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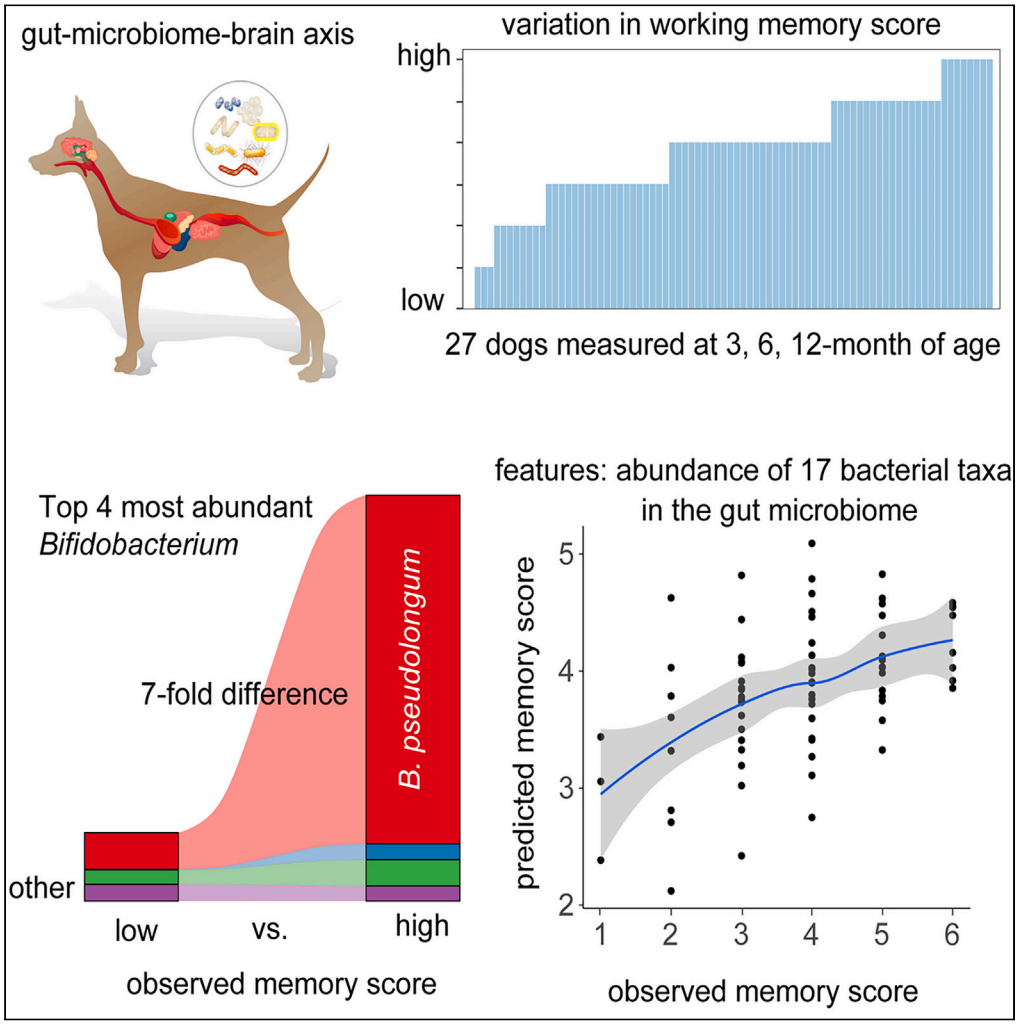

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Highlights

There are profound variations in short-term memory performance in dog puppies

Bifidobacterium pseudolongum in microbiome is linked to enhanced memory performance

Age, litter identity, sex have minimal predictive value for puppy memory performance

17 microbes in the gut microbiome are highly predictive for memory performance

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Associations between memory performance and *Bifidobacterium pseudolongum* abundance in the canine gut microbiome

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SUMMARY

Memory has been identified as the least heritable cognitive trait in canines, suggesting a significant influence of non-genetic factors. We observed a trend that overall memory scores (OMS) improve with age in a cohort of 27 young dogs, but considerable plasticity exists. Employing linear discriminant analysis of gut microbiome data from dogs exhibiting low and high OMS, a single bacterial species, *Bifidobacterium pseudolongum*, was identified and confirmed to be correlated with elevated OMS. Subsequent analysis using a random forest regression model revealed that sex, litter, and breed identity had minimal predictive importance. Age had some predictive value but failed to achieve statistical significance in this dataset. In sharp contrast, the abundance of 17 bacterial taxa in the microbiome showed a stronger predictive capacity for memory performance. Our findings provide insights into microbiome underpinnings of mammalian cognitive functions and suggest avenues for developing psychobiotics to enhance canine memory and learning.

INTRODUCTION

Working memory in non-humans is defined as the short-term memory of stimuli within a specific experimental trial or session.[1,2] It lasts from a couple of seconds to a few minutes, with dogs displaying an estimated memory performance half-life of 71 s.[3] A two-timepoint study of puppy (8 weeks old) and adult dog (21 months old) cognitive traits suggested that working memory improved with age, but puppy memory performance did not predict adult performance.[4] In addition, memory is less heritable ($h^2 = 0.17$) than other cognitive traits, such as inhibitory control in dogs ($h^2 = 0.70$).[5] The rearing environment, including diet and socialization, may affect the development and performance of canine memory.[6–9] Training can improve odor memory in working dogs.[10,11] Neuroplastic adaptations during the juvenile stage and even at later stages can have a profound impact on memory and cognition.[12] Collectively, these reports suggest plasticity in canine memory performance, indicating the potential influence of non-genetic factors.

In mammals, behavior-related traits are heavily influenced by the gut microbiota[13,14] through the microbiota-gut-brain axis.[15] Gut microbiota can produce neurotransmitters, affect the central nervous system, and affect human cognition and behavior,[16] especially in stress-related psychiatric diseases such as depression, anxiety, and even autism.[13,17,18] Recent metagenomic studies using 16S rDNA amplicon sequencing in dogs have revealed a correlation between aggression and microbiome composition in pit bull-type rescue dogs ($N = 31$)[19] and mixed breeds ($N = 42$).[20] A recent study involving 29 pet dogs found that improved memory was linked to a reduced abundance of Actinobacteria, while none of the lower taxonomic levels were significant.[21] The study by Kubinyi et al. provided insights into the connection between the gut microbiome and memory. However, studies that utilized client-owned dogs only detected differences at the phylum level and were affected by confounding factors, such as varying age, diet, breed, housing, and body conditions, which significantly reduced the statistical power.

