Review paper

SPIROPLASMAS AS CAUSATIVE AGENTS OF HONEY BEES DISEASES

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A b s t r a c t

Spiroplasmas are bacteria that play a significant role in the development of bee diseases. Infections of bees with spiroplasmas have been registered in Europe, America and Asia, and such infections occur through contaminated food, the sting of *Varroa destructor*, and vertically. The most important species that cause bee spiroplasmosis are conditionally pathogenic bacteria *Spiroplasma apis* and *Spiroplasma melliferum*. *S. apis* causes the neurological "May disease" which occurs in France, and *S. melliferum* causes the bee crawling disease which occurs in China; these two diseases stand out based on the specific symptoms and place of occurrence. Spiroplasmas are widely distributed in nature and relatively resistant to desiccation and the action of other external factors. The most important reservoir of spiroplasmas are insects, where they normally inhabit the digestive tract and have a beneficial role for the host, but some can also be pathogenic. All sources of spiroplasmas in the environment are still not known and insufficiently studied. Considering the increasing negative impact of non-specific factors affecting bees, the risk of diseases caused by spiroplasmas is increasing. In order to prevent the occurrence of diseases caused by spiroplasmas, it is important to study further the sources of spiroplasmas and other factors significant for the occurrence of infection and to implement appropriate measures of good beekeeping practice, good veterinary practice and to strengthen the immune system of bee colonies.

Keywords: honey bee, May disease, *Spiroplasma apis*, *Spiroplasma melliferum*, spiroplasmosis

INTRODUCTION

Spiroplasmas are bacteria that play a significant role in the development of bee diseases. Infection of bees with these microorganisms occurs through contaminated food, and a vertical way of transmission in insects has also been established (Moore & Ballinger, 2023). The possibility of their transmission to bees through the sting of the mite *Varroa destructor* also exists, because spiroplasmas were isolated from this mite (Hubert et al., 2015; Hubert et al., 2016; Koç, 2024). Infections of bees with spiroplasmas have been registered in Europe, America and Asia (Schwarz et al., 2014; Zheng & Chen, 2014; Pavlović et al., 2023).

Spiroplasma apis (serological group IV, Apis clade) and *Spiroplasma melliferum* (serological group I-2, Citri clade) are species that cause honey bee diseases, called spiroplasmosis (Clark, 1977, 1978; Mouches et al., 1982; Mouches et al., 1983; Clark & Whitcomb 1984; Mouches et al., 1984; Gasparich et al., 2004). While direct effects of *S. apis* infections on honeybees have been established (Mouches et al., 1982, 1983), infections caused by *Spiroplasma melliferum* are less well characterized and clear (Clark, 1977, 1978; Clark & Whitcomb, 1984). From the spiroplasmas caused by *S. apis*, the neurological "May disease" occuring in southwestern France stands out due to its specificity of symptoms, time and place of occurrence (Mouches et al., 1982; Mouches et al., 1983; Bové, 1984; Mouches et al., 1984, Gasparich et al., 2004; Schwarz et al., 2014). *S. melliferum* also causes neurological diseases that end in bee death but the symptoms of the disease are less specific and clear compared to the diseases caused by *S. apis* (Clark, 1977; Clark et al., 1985; Tully et al., 1987; Williamson et al., 1998; Meeus et al., 2012; Schwarz et al., 2014). Among the diseases caused by *S. melliferum*, the bee crawling disease occurring in China stands out due to its specificity of symptoms and place of occurrence (Yang et al., 2017).

Spiroplasmas are most often found as commensals in the bee's intestines, but some species can pass the intestinal wall, reproduce in the hemolymph, leading to the appearance of

nervous symptoms and the death of the bees. Spiroplasmas are most often found in the intestines, less often in the hemolymph, and occasionally in the salivary glands and other organs (Bové, 1997). Spiroplasmas are important phyto and insect pathogens and they are considered to play a role in the development of transmissible spongiform encephalopathies (TSE) in humans and animals (Reyes & Hoenig, 1981; Bastian et al., 2001; Gasparich, 2002; Nunan et al., 2005; Regassa & Gasparich, 2006; Bastian et al., 2012; Bastian, 2014; Cacciola et al., 2017). Not all the spiroplasma reservoirs in nature are known, and thus further research should be done in order to understand and prevent the diseases they cause in plants, animals and humans (Bastian et al., 2012). Spiroplasmas show great variation in mode of transmission, tissue tropism and effects on insects, so they can be intestinal commensals, pathogens and maternally transmitted symbionts (Ballinger & Perlman, 2019). Due to its resistance to the external environment and ability to form biofilms on various surfaces, further investigation of such spiroplasma sources in the environment as pollen, nectar, plant, soil, diseased and dead bees, bee brood, beekeeping equipment, would be useful to understand the sources of the infection bee with spiroplasmas (Tully & Whitcomb, 1992; Bastian et al., 2012).

