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STUDY OF THE INTESTINAL MICROBIOTA IN RELAPSING REMITTING MULTIPLE SCLEROSIS TOWARDS NEW POTENTIAL TREATMENTS

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ABSTRACT

Multiple sclerosis (MS) is a chronic demyelinating inflammatory disease of the central nervous system (CNS). The most frequent form is represented by the relapsing-remitting one (RRMS). Studies revealed that many genes associated with the differentiation, activation, and proliferation of CD4+ helper T cells are linked to MS susceptibility. In particular, the most involved are CD4+ T helper 1 (Th1) and T helper 17 (Th17) lymphocytes. Although the etiology and pathogenesis of MS remains unclear, it is known that the cause of MS is multifactorial and includes genetic predisposition together with environmental factors. Among the latter ones, bacterial or viral infections are listed. In the last years, evidence showed that MS patients have an altered gut microbiota (intestinal dysbiosis) and that specific bacteria could be used to relieve disease symptoms in a mouse model.

Since several studies have shown that probiotics (live microorganisms promoted with claims that they provide health benefits) and prebiotics (high-fiber foods that act as food for human microflora) can modulate microbial community and improve gastrointestinal symptoms and multi-organ inflammation in different pathologies including MS, the aims of this thesis were to study the gut microbiota in Sicilian MS patients to identify the bacteria inducing intestinal dysbiosis. Moreover, given the correlation between the onset of multiple sclerosis and infectious diseases, the spread of two pathogens (*Bartonella henselae* and *Mycoplasma mycoides*) related to MS in the environment and their presence in the MS patients were evaluated. Eventually, the biological activities of root extracts of the *Helleborus bocconei* plants, endemic in Sicily, were studied to understand whether these molecules may possess anti-inflammatory activity in MS.

The main results of this research project are shortly listed below.

The microbiota study revealed a higher abundance in MS patients of bacterial species correlated with the inflammatory state: *Ruminococcus lactaris, Alistipes putredinis*, *Gemmiger formicilis* and *Bacteroides caccae*.

Although *B. henselae* and *M. agalactiae* were isolated in the analyzed environments, they were not found in MS patients.

H. bocconei extracts induced a reduction in IL-1β and iNOS gene expression of peripheral blood mononuclear cells (PBMCs) suggesting their potential use as mediators of the inflammatory response. Finally, *H. bocconei* butanol extracts showed potential anti-viral

activity against the Herpes virus, one of the main risk factors of MS. Thus, *Helleborus* extracts could be exploited as a therapy to reduce the inflammatory state of MS patients.

INTRODUCTION

1. Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic demyelinating disease due to an autoimmune-based inflammatory process. The name comes from the Greek *skleros* (hardened) and *multus* (many) and indicates a lesion area formation called central nervous system plaques. It is the most frequent demyelinating pathology, preferably affecting younger and female subjects (Bergamaschi et al., 2007).

Multiple Sclerosis is acquired and multifocal, and determines the destruction of myelin, a substance with a lamellar structure formed by lipids and proteins, which surrounds the neuronal axons acting as an insulator. The myelin first swells and then fragments causing loss of saltatory conduction with consequent blocking or slowing of the nerve impulse.

Myelin formation sheath is entrusted to the oligodendrocytes, at the Central Nervous System (CNS) level, and to Schwann cells at the Peripheral Nervous System (PNS) level (Poitelon et al., 2020). In MS, the demyelinating process predominantly involves the NCS. In the early stages of the pathological course, there is a recovery of the neurological deficit due to the inflammation resolution and the functional reorganization of the axon with remyelination. In the later stages of the disease, clinical symptoms and signs appear: this is thought to be due to "axonal damage" (Bassem et al., 2018 and Bergamaschi et al., 2007).

Symptoms of MS patients are numerous and variable, affecting many systems and organs. Multiple Sclerosis is defined as an "extreme variability" pathology because undergoes alterations in the clinical course of the same subject.

Due to the great variety of symptoms, numerous clinical evaluation scales have been devised. To date, EDSS (Expanded Disability Status Scale, developed in the 1950s by John Kurtzke), is the scale used internationally which provides a numerical range from 0 to 10, including half-point: 0 value corresponds to the absence of symptoms while a 10 value corresponds to death caused by this pathology.

The EDSS score is determined by the walking ability and the scores relating to eight functional systems (SF) shown below: pyramidal, cerebellar, brainstem, sensory, sphincter, visual, cerebral and "others". Four levels of pathology intensity can be defined (Kurtzke et al., 1983) (Figure 1):

-**Level I** (EDSS from 0.0 to 2.5): no disability or minimal disability in two functional systems;

-**Level II** (EDSS from 3.0 to 5.5): from moderate disability to disability that prevents a complete daily activity;

-**Level III** (EDSS from 6.0 to 7.5): characterized by unilateral assistance to walk about 100 meters in a wheelchair;

-**Level IV** (EDSS from 8.0 to 9.5): condition varies from bed-bound to bed-bound and totally dependent.

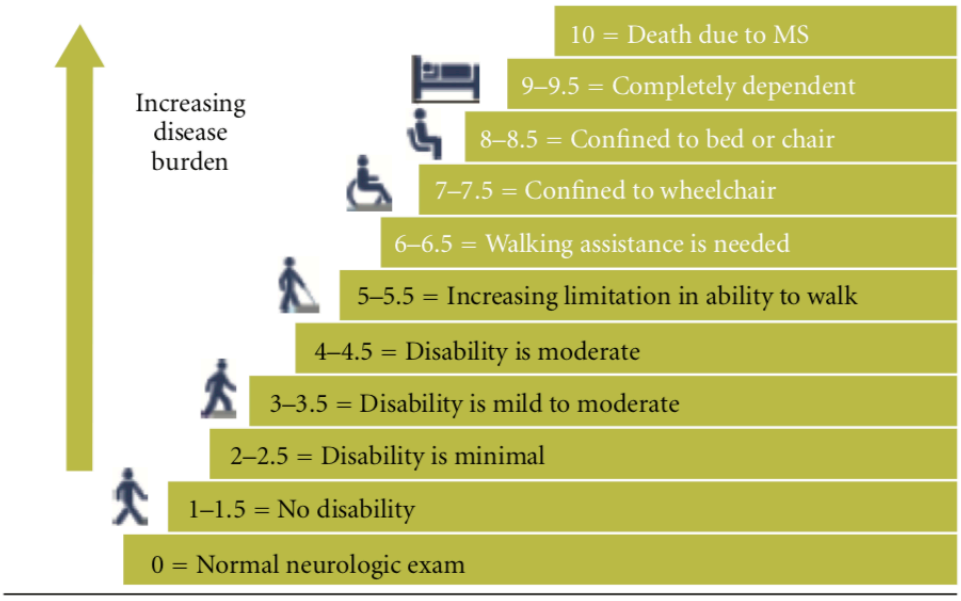


Figure 1: Scaling the progression of disability. EDSS score in MS. Adopted by Singh et al., 2012

Since MS can follow numerous clinical courses, in 1996 the NMSS (National Advisory Committee for the Clinical Trials of Multiple Sclerosis) defined different clinical subtypes (Figure 2):

- **Relapsing-Remitting Form** (MS-RR) which accounts for 80% of MS cases at the onset. It is characterized by acute attacks (relapses or poussées) whose duration can vary by a few weeks or months, followed by total or partial symptomatologic regression. About 50% of these patients can progress to the SM-SP form over the next 10 years;

-**Secondary Progressive Form** (SP) is present in 35% of MS cases, it is characterized by continuous progression, in which clinical symptoms worsen;

- **Primary Progressive Form** (PP) which represents the 10% of the cases and is characterized by the absence of acute exacerbation, there is only a slow functional decline and it is mainly late forms;

- **Progressive relapse form** (PR) represented by 10% of MS cases, patients have a constant worsening since the disease onset and symptoms are severe, with or without remissions.

Furthermore, the benign and malignant forms of MS have also been defined.

In addition, the CIS (Clinically Isolated Syndrome) and the RIS (Radiologically Isolated Syndrome) are included. CIS refers to a first episode of neurological symptoms with features of demyelination and inflammation, which does not always develop into MS. It is necessary to wait for other episodes to confirm a diagnosis of MS: symptoms can be different according to the location of the lesions in the CNS and are divided into typical and atypical (Lublin et al., 2014). The RIS defines a relevant cohort of individuals routinely encountered in clinical practice who are at risk for future demyelinating events. One-third of RIS patients experience their first clinical event, typical of RRMS, after 5 years, while another third show new brain lesions on follow-up scans (Okuda et al., 2014).

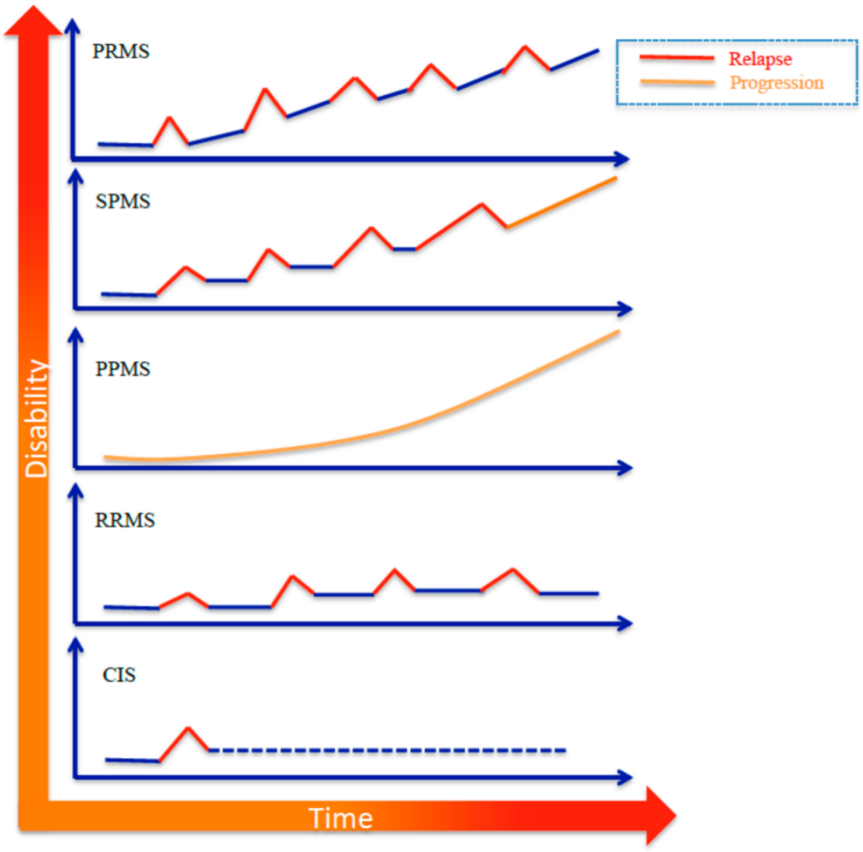


Figure 2: Different clinical MS subtypes basing on symptom patterns. Adopted by Lo Sasso et al., 2019

Etiology

Multiple Sclerosis is an extremely heterogeneous and multifactorial disease, caused by both genetic and environmental factors (Figure 3).

MS is not considered a hereditary disease, but Yamout and Alroughani (2018) showed family predisposition. In fact, the risk among first degree relatives of MS patients is 2% higher than in the general population: siblings present a 20 times greater risk while children a 10 times greater risk. Several environmental factors were proposed as risk factors, such as ultraviolet radiation (UVR), low levels of vitamin D, smoking and obesity, hypothetically through their influence on inflammation and immune functions (Schreiner et al., 2018; Rosso et al., 2020). Recently, it was proposed that sleep deprivation at younger ages might increase the risk of developing MS (Bishir et al., 2020).

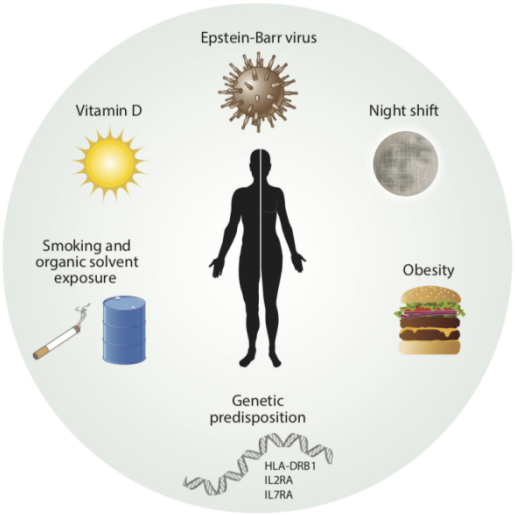


Figure 3: Genetic and environmental factors contribute to multiple sclerosis (MS) pathology. Adopted by Rodríguez Murúa et al., 2022

Regarding the environmental factors contributing to the pathogenesis, several pathogens were proposed as triggers for MS such as viruses belonging to the Herpesviridae family and bacterial pathogens. *Epstein-Barr* virus and human herpes virus 6 (Voumvourakis et al., 2010; Castellazzi et al., 2014; Guan et al., 2019) in the plasma and cerebrospinal fluid of subjects with MS more frequently were isolated. Other viruses thought to be related to the disease onset are human endogenous retroviruses (HERV), whose activation as transposable elements of the human genome could influence disease progression (Saleh et al., 2019).

Regarding bacteria, *Spirochetes, Campylobacter, Mycoplasma, Chlamydia, Bartonella, Mycobacteria* and *Streptococcus* were linked to the MS development, although to date these pathogens have not been isolated directly from patients' cerebrospinal fluid (Lindsey et al.,

2008). *Bartonella henselae* is a slow-growing and facultative intracellular pathogen, mainly transmitted by arthropod vectors or by cat scratch, correlated with a spectrum of neurological manifestations, including ischemic stroke, cerebral arteritis, transverse myelitis, radiculitis, and fatal encephalitis, in patients with cat scratch disease (CSD) (Sendi et al., 2017). Chronic neurological or neurocognitive syndromes associated with persistent *Bartonella* bacteremia are less characterized. Also, different *Mycoplasmas* were found in the brain of animals and humans (Rosales et al., 2017). *Mycoplasma pneumonia* was reported as the CNS invaders (Abramovitz et al., 1987) and is known to induce demyelination, at least in the periphery (Greenlee 2000).
Moreover, several antigens could contribute to the onset or exacerbation of the disease: *Chlamydia pneumoniae* was associated with the onset of MS, while contact with *Staphylococcus aureus* toxins was associated with the onset and/or exacerbation of the disease (Fainardi et al., 2009; Libbey et al., 2014).
Contrarily, helminthic infections (such as *Schistosoma mansoni, Fasciola hepatica, Hymenolepis nana, Trichuris trichiura, Ascaris lumbricoides, Strongyloides stercolaris, Enterobius vermicularis)* were reported as potentially protective against MS development (Libbey et al., 2014). It was found that MS patients with helminthic infections had increased IL-10 and TGF-β and decreased IL-12 and IFN-γ secreting cells, compared to MS patients without helminthic infection (Correale and Farez 2007).

MS and Immunity

Multiple Sclerosis is an autoimmune inflammatory disease in which various genetic and environmental factors determine the activation of lymphocyte clones of the immune system, which become self-reactive CD4+ type T cells capable of recognizing components of our organism as non-self. These CD4+ self-reactive T cells, with the pro-inflammatory Th1 phenotype, release inflammatory cytokines including IL-4, TNFα, IFNγ which determine the increase in the adhesion molecules expression, in particular integrins such as VLA-4 or LFA-1. Macrophages and B cells are also involved in this process (Radandish et al., 2021). Integrins bind to the endothelial membrane of vessels, and their binding with their counterparts, I-CAM molecules (including V-CAM1 and I-CAM1), determines the lytic enzymes release: matrix metalloproteases and gelatinase. These enzymes digest collagen and fibronectin, break the junctions present in the endothelium, alter the permeability of the blood-brain barrier and determine the diapedesis process (Bergamaschi et al., 2007).
In the CNS parenchyma, T lymphocytes recognize the myelinated self-antigens presented by

macrophages and microglia, immune cells responsible for the CNS defense. Here the inflammatory process will take place, which can lead to cell-mediated or antibody-mediated damage with immunoglobulins production. Macrophages attack the myelin sheath, leaving the surface of the axon uncovered, and release nitric oxide (NO), glutamate, and oxygen free radicals (ROS). The initiation of autoimmune response involves an increase in the blood-brain barrier permeability, with a greater recall of inflammatory cells from the periphery mediated by chemokines (CCL5 and MIP-1 α) including: cytotoxic CD8+ T lymphocytes (fundamental in the constitution of inflammatory plaques), microglia, macrophages, astrocytes. T lymphocytes also stimulate B lymphocytes to produce plasma cells and Ig antibodies, act as antigen-presenting cells for self-reactive T lymphocytes and release pro-inflammatory cytokines (Frohman et al., 2006 and Martin et al., 2005).

In particular, IL-1β signaling play an important role in neuroinflammation in MS and EAE contributing to the onset and progression of EAE and MS. IL-1β is a pleiotropic cytokine that can activate microglia and astrocytes and lead to the downstream synthesis of other proinflammatory and chemotactic mediators within the CNS (Shaftel et al. 2008), whereas in the periphery, IL-1β can lead to the expansion of encephalitogenic T cells (Dinarello 2011).

In parallel, immunomodulatory elements are activated, and begin to limit inflammation and initiate repair, which often results in at least partial remyelination and clinical remission (Regulatory arm) (Figure 4).

Among cytokines IL-10, IL-27, IL-35, and especially type I interferons have been associated with disease amelioration.

Was reported that also the complement system may play a role in disease pathogenesis as its effects are not limited to the removal of debris, but also to the processes related to survival cascades (Tatomir et al., 2017). Astrocytes secrete complement proteins when stimulated by TNF α, IL-1β, and IL-8 (Papiri et al., 2023).

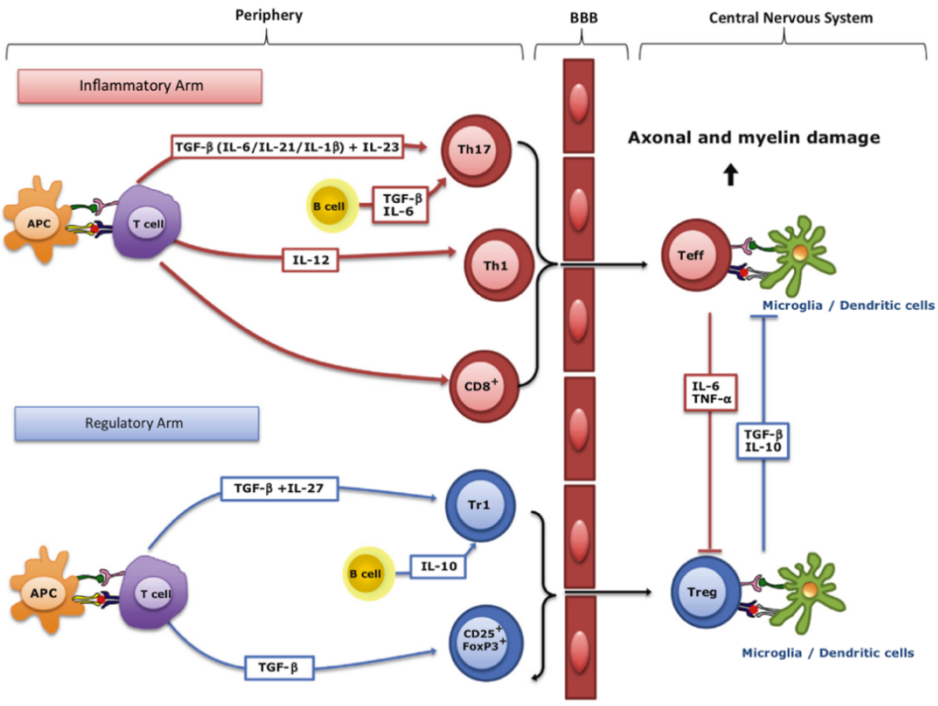


Figure 4: Immune mechanisms in multiple sclerosis: The developing of MS lesion begins with the activation of autoreactive CD4 T cells in the periphery, which then transmigrate through the blood–brain barrier into the CNS. Once in the CNS they are locally reactivated by APCs (Antigen-Presenting Cells) and recruit additional T cells and macrophages to establish the inflammatory lesion (Inflammatory arm). These cells mediate myelin, oligodendrocyte, and axon damage, leading to neurologic dysfunction. In parallel, immunomodulatory elements are activated, and begin to limit inflammation and initiate repair, which often results in at least partial remyelination and clinical remission (Regulatory arm). Adapted by Regev K and Weiner (2016)

Gut microbiota and neurodegenerative diseases

In recent years, the "intestine-brain axis" concept has been increasingly defined. The intestine relates to CNS through the sympathetic and parasympathetic nervous system, and CNS communicates with the muscular and mucous layer of the intestine through the autonomous afferent and efferent. In this way, our CNS can modulate motility, immunity, mucus secretion and intestinal permeability (Mitrea et al., 2022). The intestine is also able to communicate with the hypothalamus-pituitary-adrenal axis: under environmental stimuli, the hypothalamus releases the CRH (corticotropin-releasing hormone) which through the bloodstream reaches the pituitary, stimulating the release of the adrenocorticotropic hormone which will promote the cortisol release from adrenal glands. Finally, cortisol acts directly in the intestine, interrupting its permeability (Sarkar et al., 2019) (Figure 5).

The healthy individual intestine comprises millions of microorganisms living in symbiosis with our body and carrying out beneficial activities for it. This community of microorganisms is called intestinal microbiota and it consists of about 10^{14} bacteria per g with at least 400 different species and constitutes about 1-2 kg of an individual's body weight. Along the gastrointestinal tract, the bacterial population varies and gradually increases: in the proximal portions, represented by duodenum and jejunum, there are 10^5 CFU/g, and the most represented genera are *Streptococcus*, *Lactobacillus*, *Bacteroides* and *Bifidobacterium*. Continuing towards the ileal area, the bacterial content increases up to 10^9 CFU/g and beyond the previous genera also *Veillonella* spp, *Clotridium* spp and *Enterococcus faecalis* are present (Thursby and Juge, 2017). The microbiota is involved in many activities related to the immune system and therefore to the CNS, and also to the production of neuroactive molecules and metabolites can be involved in the development of neurodegenerative disorders (Alzheimer's disease, Parkinson's disease and Multiple Sclerosis) (Liu et al., 2022). Neurodegenerative diseases affect the central nervous system causing death and damage to neurons, resulting in behavioral, cognitive and motor deficits.

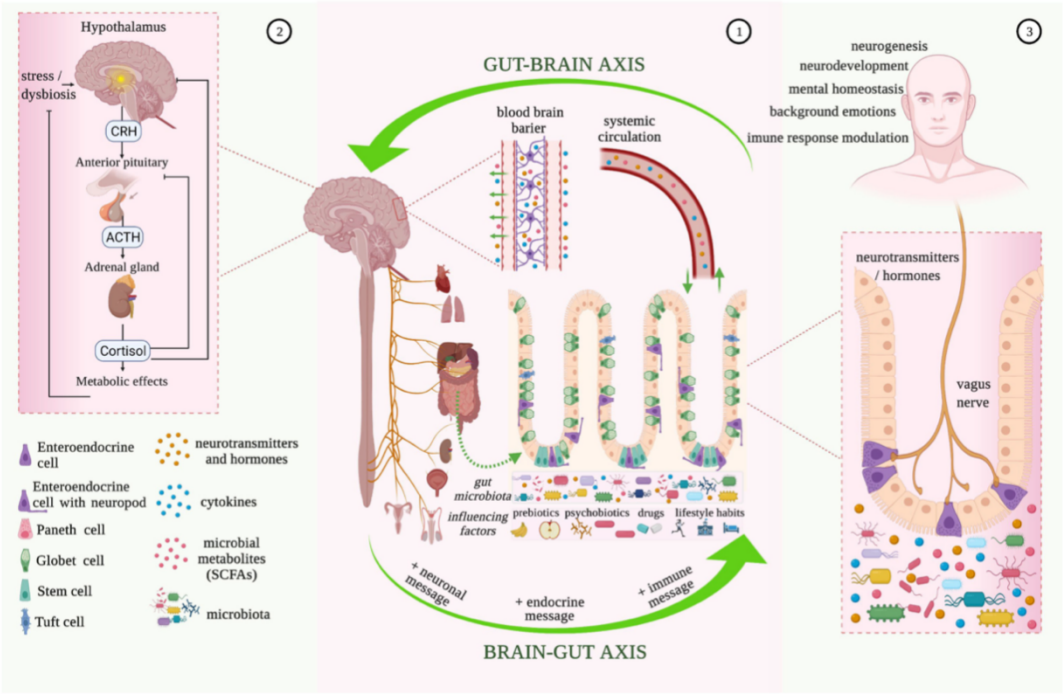


Figure 5: Microbiota-gut-brain bidirectional relationship. **1.** The vagus nerve transfers information on the state of the digestive system to the brain through sensory fibers. **2.** Hypothalamic-pituitary-adrenal (HPA) axis activation is characterized by the release of corticotropin-releasing hormone (CRH) from the hypothalamus. **3.** Enteroendocrine cells with neuropods are one of the most important influencers of bidirectional brain-gut communication. Adopted by Mitrea et al., 2022.

Subjects suffering from neurodegenerative diseases present a state of "intestinal dysbiosis: an alteration of the intestinal microbial flora balance can be related to an immune system imbalance with repercussions at the CNS level. Therefore, it is essential to study the microbiota role in MS patients to improve clinical symptoms (Mitrea et al., 2022).

The microbiota is variable, it changes both during the growth and between individuals. It develops following birth, in fact, during intrauterine life the fetus is sterile. Only at the time of birth, when the newborn comes into contact with the mother's vaginal microbiota (natural birth) or skin (cesarean delivery), and with the external environment, it will be colonized by bacteria. The intestinal microbiota of a newborn varies from birth, mainly composed of aerobic microorganisms, to a week of life when it begins to populate from anaerobic bacteria (Cresci et al., 2015). The intestinal microbiota is essential for healthy digestion and for maintaining homeostasis. It performs various functions that can be classified into:

-Structural: intestinal bacteria promote the development of intestinal villi and crypts, the permeability of tight junctions, the growth, differentiation, and regulation of epithelial cells;

-Protective: prevents the pathogenic bacteria colonization by producing antimicrobial substances and preventing the use of nutrients, altering the intestinal pH, keeping the mucous barrier intact, and activates innate and adaptive immunity;

- Immune: the microbiota is able to collaborate with the GALT (lymphoid tissue associated with the intestine), expanding the number of lymphocytes in the mucous membranes and increasing the size of the germinal center at the lymph node level, and also increases the production of immunoglobulins in the intestine;

- Metabolic: intestinal bacteria promote the digestion of complex carbohydrates, produce enzymes and metabolites essential for nutrient digestion and are involved in the production of vitamins of group B12 and K, in the biosynthesis of amino acids, in the biotransformation of bile acids and in the production of short-chain fatty acids (SCFA) (Capurso et al., 2016).

The intestinal microbiota undergoes variations from year to year, and is influenced by many external factors including: diet, environmental conditions, use of antibiotics, pathological states, geographical location and stress (Cresci et al., 2015).

Gut microbiota in MS

Studies carried out on transgenic rodents with experimental autoimmune encephalomyelitis (EAE) showed that alterations in the intestinal flora are a risk factor for the onset of autoimmune diseases, in particular for MS (Yokote et al., 2008; Lee et al., 2011, Berer et al., 2011;

Cekanaviciute et al., 2017). The transplantation of fecal material from patients with MS triggered an exacerbation of EAE symptoms in rodents, while this effect was not registered when the transplantation of fecal material from healthy controls was performed (Berer et al., 2017).

A study showed a high amount of Th 17 cells in the intestinal mucosa of MS patients related to an increase in *Streptococcus oralis* and *S. mitis*, of which inflammatory capacities were highlighted, and a reduced amount of *Prevotella*, which can reduce the differentiation of lymphocytes into Th17 cells (Cosorich et al., 2017).

The dominant phyla found in the human gut microbiota are Firmicutes (19.8-65.5%), Bacteroidetes (0.1-65%), Actinobacteria (1.1-32.5%) and Proteobacteria (0.2-21%) with a very large range of abundance (Arumugam et al., 2011). Generally, MS patients are characterized by gut dysbiosis compared to healthy controls (Cantarel, et al. 2015, Miyakemet al. 2015, Jangi, et al. 2016, Tremlett et al. 2016, Berer et al. 2017, Cosorich et al. 2017, Ling et al. 2020, Reynders et al. 2020). In principle, the intestinal microbiota of RRMS patients is characterized by a reduction of Bacteroidetes phylum with low percentages of *Prevotella, Parabacteroides* and *Bacteroides*, a decrease in Actinobacteria and Proteobacteria phyla (low percentages of *Sutterella*, but greater abundance of *Acinetobacter, Pseudomonas* and *Mycoplana*), and an increase in Firmicutes phylum with higher percentages of *Akkermansia* and *Dorea* (Shahi et al., 2017 and Sanchez et al., 2020).

Classical microbiology methods are essential for the identification of bacteria that may have anti-inflammatory activity and can be useful for the formulation of probiotics to test on mouse EAE model organisms. Liu et al. (2018) have shown that the administration of probiotics modifies the microbial community and can improve inflammatory and intestinal symptoms in various autoimmune and inflammatory diseases including MS.

Diet plays a fundamental role in the intestinal microbiota study: a diet variation (strictly animal or strictly vegetable based) can alter the intestinal microbiota composition, even within 24 hours (Singh et al., 2017).

Diet can affect the gut microbiota in two ways: directly by providing substrates that promote the growth of some microorganisms (including non-digestible carbohydrates such as resistant starch, inulin and cellulose), and/or inhibit the growth of others; and indirectly influencing the intestinal barrier function, the production of secondary products and the immunity of the subject (Sanchez et al., 2020).

The Western-style high-fat diet administration (with increased free fatty acids in the blood), or a diet rich in salts causes an increase in severity in the EAE mice, favoring the action of

macrophages and T cells (Chu et al., 2018). Low-caloric diets can improve the symptoms of EAE in murine organisms (Valburg et al., 2021).

2. Towards natural treatments for Multiple Sclerosis

Although several drugs are available for MS treatment, some drugs are not safe in the long run as they cause serious side effects. Therefore, in recent years, studies were increased to identify therapies based on probiotics or herbal supplements (Leibowitz et al., 2016).

Since the gut microbiota is altered in MS, the use of probiotics may advantageously induce the anti-inflammatory peripheral immune response in MS patients (Berer et al., 2011).

Probiotics include viable microbial species that can modulate host immune responses in a healthy way by producing antimicrobial agents, such as bacteriocins (Itoh et al., 1995) or by producing secondary metabolites (such as SCFA) (Morrison and Preston, 2016).

Probiotics, such as *Bifidobacterium* and *Lactobacillus,* decreased the inflammatory state with a reduction of IL-17 in mouse models with EAE. A technique that was gained ground in recent years is the use of fecal microbiota transplantation: it could improve walking in RRMS patients and reduce neurological symptoms (Chu et al., 2018). Different combinations of probiotics stimulate components of the immune response in different ways.

