The microbiota of Malaysian fermented fish sauce

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ABSTRACT

Budu is a Malaysian fermented anchovy sauce produced by immersing small fishes into brine solution for 6 to 18 months. Fermentation of the anchovy sauce is catalyzed by microbial enzymes, but little is known about the microbial community in Budu. A better understanding of the Budu microbiota is necessary to better control the quality, consistency and biological safety of this fermentation product. In this study, we sampled 20 bottles of Budu produced by 7 different manufacturers and analyzed their microbiota based on V3-V4 16S rRNA amplicon sequencing, at the time of opening as well as 3- and 7-months post-opening. In most samples, *Tetragenococcus* appears to be the most prevalent genus detected making up an average of 38.77 % relative abundance per sample (median = 19.56%, min = 0%, max = 98.62%). Furthermore, we also detected possible spoilage microbial genera such as *Xanthomonas, Acinetobacter, Pseudomonas, Halanaerobium,* and *Psychrobacter*. The microbial composition of majority of samples appears to be relatively stable even after seven months of bottle opening and storage at room temperature. Samples from two brands however showed a sudden increase in the number of reads belonging to the genus *Chromobacterium* in the 7th month.

Keywords: Budu, 16S sequencing, Fish sauce microbiome, Fermented food

INTRODUCTION

Fermentation is a traditional food preservation method which grants the food attractive qualities in the form of longer shelf life and appealing gastronomical value (Caplice and Fitzgerald 1999). A reproducible formation of microbial communities is imperative in maintaining the qualities of fermented food. Thus, backslopping, a practice of inserting a small portion of the previous batch of fermented food into a newer one, is commonplace to preserve the existing microbial community. Such a practice generally maintains the desirable features of the previous batch such as increased production yield and sensory properties (Kim, Jeong et al. 2018). With an ever-increasing global demand for fermented products, modifications and possibly compromises in the fermentation approach must be made to speed up production. Without proper monitoring, such changes will translate to poor fermentation practices that can lead to foodborne outbreaks. For example, the shift to the use of plastic containers in the fermentation of aquatic game promoted the growth of anaerobic *Clostridium* and lead to the endemic foodborne botulism in Alaska (Shaffer, Wainwright et al. 1990, Fagan, McLaughlin et al. 2011). Another example is the annual feseekh botulism in Egypt which was caused by poor fermentation practices by type E toxins produced by *Clostridium botulinum* (Hus and Petersen 1980, El Sheikha, Ray et al. 2014).

Budu is a popular fermented anchovy sauce in Northeast Malaysia. It is prepared by immersing anchovies in brine solution containing natural flavor-enhancing natural ingredients and left to ferment in earthen containers for 6 to 12 months (Rosma, Afiza et al. 2009). All microbiological studies of Budu to date are culture-based. For example, Sim, Chye et al. (2009) reported a bacterial succession from *Micrococcus*- to *Staphylococcus arlettae*-dominated microbiota based on total plate count on 10% NaCl plate count agar cultured for 2-14 days at 37°C, combined with species identification based on carbon utilization using Biolog Microlog Database software. In addition, they also uncovered the presence of yeast species such as *Candida glabrata* and *Saccharomyces cerevisiae* based on culturing on yeast-malt extract agar, incubated for 72 hours at 25°C. Successive microbiological studies on Budu revealed a few other culturable bacteria such as *Bacillus amyloliquefaciens* FS-05 and *Lactobacillus planatarum* which are able to produce glutamic acid, associated with "umami taste" ascribed to fermented fish sauce (Zaman, Bakar et al. 2010, Zareian, Ebrahimpour et al. 2012). A recent study on Malaysian fermented foods illuminated the potential of *Bacillus* sp. in producing biosurfactants that inhibit growth of pathogenic bacteria (Mohd Isa, Shamsudin et al. 2020).

While useful in gaining the first insights into the microbial structure of the Budu environment, culture-dependent methods are generally biased towards microbes that can grow optimally under the culture condition chosen by the researchers. To date, no research based on culture-independent methods was done to gain an improved insight into the Budu microbiota. Such information is likely more useful in understanding the microbial composition within Budu and the interactions occurring between resident bacteria, leading to differences in fermented food quality (Devanthi, Linforth et al. 2018).

More importantly, the anchovy species in Budu like *Sardinella* spp. is rich in purine (2,258.91 mg/kg) (Li, Ren et al. 2019), which is implicated with the high gout incidence in Malaysia (Paul and James 2017). Differences in the microbial fermentation process during the manufacturing of Budu may also present a potential health risk to its consumers due to the uncontrolled release of purine as a by-product of fish protein degradation. Prolonged fermentation of the fish can also lead to conversion of glutamic acid available in the fish protein into more purine (Tungkawachara, Park et al. 2003). Rosma, Afiza et al. (2009) sampled twelve Budu samples from twelve different producers and revealed that seven of them contained histamine content exceeding 50 mg/100 g sample, which is the FDA limit. Unchecked consumption of histamine-rich food can lead to scombroid food poisoning, the symptoms which are breathing difficulties and irregular heartbeats (Wilson, Musto et al. 2012, Tortorella, Masciari et al. 2014).

In this study, we report the microbial community of retail Budu of different brands at different sampling periods. We also explored the use of microbial interaction networks modeled on 16S abundance information.

MATERIALS AND METHODS

Sample collection

Twenty bottles of Budu from different brands were purchased from shops in Kelantan, Malaysia, at five different time points (July 2016, October 2016, November 2016, March 2017 and April 2017). These twenty bottles were sampled at three different periods (24th April 2017, 24th July 2017 and 20th November 2017), amounting a total of 60 samples. The

samples were stored at room temperature to emulate typical storage condition. The sample metadata is shown in Table 1. Samples were named according to the following format:<brand><bottle number>_<months since last opened>

DNA extraction and sequencing

200 µl of the sample was poured into 1.5 ml Eppendorf tubes and centrifuged at 14 800 rpm for 15 minutes to remove supernatant. Samples were resuspended in 400 uL of 20 mM Tris-EDTA buffer and added with 100 µm silica beads (OPS Diagnostics LLC, Lebanon, NJ, USA). The sample mixture was later subjected to bead beating using Vortex Genie 2 Mo Bio (Carlsbad, CA, USA) at 3200 rpm for 1 hour. They were later added with 20 ul of SDS and 20 ul of Proteinase K and incubated at 55°C for 1 hour. The samples were added with 100 ul of saturated KCl solution and were later incubated on ice for 5 minutes. Total DNA was extracted using chloroform-isopropanol precipitation and purified with Agencourt AMPure XP beads (Beckman Coulter, Inc., Indianapolis, IN, USA). The V3-V4 region of the 16S rRNA gene was amplified using the forward primer 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and reverse primer 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3' containing partial Illumina Nextera adapter sequence (Klindworth, Pruesse et al. 2012). PCR reaction and barcode incorporation were performed as previously described (Watts, Spurr et al. 2017). DNA libraries were normalized, pooled, denatured and sequenced on the Illumina MiSeq (Illumina, San Diego, CA, USA) in Monash University Malaysia Genomics Facility, using the 2 × 250 bp or 2 × 300 bp run configuration.

Sequence data analysis, phylogenetic tree construction, taxonomic assignment and generation of feature table

The forward and reverse PCR primer sequences were trimmed off using Cutadapt version 1.16 with default parameters (Martin 2011). Paired-end sequences were merged using fastq_mergepairs, and quality-filtered using fastq_filter (-fastq_maxee: 1.0, -fastq_minlen: 300) in USearch v10.0.240_i86linux32 (Edgar and Flyvbjerg 2015). High-quality sequences were then denoised to create amplicon sequence variants (ASVs) (Supplementary File 1).

ASVs were aligned using MAFFT, masking unconserved and highly gapped sites (Lane 1991, Katoh and Standley 2013). A phylogenetic tree was constructed from aligned ASV sequences and rooted at midpoint using FastTree version 2.2.10 plugin in QIIME2 version 2018.6 (Price, Dehal et al. 2010, Bolyen, Rideout et al. 2018).

16S V3-V4 Naive-Bayes classifier was trained on V3-V4-trimmed 16S sequences of the SILVA 132 release, using q2-classifier plugin in Qiime2 (Pedregosa, Varoquaux et al. 2011, Bokulich, Kaehler et al. 2018). Sequence trimming on SILVA reference sequences was done using same primer sequences and parameters applied on the raw sequencing data. The ASV sequences were then taxonomically assigned using the trained classifier (Supplementary File 2).

ASVs generated were used as reference sequences to create a raw feature table, using UNoise3 in USearch (Edgar 2016). The raw feature table was further processed by filtering out chloroplast and mitochondrial ASVs (see below for a description on taxonomic assignment) and rarefying to 6,000 reads per sample for downstream analyses (Supplementary File 3).

Alpha diversity indices calculation and rarefaction plots

Species richness indices (observed ASVs and Faith PD) were calculated in QIIME2 and plotted using matplotlib 3.0.2 (Hunter 2007, Bokulich, Kaehler et al. 2018). The trajectory of species richness indices at increasing sequencing depths was calculated and displayed in rarefaction plots. All calculations of Alpha diversity indices were done at ten iterations per sequencing depth. Wilcoxon paired tests were done to make comparisons of species richness between sampling batches, as implemented in DABEST-python library (Ho, Tumkaya et al. 2019). Since there is only one bottle for brand F and G, samples from these brands were not included in statistical comparisons.

Principal coordinates analysis and relative differential abundance analysis

Beta diversity was measured based on Bray-Curtis to calculate distances between samples (Lozupone, Lladser et al. 2011). Principal Coordinates Analysis (PCoA) plots were then produced from these distance values using R *phyloseq* package (McMurdie and Holmes 2013). ASV- and genus-level comparisons between sampling batches were done using QIIME2 plugin ANCOM (Mandal, Van Treuren et al. 2015).

Microbial association network

SPIEC-EASI 0.1.4 was used to predicting microbial association network (Kurtz, Müller et al. 2015). Since SPIEC-EASI applies its own normalization, the mitochondria and chloroplast ASVs-filtered unrarefied feature table was used as the input. Prior to network construction, prevalence filtering was applied using seqtime at the minimum occurrence of 30 samples (Faust, Bauchinger et al. 2018).

Meinshausen-Bühlmann neighborhood selection model was used to model the microbial interaction network at 10,000 replications (Meinshausen and Bühlmann 2006). Network model was selected at a variability threshold of 0.05% using StARS (Liu, Roeder et al. 2010). R *igraph* and *ggnet* packages were used for network visualization and network properties (Csardi and Nepusz 2006, Schloerke, Crowley et al. 2011). To find for important nodes in the graph, degree centrality and betweenness centrality were two node centrality measures computed from the predicted network. Degree centrality weighs a node's importance by counting the number of edges linked to the node, while betweenness centrality evaluates the geodesic distances from all node pairs that are passing through the particular node. High degree centrality suggests a role as a keystone species, while high betweenness centrality predicts its importance in maintaining the structure of the interaction network.

RESULTS

Five major bacterial phyla dominated the Malaysian Budu microbiota

Firmicutes, Proteobacteria, Halanaerobiaeota, Actinobacteria, and Bacteroidetes were the top five most abundant phyla of the entire sample collection (Figure 1) with an average relative abundance of 60.26%, 22.86%, 10.60%, 3.91%, and 1.91% respectively, while other lesser phyla contribute less than 0.5% of the rarefied observation table. The cumulative relative abundance contributed by these top five phyla in each sample ranges from 95.75% to 100.00%.

We observed a fairly distinct phyla composition among and within different brands and manufacturing batches. For example, all samples from Brand D were consistently dominated

by Firmicutes, making up an average relative abundance of 93% in each sample. On the contrary, some samples as A1 consist of all the top five at uneven distribution. The inconsistency in phyla distribution was also apparent within brand C with Halanaerobiaeota making up a substantial portion from 18.8% to 48.4% in C1, C3 (except sample C3_0) and C4 samples but was markedly smaller in C2 samples (average = 2.2%).

Alpha and Beta Diversity of Certain Budu Samples Display Variations, even for Samples within Similar Brands

Some Budu samples within a brand had greatly different species richness (Figure 2). A2 samples had higher observed ASVs and Faith PD measures compared to A1 and A3. The same was observed in brand E samples; E1 and E3 had greater alpha diversity over E2 and E4. Meanwhile, samples from brand D have alpha diversity indices which were low and close to one another. The great discrepancy of species richness in bottles within the same brand, such as brand A (Figure 2) is congruent with variation in phyla distribution and genera distribution (Figure 1). The rarefaction curves per brand, by observed ASVs and Faith PD indices are provided in Supplementary Figure 1.

There were also apparent changes in alpha diversity across sampling batches. After 3 months, increase in observed ASVs and Faith PD were generally observed in most bottles of brand A, C and E. The distribution of these shifts can go beyond the mean alpha diversity at month 0, which we used as control value. Distribution of alpha diversity values of brand A, C and E were higher than their respective control values. It can also be seen that in some of these bottles between month 3 and month 7, the alpha diversity indices lowered down while a few steadily increased. There are also shifts in species richness in brand B samples, but the trend is less consistent compared to the other brands.

Principal component analysis (PCoA) of Bray Curtis beta diversity metrics was used to observe the overall trend of bacterial diversity (Figure 3). The clustering of the samples in the PCoA mirrored the phyla distribution of samples from individual bottles, irrespective of their brands. For example, there are three sub clusters for sample A. Each cluster consists of three points correspond to three samples per bottle, suggesting that differences between the three bottles were captured. Sub clusters of brand B samples were also observed, which provide further evidence that the Budu microbiome of a brand was inconsistent between different bottles. A tight clustering of all brand D samples in the PCoA, their closely similar alpha diversity values and their consistent phyla composition suggest that the microbiome of brand D samples were similar.

Core ASVs and genera in fermented fish sauce display unique abundance and prevalence

There are eight core ASVs found in at least half of our sample collection (Figure 4), none of the core ASVs were 100% prevalent. ASV4, ASV3, and ASV7 were assigned under *Tetragenococcus halophilus* subsp. halophilus, although detected at different prevalence. Meanwhile, ASV1 was assigned as *Tetragenococcus muriaticus* and was the most abundant ASV found in our Budu samples. It was apparently abundant in samples from brand D.

