Short-communication: intra- and inter-individual milk microbiota variability in healthy and infected water buffalo udder quarters

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Abstract

The concept that ruminant mammary gland quarters are anatomically and physiologically unrelated has been recently challenged by immunological evidences. How this interdependence reflects on individual quarter milk microbiota is unknown. The aim of the present study was to cover this gap by investigating the interdependence of quarters among the same mammary gland at the milk microbiota level using next generation sequencing of V4-16S rRNA gene. A total number of 52 samples was included in this study and classified as healthy or affected by subclinical mastitis. DNA extraction, amplification of the V4-16S rRNA gene and sequencing using Ion Torrent Personal Genome Machine were carried out. We found that the most stable phylum across healthy or subclinical mastitis affected quarters was represented by *Bacteroidetes*. At family level, the relative abundance of *Propionibacteriaceae* showed the greatest stability, followed by and *Corynebacteriaceae* and *Aerococcaceae*. On the contrary, *Firmicutes* and *Proteobacteria* were the most variable phyla in both healthy and subclinical mastitis affected quarter milk samples. Interestingly, the intra-individual variability was lower than the inter-individual one. The present findings further support at milk microbiota level the hypothesis of the interdependence of quarters, as previously demonstrated following immunological studies, suggesting that individual factors (e.g. immunity, genetics) may have a role in modulating milk microbiota.

Mammary gland quarters within dairy cows have been regarded as independent of each other, given the background that each quarter has its own vascular system, nerve supply, and suspensory apparatus (Berry and Meaney, 2006; Akers and Nickerson, 2011). Preliminary investigations on immune related cells suggesting that mammary gland quarters do not act independently during mastitis (Merle et al., 2007) were further confirmed by the evidence that the infection of one udder quarter influences also other uninfected quarters (Mitterhuemer et al., 2010; Jensen et al., 2013). More recent studies demonstrated that quarters of infected udders influence the percentage of B cells and the expression of adhesion molecules in neutrophils of uninfected quarters (Blagitz et al., 2015).

To the best of our knowledge, the difference in bacterial taxonomy between quarters within the same udder has not been investigated yet, except in human breast milk, where high intra-individual similarity between individuals was demonstrated (Avershina et al., 2018). Culture-independent methodologies relying on high-throughput DNA sequencing of 16S (Next Generation Sequencing – NGS) are currently applied to describe the relationship between resident microbial population and the development of mastitis and allow for an in depth description of species that cannot be cultured (Oikonomou et al., 2012; Bicalho, 2014; Lima et al., 2018), and are regarded as the ideal techniques to identify differences between quarter milk microbiota.

The domestic water buffalo (*Bubalus bubalis*) provides a significant amount of global milk production and is the major milk producing animal in several countries, such as India and Pakistan (Fao, 2016). Water buffalo udder quarters are regarded as anatomically and physiologically independent to the others within the same mammary gland, as in cow (Thomas et al., 2004; Ambord et al., 2010). How this anatomical independence is related to immunological and microbiological status is unknown. Starting from previous results about water buffalo milk microbiota (Catozzi et al., 2017), this study aimed to elucidate the interdependence of quarters by investigating the variability of milk microbiota in composition and structure between healthy quarters within the same udder. In order to assess whether modification in unhealthy status, such as mild inflammation, reflects on the

other quarters, the composition of microbiota in milk from animals affected by subclinical mastitis was also determined.

Water buffalo quarter milk samples were collected from healthy (H) and subclinical mastitis affected (SM) quarters. Sixteen animals were enrolled from the same farm, in order to reduce the microbiota variability due to different management and feeding regimen, and were homogenous for parity (from second to fourth milking) and stage of lactation (mid lactation). A total of 52 milk quarter samples, of which 18 healthy (from 6 animals) and 34 affected by subclinical mastitis (from 11 animals), were collected. Healthy quarters were characterized by absence of clinical symptoms, negative microbiological culture for mastitis pathogens and a somatic cell count (SCC) lower than 200,000 cells/ml; subclinical mastitis samples were defined by absence of clinical symptoms, positive microbiological culture for mastitis pathogens and/or SCC higher than 200,000 cells/ml. The list of animals enrolled anmd the details of their clinical status is presented in Supplementary Table 1.