A mix of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* has been reported to modify the gut microbiota in MS by regulating intermediate monocytes (Tankou et al., 2018), while *Lactobacillus reuteri* (He et al., 2019) or a mixture of *Lactobacillus paracasei* and *L. plantarum* reduce CNS inflammation by inhibition of the pro-inflammatory cytokines Th1 and Th17 (Chae et al., 2012).

Furthermore, it was observed that *Streptococcus thermophilus* slows down the pro-inflammatory process by intervening in Th1/Th17 polarization (Kwon et al., 2013) and in the MS-induced mouse model it inhibited the secretion of the pro-inflammatory cytokines IL-1β and IFN- γ and enhanced the secretion of the anti-inflammatory cytokines IL-4, IL-5, IL-10 (Dargahi et al. 2020).

Lactobacillus acidophilus, *Lactobacillus casei*, *Bifidobacterium bifidum* and *Lactobacillus fermentum* oral administration inhibits IL-8 and TNF-α gene expression in MS patients (Tamtaji et al., 2017). Finally, the combined effect of *Lactobacillus plantarum* A7 and *Bifidobacterium animalis* strains inhibits the progression of MS by regulating the infiltration of inflammatory T cells into the CNS (Salehipour et al., 2017).

As for medicinal plants, they were reported to reduce neuronal inflammation and improve sleep quality, relieve muscle stiffness and reduce bladder problems (Mammana et al. 2018).

Herbal therapies used to treat MS take advantage of bioactive compounds found naturally in plants and act as prebiotics.

Cannabis extracts were observed to show relief from pain and muscle stiffness in MS patients (Wade et al., 2004; Zajicek et al., 2012) while *Andrographis paniculata* minimizes MS-associated fatigue (Bertoglio et al., 2016).

Curcumin extracts protect axons from degeneration (Tegenge et al., 2012) and reduce blood brain barrier damage, active inflammation and demyelination through increased remyelination and reduction of oxidative stress (Mohajeri et al., 2015). The treatment with the leaf extract of *Dendropanax morbiferus* also intervenes in the improvement of the regeneration of the oligodendrocytes (Kim et al., 2019).

Furthermore, *Crocus sativus* L. extracts were reported to inhibit the progression of MS by limiting oxidative stress and leukocyte infiltration in the central nervous system (Ghazavi et al., 2009). *Terminalia ferdinandiana* fruit extracts inhibit the growth of bacterial species that trigger the autoimmune response (Sirdaarta et al., 2015)

Regarding plants modulating the immune response, ginger extract was shown to modulate IL-27 and IL-33 expression by the reduced infiltration of inflammatory immune cells into the CNS (Jafarzadeh et al., 2014), while the *Scrophularia megalantha* extract inhibits the IFN-γ and IL-17 secretion and increases the IL-10 release (Azadmehr et al., 2019).

3. *Helleborus* spp. and its properties

The genus *Helleborus* spp., belonging to *Ranunculaceae*, comprises around 20 species and includes perennial herbs native to Europe and Asia (Tutin, 2010). *Helleborus* spp. was used for the treatment of mental conditions in Greco-Roman antiquity and its name comes from the Greek "Nourishment that kills", probably for the high toxicity of the plant, poisonous both by ingestion and for external use (Olivieri et al., 2017).

Roots, rhizomes and leaves are still used in traditional medicine to treat humans and animals. The rhizomes contain starch granules and oleosomes accumulate the largest amount of metabolites that can have a broad spectrum of pharmacological and therapeutic effects (Maior M.C. & Dobroță, 2013): these plants are rich in structurally diverse active compounds, such as cardiac glycosides, steroidal saponins, ecdysones, and protoanemonin (Balázs et al., 2020, Challinor et al. 2012; Maior and Dobrotaˇ2013). The pharmacological effects of Helleborus species included anti-rheumatic (Brillatz et al, 2020), anti-inflammatory (Horstmann et al. 2008; Littmann et al. 2008; Neacsu et al. 2010;), anti-cancer (Vochita et al. 2011; Cakar et al.

2011), anti-diabetic, anti-bacterial (Puglisi et al., 2009), and antioxidant properties (Paun-Roman et al. 2010; Apetrei et al. 2011).

In particular, the *Helleborus* species use was reported for tooth pain relief (*H. foetidus*), abortion (*H. orientalis*), anti-cystitis (*H. thibetanus*), skin diseases (*H. odorus*), and sore joints (*H. niger*) (Maior and Dobrotă, 2013; Zhang et al., 2017).

In Italy was used in Veterinary medicine, predominantly: *H. foetidus* and *H. viridis* were used to treat many kinds of disease in pigs, cows, sheep, mules and donkeys (Scherrer et al., 2005; Cornara et al., 2009) and in particular, *H. bocconei subsp. intermedius* in Sicily (Figure 6) was widely used to cure pneumonia and bronchitis in cattle (Raimondo and Lentini, 1990). For this purpose, small incisions are made with small pieces of dry root on the skin of the sick animals and was reported that, if the infected material and the root are removed, 5–6 days later the animal recovers completely (Puglisi et al., 2009).

Figure 6: *H. bocconei* subsp. *Intermedius,* an endemic plant in Sicily and in Calabria

Relating the immunomodulatory activity of this plant, studies on of *Helleborus purpurascens* root on sheep, showed a significant increase of lymphocytes and neutrophils more than 4-fold compared to controls, after 96h of treatment (Nueleanu, 2007).

Moreover, was reported that a fraction of *H. purpurascens*, called HP12 (*H. Purpurascens* 12), was capable to inhibit T-cell proliferation and, when used together to cyclosporine A (CsA), had a potential synergistic effect in the immunosuppressive activity (Terness et al., 1999).

Another compound, extracted and purified from *H. purpurascens* roots, MCS-18 was showed implicat in the immunomodulatory response: induce significant in vitro up-regulation of the immune modulatory cytokines IL-10 and TGF-β (Szegli et al., 2005), efficiently down-regulates T-cell-dependent antibody formation in mice (Kerek et al., 2007) and reduced B-cell proliferation and immunoglobulin production (Littmann et al., 2008). Moreover, was reported that MCS-18 strongly reduced the paralysis associated with the EAE mice, was able to inhibit the expression of typical molecules of mature dendritic cells (DC) and reduced leukocyte infiltration in the brain and in the spinal cord (Horstmann 2007).

Also that *Helleborus* species can be toxic was reported, when incorrect dosage is used. Toxic features of hellebores are determined mostly by the aglycons of cardiac steroids and by protoanemonin (a toxic γ–lactone). Acute toxicity effects on the central nervous system include lethargy, confusion and weakness and was mainly associated with alkaloids, glycosides and saponosides present in this plant. Chronic toxicity due to hellebore poisoning is difficult to diagnose and less obvious. It could determine anorexia, weight loss, neuropsychiatric disorders (confusion, drowsiness, headaches, delirium, and hallucinations) and visual disturbances (Maior M.C. and Dobrotă, 2013).

MAIN OBJECTIVES

In this thesis, the intestinal microbiota of RRMS patients and healthy control subjects, living in Sicily, was characterized to identify bacteria that induce the intestinal dysbiosis or that could improve gastrointestinal symptoms and multi-organ inflammation in MS.

Moreover, given the correlation between the onset of multiple sclerosis and infectious diseases, the spread of two pathogens (*Bartonella henselae* and *Mycoplasma mycoides*) related to MS in the environment and their presence in the MS patients were evaluated.

Finally, the biological activities of root extracts of the *Helleborus bocconei* plant, endemic in Sicily, were studied to investigate whether these molecules may possess anti-inflammatory activity in MS.

MATERIAL AND METHODS

1. RRMS MICROBITA

Study population

In this study, 52 subjects were recruited. Exclusion criteria included the diagnosis of a significant neurological disease other than MS, the use of antibiotics or anti-inflammatory drugs in the last 6 months, excessive alcohol intake or drug abuse, and investigator uncertainty regarding the willingness or ability of the patient to understanding the protocol of faecal sample collection (Figure 7).

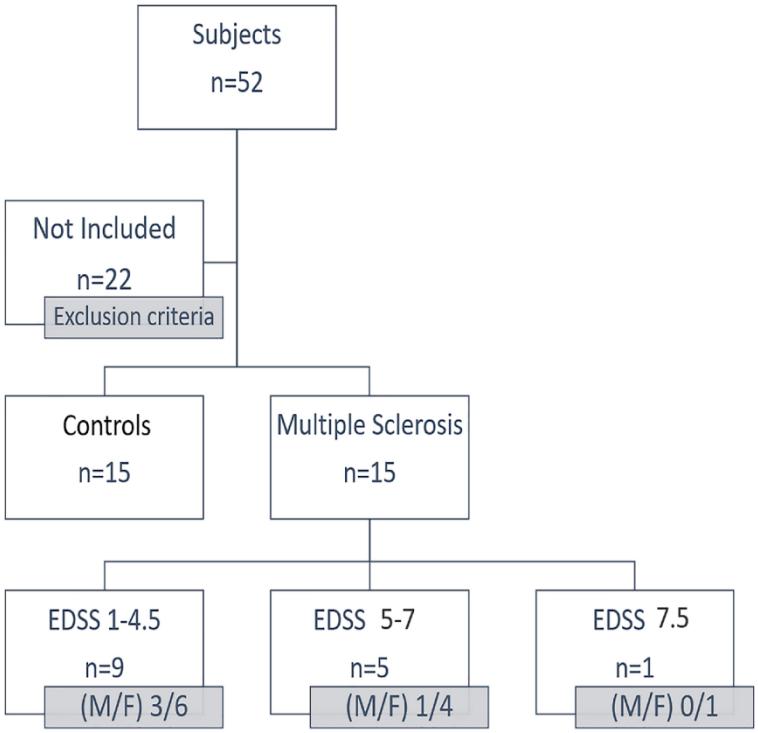


Figure 7: Scheme of recruitment of the subjects of this study. Thirty subjects were recruited: n = 15 controls and n = 15 MS patients. These patients were classified according to the EDSS value into three groups: EDSS 1–4.5 (9 patients), EDSS 5–7 (5 patients), EDSS 7.5 (1 patient).

Participants were recruited from 2 MS centres: Alcamo (TP) and Palermo (PA) of Sicily. To reduce the dietary variability, a household relative (wife/husband/daughter) for each MS patient was recruited. All the participants were requested to follow the same diet in the two weeks before the faecal collection. The patients and the controls coming from the same town were subjected to the same climatic and environmental conditions (temperature, distance from the sea, smog, pollution). The Ethics Committees of each participating centre reviewed and approved the protocol. The study was conducted following the Declaration of Helsinki. The

Ethics Committees number for this study is 7/2019. Fifteen MS patients, aged 28–66 years and with an Expanded Disability Status Scale (EDSS) between 0 and 7, were included together with their relative controls. Faecal samples from each subject were collected in two different tubes: one containing RNA Later in order to avoid alterations of nucleic acids for microbiome study; and one containing a 20% glycerol solution in Phosphate Buffered Saline (PBS) to maintain the samples at -20°C so as to also favour the isolation of anaerobic microorganisms and to improve the sample preservation following microbiological analyses.

Data of the MS patients and the controls are summarized in Table 1.

Table 1 Subjects of the study were grouped according to sex, age, and EDSS.

Cohort	**n. subjects**	**ID Family**	**Sex (M/F)**	**Age range**	**EDSS**
Controls	15	-	8/7	21-69	-
RRMS	9	1,**2**,6,**8**,11, 12,13,14,**15**	3/6	28-65	1-4.5
RRMS	5	4,5,7,9,10	1/4	54-66	5-7
PPMS	1	3	0/1	57	7.5

ID family is the number to identify the MS patient and the relative. 11S and 12S were collected from two naïve MS patients, and samples 2, 8 and 15 derived from relatives of the same sex (2 and 15 are mother and daughter; 8 are sisters), in which one is affected by MS.

Microbiological analysis

Each stool sample collected in 20% glycerol solution was sown on various culture media and different growth conditions (temperature and aerobic/anaerobic environment) in order to carry out the isolation of the bacterial strains belonging at the main phyla of the intestinal microbiota.

The different culture media used for the isolation of bacteria belonging to the main phyla and the growth conditions (temperature and aerobic/anaerobic environment) were indicated in Table 2.

Once the growth of the microorganisms had been ascertained, the colonies were isolated and purified in order to submit them to morphological analysis and Gram staining, following the procedure of the Smith and Hussey protocol (2005).

Oxidase and catalase tests were performed on 63 colonies. For the oxidase test, the DrySlide Oxidase kit (Thermo Fisher Scientific) was used to verify the presence of activity of cytochrome C oxidase in tested bacteria. Hydrogen peroxide for the catalase test was used to verify the presence of the catalase enzyme.

Table 2: Culture media used for seeding faecal samples from RRMS patients and controls: phyla and species sought, incubation temperature and growth environment (aerobic/anaerobic).

Colture media	Phyla	Bacterial species	Temperature	Environment
Blood Agar	*Firmicutes*	*Streptococcus, Staphylococcus*	37°C	Aerobic and anaerobic
	Verrucomicrobia	*Akkermasia*		
Mannitol Soya Flour (FSM)	*Actinobacteria*	*Streptomyces* spp.	37°C	Aerobic
Mannitol Salt Agar (MSA)	*Firmicutes*	*Staphylococcus* spp.	37°C	Aerobic
Starch-Casein Agar	*Actinobacteria*	*Streptomyces* spp.	30°C	Aerobic
MacConkey Agar	*Proteobacteria*	*Enterobacteriacae and* Gram negative bacilli	37°C	Aerobic
Anacker-Ordal Agar	*Bacteroidetes*	*Flavobacterium*	30°C	Aerobic and anaerobic
De Man, Rogosa e Sharp Agar (MRS)	*Firmicutes*	*Lactobacillus* spp.	30°C	Aerobic

Bacterial identification by molecular assay

Genomic DNA was extracted from pure colonies using the commercial PureLink® Genomic DNA Kit (Invitrogen, Thermo Fisher Scientific, Italy) following the manufacturer's instruction. The 16S ribosomal rRNA gene was amplified by conventional PCR utilizing the universal primers UNF (5'-GAGTTTGATCCTGGCTCAG-3') and UNR (5'-GGACTACCAGGGTATCTAAT-3') targeting bases 9–27 and 805–786 of the 16S rRNA gene (Alexeeva et al., 2006). The amplicons were detected using a 1.5% agarose gel and the fragment size was estimated using a DNA molecular weight marker (TrackIt™ 100 bp DNA Ladder, Thermo Fisher Scientific). Control reactions were done in the absence of template DNA to rule out contaminations during PCR.

PCR products, before being sequenced, were purified using the QIAquick PCR Purification Kit (Qiagen) following the procedure indicated by the company and were quantified by Nanodrop. The sequencing was carried out by B.M.R. Genomics Srl and the obtained nucleotide sequences were analyzed by NCBI nucleotide BLAST software.

Genomic DNA extraction, PCR amplification and sequencing

Faecal samples were collected in tubes containing RNA Later and maintained at 4 °C within 24h of receipt. Metagenomic DNA was extracted using the QIAmp DNA Stool Mini Kit (Qiagen), following the manufacturer's instructions. After quantification by NanoDrop 2000c

spectrophotometer (ThermoFischer Scientific), DNA was diluted to obtain a concentration of 50 ng/µl and used as a template to amplify the region V3-V4 of the 16S rDNA gene using primers described previously (Takahashi et al., 2016). Amplification products were sequenced in one 300-bp paired-end run on an Illumina MiSeq platform (BMR Genomics srl, Padova, Italy).

Raw data processing

The raw 16S rDNA data were processed using QIIME2 environment (Bolyen, Rideout et al. 2019) (https://qiime2.org) as paired-end sequences. DADA2 plug-in was used to filter, remove chimaeras and denoise all produced sequences to obtain the Operational Taxonomic Units (OTUs) and the Amplicon Sequence Variants (ASVs). After removing singletons, the taxonomy of each OTU was determined, with a 97% similarity level, through the implementation of the SINA classifier (using the SILVA dataset available at https://www.arb-silva.de/ngs/) (Pruesse, Peplies et al. 2012). For ASVs the alignment was performed by the Greengenes reference database at 99% sequence similarity (Prodan et al., 2020).

Unclassified OTUs were not assigned. Rarefaction analysis was carried out plotting the number of the observed OTUs against the total number of filtered reads for each sample. Sequences were deposited in GenBank (BioProject ID: PRJNA684124).

For each sample, the number of observed OTUs and the percentages of relative abundances of phyla, orders, classes, and families were determined. Similarities among the studied samples were observed using Principal coordinate analysis (PCoA) through the implementation of software package PRIMER 6 (Clarke and Gorley 2006). The analysis was based on the Bray-Curtis distance matrix. Furthermore, METAGENassist (Arndt, Xia et al. 2012) was used to distinguish the microbial species based on their metabolic activity.

Alpha diversity, Abundance-based Coverage Estimator (ACE), Chao1, Shannon-Wiener diversity, H', and Simpson index, 1-D, and evenness, e (equitability assumes a value between 0 and 1 with 1 being complete evenness), were estimated to determine the specific faecal microbial richness and diversity. Good's coverage was estimated to evaluate the completeness of sampling.

2. PATHOGENS CORRELATED WITH MULTIPLE SCLEROSIS

Molecular evidence of *Bartonella henselae* in stray cats

Sampling

Blood samples were collected into Ethylenediaminetetraacetic acid (EDTA) tubes from censused stray European race cats belonging to registered colonies of Palermo. The sample size was determined using Winepi software considering an expected prevalence of 50%, with 5% precision at the 95% confidence level (as no other epidemiological data were available). According to Winepi, the minimum number of required samples was 270 animals. Blood samples from 429 cats were collected.

Molecular diagnosis

Whole blood into an EDTA tube was collected and used to extract DNA. The DNA was extracted using the commercial kitMagMAX CORE (Thermo Fisher Scientific), following the manufacturer's protocol. A screening on blood samples was carried out by real-time PCR in order to check for the presence of *B. henselae* DNA. The real-time PCR protocol proposed by Valtierra (2016) was used, but a qualitative rather than a quantitative approach was carried out. Real-time PCR was performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad), amplifying a 183-base pair product of the specific 16S-23S intergenic region of *B. henselae* ribosomal RNA (Alamán Valtierra et al, 2016) DNA was added to a reaction mix containing 1× of SSo advanced Universal Sybr Green Supermix (Bio-Rad) and 0.5 µl of each primer (10 µM) (B_hen F 5'- TGTCATCAGAAAGGGCTATT-3' and B_hen R 5'-CAAAACAAAGTGCAAAACAA-3') up to a final volume of 25 µl. The instrument program consisted of an initial denaturation of 3 min at 95 °C followed by 40 cycles of amplification, which included a denaturation step at 95 °C for 10 s and an annealing/extension step at 60 °C for 30 s. The final step consisted in 0.5 °C increment from 65 to 95 °C for 2 s/step to analyze the melting curve.

Detection of *Mycoplasma* in ticks collected in Sicilian sheep and goats

Sampling

Three different Sicilian dairy farms of sheep and goats were selected for the study. The farms were located in the provinces of Palermo (North-West of Sicily) (farms A and B) and Messina (North-East of Sicily) (farm C). In these locations of Sicily, late spring and early summer

represent the main periods of tick activity. Therefore, 152 ticks were collected from 25 lactating sheep and kept alive for a week at room temperature, in order to allow the ectoparasites to cleanse themselves of any ingested blood. Each tick was bathed in 70% ethanol for 5 min and divided lengthwise into two parts in sterile Petri dishes under the stereomicroscope, using sterile forceps and scalpels: one half was screened by cultural and molecular methods, with the remaining half kept in alcohol, pending further investigation. Ticks belonging to the same species and stage, and derived from the same animal, were analyzed in pools.

Molecular analysis

Two hundred microliter of each pool or singular tick homogenate were used to tha DNA extraction using the Quick-DNA Miniprep Plus Kit (Zymo Research), following the manufacturer's instructions. To confirm the presence of *Mycoplasma* DNA, a commercial TaqMan real-time PCR was performed (VetMAX M. agalactiae and *M. mycoides* Kit of Thermo Fisher Scientific). Real-time PCR was performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad).

All of the cultures showing *Mycoplasma*-like colonies had the DNA extracted, followed by PCR and sequencing to confirm microbiological observations. A semi nested- PCR using general and operon-specific primers was performed to amplify the 16S rRNA gene. The first amplification was performed with the primers U1 (5′-GTTTGATCCTGG CTCAGGCYDAAC-3′)/U8(5′-GAAAGGAGGTRWTCCAYCCSCAC-3′). The second ampli- fication was performed using primers U1/U5 (5′-CTTGTGCGGGTCCCCGTCAATTC-3′), and the other with primers U2 (5′-CCAGACTCCTACGGGAGGCAGC-3′)/U8 (Pettersson et al., 1996). Amplicons were purified by the QIAquick Gel Extraction Kit (Qiagen), quantified, and sent for sequencing (BMR Genomics srl., Padova, Italy). The obtained sequences were aligned using the Bioedit software (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA) and analyzed for nucleotide sequence identity. Then, they were compared with the reference strains in the GenBank database using the Basic Local Alignment Search Tool (BLAST). The obtained sequences were deposited on the GenBank database (MZ621182, MZ621183, MZ621184, MZ621185, MZ621186).

B. henselae and *M. mycoides* detection in MS patients and controls

All DNAs extracted for metagenomic analysis were used to evaluate the presence/absence of both *Bartonella henselae* and *Mycoplasma mycoides* pathogens in MS patients and controls. The real-time PCR protocol proposed by Valtierra (2016) was used to search the *B. henselae*

DNA. Regarding *M mycoides* the VetMAX M. agalactiae and *M. mycoides* Kit (Thermo Fisher Scientific) was used according the manufacturer's instructions.

3. BIOLOGICAL ACTIVITIES OF *HELLEBORUS BOCCONEI* PLANT

Plant collection and extracts preparation

H. bocconei bocconei plants were harvested in the area around Nicolosi (Catania, Italy) (Figure 8). Roots were air dried at room temperature for a period of 30 days afterwards were washed, powdered and stored at room temperature until the extraction.

Figure 8: *H. bocconei* collected in Nicolosi

Thirty-five grams of *Helleborus* roots were extracted sequentially with two 350 ml aliquots of methanol (MeOH) each, placing the root powder to shake for 3h at room temperature and protected from light.

After the first extraction, the decanted suspension was filtered and the solid part was subjected again to extraction. The two extracts were combined and the organic solvent was removed in the rotavapor maintaining the water bath temperature at 30°C. Finally, nitrogen was blown and 10g of clear brown oil were obtained.

The obtained extract was partitioned between n-BuOH and H_2O in order to remove sugar compounds as described by Rosselli et al. 2009.

The extracts preparation was carried out in collaboration with Prof. Raimondi Valeria of the Università degli Studi di Palermo and Prof. Pinto of the Università degli Studi di Milano.

All the three extracts (methanolic, butanolic and water) were used to study the biological activities of *H. bocconei* roots.

Antibacterial assay

All the three extracts on bacteria isolated from feces of MS controls and on pathogenic strains were tested. Among the strains isolated from MS controls, the three extracts were tested on *Sutterella* spp., *Pediococcus pentasaceus*, *Bacillus safensis* and *Enterococcus faecium*.

H. bocconei extracts were tested on several Gram negative bacteria (*Escherichia coli ATCC 25922, Escherichia coli ATCC 33150, Salmonella enteritidis ATCC 13076, Salmonella typhimurium ATCC 14028, Yersiina enterocolitica ATCC 23715, Pseudomonas aeruginosa ATCC 10145, Listeria monocytogenes ATCC 7644),* Gram positive bacteria (*Staphylococcus aureus* ATCC 25923, *Streptococcus agalactiae* ATCC 27956, *Streptococcus dysgalactiae* ATCC 9226, *Streptococcus pyogenes* ATCC 19615, *Streptococcus uberis* ATCC 19436) and one Mollicutes (*Mycoplasma capricolum*).

According CLSI protocol, 10 µl of a 0.5 McFarland suspension of each bacterial strain in 10 ml of Muller Hinton (MH) broth were inoculated. Serial dilutions of the three *Helleborus* extracts (from 80.000 µg/ml to 15µg/ml) were added to 100 µl of bacterial suspension previously plated on a 96 wells microplate. Muller Hinton broth was used as negative control while single strains in MH broth with and without 1% DMSO as positive controls were used. Plates were incubated at 37°C for 24-48h. After 24-48h, serial dilutions of each sample were performed and 10 µl were sowed on plate count agar (PCA) in order to perform the vital count. On *Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076, *Pseudomonas aeruginosa* ATCC 10145 the anti-biofilm assay was performed by cristal violet method according to Pourhajibagher (2016). An optical densities (OD) value of Muller Hinton Broth as a negative control (ODc) was used to establish interpretation criteria of biofilm-forming ability by the treated and non-treated strains, which were classified as follows: $4 \times ODc < OD$ strong biofilm producer, $2 \times ODc < OD \leq 4 \times ODc$ moderate biofilm producers, $ODc < OD \leq 2 \times ODc$ weak biofilm producers, and $0.004OD \leq ODc$ no biofilm producer.

Moreover, since the methanolic and butanolic extracts seemed the most promising, in order to analyze their antibacterial activity, they were also tested on 16 Streptococci strains (4 S. *agalactiae, 4 S. dysgalactiae, 4 S. uberis and 4 S. pyogenes*) isolated from the field. A MIC with *Helleborus* extracts and a MIC using commercial plates (BOPO6F Sensititre plate, Thermofisher) containing 18 lyophilized antimicrobials, were conducted in parallel in order to evaluate the antibiotic resistances of these field strains. The BOPO6F plate contained: Ceftiofur (XNL) (0.25-8µg/ml), Tiamulin (TIA) (0.5–32µg/l), Chlortetracycline (CTET) (0.5–8µg/ml), Gentamicin (GEN) (1–16µg/ml), Phlorphenicol (FFN) (0.25–8µg/ml), Oxytetracycline (OXY) (0.5–8µg/ml), Penicillin (PEN) (0.12–8 µg/mL), Ampicillin (AMP) (0.25–16µg/ml),

Danofloxacin (DANO) (0.12–1 μg/ml), Sulfadimethoxin (SDM) (256 μg/ml), Neomycin (NEO) (4–32 μg/ml), Trimethoprim / sulfamethoxazole SXT) (2/38 μg/ml), Spectinomycin (SPE) (8–64 μg/ml), Tylosin tartrate (TYLT) (0.5–32 μg/ml), Tulathromycin (TUL) (1–64 μg/ml), Tilmicosin (TIL) (4–64 μg/ml), Clindamycin (CLI) (0.25–16 μg/ml) and Enrofloxacin (ENR) (0.12–2 μg/ml). Bacterial suspension (200 μl) was added to each well. Two wells antibiotics free were used as controls: 200 μl of sterile broth (K-) were added for the negative control while 200 μl of bacterial suspension was added for the positive control (K +). Plates were incubated at 37 ° C and observed daily, for up to 5 days, using the Thermo Scientific ™ Sensititre ™ Manual Viewbox. The growth wells showed a color change from red to yellow (such as K +) to determinate the Minimum inhibitory concentration (MIC) values.

PBMCs isolation and cytotoxicity assay

In order to study the immunomodulatory activity of *H. bocconei*, the three extracts were used to study their cytotoxicity on Peripheral Blood Mononuclear Cells (PBMCs).

For this purpose, 2 ml of human blood were diluted with 2 ml of PBS without calcium and without magnesium. The diluted blood was carefully stratifyied on 3 ml of Lymphosep and centrifuge at 400xg at room temperature for 20 minutes.

The upper layer was aspirated, twice volume of PBS was added and the sample was centrifuge at 200xg for 10 minutes at room temperature. The supernatant was aspirate and discard, and cells were washed twice with PBS. Cells were resuspended at the concentration of 1×106/μl in complete medium composed of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 1% penicillin/streptomycin, 10 mM HEPES, and 1 mM L-glutamine and incubated in a 96-well plate for 24 h at 37°C in a humidified 5% CO2 atmosphere. After 24h, the three Helleborus extracts, at concentrations from 10 μg/ml to 0.07 μg/ml were added.

After 24h 20 μl of Cell Titer 96 Aqueous One Solution reagent (Promega) were added to each well and the plate was additionally incubated for 4 h at 37°C in a humidified atmosphere containing 5% CO2. Plates were read by an absorbance microplate reader at the wavelength of 492 nm (Ciaglia et al., 2017).

Hemocompatibility test

In order to perform hemolysis test, as soon as possible after phlebotomy, the blood sample was centrifuged at 1700×g for 5 min. The supernatant was removed by aspiration and a washing step with PBS pH 7 was repeated three times. After final aspiration, the remaining pellet was diluted 1:100 in PBS and 250 µl of blood sample was mixed with 250 µL of *Helleborus* extracts (0.62, 0.31 and 0.15μg/ml for butanol and methanol extracts, while 5, 2.5 and 1.25 μg/ml for water extract) or detergent (20% of TritonX100 as positive control of hemolysis) in an Eppendorf tube. Samples were subsequently incubated at 37 °C for 3 h, following gentle inversion of the tubes every 30 min. After the incubation, samples were centrifugated at 500 g for 5 min at room temperature and 100 µl of the supernatants were transferred to transparent 96-well plates. Finally, absorbance of the supernatants was measured at a wavelength of 490 nm. Hemolysis was reported as the percentage of the hemoglobin liberated from the erythrocytes, measured as mean absorbance values corrected by absorbance values of untreated test items, as compared to positive control (100% lysis) (Zin et al., 2020; Sæbø et al., 2023).

LPS stimulation and gene expression analysis

Once the concentrations of each compound non-cytotoxic for PBMCs were established, cells were stimulated with bacterial LPS.

PBMCs were cultured in 24-well flat-bottom plates at 2.5×10^5 cells per well in RPMI medium. After 24h, *Helleborus* extracts at different concentrations (0.31 and 0.62 μg/ml for butanol, 0.07 and 0.15 μg/ml for methanol extract, and 0.62 and 1.25 μg/ml for water extract) were added to PBMCs. The PBMCs were either unstimulated or stimulated by LPS 1µM for 24 h at 37°C in a humidified 5% CO2 atmosphere.

After 24h of LPS stimulation, cells were collected, centrifugated at 2000xg for 10 min and the obtained pellet used for the RNA extraction.

Total RNA was extracted by High Pure RNA Isolation Kit (Roche), following the manufacturer's instructions, and 0.3 micrograms were used to synthesize the first strand cDNA using the iScript™ cDNA Synthesis Kit (Biorad). Synthesized cDNAs (2μl) were used to amplify IL1β, IL17, TNFα, iNOS, TGFβ 1 and γINF by SsoAdvanced Universal SYBR Green Supermix (Biorad). Primer sequences were reported in Table 3 Gene expression was normalized to β-actin and all data were analyzed by GraphPad software.

