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Research article

Transitions in bacterial communities across two fermentation-based virgin coconut oil (VCO) production processes

Zomesh A. Maini *, Crisanto M. Lopez

Department of Biology, School of Science & Engineering, Loyola Schools, Ateneo de Manila University, Philippines

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ABSTRACT

Despite being one of the most used methods of virgin coconut oil (VCO) production, there is no metagenomic study that details the bacterial community shifts during fermentation-based VCO production. The identification and quantification of bacteria associated with coconut milk fermentation is useful for detecting the dominant microbial genera actively involved in VCO production which remains largely undescribed. Describing the constitutive microbial genera involved in this traditional fermentation practice can be used as a preliminary basis for improving industrial practices and developing better fermentation procedures. In this study, we utilized 16S rRNA metagenomic sequencing to trace the transitions in microbial community profiles as coconut milk is fermented to release VCO in two VCO production lines. The results show that difference in the microbiome composition between the different processing steps examined in this work was mainly due to the abundance of the *Leuconostoc* genus in the raw materials and its decline and transition into the lactic acid bacteria groups *Weissella*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, and *Streptococcus* during the latter stages of fermentation. A total of 17 genera with relative abundances greater than 0.01% constitute the core microbiome of the two processing lines and account for 74%–97% of the microbial abundance in all coconut-derived samples. Significant correlations were shown through an analysis of the Spearman's rank between and within the microbial composition and pH at the genus level. The results of the present study show that the dynamics of VCO fermentation rely on the shifts in abundances of various members of the Lactobacillales order.

1. Introduction

Virgin Coconut Oil (VCO) is a coconut-derived edible oil popularized by references commending its various benefits for human health (Varma et al., 2019; Wallace, 2019; Chinwong et al., 2017; Nevin and Rajamohan, 2004). VCO is extracted from the milk of *Cocos nucifera* L. (family Arecaceae) meat or kernel without the use of any chemical treatment or modification such as refining, bleaching, or deodorizing which are utilized in the production of commercial coconut copra oil (RCO) (Srivastava et al., 2018). Despite having similar fatty acid profiles when compared with RCO, VCO retains a higher content of preserved bioactive compounds that are lost during the refining process for RCO (da Silva Lima and Block, 2019). Because of these nutritional qualities, VCO has steadily occupied a growing niche in the nutraceutical industry, being valued as a healthy functional food and oil (Ng et al., 2021; Selvaraj et al., 2020; Suryani et al., 2020; Marina et al., 2009).

Apart from its dietary, cosmetic, and industrial benefits, VCO has been documented to have antibacterial, antifungal, and antiviral properties (Nasir et al., 2018). The current COVID19 crisis has even spurred research on VCO as a potential adjuvant therapy for SARS-CoV 2, with promising results that include a lowering of C-reactive protein levels among suspect and probable cases of COVID-19 (Angeles-Agdeppa et al., 2021). As such, VCO remains an important commodity whose production is of vital significance for nations in the tropics.

Among the different methods of producing VCO, "natural" or bacterial fermentation is a favored method for preservation of its innate characteristics and quality (Mesias and Tan, 2014). The microbial fermentation of coconut milk produces VCO with desirable characteristics such as low rancidity, a longer shelf life, and a distinct coconut aroma (Masyithah, 2017). This process has also gained popularity with VCO producers since it requires the least amount of financial investment, energy input, and labor among all the VCO production procedures which increase its suitability for utilization by microenterprises run by households and small communities (Manohar et al., 2007).

The fermentation of coconut milk uses the acid-producing metabolism of its endogenous microorganisms to disrupt the water-oil

* Corresponding author.
E-mail address: zmaini@ateneo.edu (Z.A. Maini).

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microstructure of the coconut milk emulsion, leading to the liberation and eventual separation of VCO from water, carbohydrates, and proteins (Handayani et al., 2009). While microorganisms such as lactic acid bacteria (LABs) have routinely been used as inocula to induce fermentation and increase VCO yields (Narayanankutty et al., 2018), there has been no high-throughput study that describes and enumerates the microbial communities responsible for the fermentative processes for VCO. Tracking the changes in the bacterial composition present during VCO fermentation is important to identify which genera contribute to VCO liberation.

Describing the constitutive microbial genera involved in VCO fermentation can aid in the selection of microorganisms that can be used for improving its industrial and commercial production through assisted fermentation using culture additives. It can also be a basis for the optimization of fermentation parameters based on the identity of the participating bacterial assemblages. Additionally, microbial contaminants present in the different stages of production which can potentially change VCO quality can also be identified. Thus, the aim of this study was to track the microbial community profile changes at the genus level in two VCO production lines that utilize the natural fermentation method.

2. Materials and methods

2.1. Facility process description

Two facilities that utilized the natural fermentation method to produce commercially sold VCO were visited. As per agreement with the owners of each production facility, standard Non-Disclosure Agreements were signed by both parties to protect the anonymity and location of the VCO production companies from which the samples were collected.

The first processing plant (FP1) received de-husked nuts which were inspected, sorted, then manually split open directly without prior washing. Upon splitting, nuts that passed the visual inspection of the splitters had their meat manually grated. Grated coconut meat was collected and manually fed into a stainless-steel milk presser. Collected milk was filtered through porous cloth and emptied into high-density polyethylene (HDPE) covered drums where it was mixed with water at a 1:1 ratio and allowed to ferment for 17h–24 h at 28–30 °C. After the fermentation, raw VCO was found as the second layer in the fermentation drum, with the first layer being fermented solids and a third layer of fermentation water and skim milk. The bottom of the drum contained curd, gum, and other fermentation solids similar in nature and constitution to the first layer previously described. The raw VCO in the second layer was collected with a hand-held dipper and transferred into HDPE holding drums where it was allowed to settle for 12h. After settling, the raw VCO was filtered through a 1-micron filter bag (ZO-Clean, ZPO-01-P3E, China) and allowed to settle again for 12h. This settled oil was centrifuged to remove the remaining moisture and to increase clarity. The centrifuged VCO was allowed to settle again for 48 h and was then vacuum dried and packed using a pneumatic filling machine into Polyethylene terephthalate (PET) bottles for storage or sale.

The second facility (FP2) received nuts with the husk intact. These were manually de-husked, inspected, sorted, and cracked open. The split nuts were re-inspected by the graters who grated the meat manually. The grated meat was collected and was pressed for milk using a stainless-steel milk presser. Extracted milk was passed through a sterilized cheesecloth into HDPE pails. This was then mixed with heated water (85–90 °C) at a 1:1 ratio until a temperature of 35–40 °C was reached. The mixture was allowed to ferment for 18 h at 35–40 °C inside the HDPE-covered pails. After the fermentation, raw VCO was found as a second layer in between a top layer of fermented curd and solids and a bottom layer of fermentation water, skim milk, and some curd. The raw VCO was collected using a steel ladle and was allowed to settle for another 2 h. The settled oil was centrifuged, mixed with activated carbon, and passed through a pressure filter. The pressure filtered oil was allowed to settle for 12 h, after which it was passed through a vacuum evaporator to remove remaining moisture and clarify the oil. The VCO was left to rest in dehumidified conditions for 12h, and then was manually packed into PET bottles for storage or sale.