Mammary glands were disinfected, first three strains of milk were discarded and gloves were changed after every milk collection in order to rule out any contamination. Milk samples were collected, immediately refrigerated and delivered to the laboratory for microbiological and SCC analysis.

Microbiological culture tests and SCC were performed as previously reported (Catozzi et al., 2017). The DNA extraction was carried out as previously reported as well (Catozzi et al., 2017). Briefly, one ml of milk was centrifuged at room temperature at 16,100 rcf for 20 minutes. Fat and supernatant were removed and the remaining pellet was resuspended with 250ul of the Power Bead Tube of the DNeasy Power Soil Kit (QIAGEN) used to extract bacterial DNA, according to the manufacturer's instructions. V4 region of 16S rRNA gene was amplified for each sample. The forward primer was 5' – CCATCTCATCCCTGCGTGTCTCCGAC**TCAG***NNNNNNNNNNNNNNNN***GAT**GTGYCAGC MGCCGCGGTAA – 3', and composed of the adapter linker, the key, the sample-specific barcode and the 515F forward primer. The reverse primer was 5' – CCTCTCTATGGGCAGTCGGTGATGGACTACNVGGGTWTCTAAT – 3', composed of the

adapter linker and the R806 reverse primer. The Thermo Scientific Phusion Hot Start II High-Fidelity DNA polymerase kit was used to perform V4 PCR (Catozzi et al., 2017). Next-generation sequencing was carried out using an Ion Torrent Personal Genome Machine with the Ion 318 Chip Kit v2 (Thermo Fisher Scientific, Weltham, Massachusetts, U.S.A), following manufacturer's instructions. Raw sequences have been submitted to NCBI under Bioproject accession number PRJNA492401. Reads were demultiplexed and analysed using Quantitative Insight Into Microbial Ecology 2 software (QIIME 2; https://qiime2.org) (Caporaso et al., 2011). Briefly, DADA2 was used as quality filtering method in order to denoise, dereplicate single-end sequences and remove chimeras (Callahan et al., 2016); a truncation length of 245 bases was used. After that, the units of observation, composed of unique sequences namely Amplicon Sequence Variants (ASVs), were used to classify and assign taxonomy by Greengenes 13.8 (DeSantis et al., 2006) at 99% of Operational Taxonomic Units (OTUs) identity and trimmed to V4 region as reference database. The filtered feature table was used to perform the downstream analysis. Taxonomic analysis was performed for each sample or sample group at phylum and family level with a relative abundance of at least 1%. Results and taxonomic classification are presented in Figure 1 at phylum (Panel A) and family (Panel B) level and Table S2. It was found that *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* predominate the milk microbiota at phylum level (Table S2, Panel A), whereas *Aerococcaceae*, *Corynebacteriaceae*, *Moraxellaceae*, *Staphylococcaceae* and *Propionibacteriaceae* provided the most abundant taxa at family level (Table S2, Panel B). The relative abundance of phyla found in healthy milk were largely comparable with those previously reported (Catozzi et al., 2017), in particular for what concerns *Bacteroidetes* and *Proteobacteria*. On the contrary, the relative abundance of *Actinobacteria* was found to be increased (27.9% as compared to 12.04%) and *Firmicute*s were found to be decreased (37% as compared to 57.7%). At family level, the relative abundance of *Aerococcaceae* and *Corynebacteriaceae* were similar to previous reports (Catozzi et al., 2017) whereas *Moraxiellaceae* and *Staphylococcaceae* were decreased (7.6% and 9.2% as compared to 18% and 16%, respectively). On the contrary, *Propionibacteria* were increased (8% as compared to 2%).

