventional approach overlooks the distinct ecological and physiological niches present across different ages, juvenile animals undergo rapid immune and microbiota development, while mature and adult dogs exhibit distinct metabolic pressures and microbial stabilization ^[8,9]. Therefore, evaluating host-derived bacterial candidates associated with specific life stages represents a biologically rational strategy to better match host physiology.

Although culture-independent high-throughput sequencing has greatly expanded our understanding of the canine microbiome architecture, it primarily detects relative gene abundance and includes non-culturable or non-viable fractions ^[1]. In contrast, culture-dependent isolation remains indispensable for functional probiotic development, as viable, robust, and metabolically active bacterial strains are strictly required for *in vitro* stress screening, formulation, and eventual biological delivery ^[11,12].

Based on these considerations, we hypothesized that canine fecal microbiota from different life stages harbors distinct cultivable bacterial candidates displaying differential, life-stage-associated phenotypic and stress tolerance characteristics. In this study, we aimed to isolate, screen, and presumptively characterize potential probiotic candidates from healthy dogs across three distinct life stages (puppy, young, and adult). A multi-stage *in vitro* screening cascade-including gastric acid tolerance, bile resistance, safety profiling, and growth performance under combined stress-was employed to establish a characterized collection of 30 candidate strains (10 per age cohort) and to evaluate their stage-associated biochemical profiles.

MATERIALS AND METHODS

Ethical Statement

All procedures involving animals were approved by the Kafkas University Local Ethics Committee on Animal Experiments Local Ethics Committee on Animal Experiments (KAÜ-HADYEK 2026/083).

Sample Collection

Fresh fecal samples were collected from nine healthy shepherd dogs in Kars region, northeastern Türkiye. These dogs, raised in traditional rural settings with consistent environmental exposure and diet, were selected to minimize external variables. A total of nine healthy dogs were enrolled in the study and equally divided into three age groups (three dogs per group): puppies (<6 months), young dogs (6-18 months), and adult dogs (>18 months). Breed and sex were not primary stratification factors, as the study focused on developmental dynamics. All dogs met inclusion criteria, showing no history of gastrointestinal issues, up-to-date on antiparasitic treatments, and no antimicrobial or anti-inflammatory drug use for at least four months prior.

Immediately after defecation, naturally voided fecal samples were collected. To minimize peripheral and soil contamination, samples were carefully taken from the internal core of the fecal mass. Collected fresh fecal samples were transferred into sterile tubes, rapidly frozen in liquid nitrogen, stored at -80°C.

Isolation and enrichment of acid-tolerant bacteria

For the initial recovery and enrichment of acid-tolerant microbial populations, frozen fecal samples were thawed at 4°C, and 1.0 g from each sample was homogenized in 9.0 mL of sterile Maximum Recovery Diluent (MRD; 1:10, w/v). To simulate early gastric stress and preferentially enrich intrinsically acid-tolerant bacteria, the suspensions were inoculated into seven different liquid media: MRS Broth (Sigma-Aldrich, 69966), M17 Broth (Sigma-Aldrich, 56156), BSM Broth (Sigma-Aldrich, 90273) supplemented with BSM supplement (Sigma-Aldrich, 83055), Brain Heart Infusion (BHI) Broth (Sigma-Aldrich, 75917), Universal Pre-Enrichment Broth (Sigma-Aldrich, 91366), Modified Tryptone Soy Broth (Sigma-Aldrich, 76704), and Nutrient Broth (Sigma-Aldrich, 70122). Using multiple formulations was intended to capture phenotypic heterogeneity and to avoid selective recovery bias toward any single bacterial group. All media were adjusted to pH 3.2 with 1.0 M HCl prior to inoculation. Ten milliliters of each broth was inoculated separately with a 100 µL aliquot of the MRD suspension from each of the nine stool samples (nine tubes per medium; 63 enrichment cultures in total). The enrichment tubes were incubated statically at 37°C for 12 h under microaerophilic conditions and then stored at 4°C until plating.

