

UNIVERSITA' DEGLI STUDI DI NAPOLI FEDERICO II

Dipartimento di Medicina Veterinaria e Produzioni
Animali



ALTERED MICROBIOME AND METABOLOME PROFILING IN FEARFUL COMPANION DOGS: AN EXPLORATORY STUDY

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Doctoral dissertation in Veterinary Sciences
XXXVII cycle

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ABSTRACT

Behavioral dysfunctions in dogs represent one of the main social concerns, since they can endanger animals and human-dog relationship. They can bring about signs of stress or fear in dogs, either in unique situations or in multiple settings, apart from the presence of scaring stimuli (human, animal, place, scent, auditory stimuli, object). Accordingly, alongside with genetic susceptibility, generalized fear can be triggered by environmental factors, traumatic experiences, and medical conditions. Behavioral therapy, context-suited management and neurochemical manipulation, through nutrition, supplements, medicines, and pheromones, are the mainstays of the currently accessible treatments. Recent and growing evidence in humans and animals highlight the importance of the gut-brain axis in the modulation of the brain physiology and behavior. Therefore, during my PhD programme, taking advantage of high-throughput approaches, we sought to investigate the impact of the generalized fear upon microbiota, metabolome and lipidomic signatures in family dogs. Microbiota analysis from the stool of the dogs diagnosed with fear showed a differential abundance of taxa belonging to Proteobacteria and Firmicutes Phyla, when compared to the healthy control group. In addition, serum metabolome profiling documented alterations of molecules associated with GABA and glutamate neurotransmission, as well as bile acids metabolism, in the case group. Finally, the patients showed altered levels of phosphatidylcholines, which are regarded as key building block of the cell membranes and relevant players of the brain function as well. Overall, our preliminary study highlighted an intriguing role for the microbiota-metabolome-lipidome network in the generalized fear, thus shedding light on a better understanding about the pathophysiology of mood-related dysfunctions affecting companion animals and paving the way for more effective therapeutical approaches.

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INTRODUCTION

Emotions in brain and gut

Non-physiological behaviors are common among dogs, and often stem from emotional dysfunctions. Like in humans, dogs can experience a variety of emotions, such as joy, sadness, loneliness, fear and anger (Siniscalchi et al., 2018;d'Ingeo et al., 2021). Every emotional state is mainly based on a feeling condition and arousal level as well and represents a measure of mood profile and wellbeing. Feeling states range from a high happiness to sadness conditions, that can change very quickly from one moment to another. On the other hand, negative moods can lead to welfare problems in dogs and contribute to behavioral problems. Indeed, aggression, excessive barking, or restlessness can often lead to compulsive and destructive behaviors that, if left unresolved, can structure into fully-fledged pathologies, feeding the vicious cycle of anxiety. Anxiety belongs to the wide range of mood-related disorders affecting both humans and animals, and it is characterized by altered emotions triggered by the anticipation of a stressful stimulus (Martin et al., 2009). In dogs, anxiety can stem from a variety of factors, including fear, separation, pathological aging and pain (Mills et al., 2020;Landsberg et al., 2023). It has estimated that separation-related anxiety affects up to 56% of dogs worldwide, who can't feel comfortable once left alone by the family members, thus causing dogs to bark or urinate and defecate in the house (Pierantoni et al., 2022). Apart from the pathological conditions, such as cognitive and canine dysfunction syndromes, which both negatively impact on mood-related behavior, anxiety may also occur in elderly dogs (Landsberg et al., 2023). Accordingly, during aging, dogs may experience a potential decrease in their senses of hearing, sight and smell, all features that plunge them in a severe startle behavior. Moreover, anxiety may be situational (context-specific) and manifest only in specific contexts, such as being examined by a veterinarian or being left alone. Of note, fear can lead to anxiety without the dissolution of fear itself, meaning that they both may coexist in the same individual. Fear-induced anxiety generally relies on loud noises, strange people or animals, visual stimuli and new environments, thereby pets may experience many symptoms, ranging from panting and salivation, tucked tail, lowered ears, to piloerection, vocalization, or displacement behaviors. At present, most therapeutic approaches to cope with fearful behaviors in dogs rely on selective serotonin reuptake inhibitors (SSRIs), like fluoxetine, fluvoxamine and paroxetine, together with behavioral rehabilitation treatment (Landsberg et al., 2023). They mainly inhibit the reuptake of serotonin and increase serotonergic neurotransmission by allowing serotonin

molecules to have a long-lasting effect. Unfortunately, with prolonged use, there is a down-regulation of serotonin receptors, and the therapeutic efficacy wears off overtime. In addition, side effects associated with SSRIs, including drowsiness, decreased appetite, vomiting, diarrhea, and restlessness, scare caregivers; in fact, pet parents are often concerned about the use of psychotropic drugs on their pets and prefer the administration of supplements along with other interventions (Riggio, 2017). Thus, behavioural treatment plans should adopt an integrated approach that incorporates medications, environmental management, and behavioral modification, in accordance with the needs and personality traits of patients and caregivers. In recent years, much emphasis is being placed on the connection between brain, behavior, and gut microbiota, which is regarded as one of the crucial players in the onset and severity of stress-related disorders, through different mechanisms, such as the enteric nervous system, the sympathetic nervous system, the hypothalamic-pituitary-adrenal (HPA) axis, immune signaling and gut bacterial metabolites (Dinan and Cryan, 2017). For example, several studies highlighted benefits of probiotics to preserve or restore a healthy condition, through the microbiota-gut-brain axis (Mondo et al., 2019). It seems that probiotics alleviate psychological disorders by acting on multiple fronts, namely protecting the gut barrier, improving mucosal and systemic immunity, balancing the function of the hypothalamic-pituitary-adrenal axis, influencing neurotransmitters and levels of neurotrophic factors from the brain (Sacoer et al., 2024). Therefore, it is not surprising that Yu-Min Yeh and collaborators, in a recent study reported that the canine aggression and separation anxiety were likely to be ameliorated by effects of *Lactiplantibacillus plantarum* and decreased plasma serotonin turnover ratio with separation anxiety, opening wide the doors of “psychobiotics”, as novel class of psychotropic even in the context of companion animals (Dinan et al., 2013; Yu-Min et al., 2022). This suggests that clinicians should focus on investigating the gut wellbeing of their patients with behavioral disorders, and consider new strategies based on a combination of nutraceuticals, diet, prebiotics/probiotics, along with behavioral rehabilitation. To get there, it is first necessary to gain a detailed and deep understanding of the gut-brain axis.

Gut-microbiota-brain axis

In mammals, the gastrointestinal (GI) tract is populated by a huge variety of symbiotic microorganisms, at first acquired from maternal microbiota during pregnancy, influenced by mothers' diet and delivery methods (Stinson et al., 2019). Accordingly, studies in 91 pregnant women showed a significant increase of *Enterococcus*, alongside with lower levels of both *Enterobacter* and *Streptococcus* during the early phases of gestation, when the dams generally provide suitable microbes to the baby (Koren et al., 2012). Moreover, bacteria belonging to Bacteroidetes and Actinobacteria phyla are precociously transmitted to infants through the natural childbirth, since caesarean section or antibiotic exposure around birth were found to be causative of a higher incidence of pathogen colonisation and immune-related disorders in children (Browne et al., 2022). In general, healthy dogs' microbiomes shows similarities to humans (Swanson et al., 2011), likely due to a shared environment and a relevant starch-enriched diet, in comparison to cat whose predominant taxa resemble those typically found in the gut of other mammals (Song et al., 2013; Deng and Swanson, 2015; Coelho et al., 2018; Alessandri et al., 2019; Lee et al., 2022). Studies so far performed, about GI microbiota in mouse models, aimed at evaluating the gut biogeography, characterized by spatial distribution of specific phyla which, depending on chemical gradients, nutrition availability and intestine immunity as well, are differentially distributed between fore and hindgut (Tropini et al., 2017). In keeping with that, distal gut compartments generally contain higher microbial diversity than small intestine and stomach, which harbours an acid resistant flora of aerobic and anaerobic bacteria, populated by different species of *Helicobacter*, followed by *Lactobacillus spp.*, *Streptococcus spp.* and *Clostridium spp.* (Buddington, 2003). On the other hand, aerobic microorganisms (*Clostridia*, *Lactobacillus* and Proteobacteria) inhabit small intestine, whereas the anaerobic ones, such as *Clostridiales*, *Bacteroides*, *Prevotella* and *Fusobacteria*, are in the large intestine of both cats and dogs (Handl et al., 2011; Suchodolski, 2011; Honneffer et al., 2014). Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, and Actinobacteria phyla build up more than 99% of all intestinal bacteria in dogs, while at the genus level, the GI microbiota has been reported to be dominated by *Fusobacterium*, *Bacteroides*, and *Prevotella* (Swanson et al., 2011; Deng and Swanson, 2015; You and Kim, 2021). Research studies about GI microbiota in both humans and animals often do not account for the microbiota diversity within specific sections of the gut, thus representing a generalized profiling of the whole GI tract. In this framework, canine faecal samples could nevertheless provide a proper snapshot of the most

relevant gut microbiota taxa composition compared to humans, since they have a more rapid transit time, with a shorter stay within mucosa membranes (Vazquez-Baeza et al., 2016;Pilla and Suchodolski, 2019). In mammals, GI microbiota profiling can be modulated by several factors, such as sex, age, breed, nutritional status, gastrointestinal disease, body condition score, reproductive status and environment, according to different species (Honneffer et al., 2014;Hasan and Yang, 2019;Pilla and Suchodolski, 2019;Reddy et al., 2019;Kim et al., 2020;Scarsella et al., 2020;Caterino et al., 2021;You and Kim, 2021;Craddock et al., 2022;Thomson et al., 2022). Moreover, several pharmacological treatments with pump inhibitors, antidepressants, glucocorticoids, or antibiotics can even have detrimental impact upon mammalian GI microbiota (Gronvold et al., 2010;Garcia-Mazcorro et al., 2012;Sylvia et al., 2017;Atherly et al., 2019;Maier et al., 2021;Zhang et al., 2021). Besides its crucial role in digestion, several reports highlighted the importance of the GI microbiota in integrating endocrine, immune, and neural pathways. In this respect, the bidirectional activity of the gut-brain-immune axis allows the brain to modulate the GI activity, whereas GI tract itself can shape central nervous system (CNS) functioning. Disruption of the gut-brain- axis homeostasis can be associated with the onset and severity of behavioral disorders, in human and veterinary medicine (Mondo et al., 2019;Wernimont et al., 2020;Chen et al., 2021;Sacoer et al., 2024); such a gut-brain mutual communication system is exploited by the ability of the microbiota to synthesise specific metabolites and neurotransmitters, including serotonin, dopamine, acetylcholine, GABA and glutamate, which might influence mood-related behaviors, alongside with emotion, motivation, reward and cognition (Forsythe et al., 2010;Barrett et al., 2012;Carabotti et al., 2015;Fung et al., 2017;Strandwitz, 2018;Fung, 2020;Kar et al., 2022). The mechanisms involved in this regulation are summarized in Fig. 1, as previously reported (Sacoer et al., 2024).