114 The number of studies carried out in milk water buffalo is too limited to identify a common healthy
115 and unhealthy microbiota. In bovine milk, beside those related to inflammation (Bicalho, 2014;
116 Catozzi et al., 2017; Lima et al., 2017), variations in milk microbiota have been linked not only to
117 antibiotic treatment (Ganda et al., 2016, 2017), as expected, but also to lactation stage, weather
118 conditions and diet supplementation (Chaves Lopez et al., 2016; Li et al., 2018), suggesting the
119 presence of a wide range of factors and sources influencing the milk microbial community
120 (Derakhshani et al., 2018). Therefore, possible variations of the relative abundance at phyla and
121 family levels found to this study as compared to previous ones could be related to different
122 management conditions.
123 Beta diversity, which is a measure of the differences occurring between samples by estimating how
124 many taxa they share, was performed using qualitative and quantitative approaches (unweighted and
125 weighted UniFrac distances matrices, respectively). Diversity analysis was assessed using a depth of
126 17500 sequences per sample. Abundancies for phyla and families were represented using the
127 CIRCOS software (http://circos.ca/software/) (Connors et al., 2009). Wilcoxon signed pairwise test
128 was performed for unpaired comparisons among beta diversity matrices from quarters within the same
129 animal and between different animals using pairwise.wilcox.test function in R (http://www.R-
130 project.org). After false Discovery rate (FDR) correction, comparisons were considered statistically
131 significant were $p < 0.05$. Detailed workflow used in QIIME and in R is shown in Supplementary file
132 1.
133 The individual variation in the amount of the most abundant phyla (Panel A) and families (Panel B)
134 are reported in Figure 2 and Table S3. The violin plot indicates the range of standard deviations (SD)
135 of the main taxa, through which it is possible to evaluate the range of intra-individual variability for
136 all animals.
137 *Proteobacteria* and *Firmicutes* showed the highest variation in H (15% and 13%, respectively) and
138 SM samples (22% and 28%, respectively) (Table S2, Panel A). At individual level, *Firmicutes*
139 represented the most variable phylum in SM samples (SD mean of 19% ranging from 3.1% to 30.6%),

as compared to the healthy ones (SD mean of 9% ranging from 2.6% to 21.2%), followed by *Proteobacteria* (SD mean of 12% and 9% for H and SM samples, respectively). This result may potentially explain the differences found in microbiota from previous reports (Catozzi et al., 2017). The other main phyla, namely *Acidobacteria* and *Bacteroidetes*, were more stable, reaching a SD mean lower than 8% in H quarters (Table S3, Panel A). *Staphylococcaceae* and *Moraxellaceae* were the most variable families for SM and H samples with a SD of 34% and 16%, respectively (Table S2, Panel B). At individual level, these families showed the greatest variability in H samples (*Moraxellaceae* with a SD mean of 10% ranging from 0.9 to 37%) and SM samples (*Staphylococcaceae* with a SD mean of 28% ranging from 2.5 to 49%; Table S3, Panel B), whereas *Propionibacteriaceae, Corynebacteriaceae* and *Aerococcaceae* were the most stable. We found that the most stable phylum across healthy or subclinical mastitis affected quarters was represented by *Bacteroidetes*. At family level, the relative abundance of *Propionibacteriaceae* showed the greatest stability, followed by and *Corynebacteriaceae* and *Aerococcaceae*. On the contrary, *Firmicutes* and *Proteobacteria* were the most variable phyla in both healthy and subclinical mastitis affected quarter milk samples; as expected, the families *Staphylococcaceae* and *Moraxellaceae* showed the greatest variation in relative abundance. At family level, the mean and median variability within animals was always lower than 6%, with the exception of *Staphylococcaceae* and *Moraxellaceae*.