Table 3: Primer sequences used for gene expression analysis

Gene	Sequence (5'-3')	Reference
IL-1β	F: CCTGTCCTGCGTGTTGAAAGA R: GGGAACTGGGCAGACTCAAA	Boeuf et al., 2005
TGF-β1	F: CAAGGGCTACCATGCCAACT R: AGGGCCAGGACCTTGCTG	
TNF-α	F: CCCCAGGGACCTCTCTCTAATC R: GGTTTGCTACAACATGGGCTACA	
yINF	F: GTTTTGGGTTCTCTTGGCTGTTA R: AAAAGAGTTCCATTATCCGCTACATC	
IL17	F:GGTTTGACTGAGTACCAATTTGC R: AAATTCCCAAGCCCAGAATC	Mardegan et al., 2017
iNOS	F: TTCAGTATCACAACCTCAGCAAG R: TGGACCTGCAAGTTAAAATCCC	Miao et al., 2020
β actin	F: CATGTACGTTGCTATCCAGGC R: CTCCTTAATGTCACGCACGAT	

Cytotoxicity assay and anti-viral activity against *Herpes* virus

In order to evaluate the capability of the three *H. bocconei* to act as anti-viral, their cytotoxicity on MDBK (useful to cultivate BHV-1 and CpHV-1), RK13 (useful to cultivate EHV-1) cell lines were tested.

MDBK (Mardin Darby Bovine Kidney) and RK13 (Rabbit Kidney Ephitelial Cell) cell lines, were maintained in Eagle's minimal essential medium (MEM), plus 10% fetal bovine serum (FBS) and 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 0.25 µg/ml amphotericin B.

One hundred microliters of 1×10^4 cell suspension were plated into each well of a 96-well culture plate and incubated at 37°C and 5% CO_2 atmosphere.

After 24h, concentrations of the three *Helleborus* extracts (methanol, butanol and water) from 3.2 mg/ml to 0.0025 µg/ml were tested. Phenol (0.5%) was used as a positive control of cytotoxicity in all the experiments. Cell growth, cell morphology and cell viability were evaluated at 24 and 48 h, and were used as parameters to determine the cytotoxicity of these substances. After the period of incubation, the cell viability was evaluated by measuring 3-(4,5dimethylthiazol-2yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), using the commercially available kit (Cell Titer 96 Aqueous One solution Cell proliferation Assay, Promega) according to the manufacturer's instructions. The Cell Titer 96 Aqueous One Solution Reagent (20 µl) was added, and the plate was cultured for 4 h at 37 °C in a humidified 5% CO_2 atmosphere. Plates were read on an absorbance microplate reader

(Sunrise™, Tecan), complemented by universal reader control and data analysis software add-on (Magellan™, Tecan), at a wavelength of 492 nm. The percentage of cell survival was calculated. The experiment was conducted in triplicate.

Non cytotoxic concentrations of each extract (0.31 and 0.62 µg/ml for butanol extract, 0.62 and 1.2 µg/ml for both methanolic and water extracts) were tested to assess anti-BHV-1, anti-CpHV-1 and anti-EHV-1 activity. To elucidate the antiviral action mode, cells were incubated with the extracts for 72 h, following 3 experimental conditions: before and after viral infection, simultaneous infection (Boubaker et al., 2014).

Pre-treatment of cell cultures was performed by exposing the cell monolayers to the different concentrations of *Helleborus* compounds in maintenance medium (200 µl) for 2h at 37°C. After treatment, medium was removed and the cell monolayer was infected with 200 µl of BHV-1, CpHV-1 or EHV-1 suspension at 10^2 Tissue Culture Infective Dose 50% (TCID50) ml^{-1} of virus and observed for viral cytopathic activity for 72 h of incubation in 5% CO_2 atmosphere.

Regarding the Post-infection assay, 200 µl of viral suspension at 10^2 TCID50 ml^{-1} in culture medium were adsorbed to MDBK or RK13 cells for 2 hours at 37°C. After 2h, the medium was replaced with 200 µl of MEM containing the non-cytotoxic concentrations of each extract of *Helleborus bocconei* and incubated for 72 h at 37°C and 5% CO_2 atmosphere.

In the inhibition of virus attachment (simultaneous infection and treatment) assay, virus suspensions at a viral concentration of 10^2 TCID50 ml^{-1} of virus were mixed (v/v: 100 µl) with different concentrations of *Helleborus* extracts at room temperature: cells were incubated immediately with 200 µl of the mixtures for 72 h at 37°C and 5% CO_2 atmosphere.

For all the assays the controls consisted of: untreated infected with virus cells for virus control, treated uninfected cells for plant extract controls and untreated uninfected cells for cell control. After 72 h, cell morphology was observed by microscope and viability was determined as previously described by Cell Titer 96 Aqueous One Solution.

RESULTS

1. RRMS MICROBIOTA

Characterization of bacteria by microbiological analysis

To carry out the isolation of the bacterial strains belonging at the main phyla of the intestinal microbiota, each stool sample was sown on various culture media and incubated at different growth conditions (temperature and aerobic/anaerobic environment).

By microbiological analyses, 81 colonies were isolated from the feces of RRMS subjects and 100 from their healthy relatives, used as controls. Purely isolated colonies were subjected to morphological analysis by observing shape, appearance, color, margin, gloss, opacity and size (Figure 9).

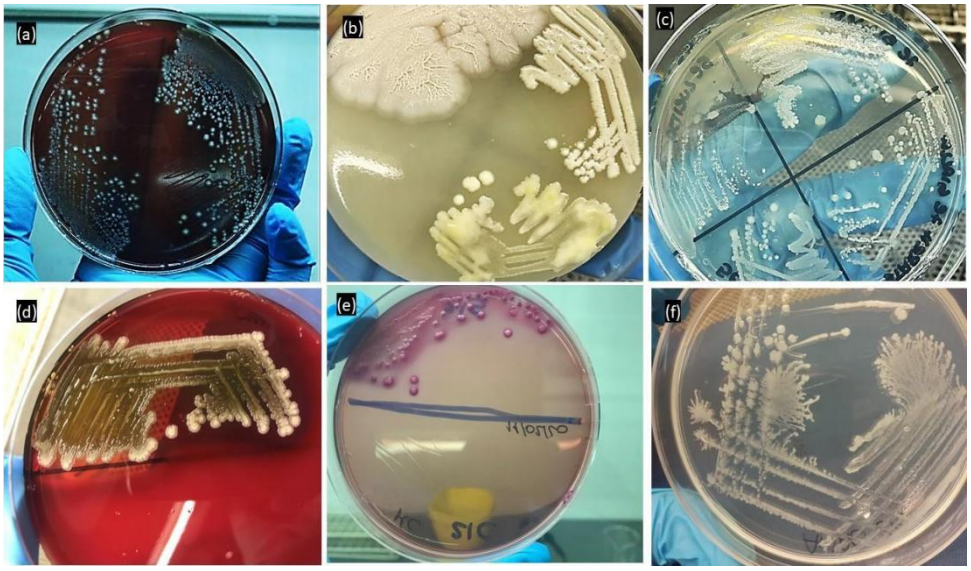


Figure 9: Morphological analysis of colonies isolated from faecal samples of RRMS patients and controls. (a) Blood agar medium with white, glossy, small colonies; (b) Mannitol Soya Flour medium with three different morphologies; (c) Starch-Casein Agar medium with two different morphologies; (d) Blood Agar medium, jagged margin colony with α-hemolysis. (e) MacConkey land; (f) Anacker-Ordal Agar medium.

After morphological analysis, strains were divided for similarity and 76 strains were selected and subjected to Gram staining, oxidase and catalase tests. Seventy colonies were Gram positive and 6 Gram negative.

Colonies were mostly Gram positive bacteria (27/29 isolated from RRMS patients, and 43/47 isolated from control subjects).

Subsequently, the biochemical characteristics of 63 colonies by catalase assay and oxidase assay were evaluated. Data obtained are summarized in Table 4.

Table 4. Data obtained from catalase and oxidase assays performed on 63 colonies isolated from faecal samples of patients affected by RRMS and control subjects

Oxidase/Catalase	N. colonies MS-RR	N. colonies control	Tot.
Oxidase +/Catalase +	4	7	11
Oxidase +/Catalase -	-	-	-
Oxidase -/Catalase +	7	17	24
Oxidase -/Catalase -	16	12	28

Bacterial identification by 16S rDNA sequencing

To confirm microbiological results, 46 samples, selected on the basis of their morphology, were subjected to PCR of the 16S rDNA gene, followed by sequencing and sequence analysis.
DNA extraction from pure colonies was carried out to amplify the 16S rDNA gene by PCR, and amplicons of approximately 1.5 kb were visualized on a 1% agarose gel (Figure 10).

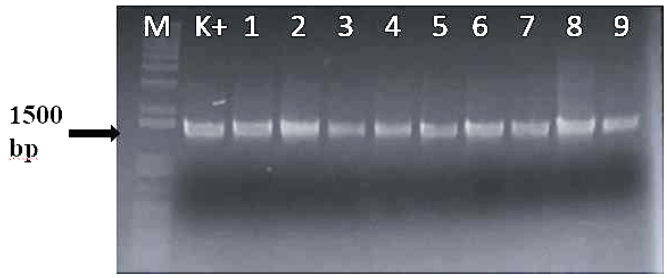


Figure 10: Electrophoretic analysis of 16S rDNA amplification products from RRMS patients and control subjects on 1% agarose gel; (M= marker 1kb plus-Invitrogen; K+= positive control; 2 to 9 fecal samples from patients and controls; K-= negative control).

The PCR products were purified, quantified and sent to BMR Genomics for Sanger sequencing. The analysis of the sequences using the Blast software allowed the identification of 6 bacterial genera: *Enterococcus* spp., *Bacillus* spp., *Pediococcus* spp., *Sutterella* spp., *Clostridium* spp. and *Escherichia*. In particular, 15 *Enterococcus faecium* colonies, 5 *Enterococcus mundii*, 4 colonies of *Bacillus safensis*, 6 colonies of *Bacillus subtilis* and 8 colonies of *Bacillus altitudinis*, 1 *Pediococcus pentaceus*, 1 *Sutterella* spp., 2 *Clostridium perfringens*, 4 colonies

of *Escherichia coli* were identified. A 97-99% sequence identity was observed, except for *Bacillus safensis* and *Sutterella* (95%).

The data obtained were grouped in Table 5.

Table 5: Bacterial species identified by 16S rDNA sequencing in stool samples of RRMS patients and control subjects.

Bacteria species	N. colonies RRMS	N. colonies control
E. faecium	7	8
E. mundtii	2	3
B. safensis	1	3
B. subtilis	3	3
B. altitudinis	3	5
P. pentaceus	0	1
Sutterella	0	1
C. perfringens	2	0
E. coli	2	2

Microbiota analysis

The microbiota study was conducted on stool samples of RRMS patients and control subjects. The DNA was used to amplify an approximately 500 bp fragment of 16S rDNA (Figure 11).

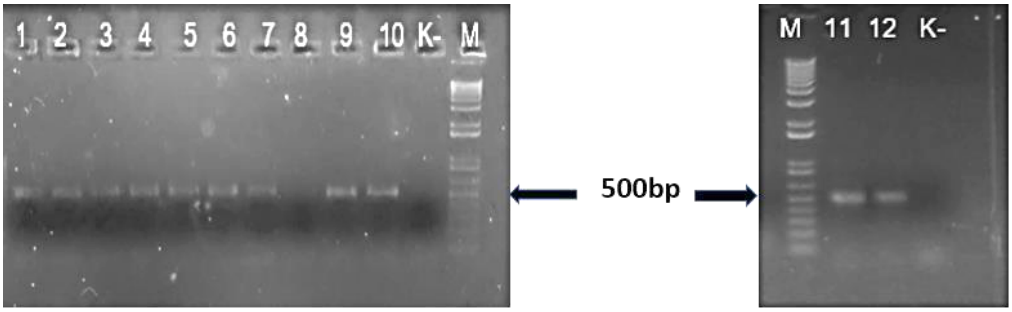


Figure 11: Visualization of metagenomic DNA extracted from faecal samples of RRMS patients and control subjects on 1.5% agarose gel. (M= marker 1Kb plus-Invitrogen; K-= negative control; 1 to 12 samples).

Obtained amplicons were sequenced at BMR Genomics srl. The bioinformatic analysis allowed to assign OTUs to sequences, and to study the diversity indices.

Bacterial communities' diversity

Microbiota analysis of faecal samples of 15 MS patients and 15 controls showed that MS samples (hereafter indicated with number-S) contained between 105 and 283 OTUs for a total of 5510 corresponding to 13 bacterial phyla, 20 classes, 39 orders and 62 families. Control samples (indicated with number-C) contained between 82 and 384 OTUs for a total of 5600 that allowed to identify 15 phyla, 25 classes, 54 orders and 87 families. Good's coverage, used to estimate the completeness of sampling, showed a high level (0.99–1.00) in the identification of bacterial groups.

The analysis of various diversity indices, i.e. the abundance - based richness estimators (Chao1 and ACE) did not reveal significant differences between the two groups, indicating good diversity. Bacterial diversity estimated by the Shannon-Wiener index varied from 2.13 to 2.80 in S samples, and 2.03 to 3.13 in C samples indicating similar diversity values between the two studied groups. Simpson index (0.09 - 0.21 in S, 0.07 - 0.20 in C) and evenness (0.65 - 0.81 in S, 0.68 - 0.85 in C) revealed no significant difference between MS and control subjects (Table 6).

Table 6: Diversity indices of the studied samples.

Sample	S	Good's coverage	Chao1	ACE	α	1-D	H'	e
1S	27	1.00	96.87	89.33	6.14	0.14	2.48	0.75
1C	60	0.99	99.39	90.94	5.7	0.08	3.13	0.76
2S	29	0.99	102.45	93.46	8.34	0.13	2.51	0.74
2C	20	1.00	102.76	94.44	5.65	0.14	2.32	0.77
3S	31	1.00	104.16	95.97	3.12	0.09	2.80	0.81
3C	21	0.99	106.54	97.32	10.9	0.18	2.14	0.70
4S	35	0.99	107.76	98.98	7.51	0.13	2.55	0.71
4C	39	1.00	111.43	101.82	5.79	0.10	2.78	0.76
5S	31	0.99	115.32	104.3	7.96	0.16	2.41	0.70
5C	26	1.00	117	105.95	6.53	0.10	2.61	0.80
6S	23	0.99	117.71	106.6	4.56	0.19	2.16	0.69
6C	19	1.00	117.52	107.63	5.31	0.20	2.07	0.70
7S	25	1.00	117.46	108.67	5.28	0.14	2.31	0.71
7C	32	1.00	118.21	109.54	6.40	0.14	2.50	0.72
8S	26	0.99	118.66	110.2	9.03	0.12	2.51	0.77
8C	25	1.00	119.18	111.56	5.92	0.15	2.28	0.70
9S	24	0.99	121.68	113.24	7.48	0.13	2.47	0.77
9C	29	1.00	122.43	114.51	4.51	0.07	2.86	0.85
10S	29	0.99	123.61	115.65	6.55	0.13	2.48	0.73
10C	21	1.00	126.93	118.21	6.61	0.16	2.24	0.73
11S	26	0.99	43.27	41.56	5.73	0.21	2.13	0.65

11C	20	1.00	59.09	50.84	6	0.20	2.03	0.68
12S	31	0.99	127.94	119.14	8.58	0.17	2.42	0.70
12C	35	0.99	128.72	119.86	6.82	0.13	2.61	0.73
13S	29	1.00	68.26	61.7	7.03	0.13	2.49	0.74
13C	28	1.00	73.39	69.46	6.42	0.17	2.28	0.68
14S	29	1.00	80.15	74.66	8.31	0.20	2.21	0.65
14C	21	0.99	82.23	78.6	3.85	0.17	2.17	0.71
15S	32	0.99	86.94	84.29	8.81	0.12	2.59	0.74
15C	29	1.00	91.01	85.36	7	0.14	2.45	0.72

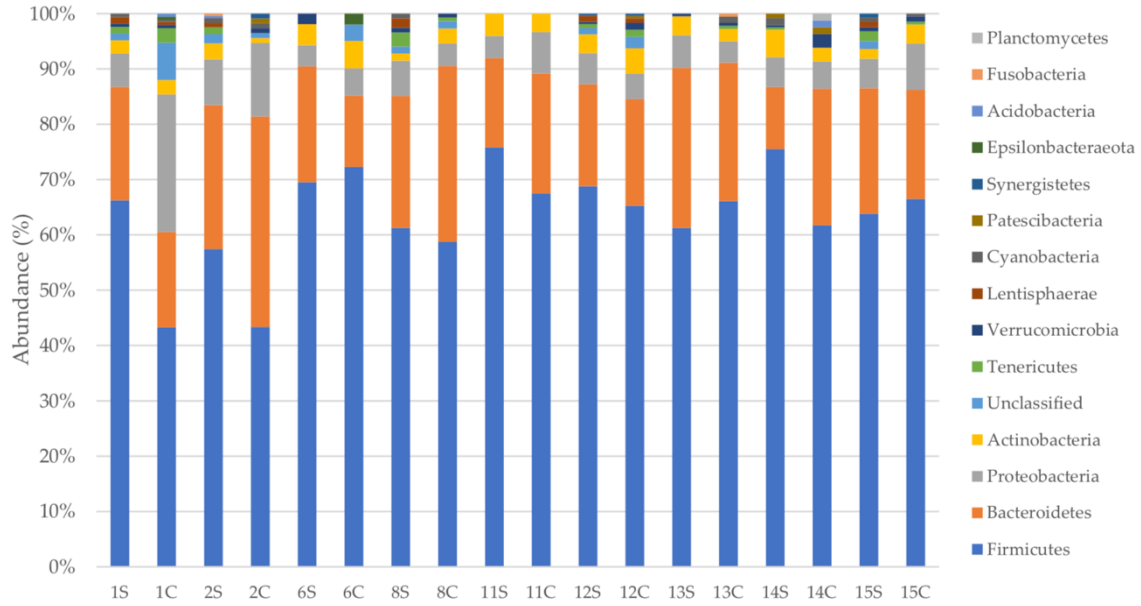
S: total number of families; Chao1 and ACE: abundance-based richness estimators; α: alpha diversity; 1-D: Simpson's index; H': Shannon-Wiener diversity; e: evenness. White and grey lines indicate RRMS patients' and controls' indices, respectively. Patients are indicated as number-S, controls as number-C.

Faecal bacterial communities' taxonomic composition

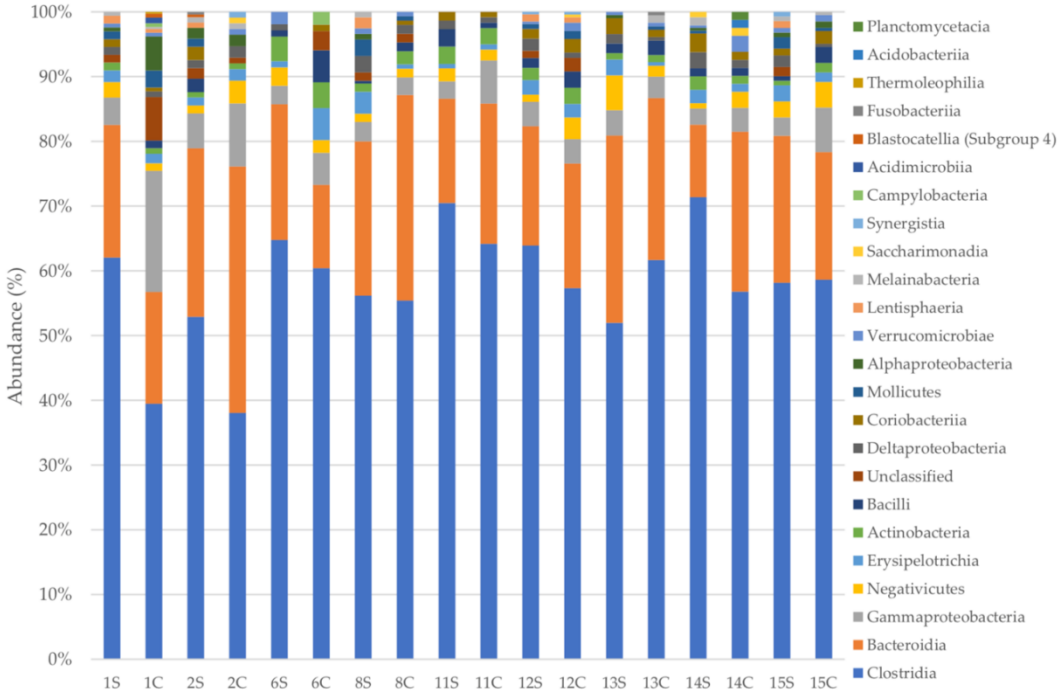
Taxonomic analysis was performed for phyla, orders, classes and families and the results are presented on the basis of the EDSS (1–4.5 and 5–7) of MS patients compared to the household relatives (Figures 12 and 13). Since only a PPMS patient was recruited, it was excluded from the study.

In particular, taxonomic classification revealed that the most dominant phylum in stool samples of MS patients was Firmicutes with an average relative abundance of 65.6% ± 6 followed by Bacteroidetes (22% ± 5), Proteobacteria (5.5% ± 1.4), and Actinobacteria (3% ± 1.1). Data from control samples revealed a lower mean abundance of Firmicutes (59% ± 8.7) and a higher mean percentage of Bacteroidetes (25.3% ± 7) and Proteobacteria (8.4% ± 5.6). Regarding Actinobacteria, no significant difference (3% ± 1.2 in controls) was registered. Minor phyla, such as Tenericutes and Lentisphaerae, were more present in MS patients (0.9% ± 1.09 and 0.6% ± 0.61, respectively) than control subjects (0.43% ± 0.7 and 0.1% ± 0.26, respectively).

(**A**)


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Abundance (%)
1S 1C 2S 2C 6S 6C 8S 8C 11S 11C 12S 12C 13S 13C 14S 14C 15S 15C
Planctomycetes
Fusobacteria
Acidobacteria
Epsilonbacteraeota
Synergistetes
Patescibacteria
Cyanobacteria
Lentisphaerae
Verrucomicrobia
Tenericutes
Unclassified
Actinobacteria
Proteobacteria
Bacteroidetes
Firmicutes

(**B**)


100%
90%
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60%
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40%
30%
20%
10%
0%
Abundance (%)
1S 1C 2S 2C 6S 6C 8S 8C 11S 11C 12S 12C 13S 13C 14S 14C 15S 15C
Planctomycetacia
Acidobacteriia
Thermoleophilia
Fusobacteriia
Blastocatellia (Subgroup 4)
Acidimicrobiia
Campylobacteria
Synergistia
Saccharimonadia
Melainabacteria
Lentisphaeria
Verrucomicrobiae
Alphaproteobacteria
Mollicutes
Coriobacteriia
Deltaproteobacteria
Unclassified
Bacilli
Actinobacteria
Erysipelotrichia
Negativicutes
Gammaproteobacteria
Bacteroidia
Clostridia

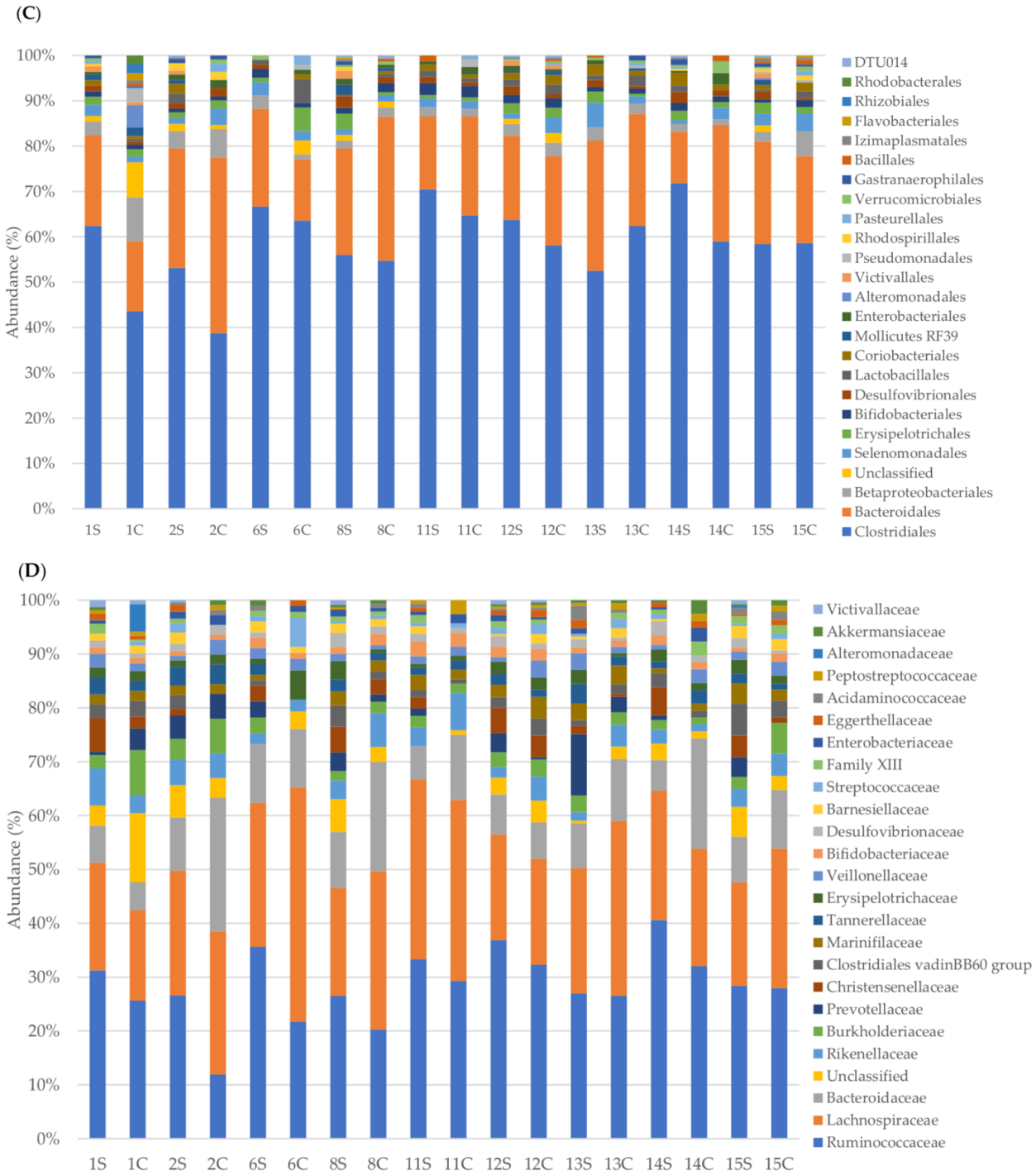
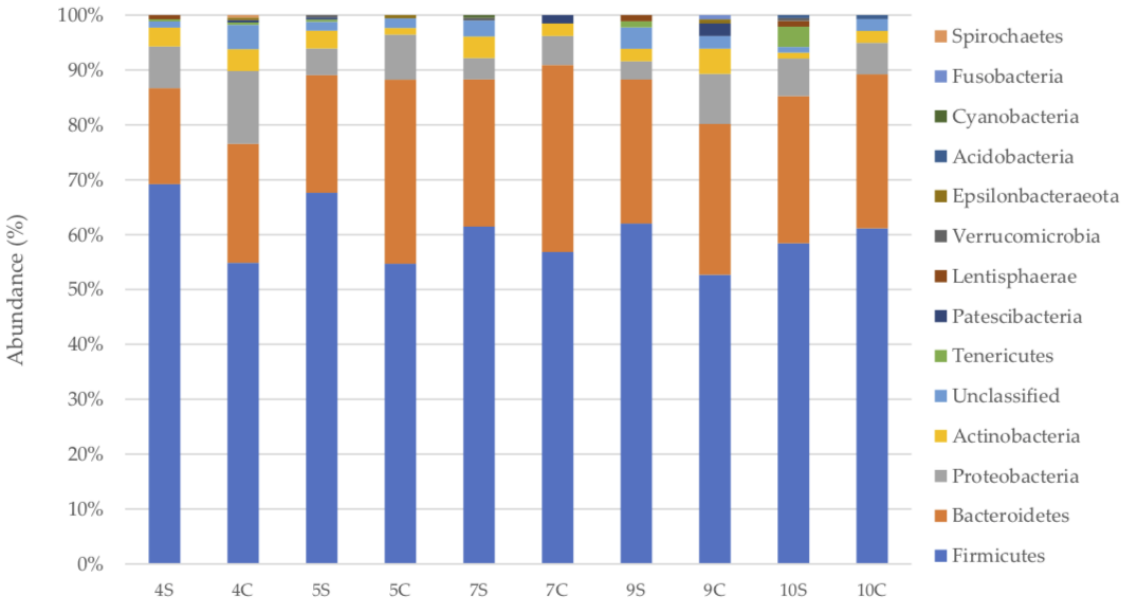
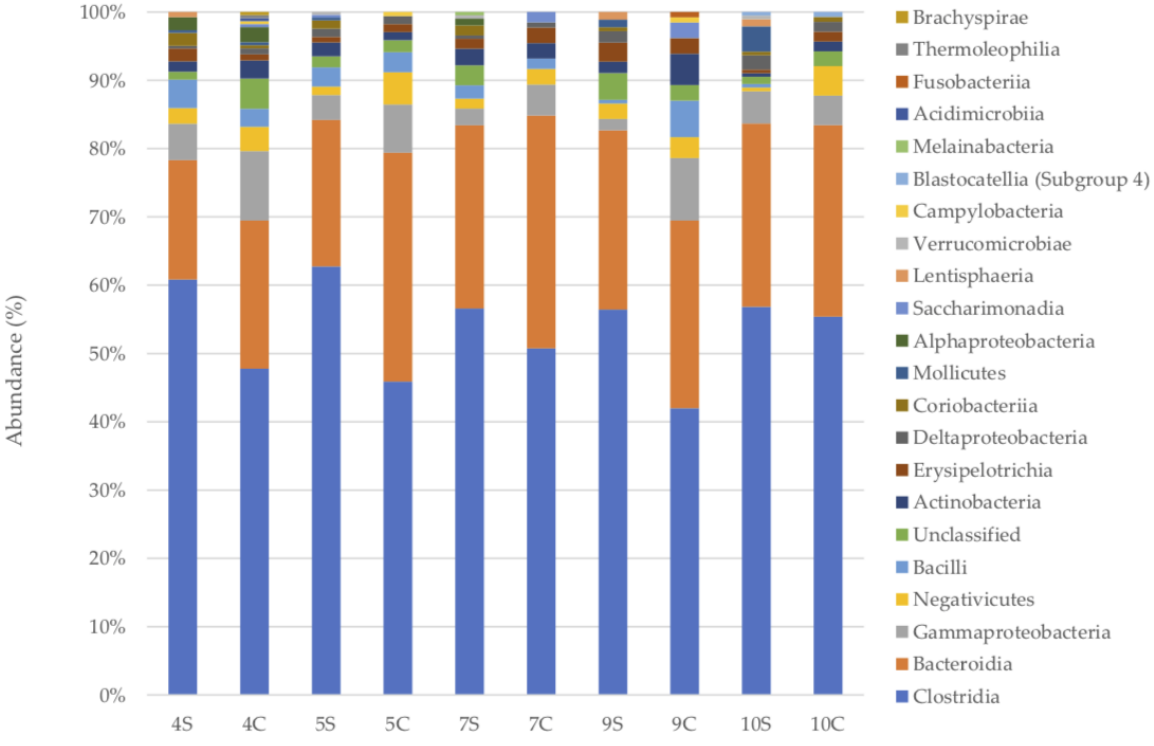


Figure 12: Relative abundance (%) of faecal bacterial communities in MS patients with EDSS 1–4.5 and controls. Bacterial communities were studied at the phyla (A), classes (B), orders (C), and families (D) levels.