Each enrichment culture was subsequently plated onto both de Man, Rogosa and Sharpe (MRS) Agar (Oxoid, CM0361B) and M17 Agar (Oxoid, CM0785B), resulting in a total of 126 plates. MRS Agar plates were incubated at 37°C for 48 h under microaerophilic conditions, whereas M17 Agar plates were incubated at 37°C for 48 h under aerobic conditions. From each plate, 10±2 colonies were randomly selected and purified by re-streaking on the same medium. The purified isolates were then inoculated onto Triple Sugar Iron (TSI) Agar slants (Oxoid, CM0277B) and incubated aerobically for 24-48 h^[11,12]. Isolates showing an acid butt/acid slant reaction without H₂S production were considered presumptive acid-tolerant bacteria, and 1000 of these were selected and maintained as stock cultures for subsequent analyses. All purified isolates were suspended in Brain Heart Infusion broth (Oxoid, CM1135B) containing 20% (v/v) glycerol and stored at -80°C until further analysis.

Acid Shock and Bile Salt Tolerance Assays

Acute acid resistance under simulated extreme gastric conditions was evaluated by exposing bacterial cell suspensions (adjusted to 0.5 McFarland standard; ~1.5 × 10⁸ CFU/mL in sterile saline) to MRD adjusted to pH 2.0 using 0.1 M HCl. Bacterial suspensions were incubated at 37°C for 15 min under microaerophilic conditions. This exposure served as a targeted acute shock challenge to select highly resilient candidates following

preliminary acid enrichment. Bacterial viability was assessed by subsequent spot-inoculation and growth monitoring on fresh MRS agar plates incubated at 37°C for 48 h under microaerophilic conditions. Bile tolerance was determined by inoculating surviving acid-tolerant cultures into MRD supplemented with 3.0% (w/v) porcine bile extract (Sigma-Aldrich, B8631). Inoculated cultures were incubated at 37°C for 1 h. Tolerance was confirmed by subculturing onto MRS agar, representing a stringent physiological challenge ensuring survival across the small intestinal barrier.

Hemolytic Activity and Antibiotic Resistance

Candidate strains were evaluated for hemolytic activity on Blood Agar containing 5-7% defibrinated sheep blood (Oxoid, SR0051C). Following incubation at 37°C for 48 h under microaerophilic conditions, non-hemolytic strains were selected for further evaluation.

Antibiotic resistance profiles were assessed utilizing disk diffusion assays on Mueller-Hinton agar (Oxoid CM0337B), adhering to Clinical and Laboratory Standards Institute (CLSI) guidelines. This was complemented by growth evaluations in broth media containing clinically relevant antibiotics (clindamycin (Oxoid, 0064B), ciprofloxacin (Oxoid, 0425B), vancomycin (Oxoid, 0058B), azithromycin (Oxoid, 0906B), methicillin (Oxoid, 0043B), penicillin (Oxoid, 0029B), and imipenem (Oxoid, 0455B)). The observed profiles were subsequently analyzed to distinguish likely intrinsic, non-transmissible resistance characteristics (physiological vancomycin resistance in certain lactobacilli) from potential acquired resistance issues.

Antimicrobial Antagonism and Growth Under Combined Stress

For the isolation of presumptive *E. coli*, 100 µL aliquots of appropriate dilutions were spread onto Tryptone Bile X-glucuronide (TBX) Agar (Oxoid, CM0945B). Plates were incubated aerobically at 44°C for 18-24 h. Blue-green colonies, indicative of β-glucuronidase activity, were considered presumptive *E. coli*. From each plate, five typical colonies were randomly selected and purified by re-streaking on the same medium. Purified isolates were screened by Gram staining and the indole test, and isolates identified as Gram-negative, indole-positive rods were retained as presumptive *E. coli*^[13].

