

Supplementary Material

Environment or genetic isolation? An atypical intestinal microbiota in the Maltese honey bee
Apis mellifera subsp. *ruttneri*

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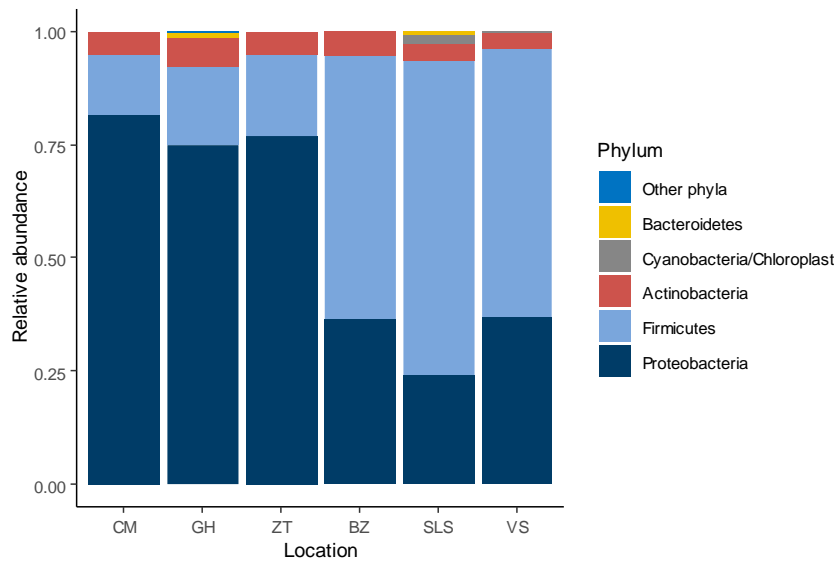
†These authors contributed equally to this work and share first authorship

1 Supplementary Figures and Tables

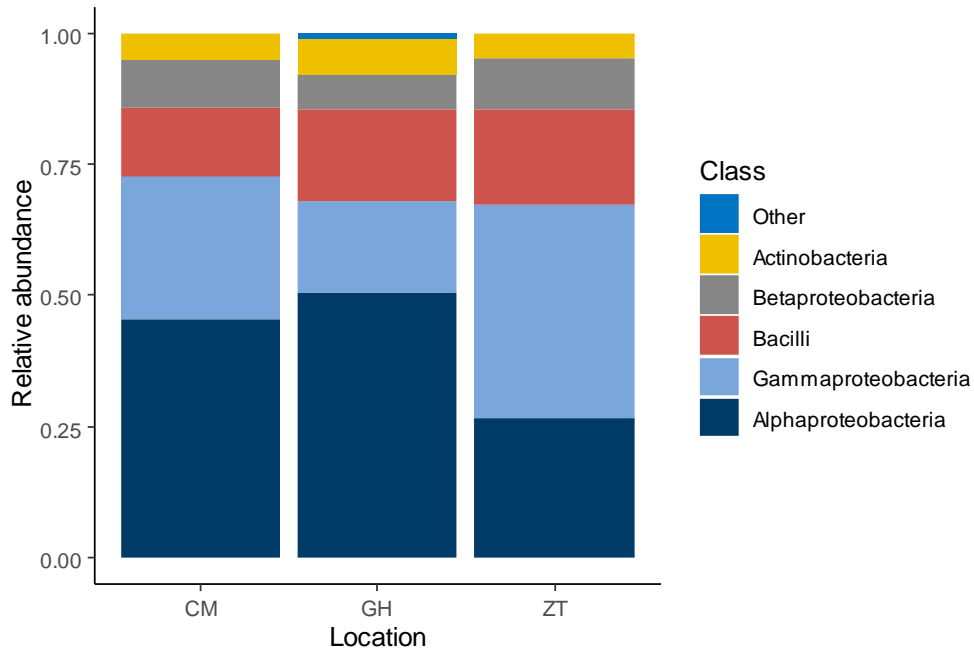
1.1 Supplementary Figures



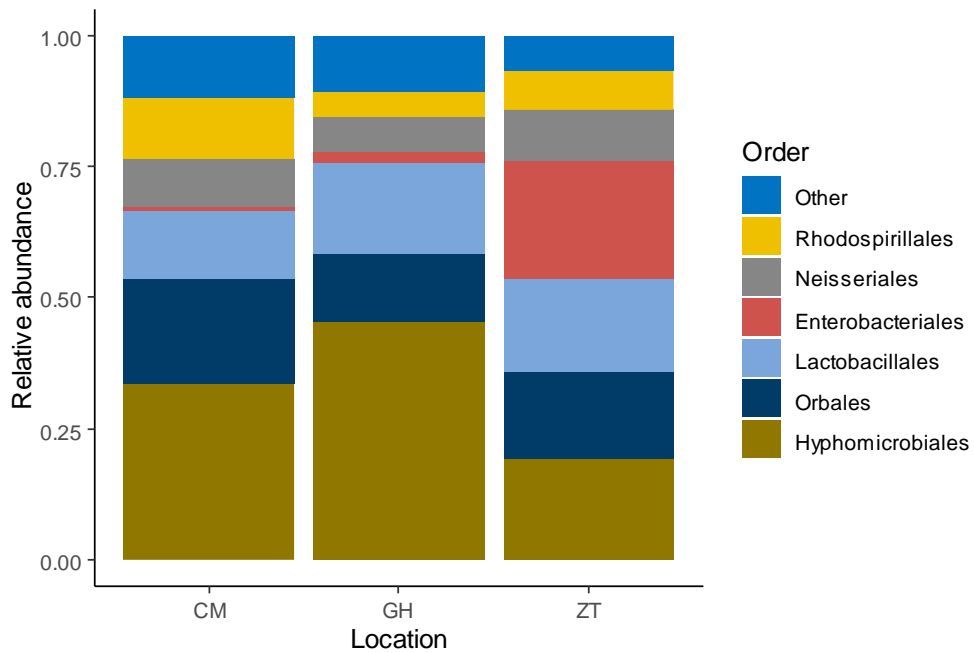
Supplementary Figure 1A-1B. Up until the 1950's, terracotta hives populated with *Apis mellifera ruttneri* were typical of the Maltese Island. This apiary belongs to Mario Sant, a beekeeper promoting Maltese honey bee conservation.



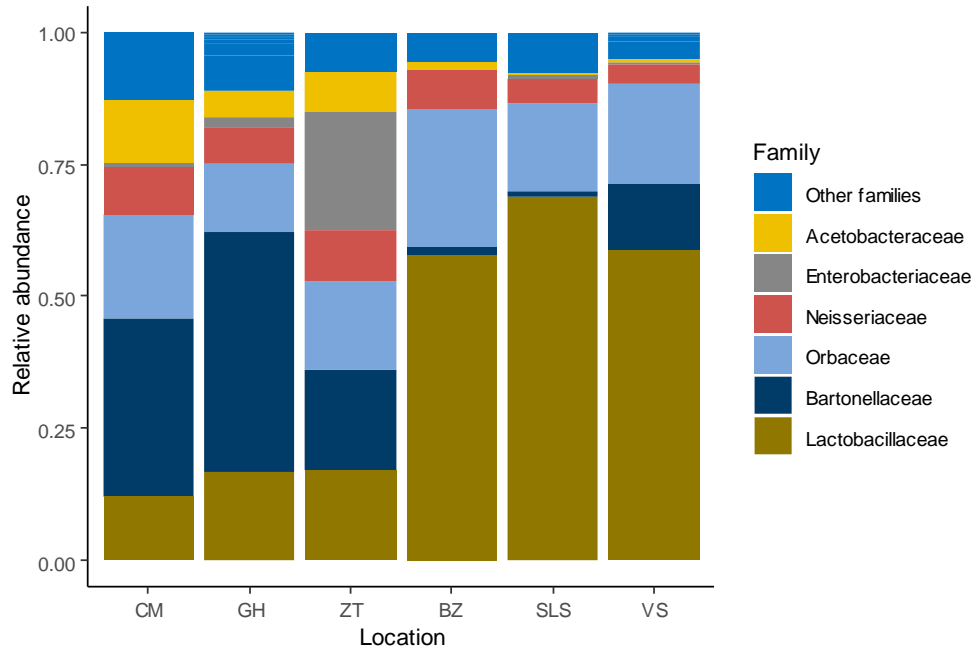
Supplementary Figure 2. Bar chart showing the relative abundance of bacterial phyla in the sampling sites in Malta and Italy: [CM] University of Malta – Campus Msida, [GH] Ghargħur, [ZT] Żeitun, [BZ] Bozen, [SLS] San Lazzaro di Savena, [VS] Valsamoggia.