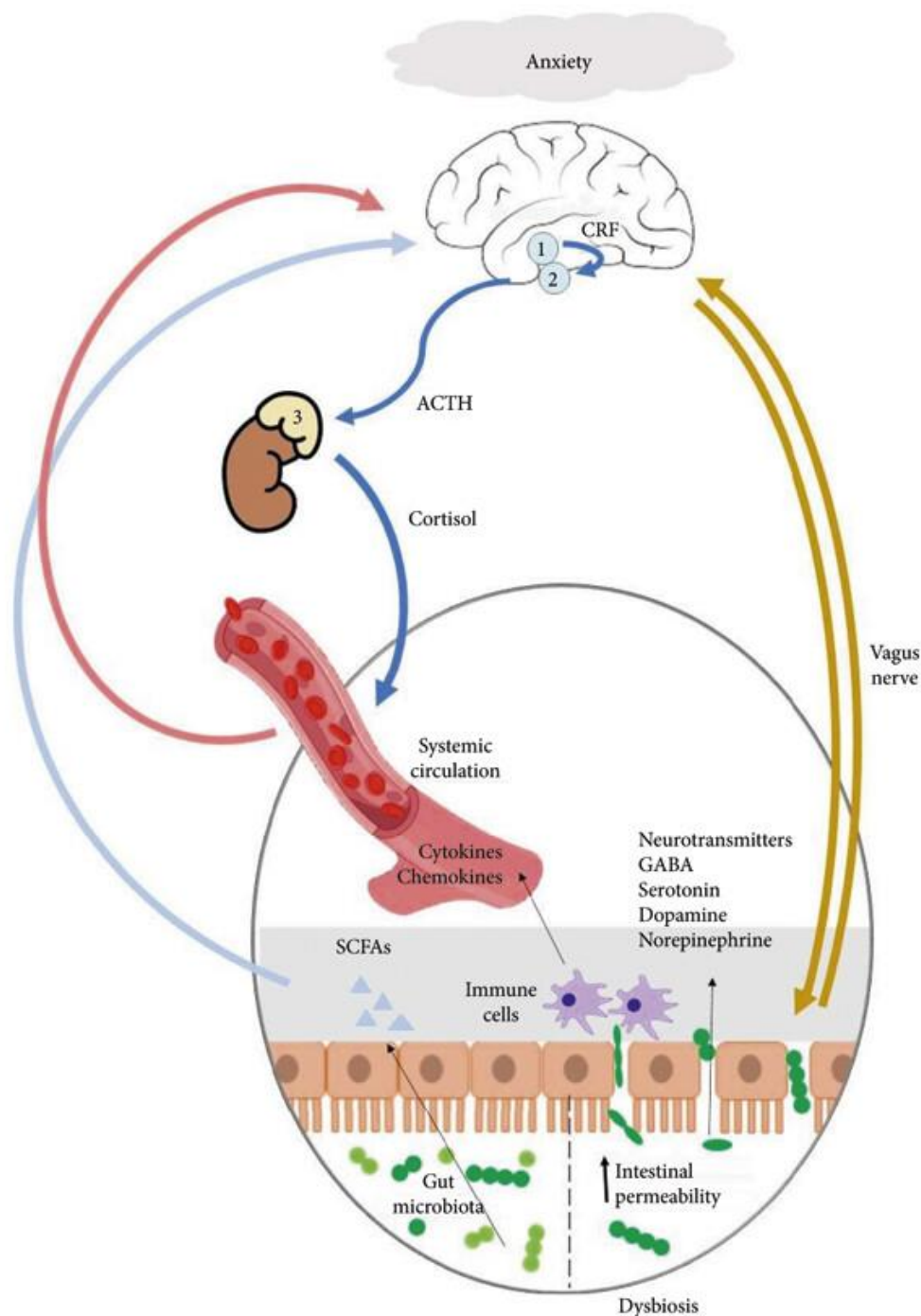


Figure 1. Mechanisms involved in the GBA. The hyperactivation of the HPA axis in anxiety disorders might result in the release of cortisol into the circulatory system. Microbiota can cross the intestinal barrier in an inflammatory setting that is marked by dysbiosis in the gut and increased intestinal permeability. Furthermore, the gut microbiota itself may create metabolites like SCFAs and neurotransmitters that might directly affect mental health, in addition to immune cells producing proinflammatory cytokines. 1–hypothalamus, 2–pituitary gland, 3–adrenal gland, ACTH–adrenocorticotropic hormone, CRF–corticotropin–releasing factor, and SCFAs–short chain fatty acids.

It is not surprising that irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) were linked to changes in the microbiota-gut-brain axis (Rousseaux et al., 2007;Hillestad et al., 2022). Additionally, these intestinal disorders appeared to be associated with cognitive impairments, such as anxiety, depression, as well as changes in bowel transit and permeability, inflammation, infectious diseases and, more in general, gut dysbiosis (Ringel and Ringel-Kulka, 2015). Therefore, questioning whether the microbiota modulates dog's behavior and vice versa will be helpful. Kirchoff and colleagues (2019) found a clustering of faecal taxa in 21 dogs with aggressive conspecific behavior, thus assuming that gut microbiome profiling might be considered a useful tool for a diagnostic assessment of aggressive behaviors (Kirchoff et al., 2019). Further investigations in a small sample of fearful, aggressive, and anxious dogs showed a different structure and composition of the faecal microbiota in dogs with behavioral issues, when compared to healthy controls (Mondo et al., 2020a;Cannas et al., 2021).

Metabolomics and lipidomics approaches in patients with altered behavior

The behavioral tests and questionnaires used in current methodologies seem to correlate well, but they have inherent limitations owing to their time and subjectivity, respectively. There is a need for new objective measurements such as physiological biomarkers, which could help not just phenotyping but could also relate to the underlying impacted molecular pathways. Metabolomics is one example of a high-throughput omics technology that may make it easier to find biomarkers for study, diagnosis, and treatment alternatives. Non-targeted metabolite profiling is a hypothesis-free method that has been effectively used to discover the genetic and environmental components of disease. It can detect molecular biosignatures (Puurunen et al., 2016). For instance, alterations in glutamine and arginine metabolism have been identified by metabolic profiling of schizophrenia, which may indicate a genetic tendency to this neuropsychiatric condition (He et al., 2012). Thus, metabolome is a complete set of low-molecular weight metabolites occurring in a peculiar biological system. Metabolomics applications are typically divided to either targeted or nontargeted approaches (Dunn, 2008; Kaddurah-Daouk and Krishnan, 2009). Targeted metabolomics refers to analysis of a particular and predetermined set of known metabolites which are of interest due to specific biochemical hypothesis or previous literature. In contrast, non-targeted metabolomics is an unbiased and hypothesis-free method aiming to measure as many metabolites as possible from a given sample with the chosen analytical instrumentation. Mass spectrometry- and nuclear magnetic resonance-based metabolomics approaches provide an accurate identification and quantification of small molecules, thus allowing to draw a metabolic fingerprinting that may reflect systemic changes (Roviello et al., 2014; Costanzo et al., 2022a), and disclose the intricate metabolic interactions that take place between gut microorganisms and their hosts (Lamichhane et al., 2018). Therefore, metabolomics offers a large-scale analysis of the current metabolic state (Fig. 2).

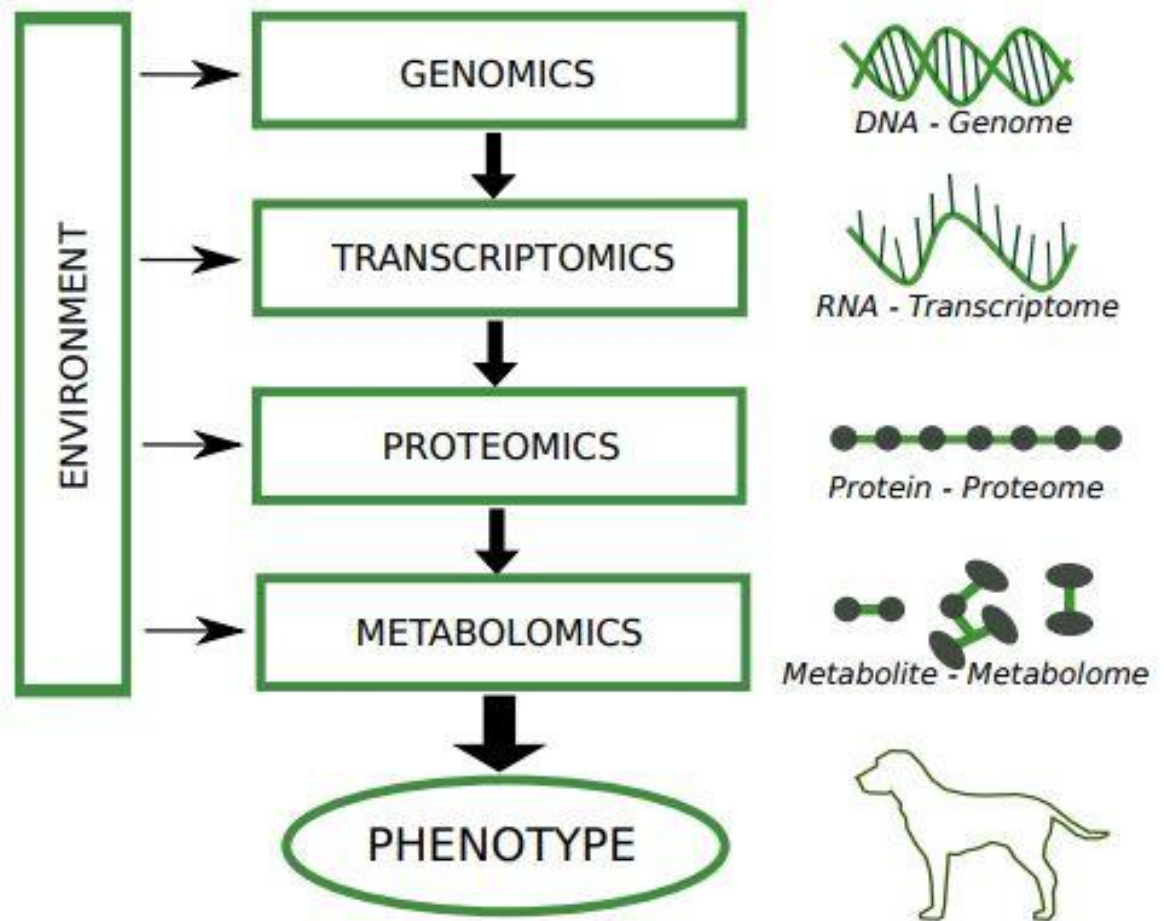


Figure 2. The omics- cascade. The transcriptome, proteome, and metabolome levels may show the effects of environmental variables interacting with the DNA. Metabolomics represents the endpoint of the cascade and thus is closer to phenotype providing real-time information about what has happened and is currently happening in an organism. Therefore, metabolomics has the most potential to identify modifications associated with states of health and disease; nevertheless, to acquire the most complete biological understanding of complex systems, data from all omics levels is required. Figure by Puruunen et al.2018.

As a complementary approach to metabolomics, lipidomic-defined as the full characterization of lipid molecular species- is a potent approach for seeking considerable insights of disease-induced lipid metabolism changes, unravelling biochemical pathogenesis mechanisms of certain disease and exploring potential biomarkers (Adibhatla et al., 2006;Hu et al., 2009;Holcapek, 2015). Lipids are a major group of biomolecules due to their crucial physiological roles as energy-storing enzyme cofactors, emulsifying agents, vitamins, hormones, intercellular messengers and structural components of cell membranes (Fahy et al., 2005). They can be categorized structurally into eight groups: fatty acyls, glycerolipids, saccharolipids, polyketides, sterol lipids, prenol lipids, sphingolipids and glycerophospholipids (PLs). PLs are subdivided into different classes according to the head group: phosphatidylcholines (PCs), phosphatidyl-ethanolamines (PEs), phosphatidylinositols (PIs), phosphatidylserines (PSs), phosphatidyl-glycerols (PGs) and phosphatidic acids (PAs) (Fahy E et al., 2005). In the brain, phosphatidylcholines (PC) have several crucial functions:

- Membrane fluidity: PC help maintain the proper fluidity of cell membranes, allowing substances and signals to pass between nerve cells.
- Synthesis of neurotransmitters: PC are involved in the synthesis of certain neurotransmitters, such as acetylcholine, which plays an important role in memory, learning and muscle control.
- Neuronal protection: PC may have a protective effect on neurons, helping to prevent damage caused by oxidative stress and other toxic substances (Muller et al., 2015).