There are fourteen core genera found in at least half of our sample collection, *Tetragenococcus* being the most prevalent genus (96.7%), as shown in Figure 4. *Tetragenococcus* and *Halanaerobium* are the only two genera that can reach an abundance of over 50% of reads per sample – this was observed in 21 samples (brands A, B, D and G) and 3 samples (brand C) respectively. Meanwhile, the abundance of *Comamonas* was at most

1.22 % of reads per sample, despite being present in half of the samples. None of the core genera are 100% prevalent. This shows that increasing prevalence of genus in the samples is not predictive of its relative abundance. Another example is the genus Weisella which was 50% prevalent in all samples, yet it made up to 20% of the genera composition in four samples - this degree of abundance level was not observed in several higher prevalence genera such as *Acinetobacter*, *Pseudomonas*, *Psychrobacter*, *Corynebacterium_1*, *Lentibacillus*, *Kocuria*, *Brevibacterium*, and *Paracoccus*.

Tetragenococcus is the most abundant genus, occupying up to 98.6% in sample G1_0. Within *Tetragenococcus*, we detected two different species which are *Tetragenococcus muriaticus* and *Tetragenococcus halophilus* subsp. halophilus. *T. muriaticus* composition was consistently high in brand D, while *T. halophilus* subsp. halophilus was especially abundant in some bottles of brand A, B and G. We also observed the co-presence of both *Tetragenococcus* species in some samples such as C1 samples and samples from brand F. Only two samples did not contain *Tetragenococcus* which are B2_7 and C3_3.

Significant differential abundance of *Chromobacterium* across different sampling batches

A comparison at genus level between sample batches revealed *Chromobacterium* as the only genus with significantly differential abundance. It was abundant in several samples from the third sampling batch (W-statistic = 399) (Figure 5). Apart from sample A1_7, *Chromobacterium* was found in all third sampling batch of brand D and E samples. Comparison at ASV level between sampling batches resulted in no significantly differential ASV distribution.

Microbial abundance interaction network

After prevalence filtering of 30 samples (50%) and network construction, there were a total of 71 nodes in the predicted microbial interaction network (Figure 6). To normalize the 16S count data, SPIEC-EASI avoids rarefaction and instead multiply counts by ratio of ideal sampling depth to total counts in sample. This might explain the greater number of ASVs retained after prevalence filtering as compared to core ASVs determined from rarefied table (Figure 4). Supplementary figure 2 displays the prevalence of microbial classes in the unrarefied feature table.

A total of 99 edges was predicted in the interaction network, 73.7% were positive associations, and 26.3% were negative associations. Several isolated subclusters formed such as a small cluster consisting of four nodes (ASV4, ASV7, ASV10 and ASV11); all four nodes were assigned as *Tetragenococcus halophilus* subsp. Halophilus. The isolation of this cluster might imply lack of interaction between *Tetragenococcus halophilus* subsp. Halophilus with the rest of the Budu inhabitants. The nodes are not fully connected with each other, possibly because of the conditional independence implemented in SPIEC-EASI to prevent spurious links. Positive associations were predicted for edges ASV4-ASV7 and ASV7-ASV11. However, a negative association formed between ASV4 and ASV10.

Degree centrality and betweenness centrality were two node centrality measures computed from the predicted interaction network. Distribution of these scores for the predicted interaction network can be observed in Supplementary Figure 3. Majority of the nodes had very low degrees and possessed low betweenness centrality scores. ASV45 (*Enterobacteriaceae*) boasted the highest degree centrality (degrees = 10) and betweenness centrality (score = 466.72).

There are interesting patterns that can be observed from the network. Several nodes of similar taxonomic assignment were predicted to interact with each other such as ASV1-ASV6 (*Tetragenococcus muriaticus*), ASV47-ASV77-ASV228 (*Acinetobacter*), ASV20-ASV28-ASV53 (*Staphylococcus*), ASV216-ASV133(*Psychrobacter*) and ASV27-ASV66 (*Kocuria*). Coabundance between these nodes were expected due to 16S copy numbers of slight nucleotide variations originating from the same bacterial genome. There are also patterns of network assortativity, which was most apparent for the Actinobacteria nodes surrounding ASV30 which is assigned as *Brevibacterium*. Supplementary figure 4 displays the adjacency matrix of predicted pairwise interaction between ASVs. ASV30 formed a number of positive links with *Corynebacterium* nodes which belonged to the Actinobacteria class.

DISCUSSION

Fermented fish sauce is very popular in the South East Asian region as a food condiment, eaten with rice and fish or as a cooking ingredient. However, research on the fermented fish sauce is not as advanced as fermented dairy products like yogurt and cheese, which benefited from years of research. The advanced research on European fermented food translated to increased production and its popularity as a dietary source of live microbes promoting health beyond basic nutrition (Rezac, Kok et al. 2018). Several microbiome undertakings have been done on fermented fish sauce in Korea (Jung, Lee et al. 2013, Lee, Jung et al. 2014), and none so far was done on Budu. The fermentation of the Budu is highly dependent on the microbial activity since the high salt concentration inhibits endogenous fish enzymes like cathepsins, but activates halophilic bacterial proteinases which hydrolyze fish protein and releases volatile fatty acids (Tungkawachara, Park et al. 2003, Yongsawatdigul, Rodtong et al. 2007, Sinsuwan, Rodtong et al. 2008, Mohamed, Man et al. 2012, Duan, Hu et al. 2016). Consumption of Budu can potentially be detrimental to health due to its high histamine and purine content (Rosma, Afiza et al. 2009, Mohd, Gupta et al. 2011). Understanding the microbial composition empowers the producers to supply beneficial bacteria that can reduce the hazard in Budu consumption. Thus, we took it upon ourselves to survey the bacterial diversity of Budu of different brands. Our findings provided evidence of inconsistent microbial diversity within similar brands, suggesting a lack of quality control in Budu production. We also observed bacterial genera that are commonly associated with food spoilage such as *Xanthomonas, Acinetobacter, Pseudomonas, Halanaerobium,* and *Psychrobacter*.

Budu samples were predominated by five bacterial phyla which are Firmicutes, Proteobacteria, Halanaerobiaeota, Actinobacteria, and Bacteroidetes (Figure ~). The combined composition of the top five most abundant phyla composing the Budu microbiome ranged from 95.75% to 100.00%. The consistency of the phyla distribution of samples of the same bottles across sampling period suggests the stability of bacterial community after fermentation, which is evident from the PCoA in Figure ~. This contrasts the shift in bacterial composition during the fermentation of fish sauce; from a Proteobacteria-dominated microbiome to a Firmicutes-dominated microbiome (Jung, Lee et al. 2013, Egerton, Culloty et al. 2018). However, we observed huge distances between bottles of similar brands like brand A and E. Such variation was also reflected in discrepancy in alpha diversity measures (Figure ~: Alpha rarefaction plots). Fermentation under uncontrolled conditions may lead to these inconsistencies and is a major obstacle to commercial production with uniform, premium quality (Jung, Lee et al. 2016). Budu is generally produced by small business owners, thus the production practices were not standardised. Meanwhile, brand D samples

demonstrated limited alpha diversity variation and consistent microbial composition between the four purchasing batches. These samples are dominated by *Tetragenococcus muriaticus* which make up over 90% of the reads per sample. The same expiry months of all brand D samples might easily suggest that all brand D samples originate from the same production batch, although only D3 samples contain colouring, but not in D2 and D1 samples.

There were fourteen core genera of our Budu samples were identified (Figure ~), seven of which were amongst the top nine most abundant genera (Figure ~). Some of these genera such as *Xanthomonas*, *Acinetobacter*, *Paracoccus* and *Comamonas* were not previously found in fermented fish sauce. *Xanthomonas* and *Acinetobacter* were associated with fish and fruit spoilage respectively (Rawat 2015). Other genera were known denizens of the fish sauce and were attributed to proteolytic processes (*Pseudomonas*), degradation of biogenic amines (*Staphylococcus*, *Brevibacterium*), production of pleasant-smelling volatile fatty acids (*Staphylococcus*, *Bacillus*) and release of off-smelling organic compounds (*Halanaerobium*) (Lopetcharat, Choi et al. 2001, Yongsawatdigul, Rodtong et al. 2007, Zaman, Bakar et al. 2010, Jung, Lee et al. 2013, Lee, Jung et al. 2014). Several lactic acid bacteria (LAB) represented as the core Budu microbiome like homofermentative *Tetragenococcus*, *Pediococcus* and also heterofermentative *Weissella*, which suggests an extensive functional diversity in Budu (Caplice and Fitzgerald 1999, Hwanhlem, Buradaleng et al. 2011). LABs are known for their extensive gene repertoire for amino acid release and hydrolysis such as peptidases, amino acid permeases and oligopeptide transporters, explaining their prevalence in Budu, which is a great resource of protein (Savijoki, Ingmer et al. 2006, Makarova and Koonin 2007). Members of the LAB are also known to be spoilage bacteria (Kalschne, Womer et al. 2015).

Tetragenococcus is a tetrad-forming coccus, gram positive halophilic LAB and is the most abundant genus in our study. It was found in high-salt fermented food such as soy sauce, squid liver sauce (Satomi, Kimura et al. 1997), dried fish, salted seafood (Kim and Park 2014), fish sauce (Thongsanit, Tanasupawat et al. 2002) and fermented fish paste (Marui, Boulom et al. 2015). Despite its prevalence in numerous fermented food, to the best of our knowledge, *Tetragenococcus* was never found in the natural environment, neither found in fish gut nor saltern sources (Egerton, Culloty et al. 2018). *Tetragenococcus* was highly abundant in majority of the Budu samples; the dominance of a few microbial taxa is expected in MCoFF microbiome (Wolfe, Button et al. 2014). However, the proportion of the genus was very low or absent in a few samples such as A2, B2, B3, E1 and E3. The greater alpha diversity indices in these low-*Tetragenococcus* samples may be because these Budu were prepared without starter cultures, leading to the growth of various halophilic or halotolerant microbes that were introduced from the surrounding environment (Lee, Jung et al. 2015). We also identified two *Tetragenococcus* species which were two *Tetragenococcus* species identified which were *T. halophilus* and *T. muriaticus* (Kobayashi, Kimura et al. 2000). *Tetragenococcus muriaticus* is known to release histamine and cadaverine, while *Tetragenococcus halophilus* is known for the ability to reduce histamine and cadaverine formation (Zaman, Bakar et al. 2010, Kim, Lee et al. 2019). *T. halophilus* was also reported to contribute towards probiotic properties (Kuda, Izawa et al. 2014). The two species were reported to grow optimally at different pH level and salt concentration and utilize different types of sugar (Kobayashi, Kimura et al. 2000, Kobayashi, Kajiwara et al. 2004, Udomsil, Rodtong et al. 2010, He, Wu et al. 2016). *T. muriaticus* predominated brand D samples while *T. halophilus* was highly abundant in A3, B1, B4, and G1 samples. The clustering of samples in the weighted Unifrac PCoA was largely determined by *Tetragenococcus* abundance (Figure ~), highlighting the major role of the genus in shaping the Budu microbiome.

Halanaerobium was the only genus representing the Halanaerobiaeota. It is an obligate anaerobic halophile, which reflects the anaerobic, low water activity environment in Budu (Jung, Lee et al. 2013, Stevenson, Cray et al. 2015). It is also a potential marker for putrefaction because it's growth was correlated with an increase in acetate, butyrate and putrescine (Lee, Jung et al. 2015). Some genera that were previously reported in fish sauce were detected at very low levels in our Budu samples including *Virgibacillus, Achromobacter, Paenibacillus, Halomonas, Micrococcus, Vibrio, Flavobacterium, Serratia, Clostridium, Streptococcus* and *Lactobacillus* (Yongsawatdigul, Rodtong et al. 2007, Mah, Chang et al. 2008, Hwanhlem, Buradaleng et al. 2011, Belfiore, Raya et al. 2013, Chuon, Shiomoto et al. 2014, Lee, Jung et al. 2015). There were also bacterial genus previously reported to be found in a fish sauce but were absent in our samples, like *Filobacillus* (Hiraga, Nishikata et al. 2005).

Comparison of samples between sampling batches showed *Chromobacterium* to be significantly increased in the last sampling batch (Figure ~). This reveals that the Budu microbiome is not stagnant post-fermentation. Apart from sample A1_7, the genus was detected exclusively in samples of brand D and E by the 7th month, although in greater abundance in brand E. Since *Chromobacterium* was not detected in first and second batches, it is also possible that the genus was introduced from the surrounding into the samples while sampling from the previous sampling batches. *Chromobacterium* was previously found environmental samples (Kämpfer, Busse et al. 2009, Menezes, Tonin et al. 2015) as well as food samples such as vegetables, cheese, seafood samples (Koburger and May 1982) and fermented barley samples (Wood 2012). Septicemia/bacteremia-causing *Chromobacterium* species were also reported (Kaufman, Ceraso et al. 1986, Parajuli, Bhetwal et al. 2016). Due to the lack of genomic sequences and analyses of the *Chromobacterium* species (Santos, Costa et al. 2018), future research should address this gap of knowledge and identify the ecological significance of the genus in fermented food.

Microbes exist in communities and interact with each other through mechanisms such as production of lactic acid, the release of antimicrobials, quorum sensing, cross-feeding. The ability to broadly predict such relationships using 16S feature table is alluring, thus attempts on modeling microbial interactions were done in the form of microbial interaction network (Deng, Jiang et al. 2012, Faust, Sathirapongsasuti et al. 2012, Friedman and Alm 2012, Kurtz, Müller et al. 2015). In this study, we constructed microbial interaction networks using the Meinshausen-Bühlmann neighborhood selection model at a prevalence filtering of 30 samples (50% of samples) to reduce the computational burden and to have better generalization and interpretability (Röttjers and Faust 2018). Isolated clusters were observed including a four-nodes cluster comprising ASV4, ASV7, ASV10 and ASV11; all were assigned to *Tetragenococcus halophilus* subsp. Halophilus. Known interaction of *Tetragenococcus halophilus* was reported, in the form of antagonism with *Zygosaccharomyces rouxii* in soy sauce fermentation (Devanthi, Linforth et al. 2018). However, *Zygosaccharomyces rouxii* was not detected in Budu. The isolation of the *T. halophilus* subsp. Halophilus cluster predicts absence of interaction between *T. halophilus* with other species. Bacterial genomes commonly possess 16S in multiple copies with minimal variation, thus the four ASVs might originate from the same genome. The positive links between these ASVs suggest a degree of accuracy construed by the constructed networks, although ASV4-ASV10 was negative. Positive links between ASVs of similar taxonomic assignment were also observed such as between ASV30 and other Actinobacteria node.

