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Ana Carolina Marques Ferreira

Preclinical evaluation of the therapeutic potential of Vaccinium corymbosum L. (blueberry) leaf biomass in experimental Multiple Sclerosis

Dissertação no âmbito do Mestrado em Investigação Biomédica orientada pela Doutora Sofia Andreia Domingues Viana e coorientada pelo Doutor Flávio Nelson Fernandes Reis e apresentada à Faculdade de Medicina da Universidade de Coimbra

Setembro 2021

Faculdade de Medicina
da Universidade de Coimbra

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Abbreviations

ACC – Acetyl-CoA carboxylase

AHR – Aryl Hydrocarbon Receptor

ALP – Alkaline Phosphatase

ALT – Alanine Aminotransferase

AST – Aminotransferase

ATP – Adenosine Triphosphate

BB – Blueberry(ies)

BBB – Blood-Brain Barrier

BDNF – Brain-derived Neurotrophic Factor

BMI – Body Mass Index

BMSCs – Bone Marrow-derived Stem Cells

BrdU – BromodeoxyUridine

BW – Body Weight

CC – Corpus Callosum

CGA – Chlorogenic Acid

CIS – Clinically Isolated Syndrome

CNS – Central Nervous System

COX – Cyclooxygenase

CPZ – Cuprizone

CSF – Cerebrospinal Fluid

CVD – Cardiovascular Diseases

CYC – Cyclophosphamide

DIS – Dissemination in Space

DIT – Dissemination in Time

DMF – Dimethyl Fumarate

EAE – Experimental Autoimmune Encephalomyelitis

EBNA1 – Epstein-Barr virus Nuclear Antigen 1

EBV – Epstein-Barr Virus

EDSS – Expanded Disability Status Scale

EDTA – EthyleneDiamine Tetraacetic Acid

ENS – Enteric Nervous System

ERK – Extracellular-signal-Regulated Kinase

ESCs – Embryonic Stem cells

FGF2 – Fibroblast Growth Factor 2

FMD – Flow-Mediated Dilation

GA – Glatiramer Acetate

GD – Gadolinium

GALT – Gut-Associated Lymphoid Tissue

GGF2 – Glial Growth Factor 2

GI – Gastrointestinal

GM – Gut Microbiota

GR – Glucocorticoid Receptor

GSH – Glutathione

GSSG – Glutathione isulfide

GWAS – Genome Wide Association Studies

Hb – Hemoglobin

HDAC – Histone Deacetylase

HE – Hematoxylin & Eosin

HLA – Human Leucocyte Antigen

HMG-CoA – β-Hydroxy β-Methylglutaryl-CoA

HMOX-1 – Heme-oxygenase 1

IFNβ - Interferon beta

Ig – Immunoglobulin

IGF-1 – Insulin-like Growth Factor 1

IL – Interleukin

IBD – Inflammatory Bowel Disease

iNOS – Inducible Nitric Oxide Synthase

ITT – Insulin Tolerance Test

KB – Kluver-Barrera

KLK6 – Kallikrein 6

LXR – Liver X Receptor

MBP – Myelin Basic Protein

MCH – Mean Cell Hemoglobin

MCHC – Mean Corpuscular Hemoglobin Concentration

MCV – Mean Cell Volume

MOG – Myelin-Oligodendrocyte Glycoprotein

MPV – Mean Platelet Volume

MRI – Magnetic Resonance Imaging

MS – Multiple Sclerosis

MSRV-Env – Multiple Sclerosis associated Retrovirus Envelope protein

MSSS – Multiple Sclerosis Severity Score

NEDA – No Evidence of Disease Activity

NGF - Neurotrophic Growth Factor

NO – Nitric Oxide

NSCs – Olfactory Ensheating Cells

OCB – Oligoclonal Band

OCT – Optimal Cutting Temperature

OFT – Open Field Test

OLG – Oligodendrocyte

OPC – Oligodendrocyte Precursor Cell

PBS – Phosphate-Buffered Saline

PC – Phenolic Compound

PDGF - Platelet-Derived Growth Factor

PLP – Myelin Proteolipid Protein

PPAR – Peroxisome Proliferator-Activated Receptor

PPMS – Primary-Progressive Multiple Sclerosis

Prx – Peroxiredoxin

RBC – Red Blood Cell

RDW – Red cell Distribution Width

RIS – Radiographically Isolated Syndrome

ROS – Reactive Oxygen Species

RRMS – Relapsing-Remitting Multiple Sclerosis

RRT – RotaRod Test

SCFA – Short Chain Fatty Acid

Smo – Smoothened

SNP – Single Nucleotide Polymorphism

SOD – Superoxide Dismutase

SPMS – Secondary-Progressive Multiple Sclerosis

T2DM – Type 2 Diabetes Mellitus

TB – Toluidine Blue

TG – Triglycerides

TGF1 – Transforming Growth Factor 1

TNFα – Tumor Necrosis Factor alpha

TPC – Total Phenolic Content

TrxR – Thioredoxin

UC – Ulcerative Colitis

UVR – Ultraviolet Radiation

VDRE – Vitamin D Response Element

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Resumo

A Esclerose Múltipla (EM) consiste numa doença autoimune desmielinizante associada a incapacidade neurológica, para a qual não existem atualmente estratégias terapêuticas que visem o foco fisiopatológico: desmielinização e remielinização.

Os padrões nutricionais constituem um fator de risco para a doença e, sendo modificáveis, podem estar na base de estratégias nutracêuticas de natureza complementar.

As folhas de várias plantas são importantes fontes de compostos bioativos. É o caso das folhas senescentes dos arbustos do mirtilo, que se destacam pelas suas propriedades benéficas resultantes do seu elevado conteúdo em compostos fenólicos e fibra. No entanto, através das técnicas de processamento tradicionais, o organismo não consegue aceder a uma porção significativa destes compostos. Assim, o nosso grupo desenvolveu uma metodologia inovadora para o processamento sustentável deste tipo de folhas, dando origem a uma biomassa de folhas de mirtilo (BB) com potencial benefício para a saúde.

Após uma afincada avaliação do perfil de segurança da BB, selecionou-se uma dose de 500 mg/kg. Pretendendo-se avaliar o potencial nutracêutico da BB em EM experimental, recorreu-se ao modelo de intoxicação por cuprizona. Os resultados obtidos permitiram observar uma atividade antioxidante sistémica e central demarcada da biomassa que se refletiu numa modulação da enzima superóxido dismutase (SOD). Notavelmente, a biomassa reverteu o desequilíbrio Treg/Th17 intestinal, antecipando as suas propriedades imunomoduladoras. Adicionalmente, observou-se uma aparente promoção de mecanismos remielinizantes, evidenciada pela promoção da expressão de genes associados à mielina (MBP e PLP) bem como uma maior marcação histológica desta no cerebelo. Em suma, estes resultados evidenciam a eficácia da biomassa em modular comunicações entre o binómio intestino/cérebro que ocorrem neste modelo experimental, em particular na fase de remielinização.

As características evidenciadas neste trabalho abrem novas perspetivas para o uso da biomassa como terapia não farmacológica em EM, potencialmente através da sua integração como ingrediente de valor acrescentado em alimentos funcionais e/ou nutracêuticos com atividade prebiótica.

Palavras-chave: Biomassa de folhas de espécies *Vaccinium*; Esclerose Múltipla; Antioxidante; Imunomodulação intestinal; Remielinização.

Abstract

Multiple Sclerosis (MS) is a demyelinating autoimmune disease displaying neurological disability, for which there are still no available therapies targeting the core of the disease: demyelination and remyelination. Nutritional patterns compose one of the risk factors for MS development. These are modifiable lifestyle components that can inspire the creation of nutraceutical strategies of complementary nature.

Several plants' leaves have been revealing to be important sources of bioactive compounds, blueberry bushes' leaves composing a great example, standing out because of its expressive beneficial properties which arise from an elevated phenolic compounds' and dietary fiber contents. Nevertheless, typical processing techniques restrict the organism's access to a large portion of these components. Hence, our group developed an innovative biotechnological methodology for sustainable leaves' processing, resulting in a blueberries' leaves biomass (BB) with increased health-promoting potential.

After a thorough evaluation of the BB's safety profile in mice, a dose of 500 mg/kg was selected. To assess BB's nutraceutical potential in experimental MS, the cuprizone intoxication model was employed. The obtained results suggest a marked central/systemic antioxidant activity upon BB biomass intake, which reflected as modulated expression/activity of SOD, one key enzyme composing the biological antioxidant system. Moreover, BB biomass was able to counteract intestinal Treg/Th17 imbalance paralleling the peak of demyelination, clearly highlighting BB' immunomodulatory profile. Remyelinating properties were likewise evidenced by the increased expression of myelin-associated genes in whole brain (MBP, PLP) as well as a more intense myelin staining (KB histology) in cerebellum samples. Collectively, data presented herein points to BB biomass efficacy to modulate active gut-to brain communications in experimental MS, particularly in the remyelination phase of the disease.

The BB biomass features highlighted in this work open new avenues for its use as non-pharmacological therapy in MS, potentially through its integration as a high-added value ingredient in functional foods and/or nutraceuticals with prebiotic activity.

Keywords: *Vaccinium* spp. leaves' biomass; Multiple Sclerosis; Antioxidant; Intestinal Immunomodulation; Remyelination

Chapter 1 | **INTRODUCTION**

1.1. Multiple Sclerosis: An Overview

1.1.1. Epidemiology of Multiple Sclerosis (MS)

Multiple sclerosis (MS) is a complex and heterogenous disease of the central nervous system (CNS) that manifests progressively through brain and spinal cord dissemination in time and space, due mainly to autoimmune inflammation. [1,2] It is one of the main causes of neurological disability in young adults, composing an enormous individual, familial, social, and economic burden. [1,2]

Although supportive treatment is available, including disability management, general symptom relief and psychiatric care, there is currently no cure for MS, and the number of MS patients has been significantly increasing over the past decades. [1,2] The disease prevalence varies considerably, with high rates in Europe, North America, New Zealand and Australia, and low rates in Asia and Africa. [3] San Marino and Germany have the highest prevalence in the world (337 per 100,000 and 303 per 100,000, respectively), followed by the USA (288 per 100,00). **(Fig. 1)** [3]

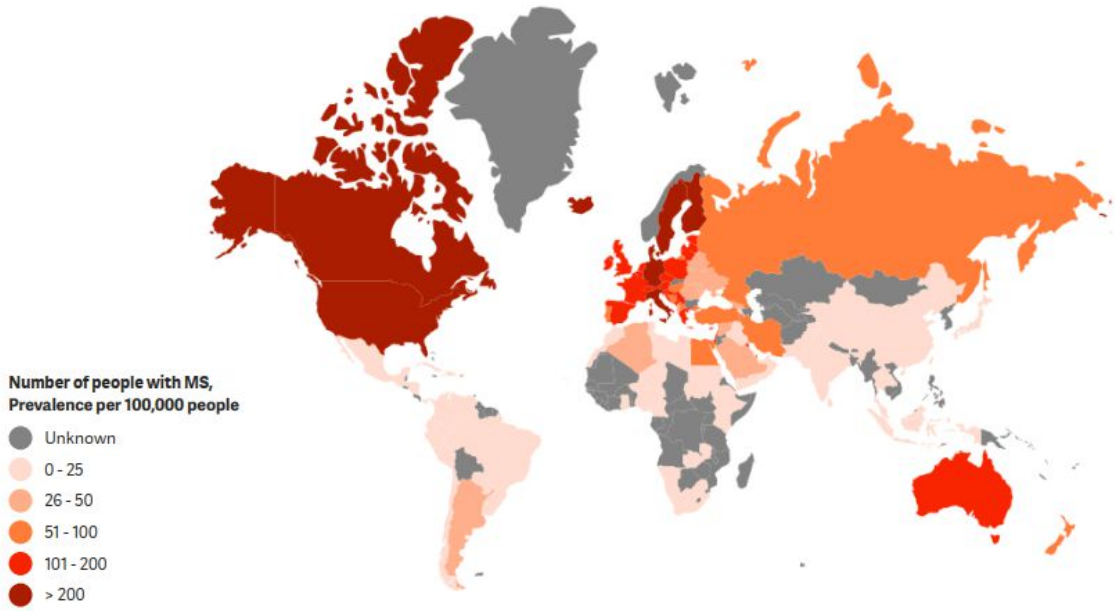


Figure 1. Number of people with MS - prevalence per 100,000 people. (Source: The Multiple Sclerosis International Federation, Atlas of MS, 3rd Edition (September 2020))

According to the MS International Federation, the number of people with MS worldwide has increased from 2.3 million in 2013 to 2.8 million in 2020, which equates to 1 to 3,000 people in the world living with MS. In fact, in countries with the highest prevalence, this numbers convert to 1 in every 300 individuals having the disease. Every five minutes, someone somewhere in the world is diagnosed with MS and its prevalence and incidence are globally increasing in a significant manner. [3] Several factors are likely to be involved in this increase, such as improved diagnosis as consequence of better access to neurologists and general healthcare, improved ability to count

the number of MS patients, as well as an augmented median life expectancy for these individuals. [2,3]

Several studies have reported differences in the incidence in prevalence of MS according to geographical location, with a positive association between latitude and risk for MS. [2,3] This effect is also observed within many countries, where people living in the north present a higher risk of developing MS comparatively to the ones living in the south. [3,4] This tendency has been partially explained by the variation in sunlight exposure and, consequently, in vitamin D availability depending on latitude [2,3] This hypothesis will be approached later on.

Although MS incidence has been increasing generally [2,5], this rise is more pronounced for women than for men, female patients being 2-3 times more frequent than male patients **(Fig.2)**. [2,5]

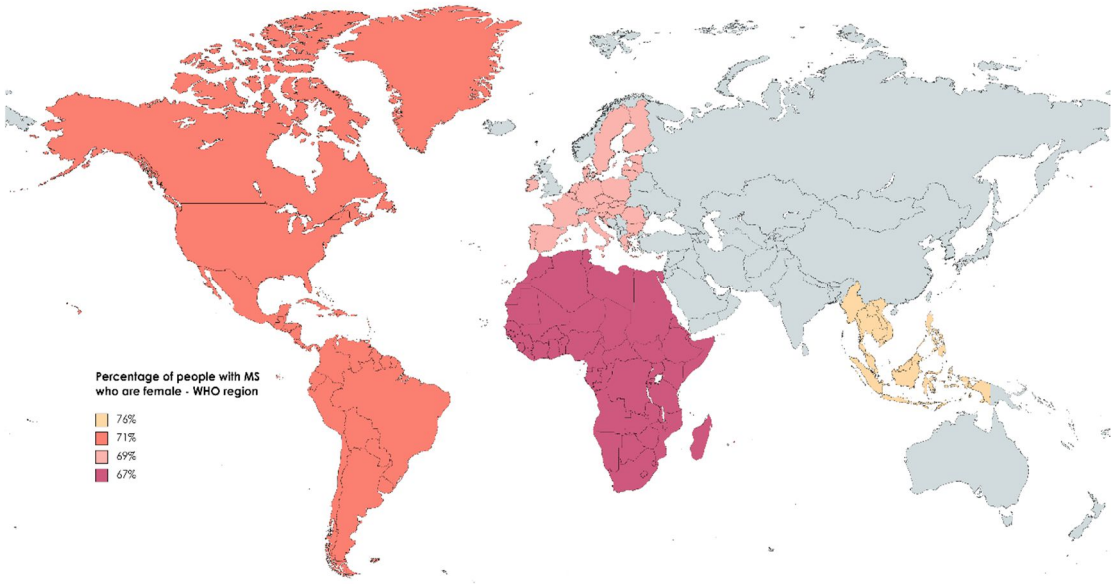


Figure 2. Percentage of people with MS who are female. (Source: The Multiple Sclerosis International Federation, Atlas of MS, 3rd Edition (September 2020))

The observed increased incidence of MS in women in the last decades might indicate change in women lifestyle for the past years. Factors such as late pregnancy, use of oral contraception, smoking, obesity, and stress have been listed as potential causes of the higher MS incidence in women nowadays. [2] MS incidence is also higher for individuals with Caucasian ancestry [1], but although the disease is less frequent for African Americans and Hispanics, it tends to have a more severe course when it occurs in these populations [1]. When it comes to age, MS incidence is low in children, increasing significantly after adolescence and reaching its peak between 25 and 35 years of age, this peak being two years anticipated in women. [6] After this period, incidence gradually reduces. [6]

MS patients present higher mortality comparatively to the general population, having a medial lifespan 6 to 14 years shorter. [2] 70 to 88% of MS patients survive up to 25 years after the disease clinical onset and the medial time between onset and decease varies from 24 to over 45 years [2]. In the last decades, MS-associated mortality has been decreasing, potentially due to treatment development. [7] The current higher life expectancy as well as reduced mortality of MS

patients are leading to a rise in disease prevalence and, therefore, to an increasing number of patients needing constant health care for this chronic condition.

1.1.2. Clinical Definition

MS disease course is described by two key phenomena – relapses and progression – which might overlap in different stages of the disease. [8,9] MS is considered to present three distinct phases, the first being the high-risk phase, the second the relapsing-remitting phase and, finally, the progressive phase, to which about 80% of patients ultimately evolve to. [8,9] Each phase is further defined as active or inactive. [9]

Relapses are transient events of neurological disability that occur in MS patients, being either symptomatic or asymptomatic. [9] A symptomatic relapse refers to a distinct, generally focal, acute or subacute event taking place in the CNS. [9] However, the emergence of new MRI lesions in the absence of symptoms is possible, being considered a case of an asymptomatic relapse. [9] Pathologically speaking, both types of relapses reflect the occurrence of inflammatory demyelination, which might, or not, be associated with axonal damage. [9] After symptomatic relapses, remission follows [9], which is a recovery phase characterized by some level of restoration of myelin. [9] Most patients completely or almost completely recover after a relapse. [9] However, in some cases there is only a partial recovery and, in rare situations, individuals never recover. [9] Patients might also experience pseudorelapses, which consist in symptom recurrence from a previous relapse or subclinical lesion, indicating that full recovery did not occur. [9] These events are promoted by stimuli of different nature, and when the stimuli are eliminated symptoms tend to disappear. [9]

The disease is considered active when new symptomatic relapses or asymptomatic MRI activity exist, including enhancing hyperintense-T_1 lesions, new hyperintense-T_2 lesions and enlarged pre-existent hyperintense-T_2 lesions. [9] On the other hand, the absence of activity for a year or more is considered "no evidence of disease activity" (NEDA), which also includes the absence of progression. [9]

Briefly, progression consists in an irreversible deterioration of the neurological function over time, for at least 6 months. [8,9] In some patients, the term pseudoprogression is employed to characterize a disability accumulation that can be disease-related or not. [9] The progressive phase of MS is established one year after the clinical documentation [9], and can be considered active or inactive based on the presence or absence of relapses before the onset of the progressive disease course, respectively. [9] Progressive phase onset is potentially age-dependent [9] and might, or not, depend on disease duration.

Regarding MS clinical course, the factors that describe the current state of the disease are activity and progression. [9] Disease activity is evidenced by clinical relapses or gadolinium (GD)-enhancing lesions, as well as new or enlarged T_2 lesions. [9] On the other hand, progression is based on clinical evidences of increased disability over time, regardless of relapses, in patients experiencing the progressive phase of the disease. [9]

MS clinical courses are usually categorized in one of three types: Relapsing-Remitting MS (RRMS), Secondary Progressive MS (SPMS) and Primary Progressive MS (PPMS). [9] Although it

is considered that the different types of MS consist of a variable clinical expression of the same disease, the possibility that they reflect different diseases with dissimilar pathophysiologic mechanisms has not been ruled out. [9] RRMS is the most common disease type, with which 85-90% of MS cases begin with. [9]

RRMS is characterized by neurologic dysfunction attacks, also designated as episodes or flares, which develop acutely but may last for days and even weeks. [1,9] After an episode, patients frequently regain their neurologic function completely in a period of time that varies from patient to patient, and between episodes neurologic function remains stable. [1,9] RRMS patient's MRI data show evidence of acute and chronic inflammation. [9,10] Acute inflammation is reflected in the emergence of new T_2 lesions and GD enhancement, while chronic inflammation is highlighted by stable and non-enhancing lesions in the brain white matter or by atrophy. [10]

The second type of MS – SPMS – initiates as a typical RRMS but, at a certain time point, the disease course is shifted, the acute episodes become less frequent and a steady deterioration of the neurological function occurs, independently of the existence of attacks. [8,10] Regarding magnetic resonance imaging (MRI), evidences of acute inflammation are less notorious, but brain atrophy becomes more prominent. [9] SPMS ultimately develops in most of RRMS patients and leads to high neurologic disability. [11]

PPMS composes only about 10% of MS cases and is characterized by a constant decline of the neurological function without acute episodes. [12] Similarly to what is observed in SPMS, PPMS patients display less evidence of active inflammation on MRI comparatively to those with RRMS. [9] PPMS patients also present a more balanced gender ratio, higher age of onset and worse prognosis when compared to those with RRMS. [10] It is possible to infer the disease course based on premature prognosis features. Early sensory symptoms are generally prognostic positive, while motor and cerebellar symptoms, premature relapses, and onset age over 40 years old are typically prognostic negative and are associated with a more aggressive and more debilitating disease course. [2]

MS diagnosis is based on two distinct neurological dysfunction episodes that occur, at least, 30 days apart from each other, in different locations of the CNS, in those with one relapse which show MRI evidence of dissemination in space (DIS) and dissemination in time (DIT). [1] DIS is characterized by one or more T_2 lesions located in, at least, two of the four areas of the CNS – periventricular, juxtacortical, infratentorial and spinal cord. [1,13] DIT is recognized by one of two criteria: (1) new T_2 lesions and/or GD-enhancing on a follow-up MRI, with reference to a basal scan, regardless of its timing; (2) Presence of GD-enhancing asymptomatic lesions simultaneously with non-enhancing lesions at any moment. [1]

In the particular case of PPMS, diagnosis criteria include one year of retrospective or perspective progression, along with two of the following three: (1) one or more T_2 lesions in at least one area of the CNS characteristic of MS; (2) two or more T_2 lesions in the spinal cord and (3) positive cerebrospinal fluid (CSF) for oligoclonal bands (OCBs) and/or elevated immunoglobulin G (IgG) levels. [1] Patients that experience a single episode and do not meet the formal criteria are considered to have Clinically Isolated Syndrome (CIS) [1,11], which is subdivided into two categories: solitary sclerosis and single-attack MS [9]. Solitary sclerosis is used to diagnose patients whose imaging results do not meet the necessary criteria for MS diagnosis,

but the displayed lesions are characteristic of MS in terms of morphology and location. [9] When a patient having solitary sclerosis reaches the criteria consistent with MS, the diagnosis might be single-attack MS. [9] Most of CIS patients present asymptomatic T_2 lesions consistent with demyelination, but there are no evidences of DIT. [1,11]

Furthermore, when patients display imaging evidence consistent with MS, but these are incidentally discovered, the diagnosis is Radiographically Isolated Syndrome (RIS). [1]

When RIS patients develop their first MS symptom, they fulfill the criteria for single-attack MS. [9] Thus, it can be considered that PPMS consists in RIS and SPMS refers to RRMS, both followed by the progressive phase.

In sum, all MS patients initiate the disease course with a high-risk period, that is determined by environmental and genetic factors. The active pre-symptomatic phase follows, and this, in its turn, is followed by the relapsing-remitting phase. Ultimately, most patients evolve to the progressive phase. Therefore, MS is a dynamic pathology divided into different phenotypic phases, each phase being associated with disability variations due to uncomplete recoveries after relapses, progression, and extrinsic factors unrelated to the disease.

1.1.3. Risk Factors

MS is a multifactorial disease, since both genetic and environmental factors are involved in disease development. [1,6,14] Among the several known risk factors for MS, the most impactful is family history. [6] As a matter of fact, the risk for individuals related to MS patients increases proportionally with the genetic similarity between them and the proband. [10] Proband's nephews and nieces present a risk 5- to 10-fold higher, but for siblings this increase is of 20- to 30-fold and 200- to 300-fold in the case of monozygotic twins. [1,5,10] Particularly, the HLA classes I and II genes have been firmly associated with MS susceptibility [1,5,14], the HLA-DRB1*15:01 being the main high-risk allele. [5,10] HLA-DRB1*15:01 variant is significantly associated with an increased risk for MS, while the HLA-A*02 variant is thought to have a protective effect against the disease. [14]

On the other hand, genome-wide association studies (GWAS) have identified approximately 110 non-HLA single nucleotide polymorphisms (SNPs) associated with the risk for MS development. [14] Virtually, all these SNPs are located near genes involved in the innate or adaptive immunity [1,2,14], which highlights the fact that MS is primarily an immune-mediated disease.

There is also evidence for a maternal effect in MS. [10] Several observations corroborate this effect, one of them being the fact that half-siblings who are concordant for MS have a doubled probability of sharing a mother than sharing a father. [15] Similarly, there is an increased risk of MS development in half-siblings that share a mother comparatively to those who share a father. [10] Moreover, there is evidence that the risk for MS in siblings that share only the mother does not differ significantly from the risk in full siblings [16]. This observation suggests that a maternal effect might potentially be a major component of MS familial aggregation, possibly resulting from mitochondrial heredity, genetic imprinting or other epigenetic and/or environmental factors. However, genetic predisposition to MS only explains a fraction of the risk for MS. Otherwise,

there would be no differences in risk between monozygotic twins among northern and southern populations for example, as it indeed happens. [17] Environmental factors and lifestyle are key aspects in disease development. Furthermore, unlike genetic factors, many of these features can be modified, having a tremendous potential for disease prevention.

1.1.3.1. Sunlight exposure and vitamin D

Several studies have reported varied incidence and prevalence values for MS according to geographical latitude. [1,2,4,10] This tendency has been partially explained by the differences in sun exposure and, consequently, vitamin D levels, at different latitudes. [18] In fact, MS prevalence maps are very similar to the global distribution maps for reduced availability of ultraviolet radiation (UVR) [10], suggesting an association between MS and sunlight exposure, the disease prevalence and incidence being increased in higher latitudes. [1,2,5,14] This observation has been the basis for innumerous studies aiming to analyze the effects of sunlight exposure and vitamin D on the risk for MS, from whose results indicate that both UVR as well as vitamin D are associated with a lower risk. [1,2,5,14] However, not all UVR-associated effects can be explained by its role in vitamin D synthesis. [19] Several studies showed a negative association between UVR exposure and susceptibility to MS, even after adjustments for vitamin D serum levels. [20,21] Preclinical evidence suggest that sunlight exposure, even independently from vitamin D, exerts a protective effect in a MS murine model of experimental autoimmune encephalomyelitis (EAE), which might be associated with UVR action on regulatory T cells and antigen-presenting dendritic cells. [21] These observations indicate that the UVR effects on MS risk are not fully dependent on vitamin D levels, but vitamin D might mediate some of them. Otherwise, oral administration of vitamin D would have no effects on MS susceptibility, which does not seem to be the case. [22] Furthermore, the intake of fatty fish, which are rich in vitamin D, is negatively associated with the risk for MS, even at higher latitudes. [23] The increased MS incidence in higher latitude locations might result from a vitamin D deficiency, since these areas lack sun exposure compared to regions of lower latitude, which is generally the primary source of vitamin D. [5] However, it is still not acknowledged if it is a cause or a consequence of MS, since MS patients might potentially spend less time on the outside due to heat intolerance. [24]

Regarding vitamin D, diverse epidemiological studies seem to confirm its protective role in the context of MS. [1,2,5,8,14] It has been reported that 1,25(OH)2D3 injection prevents EAE in animal models, while a vitamin D deficiency accelerates disease onset [5], and higher serum levels of 25(OH)D are significantly associated with a decreased incidence for MS [2]. In fact, the vitamin D response element (VDRE), to which the heterodimer composed by vitamin D, its receptor and retinoid X bind to in the nucleus, was recently identified in the promoter region adjacent to the HLA DRB1*150 allele, which is continuously associated with MS pathogenesis. [2] Additionally, besides MS, vitamin D has been associated with other autoimmune disorders, such as inflammatory bowel disease, rheumatoid arthritis and diabetes mellitus [25], which are all more common in women [26]. Therefore, gender-specific physiologic responses to vitamin D might exist. In fact, Spach and Hayes et al. [27] demonstrated that vitamin D supplementation conferred protection against EAE in normal female mice, which was not verified for males or ovariectomized

females. Considering these observations, vitamin D deficiency could be a promising candidate for the maternal factor involved in the pathogenesis of MS. This apparent gender specificity of MS might also explain the increasing incidence of the disease in women.

Interestingly, there seems to be an age-related association between vitamin D levels and risk of developing MS. [28] In a EAE model, it was demonstrated that disease incidence is affected by vitamin D levels in adolescent rats, but not during pregnancy or in adult ones. [29] However, a study analyzing a cohort of US women demonstrated that vitamin D intake during the adolescence period was not correlated to MS risk in adulthood. [30] Furthermore, neonatal levels of 25(OH)D do not show an association with the risk for MS development. [31] As the referred observations suggest, it is still not fully understood whether vitamin D and/or sunlight exposure influence the risk for MS only in specific developmental stages or if this effect occurs throughout an individual's entire lifespan.

Several mendelian randomization studies have showed an association between genetic variants affecting serum vitamin D levels and susceptibility to MS, these studies being the most significative evidence of causal relationship between vitamin D and MS. [32] However, these observations are not irrefutable since, for example, this association is not observed for African-Americans or Hispanics. [33] Therefore, it cannot be considered universally accepted.

1.1.3.2. Epstein-Barr Virus

When approaching the association between the Epstein-Barr virus (EBV) and MS, it is important to refer the "hygiene hypothesis", which states that multiple exposures to infectious agents during early childhood reduce the risk of MS development via modulation of the immune response towards regulatory and helper T cells (Th2) and attenuation of the proinflammatory activity of Th1 cells. [5] This hypothesis could partially explain MS geographical distribution. An example of infectious disease that varies age-wise in different populations is the one caused by EBV. [34] In the case of developing countries, virtually all children are infected in the first years of their life, while in developed countries many children are not infected until adolescence. [5] In fact, EBV seropositivity prevalence presents a latitude gradient parallel to the one of MS. [5] When compared to non-infected individuals, someone who is infected at infancy presents a risk for MS that is 15 times higher, this value rising to 30 when the infection occurs in adolescence or later in life. [2] EBV infection at an early age is typically asymptomatic, but when it occurs in adolescence or adulthood it frequently manifests itself as infectious mononucleosis, which has been associated with an increased risk for MS. [2,5] Particularly, if the infection results in mononucleosis, the risk for MS increases two- to threefold when compared to EBV-positive cases in which mononucleosis does not develop. [5] Therefore, there seems to be an influence of the period of life in which infection happens in the effect it has on MS, in the sense that only individuals infected in adolescence or later in life display an increased risk for disease development. In fact, evidence of an EBV infection prior to adult-onset MS is essentially 100%, and even in sporadic cases where a patient tests negative for a prior EBV infection, it can be a false negative, since antibody response is not measured for all viral antigens. [10] Certainly, the observed 100% prevalence in MS patients can not be due to a hyper immunity state of the host, since there are no increases in antibody

responses against other common pathogens. [10] Particularly, MS patients display significantly higher levels of antibodies against the EBV nuclear antigen 1 (EBNA1). [14] It is important to refer that both infectious mononucleosis and increased EBNA1 antibody titers interact with HLA MS risk genetic variants. [35,36] Particularly, there is evidence of an interaction between infectious mononucleosis (EBV infection-resulting disease) and the HLA DRB1*15:01 allele that results in a higher risk for MS. [37] In addition, Sundström et al. [36] showed that less EBNA-1 reactivity is required to increase the MS risk in individuals carrying the HLA DRB1*1501 allele when compared to the ones in which this allele was not present. Since the HLA risk alleles are involved in T cell adaptive immunity [38], these observations might point to common pathogenic pathways involved in MS pathogenesis.

