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Effect of *Lactobacillus* supplementation on seizure control, gut microbiota, and blood neurotransmitters in dogs with idiopathic epilepsy

Sylvia García-Belenguer [a,b,d,e,*], Belén Rosado [a,b,d,e], Adelaida Hernaiz [c,d,e], Jon Moral [a,b], Inmaculada Martín-Burriel [c,d,e,f,g], Jorge Palacio [a,b,d,e]

[a] Departamento de Patología Animal, Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet, 177, Zaragoza 50013, Spain
[b] Hospital Veterinario de la Universidad de Zaragoza (HVUZ), Miguel Servet, 177, Zaragoza 50013, Spain
[c] Departamento de Anatomía, Embriología y Genética Animal, Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet, 177, Zaragoza 50013, Spain
[d] Laboratorio de Genética Bioquímica de la Universidad de Zaragoza (LAGENBIO), Miguel Servet, 177, Zaragoza, 50013, Spain
[e] Instituto Universitario de Investigación Mixto Agroalimentario de Aragón (IA2) UNIZAR-CITA, Zaragoza, Spain
[f] Instituto de Investigación Sanitaria de Aragón (IIS-Aragón), Zaragoza, Spain
[g] Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Instituto de Salud Carlos III, Madrid, Spain

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ABSTRACT

Idiopathic epilepsy is one of the most common neurological diseases in dogs; however, epileptogenesis and the mechanisms underlying drug resistance remain poorly understood. This study aimed to evaluate the effects of three months of supplementation with canine-derived *Lactobacillaceae* strains on seizure frequency in dogs with drug-sensitive epilepsy (DSE) and drug-refractory epilepsy (DRE). Additionally, gut microbiota profiles and plasma levels of GABA, L-glutamate, and serotonin were analysed and compared to those of healthy control dogs.

The probiotic supplementation was associated with a reduction in both the number of seizures and seizure days in epileptic dogs. An increase in the relative abundance of gut *Lactobacillus* and plasma GABA levels did not accompany clinical improvement. However, it was associated with a significant decrease in the GABA/L-Glutamate ratio in dogs with DSE but not in those with DRE. At baseline, heterogeneity of the fecal microbial community (β-diversity) was higher in epileptic compared to controls. Following probiotic supplementation, the differences in β-diversity between control and DSE dogs were no longer observed. The probiotic had no notable effect on the control group but was associated with a significant reduction in β-diversity in dogs with DRE.

While the relative abundance of *Lactobacillus* remained unchanged after probiotic administration, some changes in gut microbiota were observed in epileptic dogs. Numerous associations were identified between seizure frequency and specific bacterial taxa with potential protective effects (e.g.,*Prevotella_9, Ligilactobacillus*) or risk effects (e.g.,*Parasutterella, Helicobacter*). These findings suggest that long-term use of *Lactobacillus* may serve as a promising adjuvant strategy for seizure control.

1. Introduction

Idiopathic epilepsy (IE) is one of the most common neurological diseases in the canine species and is also highly prevalent in human medicine (Erlen et al., 2018; Fiest et al., 2017). Beyond its high prevalence, this disease is a concern for veterinary neurologists due to its chronicity and the poor response to anti-seizure medications (ASMs) in 20–30 % of epileptic dogs (Packer et al., 2014; Postchka et al., 2023), in addition to the increased risk for adverse effects of these medications (Bhatti et al., 2015). Thus, significant knowledge gaps remain regarding the development of epileptogenesis, the influence of different modulatory factors, and the mechanisms underlying drug resistance (Packer et al., 2015; Potschka et al., 2015).

Patients with IE require lifelong treatment, as the absence of therapy is associated with an increased risk of more frequent and severe seizures. In some cases, this progression can lead to a significant decline in quality of life, which in veterinary medicine affects not only the dogs but also their owners (Wessmann et al., 2016; Hamers et al., 2022).

Currently, ASMs are a fundamental tool in the treatment of patients with epilepsy. Most of these medications work by enhancing the action of gamma-aminobutyric acid (GABA) and reducing the activity of L-glutamate (L-Glu), the main neurotransmitters involved in seizure

* Corresponding author at: Departamento de Patología Animal, Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet, 177, Zaragoza 50013, Spain.
E-mail address: sgarcia@unizar.es (S. García-Belenguer).

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generation (Podell and Hadjiconstantinou, 1997; Ellenberger et al., 2004). GABA is the main inhibitory neurotransmitter in the central nervous system and is synthesized from L-Glu by the enzyme glutamate decarboxylase (GAD), with vitamin B6 acting as a cofactor (Wang et al., 2019). In contrast, L-Glu functions as the principal excitatory neurotransmitter (Sears and Hewett, 2021). These two neurotransmitters are closely linked, as they can be readily interconverted (Mazzoli and Pessione, 2016; Wang et al., 2019). Depending on the patients' response to ASMs, epileptic dogs can be classified as drug-sensitive—when they respond well to treatment, achieving either complete seizure control or a reduction of more than 50 % in seizure frequency—or as drug-resistant/refractory, when they fail to respond or show an insufficient response (i.e., less than 50 % reduction) to a specific ASM (Bhatti et al., 2015; Potschka et al., 2015).

In some cases of canine IE, a genetic origin has been confirmed or suspected (Hülsmeyer et al., 2015). However, while genetics probably plays a significant role, other factors may also act as seizure precipitating factors in the development of epileptic seizures (Forsgård et al., 2018; Mandigers and Santifort, 2023). In this sense, increasing attention is being directed toward the role of the microbiota-gut-brain axis in the development of behavioral and neurological diseases in both humans and animals (Socała et al., 2021; Gong et al., 2021; Wang et al., 2022; Watanangura et al., 2024). Modulation of the microbiota has been suggested as a potential mechanism by which ketogenic diets reduce seizure frequency (Olson et al., 2018). Nevertheless, the relationship between these diets and gut microbiota remains poorly understood, and it is difficult to identify the specific bacterial taxa involved. Moreover, the studies published to date on diet and epilepsy in dogs are scarce and show highly variable results (Pilla et al., 2020; García-Belenguer et al., 2021, 2023; Verdoodt et al., 2022).

Supplementation with *Lactobacillus* has demonstrated neuroprotective effects in murine models (Eor et al., 2021; Abu-Elfotuth et al., 2023) and potential benefits in controlling epilepsy and its neurobehavioural comorbidities in some human clinical studies (Gómez-Eguilaz et al., 2018; Wang et al., 2022). In addition, *Lactobacillus* and *Bifidobacterium* are the most studied probiotics associated with behavioral and cognitive benefits (Stenman et al., 2020; Prajjwal et al., 2024).