The comparison between quarter milk microbiota within the same individual and between different individuals was performed using unweighted and weighted UniFrac distance matrices. Results are presented in table S4; values close to 0 are representative of high similarity; whereas, values close to 1 show a lower similarity. A box plot with statistical significant differences is presented in Figure 3. Healthy and subclinical mastitis affected quarters within individuals showed more similarity in terms of microbiota structure as compared to those between individuals. Previous studies have demonstrated the communication among quarters at immunological level (Burvenich et al., 2003; Merle et al., 2007; Jensen et al., 2013; Blagitz et al., 2015). We presented the evidence that, in water buffaloes as well, for what concerns the milk microbiota structure, the intra-individual variability was lower than the

inter-individual one in both healthy and subclinical mastitis-affected quarters. The present finding is partially consistent with what has been recently reported in human milk (Avershina et al., 2018), that demonstrated a high intra-individual similarity between microbiota of milk collected by the two mammary glands. In fact, we found that, in healthy samples, the similarity was greater in quarters within the same udder rather that between different mammary glands. The same profile was also demonstrated also in subclinical mastitis groups by means of the weighted Unifrac analysis.

Subclinical mastitis quarters showed a greater dissimilarity as compared to the healthy ones, consistently with previous studies in water buffaloes and cows (Oikonomou et al., 2014; Catozzi et al., 2017), demonstrating that the development of a disease destabilizes the microbiota rather than shifting to a determined structure (Zaneveld et al., 2017).

The new concept of hologenome, defined as the host-microbes genomes as a unit of evolution, is taking shape (Shapira, 2016), meaning that selection processes involved the genomes of both individual and microorganisms. Here, we support the presence of the quarter's interdependence at milk microbiota level, showing that the intra-individual similarity was greater than the inter-individual one.

In conclusion, the results provided in this preliminary study demonstrated that the microbiota of the four quarters of the water buffalo udder cannot be regarded as separate entities. Further investigation is required to confirm the present results in bovine species.

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Tables

Table S1: Metadata of samples, including SampleId, Status, AnimalID, Microbiological results and SCC (cells/ml x1000). H: healthy; SM: subclinical mastitis; SCC: Somatic Cell Count; CNS: Coagulase Negative Staphylococci.

Table S2: Mean, standard deviation (SD), median, minimum (min) and maximum (max) of the most abundant phyla (Panel A) and families (Panel B) with a relative abundance at least of 1%. H: healthy samples; SM: subclinical mastitis samples.

Table S3: Minimum (Min), maximum (Max), mean and median of the standard deviation (SD) of the most abundant phyla (Panel A) and families (Panel B) at individual level. Animal identification is indicated between parentheses. H: healthy samples; SM: subclinical mastitis samples.

Table S4: descriptive statistics of unweighted and weighted UniFrac distance matrices. Minimum, maximum, mean, median and standard deviation (SD) are shown.

Figures

Figure 1: Taxonomic results at phylum (panel A, relative average abundance ≥1%) and family level (panel B, relative average abundance ≥2.5%) for all animal quarters. Each slice correspond to one animal and each circle section to a quarter. The microbiological culture result for mastitis pathogens is indicated below each quarter. White quarters indicate that sample is missing

Figure 2: Variation of the standard deviation at individual level for the main phyla (Panel A) and families (Panel B). The relative average abundance was ≥1%.

Figure 3: Box plots of unweighted and weighted UniFrac distance matrices. Median (line into the box), mean (diamond shape), upper and lower quartiles (ends of the box) and highest and lowest value (extreme lines) are shown. Outiers are indicated by black points. Statistical significance are presented where $0.05 < p < 0.001$ (*) and $p < 0.001$ (**). False discovery rate correction was applied.