2.2. Sample collection and transport

Sampling points were identified for each VCO fermentation process line after observation of the production flow. Ten sampling points were identified for FP1, and eleven sampling points were identified for FP2. Detailed information on the sampling points, production phases, and sample descriptors can be found in Table 1 in the results section. Samples were collected aseptically from each of these sampling points using sterile stainless-steel dippers and forceps and stored in sterile wired sampling bags. The samples were stored and transported at 4 ± 2 °C, monitored using a digital temperature logger, and were kept at -4 ± 2 °C until these were processed.

2.3. Physicochemical measurements

Temperature was collected on-site as the samples were being taken from the production process lines using a digital thermometer. The pH of each sample was determined after these were transported to the laboratory prior to DNA extraction using an Apera PH700 pH meter (Apera Instruments LLC, Columbus, OH, USA). For non-liquid samples, portions of the mixed solid material were aseptically ground using a stainless-steel blender and filtered through sterilized gauze. The resulting filtered liquid was used for pH measurements in these cases. Three measurements of both pH and temperature were taken for each sample.

2.4. DNA extraction, 16S library preparation, and sequencing

Oil and water samples were filtered directly through a 0.22 μm polyethersulfone (PES) membrane (Membrane Solutions, Chiba-ken, Japan) to collect bacteria present (Nnadozie et al., 2015). Mixed solid samples were diluted at a 1:1 w/v with sterile peptone water (PW, 10 g peptone, 5 g NaCL per L, pH 7.2 ± 0.2). This mixture was then shaken at 200 RPM for 5 min to dislodge bacteria from solid surfaces and facilitate efficient filtration while minimizing the extraction of plant DNA. The diluted solid samples were then filtered in a similar fashion as the oil and water samples. DNA was isolated from each PES membrane using the Qiagen DNeasy PowerWater Kit (Hilden, Germany) following manufacturer instructions except for the elution step (Acharya et al., 2020). Instead of elution with 50–100 μL of the Elution Buffer, the isolated DNA was instead eluted with 30 μL of sterile Tris-EDTA (TE) buffer.

The presence of DNA in each sample was verified using agarose gel electrophoresis of DNA and standard PCR targeting the full 16S rRNA gene as previously described by Frank et al. (2008). The concentration of extracted DNA was measured using a SPECTROstar Nano UV/Vis Spectrophotometer (BMG Labtech, Saitama, Japan).

Three DNA samples per sampling point of at least 0.1 μg/mL concentrations were sent to Macrogen (Seoul, South Korea) for library preparation and sequencing following the Illumina 16S metagenomic library preparation protocol (Illumina, 2013). The quantity of the DNA sent was verified using Picogreen (Invitrogen, Waltham, MA, USA) via Victor 3 Fluorometry (Perkin-Elmer, Akron, OH, USA). The 16S rRNA variable regions V3 and V4 were amplified using the Herculase II fusion DNA polymerase Nextera XT Index Kit (Illumina, San Diego, CA, USA) using the primer pair (F), 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; and (R), 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′ (Klindworth et al., 2013). The size of the libraries produced were validated using an Agilent DNA 1000 chip in a 2100 Bioanalyzer (Agilent Technologies, Sta. Clara, CA, USA). The libraries were pooled together in equimolar ratios with a final concentration of 10 pmol/L each. These pooled samples were pair-end sequenced using the Illumina-MiSeq platform following their standard protocols (Illumina, 2013).

Table 1. FP1 and FP2 sampling points and their physicochemical properties.

Production Phase	Time elapsed (h) at collection	Sample Code	Sample Description	Sample Type	Average Temperature (°C ± 0.1) at collection	Average pH (±0.01)	16S Amplicon
Fermentation Process 1 (FP1)							
Collection of process water	N/A	A-PW	Process water	Liquid, non-oil	25.8	7.0	+
Splitting and grating of nuts	0	A-CM	Grated coconut meat	Mixed solid	28.7	6.0	+
Pressing of coconut meat	0.63	A-MLK	Pressed coconut milk	Liquid, non-oil	30.0	6.0	+
Fermentation	23.6	A-L1	Fermented solids	Mixed solid	31.5	4.6	+
Fermentation	23.6	A-L2	Raw VCO	Liquid, oil	30.2	4.8	+
Fermentation	23.6	A-L3	Fermentation water	Liquid, non-oil	31.5	4.5	+
First settling of raw VCO	36	A-PFIL	Pre-filtered VCO	Liquid, oil	28.8	5.6	+
Filtration and second settling of VCO	48.2	A-PCEN	Pre-centrifuged VCO	Liquid, oil	27.8	5.7	+
Centrifugation of double settled VCO	48.6	A-CEN	Centrifuged VCO	Liquid, oil	35.8	6.0	–
Third VCO settling, vacuum drying, and packing	97.7	A-FIN	Final VCO product	Liquid, oil	30.1	6.0	–
Fermentation Process 2 (FP2)							
Collection of process water	N/A	B-PW	Process water	Liquid, non-oil	31.5	7.0	+
Dehusking, splitting, and grating of nuts	0	B-CM	Grated coconut meat	Mixed solid	28.4	5.8	+
Pressing of coconut meat	0.78	B-MLK	Pressed coconut milk	Liquid, non-oil	29.7	6.0	+
Mixing with heated water	3.28	B-MLKW	Pressed coconut milk + water	Liquid, non-oil	37	6.1	+
Fermentation	21.8	B-L1	Fermented solids	Mixed solid	32.6	4.6	+
Fermentation	21.8	B-L2	Raw VCO	Liquid, oil	32.5	4.9	+
Fermentation	21.8	B-L3	Fermentation water	Liquid, non-oil	32.6	4.7	+
First settling and centrifugation of raw VCO	24.3	B-CEN	Centrifuged VCO	Liquid, oil	30.2	5.7	+
Filtration of settled VCO	29.8	B-FIL	Filtered VCO	Liquid, oil	29.7	6.0	–
Vacuum evaporation of filtered VCO after second settling	42.3	B-VAC	Vacuumed VCO	Liquid, oil	31.0	6.0	–
Third VCO settling and packing	54.9	B-FIN	Final VCO product	Liquid, oil	30.2	6.1	–

2.5. Microbiome sequencing analysis

All steps of the MiSeq read processing and microbiome diversity analyses were performed using various plugins of the Qualitative Insights into Microbial Ecology 2 (QIIME2) bioinformatics pipeline at standard settings unless mentioned below (Bolyen et al., 2019). The raw sequence reads for all biosamples were deposited into the NCBI Sequence Read Archive (SRA) database under BioProject PRJNA774430.