Some studies have shown that *Lactobacillus* can promote consistent changes in GABA levels and $GABA_A$ and $GABA_B$ receptors in different brain regions (Janik et al., 2016; Patterson et al., 2019). It has been also suggested that GABA produced by the gut microbiota could act as a mediator linking between gut microbiota and mental health including anxiety, depression, stress, autism or epilepsy (Braga et al., 2024).

In addition to promoting elevated levels of GABA in both the gut and brain (Braga et al., 2024; Janik et al., 2016; Patterson et al., 2019), some strains of *Lactobacillus* also appear to increase serotonin (5-hydroxytryptamine, 5-HT) levels in the colon and bloodstream (O'Mahony et al., 2015; Yano et al., 2015). Serotonin is a neurotransmitter well known for its role in regulating mood and cognition and some authors have proposed that it may possess anticonvulsant properties (Buchanan et al., 2014; Jenkins et al., 2016; Sourbron and Lagae, 2022).

This study aimed to assess the effects of a 3-month supplementation with a probiotic composed of various *Lactobacillus* strains on seizure frequency in dogs with drug-resistant epilepsy and drug-sensitive epilepsy undergoing conventional antiseizure treatment. Additionally, the study investigated changes in gut microbiota composition and blood concentrations of GABA, L-Glu, and 5-HT, comparing the results with those of healthy control dogs. We hypothesized that the probiotic supplementation would be associated with a reduction in seizures frequency and that an increase in the relative abundance of gut *Lactobacillus* and plasma GABA levels would accompany this clinical improvement.

2. Material and methods

2.1. Case selection

Sixteen dogs diagnosed with IE were recruited from the Neurology Service at the Veterinary Hospital of the University of Zaragoza (Spain). The inclusion criteria were as follows: (1) age between 2 and 12 years in the moment of the study (with age at first seizure between 1 and 6 years) and body weight between 15 and 45 kg; (2) fulfillment of at least Tier I confidence level criteria for the diagnosis of IE proposed by the International Veterinary Epilepsy Task Force (IVETF) (De Risio et al., 2015); (3) presence of generalized epileptic seizures (dog exhibiting only focal epileptic seizures or dyskinesia were excluded); (4) ongoing treatment with ASMs according to clinical criteria and IVETF recommendations (Bhatti et al., 2015) for at least six months before inclusion; and (5) absence of epilepsy induced by other causes or any other concurrent pathology. Exclusion criteria included using probiotics or antibiotics within the three months preceding the study. Dogs were excluded from the study if, during it course, if they developed concomitant pathological conditions, such as gastrointestinal symptoms requiring additional treatment.

Half of the epileptic dogs ($n = 8$) were classified as having drug-sensitive epilepsy (DSE) and were receiving monotherapy with phenobarbital. This group included dogs diagnosed with IE that either remained seizure-free with phenobarbital alone or had achieved a reduction of more than 50 % in seizure frequency and maintained a low seizure burden. The remaining dogs ($n = 8$) were classified as having drug-refractory epilepsy (DRE) and were undergoing combination therapy with phenobarbital, potassium bromide, and levetiracetam. These dogs had reached the maximum therapeutic blood concentrations of phenobarbital and required the addition of potassium bromide and levetiracetam to improve seizure control. All dogs in the DRE group had a history of cluster seizures and normal findings on magnetic resonance imaging (MRI) of the brain according to their clinical records.

In addition, a control group of healthy dogs ($n = 8$) with characteristics similar to those in the DRE group regarding age, weight, breed, and sex was selected. These dogs were recruited from local veterinarians and collaborating owners in Zaragoza.

All epileptic and control dogs lived in their home environments with their owners and were appropriately vaccinated, dewormed, and fed their usual commercial maintenance diet. Owners agreed to maintain these conditions throughout the study period. For epileptic dogs, each owner kept a seizure diary to record seizure frequency and characteristics.

2.2. Ethics statement

All dogs were privately owned and enrolled in the study only after their owners provided written informed consent. Owners were allowed to ask questions and were free to confirm or decline participation. All procedures were carried out under Project License PI67/21, approved on 22 December 2021 by the Ethics Committee for Animal Experiments of the University of Zaragoza. The care and use of control dogs complied with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

2.3. Study design and procedures

A mixed-design study with repeated measures was implemented. The procedure involved the daily administration of a probiotic supplement to the two subgroups of epileptic dogs (DSE and DRE) and the control dogs. The probiotic consisted of three live strains of *Lactobacillaceae* (formerly *Lactobacillus)* of canine origin (*Limosilactobacillus fermentum NCIMB 41636, Lacticaseibacillus plantarum NCIMB 41638, Lactiplantibacillus rhamnosus NCIMB 41640*). The daily dose was administered

according to the manufacturer's recommendations (Procanicare™, Animalcare Group plc., Barcelona, Spain) for a continuous period of three months, which is considered a long-term administration period for a probiotic.

Seizure data were collected over a nine-month period: three months prior to the start of probiotic administration (baseline), during the three-month supplementation period, and for three months after discontinuation.

Fecal and blood samples were collected from all dogs just before starting probiotic supplementation (T0) and after three months of probiotic supplementation (T3m). During the three months of supplementation, no changes were made to the ASMs regimens in the epileptic group. No modifications were made to the dogs' living conditions or diet either. In addition, data on the total number of generalized seizures and the number of days with generalized seizures were recorded over the nine-month period described above.

Fecal samples (1–3 g) were collected with sterile gloves at the time of defecation, ensuring the feces did not come into contact with the ground. Samples were frozen at −80 °C within two hours of collection to prevent bacterial growth and preserve DNA integrity.

Blood samples were consistently drawn from the jugular vein in the morning after an overnight fast. A tube of blood with EDTA for haematological analysis and another one with heparin for general biochemical analysis. These analyses were performed immediately at the Veterinary Hospital of the University of Zaragoza's laboratory, and a portion of the EDTA-plasma was preserved at −80 °C for subsequent analysis of neurotransmitters. In addition, a tube without anticoagulant was collected to analyse serum concentrations of phenobarbital and potassium bromide, as appropriate, at the beginning of the study.

2.4. Neurotransmitters analysis

EDTA-plasma concentrations of GABA, L-Glu, and serotonin were analysed in the Interlab-UMU laboratory (University of Murcia, Spain). The samples were shipped on dry ice. These analyses were performed following the bioanalysis techniques described by Fuertig et al. (2016) and Wang et al. (2019). Sample separation and analysis were performed with an HPLC/MS system consisting of an Agilent 1290Infinity II Series HPLC (Agilent Technologies, Santa Clara, CA, USA), equipped with an Automated Multisampler module and a High Speed BinaryPump, and connected to an Agilent 6550 Q-TOF Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. Experimental parameters for HPLC and Q-TOF were configured using MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.08.00). The inter and intrassay imprecision of the methods were below 15 %.