1.1.3.3. Smoking

Strong lines of evidence point to smoking as an important risk factor for MS, also affecting the disease course and progression. [39] Interestingly, the interaction between smoking and the risk for MS seems to be dose-dependent, as cumulative smoking is associated with a higher susceptibility to the disease. [14] In fact, a study performed by Hernán et al. [40] which comprised more than 200,000 women, showed that individuals who reported 25 or more pack-years of smoking displayed a risk for MS 70% higher when compared to women who had never smoked. Another study employing a cohort of Swedish construction workers reported that ever-smokers presented an increased risk for MS. [41] Regarding the effect of smoking in disease course and progression, O'Gorman et al. [42] showed that MS onset was approximately 4 years earlier in ever-smokers and Healy et al. [43] reported that when comparing three groups of MS patients (patients who continued smoking after diagnosis, patients who quitted after diagnosis and patients that never smoked), the probability of disease worsening was higher for the smoking patients when compared to the other groups, suggesting smoking exacerbates MS pathology. In fact, smoking has been associated with a faster rate of MS progression, as well as with an anticipated transition to the progressive phase of the disease. [39] A study involving RRMS patients demonstrated that the risk of transiting to a secondary progressive phase increased for smoker patients when compared to non-smoker ones. [40] Additionally, evidence show that ever smoker MS patients present a risk 3.6 times higher of transiting to the progressive phase of the disease than non-smoker patients. [40] Similarly, when compared to non-smoker patients, smokers take less time to reach an Expanded Disability Status Scale (EDSS) score of 4 and 6 and, more importantly, smoking cessation in MS patients leads to a decreased risk for EDSS progression. [44] Regarding relapses, another study reported that smoking a pack of cigarettes a day led to a 27% rise in relapse rate. [45] Regarding characteristic MS lesions, there is evidence of an effect of smoking in augmenting GD-enhancing lesions, as well as in increasing T1 and T2 lesion volume. [46] In addition, it was verified that smokers display a more severe neurodegenerative phenotype. [46]

Besides smoking, passive exposure to cigarette smoke has also been associated with an increased risk for MS. Gao et al. [47] demonstrated that EAE mice exposed to cigarette smoke for 14 days before disease induction presented worse disease scores, exacerbated microglia

activation and migration, as well as a more significative macrophage infiltration. The authors suggested that acrolein, a product of oxidative stress, might be responsible for the observed effects, since its inhibition improved the disease course. Additionally, Sundstrom et al. [48] reported that the concentration of cotinine, a nicotine metabolite detected in the blood, has a positive correlation with MS susceptibility. Furthermore, the risk for developing MS is higher in children carrying the HLA-DBR1*15 alleles when exposed to passive smoking. [49] In fact, passive smoking may explain the higher incidence of MS in women and children, since they are more prone to this kind of exposure.

Although the association between smoking and MS is well established, the basis of such relationship is difficult to define. One of the potential hypotheses is that smoking interacts with susceptibility genes, as it is seen for other autoimmune diseases such as rheumatoid arthritis. [50] In fact, several studies have analyzed the interaction between smoking and HLA-DRB1 and HLA-A genotype. Hedström et al. [51] reported a significative interaction between carriage of the HLA-DRB1*15 and the absence of HLA-A*02 in smokers, but not in non-smokers. Co-occurrence of smoking and carriage of the HLA-DRB1*15:01 allele increases the probability of developing MS approximately 14 times, which is much higher when compared to the effects of the sum of each isolated factor. A similar interaction has been observed in passive smoking situations. A study showed an association between passive smoking and both the presence of the HLA-DRB1*15 and the absence of the HLA-A*02 alleles. [52] In addition, there is evidence that children carrying the HLA-DRB1*15 alleles present a higher probability of developing MS when passively exposed to cigarette smoking. [49] These findings suggest that the effect of smoking on MS susceptibility has a dependence on the HLA genotype.

On the other hand, the interaction between smoking and MS pathology might be mediated by the lungs and their microenvironment. A study using an EAE rodent model showed that during the initial phase of the disease, T lymphocytes are not able to go through the blood-brain-barrier (BBB), only acquiring their migratory properties after passing the alveolar space, pointing to the lungs as activation spots for T cells and consequent disease induction. [53] Further research involving bronchoalveolar lavage from MS patients and healthy controls reported a significative increase in the number of alveolar macrophages, T cells proliferation markers and higher expression of antigen presentation-associated entities in smoker patients. [54] In fact, a study has reported that the use of oral tobacco is associated with lower odds for MS [55], suggesting that the observed interaction between smoking and MS might be mediated by the lung irritation caused by smoke inhalation.

1.1.3.4. Alcohol and Caffeine Consumption

Studies evaluating the role of alcohol and coffee consumption on MS have been somehow inconsistent. While some studies show no impact of these substances intake in MS [56], others suggest an inverse association between the two factors [57-59]. Hedström et al [58] demonstrated that high coffee intake is associated with a lower risk for MS and, particularly, that the immunologically active RRMS patients reporting a regular coffee consumption present a reduced risk for reaching EDSS of 6.0. [58] Furthermore, a cross-sectional analysis showed a

positive effect of coffee consumption on disease course and progression for the relapsing form of MS. [60] However, the evidence currently available are not solid enough for substantiating any recommendations concerning coffee consumption in the context of MS.

Regarding alcohol intake, case-control studies evidence a dose-dependent inverse correlation between alcohol consumption and MS, this effect being more pronounced in women in comparison to men. [59] In fact, moderate alcohol consumption during adolescence has been associated with a reduced risk for MS development among both genders. [61] Moreover, Foster et al. [62] demonstrated that drinking alcohol moderately does not have a negative influence in either EDSS or Multiple Sclerosis Severity (MSSS) scores, while Diaz-Cruz et al. [57] showed that an increased total alcohol and red wine intake were associated with lower levels of neurologic disability in MS patients.

It is also important to consider the psychological and societal roles of alcohol consumption, since it is associated to personal contact and social interactions, as well as reduced loneliness, ultimately improving the mental health and general life quality of MS patients. However, there is an elevated MS associated-depression rate, which might lead to substance abuse. [63] Therefore, substance abuse screening and advice should be a part of MS comprehensive care.

1.1.3.5. Gut Microbiota

In the last several years, several clinical studies have highlighted Gut Microbiota (GM) alterations in the context of MS, including reduced microbial diversity and altered relative abundance of certain bacterial phylum, namely Firmicutes and Bacteroidetes, among others. [64,65] It may be relevant to point out that some of these observations are evident in patients undergoing an active phase of the disease, but not for remissive ones. [66]

The observed gut dysbiosis in MS experimental models as well as clinical cases has been associated with immunoregulatory mechanisms involving the gut-to brain axis. This bidirectional route composes a communication system between the gut and CNS, which is mediated by neuronal connections as well as neuroendocrine, humoral, and immune signaling. [64] The CNS regulates several aspects of the intestinal function by promoting gut motility through an extensive innervation structure and by inducing intestinal immune cells' activity. [64] Such mechanisms are executed by numerous molecular entities, which include pro-inflammatory cytokines, neurotransmitters, and specific neuropeptides. [64] On the other hand, the intestinal epithelium, the enteric nervous system (ENS), and the gut-associated lymphatic tissue (GALT)-associated immune cells mediate signal transmission from the gut to the CNS. [64] In this way, the GM modulates several biological aspects of the host. However, at the same time, there is evidence of a modulation role of the host on the intestinal flora. For example, there are specific subtypes of T cells that respond to bacterial antigens, regulating microbial colonization in the gut. [64] These include the cluster of differentiation antigen 1d (CD1d), invariant natural killer T cells (iNKT) and γδ intraepithelial lymphocytes (γδIELs). [64] Therefore, the existence of a link between the gut-colonizing microbes, the immune system and the CNS is evident. Dysbiosis promotes a pro-inflammatory environment in the GALT and results in increased levels of gut mucosa endotoxins. [64] Translocation of bacteria and its components to the mesenteric lymphatic nodes allows

circulating T cells to activate, generating a low-grade endotoxemia. [64] Such alterations impair the integrity of the BBB, facilitating the access of pro-inflammatory molecules to the CNS, which will activate astrocytes and microglia cells, promoting neuroinflammation events. [64]

Kadowaki et al. [67] reported the involvement of the GM on the interaction between the T-cell C-C chemokine receptor type 9 (CCR9) and its ligand CCL25, which is necessary for the development of T lymphocytes in the small intestine. [68] In fact, a reduced function of the CCR9 is observed in RR-MS and SP-MS patients. [67] This results in decreased levels of CCR9+ CD4+ T cells in the peripheral blood, a feature that SP-MS patients also display. [67] The previously referred study additionally demonstrated that germ-free (GF) C57BL/6J mice present lower levels of memory CD4+ T cells, the opposite being observed for specific pathogen-free (SPF) ones after treatment with antibiotics, corroborating the influence of the GM on the activity of these cells. The authors further evaluated the effects of antibiotic therapy on an EAE mouse model, and observed a decrease in disease severity in result of such treatment, suggesting that dysbiosis-mediated modifications in the gut-immune system axis might be associated with the pathophysiology of MS. Similarly, a study evaluating the effects of antibiotic administration in EAE [69] reported a delayed disease development after oral antibiotic treatment, but no apparent effect after intraperitoneal administration, highlighting the role of the microbiome in the disease. Antibiotic-mediated protection is associated with a regulating effect of the dysfunctional T lymphocytes present in the gastrointestinal tract and the CNS, leading to decreased production of pro-inflammatory cells (Th1 and Th17) and cytokines (IFN and IL-17A) and increased secretion of FoxP3+ cells, IL-10 and IL-13. [70-72]

A study conducted by Cosorich et al. [73] aiming to compare the GM of RR-MS patients and healthy individuals found that MS patients in a relapse phase displayed increased levels of Firmicutes and a reduction in Bacteroidetes relatively to healthy individuals, as well as to remitting patients. Additionally, these patients presented lower abundancy of *Prevotella* (propionate-producing bacteria) and higher levels of *Streptococcus mitis* and *Streptococcus oralis*. Furthermore, the authors showed an inverse correlation between the relative abundance of *Prevotella* strains in the small intestine and Th17 frequency. Additionally, Mangalam et al [74] reported that treatment *with Prevotella histicola* suppresses EAE by decreasing pro-inflammatory Th1 and Th17 cells, inducing FoxP3+ CD4+ Treg cells and dendritic cells, and by inhibiting macrophage activity. Similarly to *Prevotella, Streptococcus mitis* is capable of inducing the differentiation of Th17 cells. Thus, the aforementioned study highlights a direct contribution of the microbiome to MS pathology by a mechanism involving intestinal Th17 cells' modulation.

On another note, Chen et al. [66] reported decreased *Adlercreutzia* levels in RR-MS patients. This bacterial specie is involved in phytoestrogens metabolism [65,75], specifically in converting them to monomers [65]. Thus, a reduced abundancy of *Adlercreutzia* leads to impaired phytoestrogen degradation and a consequent rise in oxidative stress and pro-inflammatory cytokine production [65], as IL-6 for example, usually elevated in MS cases [76]. Furthermore, these bacteria are also able to metabolize fiber to short chain fatty acids (SCFAs) [75], its decrease leading to less production of such important metabolites. Consequently, gut health becomes compromised since SCFAs, particularly butyrate, play an imperative role in maintaining the integrity of the intestinal barrier. [77] SCFAs are also considered anti-inflammatory and anti-

oxidative molecules [77], so decreased production of such molecules impairs the host's health at different levels. In this manner, reduced abundancy of phytoestrogen metabolizing bacteria in the gut might be associated with inflammation and demyelination, key features of MS pathophysiology.

Accordingly, a study performed by Jangi et al. [78] demonstrated that the GM of MS patients is characterized by reduced levels of *Butyricimonas*, a butyrate-producing bacteria. Besides its anti-inflammatory, antioxidant and integrity-promoting properties, butyrate is also able to induce Treg cells [79]. Thus, reduced levels of butyrate-producing bacteria in the gut are associated with several autoimmune and inflammatory diseases, including MS. Additionally, the authors found increased levels of *Methanobrevibacter* and *Akkermansia* in MS patients. *Methanobrevibacter* presents the ability to recruit human dendritic cells [80], possessing a relevant role in several inflammatory processes. In fact, a study regarding pediatric MS [81] showed that colonization with this bacterial strain led to decreased time to relapse. Similarly, *Akkermansia* bacteria present pro-inflammatory activity, which stems from its ability to degrade mucus, potentially damaging the intestinal epithelium and increasing local immune cells' exposure to microbial antigens. [82] Thus, decreased abundancy of *Butyricimonas* combined with increased levels of *Methanobrevibacter* and *Akkermansia* points to a clear pro-inflammatory pattern in the GM of MS patients.

Although it is currently widely known that GM dysbiosis is a feature of MS patients, the microbiome phenotype characteristic of the disease is not yet defined, and studies that aim to establish a causal relationship between the observed patterns in the GM of MS patients and the pathophysiology of the disease are imperative to get to the bottom of this subject. Yet, aforementioned observations regarding the role of the GM in the context of MS, dietary supplementation with functional ingredients able to promote gut health has been emerging as a potential complementary therapeutic strategy for the disease. A healthy and balanced diet in combination with probiotics and vitamins is lately being proposed as crucial to promote intestinal eubiosis. [83] Additionally, such nutrition patterns lead to increased production of anti-inflammatory mediators by the intestinal flora, such as SCFAs and microbial anti-inflammatory molecules (MAMs), as well as to a balanced Treg/Th17 ratio. [64] Furthermore, there is evidence that MS patients subjected to fecal microbiota transplantation and following modified diets experience a normalization of some microbial populations and decreased disease severity. [84]

Furthermore, MS patients often deal with bowel dysfunctions, characterized by constipation and fecal incontinence coexistence and alternation, severely impacting their quality of life and social interactions. [85] Once more, a healthy and balanced diet in combination with probiotics, vitamins, and potentially dietary supplements with functional ingredients displaying gut health-promoting properties could be useful for disease management in this context.

Therefore, it is possible that this kind of therapeutic approach alleviates MS course, making a healthy and balanced diet an imperative protective and prophylactic factor in the context of MS, particularly, but not exclusively, through its influence on the GM.

Table 1. Differences in microbial populations in MS.

Bacteria genus	Microbiota of MS patients	References
Prevotella	Decreased	[65,73]
Bacteroides	Decreased	[65,86]
Parabacteroides	Decreased	[65,87]
Mycoplana	Increased	[65,87]
Acinetobacter	Increased	[65,87]
Haemophilus	Decreased	[65,87]
Sutterella	Decreased	[65,78]
Adlercreutzia	Decreased	[65,86]
Streptococcus	Increased	[65,73]
Coprobacillus	Decreased	[65,66]
Lactobacillus	Decreased	[65,66,87]
Akkermansia	Increased	[65,87]
Clostridium	Decreased	[65,66]
Faecalibacterium	Decreased	[65,87]
Dorea	Increased	[66,87]

1.1.3.6. Nutrition and Lifestyle

There are several indications of a potential influence of dietary habits and lifestyle and the general course of MS. An example is the higher disease prevalence in Western countries less exposed to sunlight. [18] Having been verified that such effect is not observed in the correspondent latitudes in the East, it is speculated that the differentiating factor is the Western populations' lifestyle, characterized by sedentarism and unhealthy eating habits. It is now known that specific dietary factors are capable of exerting pro- or anti-inflammatory effects [88], acting directly in the metabolism, or indirectly via the GM, since it influences human metabolic and inflammatory states, and through the gut-to-brain axis it can affect the CNS [89].

Recently, it has been hypothesized that intestinal dysbiosis might be involved in the pathophysiology of MS, as previously highlighted. [64] For example, Berer et al [90] demonstrated that the GM is necessary for Myelin Oligodendrocyte Glycoprotein (MOG)-induced EAE development. Additionally, an altered GM composition in MS patients has been verified. [66] These kind of effects often lead to an imbalanced Treg/Th17 ratio [91], expression of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) [92] and compromise the intestinal barrier's integrity. [93] In fact, it has been verified that the intestinal epithelium permeability is altered in MS patients [94], and some of them also display microbial translocation from the gut to circulation. [95] It is expected that the factors causing the intestinal barrier leakage are also responsible for disrupting the BBB, which is the base of neuroinflammatory processes [96]. Therefore, it can be hypothesized that gut dysbiosis, which can derive from inadequate dietary habits, is the main association factor between nutrition and neuroinflammation.

Mielcarz and Kasper [97] reported that commensal bacteria and probiotic species play an important role in immunomodulation and are capable of reducing disease severity in EAE models,

possibly through regulating the Treg/Th17 ratio, which is altered in several autoimmune diseases, including MS. [98] Many studies, including epidemiologic analysis, pre-clinical studies and clinical trials, highlight the relevance of dietary factors on the risk for MS development as well as on the disease course. [99,100] There is growing evidence of the influence of dietary patterns on the autoimmune and inflammatory events linked to MS, resulting from an interaction between dietary molecules and transcription factors involved in immunologic and metabolic pathways. [99,100] The following paragraphs attempt to scrutinize some evidence of dietary components intake and MS course.

1.1.3.6.1. Fats

Epidemiological studies show a correlation between MS mortality and dietary fat intake, more pronounced regarding saturated fat consumption, specially from animal origin. [101] The dietary patterns followed in South Asia are characterized by low levels of saturated fatty acids (SATAs) and high intake of polyunsaturated fatty acids (PUFAs), namely Ω-3, in particular the ones derived from sea food. [101] Additionally, these regions' inhabitants consume significative amounts of fruits, vegetables, and other fiber-rich foods. [101] It can be conjectured that the rare MS prevalence in these areas is linked not only to genetic and epigenetic factors, but also to these populations' dietary habits. In fact, in the 1950's, Swank et al [102] hypothesized that the amount of fat consumed in the diet might explain the geographical variation seen for MS prevalence. By investigating MS incidence in Norway, the researchers found significative differences between regions where inland farming was performed and regions known for costal fishing, the ratio of MS cases being 4.1 in the first areas and 1.2 in the latter. These results were supported by the nutritional habits of inland farmers, whose diets were marked by high consumption of meat, butter, milk, and animal fat.

Based on these observations, Swank further evaluated the effects of a low-fat diet in MS. In the two years the patients followed a low-fat diet, the frequency of attacks was approximately half of the one observed the first year before the regimen. [103] The same individuals were then followed for nearly three decades, and beneficial effects of the low-fat diet were evident, since the group consuming 20g or less of saturated fat per day presented a mortality rate three times lower than the groups with intakes of 24 and 42g.

Another study evaluating the impact of reduced fat consumption in MS also highlighted physical and mental health improvements in patients undergoing Ω-3 supplementation. [104] However, these observations need further confirmation, since Torkildsen et al. [105] did not find beneficial effects of Ω-3 supplementation in MS activity.

In EAE animal models, it was showed that a high-fat diet resulted in more severe episodes, as well as increased disease scores, as consequence of a mechanism involving the Renin Angiotensin system. [106] However, in the experiment performed by Kim et al. [107], implementation of a ketogenic diet, which is high in fat, displayed beneficial effects, inhibiting pro-inflammatory cytokines production, leading to ventricular lesions repair, reducing atrophy, and improving the synaptic activity in the hippocampus. The existence of conflicting results is evident,

highlighting the need for further research in order to elucidate the role of fat consumption on MS course and activity.

1.1.3.6.2. Salt

Evidence of an association between an elevated dietary salt intake and exacerbation of disease course is available in EAE animal models. [108] However, the amount of salt used in these studies is equivalent to more than 500 mg/day in humans, which makes the implementation of these kind of research difficult to perform in human populations. Nevertheless, they are not inexistent. A study involving Argentine MS patients showed that individuals consuming high amounts of salt displayed an increased number of relapses and more MRI disease activity when compared to patients with a lower salt intake. [109] However, subsequent studies have not corroborated these results. [110,111] Therefore, there is still no consensus on the influence of high salt diets in the risk or prognosis of MS.

1.1.3.6.3. Dairy

Since dairy products have abundant levels of vitamin D and MS is associated with a deficiency in this vitamin, it would be expected that dairy intake would be somehow beneficial in the context of the disease. However, there is evidence of adverse effects of whole milk consumption during adolescence in MS. Munger et al [30] reported that women consuming whole milk three or more times a day presented a risk of developing MS 47% higher relatively to women who consumed less than one portion daily. It was suggested that these effects can derive from abnormal T cells responses to milk antigens. In particular, milk protein butyrophilin has been associated with MOG in EAE models and MS patients in the context of antigen mimicry. [112] Gut microbiota might also be involved in such events, since a high dairy intake can lead to a favored proliferation of specific bacterial species which, in turn, might affect the immune system. [113]

Moreover, a study performed in 2014 highlighted that MS patients who did not consume dairy reported less recent disease activity and improved general health in comparison to patients consuming these products. [114] Similarly, Fitzgerald et al. [115] verified the existence of an inverse association between dairy use and disability severity by using the North American Research Committee on MS (NARCOMS).

1.1.3.6.4. Fruits and Vegetables

Evidence suggest that a significative intake of fruit and vegetables is associated with reduced disability and MS disease activity. [114] In fact, it was reported in pediatric MS that, excepting potatoes and legumes, one-cup equivalent intake of vegetables decreases relapse risk in 50%. [116] Furthermore, a pilot study comparing the outcomes between patients following a high vegetable/low protein (HV/LP) diet and patients in a typical Western diet verified that the first group displayed lower levels of PD-1 and IL-17 positive T cells as well as an increased number of PD-L1+ anti-inflammatory monocytes. [117] Regarding gut health, the patients who adopted a

HV/LP diet presented higher abundancy of *Lachnospiraceae*, which was associated with anti-inflammatory TGFβ+ and IL-10+ Treg cells and monocytes. In fact, GM is able to ferment fiber-rich foods, such as fruits and vegetables, to SCFAs, immunomodulatory entities which induce Treg cells differentiation and reduce pro-inflammatory cytokines production. [101] In particular, butyrate administration proved to be beneficial in an animal model of MS, since it improved EAE disease course, increasing IL-10 production and Tregs Foxp3 levels. [118]

Furthermore, fruits and vegetables are an important source of tryptophan, whose metabolites stimulate the aryl hydrocarbon receptor (AhR), activating Foxp3+ Treg cells and IL-10-producing type I T cells, and modulating Th17 cell differentiation. [119] Additionally, many of these metabolites are capable of crossing the BBB, activating AhRs expressed by astrocytes, which inhibits the recruitment and activation of monocytes and microglia. [120] Therefore, tryptophan metabolites might play an important role in the neurodegenerative component of MS.

1.1.3.6.5. Physical Exercise

A lifestyle including the regular practice of physical exercise is almost linearly associated with better outcomes for several chronical health conditions, as well as decreased all-cause mortality rates. [121] This is also applicable in the context of MS.

Regarding MS animal models, it was showed that EAE mice which were able to run freely in a cage wheel displayed lower disability scores, both in the acute and progressive disease stages, effects possibly attributed to preservation of neural synapses and increased dopamine release induced by physical exercise. [122] Similarly, a study evaluating the association between consistent swimming training and EAE-mediated responses reported increased levels of Brain Derived Neurotrophic Factor (BDNF) in the brain and spinal cord in the exercised group, which can be a consequence of improved myelin repair mechanisms involving oligodendrocyte proliferation and/or regeneration. [123] Additionally, the progression of spinal cord demyelination was less pronounced in the exercised EAE mice. Other lines of evidence support the involvement of immunomodulation mechanisms in EAE improvement in result of physical exercise. Einstein et al. [124] reported that T cells transfer from EAE mice following a treadmill exercise protocol to sedentary mice reduced disease severity in the recipient animals in comparison to mice receiving cells from sedentary donor EAE mice. Likewise, in a study performed by Fainstein et al. [125] a similar experience was conducted, comparing the effects of high-intensity and moderate exercise. The authors showed the transfer of T cells from mice subjected to high-intensity exercise had a greater influence in EAE severity in sedentary recipient mice when compared to the effects caused by cells derived from mice following a moderate exercise plan. However, regarding the first study [124] the transfer of T cells did not have beneficial effects on the pathological features of EAE, highlighting the lack of a direct neuroprotective effect of physical exercise. Moreover, by evaluating the effects of both exercise protocols in *E. coli* bacterial counts and dissemination, the authors of the second study [125] suggest that exercise does not affect the innate immune response.

Regarding cuprizone (CPZ)-induced demyelination mouse models, evidence suggests that physical exercise reduces motor deficits and increases the hippocampal expression of

neurotrophic factors, as well as microglial density in the particular case of high-intensity interval training (HIIT), reducing neuroinflammation. [126]

One of the most disabling symptoms of MS is fatigue, which is experienced by the vast majority of patients. [127] Regarding the effects of physical exercise on fatigue, several studies highlight exercise-mediated improvements in MS patients. [128] Similarly to fatigue, depression is one of the most frequently MS-associated symptoms, possibly exacerbating other comorbidities such as pain, cognition, and general quality of life. [127] The available observations on the effects of physical activity on depression symptoms in the context of MS are significantly heterogenous. While some studies report exercise-induced mood improvements in MS patients [129], others fail to notice any effect on this symptom [130]. Although the information regarding the influence of exercise in cognition in MS is scarce, there is evidence of a positive association between aerobic exercise and cognitive features, particularly increased white matter integrity and faster information processing. [131] However, considering the dissimilar results obtained in the available studies on the effects of physical activity in MS patients, it is considered that there is not sufficient reliable evidence that exercise improves Health-related quality of life (HRQOL) in the MS population. [127] Thus, there is a great need for higher quality clinical trials, including similar paradigms and endpoints and which consider different disease courses and different grades of disability.

1.1.3.6.6. Obesity and Body Mass Index (BMI)

There is growing evidence of a correlation between obesity and the risk of developing MS. [132] Large cohort studies have been associating obesity in the adolescence period with the risk for MS in women. [14] Although this association showed to be more significative for BMIs above 27, even a slight excess of weight correlates with an increased risk for disease development. [14] Interestingly, adolescence seems to be the critical phase regarding body weight influence on the risk for MS in adulthood, since an elevated BMI at 10 years old, for example, is not associated with future risk for developing the disease. [133]

The relationship between obesity and MS might be mediated by deregulation of leptin, ghrelin, and adiponectin levels, which are differentially associated with BMI. [134] In fact, studies show that inducing EAE leads to increased levels of leptin. [134] In addition, animals possessing leptin-associated genetic mutations or that do not present its receptor display a higher number of Treg cells when compared to controls, and administration of soluble leptin receptor to EAE mice showed to reduce body mass, as well as CNS lesions. [135] Similarly, in situations of low leptin levels induced by fasting, EAE mice display a reduced number of effector T cells and a lower disease severity. [136] However, leptin-induced obesity composes only 2 to 3% of total obesity in the general population. [137]

On another note, low-grade inflammation is an underlying condition in obesity, leading to a rise in the production of pro-inflammatory mediators in the adipose tissue. [138] Furthermore, obesity reduces vitamin D bioavailability, which is also a cause of augmented pro-inflammatory activity. [139] Any of these mechanisms can be responsible for activating autoreactive cells of the adaptive immune system, possibly resulting in the neuroinflammation observed in MS cases.

Interestingly, the fact that the antigen-presenting cells that activate T cells are encoded by the HLA genes [140], which in turn interact with obesity [134], corroborate the hypothesis that obesity is a predisposition factor to MS, by promoting adaptive immunity processes. In fact, as it happens for smoking and the EBV virus, there is an association between the Body Mass Index (BMI) and HLA genetic variants. [133] A study performed by Hedström et al. [141] showed striking interactions between adolescent obesity and the HLADRB1*15 and HLA-A*02 variants, since it reported that individuals carrying the HLADRB1*15 allele and not the HLA-A*02 who present a high BMI, display a risk for MS approximately 14-fold higher. Evidence suggests that the presence of the HLADRB1*15 variant might amplify the inflammatory processes associated with obesity. [141] Interestingly, there seems to be a relationship between obesity and EBV infection, since it was verified that the risk for MS rises from two-fold in cases of obesity or EBV infection in an isolated manner, to 14 fold when the two factor co-occur. [142]

It is important to note that data associating obesity with MS pathophysiology is significantly relevant regarding disease prevention, particularly for high-risk individuals.

1.1.4. Pathophysiology and current pharmacological approaches

Since the pathophysiological mechanisms underlying MS present a multifactorial nature, it has not yet been possible to determine the exact cause of the disease. However, the available evidence suggests that MS pathophysiology is based on two major components: neuroinflammation and neurodegeneration. Nevertheless, research in the field of MS continues to suffer due to the lack of knowledge on the initial events leading to disease emergence and an incomplete vision of the processes responsible for its development.

1.1.4.1. Autoimmunity

The hypothesis that MS composes an autoimmune disorder is corroborated by several lines of evidence. In fact, it can be affirmed that the immunological aspect is the most well-known pathophysiological component of the disease, and its directly associated with an underlying inflammatory state. [143] Although the primary events leading to excessive immune responses remain undefined, a possible explanation includes the involvement of microbial antigens. [143] This hypothesis is designated by molecular mimicry, consisting of the activation of autoreactive T lymphocytes through cross-reactivity by viral and/or bacterial antigens structurally similar to CNS proteins, such as myelin basic protein (MBP), MOG and proteolipid protein (PLP). [143] The interaction between T cells and brain endothelial cells allows their migration to the CNS, leading to an exacerbated immune response and an extension of the number of molecules susceptible to become antigens (epitope spreading), which further fuels the inflammation. [143]

Upon receptor activation, T lymphocytes differentiate into different subtypes of effector cells, including Th1, Th2 and Th17 cells. [143] Th1 cells display a pro-inflammatory phenotype and are responsible for the production of cytokines such as TNF-α, IFN-γ, IL-12, IL-15 and IL-17. [143] The potential involvement of deregulated Th1 cells in autoimmunity development in MS is

highlighted by IFN-γ-enriched active lesions in cases of both EAE and MS. [144] On the other hand, Th2 lymphocytes are associated with protective effects and produce anti-inflammatory cytokines, including IL-4, IL-5, IL-6 and IL-13. [143] Other subtypes of T cells, such as FoxP3+, CD4 CD25+ Tregs, Tr1 and Th3 display anti-inflammatory activity. [145] Regardless, whether their effects are beneficial or prejudicial, movement of such cells to the CNS might compromise the integrity of the BBB. [143] In particular, Th17 cells have a significant ability to perform tissue infiltration, triggering a sequence of events that lead to a powerful inflammatory response [144]. This ability to cross the BBB and promote inflammation through CD4+ T cell recruitment and pro-inflammatory cytokine production confers Th17 cells a key role in the pathophysiology of MS. [73] More recently, the role of the relationship between these cells and the intestinal milieu in the immune response observed in MS has been clarified. **(Fig. 3)** It is now known that both Th17 and Treg cells are highly frequent in the intestinal tissue. [73] Notably, the GM is able to induce Th17 cell differentiation in the gut, allowing these cells to accumulate in GALT. [146] Furthermore, evidence shows that the acquisition of a myelin-reactive phenotype on the part of Th17 cells in the gut increases their pathogenicity as well as their ability to trigger immune responses in the brain. [73] Therefore, the gut might be considered as a meeting point for the immune function, microbiota, and autoimmunity mechanisms.

Several mechanisms might be responsible for the communication between the gut and the CNS, namely metabolite production by the gut bacterial population. [146] SCFAs, for example, can induce Treg cells activity through histone deacetylases (HDACs) inhibition, receptors coupled to G proteins, and dendritic cells stimulation. [146] On another hand, tryptophan metabolites act on AHR receptors as well as on Th17 cells, repressing their activity. [146]

It is important to highlight the fact that the production of the aforementioned metabolites is highly affected by the dietary lifestyle. [147] These observations are in line with a great impact of nutrition on the intestinal-specific T cells responses and, as consequence, in the CNS. Thus, dietary patterns may have therapeutic implications not only in MS but also in the context of other autoimmune disorders.

Overall, the available data suggest that Th1 and Th17 lymphocytes are the core T CD4+ cells involved in MS pathophysiology, and a deviation of T cell differentiation towards these subtypes might be in the origin of disease development. [143]

Besides T cells, there is also evidence that point to B cells as components of the pathophysiological mechanisms of MS, since during the immune response characteristic of the disease these cells are activated and cross the BBB. [143]

Together, the available observations highlight the deregulation of immune cells and their ability to reach the CNS as a key factor for MS development. However, it is imperative to define the source of such impairment and phenotypical skew in order to establish a causal relationship and potentially inhibit and/or prevent such events from occurring.

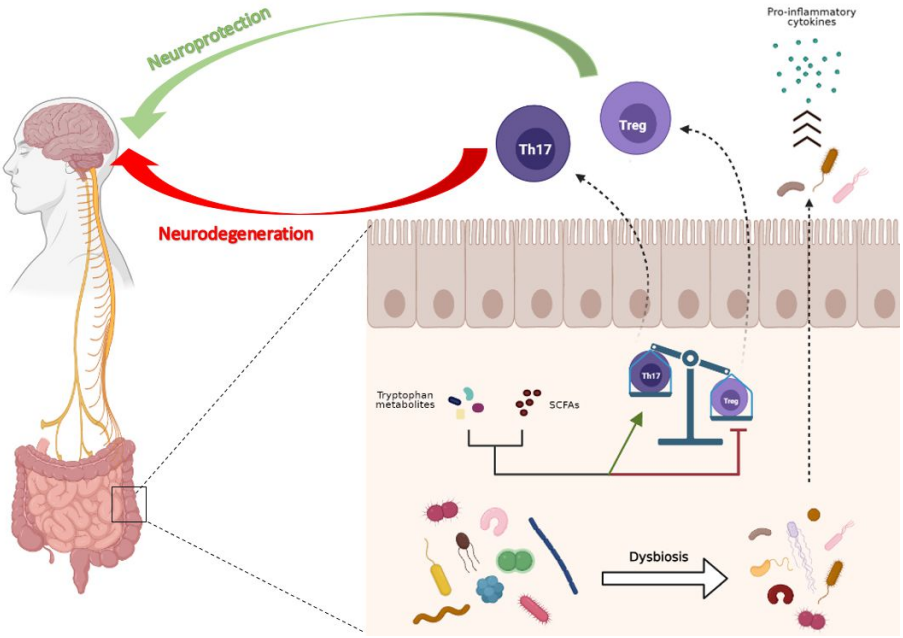


Figure 3. Treg/Th17 balance in the gut microbiota-brain crosstalk characterizing MS.