Alterations in lipid metabolism can be related to various diseases, such as obesity, diabetes, certain types of cancer, Alzheimer's disease and anxiety: in fact, phospholipids are major components of cell membranes and important signaling molecules (Muller et al., 2015) and evidence suggests that alterations in lipid profiles, including phosphatidylcholines, could be related to stress and anxiety conditions, affecting mood and behavior in companion animals, humans and mice (Faria et al., 2014;Puurunen et al., 2016;Berkecz et al., 2018). In schizophrenic patients, for example, lower levels of plasma EP and PC have been measured than in healthy controls, suggesting an involvement of lipid disorder in schizophrenia (Kaddurah-Daouk et al., 2007). However, understanding of the specific role of serum phosphatidylcholines in the context of emotional health in dogs is still limited.

AIM OF THE STUDY

The primary goal of this PhD study was to characterize the microbiota of family dog suffering from generalized fear, allowing for a descriptive overview of the phyla mainly involved in behavioral disorders. The second aim was to investigate the main alterations of metabolites and lipids present in the serum of the dogs involved, and to end by hypothesizing possible correlations between microbiota, metabolome, lipidome and behavioral disorders in dogs. Overall, our analysis highlighted an intriguing role for the microbiome-metabolome network, able to better unveil the potential pathophysiology of relational issues in companion animals and pave the way for more effective therapeutical approaches.

METHODS

Animals and sample collection

All experimental protocols were approved by the Scientific Ethic Committee for Animal Experimentation (Reference number: PG/2023/0011527), in accordance with the Italian legislative Decree (N. 26/ 2014). The blood sample was taken during routine visits, as a part of screening for health problems. Written informed consent was obtained from each owner for the publication.

Fearful dogs (case) and healthy (control) subjects (n = 8) were between one and eight years old, clustered for breed, sex, age, and weight (Table 1). Both patients and controls were owned dogs, fed with dry commercial diet containing crude protein (24-28% of total content), fat (11-18%), fiber (2,2-14%), ash (5,8-11%), and moisture (8-9%). All the enrolled subjects lived in different Italian cities, namely Caserta, Naples, Salerno (Campania Region) and Formia (Lazio Region), and did not received antibiotics four weeks before stool and blood sampling. Neither glucocorticoid, proton pump inhibitors and/or probiotics, herbal remedies and nutraceuticals were provided. One of behaviorist veterinarians carried out clinical and behavioral examination for each animal, who then categorized according to their behavioral phenotype (fearful vs control group). The diagnostic signs of generalized fear and anxiety were: hypervigilance, scanning; changes in social soliciting behavior (hiding, escape attempts); displacement behaviors – out-of-context grooming and scratching, yawning, lip licking, whining; physiologic signs (trembling, dilated pupils, hypersalivation, tachypnoea, tachycardia). The behavioural history included inadequate early environmental experiences and/or socialisation.

The animals underwent a complete blood test, which also included the evaluation of thyroid function, and protein electrophoresis, to rule out any co-morbidities, potentially related to behavioral issues (Camps et al., 2019; Amat et al., 2024). Stool and blood samples were obtained from each enrolled subject, collected and immediately frozen on dry ice, and stored at -80°C until their further processing. A part of stool samples was sent to a laboratory for a coprological examination, aiming at identifying the presence of intestinal parasites, to avoid any potential interference with the gut microbiota. The laboratory parameters assessed in the dogs were all in the ranges. The blood sample was collected during routine veterinary examinations, and as part of canine health screening; therefore, this research was conducted without any additional suffering or discomfort to the animals. All methods were carried out in accordance with relevant guidelines and regulations of Italian Guide for the Care and Use of Laboratory Animals

(Decree Law n. 26/2014, about protection of animals for scientific purposes), and all methods are reported in accordance with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Percie du Sert et al., 2020).

Table 1. Demographics of the dogs enrolled in the study.

Subject/ Breed	Group	Sex	Age (years)	Reproductive status	Weight (Kg)	BCS (1-9 scale)	City (Region)
Monky/ Mongrel	Case	Male	2	Neutered	18	4	Caserta (Campania)
Mira/ Pinscher mix-breed	Control	Female	3	Spayed	5	5	Caserta (Campania)
Cam/ Weimaraner	Control	Male	4	Intact	38	5	Salerno (Campania)
Macchia/ Mongrel	Case	Female	2	Spayed	14	4	Salerno (Campania)
Ciammarica/ Mongrel	Case	Female	5	Spayed	6	4	Salerno (Campania)
Otto/ Rottweiler	Control	Male	2.5	Intact	50	5	Salerno (Campania)
Rena/ Mongrel	Case	Female	1.5	Spayed	30	4	Formia (Lazio)
Charlotte/ Mongrel	Case	Female	7	Spayed	7	5	Formia (Lazio)
Maya/ Mongrel	Case	Female	2	Spayed	20	5	Formia (Lazio)
Rosina Mongrel	Case	Female	3	Spayed	15	4	Salerno (Campania)
Celia/ Weimaraner	Control	Female	7	Spayed	30	5	Salerno (Campania)
Muffin/ Mongrel	Case	Female	4	Spayed	19	4	Salerno (Campania)
Boris/ Shepard mix-breed	Control	Male	7	Intact	37	5	Napoli (Campania)
Liam/ Hunting mix-breed	Control	Male	5	Neutered	28	4	Napoli (Campania)
Buddy/ Mongrel	Control	Male	6	Intact	22	4	Napoli (Campania)
Heidi/ Bobtail	Control	Male	7	Neutered	50	5	Napoli (Campania)

Faecal bacterial community profiling

Genomic DNA was extracted from each collected faecal sample by using the RSC Blood DNA kit and the Maxwell RSC instrument (both from Promega, Madison, WI, USA). In detail, 100 mg of each sample were treated with 400 μ L of lysis buffer and vortexed in order to completely homogenize it. After an incubation at 95 °C in a thermomixer for 5 min at 800 rpm and a centrifugation step at 13,000 rpm for 5 min, 300 μ L of supernatant were transferred into a new tube. Subsequently, 30 μ L of proteinase K were added, the samples were vortexed and incubated at 56 °C in thermomixer for 20 min at 500 rpm. Then, the samples are loaded to the RSC cartridge to complete the extraction and eluted in 100 μ L of elution buffer. Genomic DNAs concentration and quality were analyzed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Starting from the DNA extraction, two blank/ negative samples were also included as controls and processed together with the dog's samples during each analytical step to check for any potential environmental contamination.

Illumina amplicon sequencing and bioinformatic analysis

For the microbiome composition analysis, 16S rRNA custom primers able to selectively amplify the V4-V6 hypervariable regions were used. To this end, a first-round PCR was carried out paying attention to optimize PCR mix and amplification conditions in order to avoid a non-specific products and/or primer- dimer formation(D'Argenio et al., 2022;Veneruso et al., 2023). More in detail, AmpliTaq Gold polymerase, GC enhancer (both from Thermo Fisher Scientific, Waltham, MA, USA) and 20 μ M of forward and reverse custom primers were used. Then, all PCR products were quality analysed through a 2% agarose gel electrophoretic analysis and purified by using the AMPure XP beads (Beckman Coulter, Brea, CA, USA). After a quality-check analysis on the Tape Station System with the D1000 ScreenTapes (both from Agilent Technologies, Santa Clara, CA, USA), the purified amplicons were quantified with Qubit HS (Qubit, dsDNA HS Assay, Life Technologies, Carlsbad, CA, USA) and diluted to 2 ng/ μ L to be further processed for the second-round PCR. During this step, Nextera DNA CD Indexes (Illumina, San Diego, CA, USA) were used to specifically tag each sample, and also to add the universal adapters for the following NGS reactions. Next, further AMPure XP beads-based purification and Tape Station qualitative analysis were carried out. Finally, each sample, together with the six negative controls, were quantified with the Qubit fluorometer (Life Technologies) and diluted at 4 nM. Five μ L of each diluted library and 5 μ L of each negative

control were pooled at equimolar concentrations. MiSeq reagent Kit V2.5 500 cycles (250X2) on the MiSeq instrument (Illumina, San Diego, CA, USA) was used for the sequencing reaction. The library's pool was loaded at a final concentration of 9 pM with a 30% PhiX. The FASTQ files produced by the sequencing run were subsequently analyzed by the CEINGE - Biotecnologie Avanzate Franco Salvatore Bioinformatic Facility (Naples, Italy). A first quality check analysis was conducted by using FastQC software. Next, the alignment against the reference database SILVA NR 99 v.138 enabled to the correct assignment of the OTUs (Operational Taxonomic Units). The obtained OTU and taxonomy tables were used as input files for the web-based tool Microbiome Analyst (version 2.0, last accession September 2023), that allowed for the deeper analysis of the bacterial community composition (Dhariwal et al., 2017). In particular, α diversity was evaluated applying different metrics to assess both richness and evenness; the ANOVA test was applied to verify statistically significant differences. Unweighted and weighted UniFrac distance measures were also analyzed by using the PERMANOVA test to highlight significant differences in the β diversity. Differential abundance analysis was performed using a univariate statistical test based on the EdgeR algorithm; p-values were adjusted using the FDR method (Dhariwal et al., 2017). Raw 16S rRNA sequence data were submitted to Sequence Read Archive (SRA) under BioProject accession number ID #PRJNA1181839.

Serum metabolomics and lipidomics analysis

Metabolites and lipids in blood serum from collected dogs were identified and quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and direct flow injection analysis-MS/MS (FIA-MS/MS), respectively, using a targeted metabolomics platform (Costanzo et al., 2022a; Campesi et al., 2023). In detail, 10 μ L of serum were transferred onto a 96-well plate containing the positions for blanks, phosphate buffer saline, calibrants, and quality controls (QC) according to the protocols of MxP[®] Quant 500 kit (Biocrates Life Sciences AG, Innsbruck, Austria) (Costanzo and Caterino, 2023). The mixtures were dried under N₂, incubated for 1 h in 5% phenyl isothiocyanate (PITC) and then added with 5 mM ammonium acetate in methanol to finally extract metabolites by plate centrifugation. A volume of 20 μ L was injected into the LC-MS/MS system, composed by a Triple Quad 5500 + QTRAP[®] Ready (AB Sciex, Framingham, MA, USA) coupled to a 1260 Infinity II HPLC

(Agilent, Santa Clara, CA, USA), and analyzed by multiple reaction monitoring (MRM) mode. Each sample was run by LC-MS/MS-MRM three times as technical replicates. Raw MS data were processed with the Analyst software v.1.7.1 (AB Sciex) and the MetIDQ Oxygen software (Biocrates Life Sciences AG) to integrate targeted metabolite peaks for accurate quantification with respect to labelled internal standards, and finally expressed as concentration (μM). The metabolomic analysis allowed to target 106 small molecules, including amino acids (AA) and related AA, bile acids, fatty acids, biogenic amines, carboxylic acids, hormones, indoles derivatives, alkaloids, amine oxides, cresols, vitamins, and cofactors.

For the analysis of serum lipids, the extract content eluted by centrifugation (10 μL) was diluted with 490 μL of FIA solvent. Sample volumes of 20 μL were injected into the system within the mobile phase (FIA solvent) at a flow rate of 0.03 mL/min until 1.6 min, followed by flow rates of 0.20 mL/min for 1.6 min and 0.02 mL/min for 0.20 min.

Lipid analysis was performed by FIA-MS/MS-MRM in technical triplicates to reduce the analytical variability. Quantification data were obtained using the MetIDQ Oxygen software (Biocrates Life Sciences AG). The final dataset was composed of concentration values (μM) for 523 lipids targeted by MRM, including acylcarnitines, ceramides, diacylglycerols, triacylglycerols, glycerophospholipids, sphingolipids, and cholesterol esters.