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To address the lack of microbial species resolution and the presence of confounding factors, we enrolled four litters of dogs born, reared, and trained at the Auburn University College of Veterinary Medicine's Canine Performance Sciences Program (AUCVM-CPS) colony. This program serves as a research and breeding facility for purpose-bred detection dogs. The aim was to investigate the relationship between the gut microbiome and memory performance across three timepoints. To achieve the highest resolution of the gut microbiome, we conducted whole-genome shotgun (WGS) metagenomic sequencing with more than 60 million reads per sample from feces collected near the three timepoints of the working memory test. Our study represents the most comprehensive and systematic assessment of how the microbiome correlates with canine memory performance.

RESULTS

Substantial variability was identified in canine memory performance at puppy, juvenile, and young adult stages

Memory tests were conducted with 27 dogs born and raised in the AUCVM-CPS colony at three timepoints (Table S1). The number of correct trials was recorded as the overall memory score (OMS, ranging from 0 to 6; see STAR Methods). The distribution of OMS significantly deviated from random guessing ($p < 2.2 \times 10^{-16}$, Kolmogorov-Smirnov test), confirming the presence of working memory (Figure S1A). The average OMS at the young adult stage (OMS mean $\pm$ SD = 4.15 $\pm$ 1.26 and age mean $\pm$ SD = 13.23 $\pm$ 1.50 months) was higher than that at juvenile stage (OMS mean $\pm$ SD = 3.78 $\pm$ 0.82 and age mean $\pm$ SD = 5.26 $\pm$ 0.35 months) and puppy stages (OMS mean $\pm$ SD = 3.67 $\pm$ 0.83 and age mean $\pm$ SD = 3.17 $\pm$ 0.43 months). The results indicate a trend that memory performance improves with age during puppy development. However, the difference was not statistically significant when pairwise non-parametric tests were conducted ($p > 0.05$ for all comparisons, Mann-Whitney U tests), or when or the non-parametric group was performed ($p = 0.08$, Kruskal–Wallis test; Figure S1B; Table S2). As expected, there was a slightly lower average number of correct trials (61.3%) associated with a longer (40 s) delay interval compared to the shorter (10 s) interval (67.5%), but the difference was not statistically significant ($p > 0.05$, Mann-Whitney U test and $p = 0.16$, Kruskal–Wallis test; Figure S1B). Memory scores did not differ significantly between sexes (Figure S1C) or breeds (Figure S1D). The Kruskal-Wallis test did not identify any significant differences among the litters ($p > 0.05$). When pairwise tests were performed between litters, litter B showed differences compared to litters A and C (nominal $p < 0.05$, Mann-Whitney U test; Figure S1C), but the significance did not withstand multiple testing corrections (adjusted $p > 0.05$ based on Bonferroni adjustment). All four litters were produced by genetically related breeders from the same extended pedigree, which partially controlled the genetic background. Therefore, substantial variations in memory scores cannot be explained by litter, sex, breed, or environmental effects.

Microbial diversity in canine gut microbiome was not affected by sex or litter, and was only slightly lower in 3-month puppies

To determine the influence of the gut microbiome on canine memory performance, we performed the WGS metagenomic sequencing of fecal samples collected at each memory test time point for all 27 dogs (Table S3 and STAR Methods). The five most abundant bacterial phyla were Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Fusobacteria (Figure S2A), consistent with a previous report.[22] No between-sex differences in microbial alpha diversity (Figure S2B; $p = 0.895$, Mann-Whitney U test) or beta diversity (Figure S2C; $p = 0.781$, Permutational multivariate analysis of variance, or PERMANOVA test) were observed, suggesting a lack of sexual dimorphism in the gut microbiome. When microbial diversity was compared among the three stages, 3-month puppies had an 8.6% lower alpha-diversity compared to the juvenile stage (Figure S2D; $p < 0.001$, Mann-Whitney U test) and the difference in beta diversity was significant among the stages ($p = 0.003$, PERMANOVA test), but there was no separation on the Principal Coordinates Analysis (PCoA) plot with overlapping 95% confidence intervals (Figure S2E). The microbial alpha-diversity measured by the Shannon index was not significantly different among the four litters (Figure S2F; $p > 0.05$, Mann-Whitney U test).

Linear discriminant analysis revealed a single most featured bacterial species, *Bifidobacterium pseudolongum*, is associated with memory performance

We investigated microbial diversity in the measurement groups with the same overall memory scores. The only significant deviation we found was a slightly lower alpha diversity in the OMS1 group (Figure S2G; $p < 0.05$, Mann-Whitney U test), which could be due to the small number of measurements in this group ($N = 3$; Figure 1B). Beta diversity analysis confirmed no separation based on the OMS (Figure S2H; $p = 0.200$, PERMANOVA test). To improve the power to detect microbiome differences, we defined a discovery set of eight dogs that had a high OMS (OMS = 6, high-OMS group) and eight dogs that had a low OMS (OMS $\leq$2, low-OMS group; Figure 1B). As expected, no significant differences were found in alpha diversity ($p = 0.328$, Mann-Whitney U test; Figure 1C) or beta diversity ($p = 0.363$, PERMANOVA test; Figure 1D). Collectively, these results suggest a lack of dysbiosis in the gut microbiome of healthy dogs without any medical issues. With species-level resolution from the WGS metagenomic data, we performed linear discriminant analyses (LDA) between the high-OMS and low-OMS groups on all bacterial taxonomic levels. At an LDA score cutoff of 3, the only significant species was *Bifidobacterium pseudolongum* in the high-OMS group, which drove the significance of its genus, family, and order (Figure 2A). When a less stringent LDA cutoff (LDA score <2) was used, *Bifidobacterium criceti* was associated with high memory performance (Figure 2A), whereas *Collinsella ihuae* and the genus *Holdemanella* were negatively associated with memory performance (Figure 2A).

