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“The impact of an animal protein-based diet on a murine glioma model.”

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Abbreviation table

Abbreviation	Full form (if found)
ADP	Animal-derived protein
AOAA	Aminooxiacetic acid
AP	Animal protein
APS	Adenosine-5′-phosphosulfate
BBB	Blood Brain Barrier
BSA	Bovine Serum Albumin
CAR	Chimeric antigen receptor
CBS	Cystathionine beta-synthase
CNS	Central Nervous System
CO	Carbon monoxide
CPS	Capsaicin
CPZ	Capsazepine
CSE	Cystathionine gamma-lyase
CT	Computer Tomography
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CTRL	Control

DA	dopamine
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl Sulfoxide
DRG	Dorsal root ganglion
DTNB	5,5′-dithiobis-(2-nitrobenzoic acid)
ECs	Endotelial cells
EDTA	ethylenediamine tetraacetic acid
EGFR	Epidermal Growth Factor Receptor
EMT	epithelial-mesenchymal transition
ERK	extracellular signal-regulated kinase
FBS	Fetal Bovine Serum
FMT	Fecal microbiota transplantation
FOV	Field of view
GABA	Gamma-aminobutyric acid
GB	Glioblastoma
GBA	Gut-brain axis
HCA	hierarchical clustering analysis
HFD	High-fat diet

HIV	Human immunodeficiency virus
HPA	Hypothalamic-pituitary-adrenal
5-HT	Serotonin
IBD	Inflammatory bowel diseases
IDH	Isocitrate dehydrogenase
IL	Interleukin
IVC	Individually ventilated cage
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDPI	Multidisciplinary Digital Publishing Institute
MGMT	O-6-methylguanine-DNA methyltransferase
MRI	Magnetic resonance imaging
3-MST	3-mercaptopyruvate sulfurtransferase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NF-κB	Nuclear Factor kappa-light-chain-

	enhancer of activated B
NO	Nitric oxide
NTS	Nucleus tractus solitaries
OD	Optical densities
PAG	Propargylglycine
PBS	Phosphate-buffered saline
PD-1	Programmed Death 1
PEG	Polyethylene Glycol
PFA	Paraformaldehyde
(PI3K)/AKT	Phosphoinositide 3-kinases
PLS	Partial Least Squares Discriminant Analysis
PNS	Parasympathetic nervous system
ROS	Reactive oxygen species
TERT	Telomerase Reverse Transcriptase
TMZ	Temozolomide
TNF	Tumor Necrosis Factor
VEGF	Vascular endothelial growth factor
VIP	Variable Importance in Projection

VN	Vagus nerve
VNS	Vagus nerve stimulation
WHO	World Health Organization

Summary

Glioblastoma (GB) is the most aggressive primary brain tumor in adults, characterized by high proliferation, therapy resistance, and limited survival (Koshy et al., 2012). Increasing evidence highlights the role of the gut-brain axis and microbial metabolites, such as hydrogen sulfide (H_2S), in modulating central nervous system (CNS) tumor progression (Silver et al., 2021). H_2S is endogenously produced but also derives from microbial fermentation of sulfur-containing amino acids, particularly in diets rich in red meat (Teigen et al., 2023). This study explored the impact of an animal protein diet (AP) on glioma progression in a murine orthotopic model and investigated the involvement of gut-derived H_2S in shaping tumor behavior.

Mice fed a red meat-derived protein diet displayed a distinct gut microbial composition enriched in sulfate-reducing bacteria, with significantly elevated fecal H_2S levels. Notably, glioma-bearing mice receiving this diet showed reduced tumor volume and decreased cell proliferation, as revealed by Ki67 staining, compared to control mice fed an animal-derived protein diet. Importantly, this was not accompanied by changes in body weight or gut morphology, suggesting a microbiota-driven systemic effect.

In-depth analysis of the tumor microenvironment via confocal immunofluorescence revealed a significant shift in the morphology of tumor-associated microglia/macrophages (TAMs). $Iba1^+$ cells in the red meat diet group exhibited a rounder, less ramified morphology, quantified through form factor analysis and plot profile mapping. This shape shift is indicative of an activated, pro-inflammatory phenotype, which is frequently associated with

anti-tumor responses. In contrast, mice on the animal-derived protein diet showed Iba1^{+} cells with a more branched and complex morphology, typical of a resting or tumor-supportive state. To validate the functional role of H_2S, in vitro exposure of GL261 glioma cells to sodium hydrosulfide (NaHS, an H_2S donor) led to reduced cell viability and proliferation, and induced a similar morphological rounding in both primary BV2 microglial cells, consistent with an activated state.

Conversely, inhibition of microbial H_2S biosynthesis with aminooxyacetic acid (AOAA) abolished the protective effects of the red meat diet in vivo, restoring tumor volume and reversing TAM morphology toward a less activated state. Moreover, blocking TRPV1 receptors—known to mediate vagus nerve activation by H_2S—through capsazepine treatment, abolished the antitumor effect, indicating a possible role for neuronal/neuroimmune signaling. In conclusion, these findings suggest that moderate red meat intake modulates the gut microbiota and enhances microbial H_2S production, which may exert anti-tumor effects by reshaping the glioma microenvironment through vagal TRPV1 signaling. These results open new perspectives on the gut-brain-tumor axis and highlight the potential role of diet-based interventions in GB therapy.

1. Introduction

1.1 Glioblastoma

The gliomas have been recently classified by the World Health Organization (WHO) considering the recent advances in cellular and molecular characterizations (Louis et al., 2021). The latest edition, WHO Central Nervous System (CNS) 5, introduced significant updates that enhance the importance of molecular diagnostics in the classification of CNS tumors. Despite the advancements (Czarnywojtek et al., 2023), it remains grounded to established diagnostic practices like histology and immunohistochemistry (Louis et al., 2021). In particular, the WHO CNS5 introduces new methods for CNS tumor nomenclature and grading, and underscores the value of integrated and layered diagnostic reporting. The new nomenclature for Glioblastoma (GB), is: Glioblastoma, Isocitrate dehydrogenase (IDH)-wildtype, grade 4. IDH-wildtype stands for no mutations on IDH1 or IDH2 genes, which are mutated in low grade gliomas (such as astrocytoma, IDH-mutant). Grade 4 represents the most aggressive form of the tumor, being associated to rapid growth, necrosis, vascular proliferation, and unfavorable prognosis (Louis et al., 2021) (Figure 1). The minimum criteria for the diagnosis of GB, IDH-wildtype, is the presence of at least one of the following molecular alterations: Telomerase Reverse Transcriptase (TERT) promoter mutation, Epidermal Growth Factor Receptor (EGFR) gene amplification, gain of chromosome 7 and loss of 10 (Nikolova et al., 2024). GB is one of the most common brain tumors, with a very low life expectancy (Koshy et al., 2012). Although there are numerous approaches described in vitro and in experimental models to counteract the

growth of this tumor, they are often ineffective in humans, with side effects and high relapse rates. Gliomas are tumors with multifactorial etiology, involving genetic alterations due to internal and environmental factors. Despite advances in medical research and treatment strategies, GB remains challenging to treat, with poor prognosis and limited therapeutic options (Czarnywojtek et al., 2023).

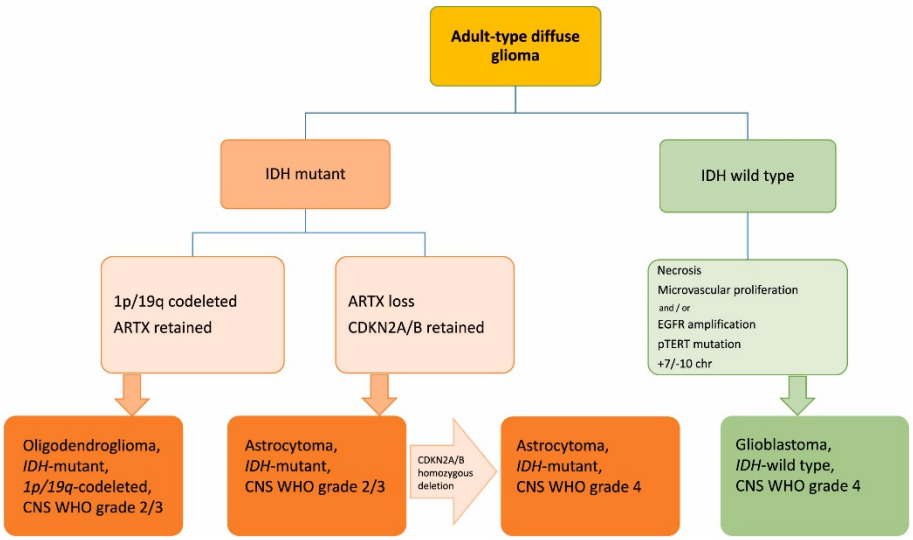


Figure 1: The 2021 WHO CNS5 classification algorithm for diagnosis of GB, IDH-wildtype (Nikolova et al., 2024).

1.1.1 Pathophysiology, clinical features and symptoms

Glioblastomas thought to arise from neuroglial stem or progenitor cells (Weller et al., 2015). According to their morphological likeness to the neuroglial cells of the central nervous system (CNS), these tumors are usually classified as astrocytomas, oligodendrogliomas, mixed oligoastrocytic gliomas or

ependymomas (Weller et al., 2015). The hallmark characteristics of GB include rapid growth, invasive behavior and significant genetic and molecular heterogeneity (Weller et al., 2015). These tumors infiltrate surrounding brain tissue, making complete surgical resection nearly impossible. Moreover, in GB, cells have different size and shape, are highly invasive, with high presence of immune infiltrates, areas of necrosis, hypoxia and inflammation (Czarnywojtek et al., 2023). From a molecular perspective, GB is characterized by frequent alterations in key signaling pathways, including epidermal growth factor receptor (EGFR) and tumor protein p53 (TP53) genes (Fan et al., 2024; Johansson et al., 2016; Vijapura et al., 2017). These mutations contribute to uncontrolled cell proliferation, resistance to apoptosis, and enhanced tumor survival. Furthermore, GBs often exhibit aberrations in metabolic pathways, relying on aerobic glycolysis (the Warburg effect) to sustain their growth (R. Zhang et al., 2023). Generally, this type of tumor does not metastasize outside the CNS, by the way there are some evidences of GB spread to the lungs, liver and other organs (Awan et al., 2015; Luan et al., 2021).

The symptoms of GB are different and depend on the tumor's size and location within the brain. Common clinical manifestations include: persistent headaches, seizures, cognitive impairment, personality or behavioral changes, focal neurological deficits, such as weakness, sensory disturbances, or visual changes (L. M. Wang et al., 2023). Diagnosis typically involves imaging studies, such as magnetic resonance imaging (MRI) with contrast, which reveals characteristic features like ring-enhancing lesions and peritumoral edema (Fan et al., 2024; L. M. Wang et al., 2023). Definitive diagnosis requires a biopsy of surgical tumor's grade and genetic profile (L. S. Hu et al., 2020).

1.1.2 Incidence and risk factors

Glioblastoma is one of the prevalent among all malignant brain tumors, approximately 50.1% (Ostrom et al., 2022). Despite its prevalence, the annual incidence is low (about 3.19 per 100000 people in developed countries) (Grech et al., 2020). Risk factors for GB are multiple and they are associated with both intrinsic and environmental factors. First of all, genetic risk factors: even though most gliomas develop sporadically without any familial predisposition, there is a significant link between gliomas and several uncommon inherited syndromes, which collectively account of less than 5% of all cases (Davis, 2018). One of these rare syndromes is the Li Fraumeni syndrome, which is linked to a TP53 gene mutations and usually patients will develop cancer before age 30 (Johansson et al., 2016; Vijapura et al., 2017).

As concern environmental risks for the onset of gliomas, many possible have been studied, but much information still remains uncertain. By the way, the exposure to moderate or high levels of medical and environmental ionizing radiation remains the only firmly established risk factor for brain tumors. This has been demonstrated in numerous studies involving atomic bomb survivors and children who received radiation therapy for benign medical conditions, as well as for the treatment of primary cancers (Braganza et al., 2012; McNeill, 2016). There is also a strong association between early age exposure to radiation and tumor development in adults, which could explain the trouble in identifying casual environmental exposures in epidemiologic studies (Inskip et al., 2016). There is also a dose-response correlation between radiation doses and risk of tumor development (Braganza et al., 2012). Last, but not least,

demographic risk factors as age and sex are relevant. In particular, GB onset strongly increases with age, where median age at diagnosis in almost 65 years, while it is extremely rare in pediatric patients (Ostrom et al., 2022; Tan et al., 2020). Moreover, males are 1.6 times more affected than females in Caucasians compared to other ethnicities (Le Rhun et al., 2019; Ostrom et al., 2022).

1.1.3 Therapeutic intervention strategies

The traditional criteria to assess treatment effectiveness and monitor disease progression of cancer is the MacDonald criteria (Sorensen et al., 2008). It is based on the use of imaging technique for detecting a brain tumor, such as computer tomography (CT) or MRI, performed with or without contrast enhancement (Weller et al., 2015). The MacDonald criteria allow to categorized the response of the tumor to a specific treatment into four categories: complete response, partial response, stable disease, and progressive disease. However, these criteria have many limitations given for example by the progression of the tumor, and the high radiographic response rates (Wick et al., 2016). By the way, despite all these criteria, a glioma can only be conclusively diagnosed through histological and molecular analysis (Louis et al., 2021). Standard treatment protocol for GB involves a combination of surgery, radiation therapy, and chemotherapy (Obrador et al., 2024). Newly, intraoperative imaging techniques have played a significant role in defining the margins of glioblastoma by enhancing the extent of resection (Fountain et al., 2021). The goal of surgery is to achieve maximal safe resection, as reducing the tumor burden correlates with prolonged survival. Complete resection is often challenging due to the infiltrative nature of GB and its proximity to critical brain region. Postoperative

radiotherapy is a cornerstone of GB treatment. It has been shown that standard fractionated radiotherapy targets residual tumor cells while minimizing damage to healthy brain tissue (De Crevoisier et al., 1997). Moreover, temozolomide (TMZ), an oral alkylating agent, is the first-line chemotherapeutic for GB. Its efficacy is enhanced in tumors with O-6-methylguanine-DNA methyltransferase (MGMT) promoter methylation, which reduces DNA repair capacity, compared to an unmethylated promoter where the MGMT gene is active and therefore the cells are able to repair the damage caused by TMZ leading to resistance to treatment (Chua et al., 2019). Despite these aggressive multimodal treatments, GB recurs in nearly all patients. Factors which contribute to poor outcomes include: blood brain barrier (BBB), which restricts the delivery of therapeutic agents to the tumor site; tumor heterogeneity of GB which complicates targeted therapy; resistance mechanisms acquired to radiation and chemotherapy (Ahmed et al., 2023; Obrador et al., 2024). To address these challenges, the scientific world is investigating innovative therapies. One of these are the targeted molecular therapies, which exploit specific genetic and molecular abnormalities in GB such as overexpression or mutation of epidermal growth factor receptor (EGFR) which is common in this type of tumor (Berger et al., 2022; Louis et al., 2021) and bevacizumab, an anti-vascular endothelial growth factor (VEGF) antibody, which reduces tumor angiogenesis and edema (Ramezani et al., 2019). Moreover, the immunotherapy which purpose is to harness the immune system to counteract GB progression. The immunotherapeutic approaches are: vaccines, such as the autologous tumor lysate-loaded dendritic cell vaccine (DCVax-L), which is a personalized therapeutic vaccine based on dendritic cells, designed to stimulate the immune system to recognize and attack GB cells using patient-specific tumor antigens and it is still under investigation (De Felice, F.

et al 2019); the chimeric antigen receptor (CAR) T-cells engineered to recognize GB antigens which seems to be promising in preclinical and early clinical trials ; the immune checkpoint inhibitors, which are agents targeting Programmed Death 1 (PD-1)/ Programmed Death-Ligand1PD-L1 or Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) pathways seek to overcome immune evasion by tumor cells (De Felice et al., 2019; Heiland et al., 2017; Shu & Li, 2020). Advances in genomics and proteomics are paving the way for personalized medicine in GB. Identifying biomarkers predictive of treatment response can guide therapy selection. For example, MGMT promoter methylation and isocitrate dehydrogenase (IDH) mutation status are critical determinants of prognosis and therapeutic sensitivity (Berger et al., 2022; Louis et al., 2021). The treatment landscape for GB remains challenging due to the tumor's aggressive nature and resistance to standard therapies. However, ongoing research into targeted therapies, immunotherapy, gene editing, and innovative drug delivery systems holds promise for improving patient outcomes. A shift toward personalized treatment strategies, guided by molecular and genetic profiling, may ultimately revolutionize the management of this devastating disease.

1.2 Gut microbiota

The gut microbiota refers to a complex, dynamic community of trillions of microorganisms, including bacteria, viruses, fungi, and protozoa, that resides in the gastrointestinal tract (Adak & Khan, 2019). Dysbiosis, an imbalance in the composition and function of the gut microbiota, has been implicated in numerous immune-related diseases. Antibiotic-induced dysbiosis can lead to increased production of pro-inflammatory cytokines like Tumor Necrosis

Factor (TNF). This excessive immune activation can predispose individuals to autoimmune or chronic inflammatory diseases (Taitz et al., 2024). Instead, human immunodeficiency virus (HIV)-1 infection alters gut microbiota, leading to immune deficiency by disrupting the intestinal barrier and promoting microbial diversity (Geng et al., 2020). Given the central role of the gut microbiota in immune modulation, therapeutic strategies targeting the microbiota hold promise for treating and preventing immune-related diseases, including cancer. These interventions include probiotics, live microorganisms which can restore microbial balance and enhance immune function; prebiotics, non-digestible fibers that promote the growth of beneficial gut bacteria enhancing short-chain fatty acids (SCFAs) production and supporting homeostasis; dietary modifications, and fecal microbiota transplantation (FMT), which involves transferring stool from a healthy donor to a recipient to restore a balanced microbiota (Belvoncikova et al., 2022; Croci et al., 2021; Kim et al., 2019; Quigley, 2019).

1.2.1 The roles of gut microbiota in the host

Despite microorganisms reside different areas of the host body, such as the skin, the mouth, and the upper respiratory tract, 90% of all microorganisms can be found in the starting sections of the small and large intestines (Kho & Lal, 2018; Singh et al., 2021). Gut microbiota is characterized by an intricate network of interrelationships that play a pivotal role in maintaining host health and well-being, influencing digestion, nutrient absorption, gut barrier homeostasis, immune system modulation, production of metabolites, and even CNS homesostasis, through the so called “gut-brain axis” (GBA), in particular mental health (Góralczyk-Bińkowska et al., 2022; Nishida et al., 2018).

1.2.2 Acquisition of nutrient fibers digestion

One of the primary roles of the gut microbiota is to aid in the digestion and breakdown of dietary components that the human digestive system cannot process on its own. In particular, dietary fibers, such as lignin, non-starchpolysaccharides, and so on, found in plant-based foods, are resistant to digestion by human enzymes but are effectively broken down by the microbiota in the colon (Anderson et al., 2009). In fact, gut bacteria have a huge set of enzymes which allow the use of these carbohydrates which are indigestible by human digestive enzymes due to their complex structure (Lombard et al., 2014). This process not only benefits the microbes themselves but also produces metabolites that profoundly influence the host's physiology. This fermentation primarly occurs in the large intestine, where microbes break down fibers into SCFAs such as acetate, propionate, and butyrate (Duncan et al., 2007). These SCFAs are prevalent and present in cecum and serve as an energy source for colonocytes, support intestinal barrier integrity, and exhibit anti-inflammatory properties (J. K. Nicholson et al., 2012; Rooks & Garrett, 2016). Moreover, a fiber-rich diet fosters microbial diversity, which is crucial for a resilient and healthy microbiota.