Metabolome and lipidome data analysis

The metabolomic and lipidomic datasets were processed using the MetaboAnalyst 6.0 tool for chemometrics and statistical analyses (Manganelli et al., 2021; Costanzo et al., 2022b). The features with more than 50% of missing values were removed, and missing values were imputed using 1/5 of minimum positive values of their corresponding variables. The dataset was normalized, log₁₀-transformed and auto-scaled. Partial Least Squares-Discriminant Analysis (PLS-DA) was performed to find the variance between the (case and control) groups and predict the class of relevant features according to the VIP (Variable importance in Projection) score. Metabolites with VIP score > 1.5 were selected as predictive of the phenotypes analyzed. Hierarchical clustering analysis and heatmap visualization were performed to check intra- and inter-group biological variability according to the relative abundance of their metabolite and lipid features. The technical triplicates of each sample were kept as individual features (not averaged) for PLS-DA and heatmap clustering also to assess the reproducibility of the data. MetaboAnalyst was also used for metabolite pathway analysis and Metabolite Set Enrichment

Analysis (MSEA) through the Over Representation Analysis (ORA) function (Xia and Wishart, 2010). For pathway enrichment analysis, the Homo sapiens KEGG was chosen as pathway library in absence of a proper database relative to dog species; matched pathways were selected according to the p-values from the pathway enrichment analysis and pathway impact values from the pathway topology analysis (i.e. the selected pathway showed concurrently $-\log(p) > 1.3$ and pathway impact > 0.1). For the enrichment of ORA terms, significant terms were selected for values of $p < 0.05$.

The normalized datasets were then exported from MetaboAnalyst to execute univariate statistical analysis within GraphPad Prism 9.0 software (Gonzalez Melo et al., 2021). In particular, volcano plot analysis was carried out to select analytes with significantly changing abundance (Difference), calculated as difference of the levels in fearful and control dogs. The significance threshold was set at $p < 0.01$. On the other hand, the significant differences for binary comparisons (between cases and controls) were evaluated for the single molecules, i.e. those significant from volcano plot analysis, using the non-normalized concentration (μM) dataset obtained by LC-MS/MS. Parametric Welch's t-test or non-parametric Mann-Whitney t-test were used as statistical tests according to the normality of the distributions, assessed by D'Agostino & Pearson test. Finally, correlation analysis between significant microbiota species and metabolites was performed computing Spearman correlation and simple linear regression. Metabolomics data have been deposited to the EMBL-EBI MetaboLights database (DOI: 10.1093/nar/gkad1045, PMID:37971328) with the identifier MTBLS11669.

RESULTS

Microbiota profiling

The sequencing reaction produced a total yield of 6.9 Gb, a cluster passing filter of 93.9% and a cluster density of 697 K/mm². All the reads detected in at least one of the six negative controls were filtered out and eliminated from the rest of the samples in a normalization-manner process. At the end of this decontamination step, an average of 235,827 reads/sample, corresponding to a total of 706 OTUs, was obtained and used for subsequent analyses. First, microbial communities' diversity measures were assessed. In particular, in order to verify both the richness and the evenness, alpha diversity was evaluated within two groups, and the ANOVA test was applied to highlight any significant difference. As shown in Fig. 3, all the different metrics used (panel A: Observed Species, panel B: Chao1, and panel C: Shannon) showed no significant differences between groups. However, a different trend could be appreciable, richness and evenness being both higher in the case than in the control group. In addition, beta diversity analysis was carried out by using both unweighted (Fig. 3D) and weighted (Fig. 3E) UniFrac distance measures, and no significant differences were found.

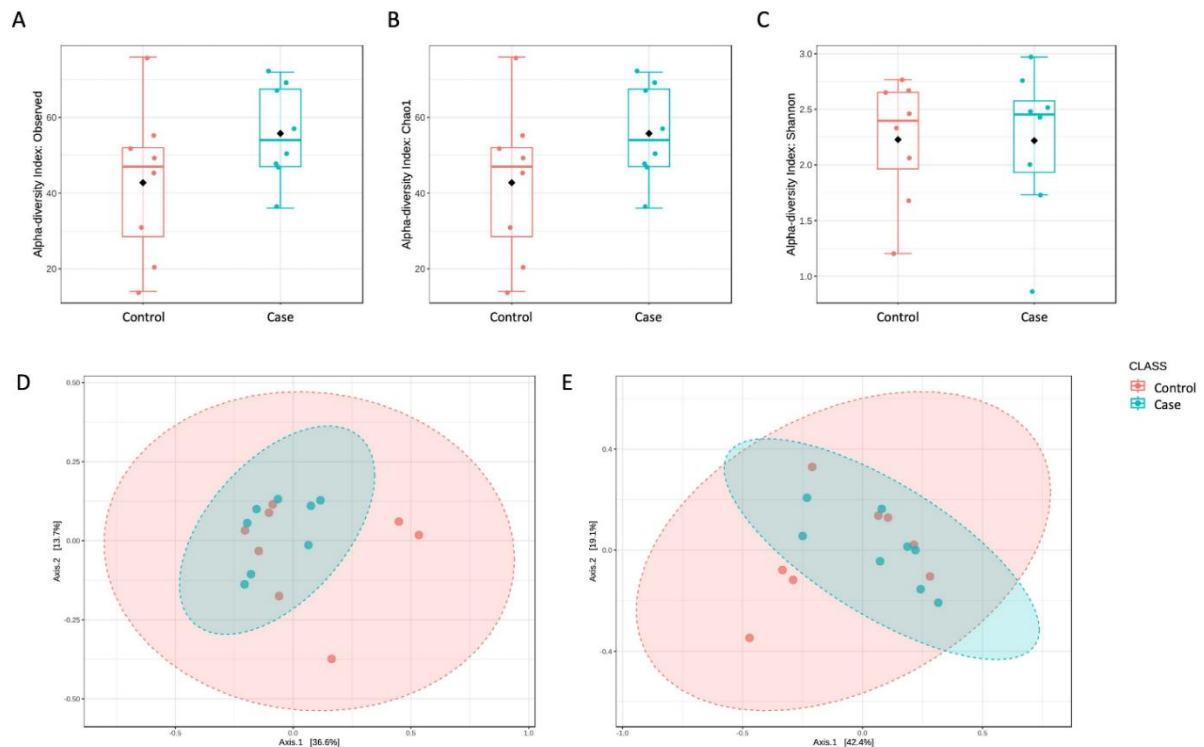


Figure 3. Diversity measures between the fearful dogs (case) and the dogs with normal behavior (control). Alpha diversity was assessed by using Observed species [(A), $p = 0.14$], Chao1 (B), $p = 0.14$ and Shannon (C), $p = 0.97$], indices and the ANOVA test. Beta diversity was also evaluated by using the unweighted (D, $p < 0.52$) and weighted (E, $p < 0.2$) UniFrac distance measures, applying the PERMANOVA test.

Although the diversity measures showed no significant differences between the two tested conditions, it can be noticed that the case group showed a reduced heterogeneity compared to the control, suggesting that the disease status may be related to common microbial features. Next, taxonomy assignment allowed to identify six phyla, four of which with an abundance > 1% in at least one of the 2 compared groups (Fig. 4A). Among these, we found a lower relative abundance of Proteobacteria in the case vs control group (3.5% vs 20.9%), while Fusobacteriota (44.9% vs 52.7%), Firmicutes (32.9% vs 40%), and Actinobacteriota (0.8% vs 3.3%) phyla were more abundant. Accordingly, the core microbiome analysis, considering a relative abundance >1% and a 20% value of sample prevalence, showed a different set of taxa characterizing the case (Fig. 4B) and the control group (Fig. 4C). At genus level, out of nine most represented taxa, we found six bacteria highly expressed in the case vs control group, namely *Cetobacterium* (10.6% vs 3.44%), *Escherichia_Shigella* (from 15.9% to 1.6%), *Lachnoclostridium* (from 7.6% to 4%), *Phascolarctobacterium* (3.7% vs 2.2%), *Ruminococcus_gnavus_group* (4.4% vs 2.5%), and *Sutterella* (4.7% vs 1.7%). On the other hand, *Fusobacterium*, *Holdemanella* and *Megamonas* were found at lower levels in the case group (*Fusobacterium*, 34.4% vs 49.2%; *Holdemanella*, 0.4% vs 4.2%; *Megamonas*, 1.4% vs 4.2%) (Fig. 4D).

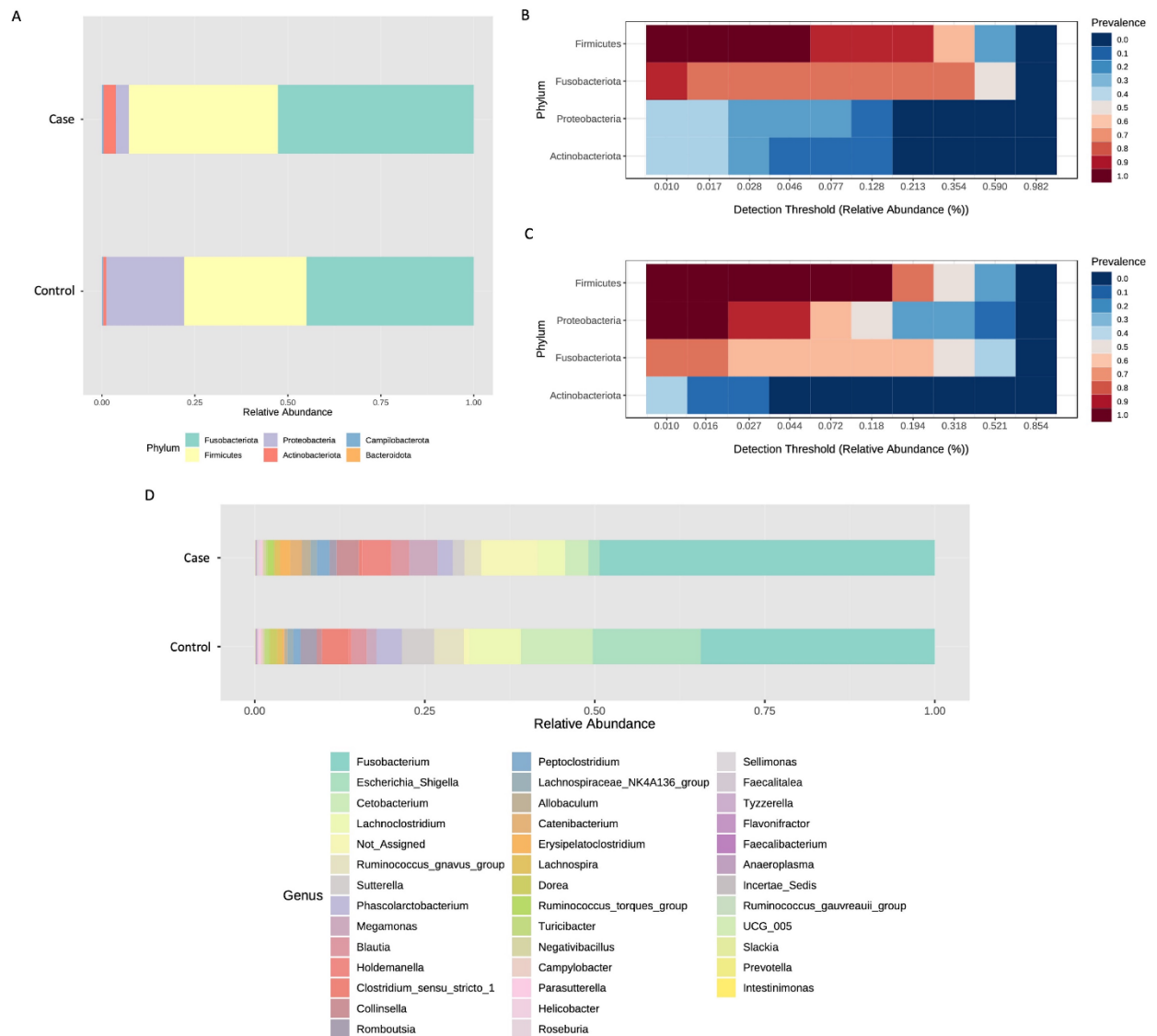


Figure 4. Taxonomic assignment highlighted different microbial taxa in case and control groups. A different bacterial composition (relative abundance, %) was highlighted at the phylum level (A) and confirmed by core microbiome analysis showing a different taxa profile in Case (B) and Control (C) groups. These differences in taxa composition were also identified at the genus level, as shown in panel (D).