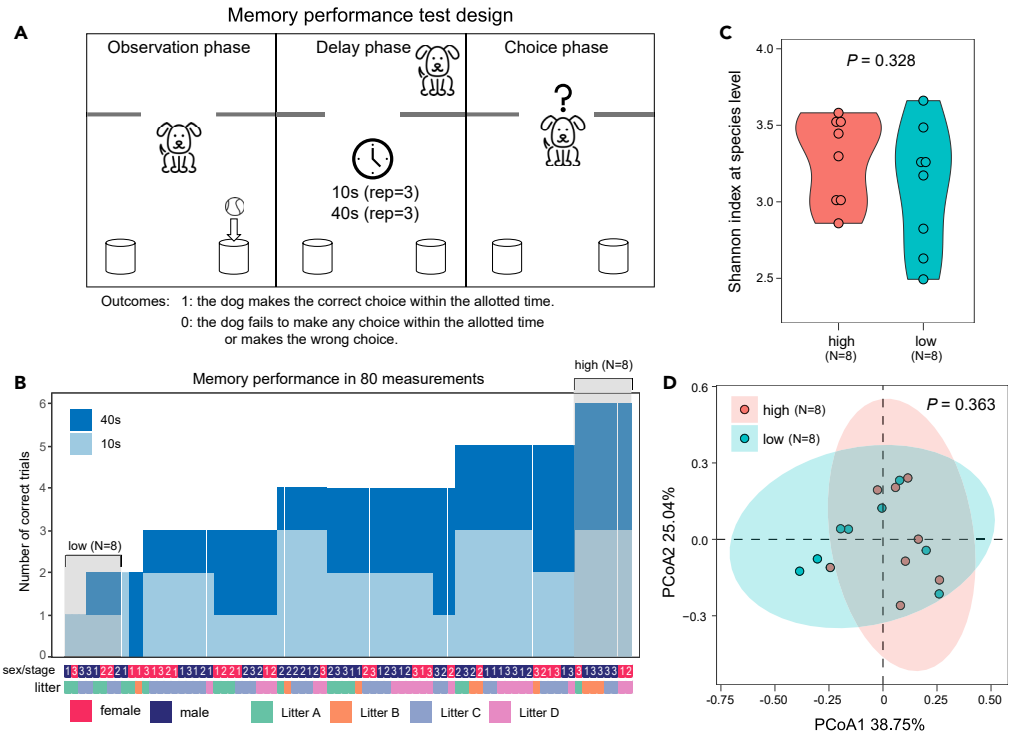

Figure 1. Memory performance in detection dogs at three developmental timepoints

(A) Schematic illustration of the working memory test.

(B) Bar plots display the number of correct trials across 80 measurements. Dark blue bars represent correct trials after a 40-s delay interval, while light blue bars represent correct trials after a 10-s delay interval. Bars within gray-shaded boxes were selected for analysis among dogs that scored high and low on the memory test.

(C) Violin plots illustrating microbial diversity (measured by Shannon index) for the high- and low-performance groups at the species level. *P*-value assessed by Mann-Whitney U test.

(D) Principal Coordinates Analysis (PCoA) plots of beta diversity between the high and low memory performance groups are generated based on the Bray-Curtis distance. *P*-value assessed by PERMANOVA test (Permutational multivariate analysis of variance).

Other bacterial taxa differ in abundance between high-OMS and low-OMS groups, in addition to *B. pseudolongum*

To identify bacterial taxa with differential abundances between the high- and low-OMS groups, we performed non-parametric tests of relative abundance between groups. Among the significant taxa, *B. pseudolongum* was the most abundant, accounting for 4.14% of the high-OMS microbiome and 0.44% of the low-OMS gut microbiome on average (Figure 2B; $p = 0.015$, Mann-Whitney U test). *B. criceti*, another bacterium positively associated with high memory performance in the LDA analysis (Figure 2A), was also significantly more abundant in the high OMS group (Figure 2C; $p = 0.028$, Mann-Whitney U test). A total of 45 species were significantly enriched in the high-OMS group (Figures 2B and 2D) and another 15 bacterial species were enriched in the low-OMS group (Figures 2C and 2E).

Confirmation of memory performance associated microbial taxa using the 80 metagenomes as a validation set

For bacterial taxa showing differences in abundance between the high- and low-OMS groups (Table S4), we quantified their relative abundance in the 80 metagenomes and correlated them with OMS to determine significant correlations in the entire dataset. A total of 32 bacterial taxa were positively correlated with memory performance (false discovery rate FDR <0.1), and eight taxa were negatively correlated (Figure 3A; Table S5). Among the 32 taxa associated with improved memory performance, nine were related to *Bifidobacterium*, including six species (*Bifidobacterium pseudolongum*, *Bifidobacterium criceti*, *Bifidobacterium anseris*, *Bifidobacterium castoris*, *Bifidobacterium dentium*, and *Bifidobacterium* sp. *GSD1FS*), *Bifidobacterium* genus, Bifidobacteriaceae family, and Bifidobacteriales order (Figure 3A). *B. pseudolongum* was the most abundant species in its genus, and it was significant at the genus, family, and order levels (Figure 3B). In contrast, the second most abundant *Bifidobacterium* species, *Bifidobacterium animalis*, was not significantly different between the high- and low-OMS groups (Figure 3B). Spearman's correlation coefficient (ρ) between *B. pseudolongum* and OMS was 0.352 ($p = 0.001$; Figure 3C), ranking as the second highest among all significant taxa (Figure 3A). Fewer taxa were negatively correlated with OMS, including *Pedobacter* sp. *ASV17* (Figures 3D and S3).