Currently, several immunomodulatory and immunosuppressive therapies are used to treat MS. Azathioprine, for example, is being used since 1963 to treat MS patients, and has shown to decrease clinical scores and delay disease progression. [148] However, it presents adverse side effects, namely gastrointestinal perturbations. [148] More recently, the use of interferon beta (IFNβ) and glatiramer acetate (GA) has been approved for MS management, being employed as combination therapy. IFNβ has shown to reduce myelin reactive T lymphocytes as well as the expansion of T cell clones, acting as an anti-proliferative agent. [148] Furthermore, it inhibits the activity of several cytokines associated with Th17 cell differentiation. [148] Similarly, GA inhibits myelin reactive T cell activation and proliferation and promotes a switch from secretion of Th1 cytokines to Th2 ones.[148] GA specific T cells have shown the ability to secrete BDNF and neurotrophic growth factor (NGF), presenting neuroprotective and reparative properties. [148]

When interferons and glatiramer acetate are not effective in MS management, immunosuppressive agents can be given to patients as monotherapy. An example is cyclophosphamide (CYC), which has shown to reduce pro-inflammatory cytokines secretion (e.g.: IFNγ, IL-12) and increase anti-inflammatory cytokine levels, such as IL-4 and IL-10 in the CSF and blood of MS patients [149], which highlights the Th2-type response induced by CYC. Moreover, unlike many of the currently approved pharmacotherapies for MS which do not penetrate the BBB, CYC displays high bioavailability in the CNS. [150] Other immunosuppressive agent which have shown to have beneficial effects on the context of MS is methotrexate, commonly used in the treatment of cancer. [148,150] Research shows that the drug reduces relapse rates in MS, along with decreasing disease progression. [151] Similar effects are observed upon treatment with mitoxantrone, a myelosuppressive agent which has proven to significantly delay disability progression. [152]

Natalizumab and fingolimod are two disease-modifying drugs used to treat RRMS, usually as a second-line treatment. [153] Natalizumab represses immune cell infiltration in the BBB, while

fingolimod inhibits lymphocytes outing from the lymph nodes and promotes an anti-inflammatory phenotype for macrophages. [153] Similarly, dimethyl fumarate (DMF) also promotes immune cells transition to an anti-inflammatory environment. [153] Monoclonal antibodies such as Alemtuzumab and Ocrelizumab have been more recently employed in MS treatment, targeting T and B cells, respectively, and causing their depletion. [153]

1.1.4.2. Demyelination and Axonal Damage

Although MS pathophysiology is complex and heterogenous, demyelination and axonal degeneration are the main characteristics of the disease. [154] Sclerotic plaques are characterized by destruction of oligodendrocytes (OLGs) and myelin sheaths, followed by recognition of myelin epitopes by immune cells, giving rise to an autoimmune episode in which T cells attack myelin and the cells that produce it even further. [155] Additionally, macrophages phagocyte and opsonize myelin, and B cells release antibodies against it. [155]

For many years, it was thought that the key factor leading to demyelinating events was the activation of peripheric autoreactive T lymphocytes, which infiltrated the CNS and triggered an excessive inflammatory response. According to this hypothesis, myelin-specific reactive T cells, B cells, macrophages and dendritic cells promote the recruitment of additional macrophages through cytokine and chemokine production, creating a cellular infiltrate that leads to the death of oligodendroglia cells and subsequent demyelination. [156] Furthermore, microglia and astrocytes are activated, resulting in axonal degeneration. [154,156] However, questions began to arise regarding such hypothesis, since there is evidence pointing to a primary neurodegenerative process occurring independently from immune mediated inflammation. [154,156] Thus, it has been suggested that besides immune cells, many other factors might be involved in the demyelination process. Evidence pointing to such hypothesis arise from studies in EAE models, in which it is reported that T cells are not capable of destroying myelin on their own, the action of B cells, antibodies and cytokines being also necessary. [154] Furthermore, myelin detachment from axons is observed in the lumbar region of the spinal cord on a pre-clinical phase of EAE, without infiltration of immune cells. [157] These observations rise the hypothesis of a new demyelination mechanism involving a primary step of myelin axonal detachment. Additionally, multilayered structures of myelin are observed in the chronic phase of the disease [154], suggesting that such excessive myelin results from OLGs dysfunction in the spinal cord.

In fact, OLGs and myelin destruction can be mediated by several mechanisms. In the particular case of OLGs death, Lucchinetti et al. [158] identified four kinds of mechanisms: (1) autoimmune demyelination mediated by T cells; (2) autoimmune demyelination mediated by B cell-produced antibodies; (3) distal oligodendrogliopathy and apoptosis and (4) primary OLGs degeneration. The authors further reported that such processes are homogenous for a unique individual but vary between different patients. Regarding antibody-mediated demyelination, there is evidence of an association between anti-MOG and anti-MBP antibodies and myelin vesicular disruption in active MS lesions [159], highlighting the potential involvement of autoantibody reactions in demyelination events. Moreover, such antibodies have been detected in the serum of RRMS patients [160], suggesting that MOG might be a target from the humoral immune response.

On another hand, innumerous diffusible factors, such as IFN-γ, TNF-α and glutamate, might be involved in the demyelination process. In the particular case of IFN-γ, studies show that its overexpression in OLGs promotes myelin destruction in a chronic manner. [161] Similarly, TNF-α overexpression has been shown to associate with progressive demyelination in animals' models of CNS inflammation. [162] However, it was established that such effect is dependent on the TNF-α receptor, since receptor I displays a cytopathic action, but receptor II presents neuroprotective properties and might promote Treg cells function. [162] Furthermore, glutamate homeostasis is found to be altered in MS patients, leading to a central accumulation of this neurotransmitter that affects oligodendrocytes, astrocytes, endothelial cells, and immune cells. [163]

More recently, a hypothesis stating that demyelination occurs not only as a result of events beginning in the systemic compartment and reaching the CNS, but also as a consequence of OLGs damage, has arisen. In fact, type III MS lesions display nuclear condensation and fragmentation of OLGs [158], suggesting that such alterations are intrinsic to these cells. More recently, a mechanism mediated by the protease Kallikrein 6 (KLK6) has been highlighted as a potential cause of demyelination in EAE models, since this protein was found upregulated in the context of the disease. [154] High levels of KLK6 are thought to be associated with myelin-related proteins degradation (eg.: MBP, MOG) as well as loss of OLGs processes. [164] By ablating the KLK6 gene in an EAE mouse model, Bando et al. [165] showed that knockout animals presented lower clinical scores, a reduced permeability of the BBB and significantly less myelin abnormalities, suggesting an involvement of the protease in MS pathogenesis. Furthermore, the fact that the referred abnormalities were observed before the formation of a cellular infiltrate suggests that KLK6 activation in OLGs might be associated with soluble molecules, such as anti-MOG autoantibodies. In fact, the authors demonstrated that cultured OLGs subjected to such antibodies suffered morphologic changes and displayed high levels of KLK6. Collectively, these observations highlight OLGs relevance for MS pathophysiology, contradicting the idea that these cells have a passive role in the demyelination process.

Axonal degeneration occurs in parallel with the demyelinating events, contributing to disability progression of MS. Demyelination promotes a series of adaptative changes on axons, including an altered ionic channels distribution through the axolemma. [159] Although this event can occasionally result in restored impulse conduction, it becomes slow and continuous or remains blocked, especially if the axons present a significative diameter. [166] Additionally, axonal excitability can increase, and ectopic impulse currents can be generated, which might relate with some of MS typical symptoms, such as spasms, ataxia, paresthesia, and pain. [159]

Although demyelination and axonal degeneration are usually paired as two simultaneous events which characterize MS, myelination does not seem to be completely necessary to maintain axonal integrity, as is suggested by the fact that *mbp* mutant mice do not present significative signs of axonal damage. [167] In fact, several lines of evidence point to OLGs as the key players in axonal protection. A study performed by Oluich et al. [168] showed that mature OLGs ablation before demyelination results in acute axonal lesions, suggesting that these cells support does not rely exclusively on myelin production, their loss having a major role in axonal degeneration. Similarly to demyelination, axonal damage processes are multifactorial events, involving several

different types of cells and mediators such as T lymphocytes, macrophages, antibodies, and free radicals, among others. [155] In fact, a study performed by Neumann et al. [169] showed that T cells and macrophages exist in close proximity to damaged axons in MS lesions, and that when neurons and T cells were cultured together *in vitro*, fixation of the T cells to axonal processes and axon transection are detected. Such effects might result from the release of toxic soluble factors, for example perforin, which is produced by CD8+ T cells [170] and that is found elevated in MS active lesions [171]. Interestingly, there is evidence of a beneficial effect of perforin expression inhibition in the MS animal model of Theiler's virus encephalopathy, since animals subject to such condition seem to be protected against neuronal loss. [172] Other molecules and pathways have been associated with axonal degeneration, nitric oxide (NO) [173], glutamate metabolism [174] and antibodies produced by B cells [155] being some examples. Thus, it is important to consider the heterogeneity of the pathogenetic mechanisms underlying axonal damage for the design of targeted therapies.

1.1.4.3. Remyelination: A Window of Opportunities

Remyelination consists of the formation of new myelin sheaths around axons residing in the CNS in response to the myelin loss characteristic of MS. [175] It is considered a reparative process, allowing the restoration of axonal conductive functions, and conferring neurologic protection. [175] Although myelin produced in these conditions shows to be morphologically and functionally different from the original myelin found before demyelinating events, the remyelination process displays significative similarities with the developmental myelin formation. [175] This process can be divided in two main phases, the first being the recruitment phase, which consists of the colonization of the lesioned areas by oligodendrocyte precursor cells (OPCs); and the second being the differentiation phase, when these cells transform into myelin-producing OLGs. [175] Both stages are associated with distinct and specific molecular patterns, which are responsible for promoting a switch on OPCs from a proliferative environment to a differentiative one. [175] Such transition is, in part, fostered by the conjoined activity of molecular entities expressed by OPCs themselves, such as Olig2 and Nkx2.2. [175] Therefore, these protein's expression levels allow to discern quiescent OPCs from active ones, facilitating the identification of lesions where remyelination is occurring as well as ones in which such process has failed. [175]

Furthermore, several growth factors have been linked to OPC proliferation, including the glial growth factor 2 (GGF2), fibroblast growth factor 2 (FGF2) and platelet-derived growth factor (PDGF), the latter being the main inducer of OPC proliferation in response to demyelination. [175] Contrarily, FGF2 inhibits this process. [175] Such as it is verified for the recruitment phase, OPC differentiation into myelinating cells involves the action of specific molecules. An example is the transcription factor Olig1. In fact, Arnet et al. [176] observed in Olig1-knockout mice that OPCs were recruited to the lesioned areas, but they would not differentiate into OLGs. Additionally, the activity of growth factors appears to be extremely relevant for the success of the remyelination process since insulin-like growth factor 1 (IGF-1) and transforming growth factor 1 (TGF1) seem to promote OPC differentiation. [177]

Due to the enormous complexity of the remyelination process and its regulation, the efficacy of such event depends on a series of factors, namely the availability of OPCs and OLGs in lesions, the interaction between these cells and axons, and the presence of astrocytes and macrophages in the demyelinated areas. [175] Giving the variety of mechanisms involved in remyelinating events, remyelination failure probably results from dysfunctions in several pathways, and not from an isolated mechanism. One of the potential causes for remyelination failure in the context of MS is the incapability of OPCs to differentiate, possibly due to the lack of promoting molecules or, alternatively, to the presence of inhibitors, as is the case of hyaluronan which accumulates in MS lesions [178]. Moreover, there is evidence showing that the presence of dysfunctional OPCs might repress lesion repopulation by functional cells, impairing the remyelination process. [179] On another hand, it can be axons that stop being responsive to remyelination [180], possibly through the expression of inhibitory factors such as PSA-NCAM, as well as alterations in proteins located at axonal nodes and perinodes. [175] Particularly, studies show that MS patients display increased cerebral levels of Notch-1 and Jagged-1 [181], molecules known by inhibiting OPC differentiation during development, the Notch-Jagged pathway being in part responsible for limiting remyelination events in the CNS. [182] Taken together, the remyelination failure observed in MS probably results from a combined deregulation of several of the cellular and molecular mechanisms needed for it to occur, and unravelling such mechanisms in the context of MS may indorse the development of potential remyelination-promoting therapeutic agents.

Strategies for inducing remyelination can be divided in two main categories: (1) promoting endogenous remyelination and (2) myelinogenic cells transplantation. [175] Antibody therapy has been also revealing to be useful in promoting myelin regenerative events through modulation of OPCs recruitment and differentiation, by preventing OLGs death and via immunomodulatory effects. [175] Additionally, several drugs have been studied regarding their ability to promote remyelination, namely through enhancing OPC differentiation, as is the case of Opicinumab, Clemastine, Benztropine, Miconazole, among others. **(Table 2)**

Table 2. Overview of the current remyelination-promoting drugs (drug repurposing)

Drug	Target	Mechanism for promoting remyelination	References
Miconazole	ERK1/2, CYP51	Promotes OPC differentiation through MEK-dependent phosphorylation of ERK1/2 and inhibition of CYP51	[183-185]
Clobetasol	GR/Smo agonist	Promotes OPC differentiation through glucocorticoid receptor (GR) signaling	[184,185]
Clemastine	M1 antagonist	Promotes OPC differentiation through antimuscarinic antagonism	[183-186]
GSK239512	H_3 receptor antagonist/inverse agonist	Promotes OPC differentiation through antimuscarinic antagonism	[183,185,186]
Temelimab	Anti- MSRV-Env antibody	Neutralizes MSRV-Env proteins, which inhibit OPC differentiation	[183,184]
Opicinumab	Anti-LINGO-1 antibody	Enhances myelin sheath production, by suppressing myelination inhibitor LINGO-1	[183,184,186]
Antisemaphorin 4D	Anti-Sema4D antibody	Neutralizes Sema4D, which inhibits OPC migration and differentiation	[186]
Quetiapine	M1 antagonist/ ERK1/2 pathway	Promotes OPC differentiation through antimuscarinic antagonism and increases MBP synthesis	[184,186]
Benztropine	M1 antagonist	Promotes OPC differentiation through antimuscarinic antagonism, inhibition of dopamine transporters and histamine receptors	[185,186]
Pioglitazone	PPARγ agonist	Promotes OPC differentiation through immunomodulation	[184]
TO9	LXR agonist	Promotes OPC differentiation through cholesterol homeostasis and Th17 differentiation suppression	[184]
GW3965	LXR agonist	Promotes OPC differentiation through cholesterol homeostasis	[184]
Simvastatin	HMG-CoA reductase inhibitor	Promotes OPC survival and differentiation through cholesterol synthesis inhibition	[186]
Biotin	Cofactor for ACC1 and ACC2	Increases myelin production through association with ACC1 and ACC2	[186]
Domperidone	D2/D3 dopamine receptor antagonist	Promotes myelin synthesis through prolactin production enhancement	[186]

ERK, extracellular-signal-regulated kinase; GR, glucocorticoid receptor; Smo, Smoothened; MSRV-Env, Multiple sclerosis associated retrovirus envelope protein; PPAR, peroxisome proliferator-activated receptor; LXR, liver X receptor; HMG-CoA, β-Hydroxy β-methylglutaryl-CoA; ACC, Acetyl-CoA carboxylase.

Another kind of therapy consists in introducing myelin-producing cells in demyelinated regions, such as OPCs, olfactory ensheating cells (NSCs), embryonic stem cells (ESCs), bone marrow-derived stem cells (BMSCs) and Schwann cells. [175]

However, despite the massive number of candidates identified as remyelination promoters, only a few have reached phase 2 of clinical studies. Thus, the search for compounds capable of diminishing the remyelination failure that characterizes MS has been intensified and it is crucial to stop or delay disease impact.

More recently, natural compounds have been emerging as efficient therapeutic agents in the context of MS, not only through symptomatology relief but also through their neuroprotective and reparative properties on the CNS. The mechanisms of action of these compounds range from promoting OPCs differentiation into OLGs and neural stem cells to increasing cellular survival, promoting remyelinating events. [187] On **Table 3**, a list of several natural compounds which have shown to have pro-remyelinating properties in animal models of MS is presented, also highlighting the probable mechanism through which they exert such effects.

Table 3. Overview of natural compounds presenting pro-remyelinating properties.

Compound	Source	Target	Mode of action	Pro-remyelinating effects	References
18β-Glycyrrhetinic acid	Licorice	Microglia cells	Inhibits inflammation-induced blockade of BDNF expression	Remyelination in the CNS	[188]
Scutellarin	Traditional Chinese medicine *Erigeron breviscapus* Hand-Mazz	Neural stem cells (NSC) Oligo-dendrocytes	Increases the survival rate of neural stem cells; Promotes differentiation of NSC into oligodendrocytes	Improved motor function of cuprizone model mice and reduced demyelination of the corpus callosum as well as NSC' apoptosis	[189]
Cannabidiol	Cannabis	BDNF	Reduces pro-inflammatory cytokines (e.g. IFN-γ and IL-17), up-regulates PPARγ, and promotes neuronal survival	Enhanced BDNF levels in the CNS	[190]
Matrine	*Sophora favescens* roots	Oligoden-docytes progenitor cells (OPCs)	Promotes OPCs proliferation; Increases oligodendrocyte number	Promotes OPCs maturation into oligodendrocytes for myelin repair in EAE.	[191]
Icariin	*Epimedium*	NGF	Increases the expression of NGF	Preventes the loss of mature oligodendrocytes and promotes remyelination as well as regeneration of axons in mice fed with cuprizone	[192]
Resveratrol	Grapes, berries, peanuts, cocoa	Olig1 MBP	Antagonizes the decrease of MBP and Olig1 induced by cuprizone	Promotes myelin regeneration in a cuprizone-induced demyelination mouse model	[193]
Nigella sativa	Flowering plant Ranunculacea family	Glial cells Neurons	Suppresses pro-inflammatory mediators	Ameliorates the clinical signs of EAE, suppresses inflammation and enhance remyelination.	[194]
Ursolic acid	Plants' leaves (e.g. rosemary, marjoram), berries and other fruits	PPARγ	Induces promyelinating neurotrophic factor CNTF in astrocytes and upregulates myelin-related gene expression during oligodendrocyte maturation.	Enhances remyelination in a cuprizone-induced demyelination model.	[195]
Diosgenin	*Dioscorea villosa*, *Trigonella foenum-graecum* and *Solanum incanum*	OPCs	Pro differentiation effects on the OPCs	Accelerates remyelination in cuprizone-treated mice has also been observed.	[196]

1.1.4.4. Peripheric and Central Metabolic Impairments

The perception of MS as a disease with a metabolic impairment component in addition to the autoimmune and inflammatory ones, allows to further comprehend some of the aspects of the disease course including the genetic susceptibility, environmental risk factors and the difference in incidence between males and females, simultaneously opening new avenues for therapy development.

Wenting et al. [197] suggested that the basis of the neurodegeneration that characterizes MS is associated with "virtual hypoglucosis" arising from mitochondrial dysfunction. This leads to perturbations in ATP production and in mitochondrial respiratory chain activity, resulting in an up-regulation of glucose and lactate transporters. Mitochondrial activity dysfunction shifts the energetic metabolism from the mitochondrial oxidative phosphorylation to aerobic glycolysis, a process named Warburg effect. [198] It is important to note that while oxidative phosphorylation yields 36 ATP molecules, aerobic glycolysis only generates two, reducing the amount of energy available for neurons to perform their functions properly. [199] In an attempt to counteract this situation, lactate uptake is stimulated through an increased expression of the monocarboxylate transporters Mct1 and Mct2. [197] In fact, there is evidence of a positive association between CSF lactate levels and disease progression. [200] The transition to an aerobic glucose metabolism aggravates MS disease course, since it induces the dysfunction of T CD4+ cells, causes neuronal cells death and it's associated with astrocytic inflammation. [199]

The Warburg effect can also be induced by deregulation of pathways such as the PPARγ and WNT/β-catenin ones, which are up-regulated in response to demyelinating events and the in neuroinflammatory conditions., respectively [199] In the specific case of MS, studies show that the WNT/β-catenin pathway is upregulated and PPARγ is decreased. [199] In fact, evidence of the beneficial effects of PPARγ agonists in MS is available. Studies performed in EAE models have reported that these molecules reduce EAE clinical signs and, accordingly, their absence exacerbate disease symptoms. [199] PPARγ stimulation reduces the inflammatory milieu and promotes remyelination, presenting a neuroprotective effect. [199] Additionally, PPARγ agonists inhibit Th17 cell differentiation, not only in animal models of MS but also in humans. [199] The WNT/β-catenin pathway, on the other hand, is associated with dysfunctional OPCs, leading to impaired repair mechanisms of the demyelinated lesions that characterize MS. [199] Taken together, the aforementioned observations highlight metabolic pathways such as oxidative phosphorylation, glycolysis, WNT/β-catenin and PPARγ as potential research targets for the development of new therapeutic strategies.

Interestingly, the potential of strategies currently used to treat metabolic syndrome in the context of MS has been evaluated. In a study performed by Negrotto et al. [201] Metformin and/or pioglitazone were administered to 50 MS patients to access their potential in reducing disease activity. 20 patients who were treated with metformin and 10 who received pioglitazone displayed a lower number of T2 and GD-enhancing lesions. Additionally, metformin decreased IFNγ and IL-17 secretion, pioglitazone having the same effect on IL-6 and TNF-α, and both increased the levels and activity of CD4+CD25+FoxP3+ regulatory T cells. Moreover, research also suggests that

including the metabolic agent cytoflavin in MS therapy increases the efficacy of conventional therapy, decreasing disease severity. [202]

1.1.4.5. Mitochondrial Dysfunction and Oxidative Stress

Oxidative stress is one of the hallmarks of MS and is involved in myelin degradation, axonal degeneration, and inflammatory events. [153] It is important to highlight that the CNS is particularly susceptible to oxidative stress due to its high oxygen demands. [203] In fact, studies involving MS patients show increased levels of oxidants in the CSF, as well as oxidative stress markers in brain tissues, specifically in degenerating neurons and oligodendrocytes undergoing apoptosis. [204] Reactive oxygen species (ROS) are associated with several events involved in the pathophysiology of MS. They mediate the migration of monocyte-derived macrophages through the BBB into the CNS, contributing to the formation of the sclerotic lesions that characterize the disease. In their turn [205], these cells are capable of producing ROS, as well as NO and pro-inflammatory cytokines, resulting in neuroinflammation. [205] Interestingly, ROS promote mitochondrial dysfunction and focal axonal degeneration also in axons with intact myelin. Besides macrophages, evidence suggests that activated microglia is one of the major sources of ROS in the CNS. [205] ROS activate immune cells and promote the release of inflammatory products, such as TNF-α, IFN-β, TRAIL, and Fas-L, inducing OLGs apoptosis. [206]

Moreover, in EAE models mitochondrial activity, dynamics, and trafficking are impaired in CNS areas displaying inflammatory infiltrates. [153] ROS produced by macrophages cause mitochondrial dysfunction and axonal damage, in both demyelinated and myelination axons. [153] Likewise, human MS active lesions are characterized by increased amounts of oxidized oligodendrocyte DNA as well as oxidized axonal phospholipids, and mitochondrial damage is limited to the lesioned section even if myelin is intact. [153] Neuronal energy consumption is mainly attributed to synaptic activity, the synaptic terminals being the preferential site of mitochondria localization. [206] Similarly, OLGs differentiation requires mitochondrial metabolism for myelin formation. [206] The pathology of MS is characterized by a number of mitochondrial abnormalities, including mitochondrial DNA (mtDNA) and protein mutations, higher production of ROS, apoptotic events and altered ionic homeostasis. [206]

Mutations in mtDNA, specifically one affecting the mitochondrial uncoupling protein 2 (UCP2), have been associated with higher susceptibility to MS. [207] Furthermore, the facts that during OLGs differentiation there is an increased mitochondrial gene expression and that this process is highly influenced by mitochondrial toxins support the crucial role of mitochondria in OLGs maturation and, consequently, myelin production. [206] Interestingly, different components of the mitochondrial respiratory chain have been associated with MS. mRNA levels of PGC-1α, for example, showed to be decreased in MS patients, which correlated with ROS production. [208] Additionally, MS patients display reduced levels of the proteins cyclooxygenase (COX)-I and COX-IV, which are components of mitochondrial oxidative phosphorylation system. [209]

Due to its higher susceptibility to oxidative damage, the brain is equipped with various antioxidant systems. These include Superoxide Dismutase (SOD) 1 and 2, catalase, heme-oxygenase 1 (HMOX-1) and thioredoxin (TrxR)- peroxiredoxin (Prx). [206] Increased levels of

these mediators are observed in MS samples. [206] Regarding reactive nitrogen species (RNS), there is evidence of increased levels of nitric oxide synthase in demyelinated areas in MS, particularly in astrocytes, macrophages, and microglia. [206] Additionally, in both animal models [210] and human MS patients [211], the inducible form of nitric oxide synthase (iNOS) is upregulated. Importantly, evidence suggests that RNS impair complex II–III and IV of the mitochondrial respiratory chain, as well as other key enzymes, being toxic to neurons and OLGs. [212] Moreover, the fact that an upregulation of some of these antioxidant systems, such as catalase, SOD and HMOX-1, ameliorates EAE disease course further supports the involvement of such enzymes in MS development. [205] Furthermore, several lines of evidence showing the beneficial effects of antioxidants and radical scavengers on EAE and MS are available, strengthening the hypothesis that ROS are key players in disease development and progression. [205]

Based on these observations, it might be logical to think of antioxidants as potential therapeutic agents, either through food or supplementation, aiming to alleviate the oxidative environment observed in MS. Therefore, antioxidant treatments might compose promising strategies to manage the disease and repress its progression. In fact, several studies reported that the intake of dietary antioxidant compounds ameliorated clinical scores and symptomatology in MS animal models. [213,214] Since the most frequent antioxidants present in the human diet are polyphenols, the following section aims to provide meaningful data in this particular field of knowledge.

1.2. Polyphenols supplementation: a plausible nutraceutic for MS management

The therapeutic potential of plant-based natural compounds and the phytochemicals composing them has been a significant point of interest in the last years. The most abundant and widely distributed bioactive molecules are polyphenolic compounds (PCs), which present a phenolic ring as their basic monomer. [215] Depending on their chemical structure, their origin and biological function, PCs can be divided in different classes, the main being flavonoids and phenolic acids. [215]

1.2.1. Flavonoids

In plants, flavonoids are responsible for the coloring and aroma of flowers and fruits [216] and the majority are found as glycosides. [215] The general structural backbone of flavonoids is C6–C3–C6, the carbon of the C ring on which the B ring is attached to being the determinator of the subgroup the compound belongs to. [215,216] When the link between the B and the C rings is in the position 3, they are isoflavones, and when this link happens in position 4 we stand before neoflavonoids. [216] Those in which the B ring is attached to the C one in the position 2 are further classified into different subgroups depending on the structure of the C ring, them being flavanones, flavanonols, flavones, flavonols, flavanols, anthocyanins and chalcones. [216] **(Figure 4)**

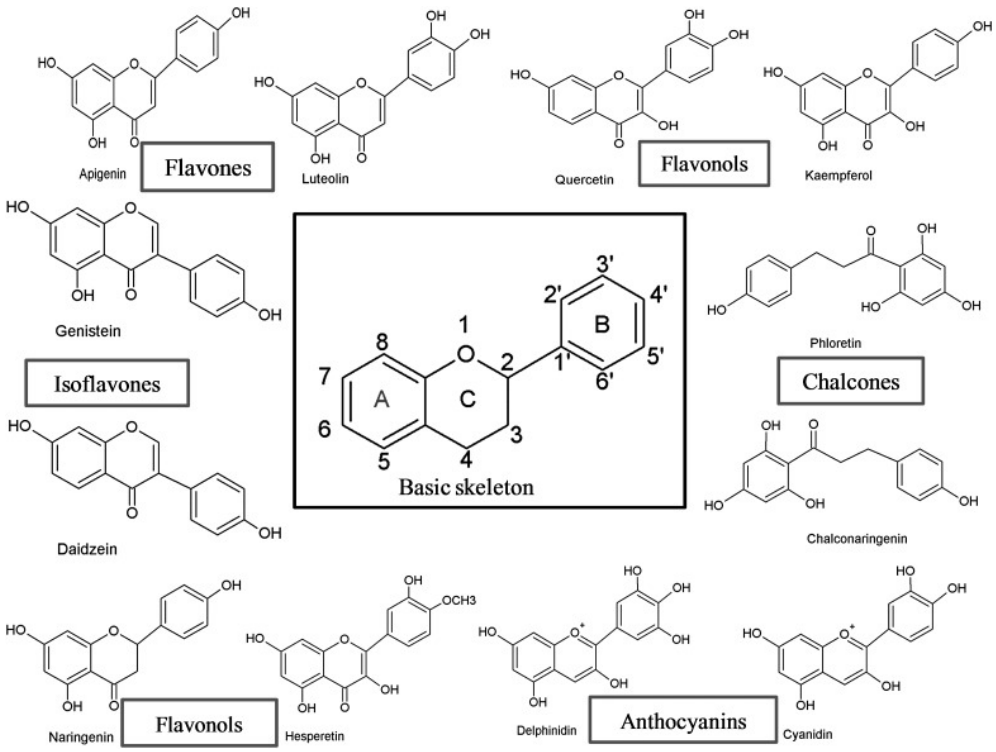


Figure 4. Chemical structure of the different classes and subgroups of flavonoids. (Taken from Panche et al [216])

1.2.1.1. Flavones

Flavones can be found in several plants, both in leaves and flowers, as well as fruits. [216] Most flavones of plant and fruit origin display a hydroxyl group in the C5 of the A ring, the hydroxylation in the other positions varying according to the taxon of the plant or fruit in question. [216]

1.2.1.2. Flavonols

Flavonols are the largest subgroup of flavonoids. [216] They are characterized by the presence of a ketone group and are the unitary blocks of proanthocyanins. [216] Like flavones, they are abundantly found in various vegetables and fruits. [216] The consumption of dietary flavonols underlies significant health benefits, especially due to their strong antioxidant properties, the most studied ones being quercetin, fisetin, myricetin and kaempferol. [216] In the case of flavonols, the hydroxyl group is positioned in the C3 of the C ring. [216]

1.2.1.3. Flavanones

As flavonols and flavones, flavanones are also generally present in fruits, particularly in citrus, in which they are responsible for the bitter flavor. [216] The difference between flavonones and flavones resides in the double bond formed between positions 2 and 3, which is saturated in flavonones. [216] This subgroup of flavonoids presents intrinsic radical-scavenging activity, which endows them with powerful antioxidant properties. [216] Additionally, flavanones have shown to

exert anti-inflammatory and lipid-lowering activity, having a great therapeutic potential. Examples of these compounds include hesperitin, isosakuratenin, naringenin and heridictyol. [216]

1.2.1.4. Isoflavonoids

Isoflavonoids are a class of flavonoids with a more limited distribution in plant-based foods, the mainly found being genistein, daidzein and glycitein. [216,217] They are structurally similar to estrogens, exerting estrogenic and anti-estrogenic activity in different tissues. [216,217] Isoflavonoids are associated with a wide range of health-beneficial effects, such as antioxidant, anti-inflammatory and immunomodulating activities. [217]

1.2.1.5. Flavanonols

Flavanonols, also known as catechins, consist of 3-hydroxy derivatives of flavanones. [216] Structurally, they present the hydroxyl group linked to the C ring in the position 3, and no double bound between this and position 2. [216] Like most flavonoids, flavononols are mainly found in fruits. [216]

1.2.1.6. Anthocyanins

Anthocyanins are the main pigments responsible for the red, purple, and blue color of plants, flowers, fruits, and certain grain varieties. [216] Anthocyanin color is dependent on the hydroxyl groups of the A and C rings, as well as on the pH. [216] The most commonly found anthocyanins are cyanidin, delphinidin, pelargonidin and peonidin. [216]

1.2.1.7. Chalcones

Chalcones differ from the remaining flavonoids because of the absence of a C ring on their backbone structure [216], being commonly referred to as open-ring or open-chain flavonoids. [216] Chalcones are abundantly found in fruits [216], the most common being chalcones phloridzin, arbutin, phloretin and chalconaringenin. [216]

It is evident that virtually all flavonoid classes display antioxidant and anti-inflammatory properties, composing potential simple and safe ways of preventing and/or treating several diseases. Studies show that flavonoids play key roles on the CNS, exerting beneficial effects in the context of different neurodegenerative diseases, such as Alzheimer's and Parkinson disease, among others. [218] The aforementioned effects possibly derive from the ability of these compounds to hinder inhibitory enzymes, such as phosphodiesterase, Ca2+ ATPase, iNOS and COX. [216,218] Additionally, flavonoids strengthen the biological antioxidant systems, and upregulate the expression of proteins involved in neuronal repair and synaptic activity. [218]

1.2.2. Phenolic Acids

Phenolic acids are PCs that possess one carboxylic acid group and can be divided into two major subtypes: benzoic acids, which present a skeletal structure C6-C1, and cinnamic acids, whose structure is C6-C3. [219] They are present in innumerous plant-based foods, such as fruits, vegetables, seeds, legumes, cereal and coffee, being mainly in a bound form, such as amides, esters and glycosides. [219] The most abundant hydroxycinnamic acid found in food is chlorogenic acid (CGA), which is an ester formed between caffeic and quinic acids. On another hand, the most common hydroxybenzoic acids are gallic, vanillic, ellagic, syringic, p-hydroxybenzoic, and protocatechuic acids. [219] These compounds might act as neuroprotective agents through radical-scavenging activity, being useful in the context of chronic diseases associated with oxidative stress. [219]

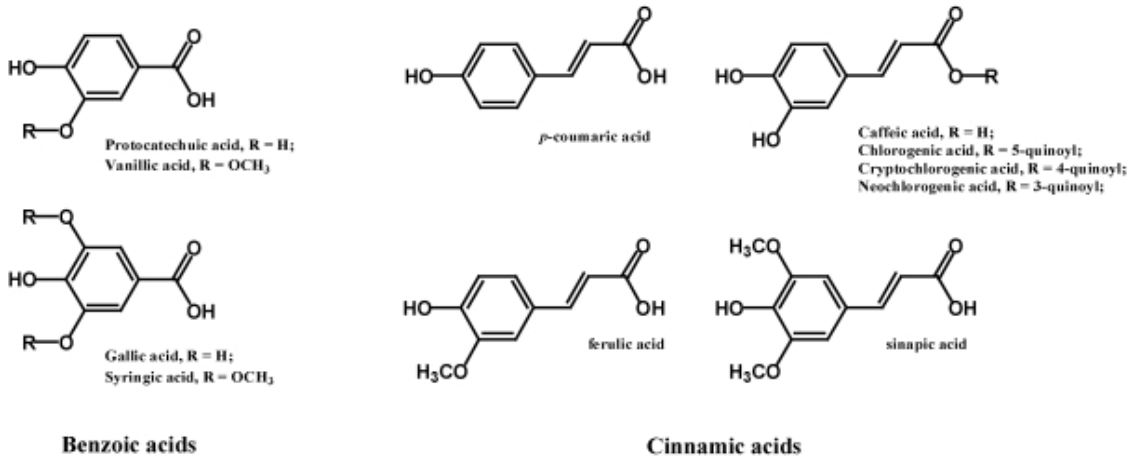


Figure 5. Chemical structure of some of the most common benzoic and cinnamic acids, the main two classes of phenolic acids. (Taken from Tsao et al [220])

1.2.3. Dietary Sources of PCs

In recent years, there has been an increased awareness of the effect of food on health, and a rise in the consumption of plant-based and natural foods occurred. PCs are closely related to the sensory and nutritional quality of fresh and processed plant-based foods and their intake is associated with potential health-promoting effects in the context of several diseases.

