UAB
Universitat Autònoma de Barcelona

THE MICROBIOTA–GUT–BRAIN AXIS IN DOGS, AND ITS RELATIONSHIP WITH AGING

Anna Fernández Pinteño

UAB
Universitat Autònoma de Barcelona

THE MICROBIOTA-GUT-BRAIN AXIS IN DOGS, AND ITS RELATIONSHIP WITH AGING

DISSERTATION TO OBTAIN THE DEGREE OF DOCTOR BY:

Anna Fernández Pinteño

UNDER THE SUPERVISION OF:

Dr. Xavier Manteca Vilanova

Dr. Anna Salas Mani

PRESENTED TO THE DEPARTMENT OF ANIMAL AND FOOD SCIENCE, FACULTY OF VETERINARY MEDICINE, UNIVERSITAT AUTÒNOMA DE BARCELONA

Bellaterra, July 2025

FACULTAT DE VETERINÀRIA

El Dr. Xavier Manteca Vilanova, catedràtic del departament de Ciència Animal i dels Aliments i la Dra. Anna Salas Mani, investigadora a l'empresa Affinity Petcare.

Certifiquen:

Que la memòria titulada 'The microbiota-gut-brain axis in dogs, and its relationship with aging' presentada per Anna Fernández Pinteño amb la finalitat d'optar al grau de doctora en Veterinària, ha estat realitzada sota la seva direcció i, considerant-la acabada, autoritzen la seva presentació perquè sigui jutjada per la comissió corresponent.

I perquè consti als efectes oportuns, signen la present a Bellaterra, en la data indicada:

Dr. Xavier Manteca Vilanova

Dra. Anna Salas Mani

“Until one has loved an animal, a part of one’s soul remains unawakened”

Anatole France

AGRAÏMENTS

Tinc molt clar que aquest treball no hauria estat possible sense el suport de totes les *persones* que m'han acompanyat. Cada conversa, cada gest i cada moment compartit ha contribuït, d'una manera o altra, a fer realitat una de les meves grans il·lusions: finalitzar un doctorat i contribuir, així, a la generació de coneixement en l'àmbit de la ciència i del benestar animal.

En l'àmbit acadèmic i professional, vull agrair molt sincerament als meus directors, en *Xavi* i l'*Anna*, la confiança i l'acompanyament durant aquesta tesi. Gràcies per haver-me guiat i pel vostre suport, especialment en els moments en què el camí s'ha fet més incert.

També vull expressar el meu agraïment als *companys i companyes d'equip*, així com als *gossos* que han participat en aquests estudis i que, d'una manera o altra, m'han permès avançar en aquest camí. La contribució d'*Affinity* en aquesta tesi ha estat clau, tant per la promoció dels estudis que es presenten, com per la meva formació durant aquests darrers quatre anys.

A nivell personal, voldria aprofitar aquesta oportunitat per expressar el meu agraïment més sincer a totes aquelles persones que han estat al meu costat i han contribuït de forma constant al meu creixement, a totes sense específicar tots els noms, molt agraïda. No es tracta només del doctorat, sinó de totes les mostres de suport que he rebut al llarg del temps i que, sense cap dubte, han fet possible assolir aquesta fita tan significativa per a mi. Específicament:

Papa, Mama, gràcies pel vostre suport incondicional; gracias por estar siempre a mi lado en todas aquellas decisiones que me han hecho y me hacen feliz. Sense cap dubte, vosaltres em vau transmetre el respecte i l'estima per la natura i els animals, així com les ganes incansables de conèixer i aprendre. Gracias infinitas.

Daniel, Ale, Paula, gràcies per ser-hi, i sobretot, gràcies per fer millor el meu present.

Mireia, Marina, gràcies per ajudar-me a seguir endavant en els moments més complicats, per fer-me sortir de la meva rutina i per compartir les vostres

experiències i perspectives. Sense cap dubte, totes dues, de manera diferent i especial, heu enriquit aquest viatge.

Miriam, Laia, Elis, Cris, Aida, gràcies per seguir-hi. Malgrat la distància, continuem construint cada dia allò que va començar quan encara no sabíem què volíem ser de grans.

Ana, gràcies per fer-me replantejar tot allò que em semblava “normal” i per fer-me qüestionar sempre el meu present. Sense saber-ho, era just el que necessitava.

Livia, gracias por estar y por acompañarme, junto con *Shey*, en esta etapa tan importante de mi vida. Infinitas gracias por darme siempre una perspectiva tan distinta, por entenderme, y sobre todo, por ayudarme en el complicado camino de relativizar.

Iaia, tito, tita, aunque no podáis leer estas líneas, allí donde estéis, gracias por todo lo que me enseñasteis. Sin duda, vuestras pérdidas han hecho este camino más difícil. Aun así, recuerdo cada día con mucho cariño el interés que mostrasteis siempre por todo lo que hacía. Gracias por tanto.

Finalment, *Scoffy*, aquest doctorat també és teu, molt teu. Gràcies per escoltar-me i per donar-me, i donar-nos, tant. No tinc cap dubte que el vincle que vam construir serà per sempre especial i irrepetible.

TABLE OF CONTENTS

ABSTRACT

Advances in veterinary medicine, preventive healthcare strategies, and increasingly personalized nutrition have contributed to extending the life span of companion animals, particularly dogs. In order to ensure not only greater longevity but also good quality of life during the aging process, it is essential to understand the underlying biological factors and mechanisms. Among these, intestinal health is emerging as a key factor, as the gastrointestinal tract is involved not only in digestion and nutrient absorption but also plays essential roles in immune modulation, metabolic regulation, and neuroendocrine signaling. These integrative functions are strongly influenced by the gut microbiota, a dynamic microbial community that changes throughout the host's life and interacts continuously with its internal and external environment. The connection between the gut microbiota and the central nervous system, known as the gut–brain axis, involves neural, hormonal, and immunological pathways that allow the gut to influence cognitive, emotional, and behavioral functions. In dogs, preliminary findings suggest that shifts in microbial composition may be associated with behavioral alterations, anxiety, or cognitive decline. However, the study of this axis, particularly in the context of aging, remains in its early stages, and more specific integrative research approaches are needed to explore the involvement of different physiological systems.

This thesis addresses this knowledge gap by evaluating indicators of intestinal health, behavior, and activity in dogs of different ages. The first study, conducted in a population of mixed-breed dogs, revealed moderate changes in selected gut health markers, particularly in microbial composition and the production of certain short-chain fatty acids. The second study, conducted in Beagle dogs, examined the relationship between intestinal health, neuroendocrine and behavioral variables, and age. In this case, age-related differences were observed in microbial composition and specific gut-derived metabolites, along with changes in behavioral patterns and activity levels.

Taken together, the results suggest that although age-associated changes in gut and behavioral parameters tend to be mild, their interactions may have relevant biological

implications. These findings may contribute to the development of tailored nutritional strategies aimed at supporting healthy aging in dogs.

RESUM

Els avenços en medicina veterinària, les estratègies de medicina preventiva i una nutrició cada vegada més personalitzada han contribuït a augmentar l'esperança de vida dels animals de companyia, i més específicament dels gossos. Per tal de garantir no només una major longevitat, sinó també una bona qualitat de vida durant el procés d'envelliment, és important conèixer els factors i mecanismes subjacents. Entre els factors biològics, la salut intestinal està emergint com un element clau, ja que el tracte gastrointestinal no només participa en la digestió i absorció de nutrients, sinó que també exerceix funcions essencials en la modulació immunitària, l'equilibri metabòlic i la senyalització neuroendocrina. Aquestes funcions estan fortament influenciades per la microbiota intestinal, una comunitat microbiana dinàmica que evoluciona al llarg de la vida de l'hoste i interactua de manera contínua amb el seu entorn intern i extern. La connexió entre la microbiota intestinal i el sistema nerviós central, coneguda com a eix intestí-cervell, implica vies neuronals, hormonals i immunològiques que permeten que l'intestí influenciï funcions cognitives, emocionals i conductuals. En gossos, alguns resultats preliminars suggereixen que els canvis en la composició de la microbiota podrien estar relacionats amb alteracions del comportament, ansietat o deteriorament cognitiu. Tanmateix, l'estudi d'aquest eix, especialment en el context de l'envelliment, encara es troba en fases inicials, i calen estudis més específics i alhora integradors dels diferents sistemes fisiològics.

Aquesta tesi aborda aquesta manca de coneixement mitjançant l'avaluació d'indicadors de salut intestinal, comportament i activitat en gossos de diferents edats. En el primer estudi, realitzat en una població de gossos de races diverses, es van observar canvis moderats en alguns marcadors intestinals, en particular en la composició microbiana i en la producció d'alguns àcids grassos de cadena curta. El segon estudi, realitzat amb gossos Beagle, va explorar la relació entre la salut intestinal, les variables neuroendocrines i conductuals, i l'edat. En aquest cas, es van identificar diferències subtils associades a l'edat en la microbiota, en alguns metabòlits intestinals derivats de l'activitat microbiana, així com en determinats comportaments i en el nivell d'activitat dels animals.

En conjunt, els resultats suggereixen que, tot i que els canvis associats a l'edat solen ser lleus, les seves interaccions poden tenir implicacions biològiques rellevants. Aquests resultats podrien contribuir al desenvolupament d'estratègies nutricionals adaptades que afavoreixin un envelliment saludable en els gossos.

RESUMEN

Los avances en medicina veterinaria, las estrategias de medicina preventiva y la nutrición cada vez más personalizada han contribuido a aumentar la esperanza de vida de los animales de compañía, y más específicamente de los perros. Para garantizar no solo una mayor longevidad en los animales de compañía, sino también una buena calidad de vida durante el proceso de envejecimiento es importante conocer los factores y mecanismos subyacentes. Entre los factores biológicos, la salud intestinal está emergiendo como un elemento clave, dado que el tracto gastrointestinal no solo participa en la digestión y absorción de nutrientes, sino que también desempeña funciones esenciales en la modulación inmunitaria, el equilibrio metabólico y la señalización neuroendocrina. Estas funciones están fuertemente influenciadas por la microbiota intestinal, una comunidad microbiana dinámica que cambia a lo largo de la vida del hospedador e interactúa de forma continua con su entorno. La conexión entre la microbiota intestinal y el sistema nervioso central, conocida como el eje intestino-cerebro, implica vías neuronales, hormonales e inmunológicas que permiten al intestino influir en funciones cognitivas, emocionales y conductuales. En perros, algunos hallazgos preliminares sugieren que los cambios en la microbiota podrían relacionarse con alteraciones del comportamiento, ansiedad o deterioro cognitivo. No obstante, el estudio de este eje, especialmente en el contexto del envejecimiento, aún se encuentra en etapas iniciales y se requieren estudios más específicos y a la vez integradores de los distintos sistemas.

Esta tesis aborda dicha laguna mediante la evaluación de indicadores de salud intestinal, comportamiento y actividad en perros de distintas edades. En el primer estudio, realizado en una población de perros de distintas razas, se observaron cambios moderados en algunos marcadores intestinales, particularmente en la composición microbiana y en la producción de algunos ácidos grasos de cadena corta. El segundo estudio, realizado en perros Beagle, exploró la relación entre salud intestinal y variables neuroendocrinas y conductuales y la edad. En este caso, se identificaron diferencias sutiles asociadas a la edad en la microbiota, algunos de los metabolitos intestinales derivados de la actividad microbiana, y en ciertas conductas y en el nivel de actividad de los animales.

En conjunto, los resultados sugieren que, aunque los cambios asociados a la edad suelen ser leves, sus interacciones pueden tener implicaciones biológicas relevantes. Estos hallazgos podrían contribuir al desarrollo de estrategias nutricionales adaptadas que favorezcan un envejecimiento saludable en los perros.

INTRODUCTION

1. GENERAL INTRODUCTION OF THE THESIS

As companion dogs increasingly reach advanced ages due to improvements in veterinary care, preventive medicine, and nutrition, understanding the biological factors that contribute to healthy aging has become a growing priority in veterinary science. Among these, intestinal health is emerging as a central determinant of overall physiological resilience. The gastrointestinal tract is not only responsible for digestion and nutrient absorption, but also plays a critical role in modulating immune responses, maintaining metabolic homeostasis, and supporting neuroendocrine signaling [1]. These integrative functions are strongly influenced by the gut microbiota, a dynamic microbial community that evolves throughout the life of the host and interacts continuously with its internal and external environment.

In dogs, recent studies have shown that the gut microbiota plays a key role in health and disease, particularly through its impact on the gastrointestinal system [2,3]. As dogs age, shifts in microbial diversity and function have been associated with increased intestinal permeability, chronic low-grade inflammation, and decreased metabolic flexibility [4,5]. These changes may contribute to frailty and other age-related health issues, although the causal mechanisms remain largely undefined.

A growing body of research has also begun to examine the influence of the gut microbiota on the central nervous system via the gut–brain axis [6]. This bidirectional communication system involves neural, hormonal, and immunological pathways that allow the gut to influence cognitive, emotional, and behavioral functions. In canine models, preliminary findings suggest that changes in microbiota composition may be linked to behavioral alterations, anxiety, and cognitive decline [7,8]. However, the study of the gut–brain axis in dogs, particularly in the context of aging, is still in its early stages, and there is a notable lack of integrative studies addressing this interaction.

The present thesis addresses this gap by investigating the relationship between gut microbiota composition, biomarkers of intestinal health, behavioral biomarkers, and aging in dogs. It aims to explore how these factors interact across different life stages, with particular emphasis on the later stages of life. By identifying patterns and associations that characterize intestinal health and microbial balance in older dogs, and by analyzing behavior-related biomarkers, this work seeks to contribute to the development of targeted nutritional or therapeutic strategies to promote healthy aging and improve the quality of life in senior canine populations.

2. THE MULTIFACTORIAL NATURE OF AGING AND KEY DRIVERS

Aging is a complex biological process that spans across all living organisms, beginning at birth and continuing throughout the lifespan. It is a multifactorial phenomenon, influenced by a multitude of genetic, environmental, and lifestyle factors, which collectively contribute to the gradual decline in an organism's ability to maintain homeostasis in the presence of internal and external stressors [9,10]. While aging manifests across various biological systems, it is marked by a complex interaction between molecular, cellular, and physiological changes. These changes unfold at multiple levels of organization, starting with biochemical alterations at the molecular level, which cascade through cellular structures, tissues, and organs, ultimately culminating in the observable signs of aging. As organisms age, the efficiency of their physiological responses diminishes, leading to a reduced capacity to adapt to stress, as well as a decline in metabolic, immune, and hormonal functions [11]. Behavioral flexibility also diminishes with age, making it more difficult to adapt to new situations or acquire new skills. Additionally, older organisms face extended recovery periods, which underscores their heightened vulnerability to diseases [12,13].

In the context of aging, these changes are not only limited to physiological functions but also extend to behavioral aspects, with older organisms exhibiting increased susceptibility to stress, altered activity patterns, and reduced responsiveness to stimuli [14,15]. The study of the aging process in dogs has received growing interest, as dogs have proven to be a valuable model for investigating human health span.

Furthermore, the human-animal bond has gained increasing importance in recent years, underscoring the need to better understand the aging process in dogs in order to support healthy aging. Despite this, research on molecular age-related changes in dogs remains limited, particularly when examining individuals at different developmental stages [16]. While some progress has been made in understanding the physiological changes associated with aging in dogs, relatively few studies have focused on identifying molecular markers that could support the early detection and monitoring of age-related alterations. Recent research highlights the need for the identification of biomarkers to track physiological aging and distinguish it from behavioral aging in dogs [17].

Aging is associated with a variety of observable phenotypic changes, such as a whitening of the coat, reduced sensory functions, and general lethargy [18]. Moreover, aging in dogs is characterized by profound alterations in crucial organ systems, including the kidneys, liver, and heart, and often results in a heightened vulnerability to chronic conditions like kidney disease and heart failure [19,20]. This physiological decline is compounded by dehydration, which exacerbates organ dysfunction and accelerates disease progression [21–23]. Beyond these physiological changes, environmental factors such as stress, exercise and nutrition have been shown to influence the aging process [24–27]. The role of these environmental factors is particularly important as they interact with biological pathways associated with inflammation and oxidative stress, both of which are key factors in the aging process [28].

One of the critical components of aging that has gained attention in recent years is the concept of dysbiosis, defined as an imbalance in the gut microbiota indicative of a reduction of microbial diversity and a loss of functional capacity [29,30]. Dysbiosis has been linked to a range of age-related diseases, such as gastrointestinal disorders, autoimmune diseases, and neurodegenerative diseases like Parkinson’s disease in humans [31,32]. The gut microbiota plays an essential role in maintaining intestinal barrier integrity and modulating immune responses, so any disruption in its composition can lead to systemic inflammation and impaired immune function [5,33]. As such, dysbiosis and the understanding of the microbiota composition and function represent an evolving area of interest in aging research, as they have the potential to

exacerbate the physical and functional decline observed in aging organisms. It is becoming increasingly clear that maintaining gut health is a crucial component in supporting health in dogs and other species [2,34–37].

3. INTESTINAL HEALTH

Gut health in dogs refers to the optimal function of the gastrointestinal tract, which encompasses efficient digestion and absorption, a stable and diverse gut microbiota, effective mucosal immunity, and the absence of gastrointestinal pathology [4,38]. In veterinary medicine, this concept also includes the animal's ability to maintain gastrointestinal homeostasis under various physiological and environmental stressors [39].

3.1. Factors influencing intestinal health

Preserving gastrointestinal health in dogs involves a complex interplay of environmental, nutritional, physiological, and microbial factors. Central to this balance is the composition and function of the intestinal microbiota, which interacts dynamically with host immunity, epithelial integrity, and metabolic activity [38]. Dietary composition plays a pivotal role in shaping the microbial ecosystem. Diets rich in indigestible fibers and prebiotic compounds (e.g. inulin, fructooligosaccharides, or certain plant-derived polysaccharides) can promote the proliferation of beneficial microbial taxa, including *Lactobacillus* and *Bifidobacterium spp*., and stimulate the production of SCFAs, which support mucosal energy metabolism and anti-inflammatory signaling [40,41].

In addition to diet, probiotic supplementation has been widely studied for its ability to stabilize the intestinal microbiota and modulate immune responses, particularly during stress or recovery from gastrointestinal disturbances. Probiotic strains such as *Enterococcus faecium* or *Lactobacillus acidophilus* have been shown to enhance local IgA production, restore microbial diversity after antibiotic use, and improve clinical outcomes in dogs with chronic enteropathies [7,42].

Beyond nutrition and biotics supplementation, non-dietary factors also influence gut health in dogs. Stress can alter gastrointestinal motility, secretory patterns, and microbial balance via the hypothalamic–pituitary–adrenal (HPA) axis, leading to increased intestinal permeability and inflammation [43]. Finally, other host-specific factors such as genetics and age can determine how the gut responds to challenges [44–46].

3.2. Age-related changes in intestinal health

Aging has been associated with a range of physiological and immunological changes affecting gastrointestinal health, potentially predisposing individuals to chronic conditions. These alterations may include reduced gastrointestinal motility, a decline in mucosal immunity, and impaired epithelial barrier function [47]. Age-related structural changes in the gastrointestinal tract, such as reduced villus height and crypt depth, may alter nutrient absorption and further compromise gut integrity [5]. Thus, these factors can collectively lead to increased intestinal permeability, allowing microbial antigens to translocate and trigger low-grade systemic inflammation [48]. This chronic, subclinical inflammatory state, referred to as inflammaging, is a hallmark of the aging process and has been linked to the gradual activation of the innate immune pathways by microbial and metabolic stimuli [49]. Additionally, studies in senior dogs have demonstrated a shift in gut-associated immune function, including decreased production of secretory immunoglobulin A (sIgA) and a diminished capacity for mucosal repair [48].

4. THE GUT MICROBIOTA

The microbiota is a vast and diverse community of microorganisms that inhabit the host, including bacteria, viruses, protozoa, fungi, and archaea [36,50]. Different anatomical sites within the host support specific microbial communities, with the gastrointestinal tract harboring the most abundant and diverse population. The gut microbiota comprises an estimated 10^{13} to 10^{14} microbial cells, encompassing more than 1,000 bacterial species. Its collective genome, the gut microbiome, is approximately 150 times larger than that of the host in most animal species [7],

including humans, where it contains over 100 times more genetic material than the human genome [51,52].

Due to this extensive genetic repertoire, the gut microbiota plays a central role in regulating numerous physiological processes. In both humans and other mammals, including dogs, these microbial communities contribute to host metabolism by aiding in the digestion of complex carbohydrates and the production of short-chain fatty acids (SCFAs), which modulate energy balance, glucose metabolism, and lipid regulation [3,53]. The microbiota also supports immune system development, contributes to mucosal immunity, and protects against pathogens. In dogs, the gut microbiome has been shown to influence immune tolerance to commensal bacteria and is implicated in the pathogenesis of conditions such as inflammatory bowel disease (IBD), allergies, and metabolic disorders [2,42,48].
Maintaining a compositionally and functionally balanced microbial ecosystem is thus essential for host health and physiological homeostasis. Disruptions in microbial community structure or function, commonly referred to as dysbiosis, may impair nutrient assimilation, compromise intestinal barrier integrity, and contribute to the development of various pathological conditions, including chronic inflammation, metabolic syndromes, and neurobehavioral disorders [54,55].

The gut microbiota interacts closely with the gastrointestinal tract through several mechanisms: it enhances nutrient absorption and fermentative capacity; it contributes to the structural maturation of the gut by promoting mucus formation and mucosal enzymatic activity; it serves as a barrier against pathogenic bacteria through competitive exclusion and antimicrobial compound production; and it is involved in the synthesis of essential vitamins, such as B-group vitamins. These functions have been described in both human and canine studies, underlining the conserved nature of these host-microbe interactions [2,3].

4.1. Factors influencing the gut microbiota

An increasing number of studies are investigating the multiple factors that influence the structure and function of the gut microbiota, given its essential role in host metabolism, immunity, and neuroendocrine signaling [56–58]. These factors are

generally categorized as extrinsic, including diet, antibiotic use, and environmental exposures, and intrinsic, such as host genetics, immune status, and age. Pathological conditions, notably chronic inflammation, metabolic syndrome, and type 2 diabetes, can also drive significant alterations in gut microbial communities, often resulting in dysbiosis [29,30]. Among these factors, age is a key determinant in shaping the gut microbiota throughout life [46].

4.2. Age-related changes in the gut microbiota

Aging is associated with reductions in microbial diversity and shifts in dominant taxa, which may compromise nutrient absorption, immune regulation, and gut–brain communication [3,59].

From infancy to senescence, the gut microbiome undergoes dynamic shifts in composition and metabolic output, which can influence health trajectories and susceptibility to disease. In humans, aging has been consistently associated with a decrease in beneficial commensals (e.g., *Faecalibacterium prausnitzii*), increased colonization by pathobionts, and reduced production of SCFAs, contributing to systemic inflammation and frailty in older adults [45,59].

Comparable patterns have been observed in dogs, highlighting the potential to develop targeted strategies that support healthy aging and improve their quality of life. This provides an opportunity to deepen our understanding of aging in dogs, ultimately leading to better care and wellbeing throughout their lifespan. In dogs, aging is linked to a decline in microbial diversity and shifts in key bacterial phyla (e.g., increased Proteobacteria, decreased Firmicutes), with potential impacts on nutrient absorption, immune function, and behavior [5,48]. Badal et al. [59] provided a detailed overview of how aging influences the gut microbiome, highlighting changes in microbial taxa, metabolic pathways, and bioactive compounds that may modulate inflammation, cognitive function, and longevity.

5. BIOMARKERS OF INTESTINAL HEALTH

The evaluation of intestinal health increasingly incorporates the use of specific biomarkers that represent functional and structural aspects of the gastrointestinal system. These markers offer valuable insights into processes such as mucosal integrity, immune activation, microbial composition, and metabolic activity. In canine studies, biomarkers have been identified in various biological matrices, including faeces, blood, intestinal content, and mucosal tissue [48]. Faecal biomarkers are widely favored due to their non-invasive collection and utility in assessing inflammation, immune responses, microbiota and microbiota-derived metabolites. However, other matrices can provide complementary insights into gastrointestinal physiology, pathology and therapeutic strategies.

5.1. Faecal microbiota composition

The **composition of the fecal microbiota** is a well-established marker of intestinal health in dogs. It is commonly assessed through 16S rRNA gene sequencing, which provides insights into the taxonomic structure and diversity of microbial communities. Quantitative PCR (qPCR) is often used to target and quantify specific taxa known to be associated either with eubiotic or dysbiotic states. In dogs, qPCR-based quantification of total bacteria and seven bacterial taxa (*Faecalibacterium spp., Turicibacter spp., Streptococcus spp., Escherichia coli, Blautia spp., Fusobacterium spp.,* and *Clostridium hiranonis*) is integrated into a clinically validated diagnostic tool known as the **Canine Dysbiosis Index (DI)**. Developed and validated by AlShawaqfeh et al. [60], the DI provides a single numerical score that reflects the degree of microbial imbalance, supporting diagnosis and monitoring of chronic enteropathies. Other taxa such as *Bifidobacterium, Bacteroides*, and *Lactobacillus* are frequently studied for their health-associated roles [46,61,62] .

Beyond taxonomic profiling, advanced sequencing methods such as shotgun metagenomics and functional metagenomics have enabled deeper exploration of microbial gene content and metabolic potential [3]. These approaches reveal alterations in key functional pathways (e.g. bile acid metabolism, SCFAs biosynthesis, and vitamin production) providing critical insight into host–microbiota

interactions and their relevance to health and disease [40].

5.2. Microbial diversity indices

Microbial diversity indices are quantitative tools used to describe the complexity of microbial communities, and they are widely applied in microbiome research. These indices capture essential ecological characteristics such as species richness (the total number of distinct taxa), evenness (how uniformly taxa are represented), and phylogenetic diversity (evolutionary relationships among taxa). The data for these indices are derived from high-throughput sequencing methods such as 16S rRNA gene amplicon sequencing or whole-genome shotgun metagenomics [40,63,64]. After sequencing, reads are clustered into operational taxonomic units (OTUs) or amplicon sequence variants (ASVs). These are then processed with bioinformatic pipelines (e.g. QIIME2 or DADA2) to calculate diversity indices [64,65].

Alpha diversity assesses the variety and distribution of microbial taxa within a single sample or environment. It provides insight into ecological richness (the number of distinct taxa), evenness (the balance of microbial communities in a relative abundance) or both within the sample [66]. From an ecological perspective, different indices are used to quantify alpha diversity [67]:

- Observed OTUs/ASVs refers to a simple count of Operational Taxonomic Units (OTUs) or Amplicon Sequence Variants (ASVs) present in the sample, accounting for the species richness.
- Chao1 estimates richness adjusting by undetected species.
- Shannon Index captures both richness and evenness, indicating how balanced the community is.
- Simpson Index emphasizes dominant species, reflecting community evenness and the dominance of specific taxa.

Healthy individuals typically exhibit greater alpha diversity than those affected by pathological conditions. In veterinary research, dogs with chronic gastrointestinal disorders consistently demonstrate reduced alpha diversity compared to healthy controls, indicating a dysbiotic state associated with disease [2,36,48].

Beta diversity, which refers to the diversity between samples, is widely used to detect shifts in microbiota composition resulting from internal or external factors affecting the microbial ecosystem. It is commonly assessed using the following metrics:

- Bray-Curtis dissimilarity compares the abundance of microbial taxa between two communities, providing a quantitative measure of how similar or different their compositions are [40,68].
- UniFrac distances incorporate phylogenetic information, offering insights into the evolutionary relationships among microbial taxa [69]. The unweighted UniFrac distance is sensitive to the presence or absence of rare taxa, while the weighted UniFrac distance emphasizes differences in the relative abundance of dominant taxa [70].
- Jaccard Index measures the similarity between communities based solely on the presence or absence of taxa. However, it does not consider their relative abundance or phylogenetic relatedness [2].

These metrics are often applied in studies involving dogs, where differences in beta diversity have been used to distinguish microbial profiles linked to chronic enteropathies, dietary patterns, and probiotic treatments [40,60]. Visualization methods such as Principal Coordinates Analysis (PCoA) are commonly used to interpret and present these differences across experimental groups [71].

5.3. Metabolic biomarkers

SCFAs can be broadly divided into straight-chain and branched-chain forms. Straight-chain SCFAs, including acetate (C2), propionate (C3), and butyrate (C4), are the most abundant in the gut and arise mainly from saccharolytic fermentation. These compounds play multifaceted roles in gastrointestinal health. Acetate is the most prevalent SCFA, readily absorbed and distributed systemically, where it serves as a substrate for lipogenesis and peripheral energy metabolism. Propionate is primarily utilized by the liver and contributes to gluconeogenesis, whereas butyrate is the preferred energy source for colonocytes and is essential for the maintenance of epithelial integrity through promotion of tight junction assembly and modulation of

mucosal immune responses [72,73]. Beyond their metabolic functions, SCFAs also exert anti-inflammatory effects and contribute to gut microbial homeostasis by lowering the luminal pH, thereby suppressing the growth of potentially pathogenic bacteria and favoring the proliferation of beneficial taxa [40,74]. Research in dogs with gastrointestinal disorders has shown reductions in butyrate and total SCFA concentrations, for example correlating with mucosal inflammation and microbial imbalance [2]. Furthermore, dietary interventions involving prebiotics and probiotics have been demonstrated to modulate SCFA production and are being explored as therapeutic strategies [40].

Branched-chain fatty acids (BCFAs), including isobutyrate (C4) and isovalerate (C5), are primarily produced through microbial fermentation of branched-chain amino acids such as valine, leucine, and isoleucine. In canine studies, these BCFAs have been identified as significant byproducts of protein fermentation in the gut [75]. For instance, research has demonstrated that isobutyrate and isovalerate are the predominant BCFAs found in the feces of dogs, directly correlating with dietary protein content and amino acid concentrations [76]. Valerate (C5), is occasionally grouped with BCFAs due to its similar structure; however, its origin is more complex. Unlike isobutyrate and isovalerate, valerate may result from both saccharolytic and proteolytic fermentation, depending on the substrate and microbial composition present in the colon. Specifically, it can be derived from the microbial metabolism of carbohydrates such as inulin and resistant starches, as well as from amino acids like lysine or ornithine under proteolytic conditions [75].

In addition to SCFAs and BCFAs, other microbial-derived metabolites have gained interest as functional indicators of gut health. **Volatile organic compounds (VOCs)**, including indoles, phenols, and sulfur-containing compounds, are produced during the microbial fermentation of amino acids. These metabolites can be measured in feces and are emerging as non-invasive markers of microbial metabolic activity, with potential applications in the detection of dysbiosis, dietary responsiveness, and gastrointestinal inflammation [77,78] .

Other microbial-derived metabolites of relevance include **bile acids**, which are modified by intestinal bacteria through deconjugation and transformation processes and play key roles in host lipid metabolism, intestinal barrier function, and immune

regulation [79].

5.4. Inflammatory and immune function biomarkers

Biomarkers indicative of intestinal and systemic inflammation, as well as mucosal immune activation, are valuable tools for assessing gastrointestinal health in dogs. In the following section, several key biomarkers will be further explained. Although these represent some of the most frequently studied markers, other analytes may also provide relevant insights into gastrointestinal and immune function.

Calprotectin (cCP) is a cytosolic protein complex belonging to the S100 family, predominantly secreted by activated neutrophils and macrophages during inflammatory responses [80]. It plays an important role in innate immunity and serves as a reliable marker of mucosal inflammation [81]. In dogs, calprotectin can be measured in feces and serum, offering complementary insights into gastrointestinal inflammatory processes.

Fecal cCP reflects neutrophil infiltration within the intestinal lumen and is considered a reliable, non-invasive marker for diagnosing and monitoring chronic inflammatory enteropathies (CIE). Elevated fecal concentrations have been associated with disease severity and response to therapy in canine inflammatory bowel disease (IBD) [82]. On the other hand, serum cCP may reflect systemic inflammatory activity or extraintestinal involvement, and is being explored as a supportive marker in the broader assessment of chronic intestinal disease in dogs. In a key study by Heilmann et al. [83], serum calprotectin concentrations were found to be significantly elevated in dogs with idiopathic IBD compared to healthy controls. Despite this, serum levels did not significantly correlate with clinical activity indices, histopathological scores, or serum C-reactive protein (CRP) concentrations.

Lactoferrin is an iron-binding glycoprotein primarily released by activated neutrophils during inflammatory responses. Its presence in feces reflects local neutrophilic infiltration of the gastrointestinal tract and has been proposed as a supportive biomarker for diagnosing several intestinal inflammatory conditions in dogs [84]. In contrast, serum lactoferrin may be more closely associated with systemic inflammatory responses. A recent study by Maden and Gülersoy [85]

evaluated both serum and fecal concentrations of lactoferrin, S100A12, and CRP in dogs with infectious and non-infectious diarrhea. The results suggested that serum lactoferrin, in particular, could be useful in distinguishing bacterial from non-bacterial causes of diarrhea in dogs, highlighting its potential as a systemic inflammatory biomarker.

Immunoglobulin A (IgA) serves as an indicator of gut health in dogs. While IgA levels in serum may indicate overall immune health, IgA measured in faeces are more directly linked to mucosal immune function, reflecting secretory activity by gut-associated B cells. In dogs, variations in fecal IgA levels have been associated with changes in gut immune status, with increases observed in response to enteric infections and other antigenic stimuli [86]. Conversely, decreased fecal IgA concentrations have been reported in dogs with inflammatory bowel disease (IBD), suggesting a compromised mucosal immune response in such conditions [87].

CRP is a major acute-phase protein in dogs, synthesized predominantly by hepatocytes in response to pro-inflammatory cytokines such as interleukin-6. Its circulating levels rise rapidly in the presence of systemic inflammation, infection, tissue injury, or neoplasia, making it a valuable general marker of inflammatory activity [88]. In the context of canine chronic enteropathies, CRP has been explored for its diagnostic and prognostic utility [89]. Elevated serum CRP concentrations have been consistently reported in dogs with IBD when compared to healthy controls or to dogs undergoing antibiotic or corticosteroid therapy [90]. Although CRP is a responsive marker of systemic inflammation, its relationship with clinical severity indices and histopathological findings in dogs with CE has yielded inconsistent results. Several studies have reported only weak or non-significant correlations, suggesting that CRP alone may not reliably reflect gastrointestinal-specific inflammatory activity [90,91].

Haptoglobin (HP) is a widely used moderate acute-phase protein and systemic inflammatory marker in veterinary medicine, particularly in the assessment of inflammatory conditions. In dogs, HP concentrations increase in response to both acute and chronic inflammatory stimuli, including infections, immune-mediated disorders, and gastrointestinal diseases [92]. Although HP shows a slower response compared to CRP, it can offer complementary diagnostic information, especially in

the evaluation of chronic inflammatory disorders such as IBD and other enteropathies [93,94].

5.5. Intestinal barrier integrity biomarkers

Biomarkers assessing the integrity and permeability of the intestinal barrier are essential for understanding gastrointestinal dysfunction in dogs, particularly in the context of chronic enteropathies, protein-losing enteropathies (PLE), and IBD. This section describes several biomarkers involved in the assessment of epithelial disruption and tight junction dysfunction, which are key features of impaired intestinal barrier integrity.

Zonulin is a protein synthesized in the intestinal and liver cells that regulates intestinal permeability by modulating tight junctions between epithelial cells [95]. It has been extensively investigated in human medicine as a marker of intestinal barrier dysfunction, where increased levels in serum and feces are associated with various inflammatory and autoimmune conditions [96]. In veterinary medicine, particularly in canine gastroenterology, the relevance of zonulin is emerging. Recent studies have demonstrated elevated fecal zonulin concentrations in dogs with CE, suggesting its potential utility in assessing mucosal barrier integrity and intestinal permeability [97].

Alpha-1-antitrypsin (A1AT) is a protease inhibitor normally present in serum but resistant to degradation in the gastrointestinal tract. When detected in feces, A1AT reflects increased permeability and protein loss across the mucosal barrier. It is commonly used as a marker of protein-losing enteropathy in dogs and correlates with clinical severity and gastric and duodenal histologic abnormalities [98,99]. Because A1AT is stable in feces and not degraded by digestive enzymes, it provides a reliable estimate of intestinal protein leakage.

5.6. Other functional gut health biomarkers

Other gut health biomarkers commonly associated with aging include cobalamin, folate, and methylmalonic acid (MMA). These three functional biomarkers have also

been evaluated to diagnose chronic enteropathies in dogs [89].

Vitamin B9 (folate) and B12 (cobalamin) levels remain valuable indicators of absorptive capacity and microbial metabolism. **Folate** is endogenously produced by certain commensal bacteria in the colon, though its absorption takes place primarily in the proximal small intestine. Thus, folate's absorption is host-driven but its systemic availability is influenced by microbial synthesis, competition and the ecosystem balance. Decreased serum folate may result from small intestinal malabsorption, alterations in microbial synthesis, or microbial overutilization, particularly under dysbiotic conditions [38].

Cobalamin absorption, in contrast, is more complex and involves both host and microbial factors. It is mainly present in animal origin ingredients and in smaller amounts can be produced by the gut microbiota. Dietary B12 is initially released from food proteins in the stomach and binds to haptocorrin. In the duodenum, pancreatic enzymes degrade haptocorrin, allowing cobalamin to bind to intrinsic factor (IF), a glycoprotein secreted by gastric parietal cells. This B12–IF complex is then specifically absorbed in the distal ileum via receptor-mediated endocytosis [100]. Disruption at any point in this process, such as ileal inflammation, exocrine pancreatic insufficiency, or bacterial overgrowth that competes for luminal B12, can result in systemic cobalamin deficiency. In dogs, low serum cobalamin is frequently associated with chronic enteropathies and exocrine pancreatic insufficiency, specially in older stages of life [89,101].

MMA is a metabolic intermediate that accumulates when intracellular levels of cobalamin are inadequate. The conversion of methylmalonyl-CoA to succinyl-CoA is dependent on adenosylcobalamin, a bioactive form of B12. Therefore, MMA is considered a sensitive and specific functional biomarker of tissue-level cobalamin status. In dogs with CE, elevated serum MMA may be detected even in the presence of normal serum cobalamin concentrations, making it a valuable tool for the early detection of functional B12 deficiency [100].

6. BEHAVIORAL INDICATORS ASSOCIATED WITH INTESTINAL HEALTH

Behavioral indicators are increasingly recognized as non-invasive markers of gut health and overall well-being in dogs. The gut–brain axis refers to the bidirectional communication system that integrates the central nervous system, enteric nervous system, immune signaling pathways, and the gut microbiota, and it plays a fundamental role in regulating both gastrointestinal function and behavioral responses [6]. The assessment of neuroactive compounds, behavioral and activity patterns, and physiological stress markers provides a multidimensional approach to evaluating the gut–brain axis in canine health.

6.1. Neuroactive metabolites

Tryptophan–kynurenine pathway-metabolites, which link gut metabolism to immune and neurological function, are gaining relevance in both human and canine gut–brain axis research. Altered serum concentrations of **tryptophan**, **kynurenine**, and **kynurenic acid** may reflect microbial catabolism, host inflammation, and neuroimmune modulation [102].

Tryptophan metabolism is a central link between gut health, immune function, and neurobehavior. Through microbial and host enzymatic pathways, tryptophan is converted into several neuroactive metabolites, including kynurenine and kynurenic acid, which can influence inflammation and neurotransmission. Altered serum levels of these metabolites have been associated with gastrointestinal inflammation, microbial dysbiosis, and behavioral disturbances such as anxiety or depression [102–105]. In dogs, emerging research suggests that gut microbiota alterations toward dysbiosis are associated with inflammatory processes and activation of the hypothalamic-pituitary-adrenal (HPA) axis [104,105].