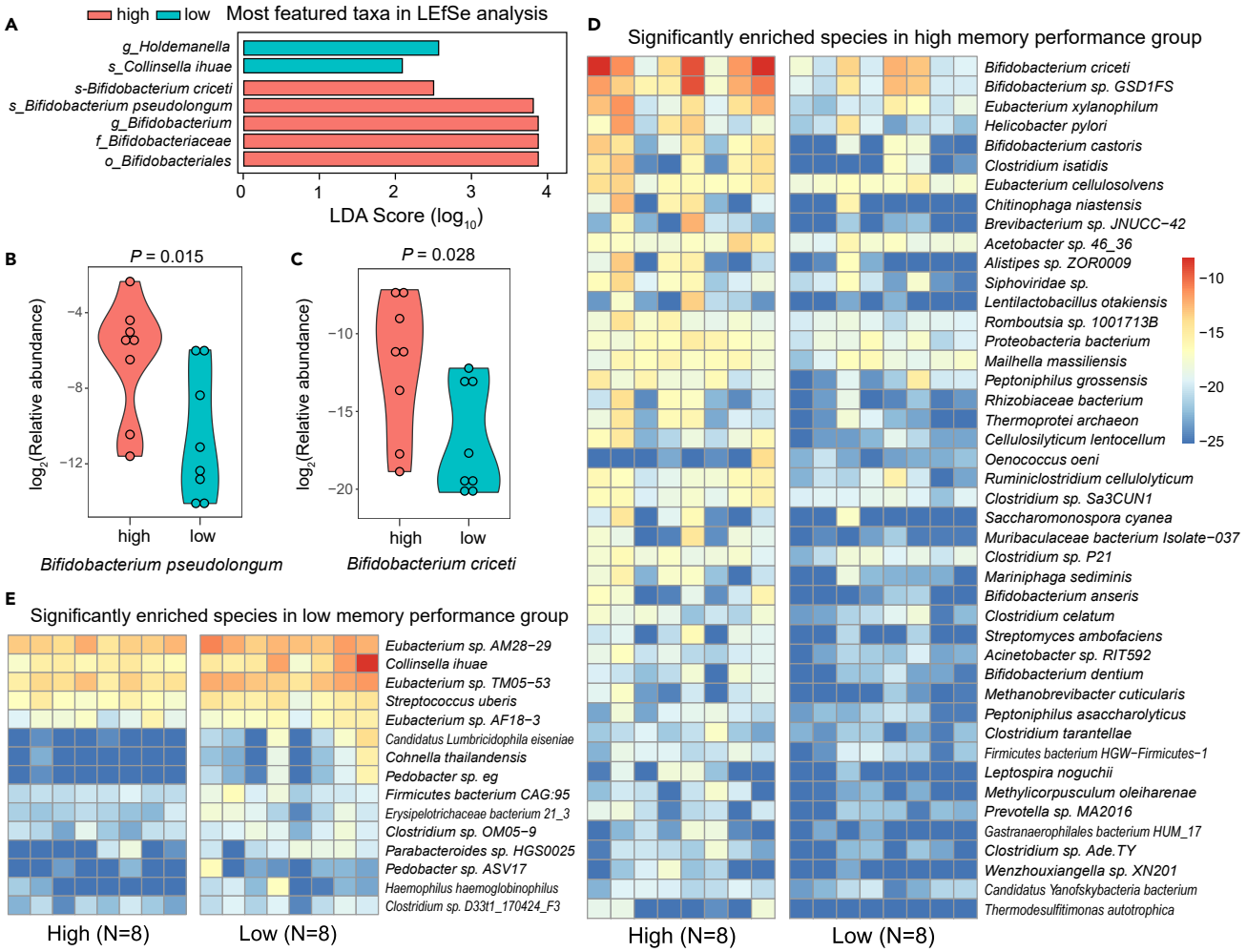

Figure 2. Microbial composition differences in the canine gut microbiome between the high and low memory performance groups

(A) Linear Discriminant Analysis (LDA) plots show the most featured taxa in dogs that scored high and low on the memory test at the order (o_), family (f_), genus (g_), and species (s_) levels.

(B and C) Violin plots illustrating the relative abundance of *Bifidobacterium pseudolongum* (B) and *Bifidobacterium criceti* (C) in dogs that scored high and low on the memory test. *P*-value assessed by Mann-Whitney U test.

(D and E) Heatmaps illustrating the abundance of bacterial species enriched in dogs that scored high (D) and low (E) on the working memory test.

Quantitative polymerase chain reaction validation confirmed that changes in *Bifidobacterium pseudolongum* abundance is associated with differences in working memory performance in the same dogs

B. pseudolongum was associated with improved canine memory function in LDA analysis, differential abundance analysis, and correlation analysis. It is also abundant in the canine microbiome. To validate this intriguing finding using an independent technique, we performed the qPCR quantification of the *nusA* gene in *B. pseudolongum* using *Prevotella copri* as a positive control, whose abundance was stable across all samples (see STAR Methods). We observed high concordance between the qPCR measurements and WGS metagenomic sequencing results ($\rho = 0.73$, $p < 0.0001$; Figure 4A), confirming high reproducibility and a significant positive correlation between *B. pseudolongum* and OMS ($\rho = 0.34$, $p = 0.005$; Figure 4B). If the presence of *B. pseudolongum* were causal to the improved OMS, for the same dog with variable OMS, we expect to observe significant changes in *B. pseudolongum* abundance across different timepoints. A total of 21 discordant pairs (OMS >3 and OMS ≤3) were identified, and the *B. pseudolongum* abundance was significantly higher at the high OMS time point (mean = 4.12%) than at the low OMS time point (mean = 0.55%; $p < 0.05$, Wilcoxon signed rank test), which implied a potential causal relationship between *B. pseudolongum* and working memory performance (Figures 3E–3G).

Predictability of microbiome composition for working memory performance using random forest regression

A predictive model was developed to predict working memory performance, with microbiome composition serving as a feature in a random forest regression model. The initial model included 36 bacterial taxa showing a significant correlation with memory scores (Table S5), as well as