The interpretation of microbial interaction network and it's application in a biological context is still limited. For example, *Bacillus* is both abundant and prevalent in our study. However, the salt concentration in Budu, by law has to be at least 15% (Mohamed, Man et al. 2012). With that salt concentration, *B. subtilis* of marine origin has inhibited growth; thus it could be that the 16S sequence of *Bacillus* was amplified from spore particles (Fukui, Yoshida et al. 2012), and that any interactions with *B. subtilis* are merely spurious links. Research in microbial interaction network is also hampered by the lack of gold standard, thus it is difficult to disentangle true interactions from strong environmental effect. Budu easily lends itself as a tractable microbial system which is replicable and manipulable, making it a potential gold standard for this research effort (Wolfe and Dutton 2015, Wolfe 2018). For example, it was discovered that abundance of LAB is contrained by establishment limitation; which is practical information from a fermented food production perspective (Miller, Kearns et al. 2019).A bottle of Budu costs approximately $0.20, thus it is economically feasible to scale up sampling and benchmark current and future microbial interaction network models.

In conclusion, we observed inconsistent bacterial community in some brands of Budu. We also discovered abundant and prevalent genera of Budu, some of which were not previously found in Budu, while some were also associated with food spoilage. *Tetragenococcus* was the most abundant in our sample collection, and the two species detected are *Tetragenococcus muriaticus* and *Tetragenococcus halophilus*. While *Tetragenococcus* was a common dweller in fermented fish sauce and other fermented seafood, some of our Budu samples were devoid of the genus, which suggests that *Tetragenococcus* is not a necessary component of fish sauce microbiome. This also points out towards a greater diversity of bacteria that are as capable as *Tetragenococcus* in fulfilling its role in fermentation, opening up a wider array of microbes as a candidate starter culture of Budu. We also detected *Chromobacterium* as the only genera that significantly increased in the last sampling batch, though it's ecological role and potential source is not yet clear. We also attempted to model the microbial interaction using 16S abundance data, although its interpretation is still quite limited. With the increasing popularity of fermented food and the descending sequencing cost, microbiome assessment through DNA sequencing will be, and is now, a viable, cost effective method in quality assurance of fermented food.

DATA ACCESS

All FastQ raw data can be accessed through SRA accession no.: SRP193353, or through NCBI BioProject under BioProject ID: PRJNA534025.

CONFLICT OF INTEREST

Authors declare that the research was conducted in the absence of any commercial or financial relationship that could be perceived as a potential conflict of interest.

FUNDING

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AUTHOR CONTRIBUTION

HMG conceived the experiment. MZ obtained samples. HMG and MZ performed sequencing, data analysis, interpreted the results and wrote the manuscript.

SUPPLEMENTAL MATERIAL

Figure 1: Composition of top five most abundant phyla

Figure 2: Trajectory of alpha diversity measures (Observed ASVs and Faith Phylogenetic Diversity) across sampling periods, and comparison of alpha diversity measures against control range (sampling month 0)

Figure 3: Principal Coordinates Analysis (PCoA) plot based on Bray Curtis Beta Diversity metric. Arrows indicate the trajectory of samples across sampling batches.

Figure 4: Heatmap displaying abundance of ASV and genera that are at least 50% prevalent in Budu sample collection. Deeper colors indicate higher abundance and is scaled with log values of relative abundance

Figure 5: A) Volcano plot showing significant genus distribution. Chromobacterium is marked in red. Two genera, Barnisiella and [Ruminococcus]_torques_group have higher clr value, although both possess lower W-statistics value and were present at very low relative abundances - thus they were not differentially significant. B) *Chromobacterium* distribution across all samples from the third sampling batch. Chromobacterium was not detected from the earlier two sampling batches.

Figure 6: Predicted interaction network based on Meinshausen-Bühlmann neighborhood selection model. Nodes represent ASVs, coloured based on class taxonomic assignment, and node size was given based on relative abundance, scaled logarithmically. Green edges indicate positive interaction, while red edges indicate negative interaction

Table 1: Sample Metadata of 20 bottles of Budu

Supplementary File 1: ASV sequences in fasta format

Supplementary File 2: Taxonomic assignment table of individual ASVs using q2-classifier

Supplementary File 3: Observation table of all samples after filtering out ASVs of non-microbial, rarefied at 6000 reads per sample

Supplementary Figure 1: Alpha rarefaction curves based on observed ASVs and Faith Phylogenetic Diversity, faceted by brand and sampling period. The interquartile ranges are shown. Circles indicate outlier alpha diversity calculations

Supplementary Figure 2: Prevalence of all detected microbial classes. Only nine bacterial classes that were adequately prevalent for downstream microbial interaction network prediction using SPIEC-EASI

Supplementary Figure 3: A) Degree distribution of the predicted microbial interaction network. B) Node betweenness score distribution of the predicted microbial interaction network.

Supplementary Figure 4: Adjacency Matrix Plot from the SPIEC-EASI predicted interaction network

Samples
A1_0
A1_3
A1_7
A2_0
A2_3
A2_7
A3_0
A3_3
A3_7
B1_0
B1_3
B1_7
B2_0
B2_3
B2_7
B3_0
B3_3
B3_7
B4_0
B4_3
B4_7
C1_0
C1_3
C1_7
C2_0
C2_3
C2_7
C3_0
C3_3
C3_7
C4_0
C4_3
C4_7
D1_0
D1_3
D1_7
D2_0
D2_3
D2_7
D3_0
D3_3
D3_7
E1_0
E1_3
E1_7
E2_0
E2_3
E2_7
E3_0
E3_3
E3_7
E4_0
E4_3
E4_7
F1_0
F1_3
F1_7
G1_0
G1_3
G1_7
0
20
40
60
80
100
Proportion (%)
k__Bacteria;p__Firmicutes
k__Bacteria;p__Proteobacteria
k__Bacteria;p__Halanaerobiaeota
k__Bacteria;p__Actinobacteria
k__Bacteria;p__Bacteroidetes

Figure 1

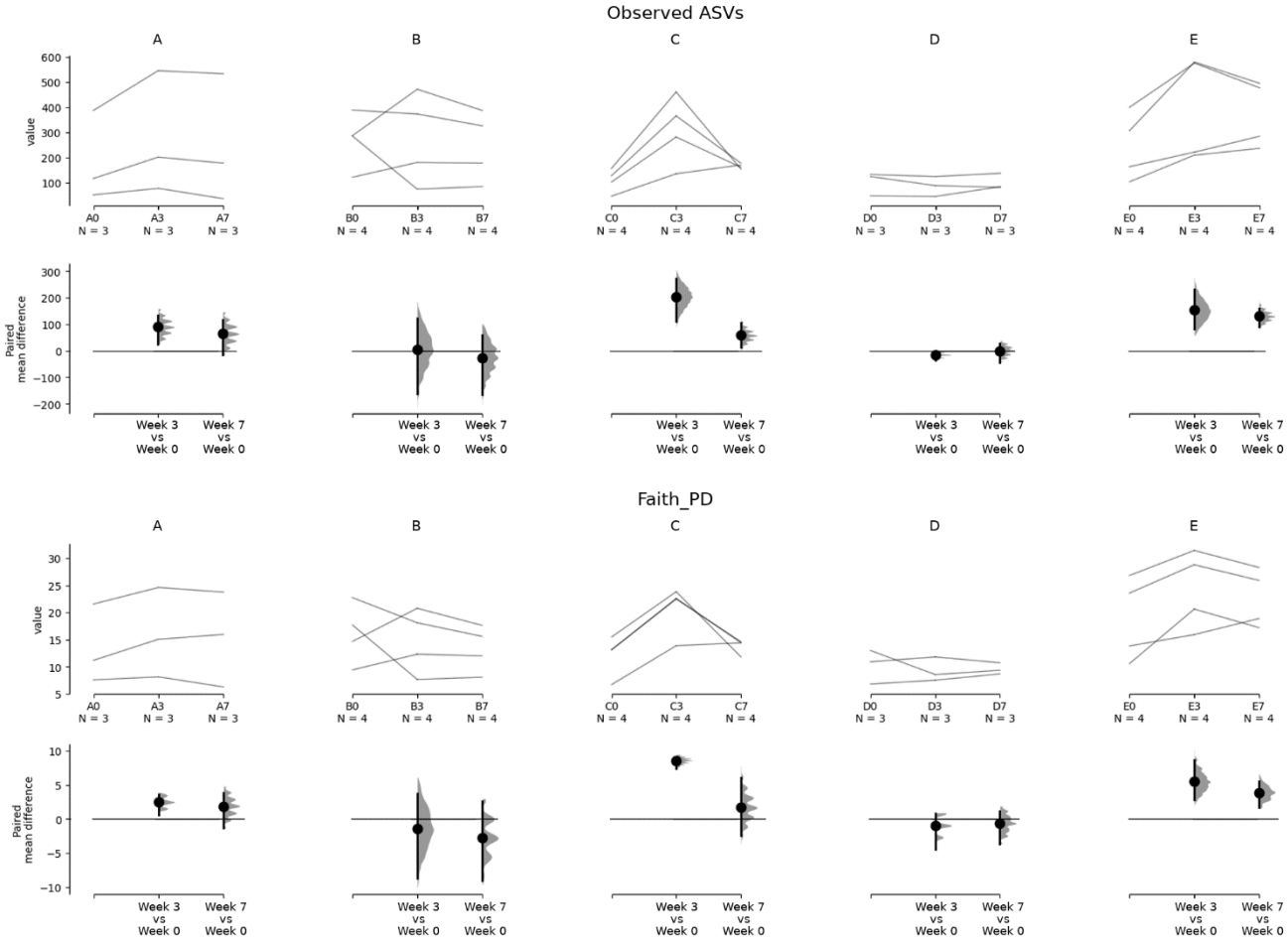

Observed ASVs
A
B
C
D
E
value
A0
N = 3
A3
N = 3
A7
N = 3
B0
N = 4
B3
N = 4
B7
N = 4
C0
N = 4
C3
N = 4
C7
N = 4
D0
N = 3
D3
N = 3
D7
N = 3
E0
N = 4
E3
N = 4
E7
N = 4
Paired
mean difference
Week 3
vs
Week 0
Week 7
vs
Week 0
Faith_PD

Figure 2

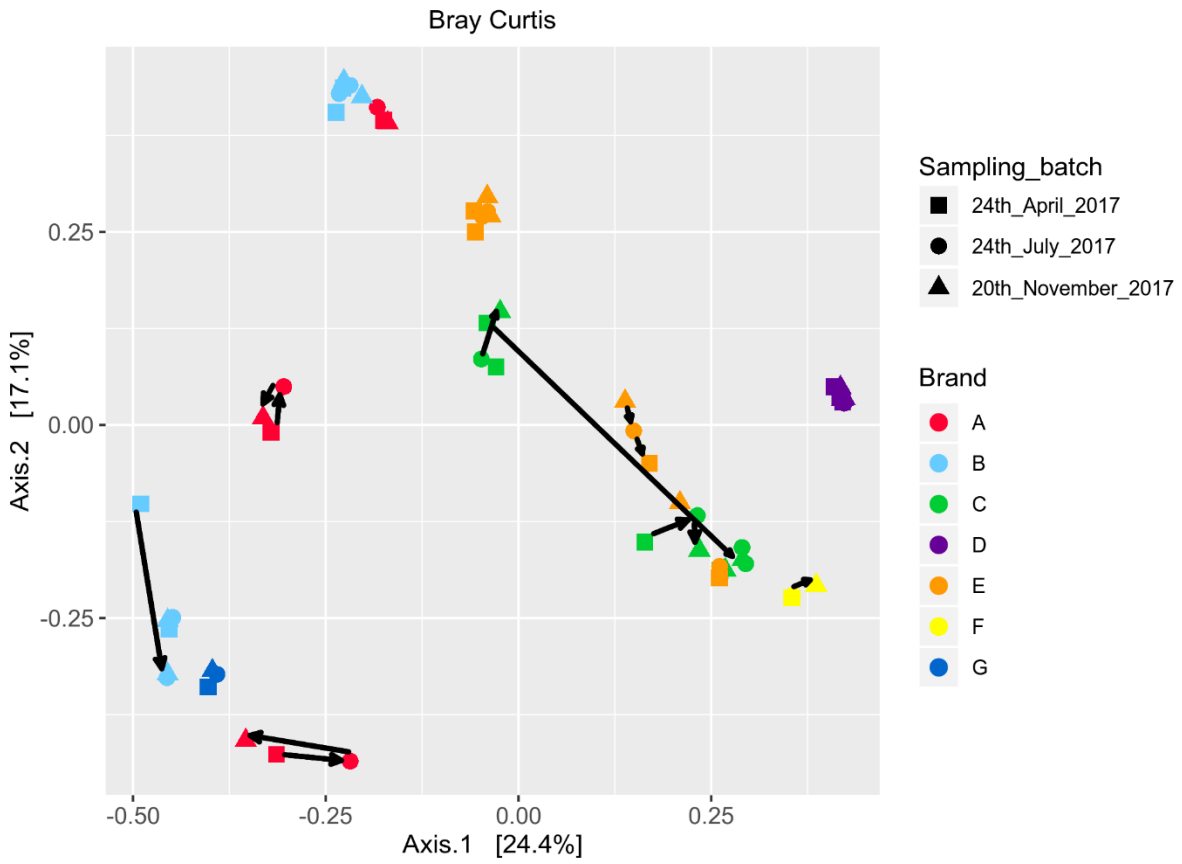

Bray Curtis
0.25
0.00
-0.25
Axis.2 [17.1%]
-0.50
-0.25
0.00
0.25
Axis.1 [24.4%]
Sampling_batch
24th_April_2017
24th_July_2017
20th_November_2017
Brand
A
B
C
D
E
F
G