PCs are secondary metabolites produced by plants that function as chemical defense agents. In addition, they are involved in reproduction and plant-to-plant interaction [221], as they modulate sensorial properties (smell, color, and taste) and are involved in pollination, germinative processes, among others. [222]

Among the health-promoting phytochemicals present in cereals, PCs are frequently found in their free or bound form. [221] Phenolic acids such as hydroxybenzoic, coumaric, ferulic, gallic, sinapic, vanillic, and syringic acid are found in different types of grains and cereal, including wheat, oats, rice, corn, and triticale, among others. [223] Although phenolic acids are the predominant PC class in cereals, flavonoids are also present. [216] Ultimately, the phenolic composition depends greatly on the type and variety of cereal. [221]

Seeds and nuts are also among the richest sources of polyphenols. [224] Cocoa also displays high polyphenol content, mainly catechins and proanthocyanidins. [224] Interestingly, the richest sources of polyphenols are spices and herbs. [224] Cloves, for example, are abundant in PCs, such as eugenol. [224] Significant amounts of flavones are found in dried herbs, including peppermint and oregano, which are also rich in hydroxycinnamic acids. [224]

Vegetables and legumes are other major sources of PCs. Interestingly, the two main vegetable sources of PCs are green and black olives, followed by artichokes and red and green chicory, which are all rich in CGA. [224] Other vegetables, such as onions, tomato, lettuce, carrots, spinach, broccoli, potatoes, and many more are also relevant sources of dietary PCs. [221,222,224]

Regarding beverages, the one ranked in first on the subject of PCs content is coffee, which is highly rich in CGA. [224] Coffee is followed by black and green teas, which are abundant in proanthocyanidins, catechins, and theaflavins; and red wine, displaying significant amounts of catechins, hydroxycinnamic acids, anthocyanins, and proanthocyanidins. [224] Some fruit juices are also found further down the list, presenting lower amounts of PCs. [224]

PCs are also broadly found in a wide variety of fruits. [224] Apples are one of the fruits most rich in PCs, the four most abundant subgroups in them being flavanols, flavonols, phenolic acids and dihydrochalcones. [221] In particular, significant amounts of CGA, phloretin, epicatechin, quercetin and procyanidin are found in apples. [221] Mangos are also known by their high PCs content, both in the pulp and peel, such as gallic, protocatechuic, chlorogenic, syringic and vanillic acids. [221] Flavonoids are also present in mango, including kaempferol, quercetin and rutin. [221] Citrus fruits are also great sources of PCs, namely cinnamic acid derivatives, coumarins, and flavonoids. [221] The main flavonoids found in this kind of fruit are flavanones, the most frequently present being eriocitrin, hesperidin, nariturin, and naringin. [216,221]

Notably, a number of berries appear at the top of the list when it comes to fruits rich in PCs, the main subgroup found in this kind of fruit being anthocyanins. [224] These compounds are responsible for the coloring and aroma of the fruits in which they are found in. [220] Thus, as expected, the berries with higher anthocyanin content are the ones displaying a darker color: black chokeberries, black elderberries, blueberries, and blackcurrants. [224] The most widespread anthocyanidins in berries are delphinidin, peonidin, cyanidin, pelargonidin, petunidin, and malvidin. [220] Among berry fruits, blueberries are considered a major source of PCs and are praised for their high antioxidant properties. It is important to note that between different blueberry types and within other *Vaccinium* species, there are significant differences in the phenolic content and antioxidant activity. [225] Flavonoids and phenolic acids are the two major classes of PCs in blueberries, the main flavonoids being anthocyanins and flavonols (e.g. quercetin). [226] Phenolic acids found in blueberries include chlorogenic, caffeic, p-hydroxybenzoic, p-coumaric, and ferulic acids [227], CGA being the predominant one. [228]

1.2.4. Bioavailability and Bioactivity of PCs

In innumerous occasions, PCs have shown to exert chief biological activity *in vitro*. [229] However, when it comes to *in vivo* assays, these compounds do not show similar effects [230], which could be in part explained by their low bioavailability. [229,230] Bioavailability is commonly defined as the fraction of a certain compound that reaches the systemic circulation as well as target tissues, where it will exert its biological action. [231] Pharmacokinetic studies show that PCs classes vary in terms of bioavailability and can be placed as follows: phenolic acids > isoflavones > flavonols > catechins > flavanones, proanthocyanidins > anthocyanins. [229-231] The overall bioavailability of PCs is determined mainly by their chemical structure, their absorption, deposition, metabolism and excretion (ADME), form of administration, and food matrix, among others. [230]

External factors, including climatic conditions, culture types and the degree of ripeness, may also affect the content of PCs in plants and fruits. [231] Furthermore, food processing related factors such as thermal treatments influence PCs content, their absorption and consequently their bioactivity, the same happening for preparing/cooking methods. [231] For example, carrots completely lose their PCs when boiled, but maintain some when fried. [232] The storage of food products may also affect PCs bioavailability, evidence showing that long periods of storage for fruits such as apples and raspberries, and vegetables such as broccoli, result in significative changes in their polyphenolic content. [231] For example, apples stored for 4 months displayed increased levels of CGA, upped from 101 to 144 mg/kg of fresh weight. [233] Additionally, food processing such as homogenization mechanisms influences the PCs composition in different vegetables and fruits. Tomato paste, for example, displays a different PCs content when compared to raw tomatoes. [234] Food related factors, such as macro and micronutrients, can as well modify PCs bioavailability and bioactivity, since the presence of fat, carbs and protein can often influence the way PCs are absorbed. Fats, for instance, have shown to increase the digestibility of some PCs, such as procyanidins. [235] Moreover, it has been recently suggested that the association between PCs and fiber delays their absorption through the gastrointestinal (GI) tract, potentially optimizing their assimilation. [236]

On another hand, PCs own lipophilicity, molecular weight and chemical structure strongly dictates their availability and activity. [230] As previously referred, the majority of PCs found in food are in glycosylated forms, in which the sugar group is known as the glycone and the non-sugar group as the aglycone. [231] The type of the sugar in the glycoside will determine the rate and extent of intestinal absorption of the PCs. [231] In order to be absorbed, this glycosylated form often undergoes hydrolysis by intestinal enzymes or the intestinal microbiota. [231] Anthocyanins compose an exception to this premise since they are detected in circulation as intact glycosides. [237]

To exert their bioactivity, PCs must be delivered to the GI and absorbed, reach circulation and, posteriorly, the target tissues. In general, PCs display low bioavailability, due to factors such as decreased solubility, the interaction with the food matrix, difficulties in membrane-crossing, as well as their extensive hepatic and intestinal metabolism and rapid clearance. [230] PCs must first be released from the food matrix through mechanical, chemical, and enzymatic mechanisms to facilitate their absorption along the GI tract, which begins in the oral cavity, with mastication and

salivation. [230,238] Mastication disrupts PCs from the food matrix, allowing the release of their components and increasing the surface area for the action of digestive enzymes present in saliva [230,238]. Glycosylated PCs metabolism initiates immediately in the oral cavity, as they come in contact with the oral microflora glycosidase enzymes. [239] In fact, Kamonpatana et al. [240] reported that anthocyanins present in fruit extracts rich in human saliva were partially metabolized by the bacterial population colonizing the oral space. The bolus formed in the mouth is then transported to the stomach, where it will be subjected to physical and chemical digestive events, which will also promote the further breakdown of PCs and the release of their constituents and help their separation from the food matrix. [238] While the aglycones are absorbed through passive diffusion, it is believed that the glycone portion crosses the epithelial cell wall through carriers such as P-glycoprotein and cotransporters for SGLT1. [230]

Although the stomach is not the main site for absorption, some small PCs loosely bound to the food matrix can be readily absorbed by gastric cells. [238] However, many polyphenols reach the intestine in their intact forms. [230] As a matter of fact, the major sites of PCs absorption and transformation are the duodenum and jejunum. [238] However, there is evidence that some PCs are lost in the duodenum due to its alkaline environment, possibly accounting for the low bioavailability of some of these compounds. [241] In the small intestine, PCs are metabolized by intestinal enzymes as well as enzymes produced by the microbiota. [230] The ones that are not absorbed in the small intestine reach the colon where they undergo substantial structural modifications, glycosides being hydrolyzed into aglycones that are posteriorly extensively metabolized to aromatic acids. [231] These are further metabolized and give rise to microbial metabolites which are absorbed and conjugated with glycine, glucuronic acid, or sulfate. [231] This conjugation process occurs in the small intestine but mostly in the liver, and aims to restrict PCs potential toxic activity and facilitate their biliary and urinary elimination by increasing their solubility and molecular weight. [231] From the colon, conjugated PCs are transported to the liver through the portal vein, where they are further metabolized into more polar molecules in order to facilitate their excretion. [239] These processes can be categorized in two phases: phase I, which includes hydrolysis, oxidation and reduction reactions mediated by CYP450 enzymes; and phase II, which comprises hydrophilicity-enhancing reactions that promote the compounds elimination. [239] The PCs displaying a high level of conjugation and significant size are excreted in the bile, while small and less conjugated ones are eliminated in the urine. [230]

On another hand, PCs and their metabolites can enter the bloodstream and bind to plasma proteins, such as albumin. [230,239] Since the conjugation mechanisms are usually highly efficient, glycosides are normally absent in the circulation or, if present, they are found in very small amounts. [230] Then, PCs are able to reach target tissues, namely those in which they are further metabolized. [242] Remaining PCs are secreted to the duodenum through the biliary route, where they may be reabsorbed, extending the presence of these compounds within the body. [242]

As can be seen, PCs are subjected to a significant degree of transformation along their journey through de GI tract. As consequence, a single PC is able to generate several different metabolites displaying different activities and properties relatively to the original compound.

1.3. PCs: A Focus on Blueberries

1.3.1. Blueberry PCs Bioavailability

Blueberries (BBs) display a wide range of PCs that interact with the food matrix through their passage along the GI tract, which will affect their stability, absorption, and metabolism, while also providing nutrients to the colon. With the goal of investigating BB PCs metabolism, Correa-Betanzo et al. [243] employed an *in vitro* gastrointestinal model simulating the digestive process through the GI tract, as well as a colonic fermentation step, and evaluated the breakdown of the PCs found in BBs, in order to evaluate the stability of these compounds and their metabolites. The study suggested that gastric digestion leads to a 50% decrease of the original anthocyanin levels, which drop even further to 10-15% during intestinal digestion and to 2-3% after fermentation in the colon.

Researchers observed substantial amounts of phenolic acids formed after colonic fermentation of anthocyanins, including kaempferol-3-rhamnoside, rhamnetin, protocatechuic acid, syringic acid, cinnamic acid, and caffeic acid. A decrease in CGA levels was verified along with the generation of caffeic acid, which makes sense since caffeic acid results from the hydrolysis of CGA by colonic microbiota. [244] Thus, the digestive process resulted in structural and chemical alterations of anthocyanins, culminating in the formation of simple phenolic compounds that differed from the ones present in the original extract. However, acetylated derivatives of anthocyanins, such as malvidin and delphinidin, maintained a significant degree of stability, retaining nearly half of their original amount, allowing to conclude that fruits rich in acetylated PCs provide increased quantities of anthocyanins to the colon. Furthermore, anthocyanins possessing more sugar molecules displayed lower stability relatively to monoglucosides.

When evaluating the radical-scavenging activity of BB PCs, the authors reported that such activity was not altered during gastric digestion but was decreased in around 50% after passing the intestine, dropping even more during colonic fermentation. These observations suggest that PCs displaying significant antioxidant properties must be absorbed before reaching the colon to become available in target tissues. Similarly, the anti-proliferative activity of BB PCs was not decreased in gastric digestion but displayed a significant reduction after *in vitro* intestinal digestion and colonic fermentation, once more suggesting that once metabolized, the antioxidant and antiproliferative activities of BB anthocyanins may be impaired. Overall, the results suggest that colonic fermentation alters the biological activity of BB PCs, giving rise to metabolites which display lower antioxidant activity as well as reduced cell growth inhibition potential.

A similar study was performed by Zhong et al. [245] in which human subjects ingested a wild blueberry (WBB) beverage consisting of 25g of freeze dried WBB powder along with two meals and collected plasma over 24h in order to quantify and characterize the present PCs as well as their metabolites. Parent compounds, namely anthocyanins and CGA, and several metabolites showed a peak in plasma between 15 minutes and 8 hours, displaying a bi-phasic response indicative of enterohepatic circulation. The total availability of anthocyanins that maintained their C6-C3-C6 structure was around 1.1%. Specifically, parent anthocyanins and CGA peaked approximately 2 hours after ingestion, while metabolites such as glucuronide conjugates of

peonidin, delphinidin, cyanidin and petunidin peaked at 2.6, 6.3, 7 and 8.8 hours, respectively. On another hand, phenolic acid metabolites peaked between 0.5 and 24 hours, suggesting that some compounds display an early phase response, while others are characterized by a late phase response, being available to the GI tract and target tissues at different time points. Three mechanisms are referred by the authors as possible explanations to this response pattern: enteric cycling, local recycling, or enterohepatic circulation. However, the existence of a second peak after a second meal points more to the enterohepatic circulation hypothesis, since it coincides with gall bladder contraction delivering a bolus to the small intestine. The bi-phasic pattern was exhibited by three of five anthocyanins – delphinidin, cyanidin and petunidin, which might be related to polarity, since these three anthocyanins present the most polar B rings. These observations highlight the complex metabolic processing of anthocyanins, involving several passages through the liver and small intestine before excretion, giving rise to a wide range of metabolites which interact with target tissues at different timepoints and different physiological states during the day.

Regarding CGA, this compound displayed a bioavailability of 0.22%, which might be explained by its rapid breakdown to caffeic and quinic acid. Furthermore, biotransformation events of CGA lead to the production of ferulic acid, which was found elevated in the plasma, as well as its conjugated form, in the first hours after WWB beverage ingestion, indicating that most of the CGA was converted into its main metabolites.

In conclusion, these studies and similar ones emphasize the complexity of BB PCs metabolism throughout the GI tract, highlighting the different factors influencing their bioactivity as well as bioavailability. Besides enzymes and transporters of the human host, the microbial population colonizing the intestine also plays a critical role in the bioavailability of these compounds, promoting their metabolization into products with varied biological activity and that might be directly associated with a reduced risk of developing diseases through their multiple health-promoting properties.

1.3.2. Blueberries' Therapeutic Potential

A growing body of positive scientific evidence on the health benefits of BBs is arising from human observational and clinical research, as well as *in vitro* and animal models. Epidemiological studies report a positive association between regular and moderate BBs consumption and a decreased risk of cardiovascular conditions, type 2 diabetes (T2DM), obesity and other metabolic impairments, as well as neurological pathologies. [246]

1.3.2.1. Cardiovascular Health

Pre-clinical and clinical evidence point to a strong association between BB intake and cardiovascular health, with a consumption of at least once weekly showing to significantly decrease the risk for cardiovascular diseases (CVD). [247] DeFuria et al. [248] reported that mice fed with a high-fat diet supplemented with BB powder displayed an attenuated expression of inflammatory genes and decreased risk factors for CVD, such as insulin resistance and hyperglycemia. Similarly,

findings from the Nurse's Health Study (NHS) allowed to find an association between the intake of BBs and strawberries and a lower risk for myocardial infarction. [249] Moreover, a study investigating the effects of daily wild BB consumption on flow-mediated dilation (FMD), showed that FMD increased in the treated group, suggesting that daily consumption of BB might improve FMD in healthy individuals. [250] Most importantly, in a high CVD risk population, the intake of 26g of freeze-dried BB for six months resulted in increased FMD in 1.4%. [251] Overall, the aforementioned observations regarding cardiovascular health benefits of BBs are promising, and BB PCs show potential to improve it through various well-established markers.

1.3.2.2. Diabetes, Obesity and Overall Metabolic Health

Epidemiological evidence suggests that incorporating BBs into the diet might lower the risk of developing diseases such as T2DM, since they promote improved insulin resistance as well as glucose tolerance. [252] Several studies performed in animal models have demonstrated the anti-diabetic effects of BB anthocyanins. For example, rodents subjected to a high-fat diet supplemented with BBs showed improved insulin resistance and glucose tolerance. [253] Furthermore, DeFuria et al. [248] reported that mice consuming a 60% high-fat diet with 4% of BB for eight weeks displayed lower levels of plasmatic glucose during insulin tolerance tests (ITT) tests in comparison to mice subjected to the high-fat diet only. Regarding human studies, Youdim et al. [252] demonstrated that the consumption of a BB smoothie for six weeks increased insulin sensitivity in prediabetic subjects comparatively to the placebo group.

There is also evidence of beneficial effects of BB intake on BMI, total body weight and total fat weight. Vuong et al. [254] reported a reduction in weight gain in obese KKAy mice after incorporating BB juice in the animals drinking water, along with improved glucose tolerance and insulin sensitivity. Specifically, BB anthocyanins have shown to decrease bodyweight in dietary-induced models of obesity, as well as blood glucose levels, serum triglyceride and total cholesterol levels, highlighting a hypoglycemic activity. [255]

The aforementioned results suggest that BBs as well as the compounds composing them are potential contributors to the regulation of glucose and lipid metabolism, possibly being helpful in the context of several metabolic disorders, such as obesity and T2DM.

1.3.2.3. Gut Health

The GI tract is colonized by an array of microbes that exert important roles in metabolic and immune responses. The composition of this microbial population is influenced by the host's diet and can significantly impact its overall health. Lacombe et al. [256] aimed to determine the effects of lowbush wild BB (LWB) dietary enrichment on the composition of the colonic microbiota, as well as their ability to improve gut health. The LWB-enriched diet promoted a significant reduction in the relative abundance of *Lactobacillus* and *Enterococcus*, and increased the relative abundance of the phylum *Actinobacteria*, the order *Actinomycetales*, and genera of the *Bifidobacteriaceae* and *Coriobacteriaceae* families, suggesting a prebiotic potential for LWB. LWB subjected animals also displayed a 20% increase in xenobiotic degradation. Moreover, evidence shows that adding a blueberry extract to faecal bacteria cultures derived from healthy

human volunteers leads to growth of beneficial bacterial species, such as *Lactobacillus rhamnosus* and *Bifidobacterium breve*. [257]

Rodríguez-Daza et al. [258] observed that the administration of a wild BB extract (WBE) to high-fat high-sucrose (HFHS)-diet induced obese mice lead to improved glucose tolerance possibly through a modulation of bacterial composition, namely *Coriobacteriaceae* and *Verrucomicrobiaceae*, as well as by maintaining the mucus layer in the colon of a diet-induced obesity and insulin resistance murine model. Similarly, a study investigating the potential effects of a BB polyphenol extract (PPE) on high-fat diet-induced obesity demonstrated that PPE inhibited body weight gain and promoted a normal lipid metabolism, by impacting the gut microbiota composition, modulating specific bacteria such as *Proteobacteria*, *Deferribacteres, Bifidobacterium; Helicbacter, Prevotella, Actinobacteria; Desulfobrivio, Flexispira* and *Adlercreutzia*. [259]

Therefore, the available literature suggests that BB polyphenols, as natural active ingredients, may act as prebiotics that modulate the gut microbiota, targeting consequent complications, such as obesity, intestinal and extra-intestinal disorders such as neurodegenerative ones.

1.3.2.4. Neurodegeneration

Neurodegenerative diseases, such as Alzheimer's, Parkinson, and Huntington disease, just to name a few, compose one of the most important public health concerns, since their frequency is increasing worldwide, along with consequent higher morbidity and mortality rates. Although significant progress has been made in the understanding of such conditions, effective therapeutic strategies are still lacking. PCs, due to their strong antioxidant, anti-inflammatory, immunomodulatory and neuroprotective properties, may constitute great opportunities for prevention and/or treatment of several neurodegenerative diseases.

It is important to note that the beneficial effects of PCs, namely the ones present in BBs, is not limited to their antioxidant properties, which is supported by the fact that these compounds display a variety of target tissues. [260] In fact, BB PCs are able to reach CNS, which might explain the effects of such compounds in the field of neurosciences. In fact, a study in which BBs were administered to pigs for four weeks reported that, among the examined tissues (brain, eye and liver), anthocyanins were found in increased concentrations in the brain [261], highlighting their ability to cross the BBB and affinity to the cerebral tissue. Furthermore, blueberry anthocyanins have been detected in specific regions of the brain, such as the hippocampus, cortex, striatum, and cerebellum, which are associated with memory and learning functions. [262]

The antioxidant activity of blueberry PCs in the area of neurology is strongly associated with a decrease in ROS production and their effects, such as lipid peroxidation. [260] Furthermore, an increased activity of the antioxidant enzymes catalase and SOD has been reported as consequence of blueberry supplementation. [263] Moreover, blueberry extracts have shown the ability to protect against β-amyloid neurotoxicity and reverse the decrease of intracellular glutathione caused by β-amyloid *in vitro* [264], as well as restore the ATP availability and the synaptic activity in the hippocampus [265]. Additionally, they also inhibit β-amyloid aggregation,

preventing the formation of neurofibrils. [265] Blueberry anthocyanins also play a key role against neurodegeneration though their anti-inflammatory effects. Microglia, which upon activation is responsible for the release of several pro-inflammatory cytokines, has shown to be repressed by blueberry anthocyanins in animal studies, resulting in decreased production of cytokines, such as TNF-α and IL-1β, as well as ROS, and reduced expression of enzymes involved in inflammation, including NOS and COX. [266] Such effects are believed to result from the suppression of NF-κB activation. [260]

Of note, blueberry polyphenols are positively correlated with cognitive performance, memory function and synaptic plasticity, by increasing hippocampal neurogenesis, activating extracellular signal-related kinases (ERK), IGF-1 levels and/or regulating calcium homeostasis and stress signaling. [267] Since they are AhR agonists [268], several studies evaluated the effects of different flavonoids in MS animal models. These revealed promising results, highlighting remyelinating and neuroprotective effects. [269-271] Despite these observations, it is important to point out that the referred studies are, mainly, relative to flavonoid compounds and not actual foods. Nevertheless, the beneficial effects of phenolic-rich foods have been reported in the context of neurodegenerative diseases, namely spinach, strawberries, and other berries. [272] Focusing on MS, several preclinical studies highlight the capacity of PCs to interfere with key cellular targets of disease pathophysiology, promoting an increased expression of myelin proteolipid protein (PLP) [273], myelin basic protein (MBP) [274] and oligodendrocyte transcription factor 1 (Olig1) [193,273], along with a decreased number of recruited T and B cells [274], inhibition of glia activation and subsequent inflammatory factors release [274]. In experimental MS, blueberry-derived PCs (e.g. ellagic acid, resveratrol) have demonstrated to improve key pathological hallmarks. [193,273,275] Remarkably, Xin and colleagues demonstrated that a blueberry-enriched diet reduced experimental MS incidence by more than 50%, promoting myelin preservation and improving clinical motor scores. [276] Collectively, these data extol the therapeutic potential of blueberry PCs in the context of MS.

1.4. Blueberries: Fruits VS Leaves

The advantageous effects of PCs present in *Vaccinium* spp. fruits, particularly blueberries, in CNS diseases are evident. However, some studies demonstrate that besides the fruit itself, the leaf of the *Vaccinium* genus displays increased phenolic content and enriched antioxidant activity, displaying a great potential in the nutraceutical field. [277] A study performed by Stefănescu et al. [278] compared the chemical and biological profiles of leaves from six blueberry varieties, and discovered that feruloylquinic acid was the most abundant PC identified, followed by rutin. However, other studies demonstrate that blueberry leaves are predominantly rich in CGA [277], also presenting significant amounts of quercetin glycosides such as rutin [279]. They also reported that most of the leaf extracts displayed strong antioxidant and antimicrobial activities. Importantly, several lines of research show that blueberry leaves possess a higher PC content as well as stronger antioxidant capacity when compared to the fruit. [280]

Moreover, evidence shows that leaf extracts from blueberry plants exert neuroprotective effects, namely on cultured rat glial cells and neurons intoxicated with glutamate. [281] A possible

explanation for such effect is that anthocyanins present in the leaves are able to reduce the amount of inflammatory mediators that are released, which in turn alleviates the damage caused by oxidative stress. It is important to highlight that these kinds of leaves are not humanly edible, and in most cases their traditional use is limited to infusions and decoctions. However, such methodologies are associated with several constraints, namely in regard to PCs, since they are thermolabile compounds and, therefore, display high degradation patterns. On another hand, the bioavailability of tea phytochemicals is relatively low, only giving the organism access to a limited amount of the available PCs. [282] Finally, blueberry leaves' extracts and/or traditionally made teas discards great amounts of vegetable raw material with a very promising composition in terms of high-molecular weight polyphenols as well as dietary fiber with prominent prebiotic and immunomodulatory effects. [283,284]

In order to counteract such limitations, our research group has been working on the optimization of a biotechnological approach for leaves processing, aiming to obtain an innovative biomass – BB biomass – displaying a superior diversity of bioactive compounds, not only PCs but also dietary fiber, with great health-promoting potential (please see Chapter II).

Chapter II | **HYPOTHESIS AND AIMS**

2.1. Hypothesis and Aims

Neurodegenerative diseases compose one of the main public health problems nowadays. Particularly, MS is one of the leading causes of neurological disability in young adults, having an enormous personal, social, and economic impact.

In the last years, the central role of dietary therapy in the management of chronic diseases has been consistently emphasized. [285,286] It is worth noting that the PCs and dietary fiber found in a panoply of plant-based foods have been showing marked therapeutic value [287,288], namely in the framework of neurologic disorders such as MS [269-272]. Available clinical evidence show that targeted dietary interventions exert positive effects on controlling MS symptomatology and relapse. [289,290] For instance, plant-based diets with an enriched PCs content and/or dietary supplements of prebiotics/probiotics can act on multiple compartments of the gut-immune-CNS axis, allowing the maintenance of a healthy symbiotic gut microbiota, increasing autoimmunity tolerance while refraining oxidative stress/inflammation, ultimately improving patient wellness. [290-293]

Blueberry bush's leaves, particularly the senescent ones, have been drawing attention due to their higher PCs content as well as superior antioxidant profile when compared to the fruit. In the last years, our research group has been working on the biotechnological processing of blueberry leaves (dried senescent leaves commercialized from distinct blueberry cultivars – Mirtilusa Lda.), aiming to obtain innovative biomasses with health-promoting potential (INOVC2020 SAAC-CENTRO-46-2016-01-5625). This upcycling strategy resorts to a sustainable environment-friendly technology, aiding in the transition to circular economy standards through the introduction of new ingredients into food chains that otherwise would remain as agricultural leftovers, in accordance with most European sustainability policies (e.g. European Green Deal, Zero Waste project). Previous results of our team openly demonstrate that the resultant BB biomass displays an impressive antioxidant potential (38-fold superior) and higher total phenolic content (TPC, 38-fold superior) when compared to the fruit. Chlorogenic acid, quercetin and rutin (quercetin-3-O-rutinoside) were found the main soluble PC's. As expected, BB biomass also presents an enriched content of dietary fiber (5-fold superior when compared to the fruit; Unpublished data - Manuscript under preparation). Collectively, these features hint for a new functional ingredient with prebiotic potential attained from blueberry cultivars' agrowastes - senescent leaves – with low commercial value. To broaden the elucidation of BB biomass' health potential, this work aimed to further assess:

i) <u>BB biomass safety profile</u>

Since there is evidence of toxic effects resulting from high-dose PCs administration [294], and because BB biomass holds the potential to constitute a new dietary supplement in the future, it is imperative to evaluate its safety profile. To do so, three different doses of the BB biomass were orally administered to healthy C57BL/6 in a sub-chronic regime (28 days) [295], and classic toxicity markers such as body weight, food and water consumption, glycemia and behavior (exploratory demeanor, anxiety, memory, motor coordination and learning abilities) were

assessed. Hematological and biochemical parameters were also determined, and kidney and liver morphology and function were evaluated. Moreover, we took this opportunity to evaluate the impact of BB biomass on gut health, namely through the modulation of Treg/Th17 balance in gut-associated lymphoid tissue (GALT) – a well-known readout of prebiotic activity. [296-298]

ii) BB biomass' nutraceutical potential in MS

In order to assess the putative nutraceutical potential of BB biomass in MS, the animal model of cuprizone (CPZ) intoxication was employed. Although the most frequently used MS model is the Experimental Autoimmune Encephalomyelitis (EAE), the CPZ one was selected due to the fact that it allows for spontaneous remyelination to occur - a central focus of current MS research as currently approved drugs significantly decrease the risk of new relapses but have virtually no impact on remyelination. [186] Identically to the first assay, body weight, food and water consumption were monitored throughout the entire protocol. In the gut, Treg/Th17 balance was evaluated, a key pathophysiological component of the disease. Brain gene expression of myelin components as well as histomorphology were carefully assessed in order to evaluate the demyelinating effects of CPZ as well as the potential myelination-promoting properties of the BB biomass. The effects of CPZ in the biological antioxidant systems as well as the antioxidant performance of the BB biomass were further evaluated through Superoxide dismutase (SOD) isoforms 1/2 gene expression and SOD enzymatic activity in the gut, serum, and brain.