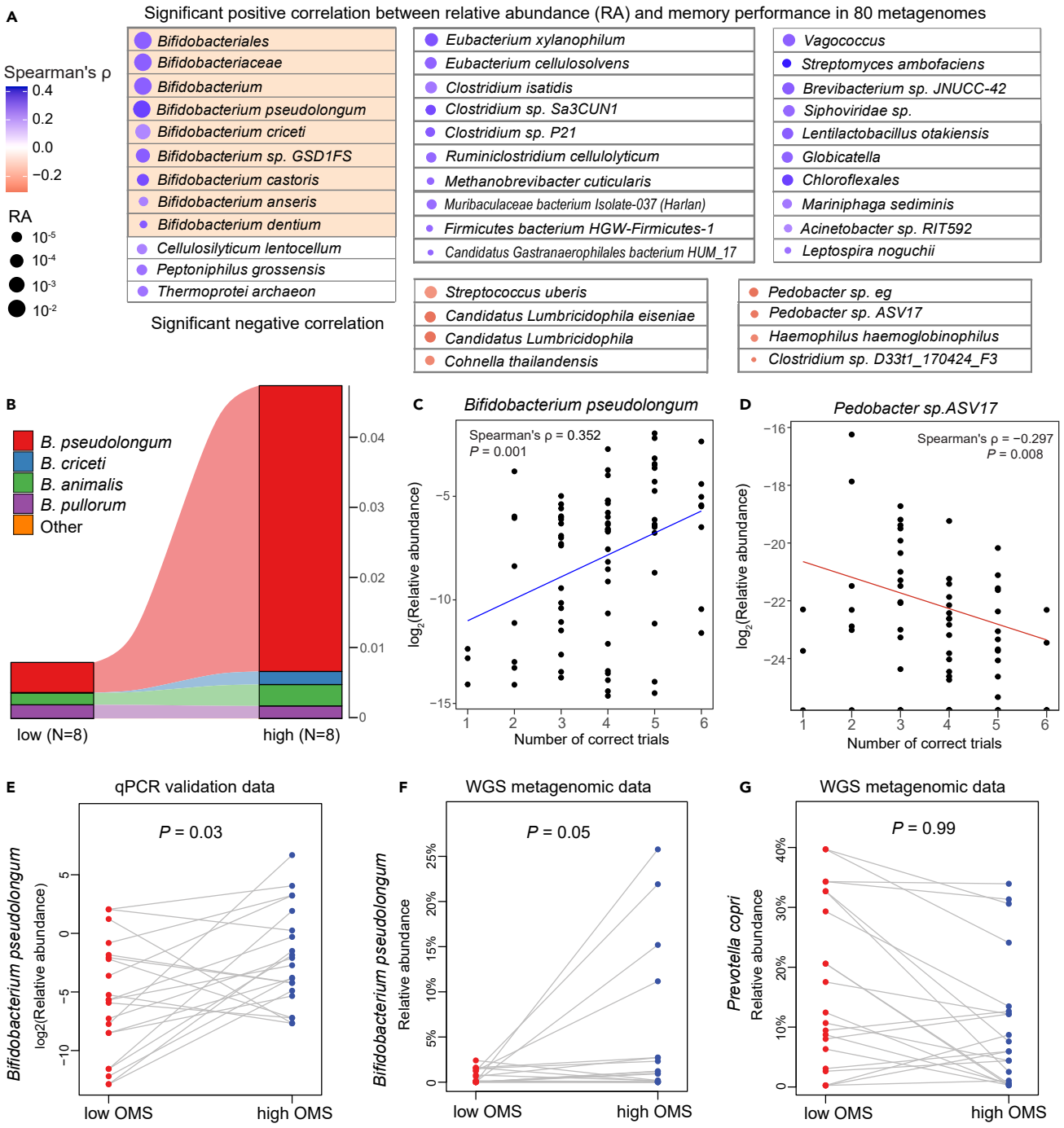

Figure 3. Correlation between memory performance and bacterial taxa abundance in 80 metagenomes

(A) Significant positive and negative correlations were found between the overall memory score (OMS) and taxa abundance ($p < 0.05$, FDR <0.10, Spearman's rank correlation test). The color of the dots represents the value of the Spearman correlation coefficient, and the dot is proportional to the relative abundance.

(B) Alluvial plots demonstrate a significant increase in *Bifidobacterium pseudolongum* and *Bifidobacterium criceti* in the high-OMS group. No significant differences were observed among the other *Bifidobacterium* species.

(C and D) Scatterplots between OMS and the relative abundance of *B. pseudolongum* (C) and *Pedobacter sp. ASV17* (D).

(E–G) Plots of relative bacterial abundance at 21 high-OMS and low-OMS pairs of time points in the same dog for qPCR (E) and whole-genome sequencing (WGS) metagenomic quantification (F) of *B. pseudolongum* and WGS metagenomic quantification of the control species *Prevotella copri* (G). *P*-value assessed by Wilcoxon signed rank test.

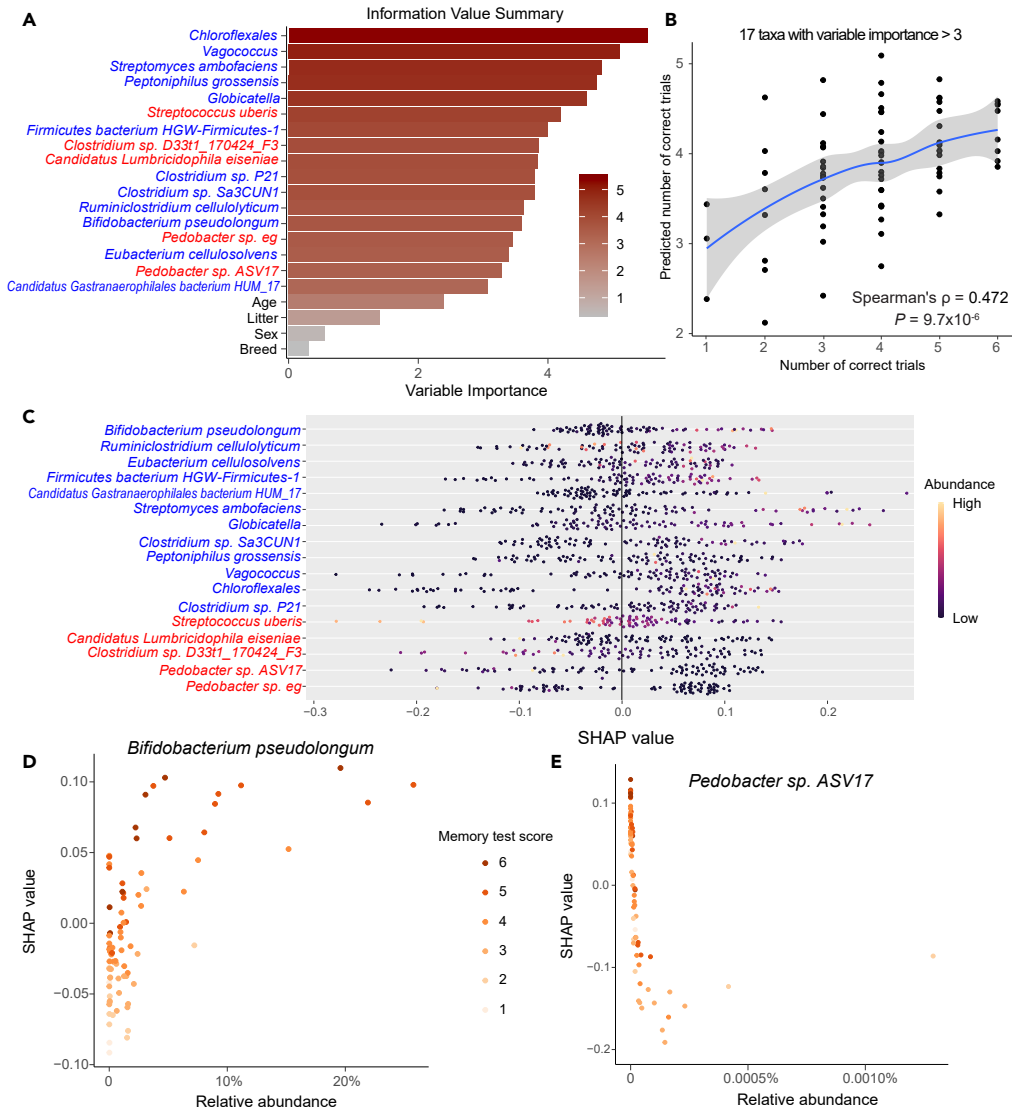

Figure 4. Variable importance and predictability modeled by random forest regression using 31 bacterial taxa with significant correlation with memory performance

(A) Feature importance scores computed using random forest.

(B) Scatterplot of the observed memory score (*x*-axis) and predicted memory scores (*y* axis) using 17 taxa in the random forest regression. The fitted line is plotted in blue, with the confidence interval in gray. *P*-value assessed by Spearman's rank correlation test.

(C) The impact of bacterial taxa (importance value >3) on the overall memory score is summarized by SHAP values. Color represents the relative abundance of bacterial taxa.