312 **Table S1**: Metadata of samples, including SampleId, Status, AnimalID, Microbiological results and

313 SCC (cells/ml x1000). H: healthy; SM: subclinical mastitis; SCC: Somatic Cell Count; CNS:

314 Coagulase Negative Staphylococci. NA: not available

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SampleID	Status	AnimaID	Quarter	Microbiological result	SCC (cells/ml x 1000)
1	H	1	Front right	Negative	13
101	SM	20	Posterior left	S. aureus	1436
12	SM	4	Front right	S. aureus	378
13	SM	4	Posterior right	S. aureus	28
14	SM	4	Posterior left	S. aureus	394
16	SM	6	Front right	CNS	1311
17	H	6	Front left	Negative	16
18R	H	6	Posterior right	Negative	20
19	SM	6	Posterior left	S. aureus	1172
2	H	1	Front left	Negative	93
20	H	7	Front right	Negative	20
21	H	7	Front left	Negative	26
23	H	7	Posterior left	Negative	12
24	SM	8	Front left	S. aureus	473
25	SM	8	Posterior right	CNS	698
26	SM	8	Posterior left	CNS	268
28	H	10	Front right	Negative	35
29R	H	10	Front left	Negative	28
3	H	1	Posterior right	Negative	229
31R	H	10	Posterior left	Negative	39
32	SM	11	Front right	CNS	225
5	SM	2	Front right	S. aureus	136
52R	SM	11	Posterior right	S. aureus	93
53	SM	11	Posterior left	S. aureus	879
55R	H	13	Front left	Negative	44
56	H	13	Posterior right	Negative	35
57	H	13	Posterior left	Negative	35
58	SM	14	Front left	CNS-S.agalactiae	1500
59	SM	14	Posterior right	S. agalactiae	7516
6	SM	2	Front left	S. aureus	16

60	SM	14	Posterior left	S.aureus-S.agalactiae	3880
61	SM	15	Front right	CNS	36
62	SM	15	Front left	S. aureus	180
64R	SM	15	Posterior right	CNS	32
65R	SM	15	Posterior left	S. aureus	323
66R	H	16	Front right	Negative	62
67	H	16	Front left	Negative	40
68R	H	16	Posterior right	Negative	35
69R	H	16	Posterior left	Negative	53
7	SM	2	Posterior right	S.aureus-S.agalactiae	1753
70R	SM	17	Front right	CNS	40
71	SM	17	Front left	S. aureus	409
72R	SM	17	Posterior right	CNS-S.agalactiae	272
73R	SM	17	Posterior left	S. agalactiae	245
74	SM	18	Front right	S. aureus	836
76	SM	18	Front left	S. aureus	3090
77R	SM	18	Posterior right	S. aureus	NA
79	SM	19	Front left	S. aureus	831
8	SM	2	Posterior left	S. aureus	634
80	SM	19	Posterior right	S. aureus	494
81	SM	19	Posterior left	S. aureus	1973
83	SM	20	Front left	S. aureus	195

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Table S2: Mean, standard deviation (SD), median, minimum (min) and maximum (max) of the most abundant phyla (Panel A) and families (Panel B) with a relative abundance at least of 1%.

H: healthy samples; SM: subclinical mastitis samples.

A

Phylum	Status	mean	SD	median	min	max
p__Actinobacteria	**H**	27.9%	11.0%	27.0%	8.2%	53.1%
	SM	12.1%	8.7%	10.0%	1.2%	36.0%
p__Bacteroidetes	**H**	6.5%	4.6%	5.9%	0.0%	16.2%
	SM	4.5%	6.1%	1.2%	0.0%	21.8%
p__Firmicutes	**H**	37.0%	13.0%	35.8%	12.6%	55.2%
	SM	58.2%	28.4%	59.2%	8.3%	97.6%
p__Proteobacteria	**H**	24.9%	15.2%	22.2%	6.6%	73.2%
	SM	21.5%	21.9%	13.0%	0.8%	85.2%

B

Families	Status	mean	SD	median	min	max
f__Aerococcaceae	**H**	10.9%	7.4%	10.0%	0.0%	22.3%
	SM	5.7%	7.4%	2.0%	0.0%	24.8%
f__Corynebacteriaceae	**H**	9.4%	5.1%	8.8%	2.6%	19.1%
	SM	4.7%	4.6%	3.1%	0.0%	17.9%
f__Moraxellaceae	**H**	7.6%	16.3%	2.1%	0.0%	68.2%
	SM	6.4%	13.3%	1.5%	0.0%	65.5%
f__Propionibacteriaceae	**H**	8.1%	7.0%	5.8%	0.3%	23.9%
	SM	2.5%	2.5%	1.6%	0.2%	11.2%
f__Staphylococcaceae	**H**	9.2%	5.7%	7.8%	1.7%	23.2%
	SM	37.8%	34.7%	30.2%	0.0%	96.6%