2.5. Fecal microbiota analysis and sequencing of bacterial 16S rRNA gene

Microbiota analyses were carried out by EXOPOL S.L. (Zaragoza, Spain). For these analyses, DNA was purified from the fecal samples, and the V3-V4 hypervariable regions of the bacterial DNA encoding the 16S rRNA subunit were amplified using specific primers. Sequencing was performed on an Illumina platform, with library preparation carried out following the manufacturer's instructions.

Assembly of the pair-end sequences was performed using FLASH (V1.2. 1 1, http://ccb.jhu.edu/software/FLASH/) (Magoč and Salzberg, 2011). Quality filtering of the sequences was conducted using fastp (V0.23 0.1) (Bokulich et al., 2013). Chimeras were detected and removed using the Silva database in combination with the Vsearch package (V2.16.0, https://github.com/torognes/vsearch) (Edgar et al., 2011). Denoising and generation of amplicon sequence variants (ASVs) were performed with DADA2 (Callahan et al., 2016). Taxonomic assignment of each ASV was conducted using the Silva 138.1 database (Quast et al., 2013) with the QIIME2 plugins, version 2022.02 (Bolyen et al., 2019).

Alpha-rarefaction curves and alpha-diversity indices were calculated with the QIIME2 plugins version 2022.02 (Bolyen et al., 2019). Alpha diversity is defined as the average species diversity across different sites or habitats within a local scale. There are different metrics or methods for estimating alpha diversity, each of which may consider the presence or absence of taxa, take into account quantity, or assess the evenness of taxa across different samples. The alpha diversity indices calculated in this report are as follows: (1) Species richness (or ASV richness); (2) Simpson's Diversity Index (takes into account the presence of different ASVs, as well as their relative abundance) (Simpson, 1949); (3) Shannon's Index (considers both the presence of different ASVs and evenness) (Shannon, 1948); (4) Pielou Index (provides information about the evenness of species abundance distribution within a community) (Kohn and Pielou, 1975).

Beta diversity assesses the differences between microbial communities. The Bray-Curtis measure quantifies dissimilarity in terms of relative species abundance, while Weighted UniFrac measures differences by taking into account phylogenetic information and weighted species abundance (Bolyen et al., 2019). The distance to the centroid measure was also calculated, which allows for the assessment of sample dispersion in principal coordinate space. This measure quantifies how clustered or dispersed the communities are around a central representative point, providing information on the heterogeneity of the microbial community in the dataset (Oksanen et al., 2013).

2.6. Bioinformatics and statistical analysis

Alpha diversity comparisons were performed with the Wilcoxon signed-rank test via the R package ggpubr v0.4.0 (Kassambara, 2020).

Weighted Unifrac and Bray-Curtis distance matrices for beta diversity analysis were generated using QIIME2 (Bolyen et al., 2019) and the R package vegan v2.5.6 (Oksanen et al., 2013), respectively. Principal Coordinate Analysis (PCoA) plots were created using the "pcoa" function from the ecodist v2.0.9 package (Goslee and Urban, 2007). Dispersion within groups was assessed using the "betadisper" function. The influence of experimental group variables on sample dissimilarities was determined by permutational multivariate analysis of variance (PERMANOVA) with the "adonis2" function (pairwise adonis) from the vegan package (Oksanen et al., 2013).

Associations between taxonomic abundance matrices at the phylum, family, and genus levels and the variables under study were analysed using the Wilcoxon signed-rank test. The *p*-values were adjusted according to the Benjamini and Hochberg (1995) method, with significance set at $p < 0.05$.

Due to the non-normal distribution of bacterial relative abundances in some variables and the small sample size, non-parametric statistical tests were used. A one-tailed Wilcoxon matched-paired signed ranks test was employed to compare two-time points (T0 and T3m) within each study group, including healthy and epileptic dogs as well as DSE and DRE epileptic subgroups. A one-tailed Mann-Whitney *U* test and Krukal-Wallis H test were used to compare groups at a single time point (e.g. Control vs. Epileptic or Control vs. DSE, and vs. DRE, respectively).

Finally, correlations among all parameters were assessed using the Spearman rank correlation coefficient.

All graphical representations were produced using the ggplot2 v3.4.0 package (Wickham, 2016). Statistical analyses were conducted in R v4.3.1 (R core team, 2021).

3. Results

3.1. Demographic data and clinical outcomes

Table 1 presents the demographic data of the dogs in the three study groups (i.e., DSE, DRE, and controls). There were no significant differences in age and body weight between the groups. Among all epileptic dogs, the sex distribution was balanced; however, the DRE group

Table 1
Demographics characteristics of all study groups and treatment data for epileptic dogs.

	DSE (n = 8)	DRE (n = 8)	Controls (n = 8)
Age (years) mean (±SD)	4.69 (2.07)	6.00 (3.58)	5.88 (3.10)
Body weight (kg) mean (±SD)	25.35 (6.89)	24.87 (8.08)	24.81 (7.93)
Males n (%)	6 (75 %)	2 (25 %)	2 (25 %)
Neutered males n (%)	4 (50 %)	0 (0 %)	1 (12.5 %)
Females n (%)	2 (25 %)	6 (75 %)	6 (75 %)
Spayed females n (%)	1 (12.5 %)	4 (50 %)	3 (37.5 %)
Breeds (n)	Beagle (1)	Belgian Shepherd (1)	German Shepherd (1)
	Border Collie (1)	Border Collie (3)	Border Collie (3)
	Golden retriever (2)	Spaniel Breton (1)	Spaniel Breton (1)
	Siberian Husky (1)	Mongrel dogs (3)	Mongrel dogs (3)
	Spanish Water Dog (1) Mongrel dogs (2)		
Treatment			
Baseline ASM concentrations (µg/ml)	Phenobarbital (18.55 ± 3.19)	Phenobarbital (31.32 ± 3.2)	None
(mean ± sd)		Potassium bromide (927.94 ± 64.98)	

DSE: drug-sensitive epilepsy; DRE: drug-refractory epilepsy; ASM: anti-seizure medication.

included more females, which were paired accordingly with the control group. Neutering status was similar across groups, with half of the dogs in the DRE and control groups being neutered, compared to 62.5 % in the DSE group.

Table 2 show the number of seizures and days with seizures recorded during the three months before the administration of the probiotic, the three months of probiotic supplementation, and the three months following its discontinuation. During probiotic supplementation, both the DRE and DSE groups showed a significant reduction in the number of seizures ($p = 0.012$ and $p = 0.042$, respectively) and days with seizures ($p = 0.027$ and p = 0.042, respectively). Three months after stopping the probiotic, a slight increase was observed in both the number of seizures and days with seizures; however, these values remained below the averages recorded before probiotic administration.