Paired-end raw reads were demultiplexed using the q2-demux plugin and then joined, denoised, and dereplicated using the DADA2 plugin (Callahan et al., 2016). The DADA2 plugin was also used to remove chimera sequences. Clustering and annotation of filtered sequences into the respective operational taxonomic units (OTUs) at 97% sequence similarity was performed using a classify-sklearn naïve Bayes taxonomy classifier (Pedregosa et al., 2011) via the q2-feature-classifier plugin (Bokulich et al., 2018) trained against 16S OTU full-length sequences found in the SILVA database (Quast et al., 2012). The representative sequences were aligned using MAFFT (Katoh and Standley, 2013) and were used to build a phylogenetic diversity tree using FastTree2 (Price et al., 2010). Contaminating chloroplast and mitochondrial sequences were filtered out of the resulting feature table. The feature table and phylogenetic tree were utilized for the alpha-and beta-diversity analyses.

The alpha- and beta-diversity analyses were performed using the q2-diversity plugin after 20 rarefactions and were computed at the sampling depth with the lowest feature count (22,310 reads) to retain all samples in the analysis. Apart from the observed OTUs, the alpha-diversity measures used were the Shannon Diversity Index (Shannon, 1948), Simpson's Diversity Index (Simpson, 1949), Chao1 Abundance Estimator (Chao, 1984), and Faith's Phylogenetic Diversity Index (Faith, 1992). The beta-diversity measures used were the Jaccard Similarity Index (Jaccard, 1912), Bray-Curtis Dissimilarity Index (Bray and Curtis, 1957), and both weighted and unweighted UniFrac Indices (Lozupone et al., 2007). Beta-diversity principal coordinate analysis (PCoA) plots were initially visualized via the EMPeror plugin (Vázquez-Baeza et al., 2013). All graphs were plotted on GraphPad Prism v6.01 (GraphPad, La Jolla, CA, USA). Heatmaps were generated using HeatMapper (Babicki et al., 2016). Correlation analysis and the correlograms representing the Spearman's rank correlation coefficient (r) matrices were generated using PAleontological STatistics Software (PAST) v4.03 (Hammer et al., 2001).

2.6. Statistical analysis

All statistical tests were performed using GraphPad Prism v6.01 and PAST v4.03. All data were tested for normality using the Shapiro-Wilk test prior to analysis. One-way and two-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparisons test was used for normally distributed data. For non-normally distributed data, non-parametric Kruskal–Wallis pairwise tests were performed. Differences inferred from the statistical tests were considered significant when the P value was less than 0.05.

3. Results

3.1. Sample phyisco-chemical qualities

The sampling points selected for both fermentation processes can be seen in Table 1. DNA was successfully isolated from all samples of FP1 except A-CEN and A-FIN, and from all samples of FP2 except B-FIL, B-VAC, and B-FIN. Standard 16S rRNA DNA PCR did not result in an amplification product for these samples and were thus not included in the subsequent metagenomic assessments.

3.2. Alpha- and beta-diversity analyses

Variations in the richness and diversity of the microbiome in the different processing steps for VCO for FP1 and FP2 were seen via an

analysis of the total number of OTUs (Figure 1A and B) and various alpha-diversity metrics (Figure 1C) computed for the global dataset.

Alpha rarefaction curves of the number of OTUs at 97% identity similarity (Figure 1A) demonstrated a good coverage of sequencing depth levelling at approximately 15,000 reads, showing differences in microbial composition between the various stages of FP1 and FP2. Fermented products (L1, L2, L3, A-PFIL, A-PCEN, B-CEN) generally possess more OTUs than unfermented raw materials (PW, CM, MLK, B-MLKW) except for B-CM and B-MLKW. The alpha rarefaction plot of the observed OTUs suggests that further sequencing will not lead to more OTUs being discovered.

The observed OTUs (Figure 1B) and alpha-diversity metrics (Figure 1C) showed differences in alpha-diversity between different samples from within each fermentation process, with raw materials generally having less alpha-diversity compared to fermented products. This is to be expected since raw materials had less environmental exposure and time for microbial growth compared to fermented samples.

A comparison of the sample microbial diversities between similar sampling points shows that there is a significant difference ($P < 0.05$) for PW, CM, and MLK between FP1 and FP2 for the observed OTUs and all alpha-diversity indices (Figure 1C). Raw VCO sample (L2) alpha-diversity between FP1 and FP2 are statistically significantly different at $P < 0.05$ for the Shannon and Simpson's indices, but not for Chao1 and Faith PD. Fermentation water (L3) sample alpha-diversities are significantly different ($P < 0.05$) between FP1 and FP2 for all alpha-diversity measures apart from the Simpson's index. These results suggest that similar samples from different process facilities have different microbial community compositions despite originating from the same substrate type.

The beta-diversity assessment utilizing different indices showed variations in composition of microbial communities between sampling points through differences in their ordination (Figure 2A-D). The Jaccard Similarity (Figure 2A), Bray-Curtis dis-similarity (Figure 2B), and Unweighted UniFrac (Figure 2C) clustered all fermented products together for FP1 and FP2 separately based on processing line of origin. Weighted UniFrac (Figure 2D) did not cluster samples according to process of origin but instead clustered all FP1 and FP2 samples into 3 groups based on shared pH. Samples clustered according to shared pH for the Bray-Curtis dissimilarity as well, with groups generally formed between samples with pH $\leq$ 5.8, and samples that have a pH $>$ 5.8, suggesting a pH-influenced assemblage of microbial communities.