(A)


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Abundance (%)
4S
4C
5S
5C
7S
7C
9S
9C
10S
10C
Spirochaetes
Fusobacteria
Cyanobacteria
Acidobacteria
Epsilonbacteraeota
Verrucomicrobia
Lentisphaerae
Patescibacteria
Tenericutes
Unclassified
Actinobacteria
Proteobacteria
Bacteroidetes
Firmicutes

(B)


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Abundance (%)
4S
4C
5S
5C
7S
7C
9S
9C
10S
10C
Brachyspirae
Thermoleophilia
Fusobacteriia
Acidimicrobiia
Melainabacteria
Blastocatellia (Subgroup 4)
Campylobacteria
Verrucomicrobiae
Lentisphaeria
Saccharimonadia
Alphaproteobacteria
Mollicutes
Coriobacteriia
Deltaproteobacteria
Erysipelotrichia
Actinobacteria
Unclassified
Bacilli
Negativicutes
Gammaproteobacteria
Bacteroidia
Clostridia

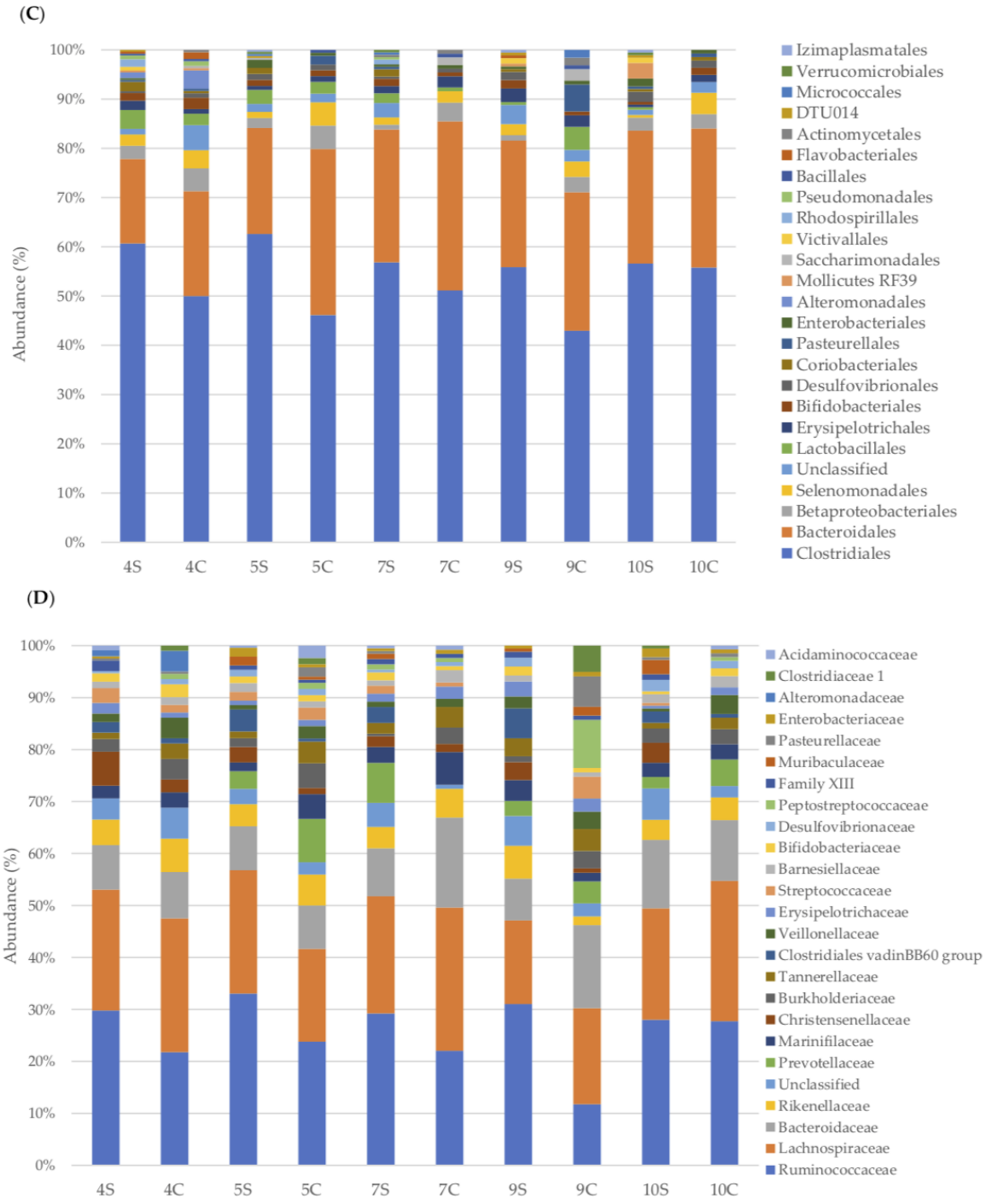


Figure 13: Relative abundance (%) of faecal bacterial communities in MS patients with EDSS 5–7 and controls. Bacterial communities were studied at the phyla (A), classes (B), orders (C), and families (D) levels.

Taxonomic classification of bacterial families revealed that the most dominant family in faecal samples of MS patients was *Ruminococcaceae* (30% ± 4), followed by *Lachnospiraceae* (22% ± 3.8), *Bacteroidaceae* (8.3% ± 1.9) and *Rikenellaceae* (3.6% ± 21.5). Control subjects revealed a lower mean abundance of *Ruminococcaceae* (22.5% ±

6.2) and a higher mean percentage of *Lachnospiraceae* (24.7% ± 7), *Bacteroidaceae* (12.5% ± 5.6) and *Rikenellaceae* (4.1% ± 1.7). Bacteria belonging to other families (*Christensenellaceae, Clostridiales, Prevotellaceae* and so on) were minor components and were not found in all samples. The relative abundance of specific bacterial families in MS patients compared to the healthy relatives is reported in Figure 14, Figure 15 and Figure 16 according to EDSS values (Tables 7, 8 and 9).

Table 7: Relative abundance (%) of family-specific 16S rRNA gene amplicon sequences in EDSS 1-4.5 group. The rows with statistically significant differences (p≤0.05) are indicated with *.

Family	**MS**	**C**	***p-value***
Ruminococcaceae	30.55	23.65	0.019*
Bacteroidaceae	7.93	12.96	0.004*
Burkholderiaceae	2.45	3.16	0.403
Prevotellaceae	3.43	1.35	0.093
Christensenellaceae	3.52	1.08	0.004*
Clostridiales vadinBB60 group	2.45	1.48	0.141
Tannerellaceae	2.22	1.60	0.164
Veillonellaceae	1.52	1.90	0.312
Desulfovibrionaceae	1.66	0.85	0.007*
Family XIII	1.02	0.87	0.647
Akkermansiaceae	0.40	0.67	0.350

Table 8: Relative abundance (%) of family-specific 16S rRNA gene amplicon sequences in EDSS 5-7 group. The rows with statistically significant differences (p≤0.05) are indicated with *.

Family	**MS**	**C**	***p-value***
Ruminococcaceae	28.83	20.45	0.020*
Bacteroidaceae	9.06	11.78	0.198
Burkholderiaceae	1.63	3.44	0.007*
Prevotellaceae	3.09	3.42	0.872
Christensenellaceae	3.58	1.13	0.014*
Clostridiales vadinBB60 group	3.31	0.44	0.003*
Tannerellaceae	1.74	3.31	0.026*
Veillonellaceae	1.21	2.81	0.012*
Desulfovibrionaceae	1.17	0.85	0.458
Family XIII	1.17	0.42	0.019*
Akkermansiaceae	0.28	0.00	0.042*

Table 9: Relative abundance (%) of family-specific 16S rRNA gene amplicon sequences in patient with EDSS 7.5. The rows with statistically significant differences (p≤0.05) are indicated with *****.

Family	**MS**	**C**	***p-value***
Ruminococcaceae	9.28	32.75	<0.00001*
Bacteroidaceae	13.40	8.30	0.156
Christensenellaceae	0.00	5.24	0.022*
Tannerellaceae	4.12	3.06	0.625
Burkholderiaceae	3.09	2.18	0.627
Prevotellaceae	1.03	2.18	0.479
Alteromonadaceae	5.15	0.44	0.004*
Clostridiales vadinBB60 group	0.00	1.75	0.190
Pseudomonadaceae	4.12	0.00	0.002*
Desulfovibrionaceae	1.03	0.87	0.891
Bifidobacteriaceae	2.06	0.00	0.029*
Spirosomaceae	2.06	0.00	0.029*

Among *Firmicutes, Ruminococcaceae, Christensenellaceae, Veillonellaceae*, Family XIII and *Clostridiales* vadin BB60 group families showed statistically significant differences among MS patients and their controls. Specifically, *Veillonellaceae* increased in MS controls than MS patients with EDSS 5–7. *Ruminococcaceae* and *Christensenellaceae* were increased in the MS patients with both the EDSS of 1–4.5 and 5–7 in respect to their controls. The differences of mean average of *Veillonellaceae* and Family XIII were significant only for EDSS 5–7 (Figure 14).

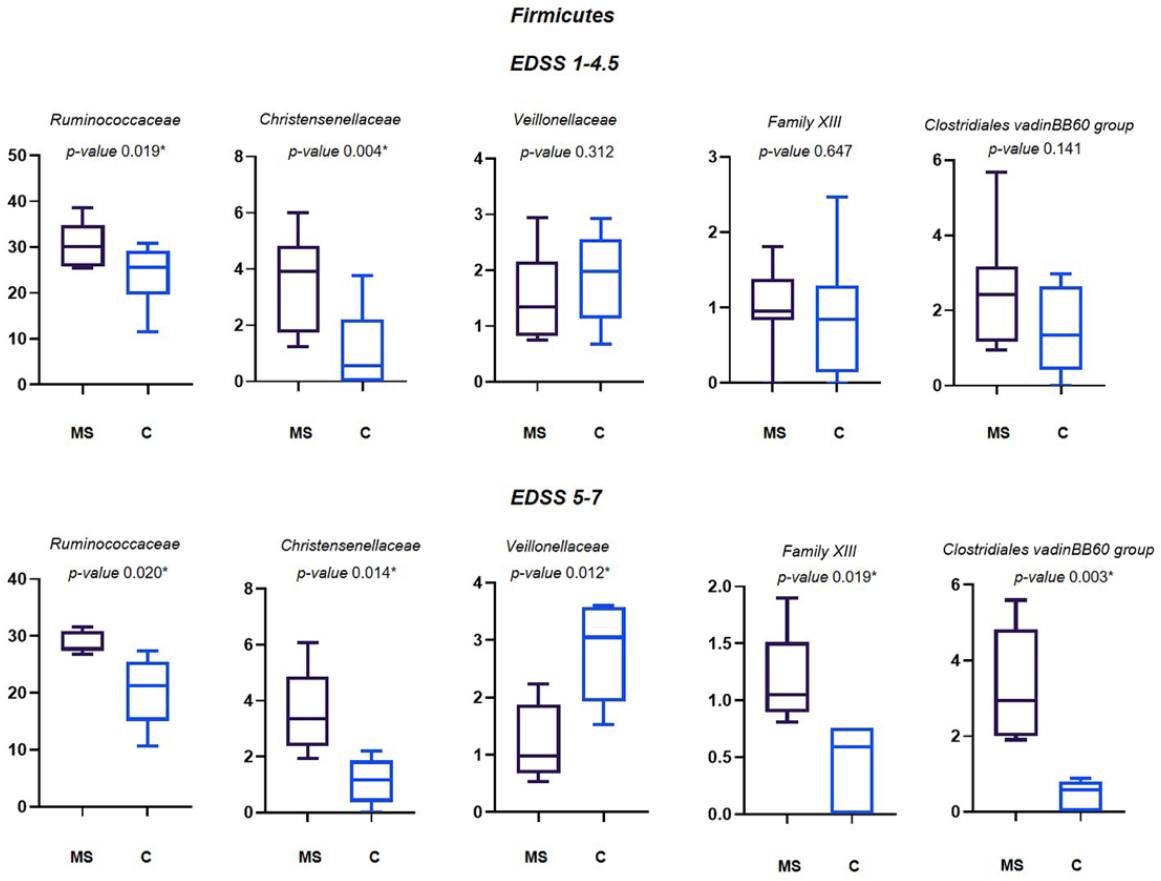


Figure 14: Relative abundance (%) of the main families of Firmicutes differently abundant in MS patients and their controls. Means ± SEM of the relative abundance of Ruminococcaceae, Christensenellaceae, Veillonellaceae, Family XIII and Clostridiales vadin BB60 group families, detected on faecal samples of MS patients with EDSS 1–4.5 and EDSS 5–7, and their relatives. The central line indicates the median value. Families with statistically significant differences ($p \leq 0.05$) are indicated with *.

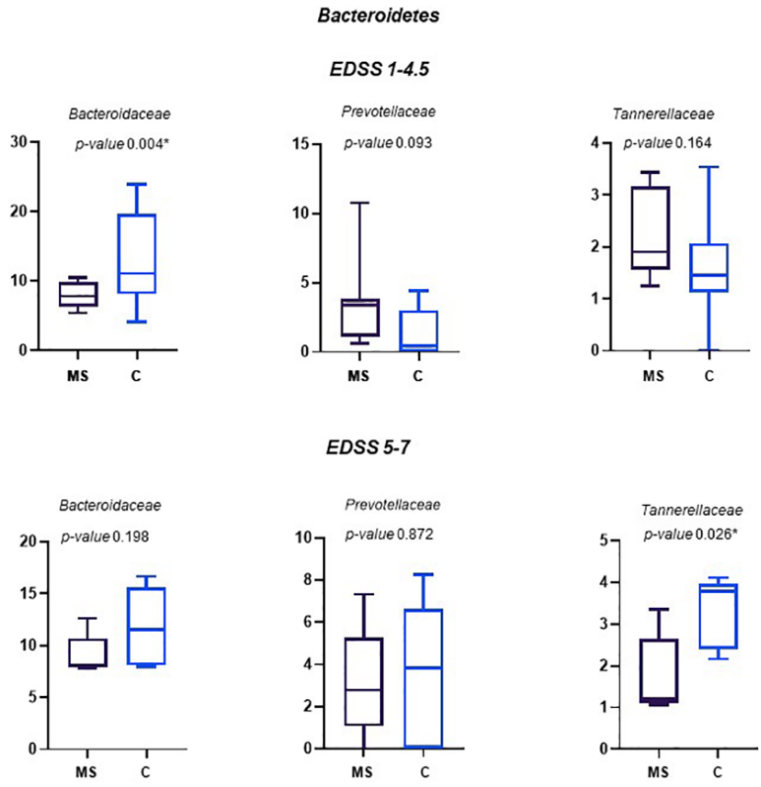


Figure 15: Relative abundance (%) of the main families of Bacteroidetes differently abundant in MS patients and their controls. Means ± SEM of the relative abundance of *Bacteroidaceae, Prevotellaceae and Tannerellaceae* families, detected on faecal samples of MS patients with EDSS 1-–4.5 and EDSS 5–7, and their relatives. The central line indicates the median value. Families with statistically significant differences ($p \leq 0.05$) are indicated with *.

A significant different abundance of *Desulfovibrionaceae* was found for MS patients with EDSS 1–4.5, while *Burkholderiaceae* and *Akkermansiaceae* were significantly different in abundance for MS patients with EDSS 5–7; *Burkholderiaceae* were decreased while *Desulfovibrionaceae* and *Akkermansiaceae* increased in MS patients (Figure 16).

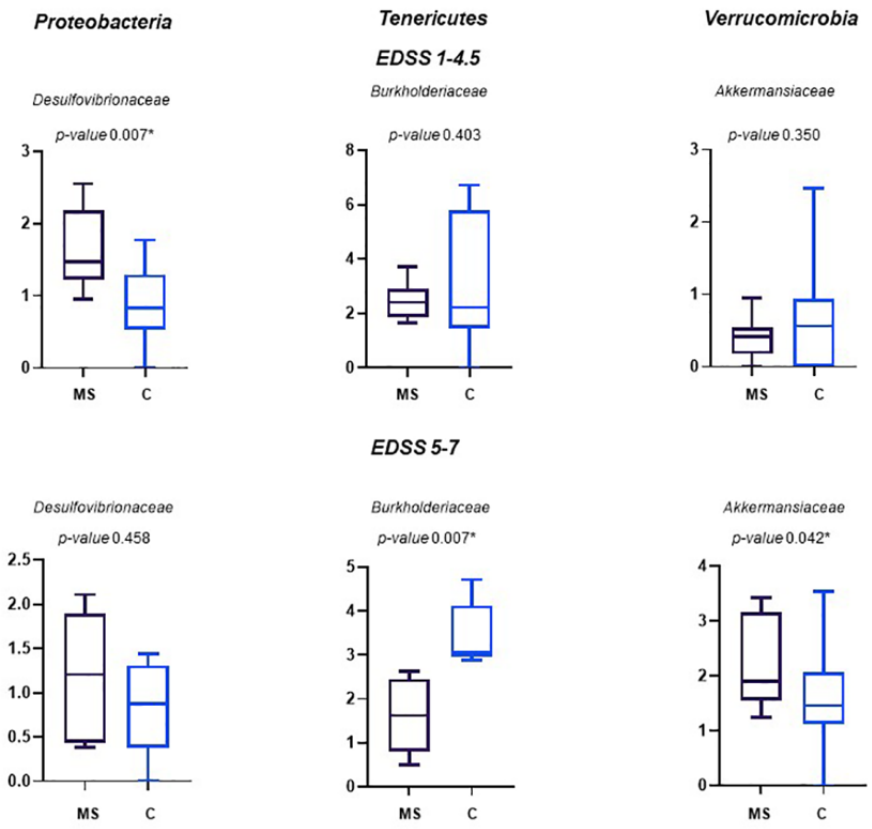


Figure 16: Relative abundance (%) of the main families of *Proteobacteria, Tenericutes* and *Verrucomicrobia* differently abundant in MS patients and their controls. Means ± SEM of the relative abundance of *Desulfovibrionaceae, Burkholderiaceae* and *Akkermansiaceae* families, detected on faecal samples of MS patients with EDSS 1–4.5 and EDSS 5–7, and their relative controls. The central line indicates the median value. Families with statistically significant differences ($p \leq 0.05$) are indicated with *.

As written above, the samples did not display different microbial diversity. Interestingly, when the MS patients and the controls were analysed, the PCoA plot showed that 11 out of the 14 samples collected from MS patients had a more similar microbial composition than the healthy controls (6 out of the 14) (Figure 17). Following this comprehensive analysis, we applied PCoA to the gut microbiome of MS patients with EDSS 1–4.5 and 5–7 and that of their household relatives. Interestingly, we found that all of the MS samples with EDSS 1–4.5 clustered and that most of the controls fell in the same cluster (Figure 17B), while MS samples with EDSS 5–7 formed a distinct cluster from their controls (Figure 17C).

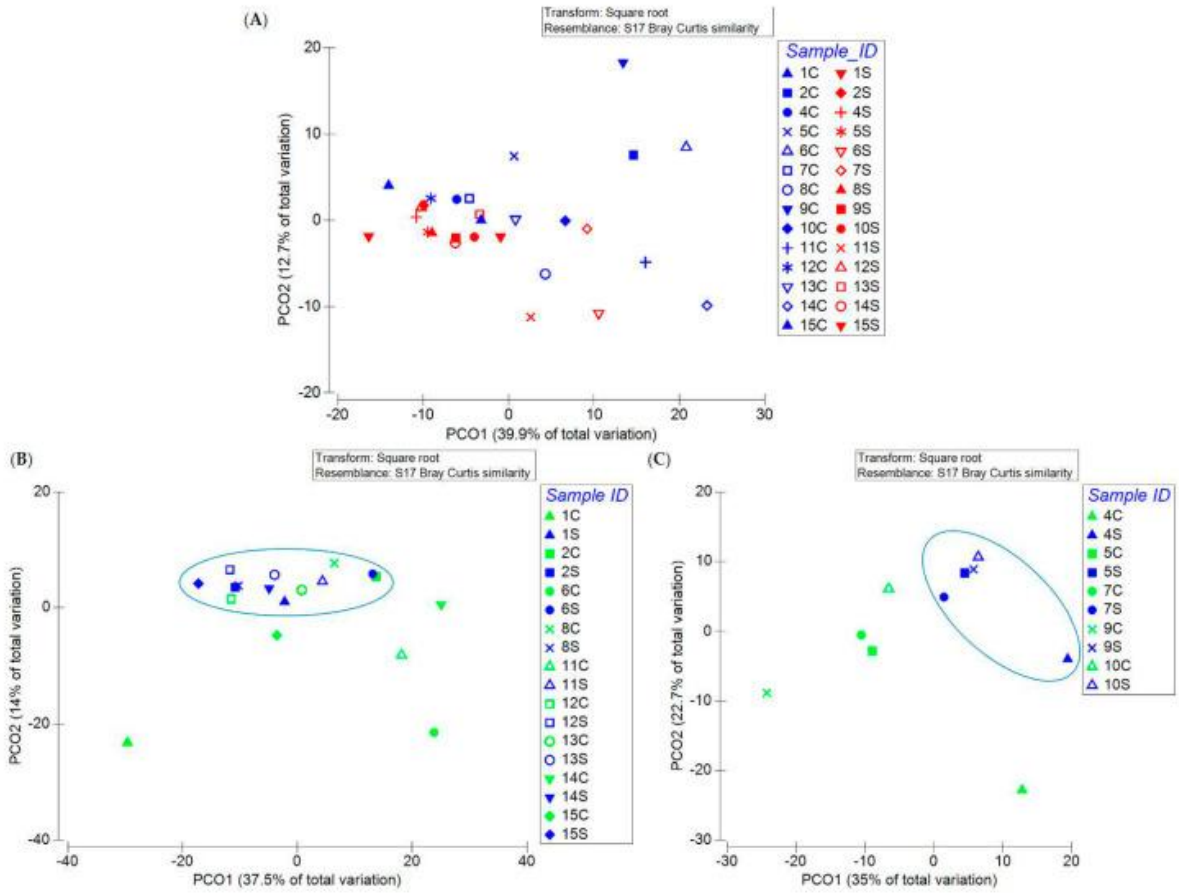


Figure 17: Principle coordinate analysis (PCoA) plot of all the MS patients (A), of MS patients with EDSS 1–4.5 (B) and of MS patients with EDSS 5–7 (C) and their relative controls. Circles indicate the samples forming a cluster.

Phenotypic and metabolic inference

The microbial metabolic profile was analyzed by METAGENassist. All samples contained bacteria with the metabolic potential to oxidize ammonia, to degrade cellulose and xylan, to reduce sulfide and nitrite, and to dehalogenate and fix nitrogen. Conversely, a few samples contained bacteria with atrazine metabolism (1S, 1C, 3S, 4S, 4C, 11C, 13C), able to degrade chitin (5S, 6S, 6C, 7S, 7C), denitrify, degrade lignin and oxidize sulfur (1S, 1C) (Figures 18 and 19). Bacteria able to oxidize ammonia, degrade cellulose and xylan, and dehalogenate were more present in controls than MS subjects. Conversely, bacteria able to reduce sulfates and oxidize sulfides were more present in MS patients.

Data about microbiota study were reported in the manuscript titled “Comparison of the Intestinal Microbiome of Italian Patients with Multiple Sclerosis and Their Household Relatives” published on Life journal (doi: 10.3390/life11070620).

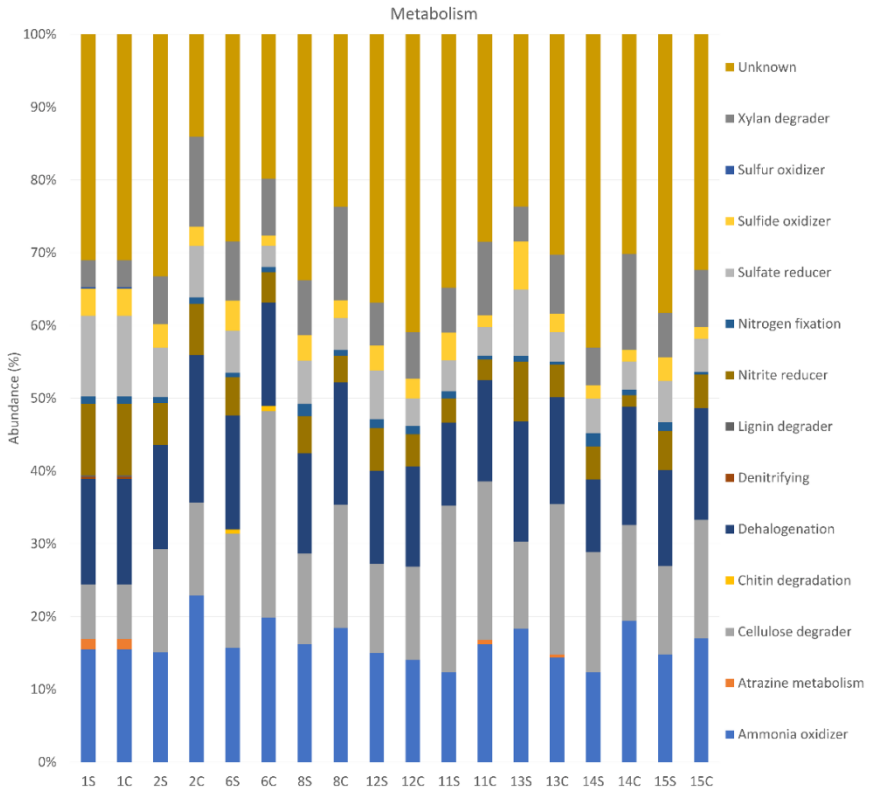


Figure 18: Metabolic activities of microbial community present in MS patients with EDSS 1-4.5 and controls.

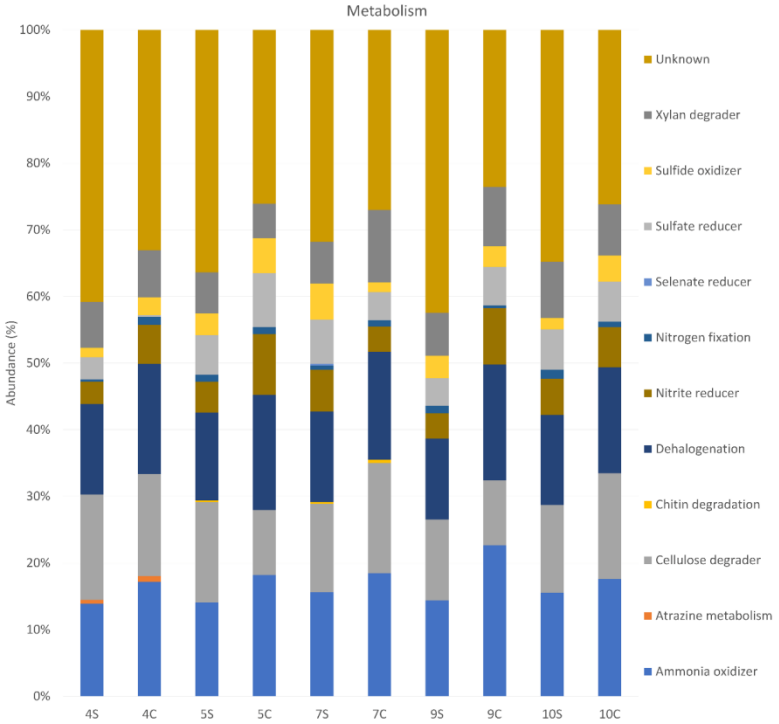


Figure 19: Metabolic activities of microbial community present in MS patients with EDSS 5-7 and controls.

OTUs and ASVs comparison

The comparison between OTUs and ASVs demonstrated that for each sample, the ASVs analysis allowed to assign not only relative abundances of phyla, orders, classes, and families, but also to determine genera and species. It is also evident that the ASVs number for phyla, classes, orders and families is much lower than those found by analyzing OTUs (Table 10).

Table 10: Comparison of ASV and OTU number obtained from the two type of analysis.

	N° ASVs	***N° OTUs***
Phyla	7	15
Classes	15	24
Orders	17	57
Families	24	94

Firmicutes, Bacteroidetes, Proteobacteria and *Actinobacteria* were the four dominant phyla also in ASVs analysis, with *Firmicutes* increased and the other decreased in RRMS patients compared to healthy controls. ASV analysis showed a larger *Verrucomicrobia* relative abundance (2.23% in patients with EDSS 1-4.5 and 1.18% in patients with EDSS5-7) than the OTU analysis (0.59% in patients with EDSS 1-4.5 and 0.28% in patients with EDSS5-7)
At family level, ASV analysis revealed a relative abundance of *Bacteroidaceae* higher than that of *Ruminococcaceae*, contrary to what was observed with OTUs in patients with EDSS 1-4.5 and 5-7. However, *Ruminococcaceae* were more abundant in RRMS patients, and *Bacteroidaceae* more abundant in controls in both the analyses (Table 11).

Table 11: Relative abundance (%) of the most abundant family-specific 16S rRNA gene amplicon sequences in EDSS 1-4.5 group obtained by analyzing OTUs and ASVs. The rows with statistically significant differences ($p \leq 0.05$) are indicated with *.

OTU				**ASV**			
Family	**MS**	**C**	***p-value***	**Family**	**MS**	**C**	***p-value***
Ruminococcaceae	30.55	23.65	0.019*	Ruminococcaceae	19.44	14.63	0.229
Bacteroidaceae	7.93	12.96	0.004*	Bacteroidaceae	17.25	35.28	0.026*

Another difference between the two approaches lies in the significance of families' relative abundances between patients and controls.

In patients with EDSS 1-4.5, the analysis of the OTUs showed that families with a significant difference were *Ruminococcaceae* (*p-value* 0.019), *Bacteroidaceae* (*p-value* 0.004), *Desulfovibrionaceae* (*p-value* 0.007) and *Christensenellaceae* (*p-value* 0.007).
Contrarily, the ASV analysis showed that the families with a significant difference were only *Bacteroidaceae* (*p-value* 0.026) and *Christensenellaceae* (*p-value* 0.048) (Figure 20).