Table 2
Number of seizures and days with seizures in epileptic dogs during the three-month study periods: before, during, and after probiotic supplementation.

	DSE (n = 8)			DRE (n = 8)		
	Mean (SD)	Min-Max	Median	Mean (SD)	Min-Max	Median
3 m before probiotic						
Seizure number	1.38 (1.51)[a]	0–4	1	12.25 (7.96)[a]	4–27	9
Days with seizures	1.38 (1.51)[a]	0–4	1	5.25 (2.43)[a]	2–8	6
3 m during probiotic						
Seizure number	0.00 (0.00)[b]	0–0	0	4.63 (3.3)[b]	0–10	5
Days with seizures	0.00 (0.00)[b]	0–0	0	2.50 (1.30)[b]	0–4	2
3 m after probiotic						
Seizure number	0.38 (1.06)[b]	0–3	0	9.00 (7.4)[c]	0–24	8
Days with seizures	0.38 (1.06)[b]	0–3	0	3.86 (2.03)	0–6	4

DSE: drug-sensitive epilepsy; DRE: drug-refractory epilepsy.
Different letters indicate significant differences time points within each group ($p < 0.01$).

3.2. Neurotransmitter concentrations before and after probiotic supplementation

Probiotic administration (T3m) did not induce significant changes in neurotransmitter plasma levels in either the control group or the total group of epileptic dogs (Table 3). However, when the epileptic group was divided into DSE and DRE, changes were observed in the DSE group. Specifically, after probiotic administration, DSE dogs showed a significant increase in L-Glu (T0: 946.11 ± 262.31 ng/ml vs. T3m: 1087.76 ± 316.18 ng/ml, $p < 0.036$) and a trend toward decreased GABA (T0: 3885.48 ± 4046.08 ng/ml vs. T3m: 1624.05 ± 607.84 ng/ml, $p = 0.069$) (Table 4). These changes significantly reduced the GABA/L-Glu ratio (4.59 ± 5.26 vs. 1.56 ± 0.67, p = 0.012) (Fig. 1).

The probiotic did not alter the GABA/L-Glu ratio in the control group but did affect the epileptic group. In the DSE group, GABA levels decreased while L-Glu levels increased, resulting in a GABA/L-Glu ratio comparable to that of the control group (DSE vs C: 1.56 ± 0.67 vs 1.50 ± 0.71). In contrast, the DRE group maintained elevated GABA levels and lower L-Glu levels compared to controls, with a persistently high GABA/L-Glu ratio (5.26 ± 6.12). Despite the large difference in mean values, this ratio did not differ significantly between the DRE, DSE, and control groups, likely due to the high variability within the refractory group (Fig. 1).

Under basal conditions (T0), no differences were observed between groups. However, a sex effect was noted for L-Glu concentrations, with females ($n = 14$) showing significantly higher levels than males ($n = 10$) (1109.41 ± 206.97 ng/ml vs. 863.79 ± 122.78 ng/ml, $p = 0.004$).

Significant differences in the GABA/L-Glu ratio were detected between the total group of epileptic dogs and the control group at T0 ($p = 0.013$), with the epileptic group exhibiting a higher ratio (4.24 ± 4.55 vs. 1.29 ± 0.40). These differences were observed in both subgroups of epileptic dogs (DSE vs controls: 4.59 + 5.26 vs. 1.29 ± 0.40, $p = 0.035$; DRE vs controls: 3.90 ± 4.06 vs.1.29 ± 0.40, $p = 0.028$). The elevated ratio in epileptic dogs, both in DSE and DRE groups, was due to higher plasma GABA levels at T0 compared to controls. In contrast, control dogs presented balanced concentrations of GABA and L-Glu, with a GABA/L-Glu ratio close to 1 at both T0 and T3 m (1.29 ± 0.40 vs. 1.50 ± 0.71).

Serotonin levels did not differ between groups or study times (i.e., T0 vs. T3m).

Due to a technical issue with one of the blood samples from a control dog, neurotransmitter analysis could not be performed for that sample. Consequently, analyses for the control group were conducted on seven dogs instead of eight.

Table 3
Neurotransmitter concentrations in the epileptic and control groups before (T0) and after supplementation with probiotic for 3 months (T3m).

	Epileptic (n = 16)		Control (n = 7)	
	T0 Mean (SD)	T3m Mean (SD)	T0 Mean (SD)	T3m Mean (SD)
GABA (ng/ml)	3736.72 (3628.08)	2957.45 (3706.98)	1414.86 (368.84)	1413.28 (554.96)
L-Glu (ng/ml)	952.05 (200.28)	971.01 (277.33)	1118.20 (205.99)	1024.30 (309.75)
GABA/L-Glu ratio	4.24 (4.55)	3.41 (4.62)	1.29 (0.40)	1.50 (0.71)
Serotonin (ng/ml)	835.74 (1451.75)	384.23 (338.49)	1775.62 (1787.39)	2985.20 (4008.64)

Table 4
Neurotransmitter concentrations in dogs with drug-sensitive epilepsy (DSE) and drug-refractory epilepsy (DRE) before (T0) and after supplementation with probiotic for 3 months (T3m).

	DSE ($n = 8$)		DRE (n = 8)	
	T0 Mean (SD)	T3m Mean (SD)	T0 Mean (SD)	T3m Mean (SD)
GABA (ng/ml)	3885.48 (4046.08)	1624.05 (607.84)	3587.95 (3432.92)	4290.84 (5001.31)
L-Glu (ng/ml)	946.12 (262.31)	1087.77 (316.19)	957.99 (130.63)	854.24 (183.50)
GABA/L-Glu ratio	4.59 (5.26)	1.56 (0.67)	3.90 (4.06)	5.26 (6.12)
Serotonin (ng/ml)	1314.69 (1985.61)	440.31 (382.32)	356.77 (222,00)	328.15 (303.57)

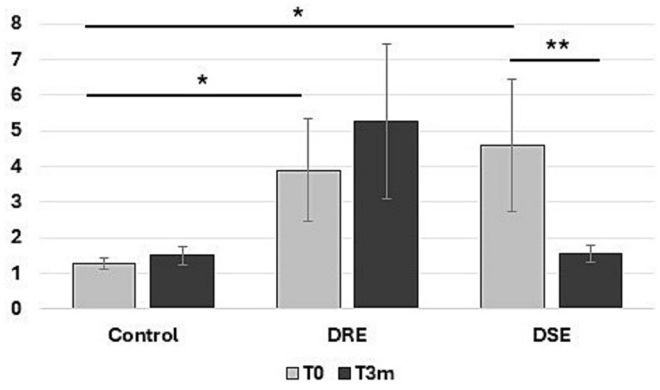

Fig. 1. GABA/L-Glu ratio in controls and dogs with drug-resistant epilepsy (DRE), and drug-sensitive epilepsy (DSE) at the beginning of the study (T0, grey bars) and after three months of probiotic supplementation (T3m, black bars). Significance levels: *p < 0.05, **$p < 0.01$.