Table S3: Minimum (Min), maximum (Max), mean and median of the standard deviation (SD) of the most abundant phyla (Panel A) and families (Panel B) at individual level. Animal identification is indicated between parentheses. H: healthy samples; SM: subclinical mastitis samples.

A

Phylum	SD	H	SM
p__Actinobacteria	**Min**	3.3% (1)	1.5% (19)
	Max	10% (6)	14.4% (20)
	Mean	7.6%	6.8%
	Median	8.4%	6.9%
p__Bacteroidetes	**Min**	1.4% (6)	0.2% (19)
	Max	5.3% (1)	9.5% (18)
	Mean	3.0%	3.9%
	Median	2.4%	2.9%
p__Firmicutes	**Min**	2.6% (6)	3.1% (19)
	Max	21.2% (13)	30.6% (14)
	Mean	8.6%	19.0%
	Median	5.7%	19.8%
p__Proteobacteria	**Min**	2.7% (1)	1.9% (19)
	Max	32.1% (13)	32.4% (14)
	Mean	11.9%	9.0%
	Median	9.4%	6.9%

B

Families	SD	H	SM
f__Aerococcaceae	**Min**	0.9% (7)	0.05% (17)
	Max	10.5% (13)	12.1% (20)
	Mean	5.7%	5.3%
	Median	6.2%	5.1%
f__Corynebacteriaceae	**Min**	2% (6)	0.9% (18)
	Max	8.5% (7)	10% (20)
	Mean	5.3%	3.9%
	Median	5.4%	4.3%
f__Moraxellaceae	**Min**	0.9% (16)	0.2% (8)
	Max	36.6% (13)	33% (14)
	Mean	10.3%	6.8%
	Median	3.4%	1.7%
f__Propionibacteriaceae	**Min**	1.1% (13)	0.2% (20)
	Max	5.3% (16)	4.9% (15)
	Mean	2.9%	1.6%
	Median	2.7%	0.8%
f__Staphylococcaceae	**Min**	2.6% (6)	2.5% (14)
	Max	10.1% (13)	48.9% (4)
	Mean	5.4%	27.8%
	Median	4.7%	26.7%

Table S4: descriptive statistics of unweighted and weighted UniFrac distance matrices. Minimum, maximum, mean, median and standard deviation (SD) are shown.

	Unweighted UniFrac distance matrices				**Weighted UniFrac distance matrices**			
	Healthy within	**Healthy between**	**Subclinical mastitis within**	**Subclinical mastitis between**	**Healthy within**	**Healthy between**	**Subclinical mastitis within**	**Subclinical mastitis between**
Min	0.35	0.41	0.24	0.34	0.17	0.16	0.02	0.02
Max	0.75	0.88	0.8	0.89	0.36	0.45	0.43	0.56
Mean	0.56	0.63	0.6	0.65	0.24	0.28	0.26	0.31
Median	0.57	0.6	0.64	0.66	0.24	0.27	0.27	0.33
SD	0.1	0.1	0.12	0.1	0.05	0.06	0.1	0.12