Then, to verify if these differences are statistically significant, we performed a differential abundance analysis. In particular, EdgeR analysis allowed us to identify a lower abundance of *Gammaproteobacteria* and *Dorea* in fearful patients (Fig. 5A, B), who also displayed higher levels of *Erysipelatoclostridiaceae* and *Peptostreptococcales Tissierellales* (Fig. 5C, D), when compared to the healthy controls.

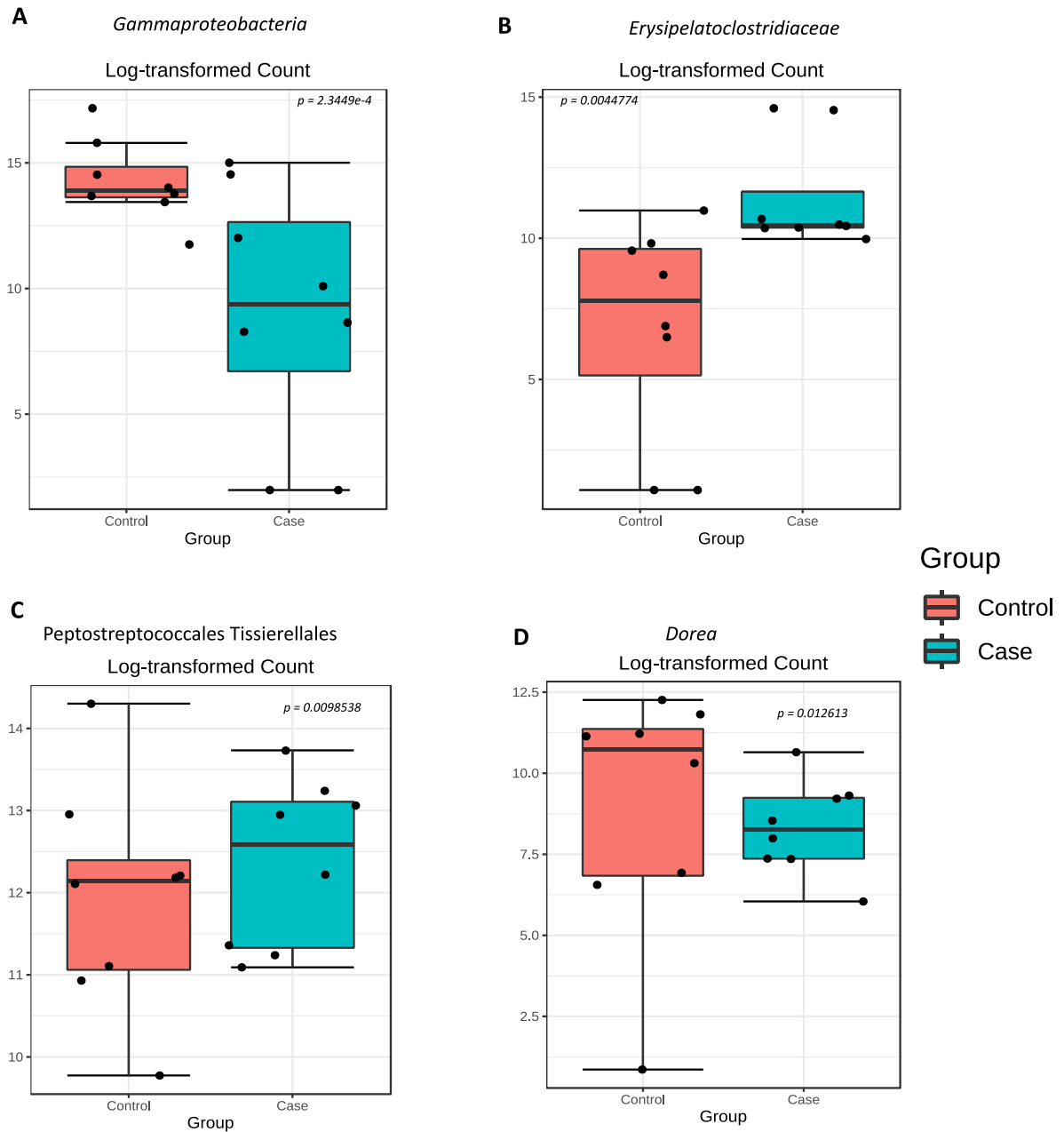


Figure 5. Significantly different taxa identified between Case and Control groups by EdgeR analysis (adjusted p-value <0.05). Among the significantly different taxa we found that the *Gammaproteobacteria* class was less abundant in the cases respect to the controls (A). The Firmicutes-related taxa, *Erysipelatoclostridiaceae* family (B) and *Peptostreptococcales Tissierellales* order (C) were more abundant in the cases, when compared the controls. At genus level, the Firmicutes-belonging *Dorea* taxon was reduced in the case group (D).

Metabolome profiling

To further investigate the systemic changes affecting fearful dogs, a metabolome analysis was performed on the blood serum samples, using a targeted LC-MS/MS approach. Univariate and multivariate statistical analyses were employed to select the most significant metabolic alterations in fearful animals. Specifically, the variances between case compared to control group revealed a good spatial separation according to the PLS-DA, with variances of principal component 1 (PC1) and 2 (PC2) of 6.2% and 5.1% (Fig. 6A). The Variable Importance in Projection (VIP) score was used to identify those metabolites (VIP >1.5), taurodeoxycholic acid (TDCA) and glutamine (Gln) as examples, whose levels can strongly discriminate fearful dogs versus controls, highlighting metabolic abnormalities likely connected with the fearful phenotype (Fig. 6B). Also, the hierarchical clustering of quantified metabolites reported in the heatmap displayed a distinct separation pattern between cases and controls (Fig. 6C). Univariate statistics performed by volcano plot analysis highlighted eleven metabolites with differential abundance in fearful dogs (Fig. 6D), many of which overlap with VIP metabolites.

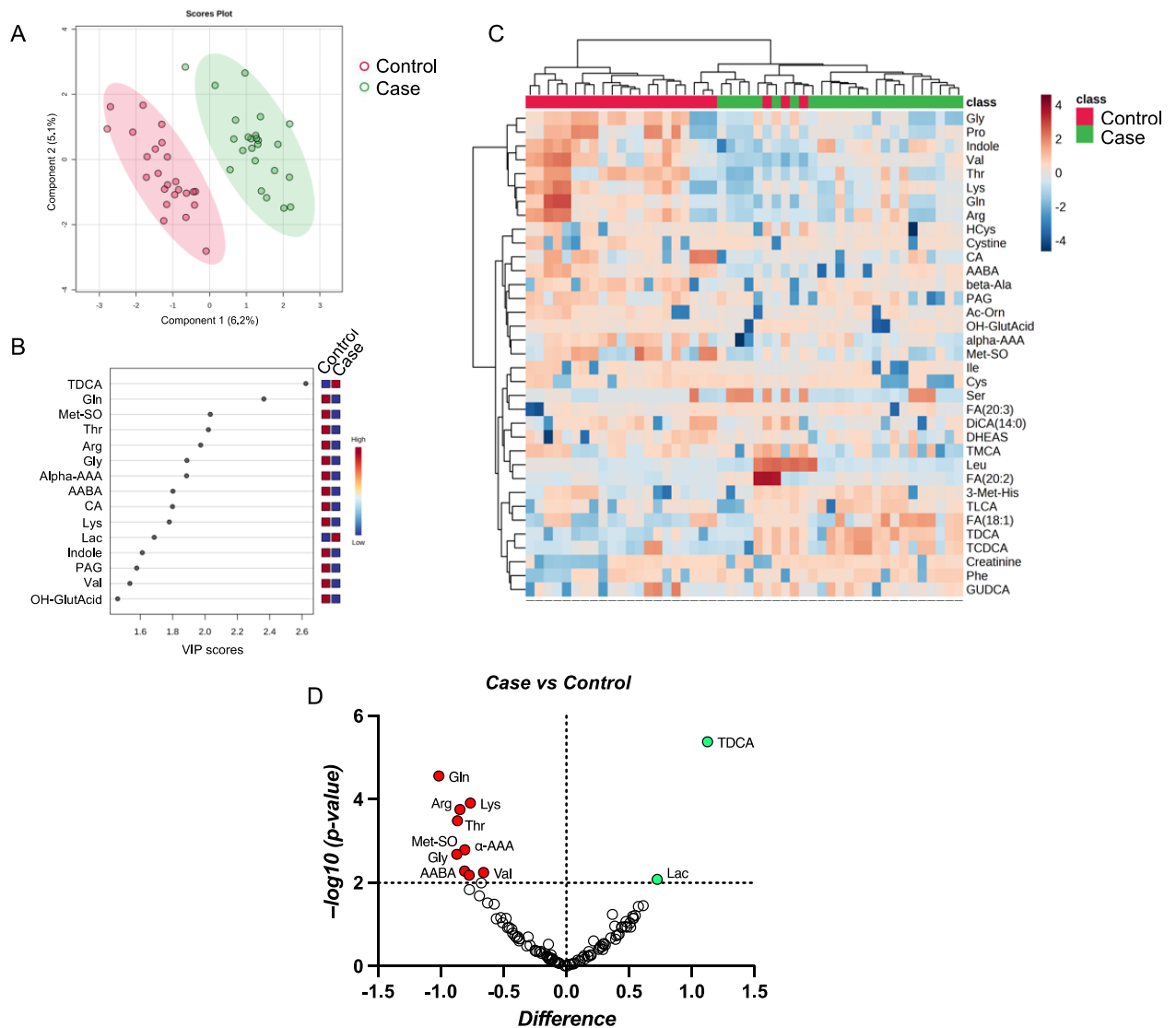


Figure 6. Comparative metabolome analysis of case (fearful dogs) versus controls. (A) PLS-DA analysis was performed using normalized metabolite levels from cases (green) and controls (red); (B) The top 15 discriminant features identified with values of VIP scores >1.5 are reported. (C) Heatmap showing metabolites concentrations in each group and replicates. The intensity of the colored boxes represents the relative abundance of each molecule, whereas metabolite concentrations were normalized, $\log_{10}$ -transformed, and auto-scaled. (D) Volcano plot analysis of metabolites significantly different in the comparison case vs control. The red and green dots represent the significant increased and decreased metabolites, respectively. Non-colored dots refer to all the molecules identified in the dataset whose relative abundance is not significantly different between groups.

Quantitatively altered metabolites are listed in Table 2 in the order from the less abundant to the most abundant, along with their corresponding P-value, $-\log_{10}(\text{P-value})$, difference, standard error (SE) of difference, and regulation trend.

Table 2. Significant metabolites found differentially abundant in fearful dogs versus controls.