3.3. Microbiome structure of FP1 and FP2

There were noticeable changes observed in terms of the relative abundances (RA%) of the different bacterial taxa across the different steps of both FP1 and FP2 at various taxonomic levels (Figure A1). Examination of the genus-level microbial distribution provided resolution to these differences in the major microbial groups present in each sample (Figure 3A and B). The heatmap of hierarchical clustering of all identified microbial taxa at the genus level revealed substantial differences in microbial composition for each sample within and between FP1

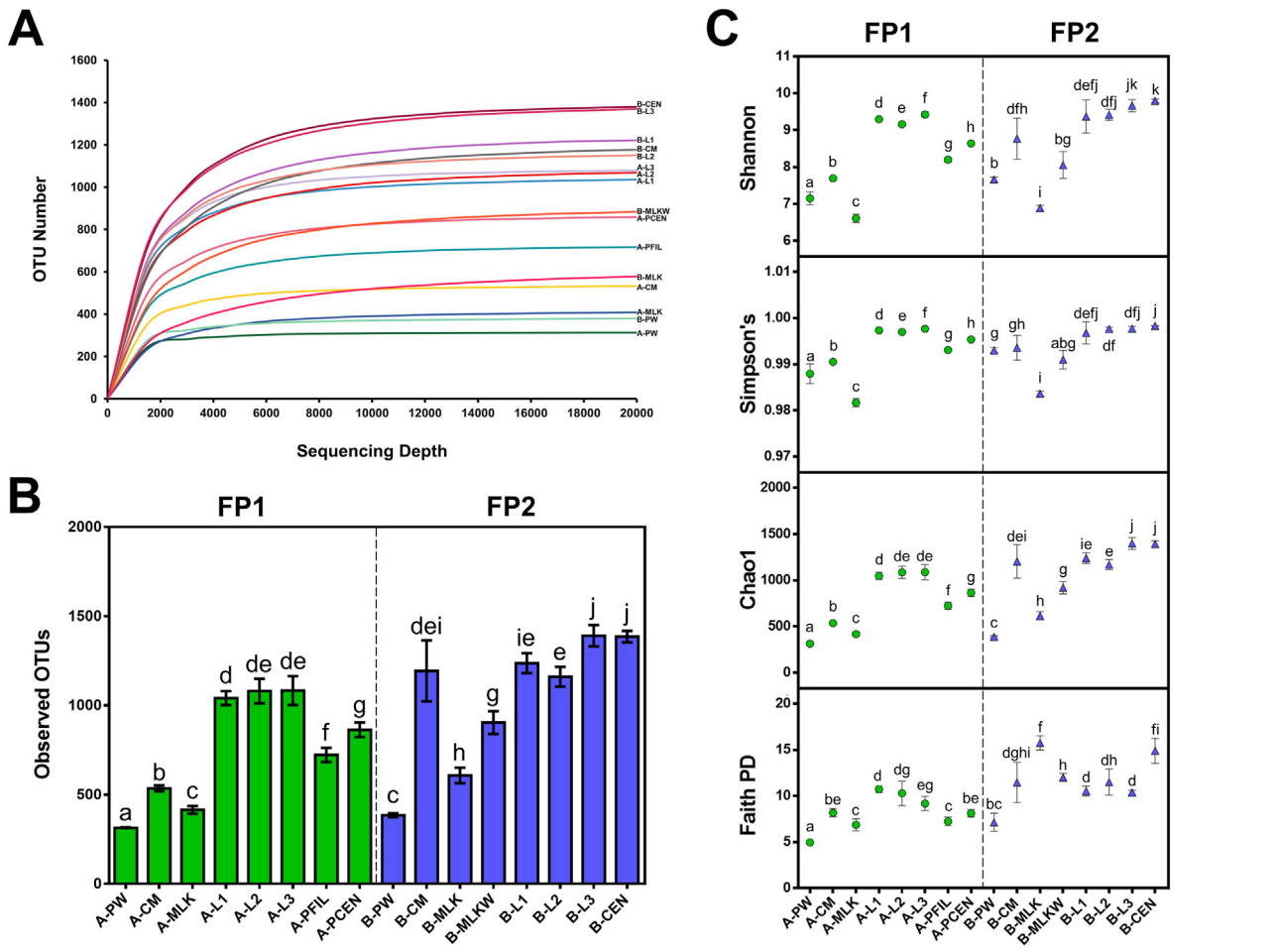

Figure 1. Rarefaction curves and Alpha Diversity measures for FP1 and FP2. (A) Alpha rarefaction curve of the 16S V3-V4 reads against the assigned OTUs to evaluate the impact of sequencing depth on OTU number. (B) Number of observed OTUs. (C) Alpha Diversity Index measurements for Shannon Diversity, Simpson's Diversity, Chao1 Abundance Estimator, and Faith Phylogenetic Diversity. Means that share at least one letter are not statistically significant at $P < 0.05$. Error bars represent the standard deviation.