Chapter III | **MATERIALS AND METHODS**

3.1. Evaluation of BB biomass' safety profile – Experimental Setting I

Even though PCs are better known for their beneficial biological effects, their dose-dependent dual mode of action (pro- versus antioxidant) is an ongoing debate [299,300]. Therefore, when studying the effects of a polyphenolic supplementation **in vivo**, it is paramount to assess its safety profile. Thus, the first task consisted of verifying the lack of systemic toxicity once BB biomass was orally delivered in a sub-chronic regimen, in three different doses.

The doses of BB biomass to be tested were selected based on a compromise between i) available literature of the most frequently administered doses of main PCs (CGA, rutin) [301-305] and ii) previous research from our team, demonstrating renal impairments upon chronic consumption of 25g/Kg of blueberry juice (fruit), a dose equivalent to a daily intake of 50.5mg TPC. Since BB fruit and BB biomass doses may not be linearly equivalent due to significant differences in PCs composition, food matrices, PCs' bioavailability and antioxidant profile, we established that the maximum dose to be tested should be significantly below the threshold of 50.5 mg TPC. Therefore, BB biomass doses corresponding to 1.25%, 12.5% and 25% of the referred value were selected, giving rise to a low dose of 50 mg/kg (BB1), an intermediate dose of 500 mg/kg (BB2) and a high dose of 1000 mg/kg (BB3). **(Table 4)**

Table 4. Total phenolic content of BB biomass doses established to assess the safety profile

	BB biomass 50mg/Kg (BB1)	**BB biomass 500mg/Kg (BB2)**	**BB biomass 1000mg/Kg (BB3)**
TPC (505mg GAE/g BB biomass)	0.631	6.300	12.625

3.1.1. Animals and treatments

Thirty-two healthy C57BL/6J mice (adult male, ~25g) were housed in the animal facility of Coimbra Institute for Clinical and Biomedical Research (iCBR), Faculty of Medicine, University of Coimbra, four *per* cage, under controlled environmental conditions with day/night cycles of 12 h, temperature 22±1 °C and relative humidity 50-60% with food and tap water supplied *ad libitum*. Following an acclimation period of 2 weeks, mice were randomly divided into four experimental groups and were daily exposed to: i) BB1 (low dose, p.o., n=8), ii) BB2 (intermediate dose, *p.o.*, n=8); iii) BB3 (high dose, *p.o.*, n=8); and iv) Vehicle (control, *p.o.*, n=8) for a period of 4 weeks (Fig. 6). All animals were monitored once a week for fluid and food intake, body weight and glycemic values. At week 3 (W3), animals were subjected to behavior tests, namely the Open Field Test (OFT) and the RotaRod Test (RRT), in order to evaluate their locomotor ability, coordination, anxiety levels, and explorative behavior. Metabolic cages were also employed (n=8/ experimental group) to obtain 24-hour urine parameters. At the end, animals

were anaesthetized, and blood was drawn by cardiac puncture for hematological and biochemical analyses. After cervical dislocation, the main organs were weighed for relative weight determination.

Animal experiments were conducted according to the National and European Communities Council Directives of Animal Care and received approval (# 12/2018) by the local (iCBR) Animal Welfare Body (ORBEA).

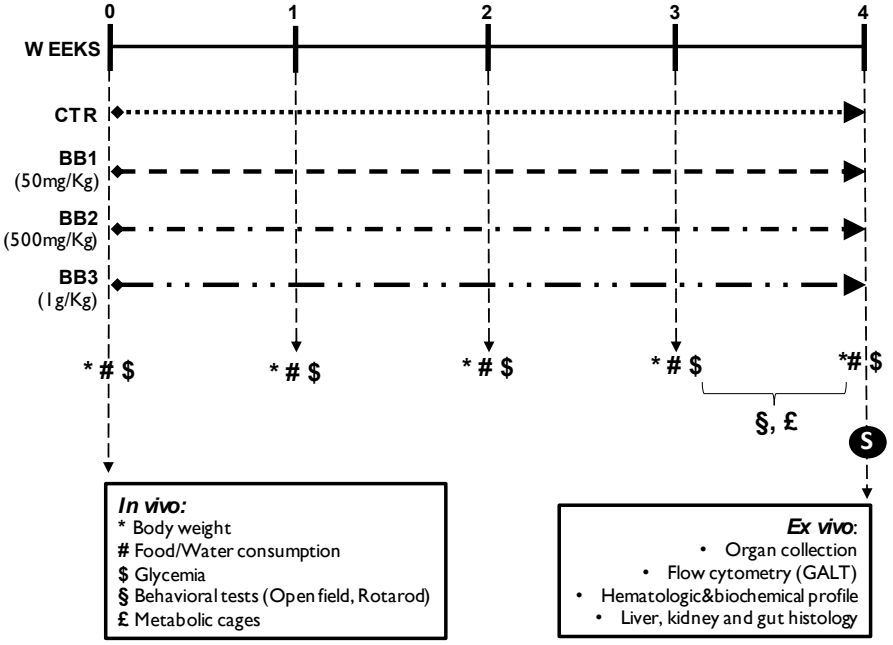


Figure 6. Experimental design I. Thirty-two C57BL/6J healthy mice were distributed among four groups (8 animals per experimental group) for sub-chronic toxicity assessment of BB biomass (50, 500 or 1000 mg/Kg BW). For 28 days (4 weeks), animals consumed BB daily through a semi-solid inert vehicle for voluntary oral consumption (Pill, patent pending n° PCT/IB2021/053124). Abbreviations: BB, blueberry leaves biomass; S, sacrifice; GALT, gut-associated lymphoid tissue.

3.1.2. *In vivo* monitoring

3.1.2.1. Body weight

Body weight (BW) was carefully monitored before study commencement, once weekly during the study and the day before the sacrifices, using an analytical balance (CQT 2000 Core® Portable Compact Balance, Adam Equipment, USA).

3.1.2.2. Mortality and toxic signs

Classical signs of toxicity (e.g. changes in skin, hair, mucous membranes, eyes, and general behavioral manifestations) and any injury or illness were conducted once daily until the end of experiments.

3.1.2.3. Food and liquid consumption

Food and water intake measurements were recorded once a week for the 4 weeks of treatment, resorting to an analytical balance (CQT 2000 Core® Portable Compact Balance, Adam Equipment, USA). Food consumption was calculated by the difference in weight between the feed placed into the cage and the remaining feed at the end of each 7 days. Similarly, beverage consumption was estimated by the difference in volume between the water initially placed in the bottle and the remaining water after one week.

3.1.2.4. Glycemic profile

Blood samples were collected from the tail vein and occasional measurements occurred once weekly. The glucose levels were measured using a portable commercial glucometer kit (GlucoMen® aero 2K, A.MENARINI diagnostics).

3.1.2.5. Behavior

A total of two behavioral tests were distributed for 4 days at W3. All the tests took place during the light phase of the day and the animals were brought to the testing room 30 min before the trials. All tests were conducted in a low–intensity light environment, and the devices were cleaned between experiments with ethanol (Aga®, Prior Velho, Portugal) at 60% (w/v).

3.1.2.5.1. Open Field test

Motor ability, anxiety, explorative behavior, and habituation patterns were measured in an OF apparatus, consisting of a 45x45cm light-grey box surrounded by walls with a height of 40cm. Two areas were defined: the peripheral zone (PZ), located 10 cm from the walls, and the central zone (CZ), comprising the remaining area. Mice were placed in the center of the arena at the beginning of the test and allowed to move freely for 15 minutes. Total travelled distance during the test was calculated, as well as the central travelled distance. These calculations were made considering the whole 15 minutes period, as well as 3-time segments (5, 10 and 15 minutes), in order to evaluate the animals' habituation pattern to the new environment. The open field test (OFT) was recorded with a Microsoft® LifeCam Cinema and results were analyzed using the behavior tracking software Any-Maze®.

3.1.2.5.2. Rotarod test

Motor coordination, balance and learning ability were evaluated through a series of four protocols of increasing difficulty executed in a speed-regulated Rotarod device (Panlab Harvard Apparatus®, Cornella, Spain). The first protocol consisted of 5 rotations per minute (rpm) for 60 seconds (s); the second of 10 rpm during 60s; the third of 10 rpm during 60s followed by 15 rpm during 100s; and the last protocol consisted of 15 rpm during 60s followed by 25 rpm during 100s.

The speed transition had place in a 10s-period with an increase of 1 rpm/s. Animals were placed on the rotating cylinder at a 45° angle, and the number of falls as well as the time the animal stayed on the cylinder were recorded.

3.1.2.6. Sample collection

At the end of W4, animals were anaesthetized by intraperitoneal injection of ketamine chloride (1 g/mL; Imalgene®) in chlorpromazine 2.5% (Largactil®). Blood was immediately collected through cardiac puncture to EDTA containing tubes (BD Vacutainer SST II 47 Advance) and non-heparinized tubes for hematological and biochemical analysis, respectively, and then were centrifuged at 3500 rpm for 15 minutes (4 °C) and stored at -20 °C. Upon sacrifice, mice were transcardiacly perfused with ice-cold PBS1x and kidneys, liver, brain, stomach, gut, thymus, pancreas, heart, adrenal glands, and spleen were isolated, washed, weighted, and observed for any gross lesions. Samples were immediately processed for flow cytometry (see 3.1.3.8.), snap frozen in liquid nitrogen for biochemical assays or kept in a 10% neutral buffered solution to be used for histological analysis.

Samples were stored at -80 °C until analyses were performed.

3.1.3. Ex-vivo analysis

3.1.3.1. Organ weight

At the end of the dosing period, all the animals were euthanized. Different organs, namely the kidneys, liver, brain, stomach, gut, thymus, pancreas, heart, adrenal glands, and spleen were carefully dissected out and weighed in grams (absolute organ weight). The relative organ weight of each animal was then calculated as follows:

$$\text{Relative Organ Weight} = \frac{\text{Absolute Organ Weight (g)}}{\text{Mouse Body Weight (g)}}$$

3.1.3.2. Lipid profile

Serum triglycerides (TGs) levels were assessed by colorimetric methods using the kit TG PAP 1000 (bioMérieux, Lyon, France), through an automatic analyzer (Hitachi 717, Roche Diagnostics Inc., Mannheim, Germany).

Hepatic TGs levels were assessed by an enzymatic colorimetric assay using a commercial kit (Ref.1155010, Triglycerides MR, Cromatest®, Linear Chemicals, Barcelona, Spain). Briefly, 50 mg of frozen tissue were homogenized in 1 mL of isopropanol. The homogenate was sonicated and then centrifuged at 3000 rpm for 5 min at 4 °C, and the supernatant was analyzed following the manufacturer's instructions.

3.1.3.3. Histopathological analysis

Hematoxylin and Eosin (HE) staining of the liver, kidneys and gut was performed as described below. Image acquisition was carried out by light microscopy with a Zeiss microscope Mod. Axioplan 332 2 (Zeiss, Jena, Germany).

Tissue samples were formalin-fixed and embedded in paraffin wax (n=4/ experimental group). One HE-stained cryosection (5 µm) from each block was reviewed. Tissue sections were deparaffinized in xylene and hydrated to a decrescent series of ethanol until distilled water. Thereafter, the tissue sections were immersed in hematoxylin stain solution, Gill 1 (Sigma Aldrich; Missouri, USA) for 2 minutes and washed in tap water. Then, they were counterstained with 0,5% aqueous eosin (Sigma Aldrich; Missouri, USA) for 30 seconds and after that dehydrated, cleared, and mounted. All samples were examined by light microscopy using a Zeiss microscope Mod. Axioplan 332 2 (Zeiss, Jena, Germany).

3.1.3.4. Renal function

The following biochemical parameters were evaluated in serum by validated automated methods and equipment (Hitachi 717, Roche Diagnostics Inc., MA, USA): urea, creatinine, and uric acid.

Urinary creatinine, uric acid and urea concentrations were evaluated in 24-hour as well as occasional urine (Cobas Integra 400 plus, Roche®, Amadora, Portugal).

3.1.3.5. Liver function

The following biochemical parameters were evaluated in serum by validated automated methods and equipment (Hitachi 717, Roche Diagnostics Inc., MA, USA): Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and Alkaline Phosphatase (ALP), Total Proteins and Albumin.

3.1.3.6. Hematological analysis

Hematological parameters were evaluated in blood (n=4/experimental group) by validated automated methods and equipment (Hitachi 717, Roche Diagnostics Inc., MA, USA). Mean cell volume (MCV) indicated the volume of the average red cell in a sample expressed in femtoliters (fL) and calculated by using the formula: MCV = packed-cell volume/RBC × 10. Mean cell hemoglobin (MCH) represented the absolute amount of Hb in the average red cell in a sample in picograms per cell and was calculated from the Hb and the RBC using the following formula: MCH = (Hb × 10)/RBC. Mean corpuscular hemoglobin concentration (MCHC) is the average Hb concentration in the RBCs, expressed as the amount of Hb per deciliter of red cells (g/dL) and calculated as follows: MCHC = Hb/packed-cell volume. The hematocrit is the ratio of RBCs to plasma and is expressed as a percentage of the whole blood volume. It is calculated from the RBC count and the MCV as follows: hematocrit = (RBC × MCV)/10. Red cell distribution width (RDW)

is a measure of the heterogeneity of the RBC population. The RDW is derived from the RBC histogram using the width of the RBC distribution at 50% of the peak height. The mean platelet volume (MPV) is derived from the platelets histogram after the platelets count has been determined and is expressed in fL.

3.1.3.7. Superoxide dismutase assay

Serum SOD levels were determined by colorimetric methods, using the Superoxide Dismutase (SOD) Colorimetric Activity Kit (EIASODC, ThermoFisher Scientific), which is designed to measure all types of SOD activity (i.e., Cu/Zn, Mn and Fe superoxide dismutases). Briefly, blood samples were diluted 1:5 in PBS1X. The homogenate was centrifuged at 1500 g for 10 minutes at 4°C, and the supernatant was analyzed following the manufacturer's instructions.

3.1.3.8. Flow cytometry

Peyer's patches (PP) were excised from the intestinal surface, which was previously mechanically cleaned out of content. Collected PP were thoroughly washed in PBS to remove excess mucus. PP were subjected to mechanical disruption in 1x PBS and filtered, by applying mild pressure through a 70-µm nylon cell strainer (Corning Cat. No. 352235), to obtain PP single cell suspensions.

The identification of Tregs (CD45+CD3+CD4+FoxP3+CD25+) and Th17 cells (CD45+CD3+CD4+IL-17+RORγt+) within the single cell suspensions of PP was assessed by flow cytometry using the BD PharmingenTM extracellular antibodies anti-CD45 (APC-CyTM7 Rat Anti-Mouse CD45, Nr. 557659), anti-CD3 (FITC Hamster Anti-Mouse CD3e, Nr. 553062), anti-CD4 (PE-CyTM Rat Anti-Mouse CD4, Nr. 561099) and anti-CD25 (PE Rat Anti-Mouse CD25, Nr. 553866), and the intracellular antibodies anti-FoxP3 (Alexa Fluor®647 Rat Anti-Mouse Foxp3, Nr. 560401), anti-IL17 (BV510 Rat Anti-Mouse IL-17A, Nr. 564168) and anti-RORγt (BV421 Mouse Anti-Mouse RORγt, Nr. 562894).

Cell suspensions were counted in the hematological counter (Coulter AcT diff, Beckman Coulter, Pasadena, CA, USA) and the concentration adjusted to 10 million cells/mL in 1x PBS. Then, 1x106 cells were incubated with extracellular antibodies for 20 minutes at RT in the dark. Cells were washed with BD PharmingenTM Stain Buffer (FBS, Cat. No. 554656) and centrifuged at 250 x g for 10 minutes. Fixation was performed by gently resuspending the pellets in a residual volume of staining buffer and adding freshly prepared cold 1X BD PharmingenTM Fixation Buffer, followed by a 30-minute incubation at 4°C in the dark. After a 5-minute centrifugation at 500 x g, cells were washed, by resuspending each pellet in pre-warmed 1x BD PharmingenTM Permeabilization Buffer, and centrifuged again in the same conditions, after which the permeabilization buffer was removed. To permeabilize the cells, pellets were once more resuspended in pre-warmed 1x BD PharmingenTM Permeabilization Buffer and incubated for 30 minutes at 37°C in the dark. Another 5-minute centrifugation at 500 x g was performed and the buffer removed. Cells were posteriorly washed with BD PharmingenTM Stain Buffer (FBS) and centrifuged in the previously referred conditions. Buffer was removed and the intracellular

antibodies diluted in BD PharmingenTM Stain Buffer (FBS) at appropriate concentrations were added to resuspend the pellets and incubated for 20 minutes at RT in the dark. Cells were washed as described above two more times, resuspended in BD PharmingenTM Stain Buffer (FBS), acquired in the BD FACSCanto II eight-color flow cytometer (BD Biosciences, San Jose, CA, USA) and visualized with the BD FACSDiva™ Software (BD Biosciences, San Jose, CA, USA). At least 15,000 to 25,000 CD4 T cells were acquired.

All the data were analyzed with FlowJo™ Software v.10.7 (BD Life Sciences, Ashland, OR, USA). The gating strategy started with the exclusion of doublets based on size parameters (FSC-W and FSC-A). Gated on single cells, the lymphocytes were identified by the positivity for CD45 and low complexity (SSC-A). Within lymphocytes, positive cells for CD3 molecule were identified as T cells, and gated on T cells, CD4 T cells were identified by the positivity for the CD4 molecule. After discrimination of CD4 T cells, we proceeded to the identification of Treg cells and Th17 cells, through the positive signal of FoxP3 and CD25 or IL-17 and RORγt, respectively.

3.1.4. Statistical analysis

Results were expressed as means ± standard errors of the mean (S.E.M.) using GraphPad Prism® software, version 8.2.1 (GraphPad Software, Inc., La Jolla, CA, USA). The distribution of continuous variables was analyzed using the Kolmogorov-Smirnov test to assess significant deviations from normality. One-way analysis of variance (ANOVA, followed by Bonferroni's test for multiple comparisons) or the nonparametric Kruskal-Wallis test (followed by the Dunn's test for multiple comparisons) were used for normally or non-normally distributed data, respectively. Repeated measures ANOVA, followed by Bonferroni post-hoc test, were used to compare parameters evolution during the experimental period. A p value <0.05 was considered statistically significant.

3.2. Evaluation of BB biomass' therapeutic potential in experimental MS – Experimental Setting II

The therapeutic BB biomass potential was evaluated in the cuprizone (CPZ)-intoxicated animal model of MS. Animals were treated with 0.2% CPZ in order to induce demyelination, and the effects of BB biomass in demyelination and remyelination were assessed. BB biomass was administered in a 500 mg/kg dose, since this was an amount displaying lack of toxic effects, simultaneously exhibiting hypolipidemic and antioxidant properties along with gut immunomodulatory effects.

The CPZ-induced MS model is a toxic demyelination model, where animals are fed with bis-cyclohexanone oxaldihydrazone (cuprizone), which chelates copper, inhibiting copper-dependent enzymes. [306] Several studies have attempted to determine the most adequate dose of CPZ able to generate significative changes in the CNS, considerable demyelination being observed at concentrations ranging from 0.2 to 0.6%. [307] However, doses higher than 0.2% have shown to increase the animals' mortality rate [307], which makes this number the preferred concentration in animal experimentation.

Until recently, it was thought that CPZ predominantly affected the corpus callosum (CC) and the superior cerebellar peduncles. [291] Nevertheless, new studies revealed that CPZ-induced demyelination is extended to other CNS regions, such as the brainstem, basal ganglia, cortex, hippocampus, and hypothalamus, to name a few. [292] Moreover, there is also evidence of frequent CPZ-induced demyelination in the cerebellum. In fact, a study performed by Oakden et al. [294] suggests that demyelination has a cerebellar origin and then progresses forward into the brain.

Although CPZ-induced demyelination is mainly notorious in the CC, there is also evidence of frequent effects in the cerebellum. In fact, a study performed by Oakden et al. [309] suggests that demyelination has a cerebellar origin and then progresses forward into the brain.

Only a few days after CPZ administration, OLGs apoptosis starts to occur, as well as the downregulation of myelin-related genes. [307,308] However, demyelination is not evident until around week 5 after the administration has started. [307,308] Besides oligodendroglia cell death, CPZ effects also include activation of microglia cells, myelin phagocytosis, astrocyte reactivity and grey matter demyelination. [307,308] Furthermore, T and B lymphocytes are believed to play a non-dominant role during CPZ-induced demyelinating events. [308]

Megamitochondria formation in the liver has been observed as a consequence of CPZ administration, due to increased levels of ROS and possibly impaired activity of cytochrome oxidase [288] In fact, CPZ has shown to decrease the activity of this enzyme in the brain, as well as monoamine oxidase (MAO) and the complexes I, II and III of the mitochondrial respiratory chain. [288] Inhibition of these complexes, in conjugation with uncoupling of oxidative phosphorylation and consequent $O2^{\bullet-}$ production resulting from CPZ treatment impose an increased oxidative stress on OLGs. [291] In fact, evidence indicates that CPZ intoxication triggers apoptosis in mature OLGs through increased oxidative stress. [288] Furthermore, CPZ has shown to inhibit the activity of enzymes composing the antioxidant system, such as SOD and catalase (CAT), to name a few. [291,292]

Myelin lipid metabolism is likewise affected by CPZ administration, since the toxin increases phospholipase A2 and plasmogenase activities, leading to degradation of myelin sheaths and production of arachidonic acid (AA), which is a key intermediate of proinflammatory signaling. [307] AA is metabolized by COX enzymes, forming prostaglandin H2 and consequently prostaglandin E2. [307] Additionally, cerebrosides and cholesterol, which are also important myelin constituents, are significantly reduced by the increased activity of plasmogenase, leading to myelin vacuolation. [307] Furthermore, CPZ induces the release of pro-inflammatory cytokines by microglia and astrocytes, such as IL-6, IL-17 and TNF-α, and upregulates iNOS expression. [307]

If CPZ administration is suspended, spontaneous remyelination takes place [306-308], which makes the CPZ model excellent for the study of agents with potential to prevent demyelination and/or promote remyelination.

3.2.1. Animals and treatments

A total of 60 C57BL/6J male mice were acquired from Charles River Laboratories, Barcelona, Spain. They were housed in the animal facility of Coimbra Institute for Clinical and Biomedical Research (iCBR), Faculty of Medicine, University of Coimbra, four *per* cage, under controlled environmental conditions with day/night cycles of 12 h, temperature 22±1 °C and relative humidity 50-60% with food and tap water supplied *ad libitum*. After 2 weeks of acclimatization, the animals were randomly divided into six groups (10 animals per experimental group): controls (CTR), which received vehicles only for 5 and 7 weeks, respectively; animals receiving CPZ only during 5 weeks and sacrificed at the end of week five (CPZ W5) (demyelination peak); animals receiving CPZ for 5 weeks and water for 2 more weeks until sacrificed (CPZ W7) (early remyelination); animals receiving CPZ for 2.5 weeks and BB biomass until the end of W5 (CPZ W5+BB) and animals receiving CPZ for 5 weeks and water for 2 more weeks until sacrificed, with the BB biomass treatment starting between W2 and W3 (CPZ W7+BB). (Fig. 7) CPZ 0.2% m/v was administered daily by oral gavage (dissolved in methylcellulose 1%, w/v), and the BB biomass at a dose of 500 mg/kg was given through a semi-solid vehicle for voluntary consumption (Pill, Patent pending n° PCT/IB2021/053124).

Animal experiments were conducted according to the National and European Communities Council Directives of Animal Care and received approval (#12/2018) by the local (iCBR) Animal Welfare Body (ORBEA).

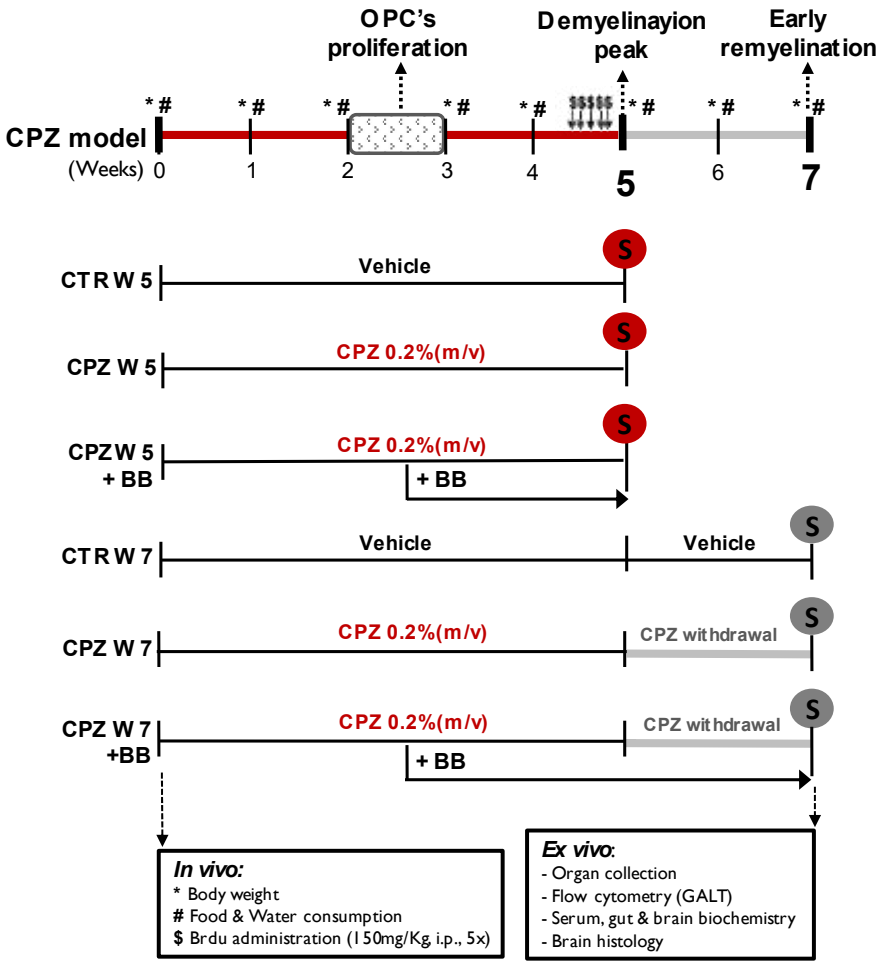


Figure 7. Experimental design II. Sixty C57BL/6J adult healthy mice were distributed among six groups (10 animals per experimental group). CTR W5/W7 groups were submitted to force-fed gavage of vehicle (methylcellulose, 1% w/v), daily, for 5/7 weeks. CPZ W5 group was submitted to force-fed gavage of CPZ 0.2% (dissolved in methylcellulose 1%, w/v) for 5 weeks to induce demyelination. To study the early remyelination period, CPZ W7 group was submitted to force-fed gavage of CPZ 0.2% (dissolved in methylcellulose 1%, w/v) for 5 weeks followed by CPZ withdrawal for 2 weeks more. To assess BB biomass therapeutic potential in experimental MS, BB (500mg/Kg) was Pill-dosed daily, from week 2.5 (onset of OPC's proliferation) to the end of the experiments (W5 or W7). All experimental groups were daily dosed with the semi-solid vehicle (Empty Pill: CTR W5, CTR W7, CPZ W5, CPZ W7; BB-PILL: CPZ W5+BB, CPZ W7+BB - Patent pending n° PCT/IB2021/053124). Abbreviations: CPZ, cuprizone; OPC's, oligodendrocytes progenitor cells; BB, blueberry leaves biomass; S, sacrifice; GALT, gut-associated lymphoid tissue.

3.2.2. *In vivo* monitoring

3.2.2.1. Body weight

Body weight (BW) was carefully monitored as previously described (section 3.1.2.1.).

3.2.2.2. Mortality and toxic signs

Classical signs of toxicity (e.g. changes in skin, hair, mucous membranes, eyes, and general behavioral manifestations) and any injury or illness were conducted once daily until the end of experiments.

3.2.2.3. Food and liquid consumption

Food and water intake measurements were recorded once a week throughout the entire treatment period, resorting to an analytical balance (CQT 2000 Core® Portable Compact Balance, Adam Equipment, USA). Food consumption was calculated by the difference in weight between the feed placed into the cage and the remaining feed at the end of each 7 days. Similarly, beverage consumption was estimated by the difference in volume between the water initially placed in the bottle and the remaining water after one week.

3.2.2.4. BrdU administration

Cell proliferation was measured by the incorporation of the thymidine analogue Bromodeoxyuridine (BrdU), which is incorporated into the DNA of dividing cells in immunohistochemically detectable quantities during the S phase of cell division [296]. Four animals of each experimental group received intraperitoneal injections of BrdU (Sigma, St Louis, MO, USA) at a concentration of 150 mg/kg daily for 5 consecutive days, starting at the 5 days before the end of W5. This timepoint will allow us to discriminate the effect of BB biomass treatment in newly generated BrdU-labeled cells (future studies).

3.2.2.5. Sample collection

At the end of weeks 5 and 7, animals were anaesthetized by intraperitoneal injection of ketamine chloride (1 g/mL; Imalgene®) in chlorpromazine 2.5% (Largactil®). Blood was immediately collected through cardiac puncture to serum tubes (BD Vacutainer SST II 47 Advance) and then centrifuged at 3500 rpm for 15 minutes (4 °C) and stored at -20 °C. Upon sacrifice, mice were transcardiacly perfused with ice-cold PBS1x and gut and brain were isolated, washed and weighted. Brains were divided into left and right hemispheres (WB) following excision of left and right cerebella (CB). Left hemispheres (n=5) were stored in OCT CryoMatrix (6769006, ThermoScientific) for fluorescence microscopy or 10% neutral buffered solution (n=5) to be used for histological analysis. Right hemispheres (n=5) were immersed in RNA latter (R-0901, Sigma Aldrich) for gene expression analysis or snap frozen in liquid nitrogen for biochemical assays (n=5). Following Peyer patches excision for flow cytometry (see 3.1.3.8), small intestine and colon were divided into four sections and properly stored for gene expression, biochemical assays, fluorescence microscopy and histology.

Samples were stored at -80 °C until analyses were performed.

3.2.3. *Ex vivo* analysis

3.2.3.1. Antioxidant activity: SOD assay

SOD levels in serum, gut and brain were determined by colorimetric methods, using the Superoxide Dismutase (SOD) Colorimetric Activity Kit (EIASODC, ThermoFisher Scientific), which is designed to measure all types of SOD activity (i.e., Cu/Zn, Mn and Fe superoxide dismutases). Briefly, blood samples were diluted 1:5 in PBS1X. The homogenate was centrifuged at 1500 g for 10 minutes at 4 °C, and the supernatant was analyzed following the manufacturer's instructions. For the gut and the brain, approximately 50 mg of tissue was weighed and sonicated in PBS1X. The homogenates were then diluted in 1:5 PBS1X and centrifuged at 1500 g for 10 minutes at 4 °C. The supernatant was analyzed following the manufacturer's instructions.

3.2.3.2. Gene expression analysis

3.2.3.2.1 RNA extraction

For brain samples, RNA was extracted from 30-50 mg of frozen tissue (preserved in RNA later Stabilization Solution, R-0901, Sigma Aldrich) using the the NZY Total RNA Isolation Kit (nzytech, MB13402), according to the manufacturer's instructions. RNA concentrations were determined (NanoDrop® ND-1000 Spectrophotometer). Samples were stored at -80°C until subsequent analysis.

3.1.2.2.2. cDNA synthesis

Synthesis of complementary Deoxyribonucleic acid (cDNA) was performed using a Xpert cDNA Synthesis Mastermix (GK81.0100, Lot. 7E2709A, GRISP). For each tube, it was pipetted the volume corresponding to 2 µg RNA, 10 µL of Mastermix and water (to a final volume of 19 µL. Then, in the thermocycler (1861096, T100TM Thermal Cycler, Bio-Rad) cDNA was synthesized following the Xpert cDNA Synthesis Mastermix protocol. Samples were stored at -20 °C.

3.1.2.2.3. RT-PCR

A mixture was prepared containing 10 µL of Sybr Green (iTaq Universal SYBR Green Supermix 1725124, Bio-Rad), 0.4 µL of mix primers (Table 4) and 7.6 µL of autoclaved water. 18 µL of this mixture and 2 µL of the sample were transferred into each well. Realtime polymerase chain reaction (RT-PCR) protocol consisted of 1 cycle for initial denaturation (10 min at 95°C), followed by 40 cycles comprising the following steps: 15s, 95 °C; 45s, 58 or 60 °C; 30 s at 72 °C. Standardization was achieved with GeNorm algorithm, where gene stability was attained with Hypoxanthine Phosphoribosyltransferase (HPRT) and Glyceraldehyde 3-phosphate

Dehydrogenase (GAPDH). The relative expression ratio of each of the target gene was computed on the basis of ΔΔCt ($2^{-\Delta\Delta C_P}$) values. Results are expressed as percentage of control.