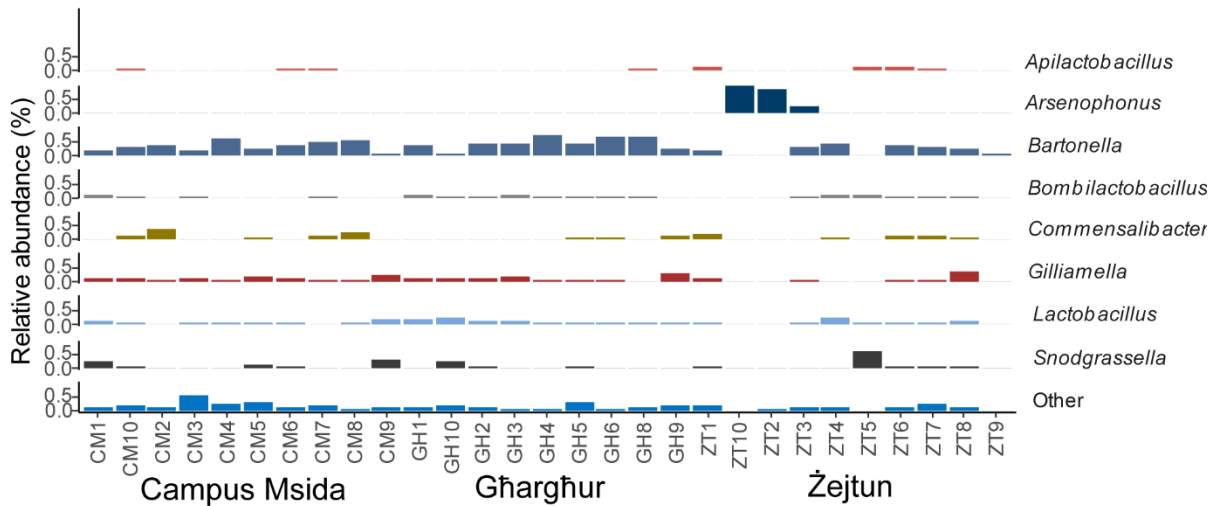
Supplementary Figure 3. Bar chart showing the relative abundance of bacterial classes in the sampling sites in Malta and Italy: [CM] University of Malta – Campus Msida, [GH] Għargħur, [ZT] Żeitun, [BZ] Bozen, [SLS] San Lazzaro di Savena, [VS] Valsamoggia.



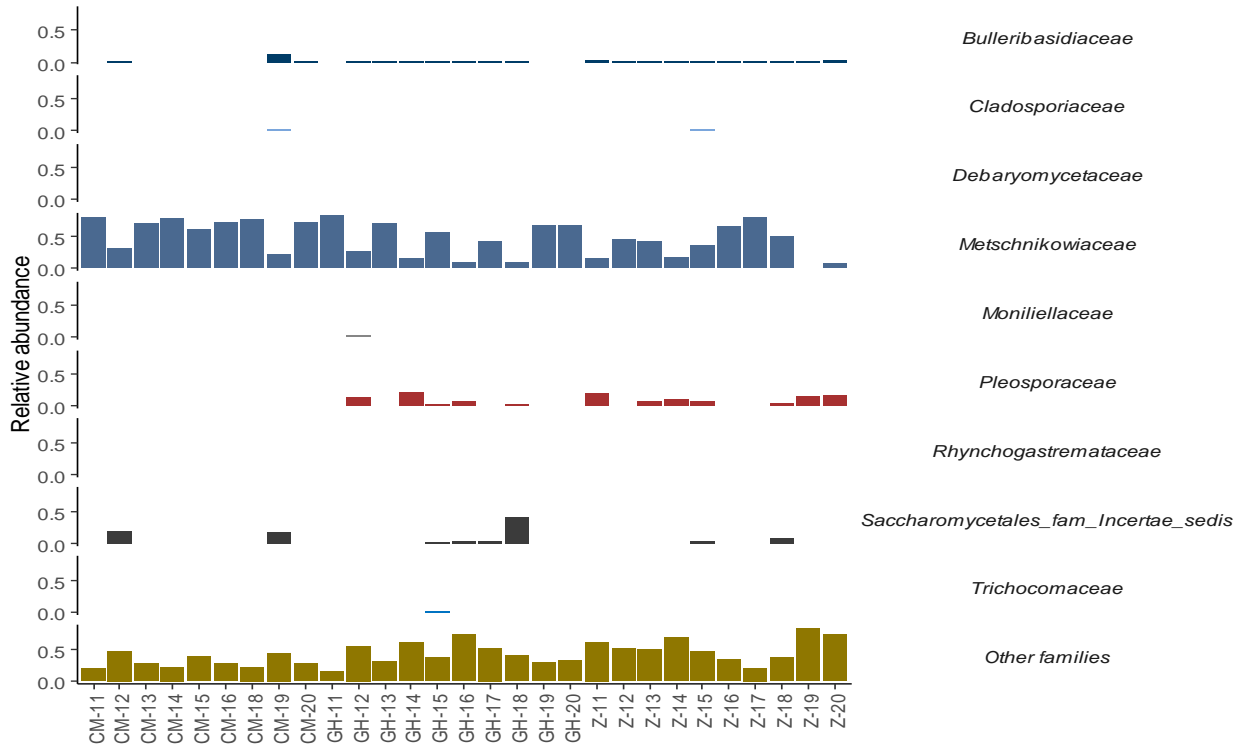
Supplementary Figure 4. Bar chart showing the relative abundance of bacterial orders in the sampling sites in Malta and Italy: [CM] University of Malta – Campus Msida, [GH] Għargħur, [ZT] Żeitun, [BZ] Bozen, [SLS] San Lazzaro di Savena, [VS] Valsamoggia.