Metabolite	P-value	$-\log_{10}(\text{P-value})$	Difference	SE of difference	Regulation
Glutamine (Gln)	0.000028	4.561	-1.015	0.1947	Down
Glycine (Gly)	0.00523	2.281	-0.8107	0.2628	Down
α -Aminobutyric acid (AABA)	0.006575	2.182	-0.7736	0.2589	Down
α -Aminoadipic acid (α -AAA)	0.00161	2.793	-0.8099	0.2266	Down
Lysine (Lys)	0.000123	3.91	-0.764	0.1657	Down
Methionine sulfoxide (Met-SO)	0.002083	2.681	-0.873	0.2517	Down
Threonine (Thr)	0.000327	3.486	-0.8679	0.2057	Down
Arginine (Arg)	0.000174	3.759	-0.8473	0.1895	Down
Valine (Val)	0.005669	2.247	-0.6594	0.2161	Down
Lactic acid (Lac)	0.008264	2.083	0.724	0.2505	Up
Taurodeoxycholic acid (TDCA)	0.000004	5.383	1.127	0.188	Up

To further validate the abundance trends in each sample group for the selected metabolites, we analyzed them individually using raw concentrations from the LC-MS/MS dataset, confirming the trends of regulation and statistical significance (Fig. 7A). Additionally, we performed pathway analysis on significant metabolites to enrich dysregulated pathways associated to the fearful phenotype. Among them, we selected those with concurrently highest impact and lowest p-value, namely ‘glycine, serine and threonine metabolism’, ‘lysine degradation’, ‘glyoxylate and dicarboxylate metabolism’ (Fig. 7B). MSEA performed through ORA analysis revealed biologically meaningful terms associated with the differential metabolome of fearful dogs. ORA analysis enriched as significant ($p < 0.05$) terms: ‘glycine and serine metabolism’, ‘carnitine synthesis’, ‘urea cycle’, ‘lysine degradation’, ‘ammonia recycling’, and ‘aspartate metabolism’ (Fig. 7C).

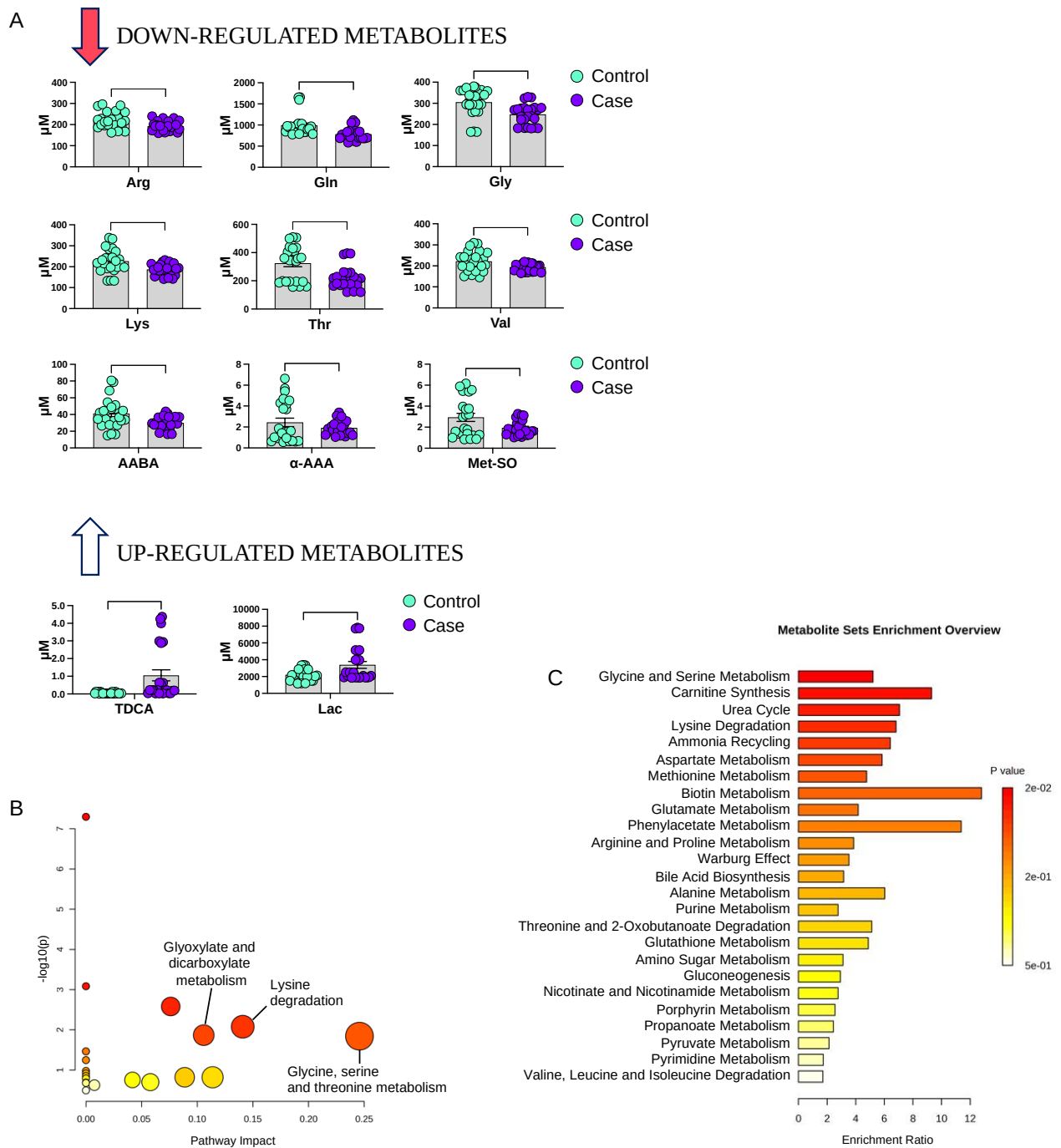


Figure 7. Statistical analysis and bioinformatic pathway enrichment of single metabolites from the differential metabolome. (A) The abundances of single metabolites were evaluated in case vs control by parametric or non-parametric t-test. The plots report the analyte concentrations (means $\pm$ SEM on the bar, dots are all the measurements in replicates). The significance of statistical analysis is referred to as: * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. (B) Pathway analysis plot with details of the significant pathways enriched by the analysis. (C) Overview of MSEA terms enriched with the over representation analysis (ORA) of significant metabolites.

Nonetheless, to assess whether faecal species and circulating metabolites varied with the same trends in fearful animals, we performed a correlation analysis using both types of features. Significant microbiota species were correlated with the global metabolome, highlighting significant correlations (Fig. 8). Interestingly, bile acids (namely TDCA, GUDCA, GLCA) were positively correlated with the *Peptostreptococcales Tissierellales* (Fig. 8A), whereas some amino acids (Val, Thr, Lys) were associated to the *Dorea* (Fig. 8B). Remarkably, many of these molecules were found as significant from both volcano plot and VIP analyses.

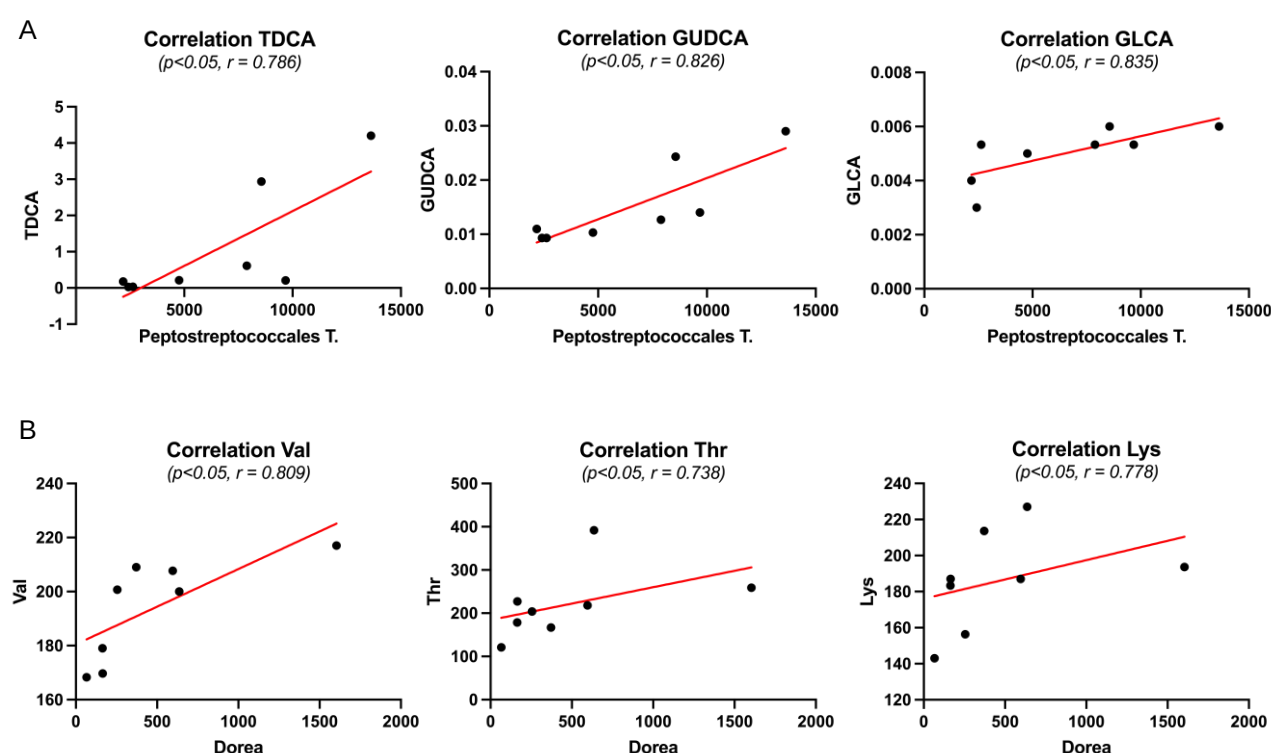


Figure 8. Correlation analysis of the differential microbiome with the global metabolome. Significantly correlating metabolites were reported for (A) *Peptostreptococcales T* and (B) *Dorea* organisms. The correlation plots report the computed r coefficient and the statistical significance (p). TDCA: Taurodeoxycholic acid, GUDCA: Glycoursodeoxycholic acid, GLCA: Glycolithocholic acid, Val: Valine, Thr: Threonine, Lys: Lysine.

Lipidomic profiling

Metabolic changes in serum of fearful dogs were also assessed by FIA-MS/MS using a targeted lipidomic approach. Globally, the platform used allowed the quantification of 523 lipids. The sample variances in case and control groups were visualized by PLS-DA, which showed groups segregation according to the lipidomic features (PC1 10.1%; PC2 3.6%) (Fig. 9A). The hierarchical clustering performed by heatmap analysis highlighted the 40 most significant lipids by T-test, showing higher variance of the quantitative features for the controls than cases (Fig. 9B). Then, volcano plot analysis was performed to select the differentially abundant lipids in the binary comparison case vs control (Fig. 9C). Specifically, 26 lipids were found to be significantly dysregulated in the case group, including 4 down-regulated and 22 up-regulated lipids. The quantitative trends of differential lipids are reported in Fig.9D. Table 3 reports the significant lipids in the order from the less abundant to the most abundant with details of statistics, including P-value, $-\log_{10}(\text{P-value})$, difference, standard error (SE) of difference, and regulation trend.