Table 5. Primer sequences and real-time PCR conditions

Gene	Primer Sequence		Temp. (ºC)
	Forward	Reverse	
GAPDH	CGA CTT CAA CAG CAA CTC	TGT AGC CGT ATT CAT TGT	58
HPRT	TCC ATT CCT ATG ACT GTA	CAT CTC CAC CAA TAA CTT	58
MBP	GCC TGT CCC TCA GCA GAT TT	GTC GTA GGC CCC CTT GAA TC	58
SOD-1	AAC CAG TTG TGT TGT CAG GAC	CCA CCA TGT TTC TTA GAG TGA	60
SOD-2	CAG ACC TGC CTT ACG ACT ATG	CTC GGT GGC GTT GAG ATT GTT	60
PLP	CAG GCA GAT CTT TGG CGA	TGA TGC CCA CAA ACG TTG	60

3.2.3.3. Brain histopathological analysis

Kluver-Barrera (KB), Toluidine Blue (TB) and Hematoxylin&Eosin (HE) staining of brain tissue were performed. Two cryosections (5 µm) from one block of each experimental group was reviewed (preliminary analysis - cerebellum). Image acquisition was carried out by light microscopy with a Zeiss microscope Mod. Axioplan 332 2 (Zeiss, Jena, Germany).

3.2.3.3.1. Kluver-Barrera

KB is a double staining method involving cresyl violet and Luxol Fast Blue (LFB) stain of myelin sheaths, which acquire a navy-blue tone, while demyelinated areas are unstained or the color less pronounced.

Tissue samples were formalin-fixed and embedded in paraffin wax. Tissue sections were deparaffinized in xylene and hydrated with ethanol, followed by overnight staining with 0.1% Luxol Fast Blue solution. Thereafter, the tissue sections were washed in tap water and differentiated with 0.05% lithium carbonate solution (Sigma Aldrich; Missouri, USA) and 70% ethanol. After another washing step, sections were stained with 0.1% Cresyl Violet solution (Sigma Aldrich; Missouri, USA) for 10 minutes and two more steps of differentiation followed, with 95% ethanol and 10% Acetic Acid solution (Sigma Aldrich; Missouri, USA). Finally, sections were dehydrated, cleared, and mounted.

3.2.3.3.2. Toluidine blue

TB is well-known for staining nervous tissue, particularly to stain neurons and glia cells. Tissue samples were were formalin-fixed and embedded in paraffin wax. Tissue sections were deparaffinized in xylene and hydrated with ethanol. Sections were pretreated with 85% formic acid solution for 10 minutes. Slides were washed thoroughly with tap water, incubated in a 3:1 solution of 100% ethanol and glacial acetic acid for 10 minutes and washed again with tap water. Then, sections were incubated in toluidine blue-O solution for 5–10 min and washed with tap water for another 5 min. Finally, sections were dehydrated, cleared, and mounted.

3.2.3.3.3. Hematoxylin & Eosin

Tissue samples were formalin-fixed and embedded in paraffin wax (n=2 each experimental group). One HE-stained cryosection (5 µm) from each block was reviewed. Tissue sections were deparaffinized in xylene and hydrated to a decrescent series of ethanol until distilled water. Thereafter, the tissue sections were immersed in haematoxylin stain Solution, Gill 1 (Sigma Aldrich; Missouri, USA) for 2 minutes and washed in tap water. Then, they were counterstained with 0,5% aqueous eosin (Sigma Aldrich; Missouri, USA) for 30 seconds and after that dehydrated, cleared, and mounted.

3.2.3.4. Flow cytometry

The percentage of Treg and Th17 cells in the gut was assessed as previously described (3.1.3.8.).

3.2.4. Statistical analysis

Results were expressed as means ± standard errors of the mean (S.E.M.) and analyzed as previously described (3.1.4.).

Chapter IV | **RESULTS**

4.1. Evaluation of BB biomass' safety profile – Experimental Setting I

4.1.1. Body Weight

Body weight (BW) was monitored weekly during the 4 weeks of the study **(Fig. 8)**. Similar values were recorded across all experimental groups. Thus, it can be said that overall BW was not affected by the biomass in any dose.

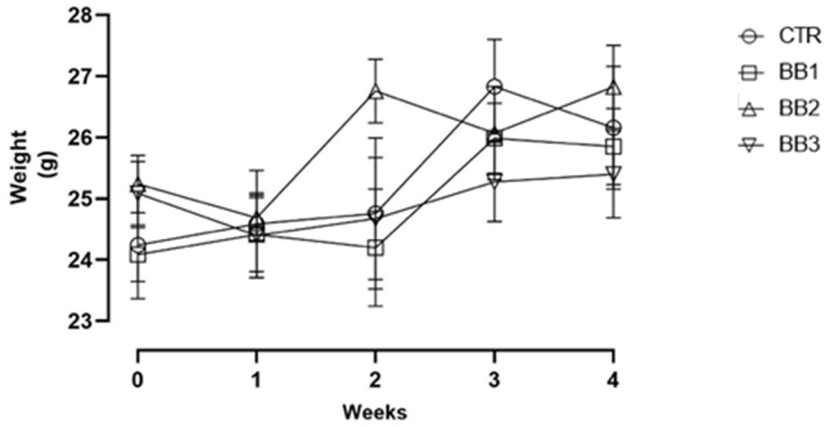


Figure 8. Body weight evolution (Experimental Setting I). Results are expressed as mean ± S.E.M of 8 animals per group.

4.1.2. Mortality and toxic signs

Daily oral administration of the BB biomass for 28 days did not produce any symptoms of toxicity in mice, including the highest dose tested at 1 g/kg body weight. No deaths or obvious clinical signs were found in any groups throughout the study. None of the animals showed signs of toxicity in their skin, fur, eyes, sleep, salivation, diarrhea, and general behavior.

4.1.3. Food and water intake

Food and water consumption were also evaluated for the 4 weeks of treatment. **(Fig. 9)** No significant differences were observed across the different experimental groups regarding food intake **(Fig. 9A)**, as well as water consumption **(Fig. 9B)**.

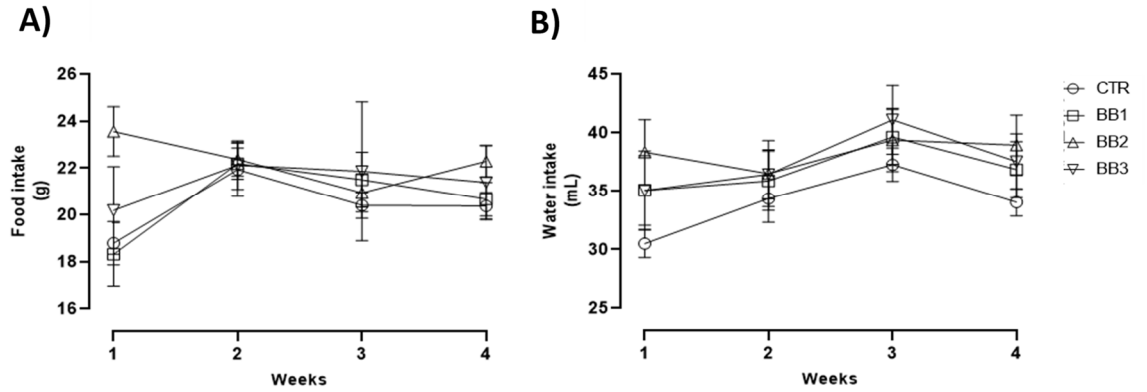


Figure 9. Food and water intake evolution (Experimental Setting I). A) Evolution of food intake at W1-W4 of BB biomass administration. **B)** Evolution of water intake at W1-W4 of BB biomass administration. Results are expressed as mean ± S.E.M of 8 animals per group.

4.1.4. Glycemic profile

To evaluate the effects of BB biomass on the glycemic profile, blood glycemia was measured once a week throughout the 4 weeks of treatment. **(Fig. 10)** There were no significant differences in glucose concentration across all experiment groups.

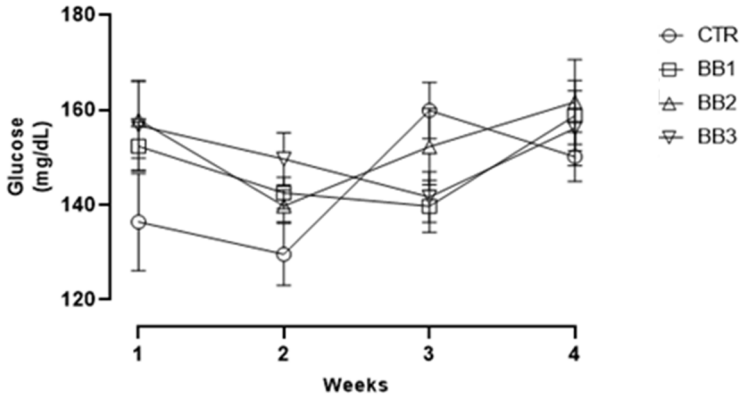


Figure 10. Glycemic profile (Experimental Setting 1). Evolution of blood glucose values between W1 and W4 of BB biomass administration. Results are expressed as mean ± S.E.M of 8 animals per group.

4.1.5. Behavior tests

4.1.5.1. Open Field test

Open field test (OFT) studied the animals' locomotor ability and their anxiety levels by measuring the total traveled distance **(Fig. 11A)** and the percentage of distance traveled in the center and periphery of the field **(Fig. 11B)**. These variables were analyzed considering the whole 15-min test. The former analysis showed no differences between the CTR and BB groups, suggesting the assessed behavioral parameters were not affected by the BB biomass.

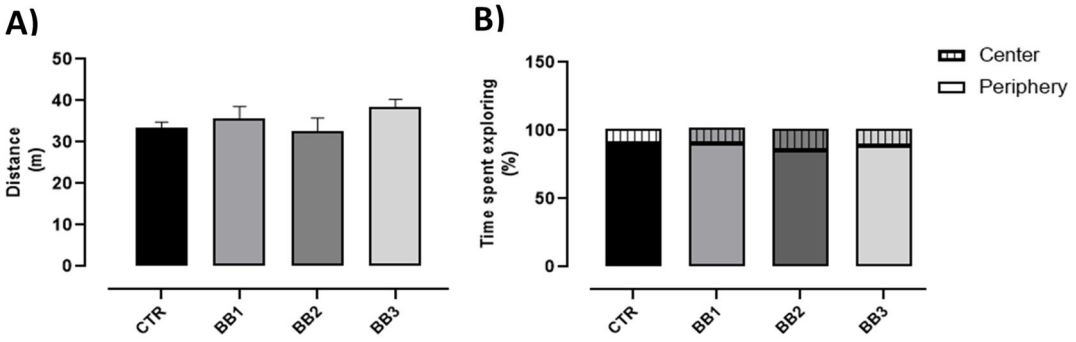


Figure 11. Open Field behavior test (Experimental Setting I). A) Total distance travelled during the 15 minutes of the OF assay. **B)** Time spent exploring between the center and the periphery of the arena. Results are expressed as mean ± S.E.M.

4.1.5.2. Rotarod test

Animals given the BB biomass displayed preserved locomotor and motor coordination abilities, as there were no significant differences in the number of falls (Fig.12A) as well as time spent on the device (Fig. 12B) between treated (BB1, BB2 and BB3) and control animals (CTR) in any assay.

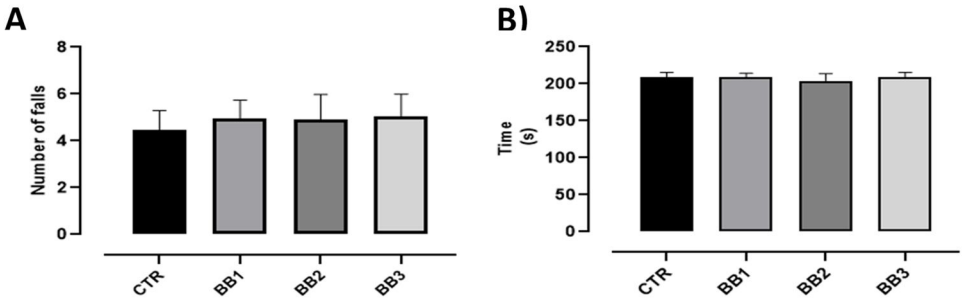


Figure 12. Rotarod behavior test (Experimental Setting I). **A)** Number of falls recorded on the Rotarod behavior test. **B)** Time spent on the Rotarod device. Results are expressed as mean ± S.E.M of 8 animals per group.

4.1.6. Organ weight

The relative organ weight of each organ recorded at necropsy. Treatment groups did not show a significant difference ($p>0.05$, when compared to CTR) (**Table 6**).

Table 6. Relative organ weight (Experimental Setting I)

	CTR (Vehicle)	BB1 (50 mg/Kg)	BB2 (500 mg/Kg)	BB3 (1 g/Kg)
Right Kidney (g)	0.650 ± 0.042	0.636 ± 0.033	0.617 ± 0.021	0.621 ± 0.013
Left Kidney (g)	0.705 ±0.029	0.753 ± 0.037	0.696 ± 0.044	0.720 ± 0.025
Liver (g)	5.642 ± 0.353	5.431 ± 0.214	4.526 ± 0.264	4.976 ± 0.271
Heart (g)	0.481 ± 0.010	0.540 ± 0.019	0.462 ± 0.007	0.492 ± 0.012
Brain (g)	1.678 ± 0.040	1.765 ± 0.053	1.653 ± 0.047	1.749 ± 0.048
Colon (g)	1.079 ± 0.063	1.063 ± 0.040	1.062 ± 0.062	1.002 ± 0.030
Stomach (g)	0.859 ± 0.054	0.898 ± 0.017	0.802 ± 0.022	0.875 ± 0.053
Pancreas (g)	1.221 ± 0.069	1.409 ± 0.053	1.234 ± 0.110	1.149 ± 0.067
Adrenals (g)	0.009 ± 0.001	0.012 ± 0.003	0.010 ± 0.002	0.012 ± 0.003
Spleen (g)	0.340 ± 0.052	0.363 ± 0.066	0.298 ± 0.026	0.315 ± 0.022
Thymus (g)	0.207 ± 0.013	0.234 ± 0.023	0.173 ± 0.008	0.195 ± 0.012

Results are expressed as mean ± S.E.M of 8 animals per group.

4.1.7. Biochemical analysis

The effects of sub-chronic administration of BB biomass on biochemical parameters are presented in **Table 7**. No statistically significant differences in the liver function parameters such as ALT, AST, and ALP were observed. Likewise, no relevant changes were found in total proteins and albumin. However, kidney function parameters, namely urea, uric acid and creatinine in the urine were significantly decreased relatively to controls ($p<0.05$, when compared to CTR). These results led to the exclusion of the BB3 dose as a safe amount.

Table 7. Biochemical parameters in the sub-chronic oral toxicity study (Experimental Setting I)

		CTR (Vehicle)	BB1 (50mg/Kg)	BB2 (500mg/Kg)	BB3 (1g/Kg)
Urine Parameters	Urea (mg/dL)	9189.8 ± 619.2	7661.3 ± 1419.4	9400.8 ± 1096.4	6008.3 ± 813.8*
	Creatinin (mg/dL)	65.0 ± 3.4	51.1 ± 6.0	62.9 ± 6.4	43.0 ± 3.0 *
	Uric Acid (mg/dL)	12.8 ± 0.8	11.9 ± 2.0	10.8 ± 0.7	6.7 ± 1.2 *
Serum Parameters	Urea (mg/dL)	56.6 ± 2.3	51.0 ± 1.7	59.0 ± 2.9	54.8 ± 7.6
	Creatinin (mg/dL)	0.5 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
	Uric Acid (mg/dL)	3.0 ± 0.3	2.9 ± 0.2	3.0 ± 0.3	2.3 ± 0.2
Serum Biochemistry	AST (U/L)	56.7 ± 2.3	51.0 ± 1.7	59.0 ± 2.9	54.8 ± 7.6
	ALT (U/L)	19.3 ± 2.6	25.0 ± 4.6	17.3 ± 1.6	18.8 ± 5.4
	ALP (U/L)	62.7 ± 28.9	76.5 ± 5.2	70.5 ± 12.5	62.8 ± 7.2
	Total proteins (mg/dL)	4.0 ± 0.1	4.0 ± 0.1	4.0 ± 0.1	4.3 ± 0.2
	Albumin (mg/dL)	2.9 ± 0.0	2.9 ± 0.1	3.0 ± 0.3	3.2 ± 0.2

Results are expressed as mean ± S.E.M of 4 animals per group. One-way ANOVA * $p<0.05$ vs CTR.

4.1.8. Hematological parameters

The effects of sub-chronic administration of BB biomass on hematological parameters are presented in **Table 8**. Most hematological parameters regarding white blood cells and platelets number in treated mice were not significantly different from the controls. However, significant differences were observed between the BB3 group and the CTR one regarding erythrocytes, hemoglobin and hematocrit values, the treated animals presenting increased numbers of the referred cells, hemoglobin concentrations and hematocrit percentage. (* $p<0.05$, when compared to CTR).

Table 8. Hematological parameters in the sub-chronic oral toxicity study (Experimental Setting I).

	CTR (Vehicle)	BB1 (50mg/Kg)	BB2 (500mg/Kg)	BB3 (1g/Kg)
Erythrocytes (T/L)	6.9 ± 1.0	7.6 ± 1.4	7.6 ± 0.5	8.1 ± 0.4*
Hemoglobin (g/dL)	10.7 ± 1.5	11.5 ± 1.9	11.8 ± 0.8	13.0 ± 0.6*
Hematocrit (%)	33.8 ± 2.3	35.8 ± 2.5	36.2 ± 2.8	40.3 ± 1.6*
MCV (fL)	49.6 ± 1.8	49.1 ± 1.4	46.7 ± 0.6	48.6 ± 0.9
Total MCH (pg)	15.6 ± 0.0	15.8 ± 0.4	15.3 ± 0.2	15.5 ± 0.4
MCHC (g/dL)	32.4 ± 0.3	32.2 ± 0.1	33.0 ± 0.3	32.2 ± 0.2
RDW (%)	17.0 ± 0.3	16.0 ± 0.4	16.0 ± 0.3	16.2 ± 0.3
Leucocytes (G/L)	3.6 ± 0.7	3.7 ± 0.6	3.7 ± 1.0	2.8 ± 0.6
Neutrophils (%)	21.0 ± 7.7	12.0 ± 2.2	25.2 ± 11.6	12.0 ± 1.0
Lymphocytes (%)	77.2 ± 8.9	85.5 ± 1.3	74.0 ± 11.7	88.0 ± 1.0
Monocytes (%)	1.2 ± 1.2	2.5 ± 1.5	1.5 ± 0.2	1.6 ± 0.2
Eosinophils (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Basophils (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Platelets (G/L)	923.3 ± 166.1	876.0 ± 35.8	873.5 ± 139.3	874.5 ± 85.4
MPV (fL)	5.6 ± 0.1	5.3 ± 0.1	5.6 ± 0.0	5.2 ± 0.1

Results are expressed as mean ± S.E.M of 4 animals per group. One-way ANOVA * $p<0.05$ vs CTR.

4.1.9. Liver and kidney histomorphology

Macroscopic examination of organs of treated mice revealed no abnormalities in terms of color or texture when compared with the organs of the control group. Moreover, light microscopy examination of sections of liver and kidney **(Fig. 13)** of control and treated groups showed a normal histology and absence of any gross pathological lesions.

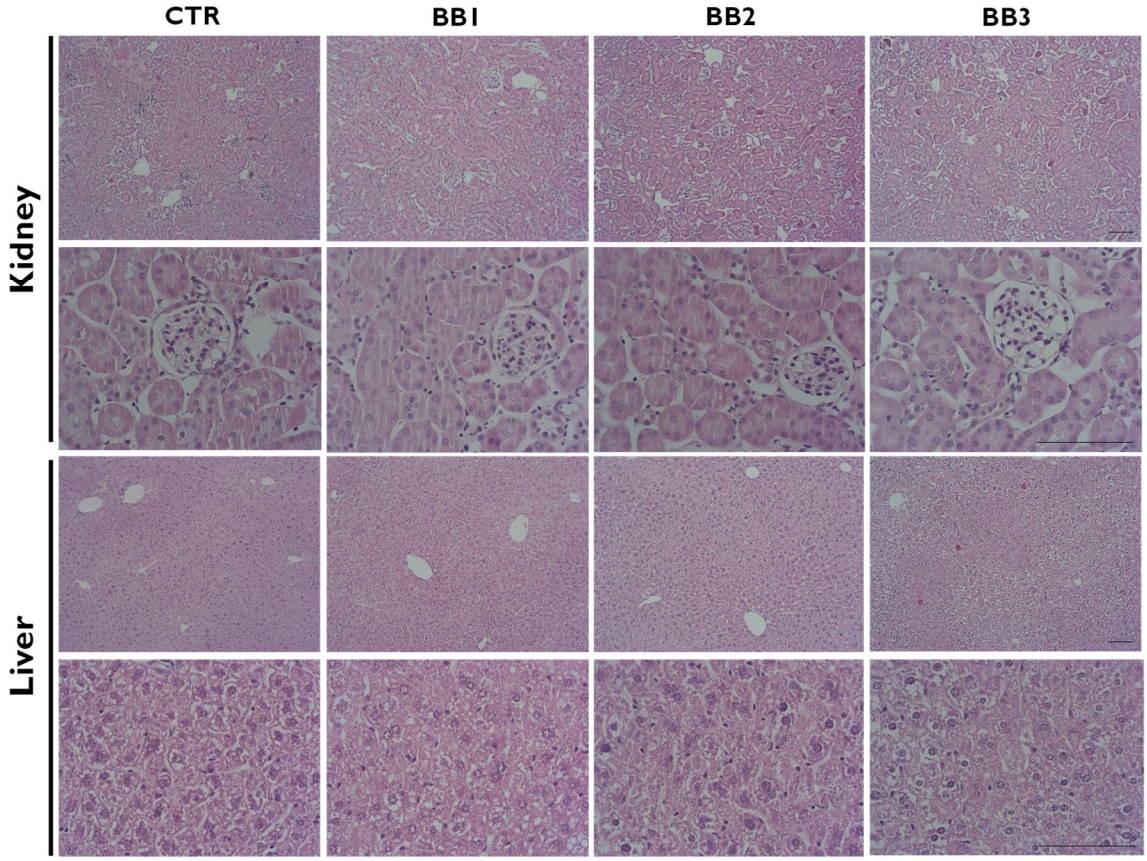


Figure 13. Histopathological examination of kidneys and liver of mice in the sub-chronic oral toxicity study (Experimental Setting I). Representative images of renal and hepatic morphologic features of all experimental groups. (H&E, Scale bar = 100 μm)

4.1.10. Lipid profile

Serum and hepatic TGs were further determined. **(Fig. 14)** Serum TGs levels display a significant decrease in both the BB2 and BB3 groups relatively to the controls ($p<0.05$, when compared to CTR). **(Fig.14A**) On another hand, TGs in the liver were significantly increased in the BB3 group ($p<0.05$, when compared to CTR). **(Fig.14B)**

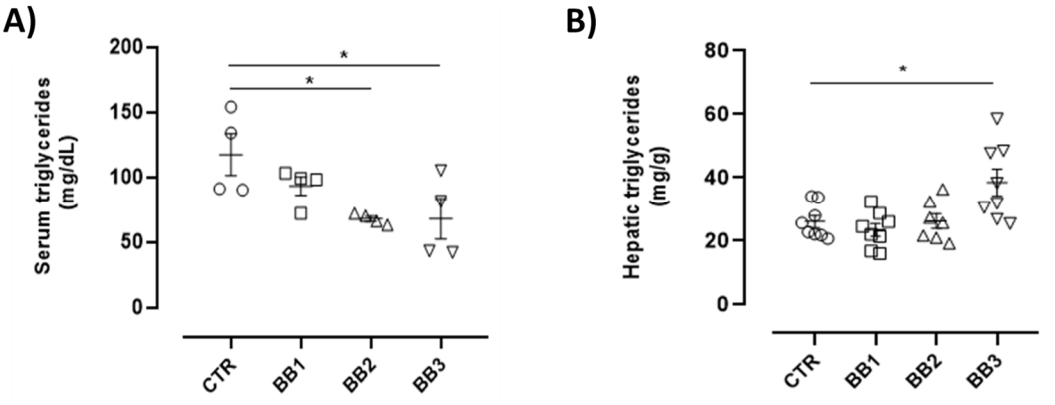
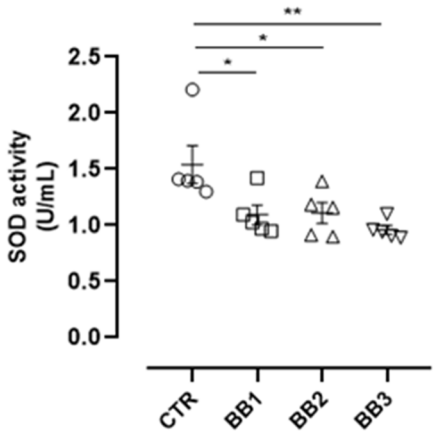


Figure 14. Triglycerides profile (Experimental Setting I). A) Serum triglyceride levels. **B).** Hepatic triglyceride levels. Results are expressed as mean ± S.E.M of 4-8 animals per group. One-way ANOVA * $p<0.05$ vs CTR.

4.1.11. SOD activity

SOD activity in the serum of mice administered BB biomass is showed in **Fig. 15.** The treated groups display a statistically significant difference in serum SOD activity relatively to the CTR group, the most relevant decrease being observed for the BB3 group ($p<0.01$, when compared to CTR), while groups BB1 and BB2 exhibit equivalent effects ($p<0.05$, when compared to CTR).



Fgure 15. SOD activity in the serum (Experimental Setting I). Results are expressed as mean ± S.E.M of 4-5 animals per group. One-way ANOVA * $p<0.05$ vs CTR; ** $p<0.01$ vs CTR.

4.1.12. Gut immunomodulation

In order to determine if the BB biomass displayed immunomodulatory properties in the intestinal mucosa, Treg/Th17 balance in the gut was assessed through flow cytometry. Antibodies against FoxP3 and CD25, as well as anti-IL17 and anti-RORγt were employed for Treg and Th17 cells identification, respectively. The obtained results are shown in **Fig. 16A.** A trend to increased percentage of CD25+ FoxP3+ IL-17- Treg cells between the controls and the BB group was observed. However, relatively to controls, mice consuming the biomass displayed a significantly lower percentage of RORγt+ IL17+ FoxP3-, evidencing the impact of BB consumption on Th17 cells. These changes paralleled a regular gut morphology (colon), evaluated by morphometric analysis (H&E). Villus height and crypt depth were apparently similar between groups. **(Fig. 16B)**

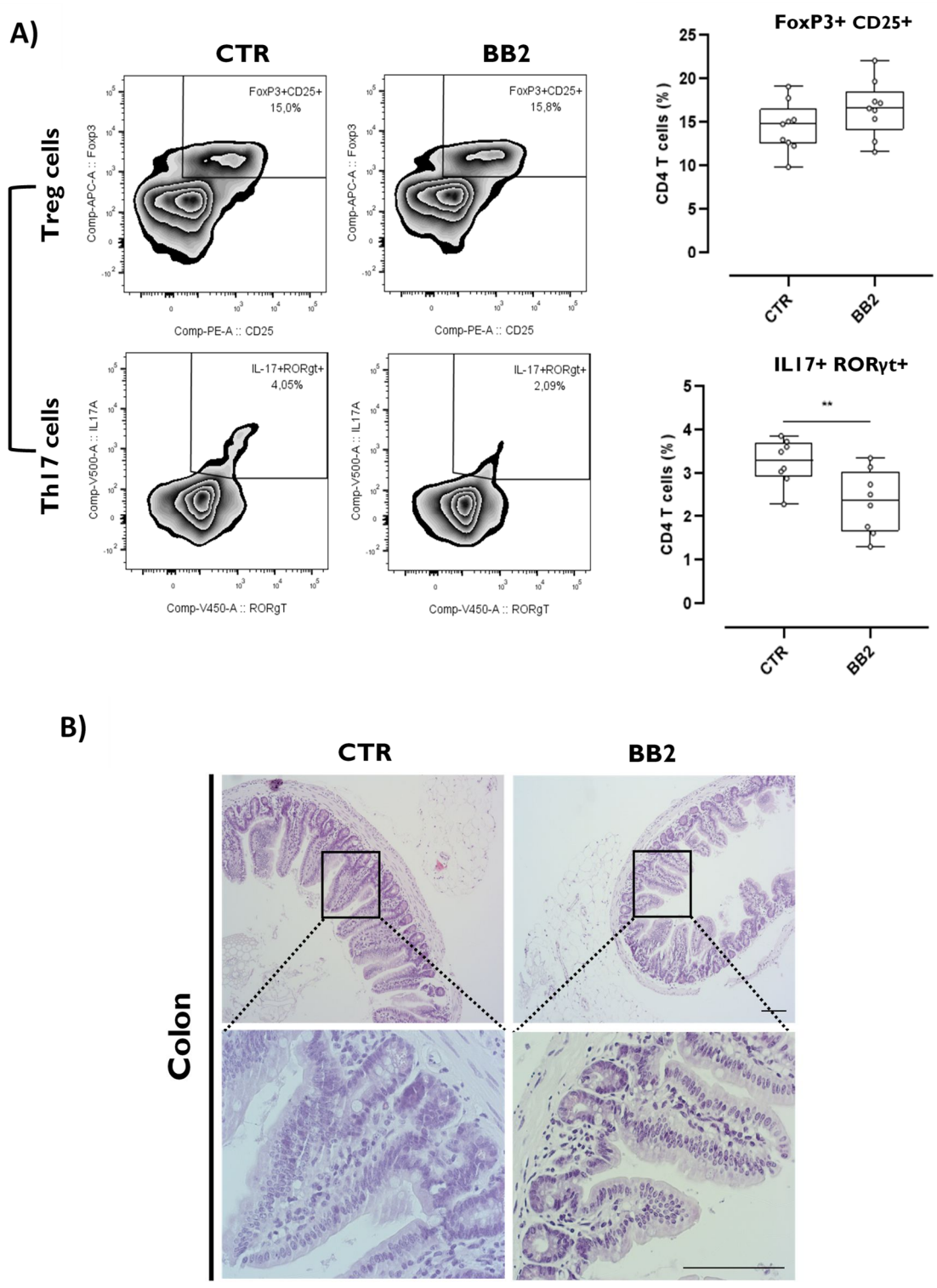


Figure 16. Gut health (Experimental Setting I). A) Gut immunomodulatory profile upon BB2 administration. Flow cytometric detection of T cell subsets: upper SSC/FSC plots show the gating of Treg population (CD4+FoxP3+CD25+ cell subset) and lower SSC/FSC plots show the gating of Th17 population (CD4+IL17+RORγt+ cell subset). Results are expressed as individual values of 8 animals per group. Student's t test ** $p<0.01$ vs CTR. **B)** Representative images of gut morphologic features upon BB2 administration (H&E, Scale bar = 100 μm)

4.2. Evaluation of BB biomass' therapeutic potential in experimental MS – Experimental Setting II

4.2.1. Body weight

BW was monitored weekly during the entire study period, which was 5 weeks to assess demyelination and 7 weeks for remyelination (Fig. 17A, 17C). Regarding the five-week experiment, BW was considerably consistent throughout the five weeks of treatment within each one of the experimental groups (CTR, CPZ W5 and CPZ W5+BB), and on the first two weeks there were no differences in BW across all of them. However, at W3, a significant difference in BW was observed between CPZ W5 animals and controls ($p<0.05$, when compared to CTR). In the following week, these animals recovered in terms of weight, but on the last week of treatment their weight dropped again, once more becoming statistically different from the values observed in the controls ($p<0.01$, when compared to CTR). Significant differences between the groups CPZ W5 and CPZ W5+BB were not observed at any timepoint.

When evaluating the weight difference between W5 and W0 (Fig.17B), the CTR group displayed a much more significant variation in BW than the CPZ-fed groups. Furthermore, the animals composing the CPZ W5 group had the lowest increase in weight, which might be a result of CPZ intoxication.

Regarding the animals subjected to the seven-week treatment, BW increased from W1 to W7 for all groups **(Fig. 17C)** CPZ-treated animals did not gain as much weight as the ones from the CTR group, which might be a consequence of CPZ treatment. No significant differences in BW were observed between groups at any point.

When evaluating the weight difference between W7 and W0 (Fig.17D), there are no statistically significant differences between the three experimental units, nevertheless the CPZ-treated groups gained considerably less weight when compared to controls, the lower values being reached in the CPZ W7 group.