(D and E) Scatterplot of SHAP values and the relative abundance of *B. pseudolongum* (D) and *Pedobacter sp. ASV17* (E). The dots were color-coded based on the overall memory score.

additional variables such as age, sex, litter, and breed. A total of 17 taxa had high discriminatory power (importance value greater than 3; Figure 4A), and they were included in the final predictive model. Among the four non-microbiome variables, age has moderate predictive value, which is less important than the microbes (Figure 4A). Litter, sex, and breed were found to have negligible discriminatory capabilities (Figure 4A). The predicted memory score using 17 microbiome features was significantly correlated with the observed memory score (ρ = 0.472, $p < 0.0001$; Figure 4B). SHAP values (SHapley Additive exPlanations) were then used to interpret the predictive model (Figure 4C). Among the final list of predictive taxa, a higher OMS was associated with an increased abundance of *B. pseudolongum* (Figure 4D) and decreased abundance of *Pedobacter* sp. *ASV17* (Figure 4E).

DISCUSSION

Domestic dogs share a common social and environmental evolutionary history with humans. They possess social and cognitive skills that are functionally analogous to humans, making them an ideal model for translational and comparative research in cognitive neuroscience and behavioral genetics.[23,24] In addition, these skills are particularly important for purpose-bred working dogs (e.g., detection dogs) to be successful in their work.[25,26] However, cognitive and behavioral studies are challenging because these traits are heavily affected by the environment, the experimenter, and other confounding factors. To address this, we measured the working memory performance of 27 candidate detection dogs that were raised and maintained in a controlled research setting. We observed significant variability in memory performance, even in the same dog, across different time points. A trend of memory performance improvement with age has been identified ($p = 0.08$), which is consistent with previous studies.[4] Dog's age cannot explain the majority of phenotypic variation in canine memory capability, reflecting functional plasticity.

The animal gut microbiome modulates brain function and cognitive abilities through the microbiota-gut-brain axis.[15] However, the gut microbiome composition is affected by diet, age, household environment, and host genetics. Previous studies have lacked sufficient resolution to identify differences in microbial species. Using fecal samples collected from age-matched detection dog candidates in the same extended pedigree and fed the same diet, we performed WGS metagenomic analysis to quantify microbial abundance. This approach aimed to enhance statistical power for identifying microbiome correlates of canine memory performance.

The phylum Actinobacteria was negatively associated with canine memory performance in 29 dogs with a large age range (3–13 years old, mean age 9.7) from diverse households and diverse breed backgrounds.[21] Since no specific bacterial species were significant in Kubinyi et al., it was unclear which microbes were driving the differences and whether the findings in phylum abundance variation were due to differences in age, sex, diet, breed, or other confounding factors. In our study, we focused on young dogs and did not find any significant correlation between memory performance and microbial diversity or phylum level composition. The lack of significant shifts in overall diversity and phylum abundance indicates that memory performance plasticity in young dogs is not caused by dysbiosis in the gut microbiome. This is expected because all the animals enrolled in this study were healthy dogs without any gastrointestinal (GI) issues. Because of the species resolution of WGS metagenomic sequencing and the experimental design controlling confounding factors, we successfully identified 41 microbial taxa significantly correlated with canine memory performance. This discovery unveils, for the first time, the influence of the microbiome on canine cognition at the microbial species level for the first time.

In specialized settings, such as military working dog (MWD) populations, there are inherent challenges in controlling confounding factors, such as sex, age, body condition scores (BCS), breeds, and GI issues. A larger sample size is required to achieve meaningful microbiome discovery. A recent study conducted by Craddock et al.,[27] which involved 134 MWDs spanning five different breeds, successfully revealed correlations between microbiome composition and behavioral attributes, including aggression, motivation, obedience, sociability, and BCS. Notably, these statistical associations were predominantly identified at the genus level or within species groups that were linked to specific metabolic pathways. Enhancing the metagenomic sequencing yield (~10 million reads per sample in the study by Craddock et al.) or increasing the sample size could potentially enhance the ability to identify precise microbiome correlations at the species level.

Among the microbiome correlates of memory performance that we discovered, the single most striking finding was that of *B. pseudolongum*. This particular species stands out as the only significant taxon identified through the linear discriminant analysis, which couples statistical significance with effect relevance. The positive correlation between the relative abundance of *B. pseudolongum* and improved memory score was subsequently validated using qPCR assays. As a well-known genus of probiotics,[28] *Bifidobacterium* produces lactic and acetic acids through fermentation, providing benefits to host gastrointestinal health.[29] *B. pseudolongum* is the immediate outgroup of *B. animalis* clade,[30,31] *B. animalis* did not show significant differences between the high-OMS and low-OMS groups. This implies a species-specific phenomenon, rather than an overarching genus effect. Intriguingly, in a previous study, *Bifidobacterium longum 1714*, a remotely related *Bifidobacterium* species, was linked to reduced stress and enhanced memory in human subjects.[32] Further investigations suggest that the reduction of social stress is achieved by neural modulation,[33] paving the way for the potential application of psychobiotics in enhancing human memory and ameliorating stress.[34] Nevertheless, in the canine microbiome, *B. longum* manifested a considerably lower mean abundance (0.02%) than *B. pseudolongum* (2.14%) and was not associated with working memory ($p = 0.17$) in our study. This distinction is not unexpected, given that the physiology is different between humans and canines. Therefore, findings centered on humans may not be directly to the canine microbiome.

Since dogs were domesticated ~15,000 years ago, they have become valuable partners working with humans in hunting, guarding, herding, guiding, and odor detection. Beyond their working capabilities, dogs provide humans with loyal companionship. Improving memory abilities in puppies will help them learn better and quicker, which can significantly enhance dog socialization and training.[25,35] In senior dogs, memory loss is commonly observed as part of the canine cognitive dysfunction (CCD) syndrome, which is a progressive neurological and behavioral disease associated with aging.[36,37] Improving memory function will help both puppy development and longevity in dogs. Our results will inform the development of probiotics and fecal microbiota transplantation (FMT) techniques to improve canine memory and cognition during puppy development. Whether our findings will apply in older dogs remain unclear and future studies are needed to explore it.

Limitations of the study

One limitation of this study is that the link between *Bifidobacterium pseudolongum* abundance and working memory is merely a correlation. Additional experiments are needed to determine whether *Bifidobacterium pseudolongum* can improve working memory. We tried to control

the breed effect and only included two breeds in this study. This study cannot and is not designed to detect any breed-specific effect. Our study was performed in puppies and young dogs, whether the results translate to older dogs remained to be determined.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109611.

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AUTHOR CONTRIBUTIONS

XW and LL contributed to the conceptualization and design of the study. LL, SK, and JGS performed the cognitive tests and analyses; PSH, RRW, MS, and LPW contributed to animal breeding, maintenance, and fecal sample collection; XM, WC, and YZ performed the DNA extraction and metagenomic sequencing library preparation; XM, JZ, and XW performed the metagenomic data analyses. XW, LPW, and SBP provided the samples, resources, analysis tools, advice, and supervision. XM and XW wrote the first draft of the article, and JZ and SK wrote the sections of the article. All authors contributed to article revision and read and approved the submitted version.