6.2. Endocrine biomarkers

Endocrine biomarkers such as cortisol, oxytocin, and thyroxine (TT4) play crucial

roles in regulating physiological and behavioral responses in dogs. The role of endocrine markers in canine physiology and behavior has been demonstrated in studies investigating stress responses, metabolic function, and social bonding [106–109]. Recent work has emphasized the potential of neuroendocrine markers like cortisol and oxytocin to reflect gut–brain interactions and behavioral modulation in both animals and humans [107,110].

Oxytocin is a neuropeptide hormone known for its roles in social bonding, emotional regulation, and stress buffering. It is also increasingly implicated in gut–brain axis signaling. Microorganisms in the gut can influence brain function by signaling through the vagus nerve, thereby affecting the synthesis and release of oxytocin [111]. Studies in both rodents and humans have demonstrated that gut microbiota composition can influence oxytocin release through multiple mechanisms, including activation of vagal afferent pathways, modulation of cytokine signaling cascades, and interaction with microbial metabolites such as SCFAs and tryptophan-derived catabolites [111–114]. Although canine-specific research remains limited, preliminary studies in murine models have shown that certain bacterial strains isolated from dogs can influence oxytocin levels in the host [115].

Cortisol is a key glucocorticoid hormone secreted by the adrenal glands that supports a wide range of physiological functions. Its release is carefully regulated by the suprachiasmatic nucleus, which governs the circadian rhythm, and is an essential part of the hypothalamic pituitary adrenal axis. This neuroendocrine system plays a central role in coordinating stress responses and maintaining physiological balance. Disruptions in cortisol regulation, whether caused by chronic stress, illness, or aging, can have significant effects on multiple body systems [116]. In dogs, elevated cortisol concentrations have been linked to behavioral changes such as anxiety, aggression, and hypervigilance [117–119]. Interventions targeting the gut microbiota, such as prebiotic and probiotic administration, have been shown to reduce salivary or fecal cortisol, and improve behavior in dogs exposed to environmental stressors [119–121].

TT4 is a key marker of thyroid function in dogs, reflecting the circulating levels of both protein-bound and free thyroxine (T4). As a product of the thyroid gland, T4 plays a central role in regulating basal metabolic rate, thermoregulation, and the

development and maintenance of various tissues, including the brain. Thyroid hormones are essential for normal neurological function, and their dysregulation has been associated with cognitive and behavioral changes in dogs [122,123].

Although TT4 is not traditionally classified as a neuroactive compound, its systemic influence on neural metabolism and mood regulation supports its inclusion as a neuroendocrine biomarker. Thyroid hormones can modulate neurotransmitter systems, including serotonin and dopamine pathways, and can affect brain development and plasticity [124,125]. In aging dogs, TT4 assessment may provide valuable information not only about metabolic status but also about potential neuroendocrine imbalances that could underlie changes in behavior, mood, and cognition.

6.3. Behavioral observations

Behavioral changes can offer real-time, non-invasive insight into intestinal and overall health status in dogs. Behavioral assessment tools, such as questionnaires, ethograms and standardized clinical behavior scales, are being increasingly used in veterinary research and medicine to quantify these behavioral changes [126–128]. Signs such as reduced playfulness, increased aggression, fear responses, and social withdrawal may reflect discomfort or dysregulation in gut–brain axis function [119].

6.4. Activity measures

Monitoring physical activity has become an increasingly important approach in canine health research, offering objective, continuous insights into behavior, energy expenditure, and physiological state [129,130]. Wearable activity trackers, such as accelerometers, have become valuable tools for monitoring canine behavior and overall health. Devices like the Actical® and ActiGraph® are commercially available, wrist- or collar-mounted accelerometers designed to measure movement across one or more axes. Both devices are frequently used in veterinary research and clinical studies to objectively quantify physical activity in dogs [131]. Actical® (Philips

Respironics) is an omnidirectional accelerometer that detects motion intensity and duration, and is commonly used for general activity monitoring in companion animals. ActiGraph® (ActiGraph LLC) is a tri-axial accelerometer originally developed for human activity tracking and validated in canine studies for research requiring high-resolution motion data [132].

Notably, age-related differences in activity patterns have been consistently observed, with younger dogs displaying higher frequencies of dynamic behaviors and shorter rest periods compared to older individuals [24,133]. Beyond their role in general activity monitoring, these wearable devices also offer potential for studying GBA interactions since changes in activity levels may reflect alterations in gut–brain communication. For example, dogs with gastrointestinal disturbances tend to exhibit reduced physical activity and prolonged rest, whereas those receiving microbiota-targeted interventions (e.g., probiotics or dietary fibers) may show enhanced exploratory behavior and increased movement [104,119]. Therefore, objective activity tracking using accelerometry can serve as a non-invasive proxy for both physical and neurobehavioral health, contributing valuable insights into GBA function in dogs.

7. THE GUT-BRAIN AXIS

Growing evidence highlights the dynamic, bidirectional communication between the gastrointestinal tract and the central nervous system; this relationship is already widely recognized as the gut–brain axis [8,77,112,116,119,134,135].

The gut microbiota plays a central role in this system, exerting effects on host physiology through immune, neural, endocrine, and metabolic pathways [136–138]. Key microbial metabolites, such as short-chain fatty acids (SCFAs), serotonin, dopamine, gamma-aminobutyric acid (GABA), and tryptophan, have been shown to influence both local gut epithelial function and systemic signaling via the bloodstream [139].

This axis operates through multiple routes, including neural connections (particularly the vagus nerve), the hypothalamic–pituitary–adrenal (HPA) axis, and the circulatory system. The gut microbiota can affect brain function, while the central nervous

system can reciprocally modulate microbial composition and activity by altering gut motility, permeability, mucus secretion, and hormone release [138,139]. This continuous exchange is crucial for maintaining homeostasis and is implicated in several systemic conditions.

Alterations in gut microbial communities (dysbiosis), can disrupt this communication. Dysbiosis may lead to changes in gut permeability and immune activation, which could compromise the blood–brain barrier and contribute to gastrointestinal or neurological dysfunctions [140]. In humans, dysfunction of the GBA has been linked to psychiatric and neurodegenerative disorders such as depression, anxiety, autism spectrum disorders, and Alzheimer's and Parkinson's diseases [52,141–143].

Although still limited, research on this topic in dogs has been expanding in recent years. Recent studies suggest functional parallels between the dog microbiome and those of other mammals [144,145]. For example, Li et al. [146] demonstrated that gut microbiota modulates age-related cognitive decline in rats. In dogs, distinct gut microbial profiles have been associated with behavioral traits, including aggression in shelter dogs [120,147] and specific behaviors such as motivation and sociability in working dogs [120,147][148]. Moreover, the therapeutic potential of probiotics and prebiotics in managing anxiety-related behaviors has been explored [119].

Neurodegenerative diseases in dogs, such as canine cognitive dysfunction and multiple system degeneration, share pathological similarities with Alzheimer's and Parkinson's disease in humans [143]. A recent review further synthesized the current evidence on gut microbiota and behavioral disorders in dogs, drawing comparisons with findings in rodent and human studies [104]. However, despite these advances, research focusing on the GBA and gut microbiota in elderly dogs remains scarce, presenting a critical gap in the field.

There is increasing evidence supporting a continuous, bidirectional communication between the gut and the central nervous system, commonly referred to as the gut-brain axis (GBA). Within this axis, the gut microbiota and its metabolites influence host physiology via neural, endocrine, immune, and metabolic pathways. Dysbiosis may alter this communication by modifying microbial composition and metabolic output, potentially affecting gut permeability and compromising the blood-brain barrier, thereby contributing to neurological and gastrointestinal

pathologies [134]. Conversely, the central nervous system can regulate microbial community dynamics through the autonomic nervous system by modulating gastrointestinal motility, secretion, and mucosal permeability. While most mechanistic insights originate from studies in rodents and humans, emerging evidence indicates that the canine gut microbiota exhibits some comparable functional characteristics. This growing understanding offers valuable opportunities to improve canine health through microbiome-targeted strategies [144,145].

7.1. Factors influencing the gut-brain axis

The gut–brain axis is shaped by a complex network of factors that influence both microbial composition and neural signaling. Diet is one of the most powerful modulators, as it directly alters microbial diversity and metabolite production, including short-chain fatty acids (SCFAs) and neuroactive compounds [138,139]. Additionally, stress and psychological states activate the hypothalamic–pituitary–adrenal (HPA) axis, increasing cortisol levels that in turn affect gut permeability, motility, and microbial structure [138].

Other relevant modulators include antibiotic exposure, infections, immune status, and host genetics. These factors can impact neuroimmune signaling pathways and the production of neurotransmitters such as serotonin or GABA, either promoting homeostasis or driving pathological responses [136,137,139]. Physical activity and environmental enrichment has also been shown to influence GBA integrity, possibly by reducing systemic inflammation, the immune response and supporting microbial resilience [149,150].

In dogs, as in humans, these environmental and physiological inputs may contribute to behavioral variations and influence susceptibility to stress-related or inflammatory conditions via the GBA. However, research in companion animals remains limited compared to rodent or human models.

7.2. Age-related changes in the gut-brain axis

Aging is associated with profound changes in gut microbiota composition and

functionality, often characterized by a decline in microbial diversity and an increase in pro-inflammatory taxa. These alterations, coupled with age-related immune dysregulation and metabolic changes, can disrupt gut barrier integrity and compromise communication within the gut–brain axis [146].

In rodent models, age-related microbial dysbiosis has been linked to neuroinflammation and cognitive decline. For example, Li et al. [146] demonstrated that changes in gut microbiota modulated memory and learning performance in aging rats. In humans, similar associations have been observed, where gut microbial profiles correlate with frailty, depression, and neurodegenerative conditions such as Alzheimer's disease [141–143,151].

In dogs, age-related cognitive dysfunction (also known as canine cognitive dysfunction syndrome) shares many features with human Alzheimer's disease, including beta-amyloid deposition and behavioral deterioration [143]. Despite this, the role of the gut microbiota in canine aging and cognitive decline remains underexplored. Identifying microbial signatures associated with aging and their influence on the GBA could offer new preventive or therapeutic strategies to improve the quality of life in senior dogs.

OBJECTIVES

The primary aim of this thesis is to investigate how aging affects intestinal health in dogs and to examine the potential associations between gut-related physiological changes and behavioral alterations. By characterizing the age-associated shifts in microbiota composition, intestinal biomarkers, and behavior, this work aims to contribute to a better understanding of the gut–brain axis and its role in canine healthy aging.

Study 1: Age-Associated Changes in Intestinal Health Biomarkers in Dogs

- To investigate differences in the intestinal microbiota and intestinal health based on fecal biomarkers in a population of dogs of different ages.
- To study changes in intestinal health biomarkers throughout the aging process in order to consider early interventions that promote healthy aging in dogs.

Study 2: Age-Related Changes in Gut Health and Behavioral Biomarkers in a Beagle Dog Population

- To examine the effects of age on intestinal biomarkers and behavioral indicators in a controlled population of Beagle dogs.
- To investigate correlations between intestinal health biomarkers and behavioral indicators in Beagle dogs.
- To better understand the gut–brain axis in dogs and pave the way for defining targeted interventions according to the needs of different age groups.

CHAPTER I

Article

Age-associated changes in intestinal health biomarkers in dogs

Anna Fernández-Pinteño[1]*, Rachel Pilla[2], Xavier Manteca[3], Jan Suchodolski[2], Celina Torre[1] and Anna Salas-Mani[1]

[1] Department of Research and Development, Affinity Petcare, L'Hospitalet de Llobregat, Spain

[2] Gastrointestinal Laboratory, Department of Small Animal Clinical Sciences, Texas A&M University, College Station, TX, United States,

[3] School of Veterinary Science, Universitat Autònoma de Barcelona, Bellaterra, Spain

This chapter has been published in Frontiers in Veterinary Science.

Citation: Fernández-Pinteño A, Pilla R, Manteca X, Suchodolski J, Torre C and Salas-Mani A (2023) Age-associated changes in intestinal health biomarkers in dogs. Front. Vet. Sci. 10:1213287. doi: 10.3389/fvets.2023.1213287

ABSTRACT

The gut microbiome is critical for maintaining host health. In healthy humans, the aging process is one of the main factors modulating the changes in the intestinal microbiota. However, little is known about the relationship between gut health, microbiota, and the aging process in dogs. The present study aims to explore the differences in the intestinal microbiota and intestinal health based on fecal biomarkers in a population of dogs of different ages. The study involved 106 dogs of different breeds aged between 0.2 and 15 years categorized as senior (> 7 years; n = 40), adult (2–7 years; n = 50), and junior (< 2 years; n = 16). Fecal samples were collected during the same period at the same facilities. The analysis included the following gut health indicators: 16S rRNA gene sequencing to investigate the differences in the fecal microbiota; qPCR to determine the dysbiosis index; fecal short-chain fatty acid concentrations; fecal calprotectin; and immunoglobulin A. Beta diversity analysis revealed a significant difference with a small effect size ($p = 0.003$; $R = 0.087$) among age categories based on the unweighted UniFrac metric, but no significance was observed based on the weighted UniFrac metric or Bray–Curtis distances. There were no significant differences in the alpha diversity measures or the fecal dysbiosis index among age categories. Senior dogs had significantly higher relative abundance proportions in phyla Bacteroidota and Pseudomonadota and the genus *Faecalibacterium*, but not on qPCR analysis. At the family level, Ruminococcaceae, Uncl. Clostridiales.1, Veillonellaceae, Prevotellaceae, Succinivibrionaceae, and Bacteroidaceae abundances were higher in the senior category than in the adult and/or junior categories. Relative proportions, but not concentrations of fecal acetate, were higher in the senior category, while butyrate, isovaleric acid, and valeric acid were lower. The valeric acid concentration was significantly lower in the senior category than in the adult category. Calprotectin and immunoglobulin A levels did not differ significantly across groups. In conclusion, this study observed multiple minor changes in the fecal microbiota composition and the relative amount of short-chain fatty acids in dogs among different age groups, but studies in larger populations representative of all ages are warranted to refine the present results.

Keywords: fecal microbiota, canine, aging, intestinal health, nutrition

1. INTRODUCTION

The gut microbiome is defined as the entire collection of microorganisms living in the gastrointestinal tract, dominated by bacteria and complemented by commensal populations of fungi, viruses, archaea, and protists (1). It has the largest microbiota population in the body when compared to other colonized organs. The intestinal microbiota is estimated to be composed of $10^{13}10^{14}$ cells (2), and its genome is 150 times larger than the human genome.

The gut microbiome plays an important role in the physiological and pathological state of its host. It participates in multiple metabolic functions, protects against pathogens, and plays a crucial role in the immune response. These processes are critical to maintaining the health status of the host (3). The composition of the microbiota is crucial to maintaining balanced gut functionality. An imbalanced or disrupted microbiota is related to several intestinal and extraintestinal diseases (4).

There is an increasing number of studies evaluating the different elements modulating the intestinal microbiota (5–8). The main factors can be classified as external and internal, including, for example, diet and pharmacological treatments and age and genetics, respectively. In addition, pathological disorders (e.g., inflammation and type 2 diabetes) may induce deviations in the gut microbiota, causing an imbalance in the gut microbiota, defined as dysbiosis. Among all these factors, age is one of the most important variables that must be considered when studying the evolution and changes of the gut microbiota.

Aging and its relationship with gut health and gut microbiota are currently being explored in mammals, and they are already well-established in humans. Badal et al. (9) summarized the current knowledge of the human gut microbiota, considering composition, function, and metabolic products from the microbiota in relation to aging and lifespan. Changes in the human microbiota related to age have been described and are apparently associated with the host's health scenario. For example, bifidobacteria belonging to members of the genus *Bifidobacterium*, which are considered a beneficial bacterial group, decrease during the shift from middle age to old age. Contrarily, *Clostridium perfringens*, lactobacilli, enterococci, and Enterobacteriaceae increase during the aging process (10). In contrast to the human microbiota, bifidobacteria may not play an important role in dog gut health, according

to the results reported by Masuoka et al. (10), in which bifidobacteria were only found in half of the youngest dogs and none of the adult dogs sampled, although differences in methodologies need to be considered when interpreting these results.

The existence of a core microbiota in healthy dogs has already been described (11). Moreover, Ziese and Suchodolski (12) associated shifts in the canine fecal microbiota with certain pathologies. Garrigues et al. (13) recently reviewed the development of the gut microbiota during the early stages of canine life, supporting observed changes in bacterial communities from day 2 of age to up to 52 weeks. On day 2 after birth, gut microbiota richness increases, and from day 2 to 21, Bacillota predominance is substituted by a codominance of Bacillota, Bacteroidota, and Fusobacteriota (14). Puppies in the first few weeks of life have immature microbiota, characterized by an increased dysbiosis index (DI), *Clostridium difficile* abundance, and decreased *Clostridium hiranonis* compared to adult dogs (15). Approximately after 4–6 months of age, the microbiota resembles that observed in adult dogs and remains largely stable in adulthood (13). However, the relationship between the intestinal microbiota, gut health conditions, and the aging process in older dogs is not well established. Studies in this field are needed first to understand this relationship and then to explore effective strategies to improve the quality of the aging process in dogs from this perspective.

The present study aimed to investigate the differences in the intestinal microbiota and intestinal health based on fecal biomarkers in a population of dogs of different ages, with a special focus on the senior category.

2. MATERIALS AND METHODS

All samples collected from dogs were in accordance with the 2010/63/UE directive, and any additional procedures to the dogs' daily routine were undertaken. Before the study, the protocol was shared with the Affinity Ethical Committee to ensure good practices and the animals' welfare. All dogs were housed at the Affinity Nutrition Center (Barcelona, Spain).

2.1. Animals

The study involved 106 multi-breed dogs (n = 53 male dogs; n = 53 female dogs) aged between 0.2 and 15.0 years (mean age = 6.3 years). The dog breeds that participated in the study were as follows: Beagle, Bichon Maltese, Jack Russell Terrier, Boxer, German Shorthaired Pointer, Poodle, Chihuahua, Cocker Spaniel, Dalmatian, German Shepherd, Spanish Water Dog, Miniature Pinscher, Andalusian Podenco, Pointer, Pomeranian, Miniature,Schnauzer, Shih Tzu, Brittany Spaniel, and Yorkshire Terrier [detailed information is in the Supplementary Table 1. Animals were grouped into junior (J), adult (A), and senior (S) categories, considering the respective age ranges: up to 2 years, from 2 to 7 years, and over 7 years (Table 1).

Animals lived in kennels in groups of between two and seven individuals, depending on the size of the dogs. Dogs living in pairs shared an area of 15 m2, whereas those living in larger groups shared an area of 57–60 m2. All the animals had free access to the outdoor and indoor parts of their kennels, and they spent between 2 and 4 h per day in a bigger outdoor park where they could socialize with a larger group of dogs. The water supply was ad libitum. Non-specific diets were manufactured and offered because of the study. Dogs were fed commercially available, dry-complete, and balanced diets adequate to their needs. Furthermore, 54% of dogs were fed a constant diet, whereas the diet was changed for 46% of dogs before and during the collection period. All diets met all requirements for protein, fat, vitamins, and minerals recommended by FEDIAF (16). Most of the dogs in the study (89%) were fed diets formulated with the following composition range: 7–8% moisture, 22–30% protein, 15– 21% fat, and 2–5% fiber. The rest of the dogs (11%) were out of range because of the protein, fat, and/or fiber composition [detailed information is in the Supplementary Table 2]. Mineral and vitamin levels were similar in all the diets that were present throughout the study. The inclusion criteria for enrollment were generally healthy animals and comprised sterilized male and female dogs of all pure breeds and ages. Based on the literature, the exclusion criteria included the presence of clinical disease or any pharmacological treatment potentially interfering with the gut microbiota and/or intestinal health. Specifically, no antibiotics, NSAIDs, PPIs, prebiotics, probiotics, or deworming treatments were administered during or 4 weeks before the sampling. None of the animals participating in this study were fed diets containing probiotics. Dogs with watery, soft, or unformed stools in three

consecutive samples were excluded from the study. No clinical signs compatible with gastrointestinal disorders (such as vomiting, regurgitation, and hyporexia) were observed in any of the animals participating in the study.

Table 1. Descriptive information regarding the age distribution of dogs sorted by age category.

	Junior	Adult	Senior
	n= 16	*n*= 50	*n*= 40
Age (in years)	0.92 [0.23; 1.14]	5.00 [4.58; 7.11]	8.25 [8.15; 9.06]

2.2. Fecal collection

Samples were collected during 3 months (March, April, and May) without interfering with the dogs' daily routines. Feces were collected through direct observation while the animals were kept in their kennels or outdoor recreational areas. The majority of samples were obtained during the first month, but due to the volume of samples and the physiologically atypical routines of some animals, the collection extended up to 3 months. In all cases, individualizing the animals and/or using cages to collect the samples were avoided.

One stool sample was collected per dog, and it was first scored according to an adapted five-point scale of fecal consistency (0 = watery stool; 25 = soft unformed stool; 50 = soft formed and moist stool retaining shape when being collected; 75 = hard formed stool remaining shaped but soft; and 100 = hard dry stool). The scoring system was adapted from the five-point scale previously described by Strickling et al. (17).

If fecal consistency was equal to or lower than 50, the sample was not collected, and the researchers waited for a higher fecal score. The entire stool or enough quantity to fill the sterile fecal collection tube with 250 ml was taken. External contaminants (stones, grass, sand, etc.) were discarded. All samples were processed within 4 h after the deposition. All the materials used, from sample collection to separation into aliquots, were sterile and meant for single use.

Feces were divided into two different aliquots according to the analysis to be conducted. For the microbiota analysis, between 0.5 and 1 g of feces were prepared

in a 1.5-ml sterile, RNAse-free tube. For short-chain fatty acid (SCFA) analysis, calprotectin (cCP) and immunoglobulin A (IgA) were filled in one sterile stool tube with 5 g of feces. Both tubes were stored at −80◦C before being sent and analyzed at the Small Animal Clinical Sciences Department at Texas A&M University (College Station, TX).

2.3. Fecal biomarkers

2.3.1. Microbiota analysis

Illumina sequencing of the bacterial 16S rRNA genes was performed using primers 515F (5′GTGYCAGCMGCCGCGGTAA) (18) to 806RB (5′-GGACTACNVGGGTWTCTAAT) (19) at the MR DNA laboratory (Shallowater, TX, USA). Sequences were processed and analyzed using the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) (20) v 2021.8 pipeline. The raw sequences were uploaded to the NCBI Sequence Read Archive under the BioProject identification PRJNA901473. In brief, the sequences were demultiplexed, and the ASV table was created using DADA2 (21). Before downstream analysis, sequences assigned as chloroplast, mitochondria, and low abundance ASVs, containing < 0.01% of the total reads in the dataset, were removed. All samples were rarefied to even sequencing depth, based on the lowest read depth of samples, to 21,025 sequences per sample.

Alpha diversity was measured with the Chao1 (richness) and Shannon diversity metrics within QIIME2. Beta diversity was evaluated with the unweighted and weighted phylogeny-based UniFrac (22) distance metric (measures that consider phylogenetic information) and the Bray-Curtis distance metric and visualized using Principal Coordinate Analysis (PCoA) plots, generated within QIIME2.

The dysbiosis index (logDNA) is based on a validated algorithm that considers a panel of eight bacterial groups identified by a Quantitative PCR assay for total bacteria, *Faecalibacterium* spp., *Turicibacter* spp., *Escherichia coli*, *Streptococcus* spp., *Blautia* spp., *Fusobacterium* spp., and *Clostridium hiranonis* (23). A DI of < 0 was defined as normal, a DI between 0 and 2 was defined as mild to moderate microbiota shift, and a DI of > 2 was considered significant dysbiosis.

2.3.2. Intestinal health indicators

Concentrations of SCFAs (acetate, propionate, butyrate, isobutyric acid, isovaleric acid, and valeric acid) in feces were measured using a stable isotope dilution gas chromatography-mass spectrometry (GC-MS) assay with some modifications (24). To consider the difference in water content between fecal samples, the final concentrations of fecal SCFAs were adjusted by fecal dry matter (DM) and expressed as $_{\mu}$mol/g of fecal DM (25). The fecal IgA (mg/g) was quantified by using the commercial ELISA kit for canine IgA determination (Bethyl Laboratories, Montgomery, TX, USA). The quantification of the fecal cCP (ng/g) was also analyzed by using the ELISA kit previously validated in dogs (26, 27).

2.4. Statistical analysis

For the statistical analysis of microbiota data, an analysis of similarity (ANOSIM) test within the PRIMER 7 software package (PRIMER-E Ltd., Luton, UK) was performed to analyze significant differences in microbial communities and the size effect (R-values between 0 and 1; a higher R-value indicates a larger size effect) among age groups (J, A, and S). All datasets were tested for normality by performing the Shapiro– Wilk test (JMP Pro 11, SAS Software Inc.). The Kruskal– Wallis test was performed (Prism v. 9, GraphPad Software Inc.), followed by a post-hoc Dunn's multiple comparison tests, to determine the age group differences in bacterial taxa (including phylum, class, order, family, genus, and species). All p-values were adjusted for multiple comparisons using Benjamini and Hochberg's False Discovery Rate (28) at each taxonomic level, and an adjusted p-value of < 0.05 (q-value) was considered statistically significant.

For the general statistical analysis, the model included demographic information as an explanatory variable and fecal indicators as response variables. The primary explanatory variable was age, which was grouped into the three different categories described. The potential explanatory variables included the following: body weight, sex, "feeding routine," "housing," and "breed." Two categories were established for feeding routines: rotation (R), if animals were changing the type of diet in periods shorter than 2 weeks, and stable (S), if animals were eating the same diet for at least

4 weeks. No animals had a change of diet in the 2–4 week range. Each animal was only assigned to one category during the whole study. Housing was the variable defined to identify the three different buildings where animals were allocated. Finally, as for the breed, the dogs were categorized into two groups: beagles and non-beagle dogs. The different breeds were not statistically analyzed due to the high number of different breeds but due to the low number of dogs within each breed; however, the dog size was introduced in the statistical analysis to reduce the heterogeneity of dogs participating in the study. The response variables considered in the general statistical analysis were as follows: fecal consistency; SCFA concentration (acetate, propionate, butyrate, isobutyric acid, isovaleric acid, valeric acid, and total SCFA); SCFA relative amounts (acetate, propionate, butyrate, isobutyric acid, and isovaleric acid); calprotectin; immunoglobulin A; alpha diversity (Chao 1 and Shannon diversity); and finally, from qPCR, total bacteria, *Faecalibacterium, Turicibacter, Streptococcus, E. coli, Blautia, Fusobacterium, Clostridium hiranonis, Bifidobacterium, Bacteroides, Lactobacillus,* and dysbiosis index.

For the general statistical analysis, a first summary statistic was performed, in which quantitative variables were analyzed using the mean and standard deviation. Qualitative variables were tested using relative and absolute frequencies. The existence of differences among age groups was tested by performing the appropriate tests (ANOVA and Kruskal–Wallis test), considering the equality among groups' null hypothesis. The compliance of the application criteria was assessed by performing the Shapiro–Wilk normality test. The relationship between quantitative variables was analyzed using Spearman's correlation.

To analyze the relationship between the age categories and fecal markers, the appropriate linear model was considered, including potential explanatory variables such as body weight, sex, feeding routines, and housing breed. Estimated means (emmeans) for age categories were calculated using the adjusted model. Pairwise post-hoc comparisons were also performed, and the model validation was analyzed by performing a graphical residual analysis. For the general statistical analyses, differences were considered significant with a p-value of < 0.05.

3. RESULTS

3.1. Quantitative real-time PCR analysis and dysbiosis index

The results from the qPCR analysis are summarized in Table 2. *Bifidobacterium* abundance was different between J and A, being higher in A. However, no difference was found when considering S vs. J and A. The calculated dysbiosis index was not different among the age categories (Figure 1). In J, 0 of 16 (0%) dogs had a DI of > 0, while in A and S, 8 of 50 (16%) and 5 of 40 (13%) dogs had a DI of > 0.

Table 2. Results of the quantitative real-time PCR (abundance expressed as LogDNA) and values of the calculated dysbiosis index (ratio).

	Junior	Adult	Senior	
	emmeans ± SE	emmeans ± SE	emmeans ± SE	*p*-value
Dysbiosis index	−4.51 ± 0.642	−3.98 ± 0.370	−3.49 ± 0.518	0.468
Universal LogDNA	10.9 ± 0.067	11.0 ± 0.038	11.0 ± 0.054	0.723
Faecalibacterium	5.43 ± 0.285	5.44 ± 0.164	5.76 ± 0.230	0.476
Turicibacter	7.23 ± 0.237	7.74 ± 0.137	7.53 ± 0.192	0.125
Streptococcus	4.57 ± 0.406	5.10 ± 0.235	5.31 ± 0.328	0.362
E. coli	4.89 ± 0.441	5.12 ± 0.254	5.26 ± 0.356	0.814
Blautia	10.7 ± 0.088	10.5 ± 0.051	10.6 ± 0.071	0.124
Fusobacterium	8.91 ± 0.189	8.81 ± 0.109	8.91 ± 0.152	0.774
Clostridium hiranonis	6.80 ± 0.91	6.86 ± 0.523	6.81 ± 0.073	0.729
Bifidobacterium	4.09 ± 0.530^{b}	6.04 ± 0.306^{a}	5.35 ± 0.428^{a,b}	0.004
Bacteroides	5.72 ± 0.195	5.84 ± 0.113	6.00 ± 0.158	0.525
Lactobacillus	4.88 ± 0.454	5.58 ± 0.262	6.11 ± 0.367	0.118

Different superscript letters indicate significant differences between groups (p-value < 0.05).

Figure 1. A representative plot of the dysbiosis index by age category.

3.2. 16s rRNA sequencing

A total of 3,856,065 quality bacterial 16S rRNA sequences were obtained from the 106 fecal samples analyzed. The range count per sample was between 21,030 and 60,827 (median: 33,688 and mean: 36,378). The beta diversity was analyzed by the weighted and unweighted UniFrac distances and the Bray–Curtis distance. The unweighted UniFrac analysis of similarities, which only considers the presence or absence of individual taxa, revealed significant differences in the microbial communities among age categories (PCoA plot shown in Figure 2). However, no significance was observed based on weighted UniFrac metric or Bray–Curtis distances (Table 3). Alpha diversity indices (Chao1 and Shannon) were not significantly different among the age groups (Figures 3, 4).

Figure 2. 3D beta diversity patterns of canine faecal microbiota comparing Adult (red), Junior (blue) and Senior (green) categories based on unweighted UniFrac distance.

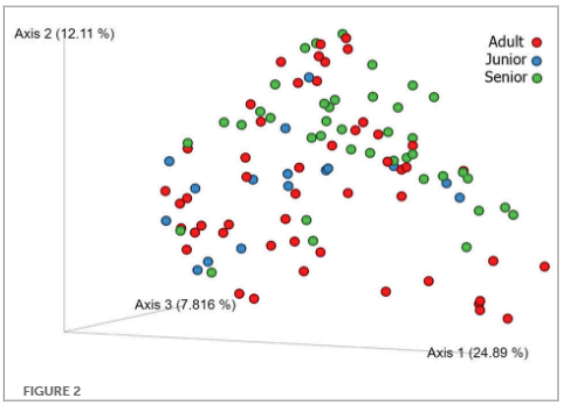
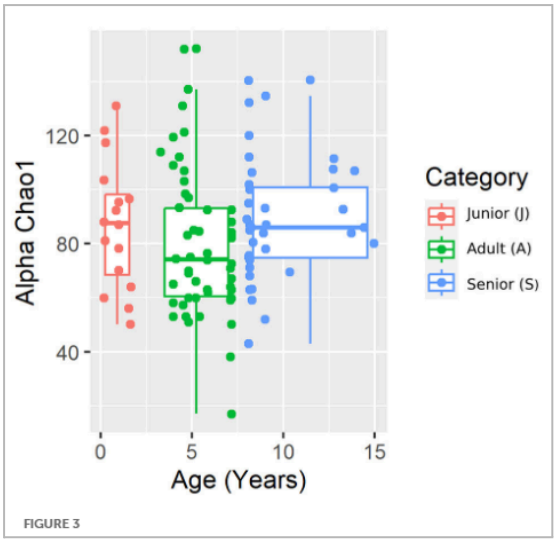


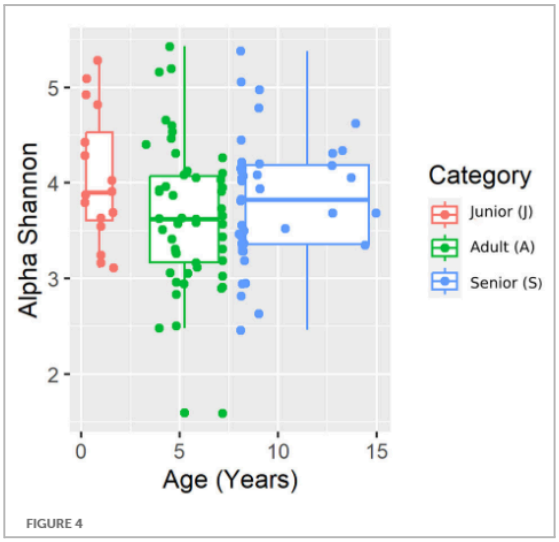
Table 3. Results of beta diversity analysis including unweighted UniFrac, weighted UniFrac, and Bray–Curtis distances.

	Senior vs. Adult vs. Junior		Senior vs. Adult		Senior vs. Junior		Junior vs. Adult	
	R	*p*-value	*R*	*p*-value	*R*	*p*-value	*R*	*p*-value
Unweighted UniFrac	0.087	0.003	0.071	0.003	0.150	0.018	0.070	0.117
Weighted UniFrac	−0.023	0.759	0.047	0.024	−0.145	0.993	−0.127	0.970
Bray–Curtis	0.001	0.459	0.037	0.028	−0.077	0.863	−0.045	0.715

The table shows the sample statistic (R) and the significance level of the sample statistic (p-value < 0.05), considering the three age categories and the pairwise test comparison.

Figures 3 and 4. Alpha diversity indices by age category. Figure 3 displays the Chao1 index, and Figure 4 shows the Shannon index. In both plots, age categories are represented as Junior (J), Adult (A), and Senior (S).





3.2.1. Phylum taxonomic level

When the bacterial relative abundance was studied at the tax level, the major phylum identified was Bacillota, followed by Actinomycetota, Fusobacteriota, Bacteroidota, and Pseudomonadota. The same distribution, considering relative abundance, was described in the three age categories. Significant differences were found for the phylum Bacteroidota and Pseudomonadota, in which S had a higher abundance than A and J (Table 4). However, the corrected q-value did not reach significance.

Table 4. Fecal microbiota composition (% of relative abundance from rarefied data) at the phylum level split by age category.

Phylum	Junior		Adult		Senior		Junior vs. Adult vs. Senior	
	Median	Range	Median	Range	Median	Range	*p*-value	*q*-value
Bacteroidota	0.06[a,b]	0–3.67	0.53[a]	0–11.01	1.74[b]	0.21–12.02	0.036	0.087
Bacillota	89.60	77.20–97.52	85.28	71.62–95.08	82.02	64.30–88.92	0.052	0.087
Actinomycetota	6.87	1.47–14.05	5.35	1.66–14.13	8.20	2.60–15.70	0.569	0.569
Fusobacteriota	1.20	0.15–10.52	5.08	0.13–20.34	3.60	0.89–27.9	0.145	0.181
Pseudomonadota	**0.01[a]**	**0–0.60**	**0.27[a,b]**	**0–1.73**	**0.28[b]**	**0.04–2.14**	**0.019**	**0.087**

Significantly different results are indicated in bold (p-value < 0.05). Superscript letters indicate differences between age categories.

3.2.2. Family taxonomic level

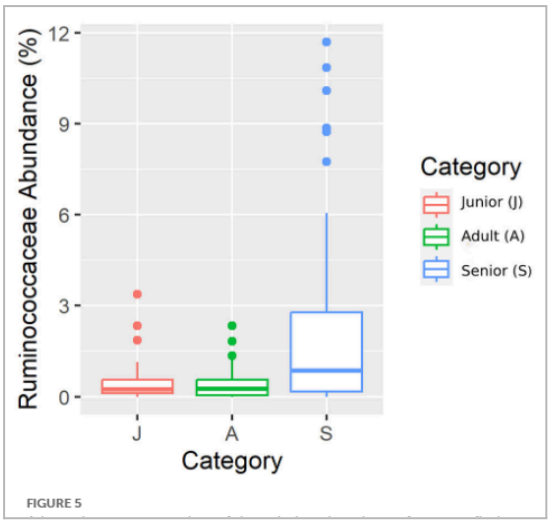
Falling into the main phylum (Bacillota), Ruminococcaceae, Uncl. Clostridiales.1, Veillonellaceae, and Lachnospiraceae families were significantly different among the age categories (Figures 5–8). Specifically, the abundance of Ruminococcaceae and Uncl. Clostridiales.1 was higher in the S category than in the A category, and Veillonellaceae showed a higher abundance in the S category than in the J category. Lachnospiraceae were significantly higher in the J category than in the A and S categories and did not differ significantly. Within the Bacteroidota phylum, the relative abundance of Bacteroidaceae and Prevotellaceae was different among the age categories; Bacteroidaceae was higher in the S category than in the A and J categories, and Prevotellaceae was higher in the S category than in the A. For Pseudomonadota, the relative abundance of Succinivibrionaceae was higher in the S category than in the A category. Finally, the Bifidobacteriaceae family (Actinomycetota) was significantly higher in the A category than in the J category; however, the adjusted q-value was not significant (Table 5).

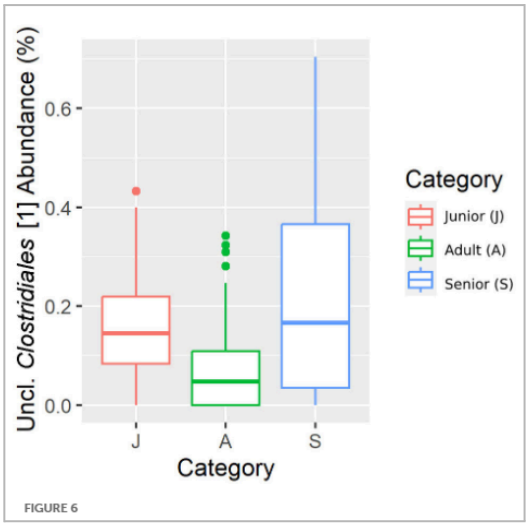
Table 5. Fecal microbiota composition (% of relative abundance from rarefied data) at the family level split by age group.