1.2.3 Gut barrier homeostasis

The gut barrier is a complex and dynamic system that separates the gut lumen from the internal environment of the body. The intestinal epithelium is regenerated every 3-5 days in humans, as mature enterocytes undergo apoptosis and exfoliation, while new cells are produced through the

proliferation of stem-cells in the crypts (Bischoff et al., 2014). The primary function of gut barrier is to regulate the selective permeability of nutrients, electrolytes, and water while preventing the translocation of harmful substances such as pathogens and toxins. The intestinal barrier comprises several layers that work together to maintain its integrity and function. First of all, physical barrier, a single layer of intestinal epithelial cells (IECs) linked together by tight junctions to regulate permeability and prevent the entry of dangerous substances. Moreover, goblet cells within the epithelium produce mucus that coats the intestinal lining, providing an additional protective layer. Furthermore, chemical barrier, characterized by molecules that neutralize pathogens ad toxins before they can breach the epithelial layer and immune barrier, which consists of immune cells, such as macrophages, dendritic cells, and lymphocytes that monitor and respond to microbial and dietary agents. Last, but not least, microbial barrier, characterized by the gut microbiota which significantly impacts the host's metabolism and energy balance. It influences the extraction of energy from food and regulates fat storage. The gut barrier can be disturbed by several features, such as, dysbiosis, incorrect nutrition, infections or diseases, antibiotics, drugs, and stress. These events alter its function leading to an increase of intestinal permeability (Aleman et al., 2023; Wells et al., 2017). "Gut permeability" means that molecules can spread, across the epithelium, from the intestinal lumen to all the body. This status of the gut barrier is also called "leaky gut" and it is usually associated with several gastrointestinal disorders, such as inflammatory bowel diseases (IBD), celiac disease and the early stages of colon cancer development, but also in general with an inflammatory state (Bischoff et al., 2014). Dysbiosis, has been associated with metabolic disorders such as obesity, diabetes, and non-alcoholic fatty liver disease. Conversely, a diverse and balanced microbiota supports metabolic

health and mitigates the risk of these conditions (Jakobsson et al., 2015). Moreover, a healthy gut microbiota serves as a barrier against pathogenic microorganisms by competing for nutrients and attachment sites, producing antimicrobial compounds, and stimulating the host's immune defences (Knoop et al., 2013). This protective role is often referred to as colonization resistance. Intestinal barrier homeostasis is maintained through a delicate interplay between epithelial cells, the immune system, and the gut microbiota. The main mechanisms include: the intestinal epithelium continuous renewal and repair, with stem cells. This process ensures the maintenance of barrier integrity, and tight junction regulation, in particular of occludins, claudins and zonula occludens (Bischoff et al., 2014; Wells et al., 2017). The tight junctions are protein complexes which control the paracellular permeability of the epithelium (Wells et al., 2017). Indeed, disruptions in these proteins can lead to the leaky gut condition. Moreover, the homeostasis of gut barrier is also maintained across microbial interaction, by producing SCFAs, which serve as an energy source for epithelial cells and enhance tight junction integrity (J. K. Nicholson et al., 2012; Rooks & Garrett, 2016). Immune surveillance, in particular of regulatory T cells (Tregs), plays a crucial role in maintaining immune tolerance to commensal bacteria and dietary agents (Wells et al., 2017). Tregs are immunosuppressive cells which, maintain immune tolerance and prevent chronic stress by restoring homeostasis and suppressing anticancer immune responses, and stimulate inflammatory cytokine production (Markman & Shiao, 2015). Strategies to support intestinal barrier homeostasis involves dietary interventions with diet rich in fibres, probiotics and prebiotics administration, pharmacological therapies by using treatments targeting inflammation, such as corticosteroids and biologics, which can help restore barrier function, lifestyle modifications, such as stress management, regular exercise, and avoiding excessive alcohol

consumption can also contribute to maintaining barrier health (Aleman et al., 2023; Sartor, 2004; Shepherd & Gibson, 2006). The intestinal barrier is a critical component of human health and disruptions to this delicate balance can have profound consequences, highlighting the importance of dietary and lifestyle choices in preserving barrier integrity. As research into the intestinal barrier continues to advance, novel therapies may emerge to address barrier dysfunction and improve health outcomes.

1.2.4 Immune system modulation

The gut microbiota plays a key role in the development, function and homeostasis of the immune system. This, intricate relationship underscores the microbiota's importance as a critical modulator of immune responses, both locally in the gut and systematically throughout the body (Lazar et al., 2018). The interaction between the gut microbiota and the immune system begins early in life and continues throughout an individual's lifetime. The colonization of the gut microbiota starts at birth, influenced by factors such as the mode of delivery, breastfeeding, and environmental exposures (Jašarević et al., 2016). From infancy, it helps train the immune system to distinguish between harmful pathogens and benign or beneficial microbes (Gensollen et al., 2016). The gut microbiota also plays a vital role in establishing immune tolerance, that is the ability of the immune system to distinguish between self and non-self, as well as between harmless antigens and harmful pathogens. Specific microbial species such as *Bacteroides fragilis* and certain *Clostridia* clusters, produce metabolites and signals, such as type I interferons, which are involved in several key immune functions, influencing immune responses (Ivashkiv & Donlin,

2014). These interferons promote the differentiation of Tregs (Ayala et al., 2024). In particular, *Bacteroides fragilis* modulate dendritic cells and Treg responses, triggers the production of immunoregulatory cytokines, such as IL-10 and IL-27 (Ayala et al., 2024). Tregs are crucial for suppressing inflammatory responses and preventing autoimmune diseases. The gut microbiota influences immune responses throughout the body and communicates with distant organs via microbial metabolites, cytokines, and immune cell trafficking (Hanus et al., 2021; Rooks & Garrett, 2016). For example, SCFAs can enter systemic circulation, modulating the function of immune cells in peripheral tissues (Wells et al., 2017). Moreover, microbial metabolites and immune mediators influence the CNS, which in turn regulates immune responses through neuroendocrine and autonomic pathways (Park & Im, 2022). This bidirectional communication highlights the interconnectedness of the gut microbiota, the immune system, and overall health.

1.2.5 Gut microbiota metabolites

The gut microbiota produces a wide array of metabolites, such as SCFAs, tryptophan and its precursors, secondary bile acid and, gases that profoundly influence host physiology, ranging from immune modulation to energy metabolism (J. Wang et al., 2023). Among these metabolites, the gasotransmitter hydrogen sulfide (H_2S) has garnered significant attention due to its dual role as both a beneficial and potentially harmful compound (L. F. Hu et al., 2007; Kushkevych et al., 2019; Ng et al., 2019). Understanding the production, functions and implications of H_2S in the context of gut microbiota metabolism is crucial for appreciating its role in health and disease.

1.2.5.1 Hydrogen sulfide (H_2S)

The gaseous molecule H_2S is produced in the gut through microbial fermentation of sulfur-containing compounds (Blachier et al., 2010). It has been recognized at first as a toxic and colorless gas with a characteristic odour of rotten eggs, able to disrupt lung and brain functions (Grieshaber & Völke, 1998; R. A. Nicholson et al., 1998). Described as the third most prevalent natural gaseous molecule, H_2S follows nitric oxide (NO) and carbon monoxide (CO) in prominence (Yong et al., 2010). The common existing forms of H_2S in mammal is gaseous or dissolved. It has been involved in several physiological and pathological processes (Szabõ, 2007). However, H_2S can be also produced by endogenous pathways (Yong et al., 2010). It is found at millimolar concentrations in human colonic lumen, while unbound sulfide, in the same district, is usually at micromolar concentrations due to fecal components capacity to bind it (Blachier et al., 2010). At low concentrations, H_2S plays important physiological roles in the gastrointestinal tract. It is involved in mucosal protection, anti-inflammatory effects and cellular signaling (Cirino et al., 2023). At mucosal level, H_2S stimulates mucus production in the gut lining, enhancing the barrier's defence against pathogens and mechanical damage (Motta et al., 2015). Moreover, by modulating immune responses, H_2S can reduce inflammation and support gut homeostasis. For example, it influences cellular inflammatory signaling pathway, such as the nuclear factor-κB pathway in cultured RAW 264.7 macrophages (L. Li et al., 2011). While, in endothelial cells (ECs), H_2S-induced S-sulfhydration and activation of mitogen-activated extracellular signal-regulated kinase 1 (ERK1) are followed by the translocation of phosphorylated ERK1/2 into the nucleus, where it stimulates the activity of Poly(ADP-ribose) polymerase-1, a nuclear protein essential for

DNA damage repair (Zhao et al., 2014). At cellular level, it acts as a signaling molecule involved in varies physiological processes, such as cell cycle, ATP synthesis, intracellular signaling and cell death, as well as pathological processes involved in cancer, including angiogenesis, tumor growth, metastasis and epithelial-mesenchymal transition (EMT) (Khattak et al., 2022). While H_2S has beneficial roles at specific low concentrations (Cirino et al., 2023), high concentrations of H_2S are cytotoxic, impairing mitochondrial function and epithelial integrity, disrupting gut homeostasis and contribute to a disease (Linden, 2014). Key pathological implication includes, epithelial damage, in particular an excess of H_2S inhibits cytochrome c oxidase in mitochondria, reducing ATP production and compromising epithelial cell viability (Cirino et al., 2023; Cooper & Brown, 2008). This can lead to a weakened intestinal barrier and increased permeability. Indeed, H_2S exacerbates inflammation by inducing oxidative stress and disrupting tight junctions in the epithelial lining (Bischoff et al., 2014).

1.2.5.1.1 Hydrogen sulfide production pathways

The endogenous production pathway of H_2S in mammals includes enzymatic and non-enzymatic pathways. The enzymatic pathway, primarily use sulfur-containing amino acids like L-cysteine and homocysteine as substrates. In particular, it involves the presence of enzymes including cystathionine beta-synthase (CBS) and cystathionine gamma-lyase **(**CSE), which are localized in the cytoplasm, and 3-mercaptopyruvate sulfurtransferase (3-MST), which is localized both in cytosol and mitochondria (Khattak et al., 2022), while the substrates can be several sulfur-containing amino acids (SAAs) gained from diet

(Magee et al., 2000) (Figure 2).

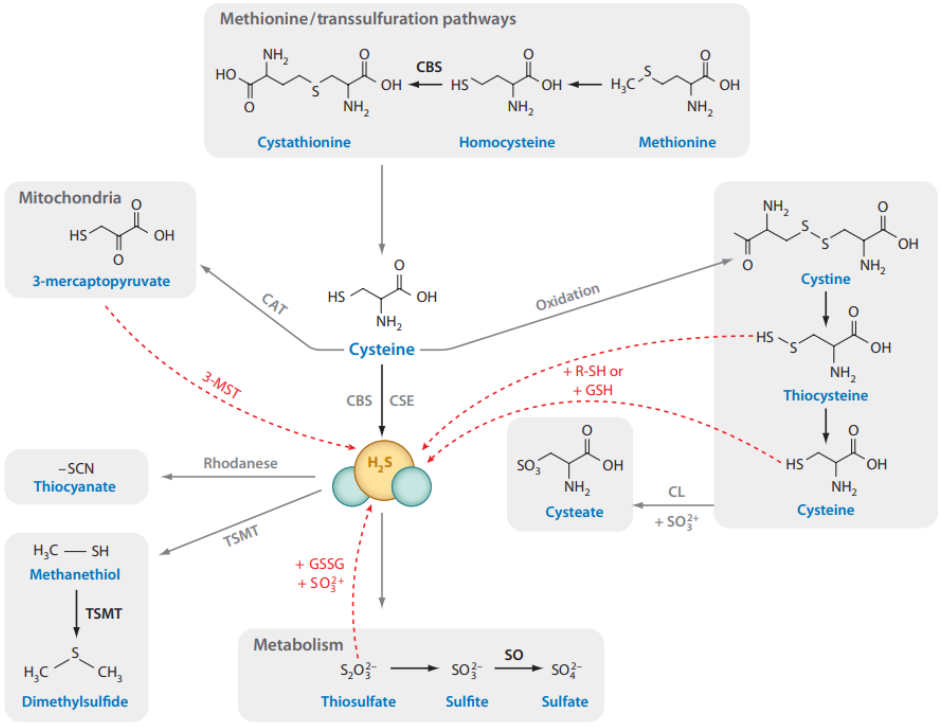


Figure 2: The biosynthesis and degradation of H_2S in mammalian cells (L. Li et al., 2011).

By the way, H_2S is not only produced by human cells but also by a wide range of microorgsnisms, particularly in anaerobic environments such as human gut (Blachier et al., 2021). This way of production is the so called non-enzymatic and microbial pathway, in particular the dissimilatory sulfate reduction pathway which is the one carried out by the sulfate-reducing bacteria (SRBs). One of the primary microbial sources of H_2S are the SRBs (Blachier et al., 2021; Linden, 2014). These are anaerobic microorganisms that thrive in environments with low oxygen availability, such as the colon. In particular, the production of H_2S in the gut involves several pathways utilized by gut microbes to metabolize

sulfur-containing compounds (Teigen et al., 2023). These pathways vary depending on the microbial species and the sulfur sources available in the diet or the host environment (Linden, 2014). Dietary sources, such as sulfate, cysteine, and taurine, are metabolized by SRBs like *Desulfovibrio* and *Desulfobacter*. These bacteria utilize sulfate as the terminal electron acceptor in anaerobic respiration. In particular, sulfate is activated to adenosine-5'-phosphosulfate (APS) by the enzyme sulfate adenylyltransferase. APS is then reduced to sulfite by APS reductase and sulfite is further reduced to H_2S by the enzyme sulfite reductase (Linden, 2014) (Figure 3). This process is highly energy-efficient for SRBs under anaerobic conditions and contributes significantly to H_2S production in the gut when dietary sulfate, derived from sulphated polysaccharides processed foods, or water, is abundant (Linden, 2014).

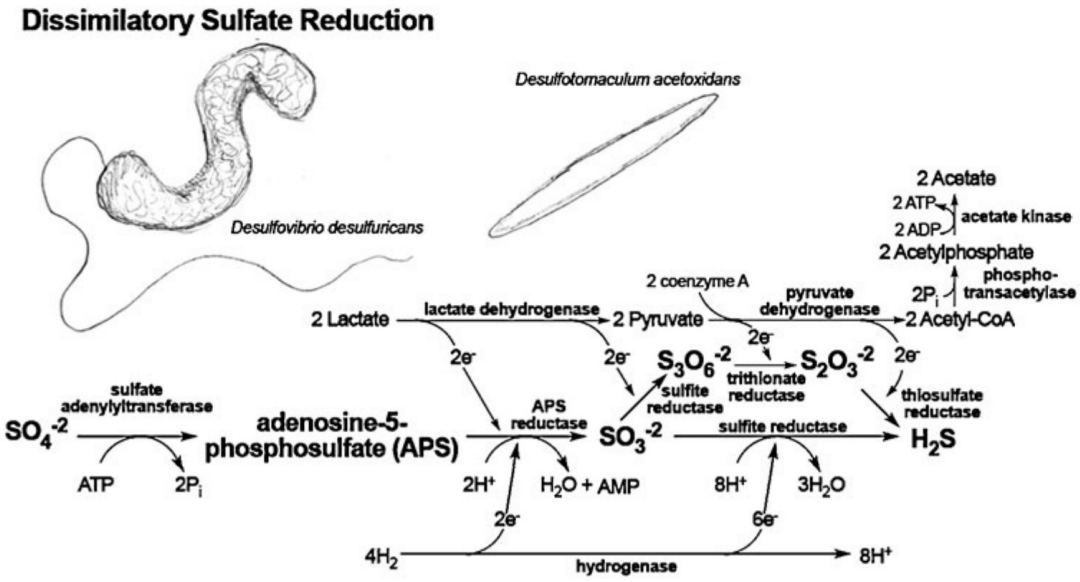


Figure 3: Bacterial dissimilatory sulfate reduction pathway for H_2S production (Linden, 2014).

In addition to SRBs, other gut microbes, including *Clostrdia* and *Escherichia coli*, can contribute to H_2S production via enzymatic pathways that degrade sulfur-containing amino acids like cysteine and homocysteine (Linden, 2014).This pathway is active in both microbes and host cells, contributing to baseline H_2S levels even in the absence of dietary sulfate. While this production is a natural part of microbial metabolism, the quantity and context of H_2S production are critical for determining its effects on the host (Chan & Wong, 2017; Cirino et al., 2023; Motta et al., 2015; Ribas et al., 2022; Xie et al., 2024). Several factors influence H_2S production, including diet (Silver et al., 2021; Teigen et al., 2023), microbiota composition, and gut environment. In particular, dietary interventions have usually the aim to reduce dietary sulfur sources, such as processed meats and sulfur-rich foods while increasing fibres intake which supports beneficial microbes that outcompete SRBs (Dordević et al., 2021; Teigen et al., 2023).

1.2.6 Gut microbiota modifiers

Gut microbiota composition and functionality are influenced by various internal and external factors, including drugs, physical activity, age, sex, stress, and dietary habits (D'Alessandro et al., 2020; Donati Zeppa et al., 2023; Fröhlich et al., 2016; Jašarević et al., 2016; X. Liu et al., 2023; Porras et al., 2018; Strasser et al., 2021). Understanding these influences provides valuable insight into strategies for maintaining or restoring a healthy gut microbiome. These factors often interact, pouring out their effects on the gut microbiota. For instance, stress combined with poor dietary habits may exacerbate dysbiosis, while exercise can mitigate some of the adverse effects of drugs. The dynamic nature

of the microbiota underscores the importance of a holistic approach to maintaining gut health.

1.2.6.1 Drugs

Drugs, in particular antibiotics as previous mentioned, are among the most potent modifiers of the gut microbiota. Antibiotics can reduce microbial diversity by eliminating both pathogenic and beneficial bacteria (Fröhlich et al., 2016) and these alterations also affect glioma growth (D'Alessandro et al., 2020). Chronic or inappropriate use may lead to dysbiosis, where the balance of microbial populations is disrupted. Other drugs, such as proton pump inhibitors (PPIs) (Jackson et al., 2016), non-steroidal anti-inflammatory drugs (NSAIDs) (Utzeri & Usai, 2017), and antidiabetic drugs like metformin (Vallianou et al., 2019), also alter the microbiota. For instance, PPIs increase the prevalence of bacteria typically found in the oral cavity within the gut, potentially contributing to gastrointestinal disorders (X. Xiao et al., 2024).

1.2.6.2 Physical activity

It is widely known that physical activity exerts a significant influence on human well-being through the release of endorphin. Furthermore, exercise also has a significant role in modifying the gut microbiota composition and regulating homeostasis and energy (Monda et al., 2017). However, the way physical activity modulates the intestinal microbiota depends on several factors such as training intensity, environment, and diet. Indeed, regular physical activity, associated with ketogenic diet, is related to an increase in microbial diversity

and higher levels of beneficial bacteria such as *Akkermansia muciniphila* and *Parabacteroides,* which are linked to improved metabolic and immune health (Campaniello et al., 2022). Conversely, a sedentary lifestyle may influence the transient evacuation time in gastrointestinal tract leading to a lower contact time between pathogens and the gastrointestinal mucus layer (Campaniello et al., 2022). Moreover, physical activity seems to be effective in reducing colon cancer development, diverticulosis, and IBD (Monda et al., 2017).

1.2.6.3 Age

The composition of the gut microbiota changes throughout the human lifespan. In particular, dynamic periods of life, such as infancy, puberty and aging, shows imbalances and high variability in microbiota composition which correlate with a high risk of develop specific age- and sex- related diseases (Jašarević et al., 2016). In infancy, the microbiota is shaped by factors such as mode of delivery (vaginal birth or caesarean section) (Blustein et al., 2013) and feeding (breast milk or formula) (Sela et al., 2008). After an early life colonization, the gut microbiome develops along childhood and puberty (Palmer et al., 2007). In this period bacterial communities are more complex and instable compared to adult ones (Hollister et al., 2015). Moreover, since during childhood gonadal hormone is quiescent, there are no sex differences in gut microbiome composition in this period (Ober et al., 2008). In adulthood, the microbiota remains relatively stable but, as previous mentioned, can be influenced by diet, lifestyle, and drugs. In older age, microbial diversity typically declines, with an increase in pro-inflammatory bacteria and a decrease in beneficial species, contributing to age-related diseases and frailty (Jašarević et al., 2016) (Figure 4).