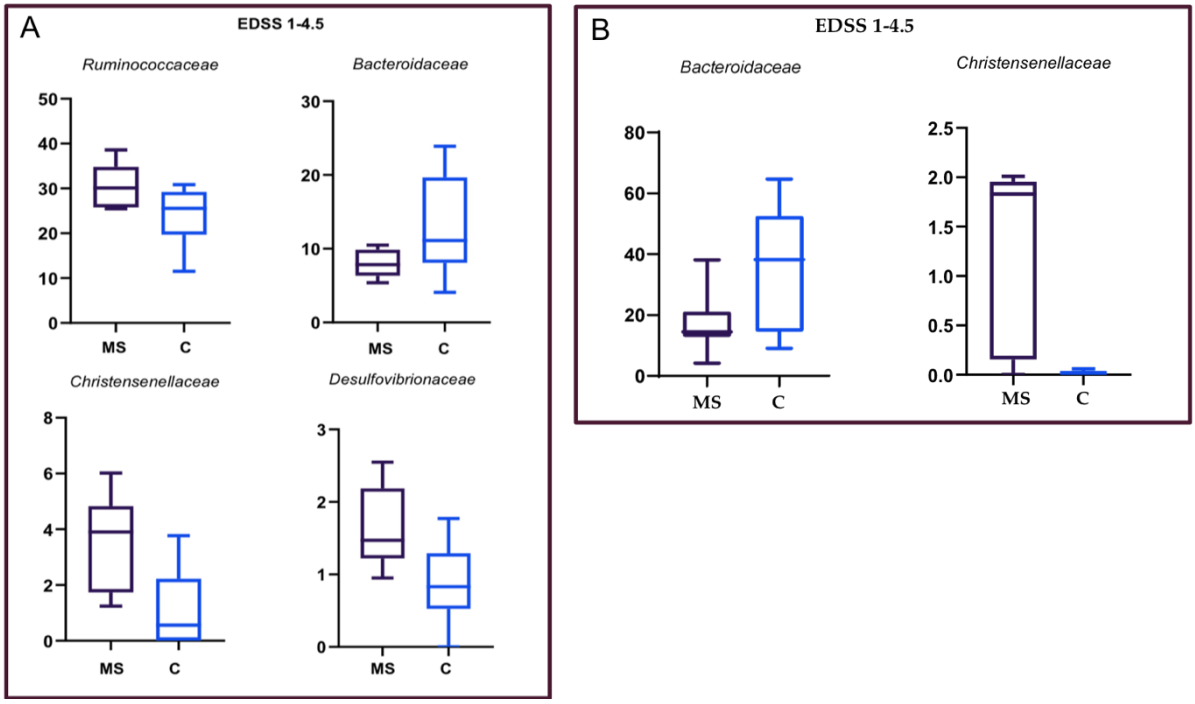


Figure 20: Relative abundance (%) of the families significantly different detected on stool samples of MS patients with EDSS 1-4.5, and their relatives, obtained by analyzing OTUs (A) and ASVs (B). The central line indicates the median value.

In patients with EDSS 5-7, the OTU analysis showed that the families with a significant difference were *Ruminococcaceae* (*p-value* 0.020), *Bulkholderiaceae* (*p-value* 0.007), *Christensenellaceae* (*p-value* 0.014), *Clostridiales* vadinBB60 (*p-value* 0.003), *Tennerellaceae* (*p-value* 0.026), *Veillonellaceae* (*p-value* 0.012), Family XIII (*p-value* 0.019) and *Akkermansiaceae* (*p-value* 0.042).
Contrarily, *Rikenellaceae* (p-value 0.030) and *Christensenellaceae* (p-value 0.025) were significantly different in ASVs analysis (Figure 21). The *Barnesiellaceae* family gave a *p-value* of 0.053.

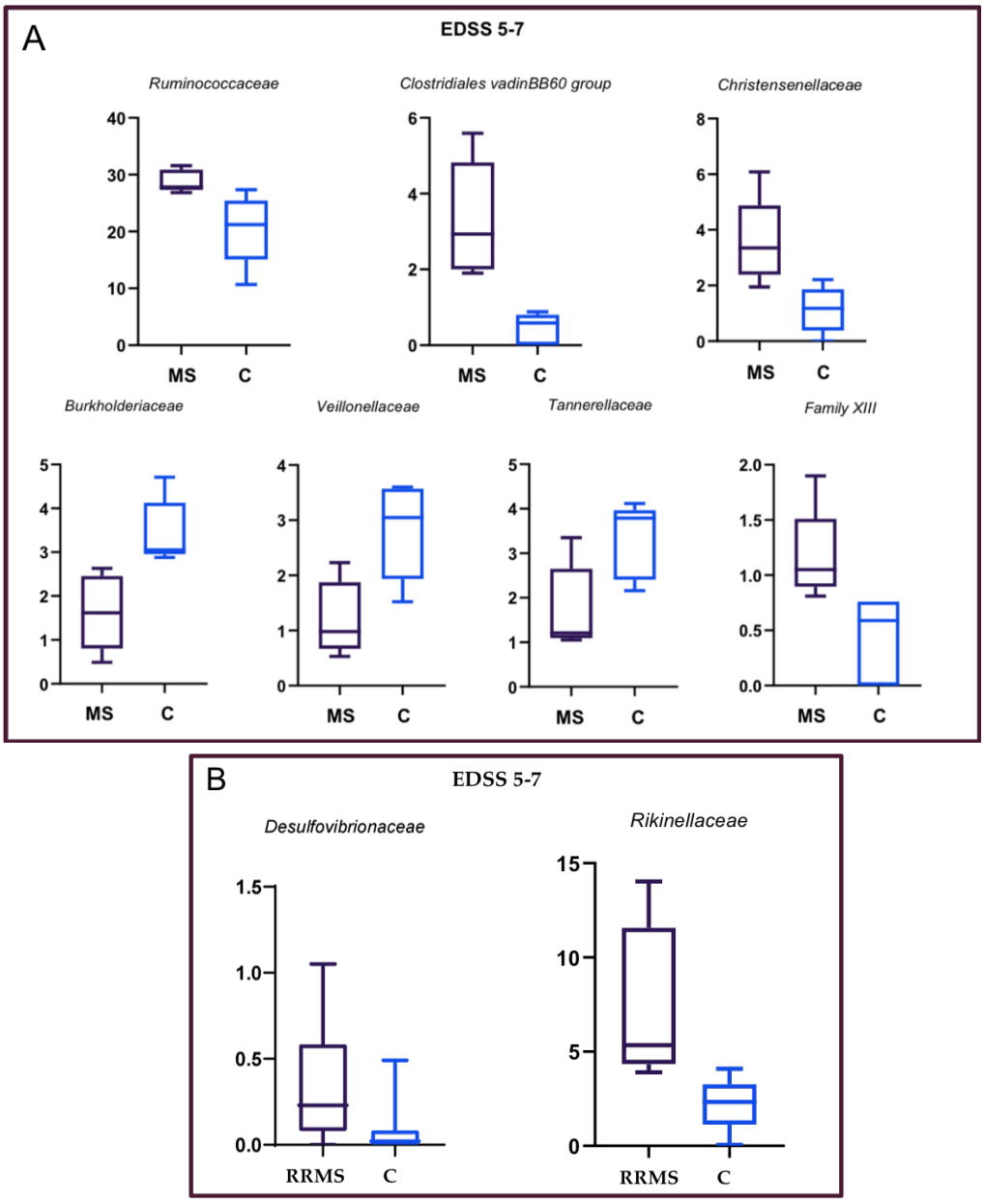


Figure 21: Relative abundance (%) of the families significantly different detected on stool samples of MS patients with EDSS 5-7, and their relatives, obtained by analyzing OTUs (A) and ASVs (B). The central line indicates the median value.

Contrarily to OTUs, the ASV analysis allowed to obtain data also regarding the relative abundances of bacterial genera and species.

In EDSS 1-4.5 patients, two significantly different genera were found between RRMS patients and controls: *Bacteroides* (*p-value* 0.03) more abundant in controls and *Oscillospira* (*p-value* 0.002) in patients.

Regarding EDSS 5-7 patients, three significantly different genera were found between patients and controls: *Prevotella* (*p-value* 0.023) more abundant in controls and *Alistipes* (*p-value* 0.019) and *Gemmiger* (*p-value* 0.032) in patients (Figure 22).

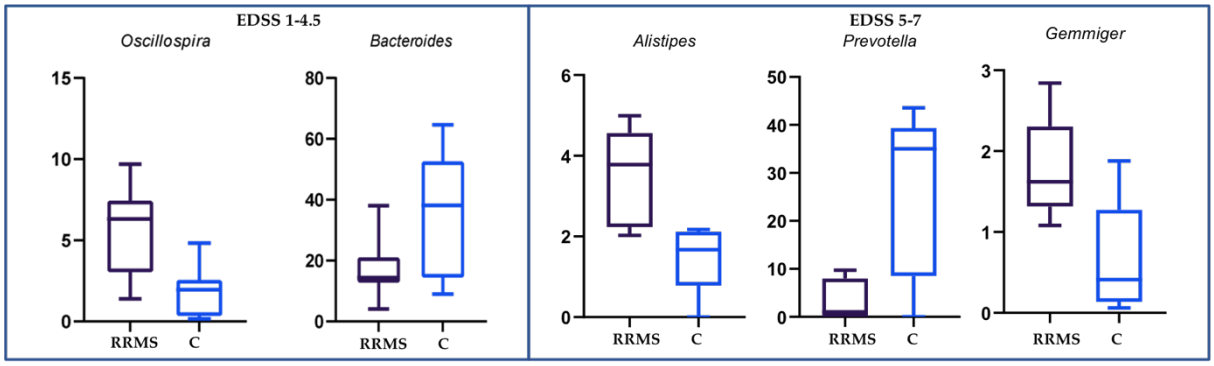


Figure 22: Relative abundance (%) of the genders significantly different detected on stool samples of MS patients with EDSS 1-4.5 and EDSS 5-7, and their controls. The central line indicates the median value.

At species level, in EDSS 1-4.5 patients only *Ruminococcus lactaris* (*p-value* 0.035) was significantly different between RRMS patients and controls. In EDSS 5-7 patients, *Alistipes putredinis* (*p-value* 0.039), *Gemmiger formicilis* (*p-value* 0.033) and *Bacteroides caccae* (*p-value* 0.036) were more significantly abundant in RRMS patients than in controls (Figure 23).

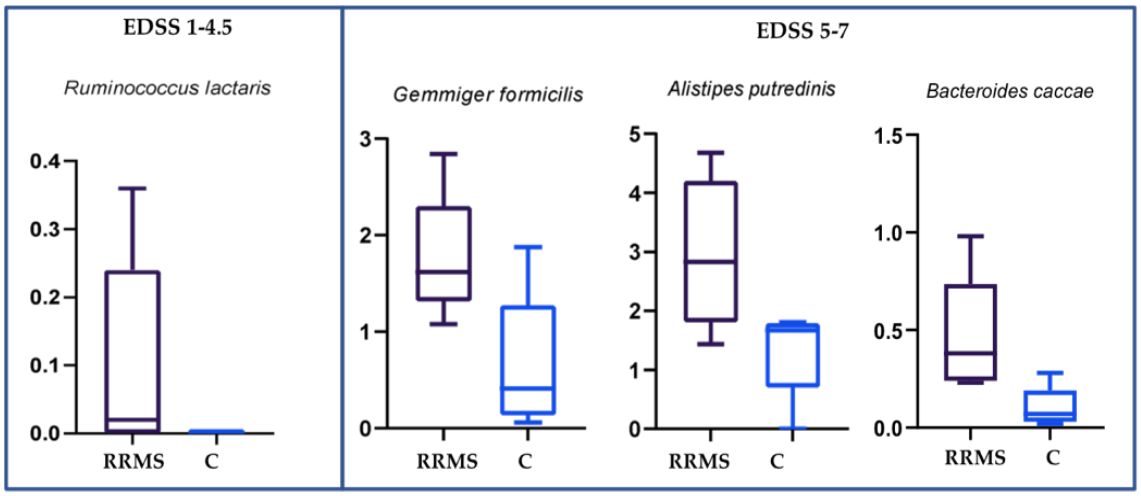


Figure 23: Relative abundance (%) of the species significantly different detected on stool samples of MS patients with EDSS 1-4.5 and EDSS 5-7, and their controls. The central line indicates the median value.

By the comparison between the two approaches used (OTU and ASV) to analyzed the raw sequences, it was possible to observe how the ASV analysis is much more specific than the

OTU and also allows to identify the genera and species involved in the dysbiosis of MS patients. Figure 25 summarizes the results obtained, highlighting that all significantly abundant bacteria in MS patients play an important role in inflammatory processes

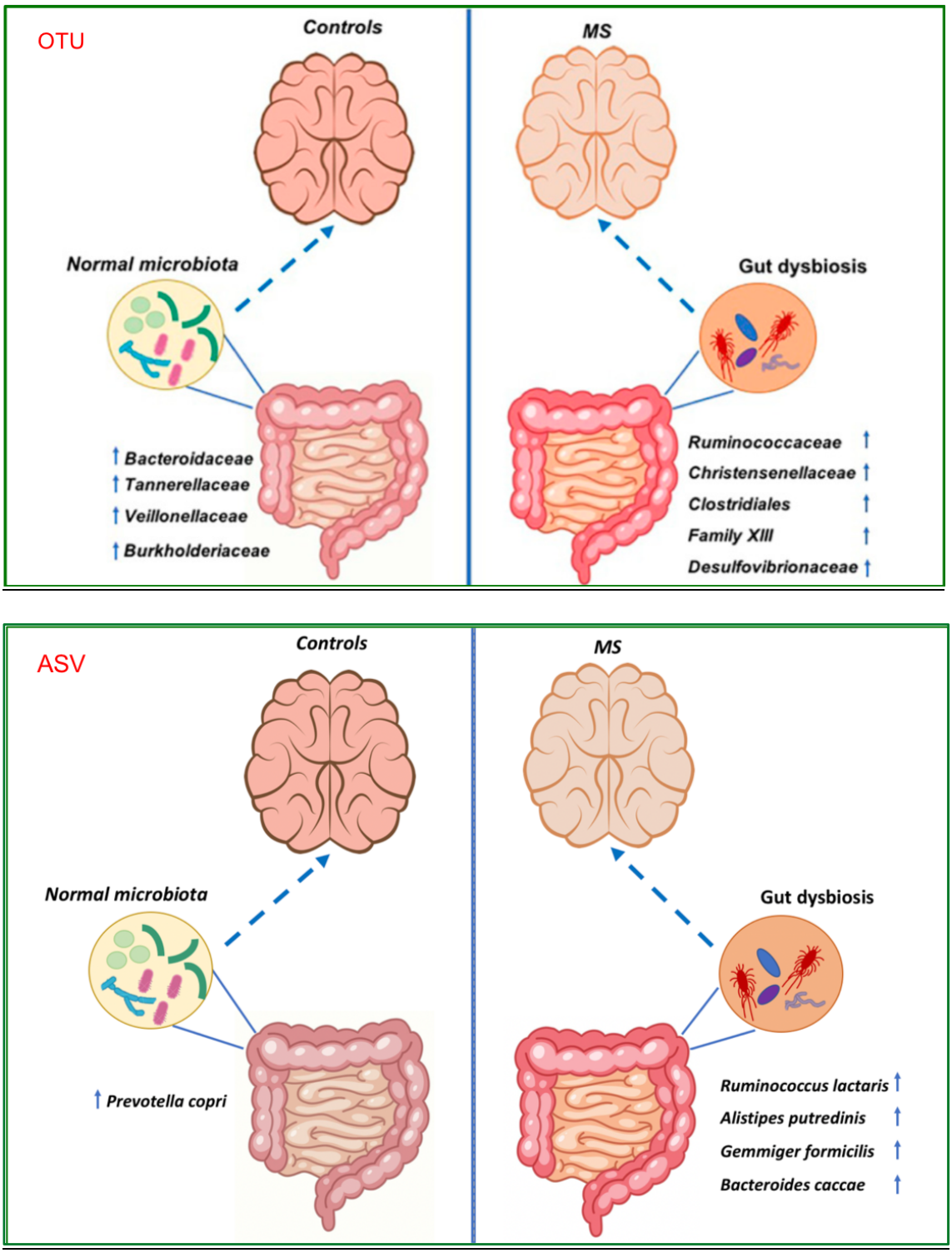


Figure 25: Scheme of bacterial variations results in control subjects and MS patients obtained using OTUs and ASVs analysis.

2. PATHOGENS CORRELATED WITH MULTIPLE SCLEROSIS

Molecular evidence of *Bartonella henselae* in stray cats and MS patients and controls

During my research period, I was involved in a screening of *Bartonella henselae* in stray cats. The manuscript titled "Serological and Molecular Evidence of *Bartonella henselae* in Stray Cats from Southern Italy" was published in the Microorganism journal (doi: 10.3390/microorganisms9050979).

A total of 429 stray cats were sampled (166 male and 263 female). Real-time PCR carried out in the whole blood of all the 429 cats revealed that 148 cats (34.5%) contained *B. henselae* DNA.

Since a great percentage of *B. henselae* positive samples was found in one of the main companion animal, *B. henselae* DNA was searched also in stool samples of MS patients and their controls. All stools samples analyzed did not show the presence of *B. henselae* DNA.

Molecular evidence of *Mycoplasma* in ticks and MS patients and controls

Since different studies reported the presence of antibodies anti-*Mycoplasma* in MS patients, a research of this pathogen was carried out.

One vector that plays an important role in the transmission of pathogens in humans are ticks. Therefore, the presence of *Mycoplsma* was sought in ticks collected from sheep and goats.

The manuscript "Detection of *Mycoplasma agalactiae* in Ticks (*Rhipicephalus bursa*) Collected by Sheep and Goats in Sicily (South-Italy), Endemic Area for Contagious Agalactia" was published in the Microorganism journal (doi: 10.3390/microorganisms9112312)

A total of 152 ticks were collected from 25 animals from three different farms to investigate the diffusion of the *Mycoplasma*. The real-time PCR analysis using the VetMAX^{TM} *M. agalactiae* and *M. mycoides* kit revealed 17 *M. agalactiae* positive pools (30.36%). A further two (3.6%) pools were positive to *M. mycoides,* while eight pools were positive to both pathogens (14.3%). Real-time PCR performed on the recovered isolates from the five *Mycoplasma* spp. positive tick pools confirmed *M. agalactiae* in each pool.

Since for the first time *M. agalactiae* and *M. mycoides* DNA was found in ticks, the same pathogens were searched also in stool samples of MS patients and their relatives' control.

All stools samples analyzed did not show the presence of *M. agalactiae* and *M. mycoides* DNA.

3. BIOLOGICAL ACTIVITIES OF *HELLEBORUS BOCCONEI* PLANT

Preparation of *Helleborus bocconei* extracts

From the extraction of 35 g of roots in methanol 10 g of oil were obtained.

The methanol extract (660 mg) was further divided into two fractions:

1. water fraction rich in carbohydrates (332.1 mg)
2. butanolic fraction (254 mg) separated by HPLC into six fractions (F1, F2, F3, F4, F5 and F6)

Chemical characterization showed that the F1 (7.1 mg), F2 (0.1 mg) and F3 (14.3 mg) fractions contain fatty acids, the F5 (26.9 mg) fraction, which is the most represented part, contains helleborosides A and B and F6 (6.4 mg) fraction contains mix ecdysteroids and traces of hellebrin (Figure 26). The F4 fraction (21.5 mg) was not characterized.

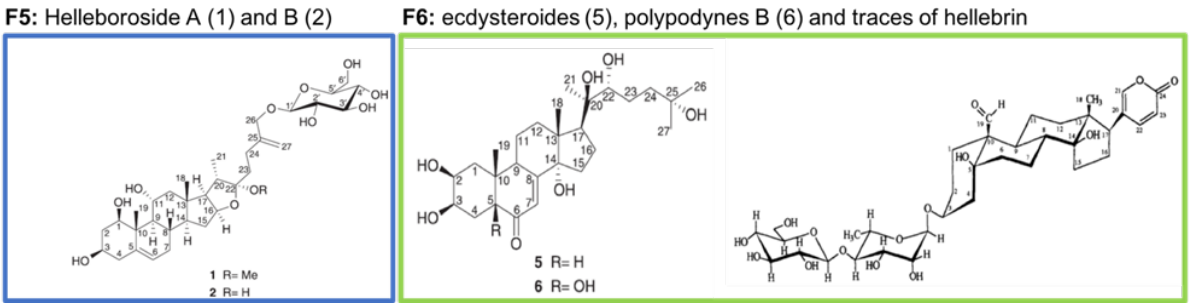


Figure 26: Structures of the most representative molecules in *H. bocconei* methanol extract

Helleborus extracts as bacterial growth modulators

All three *Helleborus bocconei* extracts (methanol, butanol and water) were tested on bacteria isolated from feces of MS controls and on pathogen strains. This study aimed to understand whether *Helleborus* extracts can promote or inhibit the growth of some key bacteria in the regulation of gut dysbiosis in MS patients.

Enterococcus faecium, *Bacillus safensis*, *Pediococcus pentaceus*, *Sutterella* spp. isolated from MS controls maintained their viability at concentrations below 16 mg/ml.

This data indicates that *Helleborus* does not modify the viability of bacteria useful in the modulation of inflammatory states characteristic in MS.

3200 µg/ml of methanol extract and 1600 µg/ml of butanol extract inhibited *M. capricolum* growth (Figure 27).

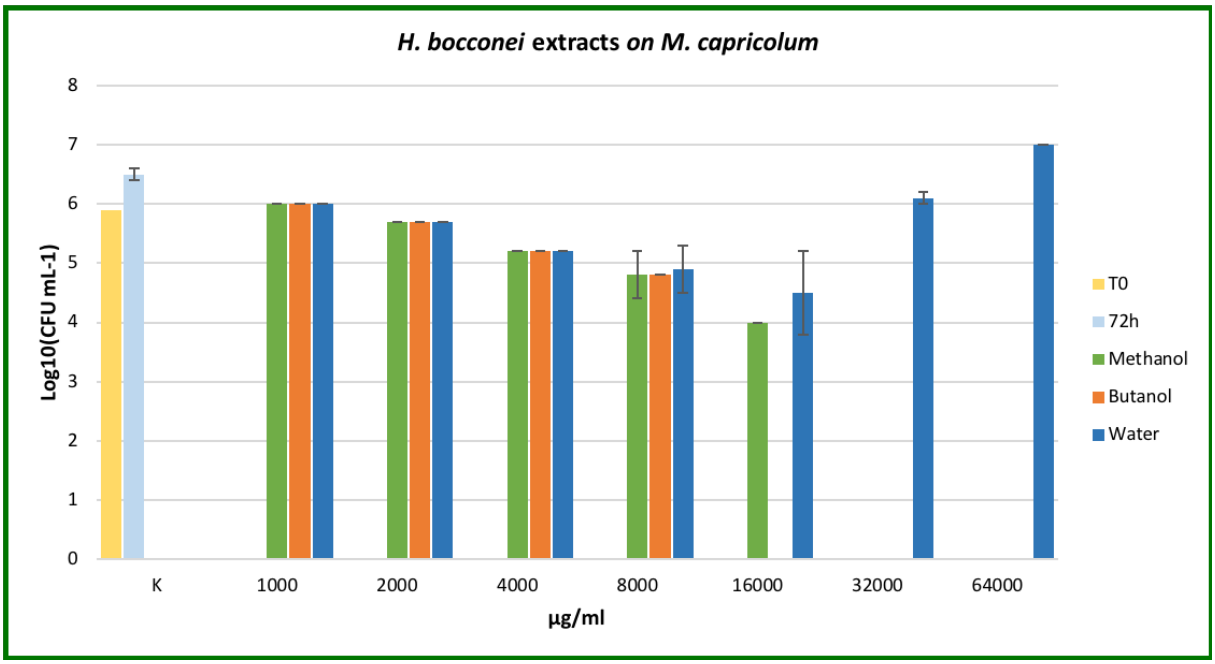


Figure 27: Antibacterial activity of *H. bocconei* extracts against *M. capricolum*. Different colors represented different treatments: yellow (*M. capricolum* untreated at T0), heavenly (*M. capricolum* untreated after 72h), green (*M. capricolum* treated with methanol extract), orange (*M. capricolum* treated with butanol fraction) and blue (*M. capricolum* treated with water fraction).

No one of the *H. bocconei* extracts tested (until 80.000 µg/ml) inhibit bacterial growth of the tested Gram-negative bacteria.

Since the butanol extract seemed to have a higher antibacterial activity than the other two extracts, as showed on *M. capricolum*, it was used to carry out tests to understand if it could have an anti-biofilm action against three Gram negative bacteria: *S. enteritidis* ATCC 13076 (weak biofilm producer), *E. coli* ATCC 25922 (moderate biofilm producer) and *P. aeruginosa* ATCC 10145 (strong biofilm producer). The highest concentration tested, 125 µg/ml, showed a 50% and a 30% reduction in the ability to create biofilms of *S. enteritidis and P. aeruginosa,* respectively. Regarding the other two pathogens, no substantial decrease in their ability to create biofilm was observed (Figure 28).

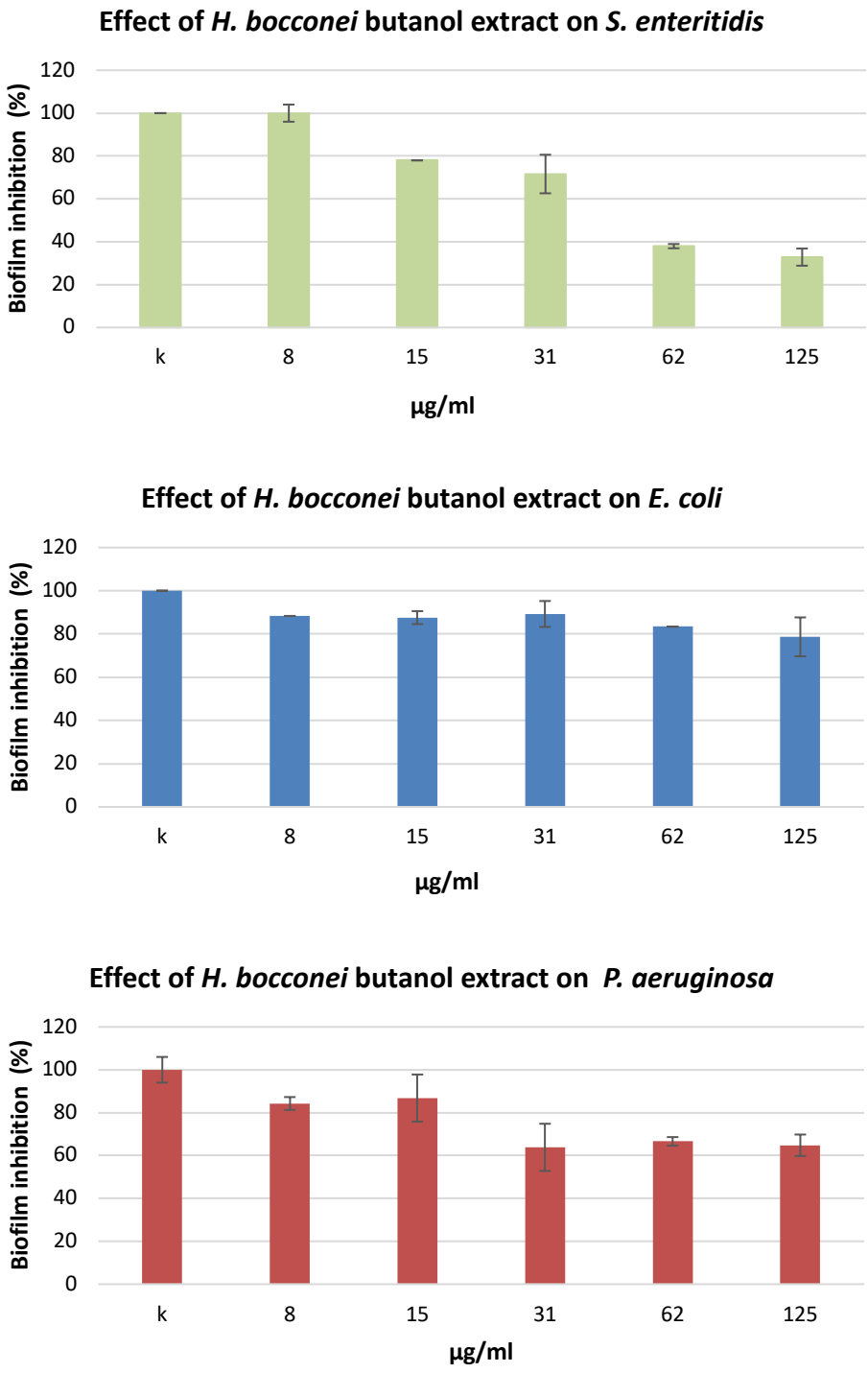


Figure 29: Antibiofilm activity of butanol *H. bocconei* extract against reference Gram negative strains: in green *S. enteritidis* ATCC 13076 (weak biofilm producer), in blue *E. coli* ATCC 25922 (moderate biofilm producer) and in red *P. aeruginosa* ATCC 10145 (strong biofilm producer).

H. bocconei extracts tested (until 80.000 µg/ml) did not inhibit b growth of the Gram positive bacteria, except Streptococci strains. In fact, butanol and methanol extracts showed an antibacterial activity against reference Streptococci from a concentration of 125 µg/ml (Figure 29 and Figure 30), while water extract had no antibacterial activity (Figure 31).

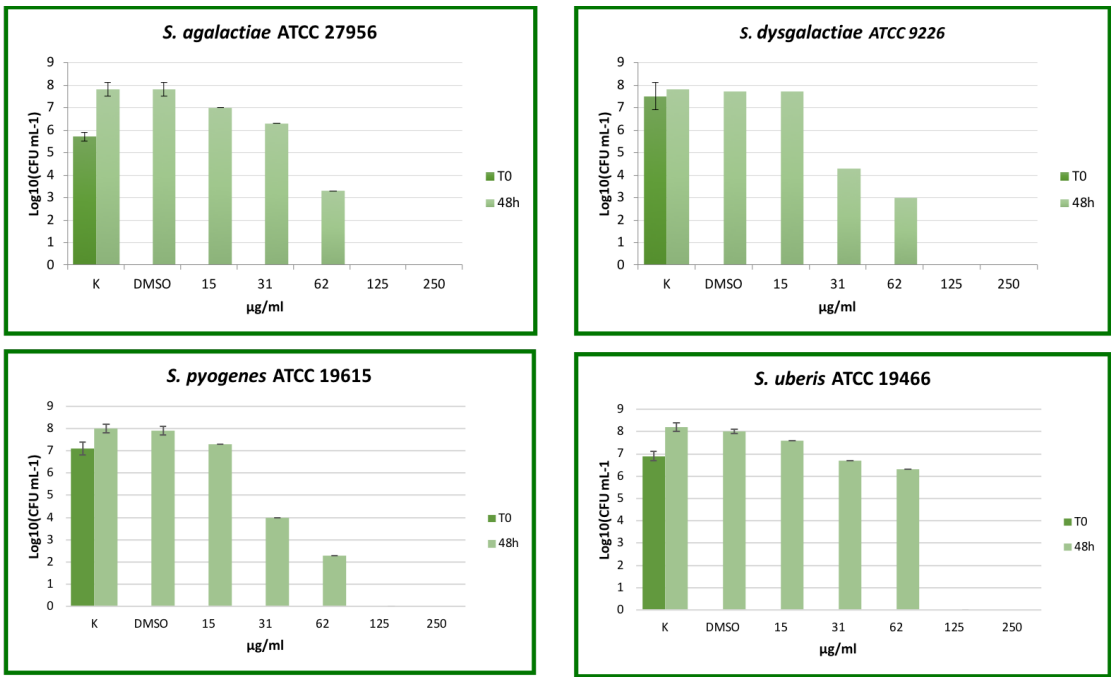


Figure 29: Antibacterial activity of methanol *H. bocconei* extract against reference Streptococci: *S. agalactiae, S. dysgalactiae, S. pyogenes* and *S. uberis*.