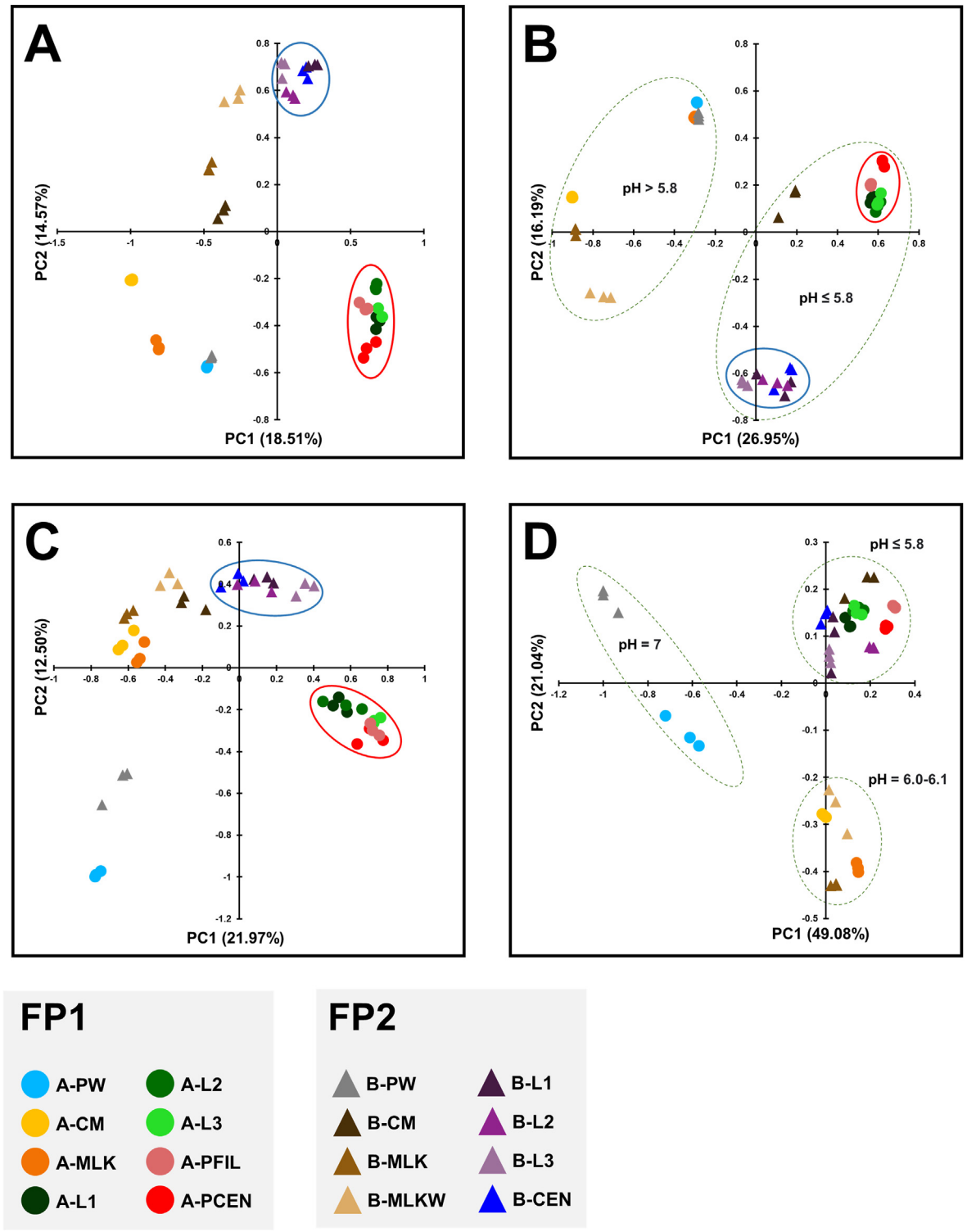

Figure 2. Principal Coordinate Analysis (PCoA) representing the beta-diversity profiles of the microbiota of FP1 and FP2. (A) Jaccard Similarity. (B) Bray-Curtis Dissimilarity. (C) Unweighted UniFrac Distance. (D) Weighted UniFrac Distance. Fermented samples from FP1 are encircled in red, and fermented samples from FP2 are encircled in blue. Green broken lines represent samples that share a similar pH range.

and FP2 (Figure 3A), with A-PW, A-MLK, A-L2, B-PW, B-MLK and B-CEN having uniquely higher numbers of certain genera compared to the rest of the samples. The most shared genera between all samples excluding A-PW and B-PW based on their relative Z-scores include *Leuconostoc*, *Weissella*, *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Serratia*, *Pantoea*, *Enterobacter*, *Stenotrophomonas*, *Bradyrhizobium*, *Kosakonia*, *Novosphingobium*, *Bacillus*, *Kurthia*, *Acinetobacter*, *Kocuria*, *Pseudomonas*, *Streptococcus*, and *Klebsiella*.

Further refining the results of the analysis of the microbiome structure, there were 17 genera detected in at least 50% of all analyzed samples with a relative abundance >0.01% which we can refer to as the "core microbiome" for FP1 and FP2 at the genus level, shown in Figure 3B. This core microbiome accounted for 74%–97% of the microbial abundance in samples excluding A-PW and B-PW. Process water samples A-PW and B-PW contain radically different genera compositions, sharing <10% of the core microbiome. The breakdown of the major genera comprising A-PW

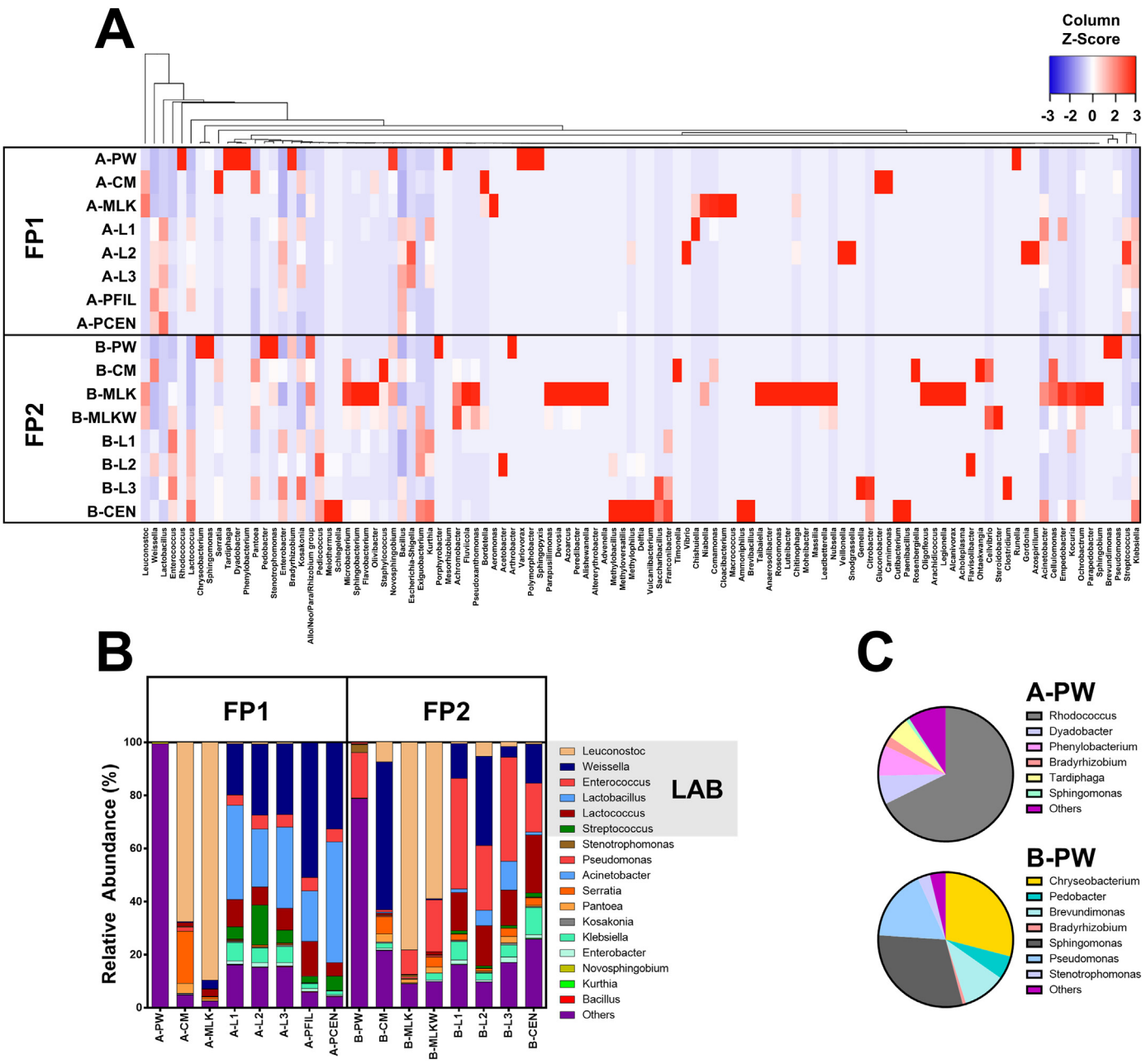

Figure 3. Microbiome structure at the genus level. (A) Heatmap of hierarchical clustering of all identified microbial taxa at the genus level using average linkage of Euclidean distance with scaling applied to columns. (B) Stacked bar graphs representing the core microbiome structure in terms of % relative abundance of all sampling points across FP1 and FP2. The core microbiome represents genera detected in at least 50% of all the sampling points having relative abundances greater than 0.01%. The six most dominant groups across all sampling points excluding A-PW and B-PW are lactic acid bacteria genera labelled LAB. (C) Pie graphs of genera with relative abundances >1% for A-PW and B-PW.

and B-PW can be seen in Figure 3C. For A-PW, the most dominant genus was *Rhodococcus* (67.7%), followed by *Phenylobacterium* (7.39%), and *Dyadobacter* (6.99%). For B-PW, the most dominant genera included *Sphingomonas* (30.1%), *Chryseobacterium* (29.1%), and *Pseudomonas* (17.2%). Water samples were expected to have these major differences as these came from two different commercial suppliers.