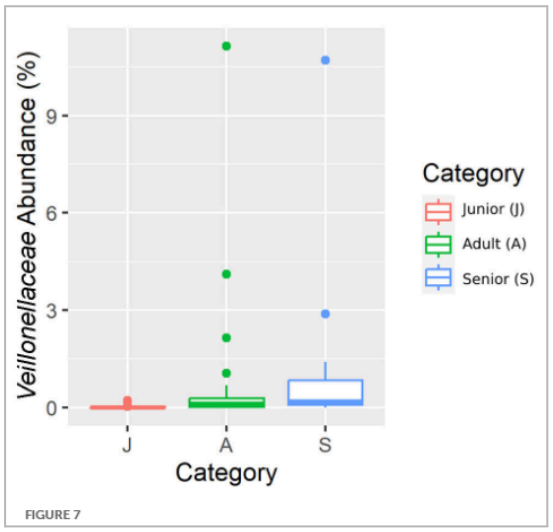
Family	Junior		Adult		Senior		Adult vs. Junior vs. Senior	
	Median	Range	Median	Range	Median	Range	*p*-value	*q*-value
Bifidobacteriaceae	**0.06**[b]	**0–0.70**	**1.43**[a]	**0–13.44**	**0.18**[a,b]	**0–4.78**	**0.024**	**0.066**
Coriobacteriaceae	6.73	1.17–14.05	3.84	0.68–13.01	7.10	0.88–15.69	0.204	0.264
[Paraprevotellaceae]	0.03	0–1.08	0.03	0–1.24	0.15	0–1.05	0.115	0.211
S24-7	0	0–0.5	0.1	0–10.8	0	0–7.8	0.201	0.264
Bacteroidaceae	**0.03**[a]	**0–1.33**	**0.07**[a]	**0–0.44**	**0.73**[b]	**0.21–3.09**	**<0.001**	**0.009**
Prevotellaceae	**0**[a,b]	**0–0.74**	**0a**	**0–0.19**	**0.03**[b]	**0–6.93**	**0.016**	**0.049**
Erysipelotrichaceae	10.01	2.18–20.99	22.31	2.25–83.44	5.58	3.71–52.05	0.199	0.264
Clostridiaceae	34.47	8.34–46.61	24.94	3.07–65.36	39.12	5.27–57.80	0.628	0.641
Uncl. *Clostridiales*.1	**0.17**[a,b]	**0–0.39**	**0.05**[a]	**0–0.28**	**0.19**[b]	**0.05–0.72**	**0.008**	**0.049**
Peptostreptococcaceae	1.17	0–11.38	0.70	0–8.22	0.23	0.03–1.46	0.094	0.189
Lachnospiraceae	**15.13**[b]	**6.16–24.89**	**7.44**[a]	**1.17–19.26**	**13.81**[a,b]	**3.66–23.45**	**0.011**	**0.049**
Turicibacteraceae	7.86	0.14–25.78	1.48	0.11–13.28	2.56	0.33–8.39	0.167	0.263
Streptococcaceae	0.23	0–16.38	1.02	0–22.00	0.19	0–14.31	0.641	0.641
Lactobacillaceae	0.69	0–67.59	0.09	0–20.13	1.01	0–47.93	0.242	0.296
Ruminococcaceae	**0.31**[a,b]	**0–3.45**	**0.25**[a]	**0.01–2.19**	**1.99**[b]	**0–11.46**	**0.014**	**0.049**
Peptococcaceae	0.32	0–0.91	0.23	0–1.82	0.17	0–0.89	0.570	0.627
Uncl. *Clostridiales*.2	0.32	0–0.51	0.29	0–0.71	0.22	0–0.48	0.494	0.572
Veillonellaceae	**0a**	**0–0.18**	**0.01**[a,b]	**0–0.2**	**0.15**[b]	**0–1.25**	**0.009**	**0.049**
Fusobacteriaceae	1.20	0.15–10.52	5.08	0.13–20.34	3.60	0.89–27.90	0.145	0.246
Succinivibrionaceae	**0**[a,b]	**0–0.51**	**0a**	**0–0.11**	**0.03**[b]	**0–0.18**	**0.013**	**0.049**
Alcaligenaceae	0	0–0.20	0.14	0–1.60	0.10	0–2.10	0.051	0.124
Enterobacteriaceae	0	0–0.04	0.02	0–1.59	0.03	0–1.01	0.094	0.189

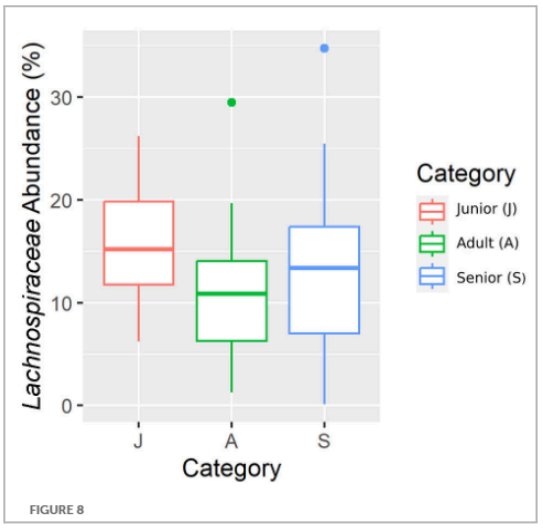
Significantly different results are indicated in bold (p-value < 0.05). Superscript letters indicate differences between age categories.

Figures 5-8. Box plot representation of the relative abundance from rarefied data of the family Ruminococcaceae (figure 5), Uncl. Clostridiales.1 (figure 6), Veillonellaceae (figure 7), and Lachnospiraceae (figure 8) families were significantly different among the age categories.









3.2.3. Genus taxonomic level

The two predominant genera identified in the dogs' population belonged to the phylum Bacillota, *Uncl. Clostridiaceae.1*, and *Allobaculum*. Within Bacillota, *Faecalibacterium*, and *Uncl. Clostridiales.3* showed the highest values in S, being significantly different when compared with A ($p = 0.006$ and 0.008, respectively). The relative abundance observed in the genus *Faecalibacterium* was 18.6 times higher in the S category than in the A category. However, the difference in *Faecalibacterium* was not confirmed by a targeted qPCR. Within this genus, the relative abundance of

F. prausnitzii was 18.4 times higher in the S category (median 1.3% of sequences) than in the A category (median 0.07%; p = 0.006 and q = 0.030). Detailed information at the species level is in the Supplementary Table 3. In relation to *Phascolarctobacterium* abundance, S was higher than the A and J categories (p = 0.011). Although the S category also showed higher values for *Ruminococcus* when compared with A (p = 0.035), the adjusted q-value did not reach significance. Contrarily, the abundance of *Eubacterium, Uncl. Erysipelotrichaceae,* and *Uncl. Lachnospiraceae* was not significantly different when the S category vs. the J and A categories were considered. However, J showed significantly higher values for the three genera when compared with the A category (p = 0.005, p = 0.001, and p = 0.002). Specifically, J showed 8.6 and 7.2 times more relative abundance in *Eubacterium* and *Uncl. Erysipelotrichacea*e genera, respectively, compared with A. Considering the *Uncl. Peptostreptococcaceae* genus, the relative abundance was higher in J than in S and A categories (p = 0.009).

The relative abundance of *Bacteroides* (phylum Bacteroidota) was 24.3 and 10.4 times higher in the S category than in the J and A categories, respectively (p < 0.001). *Prevotella* (Prevotellaceae family) was higher in S than in the A category (p = 0.016); however, the adjusted q-value was not significant. The relative abundance of *Fusobacterium* (phylum Fusobacteriota) was higher in the S category than in the A category when considering only the significance of the p-value (p = 0.045), but this was not confirmed by targeted qPCR. Detailed information at the genus level is in the Supplementary Table 4.

3.3. Fecal SCFAs, IgA, and cCP

Valeric acid was significantly lower in the S category than in the A category. However, the concentrations for total SCFA were numerically higher (but not significant) in the S category and lower in the A and J categories (Table 6). Acetate, butyrate, isovaleric acid, and valeric acid reached significant results when they were analyzed as relative percentages (Table 6). In the post-hoc analysis, the S category showed higher values than A for acetate (p < 0.001) and lower values for butyrate, isovaleric acid, and valeric acid (p = 0.001, p = 0.045, and p = 0.026, respectively). For isovaleric acid, the S category had lower values than J. Finally, no significant

differences among age categories were found for the fecal IgA and cCP concentrations (Table 7).

Table 6. Fecal SCFA values expressed as concentration (µmol/g DM) and relative percentages (%) reported by age category.

Fecal SFCA	Junior	Adult	Senior	
	emmeans ± SE	emmeans ± SE	emmeans ± SE	*p*-value
Concentration (µmol/g DM)				
Acetate	169 ± 26.1	210 ± 15.0	249 ± 21.1	0.061
Propionate	10.2 ± 1.449	12.4 ± 0.837	14.0 ± 1.171	0.149
Butyrate	52.8 ± 8.25	71.4 ± 4.76	61.2 ± 6.66	0.079
Isobutyric acid	6.91 ± 1.109	7.92 ± 0.640	7.27 ± 0.896	0.634
Isovaleric acid	9.72 ± 1.404	8.16 ± 0.811	6.69 ± 1.134	0.249
Valeric acid	**4.55 ± 2.06**^{a,b}	**8.59 ± 1.19**^{a}	**4.70 ± 1.66**^{b}	**0.049**
SCFA total	254 ± 34.7	319 ± 20.0	343 ± 28.0	0.130
Relative percentages (%)				
Acetate	**69.4 ± 1.78**^{a,b}	**66.0 ± 1.02**^{a}	**72.9 ± 1.43**^{b}	**<0.001**
Propionate	4.17 ± 0.342	4.18 ± 0.197	4.23 ± 0.276	0.986
Butyrate	**18.8 ± 1.552**^{a,b}	**22.0 ± 0.896**^{a}	**17.3 ± 1.253**^{b}	**0.003**
Isobutyric acid	2.63 ± 0.304	2.62 ± 0.175	2.22 ± 0.246	0.358
Isovaleric acid	**3.43 ± 0.359**^{a}	**2.69 ± 0.207**^{a,b}	**2.01 ± 0.290**^{b}	**0.011**
Valeric acid	**1.56 ± 0.507**^{a,b}	**2.44 ± 0.293**^{a}	**1.38 ± 0.410**^{b}	**0.043**

Significantly different results are indicated in bold (p-value < 0.05). Superscript letters indicate differences between age categories.

Table 7. Fecal cCP and IgA values by age category.

	Junior	Adult	Senior	
	emmeans ± SE	emmeans ± SE	emmeans ± SE	*p*-value
cCP (ng/g)	4.67 ± 0.564	3.90 ± 0.326	3.79 ± 0.456	0.420
IgA (mg/g)	11.5 ± 3.14	11.1 ± 1.81	12.2 ± 2.54	0.935

Different superscript letters indicate significant differences between groups (p-value < 0.05).

3.4. Correlations among qPCR, IgA, cCP,and SCFA

Considering significant correlations (rho) equal to or > 0.3, *Faecalibacterium*

abundance correlated positively with propionate and valeric acid concentrations, while *Fusobacterium* correlated positively with isobutyric concentration. *Clostridium hiranonis* correlated positively with isovaleric acid percentage, and *Bifidobacterium* correlated positively with valeric acid concentration. This genus correlated positively with the acetate relative percentage, while the correlation with the butyrate relative percentage was negative. *Bacteroides* genus correlated positively with valeric acid concentration and its relative percentage. IgA concentrations correlated negatively with isobutyric, isovaleric, and valeric acid concentrations. This indicator also correlated negatively with isobutyric and valeric absolute percentages. Correlation indexes are detailed in the Supplementary Table 5.

4. DISCUSSION

Our study supports previously published results on the relationship between the aging process and gut health and gut microbiota in dogs. Notably, we included a wider age range (between 0.2 and 15 years old) than previous studies on puppies and adult dogs. In addition, we studied our dog population from different perspectives based on the analysis of fecal microbiota together with other intestinal health biomarkers to broadly understand the overall changes. Our main focus for the discussion of the results is the senior life stage, the period when the quality of life is threatened by the aging process itself. Exploring gut health and microbiota based on fecal indicators may help find specific diet interventions to improve the quality of life of aging dogs.

To compare the selected indicators along the different life stages, this study included dogs with specific diet needs because of their age, which may be considered a confounder to understanding the results among the age categories. However, the diet, together with body weight, sex, feeding routine, housing, and breed, were considered potential explanatory variables for the general statistical analysis.

4.1. Microbiota results

Bacillota, Actinomycetota, Fusobacteriota, Bacteroidota, and Pseudomonadota were

the five main phyla found in the fecal samples analyzed in our studied population of dogs using 16S rRNA gene sequencing. Previous studies described that most bacterial sequences identified in the gut belong to these five different phyla (29–31). In this regard, previous literature may have used the old phylum terminology for Bacillota, Actinomycetota, Fusobacteriota, Bacteroidota, and Pseudomonadota, which were previously called Firmicutes, Actinobacteria, Fusobacteria, Bacterioidetes, and Proteobacteria, respectively (32). Although the names have recently changed, these phyla have been previously identified as the main contributors to gut microbial composition in dogs. More specifically, Bacillota, Bacteroidota, and Fusobacteriota were described as the three predominant phyla in the healthy canine fecal microbiome in previous studies (33, 34). However, in our study, within the three main bacterial groups, the phylum Actinomycetota was found instead of Bacteroidota. Interestingly, the distribution of relative abundance was the same across the three categories. The low abundance in Bacteroidota phyla could be because of obesity and gastrointestinal disease (35–37). Although Actinomycetota is present in the small intestine of healthy animals, the relative abundance found in feces is lower, and its increase may be associated with pathological conditions (29). Considering the health status of the population studied, obesity and gastrointestinal pathologies do not explain the low numbers obtained, and the differences may be explained by the analysis itself. The influence of methodological aspects in 16S rRNA gene sequencing on the results is important to consider, so different studies are difficult to compare. Previous studies have reported significant differences in the abundance of bacterial groups based on variations in labs and methodologies (38–40).

The Bifidobacteriaceae family is an important bacterial group for humans. Considering the results for *Bifidobacterium* in the qPCR and the sequencing analysis, this genus was present in all three age categories (senior dogs, adult dogs, and puppies), being more representative in adults and less representative in puppies. Although intestinal microbiota composition has been studied in different animal species, including dogs, the transition of the intestinal microbiota with age has not been thoroughly investigated. Masuoka et al. (10) studied a different age population, from pre-weanling puppies to senior dogs, to evaluate age-dependent differences and changes in the intestinal microbiota by employing a culture-based

method. Bifidobacteria were detected in puppies but were not present when analyzing dogs older than 3 years old (adult dogs and senior dogs). Based on the literature on human beings, the presence of this genus in the three categories should be understood as positive. Bifidobacteria is the most prevalent bacteria in infants and adults, and it is presumed to promote health benefits in the host (41). However, the extrapolation of the results among animal species should be interpreted carefully. *Bifidobacterium* abundance in the qPCR correlated positively with valeric acid concentration and acetate relative percentage. Positive correlations between *Bifidobacterium* and acetate have been previously described (42, 43), although further studies investigating links with valeric acid may be warranted.

Senior dogs had a higher relative abundance of the families Ruminococcaceae, Veillonellaceae, Bacteroidaceae, and Lachnospiraceae (phylum Bacillota). Previous studies have found decreases at this level in dogs with inflammatory bowel disease (IBD) (25, 44), but there is no clear relationship with aging. Senior dogs also had higher values in the relative percentage of acetate, one of the main products of bacterial fermentation. These two findings are in the same direction as previously published data; there is a well-known positive correlation between the bacterial groups classified in Bacillota phyla and the production of SCFA (25). However, our results supported the fact that relative percentages of butyrate and valeric acid were significantly lower in the population of senior dogs. The relative percentages are logically influenced by the high numbers in the acetate concentration. Focusing on the valeric result, the relative percentage was supported by the concentration result, which was also lower in the senior group. These findings may be interpreted as negative considering gut health since SCFA production provides an appropriate pH environment to maintain healthy microbiota, inhibits the growth of pH-sensitive pathogenic bacteria, and preserves gut integrity (45). Moreover, valeric acid has been positively associated with the gut-brain axis and cognitive function in mice (46). Therefore, senior dogs may not benefit from the mechanisms surrounding the production of SCFA that promote intestinal health and positively contribute to cognitive function.

Although *Faecalibacterium* genera were not significantly different in the qPCR analysis, *Faecalibacterium prausnitzii* was found to be higher in older dogs, which may be considered a minor positive change for this aged population. This

discrepancy may be caused by the fact that *F. prausnitzii* falls into the *Faecalibacterium* genera together with other species. Another possible reason could be that *Facecalibacterium* is frequently undetected in dogs during sequencing due to its low abundance, but it could be detected using qPCR. In addition, *Faecalibacterium* qPCR was significantly positively correlated with propionate and valeric acid concentrations, which is positive for gut health. The absence of this bacterium has been associated with gastrointestinal disorders in dogs (36) and humans (47). Focusing on the species level, *F. prausnitzii* has been clearly associated with increased fiber utilization and different fiber types in the diets of dogs (8, 48). However, the diet's fiber content was not considered a cause of the higher abundance described in the S category for *F. prausintzii*. In our study, only 5% of the dogs were out of the defined fiber composition range (2–5%), and they were similarly distributed in the three different age categories (J = 2/106; A = 2/106; and S = 1/106). From this information, considering the importance of *F. prausnitzii* within the Faecalibacterium genera, we could state that the difference found in senior dogs is a positive change in the microbiota population related to aging.

At the genus level, the two predominant groups identified belong to the phylum Bacillota (*Uncl. Clostridiaceae.1* and *Allobaculum*). These findings support previously published data on dogs, in which major taxa fell within the Bacillota group and the bacterial class Clostridia was considered the most abundant taxon (29). In our study, the *Fusobacterium* genus (within the Fusobacteriota phylum) was present in all the age populations and was specifically higher in dogs over 7 years old. This finding can be considered positive for the microbiome of senior dogs since this genus has previously been related to a healthy intestinal microbiome in dogs (13).

4.2. Beta diversity, alpha diversity, and dysbiosis index

For beta diversity, unweighted UniFrac was significantly different among age categories when ANOSIM analysis was performed. However, the size effect as estimated by the R-value was extremely small, and other distance metrics were not significant. The changes found in our population, and especially the categories defined, may not be enough to change the weighted UniFrac and Bray–Curtis beta diversity. Another possible explanation for the observed differences in the

unweighted UniFrac but not in the weighted UniFrac and Bray–Curtis beta diversity could be attributed to the fact that these two metrics consider relative abundances instead of only the presence/absence of different taxa. Furthermore, alpha diversity analysis measured by the Chao1 and Shannon indexes was not significantly different among the age groups. Our findings are consistent with previous studies. You and Kim (31) studied different individual traits in healthy dogs and also reported no differences at the age level when studying microbial diversity patterns. Interestingly, none of the dogs belonging to the junior category had a moderate or significant change in the index (DI > 0). Blake et al. (15) showed that the DI increased in younger puppies, but many dogs at 9 weeks and most dogs after 6 months of age had normal DI. In our study, the junior age ranged from 2 to 19 months; therefore, our data are consistent with previous studies, as most dogs over 6 months are expected to have a DI of < 0. From those results, we could state that, in our study, small changes in the microbiota may have occurred due to aging. To conclude with the microbiota data, the performance of the Kruskal–Wallis test for assessing differential abundance between groups could be a limitation. This statistical test considers the overall abundance between groups but not the frequency of organism identification in samples within a group. Further statistical tests for assessing differential abundance between groups (e.g., ANCOM and DESeq) could complement the information analyzed by the Kruskal–Wallis test.

4.3. Fecal SCFAs, IgA, and cCP

Fecal cCP and IgA concentrations in feces have been studied as non-invasive biomarkers of gut health in adult dogs. Fecal cCP is an indicator of intestinal inflammation in dogs (49, 50), whereas IgA is associated more with the gut immune response (51). In the present study, the fecal concentration of cCP and IgA was not related to the aging process since no differences were observed among the three age categories. Based on the literature, aged mice and humans did not show a clear relationship between age and the concentration of mucosal IgA (52). Zaine et al. (53) reported no significant differences in IgA fecal values in senior dogs compared with adults and puppies. However, 5-month-old puppies had lower IgA concentrations than adult dogs. Fecal cCP concentrations were recently correlated with age in

healthy humans in Korea (54), but to our knowledge, the results on cCP concentrations in healthy dogs of different ages are not consistent. The lack of clear reference values for fecal cCP and IgA considering the different age stages is a clear limitation to thoroughly understanding our results in relation to age and the SCFA correlation found in our study. However, in our study, age did not change the intestinal inflammation status or gut immune response based on the two indicators analyzed.

4.4. Main features

Considering the overall results of the present study, minor changes in the intestinal health biomarkers analyzed were found among the junior, adult, and senior categories. The microbiota community and SCFA production showed agedependent differences. However, the IgA and cCP were not significantly different across the three categories.

Our age category definition may be a limitation to thoroughly understanding the continuous changes of the microbiome in relation to the aging process. Significantly, no clear consensus exists about this classification in animal research or veterinary medicine (55). The definition of age categories may reduce the sensitivity of understanding whether different ages equally affect gut health and microbiota. Thus, a larger number of animals representative of all age categories may help define more categories to increase the sensitivity of these first results.

In addition, the extrapolation of this trial in a field setting study may help better explore effective strategies, such as dietary interventions, in the dog population.

5. CONCLUSIONS

In conclusion, in this study, the canine gut microbiota and certain SCFAs showed minor variations among age groups in dogs. Other intestinal health biomarkers (IgA and cCP) were similar among age groups. Considering all the parameters, we can state that, once the microbiota becomes stable in healthy dogs, minor changes occur during the aging process.

The modulation of these minor changes, considering the intestinal microbiota and short-chain fatty acid production, could improve the overall gut health during aging in dogs.

Data availability statement: The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm. nih.gov/, PRJNA901473.

Ethics statement: The animal study was reviewed and approved by Affinity Petcare Ethical Committee.

Author contributions: AF-P, AS-M, and CT designed and performed the study. AFP and RP analyzed the results and prepared the tables and graphs for inclusion in the manuscript. AF-P wrote the manuscript. Overall, all authors contributed to the paper, discussed its results, reviewed the drafts, and approved the final version submitted for publication.

Funding: This study received funding from Affinity Petcare. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

Conflict of interest: AF-P, AS-M, and CT were employed by the pet food company Affinity Petcare, which funded the study. JS and RP were employed by the Gastrointestinal Laboratory at Texas A&M University, which provides microbiome assessment on a fee-for-service basis. The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material: The Supplementary Material can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1213287/full#supplementary-material

REFERENCES

1. Matijašic M, Meštrovi´ c T, Paljetak HC, Peri´ c M, Bareši´ c A, Verbanac D.´ Gut microbiota beyond bacteria—mycobiome, virome, archaeome, and eukaryotic parasites in IBD. Int J Mol Sci. (2020) 21:2668. doi: 10.3390/ijms21082668

2. Gill SR, Pop M, DeBoy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. Science. (2006) 312:1355– 9. doi: 10.1126/science.1124234

3. Jandhyala SM, Talukdar R, Subramanyam C, Vuyyuru H, Sasikala M, Reddy DN. Role of the normal gut microbiota. World J Gastroentero. (2015) 21:8787– 803. doi: 10.3748/wjg.v21.i29.8787

4. de Vos WM, Tilg H, Hul MV, Cani PD. Gut microbiome and health: mechanistic insights. Gut. (2022) 71:1020–32. doi: 10.1136/gutjnl-2021-326789

5. Bajinka O, Darboe A, Tan Y, Abdelhalim KA, Cham LB. Gut microbiota and the human gut physiological changes. Ann Microbiol. (2020) 70:65. doi: 10.1186/s13213-020-01608-2

6. Wen L, Duffy A. Factors Influencing the gut microbiota, inflammation, and type2 diabetes. J Nutrition. (2017) 147:1468S–75S. doi: 10.3945/jn.116.240754

7. Hasan N, Yang H. Factors affecting the composition of the gut microbiota, and its modulation. PeerJ. (2019) 7:e7502. doi: 10.7717/peerj.7502

8. Pilla R, Suchodolski JS. The gut microbiome of dogs and cats, and the influence of diet. Vet Clin North Am Small Animal Pract. (2021) 51:605–21. doi: 10.1016/j.cvsm.2021.01.002

9. Badal VD, Vaccariello ED, Murray ER, Yu KE, Knight R, Jeste DV, et al.The gut microbiome, aging, and longevity: a systematic review. Nutrients. (2020) 12:3759. doi: 10.3390/nu12123759

10. Masuoka H, Shimada K, Kiyosue-Yasuda T, Kiyosue M, Oishi Y, Kimura S, et al. Transition of the intestinal microbiota of dogs with age. Biosci Microbiota Food Heal. (2016) 36:27–31. doi: 10.12938/bmfh.BMFH-2016-021

11. Suchodolski JS. Analysis of the gut microbiome in dogs and cats. Vet Clin Path. (2022) 50:6–17. doi: 10.1111/vcp.13031

12. Ziese A-L, Suchodolski JS. Impact of changes in gastrointestinal microbiota in canine and feline digestive diseases. Vet Clin North Am Small Animal Pract. (2021) 51:155–69. doi: 10.1016/j.cvsm.2020.09.004

13. Garrigues Q, Apper E, Chastant S, Mila H. Gut microbiota development in the growing dog: a dynamic process influenced by maternal, environmental and host factors. Front Vet Sci. (2022) 9:964649. doi: 10.3389/fvets.2022.964649

14. Guard BC, Mila H, Steiner JM, Mariani C, Suchodolski JS, Chastant-Maillard S. Characterization of the fecal microbiome during neonatal and early pediatric development in puppies. PLoS ONE. (2017) 12:e0175718. doi: 10.1371/journal.pone.0175718

15. Blake AB, Cigarroa A, Klein HL, Khattab MR, Keating T, Coevering PVD,et al. Developmental stages in microbiota, bile acids, and clostridial species in healthy puppies. J Vet Intern Med. (2020) 34:2345–56. doi: 10.1111/jvim.15928

16. FEDIAF. The European Pet Food Industry. Nutritional Guidelines for Complete and Complementary Pet Food for Cats and Dogs. (2021). Available online at: https://europeanpetfood.org/wp-content/uploads/2022/03/Updated-Nutritional-Guidelines.pdf

17. Strickling JA, Harmon DL, Dawson KA, Gross KL. Evaluation of oligosaccharide addition to dog diets: influences on nutrient digestion and microbial populations. Anim Feed Sci Technol. (2000) 86:205–19. doi: 10.1016/S0377-8401(00)00175-9

18. Parada AE, Needham DM, Fuhrman JA. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. Environ Microbiol. (2015) 18:1403–14. doi: 10.1111/1462-2920.13023

19. Apprill A, McNally S, Parsons R, Weber L. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. Aquat Microb Ecol. (2015) 75:129–37. doi: 10.3354/ame 01753

20. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA,et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. (2019) 37:852–7. doi: 10.1038/s41587-019-0209-9

21. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP.DADA2: high resolution sample inference from Illumina amplicon data. Nat Methods. (2016) 13:581–3. doi: 10.1038/nmeth.3869

22. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microb. (2005) 71:8228–35. doi: 10.1128/AEM.71.12.8228-8235.2005

23. AlShawaqfeh MK, Wajid B, Minamoto Y, Markel M, Lidbury JA, SteinerJM, et al. A dysbiosis index to assess microbial changes in fecal samples of dogs with chronic inflammatory enteropathy. FEMS Microbiol Ecol. (2017) 93:fix136. doi: 10.1093/femsec/fix136

24. Moreau NM, Goupry SM, Antignac JP, Monteau FJ, Bizec BJL, ChampMM, et al. Simultaneous measurement of plasma concentrations and 13Cenrichment of short-chain fatty acids, lactic acid and ketone bodies by gas chromatography coupled to mass spectrometry. J Chromatogr B. (2003) 784:395– 403. doi: 10.1016/S1570-0232(02)00827-9

25. Minamoto Y, Minamoto T, Isaiah A, Sattasathuchana P, Buono A, RangachariVR, et al. Fecal short-chain fatty acid concentrations and dysbiosis in dogs with chronic enteropathy. J Vet Intern Med. (2019) 33:1608–18. doi: 10.1111/jvim. 15520

26. Heilmann RM, Suchodolski JS, Steiner JM. Development and analytic validationof a radioimmunoassay for the quantification of canine calprotectin in serum and feces from dogs. Am J Vet Res. (2008) 69:845–53. doi: 10.2460/ajvr.69.7.845

27. Heilmann RM, Ruaux CG, Grützner N, Cranford SM, Bridges CS, Steiner JM.Biological variation of serum canine calprotectin concentrations as measured by ELISA in healthy dogs. Vet J. (2019) 247:61–4. doi: 10.1016/j.tvjl.2019.03.002

28. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and

powerful approach to multiple testing. J R Stat Soc B. (1995) 57:289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

29. Pilla R, Suchodolski JS. The role of the canine gut microbiome and metabolome in health and gastrointestinal disease. Front Vet Sci. (2020) 6:498. doi: 10.3389/fvets.2019.00498

30. Deschamps C, Humbert D, Zentek J, Denis S, Priymenko N, Apper E, et al. FromChihuahua to Saint-Bernard: how did digestion and microbiota evolve with dog sizes. Int J Biol Sci. (2022) 18:5086–102. doi: 10.7150/ijbs.72770

31. You I, Kim MJ. Comparison of gut microbiota of 96 healthy dogs by individual traits: breed, age, and body condition score. Anim Open Access J. (2021) 11:2432. doi: 10.3390/ani11082432

32. Oren A, Garrity GM. Valid publication of the names of forty-two phyla of prokaryotes. Int J Syst Evol Microbiol. (2021) 71:005056. doi: 10.1099/ijsem.0.005056

33. Hand D, Wallis C, Colyer A, Penn CW. Pyrosequencing the canine faecal microbiota: breadth and depth of biodiversity. PLoS ONE. (2013) 8:e53115. doi: 10.1371/journal.pone.0053115

34. Middelbos IS, Boler BMV, Qu A, White BA, Swanson KS, Fahey GC. Phylogenetic characterization of fecal microbial communities of dogs fed diets with or without supplemental dietary fiber using 454 pyrosequencing. PLoS ONE. (2010) 5:e9768. doi: 10.1371/journal.pone.0009768

35. Salas-Mani A, Jeusette I, Castillo I, Manuelian CL, Lionnet C, Iraculis N, et al.Fecal microbiota composition changes after a BW loss diet in Beagle dogs. J Anim Sci. (2018) 96:3102–11. doi: 10.1093/jas/sky193

36. Minamoto Y, Otoni CC, Steelman SM, Büyükleblebici O, Steiner JM, Jergens AE, et al. Alteration of the fecal microbiota and serum metabolite profiles in dogs with idiopathic inflammatory bowel disease. Gut Microbes. (2015) 6:33– 47. doi: 10.1080/19490976.2014.997612

37. Söder J, Wernersson S, Höglund K, Hagman R, Lindåse S, Dicksved J.

Composition and short-term stability of gut microbiota in lean and spontaneously overweight healthy Labrador retriever dogs. Acta Vet Scand. (2022) 64:8. doi: 10.1186/s13028-022-00628-z

38. Handl S, Dowd SE, Garcia-Mazcorro JF, Steiner JM, Suchodolski JS. Massiveparallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats. Fems Microbiol Ecol. (2011) 76:301– 10. doi: 10.1111/j.1574-6941.2011.01058.x

39. Garcia-Mazcorro JF, Lanerie DJ, Dowd SE, Paddock CG, Grützner N, SteinerJM, et al. Effect of a multi-species synbiotic formulation on fecal bacterial microbiota of healthy cats and dogs as evaluated by pyrosequencing. Fems Microbiol Ecol. (2011) 78:542–54. doi: 10.1111/j.1574-6941.2011.01185.x

40. Guard BC, Barr JW, Reddivari L, Klemashevich C, Jayaraman A, Steiner JM, et al.Characterization of microbial dysbiosis and metabolomic changes in dogs with acute diarrhea. PLoS ONE. (2015) 10:e0127259. doi: 10.1371/journal.pone.0127259

41. Mitsuoka T. History and evolution of probiotics. Jpn J Lact Acid Bact. (2011) 22:26–37. doi: 10.4109/jslab.22.26

42. Oliphant K, Allen-Vercoe E. Macronutrient metabolism by the human gutmicrobiome: major fermentation by-products and their impact on host health. Microbiome. (2019) 7:91. doi: 10.1186/s40168-019-0704-8

43. Koh A, De Vadder F, Kovatcheva-Datchary P, Bäckhed F. From dietary fiberto host physiology: short-chain fatty acids as key bacterial metabolites. Cell. (2016) 165:1332–45. doi: 10.1016/j.cell.2016.05.041

44. Suchodolski JS, Dowd SE, Wilke V, Steiner JM, Jergens AE. 16S rRNA genepyrosequencing reveals bacterial dysbiosis in the duodenum of dogs with idiopathic inflammatory bowel disease. PLoS ONE. (2012) 7:e39333. doi: 10.1371/journal.pone.0039333

45. Mackei M, Talabér R, Müller L, Sterczer Á, Fébel H, Neogrády Z, et al. Altered intestinal production of volatile fatty acids in dogs triggered by lactulose and psyllium treatment. Vet Sci. (2022) 9:206. doi: 10.3390/vetsci90 50206

46. Lai Z, Shan W, Li J, Min J, Zeng X, Zuo Z. Appropriate exercise levelattenuates gut dysbiosis and valeric acid increase to improve neuroplasticity and cognitive function after surgery in mice. Mol Psychiatr. (2021) 26:7167– 87. doi: 10.1038/s41380-021-01291-y

47. Varela E, Manichanh C, Gallart M, Torrejón A, Borruel N, Casellas F,et al. Colonisation by *Faecalibacterium prausnitzii* and maintenance of clinical remission in patients with ulcerative colitis. Aliment Pharm Therap. (2013) 38:151– 61. doi: 10.1111/apt.12365

48. Mori A, Goto A, Kibe R, Oda H, Kataoka Y, Sako T. Comparison of the effects of four commercially available prescription diet regimens on the fecal microbiome in healthy dogs. J Vet Med Sci. (2019) 81:1783–90. doi: 10.1292/jvms.19-0055

49. Grellet A, Heilmann RM, Lecoindre P, Feugier A, Day MJ, Peeters D, et al. Faecal calprotectin concentrations in adult dogs with chronic diarrhea. Am J Vet Res. (2013) 74:706–11. doi: 10.2460/ajvr.74.5.706

50. Otoni CC, Heilmann RM, García-Sancho M, Sainz A, Ackermann MR,Suchodolski JS, et al. Serologic and fecal markers to predict response to induction therapy in dogs with idiopathic inflammatory bowel disease. J Vet Intern Med. (2018) 32:999–1008. doi: 10.1111/jvim.15123

51. Grellet A, Mila H, Heilmann RM, Feugier A, Gruetzner N, Suchodolski JS, et al.Effect of age, gestation and lactation on faecal IgA and calprotectin concentrations in dogs∗. J Nutritional Sci. (2014) 3:e41. doi: 10.1017/jns.2014.44

52. Fujihashi K, McGhee JR. Mucosal immunity and tolerance in the elderly. Mech Ageing Dev. (2004) 125:889–98. doi: 10.1016/j.mad.2004.05.009

53. Zaine L, Ferreira C, de OS Gomes M, Monti M, Tortola L, Vasconcellos RS,et al. Faecal IgA concentration is influenced by age in dogs. Brit J Nutr. (2011) 106:S183–6. doi: 10.1017/S0007114511000559

54. Park SY. Age-related fecal calprotectin concentrations in healthy adults. Korean J Clin Laboratory Sci. (2020) 52:181–7. doi: 10.15324/kjcls.2020.52.3.181

55. Harvey ND. How old is my dog? identification of rational age groupings in pet

dogs based upon normative age-linked processes. Front Vet Sci. (2021) 8:643085. doi: 10.3389/fvets.2021.643085

CHAPTER II

Article

Age-Related Changes in Gut Health and Behavioral Biomarkers in a Beagle Dog Population

Anna Fernández-Pinteño[1], Rachel Pilla[2] , Jan Suchodolski[2], Emmanuelle Apper[1], Celina Torre[1], Anna Salas-Mani[1] and Xavier Manteca[3]

[1] Department of Research and Development, Affinity Petcare, 08902 L'Hospitalet de Llobregat, Spain

[2] Gastrointestinal Laboratory, Department of Small Animal Clinical Sciences, Texas A&M University, College Station, TX 77843, USA

[3] School of Veterinary Science, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

This chapter has been published in Animals.

Citation: Fernández-Pinteño, A.; Pilla, R.; Suchodolski, J.; Apper, E.; Torre, C.; Salas-Mani, A.; Manteca, X. Age-Related Changes in Gut Health and Behavioral Biomarkers in a Beagle Dog Population. Animals 2025, 15, 234. https://doi.org/10.3390/ani15020234

SIMPLE SUMMARY

The gut and its microbiome communicate with the brain through the gut–brain axis. The examination of the relationship between the gut and the brain concerning aging is particularly relevant for preserving the quality of life in senior dogs. Studies investigating this axis in dogs of different age groups remain limited. Thus, this study aims to examine multiple blood and fecal biomarkers of intestinal health, along with various behavioral indicators based on saliva, blood, observations, and activity, in a different age population (junior: < 2 y.o.; adult: 2–7 y.o.; senior: > 7 y.o.) of thirty-seven Beagle dogs. The results showed that *Bacteroides* were significantly higher in senior dogs. The relative abundance of *Faecalibacterium* and *Blautia* showed age-related trends, higher in seniors and juniors, respectively. Fecal short-chain fatty acid concentration, especially acetate, increased with age, while propionate was higher in junior dogs. For the behavioral indicators we considered, blood thyroxine concentration, playing, exploring, and total activity were higher in junior dogs. These findings suggest that the relationship between gut health and behavior varies with age. This highlights the importance of taking age into account when studying gut health and behavior. However, more research is needed to fully understand the mechanisms behind these age-related changes.

ABSTRACT

The gut and the gut microbiome communicate with the nervous system through the gut–brain axis via neuroimmune and neuroendocrine mechanisms. Despite existing research, studies exploring this link in aging dogs are limited. This study aims to examine multiple blood and fecal biomarkers of intestinal health, along with various behavioral indicators based on saliva, blood, observations, and activity, in different age populations (junior: < 2 y.o.; adult: 2–7 y.o.; senior: > 7 y.o.) of thirty-seven Beagle dogs. In our study, *Bacteroides* were significantly higher in senior dogs. The relative abundance of *Faecalibacterium* and *Blautia* showed age-related trends, higher in senior and junior dogs, respectively. Fecal short-chain fatty acid concentration, especially acetate, increased with age, while propionate was higher in junior dogs. For the behavioral indicators we considered, blood thyroxine concentration, playing, exploring, and total activity were higher in junior dogs. The differences observed between the biomarkers of gut health and behavior, particularly those significant for the age correlations, emphasize the importance of considering age-related factors when studying the gut microbiome and behavior. However, further research is needed to better understand the mechanisms and specific pathways involved in the relationship between the studied biomarkers and age.

Keywords: fecal microbiota; behavior; canine; aging; health; nutrition; gut–brain axis

1. INTRODUCTION

The gut microbiome is composed of bacteria, archaea, fungi, protozoa, and viruses inhabiting the gastrointestinal tract. These communities play a crucial role in important metabolic functions and essential immune responses that protect against pathogens and help maintain the well-being of their host, contributing to an equilibrated health status in dogs and cats [1,2].

There is increasing scientific evidence supporting the relationship between the gut microbiota and different organs and systems. Multiple studies in mammals have demonstrated that changes in the gut microbiota are associated with some pathological states, for example, inflammation, obesity, metabolic diseases [1], changes in behavior [2], and emotional states [3]. The pathways through which one body part biochemically communicates with another body part are known by the scientific community as the ‘axis’. The gut and the gut microbiota participate in specific axes through the portal vein, the nerve pathways, or through the intestinal barrier into the blood circulation. In the gut–brain axis (GBA), the gut microbiota communicates to the host nervous system through immune, neuroendocrine, and neural mechanisms. The gut microbes modulate the central nervous system (CNS) principally through neuroimmune and neuroendocrine mechanisms in which the vagus nerve and specific active metabolites are involved [4–6]. The gut microbiota can produce several neuroactive molecules, including serotonin, dopamine, gamma-aminobutyric acid (GABA), tryptophan (TRP), and short-chain fatty acids (SCFAs) [7]. The neuroactive compounds and hormones can influence locally the gut epithelium physiology and can go into the bloodstream to reach the brain. On the other hand, the brain can modulate the gut microbiota composition and function mainly through the autonomic nervous system and the hypothalamic–pituitary–adrenal (HPA) axis by changing the gut motility, the gut permeability, and the secretion of mucus and hormones [4,7].