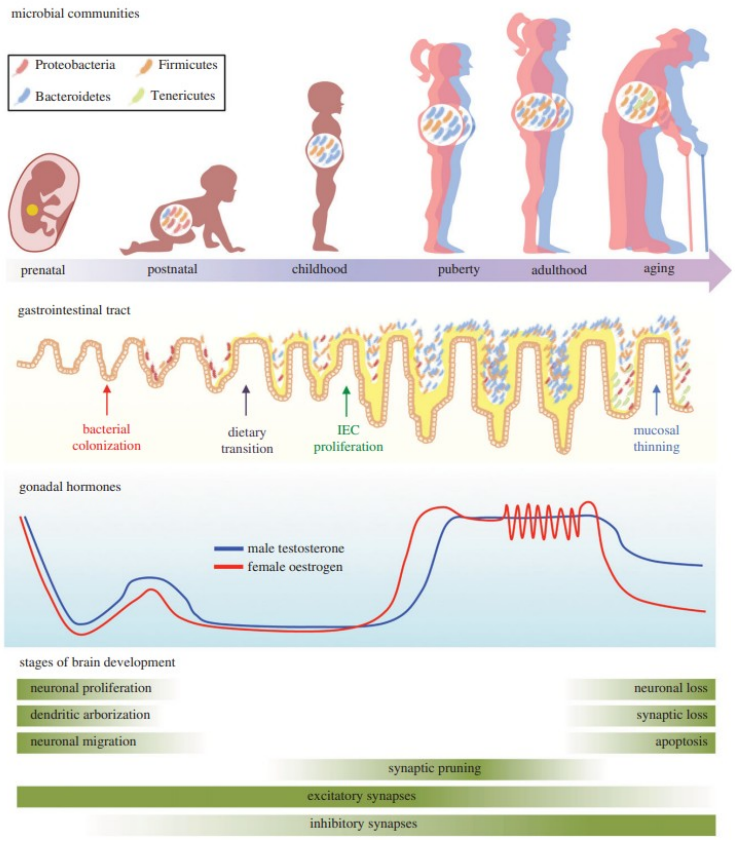


Figure 4: Microbiota modifications during lifespan (Jašarević et al., 2016).

1.2.6.4 Sex

Gut microbiota sex-related differences are well-documented across various biological fields (Ober et al., 2008). At the gastrointestinal level, males and females exhibit distinct gut microbiome compositions, which emerge at the onset of puberty (Calcaterra et al., 2022) and are influenced by multiple factors, including environment, genetics, and sex-hormones (Gomez et al., 2014; Markle et al., 2013). Preclinical studies in mice confirm that these sex-based differences arise in the post-pubertal stage and are absent before puberty (He, S. et al, 2021;

Yurkovetskiy, L. et al, 2013). Notably, several mechanisms have been proposed to explain how the gut microbiota influences sex hormone levels, including interactions with the immune system, microbial metabolites, the GBA, and chronic inflammation. At cellular level, it has been observed that progesterone promotes *Bacteroides species* and *Prevotella intermedia* growth (He et al., 2021; Kornman & Loesche, 1982). Moreover, Recently, preclinical studies demonstrated that male mice microbiota transplantation in female mice reduced the onset and severity of disease (Markle et al., 2013). However, this influence is bidirectional. Indeed, the microbiota and its metabolites play a crucial role in every stage of female fertility, pregnancy, embryo development, and the timing of delivery. By colonizing the vaginal tract, and even the endometrium placenta, the microbiota can influence reproductive health. Alterations in microbial composition have been linked to increased secretion of proinflammatory cytokines and a higher risk of preterm birth (Fettweis et al., 2019). Additionally, neonates delivered via caesarean section tend to have lower intestinal bacterial diversity, likely due to the absence of maternal microbial transfer that typically occurs during vaginal birth (Jašarević et al., 2016).

1.2.6.5 Stress

There are many shapes of stress: environmental (noise, toxicants), physical (sleep deprivation, nutrition, excessive exercise) and psychological (chronic anxiety, fear) (Gubert et al., 2020). In particular, psychological stress significantly impact the gut microbiota through the GBA, a bidirectional communication pathway between the CNS and the gastrointestinal tract, which will be explained in more depth in the next chapter. Stress alters gut motility,

increases intestinal permeability, and changes microbial composition by rapidly decrease microbial diversity and encourage the development of specific bacterial taxa (Ait-Belgnaoui et al., 2012; Gubert et al., 2020). For example, endocrine factors, such as elevated levels of cortisol can lead to gut dysbiosis and is associated to stress and depression (de Punder & Pruimboom, 2015). A stressful condition can increase intestinal barrier permeability and therefore the possible spread of bacteria and toxins across the intestinal lumen into the blood circulation (de Punder & Pruimboom, 2015). A preclinical study on germ-free mice highlight that a mild restraint stress elicits an intensified release of specific hormones, such as corticosterone and adreno-corticotropic hormone, compared to control mice, suggesting a crucial role of microbiota in the hypothalamic-pituitary-adrenal (HPA) axis development and stress response (Gubert et al., 2020). The HPA axis regulates the intestinal permeability through the release of glucocorticoids that potentiate catecholamines action (de Punder & Pruimboom, 2015). Chronic stress is also associated with a decrease in beneficial bacteria such as *Lactobacillus* (Zheng et al., 2013) and *Bifidobacterium* (Burokas et al., 2017) and, as previous mentioned, an increase in potentially pathogenic species. All these changes may exacerbate conditions such as IBD (Wlodarska et al., 2015).

1.2.6.6 Diets

Diets are one of the most powerful and modifiable factors affecting the gut microbiota (Bibbò et al., 2016). As a result, nutrient intake is essential not only for our survival and well-being but also for shaping the symbiotic microbial communities residing in the intestine. Indeed, the factors through food can

influence gut microbiota composition are quality and type. As a matter of fact, gut microbiota can be compared to a fingerprint, as it is different from individual to individual and diet is responsible for at least 20% of this variability in humans (Leeming et al., 2019). A high-fiber diet promotes the growth of beneficial bacteria that produce SCFAs (Simpson & Campbell, 2015), such as *Butyrate* and *Propionate,* which support intestinal health and immune function. Conversely, diets high in saturated fats, refined sugars, and processed foods can lead to a decrease in microbial diversity, associated to an increase in pro-inflammatory species, gut barrier disfunction and a leaky gut condition, all of which contribute to the development of an inflammatory state (Agus et al., 2016; Crawford et al., 2019; Malesza et al., 2021). Moreover, a protein diet, in particular a high-protein diet, can significantly influence the composition and functionality of the gut microbiota. Proteins consumed in the diet are primarily digested and absorbed in the small intestine, but a portion escapes digestion and reaches the colon, where it is fermented by the gut microbiota (David et al., 2014). The effects of such a diet on the microbiota depend on factors such as the protein source, overall dietary composition, and individual microbial diversity. In preclinical research, standard chow diet for mice is composed by plant-derived ingredients, such as legumens, cereals, and other vegetal by-products. The most commonly adopted formulation provides approximately 18% protein, with widely used commercial standard chow diets supplied by manufacturers including Mucedola Srl., Envigo, and LabDiet (www.mucedola.eu/en/standard-diets/; www.inotiv.com/rodent-natural-ingredient-diets; www.LabDiet.com/diets/diet-detail/RODENT). Standard chow diets are widely used for animal maintenance, but their variable composition may affect experimental outcomes. In contrast, purified and customized diets provide consistent nutrient content and can be tailored

to model specific phenotypes or disease conditions. For instance, a protein diet composed by 25% of red meat (beef powder) was used to study a mouse model of colitis, since it often alters the balance of bacterial species in the gut (D. P. Li et al., 2021). We therefore decided to adopt, also in our experiments, a diet containing a proportion of animal-derived proteins that falls within the range typically found in standard laboratory diets since it tends to modify the abundance of bacteria involved in protein fermentation, such as members of the genera *Bacteroides*, and *Alistipes* compared to a standard diet made by 20% of casein (control) (D. P. Li et al., 2021, figure 5).

When undigested proteins reach the colon, bacteria ferment them into a range of metabolites. Some of them have positive effects, such as SCFAs which support intestinal health by maintaining the integrity of the gut barrier and reducing inflammation, and other, such as H_2S at low concentration, which has a cyto- and neuro-protective effects (Chan & Wong, 2017; Cirino et al., 2023; Han et al., 2017; Motta et al., 2015; Xie et al., 2024). While, other metabolites are harmful, such as ammonia (NH_3), biogenic amines (histamine), phenols and indoles, nitrosamines and heterocyclic amines, and very high levels of H_2S (Chan & Wong, 2017; Ribas et al., 2022; Windey et al., 2012). High levels of these metabolites are linked to negative outcomes, including epithelial damage, increased gut permeability, and an elevated risk of diseases such as colorectal cancer (Giuli et al., 2023; Yue et al., 2023). According to all these studies, moderate protein consumption is essential for health and balancing protein intake with dietary fibres is crucial to promoting a healthy and diverse microbiota and mitigating potential negative effects.

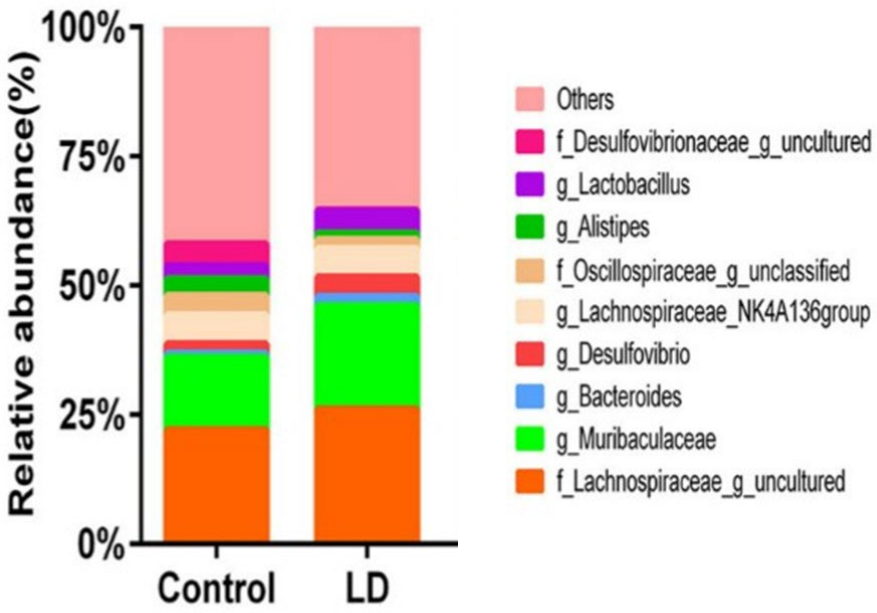


Figure 5. Alteration of gut microbiota in mice fed red meat: the genus level of composition and comparison between control diet containing casein (Control, 200g/kg) and red meat diet containing beaf (LD, 250g/kg) (figure modify by D. P. Li et al., 2021).

1.3 Gut-Brain Axis

From a traditional point of view, the gastrointestinal system and the brain have been viewed as distinct and independent entities. With the growing interest and the advancements in our understanding of the gut microbiota, it is now recognized as a key factor in both physiological health and disease conditions. Numerous psychiatric and neurodegenerative disorders have been linked to gut microbiota dysbiosis and this intrinsic connection between gut microbiota and the brain is now known as the GBA. The GBA is a complex and dynamic communication network between gastrointestinal system and CNS (Helmink et

al., 2019; Rutsch et al., 2020). This axis involves four different bidirectional pathways for communication: (I) vagus nerve (VN), (II) immune system, (III) gastrointestinal barrier, and (IV) blood stream (Aljarrah et al., 2024; Mittal et al., 2017; Verduci et al., 2020). Last route includes neural, hormonal, immune, and microbial molecules, which together influence both gut and brain health. Understanding the mechanisms and implications of the GBA has become a crucial point of research, as it is involved in many psychiatric pathologies, such as autism, neurodegenerative disorders and brain cancers that until recently were believed to be linked only to the brain (Mayer et al., 2022) (Figure 6).

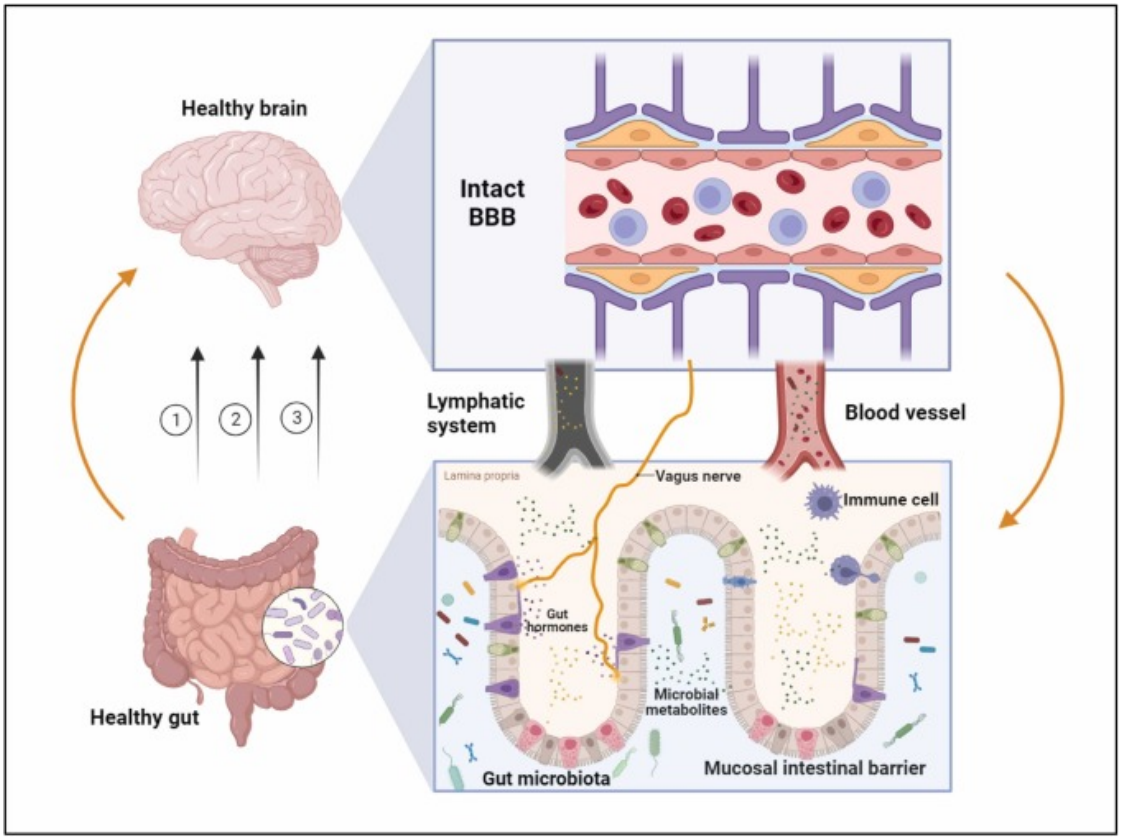


Figure 6: Bidirectional communication between brain and gut: the so-called GBA. Three way of communication: (I) blood circulation, (II) the vagus nerve, and (III) lymphatic system (immunity) (Aljarrah et al., 2024)

1.3.1 Pathways for communication: vagus nerve, immune system, gastrointestinal barrier, and blood stream

As mentioned before, brain and gut communicate in a bi-directional way through several pathways which are: VN, immune system, gastrointestinal barrier, and blood stream. In particular, the VN is the primary route for neural communication between the two systems. It contains numerous fibers that are afferent (80%) and efferent (20%) and they are distributed in the intestinal wall but not direct communicate with gut microbiota. VN transmits sensory signals from the gut to the brain and motor signals back to the gut (Shaffer et al., 2023). Indeed, study that use vagotomy in mice confirm the participation of VN to the GBA communication (Bravo et al., 2011; Reyes-Martínez et al., 2023). Gut microbiota produces various metabolites that can enter the bloodstream and travel to the brain. These include: SCFAs, neuroactive compounds (such as GABA, serotonin precursors, and dopamine-like molecules), and lipopolysaccharide (LPS) (Dehhaghi et al., 2020). Once absorbed in the gut, these substances enter the portal vein, pass through the liver, and then circulate in the systemic blood flow, where they can reach the BBB. Once the brain detects these circulating signals, they can influence: neuroinflammation, neurotransmitter production, neuronal signaling and plasticity, behavior, and mood (including anxiety, depression, and stress response) (Bassi et al., 2012). If gastrointestinal barrier is impaired, products such as LPS spread in the blood stream through leaky gut (Y. Liu et al., 2012; Reyes-Martínez et al., 2023). This phenomenon leads to a systemic inflammation that is believed to affect brain processes through cytokines that cross the BBB. Moreover, systemic inflammation impairs BBB, increasing its permeability to immune molecules

(Reyes-Martínez et al., 2023; Vitkovic et al., 2000). As concern the immune system, the activation of the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway is one of the main mechanisms involved in immune cells and inflammatory signals which stimulate brain resident immune cells (microglia, macrophages, and astrocytes) modifying neurotransmitters production (Lyu et al., 2022). In particular, it is involved in the activation of pro-inflammatory cytochine such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α).

1.3.2 Gut-Brain axis in health and disease

The GBA contributes to the regulation of mood, cognition, and gastrointestinal function. Dysregulation of this axis has been implicated in several conditions such as mental health disorders, neurodegenerative diseases, gastrointestinal disorders, and tumors (Schächtle & Rosshart, 2021). Altered gut microbiota has been also linked to depression, anxiety, and autism spectrum disorders (Schächtle & Rosshart, 2021). Probiotics and dietary interventions targeting the microbiota show promise in improving these conditions (Chudzik et al., 2021). As concern neurodegenerative diseases, it is now well known that dysbiosis and gut inflammation are emerging as contributors to diseases like Parkinson's and Alzheimer's, potentially via neuroinflammatory pathways (Schächtle & Rosshart, 2021). Moreover, conditions such as irritable bowel syndrome (Hillestad et al., 2022) are closely associated with stress and alterations in the GBA, highlighting the need for integrated therapeutic approaches. As mentioned before, disruptions in gut microbiota can strike host metabolism, wellness and immune response allowing the development of a wide range of

diseases, including melanoma, gliomas, colorectal, lung, and bladder cancer (Dehhaghi et al., 2020; D. P. Li et al., 2021), stressing the importance of holistic strategies to support its function. Continued research into the GBA will detect new opportunities for treating conditions that span the gut and brain, revolutionizing our understanding of human health.

1.3.2.1 Gut microbiota and Glioblastoma

It is widely recognized that bidirectional interactions exist between the gut microbiota and the brain (Schächtle & Rosshart, 2021), influencing metabolic and immune functions, neurogenesis, and brain development (Mehrian-Shai et al., 2019). Microbiota plays a key role in brain homeostasis. Indeed, as previous mentioned, the gut microbiota plays a crucial role in maintaining host homeostasis by regulating digestion, immune function, metabolism, and even neural signaling via the GBA. Disruptions to this microbial balance are increasingly implicated in a range of diseases, including brain cancer. Indeed, microbes regulate immune system both in healthy and brain tumor condition (D'Alessandro et al., 2020; D'alessandro et al., 2021; Rosito et al., 2024, 2025). About 20% of all cancers may also be induced by the oncogenic or oncolytic activity of microorganisms, such as human papillomaviruses, *Helicobacter pylori*, and *hepatitis B* and C viruses (Dehhaghi et al., 2020). Nevertheless, the possible association between gut microbiota and brain cancer is a new topic. Indeed, this emerging field offers novel insights into how microbial populations may influence tumor biology, treatment, responses, and overall patient outcomes. As a matter of fact, recent research has begun to explore the relationship between the gut microbiota and glioblastoma (D'Alessandro et al., 2020; Rosito

et al., 2024). In particular, it has been shown that chronic intake of antibiotics promotes glioma growth and influence treatment towards glioblastoma (D'Alessandro et al., 2020; Lyu et al., 2022; Rosito et al., 2024). Moreover, there is also evidence that glioma induces dysbiosis reducing the *Firmicutes:Bacteroidetes* ratio and increasing *Verrucomicrobia* relative abundance (Patrizz et al., 2020). Furthermore, gut bacteria have a significant influence on neurotransmitters production, such as serotonin (5-HT), and dopamine (DA) (Dicks, 2022). As concern glioblastoma, it is characterized by rapid growth, resistance to therapy, and high levels of heterogeneity within the tumor microenvironment. The immune system's interaction with glioblastoma is crucial for tumor's progression and treatment resistance. Gut microbiota can influence immune responses both locally and systematically (Dehhaghi et al., 2020). For example, certain commensal bacteria enhance the activity of dendritic cells, T cells, and natural killer cells, which are critical for anti-tumor immunity (Campbell et al., 2020). Moreover, the GBA provides a direct line of communication between the gut microbiota and the CNS. Microbial metabolites and signaling molecules, such as SCFAs, tryptophan derivatives, and microbial-associated molecular patterns, can cross the BBB or influence its integrity (Lyu et al., 2022). These molecules may affect glioblastoma by altering the tumor microenvironment, neural signaling pathways such as NF-kB, and immune cell infiltration in the CNS (Lyu et al., 2022). It is widely known that different diets modify gut microbiota composition, in particular a high-fat diet (HFD) increases cancer stem cells (CSCs) population *in vivo* in both glioma mouse and patient-derived mouse models inducing severe diseases and reducing survival, compared with low-fat diet (Silver et al., 2021). Of particular note, in this study the reduction of CSCs is associated to a higher H_2S bioavailability. The relationship between the gut microbiota and glioblastoma is a promising area

of research that highlights the link of systemic and local factors in cancer progression and treatment. By modulating inflammation, immune responses, and metabolism, the gut microbiota influences the tumor microenvironment and the efficacy of therapeutic interventions. While significant challenges remain, advancing our understanding of this relationship holds the potential to revolutionize glioblastoma treatment strategies and improve patient outcomes.