CHARACTERISTICS OF SPIROPLASMAS

Spiroplasmas are bacteria classified in the genus *Spiroplasm*a, family *Spiroplasmataceae*, order *Mycoplasmatales* (syn. *Entomoplasmatales*), class *Mollicutes,* phylum *Tenericutes* (syn. *Mycoplasmatota*) (Brown et al., 2015; Cacciola et al., 2017; Brown et al., 2018; Gasparich et al., 2020; Oren & Garrity, 2021). They are pleomorphic, with variations in size and shape - from spiral and branched non-helical strands to spherical or ovoid shapes. They most often occur in a spiral form and less often in the form of branched non-helical threads. Spiral forms are usually 100-200 nm in diameter and 3-12 μm in length, present during the exponential phase of growth, but in some species they persist during the stationary phase (Williamson et al., 1991; Harne et al., 2020). Spiroplasmas are motile and do not have a cell wall. They are covered only with a cell membrane containing cholesterol (Razin et al., 1973; Harne et al., 2020). Cytoskeletal protein fibril with a molecular mass of about 59 KDa is specific to spiroplasmas and responsible for their helical shape (Williamson, 1974; Townsend & Archer, 1983; Williamson et al., 1991; Harne et al., 2020). A cytoskeleton is attached to the cell membrane, which controls the spiral shape and mobility of the cell (Trachtenberg, 1998; Trachtenberg & Gilad, 2002; Regassa, 2014). In their spiral form, they move by flexion and twitching movements, as well as by rotation. Flagella, periplasmic fibrils, or other organelles for movement are absent (Gasparich, 2020). Spherical cells that are typically ~300 nm in diameter and also non-helical filaments are often present in the stationary phase. Some species can change from spiral to coccoid form after entering host cells. Spiroplasmas pass through a 220 nm filter (Cole et al., 1973; Davis & Worley, 1973; Davis, 1981; Ammar et al., 2004., Yu et al., 2009; Meeus et al., 2012; Duret et al., 2014; Regassa, 2014; Schwarz et al., 2014; Gerth et al., 2021). Cells divide by binary fission, with a division time of 0.7-37h. Dark-field light microscopy is commonly used to observe unstained live spiroplasmas in liquid (Boudet et al., 2018; Harne et al., 2020).

Spiroplasmas are facultatively anaerobic, hemoorganotrophic and ferment glucose. They require cholesterol for their growth and do not hydrolyze urea (Tully et al., 1987; Whitcomb et al., 1996; Rivera et al., 2013). Spiroplasmas are resistant to penicillin and rifampicin and sensitive to erythromycin and tetracycline. The optimal growth temperature varies depending on the species, from 5 to 41°C (Konai et al., 1996; Regassa et al., 2014; Williamson et al., 2015, Gasparich et al., 2020). Konai et al., (1996) examined sixty-four strains of spiroplasma from twenty-four groups and eleven subgroups. The optimal temperature for the growth of twenty-three strains of spiroplasma was 30°C, for twenty-nine strains 32°C, and for thirteen strains 37°C. Colonies on a solid medium are usually diffuse, with irregular shapes and edges, which is a consequence of cell movement. Colony characteristics significantly

depend on the concentration of agar in the nutrient medium. The size of the colonies varies from 0.1 to 4.0 mm in diameter (Gasparich, 2010; Regassa et al., 2014; Williamson et al., 2015; Gasparich et al., 2020). Primary growth requires media containing broth base for the cultivation of mycoplasma, serum and other supplements; after adaptation, they can often grow in less complex media (Konai et al., 1996; Regassa, 2014; Williamson et al., 2015; Gasparich et al., 2020; Fukatsu et al., 2024).

Spiroplasma virulence factors are host cell adhesion factors and invasion proteins (Regassa & Gasparich 2006). Spiralins-membrane proteins, lectins participate in the process of interaction of spiroplasma with hosts (Duret et al., 2003; Killiny et al., 2005; Masson et al., 2022). The fimbriae and pili-like structures observed on the cell surface of insect and plant pathogenic spiroplasmas are thought to be involved in attachment to host cells but not in movement (Gasparich et al., 2020). Spiroplasma pass the insect gut barrier and reach the hemolymph, where they multiply and kill the bee (Regassa & Gasparich, 2006). Examination of the *S. apis* B31T genome revealed a circular chromosome of 1,160,554 bp in size, and the presence of plasmids was not determined (Ku et al., 2014). In addition to chromosomal DNA, some strains of *S. melliferum* possess plasmids (Lo et al., 2013). *Spiroplasma citri* and *S. melliferum* can ferment trehalose, which is the main source of sugar and carbon in insect hemolymph (Chang & Chen 1983; Bolaños et al., 2015). Unlike the plant-pathogenic spiroplasma *S. citri*, the insect-pathogenic spiroplasma *S. melliferum* has additional genes that allow it to utilize cellobiose, chitin, and N-acetylmuramic acid. Such traits allow it to adapt to its honey bee host; the ability to degrade chitin facilitates invasion of host tissues and the ability to utilize cellobiose facillitates access to partially hydrolyzed pollen cell walls. Toxins of spiroplasmas, in addition to playing a role in pathogenesis, mediate the manipulation of the host to maintain itself as a symbiont. *Spiroplasma poulsonii* induces the killing of male *Drosophila melanogaster* using the toxic protein androcidin - Spaid toxin that damages the male X chromosome (Harumoto & Lemaitre, 2018; Arai et al., 2022; Moore et al., 2023). ETX/MTX2 toxins - β-pore forming proteins which damage cell membranes, have cytocidal and insecticidal activity, but their role in host-*Spiroplasma* interactions is not sufficiently known (Moar et al., 2017; Lassalle et al., 2020; Moore et al., 2023).

