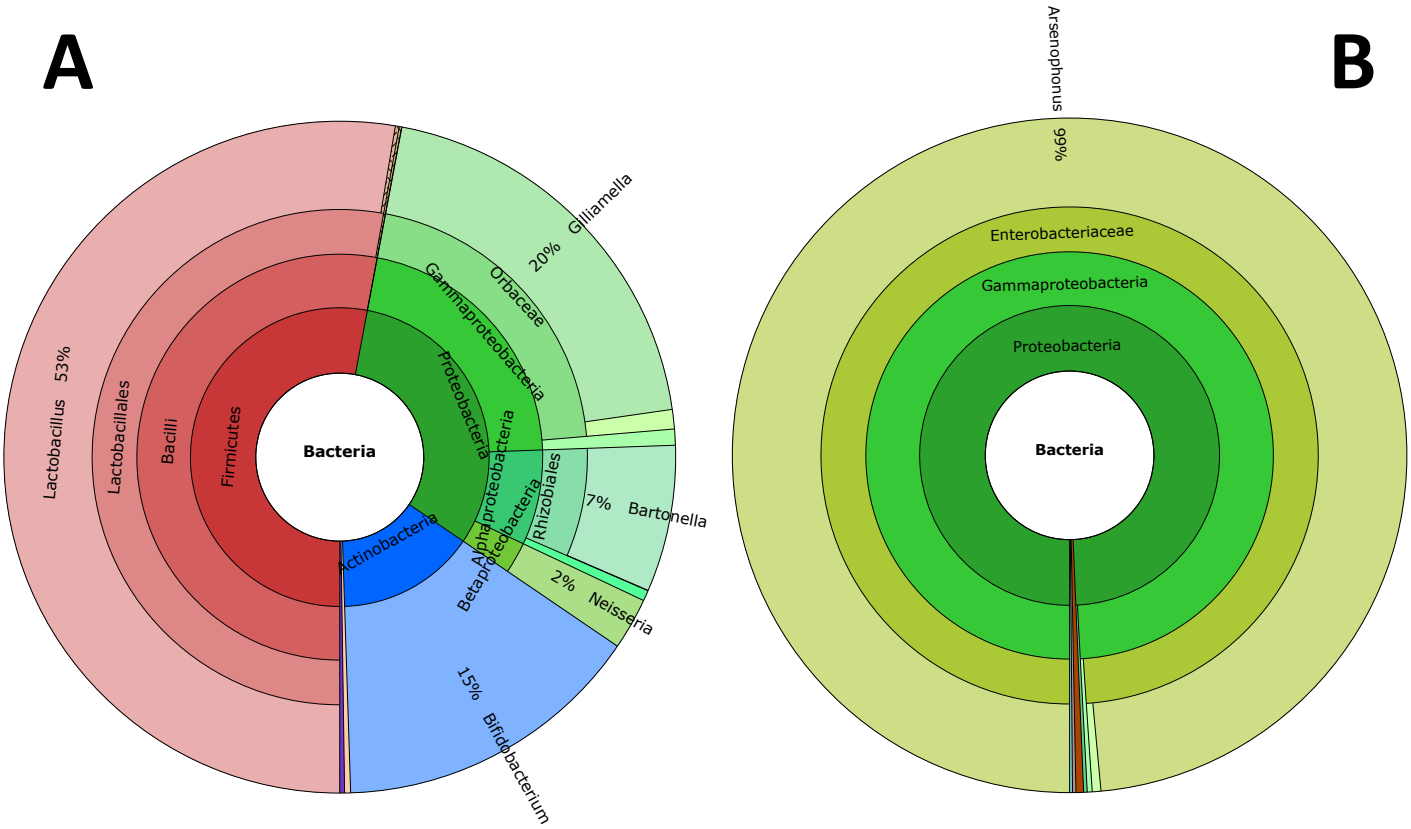
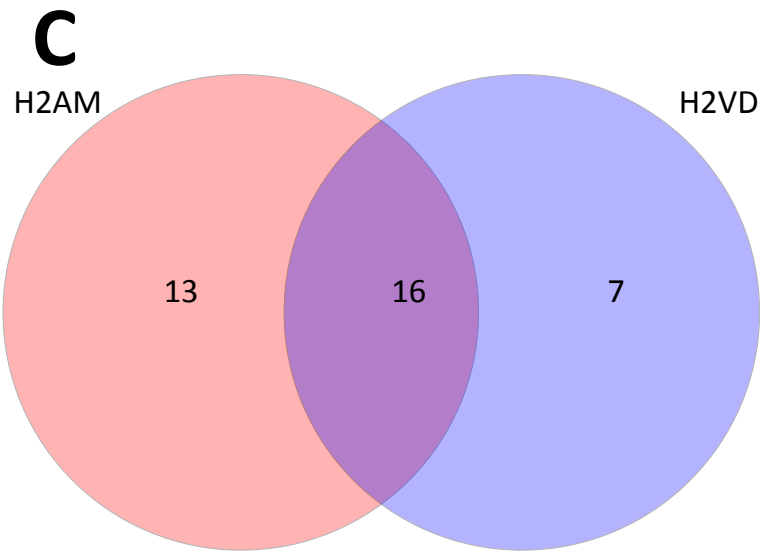
7. Přílohy

Příloha 1 – PCR detekce bakterie *Arsenophonus* u včelích matek a jejich doprovodu

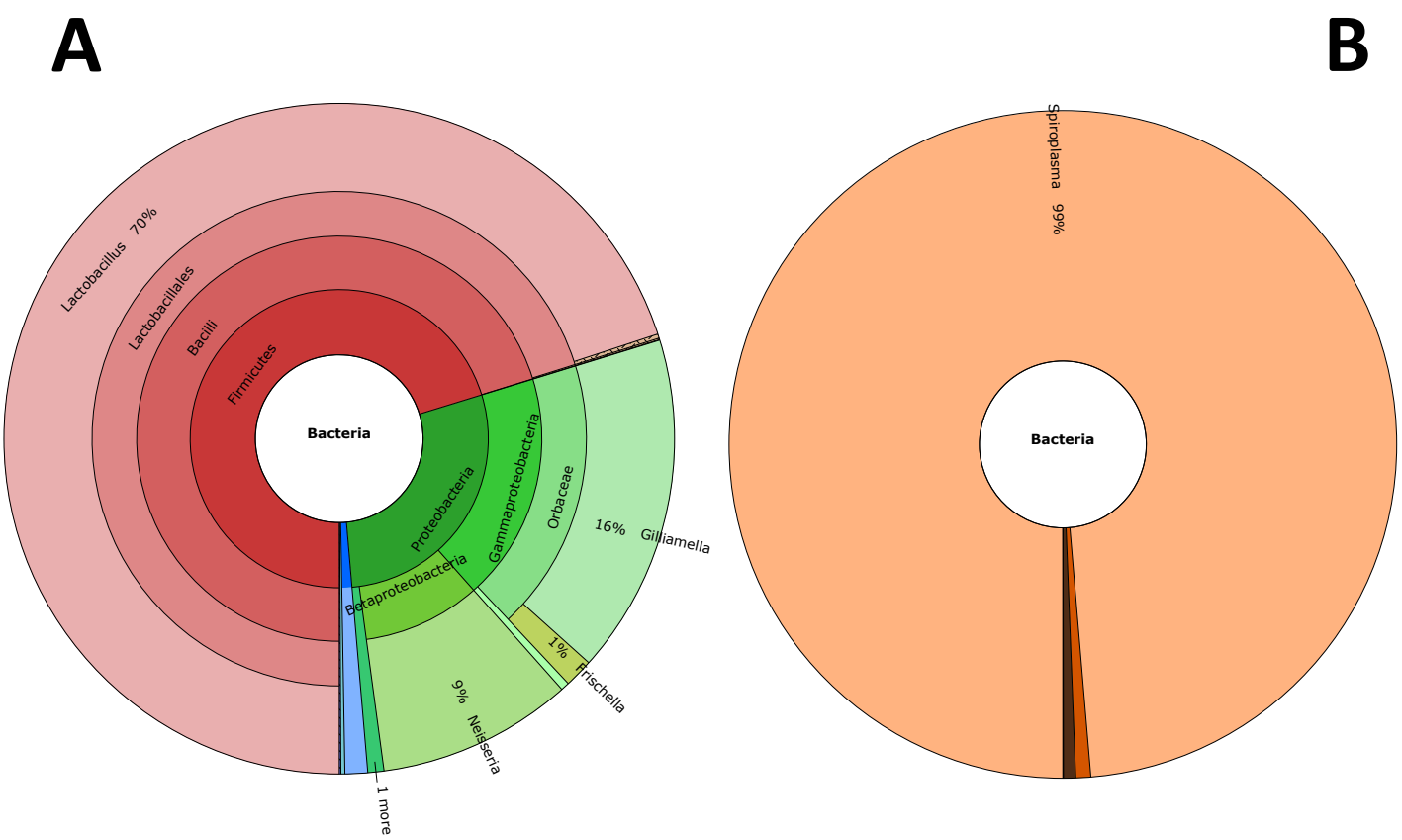
Včelstvo		**Matka/Doprovod**	***Arsenophonus***
Horní Lhota	1	M	
		D	+
Máslovice Dol	2	M	
		D	+
	3	M	
		D	
	4	M	
		D	
	5	M	
		D	
	6	M	
		D	
	7	M	
		D	
	8	M	
		D	
	9	M	
		D	
	10	M	
		D	
	11	M	
		D	
	12	M	
		D	
	13	M	
		D	
	14	M	
		D	
	15	M	
		D	
	16	M	
		D	
	17	M	
		D	+
	18	M	
		D	
	19	M	
		D	
	20	M	
		D	
	21	M	
		D	
	22	M	

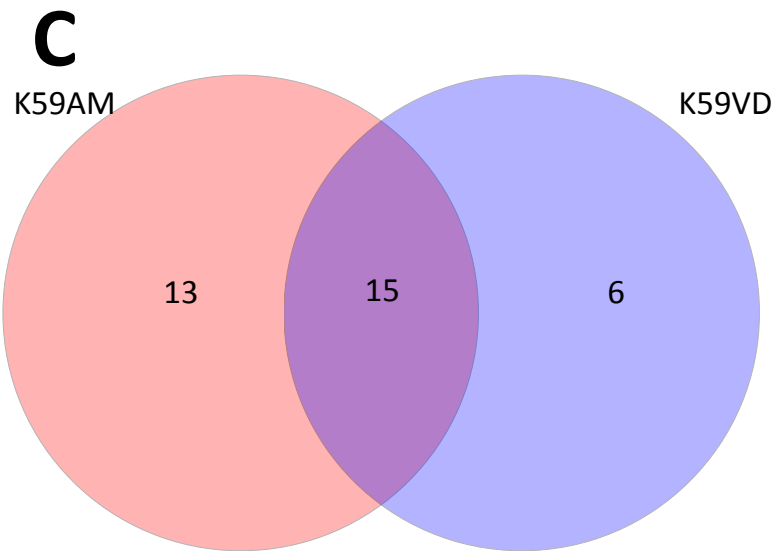
		D	
	23	M	
		D	



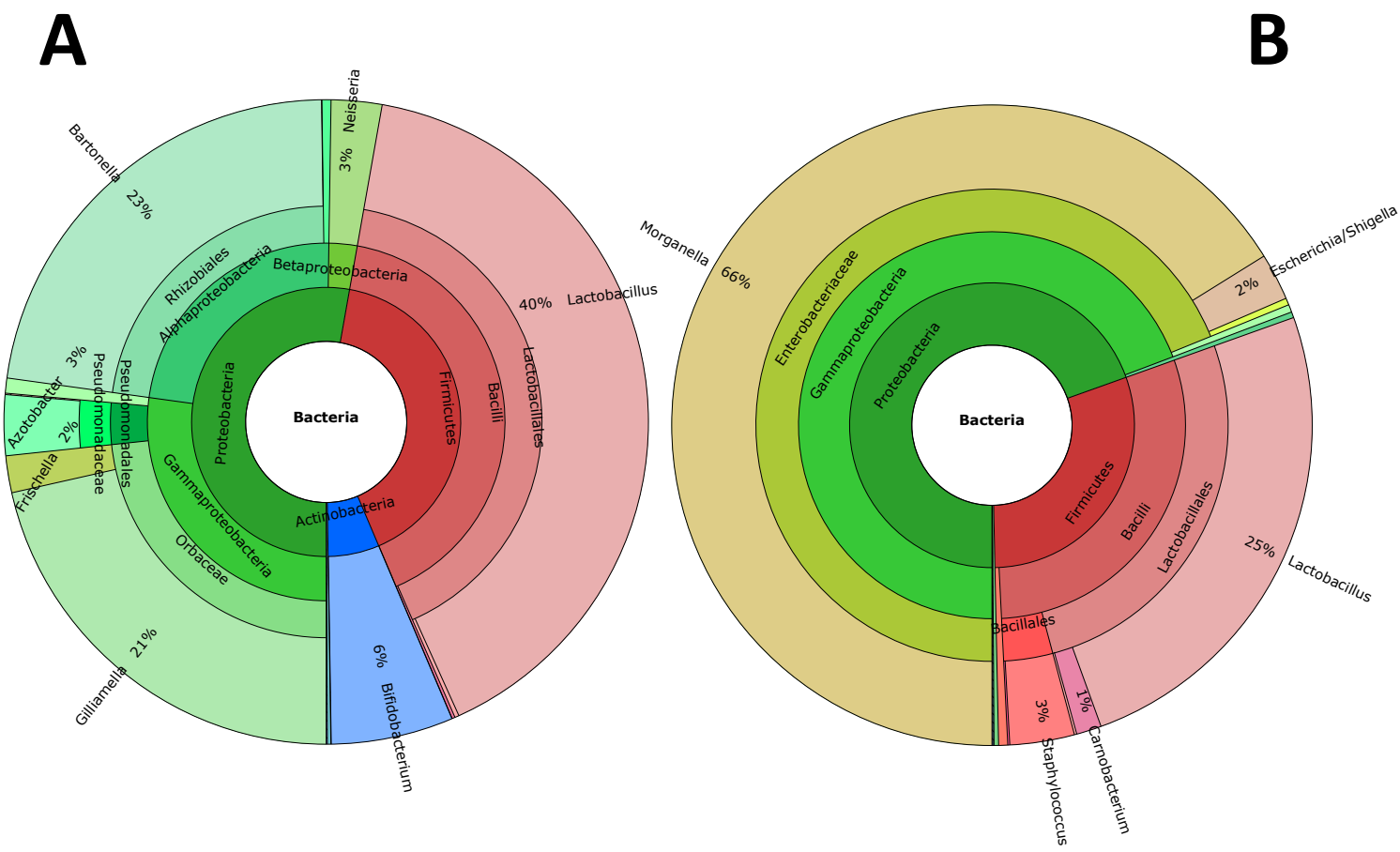


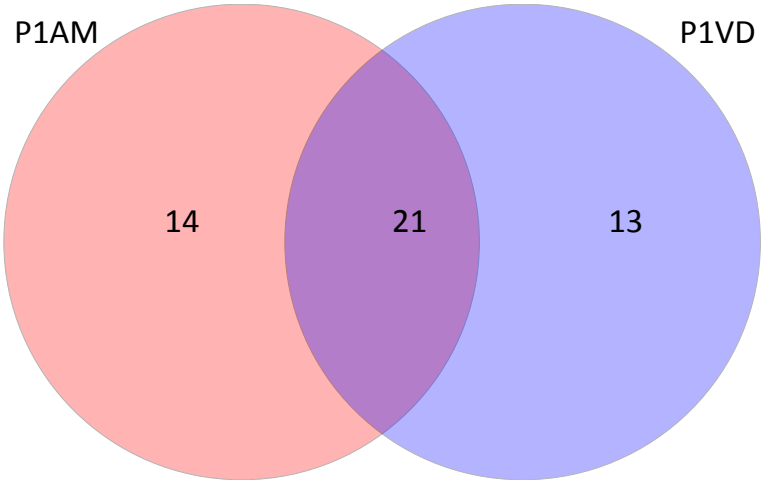
Obr. P2 - Stanoviště Horní Lhota: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště



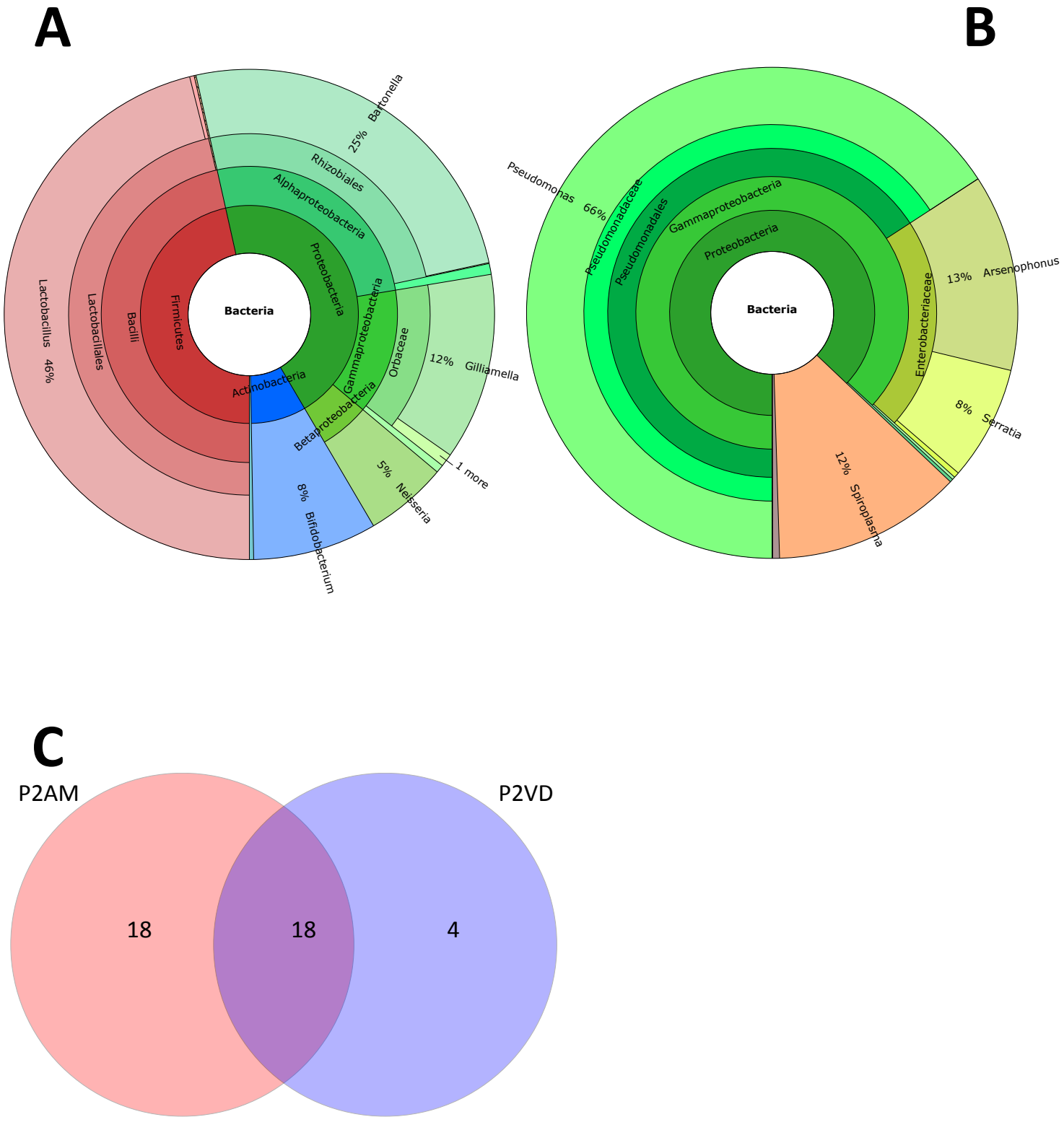


Obr. P3 - Stanoviště Kývalka: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště

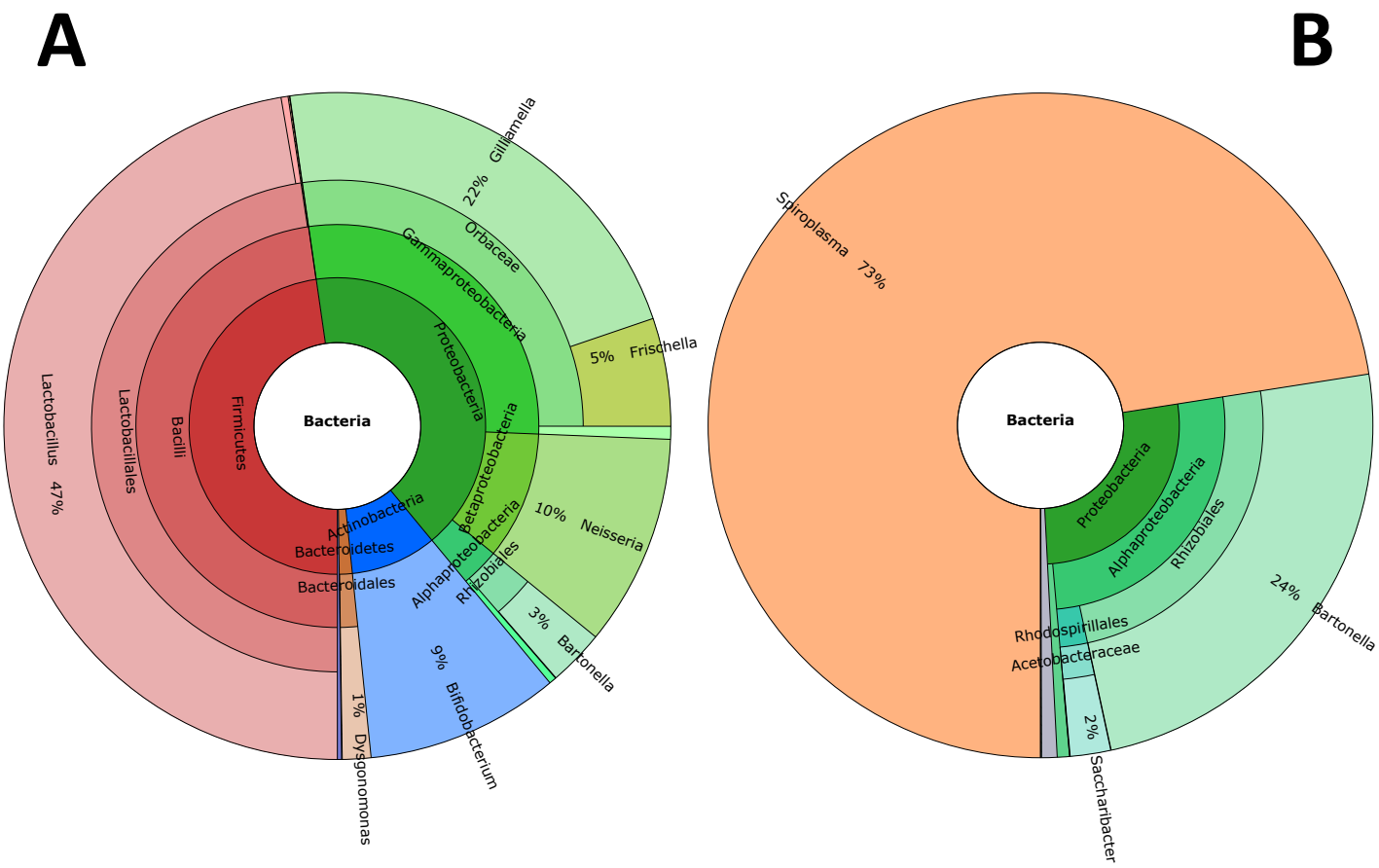


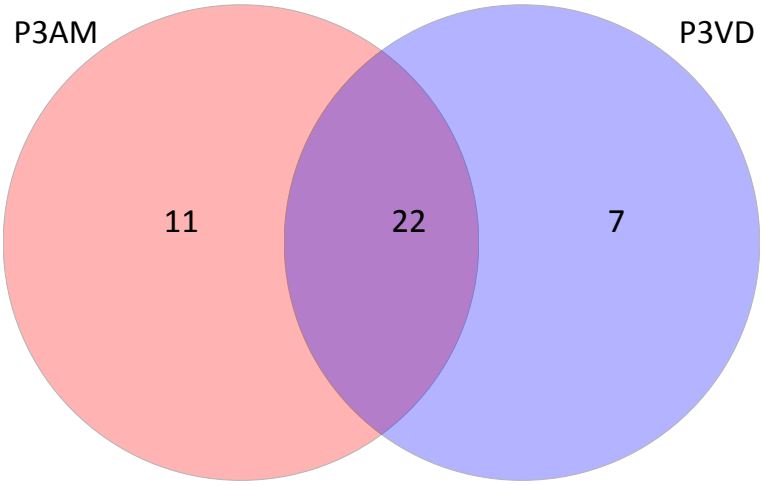


Obr. P4 - Stanoviště Postřižín 1: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště

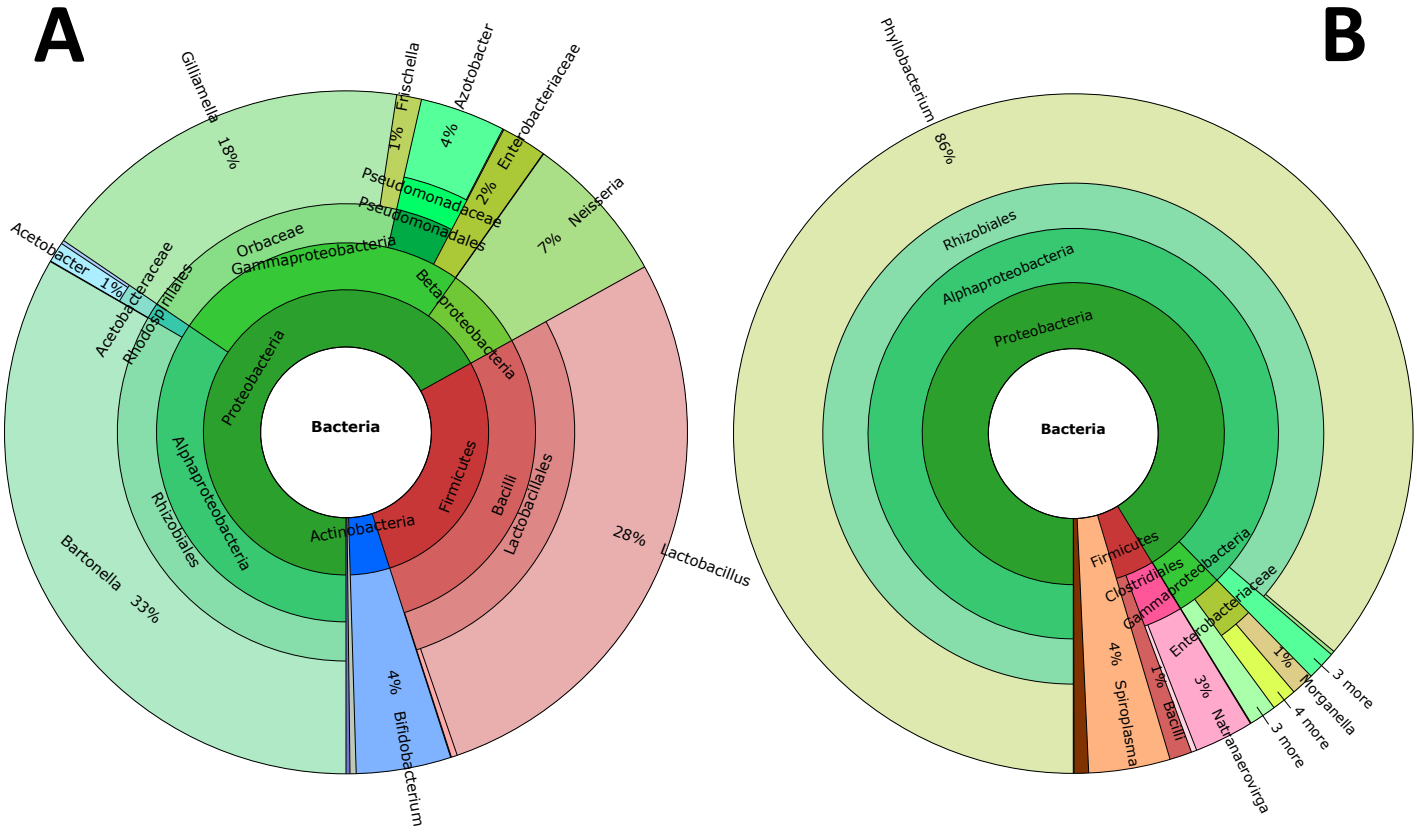


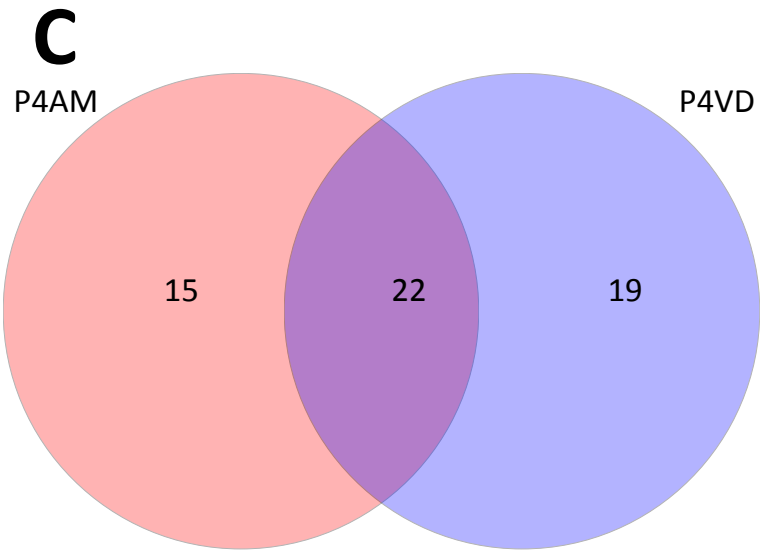
Obr. P5 - Stanoviště Postřižín 2: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště



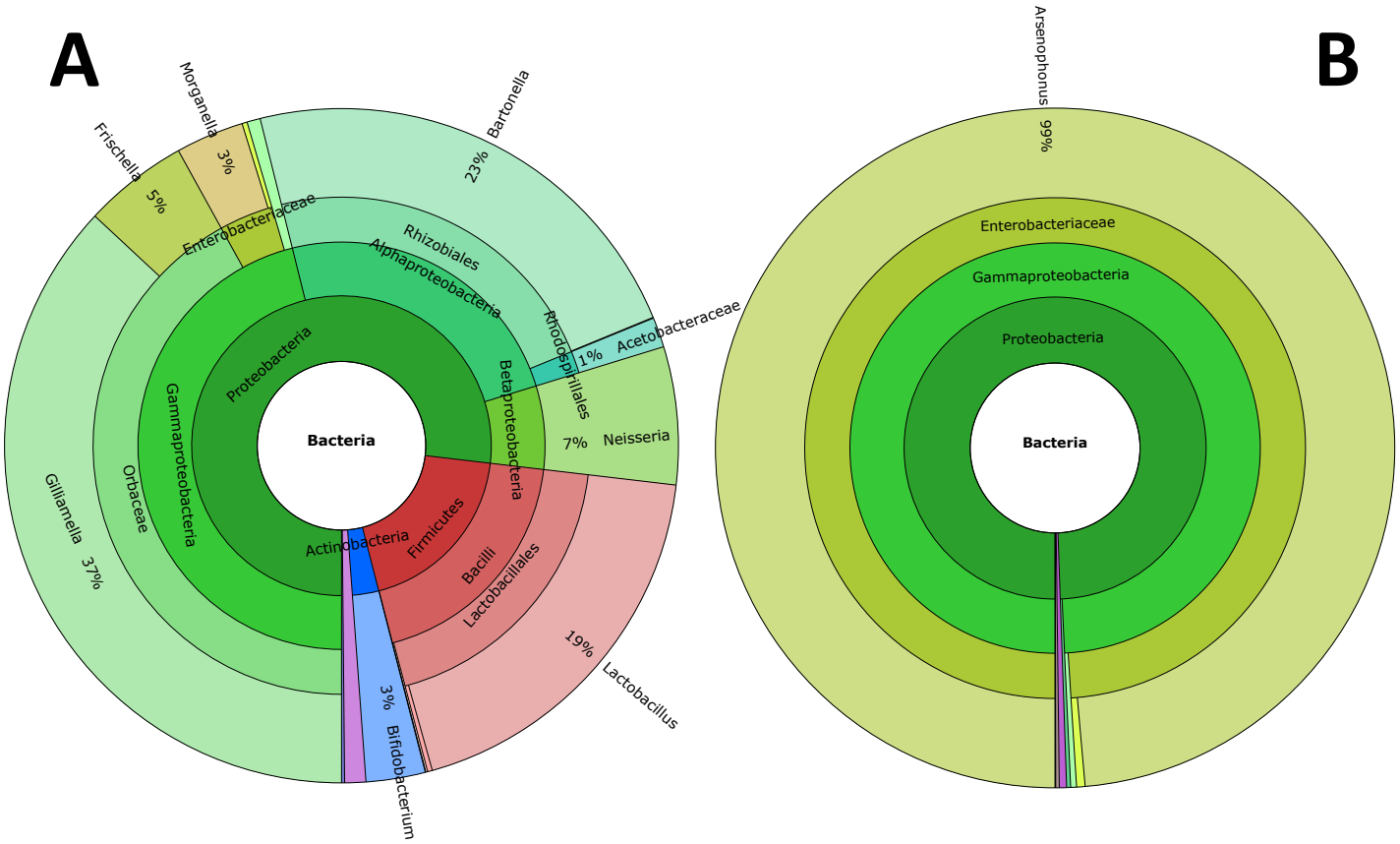


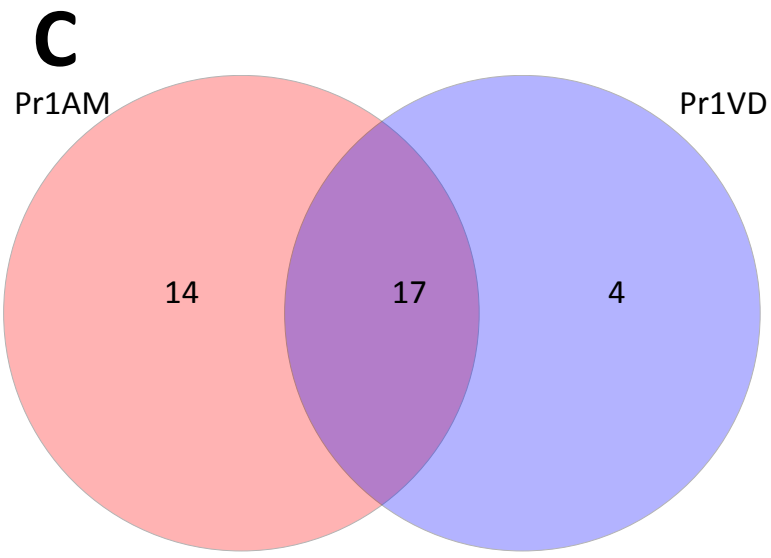
Obr. P6 - Stanoviště Postřižín 3: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště



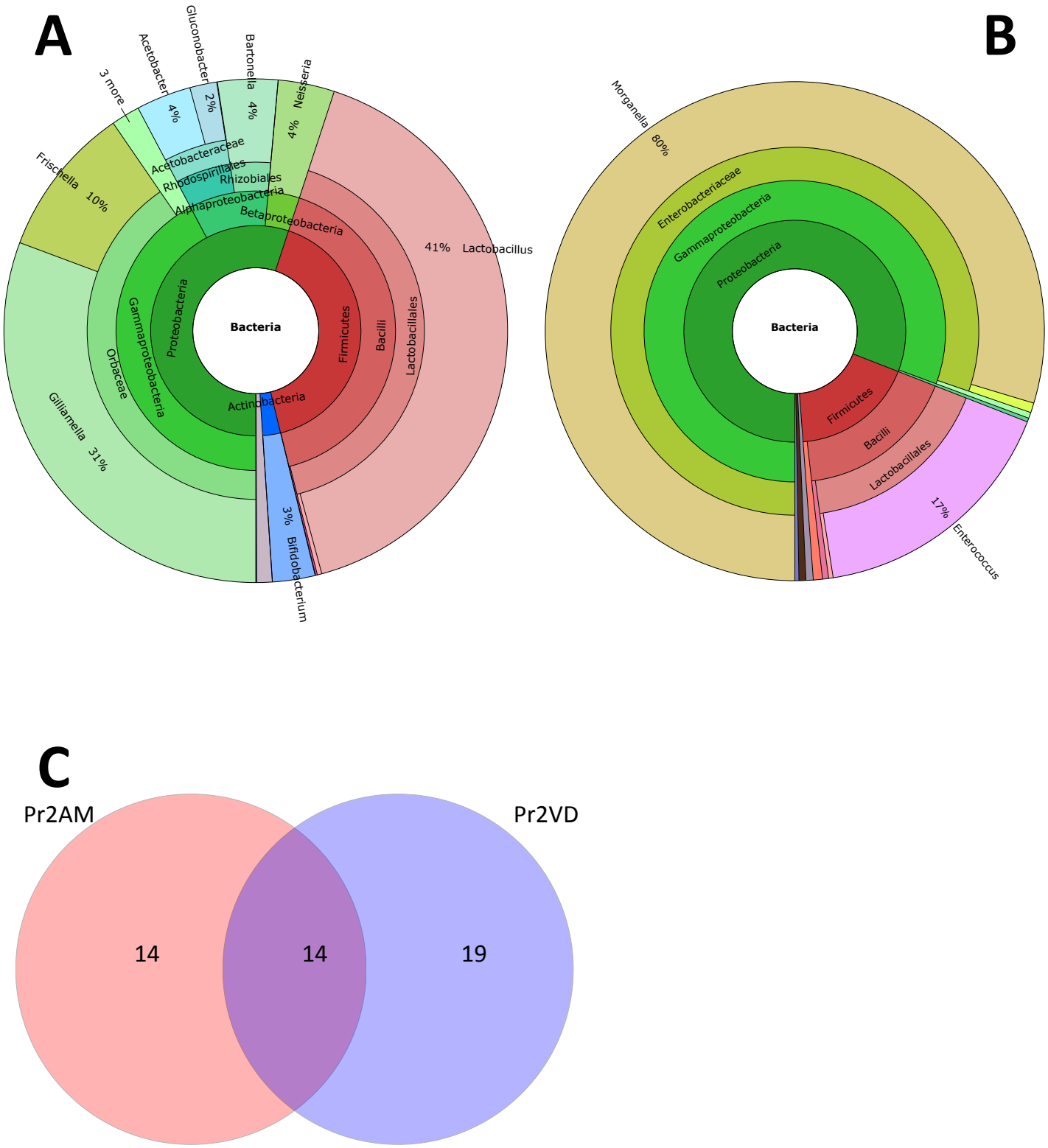


Obr. P7 - Stanoviště Postřižín 4: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště



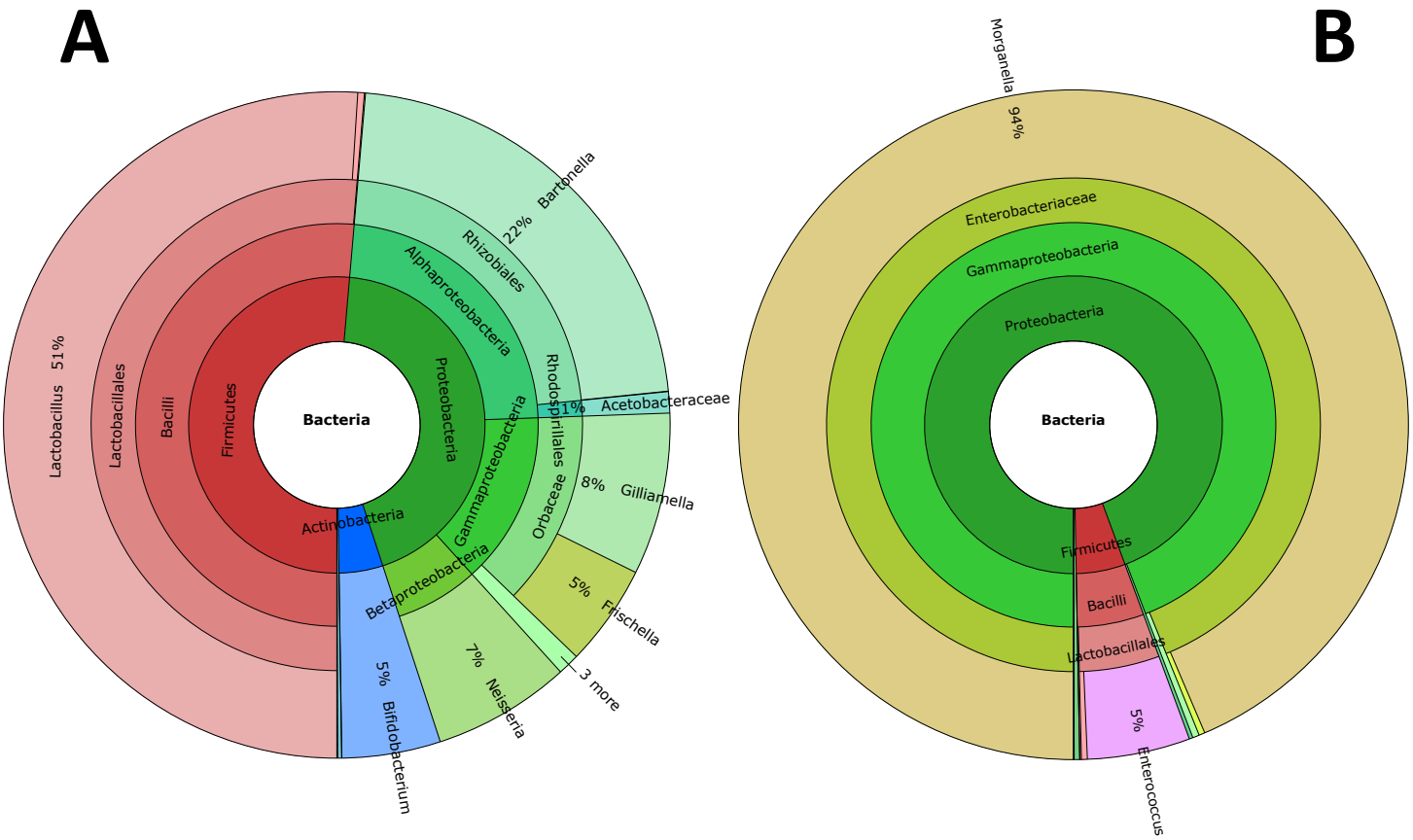


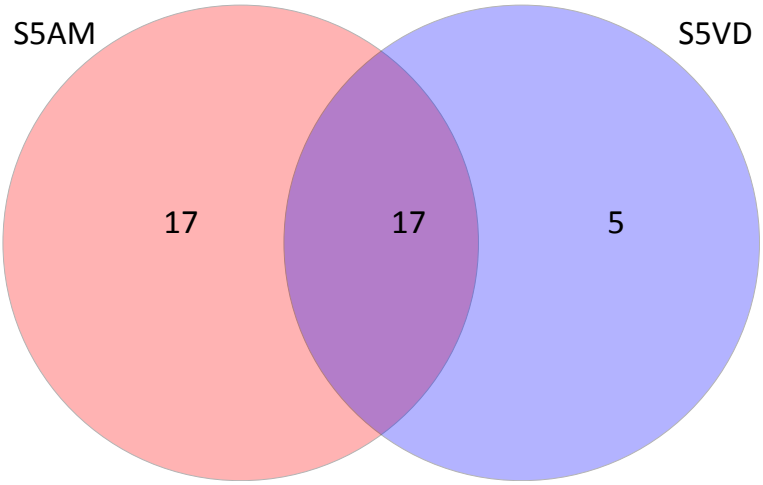
Obr. P8 - Stanoviště Přelovice 1: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště



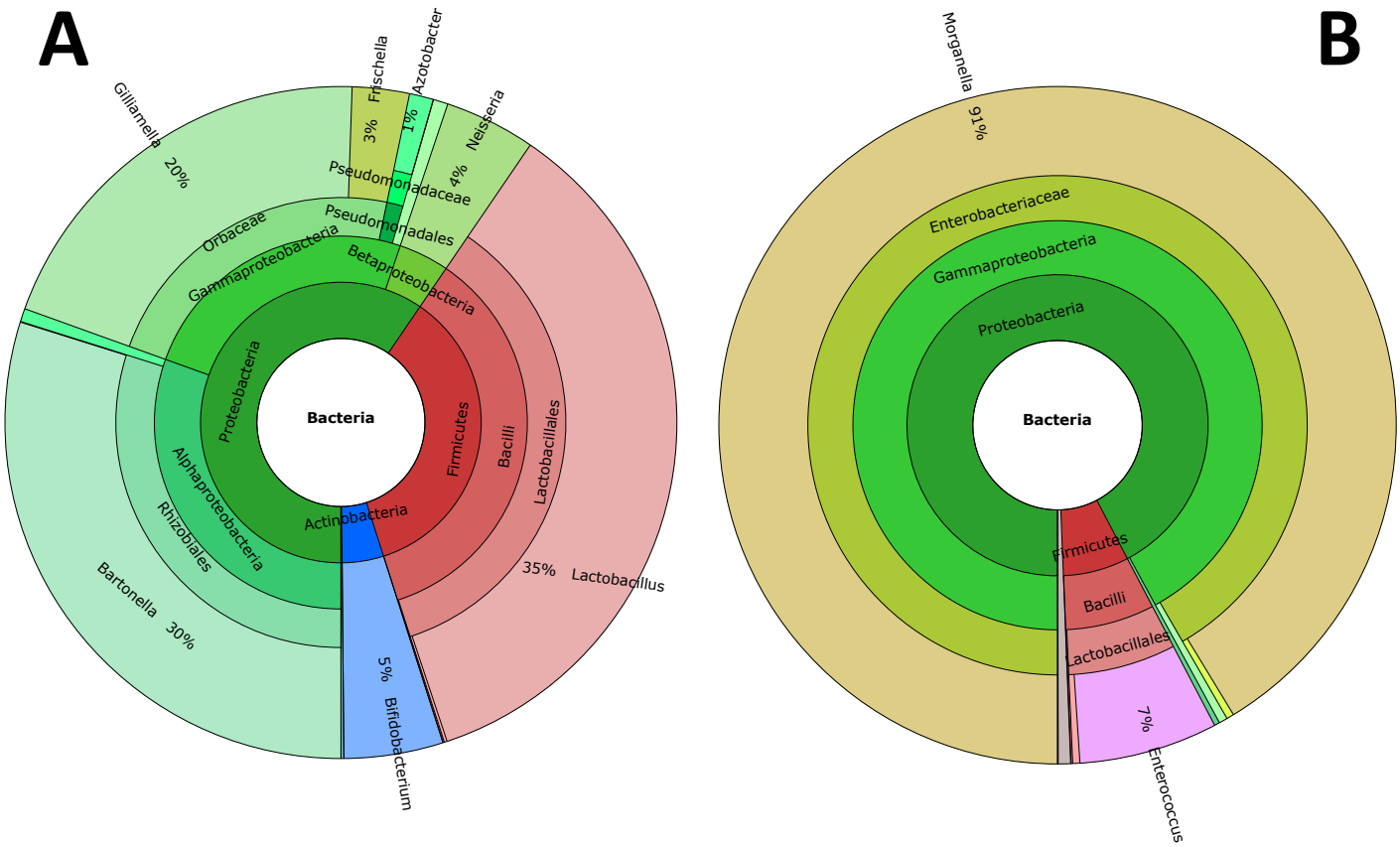
Obr. P9 - Stanoviště Přelovice 2: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště

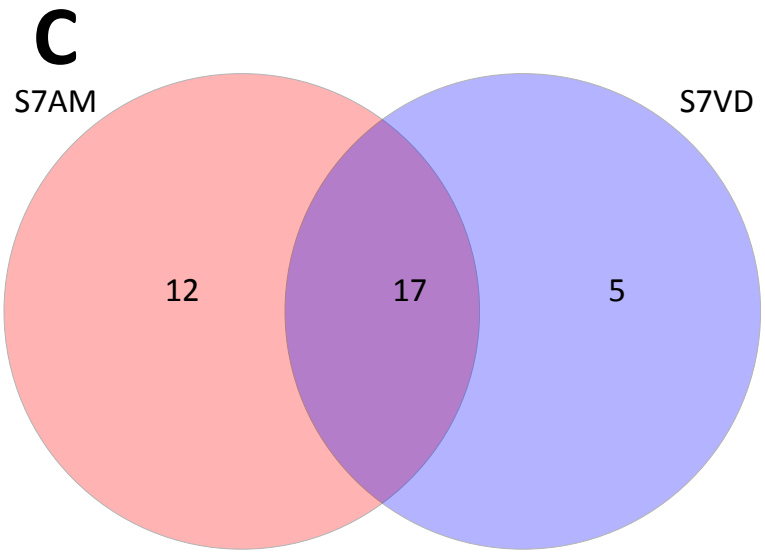
x



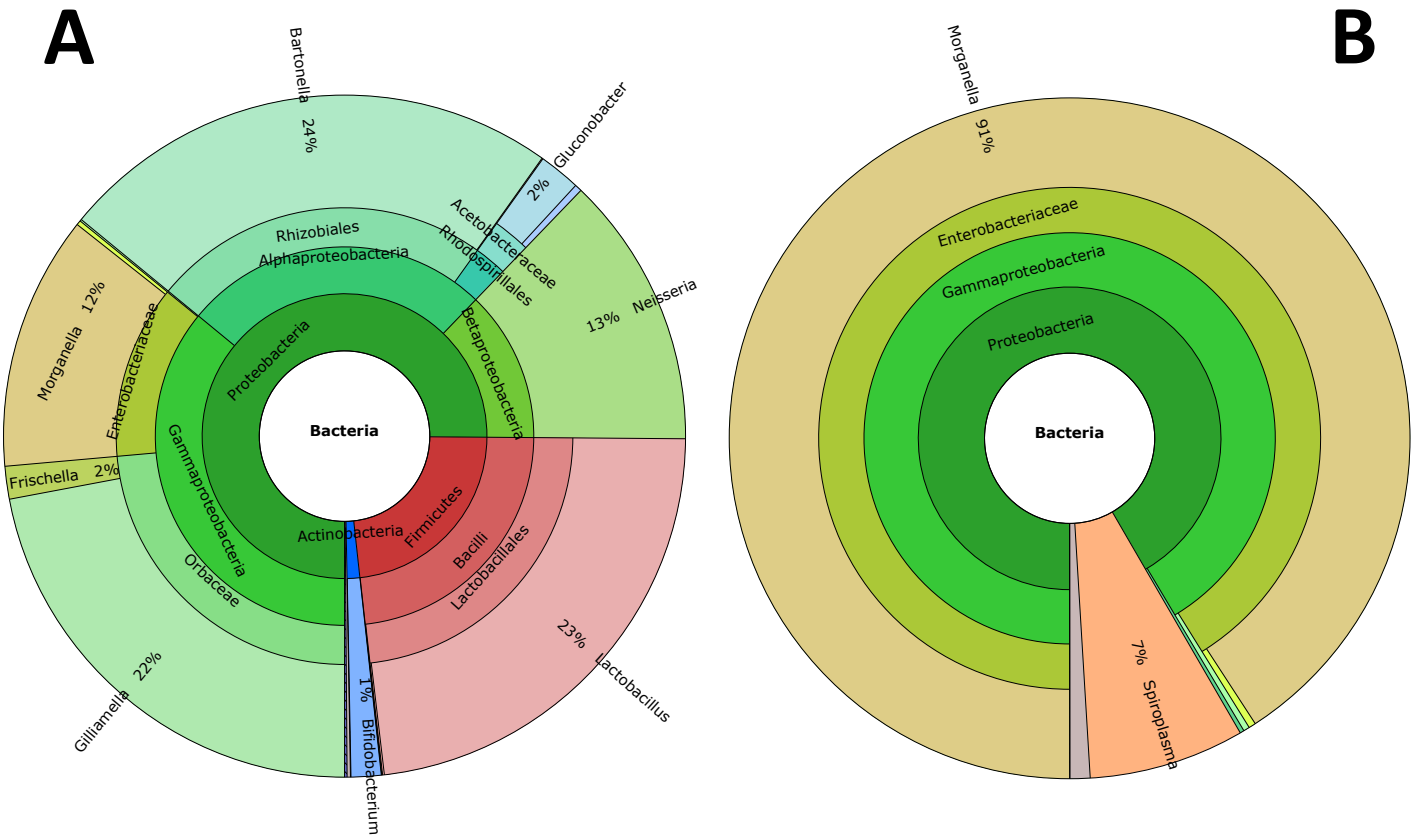


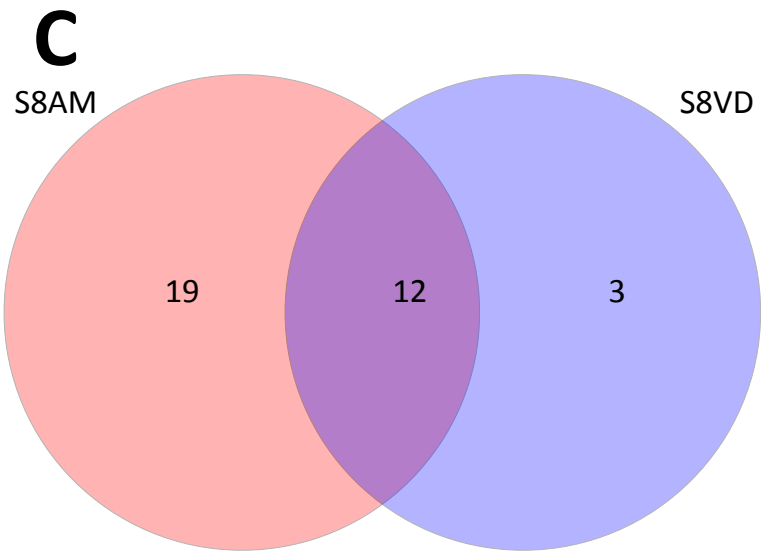
Obr. P10 - Stanoviště Hole S5: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště



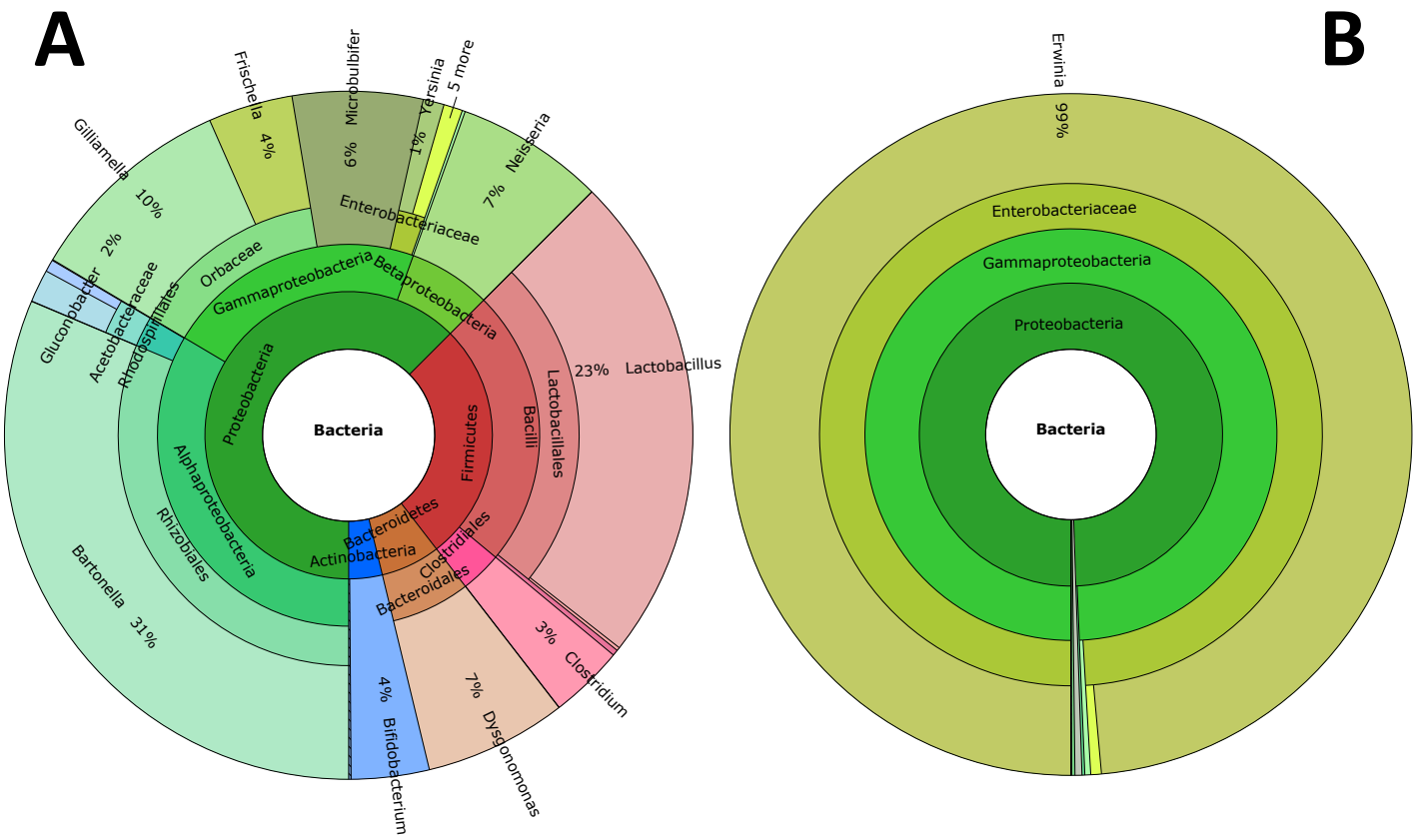


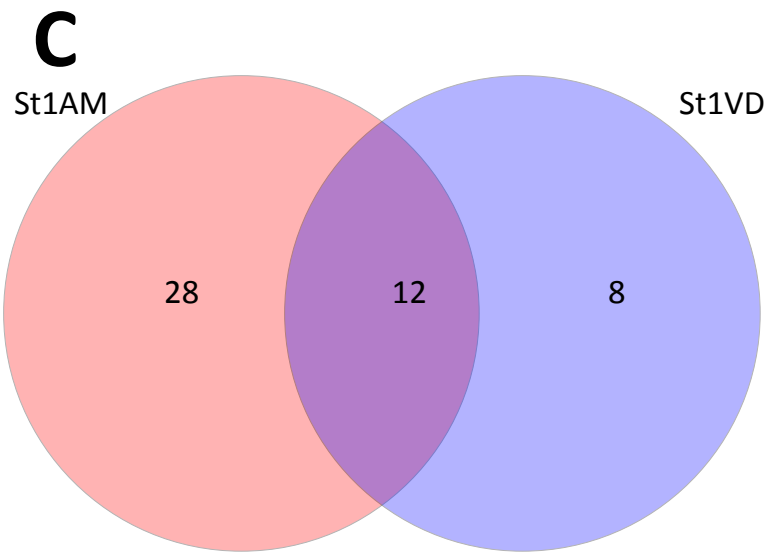
Obr. P11 - Stanoviště Hole S7: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště



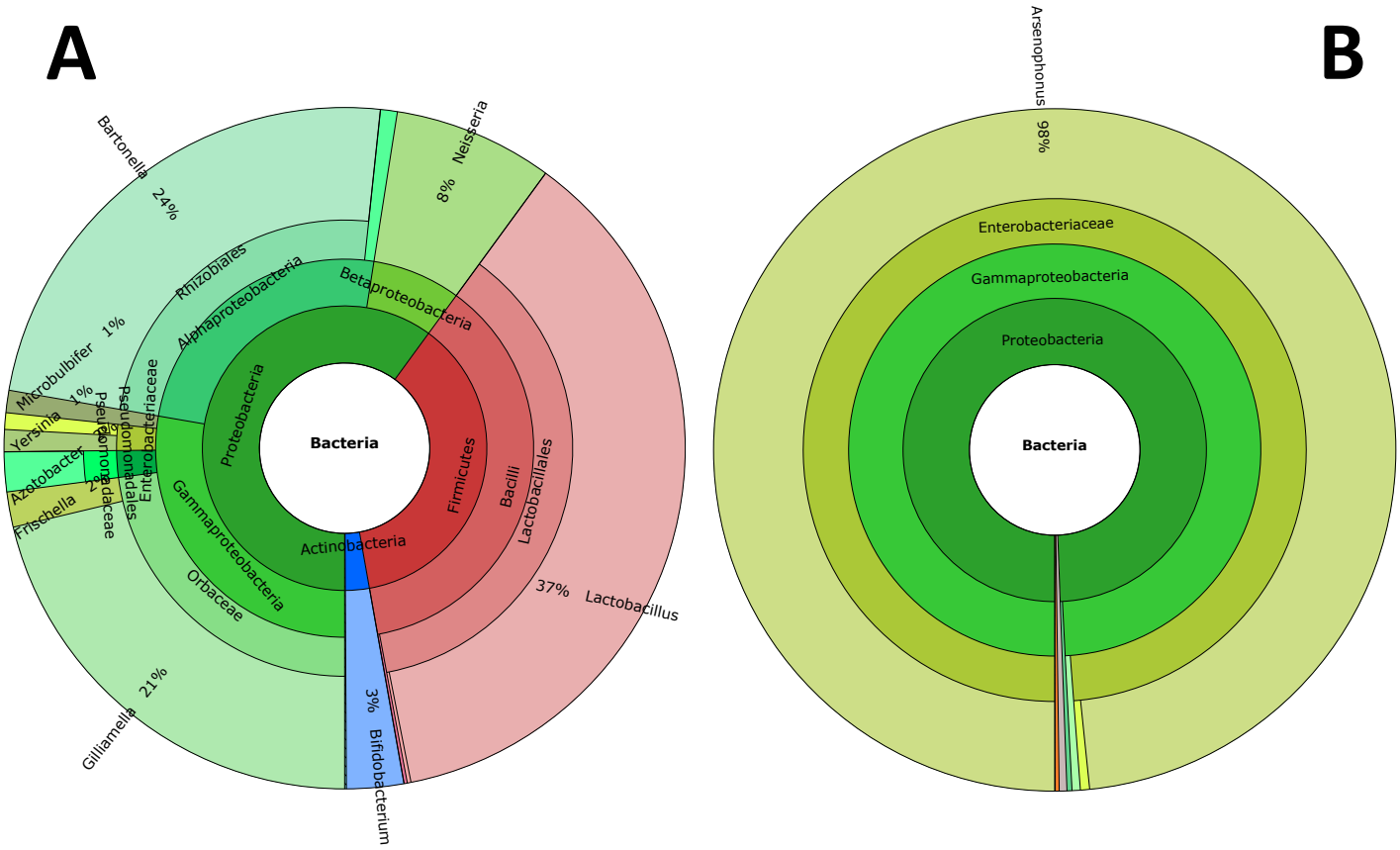


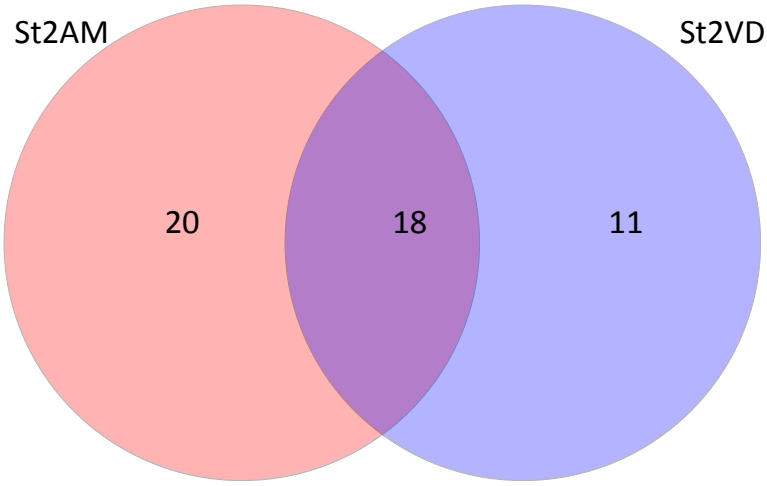
Obr. P12 - Stanoviště Hole S8: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště



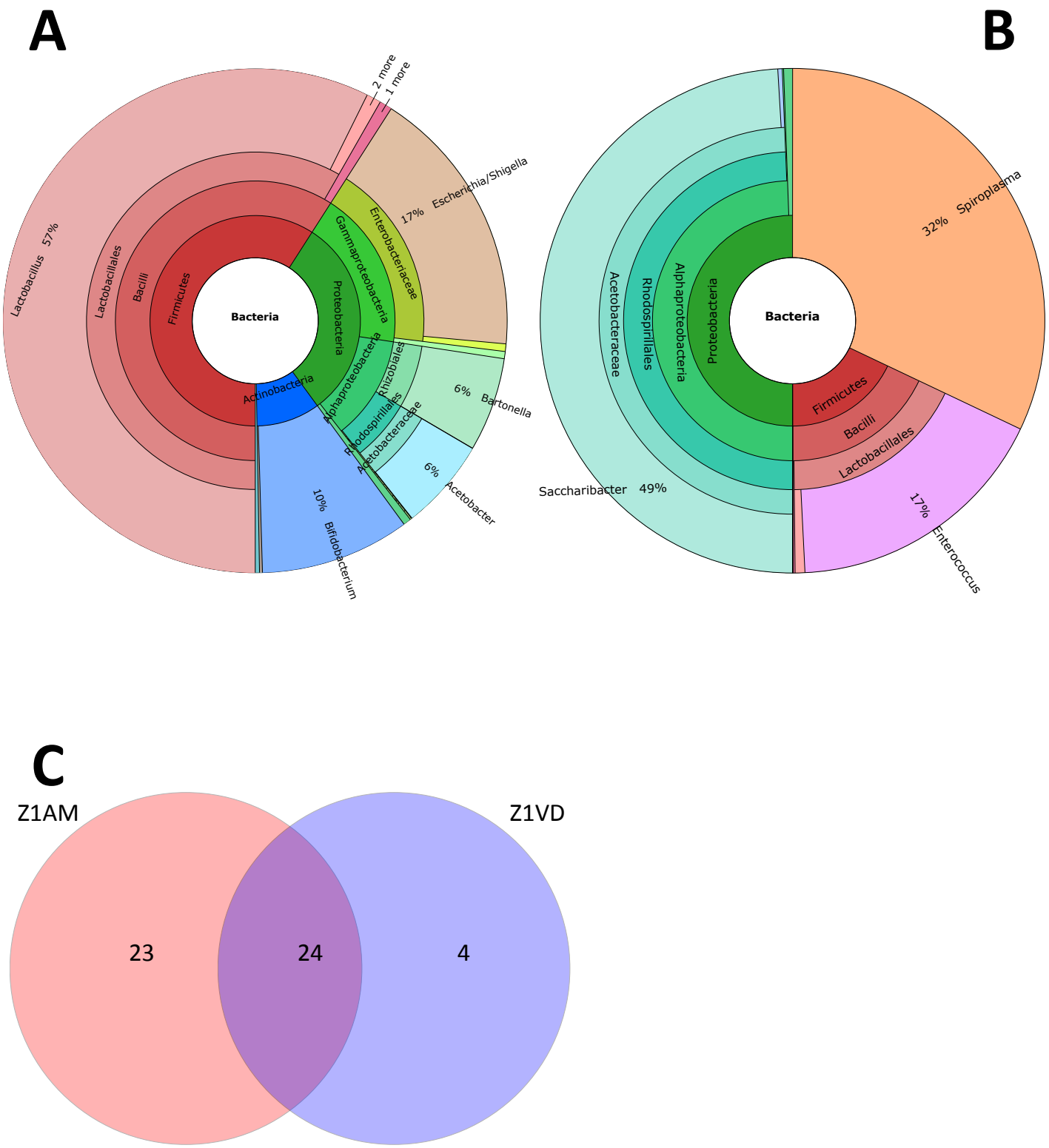


Obr. P13 - Stanoviště Střeleč 1: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště

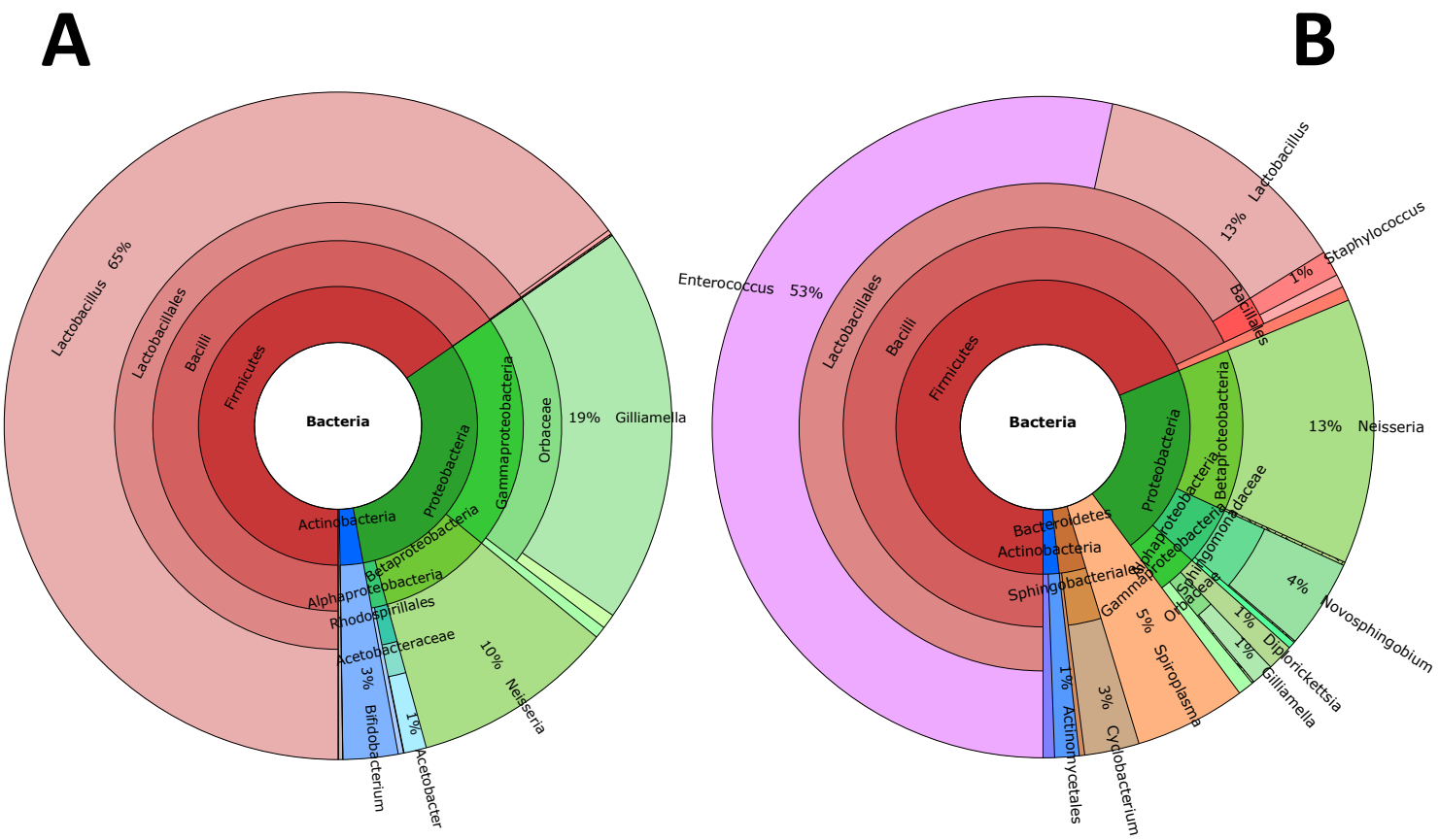


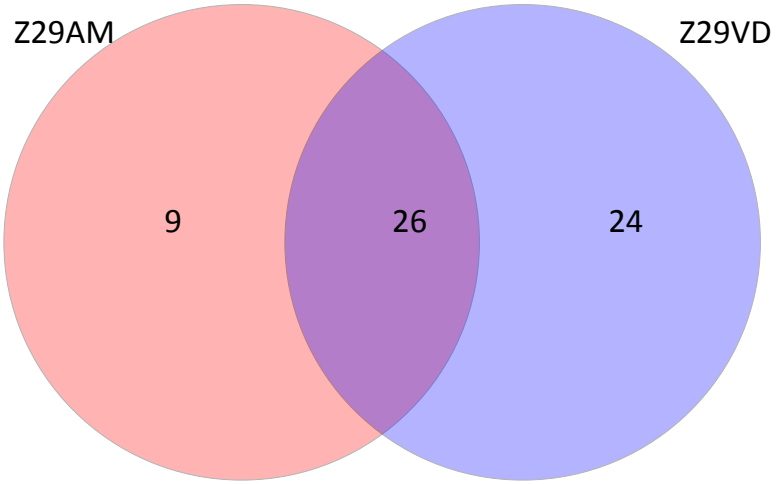


Obr. P14 - Stanoviště Střeleč 2: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště

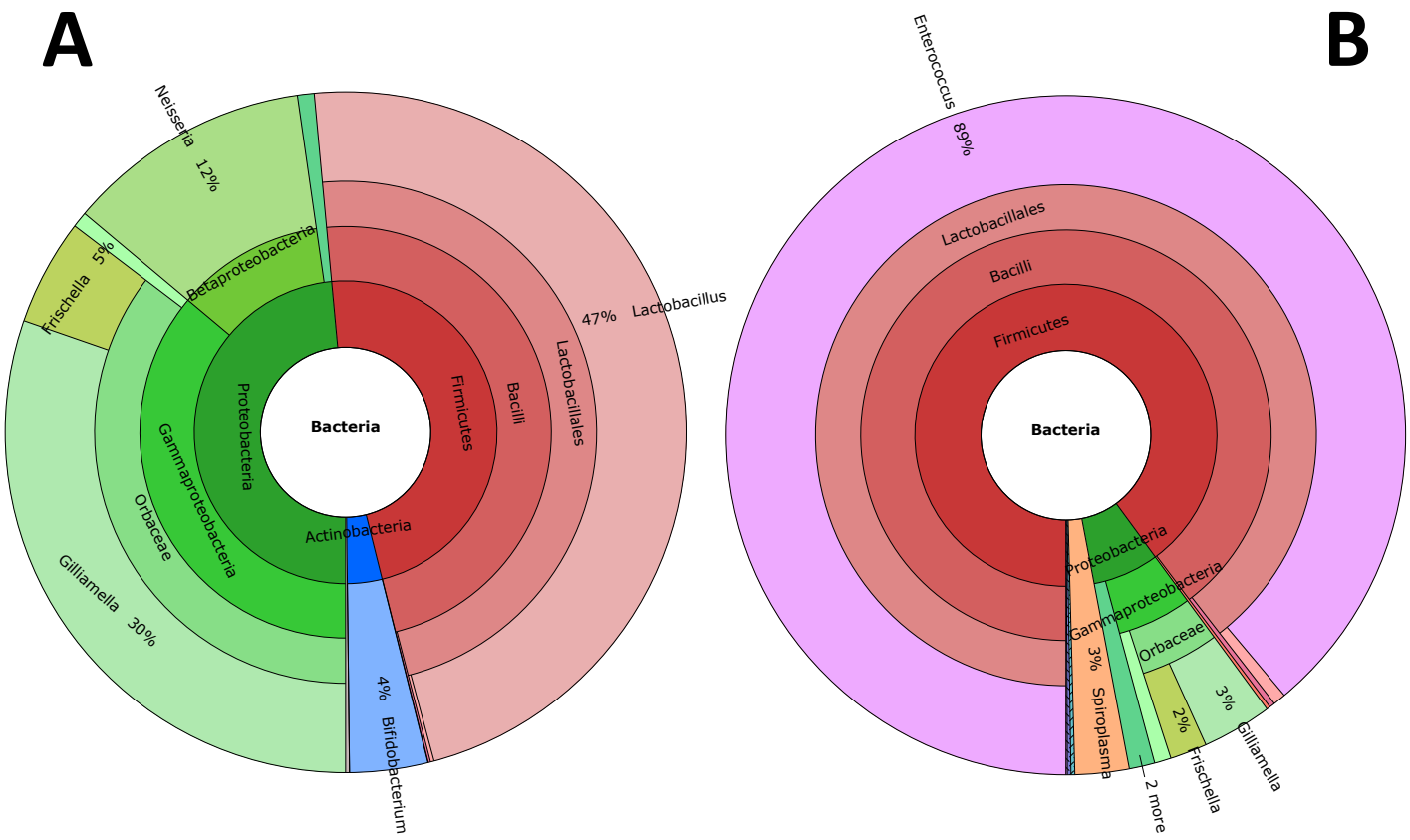


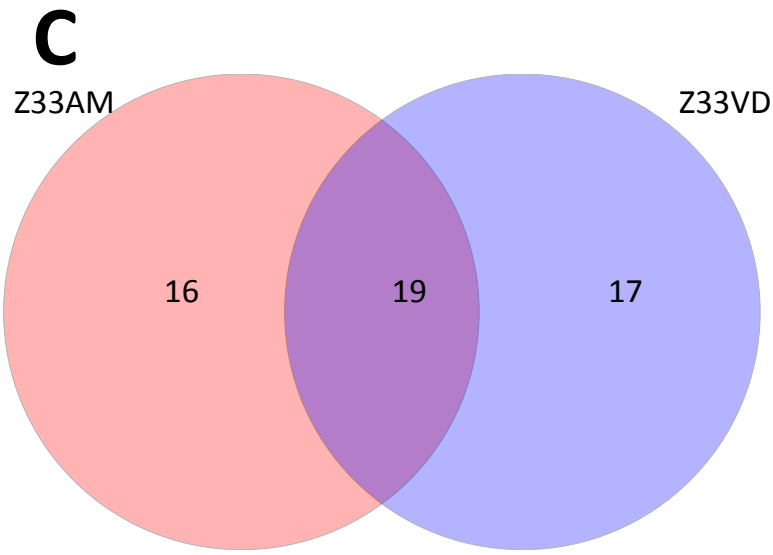
Obr. P15 - Stanoviště Štoky-Skřivánek Z1: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště



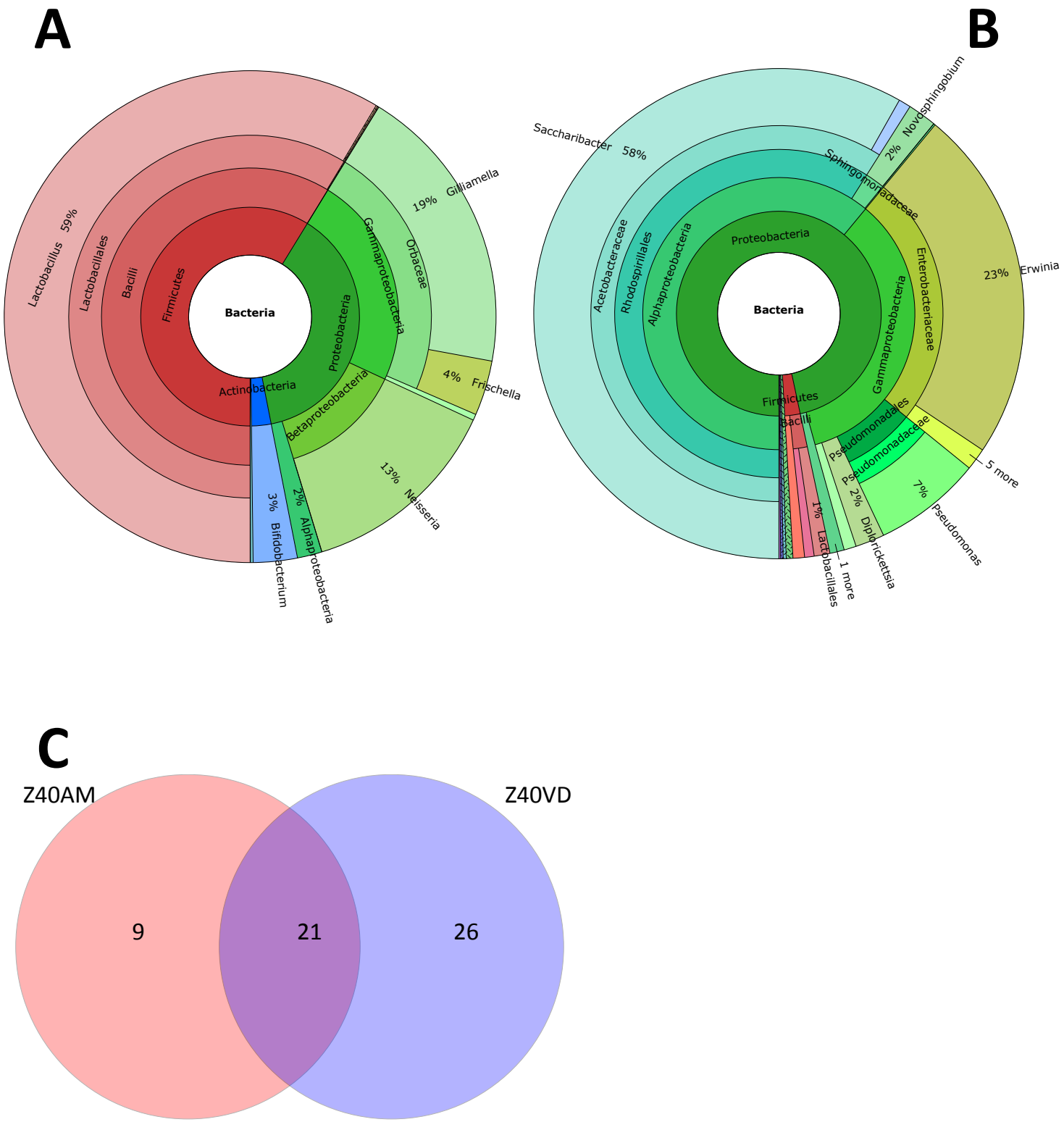


Obr. P16 - Stanoviště Štoky-Skřivánek Z29: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště

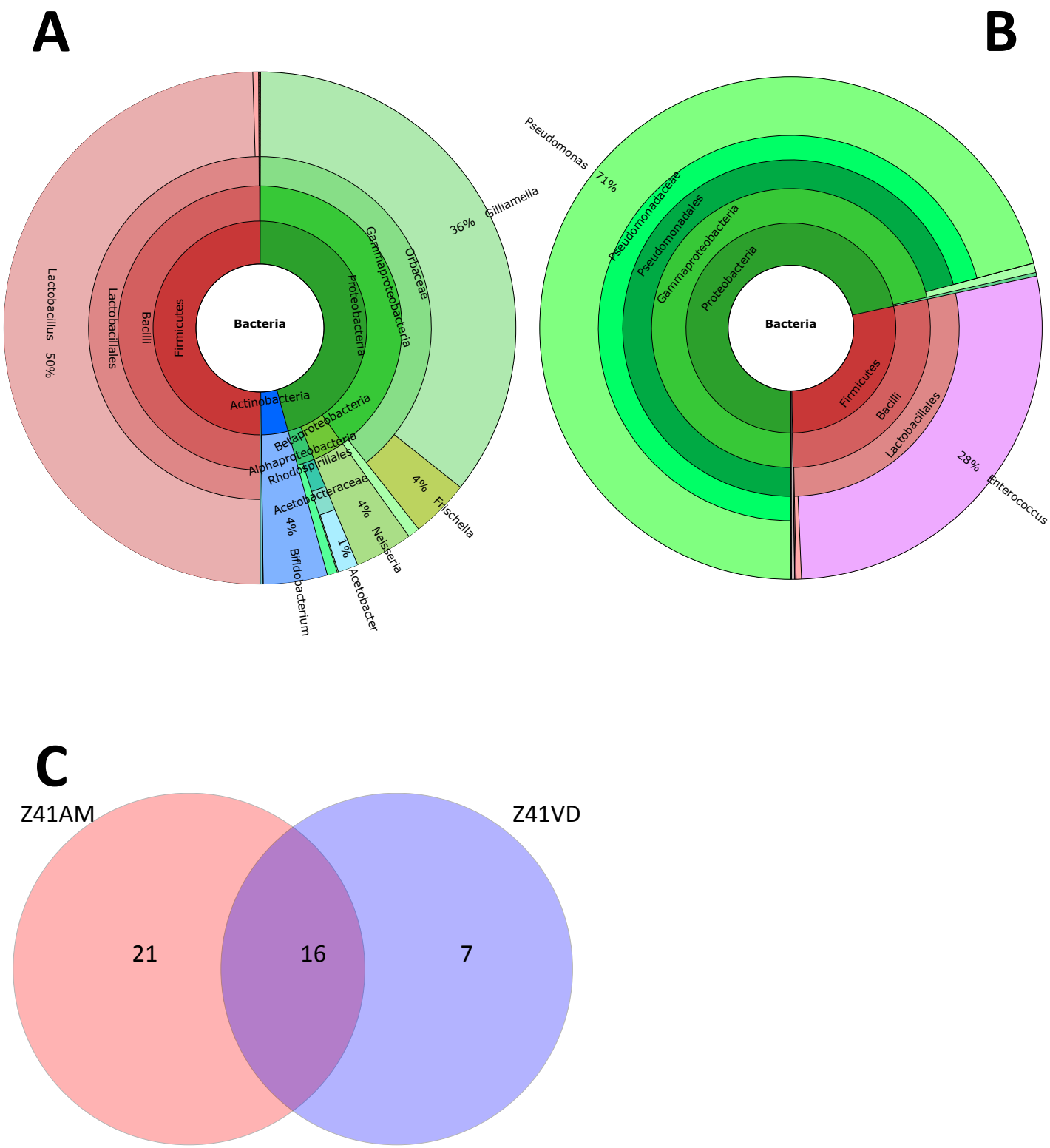




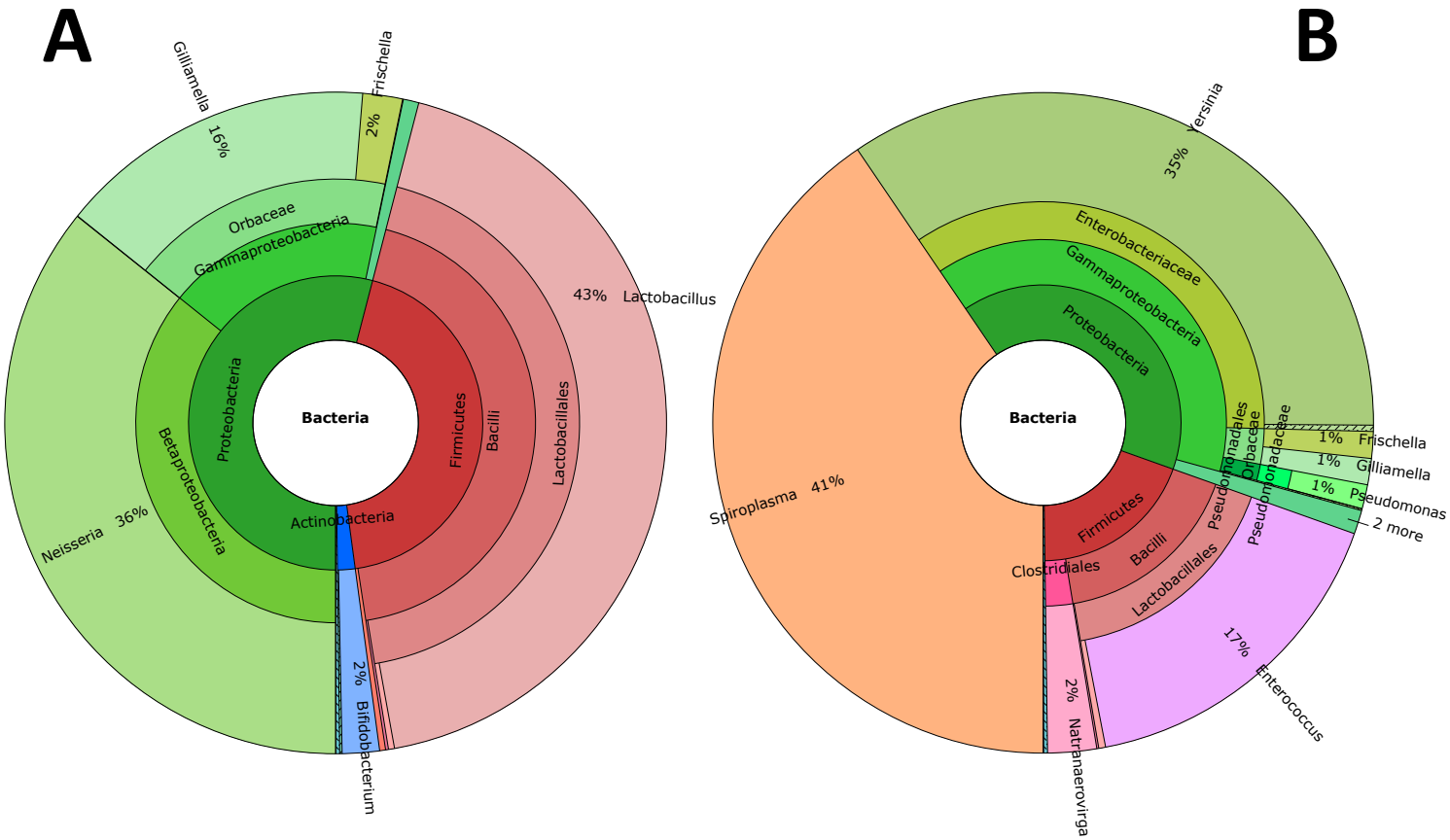
Obr. P17 - Stanoviště Štoky-Skřivánek Z33: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště

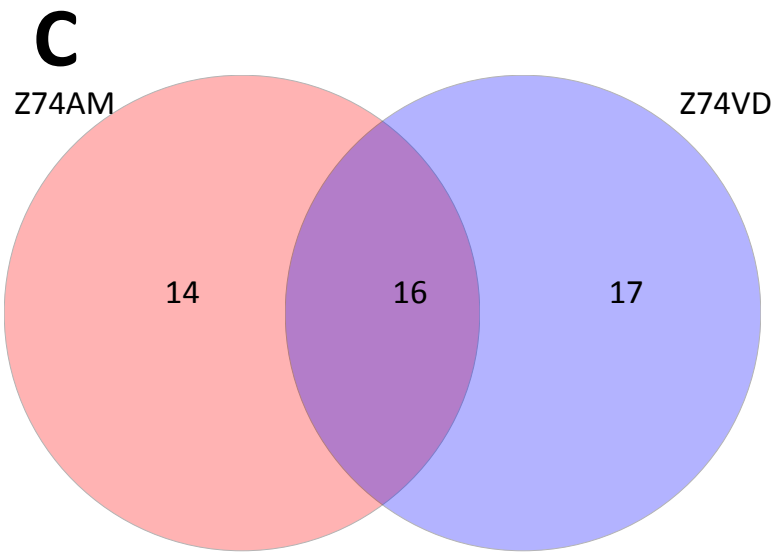


Obr. P18 - Stanoviště Štoky-Skřivánek Z40: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště

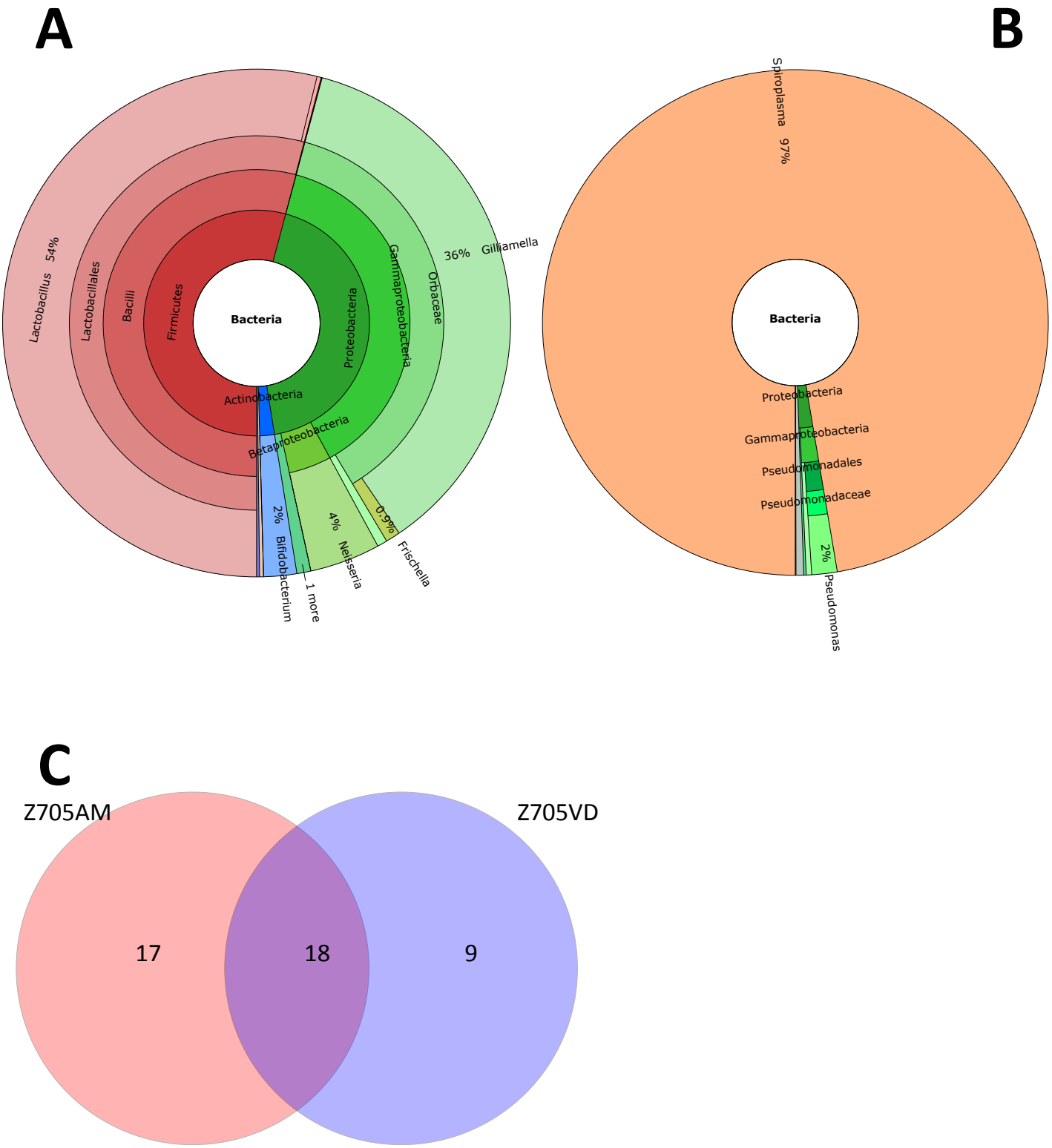


Obr. P19 - Stanoviště Štoky-Skřivánek Z41: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště

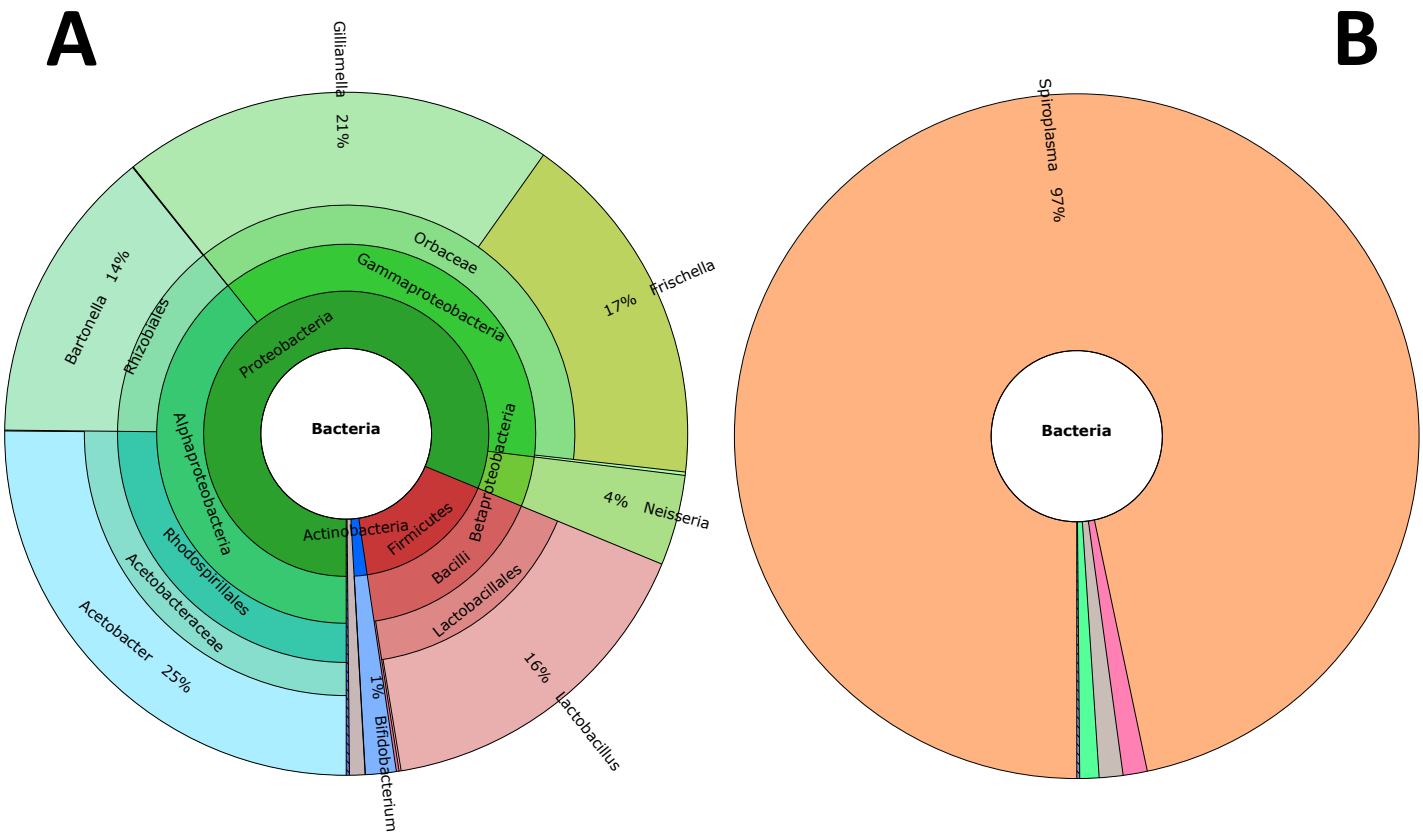


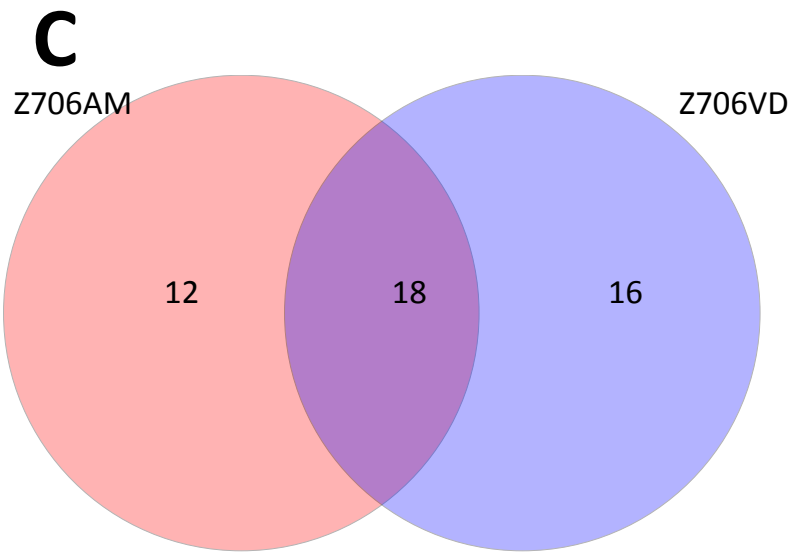


Obr. P20 - Stanoviště Štoky-Skřivánek Z74: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště

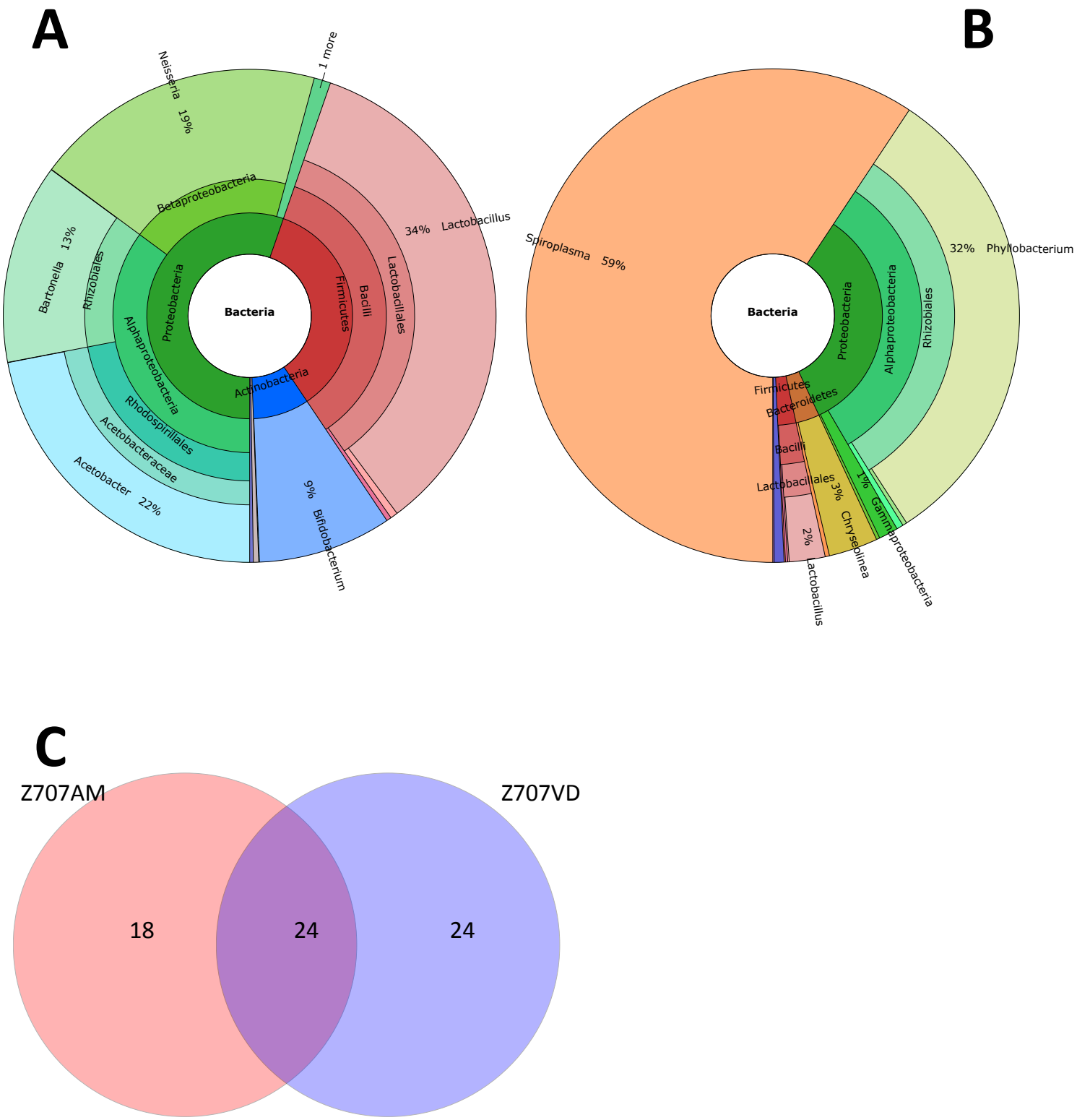


Obr. P21 - Stanoviště Štoky-Skřivánek Z705: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště

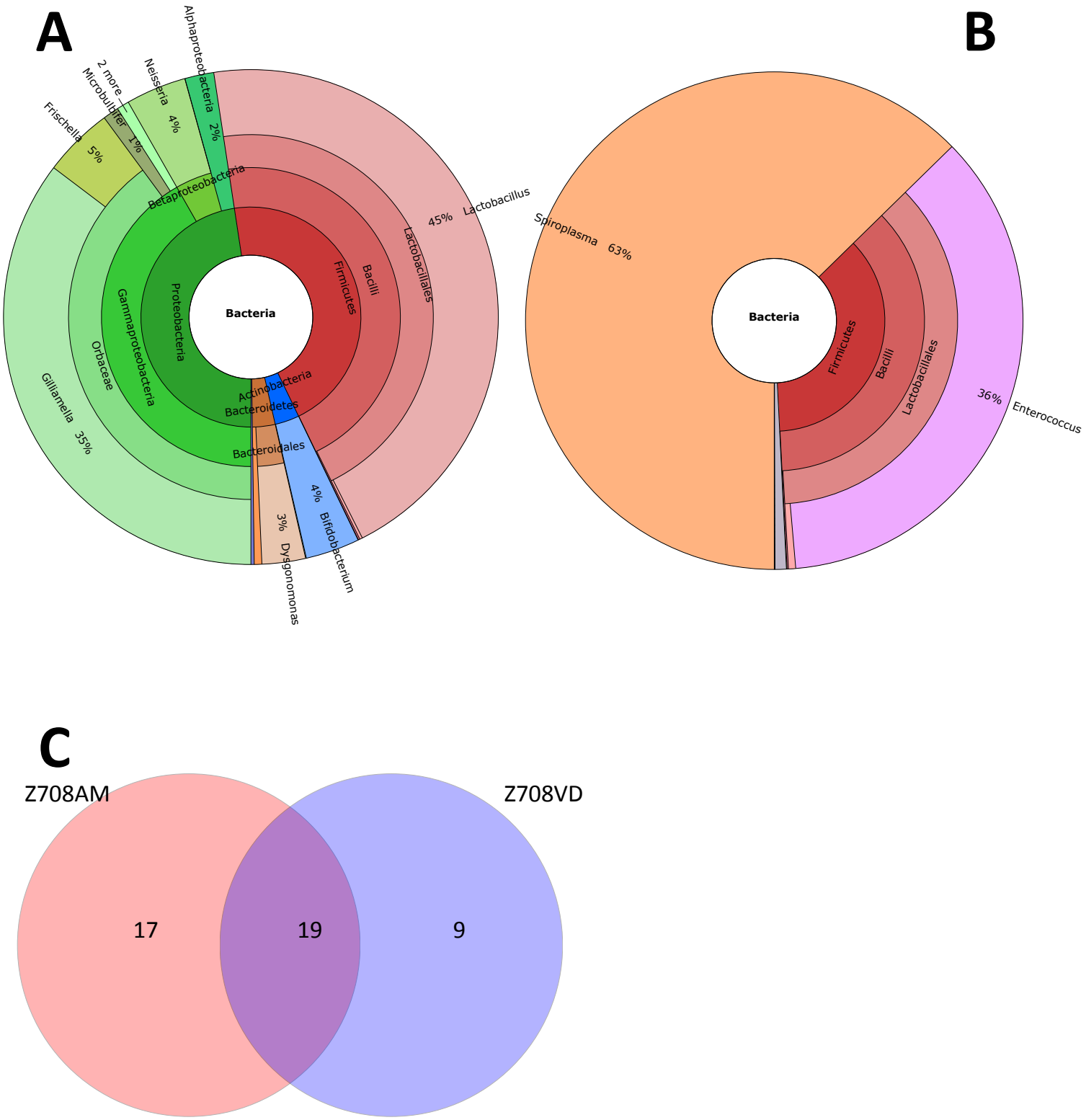




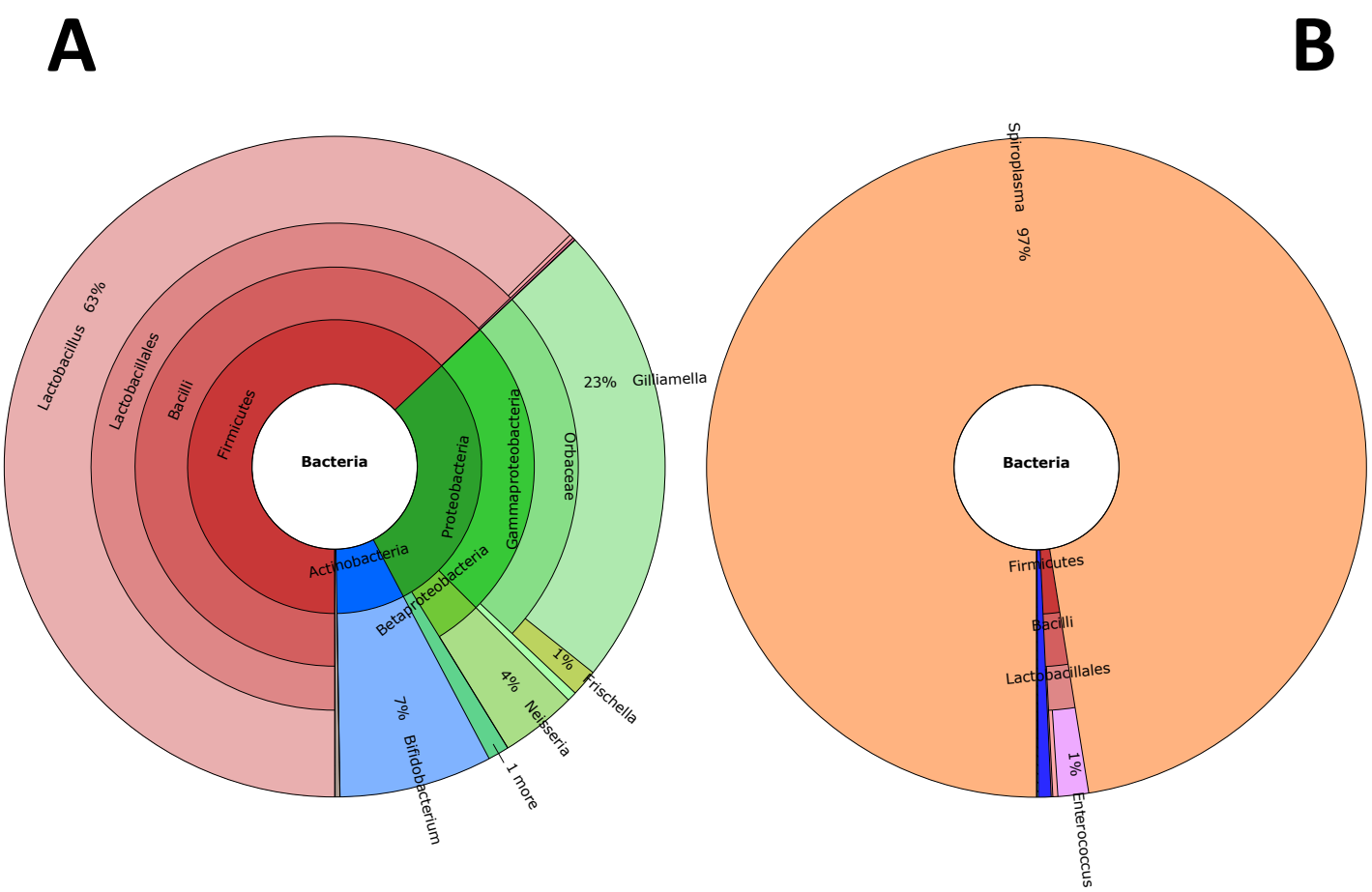
Obr. P22 - Stanoviště Štoky-Skřivánek Z706: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště

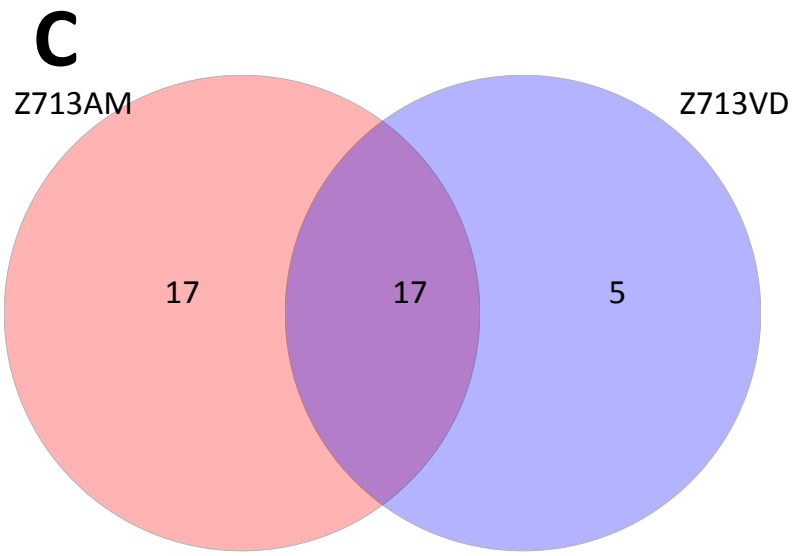


Obr. P23 - Stanoviště Štoky-Skřivánek Z707: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště

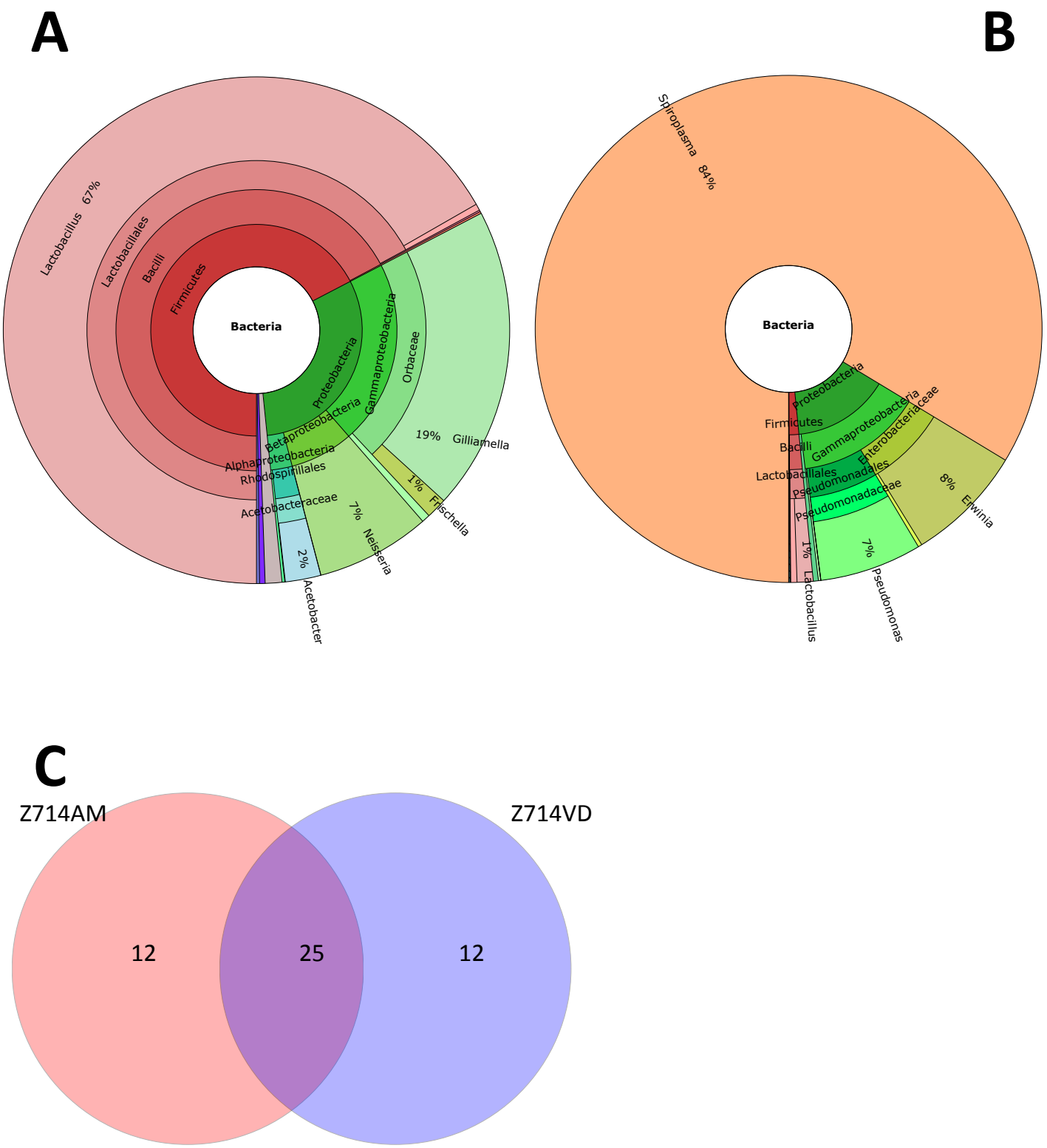


Obr. P24 - Stanoviště Štoky-Skřivánek Z708: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště

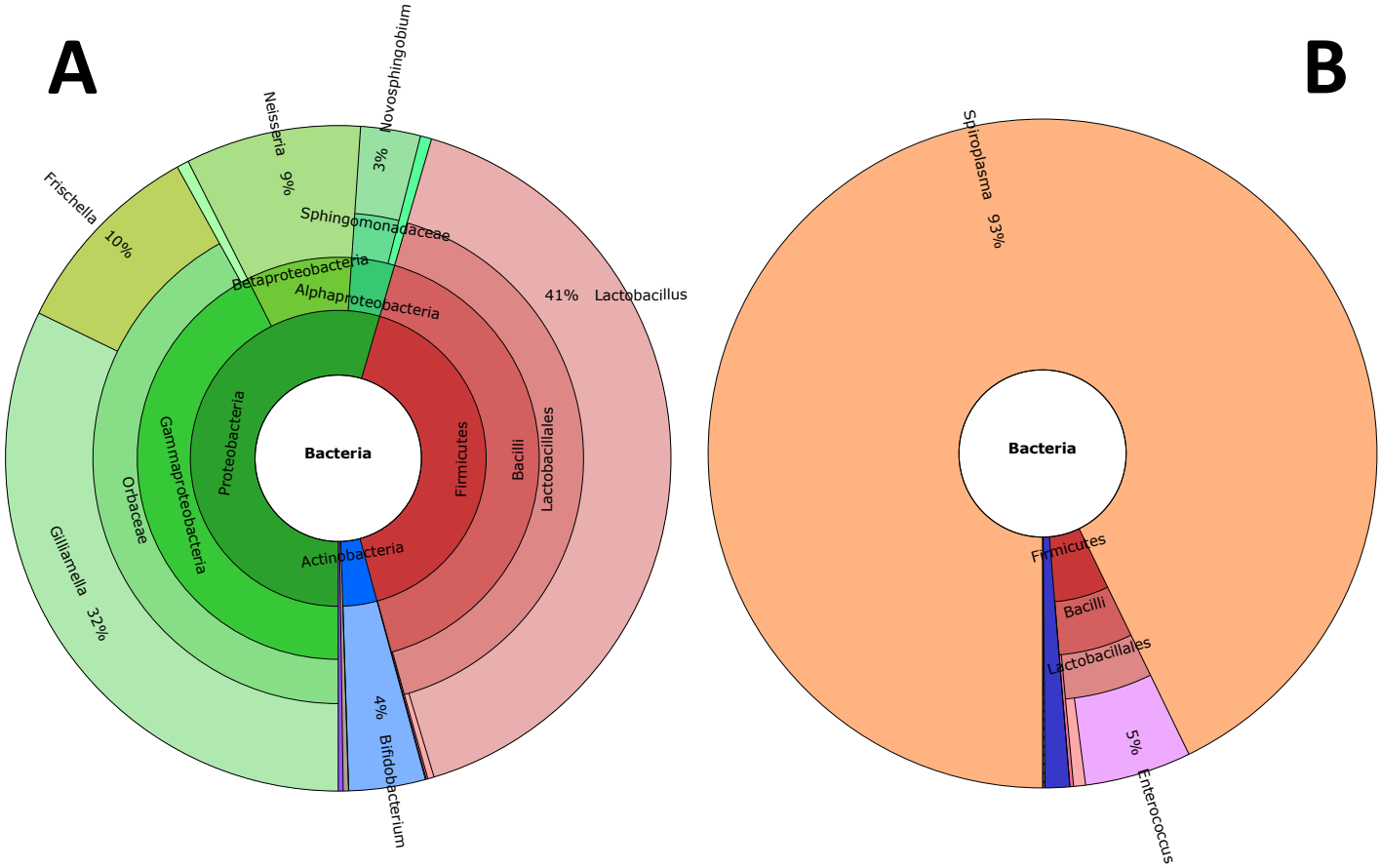


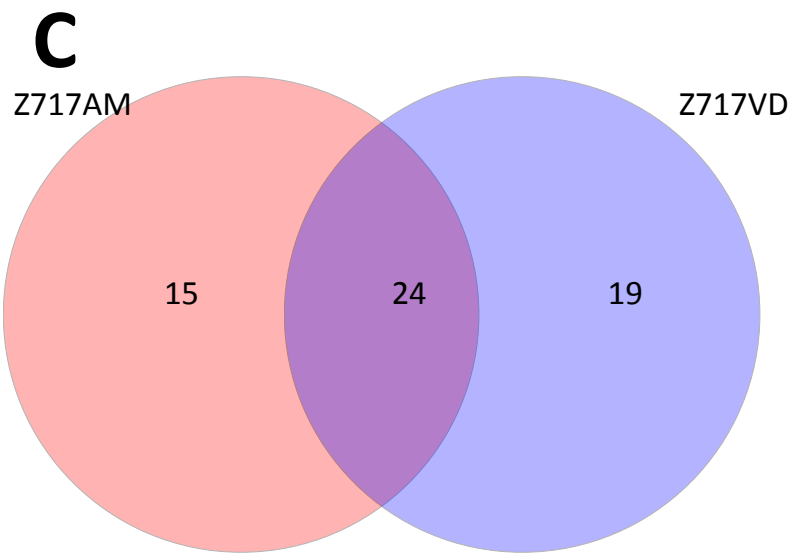


Obr. P25 - Stanoviště Štoky-Skřivánek Z713: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště



Obr. P26 - Stanoviště Štoky-Skřivánek Z714: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště





Obr. P27 - Stanoviště Štoky-Skřivánek Z717: AB - Krona zobrazení mikrobiomu v párových vzorcích včel medonosných (A) a roztočů *Varroa destructor* (B) pro jednotlivé taxony identifikované pomocí Ribosomal database project; C - Vennův diagram podobnosti pro párové vzorky z tohoto stanoviště

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ORIGINAL ARTICLE

Bacteria detected in the honeybee parasitic mite *Varroa destructor* collected from beehive winter debris

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Apis mellifera, *Arsenophonus*, bacteria feeding, diagnostics, pathogen, pathogen detection, *Spiroplasma*, *Varroa destructor*, winter wax debris.

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Abstract

Aims: The winter beehive debris containing bodies of honeybee parasitic mite *Varroa destructor* is used for veterinary diagnostics. The *Varroa* sucking honeybee haemolymph serves as a reservoir of pathogens including bacteria. Worker bees can pick up pathogens from the debris during cleaning activities and spread the infection to healthy bees within the colony. The aim of this study was to detect entomopathogenic bacteria in the *Varroa* collected from the winter beehive debris.
Methods and Results: Culture-independent approach was used to analyse the mite-associated bacterial community. Total DNA was extracted from the samples of 10 *Varroa* female individuals sampled from 27 different sites in Czechia. The 16S rRNA gene was amplified using universal bacterial primers, cloned and sequenced, resulting in a set of 596 sequences representing 29 operational taxonomic units (OTU_{97}). To confirm the presence of bacteria in *Varroa*, histological sections of the mites were observed. Undetermined bacteria were observed in the mite gut and fat tissue.
Conclusion: *Morganella* sp. was the most frequently detected taxon, followed by *Enterococcus* sp., *Pseudomonas* sp., *Rahnella* sp., *Erwinia* sp., and *Arsenophonus* sp. The honeybee putative pathogen *Spiroplasma* sp. was detected at one site and *Bartonella*-like bacteria were found at four sites. PCR-based analysis using genus-specific primers enabled detection of the following taxa: *Enterococcus*, *Bartonella*-like bacteria, *Arsenophonus* and *Spiroplasma*.
Significance and Impact of the Study: We found potentially pathogenic (*Spiroplasma*) and parasitic bacteria (*Arsenophonus*) in mites from winter beehive debris. The mites can be reservoirs of the pathogenic bacteria in the apicultures.

Introduction

In temperate regions, honeybee colonies produce either short-lived summer worker bees, which have a lifespan between 15 and 48 days, or long-lived winter worker bees, which emerge in late summer and live up to 8 months (Fluri 1990). The maintenance of honeybee longevity is critical for the survival of the honeybee colony during winter because in the temperate regions, there are major honeybee colony losses during this season (Dainat *et al.* 2012a; van Dooremalen *et al.* 2012; Erban *et al.* 2013).

In beekeeping practice, beehive debris is the most important matrix for surveying beehive health during winter. The bottom-board in apiculture is cleaned after winter acaricide treatment against *Varroa*. Thirty days after cleaning, the beehive debris on the bottom-board is collected. The collected beehive debris is screened for the most

important honeybee pathogens in veterinary laboratories under the conditions of Czechia: The pathogens included American foulbrood (AFB) (*Paenibacillus larvae*), *Nosema* spp. and *Varroa* mites (Ritter 2008; Copley *et al.* 2012; Dietemann *et al.* 2012; Alippi 2014). The beekeepers collect and deliver the beehive debris samples for investigation of the above mentioned pathogens to the accredited veterinary laboratories in Czechia according the Veterinary Law No. 166/1999.

The cosmopolitan parasitic mite *Varroa destructor*, previously classified as *Varroa jacobsoni* (Anderson and Trueman 2000), is one of the major factors responsible for the decline of the Western honeybee, *Apis mellifera* Linnaeus 1758, during winter (Amdam *et al.* 2004; vanEngelsdorp *et al.* 2009; Rosenkranz *et al.* 2010; Dietemann *et al.* 2012). Parasitic mites decrease the fitness, body weight, nutrient content and immunity of honeybees (Glinski and Jarosz 1992; Genersch and Aubert 2010; Dainat *et al.* 2012a,b). *Varro*a reproduces in the capped brood cells and sucks on the haemolymph of larvae, pupae and adult bees (Amdam *et al.* 2004; Rosenkranz *et al.* 2010). The honeybee haemolymph is composed mainly of water, inorganic salts, carbohydrates, proteins, hormones, lipids, free amino acids and macrophage-like cells (haemocytes), and offers *Varroa* a complete food source (Erban *et al.* 2014). The sucked haemolymph is stored in the mite digestive tract, including two pairs of caeca (De Ruijter and Kaas 1983). It has been shown that viruses and bacteria present in the mite digestive tract can be introduced to the honeybee haemolymph during mite feeding (Rosenkranz *et al.* 2010). The *Varroa*-honeybee virus transmission has been documented (Shen *et al.* 2005; Zhang *et al.* 2007; Gisder *et al.* 2009; Ryabov *et al.* 2014), while the transmission of bacteria is believed to be of minor importance (Rosenkranz *et al.* 2010).

Little is known about the bacterial community of *Varroa*. *Enterobacteriaceae* were found in 50–88% of the mites from the honeybee brood and workers in Poland (Glinski and Jarosz 1990). An unidentified *Rickettsia*-like organism was found in the rectum of *Varroa* using transmission microscopy (Liu and Ritter 1988). The cultured micro-organism from *V. destructor* included *Enterobacter cloacae* and *Staphylococcus albus* (Hrabak 2003). High numbers of actinobacterial genes were found in the total genomic DNA isolated from *Varroa*, while *Enterobacteriace* were not detected (Cornman *et al.* 2010). Glinski and Jarosz (1992) artificially inoculated *Varroa* mites with *Serratia marcescens* and the mites were able to transmit these bacteria into the haemolymph of drones and caused septicaemia. The most significant pathogenic bacteria in apiaries are *P. larvae* causing AFB and *Melissococcus plutonius* causing European foulbrood (EFB) (Genersch 2010). The *Varroa* mites from apiaries with high AFB infections contain the spores of *P. larvae* (De Rycke *et al.* 2002). It has been proposed that mites transmit AFB from the infected to healthy bee colonies; however, Alippi *et al.* (1995) could not demonstrate the infection of the bee colonies with *P. larvae* spores that were introduced on the body surface of *Varroa* mites. These observations open the question if there are entomopathogenic bacteria in *Varroa*.

In this study, we screened *Varroa* in the winter beehive debris for the presence of bacteria with particularly entomopathogen importance. The particular aims of this study were the following: (i) to describe the bacterial community in mites isolated from the winter beehive wax debris by culture-independent method; (ii) to prove the presence of bacteria in *Varroa* tissues by paraffin sectioning, staining and light microscopy; (iii) to collect evidence whether *Varroa* in the winter debris on the bottom-board is a possible source of entomopathogenic bacteria.

Material and methods

Mites in beehive winter wax debris

Samples of the winter hive debris were examined by a flotation technique in the accredited laboratory of the Bee Research Institute at Dol according Ritter (2008). After the examination of the samples, *Varroa* mites were collected in 96% pure ethanol. *Varroa* mites originated from the winter beehive wax debris collected at 27 sites in Czechia in December 2010 and January 2011 (Table 1). Every site represents a population of *Varroa* mites in one apiary.

DNA extraction from *Varroa* samples

Each sample contained 10 adult *Varroa* females. Before DNA extraction, 96% ethanol in each sample was replaced by phosphate buffered saline with the detergent Tween® 20 (PBST) composed of 3·2 mmol l^{-1} Na_2HPO_4, 0·5 mmol l^{-1} KH_2PO_4, 1·3 mmol l^{-1} KCl, 135 mmol l^{-1} NaCl, 0·05% Tween® 20 (cat. No. P9416, Sigma-Aldrich, Saint Louis, MO). Each sample was then washed three times with PBST and homogenized in 500 μl PBST using the Radnoti tissue grinder (cat No. 440613, Monrovia, CA). The total DNA was extracted with the Wizard® Genomic DNA Purification kit (Promega, Madison, WI) according to the manufacturer's instructions. The extracted DNA was stored in −20°C until further analysis.