For the isolation of sulfite-reducing anaerobes, 1.0 mL aliquots of appropriate dilutions were inoculated by the pour-plate method into Tryptose Sulfite Cycloserine (TSC) Agar (Oxoid, CM0587B) and overlaid with a thin layer of the same medium. Plates were incubated at 37°C for 24-48 h in anaerobic jar with GasPak/AnaeroGen system (Oxoid, AN0025A). Typical colonies, resulting from the reduction

of sulfite to sulfide, were regarded as presumptive sulfite-reducing anaerobes (*Clostridium perfringens* and related species). Representative colonies were picked, purified by re-streaking on the same medium under anaerobic conditions, and examined by Gram staining and microscopy. All purified isolates were suspended in Brain Heart Infusion broth (Oxoid, CM1135B) containing 20% (v/v) glycerol] and stored at -80°C until further analysis.

Antagonistic activities of the candidate probiotic strains against five presumptive isolates of indicator pathogens (*Escherichia coli* and sulfite-reducing anaerobic bacteria from canine fecal samples) were tested using the agar well diffusion method. Cell-free Supernatant, obtained from 24-h cultures via centrifugation and filtration, was added to 6-mm wells on pre-seeded agar plates. After 24 h of incubation at 37°C under microaerophilic conditions, diameters of growth inhibition zones were measured.

Bacterial proliferation kinetics under combined intestinal stress were assessed by inoculating isolates into Selective Enrichment Broth (SEB). SEB was prepared by adjusting the basal medium to pH 3.2 and supplementing it with 2.5% (w/v) sodium cholate (Sigma-Aldrich, C1254). Growth was monitored spectrophotometrically through optical density measurements at 600 nm (OD_{600}) taken at regular intervals over a 24-h period.

Conventional Biochemical Assays and Candidate Allocation

Microbiological verification of surviving candidates was conducted using conventional methods. Catalase activity was assessed by observing effervescence upon the application of 3% H_2O_2 to colonies on glass slides. Commercial strips were utilized for the oxidase test.

The 1000 ± 1 primary isolates that were Gram-positive, oxidase-negative, catalase-negative, and non-hemolytic were retained. Thirty of these candidate isolates per dog were randomly selected using the RAND function in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA), which assigned a random number to each eligible isolate. Isolates were ranked by these values, and the ten with the lowest values were retained. The generated values were copied and pasted as static values to ensure reproducibility of the selection. This yielded 270 isolates in total.

For each animal, 30 ± 1 isolates were selected from those showing the strongest antimicrobial activity against presumptive pathogens isolated from the same fecal sample and the most favorable antimicrobial resistance profiles. This selection cascade resulted in a finalized quota of 90 candidate strains (30 strains per age groups: P-01 to P-10 for puppies, Y-01 to Y-10 for young dogs, and A-01 to A-10 for adults) being subjected to automated VITEK® 2 GP biochemical characterization.

Phenotypic Characterization Using VITEK® 2 System

Phenotypic characterization of the 90 selected candidate strains was conducted utilizing the automated VITEK® 2 system (VITEK 2 Systems software version 9.04.4), specifically with Gram-Positive (GP) identification cards (bioMérieux, Marcy-l'Étoile, France), adhering to manufacturer guidelines. Inoculum turbidity was standardized to 0.50-0.60 McFarland units using a densitometer in 0.45% sterile saline solution. The GP cards assessed 64 miniaturized biochemical substrates, encompassing carbohydrate fermentations, enzymatic hydrolyses, and inhibitory assays. Presumptive taxonomic assignments were generated by comparison with the VITEK GP database, with identification probabilities of 90% or greater being accepted. A total of 90 isolates were grouped according to their phenotypic characteristics. Within each phenotypic group, isolates were ranked according to the inhibitory activity against presumptive pathogenic strains isolated from the same fecal samples and antimicrobial resistance profile, expressed as no or minimum inhibition zone diameter, and the no or smallest zone producing isolates were selected. In this way, 10 strains were selected from each age group (puppy, young dog and adult), yielding 30 candidate probiotic strains in total [14,15].

Statistical Analysis

Data obtained from the multi-stage phenotypic screening, biochemical substrate utilization profiles, and categorical taxa presence/absence distributions across life stages were analyzed using descriptive statistics. Categorical variables and biochemical reaction frequencies are expressed as absolute counts (n), proportions, and percentages (%). No inferential hypothesis testing was applied due to the exploratory nature of the screening design.