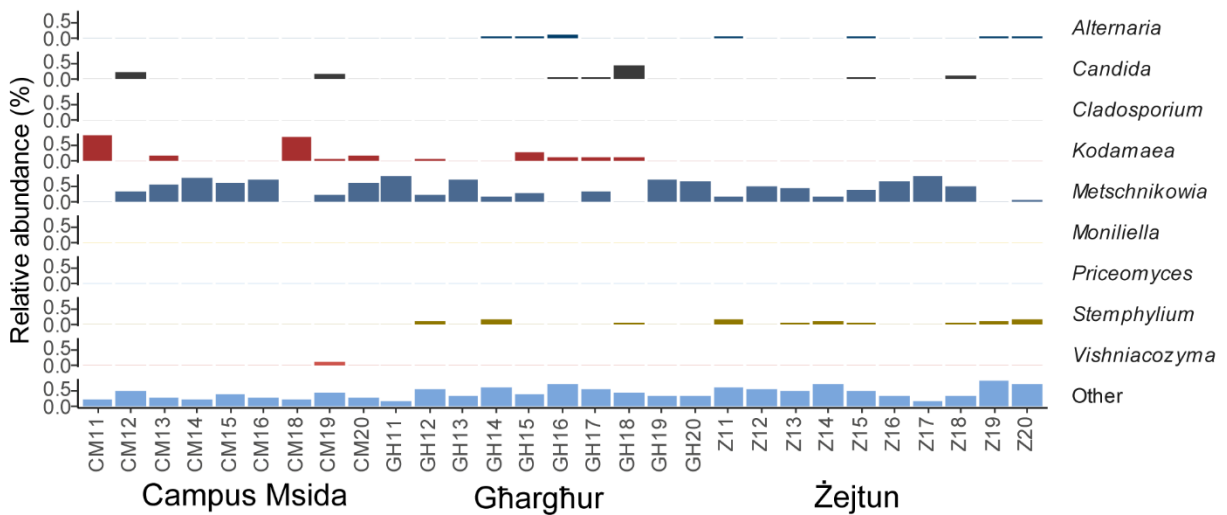
Supplementary Figure 5. Bar chart showing the relative abundance of bacterial orders in the sampling sites in Malta and Italy: [CM] University of Malta – Campus Msida, [GH] Għargħur, [ZT] Żejtun, [BZ] Bozen, [SLS] San Lazzaro di Savena, [VS] Valsamoggia.



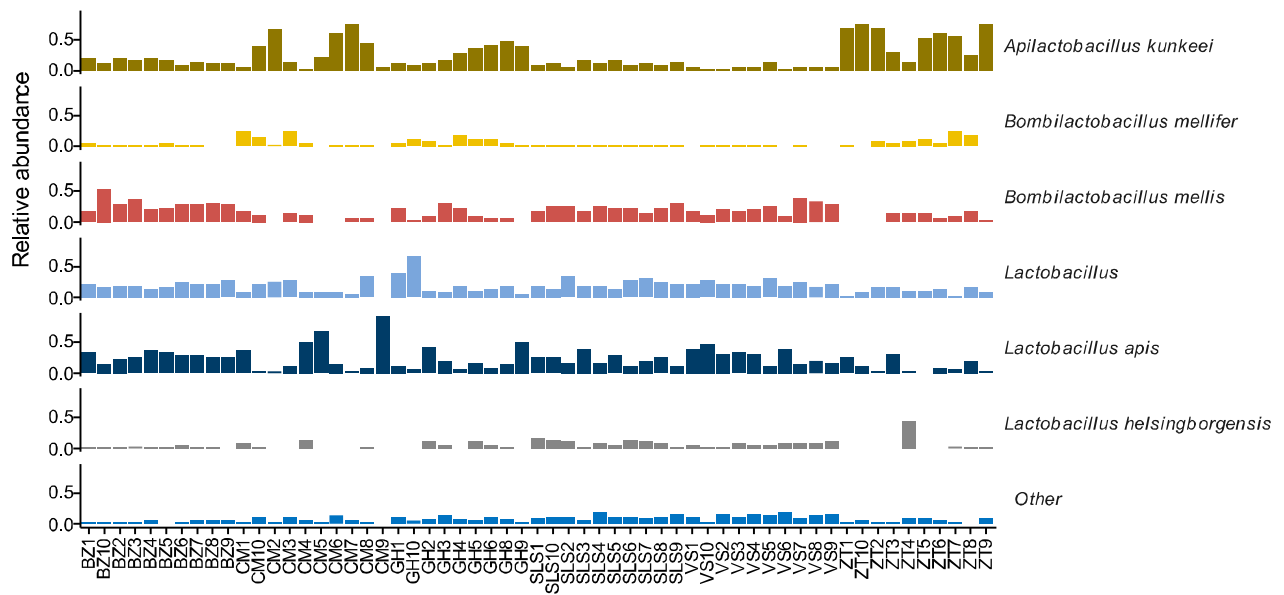
Supplementary Figure 6. Bar chart showing the relative abundance of the major genera of bacteria in every sampled honey bee gut in Malta.



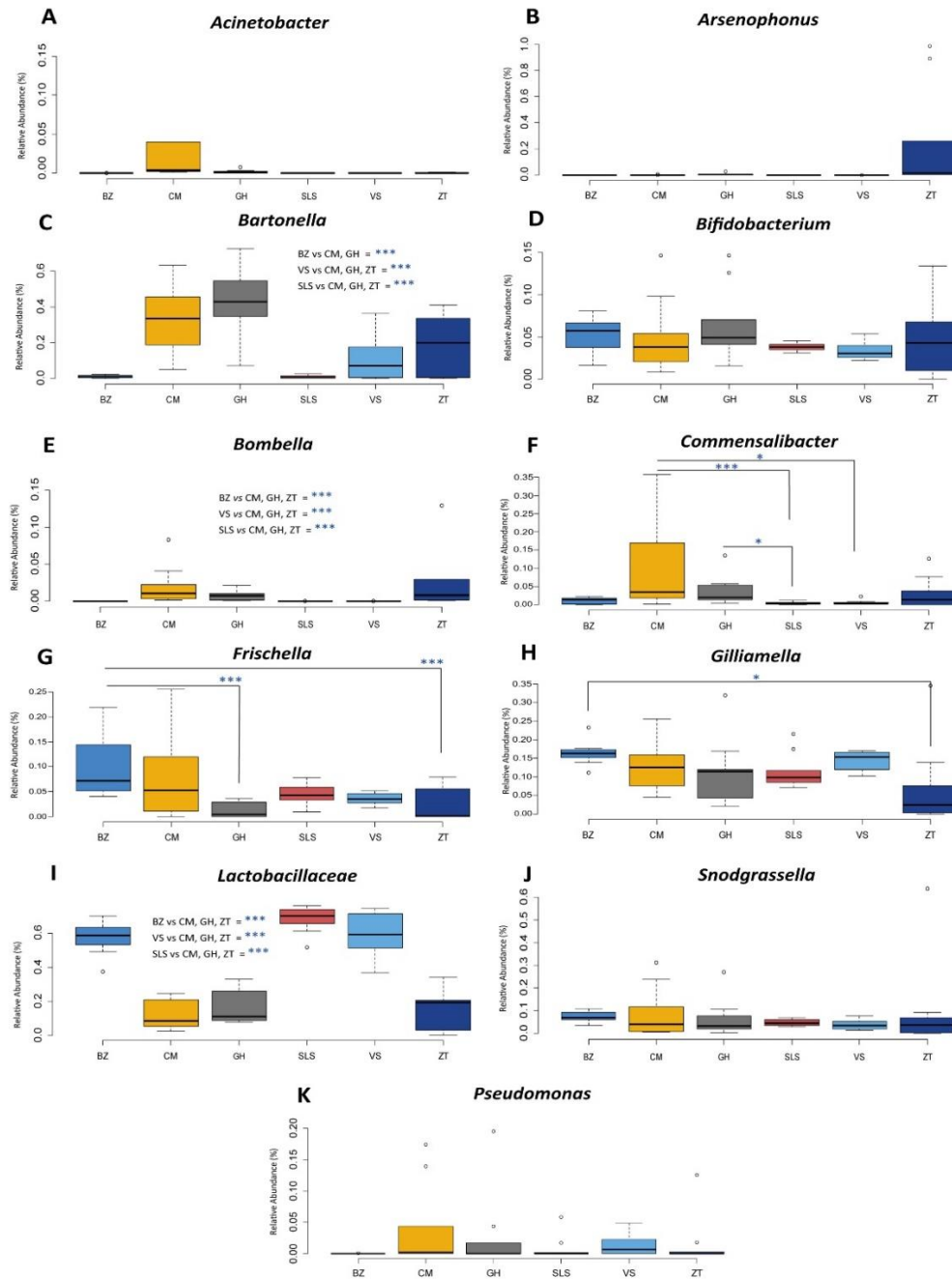
Supplementary Figure 7. Bar chart showing the relative abundance of the major families of fungi in every sampled honey bee gut in Malta.