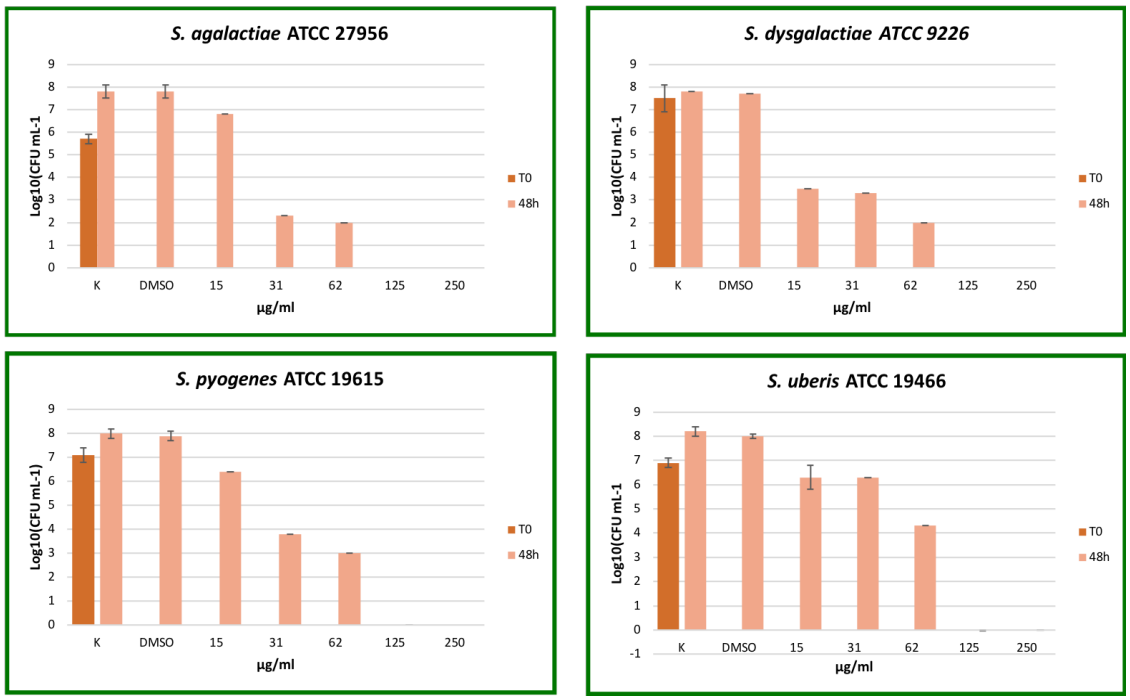


Figure 30: Antibacterial activity of butanol *H. bocconei* extract against reference Streptococci: *S. agalactiae, S. dysgalactiae, S. pyogenes* and *S. uberis*.

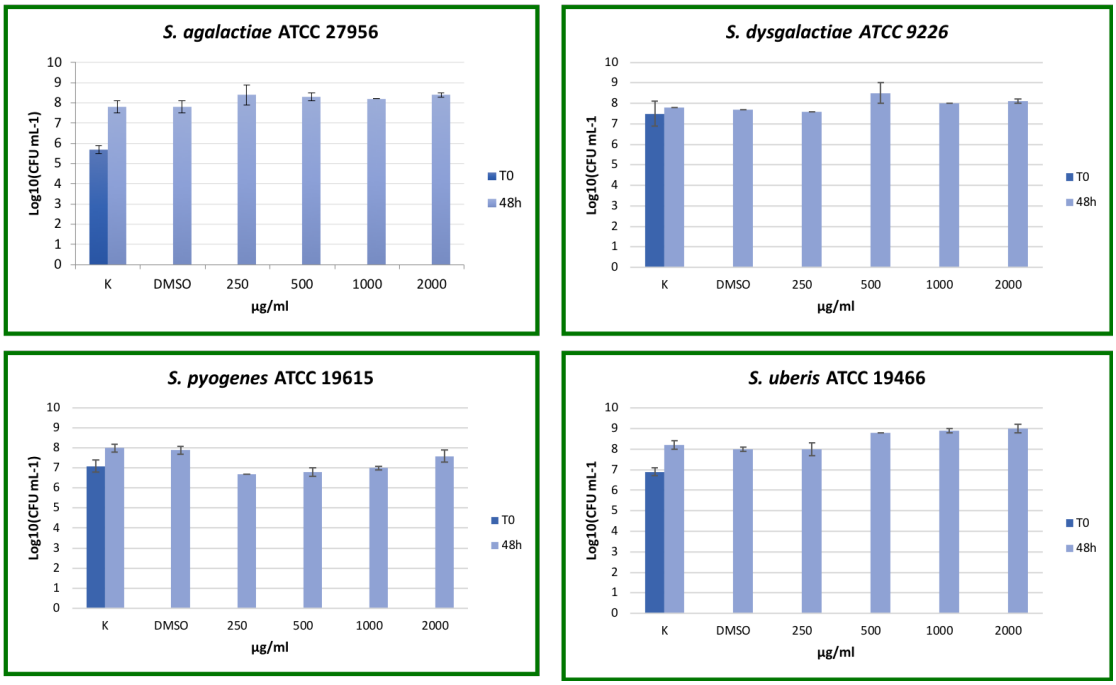


Figure 31: Antibacterial activity of water *H. bocconei* extract against reference Streptococci: *S. agalactiae, S. dysgalactiae, S. pyogenes* and *S. uberis*.

Streptococcus genera were found more represented in MS patients with disease activity (Cosorich et al., 2017). Thus, to analyze the antibacterial activity of butanol and methanol extracts, these were tested on 16 Streptococci strains (4 S. *agalactiae, 4 S. dysgalactiae, 4 S. uberis and 4 S. pyogenes*) isolated from the field. Evaluation of the Minimum Inhibitory Concentrations (MIC) of *Helleborus* extracts and 18 antimicrobials, was conducted. All field strains analyzed were resistant to both extracts at concentrations under 125 µg/ml, except for *S. agalactiae* whose viability was compromised at concentrations of 31 µg/ml of the butanol extract.

Helleborus extracts and immunomodulation

To study the immunomodulatory activity of *H. bocconei*, the three extracts (methanolic, butanol and water) were tested for their cytotoxicity on Peripheral Blood Mononuclear Cells (PBMCs) and their capability to cause hemolysis. The three *Helleborus* extracts, at concentrations from 10 µg/ml to 0.07 µg/ml, were added to PBMCs.

Concentrations higher than 2.5 µg/ml for methanol and water extracts, and 0.62 µg/ml for butanol extract were cytotoxic for human PBMCs (Figure 32).

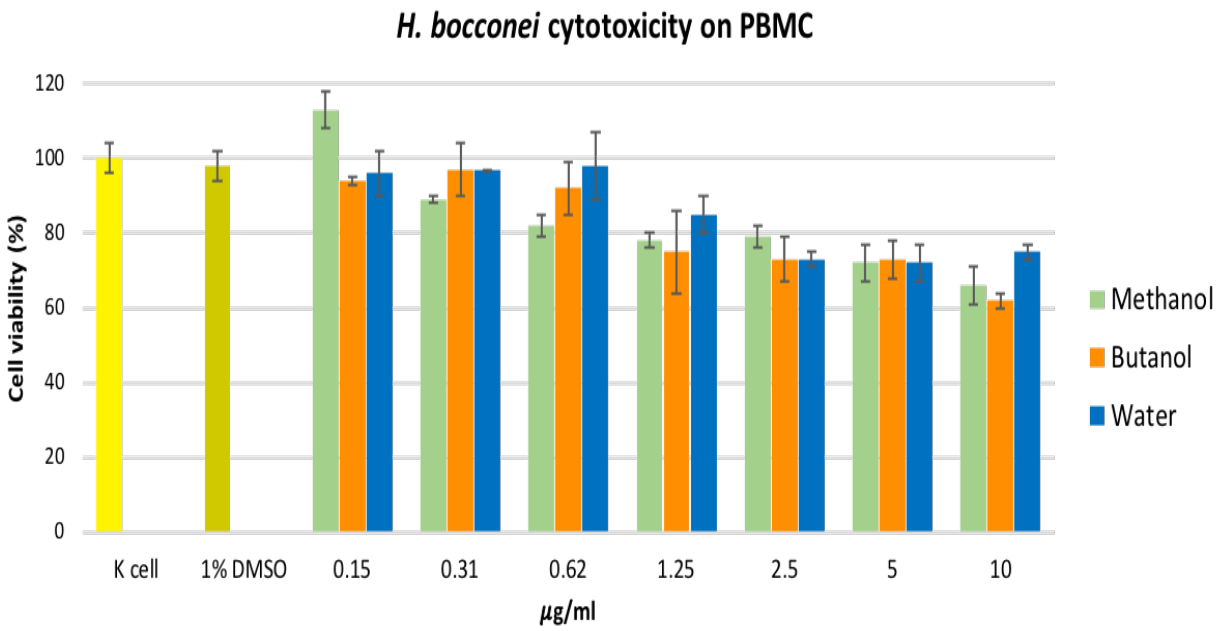


Figure 32: Cell viability of PBMCs treated with concentrations ranging from 10 µg/ml to 0.07 µg/ml of three *H. bocconei* extracts (methanol, butanol and water fractions). The yellow histogram bar reports the cell control (untreated) and the lime bar indicates cells treated with the maximum percentage of DMSO used during all experiments.

The same experiment was repeated by treating the PBMCs simultaneously with bacterial lipopolysaccharide (LPS 1 µM) and *Helleborus* extracts to evaluate the viability of cells subjected to a double stimulus (0.15 and 0.31 µg/ml for methanol extract, 0.31 and 0.62 µg/ml for the butanol fraction and 0.62 and 1.25 µg/ml for water fraction). All the tested concentrations were not cytotoxic for PBMCs (Figure 33).

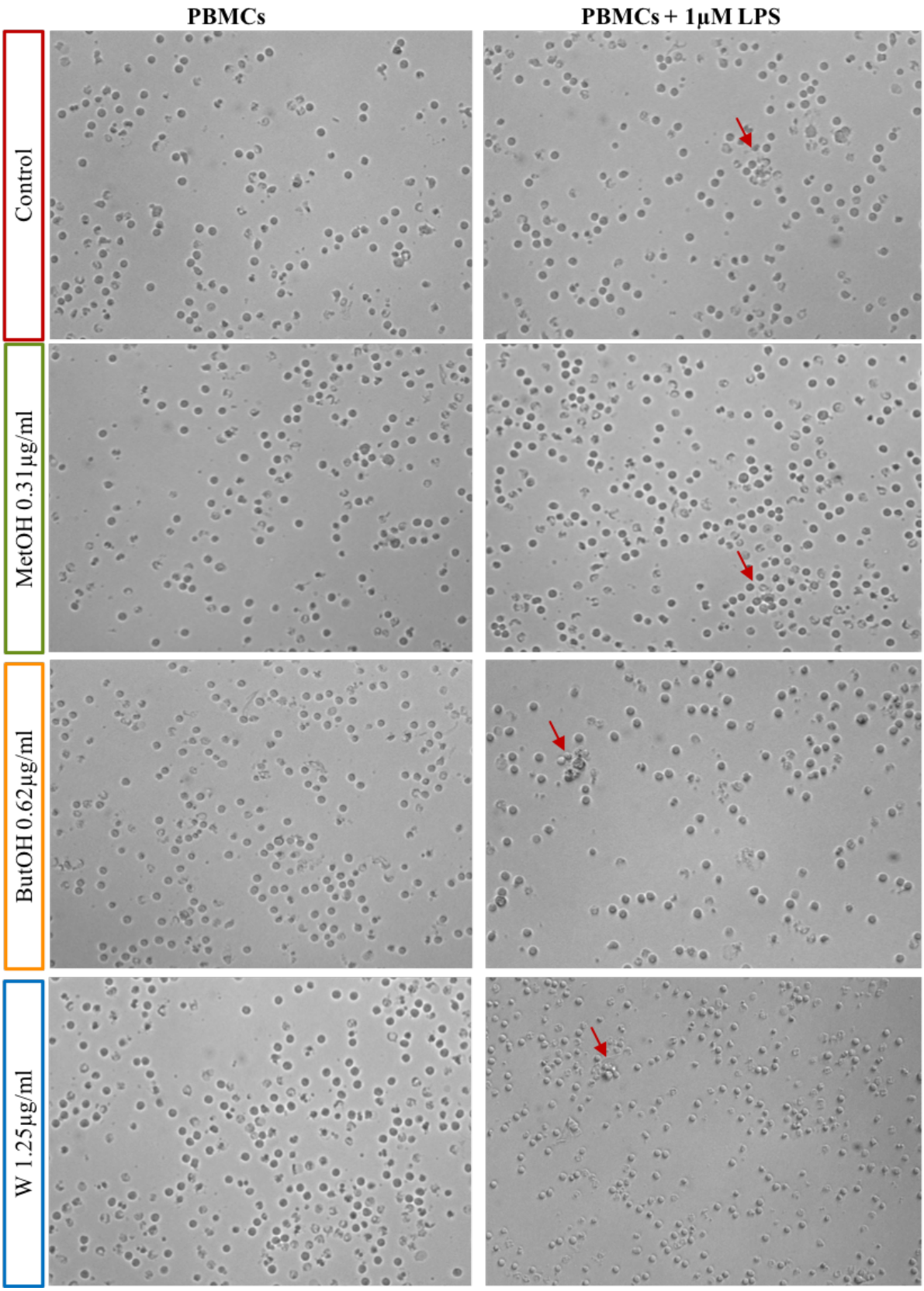


Figure 33: Representative microscopic images showing the effect of *H. bocconei* extracts with and without bacterial LPS stimulation on PBMCs after 24h. Arrows show morphological changes of PBMCs after LPS stimulation.

The biocompatibility of the *H. bocconei* extracts was evaluated by a hemolysis test performed mixing blood with *Helleborus* extracts (0.62, 0.31 and 0.15 μg/ml for butanol and methanol extracts, while 5, 2.5 and 1.25 μg/ml for water extract) or detergent (TritonX100 as positive control of hemolysis). All concentrations tested did not cause hemolysis suggesting that concentrations below 0.62 μg/ml for butanol extract and 1.25 μg/ml for methanol and water extracts can be compatible with human health (Figures 34 and 35).

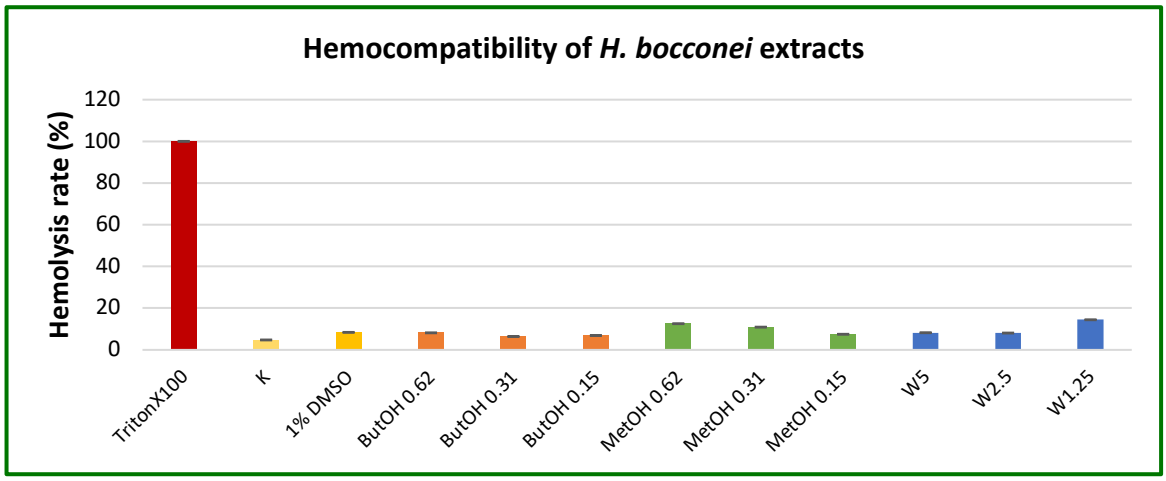


Figure 34: Hemolysis of red blood cells treated with detergent (20% TritonX100 in red), untreated (yellow), 1% DMSO (dark yellow) or *Helleborus* extracts (butanol in orange, methanol in green and water in blue)

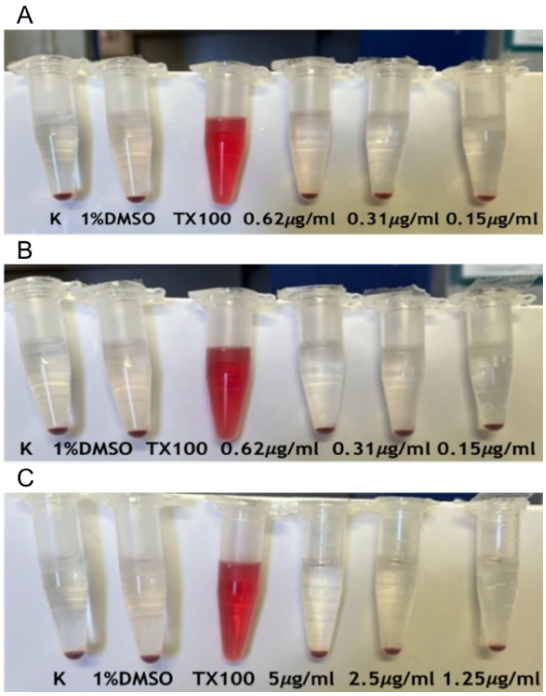


Figure 35: Hemolysis of red blood cells treated with different concentrations of *Helleborus* extracts (A: butanol, B: methanol, C water extract)

Once the non-cytotoxic concentrations of each compound were established for PBMCs, cells were stimulated with bacterial LPS. After 24 h of stimulation, cells were recovered to carry out a gene expression study of the immune system components mainly involved in MS and, more generally, in inflammatory processes.

During cell stimulation with LPS, both concentrations of butanol and aqueous extracts reduced IL1β and iNOS gene expression compared to results obtained from cells treated with LPS alone, while methanol extracts contain some components that induce gene expression of IL1β (*p-value*: 0.048 for LPS_Met0.15) and iNOS (*p-value*: 0.0038 for LPS_Met0.15) (Figure 36).

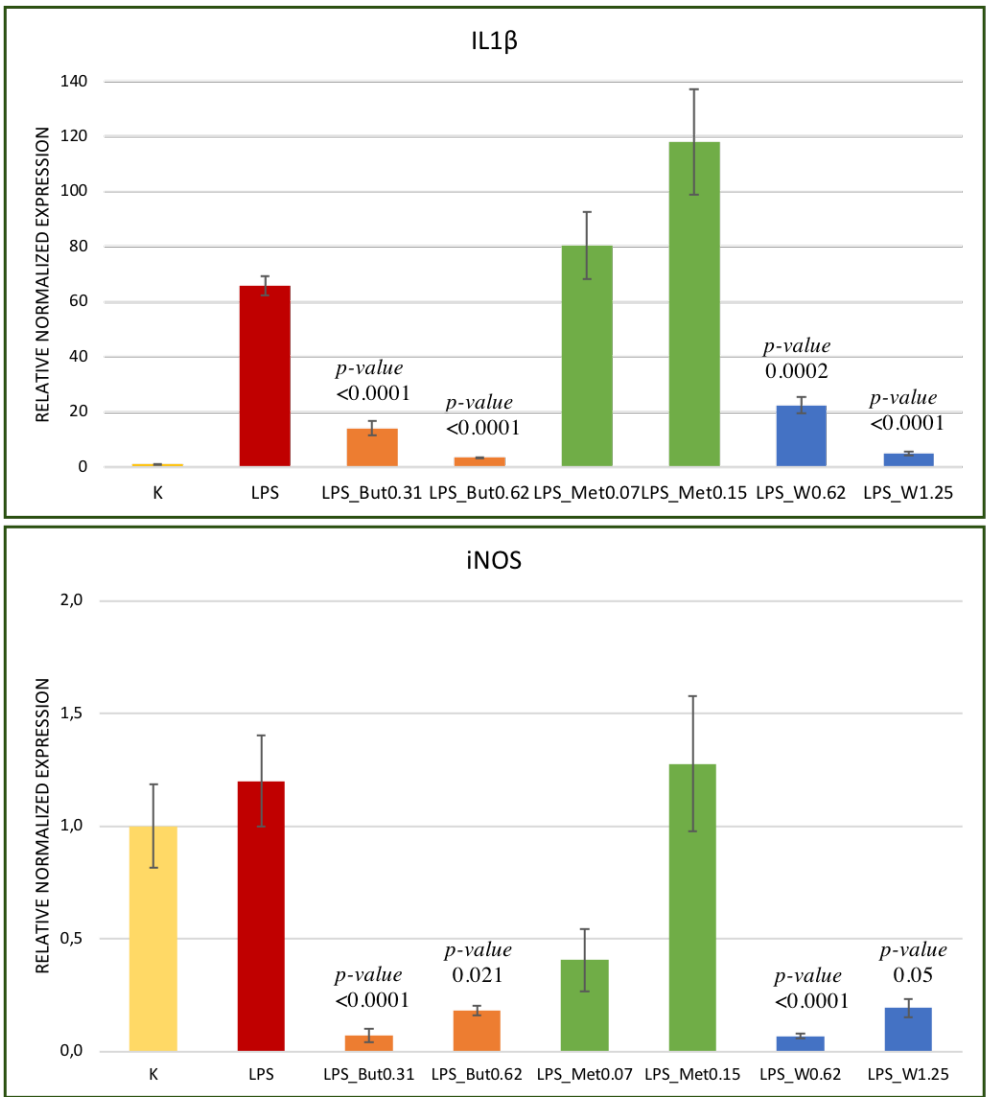


Figure 36: Comparison of IL1β and iNOS mRNA expression in the studied cell groups: K in yellow (PBMCs control untreated), LPS in red (PBMCs treated with LPS), LPS_But in orange (PBMCs treated with LPS and two concentrations of *Helleborus* butanol fraction, LPS_Met in green (PBMCs treated with LPS and two concentrations of *Helleborus* methanol fraction) and LPS_W in blue (PBMCs treated with LPS and two concentration of *Helleborus* water fraction). Cytokine mRNA expression was normalized to β-actin.

Concerning the other genes, no significant variations among the samples tested were shown even if butanol extract at 0.62 μg/ml seems to reduce TNFα gene expression (Figure 37).

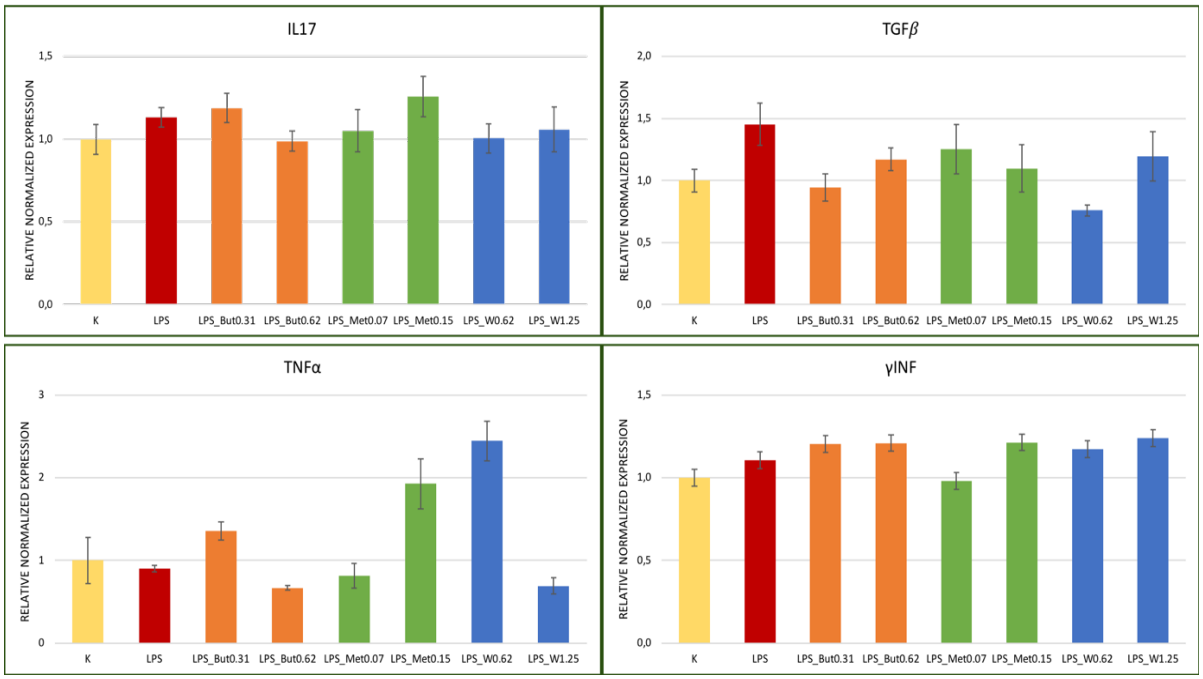


Figure 37: Comparison of cytokine mRNA expression in the studied cell groups: K in yellow (PBMCs control untreated), LPS in red (PBMCs treated with LPS), LPS_But in orange (PBMCs treated with LPS and two concentrations of *Helleborus* butanol fraction, LPS_Met in green (PBMCs treated with LPS and two concentrations of *Helleborus* methanol fraction) and LPS_W in blue (PBMCs treated with LPS and two concentration of *Helleborus* water fraction). Cytokine mRNA expression was normalized to β-actin.

Anti-viral activity of *H. bocconei* extracts

Since Herpes viruses were identified as potential triggers of MS, including Epstein-Barr virus (EBV), human herpesvirus 6 (HHV-6), cytomegalovirus (CMV) and herpes simplex virus (HSV) (Asouri et al, 2020), the anti-viral assays were carried out on Bovine (BHV-1), Caprine (CpHV-1) and Equine (EHV-1) Herpes virus. All these three virus cause rhinotracheitis, abortion and encephalitis and have a strong similarity with human Herpes viruses.

Bovine herpesvirus 1 (BHV-1) shares a number of biological properties with HSV-1 and HSV-2 (Jones, 2003). CpHV-1, like most alphaherpesviruses, after primary infection causes latent infection, usually in trigeminal and sacral ganglia as induced by Human alphaherpesvirus 2 (HSV-2) (Tempesta et al., 1999a,b, 2002). Equine herpes virus 1 (EHV-1) possesses a protein homologous to gM of HSV-1 that facilitates both entry into target cells and cell-to-cell spread (Osterrieder et al., 1996).

To evaluate the anti-viral activity of the three *H. bocconei* extracts, their cytotoxicity was tested on MDBK (useful to cultivate BHV-1 and CpHV-1) and RK13 (useful to cultivate EHV-1) cell lines. MDBK cells viability is reduced at concentrations higher than 1.25, 0.62 and 2.5 µg/ml for methanol, butanol and water extracts, respectively (Figure 38).

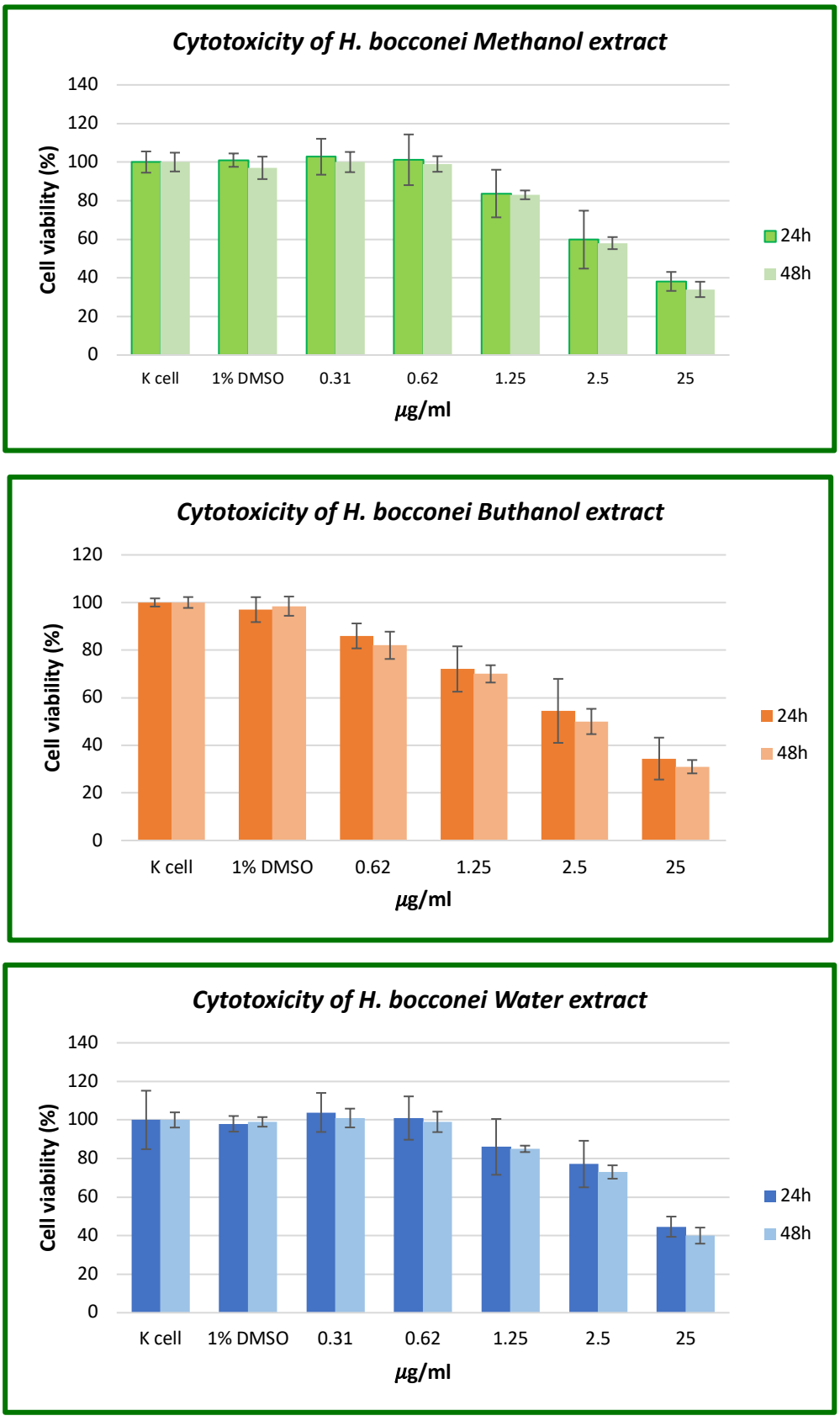


Figure 38: Cell viability of MDBK cells treated with *Helleborus* extracts: methanol extract (green), butanol extract (orange) and water extract (blue).