Spiroplasmas can create biofilms on different mica, steel, and nickel surfaces, and they stick to them with fibrillar threads and amyloid protein, which is an integral part of the biofilm. In the form of biofilms, spiroplasmas are significantly more resistant to the action of such stress factors as heat shock, and osmotic shock. Spiroplasmas in biofilms are more resistant to drying or exposure to 50% glutaraldehyde (Bastian et al., 2012). Biofilm strengthens the bonds between bacteria (Bastian et al., 2012). Spiroplasmas in the biofilm change from spiral to coccoid form and are interconnected by long (>2 μm) branched membrane nanotubules (Bastian et al., 2012). Spiroplasmas bound to clay can survive in the soil, which can be a source of infection for bees, as well as for the occurrence of TSE (Bastian et al., 2012). Spiroplasmas have been found to form biofilms on stainless steel wire (Bastian et al., 2012), which indicates the need to investigate the possibility of biofilm formation and survival of spiroplasmas on the surface of beekeeping utensils and beekeeping equipment made of this material. Spiroplasmas are able to survive on the leaf surface despite exposure to such adverse environmental conditions as desiccation (Tully & Whitcomb, 1992).

Pathogenicity of spiroplasmas for plants, animals and humans

Spiroplasmas are widely distributed in nature, but not all their habitats and sources in nature are known (Bastian et al., 2012). Spiroplasmas were originally isolated as plant pathogens, but their primary association is with arthropods, especially insects that serve as the main reservoir for spiroplasmas. *S. citri* causes citrus stubborn diseases and was the first spiroplasma to be identified and fully described (Saglio et al., 1973; Fletcher et al., 2005; Carpane, 2007; Williamson et al., 2015; Gasparich et al., 2020; Fukatsu et

al., 2024). Spiroplasmas have been isolated from a wide range of hosts, mainly insects and plants (Clark et al., 1982; Clark et al., 1984; Clark et al., 1985; Saillard et al., 1987; Regassa & Gasparich, 2006; Regassa et al., 2009; Schwarz et al., 2014). Spiroplasmas have been isolated from the surface of plant flowers and other plant parts, from the intestines and hemolymph of various insects and crustaceans, from ticks, the ovaries of insects, and the phloem sap of plants that insects feed on (Mouches et al., 1982; Mouches et al., 1984; Cisak et al., 2015; Williamson et al., 2015).

A greater number of spiroplasma species are commensals - normal inhabitants of the digestive tract of insects, spiders, ticks, mites, crustaceans or plants, while a smaller number of spiroplasma species are pathogenic for these organisms (Regassa & Gasparich, 2006; Regassa et al., 2009; Schwarz et al., 2014). In order to maintain spiroplasmas in the host organism, they must have the ability to bind to the midgut epithelial cells (Özbek et al., 2003; Ammar et al., 2004; Ashida et al., 2011; Fünfhaus et al., 2018; Zha et al., 2018). The diversity of the honey bee gut microbiota and therefore the presence of spiroplasma in the gut of bees varies significantly depending on the developmental stage of the bee, the location in the bee gut, and the honey bee species. The abundance of individual bacteria in the midgut and hindgut of adult honey bees *A. cerana* did not differ, while the differences were significant in *A. mellifera* (Ahn et al., 2012). Some strains of spiroplasma can have a positive effect on their hosts by conferring resistance against pathogens (Ballinger & Perlman, 2019; Hrdina et al., 2024). Some spiroplasma species protect their insect hosts from natural enemies, including parasitoid wasps, nematodes and pathogenic fungi (Lo et al., 2013; Cisak et al., 2015).

Spiroplasmas' relationship with hosts can also be pathogenic (Williamson et al., 1975; Mouches et al., 1982; Saillard et al., 1987; Wedincamp et al., 1996; Goodacre et al., 2006; Bi et al., 2008; Gasparich, 2010; Bastian et al., 2012; Lo et al., 2013; Watanabe et al., 2014). The pathogenicity of spiroplasmas to insects depends on their ability to penetrate the intestinal epithelium, invade the hemocoel and other host tissues and multiply in the hemolymph (Bové, 1997; Regassa & Gasparich, 2006; Gasparich, 2010; Rivera et al., 2013; Duret et al., 2014; Schwarz et al., 2014; Bolaños et al., 2015; Fünfhaus et al., 2018). Spiroplasmas are regularly found in hematophagous insects and ticks, where they multiply and are found in large numbers in the salivary glands (Longshaw, 2011; Cisak et al., 2015). However, spiroplasmas are primarily found in the hemolymph, followed by the fat body and salivary glands of insects (Steiner et al., 1984; Dera et al., 2023). Spiroplasmas can live both intracellularly and in the hemolymph of arthropods, which influences the characteristics and symptoms of the disease (Clark & Whitcomb, 1984; Eskafi et al., 1987; Mouches et al., 1984; Nunan et al., 2004; Schwarz et al., 2014). Spiroplasmas cause tremor in crustaceans, lethargic insect disease, and higher male mortality in populations of fruit flies, butterflies and insects. Spiroplasmas are pathogenic to suckling rodents and chicken embryos under experimental conditions (Regassa, 2014; Williamson et al., 2015). They can cause diseases in rodents (Tully et al., 1976; Tully et al., 1984; Bastian et al., 2012). *Spiroplasma mirum*, which was isolated from ticks, is also pathogenic to vertebrates. *S. melliferum*, in addition to being pathogenic to insects, primarily honey bees, is also pathogenic to vertebrates (Chastel et al., 1991; French, 2011).