Amplification, cloning and sequencing

PCR amplification of 16S rRNA gene fragments was used to characterize the bacterial community (Barbieri *et al.*

Table 1 Sites where the samples of *Varroa destructor* were taken. All samples are from Czechia from the winter 2010–2011

No.	Site	District	County	Coordinates	
1	Trhanov	Domazlice	Plzensky	49°25′7″	12°50′40″
2	Horni Bojanovice	Breclav	Jihomoravsky	48°57′20″	16°47′41″
3	Bulhary	Breclav	Jihomoravsky	48°49′22″	16°43′55″
4	Myslin	Pisek	Jihocesky	49°32′7″	14°1′36″
5	Pribice	Brno—venkov	Jihomoravsky	48°57′33″	16°34′40″
6	Kurdejov	Breclav	Jihomoravsky	48°57′12″	16°46′5″
7	Brod nad Dyji	Breclav	Jihomoravsky	48°52′38″	16°31′38″
8	Moravska Nova Ves	Breclav	Jihomoravsky	48°48′26″	17°0′55″
9	Hlohovec	Breclav	Jihomoravsky	48°46′21″	16°46′16″
10	Kdyně	Domazlice	Plzensky	49°23′35″	13°3′39″
11	Omice	Brno—venkov	Jihomoravsky	49°11′19″	16°27′4″
12	České Velenice	Jindrichuv Hradec	Jihocesky	48°46′47″	14°56′11″
13	Radejov	Hodonin	Jihomoravsky	48°51′56″	17°20′7″
14	Horenice	Nachod	Kralovehradecky	50°22′12″	15°54′48″
15	Klasterec nad Ohri	Chomutov	Ustecky	50°24′5″	13°10′53″
16	Obora	Jicin	Kralovehradecky	50°21′56″	15°30′47″
17	Hermanova Hut	Plzen—sever	Plzensky	49°42′37″	13°4′39″
18	Ujezdec	Jindrichuv Hradec	Jihocesky	49°12′16″	14°47′7″
19	Klenov	Jindrichuv Hradec	Jihocesky	49°11′40″	14°53′22″
20	Lom u Tachova	Tachov	Plzensky	49°49′14″	12°40′41″
21	Kruh	Semily	Liberecky	50°33′51″	15°29′33″
22	Majdalena	Jindrichuv Hradec	Jihocesky	48°57′33″	14°51′19″
23	Pravcice	Kromeriz	Zlinsky	49°19′33″	17°29′34″
24	Zeleznice	Jicin	Kralovehradecky	50°28′43″	15°22′48″
25	Kadan	Chomutov	Ustecky	50°23′9″	13°15′16″
26	Strelec	Jicin	Kralovehradecky	50°28′56″	15°15′37″
27	Kyvalka	Brno—venkov	Jihomoravsky	49°11′24″	16°26′57″

Table 2 The list of primers and reaction conditions used in the study.

Specificity	Target	Name	Primer 5′–3′	Tm (°C)	Lengt (bp)	Author
Bacteria	16S rRNA	UF	AGAGTTTGATYMTGGC	50	1400	Romero *et al.* (2006)
		UR	GYTACCTTGTTACGACTT			
Cardinium	16S rRNA	Card6	CTTAACGCTAGAACTGCGA	52	900	Kopecky *et al.* (2013)
		Card8	TCA AGCTCTACCAACTCC			
Enterococcus faecalis	*dll*	EfeF	TCAAGTACAGTTAGTCTTTATTAG	54	941	Macovei and Zurek (2006)
		EfeR	ACGATTCAAAGCTAACTGAATCAGT			
Wolbachia	16S rRNA	WpF	TTGTAGCCTGCTATGGTATAACT	52	900	O'Neill *et al.* (1992)
		WpR	GAATAGGTATGATTTTCATGT			
Bartonella-like	16S rRNA	Bart 1F	TGTCWCCGAYCCAGCCK	63	920	Kopecky *et al.* (2014)
		Bart 2R	TGT CTC CGA CCC AGC CT			
Arsenophonus	16S rRNA	ArsF	GGGTTGTAAAGTACTTTCAGTCGT	52	804	Wilkes *et al.* (2012)
/Morganella		ArsR2	GTAGCCCTRCTCGTAAGGGCC			
Arsenophonus	16S rRNA	Arsph_1F	TTGGGCCTCACACCTTC	57	1107	This study
		Arsph_1R	AGACTCCAATCCGGACTTC			
	fbaA	fbaAf	GCYGCYAAAGTTCRTTCTCC	52	659	Wilkes *et al.* (2012)
		fbaAr	CCWGAACCDCCRTGGAAAACAAAA			
	yaeT	yaeTf	GCATACGGTTCAGACGGGTTTG	52	473	
		yaeTr	GCCGAAACGCCTTCAGAAAAG			
Spiroplasma	16SRNA	BS1	AAGTCGAACGGGGTGCTT	57	975	Meeus *et al.* (2012)
		BS976	TGCACCACCTGTCTCAATGT			
Paenibacillus larvae	16S rRNA	AFB-F	CTTGTGTTTCTTTCGGGAGACGCCA	55	1106	De Graaf *et al.* (2013)
		AFB-R	TCTTAGAGTGCCCACCTCTGCG			
Melissococcus plutonius	16S rRNA	EFB-F	GAAGAGGAGTTAAAAGGCGC	55	812	Govan *et al.* (1998)
		EFB-R	TTATCTCTAAGGCGTTCAAAGG			

2001). The presence of *Cardinium*, *Wolbachia*, *Spiroplasma*, *Bartonella*-like bacteria, *Arsenophonus*, *Morganella*, *P. larvae*, *M. plutonius* and *Enterococcus faecalis* was tested using taxon-specific primers (Table 2). As a positive control, we used DNA from samples with previous positive identification based on the 16S rRNA gene (Kopecky *et al.* 2013, 2014) and the DNA extracted from *P. larvae* and *M. plutonius* obtained from the Bee Research Institute at Dol. The negative control was the double distilled water used for master mix preparation.

A total volume of 25-μl PCR reaction mixture contained 200 μmol l^{-1} dNTPs, 3 mmol l^{-1} $MgCl_2$; forward and reverse primers (100 nmol l^{-1} each), 0·5 unit *Taq* polymerase (all Promega) and 50–300 ng of the template DNA. The primers, annealing temperature and reference to amplification conditions are shown in Table 2. Amplifications were performed using the C1000 Thermal Cycler (Bio-Rad, Hercules, CA). The selected amplicons were purified with the Wizard® SV Gel and PCR product clean-up system kit (Promega). The PCR products from universal bacterial primers were cloned using pGEM®-T Easy Vector (Promega). Selected clones, roughly 28 per sample, were sequenced by Macrogen (Seoul, Korea). In addition, the products of taxon-specific primers were sequenced for validation. The obtained forward and reverse sequences were assembled with the CODONCODE ALIGNER, ver. 1.5.2 (CodonCode Corporation, Dedham, MA). The sequences were checked for chimaeras using MALLARD 1.02 (Ashelford *et al.* 2006) and PINTAIL 1.1 (Ashelford *et al.* 2005) prior to 16S rDNA library construction.

16S rRNA library

The library was formed from 596 partial 16S rRNA gene sequences (Table 3). The sequences were analysed and assigned the operational taxonomic units (OTU) defined at distance level 0·03 in MOTHUR v1.32.0 software (Schloss *et al.* 2009). The sequences were assigned to the bacterial taxa using the Ribosomal database project (RDP) naive Bayesian rRNA classifier (Wang *et al.* 2007). The consensus sequences of individual $OTUs_{97}$ were compared to the sequences in the GenBank using BLAST.

Phylogenetic analysis

The phylogenetic analysis was applied to *Bartonella*-like, *Spiroplasma* and *Arsenophonus* 16S rRNA sequences. Alignments of partial 16S rRNA gene sequences were performed using SILVA INCREMENTAL ALIGNER v.1.2.11 (Pruesse *et al.* 2012). For analysis of phylogenetic relationships, the best-fit model of nucleotide substitution was selected using JMODELTEST v.2.1.7 (Guindon and Gascuel 2003; Darriba *et al.* 2012). Based on the selection, model GTR with a proportion of invariable sites (+I), and gamma distribution with four rate categories (+G), was employed to infer phylogeny by Bayesian analysis using PHYLOBAYES-MPI, v.1.4e (Lartillot *et al.* 2009) and maximum-likelihood analysis in PHYML v.3.0 (Guindon *et al.* 2010). The resulting phylograms were finalized using MEGA 6 (Tamura *et al.* 2007).

Microanatomical sections of Varroa

For the histological study, *Varroa* mites were collected from the infested honeybee colony at the Bee Research Institute at Dol (50°12′13″N, 14°22′1″E) in the municipality of Maslovice. Mites were transferred from the honeybee pupae by a brush into Eppendorf tubes and fixed in the modified Bouin-Dubosque-Brazil fixation for 72 h (Smrz 1989). Mites were then transferred to paraffin and sectioned in 4–6 μm sections (Hubert *et al.* 1999). Sections were stained by (i) Masson's stain with periodic acid and Schiff agent (PAS) and (ii) Mann-Dominici staining. The histological sections were observed under a compound microscope Axioskop using AXIOVISON software (Carl Zeiss, Jena, Germany) and a Powershot A620 digital camera (Canon, Tokyo, Japan).

Results

Microbial community in *Varroa* from winter beehive wax debris

The 16S rDNA library contained 596 sequences (GenBank Acession Numbers KT029152–KT029747) that represented 29 OTU_{97}. The number of individual OTU_{97} ranged from 1 to 7 indicating low bacterial richness (Table 3). The sequences represented Actinobacteria: *Bifidobacterium;* Bacteroidetes: *Pedobacter*; Alphaproteobacteria: *Bartonella*-like bacteria; Firmicutes: *Enterococcus*; Gammaproteo-bacteria: *Morganella*, *Enhydrobacter*, *Pseudomonas*, *Arsenophonus* and *Erwinia*; Molicutes: *Spiroplasma*. OTU_{97} classified as *Morganella* were the most abundantly followed by *Enterococcus*, *Pseudomonas*, *Rahnella*, *Erwinia* and *Arsenophonus*. *Arsenophonus* was detected at the Strelec site only (Table 3). A potential honeybee pathogen *Spiroplasma* was detected solely at the Kadan site.

PCR with the specific primers confirmed the presence of *Spiroplasma* in one sample with a 99% similarity to *Spiroplasma apis* (NR121708). Similarly, the cloned *Spiroplasma* 16S rDNA fragment showed a high similarity (99%) to *S. apis*. It also confirmed the phylogenetic analysis of the sequences obtained in RDP (Fig. 1).

Table 3 The identified bacterial taxa in the *Varroa destructor* females from the winter wax debris collected at 27 different sites in Czechia. The sequences were identified using the Ribosomal database project (RDP) naïve Bayesian rRNA classifier (Wang *et al.* 2007). The contingency for operational taxonomic units $(OTUs)_{97}$ were Blasted in GenBank and the matches are presented

RDP-identification				GenBank-match						
Taxon			id %	Matched sequences	Acces. No.	id%	OTU_{97}	N	1	2
Actino-bacteria	Bifidobacteriales	*Bifidobacterium*	100	*Bifidobacterium indicum*	CP006018.1	99	12	7		
	Actinomycetales	*Microbacterium*	100	*Microbacterium oxydans*	KF150504.1	99	22	1		
Alphaproteo-bacteria	Rhizobiales	*Bartonella*	43–67	*Bartonella*-like	HM108389.1	99	9	15		
		Methylobacterium	100	*Methylobacterium adhaesivum*	AB698723.1	99	27	1		
	Sphingomonadales	*Sphingomonas*	100	*Sphingomonas faeni*	KC987002.1	99	20	1		
Bacilli	Lactobacillales	*Enterococcus*	100	*Enterococcus faecalis*	FJ378665.1	99	2	100		
		Lactobacillus	92–93	*Lactobacillus* sp.	HM046580.1	95	18	2		
	Bacillales	*Staphylococcus*	100	*Staphylococcus nepalensis*	JQ970476.1	99	29	1		
Betaproteo-bacteria	Burkholderiales	*Burkholderia*	100	*Burkholderia cepacia*	AB695353.1	99	24	1		
		Janthinobacterium	100	*Janthinobacterium lividum*	EU275366.1	99	19	2		
		Massilia	100	*Massilia* sp.	FM955855.1	99	17	2		
		Undibacterium	100	*Undibacterium oligocarboniphilum*	NR 117348.1	99	26	1		
Gammaproteo-bacteria	Oceanospirillales	*Carnimonas*	100	*Carnimonas nigrificans*	NR_029342.1	98	14	4		
	Pasteurellales	*Haemophilus*	100	*Haemophilus parainfluenzae*	FQ312002.1	99	25	1		
	Xanthomonadales	*Luteibacter*	100	*Luteibacter* sp.	FR714940.1	99	11	6		
	Enterobacteriales	*Morganella*	100	*Morganella* sp.	HM133591.1	99	1	264	21*	21*
		Pantoea	100	*Pantoea agglomerans*	EU047555.1	99	15	4		
		Enhydrobacter	100	*Moraxella osloensis*	NR 104936.1	99	9	19		
		Pseudomonas	100	*Pseudomonas* sp1.	AY131218.1	99	3	60		
				Pseudomonas syringae	AB001449.1	99	8	17		
				Pseudomonas lutea	NR_029103.1	99	4	4		
				Pseudomonas sp.2	AF098465.1	95	21	1		
				Pseudomonas aeruginosa	AP014622.1	99	23	1		
		Psychrobacter	100	*Psychrobacter* sp.	JX310264.1	99	28	1		
	Enterobacteriales	*Arsenophonus*	100	*Arsenophonus nasoniae*	FN545282.1	100	6	21		
		Erwinia	90–100	*Erwinia billingiae*	NR_102820.1	100	5	24		
		Serratia	69–95	*Rahnella* sp.	GU299866.1	100	4	25		
Mollicutes	Entomoplasmatales	*Spiroplasma*	100	*Spiroplasma apis*	NR_121708.1	99	10	6		
Sphingo-bacteria	Sphingobacteriales	*Pedobacter*	100	*Pedobacter cryoconitis*	NR_025534.1	99	13	4		
Total number								596	21	21

Ide, identity %.

*Indicates the confirmation of the bacterial taxa by specific primers. Detailed description of sample sites in Table 1.

Bartonella-like bacteria were confirmed at the Hlohovec and Klenov sites but not in Ujezd site. In addition, *Bartonella*-like amplicons were obtained at the Zeleznice and Kyvalka sites (Table 3). *Bartonella*-like bacteria clustered to those previously reported from honeybees (Fig. 2).

The *Arsenophonus*-specific primers ArsF and ArsR2 (Table 2) were not specific for *Arsenophonus*, and the 16S rDNA sequences were highly similar (99%) to *Morganella* sp. (KF754866), with the exception of the Strelec site, where the sequences related to *Arsenophonus nasoniae* were

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detected. To confirm the presence of *Arsenophonus,* the new primer set (*Arsph* 1F and *Arsph* 1R), for identification based on 16S rDNA, was designed (Table 2) and tested altogether with the primers for amplification of genes coding for outer membrane protein (*yaeT*) and fructose-bisphosphate aldolase class II (*fbaA*) (Wilkes *et al.* 2012). At the Strelec site, the *Arsph* 1F and *Arsph* 1R primers amplified the fragment of 16S rDNA of 1107 bp with a high similarity (99%) to *A. nasoniae* (NR042811) as confirmed by the phylogenetic analysis (Fig. 3). The products of the correct size, *yaeT* 473 bp and *fbaA* 659 bp were obtained, and the sequencing confirmed their high similarity (99%) to

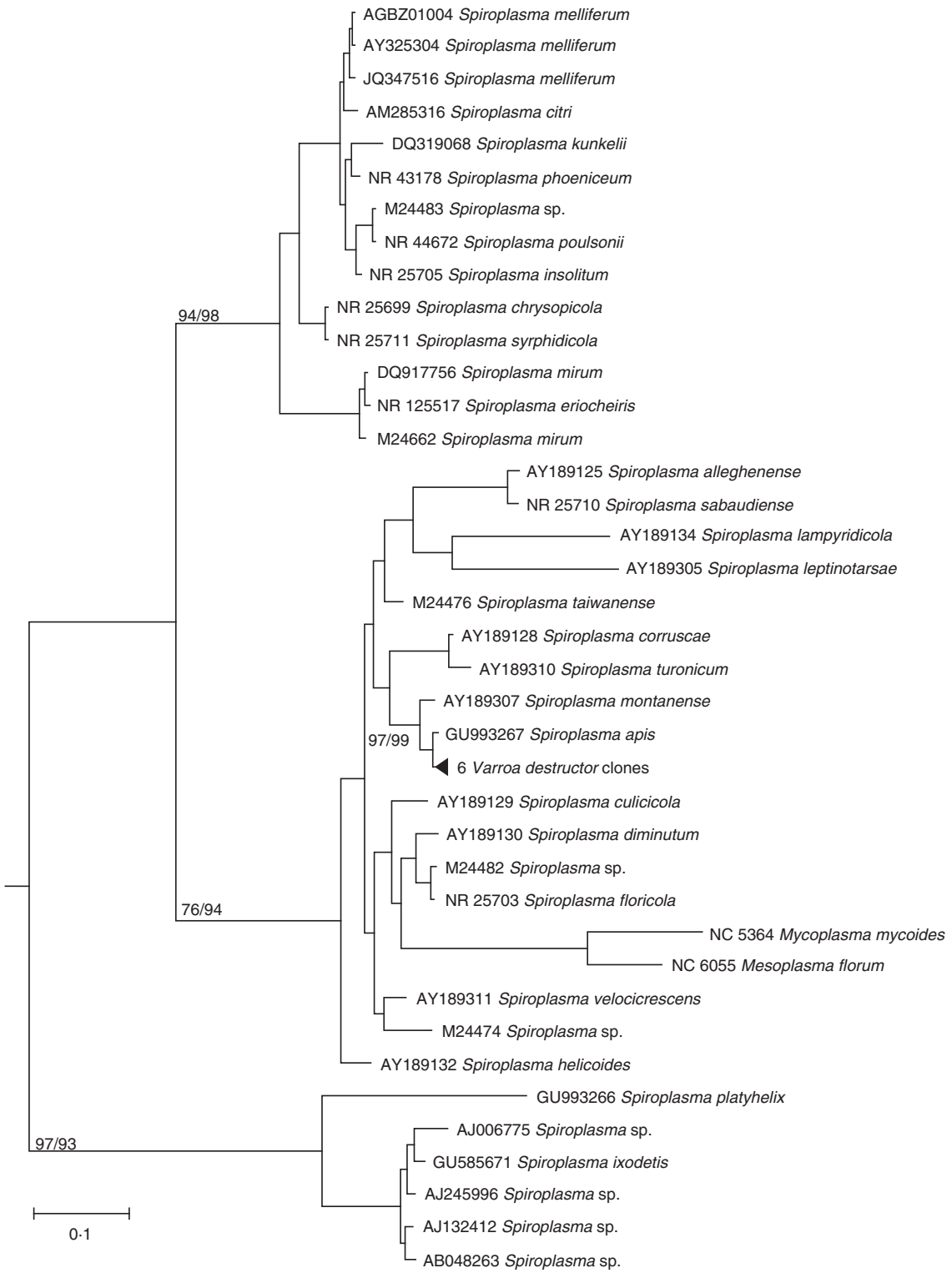

Figure 1 Phylogenetic analysis of nearly full-length 16S rRNA clones of Spiroplasma from *Varroa destructor* females collected from the winter beehive wax debris with the reference sequences available in databases. Phylogeny was inferred by Bayesian analysis. Branch lengths correspond to mean posterior estimates of evolutionary distances (scale bar, 0·1). Branch labels at selected branches indicate the Bayesian posterior probability and supporting bootstrap values from maximum-likelihood and neighbour-joining analyses. The phylograms were outgrouped using the Anaeroplasma varium 16S rRNA gene sequence NR_044663.