Figure 3

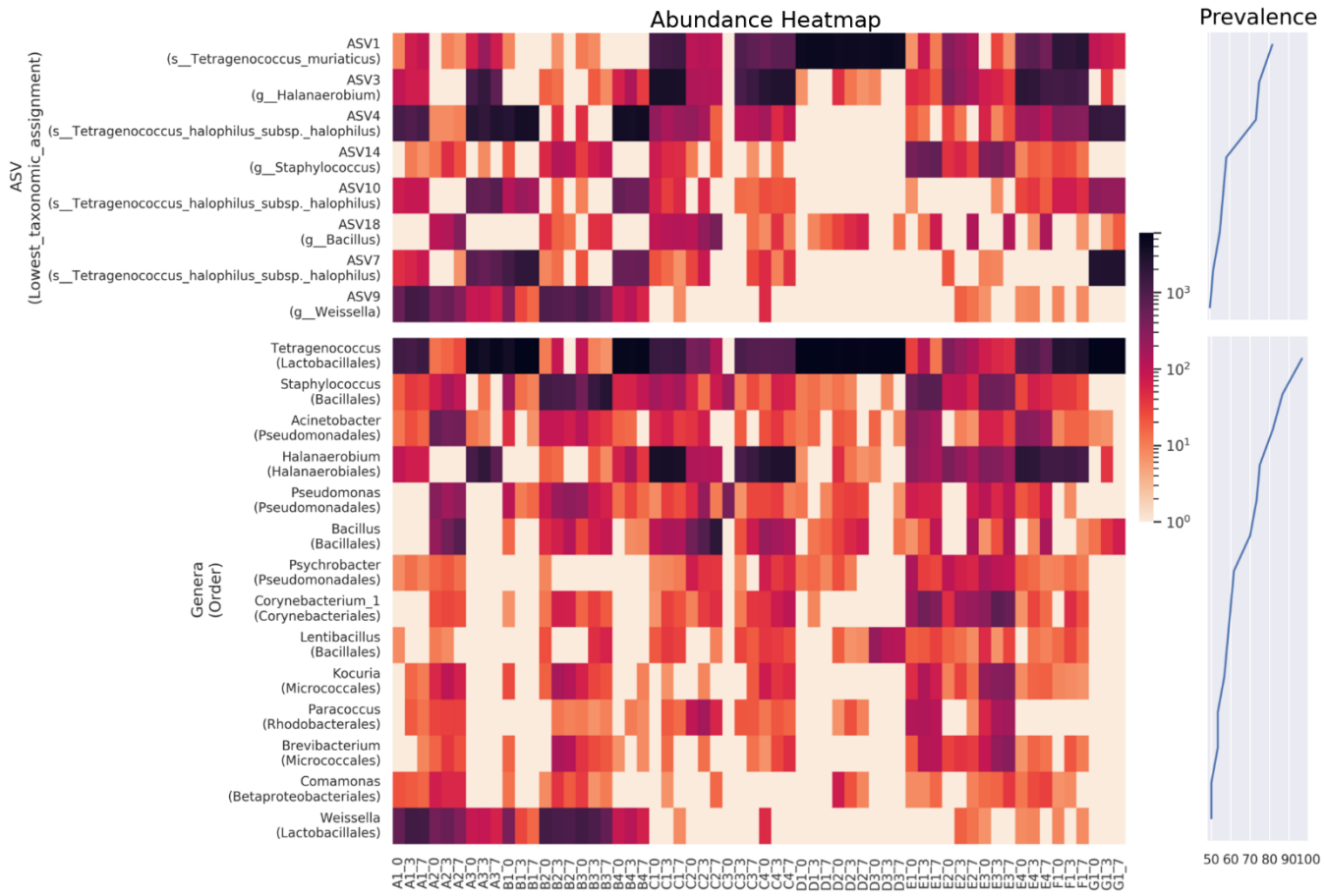

Abundance Heatmap
Prevalence
ASV
(Lowest_taxonomic_assignment)
ASV1
(s__Tetragenococcus_muriaticus)
ASV3
(g__Halanaerobium)
ASV4
(s__Tetragenococcus_halophilus_subsp._halophilus)
ASV14
(g__Staphylococcus)
ASV10
(s__Tetragenococcus_halophilus_subsp._halophilus)
ASV18
(g__Bacillus)
ASV7
(s__Tetragenococcus_halophilus_subsp._halophilus)
ASV9
(g__Weissella)
Genera
(Order)
Tetragenococcus
(Lactobacillales)
Staphylococcus
(Bacillales)
Acinetobacter
(Pseudomonadales)
Halanaerobium
(Halanaerobiales)
Pseudomonas
(Pseudomonadales)
Bacillus
(Bacillales)
Psychrobacter
(Pseudomonadales)
Corynebacterium_1
(Corynebacteriales)
Lentibacillus
(Bacillales)
Kocuria
(Micrococcales)
Paracoccus
(Rhodobacterales)
Brevibacterium
(Micrococcales)
Comamonas
(Betaproteobacteriales)
Weissella
(Lactobacillales)
10^3
10^2
10^1
10^0
50 60 70 80 90100

Figure 4

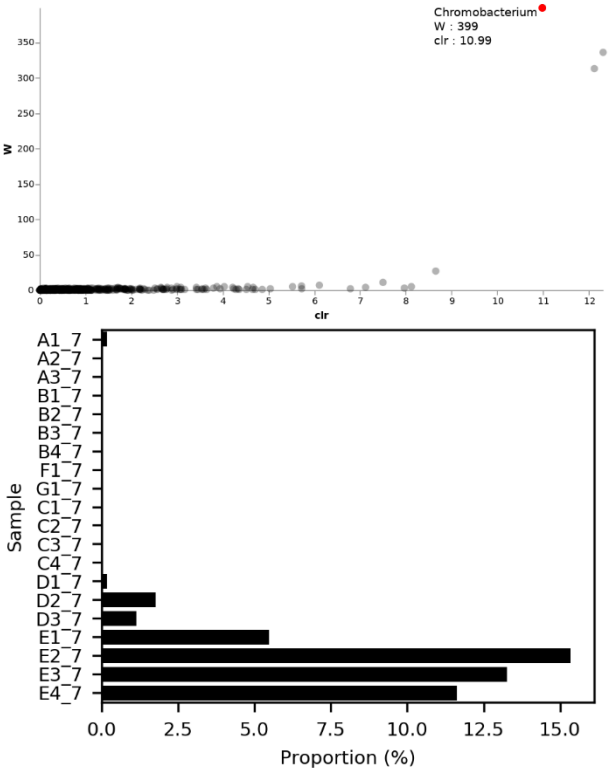

Chromobacterium
W : 399
clr : 10.99
W
clr
Sample
A1_7
A2_7
A3_7
B1_7
B2_7
B3_7
B4_7
F1_7
G1_7
C1_7
C2_7
C3_7
C4_7
D1_7
D2_7
D3_7
E1_7
E2_7
E3_7
E4_7
Proportion (%)

Figure 5

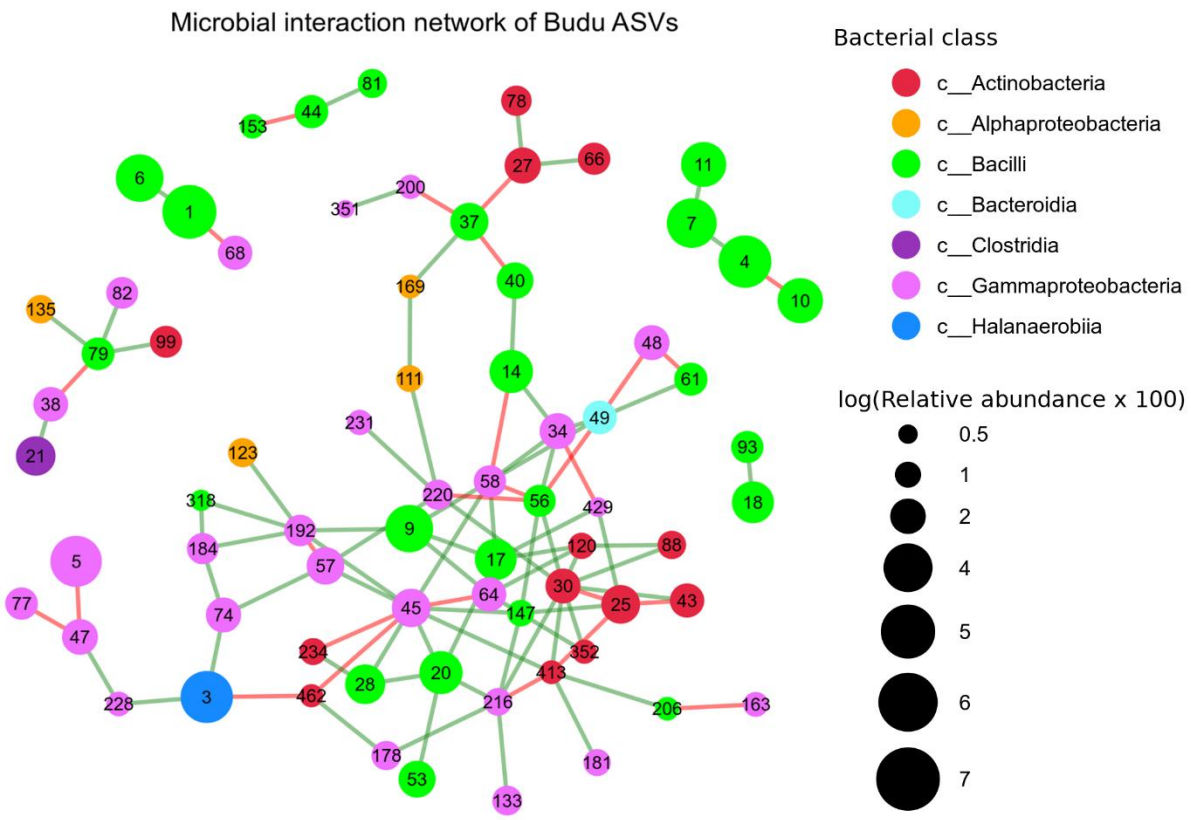
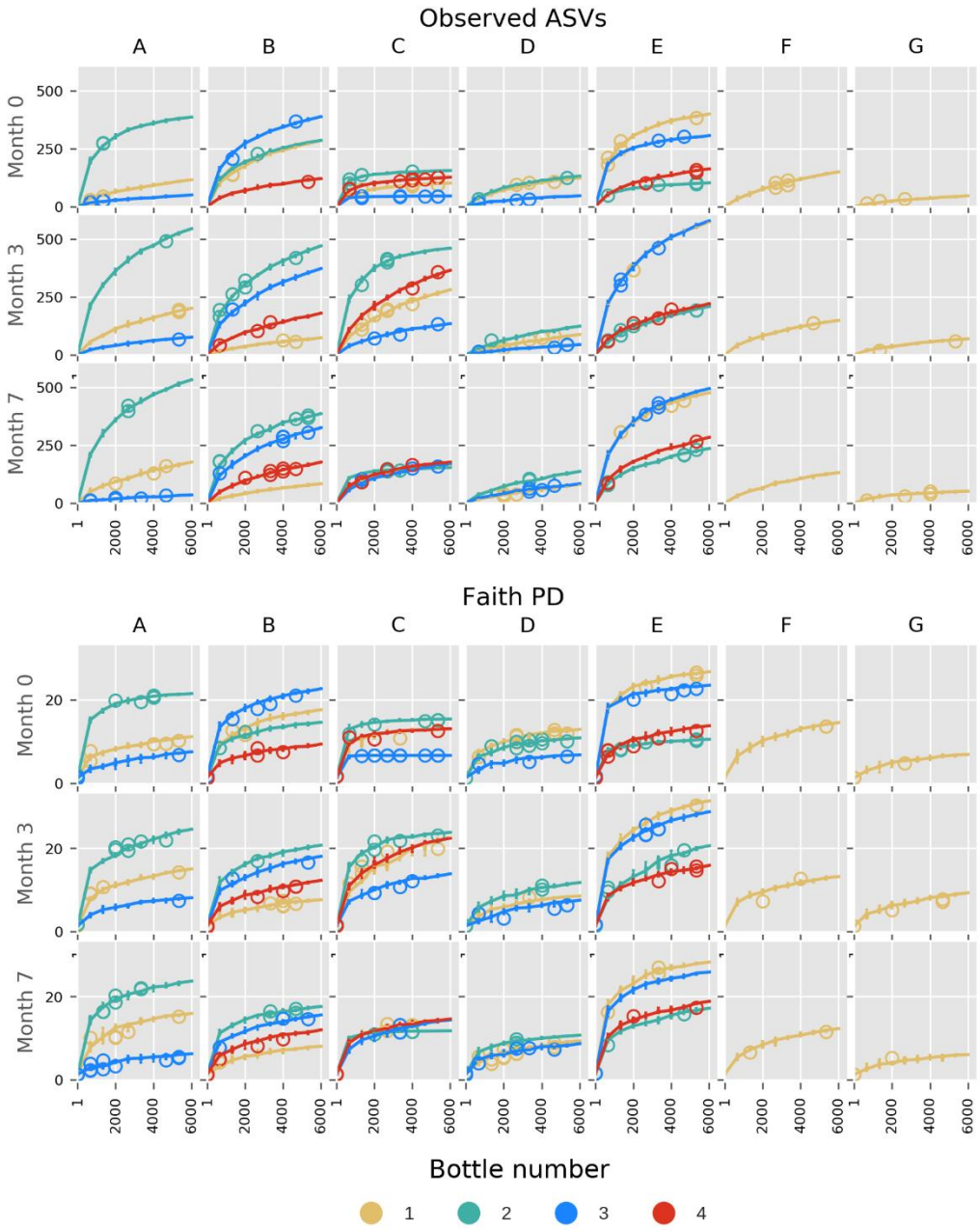
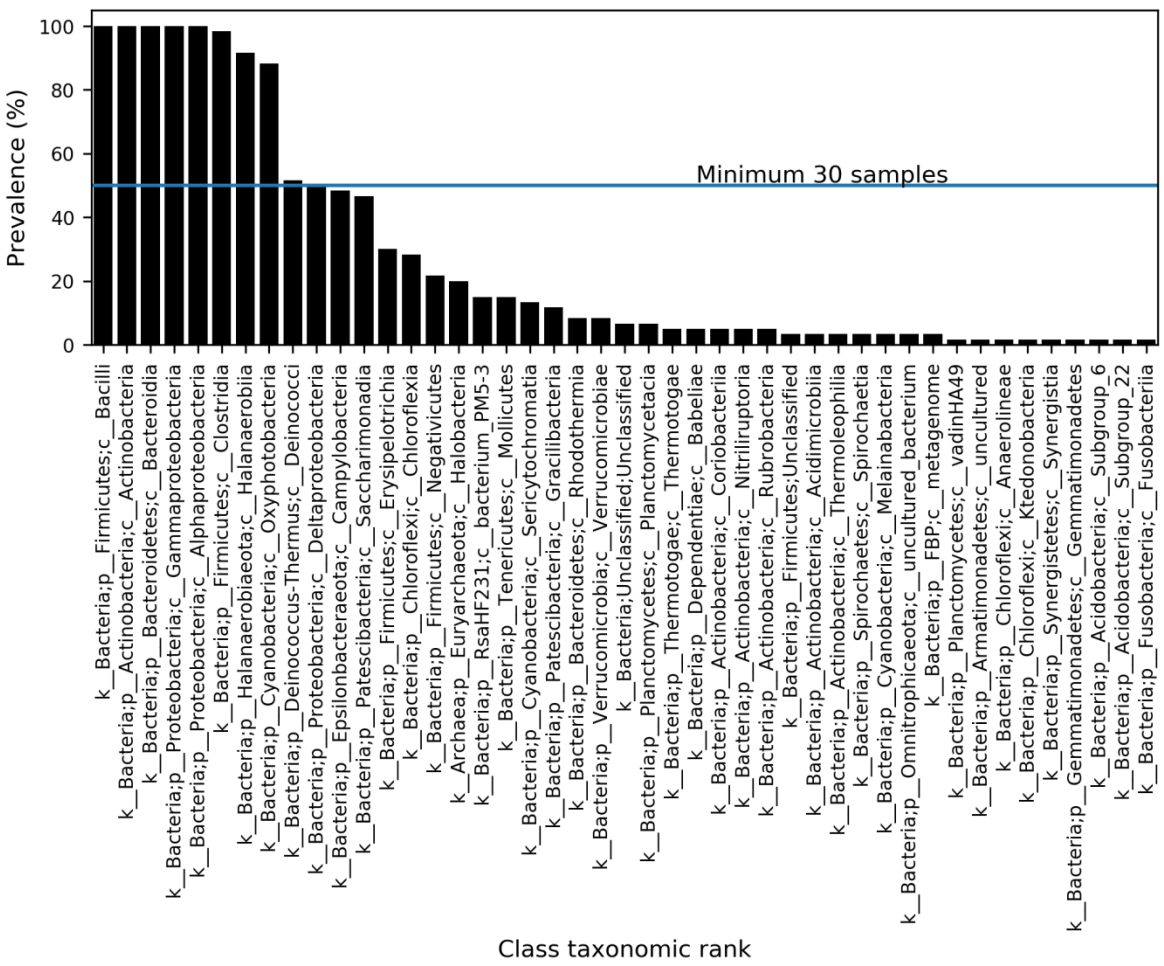