DECLARATION OF INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Dog fecal samples	This paper	N/A
Critical commercial assays		
AllPrep PowerFecal DNA/RNA kit	Qiagen	Cat #: 80244
NEBNext Ultra II DNA Library Prep Kit for Illumina	New England Biolabs	Cat #: E7645
NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1)	New England Biolabs	Cat #: E7600
NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 2)	New England Biolabs	Cat #: E7780
PerfeCTa SYBR Green FastMix, Low ROX	Quantabio	Cat #: 95074
Deposited data		
Raw whole-genome shotgun metagenomic data	This paper	NCBI: PRJNA936782
Github code	This paper	https://github.com/XuWangLab/2023_memory_and_canine_microbiome
Oligonucleotides		
The forward primer sequence for *P. copri* is GCAACACGCTGAGTACATGA, and the reverse primer sequence is CCGTGAGGTAGACGAGAATG.	This paper	N/A
The forward primer sequence for *B. pseudolongum* is AGCTTGGCCGCCAGACG, and the reverse primer sequence is TGATCGGACCTGGTGGTTCG.	This paper	N/A
Software and algorithms		
Trimmomatic (v0.36)	Bolger et al.[38]	https://doi.org/10.1093/bioinformatics/btu170
Burrows-Wheeler Aligner (BWA) (v0.7.17-r1188)	Li et al.[38]	https://doi.org/10.1093/bioinformatics/btp324
Kaiju (v1.7.3)	Menzel et al.[39]	https://doi.org/10.1038/ncomms11257
SAMtools (v1.6)	Danecek et al.[40]	https://doi.org/10.1093/gigascience/giab008
BEDTools (v2.30.0)	Quinlan et al.[41]	https://doi.org/10.1093/bioinformatics/btq033
metaBAT2 (v2.15-2)	Kang et al.[42]	https://doi.org/10.7717/peerj.7359
checkM (v1.2.2)	Parks et al.[43]	https://doi.org/10.1101/gr.186072.114
R version 4.3.2	R[44]	https://www.r-project.org
Custom code and script used in this study	This paper	https://github.com/XuWangLab/2023_memory_and_canine_microbiome
Other		
PowerLyzer24 instrument	Qiagen	Cat #: 13156
Qubit 3.0 Fluorometer	Thermo Fisher Scientific	Cat #: Q33216
NanoDrop One C Microvolume Spectrophotometer	Thermo Fisher Scientific	Cat #: ND-ONE-W
4200 TapeStation system	Agilent Technologies	Cat #: G2991BA
M220 Focused-ultrasonicator	Covaris	Cat #: 500295
Bio-Rad C1000 Touch Thermal Cycler equipped with CFX96 Real-Time PCR Detection Systems	Bio-Rad Laboratories	Cat #: 1851196

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Xu Wang (xzw0070@auburn.edu).

Materials availability

This study did not generate any new unique reagents.

Data and code availability

The whole-genome shotgun metagenomic sequencing data are available at NCBI SRA (Short Read Archive) under accession number PRJNA936782. All code used in this study is available in GitHub (https://github.com/XuWangLab/2023_memory_and_canine_microbiome) or can be found on websites of corresponding software packages. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Dogs (11 females and 16 males) enrolled in this study were born, raised, and maintained at the Auburn University College of Veterinary Medicine Canine Performance Sciences program under the same diet, training, and medical care in a controlled environment (see Table S1). All experimental animal protocols were approved by the Auburn University Institutional Animal Care and Use Committee (approved protocol number PRN-2019-3564).

Phenotypic measurement and analysis

Working memory tests were performed three times for 27 dogs at the puppy stage (3.0–3.5 months), juvenile stage (5.2–6.2 months), and young adult stage (12.8–16.0 months; see Table S2). Dogs' ability to locate a visually displaced reward (a ball) after delays of 10 or 40 s was measured using methods similar to Bray et al.,[4] with the exception that non-mnemonic cues were controlled during the delay.[10] On each trial, the handler brought the dog to the starting position. The experimenter stood 2 m away, facing the dog, in the center of two opaque plastic cups (12 × 12 × 14 cm) placed upside down, 2 m apart (Figure 1A). To begin the trial, the experimenter called the dog's name, held up the reward, placed it underneath one of the two containers, and then stepped back and turned to face the back wall. Once the reward was hidden, the handler removed the dog from the room to the hallway for the duration of the delay. At the conclusion of the delay, the handler led the dog back to the starting position in the room and then released the dog, allowing 15 s to make a choice. Once the dog chose a container (defined as the dog's snout coming within 5 cm of the container), the experimenter lifted the cup to reveal its contents. After either making a choice or 15 s lapsed, the handler led the dog back to the starting position to begin the next trial. Six trials were conducted, with a 10-s delay on trials 1–3, and a 40-s delay on trials 4–6. The number of correct trials was recorded as the overall memory score (OMS). The position of the reward (left or right) was counterbalanced throughout the session. If a dog scored more than 50% correct, an odor control test was conducted at the conclusion of the session to ensure that dogs were not locating the reward using odor cues. The odor control test was identical to the test trials, except that dogs did not witness the baiting of the container (i.e., the container was pre-baited when the dog was out of the room). A camera (GoPro Hero Session) positioned in the corner of the room recorded all trials and was used for post-session inter-reliability coding.

Statistical analysis

The effects of age, litter, sex, and breed on OMS were assessed using the Mann-Whitney U test[45] and the Kruskal-Wallis test[46] in the R software.[47] A p-value of 0.05 represents a statistically significant difference. Mean and standard deviation (SD) were plotted in Figure S1. We simulated the distribution of OMS under a null assumption that each dog makes a random choice during the memory tests, and whether the observed OMS distribution in this experience deviates from the null (no memory) was assessed by the non-parametric Kolmogorov-Smirnov test[48] implemented in R.

Fecal sample collection, microbial DNA extraction, and quality control

Fecal samples were collected on the memory test day or a few days apart (N = 10 days average), by putting a sterile P1000 pipettor tip (upside-down) into the feces immediately after the dogs defecated. The fecal samples were placed in sterile 1.5mL Eppendorf tubes and stored immediately in −80 C freezer. For microbial DNA purification, ~200 mg fecal sample was homogenized by the PowerLyzer24 instrument (Qiagen, Germantown, MD, USA) in bead-containing tubes provided in the AllPrep PowerFecal DNA/RNA kit (Qiagen, Germantown, MD, USA), and DNA samples were extracted according to the manufacturer's protocol. DNA concentrations were measured on a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA), and A260/A280 absorption ratios were assessed using a NanoDrop One C Microvolume Spectrophotometer (Thermo Scientific, Waltham, MA, USA). The size distribution was checked on TapeStation 4200 using Genomic ScreenTape (Agilent Technologies, Santa Clara, CA, USA).