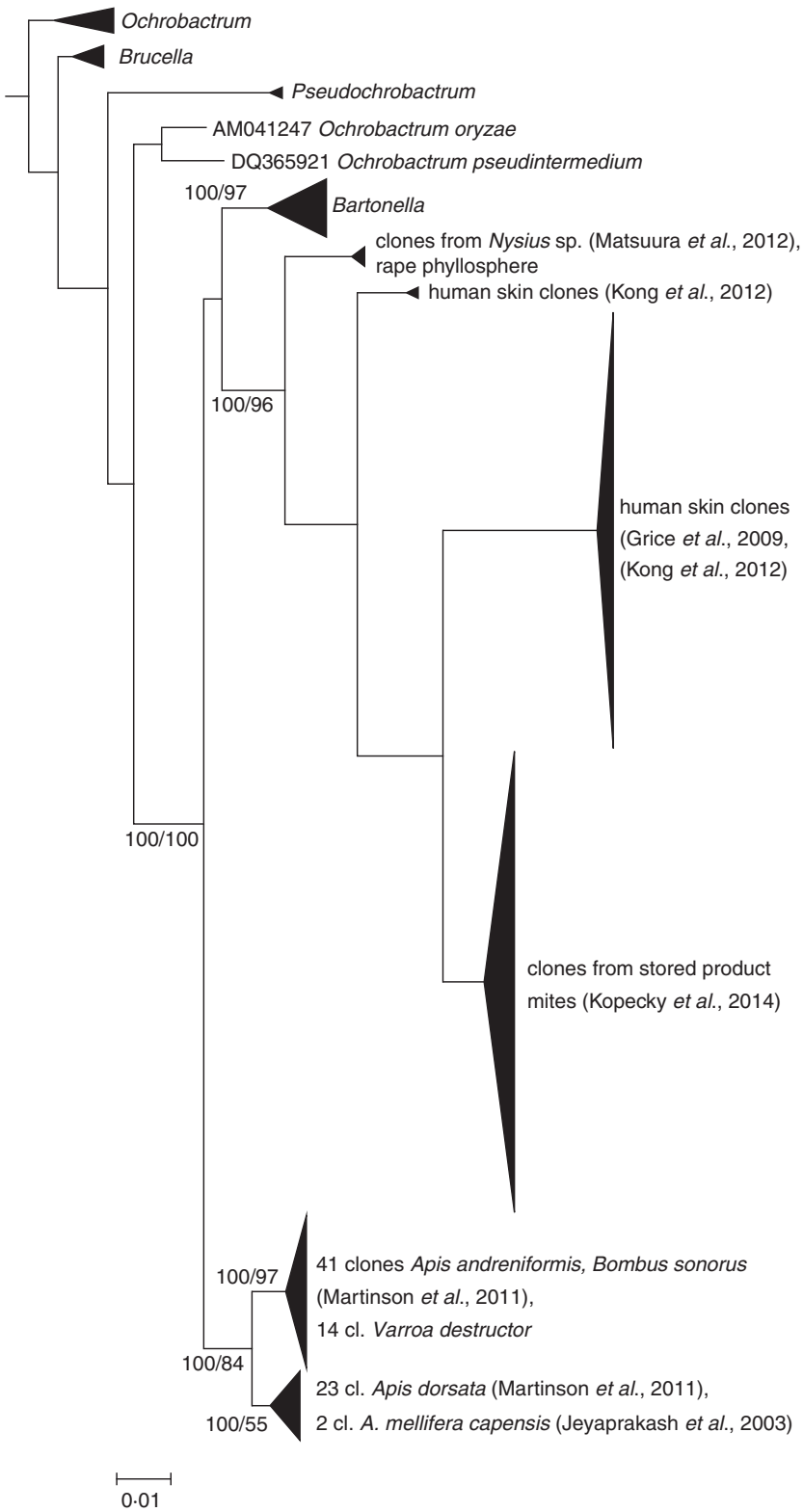

Figure 2 Phylogenetic analysis of nearly full-length 16S rRNA clones described as Bartonella-like sequences from *Varroa destructor* females from the winter wax debris with the reference sequences available in databases. Phylogeny was inferred by Bayesian analysis. Branch lengths correspond to mean posterior estimates of evolutionary distances (scale bar, 0·1). Branch labels at selected branches indicate the Bayesian posterior probability and supporting bootstrap values from maximum-likelihood and neighbour-joining analyses. The phylograms were outgrouped using the *Escherichia coli* sequence U00096.

A. nasoniae FN545250 and FN542641 respectively. *Arsenophonus* was not detected on other localities by either set of specific primers (*Arsph, yaeT, fbaA*).

The presence of *Enterococcus* was confirmed by specific primers for the amplification of the fragment of D-alanine ligase-related protein (*ddl*) (Macovei and Zurek 2006),

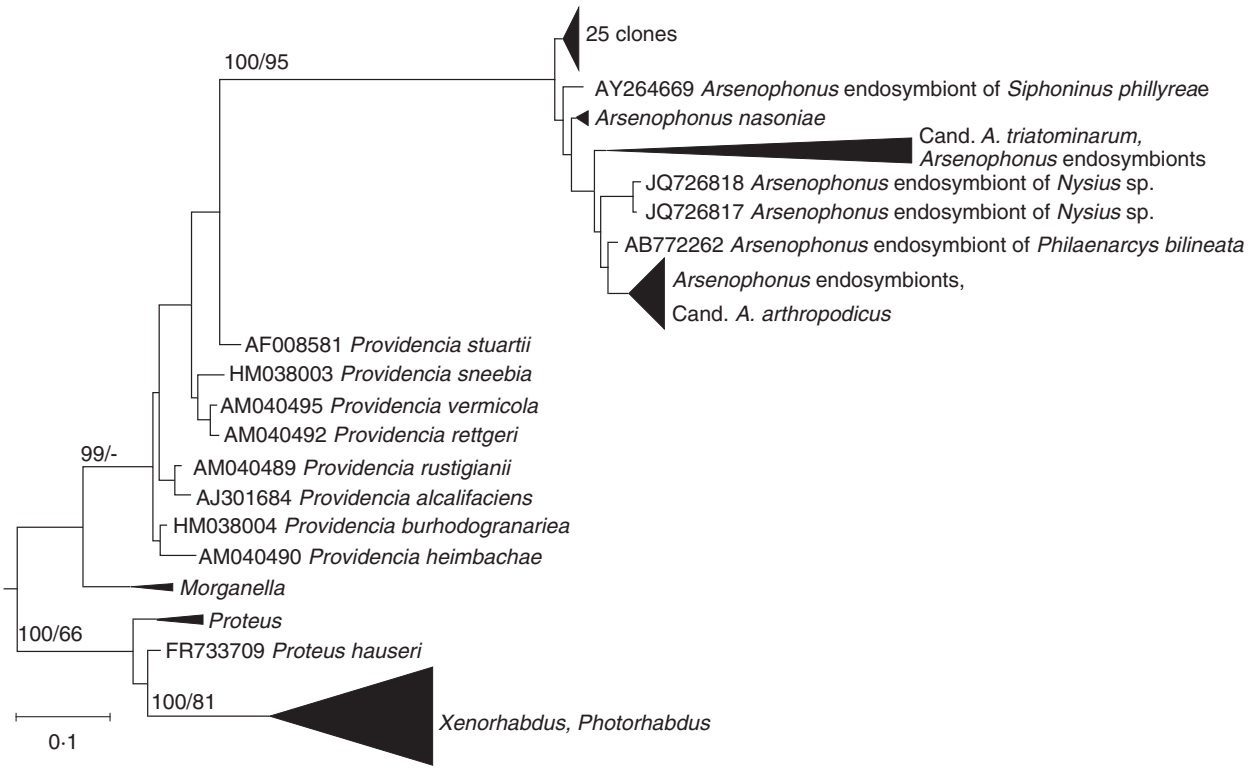

Figure 3 Phylogenetic analysis of nearly full-length 16S rRNA clones of Arsenophonus from *Varroa destructor* females from the winter beehive wax debris with the reference sequences available in databases. The phylogeny was inferred by Bayesian analysis of 16S rRNA gene sequence alignment. Branch lengths correspond to mean posterior estimates of evolutionary distances (scale bar, 0·1). Branch labels indicate the Bayesian posterior probability and supporting bootstrap values from maximum-likelihood analysis. The phylogram was outgrouped using the type strains of the remaining Enterobacteriaceae genera.

the obtained product had high similarity (99%) to *Enterococcus faecalis* (AB186032). Using the universal set or specific primers, *P. larvae*, *M. plutonius*, *Cardinium* spp. and *Wolbachia* spp. were not detected in the examined *Varroa* samples.

Histological observation of *Varroa* females

The gut of *Varroa* contained the ingested haemolymph of honeybees that formed a dark substance in the mesodeum, including caeca (Fig. 4a–c). The substances formed a compact mass which were utilized in the gut (Fig. 4b). Some particles in the gut carried coccoid bacteria, either as single cells or in small clusters (Fig. 4d). Some bacteria were also localized in the fat tissue (Fig. 5a) where they formed groups of 10–50 bacterial cells (Fig. 5b,c).

Discussion

In this study, we described the bacteria associated with *V. destructor* collected from winter beehive wax debris by the culture-independent approach. We confirmed the presence of bacteria in the gut and fat tissue. The origin of bacteria in *Varroa* can be (i) autochthonous symbiotic/parasitic bacteria in the fat body; (ii) autochthonous gut bacteria; (iii) allochtonous bacteria either from honeybee haemolymph or bee surface; (iv) contamination from the debris and/or bottom-boards. We compared the 16S rDNA sequences obtained from *Varroa* in this study to that of near full-length 16S rDNA sequences of bacteria from *A. mellifera* available in the GenBank (2584 sequences) at 97% identity level. We obtained 86 bacterial OTUs unique for *A. mellifera*, 16 OTUs shared between the two species and 6 OTUs specific for *Varroa* (Fig. 6). The rest of the OTUs were formed by single sequences, i.e. 7 for *Varroa* and 97 for *A. mellifera*. This again indicated that the bacteria in *Varroa* likely originated from *A. mellifera*. However, the used approach with 22 clones per sample cannot cover all the bacteria from the mite sample. To distinguish which bacterial taxa are allochthonous or autochthonous, a further study is necessary. The assay used here is based on the surface sterilization and cleaning of mites. We consider the sur-

face sterilization as efficiently removing the surface contamination as previously validated for stored product mites (Kopecky *et al.* 2014). The contamination of PCR should be minimized due to the presence of negative PCR control (Fenollar and Raoult 2004).

In this study, we found that *Varroa* mites were a possible reservoir of *Spiroplasma* and *Bartonella*-like bacteria. *Spiroplasma melliferum* and *S. apis* have been considered as the causative agents of neurological diseases of honeybees but also as facultative (secondary) symbionts with 33 and 54% prevalence in colonies in the USA and Brazil respectively (Schwarz *et al.* 2014). Ravoet *et al.* (2013) did not observe a correlation between the *S. melliferum* and *S. apis* and winter losses of honeybees. Flowers were suggested as the main reservoir of *Spiroplasma* and honeybees get infected in the spring and summer season (Clark 1978; Davis 1978; Mouches *et al.* 1984). It has been reported that the prevalence of *S. melliferum* in the samples from honeybee colony increased from 5% in February to 68% in May and then decreased to 25% in June and 22% in July (Zheng and Chen 2014). The sequences obtained here were highly similar to *S. apis*, but not *S. melliferum*. A possible participation of mites in spreading *Spiroplasma* to honeybees in the spring remains to be investigated.

Bartonella-like bacteria were detected in honeybees previously (Jeyaprakash *et al.* 2003; Ahn *et al.* 2012; Martinson *et al.* 2012). The related sequences were recently found in the fall and spring bees and the environment was suggested as the main source of these bacteria (Corby-Harris *et al.* 2014). In our study, the sequences of *Bartonella*-like bacteria from *Varroa* clearly clustered with those from honeybees (Fig. 4) and these bacteria likely originated from honeybees. The significance of *Bartonella*-like bacteria in *Varroa* physiology and health

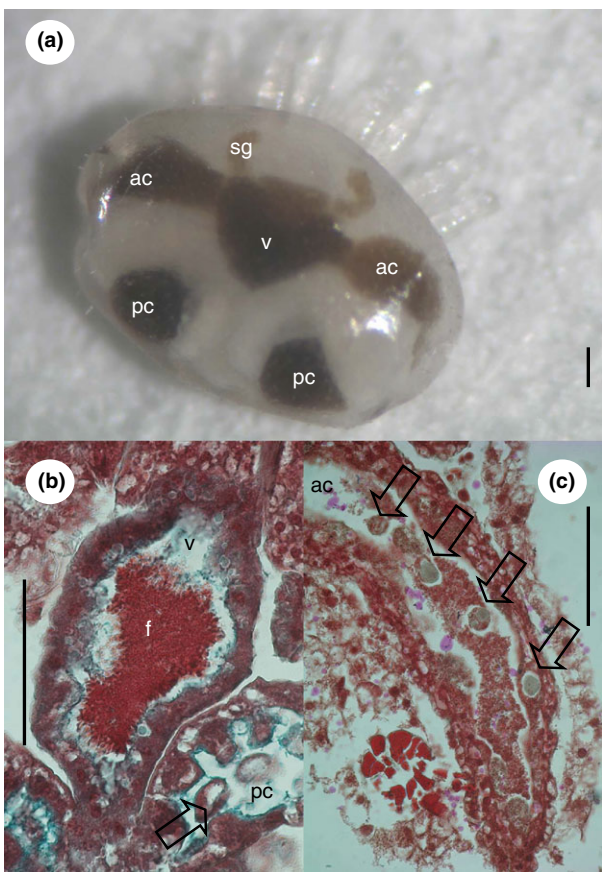

Figure 4 Digestive tract of female *Varroa destructor*; (a) overall view of the young female; (b) horizontal section of the female; arrow points to apocrine secretion in the posterior gastric caecum; (c) saggital section of the female, arrows point to secretion of the digestive enzymes in the anterior gastric caecum; Scale (a–c) 100 μm; Staining: (b, c) Masson triple stain and Periodic acid–Schiff (PAS); ac, anterior caecum; f, ingested food; v, ventriculus; pc, posterior caecum; sg, salivary gland; r, rectum.

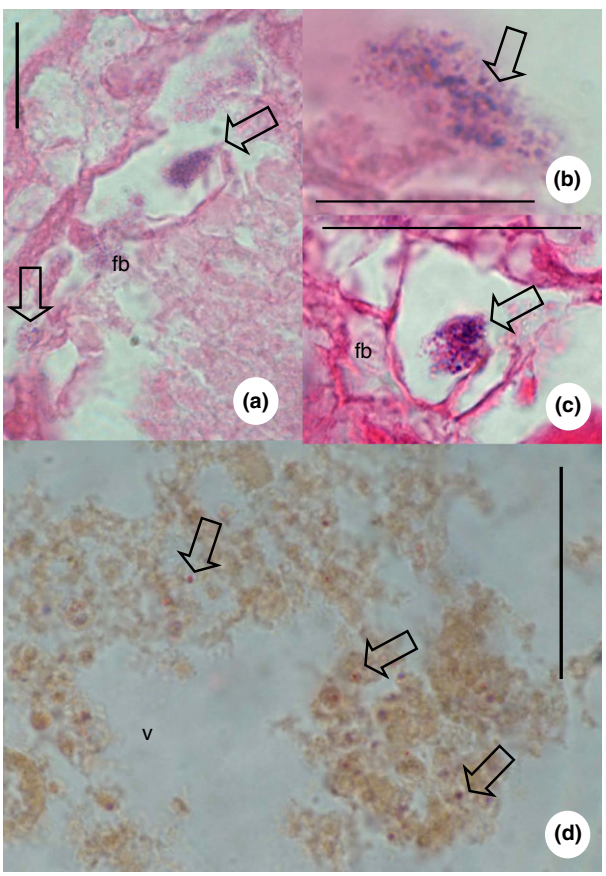

Figure 5 Identification of bacteria in the fat tissue and the digestive tract of female *Varroa destructor*; (a) overall view of the fat body with the groups of bacteria pointed by arrows; (b, c) detail of previous bacterial groups pointed by arrows; (d) bacteria (arrows) in the ingested food in the ventriculus; Scale: (a, c, d) 25 μm; (b) 10 μm; Staining: Mann-Dominici

(Kopecky *et al.* 2014) as well as the role of these mites in transmission of bacteria to bees is unknown.

In our study, we also found a high prevalence of *Ent. faecalis* in the *Varroa* gut. Enterococci are an important reservoir of antibiotic resistance traits and frequently acquire antibiotic resistance genes by horizontal gene transfer (Arias and Murray 2008). The high prevalence of *E. faecalis* in *Varroa* may have important health implications for honeybees and people. We also detected enteric bacteria *Erwinia* and *Serratia,* and these were also reported from honeybees (Babendreier *et al.* 2007; Disayathanoowat *et al.* 2012; Aizenberg-Gershtein *et al.* 2013). *Enterobacteriaceae* were commonly (up to 88%) detected in *V. destructor* (Glinski and Jarosz 1990) as well as in the digestive tract of honeybees (Anderson *et al.* 2013).

Another interesting finding in our study was the presence of *Arsenophonus* in mites from one site. *Arsenophonus* is an endosymbiont of insects (Novakova *et al.* 2009; Wilkes *et al.* 2012), but it was detected also on the body surface and in the digestive tract of honeybees (Babendreier *et al.* 2007; Aizenberg-Gershtein *et al.* 2013). In a recent study (Aizenberg-Gershtein *et al.* 2013), *Arsenophonus* sequences represented 43 and 88% of the total bacterial community in the bees captured on almond (*Amygdalus communis*) and grapefruit (*Citrus paradisi*) flowers respectively. *Arsenophonus* was also found in the crop and corbicular pollen in a honeybee colony Corby-Harris *et al.* (2014). Analyses of the microbial community of bees with the colony collapse disorder demonstrated increased numbers of bacteria related to *Arsenophonus* (Cornman *et al.* 2012) but the significance of this finding is not clear.

The most frequent sequences obtained from *Varroa* were related to *Morganella morganii*. *Morganella* was reported as most abundant in the hibernating bees (Lyapunov *et al.* 2008) but also from the spring and summer bees (Aizenberg-Gershtein *et al.* 2013). The mites likely obtained *M. morganii* during feeding on bees, especially during fall and winter.

Some bacteria are known to have a negative effect on *Varroa*. For example, *Bacillus* spp. and members of *Miccrococaceae* caused up to 54% mite mortality (Tsagou

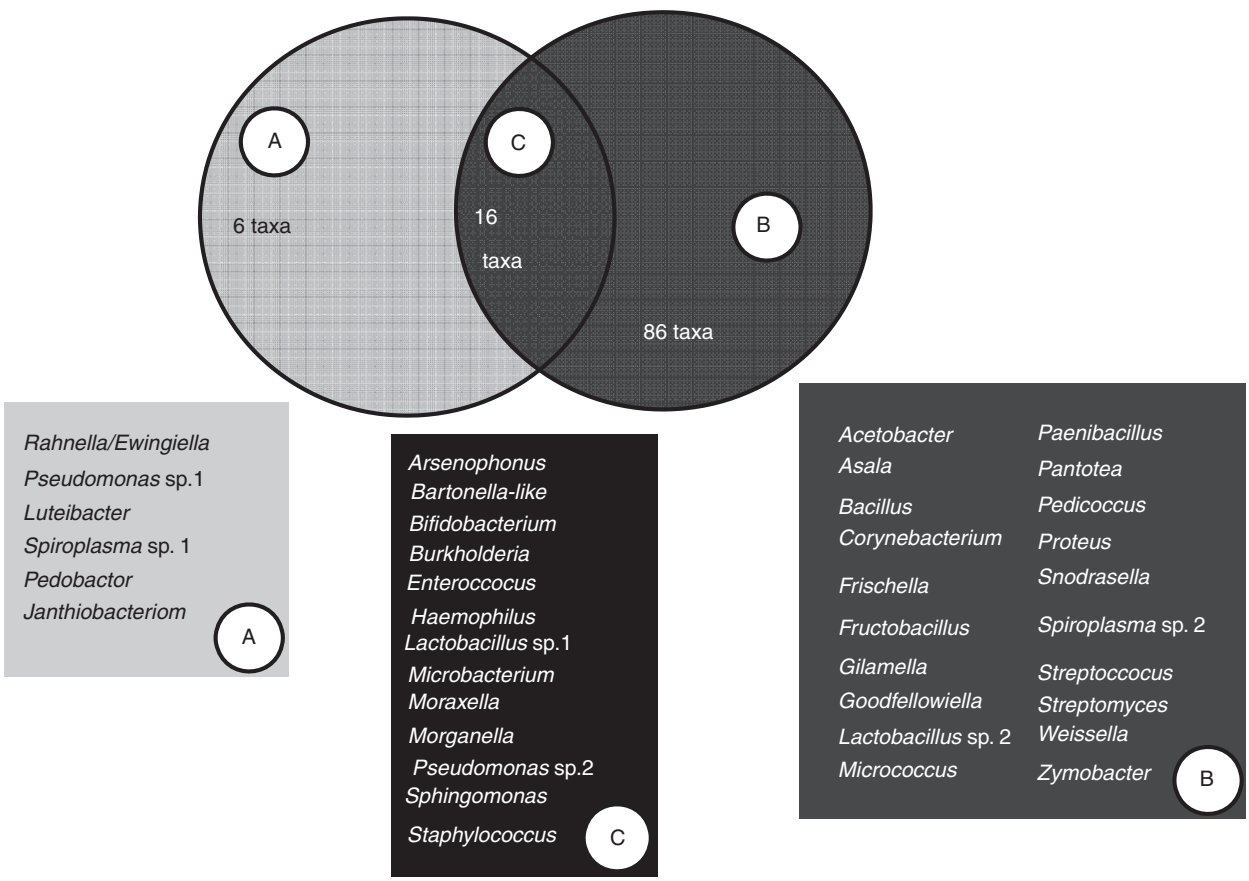

Figure 6 Venn diagram showing bacterial 16S rDNA sequences cloned from *Varroa destructor* females from the beehive winter wax debris (596 sequences) and sequences from *Apis mellifera* obtained from GenBank (2584). The operational taxonomic units (OTUs) were defined at 97% identity level. The OTUs with single sequences, 8 from *V. destructor* and 97 from *A. mellifera*, were not included in the diagram.

et al. 2004). These two groups of acaropathogenic bacteria were not detected in *Varroa* from winter beehive wax debris in our study; however, we found *Pseudomonas* as another putative acaropathogenic bacterium. *Pseudomonas putida* was used in the laboratory experiments to control the spider mite *Tetranychus urticae* (Acari: Tetranychidae). Spraying with bacteria resulted in increased egg mortality and reduction in egg hatching (Aksoy *et al.* 2008). *Pseudomonas* bacteria were also found in the gut of fall and spring honeybees (Corby-Harris *et al.* 2014). Ahn *et al.* (2012) detected *Pseudomonas* in adults but not in the larvae of honeybees.

Our study indicated the presence of entomopathogenic bacteria in *Varroa*. Although we did not prove the transmission of bacteria due to the experimental design, the results indicated that *Varroa* mites are likely reservoirs of bacteria such as *Spiroplasma*, *Arsenophonus* and *Bartonella*-like. The demonstrated detection of entomopathogenic bacteria in the mites collected from beehive winter debris is useful to identify the entomopathogens in honeybees in future. The results are relevant to honeybee health, because wintering honeybees can pick up pathogens from the beehive debris during cleaning activities, and spread the infection to healthy bees within the colony.

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Conflict of Interest

The authors declare that they have no conflict of interests.

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