Microbial interaction network of Budu ASVs
Bacterial class
c__Actinobacteria
c__Alphaproteobacteria
c__Bacilli
c__Bacteroidia
c__Clostridia
c__Gammaproteobacteria
c__Halanaerobiia
log(Relative abundance x 100)
0.5
1
2
4
5
6
7

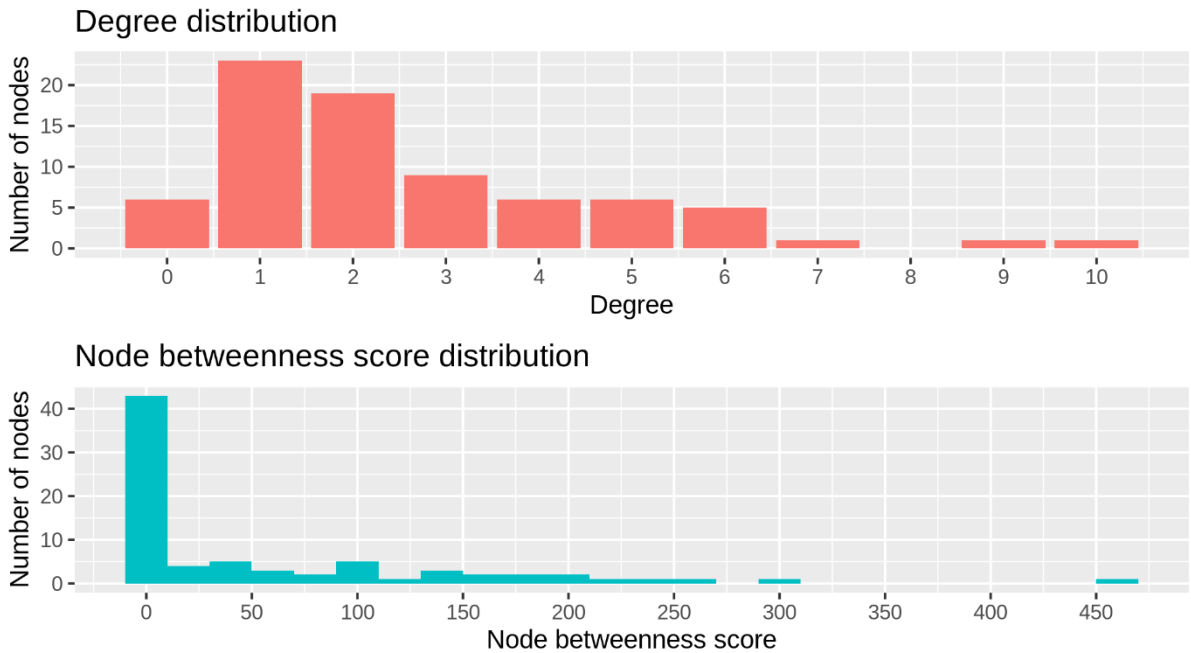
Figure 6



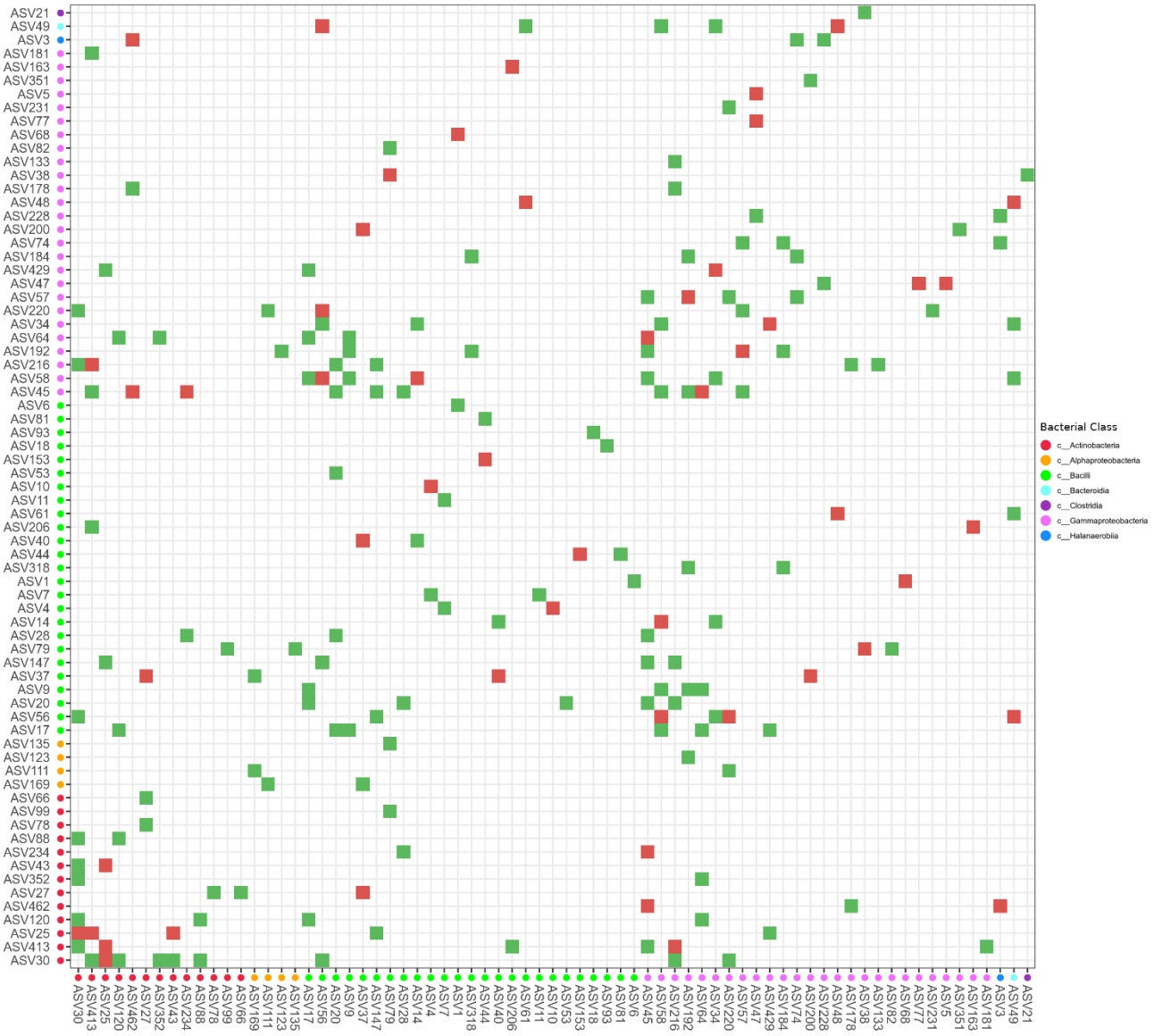
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4