RESULTS

Primary Screening and Functional Selection Cascade

From an initial isolation pool of approximately 1000 colonies recovered on selective media across the nine dogs, the multi-stage *in vitro* screening cascade progressively narrowed candidates based on gastrointestinal resilience and safety attributes. Stepwise acid enrichment and acute shock challenge (pH 2.0, 15 min) along with 3.0% bile exposure eliminated sensitive populations. Safety assessment on 5% sheep blood agar identified and excluded all hemolytic isolates, confirming non-hemolytic phenotypes in surviving candidates. A total of 90 isolates satisfied primary criteria, demonstrating Gram-positive staining, non-motile morphology, negative catalase, and negative oxidase reactions, alongside consistent acid production with predominantly negative gas and no generation in TSI slants (A/A profile). Subsequent secondary screening evaluating combined

gastrointestinal stress kinetics (in SEB at pH 4.5 + 2.5% sodium cholate), broad-spectrum antagonistic activity against *Escherichia coli* and fecal sulfite-reducing anaerobes (inhibition zones ranging from 11 to 21 mm), and non-transmissible antibiotic profiles yielded a finalized quota of 30 candidate strains (10 representative isolates per age group: P-01 to P-10 for puppies, Y-01 to Y-10 for young dogs, and A-01 to A-10 for adults) (Fig. 1).

Presumptive Phenotypic Characterization

Biochemical profiling of the 30 candidate strains via the automated VITEK® 2 GP system generated high-confidence presumptive profiles (identification probabilities ≥90%). All 30 isolates consistently exhibited classic LAB baseline attributes (oxidase-negative, catalase-negative, TSI slant/butt acid-positive [A/A], predominantly gas-negative, and H₂S-negative) (Table 1, Table 2, Table 3).

Table 1, Table 2, and Table 3 report the results of the four discriminating biochemical tests (positive/negative) for all 30 strains, which formed the basis of the preliminary grouping. Carbohydrate fermentation profiles across the four key discriminatory substrates (d-mannose [dMAN], d-trehalose [dTRE], d-sorbitol [dSOR4], and cyclodextrin [CDEX]) demonstrated distinct phenotypic heterogeneity among individual strains within each cohort (Table 1, Table 2, Table 3). The frequency of positive reactions was similar in puppy- and young dog-derived strains (17 and 16 of 40 reactions, respectively) and lower in adult-derived strains (10 of 40). D-Sorbitol fermentation was restricted to 4 of the 30 strains, whereas D-trehalose and D-mannose utilization were more widespread.

Distribution of Presumptively Identified Taxa Across Life Stages

Presumptive taxonomic assignment of the 30 candidate strains resolved 13 distinct bacterial species (Table 4). Six

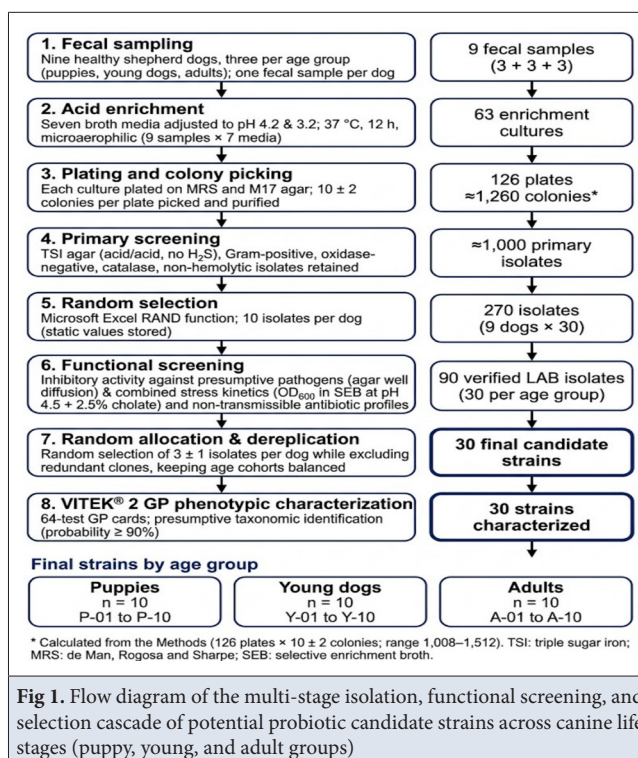


Fig 1. Flow diagram of the multi-stage isolation, functional screening, and selection cascade of potential probiotic candidate strains across canine life stages (puppy, young, and adult groups)