1.3.3 Role of vagus nerve activation by gut metabolome

The vagus nerve is the cranial nerve X and a primary component of the parasympathetic nervous system (PNS). It comprises both ascending afferents, which send sensory signals from the gut to the brain via the nucleus tractus solitaries (NTS), and descending efferents, which carry motor signals from the brain to the gut and other organs, thereby regulating digestive processes, inflammation, and immune responses (Shaffer et al., 2023). It is involved in the GBA, acting as a direct communication pathway between the gut microbiota and brain (Longo et al., 2023). Emerging researches have highlighted the significant role of the gut microbiota in modulating vagus nerve activity, and its potential impact on various neurological conditions, including GB (Brem, 2024). Interleukin-6 (IL-6), a keystone cytokine, is well known for promoting angiogenesis, immunosuppression, cell proliferation, and tumor-associated inflammation (Hanahan, 2022). Findings point out the crucial role of vagus nerve stimulation in blocking IL-6 signaling, potentially inhibiting progression of the glioma by keeping tumor cells in a quiescent state (Weissenberger et al., 2004). As previous mentioned, GBA involves neural, hormonal, immune, and

metabolic pathways. In particular, microbial metabolites -such as SCFAs, neurotransmitters, and tryptophan-derivatives- are essential in vagus nerve activation (Goswami et al., 2018). SCFAs like propionate, acetate, and butyrate, produced through fiber fermentation, influence vagal signaling by modulating gut-brain communication and reducing systemic inflammation (Moianu et al., 2023). Chronic inflammation is a hallmark of many cancers, including GB. Through the cholinergic anti-inflammatory pathway, the vagus nerve reduces both systemic and neuroinflammation (Moianu et al., 2023). The use of vagotomy procedure has been shown to increase breast cancer metastasis in mice. Conversely, vagus nerve stimulation is gaining ground as novel treatment for GB, particularly characterized by the reduction of IL-6 which enhances tumor immunity (Brem, 2024). Moreover, vagus nerve activity affects immune cells such as macrophages, regulatory T cells, and dendritic cells, fostering anti-tumor responses (Reijmen et al., 2021). Preclinical studies have shown that the gut microbiota influences liver cancer progression via vagus nerve, through tumor-associated microbiota altering hepatic immune responses and behavior (Bauer et al., 2024). Moreover, recent studies have highlighted how vagus nerve stimulation exerts a neuroprotective effect on the BBB integrity. Indeed, as previous mentioned, vagus nerve stimulation modulates inflammatory responses, regulates endothelial cell function and promote angiogenesis, all of which contribute to the preservation of the BBB integrity (Y. Wang et al., 2024). Neuroinflammation is a key contributor to BBB disruption in various neurological conditions. Microglia activation and the secretion of pro-inflammatory cytokines, IL-1β and TNF-α, may compromise the integrity of BBB, ultimately contributing to neuronal injury (Candelario-Jalil et al., 2022). Vagus nerve stimulation has been shown to attenuate neuroinflammation by inhibiting the activation of pro-inflammatory pathways, such as the NF-κB

pathway, and promoting the release of anti-inflammatory cytokines (Jelinek et al., 2024). Given that vagus nerve stimulation plays a crucial role in maintaining the BBB integrity, that GB significantly impacts the BBB, compromising its integrity and altering its permeability (Mathew-Schmitt et al., 2024), and that the gut microbiota plays a pivotal role in regulating the vagus nerve's antitumor effects (Bauer et al., 2024; Reijmen et al., 2021), enhance BBB function through vagus nerve stimulation may limit GB progression and improve treatment delivery. According to these informations, the interaction between gut microbiota, vagus nerve activation, and GB presents opportunities for novel therapeutic strategies. For instance, probiotics and prebiotics that promote beneficial gut bacteria production may enhance vagus nerve activity and modulate inflammation ameliorating many pathologies such as diabetes, depression, amyotrophic lateral sclerosis, and inflammatory bowel diseases (Franco-Robles et al., 2020). Strains like *Bifidobacterium* and *Lactobacillus* have shown efficacy in promoting anti-inflammatory pathways and supporting immune function in a context of colon inflammation (Franco-Robles et al., 2020). In particular, *Bifidobacterium* has been associated with downregulation of oncogenic pathways, such as the Mitogen-activated protein kinase (MAPK) signaling pathway, which is crucial for cancer cell survival and proliferation (Bekal et al., 2024). In mouse model of glioma, *Bifidobacterium* was found to reduce tumor volume and prolong survival by inhibiting the MAPK/Extracellular signal-regulated kinase (MAPK/ERK) cascade, a key pathway in tumor growth and progression (Fan et al., 2024). Moreover, the combination of *Bifidobacterium* and *Lactobacillus* has demonstrated significant glioma growth inhibition in mouse models, linked to modulation of the Phosphoinositide 3-kinases (PI3K)/AKT pathway and gut microbiota composition (L. Wang et al., 2022). To summarize, the gut microbiota's

influence on vagus nerve activity introduces a novel dimension to GB research. By modulating inflammation, immunity, and the tumor microenvironment, microbiota-vagus nerve interactions may hold significant potential for improving GB treatment. For this purpose, microbiota-targeted therapies and vagal modulation could become key elements in future GB management strategies.

1.3.3.1 Vagus nerve, H_2S, and cancer

The relationship between H_2S and the vagus nerve involves multiple pathways, pointing out their synergistic roles in modulating inflammation, metabolism, and neural communication (Ni et al., 2022). One of the most significant interactions between H_2S and the vagus nerve occurs in the regulation of inflammation in cerebral ischemia. The vagus nerve's anti-inflammatory effects are mediated through the release of acetylcholine, which binds to the alpha-7 nicotinic acetylcholine receptor (α7nAChR) on immune cells, suppressing the production of pro-inflammatory cytokines such as TNF-α and IL-6 (Bonaz et al., 2016; Brem, 2024). In particular, H_2S enhances the vagus nerve's anti-inflammatory functions by upregulating α7nAChR expression and activity. This interaction mediates barberine actions which reduces systemic inflammation and protects against several pathologies such as tumors, cardiovascular diseases, and metabolic syndrome (Ni et al., 2022). Moreover, in the gut, H_2S produced by microbiota or host tissues can stimulate vagal afferents, modulating brain responses to inflammation and brain injury (Ni et al., 2022). Hydrogen sulfide's neuroprotective effects are another critical aspect of its interaction with the vagus nerve. In the CNS, H_2S acts as antioxidant and anti-apoptotic agent, protecting neurons from oxidative stress and

inflammation (Xie et al., 2024). Indeed, the vagus nerve can modulate H_2S production in the brain through its interaction with specific enzymes and receptors. In particular, vagus nerve stimulation (VNS) enhances the activity of the CBS enzyme, which is responsible for H_2S synthesis in the brain (GÖNBE et al., 2022; Kamoun, 2004). This action promotes neuroprotection in conditions such as ischemic stroke, Alzheimer's disease, and Parkinson's disease (Ni et al., 2022; Xie et al., 2024). Additionally, H_2S modulates neurotransmission by enhancing the release of acetylcholine, the primary neurotransmitter of the vagus nerve (Bonaz et al., 2016). H_2S and the vagus nerve jointly mitigate inflammation, reducing disease severity and progression. In diseases such as Alzheimer's and Parkinson's, oxidative stress and neuroinflammation are key pathological features (Ni et al., 2022). The transient receptor potential vanilloid 1 (TRPV1) receptor has a key role in mediating H_2S effects on the vagus nerve. Indeed, H_2S produced by gut microbiota, is able to activate TRPV1 receptors, leading to the transmission of signals to the brain (Kimura et al., 2024; Ni et al., 2022). This GBA communication is essential for regulating various physiological processes, including neuroinflammation and neurotransmitter release. As concern the interplay between vagal nerve and GB, scientific interest has recently focused on VNS to investigate its effects on the tumor microenvironment. One emerging study by Kumaria A. et al. recently discovered that VNS could block glioma growth through glutamine neurotransmission (Kumaria & Ashkan, 2023). Another study proposes that electrical stimulation of VN, through its ability to reduce cytokine levels - particularly IL-6-, could synergize with immune checkpoint inhibitors to transform an immune-resistant ("cold") tumor into an immune-responsive ("hot") one, thereby halting GB progression (Brem, 2024). It also conceptualizes cancer as a form of immune dysautonomia, in which VNS acts as a therapeutic

reset of both systemic and local cytokine environments. According to all these informations, the interplay between VN and H_2S represents a fascinating convergence of biochemical and neural signaling pathways. Together, they modulate inflammation metabolism, and neuroprotection, offering significant therapeutic potential for a range of diseases. In particular electrical or chemical VNS will likely gain traction as a new therapy against GB.

1.4 Glioblastoma and red meat intake

Different diets are known to modify the microbiota differently by changing the proportion and type of bacteria (Beam et al., 2021; Bibbò et al., 2016; Rinninella et al., 2019). In particular, diet rich in protein, derived from red meat, modify microbiota composition. Moreover, at low doses, animal protein recently seems to have positive effects on the organism (Wagemakers et al., 2009) Diet rich in animal proteins, including red meat, is an important source of protein and essential nutrients (iron, zinc, vitamin B12). Formerly, some research has shown that red meat consumption may increase the risk of cardiovascular disease and cancer, such as colorectal, liver, lung, and esophageal cancer (Cross et al., 2007; Kontogianni et al., 2008). However, the correlation between red meat intake and glioblastoma has been explored through various studies, yielding conflicting results. While some research indicates a potential link, particularly with unprocessed red meat (Saneei et al., 2015), other studies find no significant association (Ward et al., 2018). Additionally, a diet rich in red meat and other animal proteins increase the percentage of hydrogen sulfide-producing bacteria in the gut. It has been reported that *Desulfovibrio*, *Bilophila wadsworthia* and *Fusobacterium* are the main species of bacteria produced by the intake of red meat proteins (D. P. Li et al., 2021). In addition, along with *Prevotella*, other

sulfide-producing bacteria include genera like *Fusobacterium*, *Porphyromonas*, *Selenomonas*, *Veillonella, Peptostreptococcus*, and *Eubacterium*. These bacteria are known for their ability to produce H_2S through the breakdown of sulfur-containing amino acids (Shen et al., 2010; Zhu et al., 2016). All these species use the same organic substrates, such as fatty acids (acetate, propionate, and butyrate), amino acids (glutamate, and serine), and organic acids (pyruvate, and lactate) for H_2S production and are therefore competitors of each other (Dordević et al., 2021).

1.4.1 H_2S effects on glioblastoma

Known for its regulatory roles in inflammation, oxidative stress, and cellular signaling, H_2S has garnered attention for its potential impact on cancer biology (MA et al., 2011). As reported in H_2S production pathway paragraph, the gasotransmitter is synthesized endogenously through the enzymatic activity of CBS**,** CSE**,** and 3-MST. Its effects in cancer are context-dependent, acting as both a pro- and anti-tumorigenic agent depending on concentration, cellular environment, and specific cancer type (Linden, 2014). In fact, it is shown a higher expression of CBS in tumor microenvironment, which suggests its pro-tumoral effect in the endogenous system, indeed, higher amount of H_2S and longer time of exposure seem to induce apoptosis in vivo and in vitro in colon cancer cells, but not in noncancerous fibroblast cells, which indicates the two-way of action of H_2S towards cancer (Cao et al., 2019). Such effect is validated in the experiments where NaHS (200μM), administered to the cells accelerates cell proliferation and migration in human colon cancer cell line HCT116 and colon carcinoma cell line SW480. NaHS is commonly used as the analogue of

H_2S since the letter is gaseous and is difficult to apply. As concern its pro-tumorigenic effects, H_2S supports cancer cell metabolism, and so GB progression, by promoting glycolysis and mitochondrial bioenergetics, both crucial for GB survival and rapid cell proliferation (Jin et al., 2020). It activates key enzymes in glycolysis, such as pyruvate kinase M2, thereby supporting the high energetic demands of GB cells (R.-H. Wang et al., 2023). Moreover, H_2S has been shown to have a role in promoting angiogenesis, which is one of the characteristics of cancer when large number of cells are proliferating and vessels are greatly needed to provide oxygen and nutrients to the cancer mass. In fact, H_2S stimulates endothelial cell proliferation and migration by enhancing vascular endothelial growth factor (VEGF) production, ultimately facilitating the development of abnormal and leaky blood vessels in GB (Cai et al., 2007; Papapetropoulos et al., 2009; S. Zhang et al., 2024). On the contrary, some findings suggest an anti-tumoral role for H_2S in GB models. Specifically, CSE synthesis inhibition -particularly via CSE blockade with propargylglycine (PAG)- was shown to increase tumor proliferation *in vivo*, suggesting that physiological levels of H_2S may exert tumor-suppressive effects in certain GB contexts (Silver et al., 2021). Furthermore, inhibition of H_2S synthesis, particularly inhibiting CSE production (Cao et al., 2019), has been associated with reduced angiogenesis, highlighting a potential therapeutic approach for this type of tumor (Cai et al., 2007; Papapetropoulos et al., 2009). Moreover, the gasotransmitter, at non-physiological concentrations, induces oxidative stress, by removing reactive oxygen species (ROS) and upregulating antioxidant defenses, and apoptosis in different types of cancer cells. Including GB cells, and can impair mitochondrial respiration, leading to energy crisis and cell death (Papapetropoulos et al., 2009; R.-H. Wang et al., 2023; A. Y. Xiao et al., 2019). The immunosuppressive nature of the GB microenvironment is a major barrier

to effective therapy. H_2S impacts immune cell functions in particular regulation of macrophages and inhibition of T-Cell activation. As concern the regulation of macrophages, H_2S moves macrophage polarization towards an M2 phenotype, which supports tumor growth and suppresses immune responses. Moreover, H_2S interferes with T-cell proliferation and cytokine production, contributing to immune evasion by GB cells. On the other hand, an observational study found that people who consume a diet high in animal protein and fat have changes in the composition of their microbiota, particularly an increase in common hydrogen sulfide (H_2S) producing bacteria (D. P. Li et al., 2021; Silver et al., 2021). This metabolite appears to counteract growth of different types of tumors in both in vitro and in vivo models, including GB (Silver et al., 2021).

2. Aim of the research

Lifestyle and environmental factors are known to influence tumor growth, with dietary habits playing a role in cancer development. However, the impact of red meat consumption on GB risk and progression remains unclear. A meta-analysis of 18 observational studies found a modest positive association between unprocessed red meat intake and glioma risk, primarily based on case-control studies. Notably, this association was not observed in cohort studies, and no dose-response relationship was established, indicating the need for further research to clarify these findings (Saneei et al., 2015). Red meat consumption induces changes in microbiota composition increasing H_2S production, possibly through the increased level of SRBs. H_2S has been

implicated in local conditions such as colon cancer and inflammation. However, as a lipophilic gasotransmitter, it can cross the intestinal and brain barriers, and its effects can be transferred via the GBA (vagus nerve) and influence brain function. Notably, recent studies have shown that H_2S acts as a suppressor of GB in both cell cultures and in vivo models. The aim of the research is to investigate the effect of animal protein diet in GB murine model, in particular focusing on the role of H_2S on GB progress. Moreover, taking in to account that diets are strong microbiota modifiers, we evaluated how a diet based on red meat protein changes the microbial profile and how these modifications could alter the tumor microenvironment. For these purposes in vitro and in vivo experiments were performed.

3. Materials and methods

3.1 Mice housing and treatment

The experiments reported in this study received approval from the Italian Ministry of Health (authorization n. 775/2020-PR and n. 172/2025-PR) in accordance with the guidelines on the ethical use of animals from the European Community Council Directive of September 22, 2010 (2010/63/EU), and from the Italian D.Lgs 26/2014. For our purpose we used C57BL6/N male mice supplied by Charles River Laboratories. The animals of 3 weeks old were housed (3-4 animals per cage) under a 12h light/dark cycle in Individually ventilated cage (IVC) system cages at constant temperature and relative humidity (50%) in autoclaved bedding and drinking water, and with sterilized standard chow diet ad libitum until the start of treatments. Mice were randomly assigned to the different experimental groups: animal-derived protein (ADP) diet, ADP diet

intrarectally treated with vehicle, AP diet, AP diet orally treated with AOAA, AP diet intrarectally treated with CPZ or vehicle. All the experiments in this study were conducted in accordance with the ARRIVE guidelines (Kilkenny et al., 2010), and were approved by the local animal welfare body and by the Italian Ministry of Health (authorization No. 231/2015PR) in accordance with the EC Council Directive 2010/63/EU and the Italian d.lgs.26/2014. All the efforts were done to minimize animal suffering, and to reduce the number of animals, calculating the necessary sample size before starting the experiments.

3.2 Diet composition

The diets that we used for our experiments were customized by Mucedola Srl (Italy) according to our needs. Briefly, the diets are isocaloric. The animal-protein diet is composed by 215 g/kg (17,50%) of bovine meat flour, while ADP diet holds 200 g/kg (17,50%) of casein. Body weight is constantly monitored throughout the experimental setup to confirm isocaloric state. The compositions are reported in Table 1.

	Animal-derived protein	Animal protein
Ingredient	**g/Kg**	**g/Kg**
Casein	**200**	0
Bovine meat flour	0	**215**
Corn starch	395	363
Maltodextrin	132	132
Sucrose	100	100
Cellulose	50	70
Soybean oil	70	67
Vitamin mix	15	15
Mineral mix	35	35
L-Cystine	3	3
t-Butylhydroquinone	0.014	0.014
Total	1000.014	1000.014
Gross Energy (kCal/Kg)	4600	4700
Protein (%)	**17.50**	**17.50**
Fat (%)	**7.00**	**8.50**

Table1: Diets composition and analytical components. Diets are isocaloric (around 4767 kCal/Kg), but differs one each other for the main protein, casein (200 g/kg) for ADP diet; bovine meat flour for AP diet (215 g/kg).

3.3 Orthotopic GL261 cell injection

Orthotopic GL261 cell injection Mice were anesthetized with Rompun 20 mg/ml (75 mg/kg i.p.) + Zoletil 50/ 50 mg/ml (20 mg/kg i.p.) and placed in a stereotaxic head frame. Animals were stereo-tactically injected with 7.5×10^4 GL261 cells: a median incision of ~1 cm was made; a burr hole was drilled in the skull and cells were injected in the right striatum (−2 mm lateral and +1 mm anteroposterior from the bregma). A total cell suspension of 4 μL in sterile PBS was injected with a Hamilton syringe at a rate of 1 μL/min at 3 mm depth.

3.4 Mice treatments

3.4.1 Amino-oxyacetic acid treatment

In order to deeply investigate the potential role of H_2S derived from red meat in suppressing tumor development, mice were treated with amino-oxyacetic acid (20mg/kg) in autoclaved water or autoclaved water alone (control solution). AOAA and control solutions were changed every 2 days, and continuously administrated for 2 weeks before and 3 weeks after tumor injection.