In humans, the dysfunction of the GBA and the composition of the gut microbiota are closely associated with various psychiatric pathologies, including depression, anxiety, autism, and neurodegenerative and neuroinflammatory disorders [8–11]. Li et al. [12] proved the role played by the gut microbiota in the cognitive decline associated with typical aging in rats. In dogs, scientific evidence on the relationship

between microbiota and neurodegenerative diseases remains limited, while they may impact the quality of life of both dogs and humans. However, recent studies have begun to investigate the connection between the gut–brain axis (GBA) and microbiota composition. Aggressive behavior in shelter dogs has been associated with distinct patterns in the gut microbiome [13,14]. Craddock et al. [15] studied a population of working dogs in which certain microbiome markers were related to performance (motivation, aggression, cowardice/hesitation, sociability, and obedience). Additionally, a review was recently published exploring the use of prebiotics and probiotics solutions linked to canine anxiety [16]. On the other hand, neurodegenerative disorders in dogs, such as canine cognitive dysfunction and canine multiple system degeneration, have been studied as homologous pathologies of Alzheimer's disease and Parkinson's disease in humans [10]. Interestingly, a recent review summarized the key scientific findings on the connection between behavioral disorders and the gut microbiota in dogs, including similar findings in humans and rodent models [17].

Despite growing interest in the gut–brain axis (GBA), there is a significant gap in research exploring the relationship between gut microbiota and the GBA in aged dogs. The primary aim of this study is to investigate the role of age and gut microbiota in the GBA by analyzing various biomarkers of intestinal health and behavior across different age groups of healthy Beagle dogs. Specifically, the study examines gut health through multiple blood and fecal biomarkers alongside behavioral indicators assessed through saliva, blood analysis, direct observations, and activity monitoring. These findings will contribute to the development of strategies to support gut health and overall well-being in senior dogs.

2. MATERIALS AND METHODS

2.1. Animals

This study included 37 Beagle dogs grouped in junior (J), adult (A), and senior (S) categories depending on their age: up to 2 years old (n = 10), from 2 to 7 years old (n = 16), and over 7 years old (n = 11). Both sterilized females and males were included. The inclusion criteria consisted of Beagle dogs sterilized individuals in good health. Health was evaluated through a clinical examination performed by veterinarians and a blood analysis, which included a complete blood count and a biochemical panel. The biochemical analysis included albumin, alkaline phosphatase, total bilirubin, calcium, creatine kinase, cholesterol, creatinine, fructosamine, aspartate aminotransferase, alanine aminotransferase, globulins, phosphorus, proteins, albumin/globulin ratio, reticulocyte hemoglobin content, IDEXX SDMA™, triglycerides, and urea. The exclusion criteria comprised existing clinical diseases or any pharmacological treatments potentially interfering with the studied biomarkers based on the literature. Parasitology coprological tests were conducted on a routine basis to ensure the health status of animals regarding the presence of fecal parasites and derived clinical signs. No antibiotics, prebiotics, probiotics, postbiotics, or deworming treatments were administered during the sampling period, including a four-week washout period before starting the sampling. Dogs defecating watery, soft, or unformed stools for three consecutive days were excluded from the study.

The animals were housed in the same experimental facilities (Affinity Nutrition Center, Masquefa, Spain) under similar husbandry conditions. They lived in pairs, and they had free access to the outdoor and indoor part of their kennels (15 m2). They also shared a bigger outdoor space for about four hours per day where they could socialize with a stable larger group of Beagles. During this time, the facilities were cleaned and disinfected. After the outdoor activity, the dogs came back to their kennels, and they were fed once with a commercially dry, complete, and balanced diet adequate to their needs. Daily rations were calculated following the energy requirements for each dog. The feeding routine varied between dogs: some dogs rotated their diets less than every 14 days, while others had a stable diet over time. The dogs were weighed weekly to monitor the maintenance of a stable body weight.

Non-special diets were offered because of the study. Considering the following diet composition range—7–8% moisture, 22–30% protein, 12–21% fat, and 1–5% crude fiber—mainly all the dogs (95%) ate diets within the appropriate range. Water supply was offered ad libitum 24 h per day.

2.2. Sample Collection: Feces, Blood, and Saliva

The samples collected for assessing intestinal health and behavioral markers came from feces, blood, and saliva.

Feces: Fecal samples were collected over a three-month period (March, April, and May) without disturbing the dogs' daily routines (Table 1). The animals were not individually separated or placed in cages for sample collection. Instead, the method of direct observation was employed to identify individual fecal samples. Samples were collected while the dogs were in their kennels or when they were outdoors at their parks.

Table 1. Detailed information about the number of fecal samples collected and their distribution over time by month. The samples are categorized by age group.

	Junior	Adult	Senior	Total
March	5	8	4	17
April	0	8	7	15
May	5	0	0	5
Total	10	16	11	37

A single stool sample was collected from each dog and evaluated on a five-point scale to assess fecal consistency: 0 = watery stool, 25 = soft unformed stool, 50 = soft-formed and moist stool that retains its shape when collected, 75 = hard-formed stool that remains somewhat soft, and 100 = hard dry stool. This scale has already been applied in previous research studies [18,19]. If the fecal consistency was at or below 50, the fecal score (FS) was recorded, but the sample was not collected, awaiting another sample with a higher FS. The entire stool or a sufficient amount to fill a sterile fecal collection tube of 250 mL was collected, ensuring the exclusion of any external contaminants such as stones, grass, or sand. Within 4 h after excretion,

all samples were processed using sterile, disposable materials. Following the specific requirements for the analysis, the collected fecal samples were divided into two subsamples.

The first subsample was prepared for microbiota analysis, with a minimum quantity of 0.5 g of feces introduced into a sterile 1.5 mL tube free of RNAse. The microbiota analysis was performed by Illumina sequencing of the bacterial 16S rRNA genes using primers 515F (5′ GTGYCAGCMGCCGCGGTAA) [20] and 806RB (5′ GGACTACNVGGGTWTCTAAT) [21] at the MR DNA laboratory (Shallowater, TX, USA). Sequence processing and analysis were carried out using the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) v2021.8 pipeline [22]. The sequences were demultiplexed, and an ASV table was generated using DADA2 [23]. Prior to downstream analysis, sequences classified as chloroplasts, mitochondria, and low-abundance ASVs (comprising < 0.01% of the total reads) were filtered out. All samples were rarefied to even sequencing depth, based on the lowest read depth of samples, to 21,025 sequences per sample. Alpha diversity was assessed using Chao1 (richness) and Shannon diversity indices with QIIME2. Beta diversity was calculated using both unweighted and weighted UniFrac distance metrics (phylogeny-based measures) and the Bray–Curtis dissimilarity metric, with results visualized in Principal Coordinate Analysis (PCoA) plots generated in QIIME2. For the microbiota analysis, the previous scientific nomenclature for classifying different taxa was considered; the same nomenclature was used when writing the current paper. Quantitative PCR assays were used to target total bacteria, *Faecalibacterium* spp., *Turicibacter* spp., *Escherichia coli*, *Streptococcus* spp., *Blautia* spp., *Fusobacterium* spp., and *Clostridium hiranonis*. From this panel of bacterial groups, the Dysbiosis Index (DI) was calculated. This validated algorithm was used to assess a potential intestinal dysbiosis, where a DI value of < 0 is categorized as normal, a DI between 0 and 2 indicates a mild to moderate shift in microbiota composition, and a DI > 2 is classified as significant dysbiosis [24].

The second subsample was prepared for the analysis of SCFA concentration and proportion, canine calprotectin (cCP), and immunoglobulin A (IgA) concentrations. In this case, 5 g of feces were pooled in a sterile stool tube. Both types of tubes were carefully filled and stored at −80 ◦C until they were dispatched for analysis at the Small Animal Clinical Sciences Department located at Texas A&M University,

College Station, TX, USA.

Saliva and blood: Saliva and blood samples were collected from approximately 8:30 to 12:30 am. All the dogs were previously fasted for 8 h to avoid any interference in the measurements. Initially, the dogs were moved to a familiar area close to their kennels. Within the first five minutes, saliva samples were collected by rubbing a synthetic swab specifically designed for diagnostic saliva collection (Salivette cortisol tubes; Sarstedt, Germany) on the dogs' tongue and the inside surface of their cheeks. The swab was then promptly returned to the saliva collection tube. Subsequently, blood sampling was conducted through jugular vein venipuncture. The total volume required was 5.5 mL:0.5 mL of complete blood (EDTA blood collection tube) for a complete cell blood count (CBC) analysis and 5 mL collected for analyzing various serum biomarkers.

After the sampling, the saliva and blood samples were prepared as follows for analysis. The saliva collection tubes were centrifuged at room temperature at 1420 G for 10 min.

The swab was removed, and the saliva was extracted and transferred from the tube to a 1.5 mL tube (Eppendorf). The minimum volume needed for salivary cortisol (CORT) and oxytocin (OT) analysis was 300 µL of saliva. The Eppendorf tubes were then stored at −80 ◦C until shipment to the lab (Interdisciplinary Laboratory of Clinical Analysis, Murcia, Spain). OT concentration was determined using the validated method for dogs AlphaLISA monoclonal assay [25]. CORT concentration was analyzed using the Siemens Immulite automated chemiluminescence assay.

Serum collection tubes were set at room temperature for 30 min and centrifuged at 1420 G for 10 min. The serum volume obtained after centrifugation was approximately 1.3 mL, divided into four subsamples: two 500 µL subsamples, one 200 µL subsample, and one 100 µL subsample. Three subsamples were stored at −80 ◦C. One 500 µL subsample was sent to be refrigerated, along with the EDTA blood collection tube, to Idexx laboratory (Barcelona, Spain) for a biochemistry profile and to assess thyroxine levels (TT4). The 200 µL subsample was used to analyze cobalamin (B12) and folate (B9) concentrations by using chemiluminescence assays (Immulite 2000 Vitamin B12; Folic Acid, Siemens Medical Solutions Diagnostics, Erlangen, Germany) in the Small Animal Clinical

Sciences Department located in Texas A&M University (College Station, TX, USA). In the 100 µL subsample, tryptophan and kynurenine (KYN) concentrations were determined using an L-Tryptophan ELISA kit (ImmuSmol, ref. BA E-2700, Bordeaux, France) and an L-Kynurenine ELISA kit (ImmuSmol, ref. BA E-2200, Bordeaux, France). An EMS Reader MF V.2.9-0 was used to read the ELISA plates (Clinical biochemistry laboratory at the Autonomous University of Barcelona, Barcelona, Spain). The last 500 µL subsample stored at −80 ◦C was sent to determine canine C-reactive protein (CRP) and haptoglobin (HP) concentrations by immunoturbidimetric assays validated for dogs [26,27] (Interdisciplinary Laboratory of Clinical Analysis, Murcia, Spain).

2.3. Behavioral Observations and Activity

Behavioral observations were conducted one week after the saliva and blood sampling to avoid interferences. The observations were performed by the same trained observer, and all behaviors were classified according to a predetermined ethogram (Table 2). The behavior was measured both in their kennels and in their outdoor parks, where dogs spent around 20 h and 4 h, respectively. Regarding the observations in the kennels, the animals were recorded during the morning and the afternoon. One hour was selected for each period to avoid interference with daily routines, such as feeding, outdoor time, or cleaning. Each 60 min period was divided into intervals of 10 min and scan observations were conducted for each interval. In total, twelve scans were performed per animal, and twelve behaviors were assigned to each animal. The area recorded was only the indoor one; if the animal was in the outdoor area of the kennel, the observation was considered null. The behavioral assessments in the outdoor parks were performed for 60 min and repeated on 2 different days. In total, twelve scans were performed per animal assigning twelve different behaviors to each animal. A null observation was recorded when any park structure obstructed a clear view of the animal, making it difficult to properly categorize its behavior.

Table 2. Ethogram containing the behaviors identified in the behavioral observations conducted both in the kennels and in the outdoor parks. All the behaviors described have been assessed in the kennels and in the outdoor parks.

Behavior	**Abbreviation**	**Description**
Drinking	D	The dog laps or takes up water with its tongue from the drinking bowl.
Eliminating	EL	Urinating and defecating behaviors are included in this category.
Sleeping	SL	The dog lies down, its breathing frequency decreases, and its eyes are often closed or partially closed.
Resting	R	The dog lies down but is awake. The corporal position is relaxed and not alerted.
Playing	P	Engaging in activities for entertainment involving physical movements and interactions with other inanimate objects (e.g., toys).
Exploring	EX	Investigating and interacting with the environment to gather information and satisfy curiosity. Sniffing is often present during the exploratory behavior.
Affiliative behavior	AF	Displaying actions that strengthen social bonds and promote positive interactions with conspecifics. It should be an interaction with a conspecific.
Grooming	G	Cleaning and maintaining the coat and the skin is often conducted to self-regulate (performed in a relaxed manner) or to increase social bonds between the group.
Overgrooming	OG	Excessive or compulsive self-grooming, the dog is really focused on the performance of this behavior, and the posture is tense.
Agonistic behavior	AG	Displaying behaviors associated with conflict, competition, or aggression, including growling and persecution.
Barking Defensive	BD	Vocalization is conducted together with a defensive posture that may include a lowered body, ears back, tail tucked, and a still demeanor.
Barking Offensive	BO	Vocalization is accompanied by an offensive posture that may involve a forward-leaning body position, forward-pointing ears, stiff legs, and a raised and rigid tail.

Hiding	H	Withdrawing or seeking seclusion to avoid social interactions or perceived threats. Fear and alertness behaviors are often shown.
Destructive Behavior	DB	Actions or behaviors such as chewing, digging, scratching, or other activities that result in damage to objects and structures.
Repetitive Behavior	RB	Engaging in actions or movements in a repetitive and stereotypical manner, such as pacing or circling.
High Alert	HA	Being in a state of heightened awareness and attention, often accompanied by increased vigilance and sensitivity to stimuli. The dog can be either standing or lying down.
Coprophagia	C	Consumption of dog feces.
Walking	W	Moving from one place to another with no apparent objective (e.g., chasing a dog, playing with other dogs).

For the activity measurements, a physical activity actigraphy monitor (Actical®, Philips Respironics, Bend, OR, USA) was placed on the dogs' collars for 4 days to measure their activity during three consecutive days. The first day was not recorded since this period was left for adaptation. Dogs were previously trained to wear the device. The activity was retrieved as total activity (TAC) (24 h) and then divided into diurnal activity (DAC) (from 7:00:00 to 20:59:59) and nocturnal activity (NAC) (from 21:00:00 to 6:59:59).

2.4. Definition of the Study Variables

The primary explanatory variable was defined as the age category, either junior, adult, or senior. The model was adjusted using several demographic variables, including body weight, sex, feeding routine, and housing. The feeding routine consisted of two categories: "rotation", indicating animals that changed their diet type within periods shorter than two weeks, and "stable", indicating animals that consistently consumed the same diet for at least four weeks. Each animal was exclusively assigned to one category throughout the entire study. Housing was the variable used to identify the two different buildings where animals were housed.

The response variables were classified into three groups: biomarkers of intestinal health, behavioral observations, and activity measurements. The intestinal health biomarkers measured in feces included microbiota composition using the 16S rRNA method, qPCR results for absolute quantity of total bacteria, *Faecalibacterium, Turicibacter, Streptococcus, E. coli, Blautia, Fusobacterium, Clostridium hiranonis, Bifidobacterium, Bacteroides, Lactobacillus,* and the Dysbiosis Index (DI) [27]. Within the same category of intestinal health biomarkers, alpha diversity (Chao 1 and Shannon diversity), FS, SCFA concentrations in fecal dry matter (µmol/g), SCFA relative amounts (%), cCP (ng/g), and IgA (mg/g) concentrations were also analyzed. The intestinal health biomarkers measured in blood serum included B9 (ng/mL), B12 (pg/mL), CRP (µg/mL), HP (g/L), TT4 (µg/dL), TRP (µg/mL), and KYN (ng/mL) concentrations.

For the second group of response variables, defined as behavioral indicators, salivary CORT (µg/dL), salivary OT (pg/mL) concentrations, and behavioral observations were included. Regarding the observations, the relative frequencies of the different defined behaviors were calculated for statistical analysis. Finally, activity measurements completed the set of response variables considered for statistical analysis.

2.5. Statistical Analysis

For the general statistical analysis, thirty-five dogs were considered, as the behavioral markers were missing in two senior dogs. For the descriptive statistics, quantitative variables were assessed using mean and standard deviation, while qualitative variables were examined through relative and absolute frequencies. Pearson's (or Spearman's) correlations were assessed between age, considered as a quantitative continuous variable, and the other parameters.

Then, differences among age categories were studied by applying the appropriate test according to the distribution of the variable (ANOVA; Kruskal–Wallis test), with the null hypothesis assuming equality between groups. The compliance of application criteria was verified using Shapiro–Wilk's normality test.

To investigate the relationship between age categories and the studied variables, the

appropriate linear model was performed, including body weight, sex, feeding routines, and housing as potential explanatory variables. The estimated marginal means (emmeans) and standard error (SE) for the age categories were calculated for each variable using the adjusted model. Finally, the post-hoc comparisons were performed through the Pairwise analysis. The relation between the response variables and the age as a quantitative variable was analyzed using Spearman's correlation.

The correlation between gut health biomarkers and behavioral indicators (biomarkers of behavior, behavioral observations, and activity) was evaluated using pairwise Spearman correlations. These correlations were studied considering all the animals as a group (including the three age categories) and then split by age category. This statistical approach allowed for the evaluation of potential interactions between age and the markers of interest and, more precisely, to detect different age patterns when studying the possible association between gut health biomarkers and behavioral indicators. The microbial groups included in the correlations were based on the qPCR analysis.

For the microbiota statistical analysis (16S rRNA sequencing analysis), the ANOSIM (Analysis of Similarity) test within the PRIMER 7 software package (PRIMER-E Ltd., Luton, UK) was employed to investigate the differences in microbial communities and the size effect (R-values ranging from 0 to 1, with a higher R-value indicating a larger size effect) between the defined age categories. The normality of all datasets was assessed using the Shapiro–Wilk test (JMP Pro 11, SAS Software Inc., Cary, NC, USA). Subsequently, the Kruskal–Wallis test was conducted (Prism v.9, GraphPad Software Inc., La Jolla, CA, USA), followed by post-hoc analysis using Dunn's multiple comparison test to identify age category differences in bacterial taxa. To control for multiple comparisons, all p-values were adjusted using Benjamini and Hochberg's False Discovery Rate [28] at each taxonomic level. In the statistical analyses, significance was established at a $p\text{-value} < 0.05$, while a $p\text{-value} < 0.1$ was considered a trend when the post-hoc analysis was significant ($p\text{-value} < 0.05$). The p-values derived from the 16S rRNA sequencing analysis underwent adjustment using a false discovery rate (FDR) of 0.05 (q-value).

3. RESULTS

3.1. Description of the Dog Cohort

The thirty-seven dogs in the cohort were distributed similarly between the junior and senior categories, although the number of animals in these categories was lower than in the adult category (junior: J, n = 10, adult: A, n = 16, and senior: S, n = 11). The mean body weight was slightly higher in seniors (13.4 ± 1.6 kg) compared to juniors (12.3 ± 1.5 kg) and adults (12.8 ± 1.9 kg). Across the cohort, most dogs were neutered females (72.97%), with neutered males comprising 27.03%; notably, all senior dogs were female. Regarding feeding routines, the majority followed a stable routine (64.86%), although rotational feeding was more prevalent in adults (50%) than in juniors (10%) or seniors (36.4%). Housing distribution showed more dogs living in building P (62.16%) compared to building D (37.84%), a pattern consistent across age categories (Table 3).

Table 3. Description of the dog cohort, including its demographic variables such as age, body weight, sex, feeding routine, and housing, categorized by age group. Means and standard deviation (mean ± SD) are presented for quantitative variables, and absolute numbers and relative frequencies are shown for qualitative variables.

Categories	**All**	**Junior (J)**	**Adult (A)**	**Senior (S)**
Variables	***N* = 37**	***n* = 10**	***n* = 16**	***n* = 11**
Age (y.o.)	5.5 ± 4.1	1.2 ± 0.3[a]	4.4 ± 0.5[b]	11.2 ± 2.3[c]
Body weight (kg)	12.9 ± 1.7	12.3 ± 1.5	12.8 ± 1.9	13.4 ± 1.6
Sex				
Female (neutered)	27 (72.97%)	6 (16.22%)	10 (27.03%)	11 (29.73%)
Male (neutered)	10 (27.03%)	4 (10.81%)	6 (16.22%)	0 (0%)
Feeding routine				
Rotation	13 (35.14%)	1 (2.70%)	8 (21.62%)	4 (10.81%)
Stable	24 (64.86%)	9 (24.32%)	8 (21.62%)	7 (18.92%)
Housing				
Building D	14 (37.84%)	3 (8.11%)	7 (18.92%)	4 (10.81%)
Building P	23 (62.16%)	7 (18.92%)	9 (24.32%)	7 (18.92%)

[a, b, c]: Differences between age categories are indicated using superscript letters.

3.2. Biomarkers of Intestinal Health

3.2.1. Quantitative Real-Time PCR and 16S rRNA Sequencing Analysis

The qPCR results analysis are shown in Table 4. The relative abundance of *Bacteroides* was higher in S than in A and J; these results are also supported by the 16S analysis. *Turicibacter* was significantly negatively correlated with age (rho = −0.365; p-value < 0.05), but the results were not significant in the multivariate analysis. *Faecalibacterium* and *Blautia* log DNA copies tended to differ (p-value < 0.1) according to the age category. Those results were confirmed by 16S rRNA sequencing, as relative abundances of those two bacteria significantly differed according to the age category, with *Faecalibacterium* relative abundance being higher in S compared to A (p-value = 0.002; q-value = 0.010) while *Blautia* relative abundance higher in J compared to A (p-value = 0.043).

Table 4. Results of the quantitative real-time PCR (abundance expressed as LogDNA) and values of the calculated dysbiosis index (ratio) comparing the age categories. Within each category, values are expressed as estimated marginal means (emmeans) and standard error (SE).

	Junior		Adult		Senior		
Indicators	**Emmeans**	**SE**	**Emmeans**	**SE**	**Emmeans**	**SE**	***p*-Value**
Universal Log DNA	11.04	0.08	11.08	0.06	11.03	0.10	0.858
Faecalibacterium	5.79	0.34	5.36	0.24	6.45	0.40	0.065
Turicibacter	8.25	0.20	8.01	0.14	8.00	0.24	0.593
Streptococcus	5.14	0.47	5.78	0.33	5.22	0.57	0.434
E. coli	4.90	0.46	5.78	0.32	5.32	0.55	0.267
Blautia	10.78	0.12	10.46	0.08	10.47	0.14	0.089
Fusobacterium	8.86	0.18	9.11	0.13	9.37	0.22	0.230
C. hiranonis	6.86	0.11	6.86	0.08	6.82	0.13	0.970
Bifidobacterium	4.86	0.53	6.12	0.37	6.16	0.63	0.145
Bacteroides	5.75[a]	0.11	5.93[a]	0.15	6.75[b]	0.26	0.017
Lactobacillus	5.76	0.46	5.30	0.33	5.60	0.56	0.680
Dysbiosis index	−4.26	0.72	−3.05	0.51	−4.01	0.86	0.305
Chao 1 index	74.39	9.25	84.92	6.50	98.23	11.08	0.295
Shannon index	3.65	0.26	3.60	0.18	4.16	0.31	0.276

[a,b]: Differences between age categories are indicated using superscript letters.

The dysbiosis index and the alpha diversity indices (Chao1 and Shannon) were not significantly different between age categories. The dysbiosis index values were within the normal range; only two adult dogs were within the dysbiosis index range,

defined as mild to moderate microbiota shift (DI between 0 to 2).

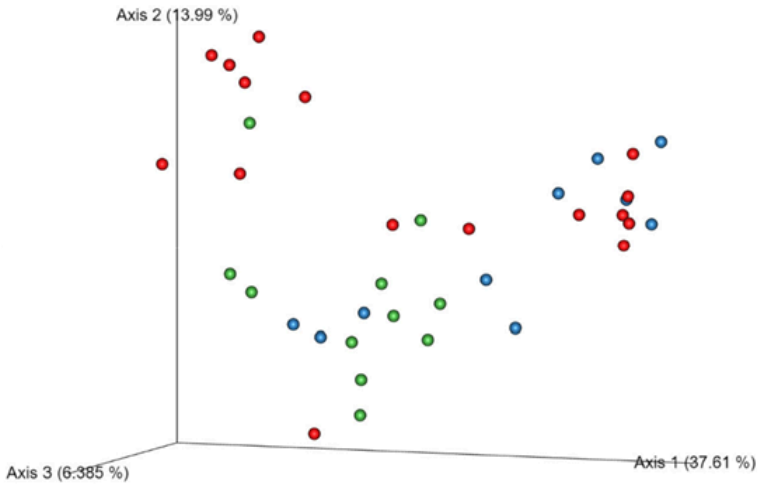
Microbial communities were significantly different based on Unweighted UniFrac analysis, which solely assesses the presence or absence of individual taxa, were significantly different between S and J categories (Figure 1) but were not significant based on the weighted Unifrac and Bray–Curtis analysis, corresponding to the results of the DI (Table 5).

Table 5. Beta diversity analysis comparing the three age categories: Senior (S), Adult (A), and Junior (J). Unweighted UniFrac, weighted UniFrac, and Bray–Curtis distances are shown.

	S vs. A vs. J		**S vs. A**		**S vs. J**		**J vs. A**	
Indicators	**R**	***p*-Value**	**R**	***p*-Value**	**R**	***p*-Value**	**R**	***p*-Value**
Unweighted UniFrac	0.105	0.036*	0.073	0.94	0.241	0.008*	0.053	0.186
Weighted UniFrac	0.053	0.112	0.048	0.173	0.053	0.136	0.069	0.128
Bray–Curtis	0.052	0.148	0.012	0.289	0.098	0.041	0.09	0.114

* *p*-value is significant when < 0.05.

Figure 1. Three-dimensional representation of unweighted Unifrac distance of the microbiota communities in the studied dog population. Different age groups are represented with different colors: green (senior), red (adult), and blue (junior).



At the phylum taxonomic level, Firmicutes, Actinobacteria, Fusobacteria, Bacteroidetes, and Proteobacteria were identified (Table 6). Firmicutes showed the highest relative abundance, while Proteobacteria exhibited the lowest. Significantly different relative abundances were observed for Actinobacteria, Bacteroidetes, and

Proteobacteria between age groups. Specifically, Bacteroidetes and Proteobacteria phyla were relatively more abundant in the oldest group, while Actinobacteria showed a lower percentage in S.

Table 6. Phylum relative abundance (%) split by age category: Junior (J), Adult (A), and Senior (S).

	Junior		Adult		Senior		J vs. A vs. S	
Phylum	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	***p*-Value**	**q-Value**
Firmicutes	85.47	66.12–97.50	81.13	63.02–96.19	83.41	56.62–99.7	0.076	0.095
Actinobacteria	8.57^{a,b}	1.53–29.13	11.32^{a}	1.50–35.84	6.69^{b}	0.17–22.1	0.005	0.010
Fusobacteria	2.29	0.17–10.49	2.47	0–20.76	3.45	0–36.85	0.265	0.265
Bacteroidetes	0.10^{a}	0–3.59	0.35 ^{a}	0–10.99	1.63^{b}	0–18.17	0.035	0.010
Proteobacteria	0.05^{a}	0–0.68	0.16^{a,b}	0–9.50	0.31^{b}	0–2.19	0.006	0.010

^{a,b}: Differences between age categories are indicated using superscript letters.

At the family level, the relative abundance of Bifidobacteriaceae (Actinobacteria) was higher in A compared to J. Bacteroidaceae, and Prevotellaceae families (Bacteroidetes) were relatively more abundant in the S category than in A and J. [Paraprevotellaceae] relative abundance was higher in S compared to A. Within Firmicutes, Lachnospiraceae, Peptococcaceae, Uncl. Clostridiales I, Ruminococcaceae, and Veillonellaceae families exhibited significantly different relative abundances between age categories. Succinivibrionaceae (Proteobacteria) were relatively more abundant in S than in A, and Alcaligenaceae was relatively higher in S compared to J. See the detailed information in the Supplementary Material (Table S1).

3.2.2. Fecal Score, SCFAs, IgA, and cCP

FS values were not significantly different between the age categories and were all within the normal range (67.46 ± 4.26; 67.82 ± 3.00; 72.15 ± 5.11; for J, A, and S, respectively).

For the SCFA concentrations, acetate was the most abundant one in the feces and it was significantly higher in S and A dogs compared to J (Table 7). Both acetate and total SCFA concentration showed significant positive correlations with age (rho = 0.358 and 0.365; p-value = 0.05). Total SCFA content was different, only showing a trend (p-value = 0.055) between groups, but was significantly different when comparing A vs. J (p-value = 0.017).

Butyrate concentration was higher in A (p-value = 0.071), being significantly different in the post-hoc analysis compared with J (p-value = 0.036).

Table 7. Results of SCFAs, IgA, and cCP split by age category. Values are expressed as estimated marginal means (emmeans) and standard error (SE). The correlation between the biomarkers and age is expressed as rho.

	Junior		Adult		Senior		J vs. A vs. S	
Indicators [1]	**Emmeans**	**SE**	**Emmeans**	**SE**	**Emmeans**	**SE**	***p*-Value**	**Rho**
cCP (ng/g)	4.82	0.75	4.14	0.53	2.81	0.90	0.262	−0.075
IgA (mg/g)	12.49	4.14	8.24	2.91	4.96	4.96	0.521	−0.197
SCFA concentrations (µmol/g DM)								
Acetate	145.92 [a]	34.99	250.29 [b]	24.61	263.29[b]	41.94	0.048	0.358*
Propionate	1.77	1.89	13.46	1.33	11.67	2.26	0.641	0.196
Butyrate	52.60	11.13	82.07	7.83	61.21	13.35	0.071	0.239
Isobutyrate	7.93	1.56	9.23	1.10	6.14	1.87	0.326	0.059
Isovalerate	11.15	2.32	8.58	1.63	8.12	2.78	0.622	0.066
Valerate	5.07	3.43	12.19	2.41	6.96	4.11	0.180	0.224
Total SCFAs	234.44	46.64	375.85	32.80	357.4	55.90	0.055	0.365*
SCFA proportions (%)								
Acetate	67.11	2.69	65.41	1.89	72.91	3.22	0.134	0.111
Propionate	4.94[a]	0.34	3.5[b]	0.24	3.35[b]	0.41	0.007	−0.197
Butyrate	19.44	1.95	22.26	1.37	17.45	2.34	0.148	0.078
Isobutyrate	3.04	0.41	2.68	0.29	1.83	0.49	0.195	−0.294
Isovalerate	3.69	0.61	2.4	0.43	2.50	0.73	0.286	−0.213
Valerate	1.78	0.96	3.45	0.67	1.96	1.15	0.250	0.013

[1] cCP: canine calprotectin; IgA: immunoglobulin A; SCFAs: short-chain fatty acids. [a,b] : Differences between age categories are indicated using superscript letters. *The rho significant values (p-value < 0.05) are marked with an asterisk.

Considering the relative proportion of SCFAs, propionate showed a significantly lower percentage in S and A compared with J (3.35%, 3.65%, and 4.94%, respectively). For the cCP and IgA analysis, no significant difference was found.

3.2.3. Serum Haptoglobin, C-Reactive Protein, Folate and Cobalamin

B9 concentration (ng/mL) was higher in S compared to A and J categories. HP concentration correlated positively with age (rho = 0.426; p-value = 0.05). On the other hand, B12 concentration was negatively correlated with age (rho = −0.424; p-value = 0.05). However, B12, CRP, and HP concentrations did not show any difference in the multivariate analysis when comparing the age categories (Table 8).

Table 8. Results of B9, B12, CRP, and HP split by age category. Values are expressed as estimated marginal means (emmeans) and standard error (SE). The correlation between the biomarkers and age is expressed as rho.

	Junior		Adult		Senior		J vs. A vs. S	
Indicators [1]	**Emmeans**	**SE**	**Emmeans**	**SE**	**Emmeans**	**SE**	***p*-Value**	**Rho**
B9 (ng/mL)	15.15 [b]	1.18	13.60[b]	0.83	20.37[a]	1.42	0.001	0.144
B12 (pg/mL)	652.39	49.85	607.19	35.06	511.99	59.75	0.226	−0.424*
CRP (µg/mL)	3.67	4.24	5.45	2.98	2.21	5.08	0.827	−0.231
HP (g/L)	1.38	0.48	1.40	0.33	2.32	0.57	0.346	0.426*

[1] B9: folate; B12: cobalamin; CRP: C-reactive protein; HP: haptoglobin. a,b: Differences between age categories are indicated using superscript letters. * The rho significant values (p-value < 0.05) are marked with an asterisk.

3.3. Biomarkers of Behavior

For the results of the behavioral indicators based on blood and saliva, no differences were found between the age categories, except for TT4, which exhibited lower levels in the A and S groups compared to the J group (Table 9).

Table 9. Results of the behavioral biomarkers split by age category. Estimated marginal means (emmeans) and standard error (SE) of TRP, KYN, KTR, CORT, OT, and TT4 are shown.

	Junior		Adult		Senior		J vs. A vs. S	
Indicators [1]	**Emmeans**	**SE**	**Emmeans**	**SE**	**Emmeans**	**SE**	***p*-Value**	**Rho**
TRP (µg/mL)	11.29	2.00	11.93	1.37	11.64	2.29	0.965	0.069
KYN (ng/mL)	646.70	101.87	770.39	69.69	957.10	116.37	0.158	0.217
KTR	61.73	15.28	79.85	10.45	97.22	17.46	0.332	0.167
CORT (µg/dL)	0.13	0.02	0.14	0.01	0.15	0.02	0.786	0.061
OT (pg/mL)	2747.32	411.39	1904.73	292.08	1991.45	481.48	0.257	−0.247
TT4 (µg/dL)	1.91 [a]	0.14	1.42 [b]	0.09	1.43 [b]	0.16	0.021	−0.241

[1] TRP: tryptophan; KYN: kynurenine; KTR: kynurenine/tryptophan ratio; CORT: cortisol; OT: oxytocin; TT4: total thyroxine. a,b: Differences between age categories are indicated using superscript letters.

3.4. Behavioral Observations and Activity

In the analysis of behavioral observations, distinct patterns were identified among the age categories (Table 10). During park observations, P behavior exhibited significantly higher levels in category J compared to both categories A and S (p-value < 0.001 and p-value = 0.001, respectively). HA behavior was more prevalent in category A than in category J (p-value = 0.005). Furthermore, when

observations were conducted in the kennels, category J displayed significantly higher levels of exploratory behavior compared to both categories A and S (p-value = 0.048 and p-value = 0.013, respectively). Regarding the activity measurements, TAC showed a negative correlation with age (rho = −0.474; p-value < 0.05). Similarly, DAC demonstrated an inverse correlation with age (rho = −0.502; p-value < 0.05). But no differences between age categories were described in the multivariate analysis.

Table 10. Results of the behavioral observations (made in the park and in the kennels) and activity measurements expressed as estimated marginal means (emmeans) and standard error (SE), split by age category. The table only shows behaviors with a relative frequency of more than 10% in the emmeans in at least one age category.

	Junior		Adult		Senior		J vs. A vs. S	
Indicators [1]	**Emmeans**	**SE**	**Emmeans**	**SE**	**Emmeans**	**SE**	***p*-Value**	**Rho**
Observations in the parks (%)								
P	16.71 [a]	2.58	2.26 [b]	1.75	2.48 [b]	3.01	<0.001	−0.435*
EX	19.68	6.10	28.77	4.13	38.09	7.13	0.170	−0.346*
HA	7.81 [a]	4.51	24.02 [b]	3.06	20.78 [b]	5.28	0.018	0.252
Observations in the kennels (%)								
EX	12.64 [a]	2.72	5.87 [b]	1.92	1.40 [b]	3.27	0.013	−0.501*
HA	6.50	3.22	11.03	2.26	8.11	3.86	0.463	0.020
Activity (bits)								
TAC	1,369,452	197,048	1,123,123	133,524	980,744	230,455	0.424	−0.474*
DAC	1,246,316	973,358	973,358	117,886	820,826	203,465	0.271	−0.502*
NAC	123,137	45,711	149,765	30,975	159,918	53,461	0.851	0.074

[1] P: playing; EX: exploring; HA: high alert; TAC: total activity; DAC: diurnal activity; NAC: nocturnal activity. a,b: Differences between age categories are indicated using superscript letters. * The rho significant values (p-value < 0.05) are marked with an asterisk.

3.5. Correlations Between Intestinal Health Biomarkers and Behavioral Indicators

The relationship between gut health biomarkers and behavioral indicators (biomarkers of behavior, behavioral observations, and activity) and the detection of different age patterns is detailed in Supplementary Material (Table S2). Considering the biomarkers of behavior: *Turicibacter* negatively correlated with TRP considering all the dogs, and this negative correlation was specifically marked in A. *Blautia* and *Clostridium hiranonis* correlated positively with thyroxine. *Lactobacillus* correlated

positively with saliva CORT concentration, while fecal acetate, propionate, butyrate, and total SCFA concentrations were negatively correlated with blood TT4 concentration. Isobutyric and isovaleric acid concentrations were negatively correlated with blood KYN and saliva CORT concentrations. Blood KYN followed the same pattern when paired with fecal cCP. And blood HP positively correlated with blood TRP concentrations, the same direction was followed within the different age categories, being significant only for A. With regard to the behavioral observations and activity, only the significant correlations influenced by age categories are discussed: the fecal DI correlated positively with DAC, following significantly the same direction in the A category. Bacteroidetes correlated negatively with TAC and DAC showing an age association in the S and A category, respectively. On the contrary, *Streptococcus* and *Blautia* positively correlated with TAC and DAC, which was also reflected when the A category was studied. *Bifidobacterium* relative abundance positively correlated with resting behavior, with a high correlation with age in the J category. FS correlated positively with grooming behavior, being significantly correlated within the J category.

4. DISCUSSION

The number of publications in the field of fecal microbiota in dogs is significantly increasing. Previously published works in canine science examine the relationship between intestinal microbiota and various pathologies, especially those related to the gastrointestinal system [29–33]. Additionally, there is a growing number of publications studying the gut microbiome related to specific ’biotic’ supplements [34–41] and different diets composition [42–44]. However, the number of studies that exploratorily investigate intestinal microbiota and biomarkers of intestinal health, as well as their relationship with other organs and systems (microbiota–gut–organ axis concept), remains limited. This publication aims to generate knowledge about the gut–brain connection in dogs, including biomarkers of intestinal health (to assess the gut part of the axis) and biomarkers of behavior (to evaluate the brain part of the axis). In addition, the study includes a cohort of healthy animals of different ages in which differences due to aging are explored.