species were consistently recovered across all three age cohorts, representing putative “core” canine probiotic candidates: *Lactiplantibacillus plantarum* (*Lactobacillus plantarum*), *Enterococcus faecium*, *Pediococcus acidilactici*, *Limosilactobacillus reuteri* (*Lactobacillus reuteri*), *Enterococcus faecalis*, and *Pediococcus pentosaceus* (Table 4).

In addition to core taxa, several candidate species displayed stage-associated distribution patterns within the examined collection. *Lactobacillus acidophilus* and *Weissella confusa* were exclusively detected among young dog isolates, whereas *Ligilactobacillus salivarius* (*Lactobacillus salivarius*) was identified in both young and adult cohorts

Table 1. Biochemical and phenotypic characteristics of selected probiotic candidates (Puppy group)

Strain ID	Species	Oxidase	Catalase	TSI (S/B)	Gas	H ₂ S	dMAN	dTRE	dSOR4	CDEX
P-01	<i>Lpb. plantarum</i>	–	–	A/A	–	–	+	+	–	+
P-02	<i>E. faecium</i>	–	–	A/A	–	–	+	–	–	–
P-03	<i>Lcb. rhamnosus</i>	–	–	A/A	–	–	–	+	+	–
P-04	<i>P. acidilactici</i>	–	–	A/A	–	–	+	+	+	–
P-05	<i>Lmb. reuteri</i>	–	–	A/A	–	–	–	–	–	–
P-06	<i>E. faecalis</i>	–	–	A/A	–	–	–	+	–	–
P-07	<i>Lcb. casei</i>	–	–	A/A	–	–	+	–	–	+
P-08	<i>W. cibaria</i>	–	–	A/A	–	–	+	+	–	–
P-09	<i>Lmb. fermentum</i>	–	–	A/A	+	–	–	+	–	+
P-10	<i>P. pentosaceus</i>	–	–	A/A	–	–	+	–	–	–

TSI (S/B): Triple Sugar Iron agar (Slant/Butt); A/A: Acid slant/Acid butt; dMAN: d-mannose; dTRE: d-trehalose; dSOR4: d-sorbitol; CDEX: Cyclodextrin; (+): positive reaction; (–): negative reaction. Identification probabilities generated by VITEK® 2 GP cards were ≥90% for all isolates

Table 2. Biochemical and phenotypic characteristics of selected probiotic candidates (Young group)

Strain ID	Species	Oxidase	Catalase	TSI (S/B)	Gas	H ₂ S	dMAN	dTRE	dSOR4	CDEX
Y-01	<i>E. faecium</i>	–	–	A/A	–	–	+	+	–	–
Y-02	<i>Lpb. plantarum</i>	–	–	A/A	–	–	–	–	–	+
Y-03	<i>L. acidophilus</i>	–	–	A/A	–	–	+	–	–	–
Y-04	<i>P. acidilactici</i>	–	–	A/A	–	–	–	+	–	+
Y-05	<i>E. faecalis</i>	–	–	A/A	–	–	+	–	–	–
Y-06	<i>Lmb. reuteri</i>	–	–	A/A	–	–	–	–	–	+
Y-07	<i>W. confusa</i>	–	–	A/A	–	–	+	+	+	–
Y-08	<i>Lcb. casei</i>	–	–	A/A	–	–	+	+	–	–
Y-09	<i>Lgb. salivarius</i>	–	–	A/A	–	–	–	–	–	–
Y-10	<i>P. pentosaceus</i>	–	–	A/A	–	–	+	+	–	+

Table 3. Biochemical and phenotypic characteristics (Adult group)