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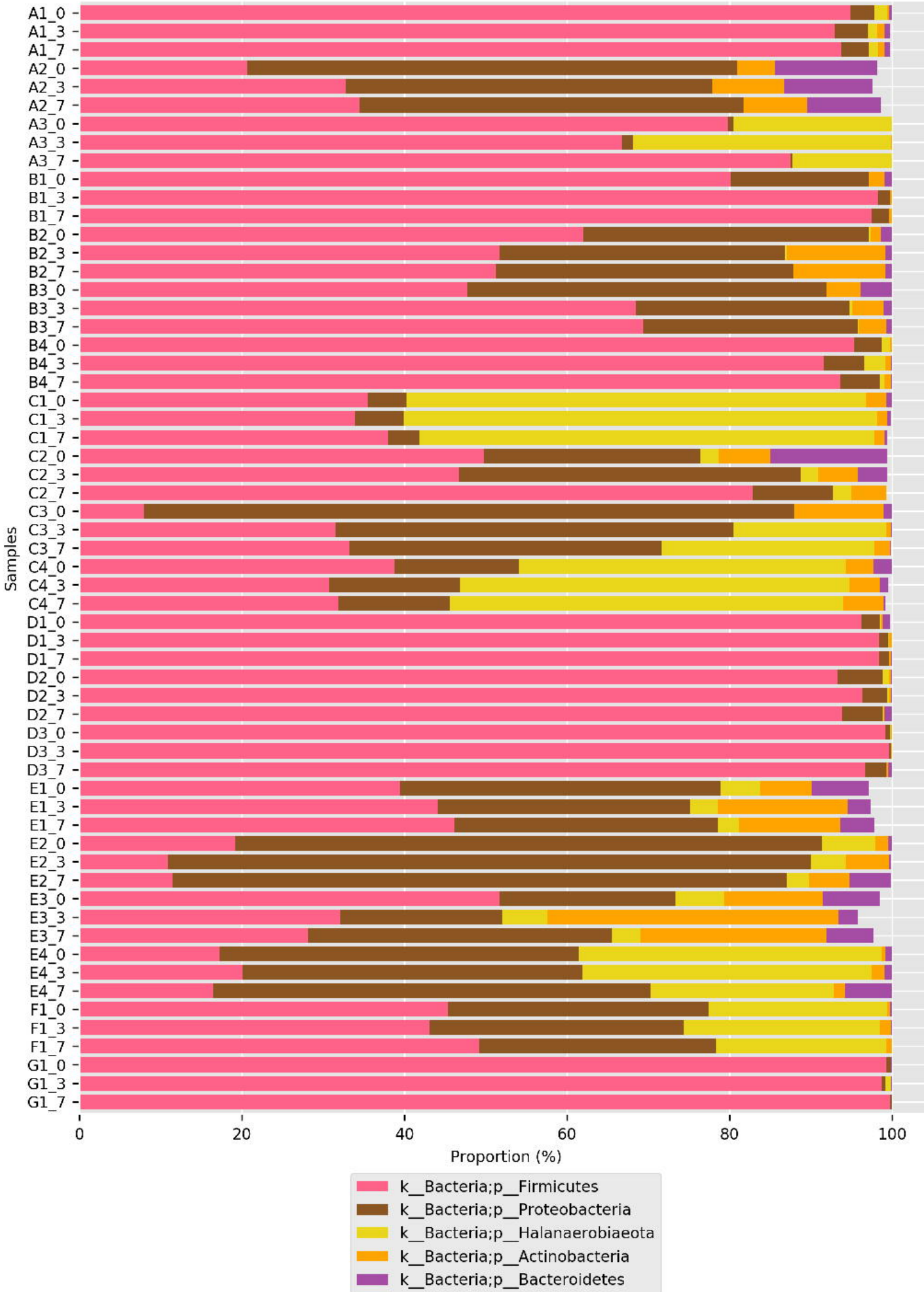
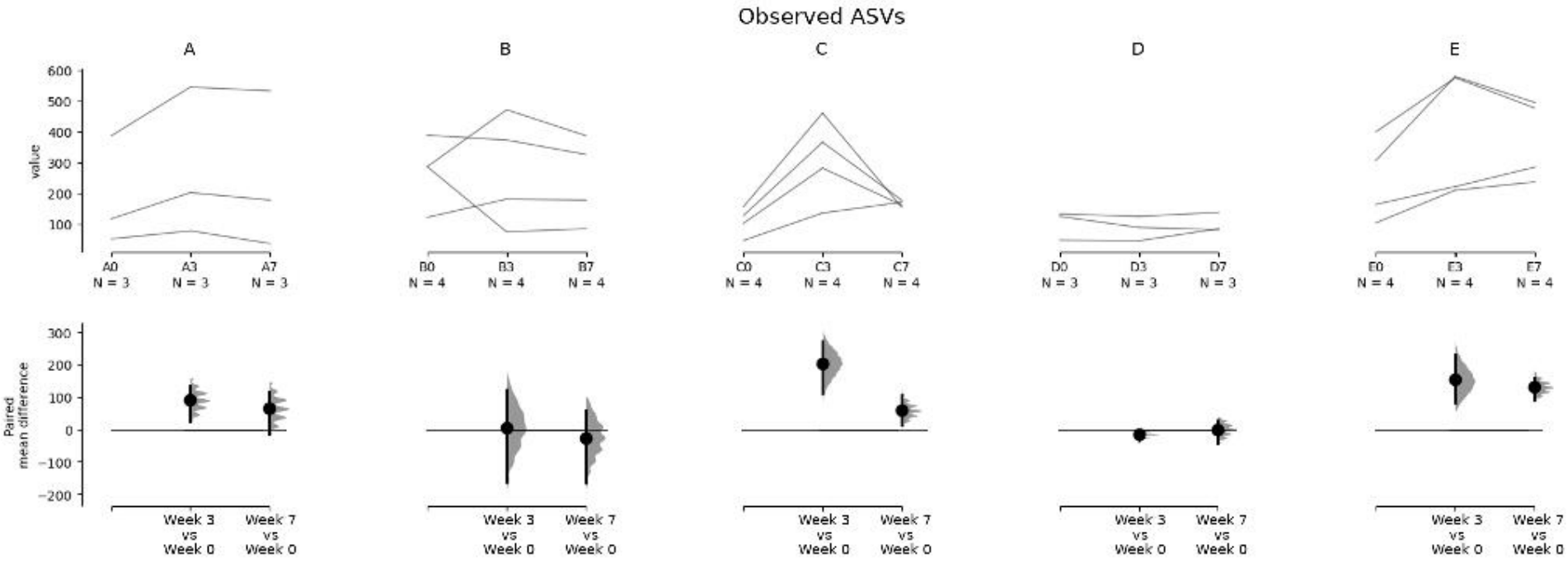
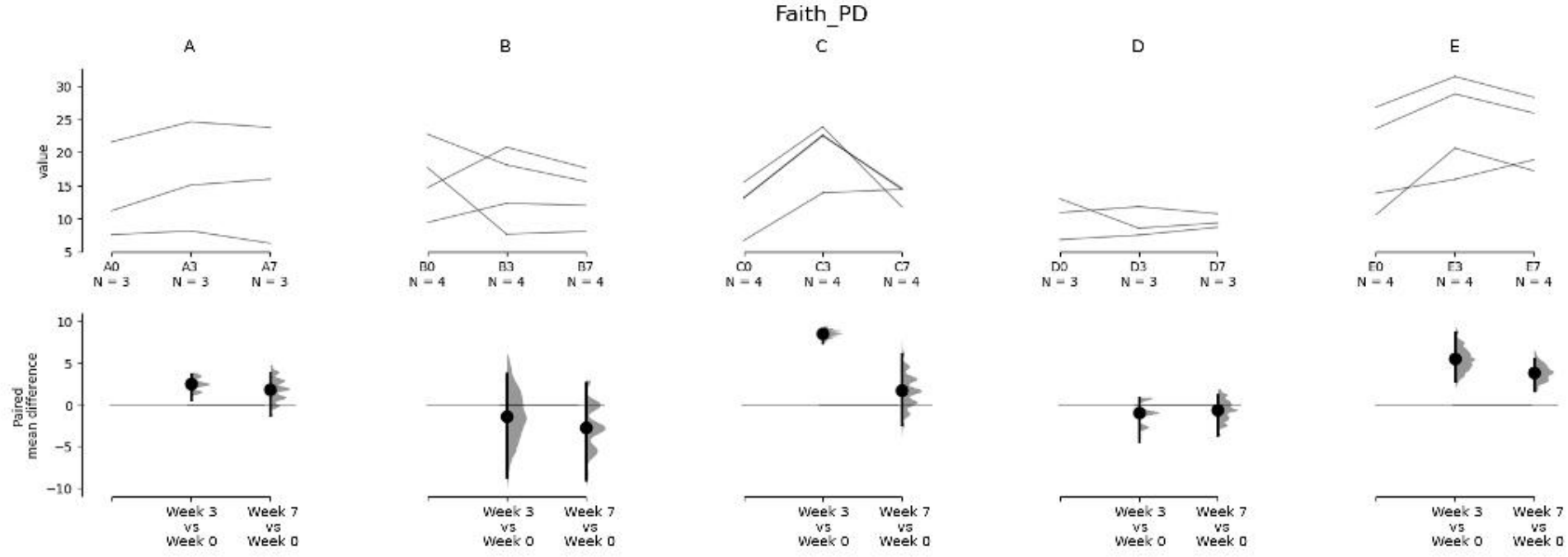
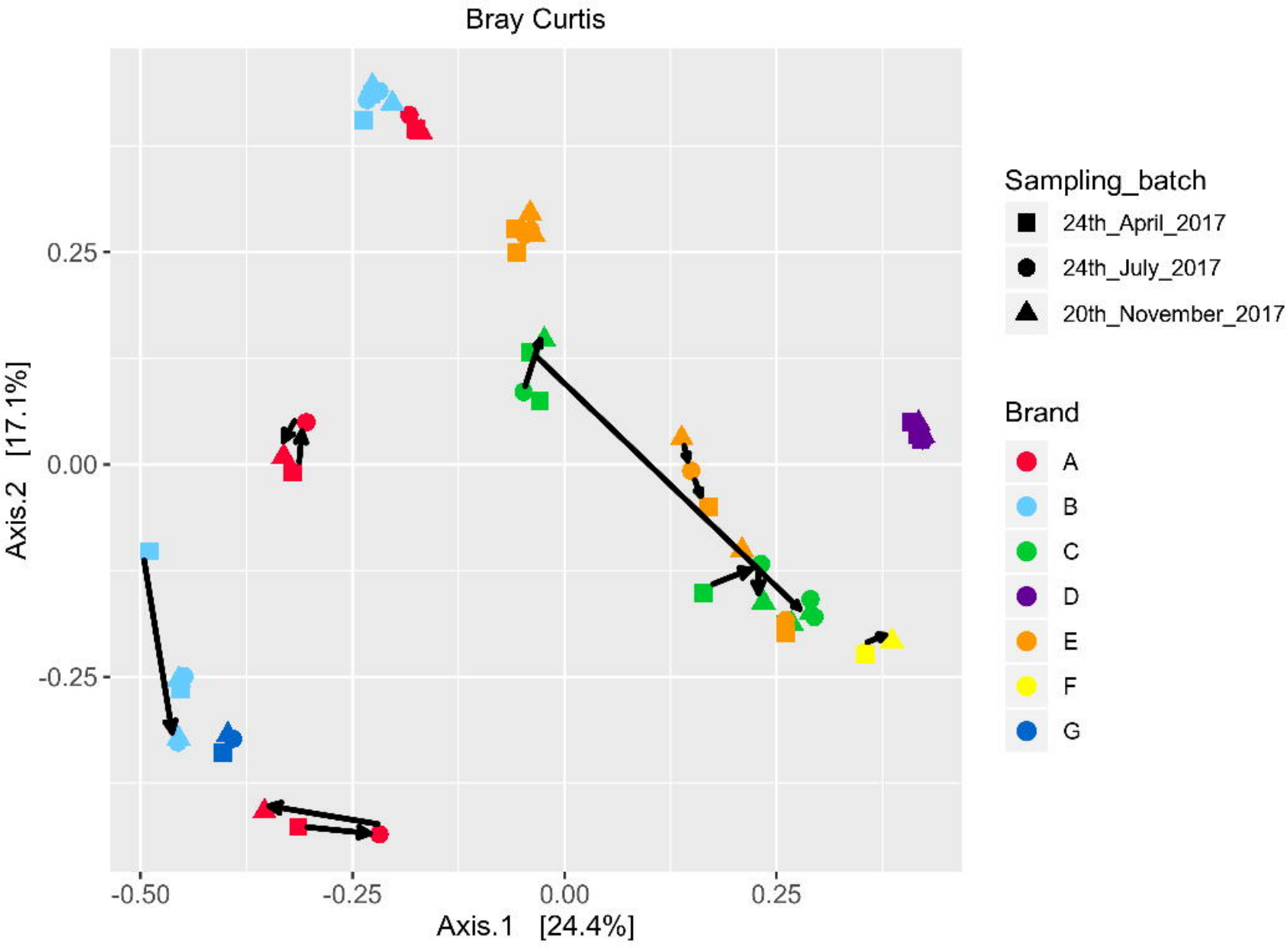
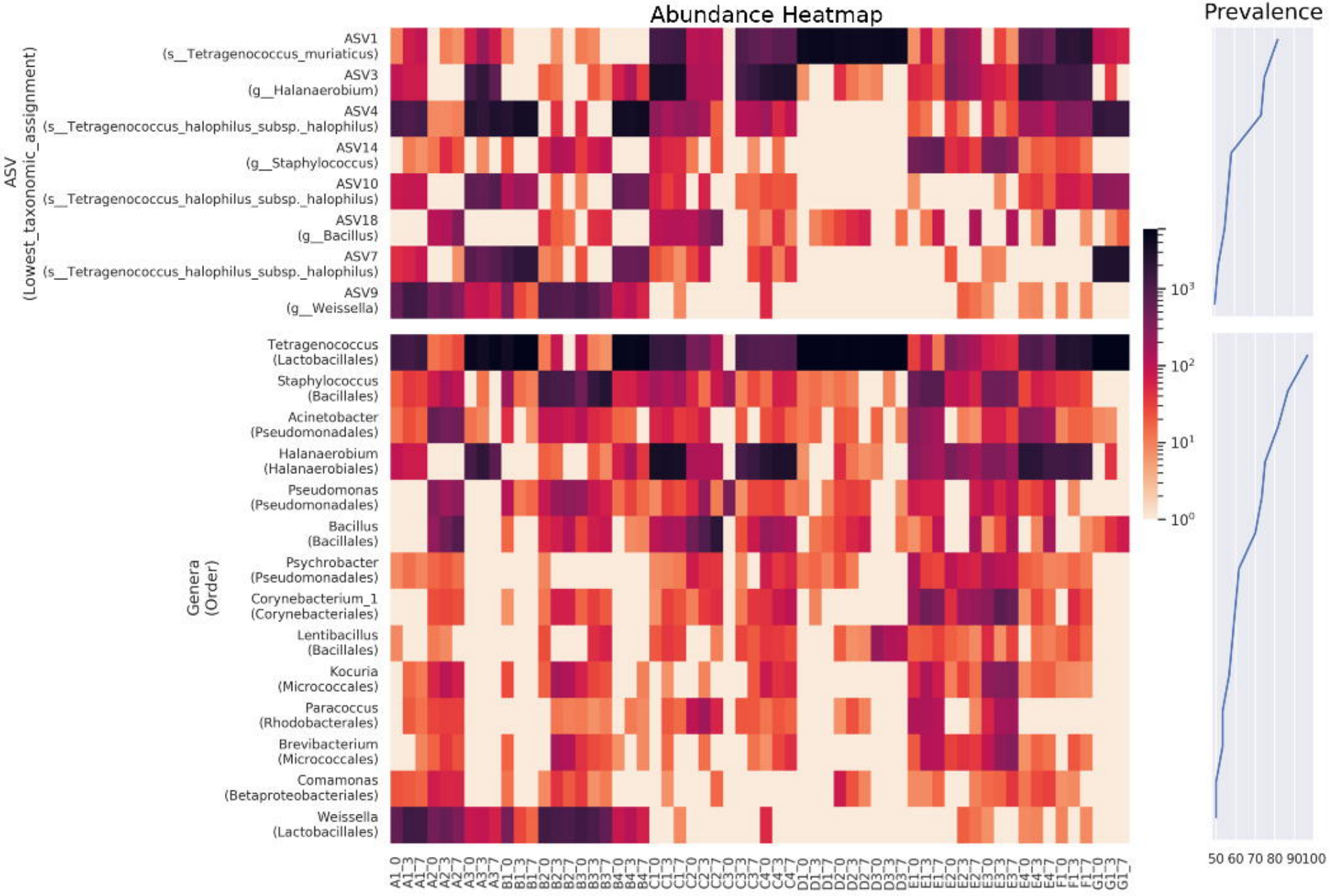
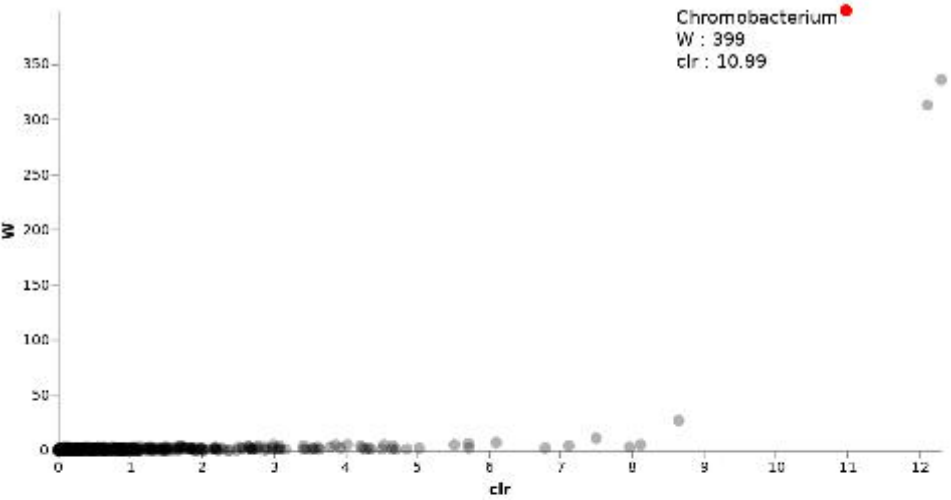

Samples
A1_0
A1_3
A1_7
A2_0
A2_3
A2_7
A3_0
A3_3
A3_7
B1_0
B1_3
B1_7
B2_0
B2_3
B2_7
B3_0
B3_3
B3_7
B4_0
B4_3
B4_7
C1_0
C1_3
C1_7
C2_0
C2_3
C2_7
C3_0
C3_3
C3_7
C4_0
C4_3
C4_7
D1_0
D1_3
D1_7
D2_0
D2_3
D2_7
D3_0
D3_3
D3_7
E1_0
E1_3
E1_7
E2_0
E2_3
E2_7
E3_0
E3_3
E3_7
E4_0
E4_3
E4_7
F1_0
F1_3
F1_7
G1_0
G1_3
G1_7
0
20
40
60
80
100
Proportion (%)
k__Bacteria;p__Firmicutes
k__Bacteria;p__Proteobacteria
k__Bacteria;p__Halanaerobiaeota
k__Bacteria;p__Actinobacteria
k__Bacteria;p__Bacteroidetes

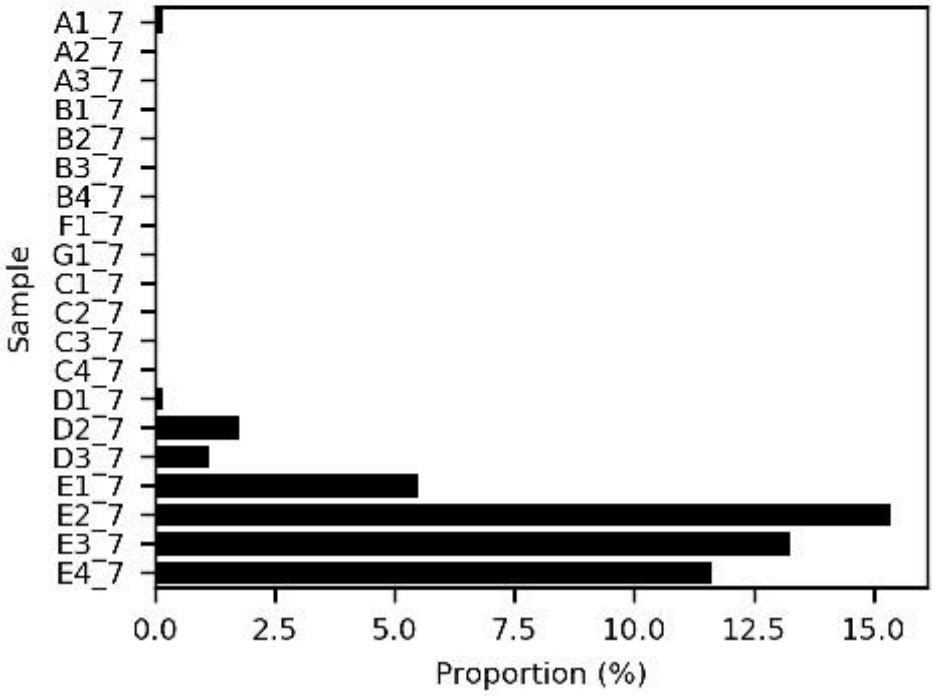

Observed ASVs
A
B
C
D
E
value
600
500
400
300
200
100
A0
N = 3
A3
N = 3
A7
N = 3
B0
N = 4
B3
N = 4
B7
N = 4
C0
N = 4
C3
N = 4
C7
N = 4
D0
N = 3
D3
N = 3
D7
N = 3
E0
N = 4
E3
N = 4
E7
N = 4
Paired
mean difference
300
200
100
0
−100
−200
Week 3
vs
Week 0
Week 7
vs
Week 0