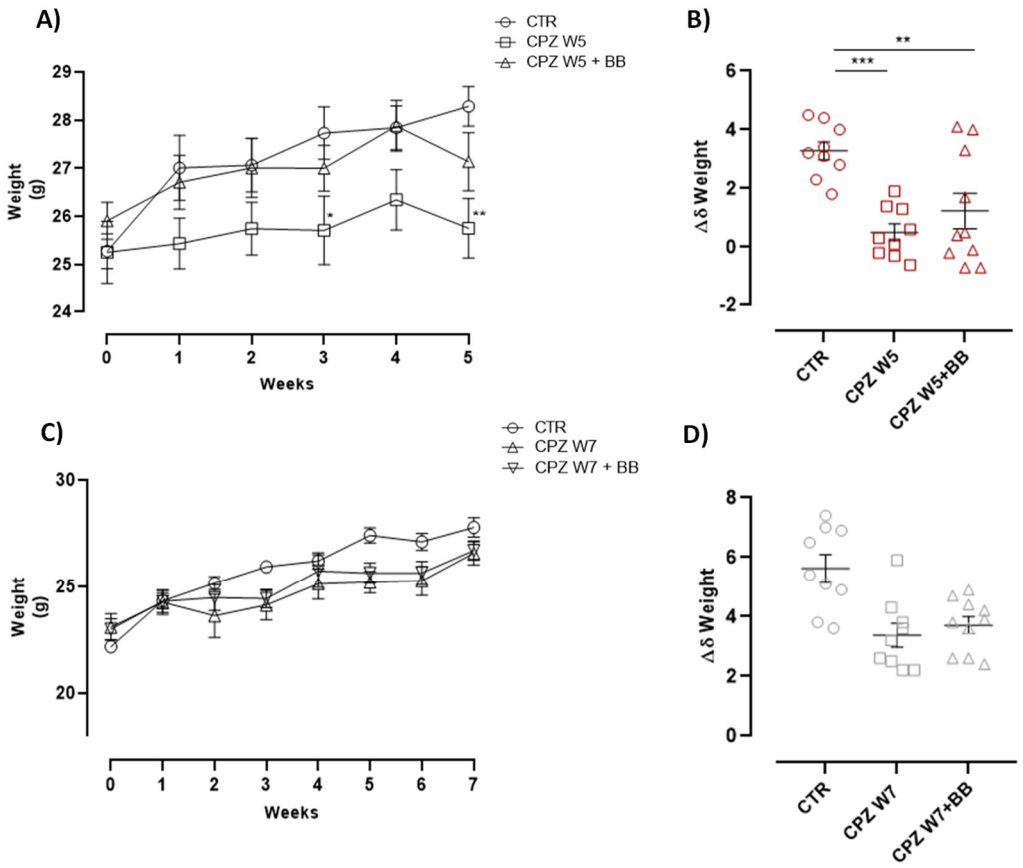


Figure 17. Body weight evolution of the experimental groups (Experimental Setting II). (A) BW evolution for the three experimental groups from W0 to W5. **(B)** BW difference between W5 and W0 for each group. **(C)** BW evolution for the three experimental groups from W0 to W7. **(D)** BW difference between W7 and W0 for each group. Results are expressed as mean ± S.E.M of 10 animals per group. One-way ANOVA * p<0.05 vs CTR; ** p<0.01 vs CTR; *** p<0.005 vs CTR.

4.2.2. Food and water consumption

Food and water consumption were also evaluated throughout the 5 and 7 weeks of treatment, respectively. When evaluating food intake at the five-week experiment (**Fig.18A**), similar values were observed between the three experimental groups throughout the entire treatment period. Food consumption increased across all groups until W3, point from which dietary consumption remained approximately consistent.

At W2 the animals from the CPZ W5 consumed significantly less food comparatively to the CTR group (p<0.05 when compared to CTR). Such outcome might have resulted from the CPZ treatment and the oral gavage, since it composes a hard procedure concerning the GI tract and could have difficulted food consumption for the animals. From W3 forward, food intake values become constant, being very similar for the three experimental groups. Water intake (**Fig.18B**) showed no significant variations between experimental groups throughout the five weeks of treatment.

Regarding food intake at the seven-week experiment **(Fig.18C)**, significant differences were observed between the experimental groups at several timepoints. At W1, a very significative difference was seen when comparing food consumption from the CTR and the CPZ W7 groups

($p<0.0001$ when compared to CTR). At W2 and W3 food intake records show increased consumption comparatively to the prior week, and values become very close between the three experimental units, pattern which is maintained until W5, timepoint in which a significant difference is observed between CTR and CPZ W7+BB animals regarding food consumption ($p<0.0005$ when compared to CPZ W7). Similarly to what happened at W3 and W4, food intake values were substantially close across all experimental groups at the final two weeks of treatment. When evaluating water intake **(Fig. 18D)**, no statistically different results were obtained. Water intake records were very similar between the three groups and were considerably consistent throughout the experiment.

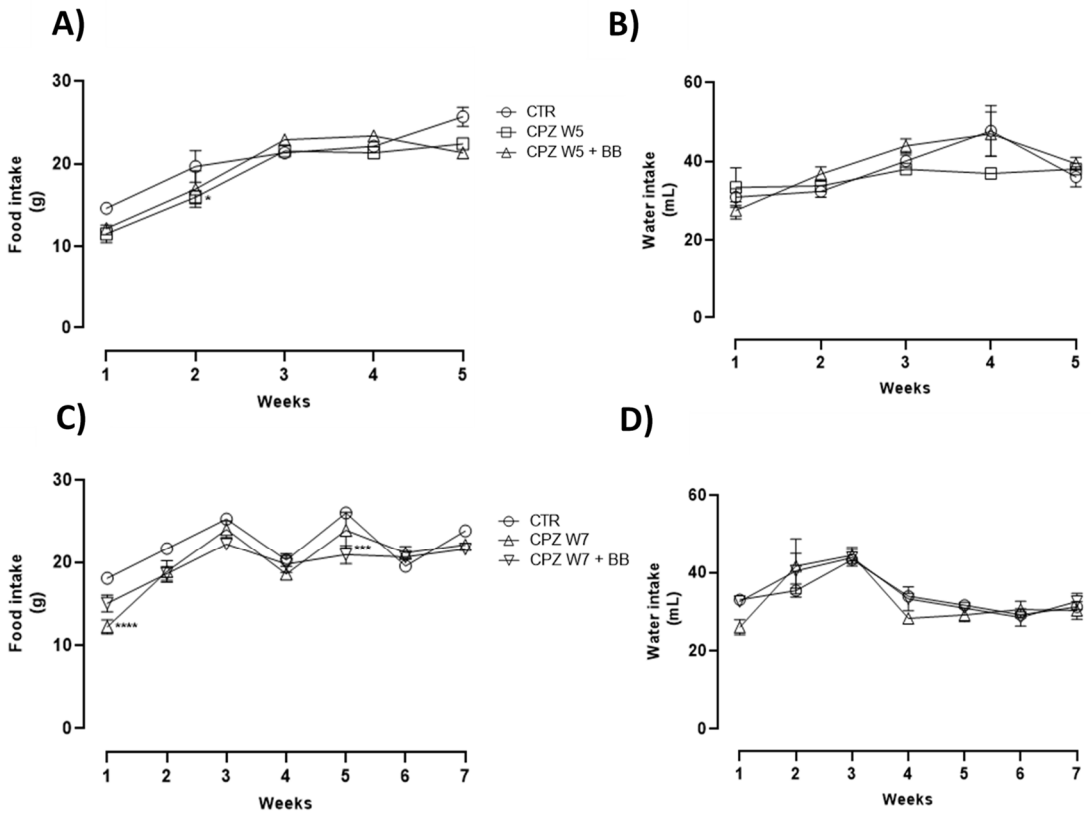


Figure 18. Food and water intake evolution (Experimental Setting II). A) Evolution of food intake at W1-W5 of CPZ administration and BB biomass treatment. **B)** Evolution of water intake at W1-W5 of CPZ administration and BB biomass treatment. **C)** Evolution of food intake at W1-W7 of CPZ administration and BB biomass treatment. **D)** Evolution of water intake at W1-W7 of CPZ administration and BB biomass treatment. Results are expressed as mean ± S.E.M of 10 animals per group. One-way ANOVA * $p<0.05$ vs CTR; *** $p<0.005$ vs CTR; **** $p<0.001$ vs CTR.

4.2.3. Antioxidant performance in the serum and gut

Serum SOD evaluated at the demyelination peak (five weeks) **(Fig.19A)** did not show to have different activity rates in the CPZ W5 group when compared to the CTR one. However, there was a significant difference in SOD activity between the animals receiving CPZ and BB biomass simultaneously and the controls ($p<0.05$ when compared to CTR). Regarding the remyelination peak (seven weeks) **(Fig.19B)**, serum SOD did not show differential activity across the three experimental groups, only presenting a mild decrease in the both CPZ-given groups when compared to the controls.

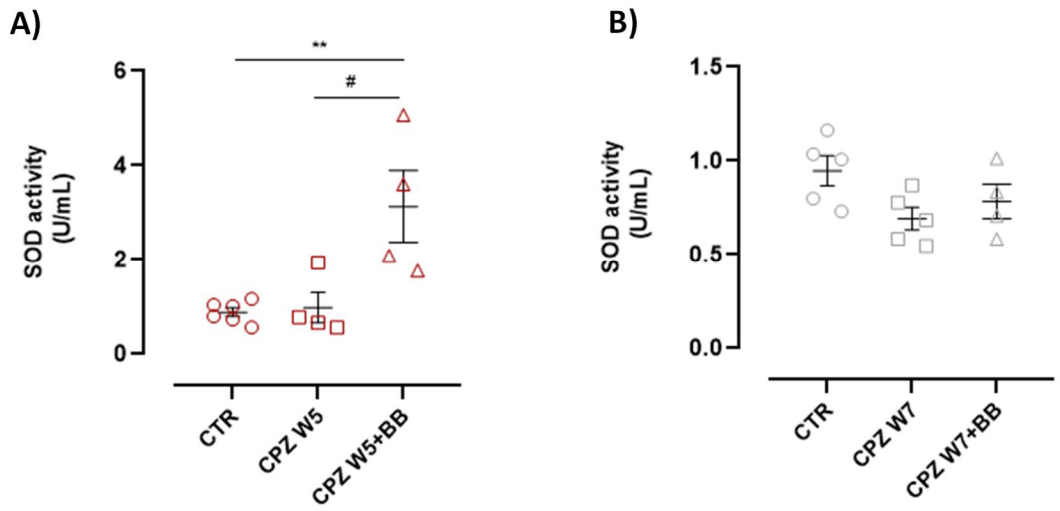


Figure 19. SOD activity in the serum (Experimental Setting II). A) SOD activity in the serum of the animals subjected to the five-week experiment. **B)** SOD activity in the serum of the animals subjected to the seven-week experiment. Results are expressed as mean ± S.E.M of 6 animals per group. One-way ANOVA * $p<0.05$ vs CTR.

Antioxidant performance in the gut was assessed resorting to SOD-1 and SOD-2 gene expression as well as total SOD activity **(Fig.20)**. Regarding the animals subjected to the five-week protocol, both SOD-1 **(Fig. 20A)** and SOD-2 **(Fig.20B)** gene expression did not show statistically significant variations in terms of expression across the three experimental groups. However, a tendency for reduced SOD-1/2 expression is observed in the CPZ-treated groups when compared to the controls. When evaluating total activity of intestinal SOD **(Fig.20C)**, although significant differences were not detected, the obtained results point to a trend in which enzymatic performance increases in the CPZ-treated groups relatively to controls, and that BB biomass further promotes this augmentation.

Concerning the seven-week experimental design, mice consuming BB biomass displayed significantly higher levels of SOD-1 **(Fig.20D)** and SOD-2 **(Fig.20E)** expression when compared to the CTR group ($p<0.05$ when compared to CTR). The same pattern is observed in terms of enzymatic activity **(Fig. 20F)**, a significant difference being seen in the CPZ- and CPZ + BB – treated animals when compared to controls ($p<0.05$ when compared to CTR).

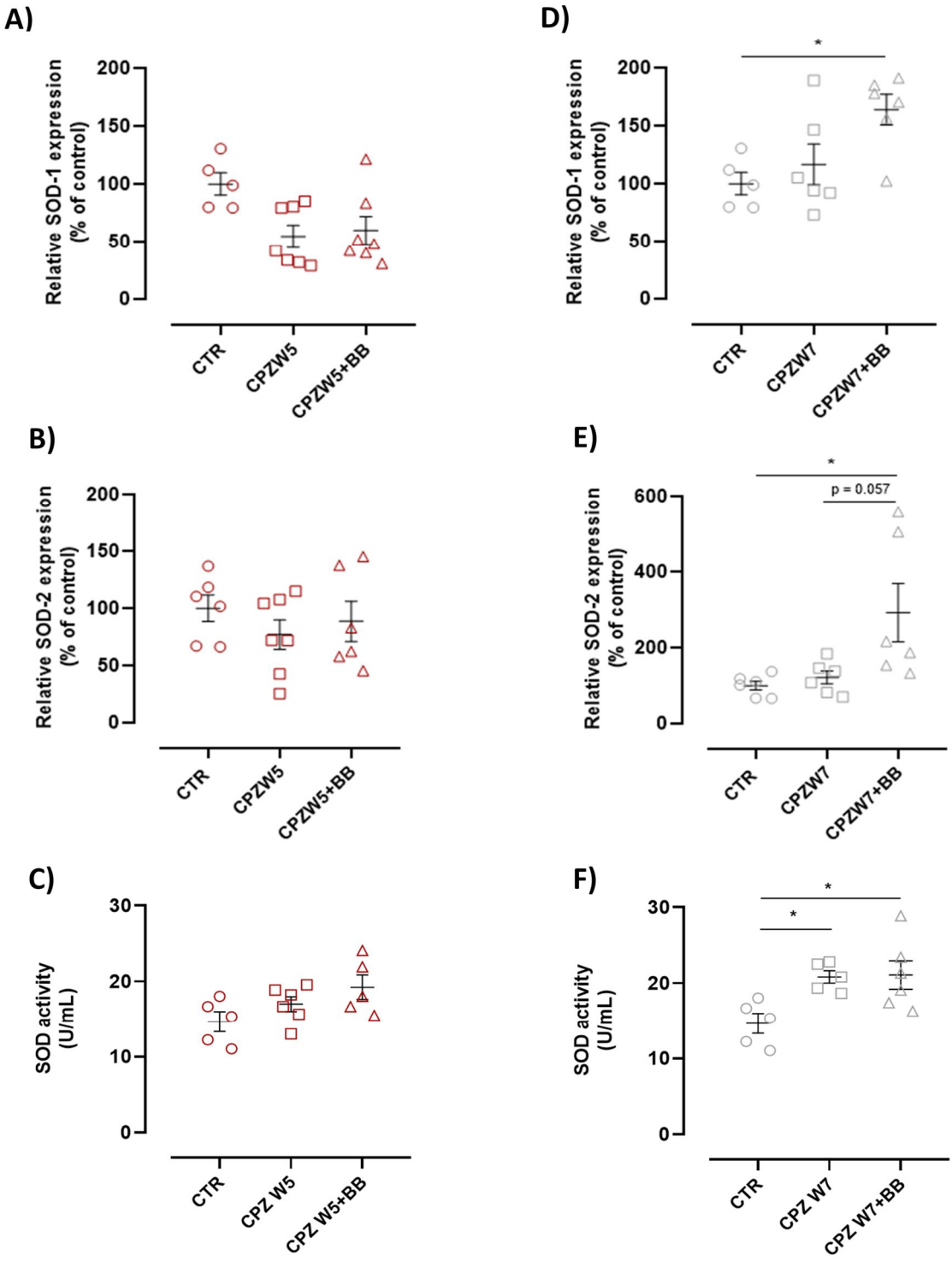


Figure 20. Antioxidant performance in the gut (Experimental Setting II). A) SOD-1 gene expression, **B)** SOD-2 gene expression, **C)** SOD activity in the small intestine of animals subjected to the five-week experiment. **D)** SOD-1 gene expression, **E)** SOD-2 gene expression, **F)** SOD activity in the small intestine of animals subjected to the seven-week experiment. Results are expressed as mean ± S.E.M of 5-7 animals per group. One-way ANOVA * $p<0.05$ vs CTR.

4.2.4. Gut immunomodulation

In order to assess the Treg/Th17 balance in the CPZ-induced MS model and the potential impact of BB biomass administration in such T cells populations, flow cytometry was performed. Antibodies against FoxP3 and CD25, as well as anti-IL17 and anti-RORγt antibodies were employed for Treg and Th17 cells identification, respectively. The obtained results are shown in **Fig. 21**.

Regarding Treg cells **(Fig. 21A)**, when compared to the controls, mice from the CPZ W5 group displayed significantly lower levels of the referred T cell population ($p<0.005$ when compared to CTR). Animals consuming BB biomass seemed to recover from the observed decrease, displaying a higher percentage of $FoxP3^+$ $CD25^+$ cells relatively to the ones treated with CPZ only ($p<0.05$ when compared to CPZ W5), reaching values near the ones seen for controls. Opposite effects were detected when analyzing Th17 cells **(Fig.21B)**. Relatively to controls, animals treated with CPZ for five weeks displayed a significantly augmented percentage of the referred cell population ($p<0.01$ when compared to CTR). Once more, the results obtained for the CPZ W5+BB group suggest that the biomass restores the $IL17^+$ $ROR\gamma t^+$ cells' percentage to numbers similar to the ones observed in controls ($p<0.05$ when compared to CPZ W5).

When evaluating the same cell populations after seven weeks of treatment, different results were noted. Concerning Treg cells **(Fig.21C)**, no significant differences were observed between experimental groups. However, Th17 cells **(Fig.21D)** displayed a significant decline in the animals treated with CPZ only comparatively to controls ($p<0.01$ when compared to CTR), these numbers returning to values close to control ones after BB administration ($p<0.05$ when compared to CPZ W7).

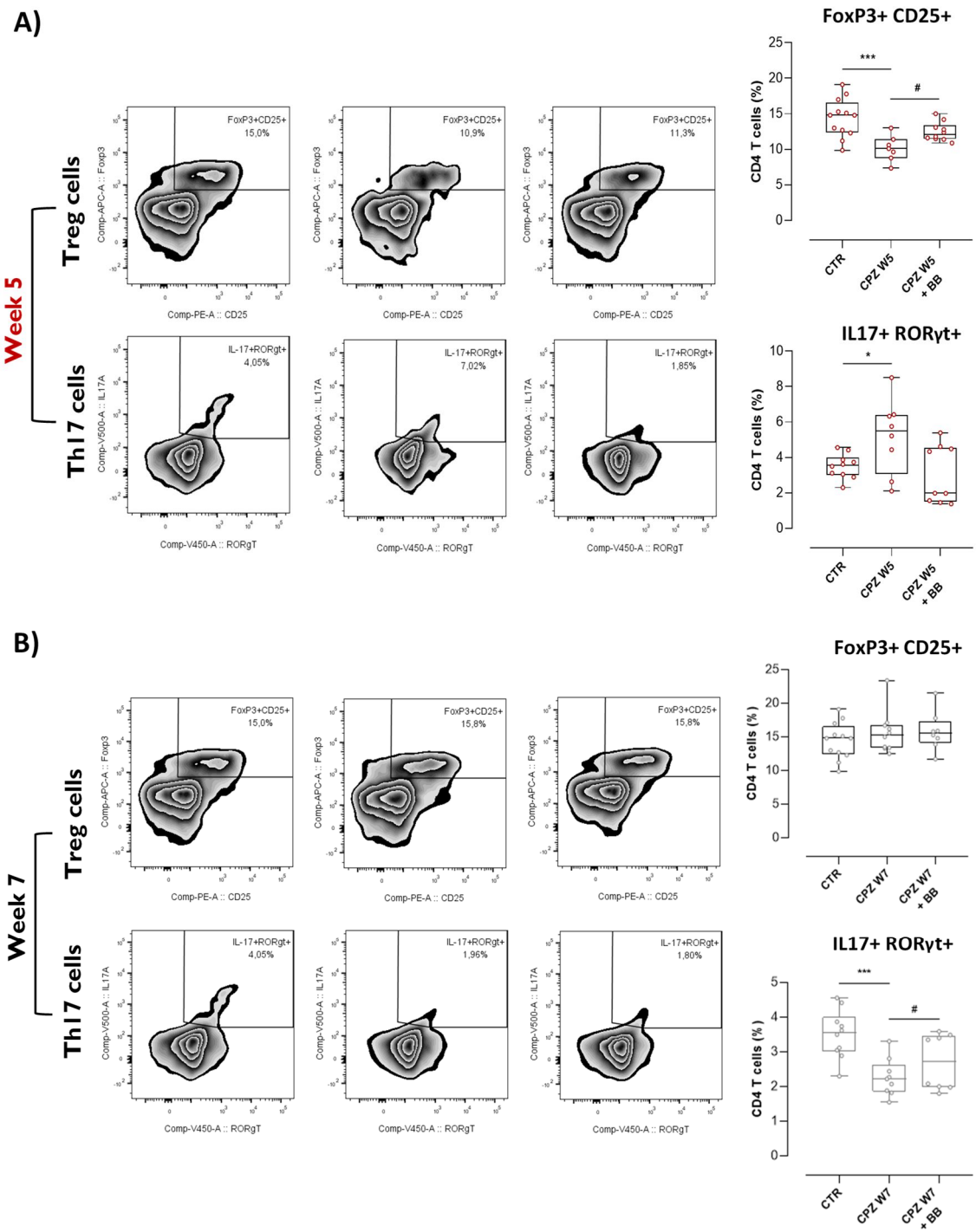


Figure 21. Gut immunomodulatory profile - Treg/Th17 balance (Experimental Setting II). A) Flow cytometric detection of T cell subsets [upper SSC/FSC plots shows the gating of Treg population (CD4+FoxP3+CD25+ cell subset) and lower SSC/FSC plots show the gating of Th17 population (CD4+IL17+RORγt+ cell subset)] of the animals subjected to the five-week experiment. **B)** Flow cytometric detection of T cell subsets [upper SSC/FSC plots shows the gating of Treg population (CD4+FoxP3+CD25+ cell subset) and lower SSC/FSC plots show the gating of Th17 population (CD4+IL17+RORγt+ cell subset)] of the animals subjected to the seven-week experiment. Results are expressed as individual values of 7-12 animals per group. One-way ANOVA * $p<0.05$ vs CTR; *** $p<0.001$ vs CTR; $^{\#}$ $p<0.05$ vs CPZ W5 or CPZ W7.

4.2.5. Antioxidant performance in the brain

Antioxidant performance in the brain was assessed resorting to SOD-1 and SOD-2 gene expression as well as total SOD activity **(Fig.22)**. When evaluating the gene expression of SOD-1 **(Fig.22A)** and SOD-2 **(Fig.22B)**, no statistically significant differences were detected between the three experimental groups composing the five-week experimental setting. Regarding SOD activity **(Fig.22C)**, although the results do not present statistical significance, a decrease is observed in the animals receiving CPZ during five weeks when compared to controls. However, in the BB biomass-treated group, there seemed to be a recovery of SOD activity to values similar to the ones observed in the CTR group.

Regarding the mice subjected to the seven-week protocol aiming to evaluate remyelination, a trend for increased SOD-1 **(Fig. 22D)** and SOD-2 **(Fig.22E)** expression is observed in the CPZ W7+BB group ($p=0.0596$ for SOD-2 when compared to CPZ W7). The enzyme's activity **(Fig. 22F)** was significantly increased in the animals consuming BB biomass when compared to the ones not consuming it ($p<0.05$ when compared to CTR and CPZ W7).

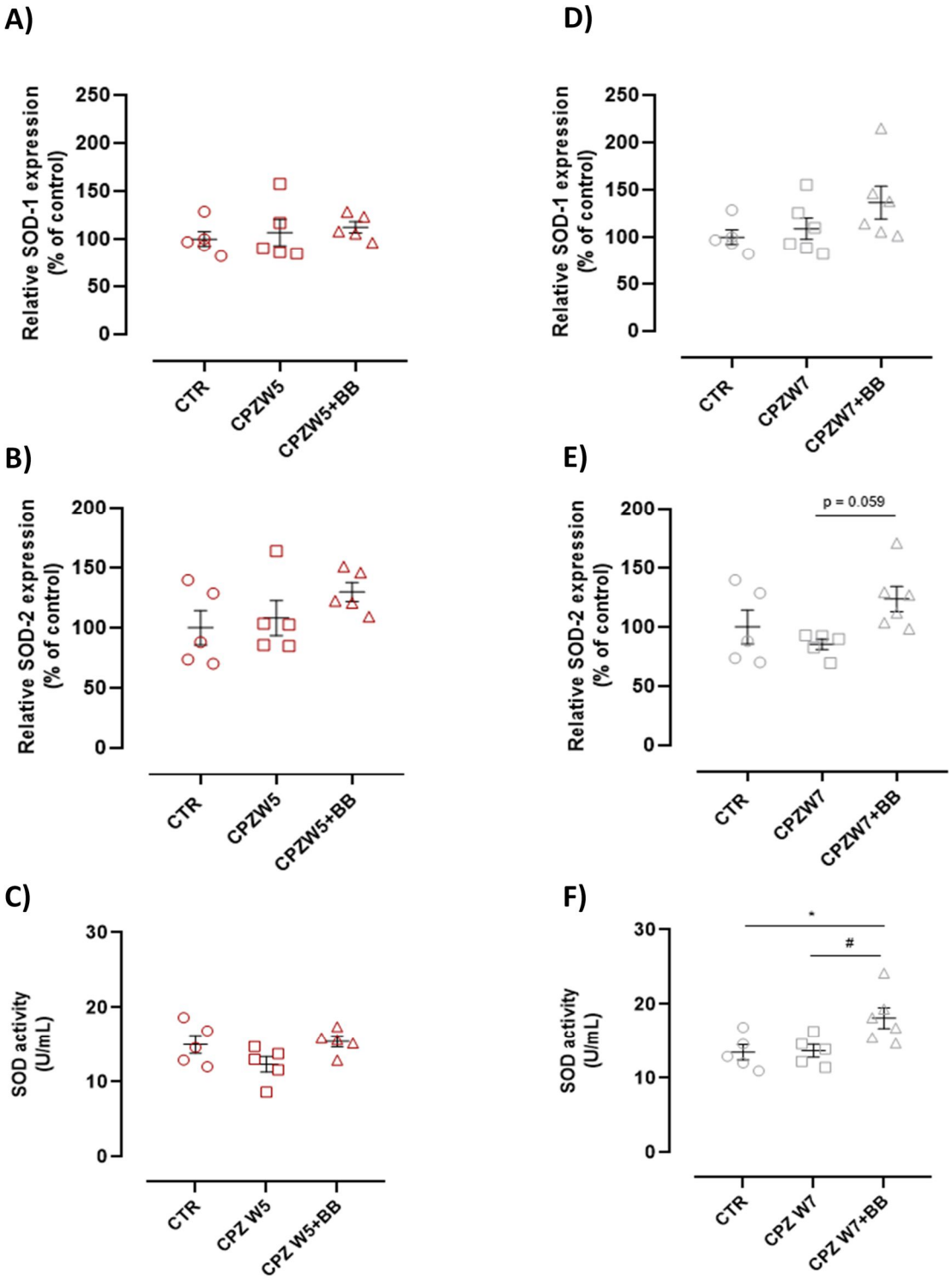


Figure 22. Antioxidant performance in the brain (Experimental Setting II). A) SOD-1 gene expression, **B)** SOD-2 gene expression, **C)** SOD activity in the brain of animals subjected to the five-week experiment. **D)** SOD-1 gene expression, **E)** SOD-2 gene expression. **F)** SOD activity in the brain of animals subjected to the seven-week experiment. Results are expressed as mean ± S.E.M of 5-6 animals per group. One-way ANOVA * $p<0.05$ vs CTR; # $p<0.05$ vs CPZ W7.

4.2.6. Brain genetic and histopathological analysis

Gene expression of MBP and PLP in the whole brain as well as myelin histology in the cerebellum were assessed in both demyelination (W5) and remyelination (W7) peaks.

MBP showed to be significantly less expressed after CPZ treatment for five weeks **(Fig. 23A)** ($p<0.05$ when compared to CTR), a trend for slightly increased expression being seen in the BB-treated group. On another hand, after the seven-week protocol, MPB expression did not display a meaningful variation in the CPZ W7 group when compared to controls but was significantly higher in the BB-treated group ($p<0.01$ when compared to CTR; $p<0.05$ when compared to CPZ W7) **(Fig. 23B)**. PLP expression exhibited a trend to decrease in the CPZ treated groups concerning the five-week experimental design **(Fig. 23C)**, such decline being statistically significant for the CPZ W5+BB group ($p<0.05$ when compared to CTR). Regarding the animals subjected to the seven-week protocol **(Fig. 23D)**, a significant enhanced expression of PLP was observed in the mice consuming the BB biomass relatively to the ones treated with CPZ only ($p<0.05$ when compared to CPZ W7).

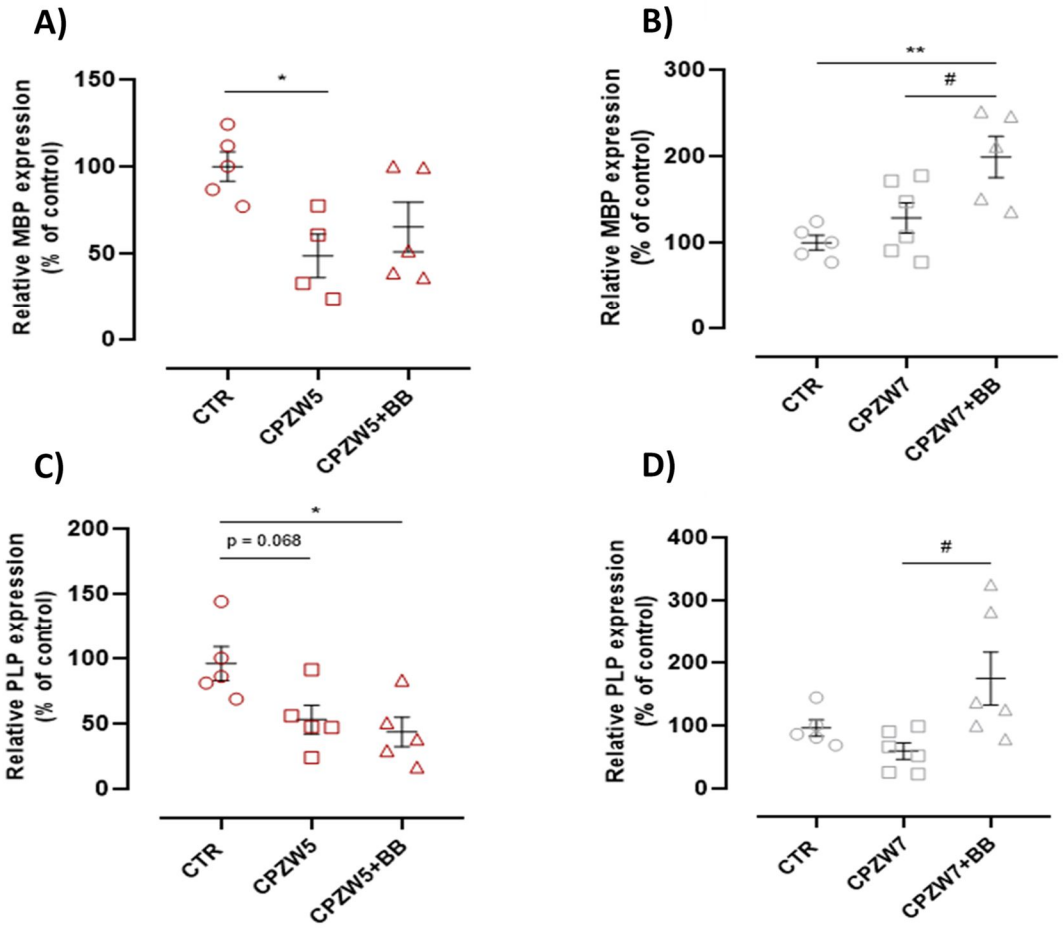


Figure 23. Analysis of the myelin basic protein (MBP) and proteolipid protein (PLP) gene expression in the brain (Experimental Setting II). A) MBP relative gene expression in the demyelination peak (W5). **B)** MBP relative gene expression in the remyelination peak (W7). **C)** PLP relative gene expression in the demyelination peak (W5). **D)** PLP relative gene expression in the remyelination peak (W7). Results are expressed as mean ± S.E.M of 5-6 animals per group. One-way ANOVA * $p<0.05$, ** $p<0.01$ vs CTR; # $p<0.05$ vs CPZ W7.