Coconut meat was dominated by *Leuconostoc* in FP1 (67.5%) and *Weissella* in FP2 (55.7%). *Leuconostoc* was also the most dominant genus in coconut milk for FP1 (89.5%) and FP2 (78.1%), as well as coconut milk diluted with water for FP2 (58.8%). The remaining sampling points for both FP1 and FP2 were composed mostly of the LAB genera *Weissella*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, and *Streptococcus* of the Lactobacillales order in various abundances. For FP1, the *Leuconostoc* in coconut meat and milk decreased substantially in quantity in fermented samples and was replaced by *Weissella* (31.2%), *Enterococcus* (4.76%), *Lactobacillus* (30.5%), *Lactococcus* (8.72%) and *Streptococcus* (6.46%). Fermented products of FP2 were dominated by *Enterococcus* (29.8%), *Weissella* (16.7%), *Lactococcus* (16.6%), *Lactobacillus* (4.75%) and *Streptococcus* (1.13%).

The core microbiome also showed that both FP1 and FP2 had >0.1 RA % for *Enterobacter*, *Klebsiella*, *Kosakonia*, *Pantoea*, *Serratia*, *Pseudomonas*, and *Stenotrophomonas* while both had RA% between 0.01 and 0.1 for *Bacillus*, *Kurthia*, *Novosphingobium*, and *Acinetobacter*. Together, these 17 genera contribute 83.8%–97.5% of the genus-level RA% of samples from FP1 and 74.1%–90.9% of samples from FP2. The total RA% contribution of LABs per sampling point can be seen in Figure 4A.

The proportion of six major LAB genera found in the samples for FP1 and FP2 in terms of RA% are seen in Figure 4B and Figure 4C

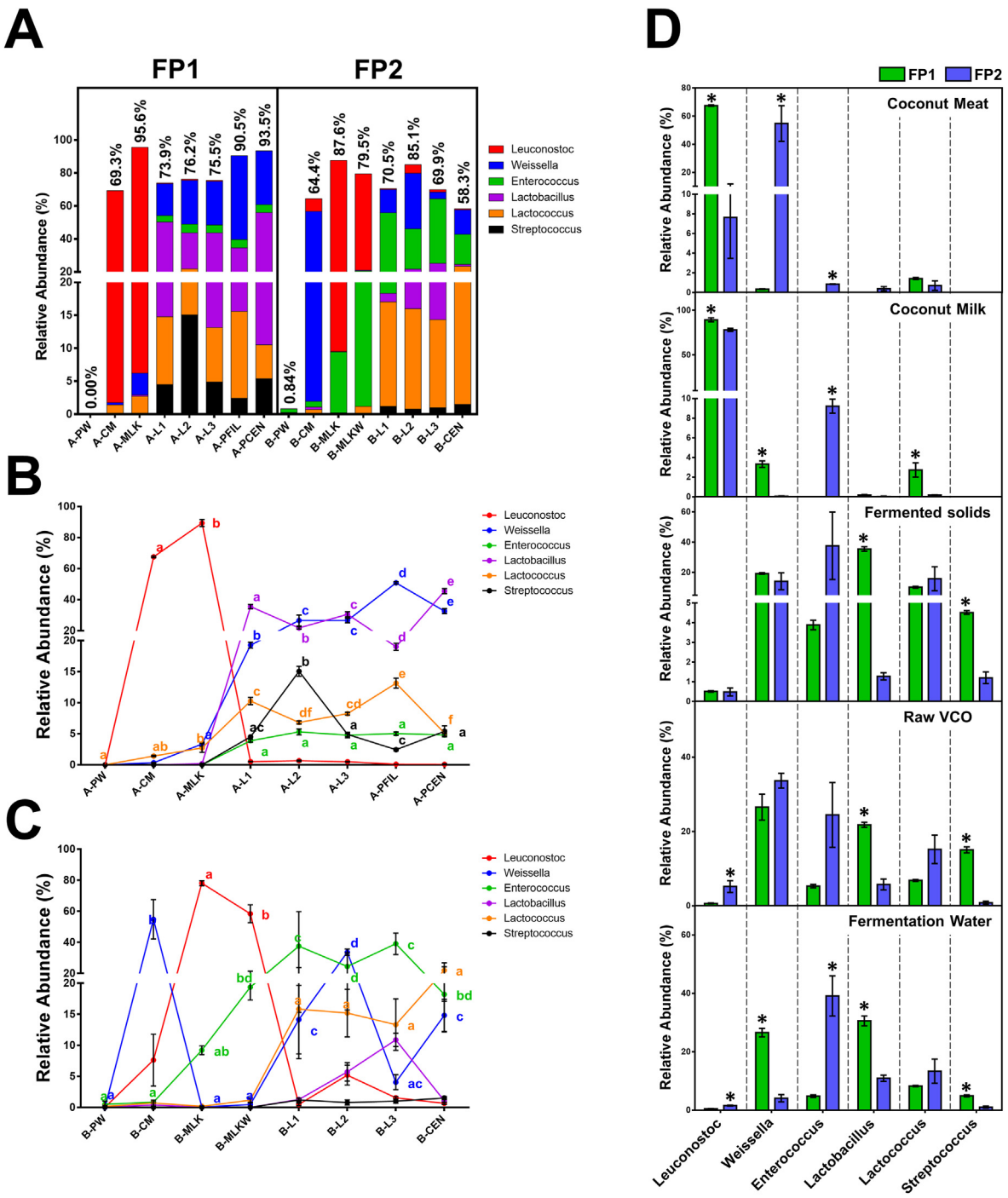

Figure 4. Lactic acid bacteria profile at the genus level for the six major LAB genera identified across all samples. (A) Stacked bar graphs representing the LAB contribution in terms of RA% of all sampling points across FP1 and FP2. (B) Statistical comparison of LAB RA% across FP1. (C) Statistical comparison of LAB RA% across FP2. For B and C, means that share at least one letter are not statistically significant at $P < 0.05$. Error bars represent the standard deviations. (D) Statistical comparison of LAB RA% between similar sampling points between FP1 and FP2. Asterisks show samples which are significantly different between similar sampling points at $P < 0.05$.

respectively. Both FP1 and FP2 showed a statistically higher ($P < 0.05$) amount of *Leuconostoc* in non-fermented raw materials than in fermented products. There was a statistically significant ($P < 0.05$) increase in the relative abundances of all LAB genera apart from *Leuconostoc* for FP1 fermented samples and a similar increase for *Weissella*, *Enterococcus*, and *Lactococcus* for FP2, showing a transition between different LAB genera as fermentation progresses.