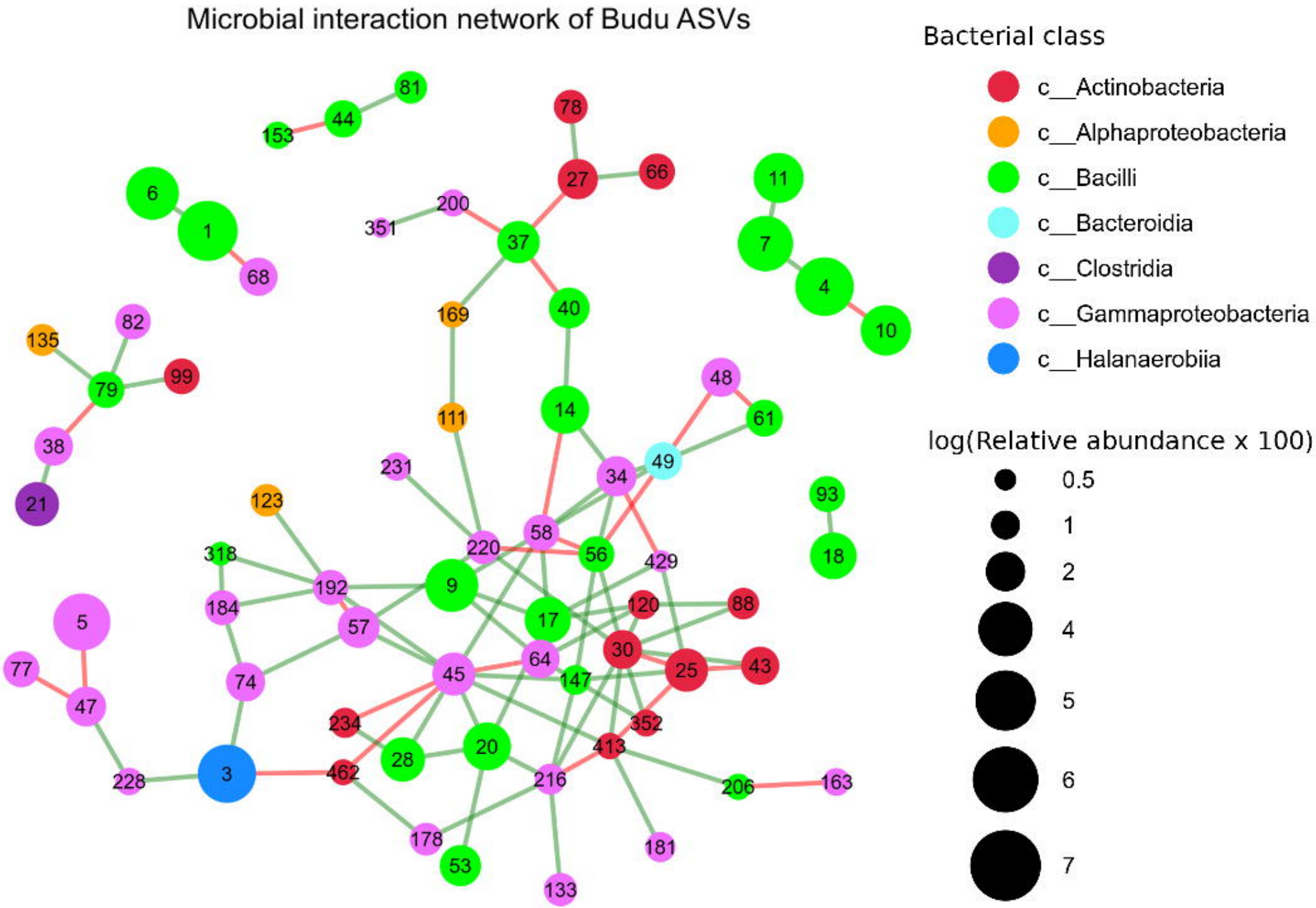

Faith_PD
A
B
C
D
E
value
30
25
20
15
10
5
A0
N = 3
A3
N = 3
A7
N = 3
B0
N = 4
B3
N = 4
B7
N = 4
C0
N = 4
C3
N = 4
C7
N = 4
D0
N = 3
D3
N = 3
D7
N = 3
E0
N = 4
E3
N = 4
E7
N = 4
Paired
mean difference
10
5
0
−5
−10
Week 3
vs
Week 0
Week 7
vs
Week 0

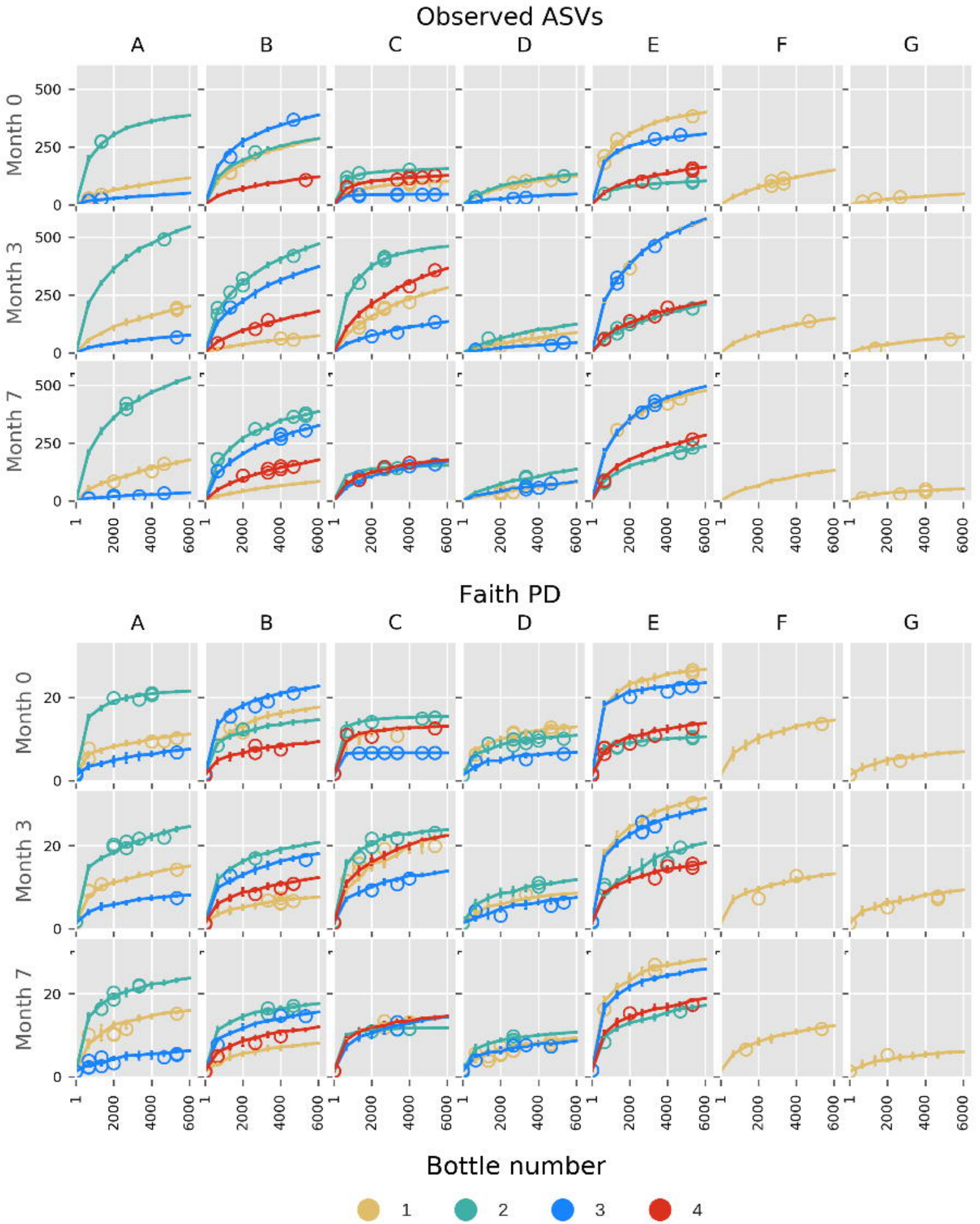

Bray Curtis
Axis.2 [17.1%]
0.25
0.00
-0.25
-0.50
-0.25
0.00
0.25
Axis.1 [24.4%]
Sampling_batch
24th_April_2017
24th_July_2017
20th_November_2017
Brand
A
B
C
D
E
F
G

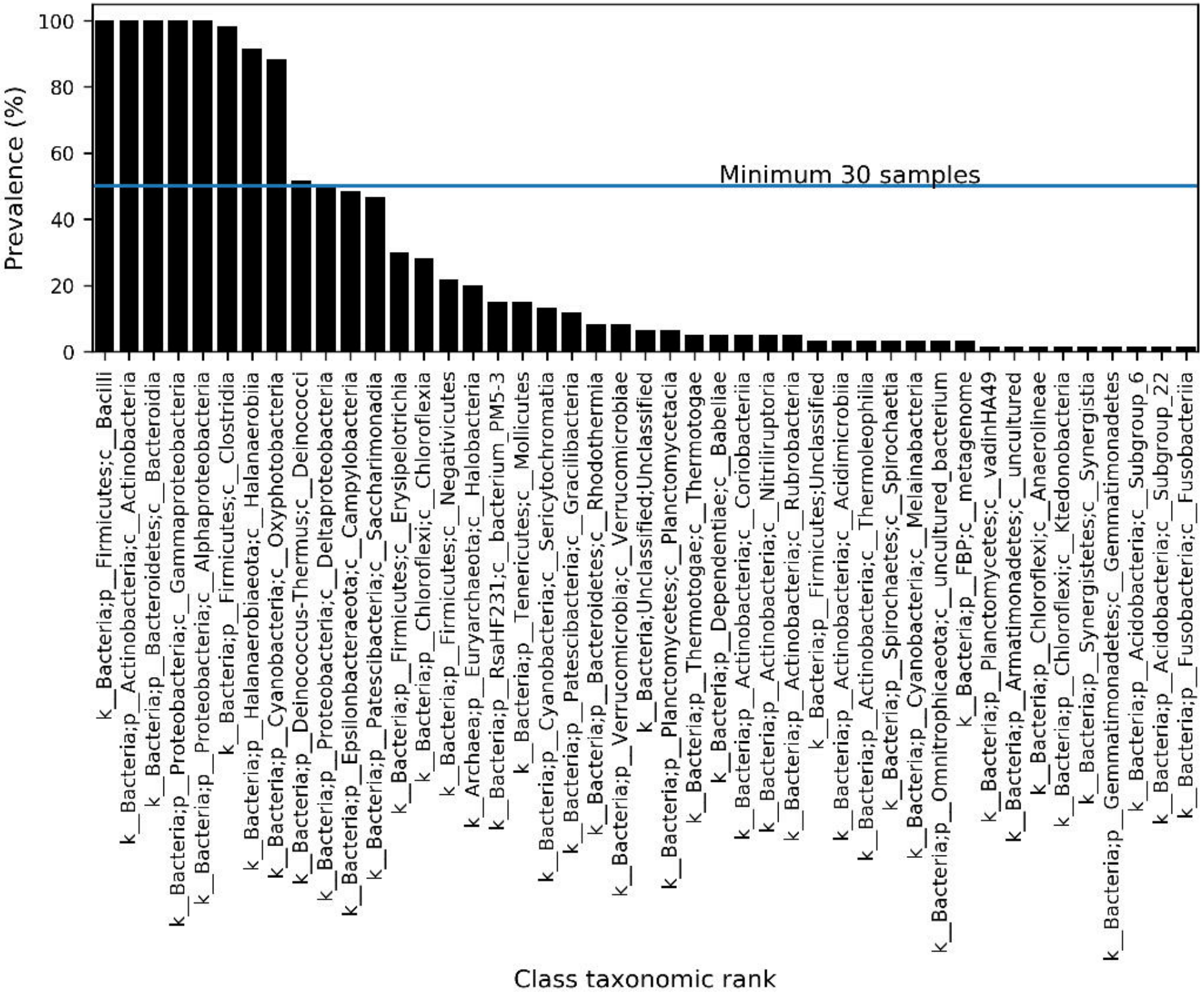

Abundance Heatmap
Prevalence
ASV
(Lowest_taxonomic_assignment)
ASV1
(s__Tetragenococcus_muriaticus)
ASV3
(g__Halanaerobium)
ASV4
(s__Tetragenococcus_halophilus_subsp._halophilus)
ASV14
(g__Staphylococcus)
ASV10
(s__Tetragenococcus_halophilus_subsp._halophilus)
ASV18
(g__Bacillus)
ASV7
(s__Tetragenococcus_halophilus_subsp._halophilus)
ASV9
(g__Weissella)
Genera
(Order)
Tetragenococcus
(Lactobacillales)
Staphylococcus
(Bacillales)
Acinetobacter
(Pseudomonadales)
Halanaerobium
(Halanaerobiales)
Pseudomonas
(Pseudomonadales)
Bacillus
(Bacillales)
Psychrobacter
(Pseudomonadales)
Corynebacterium_1
(Corynebacteriales)
Lentibacillus
(Bacillales)
Kocuria
(Micrococcales)
Paracoccus
(Rhodobacterales)
Brevibacterium
(Micrococcales)
Comamonas
(Betaproteobacteriales)
Weissella
(Lactobacillales)
10^3
10^2
10^1
10^0
50 60 70 80 90100
A1_0 A1_3 A1_7 A2_0 A2_3 A2_7 A3_0 A3_3 A3_7 B1_0 B1_3 B1_7 B2_0 B2_3 B2_7 B3_0 B3_3 B3_7 B4_0 B4_3 B4_7 C1_0 C1_3 C1_7 C2_0 C2_3 C2_7 C3_0 C3_3 C3_7 C4_0 C4_3 C4_7 D1_0 D1_3 D1_7 D2_0 D2_3 D2_7 D3_0 D3_3 D3_7 E1_0 E1_3 E1_7 E2_0 E2_3 E2_7 E3_0 E3_3 E3_7 E4_0 E4_3 E4_7 F1_0 F1_3 F1_7 G1_0 G1_3 G1_7