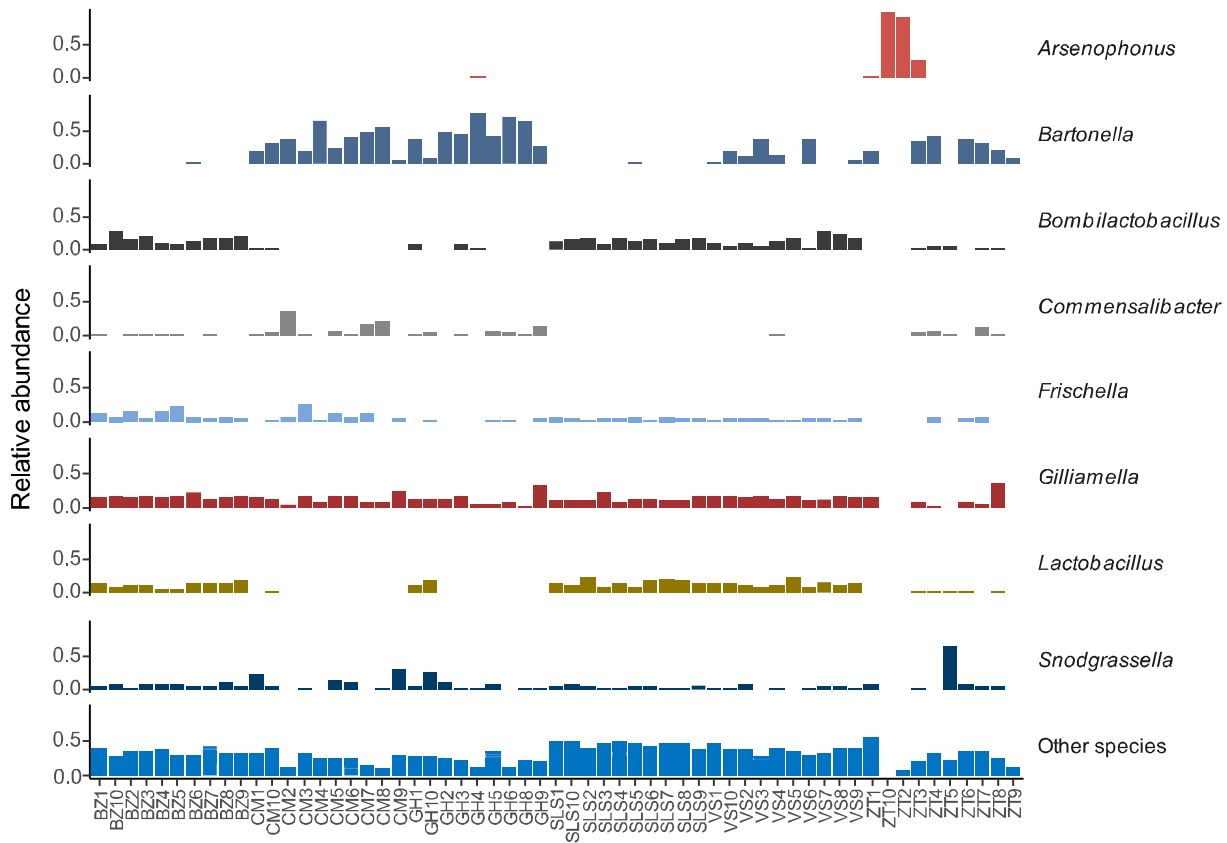
Supplementary Figure 8. Bar chart showing the relative abundance of the major genera of fungi in every sampled honey bee gut in Malta.



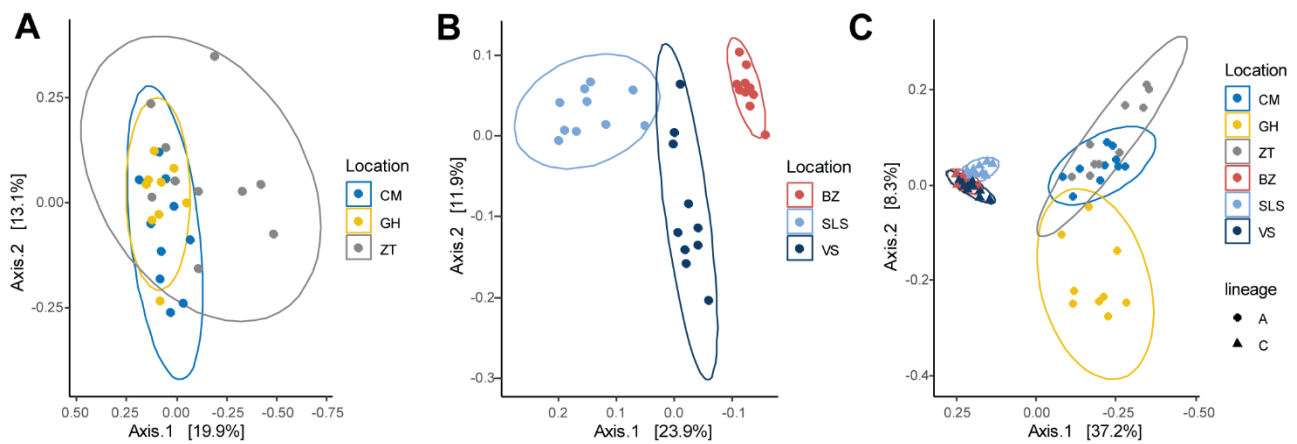
Supplementary Figure 9. Bar chart showing the relative abundance of the major species belonging to the family Lactobacillaceae in every sampled honey bee gut in Malta and Italy.