4.1. Biomarkers of Intestinal Health

4.1.1. Quantitative Real-Time PCR and 16S rRNA Sequencing Analysis

The DI showed no significant differences among the age categories. Although different age dogs were included in the study, no differences were expected between healthy individuals. This was confirmed by weighted Unifrac and Bray–Curtis analysis in the 16S rRNA gene sequencing data. Similarly, alpha diversity, which measures diversity within a specific sample, did not exhibit significant variations. This suggests that the overall microbial diversity within each group was comparable, as previously described [19]. The significant differences observed in Unweighted UniFrac between seniors and juniors imply differences in the presence of individual bacteria taxa, which may indicate potential age-related small variations in the composition of community structures.

In terms of microbiota relative abundance, Firmicutes, Actinobacteria, Fusobacteria, Bacteroidetes, and Proteobacteria were the five phyla identified. Previous research supports that the majority of bacterial sequences found in the canine gastrointestinal tract belong to these five distinct phyla [45–47]. In our study, the three main bacterial groups reported were Firmicutes, Actinobacteria, and Fusobacteria, and this distribution was observed across the three age categories. Previous studies found a different distribution in the three main phyla, with Bacteroidetes appearing in place of Actinobacteria [48,49]. Considering the characteristics of our studied population, we hypothesize that the observed differences may result from variations in the analysis methodology or specific traits of the study cohort, which share some common conditions (biotypes).

In our study, Bacteroidetes and Proteobacteria were relatively more abundant in the oldest group, whereas Actinobacteria were less abundant. Bacteroidetes are involved in the digestion of complex carbohydrates and play a role in maintaining gut health. Lower relative abundances of this phylum have been described in dogs with inflammatory bowel disease (IBD) [31]. Additionally, previous studies in senior dogs already described differences in lower relative abundance of *Bacteroides* compared to younger dogs [50,51]. In our study, this phylum exhibited a higher relative abundance in the older population, mainly caused by the results observed in the families Bacteroidaceae, Prevotellaceae, and [Paraprevotellaceae], as well as in the

genus *Bacteroides* (qPCR and 16S analysis). These findings suggest that small changes may occur in the Bacteroidetes phylum with age; however, these changes may not always follow the same direction, especially when no gastrointestinal pathologies are linked to the aged study population. Based on our findings, Proteobacteria was more abundant in the oldest category. This increase was associated with a higher relative abundance of both the Succinivibrionaceae and Alcaligenaceae families. These results are consistent with our previously published data from a larger cohort of dogs [19]. Given the association in the literature between an increase in Proteobacteria and gut inflammation [52], we hypothesize an association between the higher levels of Proteobacteria observed in older dogs and the process of inflammaging, a chronic and progressively proinflammatory state that develops in mammals as they age [53].

4.1.2. Haptoglobin, C-Reactive Protein, Folate, and Cobalamin

Serum HP, CRP, B9, and B12 serve as indicators of various physiological processes and health conditions, including inflammation disorders, infections, hepatic and cardiovascular diseases, as well as the synthesis of neuroactive compounds [54–59]. In our study, folate levels were higher in the senior category and correlated positively with age. Almost all the study dogs harbored concentrations within the normal range described by the laboratory (7.7–24.4 µg/L), and only two senior dogs had levels slightly above this range. Hyperfolatemia has been linked to intestinal inflammation in dogs, including specific conditions such as chronic enteritis [60]. Consequently, we hypothesized that the elevated levels of folate observed in older dogs could be linked to the inflammatory process associated with aging [53]. Interestingly, in our study, we found a positive correlation between folate and haptoglobin levels. In the case of haptoglobin, all values were within the normal range [23]. Although haptoglobin levels correlated with age, no differences were described between the age categories. In addition, no significant finding was described linked to the C-reactive protein analysis. Although we did not find any differences and there is limited knowledge about acute-phase proteins and aging in dogs, it is noteworthy that various acute-phase proteins, including C-reactive protein, have been reported to be higher in elderly humans compared to younger individuals [61].

In our population, the cobalamin levels were within the normal range in all groups [30,60]. The values showed a negative correlation with age, suggesting that older dogs may have reduced efficiency in their absorption. Cobalamin absorption is a complex process occurring along the gastrointestinal tract. A pathological condition in this organ may be causing hypocobalaminemia, a condition that also appears to be more prevalent in older age groups [30]. In our study, mild intestinal inflammation possibly related to aging could explain the negative correlation between cobalamin and age. Interestingly, both concomitant conditions, hypocobalaminemia and hyperfolatemia, could also be explained by bacterial consumption of cobalamin and the overproduction of folate synthesized by bacteria [62,63]. This relationship has been previously described in dogs with small intestinal dysbiosis [60]. Additionally, the measurement of methylmalonic acid could help to better understand this relation. Methylmalonic acid is implicated in key processes of metabolic reprogramming and cellular signaling pathways [64]. It is a more sensitive marker than cobalamin and has already been associated with pathologies linked to the aging process [64–66].

4.1.3. Fecal Consistency, SCFAs, Immunoglobulin A, and Calprotectin

The fecal consistency of the animals included in the study fell within the normal range, and there were no age-related differences. These results were as expected since the study was conducted in a cohort of healthy animals where gastrointestinal signs were not present.

The analysis of SCFAs was conducted mainly to assess the potential differences in the fermentation activity. In our study, acetate was the most abundant SCFA; it was significantly different between the age categories and positively correlated with age. For the total SCFA analysis, a positive correlation with age was described. The SCFA results showed a higher fermentation activity in the senior group, which was consistent with the microbiota composition, revealing an increase in the relative abundance of bacteria able to use complex carbohydrates. This higher activity may be explained by diet composition and type, a lower upper tract digestibility of macronutrients, the presence of specific pathologies, the microbiota composition, and also by age. In our study, two dogs belonging to the senior category were fed diets out of the composition defined range (7–8% moisture, 22–30% protein, 12–21% fat, and 1–5% fiber). One senior dog was fed a high-fiber diet (8.0%

moisture, 23% protein, 12% fat, and 6.5% fiber), while the other one was fed a low-protein diet (7.5% moisture, 14.5% protein, 17.5% fat and 3% fiber). All dogs were eating dry food diets. The presence of pathology or dysbiosis was not previously diagnosed in any of the dogs enrolled in this study. Finally, microbiota composition and age were identified as the major potential explanatory factors for fermentation activity in this study. For example, increased colonic butyrate concentrations have been described in senior dogs [67], as also observed in our study. Moreover, in our study, the relative abundance of *Faecalibacterium* tended to be higher in seniors, probably contributing to butyrate production due to its well-known butyrogenic capacity. In contrast, propionate levels as a percentage (relative amount) were found to be lower in senior and adult dogs compared to juniors. This condition may be explained by the relative proportion calculation. Since acetate plays an important role in this calculation, its higher concentration in seniors and the lower concentration in juniors may explain the overrepresentation of propionate when expressed as a relative amount.

Fecal concentrations of canine calprotectin and immunoglobulin A are described in the literature as non-invasive biomarkers for assessing gut health in dogs. Fecal cCP has been linked to intestinal inflammation, while IgA is associated with gut-immune homeostasis [68,69]. In our study, IgA and cCP levels showed no significant changes across different age categories. Interestingly, both biomarkers have been previously correlated with age in rodents, dogs, and humans, although the results concerning cCP remain inconsistent [70–72]. Further research is needed to determine if these two fecal biomarkers can be considered reliable indicators of aging in dogs.

4.2. Biomarkers of Behavior

Tryptophan is an essential amino acid involved in various metabolic processes, including the synthesis of neuroactive molecules such as melatonin and serotonin. kynurenine, a metabolite of tryptophan, is produced via the kynurenine pathway, which represents a major route for tryptophan metabolism [55]. Alterations in the levels of tryptophan, kynurenine and in the kynurenine/tryptophan ratio may indicate disruptions in tryptophan metabolism due to various factors, including age [73,74]. In our study, while tryptophan levels were numerically similar between the groups, both

kynurenine levels and kynurenine/tryptophan ratio increased with age. A numerical effect was observed for the kynurenine, but the intra-individual variability and the number of dogs were probably too low to detect a statistical difference. However, any of these biomarkers showed significant differences across the age groups. Thus, the relationship between age, tryptophan, kynurenine, and their ratio observed in this study differed from our expectations based on previous research [73].

Cortisol and thyroxine are bioactive compounds that participate in the HPA axis. This axis is crucial in mediating the stress response and regulating the interaction between the gut and the brain [75]. According to Taszkun et al. [76], thyroid hormone levels in healthy dogs can vary based on factors such as age, sex, breed, and physical activity. Consistent with this, our study found that TT4 levels were higher in the younger age group. Although higher cortisol levels have been associated with aging [77], our study did not observe any significant age-related changes in the dogs' cortisol levels.

Oxytocin is a neuropeptide primarily synthesized in the hypothalamus, playing a key role in various physiological and emotional processes and being linked to the gut–brain axis [78,79]. Recent research has associated oxytocin with cellular aging across several systems, including muscle, bone, skin regeneration, and hippocampal function [80–84]. In our study, we hypothesized that this biomarker would vary, particularly in the older group. However, no age-related differences were observed. We speculate that significant changes in oxytocin levels might only become apparent in dogs at the very end of their lives, possibly showing clinical signs of advanced aging. Nevertheless, the role of oxytocin in aging remains unclear and warrants further investigation, especially for its potential in anti-aging therapies.

4.3. Behavioral Observations and Activity

The gut microbiota can influence behavior and activity levels through various pathways, including neurotransmitter and metabolic production, neuroendocrine and immune modulation, and vagus nerve stimulation [3]. Assessing changes in behavior and activity may provide insights into the relationship between the gut and the brain [9,75]. Our behavioral observations suggest several age-related variations, with younger individuals generally being more active, as previously described in the literature [85,86]. Specifically, younger individuals appeared more engaged in playing

when at the park, likely taking profit from the presence of a larger group of conspecifics, and were more exploratory when being in the kennel with their mates. Conversely, high-alert behavior observed in the park was less pronounced in the junior group, which may be related to their engagement in playing.

4.4. Correlations Between Intestinal Health Biomarkers and Behavioral Indicators

The negative correlation between tryptophan and *Turicibacter* suggests a potential role for this bacterial genus in tryptophan metabolism. *Turicibacter* may be involved in the breakdown or utilization of tryptophan, potentially influencing the availability of this essential amino acid for serotonin synthesis. Various bacterial groups have been identified as key contributors to the bacterial fermentation of tryptophan in the gastrointestinal tract, producing indoles such as indole-3-acetic acid, indole-3-propionic acid, indole-3-lactic acid, indole-3-aldehyde among others [87–89]. Specifically, *Turicibacter* seemed to be involved in both the kynurenine and the indole pathways [90]. However, interestingly, no correlation was found between *Turicibacter* and the kynurenine levels. There is intense competition between serotonin, indole, and kynurenine for the available tryptophan, and this increases the complexity of understanding the tryptophan-related metabolites [90]. *Turicibacter* has also been associated with anti-inflammatory properties, influencing host bile acid and lipid metabolism and promoting the intestinal production of 5-HT [91]. All of this could have implications for behavior and cognitive function, making it particularly interesting for senior dogs due to its potential role in cognitive impairment. However, further research is needed, as the specific mechanisms underlying these relationships and their potential impact on serotonin levels are not well described in the literature.

The positive correlation between thyroxine and *Blautia* and *Clostridium hiranonis* is described for all the animals. Interestingly, within the senior category, *Clostridium hiranonis* showed a significant correlation with thyroxine levels. *Clostridium hiranonis* has an important role in maintaining a healthy microbiota in dogs [24,92]. It has been identified as a key player in the conversion of primary to secondary biliary acids, being associated with lipid metabolism and the endocrine system [46,93,94]. With all of this, we could consider a potential association between this bacterial group and

thyroid hormone levels. In our study, we observed a negative correlation between acetate, propionate, butyrate, and total SCFAs with thyroxine. The acetate correlation with thyroxine was especially important in adults and seniors, while in the case of propionate was in seniors. Although the impact of SCFAs on host thyroid function has already been described and reviewed [95], the specific mechanisms are still unclear. SCFAs have been shown to modulate thyroid hormone production and signaling, potentially through effects on the hypothalamus-pituitary-thyroid axis. This finding suggests that the gut microbiome, through SCFA production following different patterns at different life stages, may play a role in regulating thyroid hormone levels and potentially influencing behavior. In addition, the positive correlation between salivary cortisol and *Lactobacillus* suggests a potential link between this bacterial genus but unrelated to a specific life stage. *Lactobacillus* is often associated with beneficial effects on gut health and stress modulation. Specific species and strains of *Lactobacillus* have shown a positive effect on modulating salivary cortisol levels and other stress-related indicators in rats and humans [96,97]. More generally, the most extensively studied group linked to the gut–brain axis is *Lactobacillus*, mainly due to its therapeutic potential. Lactobacilli are also well known for their probiotic properties; they modulate immune responses and support a balanced microbiota [98,99]. Nevertheless, less is known about this bacteria's role in dogs, although several studies already mentioned positive effects. Of note, all four genera in which we identified different correlations with behavioral parameters belong to the Firmicutes phylum. Previous studies have already highlighted the significant roles this phylum plays in gut health, immunity, and behavior [24,87–89,91,96,97]. Those data suggest that, like in other animal species, Firmicutes and, more specifically, Lactobacillus may play functional roles in the GBA. Identifying key microbial metabolites related to key behavior is probably the next step to create putative causation between this bacterial group and the GBA. On the other hand, we found a negative correlation between cortisol and isobutyric and isovaleric acid, being especially noteworthy for the adult category. The relations described for cortisol, kynurenine, and isobutyric and isovaleric acids may suggest the existence of pathways between the microbial metabolites and the stress response in dogs. On the other hand, kynurenine correlated negatively with isobutyric and isovaleric acid, and a negative correlation was described for the senior dogs in the case of isovaleric acid. Overall, this suggests that microbial proteolytic activity-resulting metabolites

may play a role in gut–brain communication. Interestingly, in humans, isovaleric acid and cortisol had already been correlated, as well as depression and isovaleric acid [100]. The association between depressive episodes and the dysregulation of the HPA axis has been already well established [101,102].

We found a negative correlation between kynurenine and calprotectin only when we analyzed all the animals as a group, but no association was described when the two parameters were studied by age category. This correlation could be explained in the direction of having the tryptophan metabolism altered because of an inflammatory condition in which calprotectin increases while tryptophan decreases. Serum tryptophan levels were previously described as significantly lower when comparing individuals diagnosed with a specific intestinal inflammatory condition (intestinal bowel disease) with a control group [103]. Kynurenine and the kynurenine/tryptophan ratio were also altered in individuals under intestinal inflammatory conditions [104]; however, in our study, no correlation was found. On the other hand, the positive correlation found between tryptophan and haptoglobin, particularly in adults, may suggest a potential link between inflammation, tryptophan metabolism, and behavior in adult dogs. Although there was not a correlation between the kynurenine and the seric haptoglobin, the changes in haptoglobin levels could still influence tryptophan metabolism and potentially impact serotonin production, leading to behavioral changes.

Finally, it is important to outline the study's limitations, such as the number of animals that participated in the study, the fact that we conducted only a single sampling without tracking age progression, and the categorization by three age groups, which may not allow detection of changes at specific ages. Additionally, there are factors that could be influencing the results, such as the animals being fed different diets, even though most diets had a similar composition range. It would also have been beneficial to collect body composition values for the animals, in addition to their other demographic characteristics included in the study. We also acknowledge that the spayed/neutered status of the studied population introduces a potential bias by removing the influence of sexual hormones, which are indeed an important factor associated with age. This is an important point, particularly in relation to the extrapolation of our findings to the general dog population or broader canine aging studies. Acknowledging that correlations do not always imply causation, we believe

that this study provides a foundation for further exploring the relationships between behavioral parameters and intestinal health, contributing to a more comprehensive understanding of the underlying causes and mechanisms.

5. CONCLUSIONS

The overall results of the studied biomarkers of intestinal health, biomarkers of behavior, and behavioral observations suggest minor changes in the different dog age groups. The observed differences in correlations between various biomarkers of gut health and behavior, particularly within the age categories, highlight the importance of considering age-related factors when studying gut health and behavioral biomarkers. However, further research is needed to better understand the mechanisms and the specific pathways involved in the relationship between the specific studied biomarkers. The findings described in this study could have implications for the development of nutritional interventions adapted to the needs of different age groups in dogs.

Supplementary Materials: The following supporting information can be downloaded at https://www. mdpi.com/article/10.3390/ani15020234/s1. Table S1. Microbial family results organized by phylum of the 16s rRNA sequencing analysis split by age category; Table S2. Table showing the correlation between the biomarkers of intestinal health vs. the biomarkers of behavior.

Author Contributions: Conceptualization and methodology, A.F.-P., C.T., A.S.-M. and X.M.; data analysis, A.F.-P. and R.P.; study management, A.F.-P.; writing—original draft preparation, A.F.-P.; writing—review and editing, A.F.-P., R.P., J.S., E.A., C.T., X.M. and A.S.-M.; supervision, A.S.-M. and X.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Affinity Petcare.

Institutional Review Board Statement: The study was reviewed and approved by the Affinity Ethical Committee. The experimental protocol was approved by the Government of Catalonia (Reference number: CEA-OH/10006/1) following the European Union guidelines for the ethical care and handling of animals under experimental conditions.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data access is restricted to protect confidential or/and proprietary information but can be made available upon request, with permission, for the purposes of peer review.

Acknowledgments: The authors are grateful to Jose Juaquin Cerón from Murcia University for conducting the saliva and blood analyses and to the Applied Statistics Service, Faculty of Sciences, Autonomous University of Barcelona, for their support with the statistical analysis. We would also like to thank the staff of the Affinity Nutrition Center for caring for the animals and assisting with the study’s execution.

Conflicts of Interest: A.F.-P., E.A., C.T. and A.S.-M. are employees of Affinity. J.S. is an employee of the Gastrointestinal Laboratory at Texas A&M University that offers gastrointestinal function testing on a fee-for-service basis. The remaining authors declare the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

1. Boulangé, C.L.; Neves, A.L.; Chilloux, J.; Nicholson, J.K.; Dumas, M.-E. Impact of the Gut Microbiota on Inflammation, Obesity, and Metabolic Disease. Genome Med. 2016, 8, 42.

2. Hsiao, E.Y.; McBride, S.W.; Hsien, S.; Sharon, G.; Hyde, E.R.; McCue, T.; Codelli, J.A.; Chow, J.; Reisman, S.E.; Petrosino, J.F.; et al. Microbiota Modulate Behavioral and Physiological Abnormalities Associated with Neurodevelopmental Disorders. Cell 2013, 155, 1451–1463.

3. Margolis, K.G.; Cryan, J.F.; Mayer, E.A. The Microbiota-Gut-Brain Axis: From Motility to Mood. Gastroenterology 2021, 160, 1486–1501.

4. Martin, C.R.; Osadchiy, V.; Kalani, A.; Mayer, E.A. The Brain-Gut-Microbiome Axis. Cell. Mol. Gastroenterol. Hepatol. 2018, 6, 133–148.

5. Tolhurst, G.; Heffron, H.; Lam, Y.S.; Parker, H.E.; Habib, A.M.; Diakogiannaki, E.; Cameron, J.; Grosse, J.; Reimann, F.; Gribble, F.M. Short-Chain Fatty Acids Stimulate Glucagon-Like Peptide-1 Secretion via the G-Protein–Coupled Receptor FFAR2. Diabetes 2012, 61, 364–371.

6. Wikoff, W.R.; Anfora, A.T.; Liu, J.; Schultz, P.G.; Lesley, S.A.; Peters, E.C.; Siuzdak, G. Metabolomics Analysis Reveals Large Effects of Gut Microflora on Mammalian Blood Metabolites. Proc. Natl. Acad. Sci. USA 2009, 106, 3698–3703.

7. Gwak, M.-G.; Chang, S.-Y. Gut-Brain Connection: Microbiome, Gut Barrier, and Environmental Sensors. Immune Netw. 2021, 21, e20.

8. Sampson, T.R.; Debelius, J.W.; Thron, T.; Janssen, S.; Shastri, G.G.; Ilhan, Z.E.; Challis, C.; Schretter, C.E.; Rocha, S.; Gradinaru, V.; et al. Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. Cell 2016, 167, 1469–1480.e12.

9. Zhu, S.; Jiang, Y.; Xu, K.; Cui, M.; Ye, W.; Zhao, G.; Jin, L.; Chen, X. The Progress of Gut Microbiome Research Related to Brain Disorders. J. Neuroinflamm. 2020, 17, 25.

10. Ambrosini, Y.M.; Borcherding, D.; Kanthasamy, A.; Kim, H.J.; Willette, A.A.;

Jergens, A.; Allenspach, K.; Mochel, J.P. The Gut-Brain Axis in Neurodegenerative Diseases and Relevance of the Canine Model: A Review. Front. Aging Neurosci. 2019, 11, 130.

11. Jiang, C.; Li, G.; Huang, P.; Liu, Z.; Zhao, B. The Gut Microbiota and Alzheimer's Disease. J. Alzheimer's Dis. 2017, 58, 1–15.

12. Li, Y.; Ning, L.; Yin, Y.; Wang, R.; Zhang, Z.; Hao, L.; Wang, B.; Zhao, X.; Yang, X.; Yin, L.; et al. Age-Related Shifts in Gut Microbiota Contribute to Cognitive Decline in Aged Rats. Aging 2020, 12, 7801–7817.

13. Kirchoff, N.S.; Udell, M.A.R.; Sharpton, T.J. The Gut Microbiome Correlates with Conspecific Aggression in a Small Population of Rescued Dogs (Canis Familiaris). PeerJ 2019, 7, e6103.

14. Mondo, E.; Barone, M.; Soverini, M.; D'Amico, F.; Cocchi, M.; Petrulli, C.; Mattioli, M.; Marliani, G.; Candela, M.; Accorsi, P.A. Gut Microbiome Structure and Adrenocortical Activity in Dogs with Aggressive and Phobic Behavioral Disorders. Heliyon 2020, 6, e03311.

15. Craddock, H.A.; Godneva, A.; Rothschild, D.; Motro, Y.; Grinstein, D.; Lotem-Michaeli, Y.; Narkiss, T.; Segal, E.; Moran-Gilad, J. Phenotypic Correlates of the Working Dog Microbiome. npj Biofilms Microbiomes 2022, 8, 66.

16. Sacoor, C.; Marugg, J.D.; Lima, N.R.; Empadinhas, N.; Montezinho, L. Gut-Brain Axis Impact on Canine Anxiety Disorders: New Challenges for Behavioral Veterinary Medicine. Vet. Med. Int. 2024, 2024, 2856759.

17. Kiełbik, P.; Witkowska-Piłaszewicz, O. The Relationship between Canine Behavioral Disorders and Gut Microbiome and Future Therapeutic Perspectives. Animals 2024, 14, 2048.

18. Strickling, J.A.; Harmon, D.L.; Dawson, K.A.; Gross, K.L. Evaluation of Oligosaccharide Addition to Dog Diets: Influences on Nutrient Digestion and Microbial Populations. Anim. Feed Sci. Technol. 2000, 86, 205–219.

19. Fernández-Pinteño, A.; Pilla, R.; Manteca, X.; Suchodolski, J.; Torre, C.; Salas-Mani, A. Age-Associated Changes in Intestinal Health Biomarkers in Dogs.

Front. Vet. Sci. 2023, 10, 1213287.

20. Parada, A.E.; Needham, D.M.; Fuhrman, J.A. Every Base Matters: Assessing Small Subunit RRNA Primers for Marine Microbiomes with Mock Communities, Time Series and Global Field Samples. Environ. Microbiol. 2015, 18, 1403–1414.

21. Apprill, A.; McNally, S.; Parsons, R.; Weber, L. Minor Revision to V4 Region SSU RRNA 806R Gene Primer Greatly Increases Detection of SAR11 Bacterioplankton. Aquat. Microb. Ecol. 2015, 75, 129–137.

22. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2. Nat. Biotechnol. 2019, 37, 852–857.

23. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High Resolution Sample Inference from Illumina Amplicon Data. Nat. Methods 2016, 13, 581–583.

24. AlShawaqfeh, M.; Wajid, B.; Minamoto, Y.; Markel, M.; Lidbury, J.; Steiner, J.; Serpedin, E.; Suchodolski, J. A Dysbiosis Index to Assess Microbial Changes in Fecal Samples of Dogs with Chronic Inflammatory Enteropathy. FEMS Microbiol. Ecol. 2017, 93, fix136.

25. López-Arjona, M.; Mateo, S.V.; Cerón, J.J.; Martínez-Subiela, S. Changes in Salivary Oxytocin after Stroking in Dogs: Validation of Two Assays for Its Assessment. Res. Vet. Sci. 2021, 136, 527–534.

26. Muñoz-Prieto, A.; Tvarijonaviciute, A.; Escribano, D.; Martínez-Subiela, S.; Cerón, J.J. Use of Heterologous Immunoassays for Quantification of Serum Proteins: The Case of Canine C-Reactive Protein. PLoS ONE 2017, 12, e0172188.

27. Tecles, F.; Subiela, S.M.; Petrucci, G.; Panizo, C.G.; Cerón, J.J. Validation of a Commercially Available Human Immunoturbidimetric Assay for Haptoglobin Determination in Canine Serum Samples. Vet. Res. Commun. 2007, 31, 23–36.

28. Benjamini, Y.; Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J. R. Stat. Soc. Ser. B Methodol. 1995,

57, 289–300.

29. Boucard, A.-S.; Thomas, M.; Lebon, W.; Polack, B.; Florent, I.; Langella, P.; Bermúdez-Humarán, L.G. Age and Giardia Intestinalis Infection Impact Canine Gut Microbiota. Microorganisms 2021, 9, 1862.

30. Kather, S.; Grützner, N.; Kook, P.H.; Dengler, F.; Heilmann, R.M. Review of Cobalamin Status and Disorders of Cobalamin Metabolism in Dogs. J. Vet. Intern. Med. 2020, 34, 13–28.

31. Suchodolski, J.S.; Dowd, S.E.; Wilke, V.; Steiner, J.M.; Jergens, A.E. 16S RRNA Gene Pyrosequencing Reveals Bacterial Dysbiosis in the Duodenum of Dogs with Idiopathic Inflammatory Bowel Disease. PLoS ONE 2012, 7, e39333.

32. Blake, A.B.; Guard, B.C.; Honneffer, J.B.; Lidbury, J.A.; Steiner, J.M.; Suchodolski, J.S. Altered Microbiota, Fecal Lactate, and Fecal Bile Acids in Dogs with Gastrointestinal Disease. PLoS ONE 2019, 14, e0224454. [CrossRef] [PubMed]

33. Blake, A.B.; Suchodolski, J.S. Importance of Gut Microbiota for the Health and Disease of Dogs and Cats. Anim. Front. 2016, 6, 37–42.

34. Ephraim, E.; Brockman, J.A.; Jewell, D.E. A Diet Supplemented with Polyphenols, Prebiotics and Omega-3 Fatty Acids Modulates the Intestinal Microbiota and Improves the Profile of Metabolites Linked with Anxiety in Dogs. Biology 2022, 11, 976.

35. de Lima, D.C.; Souza, C.M.M.; Nakamura, N.; Mesa, D.; de Oliveira, S.G.; Félix, A.P. Dietary Supplementation with Bacillus Subtilis C-3102 Improves Gut Health Indicators and Fecal Microbiota of Dogs. Anim. Feed. Sci. Technol. 2020, 270, 114672.

36. Bastos, T.S.; Souza, C.M.M.; Legendre, H.; Richard, N.; Pilla, R.; Suchodolski, J.S.; de Oliveira, S.G.; Lesaux, A.A.; Félix, A.P. Effect of Yeast Saccharomyces Cerevisiae as a Probiotic on Diet Digestibility, Fermentative Metabolites, and Composition and Functional Potential of the Fecal Microbiota of Dogs Submitted to an Abrupt Dietary Change. Microorganisms 2023, 11, 506.

37. Cannas, S.; Tonini, B.; Belà, B.; Prinzio, R.D.; Pignataro, G.; Simone, D.D.;

Gramenzi, A. Effect of a Novel Nutraceutical Supplement (Relaxigen Pet Dog) on the Fecal Microbiome and Stress-Related Behaviors in Dogs: A Pilot Study. J. Vet. Behav. 2021, 42, 37–47.

38. Meineri, G.; Martello, E.; Atuahene, D.; Miretti, S.; Stefanon, B.; Sandri, M.; Biasato, I.; Corvaglia, M.R.; Ferrocino, I.; Cocolin, L.S. Effects of Saccharomyces Boulardii Supplementation on Nutritional Status, Fecal Parameters, Microbiota, and Mycobiota in Breeding Adult Dogs. Vet. Sci. 2022, 9, 389.

39. Perini, M.P.; Pedrinelli, V.; Marchi, P.H.; Henríquez, L.B.F.; Zafalon, R.V.A.; Vendramini, T.H.A.; Balieiro, J.C.d.C.; Brunetto, M.A. Potential Effects of Prebiotics on Gastrointestinal and Immunological Modulation in the Feeding of Healthy Dogs: A Review. Fermentation 2023, 9, 693.

40. Lin, C.-Y.; Alexander, C.; Steelman, A.J.; Warzecha, C.M.; de Godoy, M.R.C.; Swanson, K.S. Effects of a Saccharomyces Cerevisiae Fermentation Product on Fecal Characteristics, Nutrient Digestibility, Fecal Fermentative End-Products, Fecal Microbial Populations, Immune Function, and Diet Palatability in Adult Dogs. J. Anim. Sci. 2019, 97, 1586–1599.

41. Xu, H.; Huang, W.; Hou, Q.; Kwok, L.-Y.; Laga, W.; Wang, Y.; Ma, H.; Sun, Z.; Zhang, H. Oral Administration of Compound Probiotics Improved Canine Feed Intake, Weight Gain, Immunity and Intestinal Microbiota. Front. Immunol. 2019, 10, 666.

42. Pilla, R.; Suchodolski, J.S. The Gut Microbiome of Dogs and Cats, and the Influence of Diet. Vet. Clin. N. Am. Small Anim. Pract. 2021, 51, 605–621.

43. Coelho, L.P.; Kultima, J.R.; Costea, P.I.; Fournier, C.; Pan, Y.; Czarnecki-Maulden, G.; Hayward, M.R.; Forslund, S.K.; Schmidt, T.S.B.; Descombes, P.; et al. Similarity of the Dog and Human Gut Microbiomes in Gene Content and Response to Diet. Microbiome 2018, 6, 72.

44. Lin, C.-Y.; Jha, A.R.; Oba, P.M.; Yotis, S.M.; Shmalberg, J.; Honaker, R.W.; Swanson, K.S. Longitudinal Fecal Microbiome and Metabolite Data Demonstrate Rapid Shifts and Subsequent Stabilization after an Abrupt Dietary Change in Healthy Adult Dogs. Anim. Microbiome 2022, 4, 46.

45. Deschamps, C.; Humbert, D.; Zentek, J.; Denis, S.; Priymenko, N.; Apper, E.; Blanquet-Diot, S. From Chihuahua to Saint-Bernard: How Did Digestion and Microbiota Evolve with Dog Sizes. Int. J. Biol. Sci. 2022, 18, 5086–5102.

46. Pilla, R.; Suchodolski, J.S. The Role of the Canine Gut Microbiome and Metabolome in Health and Gastrointestinal Disease. Front. Vet. Sci. 2020, 6, 498.

47. You, I.; Kim, M.J. Comparison of Gut Microbiota of 96 Healthy Dogs by Individual Traits: Breed, Age, and Body Condition Score. Animals 2021, 11, 2432.

48. Middelbos, I.S.; Boler, B.M.V.; Qu, A.; White, B.A.; Swanson, K.S.; Fahey, G.C. Phylogenetic Characterization of Fecal Microbial Communities of Dogs Fed Diets with or without Supplemental Dietary Fiber Using 454 Pyrosequencing. PLoS ONE 2010, 5, e9768.

49. Hand, D.; Wallis, C.; Colyer, A.; Penn, C.W. Pyrosequencing the Canine Faecal Microbiota: Breadth and Depth of Biodiversity. PLoS ONE 2013, 8, e53115.

50. Simpson, J.M.; Martineau, B.; Jones, W.E.; Ballam, J.M.; Mackie, R.I. Characterization of Fecal Bacterial Populations in Canines: Effects of Age, Breed and Dietary Fiber. Microb. Ecol. 2002, 44, 186–197.

51. Hopkins, M.J.; Sharp, R.; Macfarlane, G.T. Age and Disease Related Changes in Intestinal Bacterial Populations Assessed by Cell Culture, 16S RRNA Abundance, and Community Cellular Fatty Acid Profiles. Gut 2001, 48, 198.

52. Minamoto, Y.; Otoni, C.C.; Steelman, S.M.; Büyükleblebici, O.; Steiner, J.M.; Jergens, A.E.; Suchodolski, J.S. Alteration of the Faecal Microbiota and Serum Metabolite Profiles in Dogs with Idiopathic Inflammatory Bowel Disease. Gut Microbes 2015, 6, 33–47.

53. Jiménez, A.G. Inflammaging in Domestic Dogs: Basal Level Concentrations of IL-6, IL-1β, and TNF-α in Serum of Healthy Dogs of Different Body Sizes and Ages. Biogerontology 2023, 24, 593–602.

54. Glick, A.; Jaffey, J.A.; Kreisler, R.; Hanzlicek, A.S.; Ringold, R. Serum C-Reactive Protein and Haptoglobin Decrease in the First Three Months of Treatment and Relative Change in Haptoglobin Predict Remission in Dogs with

Pulmonary Coccidioidomycosis. J. Am. Vet. Med. Assoc. 2024, 262, 1222–1230.

55. Heilmann, R.M.; Ruaux, C.G.; Grützner, N.; Cranford, S.M.; Bridges, C.S.; Steiner, J.M. Biological Variation of Serum Canine Calprotectin Concentrations as Measured by ELISA in Healthy Dogs. Vet. J. 2019, 247, 61–64.

56. MacKellar, M.; Vigerust, D.J. Role of Haptoglobin in Health and Disease: A Focus on Diabetes. Clin. Diabetes 2016, 34, 148–157.

57. Heilmann, R.M.; Jergens, A.E.; Ackermann, M.R.; Barr, J.W.; Suchodolski, J.S.; Steiner, J.M. Serum Calprotectin Concentrations in Dogs with Idiopathic Inflammatory Bowel Disease. Am. J. Vet. Res. 2012, 73, 1900–1907.

58. Crawford, K.; Warman, S.M.; Marques, A.I.; Yool, D.A.; Eckersall, P.D.; McCulloch, E.; Lynn, K.; Mellanby, R.J.; Gow, A.G. Serum Haptoglobin Concentrations in Dogs with Liver Disease. Vet. Rec. 2013, 173, 579.

59. Socała, K.; Doboszewska, U.; Szopa, A.; Serefko, A.; Włodarczyk, M.; Zielin´ska, A.; Poleszak, E.; Fichna, J.; Wlaz´, P. The Role of Microbiota-Gut-Brain Axis in Neuropsychiatric and Neurological Disorders. Pharmacol. Res. 2021, 172, 105840.

60. Ullal, T.V.; Marks, S.L.; Huebner, S.N.; Taylor, S.L.; Shelley, C.D. Association of Folate Concentrations with Clinical Signs and Laboratory Markers of Chronic Enteropathy in Dogs. J. Vet. Intern. Med. 2023, 37, 455–464.

61. Ballou, S.P.; Lozanski, G.B.; Hodder, S.; Rzewnicki, D.L.; Mion, L.C.; Sipe, J.D.; Ford, A.B.; Kushner, I. Quantitative and Qualitative Alterations of Acute-Phase Proteins in Healthy Elderly Persons. Age Ageing 1996, 25, 224–230.

62. Giannella, R.A.; Broitman, S.A.; Zamcheck, N. Competition Between Bacteria and Intrinsic Factor for Vitamin B12: Implications for Vitamin B12 Malabsorption in Intestinal Bacterial Overgrowth. Gastroenterology 1972, 62, 255–260.

63. German, A.J.; Day, M.J.; Ruaux, C.G.; Steiner, J.M.; Williams, D.A.; Hall, E.J. Comparison of Direct and Indirect Tests for Small Intestinal Bacterial Overgrowth and Antibiotic-Responsive Diarrhea in Dogs. J. Vet. Intern. Med. 2003, 17, 33–43.

64. Tejero, J.; Lazure, F.; Gomes, A.P. Methylmalonic Acid in Aging and Disease.

Trends Endocrinol. Metab. 2024, 35, 188–200.

65. Morris, M.S.; Jacques, P.F.; Rosenberg, I.H.; Selhub, J. Elevated Serum Methylmalonic Acid Concentrations Are Common among Elderly Americans. J. Nutr. 2002, 132, 2799–2803.

66. Gomes, A.P.; Ilter, D.; Low, V.; Endress, J.E.; Fernández-García, J.; Rosenzweig, A.; Schild, T.; Broekaert, D.; Ahmed, A.; Planque, M.; et al. Age-Induced Accumulation of Methylmalonic Acid Promotes Tumour Progression. Nature 2020, 585, 283–287.

67. Kuzmuk, K.N.; Swanson, K.S.; Tappenden, K.A.; Schook, L.B.; Fahey, G.C. Diet and Age Affect Intestinal Morphology and Large Bowel Fermentative End-Product Concentrations in Senior and Young Adult Dogs. J. Nutr. 2005, 135, 1940–1945.

68. Otoni, C.C.; Heilmann, R.M.; García-Sancho, M.; Sainz, A.; Ackermann, M.R.; Suchodolski, J.S.; Steiner, J.M.; Jergens, A.E. Serologic and Fecal Markers to Predict Response to Induction Therapy in Dogs with Idiopathic Inflammatory Bowel Disease. J. Vet. Intern. Med. 2018, 32, 999–1008.

69. Grellet, A.; Heilmann, R.M.; Lecoindre, P.; Feugier, A.; Day, M.J.; Peeters, D.; Freiche, V.; Hernandez, J.; Grandjean, D.; Suchodolski, J.S.; et al. Fecal Calprotectin Concentrations in Adult Dogs with Chronic Diarrhea. Am. J. Vet. Res. 2013, 74, 706–711. [CrossRef] 70. Fujihashi, K.; McGhee, J.R. Mucosal Immunity and Tolerance in the Elderly. Mech. Ageing Dev. 2004, 125, 889–898.

71. Park, S.Y. Age-Related Fecal Calprotectin Concentrations in Healthy Adults. Korean J. Clin. Lab. Sci. 2020, 52, 181–187.

72. Zaine, L.; Ferreira, C.; Gomes, M.d.O.S.; Monti, M.; Tortola, L.; Vasconcellos, R.S.; Carciofi, A.C. Faecal IgA Concentration Is Influenced by Age in Dogs. Br. J. Nutr. 2011, 106, S183–S186.

73. Sorgdrager, F.J.H.; Vermeiren, Y.; Faassen, M.V.; van der Ley, C.; Nollen, E.A.A.; Kema, I.P.; Deyn, P.P.D. Age-and Disease-specific Changes of the Kynurenine Pathway in Parkinson’s and Alzheimer’s Disease. J. Neurochem. 2019, 151, 656–668.

74. Gao, K.; Mu, C.; Farzi, A.; Zhu, W. Tryptophan Metabolism: A Link Between the Gut Microbiota and Brain. Adv. Nutr. 2019, 11, 709–723.

75. Dinan, T.G.; Cryan, J.F. Brain–Gut–Microbiota Axis—Mood, Metabolism and Behaviour. Nat. Rev. Gastroenterol. Hepatol. 2017, 14, 69–70.

76. Taszkun, I.; Milczak, A.; Kalisz, G.; Wilkołek, P.; Zwolska, J.; Szczepanik, M. Serum TT4, FT4 and TSH Concentrations in German Shepherd Dogs Depending on Age and Type of Work. Pol. J. Vet. Sci. 2021, 24, 63–68.