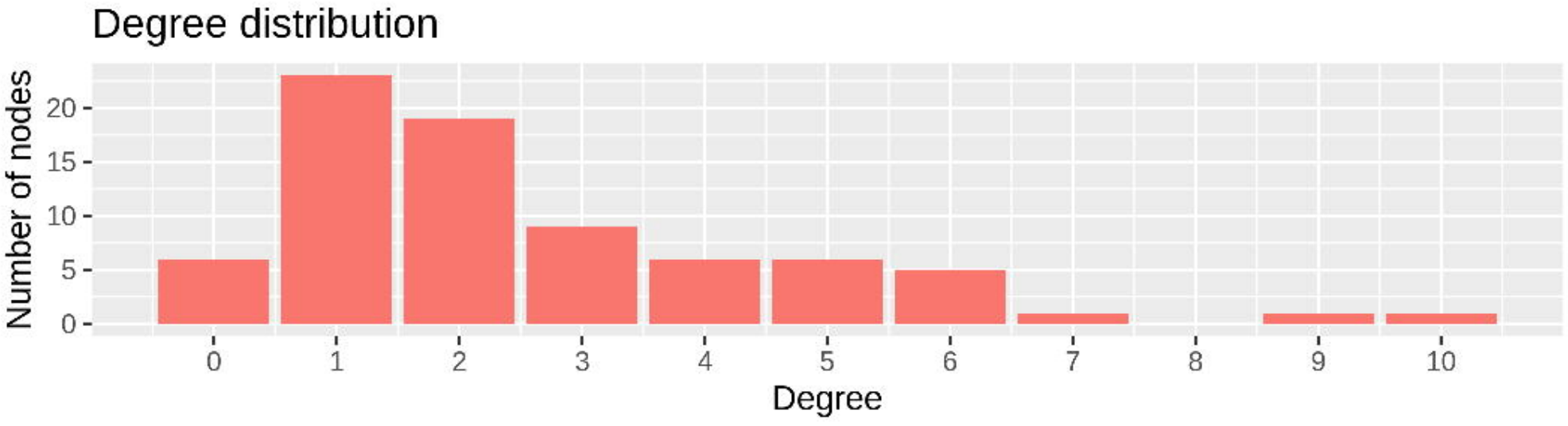

Chromobacterium
W : 399
clr : 10.99
W
clr

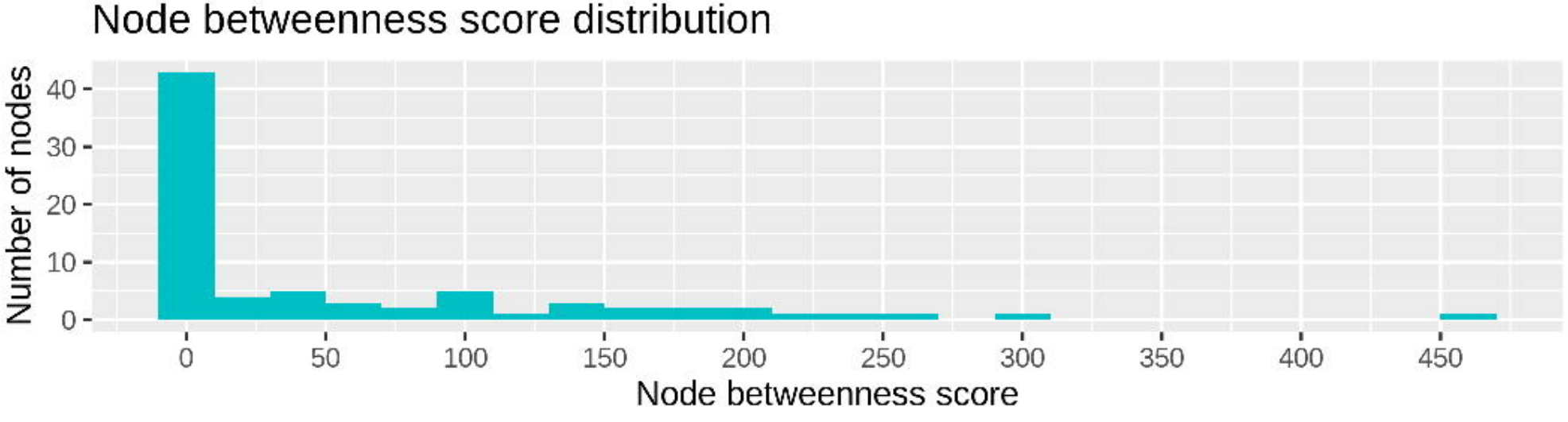

A1_7
A2_7
A3_7
B1_7
B2_7
B3_7
B4_7
F1_7
G1_7
C1_7
C2_7
C3_7
C4_7
D1_7
D2_7
D3_7
E1_7
E2_7
E3_7
E4_7
Sample
Proportion (%)

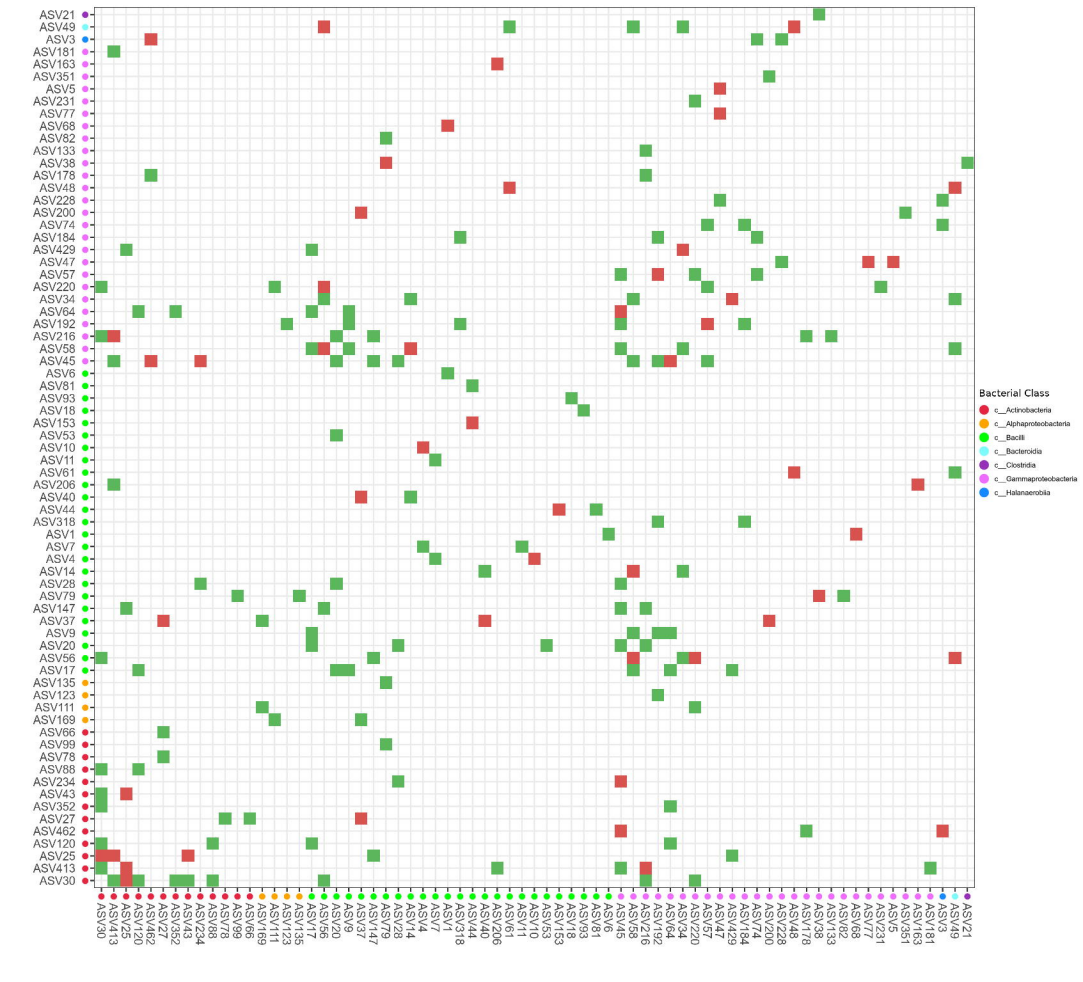

Microbial interaction network of Budu ASVs
Bacterial class
c__Actinobacteria
c__Alphaproteobacteria
c__Bacilli
c__Bacteroidia
c__Clostridia
c__Gammaproteobacteria
c__Halanaerobiia
log(Relative abundance x 100)
0.5
1
2
4
5
6
7
81
44
153
78
27
66
6
1
68
200
351
37
11
7
4
10
169
40
135
82
79
99
38
21
111
14
48
61
49
34
231
123
93
18
58
220
56
429
318
192
9
184
57
17
120
88
5
30
25
43
77
47
74
45
64
147
234
352
28
20
413
228
3
462
216
206
163
178
53
181
133


Observed ASVs
A
B
C
D
E
F
G
Month 0
Month 3
Month 7
500
250
0
1
2000
4000
6000
Faith PD
20
Bottle number
1
2
3
4


Prevalence (%)
100
80
60
40
20
0
Minimum 30 samples
k__Bacteria;p__Firmicutes;c__Bacilli
k__Bacteria;p__Actinobacteria;c__Actinobacteria
k__Bacteria;p__Bacteroidetes;c__Bacteroidia
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria
k__Bacteria;p__Firmicutes;c__Clostridia
k__Bacteria;p__Halanaerobiaeota;c__Halanaerobiia
k__Bacteria;p__Cyanobacteria;c__Oxyphotobacteria
k__Bacteria;p__Deinococcus-Thermus;c__Deinococci
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria
k__Bacteria;p__Epsilonbacteraeota;c__Campylobacteria
k__Bacteria;p__Patescibacteria;c__Saccharimonadia
k__Bacteria;p__Firmicutes;c__Erysipelotrichia
k__Bacteria;p__Chloroflexi;c__Chloroflexia
k__Bacteria;p__Firmicutes;c__Negativicutes
k__Archaea;p__Euryarchaeota;c__Halobacteria
k__Bacteria;p__RsaHF231;c__bacterium_PM5-3
k__Bacteria;p__Tenericutes;c__Mollicutes
k__Bacteria;p__Cyanobacteria;c__Sericytochromatia
k__Bacteria;p__Patescibacteria;c__Gracilibacteria
k__Bacteria;p__Bacteroidetes;c__Rhodothermia
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae
k__Bacteria;Unclassified;Unclassified
k__Bacteria;p__Planctomycetes;c__Planctomycetacia
k__Bacteria;p__Thermotogae;c__Thermotogae
k__Bacteria;p__Dependentiae;c__Babeliae
k__Bacteria;p__Actinobacteria;c__Coriobacteriia
k__Bacteria;p__Actinobacteria;c__Nitriliruptoria
k__Bacteria;p__Actinobacteria;c__Rubrobacteria
k__Bacteria;p__Firmicutes;Unclassified
k__Bacteria;p__Actinobacteria;c__Acidimicrobiia
k__Bacteria;p__Actinobacteria;c__Thermoleophilia
k__Bacteria;p__Spirochaetes;c__Spirochaetia
k__Bacteria;p__Cyanobacteria;c__Melainabacteria
k__Bacteria;p__Omnitrophicaeota;c__uncultured_bacterium
k__Bacteria;p__FBP;c__metagenome
k__Bacteria;p__Planctomycetes;c__vadinHA49
k__Bacteria;p__Armatimonadetes;c__uncultured
k__Bacteria;p__Chloroflexi;c__Anaerolineae
k__Bacteria;p__Chloroflexi;c__Ktedonobacteria
k__Bacteria;p__Synergistetes;c__Synergistia
k__Bacteria;p__Gemmatimonadetes;c__Gemmatimonadetes
k__Bacteria;p__Acidobacteria;c__Subgroup_6
k__Bacteria;p__Acidobacteria;c__Subgroup_22
k__Bacteria;p__Fusobacteria;c__Fusobacteriia
Class taxonomic rank


Degree distribution
Number of nodes
20
15
10
5
0
0
1
2
3
4
5
6
7
8
9
10
Degree


Node betweenness score distribution
Number of nodes
40
30
20
10
0
0
50
100
150
200
250
300
350
400
450
Node betweenness score


ASV21
ASV49
ASV3
ASV181
ASV163
ASV351
ASV5
ASV231
ASV77
ASV68
ASV82
ASV133
ASV38
ASV178
ASV48
ASV228
ASV200
ASV74
ASV184
ASV429
ASV47
ASV57
ASV220
ASV34
ASV64
ASV192
ASV216
ASV58
ASV45
ASV6
ASV81
ASV93
ASV18
ASV153
ASV53
ASV10
ASV11
ASV61
ASV206
ASV40
ASV44
ASV318
ASV1
ASV7
ASV4
ASV14
ASV28
ASV79
ASV147
ASV37
ASV9
ASV20
ASV56
ASV117
ASV135
ASV123
ASV111
ASV169
ASV66
ASV99
ASV78
ASV88
ASV234
ASV43
ASV352
ASV27
ASV462
ASV120
ASV25
ASV413
ASV30
ASV30
ASV413
ASV25
ASV120
ASV462
ASV27
ASV352
ASV43
ASV234
ASV88
ASV78
ASV99
ASV66
ASV169
ASV111
ASV123
ASV135
ASV117
ASV56
ASV20
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ASV79
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ASV7
ASV318
ASV44
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ASV206
ASV61
ASV11
ASV10
ASV53
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ASV18
ASV93
ASV81
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ASV58
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ASV74
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ASV48
ASV178
ASV148
ASV38
ASV133
ASV82
ASV68
ASV77
ASV231
ASV5
ASV351
ASV163
ASV181
ASV3
ASV49
ASV21
Bacterial Class
c__Actinobacteria
c__Alphaproteobacteria
c__Bacilli
c__Bacteroidia
c__Clostridia
c__Gammaproteobacteria
c__Halanaerobiia