3.3. Microbiota results

3.3.1. Characteristics of the fecal microbiota in epileptic and control dogs before probiotic supplementation

Under basal conditions (T0), no significant differences were observed in α-diversity between groups. However, significant differences in β-diversity were detected between dogs diagnosed with IE (DSE and DRE together) and healthy controls (p = 0.0156) (Table 5), with epileptic dogs exhibiting greater diversity compared to healthy animals (Fig. 2). This indicates that the microbiome of epileptic dogs is more heterogeneous than that of healthy controls.

At the phylum level, dogs with IE showed a significantly higher relative abundance of Firmicutes (45.52 ± 21.53 vs. 29.04 ± 13.40, $p = 0.023$) and a lower abundance of Bacteroidetes (25.47 ± 16.97 vs. 44.92 ± 11.14, $p = 0.008$) and Deferribacterotes (0.00 ± 0.00 vs. 0.040 ± 0.071, $p = 0.003$) compared to healthy controls. At the genus level, dogs with IE had significantly lower relative abundance of *Turicibacter* (0.06 ± 0.11 vs. 0.80 ± 0.98, p = 0.008), *Butyricicoccus* (0.034 ± 0.04 vs. 0.08 ± 0.06, $p = 0.027$), and *Prevotella_9* (8.80 ± 11.78 vs. 17.97 ± 9.85, $p = 0.037$), while they showed higher abundances of *Parasutterella* (1.13 ± 4.51 vs. 0.40 ± 0.96, $p = 0.002$) than the healthy controls.

Table 5
Statistical analysis of beta-diversity: Significant results of the PERMANOVA analysis for Bray-Curtis distance matrices between groups, comparing the different experimental groups two by two before (T0) and after supplementation with probiotic for 3 months (T3m).

Comparison	R^2	p
Control T0 vs Epileptic T0	0.080	0.0156
Control T0 vs DSE T0	0.124	0.012
Control-T0 vs DRE T0	0.105	0.033
Control T3 m vs DRE T3m	0.102	0.040

DSE: drug-sensitive epilepsy; DRE: drug-refractory epilepsy.

When the IE group was divided into DSE and DRE, the differences in β-diversity between controls and DSE ($p = 0.012$) and between controls and DRE ($p = 0.033$) were maintained, with no significant differences observed between DSE and DRE. Regarding the relative abundance of bacteria, no significant differences were found at the phylum level between DRE and DSE or in the *Prevotella_9* or *Turicibacter* genera. However, significant differences were detected in the *Parasutterella* genus, with a higher abundance observed in the DRE group comparted to the DSE or control groups (DRE: 2.26 ± 6.39 vs. DSE: 0.04 ± 0.01 or Control: 0.40 ± 0.96, $p = 0.009$).

3.3.2. Effect of the probiotic on the microbiota of epileptic and control dogs

Probiotic administration for 3 months did not result in significant changes in α-diversity, β-diversity, or the relative abundance of bacteria in the healthy control group. In contrast, in the group of dogs with IE, while the probiotic did not alter α or β-diversity, it did lead to changes in the relative abundance of specific bacteria taxa. Notably, the relative abundance of bacteria from the Firmicutes phylum decreased significantly (45.52 ± 21.54 vs. 25.79 ± 10.82, $p = 0.0156$), and there was a trend toward an increase in bacteria from the Fusobacteriales phylum (17.99 ± 19.92 vs. 25.44 ± 10.62, $p = 0.0645$). Additionally, univariate analysis revealed a significant reduction in the relative abundance of *Helicobacter* genus after probiotic supplementation (T0: 0.05 ± 0.04 vs. T3m: 0.02 ± 0.03; $p = 0.046$).

When the group of epileptic dogs was divided into DSE and DRE, the probiotic significantly reduced β-diversity in DRE group ($p = 0.05$) (Fig. 3). The Wilcoxon rank test only showed significant differences in the dispersion observed in DRE dogs before and after the probiotic treatment ($p < 0.05$). No significant differences were observed for the rest of groups. Additionally, univariate analysis showed that probiotic supplementation significantly increased the relative abundance of *Prevotella_9* genus in DSE group (12.21 ± 13.40 vs 18.83 ± 22.24, $p = 0.028$).

3.3.3. Differences in microbiota between epileptic and control dogs after probiotic supplementation

After 3 months of daily probiotic administration (T3m), comparisons of the fecal microbiota between dogs diagnosed with IE with healthy controls revealed that sick dogs continued to exhibit significantly lower relative abundances of bacteria from the *Turicibacter* (0.08 ± 0.18 vs. 0.31 ± 0.42, $p = 0.030$) and *Butyricicoecus* (0.04 ± 0.04 vs. 0.12 ± 0.13, $p = 0.044$) genera, while showing higher abundances of *Helicobacter* (1.11 ± 2.38 vs. 0.02 ± 0.03, $p = 0.024$) compared to healthy controls. No significant differences in β-diversity were detected between epileptic and control dogs at T3m.

When the group of dogs with IE was divided into DSE and DRE, no significant differences in β-diversity were found between these subgroups after 3 months of probiotic supplementation. Comparisons with healthy controls showed no differences between controls and DSE group ($p = 0.407$), but significant differences were observed between controls and the DRE group ($p = 0.040$) (Table 5).

In terms of relative abundance, no significant differences were found between the DSE group and healthy controls. However, significant differences were observed between DRE and controls, with the DRE group showing a higher abundance of bacteria from the Bacteroidaceae family (21.20 ± 8.61vs. 9.65 ± 4.60, $p = 0.035$) and a trend toward lower relative abundance of *Prevotella_9* (2.88 ± 7.25 vs. 18.56 ± 18.64, $p = 0.086$) compared to healthy controls.