3.4.2 Capsazepine treatment

In order to explore the potential involvement of TRPV1-mediated vagus nerve activation by H_2S in regulating glioma progression, mice were treated with capsazepine (CPZ, 15mg/kg) dissolved in Dimethyl Sulfoxide (DMSO) 10%, Polyethylene Glycol (PEG) 300 40%. TWEEN-80 5% and salin 45% or DMSO 10%, PEG 300 40%. TWEEN-80 5% and salin 45% alone (vehicle). Intrarectal CPZ or vehicle administration started 48h after tumor injection to allow the animals to recover. After a fasting period of approximately 4 hours, the mice were anesthetized with gas anesthesia based on isoflurane (2%) and oxygen for approximately 3 minutes. After 3 minutes, mice were placed upside down in a mice restrainer to maintain the vertical position. To correctly expose the anus, the hind legs remain inserted in the tube and the tail is stretched backwards on the resteriner's neck. A 1ml syringe is used to administer the drug, a small

polyethylene microtube with an internal diameter of 0.38mm and an external diameter of 1.09mm is inserted onto the needle. The microtube has a length of approximately 5 cm and is inserted 4 cm inside the anus, the distance necessary to reach the colon in an adult mouse weighing approximately 25g. To facilitate access of the tube into the anus, place a little Vaseline on the end. At the end of the administration, the animal is placed back in the cage waiting for it to wake up. The administrations are daily and the treatment lasts 3 weeks. During the first week the animals received double the amount of CPZ. Then followed two weeks of daily administration of a 1X concentration of the drug.

3.5 Behavioral test: Tail flick test

In order to evaluate if our treatment with CPZ modulated TRPV1 activity, we performed a tail-flick test. As shown in previous studies, modulation of TRP channles by different treatments was confirmed by a loss of sensibility to hot or cold (Joseph et al., 2019; Kimura et al., 2024) The tail-flick test Fare clic o toccare qui per immettere il testo.was performed on mice fed a standard diet after three weeks of daily administration of CPZ or Veh. Briefly, the tip of the tail was immersed in hot water (50°C), and the latency to tail withdrawal was recorded as an indicator of nociceptive response. A cut-off time of 30 seconds was set as the maximum latency allowed for tail withdrawal, after which the test was terminated to ensure animal safety. Each animal performed the test three times at regular intervals of five minutes (protocol modified by Kimura et al).

3.6 Stool collection processing

Fresh stools were collected from the animals before switching diets, before tumor inoculation an at the end of all experiments. Samples were frozen and stored to further analyses. We collected enough stools to perform metabolomic and metagenomic analysis and H_2S extraction and quantification.

3.6.1 Metabolomic and metagenomic analysis

Fecal metabolomic and metagenomic analyses provide complementary insights into gut physiology and host-microbiome interactions. Metabolomic analysis of fecal samples ocuses on the identification and quantification of small-molecule metabolites, such as SCFAs and amino acids, which reflect both microbial activity and host metabolism. Metagenomic analysis, on the other hand, relies on sequencing the collective microbial DNA present in feces, allowing the characterization of microbial diversity, taxonomic composition, and functional gene profiles. For metabolomic analysis, the stool-containing tubes were quickly cryopreserved by direct immersion in liquid nitrogen and then stored at -80°C until use (timeline fig. 8a). Indeed, for metagenomic analysis, the samples were collected in tubes containing InhibitEX buffer (from QIAmp fat DNA stool mini kit) and then processed to bacterial DNA extraction according with the QIAmp fat DNA stool mini kit (QIAGEN) manufacturer's instructions.

3.6.2 H_2S extraction and quantification

From each mouse, three pieces of fresh stool were collected in 1.5 ml Eppendorf tubes holding 200µL of extraction buffer and promptly processed at each time point. Breefly, the eppendorfs were weighed before and after the stool collection to calculate the weight of the stool. Extraction buffer consist of 20mM Tris-HCl (pH 8.0), 10mM ethylenediamine tetraacetic acid (EDTA) and 20mM zinc acetate ($Zn(OAc)_2$). The reaction is based on the use of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), prepared in ethanol at the concentration of 20mM. Samples are vortexed for 20' and afterwards centrifuged at 20,000g at 4°C for additional 20'. Supernatant were collected and used for H_2S measurement through colorimetric assay. In particular, 100µL of the supernatant was added to a 24 multiwell containing 860µl of extraction buffer and 40µl DTNB solution. After 5 minutes of incubation at room temperature, optical density is read with the spectrophotometer at the wavelength of 405nm. The standard curve is built by preparing samples of NaHS, a donor of H_2S, of 100mM, 50mM, 25mM, 12.5mM and 6.25mM respectively in 100µl buffer, then standards are added to a 24 multiwell containing 860µl buffer and 40µl DTNB. Then the linear function is calculated between the given concentration and the OD evaluated at 405nm and the slope value is obtained to calculated the unknow sample in mM (Figure 7) A white control is prepared with 960µl buffer and 40µl DTNB. For the complete protocol: Z. G. Li, 2015.

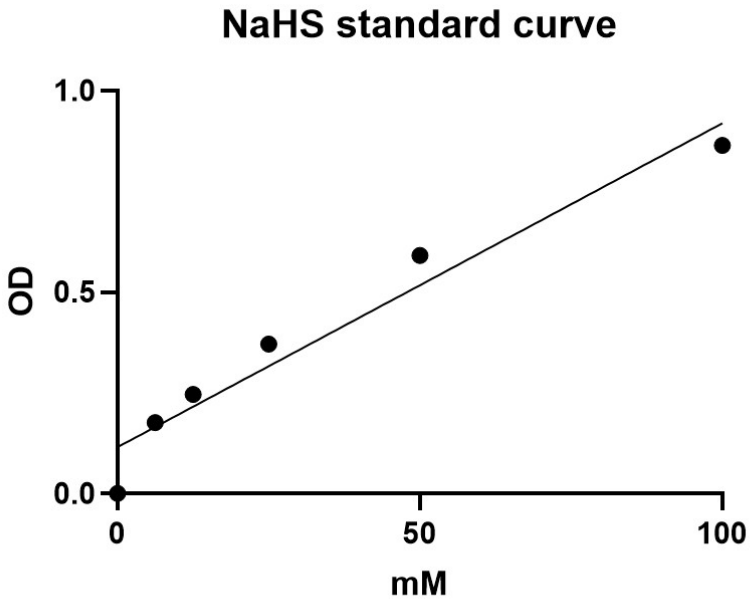


Figure 7. Standard concentration-absorption curve obtained from given concentration of NaHS

3.7 Gut microbiota evaluation

3.7.1 Library preparation and sequencing

Library preparation has been carried out using primer combination Pro341F (CCTACGGGNBGCASCAG) and Pro805R (GACTACNVGGGTATCTAATCC) to amplify the V3-V4 region of 16S rRNA. Subsequently, all samples have been sequenced in 300 paired-end with an Illumina MiSeq platform.

3.7.2 Metagenomic analysis

Data matrices were initially normalized and subsequently standardized using the QuantileTransformer and StandardScaler funtions from the Scikit-learn package (v0.20.3). Normalization with output:distribution='normal' transformed each variable into a Gaussian-like distribution. Standardization then ensured that each normalized variable had a mean of zero and unit variance. These two preprocessing steps allowed for accurate comparisons among variables with differing dynamic rangers, such as bacterial relative abundances. For microbiota analysis, alpha diversity metrics (e.g., Richness and Shannon index) were computes at the species level using Scikit-bio (v0.4.1). Beta diversity was assessed via Bray-Curtis dissimilarity and visualized using Principal Coordinate Analysis (PCoA). To evaluate group-level differences in microbial composition, ANOSIM and PERMANOVA were applied using 999 permutations, also via Scikit-bio. Unsupervised clustering was visualized through PCoA, while hierarchical clustering analysis (HCA) was performed using custom Python (v3.8.2) scripts with the Bray-Curtis metric and complete linkage method. For supervised analysis, Partial Least Squares Discriminant Analysis (PLS-DA) was implemented followed by Variable Importance in Projection (VIP) plots to highlight the most discriminant bacterial species between cohorts. Bar thickness in VIP plots indicated fold ratio of mean relative abundance, and bars without borders represented a zero mean in the compared group. Group comparisons were performed using the Mann-Whitney U test (without False Discovery Rate correction, as sample size was fixed), and Kruskal-Wallis tests were applied for multiple group comparisons. Significance was defined as $p<0.05$. We acknowledge that the statistical power may be limited by the sample size, and future studies with larger animal microbiota

composition. Analyses involving more than two groups were performed via ANOVA followed by Bonferroni-corrected pairwise comparisons.

3.8 Evaluation of the tumoral volume

Twenty-one days after tumor cell inoculation, glioma-bearing mice were sacrificed and brains were isolated, fixed in 4% buffered paraformaldehyde (PFA) and snap frozen. Coronal brain cryosections (20 μm of thickness) were prepared by using cryostat and collected every 100 μm on polarized glasses as the parallel series for immunofluorescence and histological evaluation. The histological difference in density of the tumoral cell and tumor volume was evaluated with hematoxylin–eosin staining, as detailed by the manufacturer. Briefly, hematoxylin stains mainly the nucleus and eosin stains the cytoplasm. After staining, the slides were scanned and analyzed by the ImageJ software (National Institutes of Health, Bethesda, USA). The area of tumor was circled by the border using "freehand" selection function. The number of pixels was counted by the measure function. The ratio of the area of one pixel and the area measured in reality in millimeters was converted, thus makes it possible to know the real area of the tumor on each slice. Tumor volume was calculated according to the formula (volume = $t \times \Sigma A$, where t = thickness and A = tumor area/slice).

3.9 Cell cultures

The Dulbecco's Modified Eagle's medium (DMEM) is completed with 100 U/ml of Penicillin, 100 mg/ml of Streptomycin, and 2,5 μg/ml of Amphotericin B before using. Murine glioma cells GL261 are cultured in DMEM with 20% Fetal Bovine Serum (FBS) in humified incubator in which the temperature is 37°C and the concentration of CO2 is 5%. Murine glioma cells CT2A are cultured under the same conditions but in DMEM with 10% FBS. The cells are passed with the dilution of 1 to 5 every three days. To pass the cells, the medium is discarded and a wash is done with Phosphate-buffered saline solution without Calcium and Magnesium (PBS-/-), after which 1ml of trypsin (gibco) is added and the flask is incubated for one minute. To terminate the digestion, 4ml of DMEM with 20% of FBS is added and cell gently collected.

3.10 Viability assay

GL261 cells and CT2a cells were seeded into 96-well plates ($5x10^3$ cells/ well) and exposed to different concentrations of NaHS (250 – 500 μM and 1 mM) to assess the viability of cells. Cell viability was determined by a colorimetric assay for assessing cell metabolic actibity by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) salt. MTT solution (500 μg/ml) was added to the cells in each well and the plates were incubated for 1.5 h at 37 °C in a 5% CO2 incubator. The resulting formazan crystals were dissolved by adding DMSO. Optical densities (OD) were measured at 570 nm using Tecan Infinite F Nano+ plate reader and processed using Tecan iControl 2.0 software. Cell viability data was expressed relative to the variation in absorbance over

time, by plotting the means of OD value measured minus reference (600 nm) for each condition versus time.

3.11 Immunofluorescence

3.11.1 Immunofluorescence on cells

The immunofluorescence staining was performed on $20x10^3$ GL261 and $40x10^3$ CT2a cells and 10^4 microglia cells seeded on 12 mm coverslips and fixed with 4% PFA solution Then, cells were permeabilized with 0.2% triton in PBS and blocked with Bovine Serum Albumin (BSA) 1% in PBS for 45 min at RT. Cells were then incubated with primary antibodies overnight (anti-Ki-67, Abcam, 1:1000; Alexa-Fluor 488 Phalloidin, Invitrogen, 1:150) in 0,1% of BSA-PBS. Cells were then stained with fluorophore-conjugated secondary Abs and Hoechst for nuclei visualization.

3.11.2 Immunofluorescence on brain slices

Parallel brain cryosections were used for immunofluorescence staining of GFAP and Ki-67. Briefly, sections were incubated with 3% goat serum in 0.3% Triton X-100 for 1 h at RT, and overnight at 4 °C with anti-GFAP (Novus Biologicals,1:500), anti-Ki-67 (Abcam, 1:50) in 1% goat serum, 0.1% Triton X-100. Sections were then stained with fluorophore-conjugated secondary antibodies and Hoechst for nuclei visualization.

3.11.3 Form factor to calculate cells circularity

Microglia were seeded on 12 mm glass coverslips (10^4 cells), treated as reported, fixed, permeabilized, blocked and stained with Alexa-Fluor 488 Phalloidin (Invitrogen) 1:150 for 1 hour together with Hoechst. For the form factor calculation, we used 3 different primary microglial culture preparations, 2 glass coverslips for each different conditions and we counted 20 cells for each glass coverslips randomly selected. Fluorescent images were processed using the MetaMorph 7.6.5.0 software (Molecular Device, Sunnyvale, CA, United States), and form factor was calculated according the formula: 4π area/$(\text{perimeter})^2$ [29]. Form factor is a parameter taken as 1 for round cells, and correspondingly <1 when the morphology deviates from the spherical shape.

3.12 Statistical analysis

Statistical analyses were performed using Student's t-test, one way ANOVA, or two.way ANOVA for parametric data, as specified in the corresponding figure legends. A *p*-value < 0.05 was considered statistically significant. The reported sample size (*n*) corresponds to: (i) the number of independent experiments for *in vivo* analyses, with details on the number of wells or fields of views used (e.g., for MTT viability or proliferation rate, respectively); and (ii) the number of mice used in *in vivo* experiments. Data are presented as mean ± standard error of mean (S.E.M.). All statistical analyses were conducted and converted into graphs by using GraphPad Prism version 8.0.1.

4. Results

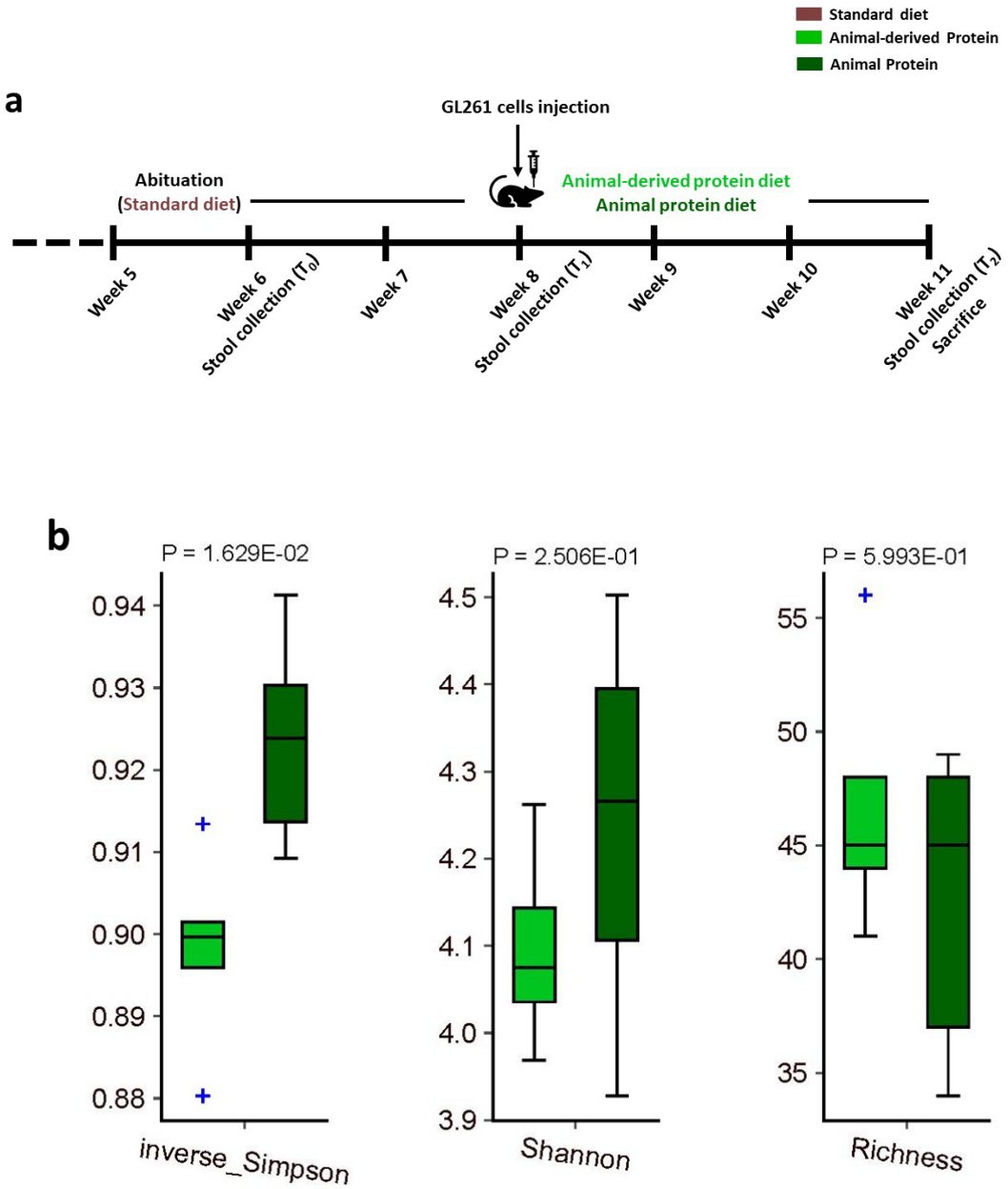
4.1 Animal protein diet modifies gut microbiota increasing fecal H_2S concentration

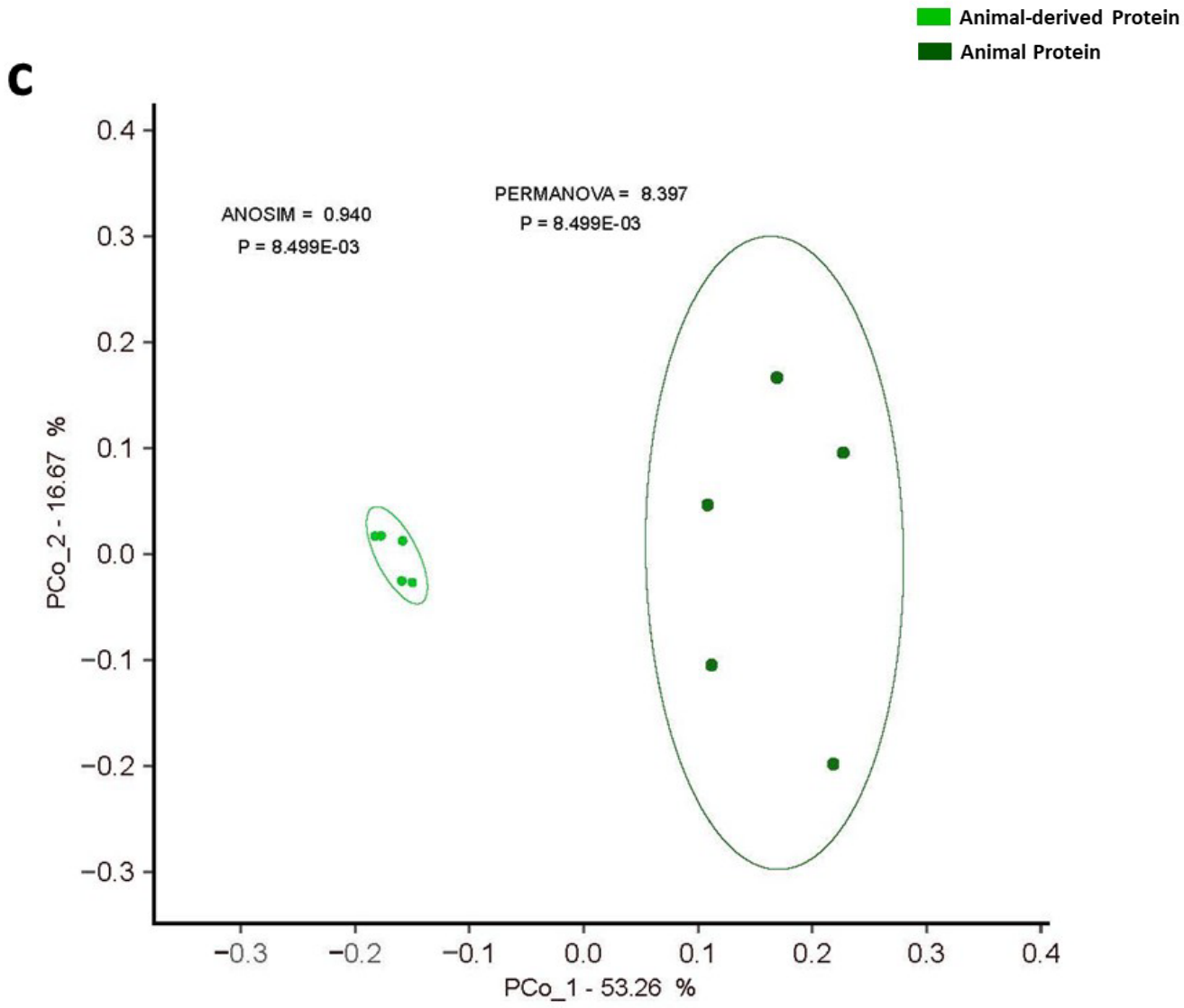
Changes in eating habits alter gut microbiota composition (Rinninella et al., 2019). To investigate if a standard red meat protein intake can affect glioma growth, we formulated two isocaloric diets: the first named ADP (control diet, containing 17.50% of casein) and AP diet (treatment diet, containing 17.50% of protein derived from dried beaf meat). Soon before changing diet, before tumor injection and before sacrifice feces were collected to evaluate gut microbiota composition and H_2S concentration in fecal water (Figure 8a). Firstly, we evaluated whether these diets modify gut microbiota composition. At this aim bacterial DNA from feces were isolated and evaluated for H_2S production at three time points and composition by ribosomal 16sRNA sequencing at the end of the experimental plan (Figure 8a). As shown in figure 8, glioma mice fed AP diet showed different microbiota compositions compared to ADP diet fed mice. In particular, alpha diversity was evaluated using three distinct metrics: inverse Simpson index, Shannon index, and richness (Figure 8b). These indices provide complementary insights into the evenness and richness of the microbial communities within each experimental group. As shown, the inverse Simpson

index (Figure 8b) was significantly increased in the AP mice compared to controls, suggesting a greater dominance balance and evenness in the microbial population following treatment. This indicate that AP diet promoted a more evenly distributed microbial community. In contrast, the Shannon index (Figure 8b), showed a modest, non-significant trend toward increased diversity in the AP mice, reflecting a more nuanced change in diversity that includes both richness and evenness. Similarly, species richness (Figure 8b), did not differ significantly between groups, suggesting that while the number of taxa present remained relatively stable, their relative abundances were redistributed under the treatment condition. An unsupervised ordination plot (Principal Coordinate Analysis, PCoA, Figure 8c) evidenced a clear separation among ADP and AP cohorts, revealing differences in the two microbiota compositions. A further confirmation of the good separation between the ADP and AP groups derives from the unsupervised Hierarchical Clusterization Analysis (HCA, Figure 8d), which evidence two definite clusters by the Fisher's exact test. To possibly identify bacterial species distinctive for ADP and AP cohorts, PLS-DA model and the VIP score were implemented (Figure 8e). At least thirteen species were distinctive for the AP group. Among them three highly enriched species *Alistipes putredinis, Bacteroides acidifaciens, and Bacteroides stercorirosoris* belong to genera producing H_2S such *Alistipes and Bacteroides. In addition, the species Aminipila butyrica*, a strictly anaerobic, is able to produce H_2S from arginine decomposition (Ueki et al., 2018). We also evaluate H_2S concentration present in fecal water by a colorimetric assay specifically described in the materials and methods. AP diet increases fecal H_2S concentration compared with ADP diet (Figure 8f), which confirm our purpose of increasing H_2S bioavailability in the gut.