Whole-genome shotgun (WGS) metagenomic library preparation and sequencing

To construct the WGS metagenomic library, 500 ng of extracted microbial DNA was used as input for each sample. The DNA samples were first fragmented to a target size of 550 bp using the M220 Focused-ultrasonicator (Covaris, Woburn, MA, USA). Subsequently, the sequencing library constructions were performed using NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA), along with adaptors and primers provided by NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1 and Set 2). The final library concentrations were measured by Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA), and size distributions were checked using TapeStation 4200 System with the D1000 ScreenTape (Agilent Technologies, Santa Clara, CA, USA). Finally, the pooled library was sent for sequencing on an Illumina NovaSeq6000 machine at 150-bp paired-end mode at Novogene (Novogene Corporation Inc., Sacramento, CA, USA) to achieve an average yield of 64 million reads per sample.

Metagenome data processing, alignment, and taxonomy annotation

For the 27 dogs included in our study, we obtained a total of 1.8 billion, 1.8 billion, and 1.5 billion Illumina reads, respectively, at the puppy stage, the juvenile stage, and the young adult stage (Table S3). The adapter reads and low-quality bases were trimmed by Trimmomatic version 0.36.[49] The host-contaminated sequences were removed by mapping the trimmed reads to the dog reference genome (CanFam3.1) using Burrows-Wheeler Aligner (BWA) version 0.7.17-r1188.[38] The viral reads and rRNA sequences were deleted in the same way. The filtered reads were then aligned to the canine gut microbiome reference contigs (accession number JARCCX000000000), which were fully annotated using Kaiju version 1.7.3[39] against the NCBI-NR database. The read counts were extracted using SAMtools version 1.6[40] and BEDTools version 2.30.0.[41] Taxonomic abundances were normalized in a relative abundance format on a scale of 0–1 for subsequent metagenomic analysis.

Microbial diversity analysis

R package vegan version 2.5.7[44] was deployed to calculate the alpha- and beta-diversities of taxonomy profiles at the species level. Alpha-diversity of the microbial community was measured by Shannon index,[50] while beta-diversity was assessed based on the Bray-Curtis dissimilarity.[51] Mann-Whitney U tests[45] were utilized to compare the alpha diversities of gut microbiomes from dogs of different litters, ages, sexes, and breeds. The cutoff for the null hypotheses was also set at 0.05. The beta diversities among gut microbiomes from different litter, age, sex, and breed groups were calculated using the Bray-Curtis distances. The beta diversity was analyzed by Principal Coordinate Analysis (PCoA) using Permutational multivariate analysis of variance (PERMANOVA) test,[52] which is a permutation-based non-parametric statistical test that tests the null hypothesis that there is no difference in distribution among groups of multiple variables. Function adonis2 from the R package vegan was used to perform the PERMANOVA tests.

Identification of the most featured bacterial taxa between high and low memory performance groups

LEfSe (Linear discriminant analysis Effect Size) version 1.1.2 was utilized to discover the most featured orders, families, genera, and species between the high and low memory performance groups. The analysis was conducted via Galaxy web application (http://huttenhower.org/galaxy) using default options (alpha = 0.05). Moreover, Mann-Whitney U tests[45] were performed on the taxonomy profiles of the high and low memory performance groups, to identify the species that exhibited significant differences in relative abundance. These biomarkers were displayed in heatmap plots, which were generated with R package pheatmap version 1.0.12.

Correlation between bacterial taxa and overall memory score

To estimate the correlation between the bacterial taxa and memory performance, we performed Spearman's correlation test on the OMS and the abundance levels of the taxa across all 80 measurements using the R software.

Random forest regression analysis

Random forest model[53] was used to predict the working memory score. The initial model included all microbiome composition, age, litter, sex, and breed as features. The importance score, which quantifies the impact of each feature on the accuracy of the model, was calculated. The most critical features with high importance scores were included in the final predictive model. R package *Ranger version 0.15.1* was used to fit the random forest regression model, and *ggplot2 version 3.4.2* was used to display the feature importance. The SHAP values[54] were then computed to interpret the predictive model. R package *shapper, DALEX2, shapviz*, and *treeshap* were used to compute and visualize the SHAP values of each feature. Positive SHAP value indicates a positive impact on the memory score, while negative SHAP indicates a negative impact. The magnitude of SHAP represents the degree of impact of each feature on the model output, with a larger magnitude indicating a greater impact on the model output.

Reconstruction of MAGs and qPCR validation of abundance differences for *Bifidobacterium pseudolongum*

To confirm the species identity of our findings and obtain bacterial genome sequences for validation, metaBAT2 version 2.15–2[42] was used to bin metagenome-assembled genomes (MAGs) from the canine gut microbiome reference microbial contigs (accession number JARCCX000000000). The genome completeness and contamination were assessed using checkM version 1.2.2.[43] To confirm the association between *B. pseudolongum* abundance and memory performance, we designed qPCR assay targeting the *nusA* gene, which is one of the universal single-copy gene families.[55] *Prevotella copri*, the most abundant species in the canine gut microbiome, was chosen as the control

species because its abundance is the most stable across 80 metagenomes. The qPCR primers were synthesized by Eurofins (Eurofins Genomics Inc., Huntsville, AL, USA) after being designed in Oligo 7 software.[56] The forward primer sequence for *P. copri* is GCAAC ACGCTGAGTACATGA, and the reverse primer sequence is CCGTGAGGTAGACGAGAATG. The forward primer sequence for *B. pseudolongum* is AGCTTGGCCGCCAGACG, and the reverse primer sequence is TGATCGGACCTGGTGGTTCG. PCR product lengths are 200 bp and 244 bp, respectively, and they were confirmed by regular PCR, electrophoresis, and visualization on 2% agarose gel.

For each qPCR reaction, 2 ng fecal DNA sample was mixed with PerfeCTa SYBR Green FastMix, Low ROX (Quantabio, Beverly, MA, USA), and nuclease-free water in a 96-well plate. The qPCR was carried out using a Bio-Rad C1000 Touch Thermal Cycler equipped with CFX96 Real-Time PCR Detection Systems (Bio-Rad Laboratories, Hercules, CA, USA). To quantify the expression level of *B. pseudolongum* in each sample, the Ct value difference between *B. pseudolongum* and *P. copri* was computed for relative quantification. Non-parametric Wilcoxon Rank-Sum test and Spearman's correlation test were performed on the $\log_2$ scale of the relative abundance to verify the WGS results.