3.4. Correlation results

The number of seizures and days with seizures showed a significant

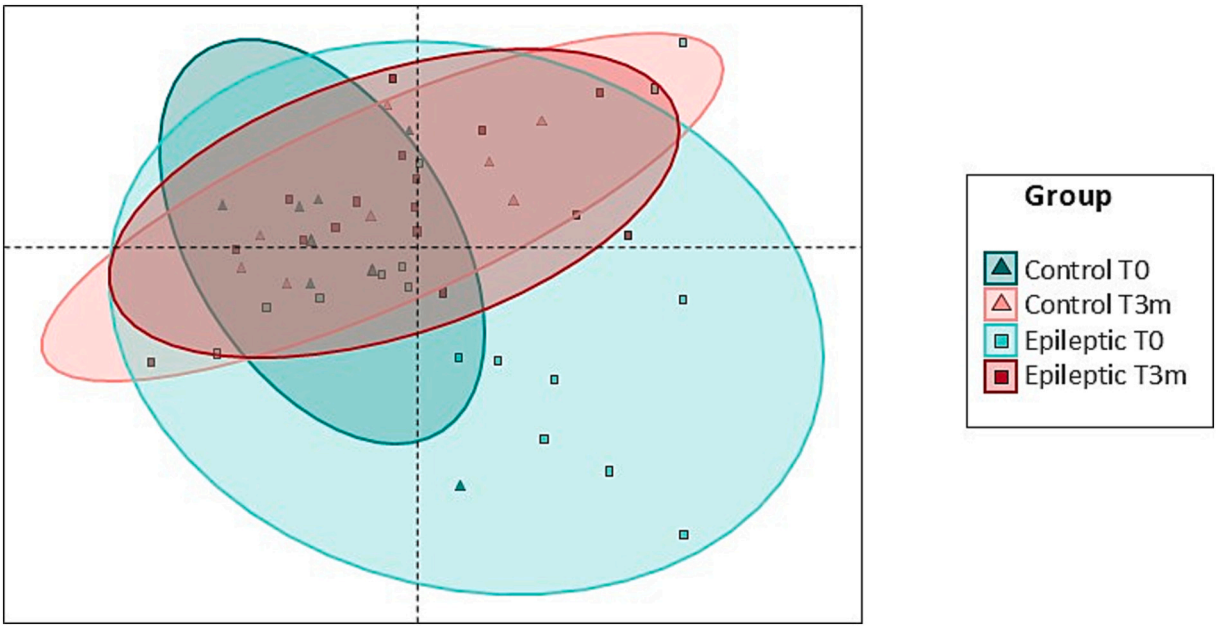

Fig. 2. β-diversity using Bray-Curtis distance matrix and weighted Unifrac represented by principal coordinate analysis (PCoA) in controls and epileptic dogs before (T0) and after probiotic supplementation during 3 months (T3m).

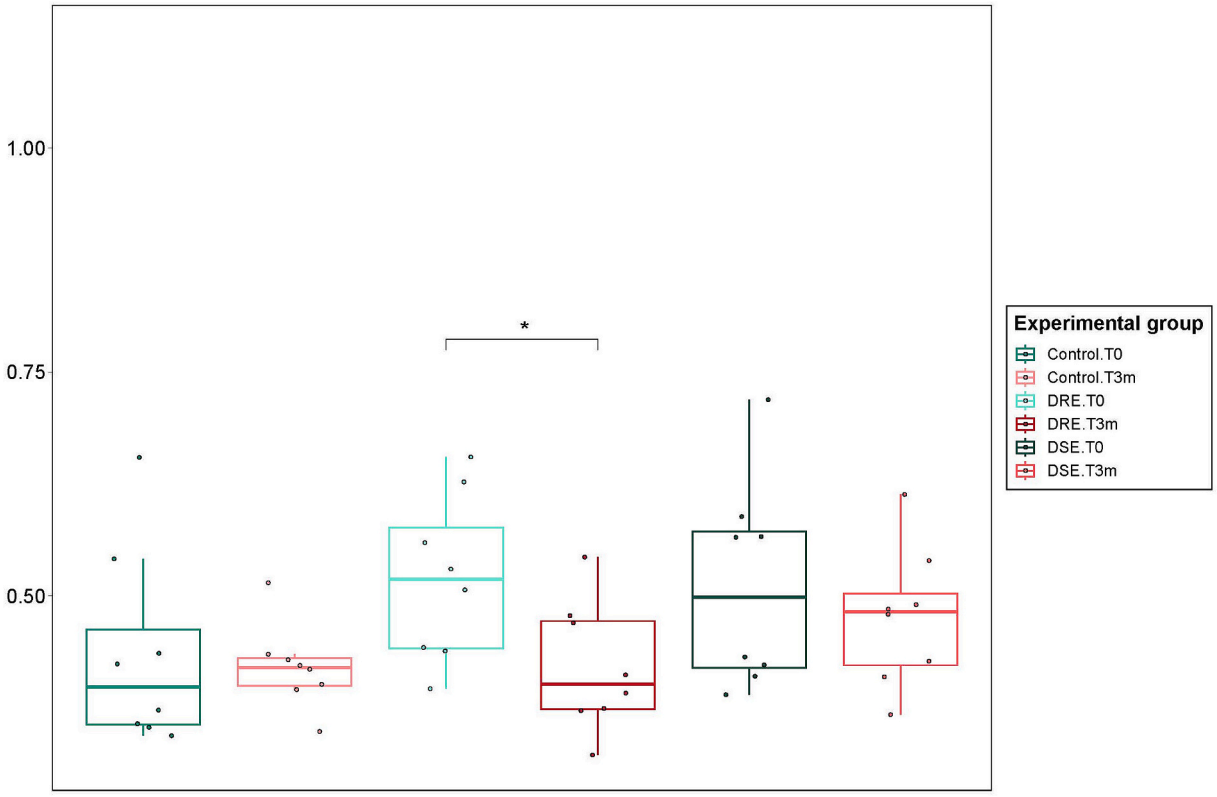

Fig. 3. β-diversity in controls, dogs with drug-resistant epilepsy (DRE), and drug-sensitive epilepsy (DSE) before (T0) and after probiotic supplementation during 3 months (T3m). Wilcoxon rank test *$p = 0.05$.

correlation ($p < 0.001$) before, during and after probiotic supplementation. Regarding neurotransmitters, a significant correlation was observed between pre- and post-treatment GABA concentrations ($R =$ 0,609; $p = 0.012$), as well as between pre- and post-treatment L-Glu concentrations (R = 0,615; $p = 0.011$). Furthermore, an association was found between the abundance of bacteria from the *Bacteroidota* phylum and GABA concentrations under basal conditions ($R = 0.532$; $p = 0.034$). An inverse relationship was observed between the abundance of bacteria from the *Parasutterella* genus and serotonin concentrations, both under basal conditions (T0: R = -0,664; $p = 0.005$) and after probiotic intervention (T3m: R = -0.532; p = 0.034). A similar inverse correlation was found for the genus *Erisipelatociostridium* at both time moments (T0: R =

-0.537; $p = 0.032$ and T3m: R = -0.495; $p = 0.051$).