77. Stamou, M.I.; Colling, C.; Dichtel, L.E. Adrenal Aging and Its Effects on the Stress Response and Immunosenescence. Maturitas 2023, 168, 13–19.

78. Jurek, B.; Neumann, I.D. The Oxytocin Receptor: From Intracellular Signaling to Behavior. Physiol. Rev. 2018, 98, 1805–1908.

79. Erdman, S.E. Oxytocin and the Microbiome. Curr. Opin. Endocr. Metab. Res. 2021, 19, 8–14.

80. Beranger, G.E.; Djedaini, M.; Battaglia, S.; Roux, C.H.; Scheideler, M.; Heymann, D.; Amri, E.-Z.; Pisani, D.F. Oxytocin Reverses Osteoporosis in a Sex-Dependent Manner. Front. Endocrinol. 2015, 6, 81.

81. Elabd, C.; Cousin, W.; Upadhyayula, P.; Chen, R.Y.; Chooljian, M.S.; Li, J.; Kung, S.; Jiang, K.P.; Conboy, I.M. Oxytocin Is an Age-Specific Circulating Hormone That Is Necessary for Muscle Maintenance and Regeneration. Nat. Commun. 2014, 5, 4082.

82. Benameur, T.; Panaro, M.A.; Porro, C. The Antiaging Role of Oxytocin. Neural Regen. Res. 2021, 16, 2413–2414.

83. Koulousakis, P.; Willems, E.; Schepers, M.; Rombaut, B.; Prickaerts, J.; Vanmierlo, T.; Hove, D. van den Exogenous Oxytocin Administration Restores Memory in Female APP/PS1 Mice. J. Alzheimer’s Dis. 2023, 96, 1207–1219.

84. Cho, S.-Y.; Kim, A.Y.; Kim, J.; Choi, D.-H.; Son, E.D.; Shin, D.W. Impact of Oxytocin on Skin Ageing. Br. J. Dermatol. 2019, 181, e148.

85. Lee, H.; Collins, D.; Creevy, K.E.; Promislow, D.E.L.; Akey, J.M.; Benton, B.;

Borenstein, E.; Castelhano, M.G.; Coleman, A.E.; Creevy, K.E.; et al. Age and Physical Activity Levels in Companion Dogs: Results From the Dog Aging Project. J. Gerontol. Ser. A 2022, 77, 1986–1993.

86. Head, E.; Callahan, H.; Cummings, B.J.; Cotman, C.W.; Ruehl, W.W.; Muggenberg, B.A.; Milgram, N.W. Open Field Activity and Human Interaction as a Function of Age and Breed in Dogs. Physiol. Behav. 1997, 62, 963–971.

87. Qi, Q.; Li, J.; Yu, B.; Moon, J.-Y.; Chai, J.C.; Merino, J.; Hu, J.; Ruiz-Canela, M.; Rebholz, C.; Wang, Z.; et al. Host and Gut Microbial Tryptophan Metabolism and Type 2 Diabetes: An Integrative Analysis of Host Genetics, Diet, Gut Microbiome and Circulating Metabolites in Cohort Studies. Gut 2022, 71, 1095–1105.

88. Sun, J.; Zhang, Y.; Kong, Y.; Ye, T.; Yu, Q.; Satyanarayanan, S.K.; Su, K.-P.; Liu, J. Microbiota-Derived Metabolite Indoles Induced Aryl Hydrocarbon Receptor Activation and Inhibited Neuroinflammation in APP/PS1 Mice. Brain Behav. Immun. 2022, 106, 76–88.

89. Vogt, N.M.; Kerby, R.L.; Dill-McFarland, K.A.; Harding, S.J.; Merluzzi, A.P.; Johnson, S.C.; Carlsson, C.M.; Asthana, S.; Zetterberg, H.; Blennow, K.; et al. Gut Microbiome Alterations in Alzheimer's Disease. Sci. Rep. 2017, 7, 13537.

90. Agus, A.; Planchais, J.; Sokol, H. Gut Microbiota Regulation of Tryptophan Metabolism in Health and Disease. Cell Host Microbe 2018, 23, 716–724.

91. Canani, R.B.; Costanzo, M.D.; Leone, L.; Pedata, M.; Meli, R.; Calignano, A. Potential Beneficial Effects of Butyrate in Intestinal and Extraintestinal Diseases. World J. Gastroenterol. 2011, 17, 1519–1528.

92. Vazquez-Medina, A.; Rodriguez-Trujillo, N.; Ayuso-Rodriguez, K.; Marini-Martinez, F.; Angeli-Morales, R.; Caussade-Silvestrini, G.; Godoy-Vitorino, F.; Chorna, N. Exploring the Interplay between Running Exercises, Microbial Diversity, and Tryptophan Metabolism along the Microbiota-Gut-Brain Axis. Front. Microbiol. 2024, 15, 1326584.

93. Chaitman, J.; Ziese, A.-L.; Pilla, R.; Minamoto, Y.; Blake, A.B.; Guard, B.C.; Isaiah, A.; Lidbury, J.A.; Steiner, J.M.; Unterer, S.; et al. Fecal Microbial and Metabolic Profiles in Dogs With Acute Diarrhea Receiving Either Fecal Microbiota

Transplantation or Oral Metronidazole. Front. Vet. Sci. 2020, 7, 192.

94. Song, Y.; Zhao, M.; Zhang, H.; Zhang, X.; Zhao, J.; Xu, J.; Gao, L. Thyroid-Stimulating Hormone Levels Are Inversely Associated With Serum Total Bile Acid Levels: A Cross-Sectional Study. Endocr. Pr. 2016, 22, 420–426.

95. Mendoza-León, M.J.; Mangalam, A.K.; Regaldiz, A.; González-Madrid, E.; Rangel-Ramírez, M.A.; Álvarez-Mardonez, O.; Vallejos, O.P.; Méndez, C.; Bueno, S.M.; Melo-González, F.; et al. Gut Microbiota Short-Chain Fatty Acids and Their Impact on the Host Thyroid Function and Diseases. Front. Endocrinol. 2023, 14, 1192216.

96. Messaoudi, M.; Lalonde, R.; Violle, N.; Javelot, H.; Desor, D.; Nejdi, A.; Bisson, J.-F.; Rougeot, C.; Pichelin, M.; Cazaubiel, M.; et al. Assessment of Psychotropic-like Properties of a Probiotic Formulation (*Lactobacillus Helveticus* R0052 and *Bifidobacterium Longum R0175*) in Rats and Human Subjects. Br. J. Nutr. 2011, 105, 755–764.

97. Rosin, S.; Xia, K.; Azcarate-Peril, M.A.; Carlson, A.L.; Propper, C.B.; Thompson, A.L.; Grewen, K.; Knickmeyer, R.C. A Preliminary Study of Gut Microbiome Variation and HPA Axis Reactivity in Healthy Infants. Psychoneuroendocrinology 2021, 124, 105046.

98. Zhou, B.; Jin, G.; Pang, X.; Mo, Q.; Bao, J.; Liu, T.; Wu, J.; Xie, R.; Liu, X.; Liu, J.; et al. Lactobacillus Rhamnosus GG Colonization in Early Life Regulates Gut-Brain Axis and Relieves Anxiety-like Behavior in Adulthood. Pharmacol. Res. 2022, 177, 106090.

99. John, S.K.; Chandrapragasam, V.; Dey, P. Impact of Gut Microbiome Lactobacillus Spp. in Brain Function and Its Medicament towards Alzheimer's Disease Pathogenesis. J. Pure Appl. Microbiol. 2021, 15, 1029–1041.

100. Szczesniak, O.; Hestad, K.A.; Hanssen, J.F.; Rudi, K. Isovaleric Acid in Stool Correlates with Human Depression. Nutr. Neurosci. 2016, 19, 279–283.

101. Barden, N. Implication of the Hypothalamic-Pituitary-Adrenal Axis in the Physiopathology of Depression. J. Psychiatry Neurosci. 2004, 29, 185–193.

102. Nickel, T.; Sonntag, A.; Schill, J.; Zobel, A.W.; Ackl, N.; Brunnauer, A.; Murck, H.; Ising, M.; Yassouridis, A.; Steiger, A.; et al. Clinical and Neurobiological Effects of Tianeptine and Paroxetine in Major Depression. J. Clin. Psychopharmacol. 2003, 23, 155–168.

103. Nikolaus, S.; Schulte, B.; Al-Massad, N.; Thieme, F.; Schulte, D.M.; Bethge, J.; Rehman, A.; Tran, F.; Aden, K.; Häsler, R.; et al. Increased Tryptophan Metabolism Is Associated With Activity of Inflammatory Bowel Diseases. Gastroenterology 2017, 153, 1504–1516.e2.

104. Ferdinande, L.; Demetter, P.; Perez-Novo, C.; Waeytens, A.; Taildeman, J.; Rottiers, I.; Rottiers, P.; Vos, M.D.; Cuvelier, C.A. Inflamed Intestinal Mucosa Features a Specific Epithelial Expression Pattern of Indoleamine 2,3-Dioxygenase. Int. J. Immunopathol. Pharmacol. 2008, 21, 289–295.

GENERAL DISCUSSION

Aging is accompanied by structural and functional alterations in the GI tract, including changes in intestinal permeability, immune function, and microbiota composition [45,47]. In humans, these age-related shifts may compromise GBA signaling and contribute to a broad range of health conditions, including inflammation and altered neurobehavioral states [110,152]. While the microbiota–gut–brain axis is well established in rodent and human models [6], research specifically addressing how aging affects gut health and neurobehavioral function in dogs remains limited, particularly regarding integrative studies focused on the GBA [29,153]. Most veterinary studies in dogs have focused on isolated aspects of gastrointestinal health, without integrating the study of behavioral indicators together with gut health biomarkers [2,4,46,54,60,154]. Furthermore, while fecal biomarkers and microbial analysis are increasingly used in veterinary diagnostics and research, their potential role as indicators of neurobehavioral and cognitive status remains largely unexplored in dogs.

The present research aims to address this knowledge gap through two complementary studies. The first study evaluates intestinal health biomarkers and microbiota composition across different age groups in a multibreed population of dogs under the same housing conditions (Affinity Nutrition Center). From this initial cohort, Beagle dogs were selected to enable a more detailed investigation of biomarkers. Beagles are widely considered the most suitable breed for experimental studies due to their genetic and physiological homogeneity, manageable size, and docile temperament. These characteristics help reduce variability related to breed differences and facilitate handling during experimental procedures.

As the second study involved partially invasive techniques (saliva and blood sampling), which required specific volumes to assess all selected indicators, Beagles represented the most appropriate choice to meet the methodological demands. Therefore, in the second study, we evaluated age-related changes in intestinal and behavioral indicators in a population of Beagle dogs, providing valuable insights into the gut–brain axis (GBA) connection.

Together, these two studies explore how aging shapes the gut ecosystem and behavioral physiology in dogs, and whether measurable changes in the gut may correlate with alterations in brain-related functions.

The better understanding of the GBA in dogs and its relation to the aging process, it may help to pave the way for defending targeted solutions depending on the dogs age increasing the chances of healthy aging. It is not merely about living longer, but about understanding how to ensure that dogs maintain their quality of life throughout the aging process.

1. AGE-RELATED CHANGES IN INTESTINAL HEALTH

Age is one of the most important intrinsic factors shaping gut microbiota composition, diversity, and function. In both humans and companion animals, the gastrointestinal microbial ecosystem undergoes dynamic changes over the life span, including shifts in taxonomic representation, microbial metabolites, and host–microbe interactions [45,110].

1.1. Faecal microbiota composition

Phylum

In the two papers of this thesis, the five predominant phyla identified in canine fecal samples were Actinomycetota (Actinobacteria), Bacillota (Firmicutes), Bacteroidota (Bacteroidetes), Fusobacteriota (Fusobacteria) and Pseudomonadota (Proteobacteria), consistent with previous reports on the canine gut microbiome [155]. However, in our data, the three most abundant phyla were Actinomycetota, Bacillota and Fusobacteriota, a distribution that remained stable across the three age categories. This contrasts with previous studies, which reported Bacteroidota among the three dominant phyla instead of Actinomycetota [156,157].

Among the five phyla, Bacillota showed the highest relative abundance, while Pseudomonadota displayed the lowest. In the Beagle population, significant differences in relative abundance were observed for Actinomycetota, Bacteroidota,

and Pseudomonadota across age groups. Actinomycetota was lower in seniors compared to adults; Bacteroidota was higher compared to the two younger age categories; and, Pseudomonadota was higher in seniors compared to juniors. In the multibreed cohort, the direction and significance of changes for Bacteroidota and Pseudomonadota was consistent.

Generally, the low abundance of Bacteroidota may be associated with conditions such as obesity and gastrointestinal disease [2,158], while the higher abundance of Actinomycetota has been linked to pathological states [48]. Considering the health status and characteristics of the two populations, along with the results, our most plausible hypothesis is that the observed differences may be attributed to cohort-specific traits, or possibly influenced by methodological variation [159–161]. The two cohorts of animals shared the same conditions, since Beagles were a subpopulation of the multibreed cohort, and the analytical procedures were the same in both studies.

Family, genus and species

In both studies included in this thesis, we observed several subtle age-associated shifts in the microbiota composition of dogs at the family, genus, and species taxonomic levels. Within the Actinomycetota phylum, Bifidobacteriaceae family and *Bifidobacterium* genus were consistently higher in adults compared to juniors in the two studied cohorts. These results contrast with previous published findings, in which Bifidobacteria was only present in puppies but not in older dogs (adults and seniors) [5]. However, this study could be biased in finding specific taxa since the results were based on culture-based methods. Moving to the human literature, Bifidobacteria is described to enhance health benefits in the host and it is the most prevalent bacteria among infants and adults [162]. From this, we could extrapolate that Bifidobacteria presence in the three categories may be understood as positive. However, the extrapolation of the results among animal species should be interpreted carefully. The abundance of *Bifidobacterium* measured in the multibreed cohort showed a positive correlation with valeric acid concentration and the relative proportion of acetate. While associations between *Bifidobacterium* and acetate have been reported previously [163,164], additional research is needed to clarify its potential relationship with valeric acid.

Ruminococcaceae and Veillonellaceae showed consistently higher relative abundance in senior dogs across both cohorts. These families falling within Bacillota taxa are well-recognized producers of SCFA, which play important roles in gut barrier integrity and immune modulation [165,166]. Additionally, the decrease in Ruminococcaceae has been previously related with inflammatory bowel disease [165,166]. Considering the two studies, more changes in the same direction were found in Bacteroiddota phylum, in which age-related increases were observed in Bacteroidaceae and Prevotellaceae families. Bacteroidota, which includes both Bacteroidaceae and Prevotellaceae, showed a notable shift with age, with increased Bacteroidaceae observed in older individuals, especially those with frailty [167].

Falling within Pseudomonadota phylum, we found Succinivibrionaceae and Alcaligenaceae families were more abundant in senior dogs. These changes contributed to an overall increase in Pseudomonadota, which has been associated with intestinal inflammation [165]. This observation could support a potential link between these microbial changes and inflammaging, a chronic, low-grade inflammatory state characteristic of senior animals as a consequence of aging [168].

Focusing on the species level, the most notable feature, due to its biological importance for gut health, was *Faecalibacterium prausnitzii*, which showed significant differences between age categories in the 16S analysis. This taxa exhibited higher relative abundance in the senior population. Additionally, in the multibreed study, we examined the correlation with SCFA levels and observed that *Faecalibacterium* was positively correlated with propionate and valeric acid concentrations. *F. prausnitzii* is a major butyrate-producing bacterium with known anti-inflammatory properties, and it has already been associated with a healthy microbiome in dogs [46]. In contrast, the absence of this bacterium has previously been linked to gastrointestinal disorders in both dogs and humans [2,169] .

Additionally, *F. prausnitzii* has been clearly associated with increased fiber fermentation and various types of dietary fiber in dogs [40,170]. However, we consider that the fiber content of the diet was not the cause of the higher abundance observed in the senior category for *F. prausnitzii*. This interpretation is based on the fact that, considering the range of diet composition (7-8% moisture, 22-30% protein, 12-21% fat and 1-5% crude fiber) nearly all dogs (95 % in the multibreed cohort and

92 % in the Beagle cohort) were fed diets within the same range. Additionally, the dogs which were out the range were similarly distributed between the age categories (J = 2/106; A = 2/106; and S = 1/106 in the multibreed cohort; and J = 2/37; A = 0/37; and S = 1/37, in the Beagles cohort).

Dysbiosis index

To further characterize microbial imbalance, we applied the DI, a validated complex algorithm that considered the abundance of seven bacterial taxa and total bacteria identified by qPCR to a reference database [60]. DI values were interpreted as follows: values < 0 were considered indicative of a normobiotic microbiota; values between 0 and 2 suggested a mild to moderate microbial imbalance; and values > 2 were indicative of a significant microbial shift [4,171]. In our first study, while the DI did not reach pathological thresholds in any age group, some adult and senior individuals exhibited higher DI values. In our second study, the DI mean values fell within the normal range; only two adult dogs were within the dysbiosis index range, defined as mild to moderate microbiota shift (DI between 0 to 2). This subtle dysregulation could be associated with age-related factors such as immune senescence, compromised mucosal barrier integrity, or altered digestive enzyme secretion [110]. Interestingly, the DI correlated with several SCFAs and behavioral markers in the Beagle cohort, including rest time and exploratory behavior, further supporting its relevance as a potential indicator of functional GBA status (see Section 3 for further discussion).

Taken together, the findings from faecal bacterial composition and the dysbiosis index support the idea that microbiota composition remains relatively stable in healthy animals despite aging. Nonetheless, subtle but biologically relevant shifts occur, involving taxa associated with short-chain fatty acid production and inflammation. In both human and animal studies, aging has been linked to subtle but biologically meaningful shifts in gut microbial diversity and structure, though results remain variable depending on methodology, diet, health status, and housing environment [29,172]. However, what appears consistent is that aging drives modest alterations in microbial composition; and these microbial signatures may represent early indicators of physiological aging in dogs, offering potential targets for dietary or biotic strategies to support healthy aging.

1.2. Microbial diversity indices

Diversity indices

Considering the two studies included in this thesis, we observed that overall microbial diversity indices remained relatively stable with age. In both studies, only the unweighted UniFrac metric for beta diversity, which considers the presence or absence of individual taxa, showed a statistically significant difference between age groups, while all other diversity metrics remained unchanged. Additionally, the alpha diversity indices did not differ significantly across age categories in any of the studies. These findings are consistent with previous published results in dogs suggesting that microbial diversity may remain unaffected by age [154]. However, other authors have reported that the canine gut microbiota appears to stabilize after early developmental stages, and may begin to exhibit a gradual decline in diversity during the senior stage [9]. In humans, research showed that aging is typically associated with a decline in microbial diversity [110]. This loss of microbial diversity has been associated with increased susceptibility to gastrointestinal and systemic diseases, primarily due to the loss of microbial resilience and functional redundancy [15,16].

Nevertheless, although microbial diversity indices did not show substantial changes in our studies, we identified minor shifts in microbial composition across age categories (as discussed in the previous subsection). Taxonomic shifts may occur without significantly altering diversity metrics but may nonetheless have physiological consequences [110,152]. These findings lead us to hypothesize that larger canine cohorts may be required to more accurately determine whether microbial diversity in dogs follows the same age-related declining trend observed in humans [110].

1.3. Metabolic biomarkers

SCFA are microbial metabolites primarily produced in the large intestine through the anaerobic fermentation of non-digestible carbohydrates, including dietary fibers and resistant starch. Although fiber fermentation is the main route for generating acetate, propionate, and butyrate, these compounds can also originate from amino acid metabolism. However, this alternative pathway is employed by less than 1% of the

colonic microbiota [173–175]. Protein fermentation generally occurs in the distal colon when carbohydrate sources are depleted, producing not only SCFAs but also potentially harmful byproducts such as ammonia, phenols, and hydrogen sulfide, along with branched-chain fatty acids (BCFAs) like isobutyric and isovaleric acid [173–175].

SCFAs act as key signaling molecules at the host–microbiota interface, influencing gastrointestinal physiology, systemic immune responses, metabolic regulation, and even neurobiological pathways through the gut–brain axis [6,164,175]. In dogs, the profile of SCFAs in feces reflects the metabolic output of the microbiota and the availability of fermentable substrates [156,176].

Major SCFAs: Acetate, Propionate, Butyrate

Among the major SCFAs, acetate is the most abundant, often constituting over 60% of total SCFA content in canine feces [156]. It is readily absorbed by colonocytes and enters systemic circulation, where it contributes to lipid synthesis, appetite regulation, and immune signaling [74,175]. Acetate consistently emerged as the most abundant SCFA in both studies. Its concentration showed an increasing trend with age and was significantly elevated in senior dogs in the Beagle study, supporting an age-related increase. This could reflect enhanced fermentative capacity or increased colonic retention of fiber substrates with aging. Notably, previous findings in older dogs and humans suggest that acetate production may increase as a compensatory response to changes in fiber fermentation efficiency or microbiota composition [45,177].

Propionate, a SCFA linked to gluconeogenesis and cholesterol metabolism, showed a higher relative percentage in junior dogs in the Beagle cohort. This higher propionate production may reflect the higher metabolic demands and energy turnover of younger animals [178]**.** However, propionate was not significantly different in absolute concentration across groups in either study. These numbers may be partly attributed to proportion-based calculations, as the higher acetate levels in seniors reduced the relative abundance of other SCFAs.

Butyrate, recognized for its anti-inflammatory effects and trophic support of the intestinal epithelium, showed a slight increase in adult dogs, though no significant

age-related differences were observed in either study. Interestingly, in the multibreed cohort, the relative percentage of butyrate was lower in senior dogs compared to adults and juniors. This reduction may result from the elevated proportion of acetate in seniors, as SCFA proportions are interdependent when expressed in relative terms. Furthermore, previous research suggests that butyrate levels can vary between dog breeds and may be influenced by factors such as body size, diet, and microbial composition [176,179], which might help explain inter-cohort variability.

Minor SCFAs and Branched-Chain Fatty Acids

Minor SCFAs, such as valeric acid, isobutyric acid, and isovaleric acid, showed subtle but potentially biologically meaningful age-related trends across both studies. While these metabolites are less abundant than major SCFAs like acetate or butyrate, they play distinctive roles in gut motility, microbial competition, and host–microbiota signaling, particularly through amino acid fermentation pathways [175].

Valeric acid consistently decreased in senior dogs across both the multibreed study and the controlled Beagle cohort, observed in absolute concentration and in relative percentage of total SCFAs. In the multibreed dataset, the reduction in valerate was statistically significant when expressed as concentration, while in the Beagle group, the most apparent change was reflected in its proportion within the SCFA profile. This finding is consistent with prior reports in humans and rodent models, where lower fecal valerate has been associated with reduced microbial diversity, decline in proteolytic bacteria, and frailty-related gut dysfunction [45,177]. Because valerate is thought to influence enteric nervous system regulation and gut muscle tone, its reduction with age may also have implications for gut–brain axis (GBA) signaling [175].

Branched-chain SCFAs, such as isobutyric and isovaleric acid—derivatives of valine and leucine metabolism—showed non-significant but generally decreasing trends in older dogs in both studies. In the Beagle cohort, the relative percentage of isovaleric acid was significantly lower in seniors compared to juniors, while in the multibreed population, decreases were observed in both relative and absolute terms.These differences may arise from breed- or diet-related factors or inter-individual variation

in protein digestion and microbial composition [156]. In addition, although the physiological roles of isobutyric and isovaleric acid in canine aging remain poorly defined, their decrease may point to shifts from proteolytic to saccharolytic fermentation, a trend frequently reported in elderly humans and associated with decreased microbial diversity and increased gut permeability [45,110].

Overall, the integrated analysis of both cohorts revealed that aging in dogs is associated with changes in the SCFA profile, characterized by elevated acetate and reduced valerate and branched SCFAs, suggesting functional shifts in the gut microbiota. These changes may have downstream consequences for immune modulation, neuromuscular function, and cognitive health, potentially mediated through GBA signaling [6,177].

1.4. Inflammatory and immune function biomarkers

Fecal calprotectin and **immunoglobulin A** are widely used as non-invasive indicators of intestinal inflammation and mucosal immune function, respectively [82,180]. In both studies, neither cCP nor IgA levels differed significantly between age groups, suggesting that intestinal inflammatory status and mucosal immunity remained stable in the absence of pathological conditions. According to existing literature, no consistent association has been observed between age and mucosal IgA levels in aged mice and humans [181,182]. Similarly, Zaine et al. [183] found no significant differences in fecal IgA concentrations between senior dogs and younger categories. However, in the same study, puppies at five months of age exhibited lower IgA levels than adult dogs [183]. Although recent studies in healthy Korean individuals have shown an age-related correlation in fecal cCP concentrations [184], evidence in dogs across different age groups remains inconclusive.

Positive acute-phase proteins (APPs), such as **C-reactive protein** and **haptoglobin**, are synthesized in response to different forms of inflammation [94]. Although haptoglobin levels were correlated with age (rho = 0.426; p-value = 0.05), no statistically significant differences were observed among the defined age groups. All measured haptoglobin values fell within the normal reference range [185]. Interestingly, our study revealed a positive correlation between haptoglobin and

folate concentrations. In contrast, C-reactive protein levels did not show significant variation between age categories. Although acute-phase proteins such as C-reactive protein are well-established markers of inflammation in dogs [94], their potential modulation with age has not been clearly established. In humans, however, several acute-phase proteins have been shown to increase with advancing age [186,187], suggesting possible interspecies similarities that deserve further exploration.

1.5. Other functional gut health biomarkers

Folate and cobalamin

The results of the Beagles study revealed significant age-associated changes in serum folate (vitamin B9) and a negative correlation between age and cobalamin (vitamin B12) concentrations, suggesting that aging may influence micronutrient homeostasis, either through altered intestinal absorption, changes in microbial metabolism, or systemic physiological shifts [188,189].

Specifically, **serum folate** levels were significantly elevated in senior dogs compared to junior and adults. This increase could be reflecting an enhanced bacterial folate synthesis in the colon due to shifts in microbiota composition, as certain taxa known to proliferate with age (e.g., *Bacteroides*) are also folate producers [190]. Another plausible explanation is a compensatory response to subclinical inflammation, as folate plays an important role in nucleotide synthesis and immune modulation [191].

Serum cobalamin concentrations showed a decreasing pattern with age. While not statistically significant across all age groups in the model, it exhibited a moderate negative correlation with age, suggesting a biologically meaningful decline. Cobalamin absorption is highly dependent on intrinsic factor secretion and intact ileal function, both of which can be impaired by age-related changes in gut integrity or low-grade inflammation [192]. Furthermore, we observed an increase in Proteobacteria in the seniors, which has been previously linked to lower cobalamin levels [193].

Of note, the simultaneous occurrence of low cobalamin and elevated folate levels could also reflect increased bacterial utilization of cobalamin and excess bacterial

folate synthesis [194]. This pattern has previously been described in dogs with bacterial overgrowth and small intestinal dysbiosis [38,195]. To better characterize this relationship, assessing methylmalonic acid could provide additional insights. Methylmalonic acid plays a role in cellular metabolic regulation and signaling pathways [196]. It is recognized as a more sensitive and accurate biomarker of cobalamin body status than cobalamin concentrations and has already been associated with several age-related pathological conditions [196–198].

In conclusion, the contrasting trends in folate and cobalamin levels with age suggest complex microbiota–host interactions that warrant further investigation. The analysis of additional biomarkers, such as methylmalonic acid, could provide a more comprehensive understanding. While elevated folate levels may result from increased microbial synthesis or inflammation-related signaling, declining cobalamin concentrations may reflect impaired absorption or competition with bacteria for uptake.

2. AGE-RELATED CHANGES IN BEHAVIOR

2.1. Neuroactive and endocrine metabolites related to gut health

Neuroactive metabolites are involved in neurophysiological processes and are increasingly studied in the context of the GBA. In our study in Beagle dogs, **kynurenine** levels and the KYN/TRP ratio exhibited a tendency to increase with age, whereas **tryptophan** concentrations remained relatively constant across groups. These findings partially align with previous research in rodents and humans, in which aging has been associated with changes in tryptophan metabolism, including increase degradation of tryptophan and elevated kynurenine levels, which are considered markers of immune activation and neuroinflammation [199,200]. The kynurenine pathway represents the principal route of TRP catabolism, accounting for approximately 95% of its systemic degradation. This metabolic cascade is predominantly initiated by the enzyme **indoleamine 2,3-dioxygenase (IDO)**, which catalyzes the first step in the conversion of TRP to KYN. IDO activity is strongly inducible by pro-inflammatory cytokines, especially interferon-γ, and is thus closely linked to immune surveillance and inflammatory states. Increased IDO expression

and activity have been documented in various contexts of chronic low-grade inflammation, a hallmark of aging known as inflammaging [201,202]. This age-associated immunological remodeling contributes to sustained activation of the kynurenine pathway, potentially leading to the accumulation of neuroactive and neurotoxic TRP metabolites, which may impact central nervous system function and behavior [201,203].

Tryptophan metabolism is regulated by both host and microbial factors and plays a critical role in intestinal and systemic homeostasis. Besides the kynurenine route, which dominates TRP catabolism, approximately 4-6% of dietary TRP is metabolized by the gut microbiota into indole and its derivatives, and a smaller fraction (1–2%) contributes to serotonin biosynthesis in the host's enterochromaffin cells [199]. Given the interplay between microbial activity, immune function, and aging, the modulation of TRP metabolic pathways, particularly via IDO and the kynurenine pathway, may represent a mechanistic link between gut microbial dynamics and age-related changes in neurophysiology and systemic inflammation [204]

Metabolites derived from tryptophan metabolism by the gut microbiota can influence both gastrointestinal function and central nervous system (CNS) activity, highlighting the microbiota’s critical role in modulating the gut–brain axis. Specifically, bacterial genera such as *Clostridium*, *Burkholderia*, *Streptomyces*, *Pseudomonas*, and *Bacillus* have been identified through *in silico* genomic analyses as harboring metabolic pathways capable of converting tryptophan into various neuroactive compounds, including indole, indoleacetic acid, quinolinate, and tryptamine [205]. With aging, shifts in the composition of the gut microbiota may lead to an increased abundance of these tryptophan-metabolizing taxa, potentially enhancing the flux through the kynurenine pathway [199,205]. This metabolic shift could result in elevated production of neurotoxic intermediates such as quinolinate and reduced synthesis of neuroprotective metabolites like serotonin and kynurenic acid [206,207]. Drawing from evidence primarily derived from studies in humans and rodents, we hypothesize that imbalances in tryptophan metabolism may impair gut–brain axis signaling and thereby contribute to age-related cognitive decline and neuroinflammatory processes.

To gain a more comprehensive understanding of tryptophan metabolism complexity and its role in behavioral and physiological aging, future studies should include a broader panel of downstream metabolites. In the present study, our analysis was limited to tryptophan and kynurenine concentrations. While these provide valuable insight into the activation of the kynurenine pathway, they do not capture the full complexity of tryptophan catabolism. The inclusion of additional metabolites, such as quinolinic acid, kynurenic acid and serotonin, would offer a more detailed perspective on the balance between neurotoxic and neuroprotective processes. Such data could help clarify how age-related alterations in tryptophan metabolism affect gut–brain axis signaling, inflammatory responses, microbiota composition, and behavior. Moreover, this knowledge could contribute to the identification of potential biomarkers and therapeutic targets to promote healthy cognitive aging.

Oxytocin, a neuropeptide involved in social behavior, bonding, and stress regulation, did not differ significantly across age groups in our study. Although a slight reduction in oxytocin levels was observed in senior dogs, this trend did not reach statistical significance. Although it is important to consider that the results were a trend, the discussion of the biological importance of oxytocin in the gut-brain deserves to go into more detail. Nevertheless, this decrease may be biologically relevant, as lower oxytocin levels in older dogs could contribute to the reduced social and exploratory behaviors observed in this group. Previous research in humans and rodents has suggested a role for oxytocin in cellular aging and hippocampal function [61,62]. Furthermore, emerging evidence supports that oxytocin may interact with the tryptophan metabolic pathway, particularly by modulating the activity of indoleamine 2,3-dioxygenase (IDO), a key enzyme that influences the balance between serotonin synthesis and the kynurenine pathway [102]. Through this mechanism, oxytocin could impact both serotonin availability and neuroinflammatory responses [208].

In aging populations, both human and animal models have suggested a decline in oxytocinergic signaling, potentially contributing to impairments in cognition, memory, and social interaction [209,210]. Although our findings do not demonstrate a clear relationship between oxytocin and chronological aging in dogs, they do not exclude the possibility of subtle functional changes that might have potential downstream effects on behavior and cognition. Importantly, oxytocin does not act in isolation but

interacts with other components of the gut–brain axis, including the HPA axis, immune mediators, and the gut microbiota. Emerging evidence suggests that oxytocin signaling may be influenced by gut microbial composition. Certain microbial taxa have been shown to affect the expression of oxytocin and its receptors through immune and vagal pathways, contributing to social behavior regulation and stress responses

Additionally, **cortisol**, a widely recognized biomarker of stress and HPA axis function, did not show significant differences between age groups in the study, though senior dogs exhibited greater variability in cortisol levels. However, greater variability in cortisol levels among senior dogs was observed descriptively, which may reflect increased heterogeneity in stress responsiveness or subtle age-related changes in HPA axis regulation. While speculative, this interpretation aligns with previous findings suggesting age-associated alterations in neuroendocrine control [211].

Cortisol is essential for normal neurodevelopment and cognitive functioning, including learning and memory, and its regulation is closely linked to the activity of the hypothalamic-pituitary-adrenal (HPA) axis. Evidence from experimental studies indicates that the gut microbiota exerts regulatory effects on the HPA axis from early life into adulthood [212]. Perturbations in this bidirectional relationship, such as those induced by stress, can disrupt the microbiota-gut-brain axis and alter HPA axis activity. Increased cortisol levels have been previously associated with impairments in cognition and behavior, potentially contributing to age-related functional decline [213]. In our study, we observed that cortisol was negatively correlated with isobutyric and isovaleric acids, particularly in adult dogs. In parallel, *Lactobacillus* abundance was linked to salivary cortisol concentration. These findings may suggest that microbial activity and microbial proteolytic metabolites may be involved in modulating the stress response, a relationship also observed in human studies on depression and HPA axis dysregulation [214,215]. Interestingly, *Lactobacillus* abundance correlated with salivary cortisol, confirming previous findings in stress-modulated gut microbiota studies [216,217]. Gut microbiota composition has been shown to influence HPA axis activity, potentially linking microbial shifts in aging dogs to changes in cortisol regulation [217,218].

Collectively, our findings support a model in which specific microbial taxa and their metabolic products interact with host endocrine pathways (HPA axis), influencing stress regulation and potentially affecting behavior and cognitive health across the lifespan.

Lastly, we investigated **thyroxine**, a key thyroid hormone involved in metabolic regulation, growth, and development, and found notable associations between endocrine status and gut health indicators. Specifically, TT4 concentrations were found to be positively associated with specific gut bacterial genera, such as *Blautia* and *Clostridium hiranonis*, both known for their roles in SCFA production and bile acid metabolism. Conversely, higher concentrations of acetate in feces were negatively associated with TT4 levels. This opposing pattern may reflect a regulatory interaction, whereby increased microbial production of acetate may affect the regulation of the HPA axis. Given that acetate can cross the blood-brain barrier and interact with central regulatory systems, it may play a role in modulating hormonal pathways at the systemic level, including those involved in thyroid regulation. *Blautia*, a genus within the Lachnospiraceae family, is generally recognized as a beneficial commensal taxon that primarily produces acetate and may also contribute to butyrate availability through metabolic cross-feeding interactions with other butyrate-producing microbes [175,219]. *Clostridium hiranonis*, meanwhile, is well characterized for its role in the 7α-dehydroxylation of primary bile acids, leading to the production of secondary bile acids such as deoxycholic acid. Additionally, this species contributes to butyrate synthesis, a metabolite essential for maintaining colonic epithelial health and regulating intestinal inflammation [62].

Overall, TT4 concentrations were higher in junior dogs, potentially reflecting increased metabolic demands during growth or age-dependent host–microbiota interactions during early developmental stages [220]. These findings align with the greater behavioral engagement we observed in junior dogs, as well as the higher activity levels registered by accelerometers in this age group.

2.2. Behavioral observations

Behavioral differences across age groups were evident. Junior dogs exhibited higher play and exploratory behaviors, while adults and seniors demonstrated increased alertness and reduced social engagement. These results are consistent with previous findings that link aging to reduced motivation and increased cautiousness in dogs [221]. In our study, the observed decline trend in oxytocin levels in seniors, although not statistically significant, could be biologically meaningful. Lower oxytocin concentrations trend in older dogs could partly underlie the reduced sociability and exploratory behavior seen in this group, suggesting a potential neuroendocrine contribution to age-related behavioral changes.

The behavioral engagement of younger dogs may reflect both neurobiological flexibility and higher energy levels [222], which could correlate with more favorable gut profiles [223]. Notably, behaviors such as play and exploration are considered indirect indicators of welfare and cognitive health [224,225]. Their decline in senior dogs may signal emerging cognitive shifts.

2.3. Activity measures

Using accelerometry (Actical®), we detected a decline in total activity and diurnal activity in older dogs. These findings are consistent with those of Lee et al. [24], who demonstrated that physical activity decreases with age in companion dogs, with potential implications for metabolic function, cognitive performance, and overall longevity. Moreover, activity reduced in aged individuals have also been associated with changes in gut microbial diversity and increased systemic inflammation, potentially implicating GBA mechanisms [6].

Although our data did not directly address all the physiological mechanisms underlying this reduction, it is plausible that the observed decline in activity is influenced by age-related decreases in mitochondrial efficiency, energy expenditure, and muscle mass. These physiological changes may act in conjunction with the behavioral alterations we observed, such as reduced play, exploratory behavior, and

social interaction, as well as the patterns we described for the neuroactive and endocrine metabolites related to gut health.

3. GUT–BRAIN AXIS DYNAMICS ACROSS AGING IN DOGS: GENERAL INSIGHTS AND INTEGRATIVE APPROACH FROM A GUT MICROBIAL PERSPECTIVE

This study examined how gut microbiota composition, microbial-derived metabolites and neuroendocrine and behavioral markers interact across different life stages in dogs. Our findings revealed age-related shifts in bacterial taxa and metabolite profiles that coincided with differences in physical activity, social behavior, and neuroendocrine biomarkers levels. Specifically, associations were observed between certain microbial genera and neuroactive or endocrine compounds such as SCFAs, tryptophan metabolites, and cortisol, suggesting that these microbial products may influence age-related changes in behavior and neuroendocrine function.

The gut microbiota is widely recognized as a key modulator of host physiology, acting through structural components such as lipopolysaccharides and peptidoglycans, and through bioactive products like SCFAs, microbial enzymes, and neurotransmitters [226–229]. These factors contribute to the regulation of intestinal barrier integrity and mucosal immune homeostasis [47,48]. Moreover, microbial interactions with enteroendocrine and neural pathways facilitate communication with the CNS, establishing the gut microbiota as a central actor in gut–brain crosstalk [217,230–232]. Emerging evidence has also shown that the gut microbiota influences hormonal regulation via modulation of the HPA axis, particularly by shaping glucocorticoid responses [212]. Disruptions to this system can affect immune function, intestinal permeability, and neurotransmitter signaling, with potential consequences for the CNS, including cognitive and emotional regulation [218,233]. In aging, these mechanisms gain particular relevance. Age-associated microbial shifts may influence the availability of neuroactive compounds such as serotonin, quinolinic acid, and indole derivatives, which have been implicated in inflammation, stress sensitivity, and behavioral adaptation. In our study, we observed that these microbial and metabolic changes correlated with both behavioral outputs

and endocrine profiles, including activity levels, sociability, and hormonal markers such as cortisol and thyroxine.