Regarding RK13 cells their viability is reduced at concentrations higher than 2.5, 0.62 and 10 µg/ml for methanol, butanol and water extract, respectively (Figure 39).

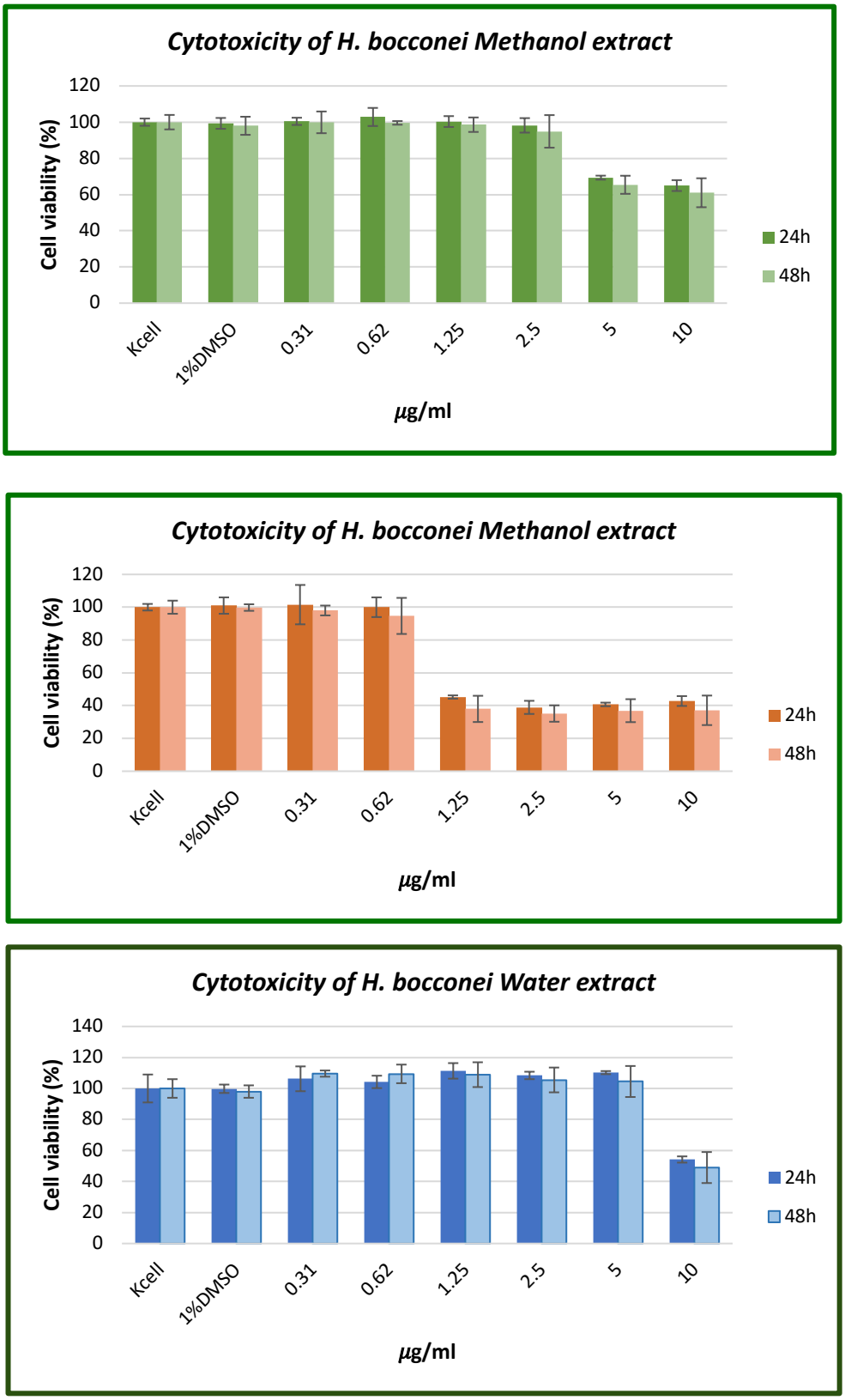


Figure 39: Cell viability of RK13 cells treated with *Helleborus* extracts: methanol extract (green), butanol extract (orange) and water extract (blue).

The non-cytotoxic concentrations of each extract (0.31 and 0.62 µg/ml for butanol extract, 0.62 and 1.2 µg/ml for both methanol and water extracts) were tested to assess their activities anti-BHV-1, anti-CpHV-1 and anti-EHV-1.

To elucidate the antiviral action mode, cells were incubated with the extracts for 72 h, following 3 experimental conditions: before and after viral infection, simultaneous infection.

MDBK infected with BHV-1 showed the cytopathic effect in the pre-treatment with all *H. bocconei* extracts (Figure 40).

Relating the post-infection assay, MDBK treated with all *H. bocconei* extracts showed a faint reduction of the cytopathic effect induced by BHV-1 .

The observation of cell morphology was in accordance with the percentage increment in MDBK viability, revealed by MTT assay, compared to the pre-infection treatment (Figure 41).

With regard to simultaneous BHV-1 infection and *H. bocconei* treatment, MDBK treated with methanol and water extracts showed a faint reduction of the cytopathic effect induced by BHV-1, while butanol extract showed an anti-viral effect (Figure 42).

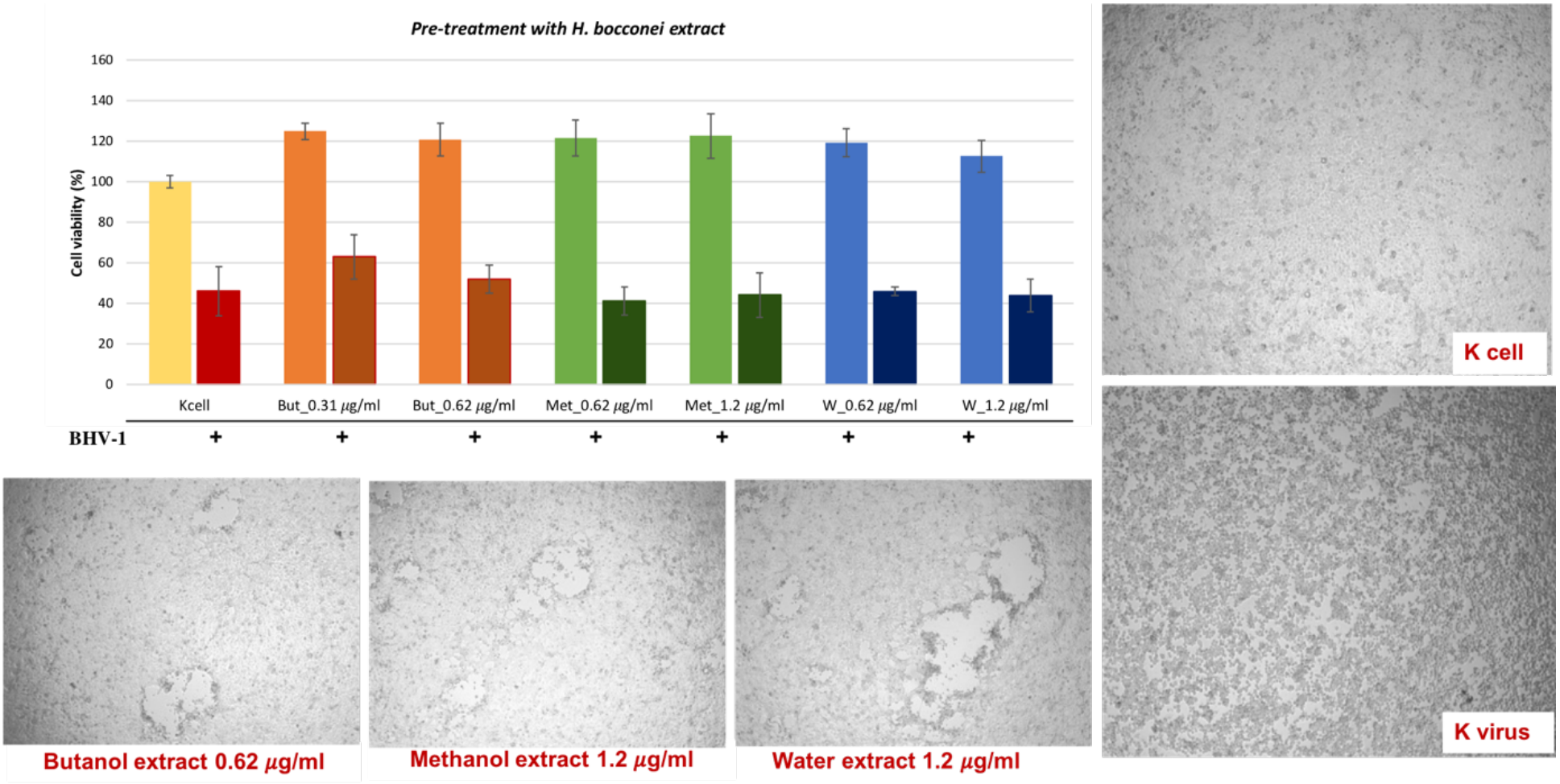


Figure 40: MDBK viability (%) and microscopic observation of cell morphology (5x) in BHV-1 infection after pre-treatment of with *Helleborus* extracts. Pictures of mmicroscopic observation are reported: cell control (untreated), virus control (cells with virus), cells with virus and butanol extract, cells with virus and methanol extract and cells with virus and water extract.

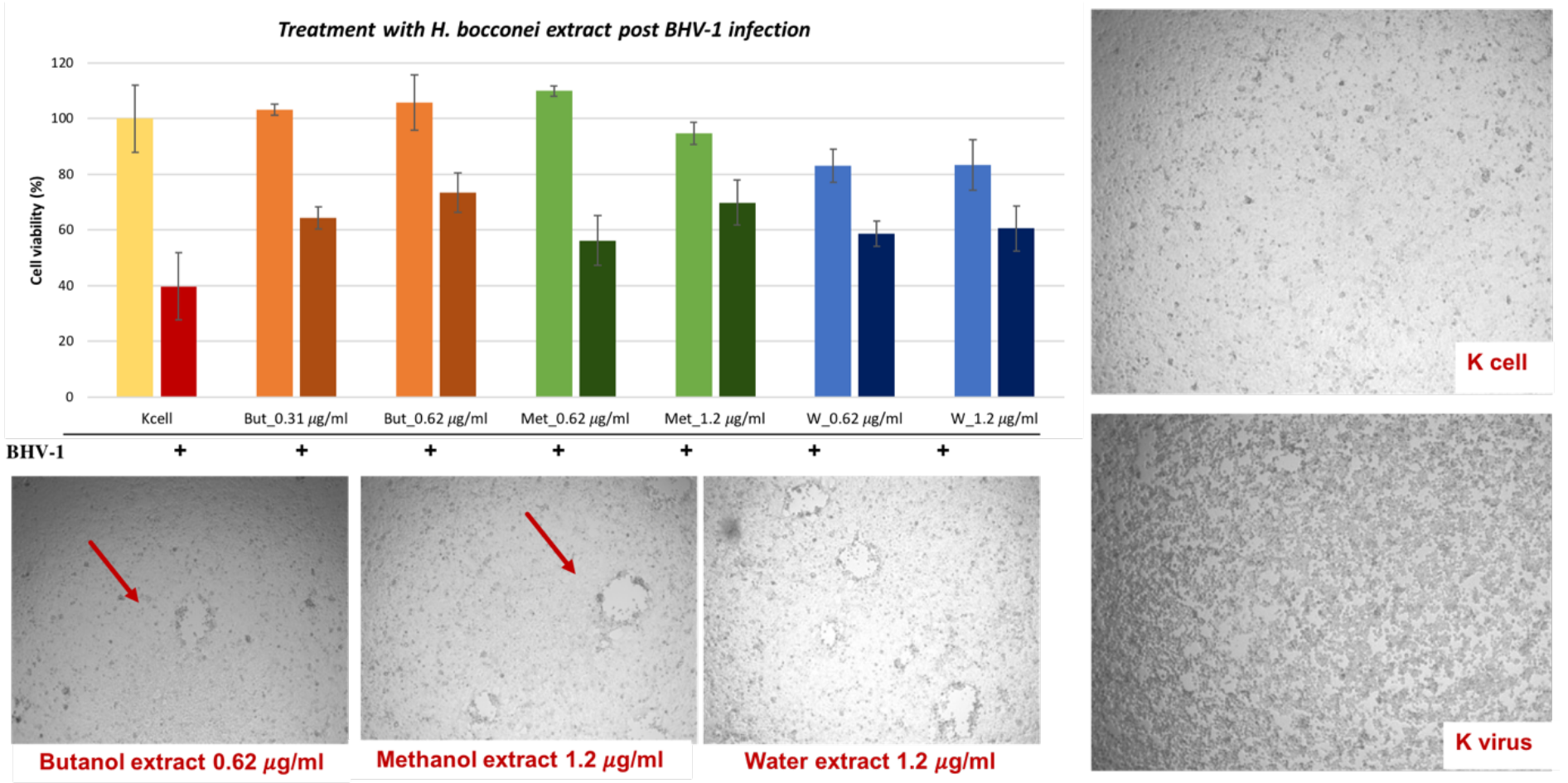


Figure 41: MDBK viability (%) and microscopic observation of cell morphology (5x) in BHV-1 infection after post-treatment of with *Helleborus* extracts. Pictures of mmicroscopic observation are reported: cell control (untreated), virus control (cells with virus), cells with virus and butanol extract, cells with virus and methanol extract and cells with virus and water extract. Arrows indicate the BHV-1 outbreak.

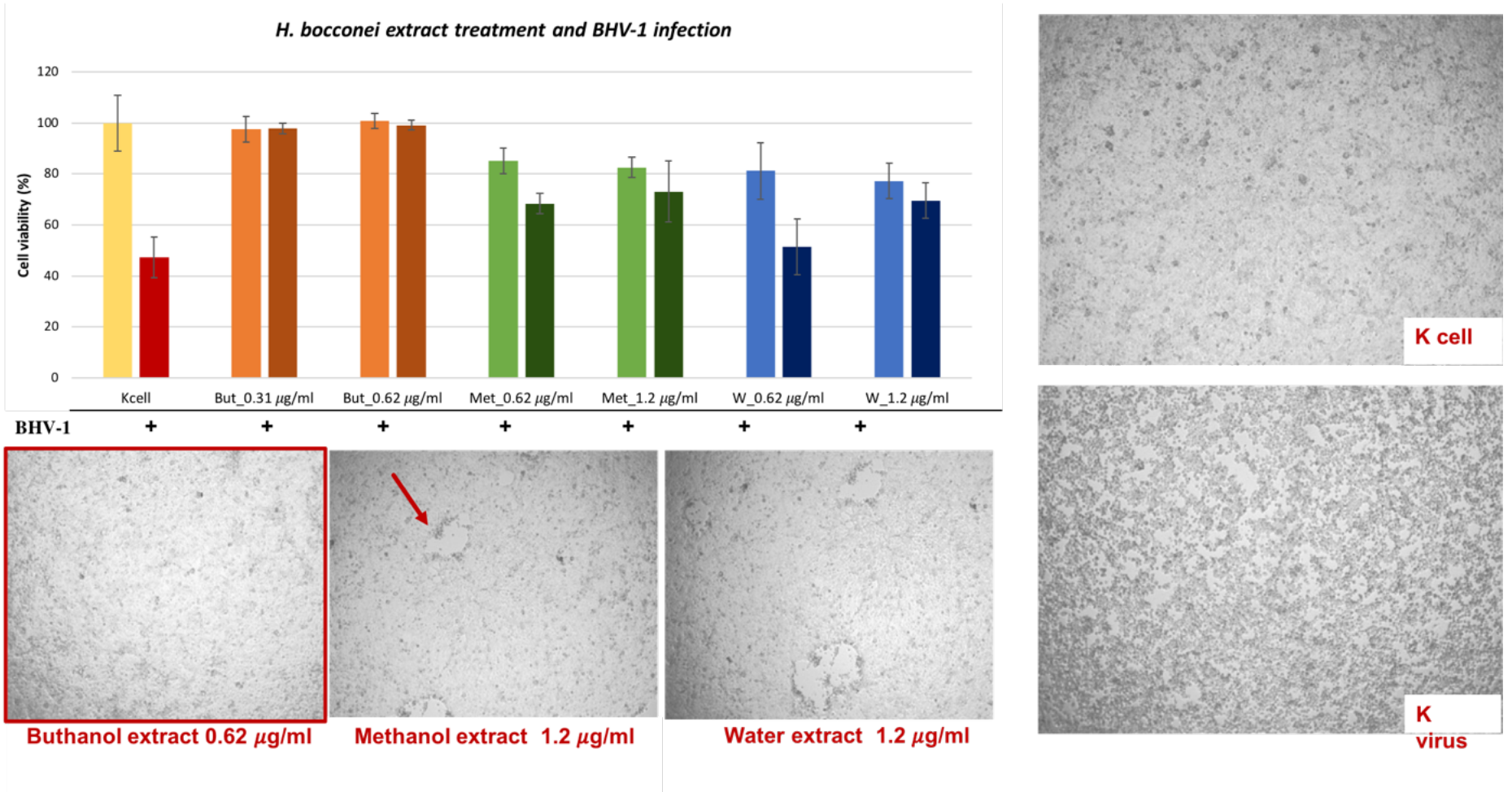


Figure 42: Cell viability (%) and microscopic observation of cell morphology (5x) in MDBK subjected to simultaneous BHV-1 infection and *H. bocconei* treatment. Pictures of mmicroscopic observation are reported: cell control (untreated), virus control (cells with virus), cells with virus and butanol extract, cells with virus and methanol extract and cells with virus and water extract. Arrows indicate the BHV-1 outbreak while the red box indicates compounds with anti-viral activity.

As reported for BHV-1, MDBK infected with CpHV-1 showed the cytopathic effect during the pre-treatment with *H. bocconei* extracts except for the butanol extract at the concentration of 0.31 μg/ml that induced a great reduction of the cytopathic effect (Figure 43).

Relating to post-infection assay, MDBK treated with *H. bocconei* methanol and water extracts showed a faint reduction of the cytopathic effect induced by CpHV-1. The butanol extract at the concentration of 0.31 μg/ml showed an anti-viral activity.

The observation of cell morphology was in accordance with the percentage increment in MDBK viability revealed by MTT assay (Figure 44).

Concerning simultaneous CpHV-1 infection and *H. bocconei* treatment, MDBK treated with methanol and water extracts showed a strong reduction of the cytopathic effect induced by CpHV-1, while butanol extract showed an anti-viral effect (Figure 45).

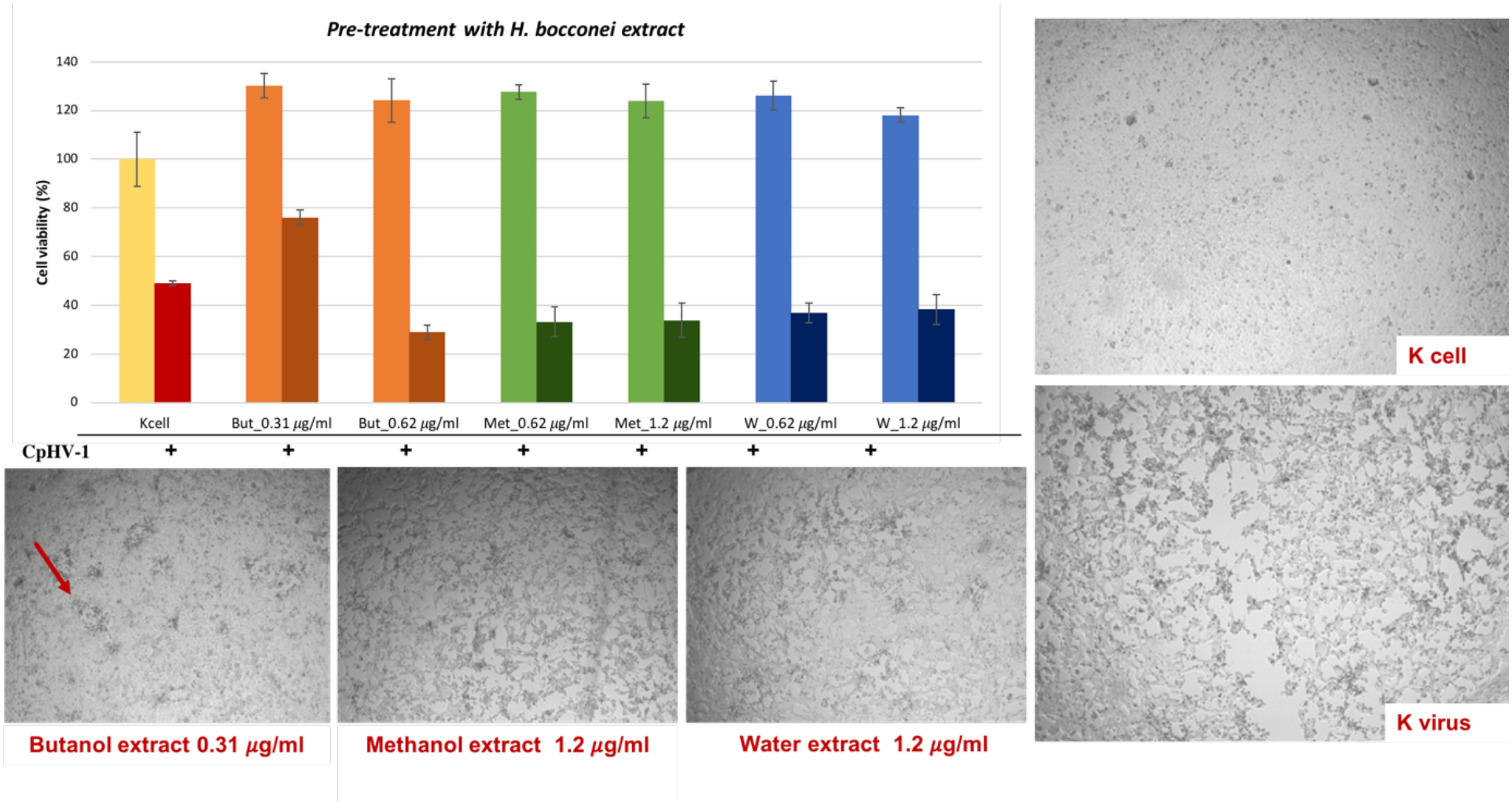


Figure 43: MDBK viability (%) and microscopic observation of cell morphology (5x) in CpHV-1 infection after pre-treatment of with *Helleborus* extracts. Pictures of mmicroscopic observation are reported: cell control (untreated), virus control (cells with virus), cells with virus and butanol extract, cells with virus and methanol extract and cells with virus and water extract. Arrows indicate the BHV-1 outbreak.

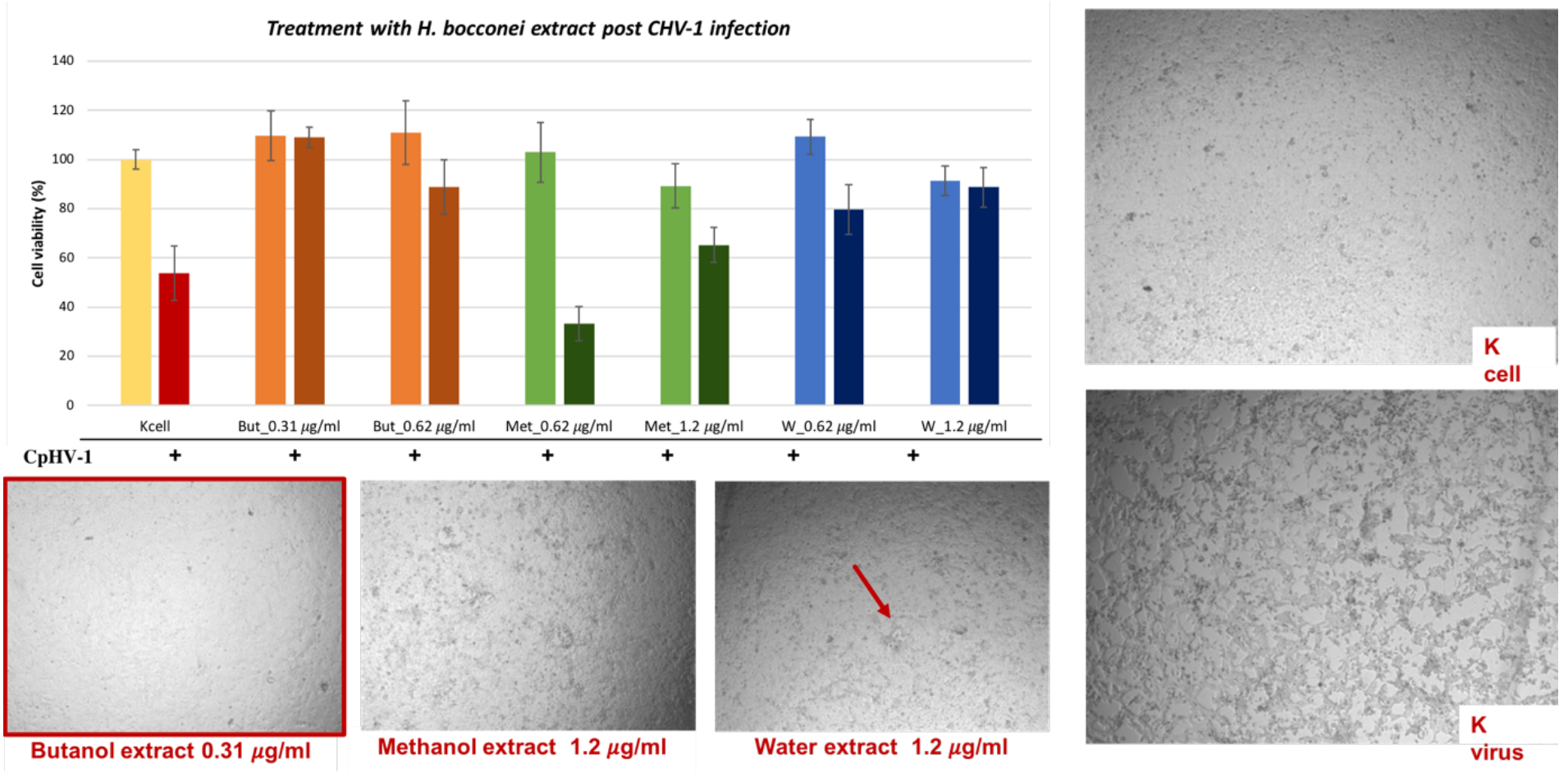


Figure 44: MDBK viability (%) and microscopic observation of cell morphology (5x) in CpHV-1 infection after post-treatment of with *Helleborus* extracts. Pictures of mmicroscopic observation are reported: cell control (untreated), virus control (cells with virus), cells with virus and butanol extract, cells with virus and methanol extract and cells with virus and water extract. Arrows indicate the CpHV-1 outbreak while the red box indicates compounds with anti-viral activity.

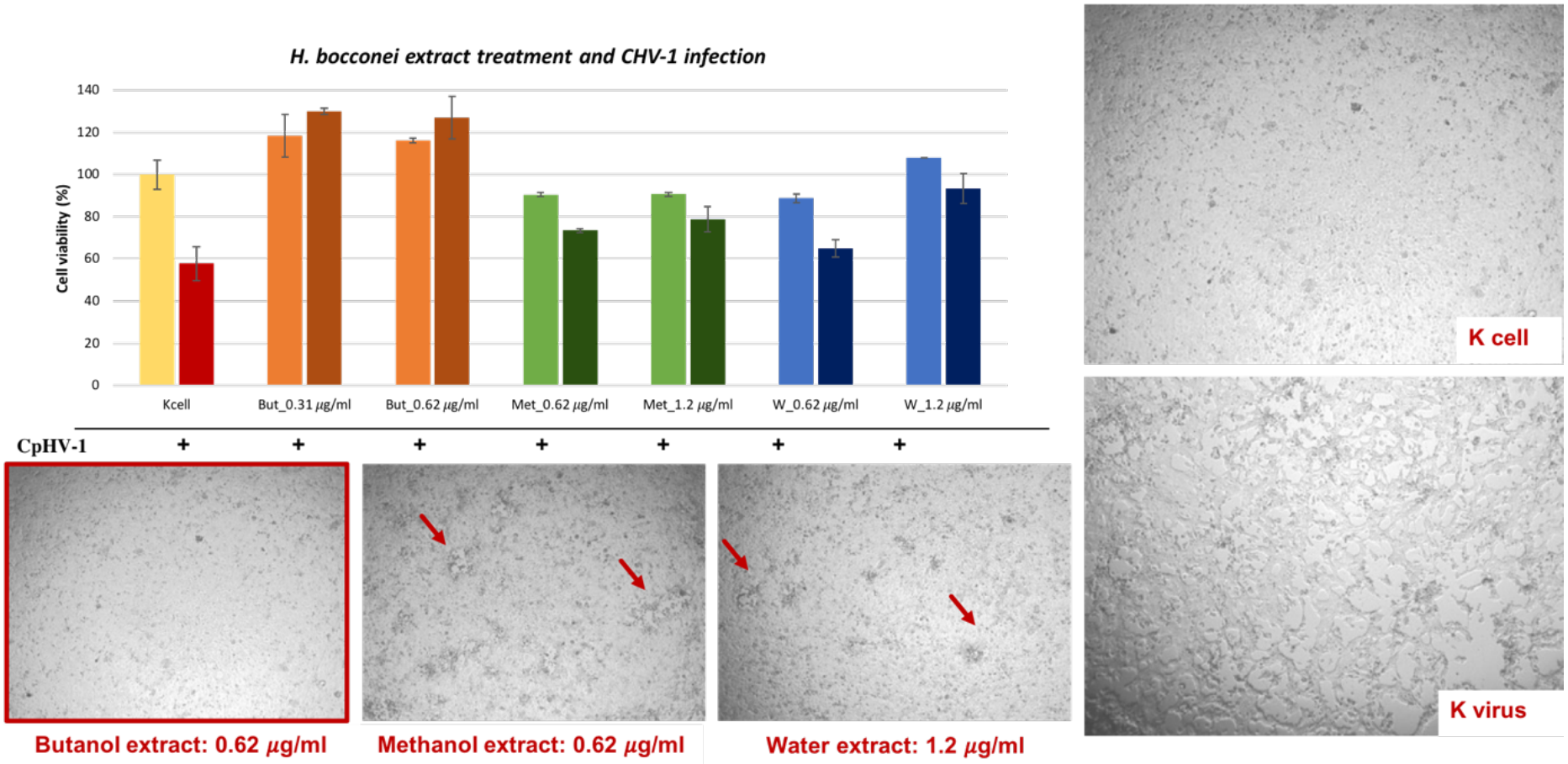


Figure 45: Cell viability (%) and microscopic observation of cell morphology (5x) in MDBK subjected to simultaneous CpHV-1 infection and *H. bocconei* treatment. Pictures of mmicroscopic observation are reported: cell control (untreated), virus control (cells with virus), cells with virus and butanol extract, cells with virus and methanol extract and cells with virus and water extract. Arrows indicate the CpHV-1 outbreak while the red box indicates compounds with anti-viral activity.