Under basal conditions, a higher abundance of bacteria from the *Xanthomonaceae* family and the *Prevotella* genus was associated with a lower number of seizures. During or after probiotic administration, a greater abundance of bacteria from the Firmicutes phylum, the *Comamonadaceae* family and the *Ligilactobacillus* and *Prevotella* genera was associated with a lower number of seizures. Conversely, a greater abundance of bacteria from *Helicobacteriaceae* and *Fusobacteriaceae* families or the *Catenisphaera* genus was associated with a higher number of seizures (Table 6).

Finally, a positive correlation was found between the dogs' weight under basal conditions and the relative abundance of bacteria from the *Acidaminococaceae* family ($R = 0.52$; $p = 0.039$).

4. Discussion

This study found that supplementation with three canine-derived *Lactobacillaceae* strains over a three-month period was associated with a reduction in both seizure frequency and the number of seizure days in dogs diagnosed with IE. Previous research has shown that probiotic bacteria can modulate the gut–brain axis and exert beneficial effects on neurological health (Lyte, 2011). Among the most extensively studied genera in this context are *Lactobacillus* and *Bifidobacterium,* which have been linked to improved cognitive function and brain health (Prajjwal et al., 2024). In murine models, for instance, probiotics containing these bacteria have shown potential in mitigating Alzheimer's-like symptoms and pathological changes, including neural damage, Aβ and tau pathology, and neuroinflammation (Xiao-hang et al., 2024).

Increasing evidence also supports the involvement of the gut microbiota in epileptogenesis (Gernone et al., 2022). In experimental epilepsy models, modulation of gut dysbiosis through prebiotics, probiotics, or synbiotics has been shown to reduce spontaneous seizures and cognitive deficits (Wang et al., 2022). In people with refractory epilepsy, probiotic supplementation—particularly with *Lactobacillus* species—has been associated with either fewer than four seizures per year (Peng et al., 2018) or with >50 % seizure reduction (Gómez-Eguilaz et al., 2018). However, despite this growing body of research, evidence remains limited, and to the best of our knowledge, this is the first study evaluating the effect of probiotics on seizure control in dogs with epilepsy.

Our results suggest that this dog-specific *Lactobacillus* formulation may exert beneficial effects in dogs with both drug-sensitive (i.e., responder) and drug-resistant (i.e., refractory) epilepsy. Notably, the reduction in seizure frequency persisted for three months after discontinuation of the probiotic, compared to baseline values recorded three months prior to supplementation. This beneficial effect could suggest a potential neuromodulatory impact of the probiotic.

While promising, these findings must be interpreted with caution due to several limitations, primarily the small sample size and relatively short study duration. Recruiting owners willing to strictly follow study protocols is challenging, and although three months may be considered long-term for probiotic supplementation, it is a short period from an epilepsy research perspective. To mitigate this, seizure data were collected three months before and three months after the intervention, resulting in a total observation time of 9 months and follow-up period of 6 months after the start of the intervention. Still, we must consider possible placebo effects, regression to the mean, and random seizure variability. Ideally, longer observation periods would be needed for more robust evaluation of changes in seizure patterns (Potschka et al., 2015; Stabile et al., 2017). Additionally, a limitation to consider is that some dogs diagnosed with DRE might have experienced changes in diet or medication dosage during the baseline phase, which were not systematically controlled and could have impacted the treatment period.

Nonetheless, the study has several notable strengths, including stringent inclusion criteria, an extended 3-month follow-up period, the inclusion of a control group, and balanced group allocation — with closely matched sex distribution between the DRE and control groups, and only the DSE group showing a higher proportion of males. This is particularly relevant given that previous studies in veterinary medicine have associated neutered male dogs with IE with a poorer prognosis (Fredsø et al., 2014), while neutered females tend to have more favorable outcomes (Packer et al., 2014).

To better understand the potential mechanisms underlying the observed clinical effects, we analysed both blood neurotransmitter concentrations and fecal microbiota. Our working hypothesis was that clinical improvement would be accompanied by an increased abundance of fecal *Lactobacillus* and elevated plasma GABA levels, given the known ability of some *Lactobacillus* strains to produce neuroactive molecules, including GABA (Lyte, 2011; Janik et al., 2016; Patterson et al., 2019; Braga et al., 2024).

Contrary to expectations, no significant changes in plasma GABA levels were observed following probiotic supplementation, and dogs with drug-sensitive epilepsy showed a significant increase in plasma L-Glu. Interestingly, analysis of the GABA/L-Glu ratio revealed distinct patterns between groups. In responder dogs (i.e., drug-sensitive epilepsy), the GABA/L-Glu ratio decreased, aligning more closely with the values observed in healthy controls and suggesting a possible normalization of the excitatory/inhibitory neurotransmitter balance. In contrast, dogs with resistant epilepsy maintained elevated GABA/L-Glu ratios throughout the study. In humans, similar findings have been reported in non-responder patients by Erdal et al. (1999), while Wang et al. (2021) observed elevated L-Glu levels in cases of drug-resistant focal epilepsy. Our data suggest that the probiotic may influence neurotransmitter dynamics differently in responder and non-responder dogs, with a potential decrease in GABA levels in responders and persistently elevated GABA concentrations in refractory cases. Although unexpected, these findings warrant further investigation. Notably, control dogs maintained a stable GABA/L-Glu ratio throughout the

Table 6
Significant correlations between relative abundance of bacteria and seizures frequency in the epileptic group before (T0) and after probiotic supplementation (T3m).

	Before probiotic	During probiotic	After probiotic
	R (p)	R (p)	R (p)
T0			
Phylum Proteobacteria			
Family Xanthomonadaceae	−0.557 (0.025)	−0.552 (0.027)	
Phylum Bacteroidota			
Genus *Prevotella*	−0.489 (0.055)		
T3m			
Phylum Firmicutes		−0.616 (0.011)	
Genus *Ligilactobacillus*	−0.666 (0.005)	−0.641 (0.007)	−0.696 (0.004)
Genus *Catenisphaera*		0.493 (0.053)	0.551 (0.033)
Phylum Proteobacteria			
Family Helicobacteriaceae			0.589 (0.021)
Genus *Helicobacter*			0.589 (0.021)
Family Comamonadaceae		−0.552 (0.027)	
Phylum Fusobacteria			
Family Fusobacteriaceae		0.564 (0.023)	
Phylum Bacteroidota			
Genus *Prevotella*	−0.644 (0.007)		−0.569 (0.027)

study.