Altogether, these data indicate that an AP diet significantly modifies gut

microbiota composition and induces an increase in H_2S concentration in feces of these mice compared to those receiving an animal-derived diet.




Animal-derived Protein
Animal Protein
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ANOSIM = 0.940
P = 8.499E-03
PERMANOVA = 8.397
P = 8.499E-03
PCo_2 - 16.67 %
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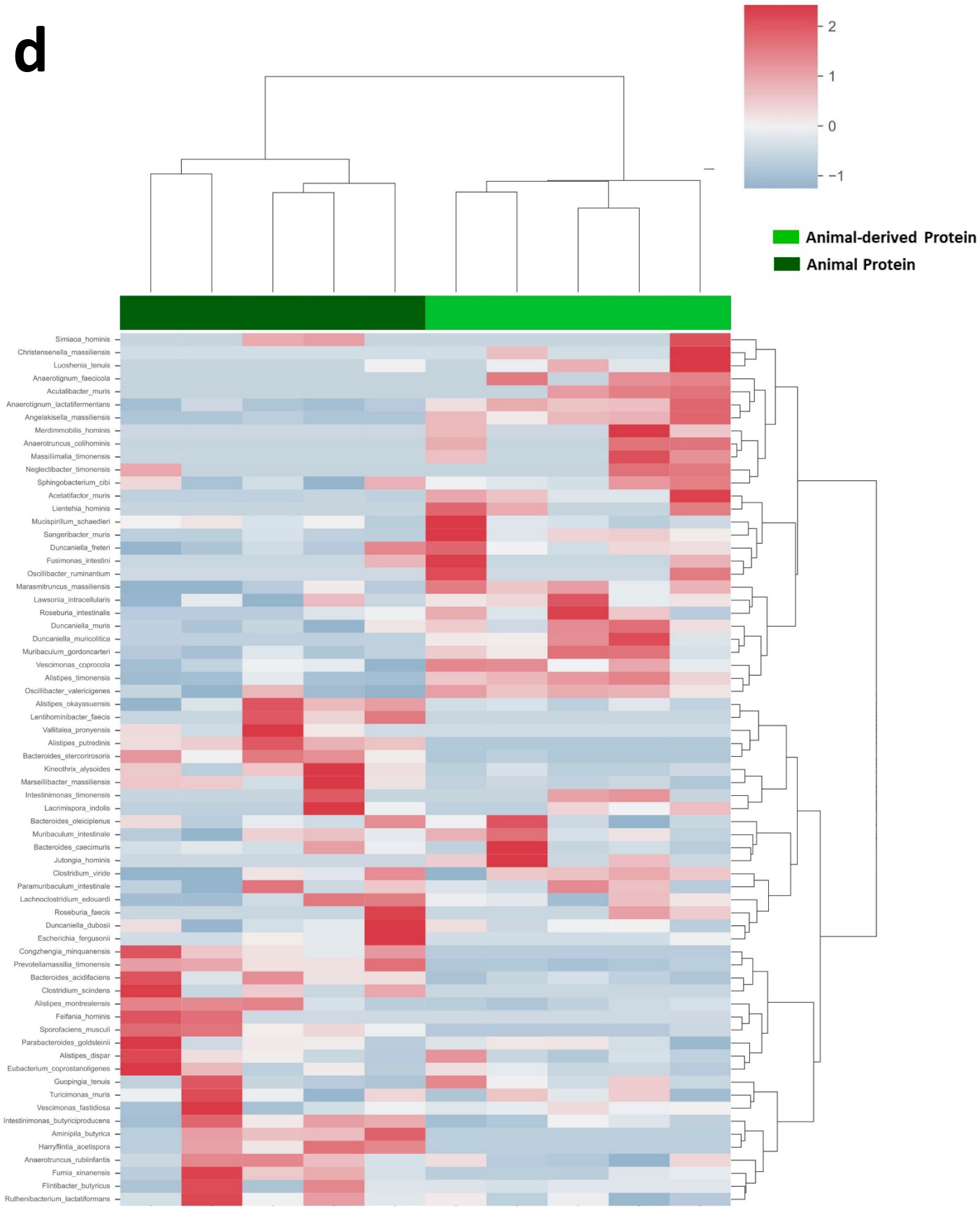
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Animal-derived Protein
Animal Protein
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Merdimmobilis_hominis
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Neglectibacter_timonensis
Sphingobacterium_cibi
Acetatifactor_muris
Lientehia_hominis
Mucispirillum_schaedleri
Sangeribacter_muris
Duncaniella_freteri
Fusimonas_intestini
Oscillibacter_ruminantium
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Lacrimispora_indolis
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Prevotellamassilia_timonensis
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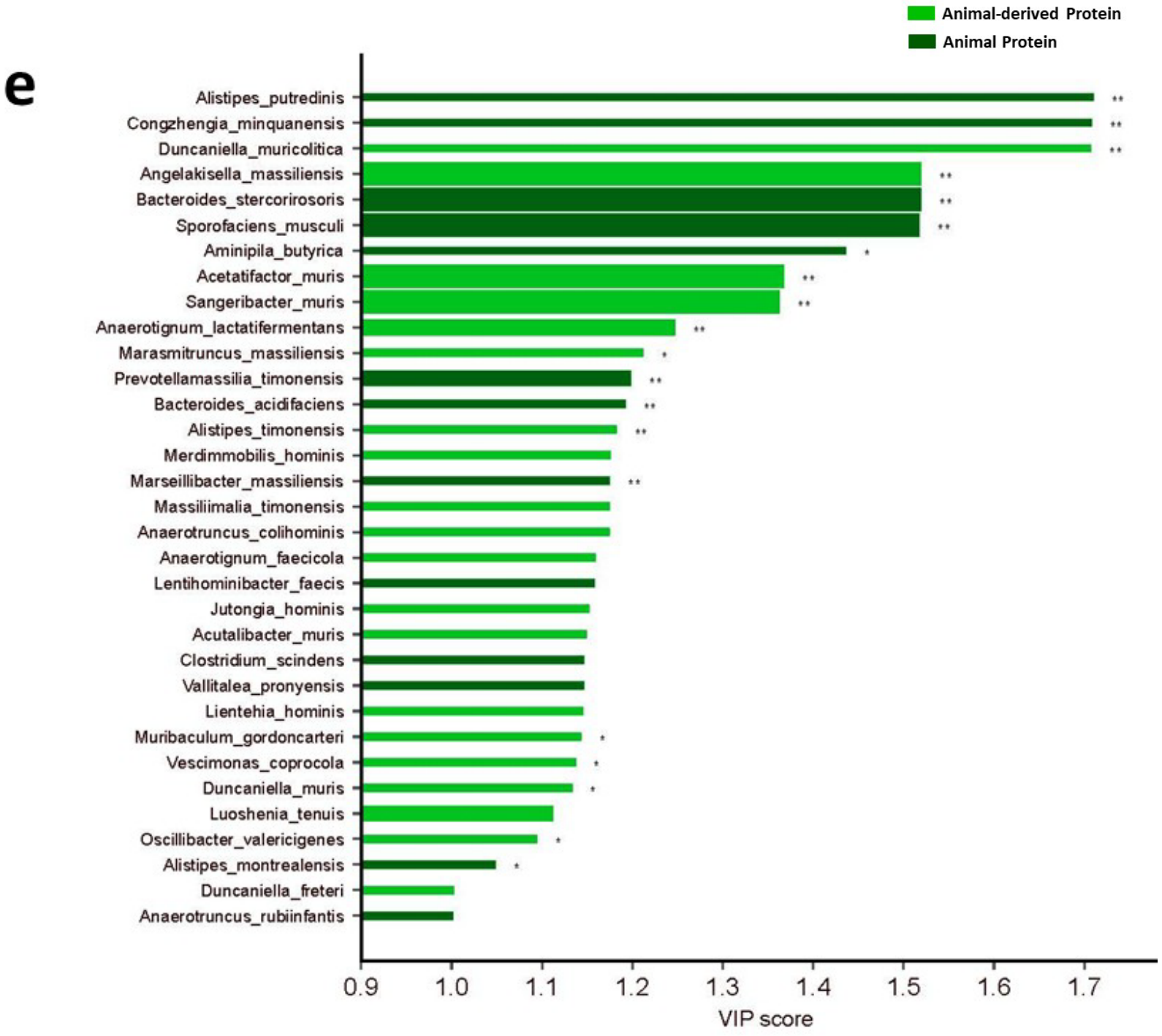
e
Animal-derived Protein
Animal Protein
Alistipes_putredinis
Congzhengia_minquanensis
Duncaniella_muricolitica
Angelakisella_massiliensis
Bacteroides_stercorirosoris
Sporofaciens_musculi
Aminipila_butyrica
Acetatifactor_muris
Sangeribacter_muris
Anaerotignum_lactatifermentans
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Prevotellamassilia_timonensis
Bacteroides_acidifaciens
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1.1
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1.5
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1.7
VIP score

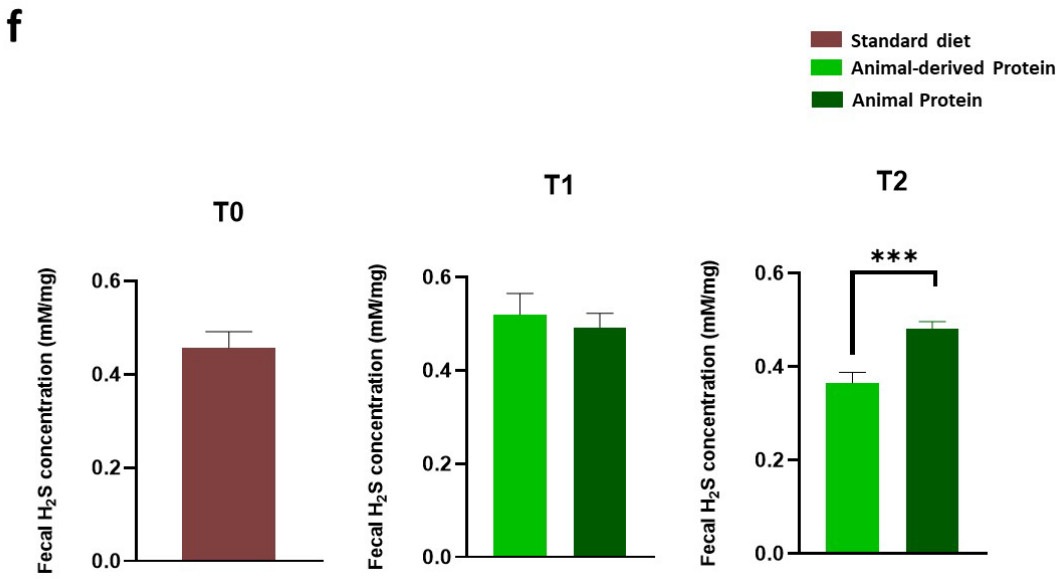


Figure 8. Microbiota composition and H_2S from faecal water: Experimental design (**a**) (week = week old). Microbiota diversity between groups is calculated by alpha diversity (**b**) shows differences in microbial population (inverse Simpson index) following treatment (p=0.016), but not in biodiversity (Shannon index, p=0.250), and richness (p=0.599) among controls (ADP, light green, n=5 biologically independent samples) and treated mice (AP, dark green, n=5 biologically independent samples). Beta-diversity (**c**), represented as PcoA, evidenced a clear separation among AP and ADP cohorts (PERMANOVA p=0.00859, ANOSIM p=0.00879). HCA (**d**) following the Bray-Curtis dissimilarity distance algorithm, both showed significant separation of ADP and AP. VIP I shows: (i) discriminant species after PLS-DA in descending order of VIP score (bar length); (ii) the highest relative abundance depending on the cohort (central bar color) and the lowest one (edge bar color); (iii) fold ratio highest vs the lowest relative abundance (bar thickness). **f.** H_2S concentration in faecal water is modify by different type of protein. During time animal-protein diet increase the concentration of H_2S. T_1 (ADP, n=15; AP, n=12), p=0.642; T_2 (ADP, n=11; AP, n=12) *** p =0.0005**,** Unpaired t-test. Data are presented as the mean ± SEM.

4.2 Animal protein diet does not impact mouse body weights and gut morphology

To verify that the two different diets were isocaloric and did not impact nutritional status, body weight of the mice was assessed weekly for the entire duration of the experiment. As shown in figure 9a, both groups of mice gained weight in the same way, without statistical differences. These results are supported by food intake rate data, evaluated two days a week during all the experiment, which confirmed that both groups intake the same amount of food, without any significant difference (Figure 9b). To investigate whether AP diet affected overall colon homeostasis, the length of the colon from the caecum to the anus and the mucosal morphology were evaluated at the end of the experiments. As shown in figure 9c, no differences were highlighted from colon measure in the two groups as well as no changes were highlighted at mucosal level in coronal section of caecum upon hematoxylin and eosin staining as reported in figure 9d.

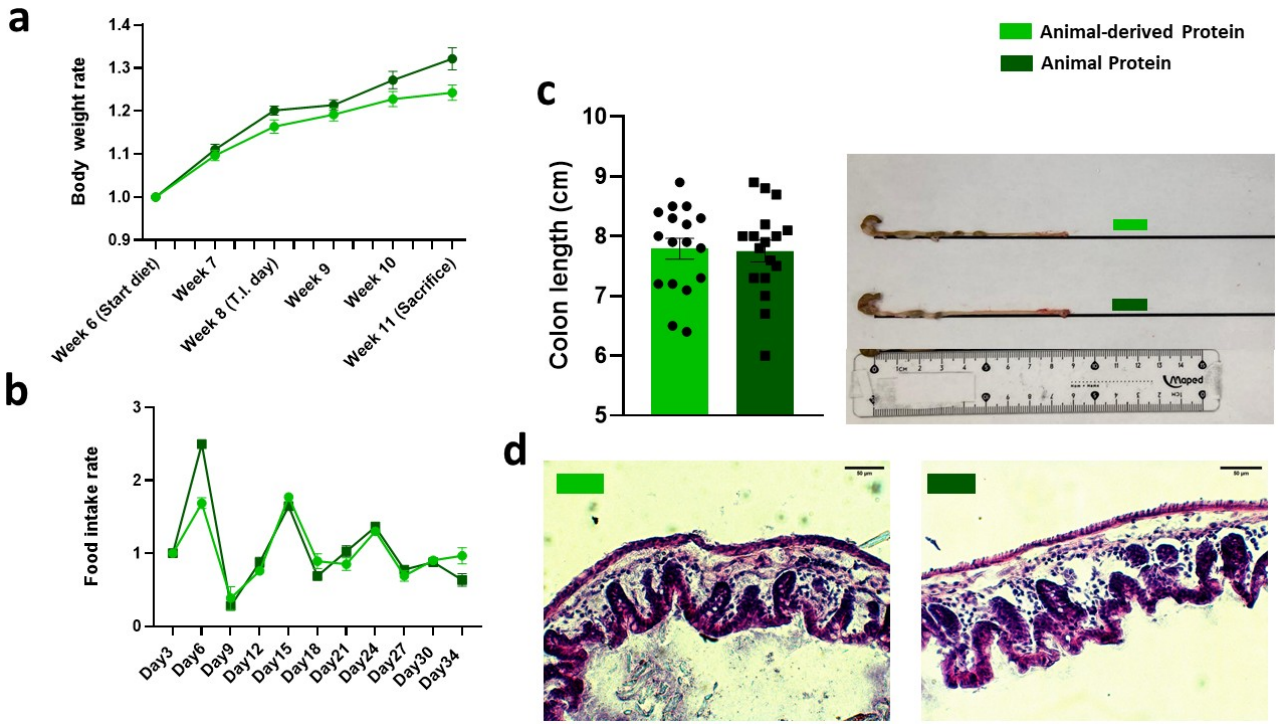


Figure 9. Murine nutritional status and gut morphology: a. Diets do not significantly affect body weight (**a**, n=17 p=0.052, Ordinary two-way ANOVA), food intake rate (**b**, n=17 p=0.742, Ordinary two-way ANOVA) along the entire experimental time (week= weeks old (**a**), or days of treatment (**b**)) between groups. **c.** Colon length was measured soon after transcardiac perfusion. Bar plot shows colon length (cm). No differences were highlighted between groups (n=17). p=0.8901, Unpaired t-test). Data are presented as the mean ± SEm. **d.** Hematoxylin and eosin staining on cecum coronal slices showed no changing at mucosa levels between groups, scale bar = 50μm.

4.3 Animal protein diet reduces glioma volume, cell proliferation and induced morphology changes in tumor associated Iba1+ cells

To assess in vivo whether red meat intake affected glioma growth, C57BL6/N mice bearing murine GL261 glioma cells were sacrificed twenty-one days after tumor inoculation (Figure 8a). Brains were isolated, fixed and frozen, and coronal brain cryosections were prepared for tumor volume evaluation and immunofluorescence staining. As reported in figure 10 a and b, mice fed the AP diet showed reduced tumor volume compared to those fed the ADP diet. To determine whether the observed reduction in tumour volume was due to a decrease in cell proliferation within the tumour mass, Ki-67 staining was performed. Sections stained with the proliferation marker and Hoechst for nuclei visualization showed that Ki-67 was expressed at higher levels in the glioma cells of ADP diet fed mice compared to protein diet mice (Figure 10b). We further investigated the effect of AP diet on tumor microenvironment evaluating by confocal immunofluorescence the morphology of tumor associated Iba1^{+} cells (microglia and macrophages), TAMs. Mice fed animal-protein diet showed a noticeable roundness of TAMs compared to ADP diet (Figure 10c). Indeed, on the graph showing the plot profile distance, in which each peak describes the shape of a single cell, it is possible to note that peaks from ADP group are more jagged than AP highlighting a more complex shape (Figure 10c). As previous described (Grimaldi et al., 2016), the rounded morphology of TAMs is usually associated with an activated state, compared to

a branched one, and this profile is often associated to a pro inflammatory/anti tumoral phenotype. These findings suggest that an AP diet may exert anti-tumor effects in glioma-bearing mice by reducing tumor cell proliferation and promoting a more activated, potentially anti-tumoral microglial/macrophage phenotype within the tumor microenvironment.