Infections of plants with spiroplasmas lead to citrus disease and corn disease, while in insects they lead to disruption of the sex ratio and mortality of honey bees (Saglio et al., 1973; Clark, 1977; Fukatsu et al., 2024; Toloy et al., 2024). One of the most important corn diseases in America is corn stunting, which is caused by *Spiroplasma kunkelii* (Whitcomb et al., 1986; Carpane, 2007; Toloy et al., 2024). Spiroplasmas that live in arthropods are transferred to plant surfaces through defecation or regurgitation of liquid on the plant surface, or introduced into the phloem sap of the plant during sucking.

The role of *Spiroplasma* spp. as opportunistic pathogens of humans should not be overlooked. Some species are associated with such animal and human diseases as TSE and Creutzfeldt-Jakob disease (Bastian et al., 2001; Gasparich, 2002;

Regassa & Gasparich, 2006; Bastian et al., 2012; Cacciola et al., 2017). Such spiroplasma infections as cataract and uveitis have also been identified in infant humans (Lorenz et al., 2002; Matet et al., 2020; Farassat et al., 2021) and systemic infections in immunocompromised patients (Aquilino et al., 2015; Etienne et al., 2018).

Prevalence and factors influencing the occurrence of spiroplasmosis in bees

S. apis and *S. melliferum* are pathogens that cause neurological diseases and increased mortality in honey bees (Clark, 1977, 1978; Mouches et al., 1982, 1984). Spiroplasma infections in honey bees have become a global problem. These infections have been registered in Europe (Ravoet et al., 2013; Ravoet et al., 2014; Cilia et al., 2022; Pavlović et al., 2023), Asia (Li et al., 2012; Yang et al., 2017) and America (Badillo et al., 2014; Schwarz et al., 2014; Zheng & Chen, 2014). *S. apis* and *S. melliferum* are not part of the typical honeybee microbiota, i.e. they are facultative symbionts, but they can be found in certain regions and weather conditions and lead to honeybee diseases (Jaenike et al., 2010; Schwarz et al., 2014).

The prevalence of infection *Apis mellifera liguistica* bee colonies with *S. apis* and *S. melliferum* was 33% in the state of Maryland (USA) and 54% Africanized honey bee *A. mellifera scutellata* colonies in eleven states of Brazil - with the most samples originating from the states of Santa Catarina and Sao Paolo (116 of 139 samples) (Schwarz et al., 2014). Annual and seasonal variations in the prevalence of *S. apis* and *S. melliferum* were found in bee colonies from the USA (temperate continental climate) and Brazil (tropical and subtropical climates) (Schwarz et al., 2014). *S. apis* and *S. melliferum* are transmitted primarily during spring and summer, thought to be via fecal contamination of flower surfaces by infected hosts (Schwarz et al., 2014). In American bee colonies, the emergence of *S. melliferum* occurs with the simultaneous peak of natural flower grazing and colony population density (Winston, 1992; Schwarz et al., 2014). Minor seasonal variations in the prevalence of *S. melliferum* in Brazil compared to the USA may be related to tropical climate conditions, where winters are mild and the temperature during all months does not drop below 18°C (Cerveny et al., 2006; Schwarz et al., 2014).

The long flowering period of plants in tropical and subtropical climate zones provides a greater possibility of transmitting spiroplasma to the honey bee (Altizer et al., 2006; Schwarz et al., 2014). *S. melliferum* grows in the range of 20 to 37°C, and the optimum temperature for growth is 32 to 35°C (Clark, 1985). Conditions that affect the annual flowering cycle of plants, higher ambient temperatures, climate, and lower rainfall, contribute to the occurrence of *S. melliferum* infection (Schwarz et al., 2014).

Bees are also infected with spiroplasmas during periods when plant flowering is minimal - during dry spring and summer, which indicates that, in addition to plants, there are other sources of bee infection with these microorganisms (Schwarz et al., 2014). When it comes to prevalence with *S. apis*, there was minimal seasonal variation and less association with flowering plants for this species in the USA (Schwarz et al., 2014). This may be related to the large temperature range in which *S. apis* can grow (5°C-41°C; optimal 30°C), but also to the existence of other sources of infection (Mouches et al., 1983; Konai et al., 1996; Schwarz et al., 2014).

The ability of spiroplasmas to withstand a wide range of environmental temperatures, as well as the temperatures of insects, ticks, and plants, significantly influences their distribution in nature (Konai et al., 1996). Since *S. apis* and *S. melliferum* are found in honeybees throughout the year in colonies in the USA and Brazil, their pathogenicity is most likely not limited to the spring period. Each of these species has its own prevalence dynamics that depend on weather, geographical and climatic conditions (Whitcomb et al., 1996; Schwarz et al., 2014). Climatic conditions play a key role in the prevalence of diseases and parasites affecting honey bees (Giliba et al., 2020; Rowland et al., 2021; Tennakoon et al., 2024). Temperature fluctuations significantly affect the survival rate of bees (Sarmad & Ryšánek, 2025; Vincze et al., 2025).