A comparison of the RA% of the different LAB groups from similar samples between FP1 and FP2 (Figure 4D) showed that statistically significant ($P < 0.05$) differences between the two processes in terms of RA% occur for *Leuconostoc* in CM, MLK, L2, and L3. *Weissella* similarly had variations in relative abundance for CM, MLK, and L3, while *Enterococcus* had differences in CM, MLK, and L3. Both *Lactobacillus* and *Streptococcus* had variations in relative abundances for L1, L2, and L3, and *Lactococcus* was only significantly different for MLK. These results show that the major microbes associated with similar substrates are different and could be influenced by factors other than the sample type itself.

3.4. Correlations between microbial taxa and physico-chemical parameters

Spearman's rank order correlation coefficient (*r*) correlograms between the different sampling points (Figure 5A) revealed that there were statistically significant ($P < 0.05$) positive correlations between all samples except with A-PW and B-PW, to which the rest of the samples had either weak negative or no correlation with.

Positive correlations between fermented products within and between both FP1 and FP2 are stronger than their correlations with raw materials from either processing line. This supports the microbial community compositions shared between raw materials and between the fermented products that are dominated by different genera under the Lactobacillales order.

Examination of the correlations between member genera of the core microbiome (Figure 5B) show that *Leuconostoc* is not positively correlated with any other LAB genus and is negatively correlated with *Streptococcus*. Positive correlations exist between all other five LAB genera except for *Enterococcus* which is only strongly correlated with *Lactococcus*. *Leuconostoc* is positively correlated with *Serratia*, *Pantoea*, and *Pseudomonas*, while other LAB genera are positively correlated with *Klebsiella*, *Enterobacter*, and *Bacillus*, but are negatively correlated with *Novosphingobium*. These correlations maybe due to the inherent environmental tolerances of the microbes, with those positively correlated with *Leuconostoc* favoring a less acidic environment, and those correlated with the other LAB groups favoring environments with lower pH.

Correlations between pH, temperature and the core microbiome show that *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Klebsiella*, *Enterobacter*, and *Kurthia* were negatively correlated with increasing pH in analyses that included (+PW) and excluded (−PW) process water samples (Figure 5C). *Novosphingobium* was positively correlated with pH, and *Enterococcus* was positively correlated with temperature in both analyses. When +PW, *Weissella* and *Novosphingobium* became negatively correlated with pH and temperature, respectively. When -PW, *Leuconostoc*, *Pantoea*, and *Pseudomonas* showed significant positive correlations with increasing pH. These results further highlight the impact of shifts in pH on the microbial assemblages found in the different samples.

4. Discussion

Enumerating the genera that are most active in the fermentation of VCO is important since these are the primary agents responsible for oil liberation from coconut milk in the natural fermentation method of VCO production. Coconut milk is an emulsion which is destabilized by microbial physiological action to release the oil from associated stabilizing proteins and lipids (Xiang et al., 2019). Thus, this study aimed to identify the major microbial genera populating pre-fermented raw materials and fermented products in two VCO production processes.

The trimmed data set identified 8 phyla, 14 classes, 39 orders, 58 families, and 109 genera distributed across 48 samples. There were significantly more OTUs in the later stages of fermentation for both FP1 and FP2 compared to the pre-fermented raw materials used except for B-CM, which could have been contaminated by handling. Together with the significantly lower measurements for the Shannon, Simpson's, and Chao1 indices, these results suggested an uneven community structure of