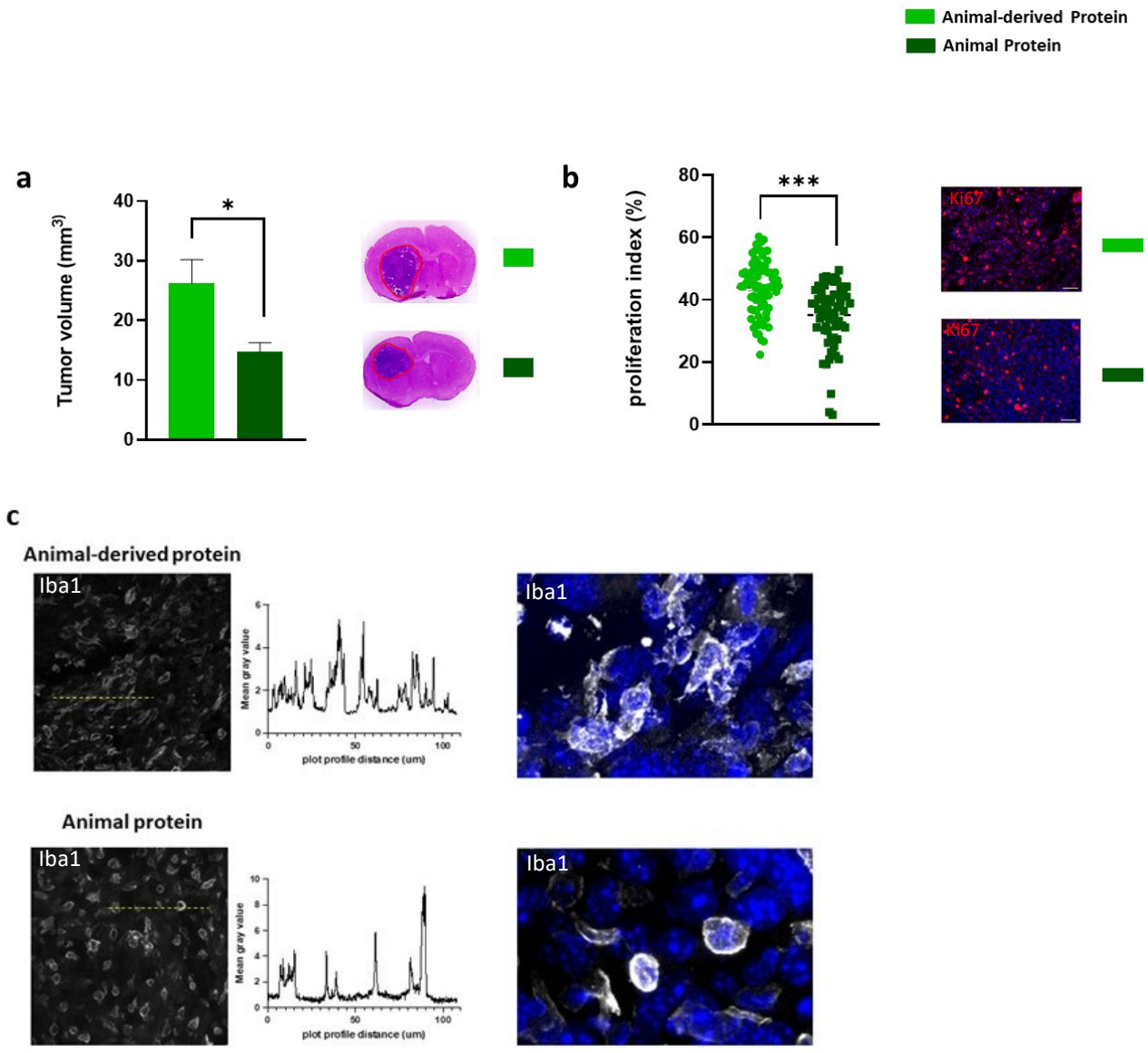


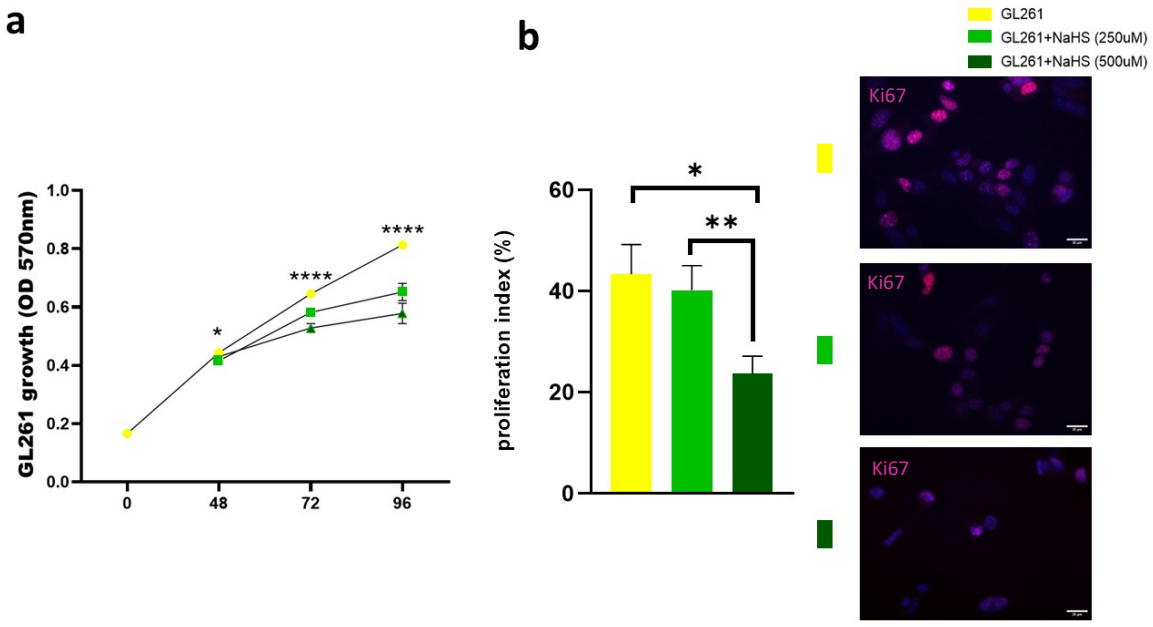
Figure 10. Effect of the Animal-protein diet on tumor growth: a. Tumor volume (mm^3) in different groups, (ADP, n= 10; AP, n=15). *p =0.0137, Unpaired t-test. **a.** Representative images

of haematoxylin and eosin-stained brain coronal slices. **b.** On the left, scatter plot showing the percentage of Ki67 positive cells in the tumor core area. The percentage of Ki67-positive cells (red) was calculated over the total number of nuclei (blue) (Ki67-positive cells/number of nuclei) (ADP: 30/15/3 field of views (FOVs)/slices/mice; AP: 30/15/3 FOVs/slices/mice). * and *** p <0.0001, Unpaired t-test. Data are presented as the mean ± SEM **b.** On the right, representative images of Ki67 (red) positive cells in tumor core area. Hoechst staining (blu) for nuclei visualization; scale bar: 50 μm. **c**. Immunofluorescent confocal images of brain slices stained for Iba1, showing TAMs within the tumor core. The left panel (10x magnification) provides an overview of the cellular distribution, while the right panel (digital zooming) highlights individual TAMs displaying distinct morphologies corresponding to either a resting (ADP) or activated (AP) state. Plot profile distance (μm) describing the shapes of the cells (center).

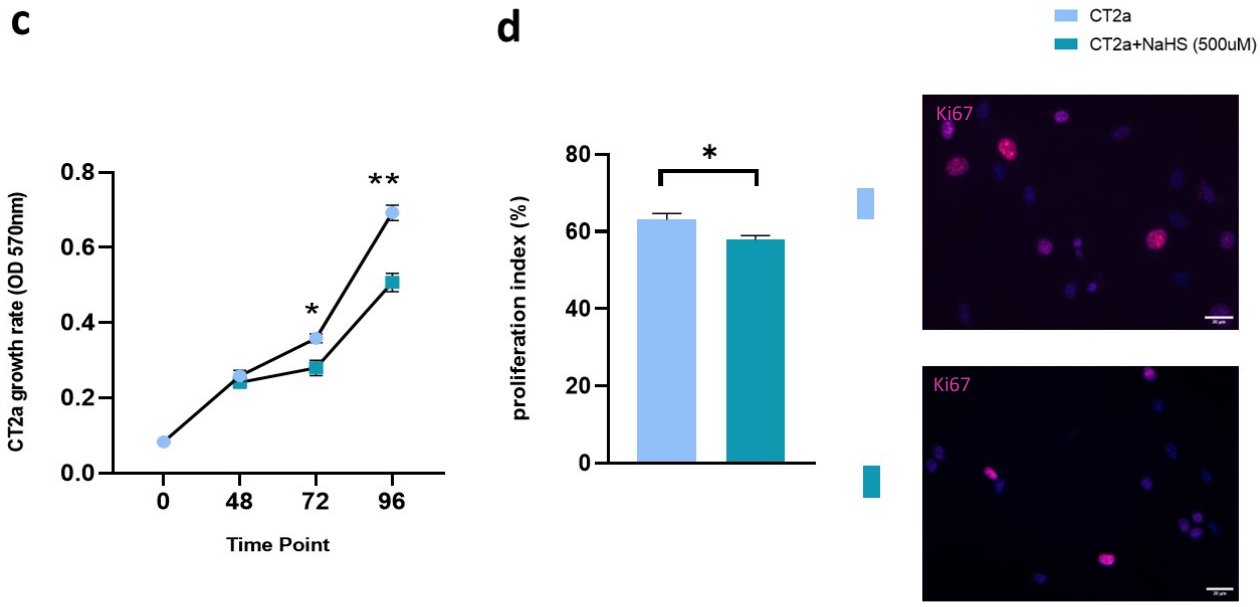
4.4 Effect of NaHS (a H_2S donor) treatment on glioma growth and microglia morphology in vitro

To evaluate whether H_2S was directly involved in tumor growth, we investigated in vitro the effect of the gasotransmitter on murine GL261 cell growth. Cells were cultured in 96 multiwell-plates, and exposed to different concentrations of NaHS (0, 250 μM and 500 μM), an inorganic salt which rapidly releases H_2S in water, at different time points (48h, 72h and 96h) to assess cell viability by MTT-assay. Treatment with H_2S decreases cell viability compared to controls at both concentrations used. In particular, GL261 treated with NaHS, showed reduced cell viability early at 48 up to 96 hours (Fig. 11a). The relation between cell viability and proliferation index observed in ex-vivo was confirmed also in vitro by Ki67 immunofluorescence staining at different

concentration previously used (ctrl, 250 μM and 500 μM, (Linden, 2014). As shown in figure 10b, NaHS at higher concentration reduced GL261 proliferation compared to both controls and NaHS at lower concentration 48 hours after treatment. The same results were obtained with a second murine glioma cell line, CT2a, treated with the higher dose of NaHS as shown in figure 11c and d. In order to assess the effect of NaHS treatment on microglia morphology we performed immunofluorescence staining on both primary and BV2 microglia cells. We used phalloidin antibody, commonly used to stain actin filaments and thus evaluate the structure of the cytoskeleton. We seeded 10^4 primary microglia and $5x10^4$ BV2 cell line, and treated both with NaHS (500μM). After 24 hours, we performed morphology analysis and evaluated the roundness of cells using the form factor formula as shown in figure 11f. Both types of cells treated with NaHS showed a more rounded morphology compared to the control one (Figure 11e and 11g) and higher circularity values. Commonly, the rounder the cell, the more likely it is to be in an activated state, tipically associated with a proinflammatory phenotype, and this morphology is often



assumed to be associated to an antitumoral effect (Grimaldi et al., 2016). Altogether, these data indicate that H_2S delivered via NaHS, reduces glioma cell viability and proliferation in vitro in two different types of GB cell lines, and promotes a morphological shift in microglia toward a more rounded, potentially proinflammatory and anti-tumoral phenotype.



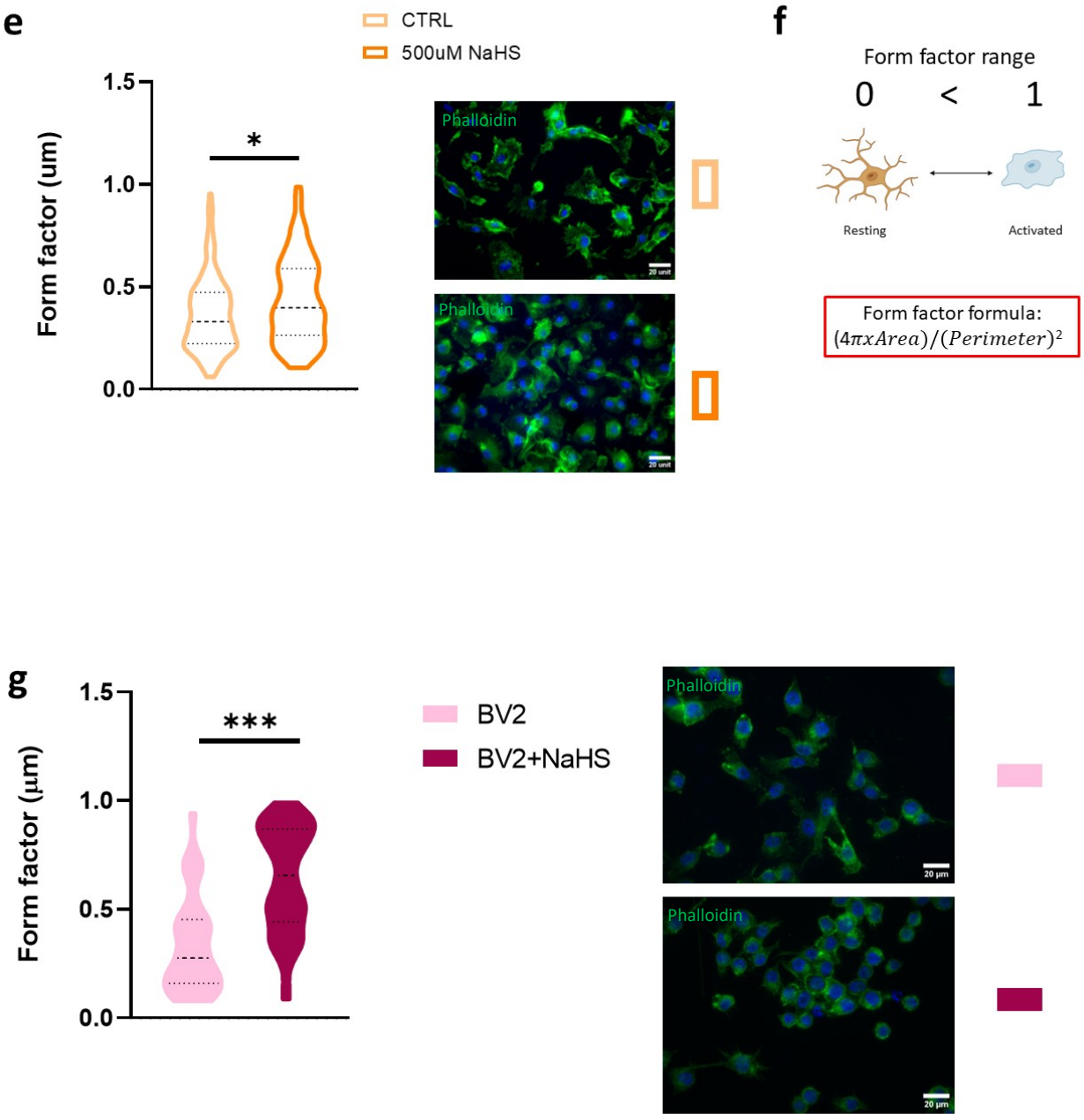


Figure 11. Effect of NaHS (a H_2S donor) treatment on microglia morphology: a. Growth curves

showing GL261 cells treated with different NaHS concentrations or vehicle. * p <0.0461 (vs 250μM 48h); **** p <0.0001 (vs 250μM or 500μM 72h and 96h), Kruskal-Wallis test (One-way ANOVA). n= 36 wells per condition, 3 independent experiments. **b.** Bar plot showing proliferation index as % of Ki67 positive GL261 cells evaluated after 48h NaHS treatment (GL261: 10/3 field of views (FOV)/wells; GL261 + NaHS (250μM): 10/3 FOV/wells; GL261 + NaHS (500μM): 10/3 FOV/wells). * p =0.02, Ordinary one-way ANOVA; ** p =0.0132, Unpaired t-test. (Right) Representative overlay images of Ki67 (purple) positive cells on total nuclei (blu); scale bar: 20 μm. **c.** Line plot showing the MTT assay on CT2a cells with (500μM) or without NaHS treatment. * p =0.001363 (vs 72h); ** p =0.000005 (vs 96h), multiple t-test – one per row. **d.** Bar plot showing proliferation index as % of Ki67 positive CT2a cells evaluated after 48h NaHS treatment (CT2a: 12/3 FOV/wells; CT2a + NaHS: 12/3 FOV/wells. * p =0.0125, Unpaired t-test. (Right) Representative overlay images of Ki67 (purple) positive cells on total nuclei (blu); scale bar: 20 μm. **e.** Violin plot showing how microglia morphology is modified by 24h treatment of NaHS (500μM) or CTRL, n =100 cells. * p =0.0042, Unpaired t-test. (Right) Microglia in the absence (CTRL) or presence of NaHS (500 μM), stained with phalloidin (green) and Hoechst (blue). Scale bar: 20 μm. **f.** Microglia morphology is calculated through *form factor formula* while *form factor value* indicates inflammation state. **g.** Violin plot showing how BV2 microglia morphology is modified by 24h treatment of NaHS (500μM) or only BV2, n =100 cells. *** p <0.0001, Unpaired t-test. (Right) BV2 in the absence (CTRL) or presence of NaHS (500 μM), stained with phalloidin (green) and Hoechst (blue). Scale bar: 20 μm. Data are presented as the mean ± SEM

4.5 Inhibition of H_2S biosynthesis by aminooxiacetic acid (AOAA) prevents anti-tumor effect of red meat consumption

To verify the hypothesis that the H_2S derived from red meat intake was involved in the reduction of tumor growth, mice were treated with AOAA, an

inhibitor of the enzyme (Petrosino et al., 2022) involved in the biosynthesis pathway of H_2S, cystathionine beta-synthase (CBS**)**, for five weeks, starting 2 weeks before glioma cell injection (Figure 12a). Mice fed AP diet and treated with AOAA (AP+ AOAA) showed larger tumor volumes than AP mice that again showed tumor volume lower than animal-derived diet (controls). The inhibition of H_2S biosynthesis did not affect tumor volume in animal-derived diet (Figure 12b, c). Preliminarily, we verified that the AOAA treatment reduced fecal H_2S concentration and, as shown in figure 12d, we reported that soon after 1 week of inhibitor administration the amount of gasotransmitter found was significantly decreased in AP+AOAA mice compared to AP ones (Figure 12d). Moreover, immunofluorescence $Iba1^+$ staining, performed on brain slices, confirmed, through form factor analysis, that AP+AOAA mice shifted TAMs toward a less rounded morphology compared to cells of AP mice, and more similar to ADP ones (Figure 12e, left). These results confirm that in the gut, increased H_2S bioavailability, upon read meat intake, could be directly involved and responsible for tumor growth modulation in glioma *in vivo* model since the gasotransmitter could pass in the circulation through the intestinal barrier to reach the brain and carry out its effect.