The percentage of identified coinfections caused by *S. melliferum* and *S. apis* in Brazil (52%) and the

USA (16.5%) indicates the need to investigate the joint impact of these bacteria on bee immunity. Spiroplasmas can suppress the host's immune response, thus increasing the host's susceptibility to infection by other microorganisms (Schwarz et al., 2014). Infections caused simultaneously by both types of spiroplasma have a stronger negative effect on the health of bees (Mouches et al., 1982). Schwarz et al., (2014) conducted studies on spiroplasma on whole bee samples, not on separate bee gut and hemolymph samples. Separate samples would have provided more precise data on the percentage of infected bees, i.e. sick bees.

Zheng and Chen (2014) also showed high variability in the prevalence of honey bee infections with *S. melliferum* in Beltsville, MD, USA during the year, with the maximum prevalence in the month of May. Colony prevalence increased from 5% in February to 68% in May, then decreased to 25% in June and 22% in July. Bees are infected through plants when collecting nectar and pollen from the infected and contaminated surface of flowers (Hanshou et al., 2008; Gasparich, 2010; Longshaw, 2011; Zheng & Chen 2014; Fünfhaus et al., 2018; Pavlović et al., 2023). Ravoet et al., (2013) first identified the presence of *S. apis* and *S. melliferum* in honey bee samples in Europe (Belgium) in the summer of 2011. *S. apis* was detected in one (0.3%) and *S. melliferum* in 16 (4.4%) of the 363 samples examined. The authors associated the finding of *S. melliferum* with 6.5% of winter losses of bee colonies. Pavlović et al., (2023) found high variation in the prevalence of infection with *S. apis* in the honey bee, with the highest prevalence in May during the one-year study period. Colony prevalence increased from 2% in March to 61% in May, then decreased to 22% in June and 20% in July.

The presence of spiroplasma should always be investigated when the three causative agents of bee diseases are present - *Nosema ceranae*, *Varroa destructor* and *Varroa destructor virus-1* (Schwarz et al., 2014). The health status of a bee colony infected with spiroplasmas will be worse if it is simultaneously attacked by parasites and viruses, due to the weakening of the immune response (Hedtke et al., 2011; Cornman et al., 2012; Schwarz & Evans 2013). Different pathogens often infect bee colonies at the same time, which makes these colonies even more vulnerable to disease (Evans & Schwarz, 2011; Lannutti et al., 2022). Spiroplasmas can be primary causes of disease or secondary - they exacerbate the effects of such causes as parasites, viruses, poor nutrition and exposure to chemicals (Maharramov, 2015). Spiroplasmas are opportunistic pathogens and prevalence does not depend only on the presence of a environment source of infection, but also on the influence of climatic factors, temperature, nutrition and immunity of bees, etc. One of the most stressful periods for a bee colony is the wintering period, when its food options are reduced (Plavša & Pavlović, 2017; Fünfhaus et al., 2018; Van Herzele et al., 2024). The occurrence and characteristics of diseases caused by *S. apis* and *S. melliferum* depend on the degree of virulence of these bacteria, the immunity of the bees, and the influence of other biotic and abiotic factors (Cox-Foster et al., 2007; van Engelsdorp et al., 2009; Evans & Schwarz, 2011). The presence of spiroplasmas in the hemolymph is a sign that the bees are infected and that they represent an reservoir in which spiroplasmas multiply. These bees excrete spiroplasmas through the secretions of their glands, as well as through feces (Costa-Leonardo & Silva de Moraes, 1985; Schwarz et al., 2014). Honeybee colonies are reservoirs of *S. apis* and *S. melliferum* throughout the year and during periods of low prevalence (Clark & Whitcomb 1984; Schwarz et al., 2014).

Pathogen species *S. apis*, as well as *S. melliferum* were detected in adjacent wild bees, which suggested a spillover effect of pathogens leading to infection in wild pollinator populations, as well as honey bees infections via wild bees (Ravoet et al., 2014; Gamboa et al., 2015; Nanetti et al., 2021; Jones et al., 2022). Microorganisms that cause disease in honey bees also replicate in wild bees and can shorten their lifespan (Piché-Mongeon & Guzman-Novoa, 2024). *S. melliferum* was isolated from two bumblebee species, *B. pratorum* and *B. pascuorum,* and *S. apis* was isolated from *B. pratorum* (Meeus et al., 2012; Schwarz et al., 2014). Cilia et al., (2022) identified seventy-two wild pollinator species as potential hosts of honey

bee pathogens. During the examination of 712 samples, of which 700 were from wild pollinators, *S. apis* was found in 2.1% and *S. melliferum* in 2.7% of samples. Twelve tested samples of honey bee A. *mellifera* were negative for the presence of *S. apis* and *S. melliferum*. These studies have indicated the circulation of honeybee pathogens in wild pollinator entomofauna in two regions in northern Italy (Cilia et al., 2022). A high prevalence of *S. apis* was found in the northeastern USA in the solitary bee *Eucera pruinosa* (75%) and bumble *B. impatiens* (73%), in a study that included hundreds of samples (Piché-Mongeon & Guzman-Novoa, 2024).