Strain ID	Species	Oxidase	Catalase	TSI (S/B)	Gas	H ₂ S	dMAN	dTRE	dSOR4	CDEX
A-01	<i>Lpb. plantarum</i>	–	–	A/A	–	–	–	–	–	–
A-02	<i>E. faecium</i>	–	–	A/A	–	–	+	+	+	–
A-03	<i>Lmb. fermentum</i>	–	–	A/A	+	–	–	–	–	+
A-04	<i>Lcb. rhamnosus</i>	–	–	A/A	–	–	+	–	–	–
A-05	<i>P. acidilactici</i>	–	–	A/A	–	–	–	–	–	+
A-06	<i>W. cibaria</i>	–	–	A/A	–	–	–	–	–	+
A-07	<i>Lmb. reuteri</i>	–	–	A/A	–	–	–	–	–	–
A-08	<i>E. faecalis</i>	–	–	A/A	–	–	–	–	–	–
A-09	<i>Lgb. salivarius</i>	–	–	A/A	–	–	+	+	–	–
A-10	<i>P. pentosaceus</i>	–	–	A/A	–	–	–	–	–	+

Table 4. Distribution of presumptively characterized candidate species across canine life stages

Species	Puppy	Young	Adult
<i>Lpb. plantarum</i>	+	+	+
<i>E. faecium</i>	+	+	+
<i>Lcb. rhamnosus</i>	+	–	+
<i>P. acidilactici</i>	+	+	+
<i>Lmb. reuteri</i>	+	+	+
<i>E. faecalis</i>	+	+	+
<i>Lcb. casei</i>	+	+	–
<i>W. cibaria</i>	+	–	+
<i>Lmb. fermentum</i>	+	–	+
<i>P. pentosaceus</i>	+	+	+
<i>L. acidophilus</i>	–	+	–
<i>W. confusa</i>	–	+	–
<i>Lgb. salivarius</i>	–	+	+

(+): Detected; (–): Not detected in the selected candidate quota

but was absent from puppies (Table 4). Conversely, *Lactocaseibacillus rhamnosus*, *Lactocaseibacillus casei*,

Limosilactobacillus fermentum, and *Weissella cibaria* were recovered in specific combinations across juvenile and mature groups (Table 4).

DISCUSSION

The present study provides a comprehensive culture-based evaluation of probiotic candidate bacteria isolated from canine fecal microbiota across three distinct life stages, revealing both taxonomic and functional differences that are highly relevant for probiotic development. The selection of 30 phenotypically distinct strains from an initial pool of approximately 1000 isolates, combined with their biochemical characterization, highlights the importance of integrating both diversity-based and function-oriented screening strategies in probiotic research.

At the genus level, the isolates obtained in this study were predominantly affiliated with LAB, including *Lactobacillus*, *Enterococcus*, *Pediococcus*, and *Weissella*. This distribution is consistent with previous culture-based studies indicating that LAB constitute the primary group of probiotic candidates isolated from canine feces [6,7]. However, when compared with sequencing-based

microbiome studies, a clear discrepancy emerges. High-throughput analyses have demonstrated that canine fecal microbiota is dominated by phyla such as *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, and *Proteobacteria*, with *Fusobacteria* often representing a major fraction [16]. In contrast, LAB typically represent a relatively minor fraction in sequencing datasets but are preferentially enriched under selective probiotic screening conditions.

This difference reflects the fundamental methodological divergence between culture-based and sequencing-based approaches. While sequencing captures the total microbial community, including non-culturable and functionally irrelevant taxa, selective culture conditions enrich for bacteria with desirable probiotic traits such as acid and bile tolerance. Therefore, the dominance of LAB in the present study confirms the effectiveness of the applied selection strategy in isolating functionally relevant probiotic candidates rather than representing the overall microbiota composition.

Importantly, the biochemical profiling of the selected strains revealed that all isolates shared core LAB characteristics, including oxidase-negative and catalase-negative reactions and a consistent acid-producing phenotype (A/A) in TSI agar. These features are well-established hallmarks of LAB and further support the reliability of the presumptive identification approach. However, despite these shared core traits, substantial differences were observed in carbohydrate utilization patterns, particularly for substrates such as mannose (dMAN), trehalose (dTRE), and sorbitol (dSOR4), which served as key discriminative features among strains.