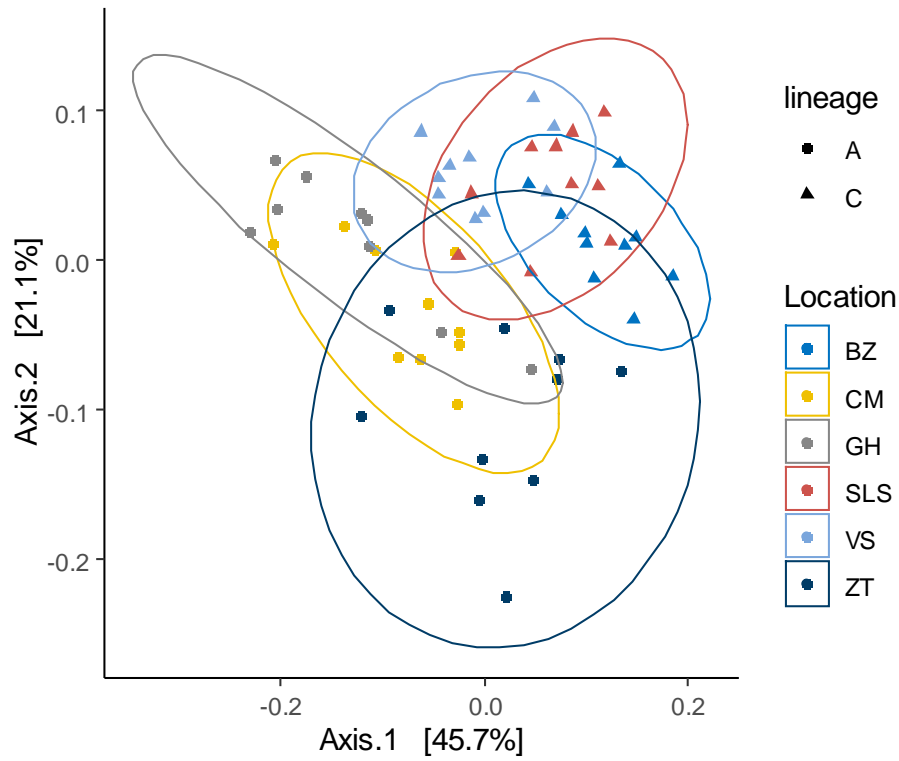
Supplementary Figure 10A – 10K. The boxplot charts show the relative abundance of the major microbial taxa populating the sampled honey bee guts: A) *Acinetobacter*; B) *Arsenophonus*; C) *Bartonella*; D) *Bifidobacterium*; E) *Bombella*; F) *Commensalibacter*; G) *Frischella*; H) *Gilliamella*; I) *Lactobacillaceae*; J) *Snodgrassella* and K) *Pseudomonas* in the different sampling sites. The sampling sites for Italy are [BZ] Bozen, South Tyrol region; [SLS] San Lazzaro di Savena, Emilia Romagna region; [VS] Valsamoggia, Emilia Romagna region, all belonging to C lineage. Sampling sites for Malta are [CM] University of Malta – Campus Msida; [GH] Għargħur; [ZT] Żeitun, all belonging to A lineage. Every sampling site account for 10 sampled honey bees. Asterisks indicate statistically significant differences (*p<0.05; ***p<0.01).



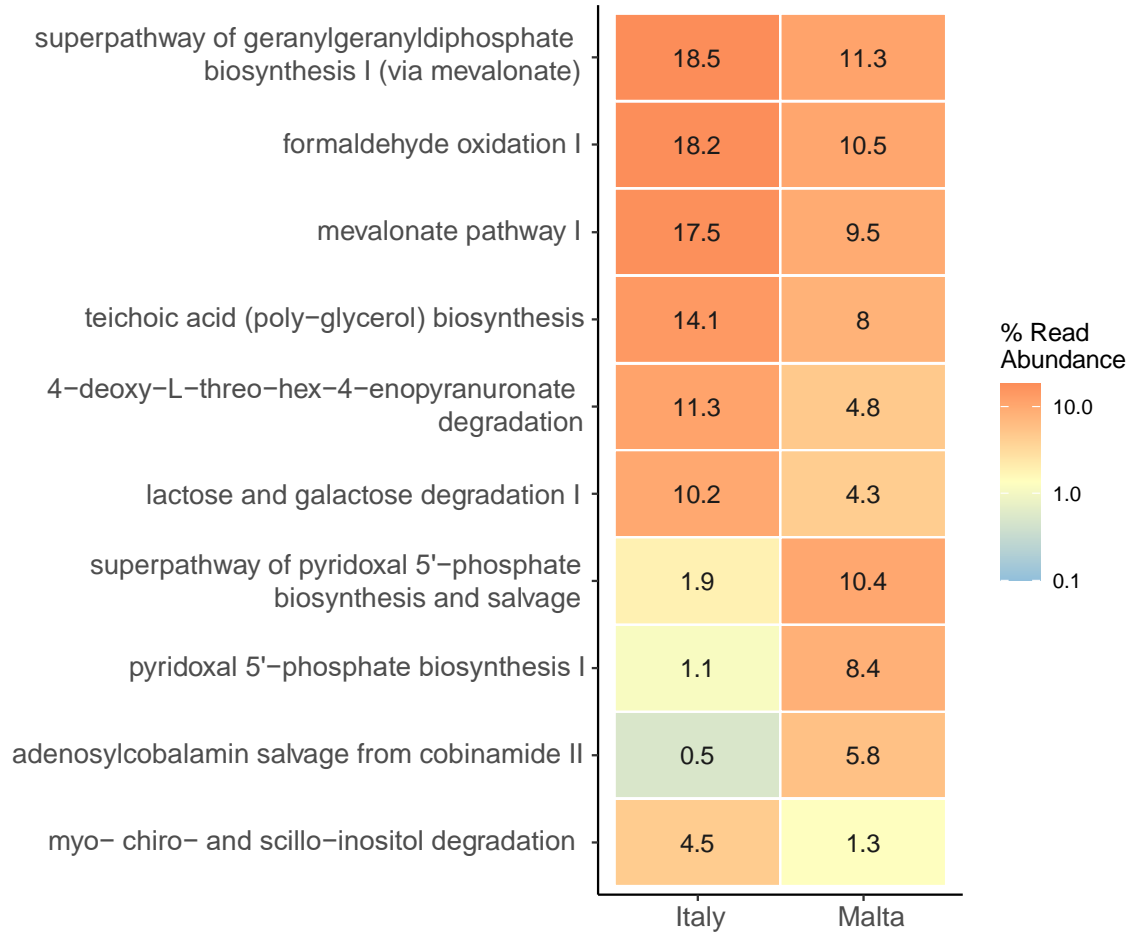
Supplementary Figure 11. Bar chart showing the relative abundance (%) of the major bacterial genera in every sampled honey bee gut in Malta and Italy.