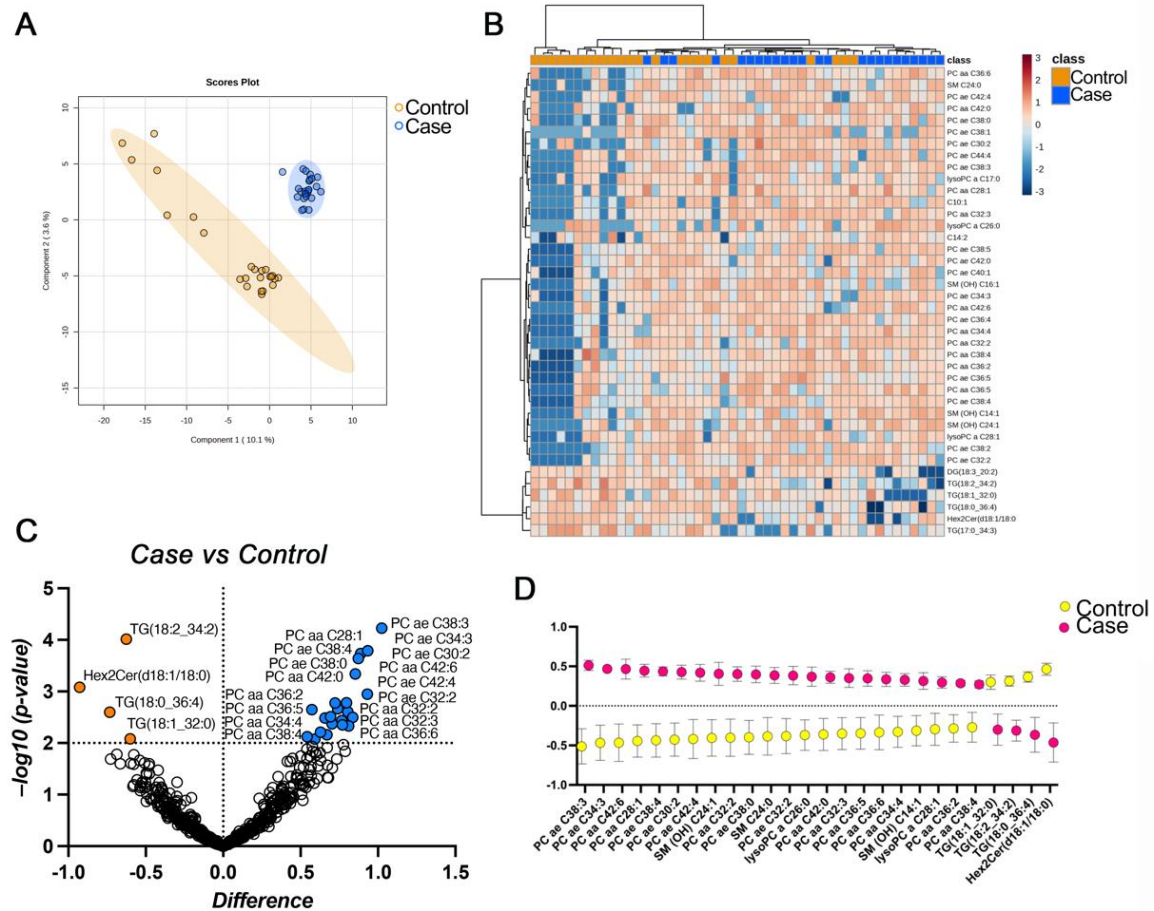


Figure 9. Comparative lipidomic analysis of case (fearful dogs) versus controls. (A) PLS-DA analysis was performed using normalized lipid levels from cases (blue) and controls (orange); (B) Heatmap showing lipid abundance in each group and replicates. The intensity of the colored boxes represents the relative abundance of each molecule, whereas lipid concentrations were normalized, $\log_{10}$ -transformed, and auto-scaled. (C) Volcano plot analysis of significantly different lipids in the comparison case vs control. The blue and orange dots represent the significant increased and decreased lipids, respectively. Non-colored dots refer to all the molecules identified in the dataset whose relative abundance is not significantly different between groups. (D) The plot shows the normalized means with SEM (standard error of the mean) for each of the lipid selected as differentially abundant.

Table 3. Significant lipids found differentially abundant in fearful dogs versus controls.

Lipid	P-value	−log10(P-value)	Difference	SE of difference	Regulation
Hex2Cer(d18:1/18:0)	0.00083	3.081	-0.9267	0.2591	Down
TG(18:0_36:4)	0.002523	2.598	-0.7332	0.2294	Down
TG(18:2_34:2)	0.000096	4.017	-0.6258	0.1465	Down
TG(18:1_32:0)	0.008309	2.08	-0.6002	0.2176	Down
PC aa C38:4	0.007551	2.122	0.542	0.1939	Up
PC aa C36:2	0.002239	2.65	0.5729	0.1769	Up
lysoPC a C28:1	0.008388	2.076	0.5927	0.2152	Up
SM (OH) C14:1	0.006154	2.211	0.6281	0.2187	Up
PC aa C34:4	0.003282	2.484	0.6571	0.2118	Up
PC aa C36:6	0.006936	2.159	0.6701	0.2371	Up
PC aa C36:5	0.003092	2.51	0.694	0.2222	Up
PC aa C32:3	0.004249	2.372	0.7029	0.2336	Up
PC aa C42:0	0.001678	2.775	0.7232	0.2167	Up
lysoPC a C26:0	0.002157	2.666	0.7395	0.2275	Up
PC ae C32:2	0.003678	2.434	0.766	0.2503	Up
SM C24:0	0.004445	2.352	0.7702	0.2574	Up
PC ae C38:0	0.001656	2.781	0.7962	0.2382	Up
PC aa C32:2	0.002473	2.607	0.8051	0.2514	Up
SM (OH) C24:1	0.00465	2.333	0.8101	0.2723	Up
PC ae C42:4	0.003172	2.499	0.8373	0.2689	Up
PC ae C30:2	0.000458	3.339	0.8534	0.2261	Up
PC ae C38:4	0.000229	3.641	0.8723	0.2181	Up
PC aa C28:1	0.000184	3.736	0.8887	0.2184	Up
PC aa C42:6	0.001126	2.949	0.9307	0.2678	Up
PC ae C34:3	0.000162	3.791	0.9337	0.2273	Up
PC ae C38:3	0.00006	4.225	1.024	0.2317	Up

DISCUSSION

Gut bacteria: the good, the bad, the fearful

In the present research, we documented the differential abundance of Proteobacteria and Firmicutes Phyla in family dogs, suffering from generalized fear. Like in humans, companion animals diagnosed with such a behavioral disorder very often experience unreasonable, intense, and even excessive fright towards tangible stimuli or situations. These reactions can eventually trigger immediate responses and concomitant autonomic arousal, turning into escape, avoidance, or defensiveness behaviors (Sherman and Mills, 2008). Despite the huge amount of driving factors, dogs can also display fearful behaviors due to the lack of socialization during their early developmental phase (3-12 weeks of age), aversive experiences, as well as pharmacologic treatments or genetic susceptibility (Seksal, 2008; Tiira and Lohi, 2015; Sarviaho et al., 2019; Craven et al., 2022; Sacchettino et al., 2023). Furthermore, fear can lead to anxiety without the dissolution of fear, meaning that both may coexist in the same individual (Landsberg et al., 2023).

Tailored therapies, which generally rely on integrated approaches (drugs and behavioral treatment), by fostering positive and emotional situations, and avoiding punishment or correction during training activities, are employed to reduce excessive fear or/ and anxiety reactions. Indeed, dogs who suffer from generalized fear and anxiety require to be pharmacologically treated with selective serotonin reuptake inhibitors (SSRIs) or benzodiazepines, that allow them to cope with such a dysfunctional situation. Unfortunately, the owners' resistance, together with occurrence of side effects, including drowsiness, paradoxical arousal, vomiting, diarrhoea, and restlessness, sometimes makes this treatment choice unfeasible. Therefore, more thoughtful research, characterized by the development of new strategies to counter mood-related dysfunction, is a way forward. Compelling and growing evidence in mammals highlighted the crucial role of the microbiota, one of the main physiologic players within the gastrointestinal tract, consisting of trillions of microbes, able to modulate the bidirectional gut-brain axis communication, namely hypothalamic–pituitary–adrenal axis (HPA) and immune system, as well as shape behavior, physiology, metabolic profile, and vulnerability to the onset and severity of CNS diseases (Cryan et al., 2019). Preclinical studies in germ-free (GF) mice documented that the lack of GI microbiota affected both physiology and development of the animals, who exhibited alterations in impaired immune systems, hormone signalling, metabolism, neurotransmission and synaptic plasticity, in specific brain

areas, such as hippocampus and amygdala (Hoban et al., 2018; Cryan et al., 2019). Moreover, GI microbiota-depleted mice, transplanted with the microbiota of aged mice, exhibited deficits in spatial learning memory, without affecting the short-term cognitive ability (D'Amato et al., 2020). Thus, the overall intestinal microbiota has been regarded as one of the main peripheral modulators of CNS physiology, either by synapsing with vagus nerve, or passing through the blood brain barrier (BBB). The composition and integrity of the BBB can be affected by a dysfunctional GI microbiota, that makes it more permeable, most likely because of the reduced expression of tight junction proteins, involved in the regulation of barrier function in endothelial tissues (Braniste et al., 2014). This, in turn, allows the access of neurotoxins within the brain, thus affecting the release of transmitters, like serotonin, dopamine, GABA, and eventually bringing about mood-related disorders (de Punder and Pruimboom, 2015; Luo et al., 2018; Winter et al., 2018; Peirce and Alvina, 2019). Human and canine GI microbiota generally share the same microbial milieu, made up of Firmicutes, Fusobacteria, Bacteroidetes, as well as the less abundant Actinobacteria and Proteobacteria phyla (Coelho et al., 2018). All of them populate the GI tract and provide essential vitamins, intestinal maturation, and development of the immune system, thereby having the ability to protect against pathogens and infections over time. Dysregulation of the GI microbiota, characterized by a relative increment of Proteobacteria, has been documented in metabolic and inflammatory disorders, so that these gram-negative microorganisms and their related taxa can be regarded as potential markers of microbiota instability (De Filippo et al., 2010; Rizzatti et al., 2017). The differential and quantitative abundance analysis, based on the EdgeR algorithm, arising from the relative abundance evaluation, allowed us to find a significant alteration of Proteobacteria-related *Gammaproteobacteria*, which were less abundant in fearful patients. On the other hand, we documented that Firmicutes-belonging taxa, including *Erysipelatoclostridiaceae* and *Peptostreptococcales Tissierellales* dominated GI tract of the case group, who also displayed reduced levels of *Dorea* bacteria. In the present research we involved patients from different locations in Italy and fed a commercial diet (similar composition), thus trying to reduce the number of intervening variables on altered behavior, such as diet and environment. Although further quantitative and more detailed studies are mandatory, we hypothesize that the lower abundance of the *Gammaproteobacteria* in fearful dogs might be linked to the pathology. In a recent study from Brunetto and colleagues, the authors found a reduction in Proteobacteria density of healthy adult dogs, who were fed with the purified yeast-derived beta-1,3/1,6-

glucans, which are well-known to stimulate both innate and adaptive immunity, supporting the optimal functioning of gut-associated lymphoid tissue and strengthening the concept of considering these microbes as metabolic or inflammatory markers in canine gut (Marchi et al., 2024). In this line, the lower amount of the Proteobacteria-related taxon found in fearful dogs might be due to a compensatory effect, which comes into play, to avoid the worsening of behavioral symptoms. On the other hand, in line with what observed in humans, showing that Proteobacteria were more abundant in males than females (Li et al., 2008), a potential gender effect underlying the reduction of *Gammaproteobacteria* population in the case dog group might be considered, since many of our healthy controls were males, compared to the most representative female patients. The higher incidence of female dogs suffering from fear and anxiety might be in accordance with data obtained in humans, showing that women are likely to develop anxiety-related disorders up to two times more than males (McLean et al., 2011). Despite the absence of functional connections between Proteobacteria and gut-brain homeostasis, our data may open the way towards further investigations, gathering a wider and heterogeneous sample size, aimed at addressing the potential role of such microorganisms in mood-related disturbances, to rule out any potential confounders between experimental groups.