Carbohydrate fermentation profiles for the four variable substrates (D-mannose, D-trehalose, D-sorbitol and cyclodextrin) differed among strains within each age group (Table 1, Table 2, Table 3). The total numbers of positive reactions were 17 in puppy-derived strains (Table 1), 16 in young dog-derived strains (Table 2) and 10 in adult-derived strains (Table 3). Cyclodextrin (CDEX) fermentation was observed in 4 of 10 adult-derived strains, compared with 3 of 10 puppy-derived strains and 4 of 10 young dog-derived strains. Isolates from young dogs showed 16 positive reactions across the four discriminatory substrates, compared with 17 in puppy-derived isolates and 10 in adult-derived isolates. The distribution of positive reactions among the four substrates (dMAN, dTRE, dSOR4 and CDEX) differed between age groups, as detailed in (Table 1, Table 2, Table 3). As no inferential statistical analysis was performed and the number of strains was small, these observations are descriptive only and should be interpreted with caution [9,17].

The antimicrobial activity observed against *Escherichia coli* and sulfite-reducing anaerobes further confirms the functional relevance of the selected strains. Similar

antagonistic effects have been reported for LAB isolated from canine sources, where probiotic supplementation reduced pathogenic bacterial loads and improved gut health parameters [4,5]. These findings reinforce the importance of combining phenotypic screening with functional assays in probiotic selection.

The findings of this study collectively offer preliminary evidence indicating that the selection of probiotic candidates could benefit from a comprehensive consideration of both host specificity and age-related dynamics. While the concept of host-adapted probiotics has been previously proposed, most commercial products remain age-independent. The present findings extend this concept by demonstrating that both taxonomic composition and phenotypic diversity of probiotic candidates may vary across age groups.

From a practical standpoint, selecting probiotic strains from age matched fecal microbiota may enhance colonization efficiency, ecological compatibility, and functional efficacy. This approach is particularly relevant for young and elderly animals, where microbiota instability increases susceptibility to dysbiosis. Therefore, age-specific probiotic formulations may represent a more effective strategy compared to conventional, generalized products.

The presence of these core taxa is in agreement with previous studies reporting that members of *Lactobacillus* and *Enterococcus* are among the most stable and commonly isolated probiotic-associated genera in dogs [6,7]. These bacteria are known for their resilience to gastrointestinal stress conditions, ability to colonize the intestinal mucosa, and functional roles in maintaining gut homeostasis [1]. Their detection across all life stages in the present study further supports their role as common candidate taxa across canine life stages.

In addition to this shared core, the distribution of some species differed among age groups. *Lactocaseibacillus casei*, *Limosilactobacillus fermentum*, and *Weissella cibaria* were each detected in two of the three age groups. As the number of animals was small and no inferential statistical analysis was performed, these distribution patterns are descriptive only, and their relationship to age-related changes in the gut microbiota cannot be established from the present data [8].

Conversely, species such as *Lactobacillus acidophilus* and *Weissella confusa* were detected only in the young group, indicating a transitional microbiota phase. This observation supports the concept that the gut microbiota of dogs evolves through intermediate stages before reaching a stable adult configuration. Similar age-dependent shifts in microbial composition have been previously reported, where specific taxa appear transiently during microbiota maturation [9,17].

Interestingly, *Ligilactobacillus salivarius* was absent in puppy samples but present in both young and adult groups, suggesting that certain probiotic-associated species may establish later in life and contribute to microbiota stabilization. This finding aligns with previous reports indicating that adult canine microbiota is more stable and less diverse compared to early-life stages, with a selective enrichment of functionally adapted taxa ^[9].

The detection of *Weissella* species in multiple age groups is also noteworthy. Although these bacteria are not typically dominant in sequencing-based canine microbiome studies, their repeated isolation in this study suggests that they may play a more important functional role than previously recognized. Recent studies have highlighted the probiotic potential of *Weissella* spp., including antimicrobial activity and immunomodulatory effects, supporting their inclusion as emerging probiotic candidates ^[17,18].