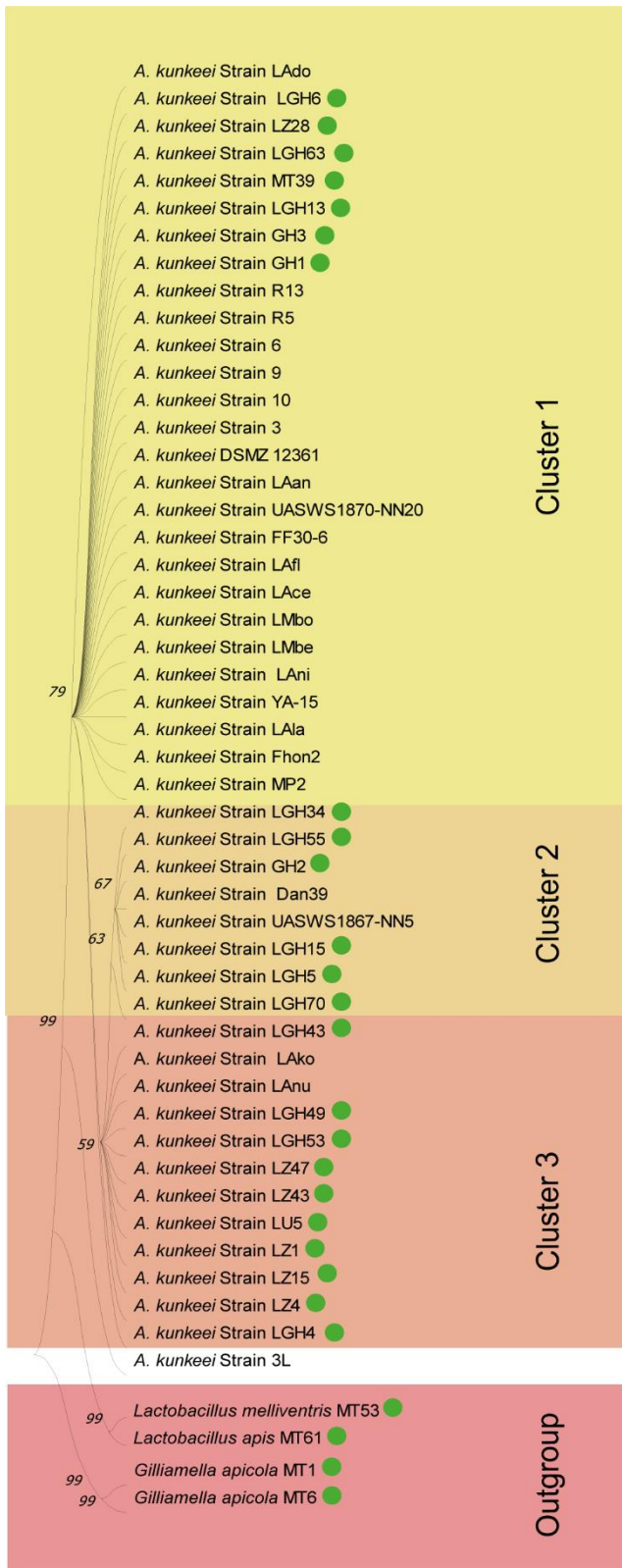
Supplementary Figure 12. A) β -diversity Bray–Curtis dissimilarity index per sampling site in Malta; B) β -diversity Bray–Curtis dissimilarity index per sampling site in Italy; C) β -diversity Bray–Curtis dissimilarity index per mitochondrial haplotype (lineage) and sampling site.



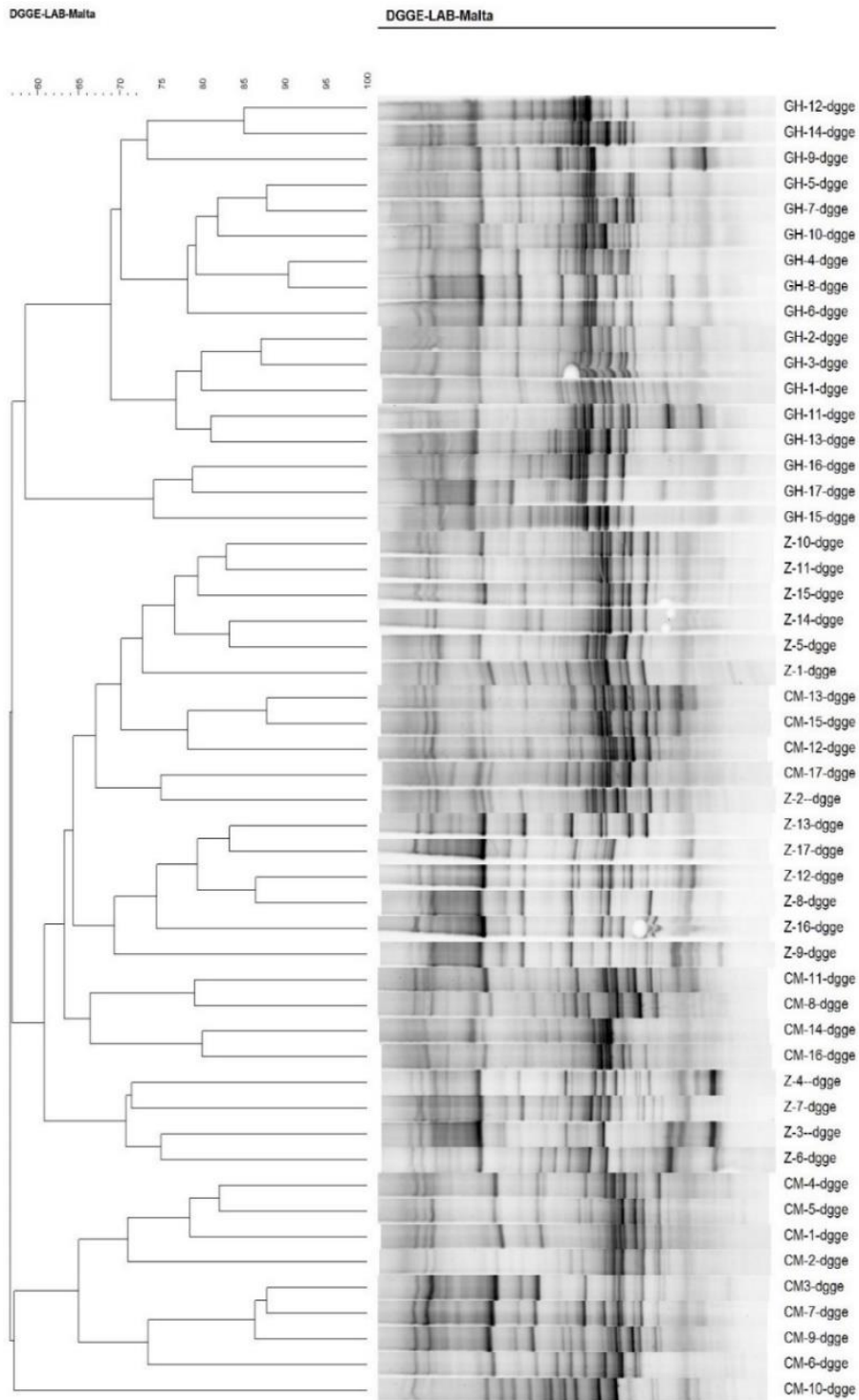
Supplementary Figure 13. PCA for PCRUST2 overall prediction of functionality, considering all the honey bees sampled both in Italy and in Malta, separates the lineage C honey bees from those of lineage A.



Supplementary Figure 14. Differential abundance heatmap highlighting significantly differentially abundant ($p < 0.05$) microbial genera between honey bees sampled in Italy (lineage C) and in Malta (lineage A), showing the relative abundance of the genus;



Supplementary Figure 16. Phylogenetic tree showing the isolated *A. kunkeei* isolated within this work (Indicated with the green dot) and other *A. kunkeei* strains retrieved from NCBI Genome RefSeq.



Supplementary Figure 17. DGGE profiles on Lactobacillaceae populating the gut of Maltese honey bees.

1.2 Supplementary Tables

Supplementary Table 1. The table reports the list of samples derived from [Alberoni et al., 2021a](#); [Alberoni et al., 2021b](#); [Baffoni et al., 2021](#) research works, the original samples name and the NCBI accession number and bio-project.