Firmicutes increased in fearful dogs

Our results documented an increase of Firmicutes-related taxa in fearful dogs, and they are in line with previous findings in shelter as well as working dogs, with higher cowardice scores, which revealed an increase of the Firmicutes-related *Lactobacilli* in the gut of the fearful animals (Mondo et al., 2020a; Craddock et al., 2022). Again, these aerotolerant anaerobic microbes, that naturally inhabit the GI tract of humans and animals, were still increased in rescued dogs, who experienced intraspecific aggression (Klein et al., 1998; Kirchoff et al., 2019). Studies from Mondo and colleagues (2020), showed that *Lactobacilli* were significantly higher in a group of German shepherds, who displayed a similar GI microbiota to that of depressed humans (Mondo et al., 2020b), or those patients who underwent vagotomy at an early age, to decrease risk of certain neurologic disorders, thus identifying the vagus nerve as a major modulatory constitutive communication pathway between the bacteria exposed to the gut and the brain (Svensson et al., 2015). *Lactobacillus Rhamnosus* was also found to reduce stress-associated corticosterone levels and anxiety-related behaviors in a mouse, together with an alteration of GABA-dependent neurotransmission (Bravo et al., 2011). Taken together, it is

conceivable that *Bacilli* could modulate several aspects of the microbiome-gut-brain axis, although care must be taken in extrapolating data, since identifying the precise mechanism underlying the observed increase of *Lactobacillus* in fearful dogs is not always immediate. Some anaerobic microbes belonging to Bacteroidetes and Firmicutes phyla generate butyrate, propionate and acetate as by-products of the indigestible polysaccharides' fermentation. These short-chain fatty acids (SCFAs), once crossing BBB, can affect cognition and mood-related behaviors (Luca et al., 2020). According to our data about a significant reduction of *Dorea* taxon in fearful dogs, lower levels of this Firmicutes-belonging genus were also previously found in human and animal with behavioral disorders (Shen et al., 2018; Kirchoff et al., 2019; Homer et al., 2023). Previous studies documented that, together with *Akkermansia* and *Ruminococcus*, *Dorea* correlated with anxiety- and depressive-like behavior, in both CD-1 and C57BL/6 mice, exposed to social defeat stress protocol, thus suggesting a potential role for such an obligate-anaerobic Gram-positive bacteria in the modulation of the gut-brain axis and behavior (McGaughey et al., 2019; Craddock et al., 2022). Anaerobic bacteria use specific amino acids that can be metabolized to acetate, or used for SCFAs production (Silva et al., 2020). In this respect, we found a direct correlation between gut *Dorea* abundance and serum levels of valine, threonine and lysine metabolites. The higher levels of the Firmicutes-related taxa found in patients well fit with serum levels of lactic acid in fearful dogs, since they produce lactic acid through fermentation of glucose, resulting in acidification of environment that can inhibit the growth of some pathogenic microorganisms (Wilson et al., 2010). It is well recognized that Firmicutes-related species are involved in the conversion process of primary to secondary bile acids (and then deconjugating all the glyco- and tauro-conjugated forms) (Jones et al., 2008; Ridlon and Bajaj, 2015), thereby resulting in an antimicrobial function, which is more potent than primary cholic acid, because of its ability to damage microbial membranes. Of note, we also found that *Peptostreptococcales Tissierellales* positively correlated with TDCA, GUDCA and GLCA metabolites, thus pointing towards multidisciplinary approaches, aimed at developing new pharmacological targets for the treatment of canine mood-related disorders.

Glutamine, glycine and α -aminoadipic acid decrease in fearful dogs

The multifunctional amino acid glutamine (Gln) is a precursor of neurotransmitter amino acids—a major excitatory neurotransmitter, glutamate (Glu), and an inhibitory neurotransmitter, γ -aminobutyric acid (GABA)—and pivotal for normal brain energy metabolism and anti-inflammatory processes (Baek et al., 2024). Gln is the most abundant non-essential amino acid within the CNS, where it is synthesized by astrocytes under normal conditions required for numerous critical biological processes. Most of the free glutamine is metabolized by the liver, kidneys, immune system and intestines, while most of the circulating glutamine comes from skeletal muscle (Baek et al., 2024).

In the brain, as part of the glutamate-glutamine cycle (Schousboe, 2019), glutamine maintains normal extracellular levels of glutamate and removes excess ammonia, which is formed as a byproduct of the glutamate-glutamine cycle, in the circulation (Cooper, 2001). Since glutamate is the most common and important excitatory neurotransmitter in the CNS, adequate activity and function of this cycle are necessary for a proper glutaminergic neurotransmission (Bergink et al., 2004).

It is not surprising that a wide range of mental disorders have been associated with disruptions in the glutamate-glutamine cycle, emphasizing the importance of adequate glutamine levels in a variety of physiological and pathological contexts (He et al., 2012; Filiou et al., 2014; Jelen et al., 2018). In a previous study by Puruunen and colleagues (Puruunen et al., 2018) fearful dogs showed an increase in plasma glutamine levels, which is not in line with our results showing downregulation of circulating glutamine. This discrepancy could be due to the different sampling enrolment. Accordingly, they studied Great Danes and German shepherd dogs, while our experimental cohort was more heterogeneous, consisting of mixed breeds and mongrel. In addition, plasma levels of glutamine (and amino acids, in general) can be influenced by various factors, such as sex, age and nutritional status. Indeed, the dogs involved in our study had an overlapping diet, which differed from that indicated in the Puruunen's study (Royal Canine Maxi Sensible).

These controversial findings also emerge in human patients with emotional and cognitive disorders, likely due to the great heterogeneity in disease stage, medications, regions of interest (brain or blood), and processing methods (Baek et al., 2024). However, recent meta-analysis reviews estimate that the amount of glutamine decreases in the blood or brain of patients with depression (Moriguchi et al., 2019), under conditions of chronic stress, inflammation, and

oxidative stress (Baek et al., 2024) or schizophrenia (Madeira et al., 2018), whereas an increase of such amino acid was reported in the brains of patients with bipolar disorder (Ino et al., 2023). We also found a significant reduction of serum α -Aminoadipic acid (AAA) levels. In animal systems, AAA is an intermediary metabolite of lysine and of tryptophan, able to antagonize neuroexcitatory activity modulated by the glutamate N-methyl-D-aspartate receptor (NMDAR) (Haugstad and Langmoen, 1997). In this respect, previous findings reported that, when injected in both pre-limbic cortex and hippocampus, it brought about depressive-like behaviors in rodents, arguing that the lack of a system able to monitor the homeostasis of the NMDAR-dependent transmission might be associated with mood-related disorders in companion animals, as well as in humans (Nasir et al., 2020).

Metabolomic results highlighted a down regulation of the amino acid glycine, which has been regarded as one of the anti-stress molecules (Shishkova et al., 2022), since low serum glycine levels have been correlated with several psychiatric disorders in humans (Zheng et al., 2012;Gagliano et al., 2022), and altered metabolism of this amino acid has also been described in the metabolomic profile of anxious rats (Zhang et al., 2011). Therefore, although this molecule has never been studied extensively in canine behavior, it does not appear surprising that the fearful dogs sampled in our study expressed significantly lower levels of this circulating amino acid.

Further studies in veterinary medicine will help to better reveal the mechanisms underlying these variations in amino acids concentration in a larger cohort of patients.

Fearful dogs exhibit increased phosphatidylcholine levels

Our results showed differentially abundant lipids (n = 26) in fearful dogs, of which 17 are phosphatidylcholine, implying that disorders of lipid metabolism may be related to fear and anxiety in dogs.

Although current hypotheses on the biological background of anxiety disorders assume dysfunctions in neurotransmitter systems and peptides, lipids nevertheless play an essential role as components of lipid membranes, determining the location and function of various receptors. Indeed, dysregulation of lipid homeostasis may contribute to the pathogenesis of major depression and anxiety (Muller et al., 2015); in fact, it has been shown that alteration of phosphatidylcholine (PC) was involved in major depressive disorder patients' plasma (Huang et al., 2021), and that plasma phosphatidylcholine, together with sphingomyelin, levels change in patients with depression (Demirkan et al., 2013). In line with our findings, previous research about major depressive disorder showed that bile acid metabolites and PCs were significantly increased in patients. Of note, PCs are being used as a diagnostic tool for depression, thus helping in a more accurate clinical evaluation of such psychiatric disorder in humans (Wang et al., 2023).

Our results are also in accord with the study of Berkecz and colleagues, which showed significant increase in the plasma concentration of lipid classes (PI, LPE, SM and PC) in the anxious mice (Berkecz et al., 2018). In particular, they pointed out that 33/35 glycerophospholipid species were up-regulated in high anxiety-related behavior group, compared to low anxiety-related one, further suggesting that plasma lipidome profiling may yield a useful biomarker in mood disorders. The altered lipidomic pattern found in our patients was independent on the diet (Dougherty et al., 1987), since case and control groups had similar nutritional plan.

This suggests an endogenous cause, i.e. altered absorption of dietary lipids or altered lipid metabolism, for the pathways involved in anxious dogs. Such an interpretation is shared by Finnish colleagues (Puurunen et al., 2016), who reported a decrease of several phospholipids in fearful Great Danes, along with an increase of plasmalogen, one of relevant free radical scavengers. Interestingly, these dogs showed a significant increase in two biomarkers of oxidative stress, hypoxanthine and indoxylsulfate, calling into question the role of neuroinflammation and oxidative stress in neuropsychiatric disorders in humans, in dogs and animal model (Rammal et al., 2008; Bauer and Teixeira, 2021; Piotti et al., 2024; Zabet et al.,

2024). Although our results are in contrast with the Puruunen's study, some interpretations could justify these different data. To begin with, we analyzed different matrices; we used serum, while they employed whole blood that, as they stated, might cause more background and eventual problems in downstream analysis, straining experimental settings, including sample processing.

In addition, we used a targeted technique, while Puruunen focused on a non-targeted approach, and this may have accounted for the different results. The targeted technique has high sensitivity and specificity, allowing us to accurately quantify a limited number of predefined metabolites, thus increasing the sensitivity and specificity of the analysis. However, the targeted technique has the disadvantage of providing a limited image of the metabolic profile compared with the nontargeted one, thus not allowing overall metabolic alterations to be identified (Ribbenstedt et al., 2018).

Furthermore, there could be the influence of diet in that, although the two groups of dogs involved in our study had an overlapping dry diet, it differed from that administered in Puruunen's study (the diets contained equally a mix of raw food, commercial dry foods, homemade food and different dietary supplements in both test groups), resulting in a different impact on phospholipid concentration. Moreover, this discrepancy can be attributed to the different cohort of dogs, since they enrolled pure-bred Great Danes, compared to the mixed breed/mongrel subjects of our study, thus allowing to not exclude the breed (genetic) effect on lipid metabolism.

We can hypothesise that the link between phosphatidylcholines and anxiety might be indirect and multifactorial. Dysregulation in the cell membrane composition, due to phosphatidylcholine alterations, may influence the transmission of nerve signals and contribute to the development of mood-related disorders, such as anxiety. On the other hand, phosphatidylcholines could help to reduce anxiety by exerting a protective action on neurons and counteracting the negative effects of stress. In this perspective, the different disease stages (acute vs. chronic) should be investigated, since phospholipids might have a protective role at an early phase, where a compensatory activity to achieve a new homeostatic balance may occur during a late disease phase.

CONCLUSIONS

At present, despite the difficulty in claiming whether gut and metabolic dysfunctions come first behavioral changes or vice versa, our data emphasized for the first time how fear (and anxiety) might be linked to significant dysregulation in both GI microbiota and blood metabolic homeostasis, in family dogs suffering from generalized fear. Despite the significant recent advances in veterinary behavioural medicine, the link between behavioral disorders and the gut microbiome, as well as the potential benefits of new treatments (i.e. probiotics, diet, fecal microbiota transplantation) in this circumstance, needs to be further elucidated and proven on a larger scale. We have only scratched the surface on both lipidomic and metabolomic profiling in fearful dogs, thereby further studies in this research field are needed, to better investigate canine behavioural disorders.

In conclusion, elucidating more physiological underpinnings of mood-related dysfunctions in dogs, by means of multi-omics approach, might represent a worth employing task in the veterinary practice.

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