Myelin histology was assessed resorting to Kluver-Barrera myelin staining. The acquired images show reduced myelinated areas in the CPZ-treated groups when compared to controls, myelin fibers displaying lower stain intensity as well as decreased thickness. However, there seems to be a recovery in myelin morphology and intensity in the animals consuming BB, especially in the remyelination peak (CPZ W7+BB). **(Fig. 24)**

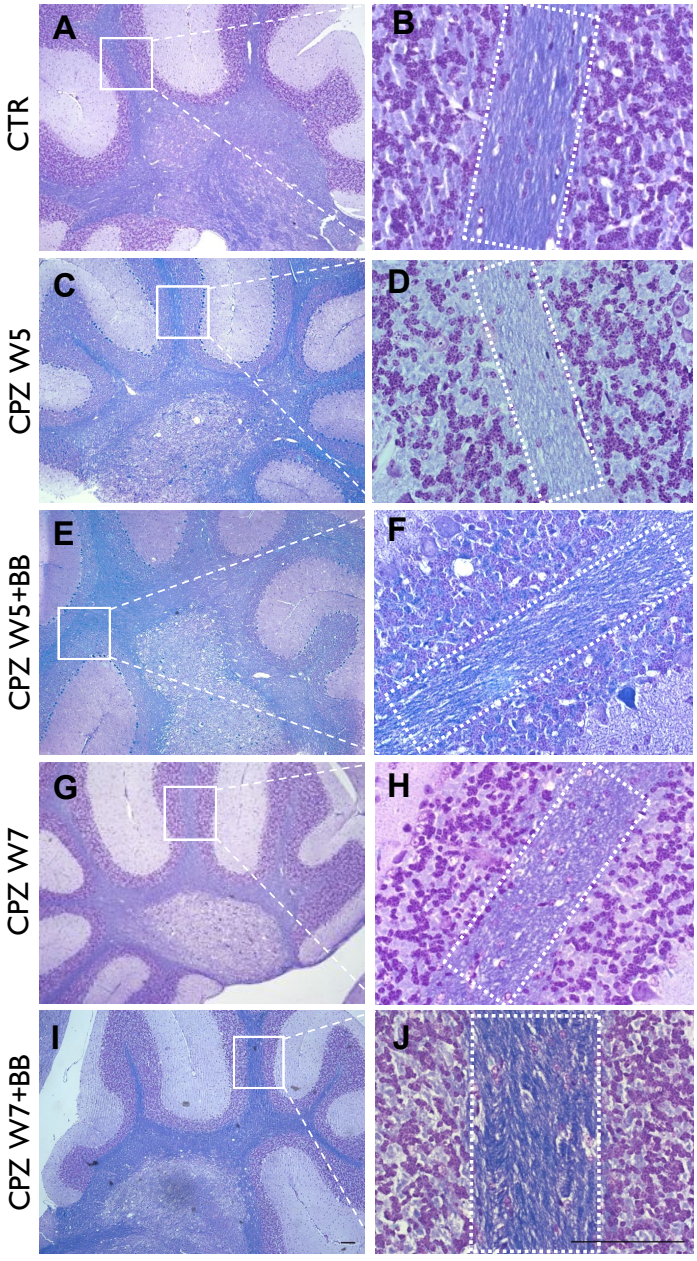


Figure 24. Kluver-Barrera (KB) staining of sagittal cerebellum slices of the control (CTR), cuprizone-fed (CPZ W5 and CPZ W7), and BB biomass-administered (CPZ W5+BB and CPZ W7+BB) groups in the cerebellum. Myelinated fibers are shown in blue, and Nissl substance and nerve cells in violet. Demyelination can be seen in the cerebellum of both CPZ only-treated groups **(C,D,G,H)**, evidenced by a reduced stain intensity as well as decreased thickness of the myelinated areas, which were considerably more visible in the CPZ W5 animals **(C,D)**. The reduced myelin content observed in the CPZ groups when compared to the controls **(A,B)** seemed to increase after BB biomass administration **(E,F,I,J)**, particularly in the CPZ W7+BB group **(I,J)**. (Scale bar = 100 μm).

Brain histomorphology was also evaluated resorting to Hematoxylin and Eosin (HE) and Toluidine Blue (TB) staining in the cerebellum **(Fig.25)**. The histopathological examination of the cerebellar tissue of CPZ-treated mice using HE stain revealed histological alterations of the cerebellar cortex layers with shrinkage and degeneration of the Purkinje and molecular layer cells. **(B-E)** Compared to the controls **(A)**, CPZ-treated mice' Purkinje cells exhibited darkly stained nuclei with eosinophilic cytoplasm and empty spaces between them, indicating cellular degeneration. The results suggest that CPZ withdrawal allowed a partial recovery of the cerebellar tissue, as evidenced by the fact that the Purkinje cells of the CPZ W7 group **(D)** are less degenerated when compared to the ones from the CPZ W5 mice **(B)**. Oral administration of BB biomass attenuated the pathological changes in the cerebellums of mice in the CPZ W5+BB **(C)** and CPZ W7+BB **(E)** groups, with these animals displaying a lower number of degenerated Purkinje cells when compared to mice treated with CPZ only. It is worth noting that such effect was much more relevant in the remyelination peak **(E)** than demyelination **(C)**.

The TB-stained photomicrograph sections from the cerebellums of the control group indicated normal histological structure of the cerebellum with the three layers of the cerebellar cortex. The Purkinje cells appeared to have regular and prominent central nuclei. The granular and molecular layers showed normal cells with darkly stained nuclei **(A)**. Photomicrograph sections from the cerebellums of the CPZ-intoxicated mice revealed alterations in the Purkinje cell layer, with the Purkinje cells appearing to have darkly stained cytoplasm with irregular nuclei **(B-E)**. The photomicrograph sections from the CPZ W5+BB **(C)** and CPZ W7+BB **(E)** groups showed partial restoration of the Purkinje cell layer, with these animals displaying a lower number of Purkinje cells when compared to mice treated with CPZ only. Once more, this outcome is much more evident in the mice allowed to recover after the five-week CPZ treatment **(E)** when compared to the ones to which CPZ was administered continuously until the end of the experiment **(C)**.

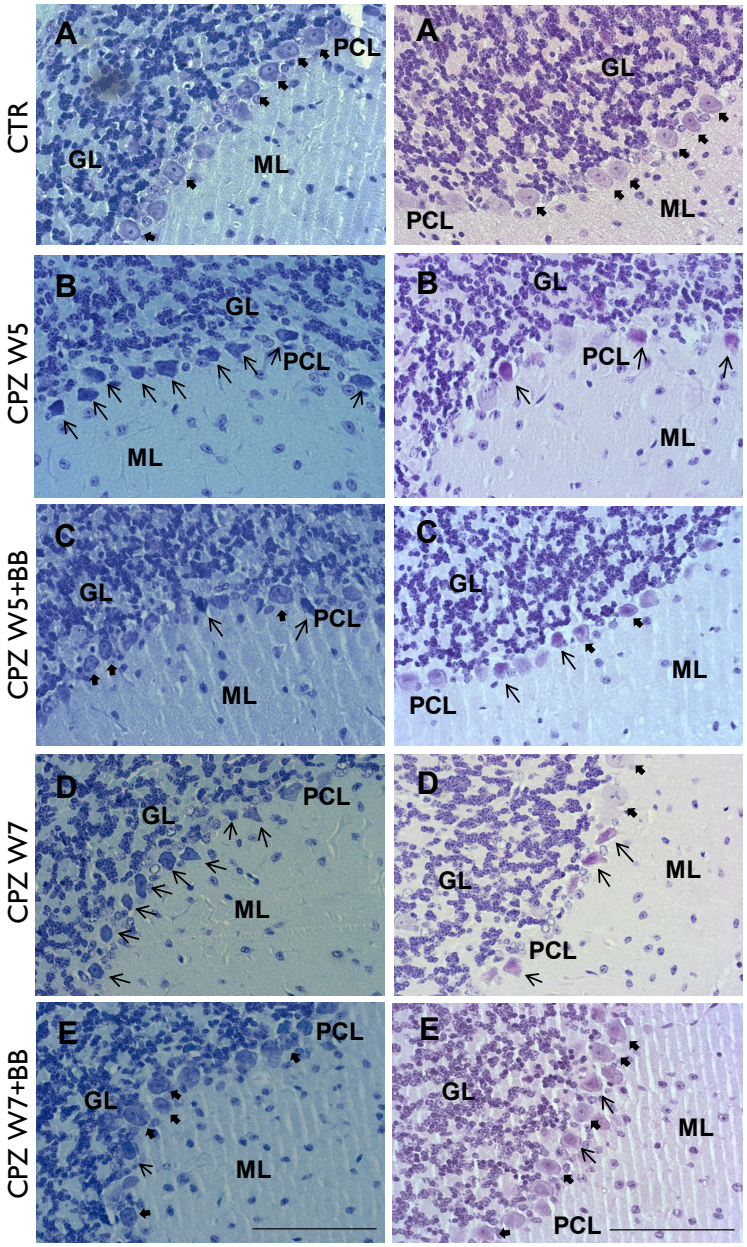


Figure 25. Toluidine blue and Hematoxylin and eosin staining of sagittal cerebellar s**ections**. **Left:** Toluidine blue staining of sagittal cerebellar slices of the control (CTL), cuprizone-fed (CPZ W5 and CPZ W7) and BB biomass-administered (CPZ W5+BB, CPZ W7+BB) groups. Control cerebellums **(A)** showed densely packed internal granular layer (GL), separated from the external molecular layer (ML) by a single-celled layer of Purkinje cells (PCL). The PCL appeared to be arranged in a linear pattern displaying regular and prominent nuclei **(large arrow)**, and the granular and molecular layers showed normal cells with darkly stained nuclei. Sections from the cerebellums of the CPZ only-treated mice **(B,D)** revealed alterations in the PCL, with cells appearing to have darkly stained cytoplasm with irregular nuclei and abnormal shape and shrinkage **(thin arrow),** such effects being more prominent in the demyelination phase **(B)** relatively to remyelination **(D)**. The cerebellums from animals consuming BB **(C,E)** displayed a decreased number of degenerated Purkinje cells when compared to CPZ-only treated ones **(B,D)**, with a more evident impact in the CPZ W7+BB group **(E)** in relation to the CPZ W5+BB **(C)**. **Right:** Hematoxylin and eosin staining of sagittal cerebellar slices of the control (CTL), cuprizone-fed (CPZ W5, CPZ W7) and BB biomass-administered (CPZ W5+BB and CPZ W7+BB) groups. Controls presented the typical histomorphology **(A)**, showing a single-celled layer of Purkinje cells (PCL) with a large pyriform shape **(large arrow)**. The CPZ groups **(B,D)** showed PCL degeneration, showing an irregular and darkly stained cytoplasm and distorted nuclei **(thin arrow)** with empty space between them, indicating cellular loss. PCL degeneration is much more significative in the CPZ W5 animals **(B)** in comparison to the CPZ W7 ones **(D)**. BB administration seemed to partially restore PCL morphology and organization, CPZ W5+BB **(C)** and CPZ W7+BB **(E)** displaying a higher number of normal-appearing Purkinje cells with a large pyriform structure **(large arrow)** when compared to CPZ-only treated groups **(B,D)**. This effect was significantly more evident in the remyelination peak **(E)** relatively to demyelination **(C)**. (Scale bar = 100 μm)

Chapter V | **DISCUSSION AND CONCLUDING REMARKS**

5.1. Discussion and concluding remarks

Its complex and heterogenous character, as well as its debilitating nature, make MS a devastating disease and hamper the development of effective therapeutic strategies. In fact, MS management is solely dependent on symptomatic treatments that do not tackle the core of the disease: demyelination along with spontaneous remyelination. Surprisingly, there are still no effective pharmacological interventions available that target the remyelinating component of the disease. Therefore, the discovery of innovative solutions that exert beneficial effects in the context of MS is a crucial step for relieving and managing the disease burden.

Nowadays, clinical and research entities, as well as the general public, value and prioritize therapeutic strategies with natural origin. Additionally, an increasing concern with lifestyle, namely dietary habits and nutritional patterns, has been observed in humankind. Consequently, the search for natural dietetic and nutraceutical supplements is rising at a considerable rate. PCs supplementation has been widely and frequently employed for several years now and has been showing to be extremely useful in several health areas. Particularly, neurodegenerative disorders have benefit greatly from the antioxidant and anti-inflammatory features of these compounds. *Vaccinium* species are extensively known by their PCs-enriched edible fruits – blueberries – which possess a well-established medicinal value. [311-313] It is worth noting that there are strong suggestions of the ability of PCs to interfere with key cellular targets of MS pathophysiology, promoting an increased expression of myelin proteolipid protein (PLP) *[273]*, myelin basic protein (MBP) *[274]* and oligodendrocyte transcription factor 1 (Olig1) *[273]* along with a decreased number of recruited T and B cells *[274]*, inhibition of glia activation and subsequent inflammatory factors release *[274]*.

Interestingly, besides the fruit, other parts of the plant have been emerging as superior sources of bioactive compounds displaying therapeutic properties, as is the case of the leaves, particularly the senescent ones. However, human intake of leaves is restricted to infusions and decoctions, due to the presence of a higher content of indigestible components (e.g. structural polysaccharides of the vegetable cell wall) that hamper phytochemicals' gastrointestinal absorption. Since PCs are thermolabile [314], these techniques are associated with several limitations, including the use of high temperatures that may foster PCs' degradation, as well as the fact that they only allow the organism to access a small portion of the available compounds. Aiming to surpass such limitations, a biotechnological methodology for leaves processing was developed, resulting in a herein employed leaves biomass with superior diversity of bioactive compounds displaying great health-promoting potential. Furthermore, this innovative methodology allows the preservation of heterosydic forms of PCs (quercetin-3-O-rutinoside - rutin), which display privileged transport across blood brain/retinal barriers and enhanced central bioavailability [315]. Since it was hypothesized that the biomass could compose an effective supplement and considering the reported dual-mode of PCs supplementation, assessing its safety profile was required (Experimental Setting I), in order to confirm the lack of systemic toxicity as well as to define an adequate dose to be employed in subsequent animal studies.

Reduction in body weight is known to be one of the most common indices to understand the toxicity profile of drugs. [316] At doses of 50 mg/kg (BB1), 500 mg/kg (BB2) and 1 g/kg (BB3), the biomass showed not to exert significative effects in terms of body weight, which is a sensitive indication of animals' general health status. After 28 days of treatment, all mice exhibited similar growth curves and regular food intake/water drinking patterns. Blood glucose measurements between the beginning and the end of the 28-day experiment were considerably similar and did not vary between groups, suggesting that glycemia was likewise not affected by BB consumption. Furthermore, findings of the OFT and RRT in this study revealed that BB biomass did not alter exploratory, motor coordination, balance, and learning ability behaviors in mice. Collectively, *in vivo* evaluation suggests no glossy toxic effect from BB biomass.

Similarly, administration of BB biomass at sub-chronic oral doses did not produce significant changes in the relative organ weight, which showed that grossly none of the organs were adversely affected. Macroscopic examination of liver, kidney and colon gross anatomy also showed regular appearance, which was further confirmed through histological analysis. Assessment of serum and urine biochemistry was also done to identify the possible alterations in renal and hepatic functions induced by the BB biomass. The lack of significant alterations in the levels of ALT, AST, ALP, which are good indicators of liver functions [317], suggested that sub-chronic administration of the biomass did not alter hepatic function. However, the highest dose (BB3 – 1 g/kg) demonstrated to alter kidney function parameters, since it significantly reduced urine concentrations of urea, uric acid, and creatinine. These results are in accordance with a previously study performed by our group in which supplementation with blueberry juice at a 25 g/kg concentration (equivaling to 50 mg/kg TPCs) resulted in altered kidney function. Although the referred dose of biomass possesses only 25% of this TPC value (12.625 mg), its conjugation with fiber may increase PCs bioavailability and bioactivity. As a matter of fact, evidence suggest that an association between PCs and fiber delays their absorption through the GI tract, optimizing the assimilation of these compounds in the colon following microflora metabolism. [236] These effects have been observed for several kinds of PCs supplementation, such as green tea. [294]

Hematologic profile didn't show significant changes in white blood cells (neutrophils, lymphocytes, and monocytes) in the BB biomass-treated animals, the first line of defense in response to infectious agents, tissue injury and inflammation [318]. On another hand, the hematopoietic system is one of the most vulnerable targets of toxic compounds, constituting an important physiological and pathological status index in both humans and animals. [318] The highest BB biomass dose significantly increased hemoglobin concentration and hematocrit percentage, as well as an ability to boost erythrocytes' levels. Data highlighting PCs ability to prevent hemoglobin oxidation is available [319] and might be one explanation for the rise in hemoglobin concentration observed in the BB3 group. Additionally, it has been demonstrated that flavonoids are capable of reducing lipid peroxidation levels, consequently preventing erythrocytes' hemolysis. [320] Furthermore, components such as luteolin-7-glucosidase and apigenin-7-glucoronide, which are present in *Vaccinium* species [321], have shown to induce human hematopoietic stem cells' differentiation towards an erythroid lineage. [322] Interestingly, a study performed by Shibuya et al. [323] reported similar effects, where acai administration to mice resulted in increased erythrocytes, hemoglobin, and hematocrit, which resulted from augmented

erythropoietin blood levels and gene expression of *Epo* in the kidney. Authors suggested that the increased erythropoietin expression happened as a response to kidney hypoxia. Such observation is extremely interesting and relevant in the context of our study, since altered kidney parameters were observed as consequence of BB administration, suggesting that this might be the origin of its putative hematopoiesis-promoting properties. Thus, assessing the presence of renal hypoxia as well as determining *Epo* expression levels in future experiments is an imperative step to unravel whether similar events are elicited by BB biomass. Overall, these results suggest a hematopoiesis-potentiating effect of the biomass, which has great interest and warrants further investigation in order to understand its underlying mechanism. Collectively, aforesaid observations led to the exclusion of BB3 as a safe dose.

Notably, BB biomass displayed marked hypolipidemic properties in the intermediate (BB2 – 500 mg/kg) and the higher (BB3 – 1 g/kg) doses, eliciting a decrease of serum TGs contents. In fact, there is evidence of the beneficial effects of berry juice intake on blood TGs content in the context of metabolic syndrome spectrum diseases, such as obesity and type 2 diabetes. [287] Particularly, blueberries have shown to exert similar hypolipidemic effects. A study performed by Stote et al. [288] highlighted the decreased serum TGs levels in men with type 2 diabetes as a result of freeze-dried blueberries consumption for 8 weeks. Furthermore, a blueberry leaves infusion has shown to decrease TGs plasma content in about 39%. [324] A similar effect has been observed after oral administration of flavonols from blueberry leaves in hyperlipidemic rats. [325] On another hand, when evaluating TGs concentration in the liver, a dose-dependent accumulation was observed, being statistically different in the group consuming BB3. Even so, these levels are still normal hepatic TGs levels seen in mice [326], being far from the ones normally observed in pathological situations and therefore were not considered as an adverse effect resulting from biomass administration. These observations suggest that the increased TGs in the livers of mice consuming 1g/kg BB biomass may not compose an adverse effect resulting from the treatment, which is further supported by the fact that no changes in liver function enzymes or organ morphology were observed. Furthermore, such outcome was only observed in the group treated with the highest BB dose, which had already been excluded from the safety range.

Aiming to assess BB biomass impact on the mice antioxidant system, SOD activity was quantified in the serum. All three doses showed to significantly decrease serum SOD activity, the most notorious impact being observed in animals consuming BB3. Possibly, such outcome is a result of the strong antioxidant properties displayed by the biomass. The presence of compounds with marked antioxidant activity potentially reduces the amount of available superoxide radicals ($O2^{-}$), decreasing the need for SOD to perform. In fact, several PCs are known to possess O2-scavenging activity. [327] Particularly, blueberries have shown to present SOD-like activity and strong antioxidant capacity against superoxide radicals [328,329], as well as blueberry leaves [330].

Upon verification of the lack of toxic effects resulting from biomass administration, and basing on its elevated fiber content, it was intended to evaluate BB's immunomodulatory properties. To do so, the Treg/Th17 balance was evaluated in the gut-associated lymphoid tissue (GALT) of control and BB2-consuming mice. Flow cytometry data revealed a significative reduction in the percentage of Th17 lymphocyte subpopulation, highlighting the immunomodulator potential of the biomass towards an anti-inflammatory profile. Whether such

outcome is a result of gut microbiota modulation due to increased production of SCFAs (entities known to be involved in T cell differentiation processes [101]) or a direct effect of PCs (known to exert direct immune-potentiating effects [319]) is still unknown. Nevertheless, several lines of evidence highlight the ability of berries, namely blueberries, to increase SCFAs production [331] in part by promoting the growth of butyrate-producing bacteria [332]. It is worth noting that, in parallel with this work, recent experiments carried by our team clearly demonstrate that the BB biomass increases fecal SCFAs production, including butyrate and propionate, in heathy conditions. Interestingly, a wealth of evidence supports the action of propionate on T-cell activity, resulting in decreased Th17 and increased Treg levels and activity, which is aligned with BB biomass' immunomodulatory profile observed in GALT upon BB biomass intake. [333] Altogether, these results highlight the advantageous nutraceutical assets of the biomass that go beyond the antioxidant potential. Thus, the apparent immunomodulatory activity of BB confers it enormous potential to be a new functional ingredient of dietary supplements for managing several pathological conditions, namely autoimmune diseases in which the gastrointestinal tract is a major component, such as Inflammatory Bowel Disease (IBD), Ulcerative Colitis (UC) and MS. Still, in order to unequivocally confirm that such effects are mediated by the GM, studies involving antibiotic use or germ-free mice would have to be designed and employed.

All the aforementioned results led to the selection of BB2 (500 mg/kg) as the dose to be employed in the following assay, consisting in the evaluation of the biomass' nutraceutical potential in experimental MS, resorting to the CPZ-induced demyelination model (Experimental Setting II). This model presents a very specific disease evolution pattern, which is characterized by a demyelination peak five weeks after initiating CPZ administration and includes a recovery period in which remyelination can occur after CPZ-treatment suspension. In this study, the defined recovery period was of two weeks, leading to a seven-week protocol. As it was performed in the Experimental Setting I, assessing the nutraceutical potential of BB biomass in the context of experimental MS included the evaluation of animal body weight, as well as food and water consumption, which compose commonly employed animal welfare parameters. Regarding body weight, animals subjected to the five-week experiment displayed very similar intragroup values throughout the entire treatment period, the controls presenting a slight weight gain between W1 and W5, while the CPZ-treated groups practically maintained their weight. Since the animals from the CPZ W5 group presented initial weight numbers close to the ones from the controls, the fact that the first animals did not experience such a relevant weight gain might be a consequence of CPZ treatment. These observations suggest that CPZ administration impaired the animals' grow curves and that BB did not influence this parameter. Additionally, in the seven-week time-point, although controls displayed a more significative weight gain, the animals from the CPZ W7 group showed a trend to gain more weight than the ones composing the CPZ W5. Such outcome was expected, since the seven-week protocol includes a recovery period, allowing the weight to be restored after dropping as consequence of CPZ administration. Neither CPZ or BB administration seemed to have directly affected water and food intake of the animals. Although the animals taking part of the five-week experiment displayed a decrease in food consumption at W2, this was a sporadic event possibly resulting from deglutition complications stemming from the oral gavage protocol. The same explanation could be in the basis of the lower food intake

seen in the CPZ W7 group at the first week of treatment. Nevertheless, mice recovered in the following weeks and maintained approximately the same consumption rate until the end of the experiment.

Additionally, the biomass demonstrated the ability to modulate the activity of the antioxidant enzyme SOD. The sub-chronic toxicity assay had already evidenced this association, showing that increasing BB concentrations reduced SOD activity in the serum, possibly due to the biomass' superoxide radicals-scavenging properties, lessening the necessity for SOD to perform. In the context of this second experimental setting, results showed an increase in serum SOD activity in the demyelination peak after BB administration, suggesting that, contrarily to the observed in a healthy condition, when CPZ intoxication is induced, BB influence on SOD activity is different. This might result from the increased ROS levels arising from CPZ treatment, which are targeted by the biomass' antioxidant activity, promoting SOD activity in order to counteract the increased oxidative stress that characterizes the disease model. Besides serum, SOD activity was also evaluated in the gut and brain.

In the gut, SOD activity variations between the three experimental groups were similar between the animals subjected to the five-week protocols, even though a trend for decreased SOD-1/2 gene expression was observed in the CPZ treated groups when compared to controls. These observations are easily explained by the inhibitory effect of CPZ on SOD. Yet, BB biomass treated animals showed a trend for increased SOD activity, a plausible response to the oxidative stress induced by CPZ. [334] Two weeks following CPZ administration (CPZ W7), CPZ intoxicated mice displayed increased SOD activity, probably an attempt to counteract the CPZ-induced oxidative stress burden, an effect further reinforced by BB biomass intake (CPZ W7+BB). Another possible and complementary explanation for increased SOD-1/2 expression and stimulated enzymatic performance as a result of biomass' administration might likewise be associated with GM modulation. As a matter of fact, it is important to refer that there is evidence of changes in GM composition in the CPZ-induced demyelination mouse model. [335] Furthermore, studies pointing to a direct influence of the intestinal microflora in SOD activity have been arising. [336,337] Therefore, GM alterations might be also associated with antioxidant enzymes' modulation, such as SOD. Likewise, evidence of the SOD-stimulating effects of SCFAs in the gut are available [338], namely for butyrate [339]. Accordingly, Nielsen et al. [340] reported increased SOD-2 expression in the colon as a result of butyrate administration, once more pointing to a potential prebiotic role of the BB as the basis of its effects on SOD.

Gut-associated lymphoid tissue is strongly implicated in the autoimmune phenomenon in MS. As a matter of fact, the acquisition of a myelin-reactive phenotype on the part of Th17 cells occurs in a great extent in the gut, increasing their pathogenicity as well as their ability to trigger the brain. [73] Taking into account the intestinal immunomodulation upon BB biomass intake observed in Experimental Setting I, we then asked whether a similar profile was observed in the cuprizone intoxication. When evaluating the Treg and Th17 percentages after CPZ treatment, we found that the demyelination peak (W5) was characterized by a notorious reduction in Tregs cells numbers along with a significant increase of Th17 population in the gut. When evaluating the same parameters in the remyelination phase (W7), Treg cells' percentage recovered to basal values and the frequency of Th17 population significantly decreased, probably a negative feedback event that

may feature compensatory mechanisms encompassing central regeneration. Strikingly, BB biomass was able to counteract aforesaid events, decreasing the percentage of Th17 cells while favoring Treg frequency in the peak of demyelination. At the remyelination phase, a more balanced Treg/Th17 was also observed. Altogether, our results clearly demonstrate the immunomodulatory properties of BB biomass in the context of debilitating autoimmune disorders, namely MS. Notably, a study evaluating the effects of propionate supplementation on MS patients reported that a 14-day treatment resulted in a significant reduction in Th17 cells as well as increased number and activity of Treg cells [291]. Thus, it is likely that gut immunomodulation elicited by BB biomass may derive from SCFAs upregulation. Whether BB biomass elicits GM modulation and fecal SCFA's elevation in cuprizone-intoxicated mice necessarily warrants further investigation.

To move forward, we attempted to unveil if BB biomass' gut-immunomodulatory properties paralleled central effects. In mice undergoing the five-week treatment, SOD activity analysis in the brain revealed a slight trend for reduction in the CPZ W5 group when compared to controls, which was recovered as a result of BB biomass administration. Such outcome could be explained by the copper chelating activity of CPZ, which inhibits the performance of Cu,Zn-SOD enzymes. Omotoso et al. [341] obtained similar results, reporting that administration of the flavonoid Kolaviron reverted the decrease in SOD activity caused by CPZ treatment. However, when evaluating SOD-1 and SOD-2 gene expression, no significative differences were observed between the three experimental groups, suggesting that CPZ might exert a post-translational effect on SOD enzymes.

Regarding the seven-week experimental design, which relatively to the five-week one included a recovery period when CPZ administration was suspended, the obtained results were considerably different. These mice brains did not show significative variations in SOD activity between the CPZ W7 and CTR groups, possibly due to the fact that these animals had a recovery phase before sacrifice, perhaps allowing the oxidative cargo to return to basal values. On the other hand, SOD activity was extremely increased in mice subjected to a long-term BB administration (4.5 weeks), once more highlighting BB biomass' central antioxidant activity *in vivo*. Evidence supporting an augmented SOD activity as a consequence of blueberry supplementation is already available and corroborate our observations. [263] Yet, the impact of BB biomass on other antioxidant enzymes in the brain deserves to be further evaluated in a near future. Finally, while in the brain BB seemed to not affect gene expression, the results obtained in the gut point to a different direction. This observation might be a consequence of a higher BB bioavailability in the gut comparatively to the brain since it is the first point of contact between the organism and the biomass.

Naturally, we then looked for readouts of myelination index. When analyzing gene expression of MBP and PLP in brain samples, similar results were obtained for both genes. The demyelination peak was characterized by a decrease in MBP and PLP expression, which was essentially maintained in the BB-consuming group. However, when assessing gene expression in the remyelination phase, PLP and MBP levels returned to basal levels at CPZ intoxicated animals, similar to what has been previously reported [342,343], while significantly augmented after BB consumption. Evidence on the expression-promoting effects of PCs on the evaluated genes are

available [344], reinforcing the assumption that the BB biomass may foster remyelination. Even though future studies assessing the corresponding protein levels are warranted, these results are in accordance with the data obtained in the KB histopathological analysis. Although still preliminary, we started to assess the myelination pattern in cerebellum - one of the most frequently CPZ-affected brain regions and recently suggested to be the origin of demyelination [309] - in which the presence of demyelinated areas in animals treated with CPZ only relatively to controls was confirmed (W5). Two weeks following CPZ withdrawal (W7), KB staining also suggests an apparent recovery process. In the BB-consuming groups, myelin staining was more evident in the seven-week experimental design, further supporting the hypothesis that the biomass might be capable of promoting remyelination. Since BB was administered at W2.5 after initiating CPZ intoxication (a timepoint in which the OPCs have just started to proliferate), our results suggest positive effects driven by BB biomass on oligodendrogenesis and remyelination. Additionally, HE and TB samples of mice undergoing CPZ treatment without BB administration evidenced cellular degeneration and neuronal damage, which was partially reverted in the CPZ+BB groups.

Interestingly, oligodendrogenesis and neurogenesis processes have been shown to be influenced by many exogenous modulators, including dietary factors. In fact, compounds such as curcumin, resveratrol, and blueberry polyphenols in particular, have shown to induce neurogenesis in the adult brain. [345] Studies evaluating blueberry supplementation in rats have also reported increased proliferation of neuronal precursor cells in the blueberry-fed animals [345]. Moreover, blueberries have shown to increase the levels of IGF-1 and its receptor IGF-1R, which compose major players in the induction and maintenance of neurogenesis. [267] Since the referred growth factor is known to promote OPC differentiation [346], it is reasonable to suggest that BB biomass may promote oligodendrogenesis through mechanisms encompassing IGF-1-elevation. Moreover, there is evidence of an increased Olig1 expression as a result of PCs administration, which is a key transcription factor involved in OLGs differentiation. [347] Therefore, it would be of interest to evaluate gene and protein expression of Olig1, as well as other markers of remyelination. Notably, a phenolic derivative from Moring oleifera leaves has shown to rescue damage caused by vanadium administration by restoring the density of mature OLGs, seemingly potentiating myelin production. [348] Collectively, these observations are in accordance with the herein obtained results and future immunohistochemistry studies on $Brdu^{+}/Olig\text{-}2^{+}$ and $Brdu^{+}/DCX^{+}$ double-labeled cells are planned to provide a fine analysis of BB biomass impact on oligodendrogenesis and neurogenesis processes, respectively.

In conclusion, this project allowed to confirm the efficacy of the CPZ-induced demyelination along with spontaneous remyelination, further fomenting the discovery of a novel component characterizing this specific animal model: altered intestinal immunity. Whether these changes present a central and systemic readout is yet to be evaluated. Nevertheless, the herein evaluated blueberry leaf biomass has shown promising central antioxidant effects as well as expressive immunomodulatory properties in the intestinal mucosa, tackling two key components implied in several pathological conditions, particularly autoimmune diseases such as MS. Moreover, it showed to exert beneficial effects on the remyelination phase of the disease, for which there are still no therapeutic interventions available. Such readouts might compose indirect effects

resulting from gut-brain communication but can likewise result from a direct consequence of heteroside-conjugated PCs composing the biomass (ex.: rutin), which display privileged transportation across the BBB and, consequently, increased central bioavailability.

The BB biomass features highlighted in this work open new avenues for its use as complementary non-pharmacological therapy in MS, potentially through its integration as a high-added value ingredient in functional foods and/or nutraceuticals. This mindset is aligned with BB's composition and production, which has the additional asset of being an environmental-friendly approach, since it is based on the use of a currently wasted blueberry cultivars' byproduct: senescent leaves. Therefore, the development of BB-based nutraceutical supplementation composes a trifecta in the sense that it: (1) presents notorious health-promoting effects, especially on the remyelination phase of MS, for which no effective therapeutic strategies currently exist; (2) leans on natural compounds and (3) is achieved resorting to a green biotechnological processing, encouraging the transition towards sustainable bioeconomy as properly demanded by society.

Chapter VI | **REFERENCES**

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