Altogether, our findings emphasize the need for integrative, multi-level approaches to investigate the gut–brain axis in aging. Combining microbiota profiling with endocrine, metabolomic, and behavioral data can provide a more complete picture of how age-related changes in gut physiology influence neuroendocrine and cognitive health. Such a systems-level perspective is essential for identifying microbial and molecular signatures of healthy aging, and for developing precision-targeted interventions, such as dietary strategies, probiotics, or prebiotics, to support emotional and behavioral resilience throughout the canine lifespan.

4. LIMITATIONS AND FUTURE RESEARCH

Despite the valuable insights generated by both studies into age-related changes in gut health and gut–brain interactions in dogs, several limitations must be acknowledged, and future research pathways identified.

4.1. Limitations

A major limitation in both studies lies in the cross-sectional design, which does not allow for the direct observation of within-individual changes over time. Although age groups were categorized as junior, adult, and senior, this approach may oversimplify the complex and gradual nature of biological aging, possibly masking critical transitions that occur between these life stages. Longitudinal studies would allow better understanding of how gut microbiota, SCFA production, and behavioral profiles evolve within the same individual, offering more precise temporal associations.

Another limitation is the absence of comprehensive dietary control across all animals in the multibreed study. Although most diets had similar nutrient profiles, even small variations in fiber source, protein quality, or fat content can substantially alter the gut microbiome composition and metabolite profiles [155]. In contrast, the Beagle study did benefit from environmental and dietary homogeneity, but this reduced external validity may limit generalization to the wider pet population.

Additionally, the relatively small sample sizes, particularly when stratified by age, reduce statistical power and may limit the detection of subtle but biologically relevant differences. For instance, although numerical changes in kynurenine levels and kynurenine/tryptophan ratios were observed, they did not reach statistical significance, likely due to intra-individual variability and the limited cohort size.

Finally, behavioral assessments, although informative, were inherently subjective and partially dependent on observer interpretation. Although standardized scoring and activity tracking tools (accelerometers) were used, more objective cognitive tests or advanced tracking of social behaviors could further improve behavioral characterization in future work.

4.2. Future research

To address these limitations, future research should emphasize longitudinal study designs with repeated measurements within the same individuals. This approach would enable the characterization of aging trajectories and the early identification of markers associated with gut or behavioral dysregulation.

Increasing the size and diversity of study populations is also essential to improve the external validity of findings, particularly in mixed-breed dogs and those living in heterogeneous home environments. Incorporating measures of body condition score, body composition, detailed dietary intake and dietary composition would provide a more comprehensive understanding of the multiple factors influencing gut and brain health.

Expanding the biomarker panel should remain a key priority. The integration of high-throughput techniques such as metabolomics, proteomics, transcriptomics and shotgun metagenomic sequencing would provide deeper insights into host–microbiota communication pathways, particularly those involved in neurotransmitter biosynthesis, immune regulation, and intestinal barrier function. Importantly, shotgun metagenomic sequencing could enable a detailed characterization of the gut microbiota at both taxonomic and functional levels, facilitating the detection of microbial species as well as the inference of their

metabolic potential. In addition, incorporating markers of gut permeability, such as zonulin or lipopolysaccharide-binding protein (LBP), together with a comprehensive profile of bile acids, could offer a more direct and integrative assessment of microbial activity and its interaction on host physiology. Primary bile acids, synthesized in the liver, are converted by gut bacteria into secondary bile acids, a transformation that reflects the metabolic functionality of the gut microbiota. Shifts in bile acid composition have been linked to alterations in microbial diversity, intestinal inflammation, and systemic metabolic disturbances [234]. Additionally, measuring key neuroactive compounds such as serotonin, quinolinic acid, and indole derivatives would be highly informative for monitoring changes in microbial metabolism that may impact the CNS regulation.

In parallel, precision nutrition strategies present a promising avenue for developing targeted interventions to support gut and brain health throughout the canine lifespan. By integrating multi-omics data with predictive modeling, it may become feasible to define individualized microbial signatures that respond optimally to specific dietary fibers, prebiotics, or other bioactive compounds [120,148]. These approaches could ultimately support tailored nutritional strategies aimed at promoting resilience and mitigating cognitive and behavioral decline with age.

Altogether, the associations reported in these studies underscore the dynamic and age-dependent relationships among gut microbial composition, microbially derived metabolites, neuroendocrine activity, and behavioral and activity outcomes. These findings highlight the importance of integrative frameworks that combine microbial, metabolic, hormonal, and behavioral data to better characterize the gut–brain axis across the aging process. Advancing our understanding of these mechanisms may inform the design of targeted interventions, including probiotic supplementation and dietary modulation, to promote healthy aging in dogs.

CONCLUSIONS

Study 1

- Aging is associated with significant modifications in some intestinal biomarkers, including those related to gut microbiota and specific SCFAs.
- Considering the studied parameters to assess gut health, we could state that once the microbiota becomes stable in healthy dogs, minor changes occur during the aging process.
- The modulation of the aged-related changes, considering the intestinal microbiota and short-chain fatty acid production, could improve the overall gut health promoting a healthy aging process in dogs.

Study 2

- The overall results of the studied biomarkers of intestinal health and behavioral indicators suggest minor changes in the different dog age groups.
- The observed differences in correlations between different biomarkers of gut health and behavior, particularly within the age categories, highlight the importance of considering age-related factors when studying the gut-brain axis.
- Further research is needed to better understand the mechanisms and the specific pathways involved in the relationship between the gut health biomarkers and the behavior in dogs.
- The findings described in this study could have implications for the development of nutritional interventions adapted to the needs of different age groups in dogs.

REFERENCES

1. Belkaid, Y.; Hand, T.W. Role of the Microbiota in Immunity and Inflammation. *Cell* **2014**, *157*, 121–141, doi:10.1016/j.cell.2014.03.011.

2. Minamoto, Y.; Otoni, C.C.; Steelman, S.M.; Büyükleblebici, O.; Steiner, J.M.; Jergens, A.E.; Suchodolski, J.S. Alteration of the Fecal Microbiota and Serum Metabolite Profiles in Dogs with Idiopathic Inflammatory Bowel Disease. *Gut Microbes* **2015**, *6*, 33–47, doi:10.1080/19490976.2014.997612.

3. Alessandri, G.; Milani, C.; Mancabelli, L.; Mangifesta, M.; Lugli, G.A.; Viappiani, A.; Duranti, S.; Turroni, F.; Ossiprandi, M.C.; Sinderen, D. van; et al. Metagenomic Dissection of the Canine Gut Microbiota: Insights into Taxonomic, Metabolic and Nutritional Features. *Environ. Microbiol.* **2019**, *21*, 1331–1343, doi:10.1111/1462-2920.14540.

4. Suchodolski, J.S. Analysis of the Gut *Microbiome* in Dogs and Cats. *Vet Clin Path* **2022**, *50*, 6–17, doi:10.1111/vcp.13031.

5. Masuoka, H.; Shimada, K.; Kiyosue-Yasuda, T.; Kiyosue, M.; Oishi, Y.; Kimura, S.; Yamada, A.; Hirayama, K. Transition of the Intestinal Microbiota of Dogs with Age. *Biosci Microbiota Food Heal* **2016**, *36*, 27–31, doi:10.12938/bmfh.bmfh-2016-021.

6. Cryan, J.F.; O'Riordan, K.J.; Sandhu, K.; Peterson, V.; Dinan, T.G. The Gut Microbiome in Neurological Disorders. *Lancet Neurol.* **2020**, *19*, 179–194, doi:10.1016/s1474-4422(19)30356-4.

7. Mondo, E.; Marliani, G.; Accorsi, P.A.; Cocchi, M.; Leone, A.D. Role of Gut Microbiota in Dog and Cat's Health and Diseases. *Open Vet J* **2019**, 9, 253–258, doi:10.4314/ovj.v9i3.10.

8. Forsythe, P.; Sudo, N.; Dinan, T.; Taylor, V.H.; Bienenstock, J. Mood and Gut Feelings. *Brain, Behav., Immun.* **2010**, *24*, 9–16, doi:10.1016/j.bbi.2009.05.058.

9. Vajányi, D.; Skurková, L.; Peťková, B.; Kottferová, L.; Kasičová, Z.; Simanová, V.; Kottferová, J. Ageing Canine Companions: Most Common Manifestations and the Impact of Selected Factors. *Appl. Anim. Behav. Sci.* **2024**, *271*, 106164, doi:10.1016/j.applanim.2024.106164.

10. Ruple, A.; MacLean, E.; Snyder-Mackler, N.; Creevy, K.E.; Promislow, D. Dog Models of Aging. *Annu. Rev. Anim. Biosci.* **2021**, *10*, 1–21, doi:10.1146/annurev-animal-051021-080937.

11. Kennedy, B.K.; Berger, S.L.; Brunet, A.; Campisi, J.; Cuervo, A.M.; Epel, E.S.;

Franceschi, C.; Lithgow, G.J.; Morimoto, R.I.; Pessin, J.E.; et al. Geroscience: Linking Aging to Chronic Disease. Cell 2014, *159*, 709–713, doi:10.1016/j.cell.2014.10.039.

12. Adav, S.S.; Wang, Y. Metabolomics Signatures of Aging: Recent Advances. *Aging Dis.* **2021**, *12*, 646–661, doi:10.14336/ad.2020.0909.

13. Saul, D.; Kosinsky, R.L. Epigenetics of Aging and Aging-Associated Diseases. *Int. J. Mol. Sci.* **2021**, *22*, 401, doi:10.3390/ijms22010401.

14. Azkona, G.; García-Belenguer, S.; Chacón, G.; Rosado, B.; León, M.; Palacio, J. Prevalence and Risk Factors of Behavioural Changes Associated with Age-related Cognitive Impairment in Geriatric Dogs. *J. Small Anim. Pr.* **2009**, *50*, 87–91, doi:10.1111/j.1748-5827.2008.00718.x.

15. Watowich, M.M.; MacLean, E.L.; Hare, B.; Call, J.; Kaminski, J.; Miklósi, Á.; Snyder-Mackler, N. Age Influences Domestic Dog Cognitive Performance Independent of Average Breed Lifespan. *Anim. Cogn.* **2020**, *23*, 795–805, doi:10.1007/s10071-020-01385-0.

16. Harvey, N.D. How Old Is My Dog? Identification of Rational Age Groupings in Pet Dogs Based Upon Normative Age-Linked Processes. *Frontiers Vet Sci* **2021**, *8*, 643085, doi:10.3389/fvets.2021.643085.

17. Moqri, M.; Herzog, C.; Poganik, J.R.; Consortium, B. of A.; Justice, J.; Belsky, D.W.; Higgins-Chen, A.; Moskalev, A.; Fuellen, G.; Cohen, A.A.; et al. Biomarkers of Aging for the Identification and Evaluation of Longevity Interventions. Cell **2023**, *186*, 3758–3775, doi:10.1016/j.cell.2023.08.003.

18. Willems, A.; Paepe, D.; Marynissen, S.; Smets, P.; Maele, I.V. de; Picavet, P.; Duchateau, L.; Daminet, S. Results of Screening of Apparently Healthy Senior and Geriatric Dogs. *J. Vet. Intern. Med.* **2017**, *31*, 81–92, doi:10.1111/jvim.14587.

19. Mosier, J.E. Effect of Aging on Body Systems of the Dog. *Vet. Clin. North Am.: Small Anim. Pr.* **1989**, *19*, 1–12, doi:10.1016/s0195-5616(89)50001-9.

20. Bellows, J.; Colitz, C.M.H.; Daristotle, L.; Ingram, D.K.; Lepine, A.; Marks, S.L.; Sanderson, S.L.; Tomlinson, J.; Zhang, J. Common Physical and Functional Changes Associated with Aging in Dogs. *J. Am. Vet. Méd. Assoc.* **2015**, *246*, 67–75, doi:10.2460/javma.246.1.67.

21. Cianciolo, R.E.; Benali, S.L.; Aresu, L. Aging in the Canine Kidney. *Vet. Pathol.* **2016**, *53*, 299–308, doi:10.1177/0300985815612153.

22. Hoskins, J.D. Liver Disease in the Geriatric Patient. Vet. Clin. North Am.: Small

Anim. Pr. **2005**, *35*, 617–634, doi:10.1016/j.cvsm.2004.12.010.

23. Hamlin, R.L. Geriatric Heart Diseases in Dogs. Vet. Clin. North Am.: Small Anim. Pr. 2005, 35, 597–615, doi:10.1016/j.cvsm.2005.01.003.

24. Lee, H.; Collins, D.; Creevy, K.E.; Promislow, D.E.L.; Akey, J.M.; Benton, B.; Borenstein, E.; Castelhano, M.G.; Coleman, A.E.; Creevy, K.E.; et al. Age and Physical Activity Levels in Companion Dogs: Results From the Dog Aging Project. *J. Gerontol.: Ser. A* **2022**, *77*, 1*9*86–1993, doi:10.1093/gerona/glac099.

25. Gürel, S.; Pak, E.N.; Tek, N.A. Aging Processes Are Affected by Energy Balance: Focused on the Effects of Nutrition and Physical Activity on Telomere Length. *Curr. Nutr. Rep.* **2024**, *13*, 264–279, doi:10.1007/s13668-024-00529-9.

26. Galkin, F.; Kovalchuk, O.; Koldasbayeva, D.; Zhavoronkov, A.; Bischof, E. Stress, Diet, Exercise: Common Environmental Factors and Their Impact on Epigenetic Age. *Ageing Res. Rev.* **2023**, *88*, 101956, doi:10.1016/j.arr.2023.101956.

27. Polsky, L.R.; Rentscher, K.E.; Carroll, J.E. Stress-Induced Biological Aging: A Review and Guide for Research Priorities. Brain, Behav., Immun. 2022, *104*, 97–109, doi:10.1016/j.bbi.2022.05.016.

28. McCune, S.; Promislow, D. Healthy, Active Aging for People and Dogs. *Front. Vet. Sci.* **2021**, *8*, 6*55*191, doi:10.3389/fvets.2021.655191.

29. Barko, P.C.; McMichael, M.A.; Swanson, K.S.; Williams, D.A. The Gastrointestinal Microbiome: A Review. *J Vet Intern Med* **2018**, *32*, 9–*25*, doi:10.1111/jvim.14875.

30. Tilg, H.; Zmora, N.; Adolph, T.E.; Elinav, E. The Intestinal Microbiota Fuelling Metabolic Inflammation. *Nat. Rev. Immunol.* **2020**, *20*, 40–54, doi:10.1038/s41577-019-0198-4.

31. Abdolmaleky, H.M.; Zhou, J.-R. Gut Microbiota Dysbiosis, Oxidative Stress, Inflammation, and Epigenetic Alterations in Metabolic Diseases. *Antioxidants* **2024**, *13*, 985, doi:10.3390/antiox13080985.

32. Mousa, W.K.; Chehadeh, F.; Husband, S. Microbial Dysbiosis in the Gut Drives Systemic Autoimmune Diseases. *Front. Immunol.* **2022**, *13*, 90*6*258, doi:10.3389/fimmu.2022.906258.

33. Bosco, N.; Noti, M. The Aging Gut Microbiome and Its Impact on Host Immunity. *Genes Immun.* **2021**, *22*, 289–303, doi:10.1038/s41435-021-00126-8.

34. Sommer, F.; Bäckhed, F. The Gut Microbiota — Masters of Host Development and Physiology. *Nat. Rev. Microbiol.* **2013**, *11*, 2*27*–238, doi:10.1038/nrmicro2974.

35. Pilla, R.; Suchodolski, J.S. The Role of the Canine Gut Microbiome and Metabolome in Health and Gastrointestinal Disease. Frontiers Vet Sci 2020, 6, 498, doi:10.3389/fvets.**2019**.00498.

36. Suchodolski, J.S. The Intestinal Microbiome in Canine Chronic Enteropathy and Implications for Extraintestinal Disorders. *Adv Small Animal Care* **2020**, *1*, *10*1–110, doi:10.1016/j.yasa.2020.07.008.

37. V., L.S.; Oluf, P. The Human Intestinal Microbiome in Health and Disease. *New Engl. J. Medicine* **2016**, *375*, 2369–2379, doi:10.1056/nejmra1600266.

38. Suchodolski, J.S. Diagnosis and Interpretation of Intestinal Dysbiosis in Dogs and Cats. Vet J 2016, 215, 30–37, doi:10.1016/j.tvjl.2016.04.011.

39. Colombino, E.; Prieto-Botella, D.; Capucchio, M.T. Gut Health in Veterinary Medicine: A Bibliometric Analysis of the Literature. *Animals* **2021**, *11*, *19*97, doi:10.*33*90/ani11071997.

40. Pilla, R.; Suchodolski, J.S. The Gut Microbiome of Dogs and Cats, and the Influence of Diet. Vet. Clin. North Am.: Small Anim. Pr. 2021, 51, 605–621, doi:10.1016/j.cvsm.2021.01.002.

41. Schmitz, S.S. Evidence‐based Use of Biotics in the Management of Gastrointestinal Disorders in Dogs and Cats. *Vet. Rec.* **2024**, *195*, 26–32, doi:10.1002/vetr.4916.

42. Garcia-Mazcorro, J.F.; Kawas, J.R.; Cassani, C.L.; Mertens-Talcott, S.; Noratto, G. Different Analysis Strategies of 16S RRNA Gene Data from Rodent Studies Generate Contrasting Views of Gut Bacterial Communities Associated with Diet, Health and Obesity. *PeerJ* **2020**, *8*, e10372, doi:10.7717/peerj.10372.

43. Hennessy, M.B. Using Hypothalamic–Pituitary–Adrenal Measures for Assessing and Reducing the Stress of Dogs in Shelters: A Review. Appl. Anim. Behav. Sci. 2013, *149*, 1–12, doi:10.1016/j.applanim.2013.09.004.

44. Goodrich, J.K.; Waters, J.L.; Poole, A.C.; Sutter, J.L.; Koren, O.; Blekhman, R.; Beaumont, M.; Van Treuren, W.; Knight, R.; Bell, J.T.; et al. Human Genetics Shape the Gut Microbiome. Cell **2014**, 159, 789–799, doi:10.1016/j.cell.2014.09.053.

45. O'Toole, P.W.; Jeffery, I.B. Gut Microbiota and Aging. *Science* **2015**, *350*, 1214–1215, doi:10.1126/science.aac8469.

46. Garrigues, Q.; Apper, E.; Chastant, S.; Mila, H. Gut Microbiota Development in the Growing Dog: A Dynamic Process Influenced by Maternal, Environmental and Host Factors. Frontiers Vet Sci 2022, 9, 964649, doi:10.3389/fvets.2022.964649.

47. Harper, E.J. Changing Perspectives on Aging and Energy Requirements: Aging and Digestive Function in Humans, Dogs and Cats 1. *J. Nutr.* **1998**, *128*, 2*632*S-2635S, doi:10.1093/jn/128.12.2632s.

48. Pilla, R.; Suchodolski, J.S. The Role of the Canine Gut Microbiome and Metabolome in Health and Gastrointestinal Disease. Front. Vet. Sci. 2020, 6, 498, doi:10.3389/fvets.**2019**.00498.

49. FRANCESCHI, C.; BONAFÈ, M.; VALENSIN, S.; OLIVIERI, F.; LUCA, M.D.; OTTAVIANI, E.; BENEDICTIS, G.D. Inflamm‐aging: An Evolutionary Perspective on Immunosenescence. *Ann. N. York Acad. Sci.* **2000**, *908*, 244–254, doi:10.1111/j.1749-6632.2000.tb06651.x.

50. Leonard, J.M.; Toro, D.D. Defining the Microbiome Components (Bacteria, Viruses, Fungi) and Microbiome Geodiversity. *Surg. Infect.* **2023**, *24*, 208–2*12*, doi:10.1089/sur.2023.014.

51. Bäckhed, F.; Ley, R.E.; Sonnenburg, J.L.; Peterson, D.A.; Gordon, J.I. Host-Bacterial Mutualism in the Human Intestine. Science **2005**, *307*, 1915–1920, doi:10.1126/science.1104816.

52. Zhu, B.; Wang, X.; Li, L. Human Gut Microbiome: The Second Genome of Human Body. *Protein Cell* **2010**, *1*, 718–725, doi:10.1007/s13238-010-0093-z.

53. Zhao, L.; Zhang, F.; Ding, X.; Wu, G.; Lam, Y.Y.; Wang, X.; Fu, H.; Xue, X.; Lu, C.; Ma, J.; et al. Gut Bacteria Selectively Promoted by Dietary Fibers Alleviate Type 2 *Diabetes*. Science **2018**, *359*, 1151–1156, doi:10.1126/science.aao5774.

54. Guard, B.C.; Mila, H.; Steiner, J.M.; Mariani, C.; Suchodolski, J.S.; Chastant-Maillard, S. Characterization of the Fecal Microbiome during Neonatal and Early Pediatric Development in Puppies. *Plos One* **2017**, *12*, e0175718, doi:10.1371/journal.pone.0175718.

55. Deng, P.; Swanson, K.S. Gut Microbiota of Humans, Dogs and Cats: Current Knowledge and Future Opportunities and Challenges. *Br. J. Nutr.* **2015**, *113*, S6–S17, doi:*10*.1017/s0007114514002943.

56. Gilbert, J.A.; Blaser, M.J.; Caporaso, J.G.; Jansson, J.K.; Lynch, S.V.; Knight, R. Current Understanding of the Human Microbiome. *Nat. Med.* **2018**, *24*, 392–400, doi:10.1038/nm.4517.

57. Rodríguez, J.M.; Murphy, K.; Stanton, C.; Ross, R.P.; Kober, O.I.; Juge, N.; Avershina, E.; Rudi, K.; Narbad, A.; Jenmalm, M.C.; et al. The Composition of the Gut Microbiota throughout Life, with an Emphasis on Early Life. *Microb. Ecol. Heal.*

Dis. **2015**, *26*, 26050, doi:10.3402/mehd.v26.26050.

58. Zierer, J.; Jackson, M.A.; Kastenmüller, G.; Mangino, M.; Long, T.; Telenti, A.; Mohney, R.P.; Small, K.S.; Bell, J.T.; Steves, C.J.; et al. The Fecal Metabolome as a Functional Readout of the Gut Microbiome. *Nat. Genet.* **2018**, *50*, 790–795, doi:10.1038/s41588-018-0135-7.

59. Badal, V.D.; Vaccariello, E.D.; Murray, E.R.; Yu, K.E.; Knight, R.; Jeste, D.V.; Nguyen, T.T. The Gut Microbiome, Aging, and Longevity: A Systematic Review. *Nutrients* **2020**, *12*, 3759, doi:10.3390/nu121*23*759.

60. AlShawaqfeh, M.; Wajid, B.; Minamoto, Y.; Markel, M.; Lidbury, J.; Steiner, J.; Serpedin, E.; Suchodolski, J. A Dysbiosis Index to Assess Microbial Changes in Fecal Samples of Dogs with Chronic Inflammatory Enteropathy. *Fems Microbiol Ecol* **2017**, *93*, fix136, doi:10.1093/femsec/fix136.

61. Grześkowiak, Ł.; Endo, A.; Beasley, S.; Salminen, S. Microbiota and Probiotics in Canine and Feline Welfare. *Anaerobe* **2015**, *34*, 14–23, doi:10.10*16*/j.anaerobe.2015.04.002.

62. Yang, Q.; Wu, Z. Gut Probiotics and Health of Dogs and Cats: Benefits, Applications, and Underlying Mechanisms. *Microorganisms* **2023**, *11*, 2452, doi:10.3390/microorganisms11102452.

63. Caporaso, J.G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Peña, A.G.; Goodrich, J.K.; Gordon, J.I.; et al. QIIME Allows Analysis of High-Throughput Community Sequencing Data. *Nat. Methods* **2010**, *7*, 335–336, doi:10.1038/nmeth.f.303.

64. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High Resolution Sample Inference from Illumina Amplicon Data. *Nat Methods* **2016**, *13*, 581–583, doi:10.1038/nmeth.3869.

65. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2. *Nat. Biotechnol.* **2019**, *37*, 852–857, doi:10.1038/s41587-019-0209-9.

66. Willis, A.D. Rarefaction, Alpha Diversity, and Statistics. *Front Microbiol* **2019**, *10*, 2407, doi:10.3389/fmicb.2019.02407.

67. Kim, B.-R.; Shin, J.; Guevarra, R.B.; Lee, J.H.; Kim, D.W.; Seol, K.-H.; Lee, J.-H.; Kim, H.B.; Isaacson, R.E. Deciphering Diversity Indices for a Better Understanding of Microbial Communities. *J. Microbiol. Biotechnol.* **2017**, *27*,

2089–2093, doi:10.4014/jmb.1709.09027.

68. Bray, J.R.; Curtis, J.T. An Ordination of the Upland Forest Communities of Southern Wisconsin. *Ecol. Monogr.* **1957**, *27*, 325–349, doi:10.2307/1942268.

69. Lozupone, C.; Knight, R. UniFrac: A New Phylogenetic Method for Comparing Microbial Communities. *Appl Environ Microb* **2005**, *71*, 8228–8235, doi:10.1128/aem.71.12.8228-8235.2005.

70. Lozupone, C.; Lladser, M.E.; Knights, D.; Stombaugh, J.; Knight, R. UniFrac: An Effective Distance Metric for Microbial Community Comparison. *ISME J.* **2010**, *5*, 169–172, doi:10.1038/ismej.2010.133.

71. Knight, R.; Vrbanac, A.; Taylor, B.C.; Aksenov, A.; Callewaert, C.; Debelius, J.; Gonzalez, A.; Kosciolek, T.; McCall, L.-I.; McDonald, D.; et al. Best Practices for Analysing Microbiomes. Nat. Rev. Microbiol. 2018, 16, 410–422, doi:10.1038/s41579-018-0029-9.

72. Besten, G. den; Lange, K.; Havinga, R.; Dijk, T.H. van; Gerding, A.; Eunen, K. van; Müller, M.; Groen, A.K.; Hooiveld, G.J.; Bakker, B.M.; et al. Gut-Derived Short-Chain Fatty Acids Are Vividly Assimilated into Host Carbohydrates and Lipids. *Am. J. Physiol.-Gastrointest. Liver Physiol.* **2013**, *305*, G900–G910, doi:10.1152/ajpgi.00265.2013.

73. Peng, L.; Li, Z.-R.; Green, R.S.; Holzmanr, I.R.; Lin, J. Butyrate Enhances the Intestinal Barrier by Facilitating Tight Junction Assembly via Activation of AMP-Activated Protein Kinase in Caco-2 Cell Monolayers 1 , 2. J. Nutr. **2009**, *139*, 1619–1625, doi:10.3945/jn.109.104638.

74. Koh, A.; De Vadder, F.; Kovatcheva-Datchary, P.; Bäckhed, F. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. Cell 2016, *165*, 1332–1345, doi:10.1016/j.cell.2016.05.041.

75. Macfarlane, S.; Macfarlane, G.T. Regulation of Short-Chain Fatty Acid Production. *Proc. Nutr. Soc.* **2003**, *62*, 67–72, doi:10.1079/pns**2002**207.

76. Kilburn-Kappeler, L.R.; Almeida, K.A.L.; Paulk, C.B.; Aldrich, C.G. Comparing the Effects of Corn Fermented Protein with Traditional Distillers Dried Grains Fed to Healthy Adult Dogs on Stool Quality, Nutrient Digestibility, and Palatability. *Front. Anim. Sci.* **2023**, *4*, 1210144, doi:10.3389/fanim.2023.1210144.

77. Dalis, C.; Mesfin, F.M.; Manohar, K.; Liu, J.; Shelley, W.C.; Brokaw, J.P.; Markel, T.A. Volatile Organic Compound Assessment as a Screening Tool for Early Detection of Gastrointestinal Diseases. Microorganisms 2023, 11, 1822,

doi:10.3390/microorganisms11071822.

78. Rossi, M.; Aggio, R.; Staudacher, H.M.; Lomer, M.C.; Lindsay, J.O.; Irving, P.; Probert, C.; Whelan, K. Volatile Organic Compounds in Feces Associate With Response to Dietary Intervention in Patients With Irritable Bowel Syndrome. *Clin. Gastroenterol. Hepatol.* **2018**, *16*, 385-391.e1, doi:10.1016/j.cgh.2017.09.055.

79. Wahlström, A.; Sayin, S.I.; Marschall, H.-U.; Bäckhed, F. Intestinal Crosstalk between Bile Acids and Microbiota and Its Impact on Host Metabolism. *Cell Metab.* **2016**, *24*, 41–50, doi:10.1016/j.cmet.2016.05.005.

80. Ehrchen, J.M.; Sunderkötter, C.; Foell, D.; Vogl, T.; Roth, J. The Endogenous Toll–like Receptor 4 Agonist S100A8/S100A9 (Calprotectin) as Innate Amplifier of Infection, Autoimmunity, and Cancer. *J. Leukoc. Biol.* **2009**, *86*, 557–566, doi:10.1189/jlb.1008647.

81. Lamb, C.A.; Mansfield, J.C. Measurement of Faecal Calprotectin and Lactoferrin in Inflammatory Bowel Disease. *Front. Gastroenterol.* **2011**, *2*, 13–18, doi:10.1136/fg.2010.001362.

82. Grellet, A.; Heilmann, R.M.; Lecoindre, P.; Feugier, A.; Day, M.J.; Peeters, D.; Freiche, V.; Hernandez, J.; Grandjean, D.; Suchodolski, J.S.; et al. Fecal Calprotectin Concentrations in Adult Dogs with Chronic Diarrhea. *Am J Vet Res* **2013**, *74*, 706–711, doi:10.2460/ajvr.74.5.706.

83. Heilmann, R.M.; Jergens, A.E.; Ackermann, M.R.; Barr, J.W.; Suchodolski, J.S.; Steiner, J.M. Serum Calprotectin Concentrations in Dogs with Idiopathic Inflammatory Bowel Disease. *Am. J. Vet. Res.* **2012**, *73*, 1900–1907, doi:10.2460/ajvr.73.12.1900.

84. Lehmann, F.S.; Burri, E.; Beglinger, C. The Role and Utility of Faecal Markers in Inflammatory Bowel Disease. *Ther. Adv. Gastroenterol.* **2015**, *8*, 23–36, doi:10.1177/1756283x14553384.

85. Maden, M.; Gülersoy, E. Serum/Faecal S100A12, CRP and Lactoferrin Can Be Used to Distinguish Infectious and Non‐ınfectious Canine Diarrhoea. *Vet. Med. Sci.* **2023**, *9*, 2485–2496, doi:10.1002/vms3.1245.

86. Oliveira, I.M.; Ribeiro, R.R.; Cysneiros, M.E.C.; Torres, L.B.; Moraes, V.R.; Ferreira, L.R.; Silva, W.P.R. da; Souza, M.R. de; Xavier, R.A.L.; Costa, P.R. dos S.; et al. Intestinal Biomarkers and Their Importance in Canine Enteropathies. *Vet. Med. Int.* **2024**, *2024*, 7409482, doi:10.1155/vmi/7409482.

87. Maeda, S.; Ohno, K.; Uchida, K.; Nakashima, K.; Fukushima, K.; Tsukamoto, A.;

Nakajima, M.; Fujino, Y.; Tsujimoto, H. Decreased Immunoglobulin A Concentrations in Feces, Duodenum, and Peripheral Blood Mononuclear *Cells* of Dogs with Inflammatory Bowel Disease. J. Vet. Intern. Med. 2013, 27, 47–55, doi:10.1111/jvim.1**2023**.

88. Nakamura, M.; Takahashi, M.; Ohno, K.; Koshino, A.; Nakashima, K.; Setoguchi, A.; Fujino, Y.; TSUJIMOTO, H. C-Reactive Protein Concentration in Dogs with Various Diseases. *J. Vet. Méd. Sci.* **2008**, *70*, *12*7, doi:10.1292/jvms.70.127.

89. Thailand, D. of C.A. and W.C., Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100,; Chantawong, P.; Thailand, R.C. for V.B. and V.P.H., Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100, Clinical Applications of Current Biomarkers in Canine Chronic Enteropathies. *Vet. Integr. Sci.* **2023**, *22*, 713–737, doi:10.12982/vis.2024.048.

90. Otoni, C.C.; Heilmann, R.M.; García-Sancho, M.; Sainz, A.; Ackermann, M.R.; Suchodolski, J.S.; Steiner, J.M.; Jergens, A.E. Serologic and Fecal Markers to Predict Response to Induction Therapy in Dogs with Idiopathic Inflammatory Bowel Disease. J. Vet. Intern. Med. 2018, 32, 999–1008, doi:10.1111/jvim.15123.

91. Jergens, A.E.; Crandell, J.; Morrison, J.A.; Deitz, K.; Pressel, M.; Ackermann, M.; Suchodolski, J.S.; Steiner, J.M.; Evans, R. Comparison of Oral Prednisone and Prednisone Combined with Metronidazole for Induction Therapy of Canine Inflammatory Bowel Disease: A Randomized-Controlled Trial. J. Vet. Intern. Med. 2010, 24, 269–277, doi:10.1111/j.1939-*1676*.2009.0447.x.

92. Gruys, E.; Toussaint, M.J.M.; Niewold, T.A.; Koopmans, S.J. Acute Phase Reaction and Acute Phase Proteins. *J. Zhejiang Univ.-Sci. B* **2005**, *6*, 1045–1056, doi:10.1631/jzus.2005.b1045.

93. Eckersall, P.D.; Bell, R. Acute Phase Proteins: Biomarkers of Infection and Inflammation in Veterinary Medicine. *Vet. J.* **2010**, *185*, 23–27, doi:10.1016/j.tvjl.2010.04.009.

94. Cerón, J.J.; Eckersall, P.D.; Martínez-Subiela, S. Acute Phase Proteins in Dogs and Cats: Current Knowledge and Future Perspectives. *Vet. Clin. Pathol.* **2005**, *34*, 85–99, doi:10.1111/j.1939-165x.2005.tb00019.x.

95. Wang, W.; Uzzau, S.; Goldblum, S.E.; Fasano, A. Human Zonulin, a Potential Modulator of Intestinal Tight Junctions. *J. Cell Sci.* **2000**, *113*, 4435–4440, doi:10.12*42*/jcs.113.24.4435.

96. Fasano, A. Zonulin and Its Regulation of Intestinal Barrier Function: The

Biological Door to Inflammation, Autoimmunity, and Cancer. *Physiol. Rev.* **2011**, *91*, 151–175, doi:10.1152/physrev.00003.2008.

97. Dinesh, N.; Slovak, J.E.; Kogan, C.; Kopper, J.J. Preliminary Evaluation of Serum Zonulin in Canine Chronic Enteropathies. J. Small Anim. Pr. 2022, 63, 679–685, doi:10.1111/jsap.13506.

98. Heilmann, R.M.; Parnell, N.K.; Grützner, N.; Mansell, J.; Berghoff, N.; Schellenberg, S.; Reusch, C.E.; Suchodolski, J.S.; Steiner, J.M. Serum and Fecal Canine A1-Proteinase Inhibitor Concentrations Reflect the Severity of Intestinal Crypt Abscesses and/or Lacteal Dilation in Dogs. Vet. J. **2016**, *207*, 131–139, doi:10.1016/j.tvjl.2015.10.042.

99. Murphy, K.F.; German, A.J.; Ruaux, C.G.; Steiner, J.M.; Williams, D.A.; Hall, E.J. Fecal A1-Proteinase Inhibitor Concentration in Dogs with Chronic Gastrointestinal Disease. Vet. Clin. Pathol. **2003**, *32*, 67–72, doi:10.1111/j.1939-165x.2003.tb00316.x.

100. Kather, S.; Grützner, N.; Kook, P.H.; Dengler, F.; Heilmann, R.M. Review of Cobalamin Status and Disorders of Cobalamin Metabolism in Dogs. J. Vet. Intern. Med. 2020, 34, 13–28, doi:10.1111/jvim.15638.

101. Soetart, N.; Rochel, D.; Drut, A.; Jaillardon, L. Serum Cobalamin and Folate as Prognostic Factors in Canine Exocrine Pancreatic Insufficiency: An Observational Cohort Study of 299 Dogs. Vet. J. **2019**, *243*, 15–20, doi:10.1016/j.tvjl.2018.11.003.

102. Agus, A.; Planchais, J.; Sokol, H. Gut Microbiota Regulation of Tryptophan Metabolism in Health and Disease. *Cell Host Microbe* **2018**, *23*, 716–724, doi:10.1016/j.chom.2018.05.003.

103. Gao, J.; Xu, K.; Liu, H.; Liu, G.; Bai, M.; Peng, C.; Li, T.; Yin, Y. Impact of the Gut Microbiota on Intestinal Immunity Mediated by Tryptophan Metabolism. *Front. Cell. Infect. Microbiol.* **2018**, 8, 13, doi:10.3389/fcimb.2018.00013.

104. Kiełbik, P.; Witkowska-Piłaszewicz, O. The Relationship between Canine Behavioral Disorders and Gut Microbiome and Future Therapeutic Perspectives. Animals 2024, 14, 2048, doi:10.3390/ani14142048.

105. Nikolaus, S.; Schulte, B.; Al-Massad, N.; Thieme, F.; Schulte, D.M.; Bethge, J.; Rehman, A.; Tran, F.; Aden, K.; Häsler, R.; et al. Increased Tryptophan Metabolism Is Associated With Activity of Inflammatory Bowel Diseases. *Gastroenterology* **2017**, *153*, 1504-1516.e2, doi:10.1053/j.gastro.2017.08.028.

106. Hrovat, A.; Keuster, T.D.; Kooistra, H.S.; Duchateau, L.; Oyama, M.A.; Peremans, K.; Daminet, S. Behavior in Dogs with Spontaneous Hypothyroidism

during Treatment with Levothyroxine. J. Vet. Intern. Med. 2019, 33, 64–71, doi:10.1111/jvim.15342.

107. Johnson, K.V.-A.; Foster, K.R. Why Does the Microbiome Affect Behaviour? Nat. Rev. Microbiol. 2018, 16, 647–655, doi:10.1038/s41579-018-0014-3.

108. MacLean, E.L.; Hare, B. Dogs Hijack the Human Bonding Pathway. Science 2015, *348*, 280–*281*, doi:10.1126/science.aab1200.

109. Mesarcova, L.; Kottferova, J.; Skurkova, L.; Leskova, L.; Kmecova, N. Analysis of Cortisol in Dog Hair - a Potential Biomarker of Chronic Stress: A Review. *Veterinární medicína* **2017**, *62*, 363–376, doi:10.17221/19/2017-vetmed.

110. Ragonnaud, E.; Biragyn, A. Gut Microbiota as the Key Controllers of "Healthy" Aging of Elderly People. *Immun. Ageing* **2021**, *18*, 2, doi:10.1186/s12979-020-00*213*-w.

111. Varian, B.J.; Weber, K.T.; Erdman, S.E. Oxytocin and the Microbiome. *Compr. Psychoneuroendocrinology* **2023**, *16*, 100205, doi:10.1016/j.cpnec.2023.100205.

112. Morais, L.H.; Schreiber, H.L.; Mazmanian, S.K. The Gut Microbiota–Brain Axis in Behaviour and Brain Disorders. Nat. Rev. Microbiol. **2021**, 19, *241*–255, doi:10.1038/s41579-020-00460-0.