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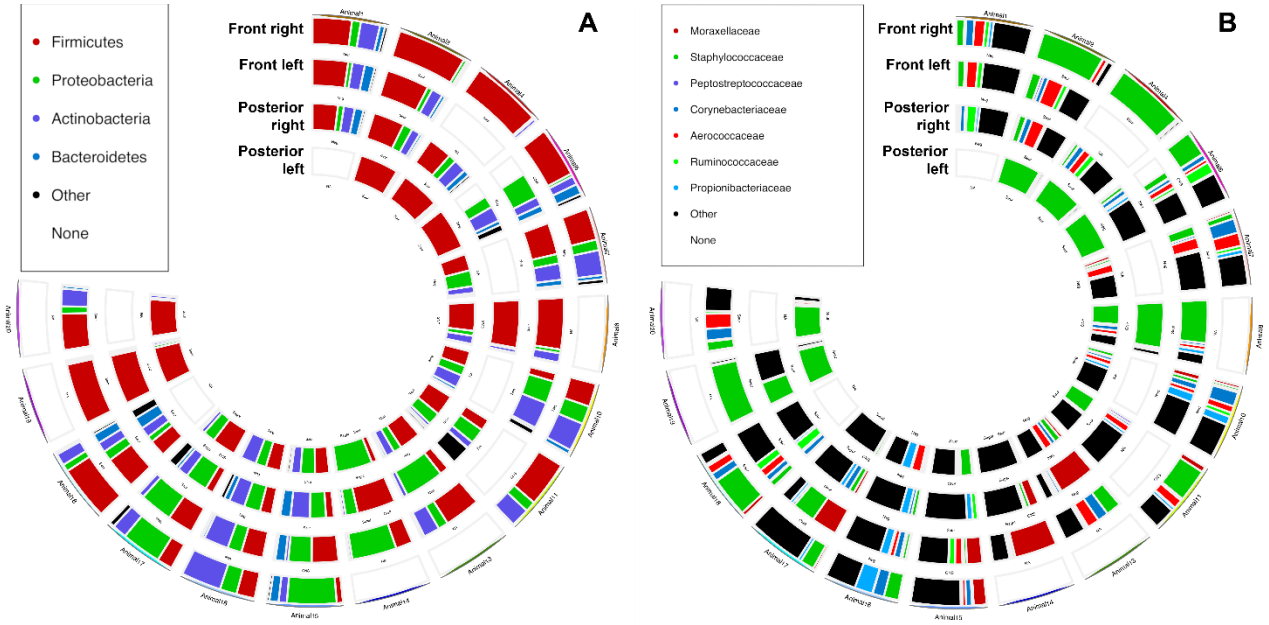