Samples name in this work	Sample name in the original works	NCBI accession	Reference
BOLZANO/BOZEN (<i>APIS MELLIFERA CARNICA</i>)			
BZ1	0.CTR.1	SAMN15728541	Alberoni et al., 2021a (NCBI bioproject PRJNA655184)
BZ2	0.CTR.2	SAMN15728542	
BZ3	0.CTR.3	SAMN15728543	
BZ4	0.CTR.4	SAMN15728544	
BZ5	0.CTR.5	SAMN15728545	
BZ6	0.IMID.1	SAMN15728552	
BZ7	0.IMID.2	SAMN15728553	
BZ8	0.IMID.3	SAMN15728554	
BZ9	0.IMID.4	SAMN15728555	
BZ10	0.IMID.5	SAMN15728556	
VALSAMOGIA (<i>APIS MELLIFERA LIGUSTICA</i>)			
VS1	T0.C1	SAMN16442367	Alberoni et al., 2021b (NCBI bioproject PRJNA669646)
VS2	T0.C2	SAMN16442368	
VS3	T0.C3	SAMN16442369	
VS4	T0.C4	SAMN16442370	
VS5	T0.C5	SAMN16442371	
VS6	T0.P1	SAMN16442385	
VS7	T0.P2	SAMN16442386	
VS8	T0.P3	SAMN16442387	
VS9	T0.P4	SAMN16442388	
VS10	T0.P5	SAMN16442389	
SAN LAZZARO DI SAVENA (<i>APIS MELLIFERA LIGUSTICA</i>)			
SLS1	T0.CA1	SAMN16442373	Baffoni et al., 2021 (NCBI bioproject PRJNA669646)
SLS2	T0.CA2	SAMN16442374	
SLS3	T0.CA3	SAMN16442375	
SLS4	T0.TN1	SAMN16442409	
SLS5	T0.TN2	SAMN16442410	
SLS6	T0.TN3	SAMN16442411	
SLS7	T4.CA1	SAMN16442428	
SLS8	T4.CA2	SAMN16442429	
SLS9	T4.CA3	SAMN16442430	
SLS10	T4.CA4	SAMN16442431	

Supplementary Table S2. Retrieved *Apilactobacillus kunkeei* 16S rRNA gene sequences from the NCBI RefSeq database.

Strain	Origin	RefSeq
<i>Apilactobacillus kunkeei</i> Fhon2	Sweden	GCF_000830375.1
<i>Apilactobacillus kunkeei</i> LAan	Sweden	GCF_019575995.1
<i>Apilactobacillus kunkeei</i> LAce	Sweden	GCF_001421115.1
<i>Apilactobacillus kunkeei</i> LAfl	Sweden	GCF_001421135.1
<i>Apilactobacillus kunkeei</i> LAko	Sweden	GCF_001281205.1
<i>Apilactobacillus kunkeei</i> LAla	Sweden	GCF_001281215.1
<i>Apilactobacillus kunkeei</i> LAni	Sweden	GCF_001281285.1
<i>Apilactobacillus kunkeei</i> YH-15	Sweden	GCF_001281265.1
<i>Apilactobacillus kunkeei</i> LAdo	Sweden	GCF_001308205.1
<i>Apilactobacillus kunkeei</i> LAnu	Sweden	GCF_001308215.1
<i>Apilactobacillus kunkeei</i> LMbe	Sweden	GCF_001308185.1
<i>Apilactobacillus kunkeei</i> LMbo	Sweden	GCF_001308195.1
<i>Apilactobacillus kunkeei</i> MP2	Cile	GCF_001314945.1
<i>Apilactobacillus kunkeei</i> FF30-6	Japan	GCF_001949975.2
<i>Apilactobacillus kunkeei</i> UASWS1867-NN5	Switzerland	GCF_005930915.1
<i>Apilactobacillus kunkeei</i> UASWS1869-NN19	Switzerland	GCF_005930925.1
<i>Apilactobacillus kunkeei</i> UASWS1870-NN20	Switzerland	GCF_005930935.1
<i>Apilactobacillus kunkeei</i> UASWS1868-NN17	Switzerland	GCF_005930975.1
<i>Apilactobacillus kunkeei</i> KA_01	USA	GCF_006493415.1
<i>Apilactobacillus kunkeei</i> 3L	USA	GCF_006493615.1
<i>Apilactobacillus kunkeei</i> R13	Germany	GCF_023218665.1
<i>Apilactobacillus kunkeei</i> R5	Germany	GCF_023218695.1
<i>Apilactobacillus kunkeei</i> 26	Germany	GCF_023218705.1
<i>Apilactobacillus kunkeei</i> 9	Germany	GCF_023218755.1
<i>Apilactobacillus kunkeei</i> 10	Germany	GCF_023218775.1
<i>Apilactobacillus kunkeei</i> 3	Germany	GCF_023218815.1

Supplementary Table 3. Relative abundance (%) per microbial taxa and sample.

	<i>Acinetobacter</i>	<i>Apilactobacillus</i>	<i>Arsenophonus</i>	<i>Bartonella</i>	<i>Bifidobacterium</i>	<i>Bombilactobacillus</i>	<i>Commensalibacter</i>	<i>Frischella</i>	<i>Gilliamella</i>	<i>Lactobacillus</i>	<i>Pseudomonas</i>	<i>Snodgrassella</i>
BZ1	0.00	0.11	0.00	0.02	0.04	0.12	0.01	0.12	0.15	0.36	0.00	0.06
BZ2	0.00	0.11	0.00	0.01	0.05	0.19	0.01	0.16	0.14	0.30	0.00	0.03
BZ3	0.00	0.09	0.00	0.01	0.05	0.23	0.02	0.05	0.18	0.30	0.00	0.07
BZ4	0.00	0.09	0.00	0.02	0.07	0.12	0.02	0.15	0.16	0.29	0.00	0.09
BZ5	0.00	0.06	0.00	0.02	0.07	0.11	0.02	0.23	0.18	0.22	0.00	0.10
BZ6	0.00	0.05	0.00	0.03	0.06	0.16	0.01	0.08	0.23	0.32	0.00	0.06
BZ7	0.00	0.09	0.00	0.02	0.07	0.21	0.02	0.04	0.11	0.39	0.00	0.06
BZ8	0.00	0.07	0.00	0.01	0.03	0.19	0.00	0.07	0.16	0.38	0.00	0.11
BZ9	0.00	0.08	0.00	0.00	0.02	0.20	0.00	0.04	0.18	0.42	0.00	0.06
BZ10	0.00	0.06	0.00	0.01	0.08	0.30	0.00	0.07	0.17	0.22	0.00	0.09
CM1	0.04	0.02	0.00	0.19	0.10	0.10	0.04	0.00	0.14	0.14	0.00	0.24
CM2	0.02	0.04	0.01	0.38	0.01	0.00	0.41	0.08	0.05	0.01	0.00	0.01
CM3	0.18	0.01	0.00	0.18	0.04	0.03	0.03	0.26	0.16	0.04	0.04	0.02
CM4	0.00	0.00	0.00	0.64	0.05	0.01	0.00	0.01	0.08	0.02	0.18	0.01
CM5	0.00	0.01	0.00	0.25	0.02	0.00	0.09	0.14	0.17	0.04	0.15	0.12
CM6	0.03	0.12	0.00	0.40	0.03	0.00	0.05	0.06	0.16	0.04	0.00	0.10
CM7	0.00	0.08	0.00	0.48	0.02	0.01	0.18	0.12	0.08	0.02	0.00	0.01
CM8	0.00	0.03	0.00	0.56	0.04	0.00	0.25	0.00	0.07	0.03	0.00	0.02
CM9	0.00	0.01	0.00	0.05	0.05	0.00	0.02	0.05	0.26	0.20	0.02	0.32
CM10	0.00	0.09	0.00	0.31	0.15	0.05	0.12	0.02	0.11	0.08	0.00	0.06
GH1	0.00	0.04	0.00	0.36	0.13	0.09	0.02	0.00	0.12	0.20	0.00	0.04
GH2	0.00	0.02	0.01	0.49	0.05	0.03	0.01	0.00	0.13	0.12	0.02	0.12
GH3	0.00	0.05	0.00	0.44	0.07	0.09	0.02	0.00	0.17	0.12	0.00	0.03
GH4	0.01	0.03	0.03	0.76	0.04	0.03	0.01	0.00	0.04	0.03	0.00	0.02
GH5	0.00	0.04	0.00	0.44	0.07	0.02	0.07	0.01	0.05	0.05	0.16	0.09
GH6	0.00	0.04	0.00	0.70	0.05	0.01	0.06	0.03	0.07	0.03	0.00	0.00
GH8	0.00	0.07	0.00	0.72	0.02	0.01	0.05	0.01	0.03	0.06	0.00	0.02
GH9	0.00	0.04	0.00	0.27	0.03	0.00	0.15	0.04	0.35	0.05	0.05	0.02
GH10	0.00	0.03	0.00	0.07	0.15	0.04	0.05	0.03	0.12	0.22	0.00	0.28
SLS1	0.00	0.07	0.00	0.01	0.04	0.13	0.00	0.08	0.10	0.52	0.00	0.05
SLS2	0.00	0.03	0.00	0.01	0.04	0.20	0.00	0.01	0.10	0.54	0.00	0.06
SLS3	0.00	0.09	0.00	0.02	0.05	0.10	0.01	0.05	0.23	0.36	0.06	0.04
SLS4	0.00	0.09	0.00	0.00	0.04	0.20	0.00	0.05	0.07	0.50	0.00	0.04
SLS5	0.00	0.11	0.00	0.03	0.03	0.16	0.01	0.07	0.12	0.38	0.02	0.07
SLS6	0.00	0.06	0.00	0.01	0.05	0.18	0.01	0.02	0.11	0.50	0.00	0.06