Characteristics of spiroplasmosis called May disease caused by *S. apis*

S. apis was isolated the first time from honey bee colonies (*A. mellifera*) affected by May disease, in the department of Landes in southwestern France (Mouches et al., 1982; Mouches et al., 1983). These pathogen was isolated from the hemolymph and gut lumen of adult honey bees (Raju et al., 1981; Mouches et al., 1982), which displayed bloated abdomens filled with undigested pollen and body quivering (Mouches et al., 1982). Beekeepers have named this disease May disease because it occurs in the May-June period in the spring (Mouches et al., 1982). However, this disease also occurs in other parts of the world (Lolin, 1991; Plavša & Pavlović, 2018; Pavlović et al., 2023); it also occurs in Montenegro, but no research has been conducted into the causes of its occurrence (Koprivica, 2011). Tomašec (1955) also describes May disease, stating that it occurs predominantly in May, but also in other spring months. Since spring infection of bees with *S. apis* has also been identified in parts of the world where May is not a spring month, such as Brazil (Schwarz et al., 2014), for the purpose of easier study and comparison, the name of "spring disease" seems more appropriate instead of May disease. Infection caused by *S. apis* is lethal to honey bees when ingested and can be spread through faecal contamination (Mouches et al., 1982; Nanetti et al., 2021).

Symptoms of the May disease disease exhibited by the affected bees were nervous disorders, loss of the ability to fly and finally high mortality in a few days. Bees do not lose hair, do not become black and shiny, as is the case with chronic paralysis of bees, which also often occurs in spring. At the peak of the disease, thousands of bees die within four to five days, with the bee population decreasing by more than 25%. Although the disease is severe, colonies usually recover spontaneously in July (Mouches et al., 1982). A high titer of *S. apis* was found in the hemolymph of dead bees (Mouches et al. 1982; Mouches et al., 1983; Ku et al., 2014).

Honey-bee May disease can be also suspected on the basis of intestinal constipation and hard consistency of feces, behavior of young nurse bees and weather conditions when the disease occurres. As a result of constipation, young nurse bees die at the age of 6-13 days (Mouches et al., 1982; Plavša & Pavlović, 2017; Fünfhaus et al., 2018; Van Herzele et al., 2024). Affected bees have a visibly swollen abdomen, try to fly, flap their wings and fall in front of the hive (Mouches et al., 1982). Thousands of diseased bees die in a very short time with convulsive movements, and dead bees are seen in front of the hive. Sick bees very often die unnoticed, far from the hive, so beekeepers often cannot see the external signs of may disease. In addition to young bees, the bee colony also perishes, because sick young bees cannot feed it (Tomašec, 1955; Mouches et al., 1984; Lolin, 1991; Plavša & Pavlović, 2017; Fünfhaus et al., 2018; Van Herzele et al., 2024).

Experiments have shown that artificial infection of honey bees with *S. apis* bacteria via injection or food can lead to classic symptoms of May disease within a few days (Mouches et al., 1982; Mouches et al., 1984). Artificial infection of honeybees with a concentration of 10^2 *S. apis* in a volume of 2.5 µl resulted in death of all infected bees within nine days. Artificial infection of bees with a concentration of 10^8 resulted in death within four days. Infected bees died more rapidly at a temperature of 38°C than at 21°C and 26°C. Higher mortality at this temperature can be associated with exposure of bees to temperature stress, considering that the optimal temperature for bees life is 20-30°C (Vincze et al., 2025). In the time period of twelve hours before death,

infected bees showed such symptoms as nervous disorders, inability to fly, and crawling. When the hemolymph of the dead bees was inoculated on BSR spiroplasma medium (Bové & Saillard, 1979), $2x10^9$ CFU of spiroplasmas were detected per bee (Mouches et al., 1982). In the hemolymph of bees fed with spiroplasma-contaminated honey, a large number of spiroplasmas were found four days after infection, and the bees also showed symptoms of nervous disorder (Mouches et al., 1982).

The large number of disorders that occur with this disease indicates that its occurrence is most likely influenced by numerous factors (Mouches et al., 1982). Inadequate nutrition, cold and rainy weather and other non-specific factors, as well as the bacterium *S. apis* participate in the emergence of May disease (Mouches et al.,1983; Plavša & Pavlović, 2017; Fünfhaus et al., 2018; Van Herzele et al., 2024). However, additional research is needed to determine the causal link between spiroplasma infection and May disease, as well as other disorders or death of honey bees. To this end, it is necessary to conduct experiments based on the infection of bees in laboratory conditions (Fünfhaus et al., 2018).

Characteristics of spiroplasmosis caused by *S. melliferum*

Spiroplasmosis caused by *S. melliferum* is less well known but is thought to lead to the death of adult bees after oral infection. Infection with *S. melliferum* has similar symptoms to infections with *S. apis*, although they are less intense (Clark, 1978; Mouches et al., 1982). *S. melliferum* is also a causative agent of neurological diseases in bees during the spring (Clark, 1978; Mouches et al., 1982; Clark et al., 1985; Nanetti et al., 2021).

S. melliferum was the first spiroplasma isolated from an insect, from honeybees in Maryland, USA in 1976. This spiroplasma was discovered in hemolymph of the honey bees in Maryland and was associated with high mortality rates of bee colonies. The organism has also been isolated from bumblebees, andrenids, anthophores, predatory flies, and butterflies (Clark et al., 1985). Spiroplasmas of group I-2 were also isolated from honey bees in southwestern France. These strains were isolated from the intestines of apparently healthy individual honey bees, but not from hemolymph (Mouches et al., 1984). However, under experimental conditions, isolate B25 of group I-2 spiroplasma was pathogenic and lethal when introduced to bees through injection or ingestion of food (Mouches et al., 1984). In the United States, spiroplasmas of group I-2 have shown a natural pathogenicity to honeybees (Clark 1977, 1978; Clark & Whitcomb, 1984).