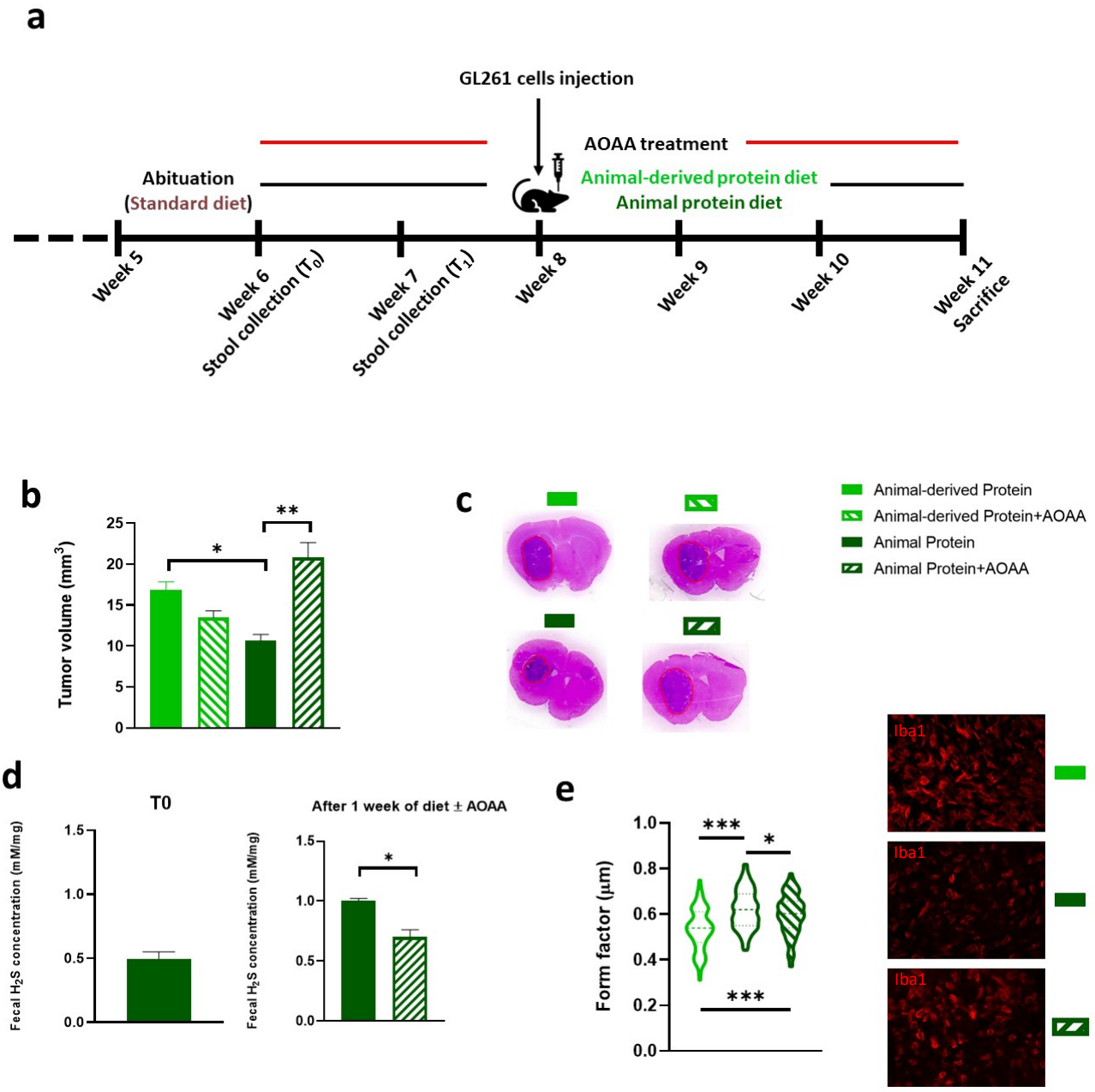


Figure 12. **Effect of Aminooxyacetic acid treatment on tumor growth: a.** Experimental design (week = week old). **b.** Tumor volume (mm^3) in different groups (ADP, n= 5; ADP+AOAA, n=5; AP, n=4; AP+AOAA, n=6). *p =0.03; ** p= 0.0004, Ordinary one-way ANOVA. **c.** Rapresentative images of hematoxylin and eosin-stained brain coronal slices. **d.** Effect of H_2S inhibition through AOAA treatment soon after one week in AP mice treated with or without AOAA (AP, n =5;

AP+AOAA, n=8). * p =0.0031, Unpaired t-test. **e.** Form factor analysis (left) and representative FOVs images (right) of Iba1 immunofluorescence, on mice brain slices, to stain $Iba1^+$ cells and to assess cell morphology differences in AP mice compared to ADP and AP+AOAA mice (10/6/3, FOVs/brain slices/mice). *** p<0.0001; * p=0.0293, Brown-Forsythe and Welch ANOVA tests (multiple comparisons). Data are presented as the mean ± SEM

4.6 TRPV1 inhibition by capsazepine (cpz) abolishes the effect of animal protein diet on tumor growth

As previously shown in a model of transient middle cerebral artery occlusion (tMCAO) in rat, gut H_2S can modulate vagal afferents trough TRPV1 activation (Kimura et al., 2024; Ni et al., 2022). TRPV1 expression in colonic afferent neurons has been well characterized in a study which demonstrated higher percentage colonic-labeled dorsal root ganglion (DRG) neurons immunoreactive to TRPV1 (Brierley et al., 2005). In order to assess the possible role of vagus nerve activation by H_2S trough TRPV1 in glioma growth, AP e ADP mice were treated intrarectally with CPZ, a TRPV1 antagonist for three weeks after tumor cell inoculation (Figure 13a). As shown, AP mice who received the inhibitor treatment showed larger tumor volumes than untreated AP mice, which as expected showed lower tumor volumes than ADP mice (Figure 13b). In order to measure the efficacy of TRPV1 blocking, the behavioral response to hot was evaluated performing tail-flick test as TRPV1 channels are also activated by noxious heat stimuli, such as above 44°C (Joseph et al., 2019). The latency (time, in seconds) before mice react to the hot water (50°C) was

recoded for CPZ and vehicle (Veh) treated. In figure 13c, CPZ mice showed significantly longer withdrawal latency compared to Veh demonstrating sustained modulation of TRPV1 expressing sensory neurons. These results support the hypothesis that H_2S could modulates tumor growth involving the afferent fibers of the vagal nerve, potentially via activation or sensitization of TRPV1 channels.

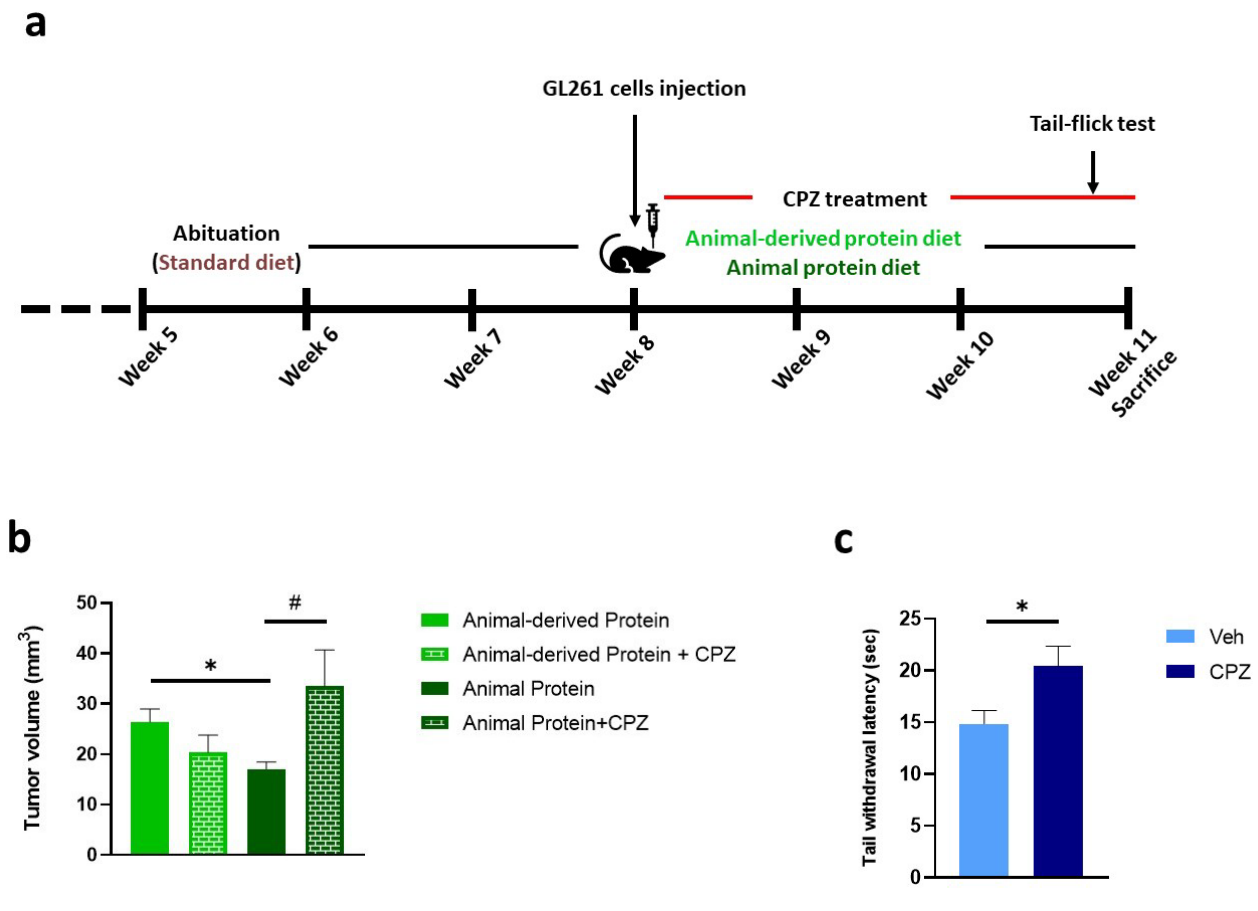


Figure 13. Effect of capsazepine treatment on glioma growth: a. Experimental design (week = week old). **b.** Tumor volume (mm^3) in different groups, n=6. *p =0.0241; # p= 0.0120, Kruskal-Wallis test (One-way ANOVA). **c.** Tail-flick test withdrawal latency (expressed in seconds) at 50°C on day 34 (Veh, n=12; CPZ, n=13). ** p=0.0273, Unpaired t-test. Data are presented as the mean ± SEM.

5. Discussion

The GBA is a bidirectional way of communication between the two systems that acts through many different mechanisms. The GBA has recently been widely studies in different experimental models, in particular due to the key role that immune cells play in regulating it (Erny et al., 2015). Gut microbiota, the sum of the microorganisms resident in the gastrointestinal tract is a relevant actor in this axis: dysbiosis and psychiatric disorders are often interconnected, suggesting that alterations in gut microbiota composition contribute to changes in brain function and potentially playing a role in the development of mental health diseases (Fröhlich et al., 2016). Gut microbiota can be modified by many factors such as antibiotic treatment, environment and diets (Cordella et al., 2021; D'Alessandro et al., 2020; Marrocco et al., 2022). In this study we investigate the effect of a red meat intake on modulation of GB tumor growth and tumor microenvironment. In preclinical studies, mice are generally maintained on standard chow diets composed primarily of plant-derived ingredients, including soybean meal, corn, wheat, and other vegetal by-products. The most common proportion of protein is approximately 18%. In the present study, Mucedola Srl costumized for us a diet containing 17% of animal protein derived from red meat. This choice was motivated by evidence showing that diets enriched in red meat proteins influence gut microbial composition by modulating the abundance of bacteria involved in protein fermentation, such as *Bacteroides* and *Alistipes* (D. P. Li et al., 2021).Indeed, previous work has

demonstrated that red meat-based protein diets can alter microbial communities and have been used in mouse models of colitis (D. P. Li et al., 2021). Moreover, the fermentation of undigested dietary proteins in the colon produces a broad spectrum of metabolites, some of which exert beneficial effects, such as SCFAs and H_2S, the latter being particularly relevant to our research as it has been implicated in shaping the TME by influencing cellular proliferation, immune cell phenotypes, and CSCs dynamics (Cirino et al., 2023; Marrocco et al., 2022; Moianu et al., 2023; Silver et al., 2021; Windey et al., 2012; Xie et al., 2024). In particular, we proposed a key role for H_2S, produced upon red meat protein diet, in modulating tumor progression by modifying GB proliferation, CSCs abundance and the phenotype of the tumor microenvironment, as described in a previous study employing a high-lipid diet (Silver et al., 2021). Indeed, the negative modulation of H_2S production through oral administration of amminooxiacetic acid (AOAA) or intrarectal capsazepine (CPZ) administration to prevent H_2S induced TRPV1 activation inhibits the antitumoral effect of the diet in vivo, suggesting in our model a potential dual mechanism of action of the gasotransmitter: direct (decreasing H_2S bioavailability) or indirect (through vagal TRPV1 activation, inhibiting the receptor or decreasing H_2S bioavailability). We here demonstrated that a red meat intake enhances H_2S concentration and modify gut microbiota, contributing to a reduction in tumor growth. It is known that sulfide generation in gut, specifically in colon, is influenced by dietary components (Magee et al., 2000). Specifically, in mice fed with animal proteins, we point out a marked modification of the gut microbiota composition as concern alpha and beta diversity. Particularly, in mice who received red meat proteins, we describe a specific increase of species belonging to genera previously found modulated in mice in similar experimental condition (D. P. Li et al., 2021). Specifically, the

following are known to be associated with the production of H_2S, among other metabolites: *Alistipes putredinis, Bacteroides acidifaciens, Bacteroides stercorirosoris*, and *Aminipila butyrica*. The latter produces almost only H_2S (D. P. Li et al., 2021; Parker et al., 2020; Shen et al., 2010; Ueki et al., 2018; Zhu et al., 2016). Moreover, we observe an increased concentration of the gasotransmitter in faeces of mice fed with AP compared to ADP diet. A positive correlation between protein intake and sulfur concentration in feces has already been observed in humans (Magee et al., 2000). Concerning the isocaloric formulation of the diet, the unchanged body weight and food intake rate between the two groups suggests that differences observed could be due to the different effects exerted by the different source of protein. Furthermore, it has been demonstrated a reduction in colon length in mice fed a medium and high percentage (50% and 75% respectively) of red meat protein diet compared to a standard diet while no differences were shown in a low-dose red meat diet (25%) compared to controls (D. P. Li et al., 2021). This last result is in line with our model where a diet containing standard percentage of red meat protein (17%) does not induced structural or morphological differences in the colon of mice that received the diet, compared to those that received an equivalent amount of ADP diet. In the context of glioma, we observed that mice who ate red meat proteins showed a significant reduction in tumor volume compared to animals that received casein protein. We hypothesized that this effect may be due to an increased production of H_2S in the gut, which can diffuse to plasma and then reach other organs, including the brain (Munteanu et al., 2024). In addition, confocal immunofluorescence analysis for $Iba1^+$ cells, exhibits clear differences between tumor infiltrating cell morphology of AP compared to ADP suggesting that the gasotransmitter could influences neuroinflammation of tumor microenvironment, possibly leading to tumor suppressive effects. These

hypotheses were previously proposed in several studies which investigated the concentration-dependent effect of H_2S on brain injury, cognitive impairment, psychiatric diseases, and glia mediated inflammatory response models (Kimura et al., 2024; Kumar & Sandhir, 2019; J.-Y. Zhang et al., 2017). The role of H_2S in reducing GB aggression has already been demonstrated by feeding mice with a high- or low-fat diet. Indeed, high-fat compared to low-fat diet decrease H_2S availability in the brain leading to an increased tumor cell growth and an increased presence of cancer stem cells (Silver et al., 2021). Here, the cytotoxic effect of H_2S on tumor cells was confirmed through cell viability and proliferation assays. These effects were observed in vitro, using two different murine glioma cell lines, the GL261 and CT2a. However, previous study demonstrated that exogenous H_2S administration, through its donor NaHS, could exert its cytotoxic effect on both immortalized and cancerous colon epithelial cell lines (respectively YAMC, and HT-29, HCT-166, and SW1116) inhibited proliferation of colon epithelial cells and migration of colon cancer cells (Linden, 2014; Wu et al., 2012). Moreover, within a brain-related context, H_2S administration prevents hypoxia-induced apoptosis in mouse hippocampal neurons (J.-Y. Zhang et al., 2017) and neuron pyroptosis, microglia/macrophage activation and inflammation in a context of ischemia-reperfusion injury (Xie et al., 2024). Since we hypothesized that exogenously produced H_2S by gut bacteria could exert an anti-tumour action, we decided to inhibit its production pathway locally. We therefore administered AOAA orally at very low dose (20mg/kg) (A. Xiao et al., 2016). AOAA is a well-known inhibitor of certain enzymes, particularly Cystathionine β-Synthase (CBS) which represents one of the rate-limiting steps of the gasotransmitter biosynthesis (A. Xiao et al., 2016). Although AOAA may cross the gut barrier, its bioavailability could vary. Indeed, it is reported that AOAA exhibits poor systemic absorption when

administered orally, primarily due to its limited intestinal permeability (A. Xiao et al., 2016). Soon after one week of treatment, we observed a significant reduction of H_2S concentration in feces in mice fed with animal-protein diet who received AOAA orally compared to those fed with the same diet, but which did not receive the treatment. Following this inhibition, we observe an increase in tumor volumes in mice receiving the treatment confirming the inhibition of the H_2S production pathway by AOAA and the key role of the gasotransmitter on tumor growth. Indeed, these experiments support our main hypothesis that gasotransmitter produced by the gut microbiota influences brain tumor growth in a direct way, possibly through blood stream transport. Lastly, considering the other ways through gut and brain communicate, we further investigated whether the vagal route was in some way involved in an indirect H_2S action on glioma growth. In particular, we investigated the effects of TRPV1 inhibition to prevent H_2S binding to the receptor, which is also localized on the afferent fibers of the vagus nerve (Hermes et al., 2016) innervating many organs, including gut (Butt et al., 2020). These receptors have numerous putative endogenous and exogenous agonists (such as Anandamide, and Capsaicin, respectively) and antagonists (such as Noradrenaline, and Capsazepine, respectively) which are being used in clinical trials (L. Li et al., 2021). For our purpose we decided to use the antagonist CPZ and successfully demonstrated that the treatment effectively blocked TRPV1. We observed that mice fed an animal-protein diet and received CPZ treatment had a higher tumour volume compared to those that only ate red meat. These results demonstrate that vagal afferent activation through TRPV1 channels could be indirectly involved in the controlling brain tumour growth. Particularly, it has been well-studied that the effect of capsaicin (CPS) alone, or combined with CPZ on vagal nerve modulation in a glioma context, depends on to the concentrations, and the duration of the treatment,

and it's a topic on which conflicting evidence can be found in the literature (Amantini et al., 2007; Bíró et al., 1998). Indeed, if on the one hand it was demonstrated that CPS, a TRPV1 agonist, at low doses has the capacity to inhibit tumor cells growth and induce cell death in several in vivo and in vitro models (e.g. C6, U87, and FC1 glioma cells) and that CPZ antagonize this effect (Amantini et al., 2007; Athanasiou et al., 2007; Bíró et al., 1998), on the other side, it has been observed that in presence of high doses and/or long-term exposure to CPS, CPZ acted as an agonist too having itself an inhibitory effect on the growth and proliferation of some tumors (Bíró et al., 1998). Based on this knowledge, further investigation is needed to elucidate the pathways through which vagus nerve contribute to tumor growth control. So far, our results suggest a mechanism whereby CPZ, antagonizing H_2S signaling through vagal afferents expressing TRPV1, influences tumor microenvironment in a way that merits detailed examination.

In conclusion, we demonstrated that consumption of an AP diet with a balanced amount of proteins resulted in a modification of gut microbiota composition in a glioma-bearing-mouse model. These changes are associated with an increased production of microbial H_2S, which we identified as a possible direct modulator of both tumor cell proliferation and tumor microenvironment remodeling. Notably, pharmacological inhibition of H_2S biosynthesis (via AOAA) or preventing TRPV1 activation (via CPZ) led to enhanced tumor growth, highlighting the critical role of microbiota-derived H_2S in glioma progression. Our findings underscore the involvement of the GBA in these processes and suggest that enhancing H_2S bioavailability could represent a novel therapeutic strategy to slow down glioblastoma growth and

potentially improve responsiveness to conventional treatments such as chemotherapy and radiotherapy.

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