SLS7	0.00	0.09	0.00	0.00	0.03	0.13	0.00	0.06	0.09	0.56	0.00	0.03
SLS8	0.00	0.06	0.00	0.00	0.04	0.18	0.00	0.04	0.09	0.56	0.00	0.03
SLS9	0.00	0.09	0.00	0.01	0.04	0.22	0.00	0.05	0.18	0.37	0.00	0.04
SLS10	0.00	0.07	0.00	0.01	0.04	0.19	0.00	0.04	0.10	0.48	0.00	0.07
VS1	0.00	0.04	0.00	0.04	0.04	0.12	0.01	0.02	0.17	0.51	0.02	0.03
VS2	0.00	0.02	0.00	0.10	0.06	0.12	0.01	0.04	0.15	0.41	0.02	0.08
VS3	0.00	0.02	0.00	0.36	0.03	0.07	0.00	0.05	0.17	0.28	0.01	0.01
VS4	0.00	0.03	0.00	0.14	0.03	0.14	0.01	0.03	0.13	0.47	0.00	0.02
VS5	0.00	0.09	0.00	0.01	0.03	0.20	0.00	0.03	0.17	0.45	0.00	0.01
VS6	0.00	0.01	0.00	0.37	0.03	0.04	0.01	0.05	0.11	0.35	0.00	0.04
VS7	0.00	0.03	0.00	0.00	0.02	0.29	0.00	0.04	0.13	0.42	0.00	0.06
VS8	0.00	0.03	0.00	0.01	0.03	0.24	0.00	0.03	0.16	0.45	0.00	0.05
VS9	0.00	0.02	0.00	0.05	0.04	0.18	0.00	0.05	0.15	0.41	0.05	0.04
VS10	0.00	0.02	0.00	0.18	0.04	0.06	0.00	0.04	0.17	0.44	0.01	0.03
ZT1	0.00	0.14	0.03	0.19	0.04	0.00	0.20	0.00	0.14	0.05	0.13	0.07
ZT2	0.00	0.02	0.90	0.01	0.01	0.00	0.03	0.00	0.00	0.01	0.02	0.00
ZT3	0.00	0.05	0.27	0.34	0.08	0.03	0.04	0.00	0.08	0.08	0.00	0.02
ZT4	0.00	0.04	0.01	0.41	0.07	0.08	0.08	0.08	0.01	0.22	0.00	0.00
ZT5	0.00	0.18	0.00	0.01	0.02	0.08	0.03	0.00	0.00	0.04	0.00	0.65
ZT6	0.00	0.13	0.02	0.37	0.05	0.03	0.13	0.06	0.07	0.05	0.00	0.09
ZT7	0.00	0.11	0.00	0.33	0.14	0.07	0.13	0.08	0.04	0.03	0.00	0.07
ZT8	0.00	0.06	0.00	0.23	0.07	0.07	0.06	0.00	0.36	0.09	0.00	0.05
ZT9	0.00	0.22	0.08	0.31	0.06	0.01	0.21	0.00	0.01	0.04	0.00	0.05
ZT10	0.00	0.00	0.99	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Supplementary Table S4. Microbial isolates (one per representative cluster in Fig.S15), identified with the sequencing of the 16S rRNA gene.

Accession Number	Sequence ID/Strain	Identification
MG601163	MT53	<i>Lactobacillus melliventris</i>
MG601164	MT1	<i>Gilliamella apicola</i>
MG601165	MT76	<i>Lactobacillus apis</i>
MG601166	MT61	<i>Lactobacillus apis</i>
MG601167	MT39	<i>Apilactobacillus kunkeei</i>
MG601168	MT11	<i>Klebsiella oxytoca</i>
MG601169	MT6	<i>Gilliamella apicola</i>
MG601170	GH1	<i>Apilactobacillus kunkeei</i>
MG601171	GH2	<i>Apilactobacillus kunkeei</i>
MG601172	GH3	<i>Apilactobacillus kunkeei</i>
MT381710	LGH12	<i>Apilactobacillus kunkeei</i>
MT381711	LGH13	<i>Apilactobacillus kunkeei</i>
MT381712	LGH15	<i>Apilactobacillus kunkeei</i>
MT381713	LGH34	<i>Apilactobacillus kunkeei</i>
MT381714	LGH35	<i>Apilactobacillus kunkeei</i>
MT381715	LGH43	<i>Apilactobacillus kunkeei</i>
MT381716	LGH49	<i>Apilactobacillus kunkeei</i>
MT381717	LGH53	<i>Apilactobacillus kunkeei</i>
MT381718	LGH55	<i>Apilactobacillus kunkeei</i>
MT381719	LGH63	<i>Apilactobacillus kunkeei</i>
MT381720	LGH70	<i>Apilactobacillus kunkeei</i>
MT381721	LU2	<i>Gilliamella apicola</i>
MT381722	LZ15	<i>Apilactobacillus kunkeei</i>
MT381723	LZ25	<i>Apilactobacillus kunkeei</i>
MT381724	LZ47	<i>Apilactobacillus kunkeei</i>
MT381725	LZ43	<i>Apilactobacillus kunkeei</i>
MT381726	LU5	<i>Apilactobacillus kunkeei</i>
MT381727	LZ1	<i>Apilactobacillus kunkeei</i>
MT381728	LZ4	<i>Apilactobacillus kunkeei</i>
MT381729	LZ28	<i>Apilactobacillus kunkeei</i>
MT381730	LZ48	<i>Gilliamella apicola</i>
MT381731	LU6	<i>Gilliamella apicola</i>
MT381732	LU12	<i>Klebsiella oxytoca</i>
MT381733	LGH4	<i>Apilactobacillus kunkeei</i>
MT381734	LGH5	<i>Apilactobacillus kunkeei</i>
MT381735	LGH6	<i>Apilactobacillus kunkeei</i>
MT381736	LZ32	<i>Apilactobacillus kunkeei</i>