S. melliferum is also one of the common causes of bee creeping disease in China. *S. melliferum* was first isolated in China from the honey bee *A. mellifera* in 1984 in bees that crawled and died near the hive, which is why the disease was called bee creeping disease (Yang et al., 2017). Bee creeping disease leads to large economic losses in beekeeping and agricultural production (Yang et al., 2017). *S. melliferum* CH-1 is capable of activating the immune response of the honey bee during the first phase of infection but also of bypassing the host's defense mechanisms at a later stage and completing its life cycle. They achieve this by reducing the levels of antimicrobial peptides in the bee's hemolymph (Yang et al., 2017).

Zoonotic potential of the bee spiroplasmosis

In addition to arthropods, human infection with *S. apis,* probably through stings of insects similar to hornets, have also been established (Etienne et al., 2018). This indicates the possibility of human infection with *S. apis* through other insects besides bees (Etienne et al., 2018). One affected person was an immunocompromised person with agammaglobulinemia (Etienne et al., 2018), and showed signs of non-specific dermatitis and arthritis (Etienne et al., 2018). Human infections with *S. apis* that were not transmitted through insect bites were also found - these infections occurred in people with lung transplants and were manifested in the form of hepatitis, and in premature infants in the form of inflammation of the choroid of the eye - uveitis (Mueller et al., 2015; Matet et al., 2020).

When it comes to *S. melliferum*, according to data from the literature, there are no registered cases of human infection. However, *S. melliferum*

A56 can replicate in both invertebrates and vertebrates (Chastel et al., 1991). The bee pathogen *S. melliferum* A56, after intracerebral inoculation in sucking mice, multiplied in a very high titer in the brain, where it remained for up to nine months. No antibodies were detected, indicating an "immunological tolerance" phenomenon. Compared to control mice, the replication of this strain resulted in certain lesions in the brain tissue as well as clinical symptoms. However, a better laboratory animal model needs to be found to study this spiroplasma as a possible cause of degenerative changes in the human brain (Chastel et al., 1991).

Spiroplasmas are associated with TSE: scrapie in sheep, chronic wasting disease (CWD) in deer, and Creutzfeldt-Jakob disease in humans (Bastian, 1979; Bastian & Foster, 2001; Bastian, 2005; Bastian et al., 2006; Bastian et al., 2007). *S. mirum* isolated from rabbit ticks, as well as spiroplasmas related to *S. mirum*, experimentally cause spongiform encephalopathy in rodents, deer, sheep, and goats (Bastian et al., 2006; Bastian et al., 2007, French, 2011; Cacciola et al., 2017). Spiroplasmas in biofilms attach to surfaces by means of curly fibrils, which are made of functional amyloid which is important for the entry of spiroplasmas into cells. The curly fibrils interact with host proteins and initiate the formation and accumulation of potentially toxic amyloid. These data justify the introduction of diagnostic tests for TSE based on the presence of spiroplasma-specific proteins or nucleic acids (Bastian, 2014). Since both *S. melliferum* and *S. mirum* reproduce well at 37°C, this is possibly the main prerequisite for spiroplasmas to invade mammalian tissues (Chastel et al., 1991, Konai et al., 1996).

The examination of 135 honey samples in Australia using the multiplex PCR method revealed a high prevalence of *S. melliferum* (70%), while *S. apis* was detected in 13% of the samples. Both species of spiroplasmas were detected in 12% of the samples. Spiroplasma infections pose a significant threat to honey bee health and should be monitored together with other stressors affecting bee populations. Detection of *Spiroplasma* DNA in honey by PCR does not provide information on whether it is DNA from living or dead *Spiroplasma* cells. In order to understand the role of contaminated honey in the transmission of infections, it is necessary to conduct research on the presence of living *Spiroplasma* and the possibility of their survival in honey. *S. melliferum* DNA was more often detected in bee samples than *S. apis* DNA (Schwarz et al., 2014) but also in honey samples (Bhasi et al., 2026). Research that would include comparative studies of bee samples, honey and the environment would be important for a better understanding of spiroplasma ecology and transmission dynamics. Given that some species of spiroplasmas have also been found to cause disease in humans, it is also important to determine the possibilities of their transmission through the consumption of contaminated honey (Bhasi et al., 2026).

Diagnostic of bee spiroplasmosis

A complicating factor in the diagnosis of spiroplasma-induced diseases is that spiroplasma-infected bees are difficult to identify by external signs. Laboratories do not routinely screen bee colonies for *S. apis* or *S. melliferum* by hemolymph culture, nor do they use the multiplex PCR technique that has recently become available (Meeus et al., 2012). In addition, specialized knowledge of dark-field microscopy is required to visualize the bacteria, but bees may die unnoticed far from the hive, making laboratory diagnosis impossible (Schwarz et al., 2014). This symptom indicates the need to consider and investigate spiroplasmas as one of the factors that lead to colony collapse disorder.