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355 Figure 1

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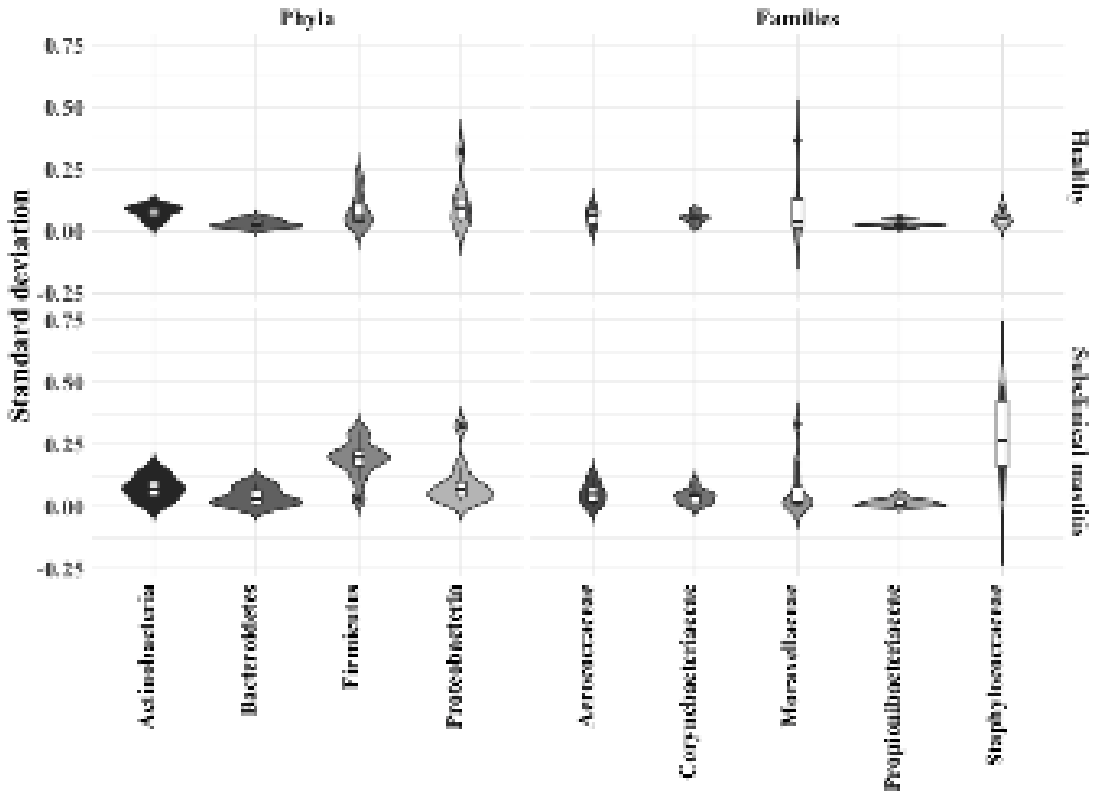
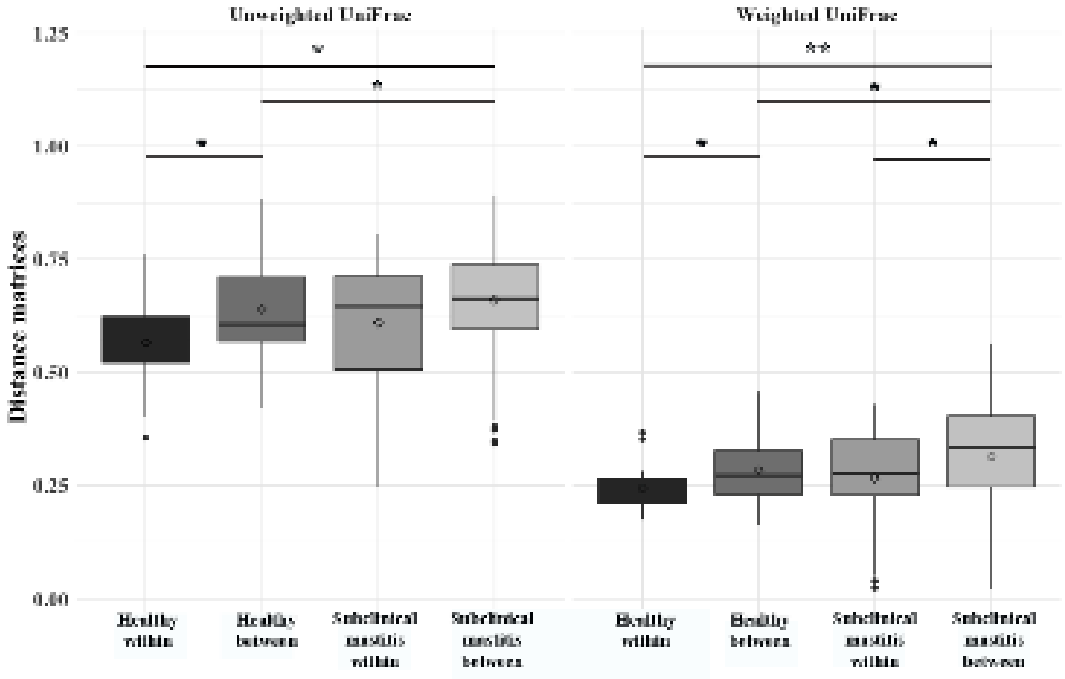

Phyla
Families
Standard deviation
0.75
0.50
0.25
0.00
-0.25
Habitat
Skeletal mass
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Aeromonadaceae
Corynebacteriaceae
Moraxellaceae
Propionibacteriaceae
Staphylococcaceae

Figure 2



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368 Figure 3