113. Dalile, B.; Oudenhove, L.V.; Vervliet, B.; Verbeke, K. The Role of Short-Chain Fatty Acids in Microbiota–Gut–Brain Communication. *Nat. Rev. Gastroenterol. Hepatol.* **2019**, *16*, 4*61*–4*78*, doi:10.1038/s41575-019-0157-3.

114. Janakiraman, M.; Krishnamoorthy, G. Emerging Role of Diet and Microbiota Interactions in Neuroinflammation. Front. Immunol. 2018, 9, 2067, doi:10.3389/fimmu.2018.02067.

115. Varian, B.J.; Levkovich, T.; Poutahidis, T.; Ibrahim, Y.M. Beneficial Dog Bacteria Up-Regulate Oxytocin and Lower Risk of Obesity. *J. Probiotics Heal.* **2016**, *4*, doi:10.4172/2329-8901.1000149.

116. Knezevic, E.; Nenic, K.; Milanovic, V.; Knezevic, N.N. The Role of Cortisol in Chronic Stress, Neurodegenerative Diseases, and Psychological Disorders. Cells 2023, 12, 2726, doi:10.3390/cells1*223*2726.

117. Beerda, B.; Schilder, M.B.H.; Hooff, J.A.R.A.M. van; Vries, H.W. de; Mol, J.A. Behavioural, Saliva Cortisol and Heart Rate Responses to Different Types of Stimuli in Dogs. Appl. Anim. Behav. Sci. 1998, 58, 365–381, doi:10.1016/s0168-1591(97)00145-7.

118. Rehn, T.; Handlin, L.; Uvnäs-Moberg, K.; Keeling, L.J. Dogs' Endocrine and

Behavioural Responses at Reunion Are Affected by How the Human Initiates Contact. *Physiol. Behav.* **2014**, *124*, 45–53, doi:10.1016/j.physbeh.2013.10.009.

119. Sacoor, C.; Marugg, J.D.; Lima, N.R.; Empadinhas, N.; Montezinho, L. Gut-Brain Axis Impact on Canine Anxiety Disorders: New Challenges for Behavioral Veterinary Medicine. Vet. Med. Int. 2024, 2024, 2856759, doi:10.1155/2024/2856759.

120. Kirchoff, N.S.; Udell, M.A.R.; Sharpton, T.J. The Gut Microbiome Correlates with Conspecific Aggression in a Small Population of Rescued Dogs (Canis Familiaris). *Peerj* **2019**, *7*, e6103, doi:10.7717/peerj.6103.

121. Fan, Z.; Bian, Z.; Huang, H.; Liu, T.; Ren, R.; Chen, X.; Zhang, X.; Wang, Y.; Deng, B.; Zhang, L. Dietary Strategies for Relieving Stress in Pet Dogs and Cats. Antioxidants 2023, 12, 545, doi:10.3390/antiox12030545.

122. Camps, T.; Amat, M.; Manteca, X. A Review of Medical Conditions and Behavioral Problems in Dogs and Cats. Animals 2019, 9, 1133, doi:10.3390/ani9121133.

123. Scott-Moncrieff, J.C. Thyroid Disorders in the Geriatric Veterinary Patient. Vet. Clin. North Am.: Small Anim. Pr. 2012, 42, 707–725, doi:10.1016/j.cvsm.2012.04.012.

124. Lee, E.-H.; Kim, S.-M.; Kim, C.-H.; Pagire, S.H.; Pagire, H.S.; Chung, H.Y.; Ahn, J.H.; Park, C.-H. Dopamine Neuron Induction and the Neuroprotective Effects of Thyroid Hormone Derivatives. *Sci. Rep.* **2019**, 9, 13659, doi:10.1038/s41598-019-49876-6.

125. Bauer, M.; Heinz, A.; Whybrow, P.C. Thyroid Hormones, Serotonin and Mood: Of Synergy and Significance in the Adult Brain. *Mol. Psychiatry* **2002**, *7*, 140–156, doi:10.1038/sj.mp.4000963.

126. Mercier, P.; Honeckman, L.; Jokela, F.; Dunham, A.E.; Overall, K.L. Using Standardized Scales to Assess Fear at Veterinary Visits: Intra- and Inter-Rater Reliability. *J. Vet. Behav.* **2023**, *62*, 12–17, doi:10.1016/j.jveb.2023.02.004.

127. Borg, J.A.M. van der; Schilder, M.B.H.; Vinke, C.M.; Vries, H. de Dominance in Domestic Dogs: A Quantitative Analysis of Its Behavioural Measures. *PLoS ONE* **2015**, *10*, e0133978, doi:10.1371/journal.pone.0133978.

128. Hsu, Y.; Serpell, J.A. Development and Validation of a Questionnaire for Measuring Behavior and Temperament Traits in Pet Dogs. J. Am. Vet. Méd. Assoc. 2003, 223, 1293–1300, doi:10.2460/javma.2003.223.1293.

129. Morrison, R.; Reilly, J.J.; Penpraze, V.; Pendlebury, E.; Yam, P.S. A 6-month Observational Study of Changes in Objectively Measured Physical Activity during Weight Loss in Dogs. J. Small Anim. Pr. 2014, 55, 566–570, doi:10.1111/jsap.12273.

130. Karimjee, K.; Harron, R.C.M.; Piercy, R.J.; Daley, M.A. A Standardised Approach to Quantifying Activity in Domestic Dogs. *R. Soc. Open Sci.* **2024**, *11*, 240119, doi:10.1098/rsos.240119.

1*31*. Kumpulainen, P.; Cardó, A.V.; Somppi, S.; Törnqvist, H.; Väätäjä, H.; Majaranta, P.; Gizatdinova, Y.; Antink, C.H.; Surakka, V.; Kujala, M.V.; et al. Dog Behaviour Classification with Movement Sensors Placed on the Harness and the Collar. Appl. Anim. Behav. Sci. 2021, 241, 105393, doi:10.1016/j.applanim.2021.105393.

132. Yam, P.S.; Penpraze, V.; Young, D.; Todd, M.S.; Cloney, A.D.; Houston-Callaghan, K.A.; Reilly, J.J. Validity, Practical Utility and Reliability of Actigraph Accelerometry for the Measurement of Habitual Physical Activity in Dogs. J. Small Anim. Pr. 2011, 52, 86–91, doi:10.1111/j.1748-5827.2010.01025.x.

133. Brown, D.C.; Boston, R.C.; Farrar, J.T. Use of an Activity Monitor to Detect Response to Treatment in Dogs with Osteoarthritis. J. Am. Vet. Méd. Assoc. 2010, 237, 66–70, doi:10.2460/javma.237.1.66.

134. Sasso, J.M.; Ammar, R.M.; Tenchov, R.; Lemmel, S.; Kelber, O.; Grieswelle, M.; Zhou, Q.A. Gut Microbiome–Brain Alliance: A Landscape View into Mental and Gastrointestinal Health and Disorders. *ACS Chem. Neurosci.* **2023**, *14*, 1717–1763, doi:10.1021/acschemneuro.3c00127.

135. Han, Y.; Wang, B.; Gao, H.; He, C.; Hua, R.; Liang, C.; Zhang, S.; Wang, Y.; Xin, S.; Xu, J. Vagus Nerve and Underlying Impact on the Gut Microbiota-Brain Axis in Behavior and Neurodegenerative Diseases. *J. Inflamm. Res.* **2022**, *15*, 6213–6230, doi:10.2147/jir.s384949.

136. Wikoff, W.R.; Anfora, A.T.; Liu, J.; Schultz, P.G.; Lesley, S.A.; Peters, E.C.; Siuzdak, G. Metabolomics Analysis Reveals Large Effects of Gut Microflora on Mammalian Blood Metabolites. *Proc. Natl. Acad. Sci.* **2009**, *106*, 3698–3703, doi:10.1073/pnas.0812874106.

137. Tolhurst, G.; Heffron, H.; Lam, Y.S.; Parker, H.E.; Habib, A.M.; Diakogiannaki, E.; Cameron, J.; Grosse, J.; Reimann, F.; Gribble, F.M. Short-Chain Fatty Acids Stimulate Glucagon-Like Peptide-1 Secretion via the G-Protein–Coupled Receptor FFAR2. Diabetes 2012, 61, 364–371, doi:10.2337/db11-1019.

138. Martin, C.R.; Osadchiy, V.; Kalani, A.; Mayer, E.A. The Brain-Gut-Microbiome Axis. *Cell Mol Gastroenterology Hepatology* **2018**, *6*, 133–148, doi:10.1016/j.jcmgh.2018.04.003.

139. Gwak, M.-G.; Chang, S.-Y. Gut-Brain Connection: Microbiome, Gut Barrier, and Environmental Sensors. *Immune Netw.* **2021**, *21*, e20, doi:10.4110/in.2021.21.e20.

140. Sorgdrager, F.J.H.; Vermeiren, Y.; Faassen, M.V.; Ley, C. van der; Nollen, E.A.A.; Kema, I.P.; Deyn, P.P.D. Age- and Disease-specific Changes of the Kynurenine Pathway in Parkinson's and Alzheimer's Disease. *J. Neurochem.* **2019**, *151*, 656–668, doi:10.1111/jnc.14843.

141. Sampson, T.R.; Debelius, J.W.; Thron, T.; Janssen, S.; Shastri, G.G.; Ilhan, Z.E.; Challis, C.; Schretter, C.E.; Rocha, S.; Gradinaru, V.; et al. Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. Cell 2016, 167, 1469-1480.e12, doi:10.1016/j.cell.2016.11.018.

142. Jiang, C.; Li, G.; Huang, P.; Liu, Z.; Zhao, B. The Gut Microbiota and Alzheimer's Disease. *J. Alzheimer's Dis.* **2017**, *Preprint*, 1–15, doi:10.3233/jad-161*14*1.

143. Ambrosini, Y.M.; Borcherding, D.; Kanthasamy, A.; Kim, H.J.; Willette, A.A.; Jergens, A.; Allenspach, K.; Mochel, J.P. The Gut-Brain Axis in Neurodegenerative Diseases and Relevance of the Canine Model: A Review. *Front Aging Neurosci* **2019**, *11*, 130, doi:10.3389/fnagi.2019.00130.

144. Benjamini, Y.; Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society: Series B (Methodological)* **1995**, doi:10.1111/j.2517-6161.1995.tb02031.x.

145. Parada, A.E.; Needham, D.M.; Fuhrman, J.A. Every Base Matters: Assessing Small Subunit RRNA Primers for Marine Microbiomes with Mock Communities, Time Series and Global Field Samples. *Environ Microbiol* **2015**, *18*, 1403–1414, doi:10.1111/1462-2920.13023.

146. Li, Y.; Ning, L.; Yin, Y.; Wang, R.; Zhang, Z.; Hao, L.; Wang, B.; Zhao, X.; Yang, X.; Yin, L.; et al. Age-Related Shifts in Gut Microbiota Contribute to Cognitive Decline in Aged Rats. *Aging Albany Ny* **2020**, *12*, 7801–7817, doi:10.18632/aging.103093.

147. Mondo, E.; Barone, M.; Soverini, M.; D'Amico, F.; Cocchi, M.; Petrulli, C.; Mattioli, M.; Marliani, G.; Candela, M.; Accorsi, P.A. Gut Microbiome Structure and Adrenocortical Activity in Dogs with Aggressive and Phobic Behavioral Disorders. *Heliyon* **2020**, *6*, e03311, doi:10.1016/j.heliyon.2020.e03311.

148. Craddock, H.A.; Godneva, A.; Rothschild, D.; Motro, Y.; Grinstein, D.; Lotem-Michaeli, Y.; Narkiss, T.; Segal, E.; Moran-Gilad, J. Phenotypic Correlates of the Working Dog Microbiome. *Npj Biofilms Microbiomes* **2022**, *8*, 66, doi:10.1038/s41522-022-00329-5.

149. Álvarez-Herms, J.; Martínez, A.O. Microbiome and Physical Activity. *Adv. Genet.* **2024**, *111*, 409–450, doi:10.1016/bs.adgen.2024.01.002.

150. Brod, S.; Gobbetti, T.; Gittens, B.; Ono, M.; Perretti, M.; D'Acquisto, F. The Impact of Environmental Enrichment on the Murine Inflammatory Immune Response. *JCI Insight* **2017**, *2*, e90723, doi:10.1172/jci.insight.90723.

151. Zhu, S.; Jiang, Y.; Xu, K.; Cui, M.; Ye, W.; Zhao, G.; Jin, L.; Chen, X. The Progress of Gut Microbiome Research Related to Brain Disorders. *J. Neuroinflammation* **2020**, *17*, 25, doi:10.1186/s12974-020-1705-z.

152. Claesson, M.J.; Jeffery, I.B.; Conde, S.; Power, S.E.; O'Connor, E.M.; Cusack, S.; Harris, H.M.B.; Coakley, M.; Lakshminarayanan, B.; O'Sullivan, O.; et al. Gut Microbiota Composition Correlates with Diet and Health in the Elderly. *Nature* **2012**, *488*, 178–184, doi:10.1038/nature11319.

153. Fernández-Pinteño, A.; Pilla, R.; Suchodolski, J.; Apper, E.; Torre, C.; Salas-Mani, A.; Manteca, X. Age-Related Changes in Gut Health and Behavioral Biomarkers in a Beagle Dog Population. Animals **2025**, *15*, 234, doi:10.3390/ani15020234.

154. You, I.; Kim, M.J. Comparison of Gut Microbiota of 96 Healthy Dogs by Individual Traits: Breed, Age, and Body Condition Score. *Animals Open Access J Mdpi* **2021**, *11*, 2432, doi:10.3390/ani11082432.

155. Strickling, J.A.; Harmon, D.L.; Dawson, K.A.; Gross, K.L. Evaluation of Oligosaccharide Addition to Dog Diets: Influences on Nutrient Digestion and Microbial Populations. *Anim. Feed Sci. Technol.* **2000**, *86*, 205–219, doi:10.1016/s0377-8401(00)00175-9.

156. Middelbos, I.S.; Boler, B.M.V.; Qu, A.; White, B.A.; Swanson, K.S.; Fahey, G.C. Phylogenetic Characterization of Fecal Microbial Communities of Dogs Fed Diets with or without Supplemental Dietary Fiber Using 454 Pyrosequencing. Plos One 2010, 5, e9768, doi:10.1371/journal.pone.0009768.

157. Hand, D.; Wallis, C.; Colyer, A.; Penn, C.W. Pyrosequencing the Canine Faecal Microbiota: Breadth and Depth of Biodiversity. Plos One 2013, 8, e53115, doi:10.1371/journal.pone.0053115.

158. Salas-Mani, A.; Jeusette, I.; Castillo, I.; Manuelian, C.L.; Lionnet, C.; Iraculis, N.; Sanchez, N.; Fernández, S.; Vilaseca, L.; Torre, C. Fecal Microbiota Composition Changes after a BW Loss Diet in Beagle Dogs. *J Anim Sci* **2018**, *96*, 3102–3111, doi:10.1093/jas/sky193.

159. Garcia-Mazcorro, J.F.; Lanerie, D.J.; Dowd, S.E.; Paddock, C.G.; Grützner, N.; Steiner, J.M.; Ivanek, R.; Suchodolski, J.S. Effect of a Multi-species Synbiotic Formulation on Fecal Bacterial Microbiota of Healthy Cats and Dogs as Evaluated by Pyrosequencing. Fems Microbiol Ecol 2011, 78, 542–554, doi:10.1111/j.1574-6941.2011.01185.x.

160. Guard, B.C.; Barr, J.W.; Reddivari, L.; Klemashevich, C.; Jayaraman, A.; Steiner, J.M.; Vanamala, J.; Suchodolski, J.S. Characterization of Microbial Dysbiosis and Metabolomic Changes in Dogs with Acute Diarrhea. Plos One 2015, 10, e0127259, doi:10.1371/journal.pone.0127259.

161. Handl, S.; Dowd, S.E.; Garcia-Mazcorro, J.F.; Steiner, J.M.; Suchodolski, J.S. Massive Parallel 16S RRNA Gene Pyrosequencing Reveals Highly Diverse Fecal Bacterial and Fungal Communities in Healthy Dogs and Cats. Fems Microbiol Ecol 2011, 76, 301–310, doi:10.1111/j.1574-6941.2011.01058.x.

162. Mitsuoka, T. History and Evolution of Probiotics. *Jpn J Lact Acid Bact* **2011**, *22*, 26–37, doi:10.4109/jslab.22.26.

163. Oliphant, K.; Allen-Vercoe, E. Macronutrient Metabolism by the Human Gut Microbiome: Major Fermentation by-Products and Their Impact on Host Health. Microbiome 2019, 7, 91, doi:10.1186/s40168-019-0704-8.

164. Koh, A.; De Vadder, F.; Kovatcheva-Datchary, P.; Bäckhed, F. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. Cell 2016, 165, 1332–1345, doi:10.1016/j.cell.2016.05.041.

165. Minamoto, Y.; Minamoto, T.; Isaiah, A.; Sattasathuchana, P.; Buono, A.; Rangachari, V.R.; McNeely, I.H.; Lidbury, J.; Steiner, J.M.; Suchodolski, J.S. Fecal Short-chain Fatty Acid Concentrations and Dysbiosis in Dogs with Chronic Enteropathy. J Vet Intern Med 2019, 33, 1608–1618, doi:10.1111/jvim.15520.

166. Suchodolski, J.S.; Dowd, S.E.; Wilke, V.; Steiner, J.M.; Jergens, A.E. 16S RRNA Gene Pyrosequencing Reveals Bacterial Dysbiosis in the Duodenum of Dogs with Idiopathic Inflammatory Bowel Disease. Plos One 2012, 7, e39333, doi:10.1371/journal.pone.0039333.

167. Cosquer, G.L.; Vergnolle, N.; Motta, J.-P. Gut Microb-Aging and Its Relevance

to Frailty Aging. *Microbes Infect.* **2024**, *26*, 105309, doi:10.1016/j.micinf.2024.105309.

168. Jiménez, A.G. Inflammaging in Domestic Dogs: Basal Level Concentrations of IL-6, IL-1β, and TNF-α in Serum of Healthy Dogs of Different Body Sizes and Ages. *Biogerontology* **2023**, *24*, 593–602, doi:10.1007/s10522-023-10037-y.

169. Varela, E.; Manichanh, C.; Gallart, M.; Torrejón, A.; Borruel, N.; Casellas, F.; Guarner, F.; Antolin, M. Colonisation by *Faecalibacterium Prausnitzii* and Maintenance of Clinical Remission in Patients with Ulcerative Colitis. *Aliment Pharm Therap* **2013**, *38*, 151–161, doi:10.1111/apt.12365.

170. Mori, A.; Goto, A.; Kibe, R.; Oda, H.; Kataoka, Y.; Sako, T. Comparison of the Effects of Four Commercially Available Prescription Diet Regimens on the Fecal Microbiome in Healthy Dogs. *J Vet Med Sci* **2019**, *81*, 1783–1790, doi:10.1292/jvms.19-0055.

171. Cagnasso, F.; Suchodolski, J.S.; Borrelli, A.; Borella, F.; Bottero, E.; Benvenuti, E.; Ferriani, R.; Tolbert, M.K.; Chen, C.-C.; Giaretta, P.R.; et al. Dysbiosis Index and Fecal Concentrations of Sterols, Long-Chain Fatty Acids and Unconjugated Bile Acids in Dogs with Inflammatory Protein-Losing Enteropathy. *Front. Microbiol.* **2024**, *15*, 1433175, doi:10.3389/fmicb.2024.1433175.

172. Odamaki, T.; Kato, K.; Sugahara, H.; Hashikura, N.; Takahashi, S.; Xiao, J.; Abe, F.; Osawa, R. Age-Related Changes in Gut Microbiota Composition from Newborn to Centenarian: A Cross-Sectional Study. *Bmc Microbiol* **2016**, *16*, 90, doi:10.1186/s12866-016-0708-5.

173. Smith, E.A.; Macfarlane, G.T. Dissimilatory Amino Acid Metabolism in Human Colonic Bacteria. Anaerobe **1997**, *3*, 327–337, doi:10.1006/anae.1997.0121.

174. Louis, P.; Flint, H.J. Formation of Propionate and Butyrate by the Human Colonic Microbiota. Environ. Microbiol. 2017, 19, 29–41, doi:10.1111/1462-2920.13589.

175. Silva, Y.P.; Bernardi, A.; Frozza, R.L. The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication. *Front. Endocrinol.* **2020**, *11*, 25, doi:10.3389/fendo.2020.00025.

176. Sandri, M.; Monego, S.D.; Conte, G.; Sgorlon, S.; Stefanon, B. Raw Meat Based Diet Influences Faecal Microbiome and End Products of Fermentation in Healthy Dogs. *BMC Vet. Res.* **2017**, *13*, 65, doi:10.1186/s12917-017-0981-z.

177. Zhang, M.; Cui, Y.; Mei, X.; Li, L.; Wang, H.; Li, Y.; Wu, Y. Effect of Dietary

Composite Probiotic Supplementation on the Microbiota of Different Oral Sites in Cats. Vet. Sci. 2024, 11, 351, doi:10.3390/vetsci11080351.

178. Besten, G. den; Eunen, K. van; Groen, A.K.; Venema, K.; Reijngoud, D.-J.; Bakker, B.M. The Role of Short-Chain Fatty Acids in the Interplay between Diet, Gut Microbiota, and Host Energy Metabolism. *J. Lipid Res.* **2013**, *54*, 2325–2340, doi:10.1194/jlr.r036012.

179. Bermingham, E.N.; Young, W.; Kittelmann, S.; Kerr, K.R.; Swanson, K.S.; Roy, N.C.; Thomas, D.G. Dietary Format Alters Fecal Bacterial Populations in the Domestic Cat (Felis Catus). *Microbiologyopen* **2013**, *2*, 173–181, doi:10.1002/mbo3.60.

180. Otoni, C.C.; Heilmann, R.M.; García-Sancho, M.; Sainz, A.; Ackermann, M.R.; Suchodolski, J.S.; Steiner, J.M.; Jergens, A.E. Serologic and Fecal Markers to Predict Response to Induction Therapy in Dogs with Idiopathic Inflammatory Bowel Disease. J Vet Intern Med 2018, 32, 999–1008, doi:10.1111/jvim.15123.

181. Fujihashi, K.; McGhee, J.R. Mucosal Immunity and Tolerance in the Elderly. *Mech Ageing Dev* **2004**, *125*, 889–898, doi:10.1016/j.mad.2004.05.009.

182. Beharka, A.A.; Paiva, S.; Leka, L.S.; Ribaya-Mercado, J.D.; Russell, R.M.; Meydani, S.N. Effect of Age on the Gastrointestinal-Associated Mucosal Immune Response of Humans. *J. Gerontol. Ser. A: Biol. Sci. Méd. Sci.* **2001**, *56*, B218–B223, doi:10.1093/gerona/56.5.b218.

183. Zaine, L.; Ferreira, C.; Gomes, M. de O.S.; Monti, M.; Tortola, L.; Vasconcellos, R.S.; Carciofi, A.C. Faecal IgA Concentration Is Influenced by Age in Dogs. *Brit J Nutr* **2011**, *106*, S183–S186, doi:10.1017/s0007114511000559.

184. Park, S.Y. Age-Related Fecal Calprotectin Concentrations in Healthy Adults. *Korean J Clin Laboratory Sci* **2020**, *52*, 181–187, doi:10.15324/kjcls.2020.52.3.181.

185. Tecles, F.; Caldín, M.; Zanella, A.; Membiela, F.; Tvarijonaviciute, A.; Subiela, S.M.; Cerón, J.J. Serum Acute Phase Protein Concentrations in Female Dogs with Mammary Tumors. *J. Vet. Diagn. Investig.* **2009**, *21*, 214–219, doi:10.1177/104063870902100206.

186. Ballou, S.P.; Lozanski, G.B.; Hodder, S.; Rzewnicki, D.L.; Mion, L.C.; Sipe, J.D.; Ford, A.B.; Kushner, I. Quantitative and Qualitative Alterations of Acute-Phase Proteins in Healthy Elderly Persons. *Age Ageing* **1996**, *25*, 224–230, doi:10.1093/ageing/25.3.224.

187. Michaud, M.; Balardy, L.; Moulis, G.; Gaudin, C.; Peyrot, C.; Vellas, B.; Cesari,

M.; Nourhashemi, F. Proinflammatory Cytokines, Aging, and Age-Related Diseases. *J. Am. Méd. Dir. Assoc.* **2013**, *14*, 877–882, doi:10.1016/j.jamda.2013.05.009.

188. Sobczyńska-Malefora, A.; Delvin, E.; McCaddon, A.; Ahmadi, K.R.; Harrington, D.J. Vitamin B12 Status in Health and Disease: A Critical Review. Diagnosis of Deficiency and Insufficiency – Clinical and Laboratory Pitfalls. *Crit. Rev. Clin. Lab. Sci.* **2021**, *58*, 399–429, doi:10.1080/10408363.2021.1885339.

189. Rémond, D.; Shahar, D.R.; Gille, D.; Pinto, P.; Kachal, J.; Peyron, M.-A.; Santos, C.N.D.; Walther, B.; Bordoni, A.; Dupont, D.; et al. Understanding the Gastrointestinal Tract of the Elderly to Develop Dietary Solutions That Prevent Malnutrition. *Oncotarget* **2015**, *6*, 13858–13898, doi:10.18632/oncotarget.4030.

190. LeBlanc, J.G.; Laiño, J.E.; Valle, M.J. del; Vannini, V.; Sinderen, D. van; Taranto, M.P.; Valdez, G.F. de; Giori, G.S. de; Sesma, F. B-Group Vitamin Production by Lactic Acid Bacteria – Current Knowledge and Potential Applications. *J. Appl. Microbiol.* **2011**, *111*, 1297–1309, doi:10.1111/j.1365-2672.2011.05157.x.

191. Courtemanche, C.; Elson-Schwab, I.; Mashiyama, S.T.; Kerry, N.; Ames, B.N. Folate Deficiency Inhibits the Proliferation of Primary Human CD8+ T Lymphocytes In Vitro. *J. Immunol.* **2004**, *173*, 3186–3192, doi:10.4049/jimmunol.173.5.3186.

192. Simpson, J.M.; Martineau, B.; Jones, W.E.; Ballam, J.M.; Mackie, R.I. Characterization of Fecal Bacterial Populations in Canines: Effects of Age, Breed and Dietary Fiber. Microb. Ecol. 2002, 44, 186–197, doi:10.1007/s00248-002-0001-z.

193. Shivappa, N.; Hebert, J.R.; Marcos, A.; Diaz, L.; Gomez, S.; Nova, E.; Michels, N.; Arouca, A.; González-Gil, E.; Frederic, G.; et al. Association between Dietary Inflammatory Index and Inflammatory Markers in the HELENA Study. *Mol. Nutr. Food Res.* **2017**, *61*, doi:10.1002/mnfr.201600707.

194. Giannella, R.A.; Broitman, S.A.; Zamcheck, N. Competition Between Bacteria and Intrinsic Factor for Vitamin B12: Implications for Vitamin B12 Malabsorption in Intestinal Bacterial Overgrowth. Gastroenterology **1972**, *62*, 255–260, doi:10.1016/s0016-5085(72)80177-x.

195. Ullal, T.V.; Marks, S.L.; Huebner, S.N.; Taylor, S.L.; Shelley, C.D. Association of Folate Concentrations with Clinical Signs and Laboratory Markers of Chronic Enteropathy in Dogs. J. Vet. Intern. Med. 2023, 37, 455–464, doi:10.1111/jvim.16681.

196. Tejero, J.; Lazure, F.; Gomes, A.P. Methylmalonic Acid in Aging and Disease. *Trends Endocrinol. Metab.* **2024**, *35*, 188–200, doi:10.1016/j.tem.2023.11.001.

197. Gomes, A.P.; Ilter, D.; Low, V.; Endress, J.E.; Fernández-García, J.;

Rosenzweig, A.; Schild, T.; Broekaert, D.; Ahmed, A.; Planque, M.; et al. Age-Induced Accumulation of Methylmalonic Acid Promotes Tumour Progression. Nature 2020, *585*, 283–287, doi:10.1038/s41586-020-2630-0.

198. Morris, M.S.; Jacques, P.F.; Rosenberg, I.H.; Selhub, J. Elevated Serum Methylmalonic Acid Concentrations Are Common among Elderly Americans. J. Nutr. 2002, 132, 2799–2803, doi:10.1093/jn/132.9.2799.

199. Gao, K.; Mu, C.; Farzi, A.; Zhu, W. Tryptophan Metabolism: A Link Between the Gut Microbiota and Brain. *Adv Nutr* **2019**, *11*, 709–723, doi:10.1093/advances/nmz127.

200. Solvang, S.-E.H.; Hodge, A.; Watne, L.O.; Cabral-Marques, O.; Nordrehaug, J.E.; Giles, G.G.; Dugué, P.-A.; Nygård, O.; Ueland, P.M.; McCann, A.; et al. Kynurenine Pathway Metabolites in the Blood and Cerebrospinal Fluid Are Associated with Human Aging. *Oxidative Med. Cell. Longev.* **2022**, *2022*, 5019752, doi:10.1155/2022/5019752.

201. Franceschi, C.; Garagnani, P.; Parini, P.; Giuliani, C.; Santoro, A. Inflammaging: A New Immune–Metabolic Viewpoint for Age-Related Diseases. *Nat. Rev. Endocrinol.* **2018**, *14*, 576–590, doi:10.1038/s41574-018-0059-4.

202. Sorgdrager, F.J.H.; Naudé, P.J.W.; Kema, I.P.; Nollen, E.A.; Deyn, P.P.D. Tryptophan Metabolism in Inflammaging: From Biomarker to Therapeutic Target. Front. Immunol. 2019, 10, 2565, doi:10.3389/fimmu.2019.02565.

203. Kennedy, P.J.; Cryan, J.F.; Dinan, T.G.; Clarke, G. Kynurenine Pathway Metabolism and the Microbiota-Gut-Brain Axis. *Neuropharmacology* **2017**, *112*, 399–412, doi:10.1016/j.neuropharm.2016.07.002.

204. Ye, X.; Li, H.; Anjum, K.; Zhong, X.; Miao, S.; Zheng, G.; Liu, W.; Li, L. Dual Role of Indoles Derived From Intestinal Microbiota on Human Health. Front. Immunol. 2022, 13, 903526, doi:10.3389/fimmu.2022.903526.

205. Kaur, H.; Bose, C.; Mande, S.S. Tryptophan Metabolism by Gut Microbiome and Gut-Brain-Axis: An in Silico Analysis. *Front. Neurosci.* **2019**, *13*, 1365, doi:10.3389/fnins.2019.01365.

206. Cao, Y.; Li, B.; Ismail, N.; Smith, K.; Li, T.; Dai, R.; Deng, Y. Neurotoxicity and Underlying Mechanisms of Endogenous Neurotoxins. Int. J. Mol. Sci. 2021, 22, 12805, doi:10.3390/ijms222312805.

207. Pathak, S.; Nadar, R.; Kim, S.; Liu, K.; Govindarajulu, M.; Cook, P.; Alexander, C.S.W.; Dhanasekaran, M.; Moore, T. The Influence of Kynurenine Metabolites on

Neurodegenerative Pathologies. Int. J. Mol. Sci. 2024, 25, 853, doi:10.3390/ijms25020853.

208. Friuli, M.; Eramo, B.; Valenza, M.; Scuderi, C.; Provensi, G.; Romano, A. Targeting the Oxytocinergic System: A Possible Pharmacological Strategy for the Treatment of Inflammation Occurring in Different Chronic Diseases. Int. J. Mol. Sci. 2021, 22, 10250, doi:10.3390/ijms221910250.

209. Audunsdottir, K.; Quintana, D.S. Oxytocin's Dynamic Role across the Lifespan. *Aging Brain* **2022**, 2, 100028, doi:10.1016/j.nbas.2021.100028.

210. Takahashi, J.; Yamada, D.; Nagano, W.; Saitoh, A. The Role of Oxytocin in Alzheimer's Disease and Its Relationship with Social Interaction. Cells 2023, 12, 2426, doi:10.3390/cells12202426.

211. Lupien, S.J.; Leon, M. de; Santi, S. de; Convit, A.; Tarshish, C.; Nair, N.P.V.; Thakur, M.; McEwen, B.S.; Hauger, R.L.; Meaney, M.J. Cortisol Levels during Human Aging Predict Hippocampal Atrophy and Memory Deficits. *Nat. Neurosci.* **1998**, *1*, 69–73, doi:10.1038/271.

212. Sudo, N.; Chida, Y.; Aiba, Y.; Sonoda, J.; Oyama, N.; Yu, X.; Kubo, C.; Koga, Y. Postnatal Microbial Colonization Programs the Hypothalamic–Pituitary–Adrenal System for Stress Response in Mice. J. Physiol. 2004, *558*, 263–275, doi:10.1113/jphysiol.2004.063388.

213. Ouanes, S.; Popp, J. High Cortisol and the Risk of Dementia and Alzheimer's Disease: A Review of the Literature. *Front. Aging Neurosci.* **2019**, *11*, 43, doi:10.3389/fnagi.2019.00043.

214. Dinan, T.G.; Cryan, J.F. Brain–Gut–Microbiota Axis — Mood, Metabolism and Behaviour. Nat. Rev. Gastroenterol. Hepatol. 2017, 14, 69–70, doi:10.1038/nrgastro.2016.200.

215. Qi, Q.; Li, J.; Yu, B.; Moon, J.-Y.; Chai, J.C.; Merino, J.; Hu, J.; Ruiz-Canela, M.; Rebholz, C.; Wang, Z.; et al. Host and Gut Microbial Tryptophan Metabolism and Type 2 Diabetes: An Integrative Analysis of Host Genetics, Diet, Gut Microbiome and Circulating Metabolites in Cohort Studies. Gut 2022, 71, 1095–1105, doi:10.1136/gutjnl-2021-324053.

216. Rosin, S.; Xia, K.; Azcarate-Peril, M.A.; Carlson, A.L.; Propper, C.B.; Thompson, A.L.; Grewen, K.; Knickmeyer, R.C. A Preliminary Study of Gut Microbiome Variation and HPA Axis Reactivity in Healthy Infants. Psychoneuroendocrinology 2021, 124, 105046,

doi:10.1016/j.psyneuen.2020.105046.

217. Foster, J.A.; Neufeld, K.-A.M. Gut–Brain Axis: How the Microbiome Influences Anxiety and Depression. *Trends Neurosci.* **2013**, *36*, 305–312, doi:10.1016/j.tins.2013.01.005.

218. O'Mahony, S.M.; Clarke, G.; Borre, Y.E.; Dinan, T.G.; Cryan, J.F. Serotonin, Tryptophan Metabolism and the Brain-Gut-Microbiome Axis. *Behav. Brain Res.* **2015**, *277*, 32–48, doi:10.1016/j.bbr.2014.07.027.

219. Culp, E.J.; Goodman, A.L. Cross-Feeding in the Gut Microbiome: Ecology and Mechanisms. Cell Host Microbe 2023, 31, 485–499, doi:10.1016/j.chom.2023.03.016.

220. Taszkun, I.; Milczak, A.; Kalisz, G.; Wilkołek, P.; Zwolska, J.; Szczepanik, M. Serum TT4, FT4 and TSH Concentrations in German Shepherd Dogs Depending on Age and Type of Work. *Pol. J. Vet. Sci.* **2021**, *24*, 63–68, doi:10.24425/pjvs.2021.136793.

221. Studzinski, C.M.; Araujo, J.A.; Milgram, N.W. The Canine Model of Human Cognitive Aging and Dementia: Pharmacological Validity of the Model for Assessment of Human Cognitive-Enhancing Drugs. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* **2005**, *29*, 489–498, doi:10.1016/j.pnpbp.2004.12.014.

222. Chopik, W.J.; Weaver, J.R. Old Dog, New Tricks: Age Differences in Dog Personality Traits, Associations with Human Personality Traits, and Links to Important Outcomes. *J. Res. Pers.* **2019**, *79*, 94–108, doi:10.1016/j.jrp.2019.01.005.

223. Montenegro, J.; Armet, A.M.; Willing, B.P.; Deehan, E.C.; Fassini, P.G.; Mota, J.F.; Walter, J.; Prado, C.M. Exploring the Influence of Gut Microbiome on Energy Metabolism in Humans. *Adv. Nutr.* **2023**, *14*, 840–857, doi:10.1016/j.advnut.2023.03.015.

224. Held, S.D.E.; Špinka, M. Animal Play and Animal Welfare. Anim. Behav. 2011, 81, 891–899, doi:10.1016/j.anbehav.2011.01.007.

225. Boissy, A.; Manteuffel, G.; Jensen, M.B.; Moe, R.O.; Spruijt, B.; Keeling, L.J.; Winckler, C.; Forkman, B.; Dimitrov, I.; Langbein, J.; et al. Assessment of Positive Emotions in Animals to Improve Their Welfare. Physiol. Behav. **2007**, *92*, 375–397, doi:10.1016/j.physbeh.2007.02.003.

226. Ahmed, S.; Regmi, P. Advancements in Gut Microbiota Research: A Critical Review. *medtigo J. Med.* **2023**, *2*, doi:10.63096/medtigo3062236.

227. Tosoni, G.; Conti, M.; Heijtz, R.D. Bacterial Peptidoglycans as Novel Signaling

Molecules from Microbiota to Brain. *Curr. Opin. Pharmacol.* **2019**, *48*, 107–113, doi:10.1016/j.coph.2019.08.003.

228. Pires, L.; Gonzalez-Paramás, A.M.; Heleno, S.A.; Calhelha, R.C. Gut Microbiota as an Endocrine Organ: Unveiling Its Role in Human Physiology and Health. *Appl. Sci.* **2024**, *14*, 9383, doi:10.3390/app14209383.

229. Cani, P.D.; Osto, M.; Geurts, L.; Everard, A. Involvement of Gut Microbiota in the Development of Low-Grade Inflammation and Type 2 Diabetes Associated with Obesity. Gut Microbes 2012, 3, 279–288, doi:10.4161/gmic.19625.

230. Collins, S.M.; Kassam, Z.; Bercik, P. The Adoptive Transfer of Behavioral Phenotype via the Intestinal Microbiota: Experimental Evidence and Clinical Implications. *Curr Opin Microbiol* **2013**, *16*, 240–245, doi:10.1016/j.mib.2013.06.004.

231. Gernone, F.; Uva, A.; Silvestrino, M.; Cavalera, M.A.; Zatelli, A. Role of Gut Microbiota through Gut–Brain Axis in Epileptogenesis: A Systematic Review of Human and Veterinary Medicine. *Biology* **2022**, *11*, 1290, doi:10.3390/biology11091290.

232. Margolis, K.G.; Cryan, J.F.; Mayer, E.A. The Microbiota-Gut-Brain Axis: From Motility to Mood. Gastroenterology 2021, 160, 1486–1501, doi:10.1053/j.gastro.2020.10.066.

233. Kelly, J.R.; Clarke, G.; Cryan, J.F.; Dinan, T.G. Brain-Gut-Microbiota Axis: Challenges for Translation in Psychiatry. *Ann. Epidemiology* **2016**, *26*, 366–372, doi:10.1016/j.annepidem.2016.02.008.

234. Yang, M.; Gu, Y.; Li, L.; Liu, T.; Song, X.; Sun, Y.; Cao, X.; Wang, B.; Jiang, K.; Cao, H. Bile Acid–Gut Microbiota Axis in Inflammatory Bowel Disease: From Bench to Bedside. Nutrients 2021, 13, 3143, doi:10.3390/nu13093143.