Regarding EHV-1, all *H. bocconei* extracts did not show an anti-viral effect against RK13 cells, both in the pre-treatment and post-treatment assays (Figure 46 and Figure 47).

Concerning the simultaneous EHV-1 infection and *H. bocconei* treatment, all the plant extracts showed an anti-viral activity at concentrations of 0.62 μg/ml for butanol and methanol and 1.2 μg/ml for water extract. The observation of cell morphology was in accordance with the percentage increment in RK13 viability revealed by MTT assay (Figure 48).

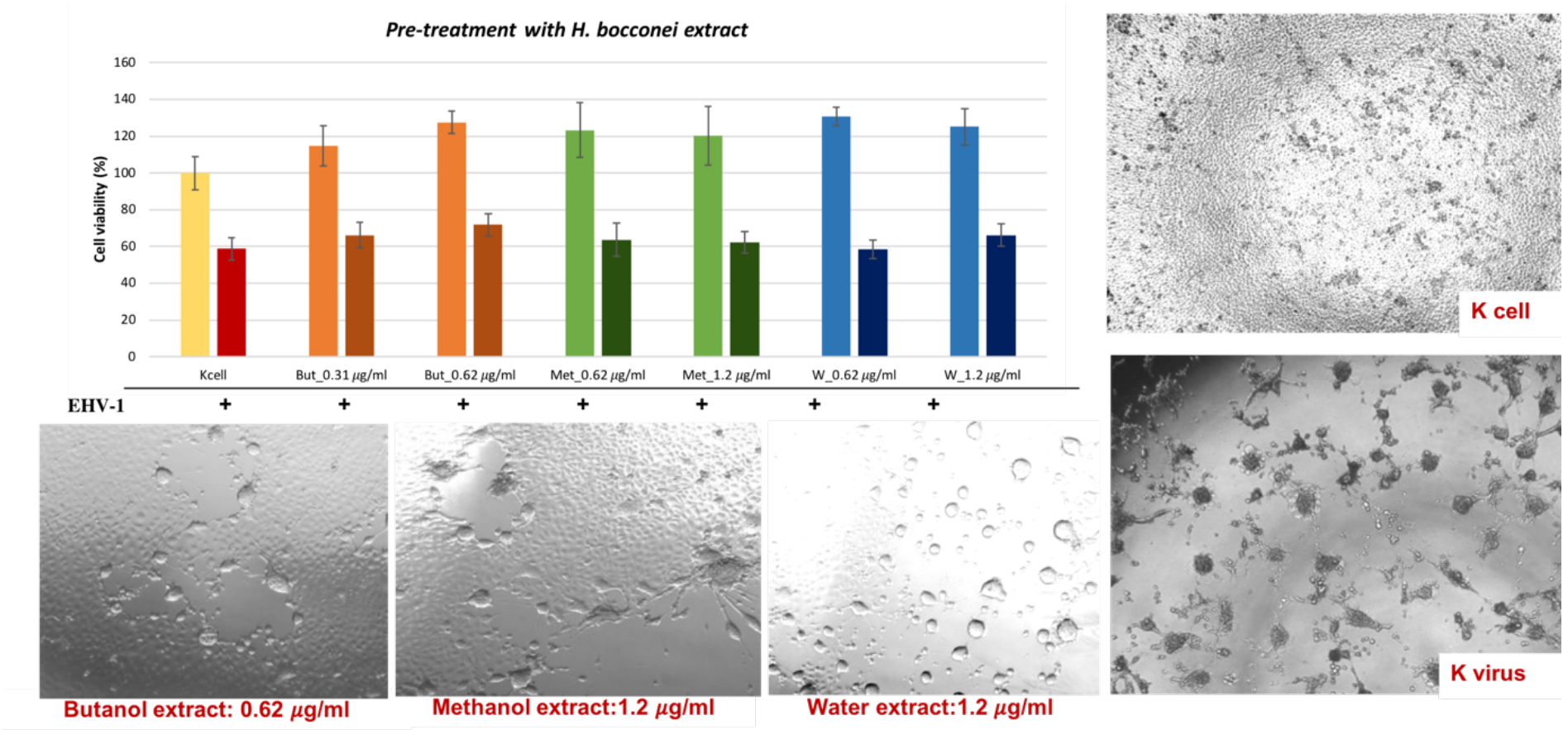


Figure 46: RK13 viability (%) and microscopic observation of cell morphology (5x) in EHV-1 infection after pre-treatment of with *Helleborus* extracts. Pictures of mmicroscopic observation are reported: cell control (untreated), virus control (cells with virus), cells with virus and butanol extract, cells with virus and methanol extract and cells with virus and water extract.

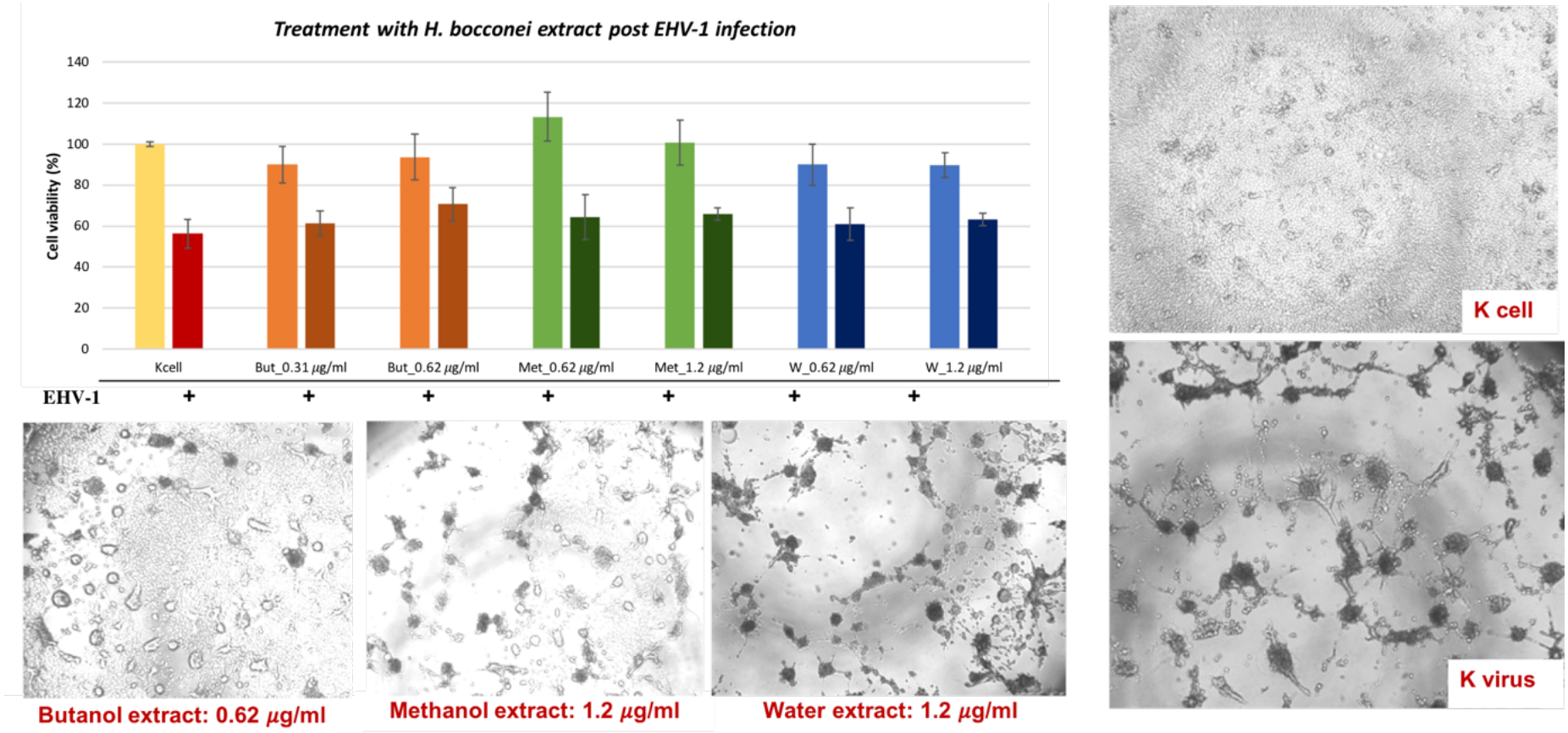


Figure 47.: RK13 viability (%) and microscopic observation of cell morphology (5x) in EHV-1 infection after post-treatment of with *Helleborus* extracts. Pictures of mmicroscopic observation are reported: cell control (untreated), virus control (cells with virus), cells with virus and butanol extract, cells with virus and methanol extract and cells with virus and water extract.

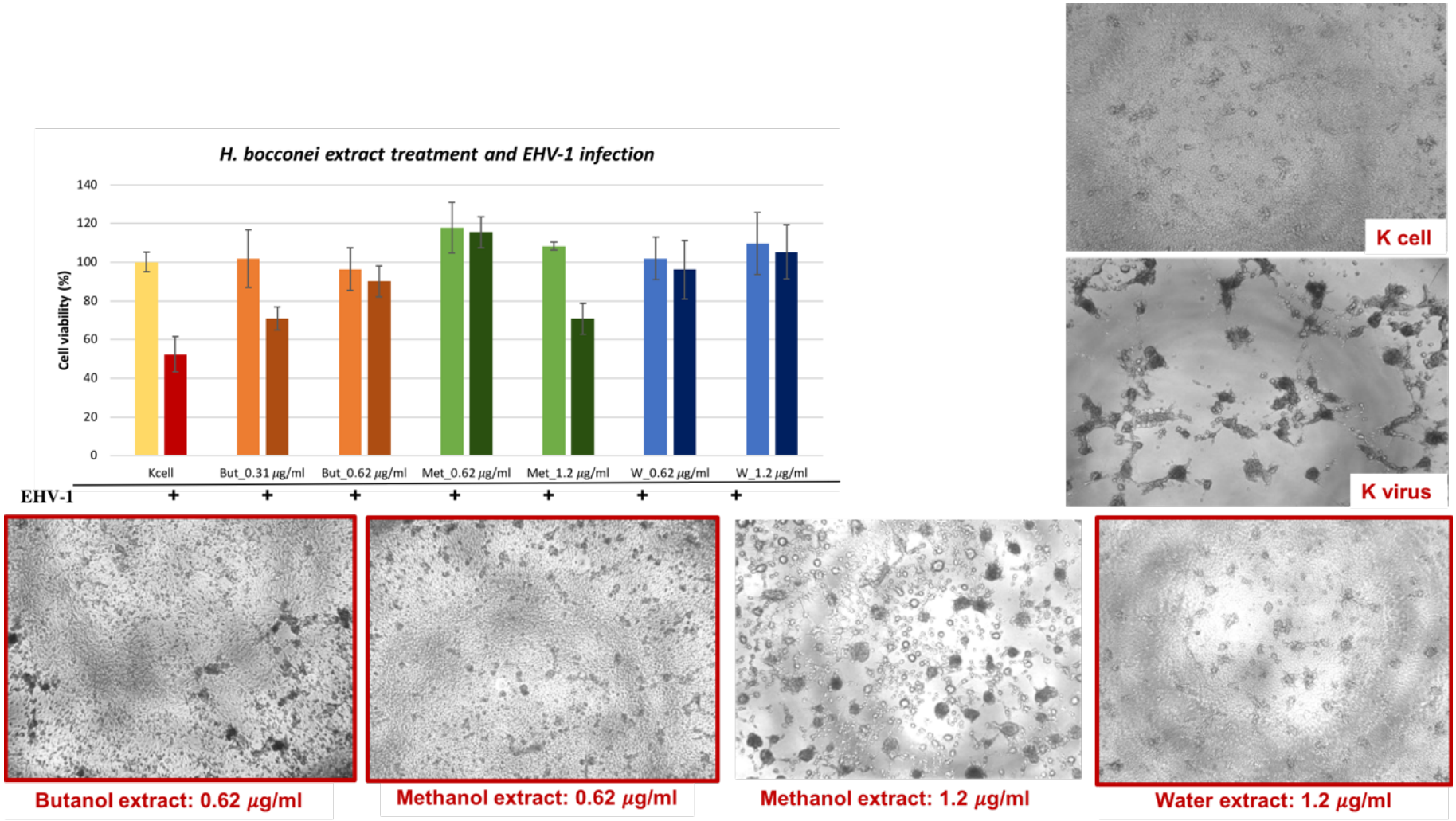


Figure 48: Cell viability (%) and microscopic observation of cell morphology (5x) in RK13 subjected to simultaneous EHV-1 infection and *H. bocconei* treatment. Pictures of mmicroscopic observation are reported: cell control (untreated), virus control (cells with virus), cells with virus and butanol extract, cells with virus and methanol extract and cells with virus and water extract. The red boxes indicate compounds with anti-viral activity.

DISCUSSION

1. MICROBIOTA ANALYSIS

During the PhD course, for the first time, the gut microbial composition of a cohort of samples coming from the same location in Sicily, sharing lifestyle and environmental factors was evaluated. Furthermore, since the onset of multiple sclerosis has been reported to be correlated with different pathogens, such as *Epstein-Barr* virus (EBV) (Asouri et al., 2020), *Brucella*, *Mycobacterium*, *Borrelia* (*Lindsey et al., 2008)* and *Mycoplasma* (Brown, 2003) infections, the presence of these last two pathogens was evaluated both in the stool samples of patients and their controls, and in potential environmental vectors: *Bartonella henselae* in cats and *Mycoplasma mycoides* in ticks. In parallel, some biological activities of *Helleborus bocconei* plant in order to evaluated its potential role in the MS therapy were studied.

Microbiota studies of individuals living in the same household may be a valid strategy to mitigate the differences due to the heterogeneity of otherwise unrelated controls and it was already demonstrated that matched household controls share similar gut, skin, and oral microbiotas (Turnbaugh et al. 2009; Song et al., 2013; Goodrich et al., 2014 Abeles et al., 2016). To the best of our knowledge, only in a few studies, samples for microbiota analysis were collected from controls from the MS patient's house (Berer et al. 2017; The iMSM Consortium 2020).

It would seem that only one study had already investigated the duodenal mucosa microbiota of Italian MS patients with an EDSS between 1 and 5, coming from Northern Italy (Cosorich et al., 2017). The main intestinal dysbiosis reported by Cosorich and colleagues was due to the increase of Bacteroidetes and Proteobacteria and the decrease of Firmicutes in MS patients differently to was found in this study: the opposite trend with the increase of Firmicutes and the decrease of Bacteroidetes and Proteobacteria.

Data on Sicilian people showed an increase of Firmicutes (59% vs. 52%), Bacteroidetes (25% vs. 10%) and Actinobacteria (3% vs. 1.4%) and a decrease of Proteobacteria (8% vs. 18%) in healthy people. These differences could be due to the different sampling origin (faecal vs. duodenal mucosa), but it could be hypothesized that sun exposure, eating habits and other environmental factors could influence the microbial composition of intestinal samples of people from Northern and Southern Italy. Therefore, these large differences show as the environment and the sampling are two important factors in the analysis of the gut microbiota.

An important observation is that the differences between household relatives and MS patients varied according to EDSS: the number of bacterial families (for OTUs), genera and species (for

ASVs) with significant differences between MS patients and controls is smaller in the group of patients with EDSS 1–4.5 than in patients with higher EDSS suggesting the degree of dysbiosis increases as the disease progresses.

Ruminococcaceae and *Christensenellaceae* are the two families whose abundance was higher in MS patients with EDSS 1–4.5 and 5–7 than in household controls, both as OTUs than ASVs. The *Ruminococcaceae* family is correlated with a pro-inflammatory situation and an increased presence of pro-inflammatory mediators, such as TNFα, IL-6, and IL-17. This family is also associated with vitamin D reduction (Ghaly et al., 2018), which can be linked to the very low levels of the vitamin reported by most patients. The *Christensenellaceae* family has been associated with Parkinson's disease (Hill-Burns et al., 2017), metabolic disorders and gastrointestinal diseases, as well as a potential indicator of mortality risk in patients with neurocritical disease (Xu et al., 2019). The increase of *Ruminococcaceae* and *Christensenellaceae* in MS patients was observed in other independent studies (Tremlett et al., 2016; Ling et al., 2020; Reynders et al., 2020).

Regarding the healthy controls, the ASV analysis highlighted an increase of relative abundance of *Bacteroidaceae* family in according with results of another study carried out in Japan (Miyake et al., 2015) and with the role assigned as anti-inflammatory. This family is able to hydrolyze starch and produce butyrate, propionate, acetate and other SCFAs, so it is assumed that is involved in an anti-inflammatory role and that it favors the differentiation of Treg cells, stimulating the production of TGF-β and IL- 10 (Saresella et al., 2017).

Desulfovibrionaceae and *Rikenellaceae* have never been found to be differently abundant in the gut microbiota of MS patients but were associated with inflammatory states.

Shahi and colleagues (2022) demonstrated that obesity in mice increased the EAE severity through the modulation of the gut microbiota by highlighting an enrichment of *Proteobacteria* and *Desulfovibrionaceae* bacteria with enrichment of sulfur metabolism, lipopolysaccharide biosynthesis, and long chain fatty acid biosynthesis (LCFA) pathways linked with inflammation. Finally, obese mice also exhibited leaky gut characteristics characterized by increased intestinal permeability, translocation of intestinal proteins, and high levels of pro-inflammatory mediators in the systemic circulation (Shahi et al., 2022). Regarding *Rikenellaceae,* this family was reported as more abundant also in Multiple System Atrophy (Engen et al., 2017) and in subjects following a high-fat diet (Wang et al., 2020).

Contrarily to OTUs, the ASV analysis allowed to obtain data also regarding the relative abundances of bacterial genera and species. While in EDSS 1-4.5 patients *Bacteroides* was more abundant in controls and *Oscillospira* in patients, in EDSS 5-7 patients *Prevotella* was

more abundant in controls and *Alistipes* and *Gemmiger* in MS patients. These data were in accordance with data found at species level: *Alistipes putredinis, Gemmiger formicilis* and *Bacteroides caccae* were more significantly abundant in RRMS patients than controls.

The *Gemmiger* species was associated with PPMS (Kozhieva et al., 2019) while the *Alistipes* genera were associated with an inflammatory state. In particular, was reported to be a potential SCFA producer (Dziarski et al., 2016; Parker et al., 2020) and to play a pro-inflammatory role in cardiovascular disease, colorectal cancer and with mental signs of depression (Parker et al., 2020 and Lang et al., 2021).

In Cosorich et al., (2017) MS patients showed a strong reduction of *Prevotellaceae* and a higher differentiation of Th17 cells, and therefore an increase of the inflammatory state. *Prevotella* was found in the gastrointestinal tract of subjects who consumed a diet rich in vegetables and has been shown to improve the metabolism of glucose, but high percentages are also associated with autoimmune, insulin-resistant diseases, intestinal inflammation and rheumatoid arthritis, suggesting its role in intestinal dysbiosis (Iljazovic et al., 2020).

Although no bacterial biodiversity was evidenced by the biodiversity indices, the PCoA analysis showed a cluster of microbiota samples of MS patients, mainly with EDSS 4.5–7, regardless of age and gender.

The bacteria present in all faecal samples are ammonia oxidizers, cellulose degraders and capable of dehalogenation, in particular, they seem more abundant in samples of control subjects. Sulphate reducing bacteria, such as *Desulfovibrionaceae*, seem more abundant in faecal samples of patients with MS.

2. PATHOGENS AND MULTIPLE SCLEROSIS

The etiology of the disease is unknown and highly debated since the genetic susceptibility, hormonal factors, environmental stimuli and the neuroimmune axis seems play a role in the disease mechanisms and in direct autoimmunity of the CNS.

Regarding the environmental factors contributing to the pathogenesis of the disease, several pathogens have been proposed as triggers for MS. All stool samples of both MS patients and healthy controls were analyzed to search *Bartonella* and *Mycoplasma* DNA. No sample tested was positive for these two pathogens. This data was in agreement with Lindsey (2008) according to which *Campylobacter, Mycoplasma, Chlamydia, Bartonella, Mycobacteria* and *Streptococcus* were not detected DNA in cerebrospinal fluid from any patients or controls (Lindsey et al., 2008). These data suggest the difficulty of isolating MS-associated pathogens

although there are data reporting the presence of antibodies against these bacteria in the serum of MS subjects (Birberfeld, 1971).

Bartonella henselae is a slow growing and facultative intracellular pathogen mainly transmitted by arthropod vectors adapted to domestic and wild mammalian reservoir hosts or following a cat scratch or bite.

The presence of anti-*B. henselae* antibodies and the DNA of *B. henselae*, in blood sample of stray cats from Palermo was assessed, in order to evaluate the prevalence of this pathogen.

However, data suggests that a high prevalence of cats could be carriers of the pathogen and therefore could be contagious.

Regarding *Mycoplasma,* a geographic correlation of the global distribution of MS with *Ixodes* tick-borne diseases was previously published (Brown 1996). Mollicutes are a group of infectious agents that infect *Ixodes* ticks but no evidence was reported for mycoplasmas pathogenic to animals but apparently not to humans. Animal infections caused by *M. neurolyticum* and *M. gallisepticum*, respectively, could serve as possible models of vascular damage in the human nervous system. Moreover, antibody anti- *M. mycoides* were found in sera of MS patients (Brown 2003). For this reason, *M. mycoides* presence was investigated in ticks as possible vectors for the spread in humans.

This preliminary study demonstrated the carriage of viable *M. agalactiae* by *R. bursa* ticks suggesting a potential role of these ticks as a reservoir or potential vector of the pathogen.

The spread of non-zoonotic pathogens is often underreported and almost studied in relation to human health. The presence of antibodies against animal pathogens in human samples should prompt further studies on how much these can affect human health, even after many years. The discovery of *M. agalactiae*, closely related to *M. mycoides*, alive in ticks opens a new scenario in the understanding of how these vectors can carry pathogens or toxins (currently monitored only for animals) capable of inducing disease in humans.

3. *HELLEBORUS BOCCONEI* BIOLOGICAL ACTIVITIES

Helleborus bocconei is a Sicilian endemic plant of which, apart from the chemical composition (Rosselli et al., 2009), only the antibacterial activity (Puglisi et al., 2009) and the cytotoxic activity against rat glioma cells were investigated.

The choice of this plant arose because in another kind of hellebore, *H. purpurascens*, the compound MCS-18 (macrocyclic carbon dioxide), related to immunomodulation in

experimental autoimmune encephalomyelitis (EAE), a mouse model for human multiple sclerosis, was described.

Regarding the antibacterial activity, data obtained on Gram negative bacteria are in agreement with Puglisi and colleagues (2009). Concerning Gram positive bacteria, no antibacterial activity was found against *S. aureus* while data of assays conducted on reference Streptococci showed an antibacterial activity of *H. bocconei* extracts at different concentrations compared to those reported by Puglisi et al. (2009): while the butanolic extract gave the same result, the concentration of methanolic and water extracts reported by Puglisi were higher and much lower, respectively. Despite the discordant data, the antibacterial activity of the butanol extract against Streptococci could support the traditional use of this plant for the treatment of respiratory diseases in cattle: a concomitant immunomodulating, immunostimulating anti-inflammatory but also antibacterial activities (Erdemoglu et al., 2003).

Although the butanol and methanolic extracts of *Helleborus* at 125 μg/ml are capable of killing Streptococci, this concentration is toxic for the PBMCs and cause haemolysis. However, these data suggest the possibility of using these extracts as disinfectants for surfaces.

On the other hand, it was observed that *Helleborus* extracts had no effect on bacteria isolated from MS controls. This data is very important as it demonstrates that *Helleborus* extracts do not modify the growth of bacteria with anti-inflammatory action (*Sutterella, Bacillus, Pediococcus*) should they be used in MS therapy.

The assay of stimulation with bacterial LPS showed that both concentrations of the butanol and aqueous extracts reduced IL1β and iNOS expression compared to results with LPS alone. In contrast, cells treated with the methanolic extract showed a pro-inflammatory immune response pattern: an increment of IL1β, TNFα, iNOS expression.

The IL-1β overproduction or chronic exposure is known to contribute to disease pathogenesis in rheumatoid arthritis, gout, inflammatory bowel disease, and type 2 diabetes, in which pharmacological inhibition of IL-1β signaling directly or indirectly limits disease progression (Dinarello et al. 2012).

The importance of IL-1 signaling, and specifically IL-1β-driven neuroinflammation in MS and EAE, was discussed for years. Growing evidence supports the hypothesis that encephalitogenic Th17 cells recruited to the CNS are critical for EAE pathogenesis, and interestingly IL-1β signaling is recognized in supporting the expansion of pathogenic Th17 cells (Sutton et al. 2006; Chung et al. 2009; El-Behi et al. 2011). The IL-1 role in demyelination and oligodendrocyte injury seems more indirect: IL-1β does not kill oligodendrocytes in pure cultures, while it is a potent activator of damaging-myelin microglia and macrophages. Furthermore, IL-1β may not

only contribute at increasing TNF release by acting on astroglia, but may also cause the progressive loss of oligodendrocytes by inducing glutamate excitotoxicity through downregulation of glial glutamate transporters (Musella et al. 2020).

A genetic composition was identified in which MS patients with a higher IL-1β over IL-1Ra (which competes for IL-1R1 and inhibits the actions of both IL-1α and IL-1β) ratio have an increased risk for relapse-onset MS and where families have a 2.2-fold risk of developing relapsing-remitting MS (RRMS) (de Jong et al. 2002). Indeed, IL-1β protein levels are elevated in chronic active lesions of MS patients (Cannella and Raine 1995). Moreover, the involvement of IL-1β in MS was reported with data correlating elevated IL-1β levels in the cerebral spinal fluid (CSF) and blood of MS patients with increased cortical lesion load and disease severity (Mellergård et al. 2010; Seppi et al. 2014).

The proinflammatory cytokine IL-1β was implicated in perpetuating immune responses and contributing to disease severity in a variety of CNS diseases ranging from multiple sclerosis, neurodegenerative diseases, traumatic brain injury, and diabetic retinopathy. Moreover, pharmacological blockade of IL-1 signaling has shown to be beneficial in some autoimmune and autoinflammatory diseases, making IL-1β a promising therapeutic target in neuroinflammatory conditions (Mendiola and Cardona, 2018).

Regarding iNOS gene expression, multiple cell types, including hypertrophic astrocytes, macrophages/ microglia, and endothelial cells, play a role in the NO production in highly acute MS lesions. Astrocytes are the predominant cell type in age lesions and have an important immunoregulatory role in CNS inflammation. The close proximity of astrocytes to the blood brain barrier and the expression of iNOS in activated astrocytes support the possibility that astrocyte may contribute to vasodilation and damage to the blood brain barrier in MS, in addition to immunoregulatory and cytotoxic roles (Liu et al, 2001).

In this context, a natural extract that would allow the significant reduction of IL-1β and iNOS mRNA expression could be useful to study a natural therapy finalized to lowering the inflammation levels typical of MS.

With regard to the anti-viral activity of *H. bocconei* extracts, this is the first time it was investigated. The anti-viral activity of *H. bocconei* extracts was tested on three Herpes viruses: BHV-1, CpHV-1 and EHV-1. Herpesviridae is a large family of enveloped DNA viruses and is spread worldwide causing health problems in humans and animals (Azab et al. 2018). In particular, several species of Herpes were described as risk factors for the development of MS.

The anti-viral test results showed that the butanol extract caused a cytopathic effect reduction in the post-infection assay, and did not allow the virus to induce cytopathic effect in cells treated simultaneously with *Helleborus* and virus.

To date, drugs used against Herpes virus inhibit viral replication or viral genome synthesis.

The lack of cytopathic effect in the cells treated simultaneously with the *Helleborus* butanol extract and with the three different viruses, suggests that the latter are unable to penetrate inside the cell. Herpes viruses employ glycosaminoglycan as initial attachment receptors during infection of their host cell. Polyphenols were reported to target Herpes glycoproteins that interact with GAGs and prevent them from associating with cell surface GAGs and subsequent binding receptors. This inhibitory effect appears to occur both against the virus, both during the viral attachment and fusion phases and in the spread of the intercellular junction of HSV-1, which is mediated by its glycoproteins. Phytochemicals that possess antiviral activity against HSV types are flavonoids, alkaloids, saponins, terpenes, quinones, lignans, polysaccharides, tannins (Sayed et al., 2018) of which *Helleborus* species are rich. Quercetin significantly reduced HSV infectivity inhibiting the expression of HSV proteins (gD, ICP0) and genes (ICP0, UL13, UL52) (Lee et al., 2017). This data could also give an answer to the result of the post-infection experiments with the butanol extract that caused a reduction of the cytopathic effect or an antiviral action against CpHV suggesting that the Helleborus extract inhibits the protein expression of the virus already penetrated into the cells.

CONCLUSION

Studies of correlation between MS and environmental factors in different geographical areas can help us to understand how environmental and climatic factors influence the prevalence of MS. The identification and characterization of microbes or microbial consortia responsible for intestinal dysbiosis in MS patients is of fundamental importance in order to identify future intervention strategies that modulate the intestinal microbiota in a rational and evidence-based way. This study, for the first time, investigated the composition of the gut microbiome of MS patients and their household relatives who came from a delimitated geographical location. Analyses conducted in this study demonstrated a state of intestinal dysbiosis in patients with MS compared to controls living under the same environmental conditions.

The preliminary data here presented need further studies in order to better investigate the role and mechanisms of action of *Helleborus bocconei* in MS therapy.

However, these data demonstrate that butanol or water extract (extracts with greater biological properties) can potentially act on different levels for the decrease of the inflammation characteristic of MS.

In fact, *Helleborus* did not negatively affect intestinal bacteria useful as probiotics (*Sutterella* and *Pediococcus*) and induce a significantly reduction of IL-1β and iNOS gene espression.

Cytokines are crucial mediators of the inflammatory response and, for this reason, they have been considered ideal targets since the early development of therapies.

Furthermore, the antiviral activity of the butanol extract of *Helleborus*, investigated for the first time, may also be of great interest in order to identify alternative therapies against Herpes viruses and other human viruses as viral epidemics and pandemics recur throughout history

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