The diagnosis of spiroplasmosis of bees is confirmed in the laboratory by microbiological examinations, isolation and identification of the bacterium spiroplasma (Tully et al., 1987; Whitcomb et al., 1996; Alippi, 1999; Shimanuki & Knox, 2000; Meeus et al., 2012; Badillo et al., 2014; Schwarz et al., 2014; Rivera et al., 2018; Harne et al., 2020; Cleary & Szalanski, 2022; Turhan & Izol, 2024). They include the examination of basic morphological features, motility, confirmation of the lack of a cell wall, as well as the determination of biochemical, serological and molecular properties. In the hemolymph of bees, *S. apis* can be observed under a dark-field microscope or

a phase-contrast microscope, with an immersion objective. Hemolymph of adult bees is sampled by piercing the intersegmental membrane behind the hips of the first legs with the help of a thin capillary tube of a Pasteur pipette. Spiroplasma can be isolated on standard mycoplasma media and in Singh's medium used for mosquito tissue culture supplemented with 20% fetal calf blood serum (Alippi, 1999; Shimanuki & Knox, 2000). Electron microscopy reveals the lack of a cell wall and determines the dimensions of the cell (Bastian et al., 2012; Meeus et al., 2012). Because spiroplasmas are difficult to detect by light microscopy, which is also impractical when spiroplasmas are present in small numbers, atomic force microscopy is recommended for observing (Badillo et al., 2014). This microscopy provides realistic three-dimensional images of the surface of various macromolecules and cells with subnanometer resolution (Singer et al., 2019). In addition, atomic force microscopy allows measurements of inter- and intramolecular forces, adhesion forces, sample elasticity and surface hardness (Morris et al., 2009; Čadež & Šegota, 2016). Given the small cell diameter (~0.15 μm) and the diffraction limit of light, super-resolution microscopy techniques are essential for spiroplasma imaging experiments (Harne et al., 2020). Electron cryotomography is recommended for studying spiroplasmas; this technique allows three-dimensional visualization of the interior of spiroplasmas in their native state - observation of protein filaments, secretion systems, chemoreceptors and protein localization (Trachtenberg et al., 2008; Tocheva et al., 2010; Harne et al., 2020; Sun et al., 2025). Using multiplex PCR, *S. apis* and *S. melliferum* can be detected and distinguished (Gasparich, 2002; Meeus et al., 2012; Schwarz et al., 2014; Cleary & Szalanski, 2022). A real-time quantitative PCR (qPCR) assay specifically detects *S. apis* (up to 10 genome copies) and *S. melliferum* (up to 100 genome copies) (Schwarz et al., 2014). Multiplex PCR was designed based on specific primers for amplification of the rpoB gene (encoding the β subunit of RNA polymerase) of *S. apis* and the spiralin gene of *S. melliferum* (Meeus et al., 2012., Lannutti et al., 2022).

The use of MALDI-TOF (Matrix-assisted laser desorption/ionization time-of-fligh) mass spectrometry in diagnostics should also be considered, which is based on detecting the presence of a specific protein secreted by spiroplasmas during infection. Methods based on detecting the presence of specific proteins secreted by spiroplasmas (antigens) - with the help of known, specific antibodies, may also be of importance in diagnostics (Shi et al., 2014). Two of these tests are ELISA (enzyme-linked immunosorbent assay) (Jordan et al., 1989., Zhang et al., 2015) and Western blotting test (Shi et al., 2014). Western blot is used in cell and molecular biology and can identify specific proteins from a complex mixture of proteins extracted from cells. The technique is based on the separation of proteins through the use of gel electrophoresis, transfer of proteins to a solid support and then the labeling and visualization of the target protein using specific antibodies (Mahmood & Yang, 2012; Sule et al., 2023).

Measures to prevent and control the occurrence of spiroplasmosis in bees

Considering the factors and circumstances that favor the occurrence of the disease, as well as its importance for the health of the bee colony, work should be conducted in this area on the implementation of preventive measures and the diagnosis of this disease (Sarwar, 2016; Bojanić Rašović, 2019, 2021, 2022; Bojanić Rašović et al., 2019; Piché-Mongeon & Guzman-Novoa, 2024). Specific therapy of bees suffering from spiroplasmosis is not carried out, but in these cases the bees' immunity must be strengthened with the implementation of adequate beekeeping and veterinary practices. Additionally, hygienic measures must be implemented in the apiary to take care of the hygiene of food, beekeeping equipment and accessories, hygiene of work in the apiary, etc. (Sarwar, 2016; Bojanić Rašović, 2019, 2021, 2022; Bojanić Rašović et al., 2019; Piché-Mongeon & Guzman-Novoa, 2024.)

CONCLUSIONS

Considering the increasingly pronounced negative impact of non-specific factors on honey bees, the danger of diseases caused by spiroplasmas is increasing. These microorganisms have confirmed to infect bees through contaminated food, *Varroa destructor* bites and a vertical method of transmission in insects. Among the honey bee spiroplasmoses, the May disease prevalent in France and bee creeping disease in China are recognized by their specific symptoms. Because of the resistance of spiroplasmas to the action of external factors, they are widely distributed in nature and the most important reservoir of spiroplasmas are insects, but there is not enough information about the all sources of spiroplasmas in nature, their spread and ecology. There is also insufficient information on the pathogenesis and characteristics of spiroplasmosis in bees. Therefore, it is necessary to continue research in these areas. Given that spiroplasmas are conditional pathogens of bees and are widespread in the environment, in order to prevent the occurrence of diseases, it is important to implement adequate measures of good beekeeping practices, good veterinary practices and strengthen the immune system of the bee colonies.

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