Maintaining a balance between GABA and L-Glu is crucial for brain homeostasis (Sears and Hewett, 2021). However, it remains unclear whether plasma concentrations of these neurotransmitters accurately reflect central nervous system levels. GABA has been reported to cross the blood-brain barrier (BBB) (Boonstra et al., 2015), and correlations between plasma and cerebrospinal fluid (CSF) concentrations have been described (Adinoff et al., 1995). Although L-Glu does not readily cross the BBB (Hawkins, 2009), some studies report positive correlations between its blood and CSF levels (Hashimoto et al., 2016; Madeira et al., 2018). BBB permeability can also be influenced by factors such as stress, age, diet, and microbiota composition (Braniste et al., 2014; Boonstra et al., 2015; Kelly et al., 2015; Mazzoli and Pessione, 2016). Additionally, neurotransmitter levels in blood and urine may be affected by epilepsy itself, pharmacological treatment, sex, reproductive status, or body condition (Wang et al., 2021; Schmidt et al., 2022). These factors may explain the higher baseline L-Glu concentrations observed in females in our study, a finding contrary to Rossetti et al. (2016), who reported an inverse relationship between L-Glu and circulating estrogen and progesterone levels. In sum, while our data suggest that the GABA/L-Glu ratio may be a useful biomarker of treatment response in epileptic dogs, the relationship between peripheral and central neurotransmitter levels remains uncertain and further studies are required to confirm this potential marker.

The gut microbiota analysis also yielded unexpected results. Contrary to our hypothesis, *Lactobacillus* supplementation did not significantly increase the relative abundance of this genus in either epileptic or control dogs. This may be due to the typically low baseline abundance of Lactobacillaceae in canine fecal samples, making it difficult to detect post-supplementation increases. Despite their low abundance, *Lactobacillus* may still exert significant neuromodulatory effects, potentially through the enteric nervous system rather than systemic absorption (Bravo et al., 2011; Braga et al., 2024). Nevertheless, Muñana et al. (2020) found no differences in *Lactobacillus* abundance between epileptic and healthy dogs.

Consistent with previous studies (Peng et al., 2018; García-Belenguer et al., 2021), epileptic dogs exhibited significantly higher β-diversity compared to controls at baseline. In humans, dysbiosis has been proposed as a contributing factor to drug resistance in epilepsy (Peng et al., 2018). Interestingly, the probiotic had no observable effect on the microbiota of healthy dogs, while it reduced β-diversity in epileptic dogs, and significantly in those with resistant epilepsy. Although this reduction was not sufficient to normalize the microbiota of this group, the differences in β-diversity between responder dogs and controls were no longer significant following supplementation.

At the genus level, *Parasutterella*, which was more abundant in epileptic dogs—particularly in the refractory group—decreased after probiotic supplementation, with an inverse correlation observed between its abundance and serotonin levels. Serotonergic modulation is emerging as a promising target in epilepsy treatment (Sourbron and Lagae, 2022).

Further analysis of the fecal microbiota revealed specific bacterial taxa potentially associated with seizure risk. Genera such as *Turicibacter*, *Butyricicoccus*, *Prevotella*_9, and *Ligilactobacillus*, along with the families *Comamonadaceae* and *Xanthomonadaceae* and the phylum *Firmicutes*, were associated with lower seizure frequency, suggesting a possible protective role. Some of these bacteria have previously been associated with beneficial effects. *Turicibacter* has been proposed as a modulator of host fat metabolism (Lynch et al., 2023), while *Butyricicoccus* may support gastrointestinal health through short-chain fatty acid activity (Geirnaert et al., 2014; Chang et al., 2020). Members of the *Prevotellaceae* family have been linked to reduced risk of meningoencephalomyelitis of unknown origin in dogs (Jeffery et al., 2017), and *Ligilactobacillus*, a genus separated from *Lactobacillus* in 2020, includes strains commonly used in probiotic formulations (Zheng et al., 2020). However, some bacteria can have both beneficial and detrimental associations depending on the context. For example, *Turicibacter* and *Prevotella* have also been linked to greater severity in Parkinson's disease (Jin et al., 2019).

Conversely, higher seizure frequency was linked to increased abundance of *Parasutterella*, *Helicobacter*, and *Catenisphaera*, as well as members of the families *Fusobacteriaceae* and *Bacteroidaceae*. The role of *Parasutterella* remains poorly understood, despite being a core component of the mammalian gut microbiota (Ju et al., 2019). While *Helicobacter* species are well known for their role in gastrointestinal pathology in humans, their relevance in canine health is considered limited (Taillieu et al., 2023). For other genera, such as *Catenisphaera*, little to no information is available on their role in animal health.

Importantly, although all dogs were fed their usual commercial maintenance diet throughout the study, a standardized diet was not used. This constitutes a limitation, as diet is known to influence both seizure activity and microbiota composition (Verdoodt et al., 2022; García-Belenguer et al., 2023).

5. Conclusions

This study provides preliminary evidence that supplementation with dog-specific *Lactobacillaceae* strains can significantly improve seizure control in dogs with idiopathic epilepsy. The beneficial effect appeared to persist beyond the administration period, suggesting a potential neuromodulatory impact of the probiotic.

Complete seizure freedom was achieved in dogs with drug-sensitive epilepsy during probiotic supplementation. In these responder dogs, probiotic also led to a significant reduction in plasma GABA/L-Glu ratio and β-diversity, normalizing both parameters to levels comparable to healthy controls. In contrast, although a significant reduction in β-diversity was also observed in drug-refractory dogs, it was insufficient to eliminate the differences compared to the control group. Although the underlying mechanisms remain unclear, changes in β-diversity and the GABA/L-Glu ratio point to possible interactions between gut microbiota and neurotransmitter balance.

Interestingly, the relative abundance of *Lactobacillus*—a known GABA-producing genus—did not increase following supplementation. However, several associations were identified between specific bacterial taxa and seizure frequency, underscoring the complexity of the gut–brain axis in epilepsy.

Despite limitations such as small sample size, short duration, and lack of dietary standardization, these findings support the potential role of probiotics as an adjunctive therapeutic strategy within a multimodal approach to canine epilepsy. Further research is warranted to validate these results and clarify the underlying mechanisms involved.

CRediT authorship contribution statement

Sylvia García-Belenguer: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Belén Rosado:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Adelaida Hernaiz:** Writing – review & editing, Methodology, Formal analysis. **Jon Moral:** Methodology, Investigation, Data curation. **Inmaculada Martín-Burriel:** Writing – review & editing, Validation, Resources, Project administration, Investigation, Funding acquisition, Formal analysis. **Jorge Palacio:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

Authors declare no conflict of interest.

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Data availability statement

The sequencing data of the microbiota analysis are available at NCBI Sequence Read Archive (SRA), BioProject ID PRJNA1197442.

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