Although the identification of isolates in the present study was based on phenotypic characterization using the VITEK 2 system, which provides rapid and standardized biochemical profiling, it is important to acknowledge that these results should be considered presumptive. Phenotypic methods may have limited resolution in distinguishing closely related species, particularly within lactic acid bacteria. Therefore, definitive taxonomic identification requires molecular approaches such as 16S rRNA gene sequencing or whole-genome sequencing. In this context, future studies are planned to validate the taxonomic identity of the selected strains using molecular techniques and to further investigate their functional properties at the genomic level. Despite this limitation, the applied selection strategy successfully enriched for physiologically relevant probiotic candidates, and the observed age-associated patterns provide a descriptive baseline at the phenotypic level.

Overall, the distribution patterns observed in this study indicate that the canine gut microbiota consists of both stable core species and taxa that reflect microbiota maturation and adaptation processes. These findings have direct implications for probiotic development. While core species may be suitable for general probiotic formulations, age-related taxa may offer additional benefits when used in targeted formulations designed for specific life stages.

The recovery of *Enterococcus* strains (*E. faecium* and *E. faecalis*) warrants careful safety consideration. While enterococci are natural commensals of the canine gut and several strains are widely used as commercial veterinary probiotics, members of this genus may harbor opportunistic pathogenic traits or transmissible antimicrobial resistance genes (particularly vancomycin resistance) ^[19,20]. All candidate strains were non-hemolytic. Phenotypic

antibiotic testing showed that (the strains lacked inhibition zones for vancomycin), a trait that is generally regarded as intrinsic in many lactobacilli and related lactic acid bacteria and is not considered transferable. However, because only phenotypic methods were used and the species identities are presumptive, it remains to be determined whether the resistance is intrinsic or acquired; genomic analyses are therefore needed to exclude mobile resistance elements and virulence factors before any practical formulation. Although all candidate strains in this study were non-hemolytic and phenotypically susceptible to clinically relevant antibiotics under CLSI criteria, future genomic investigations are mandatory to confirm the absence of mobile resistance elements and virulence factors prior to practical formulation.

This study has some limitations to consider. Firstly, the VITEK® 2 GP system used to identify bacterial strains only provides probable identifications, not definite ones. Future research should use 16S rRNA gene sequencing and whole-genome sequencing for more accurate identification. Secondly, important characteristics like how well the bacteria stick to the gut lining, survive in the digestive system, affect the immune system, or their effectiveness in living organisms were not studied. Lastly, the study only included a small number of dogs (three per life stage), making it an exploratory study. Although factors like environment and diet were controlled by using local shepherd dogs from similar rural settings, larger studies involving multiple centers and more animals are needed to confirm these initial findings.

As a result, this study demonstrates that canine fecal microbiota represents a diverse and functionally rich source of probiotic candidate bacteria, and that both taxonomic composition and metabolic profiles of candidate isolates exhibit distinct stage-associated patterns across life stages. The findings indicate supporting the concept that stage-associated probiotic selection may offer targeted functional traits compared to conventional, generic formulations.

Although the concept of age-adapted probiotics has been previously suggested, the present study contributes experimental evidence supporting this approach and highlights the need for further research in this field. Expanding such studies, particularly through molecular identification and *in vivo* validation, will be critical for developing more targeted, effective, and scientifically validated probiotic products for dogs.

Taken together, the species distribution analysis supports the concept that probiotic selection should consider not only host specificity but also age-dependent microbiota composition. Incorporating both core and age-related strains into probiotic formulations may enhance efficacy

and better align with the physiological and microbial characteristics of the host.

DECLARATIONS

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Ethical Compliance Declaration: The authors declare that the study was conducted in accordance with all applicable animal welfare and research ethics principles, institutional and national/international regulations, and the requirements of the relevant ethics committees and funding/supporting institutions, where applicable. The authors accept full responsibility for the ethical integrity of the study.

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