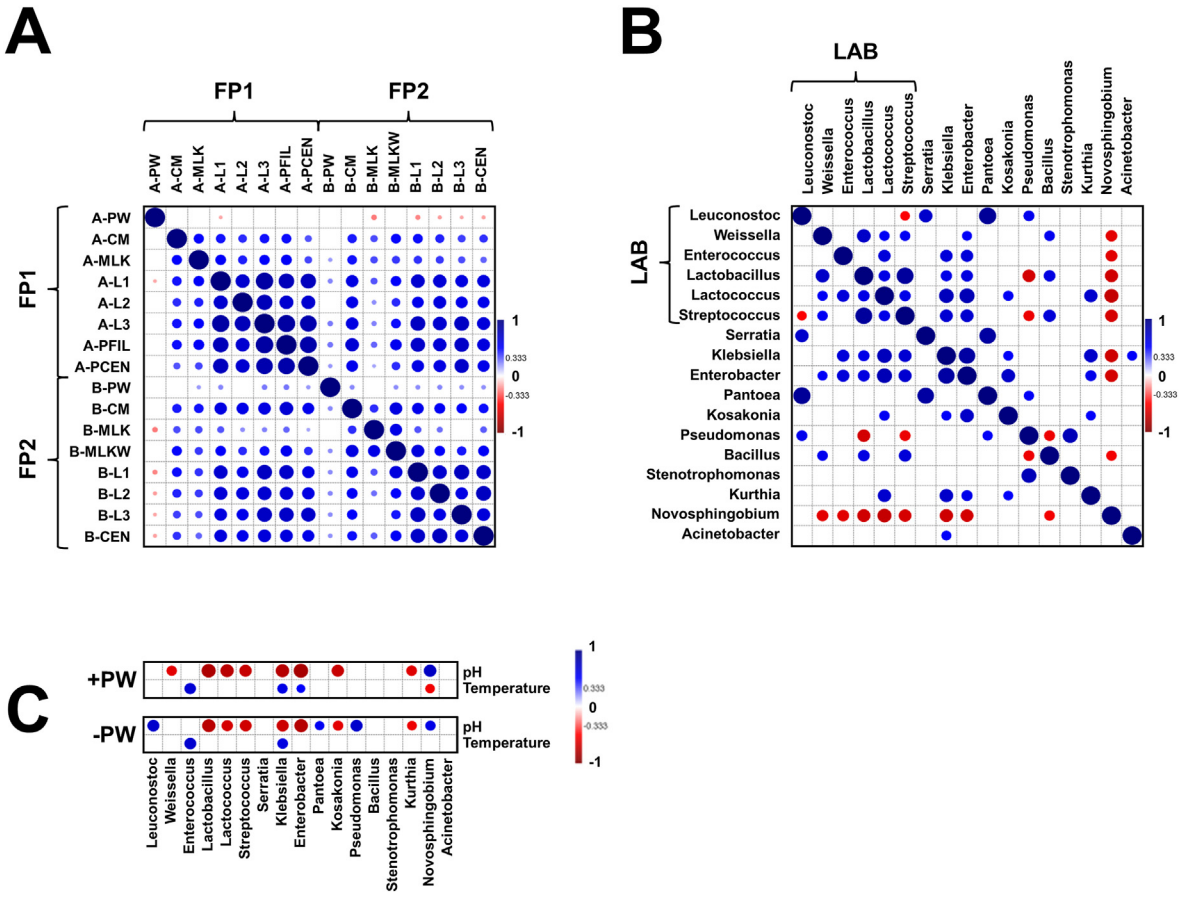

Figure 5. Spearman's rank order correlation coefficient (*r*) correlograms between various samples compared at the genus level. (A) Correlation between sampling points based on genus-level microbial composition. (B) Correlations between the core microbiome, with lactic acid bacteria labelled LAB. (C) Correlation of core microbiome with pH and temperature at collection, with A-PW and B-PW included (+PW) and excluded (-PW) from the analysis. Spherical sizes and color intensity indicate strength of correlations. Only significant ($P < 0.05$) positive (blue) and negative (red) correlations are shown in the figures.

raw material samples that could be dominated by few genera with large abundances. This was proven true by an examination of the relative abundances of microbes for these pre-fermented materials at the order level, where it was seen that CM, MLK, and MLKW were predominantly composed of members of the Lactobacillales order for both FP1 (83.2%) and FP2 (79.7%), while A-PW was composed chiefly of Corynebacteriales (67.7%), and B-PW was composed of a mixture of Sphingomonadales (30.8%) and Flavobacteriales (29.1%).

The lower alpha-diversity for raw materials may also indicate less microbial contamination, which is the goal of any processing facility. Handling coconut meat and milk and passing these through various surfaces such as those of the milk pressers and collecting drums which were done manually for both processing facilities could have introduced microbial contamination. These may have been detected in the succeeding steps once the microbes have had ample time to proliferate, leading to larger microbial diversity in the fermented products.

A major difference in the alpha-diversity pattern arises with the difference of the Faith PD metric from the rest of the alpha-diversity indices used. The Faith PD metric shows more similar alpha-diversity measures between some raw materials and fermented samples, especially for FP2. We must consider that Faith PD is phylogenetic, which calculates the diversity as a sum of branch lengths of members of the sample examined (Faith, 1992). This may lead to lesser diversity assignments for samples with closely related taxa compared to the other alpha-diversity metrics which do not consider phylogeny. Thus, the Lactobacillales which dominate all samples apart from A-PW and B-PW may lead to more significantly similar measurements for the Faith PD metric across raw materials and fermented products.

At the genus level, *Leuconostoc* accounted for 58.9%–89.6% of the microbiome for all pre-fermented samples apart from B-CM which had only 7.35% *Leuconostoc*, but had 55.7% *Weissella*, also a member of the family *Leuconostocaceae*. This dominance of the *Leuconostoc* genus in these raw materials can be explained by its wide tolerance of temperature and salinity which allow its rapid colonization of various plant matter (Rhee et al., 2011). The pre-fermented materials in FP1 and FP2 have pH and temperature conditions which easily favor *Leuconostoc* growth, considering the genus thrives optimally at ~25 °C and pH 6–7 (Endo et al., 2020). Both *Leuconostoc* and *Weissella* have been shown to be part of the core microbiome of pre-fermented coconut water (Zhang et al., 2017) and play significant roles in the early stages of fermentation in other fermented vegetable food such as kimchi (Mannaa et al., 2019), sauerkraut (Zabat et al., 2018), and idli (Mandhania et al., 2019).

The fermented samples showed significantly lower mean relative abundances for *Leuconostoc* for both FP1 (0.37%) and FP2 (1.97%). This coincided with a pH change in these samples which shifted from a pH range of 6.0–7.0 for pre-fermented samples apart from B-CM (pH 5.8) to a pH range of 4.5–4.9 for L1, L2, and L3, and a pH range of 5.6–5.7 A-PFIL, A-PCEN and B-CEN. The relationship between pH change and *Leuconostoc* decline is supported by correlational analysis using Spearman's (r), which showed a positive correlation between *Leuconostoc* abundance and increasing pH for all FP1 and FP2 samples except A-PW and B-PW. This is further demonstrated in the Bray-Curtis and Weighted UniFrac distance measurements that clustered all fermented samples that have both pH of $\leq$5.8 and consequently, *Leuconostoc* relative abundances of <1%, toget-her.

Changes in pH have been shown to be significant in the decline of *Leuconostoc* relative abundance in studies on other fermented vegetables (Zabat et al., 2018; Lee et al., 2017; Swain et al., 2014). It has been documented that the continued proliferation of *Leuconostoc* species in early stages of fermentation leads to the liberation of high amounts of CO_2 that replace oxygen, as well as the production of organic acids that lower the pH, changing the sample environmental conditions to inhibit the growth of other microbes and favor the succession of other LAB genera in later stages of fermentation (Erkmen and Bozoglu, 2016). However, since *Leuconostoc* species are sensitive to pH with growth being significantly diminished at pH < 5.0, the continued production of acid leads to their self-inhibition and decline (Endo et al., 2020).

The kind of microbial communities that succeed *Leuconostoc* during its decline as pH shifts to an acidic spectrum is heavily dependent on initial microbial load, species growth rates, and salt- and acid-tolerances of microbes that are present in the food sample (Björkroth and Holzapfel, 2006). In this study, fermented sample microbial communities were composed mostly of *Weissella*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, and *Streptococcus*, which together accounted for an average of 81.6% and 69.3% of the microbiome abundances of FP1 and FP2 respectively. The Spearman correlation analysis between these LAB groups show strong positive correlations between *Weissella*, *Lactobacillus*, *Lactococcus* and *Streptococcus*, as well as positive correlations between *Enterococcus* and *Lactococcus* for FP1 and FP2. All these genera have been documented to be major fermenters in various fermented foods (Leech et al., 2020; Rezac et al., 2018) and are commonly present in the late-stage fermentation microbiomes of other fermented vegetables (Mannaa et al., 2019; Zabat et al., 2018; Swain et al., 2014).

A comparison of pre-fermented and fermented samples showed that for FP1, statistically significant ($P < 0.05$) increases in RA% were present for *Weissella*, *Enterococcus*, *Lactobacillus*, *Lactococcus* and *Streptococcus* in fermented samples while for FP2, statistically increased RA% in fermented samples were only noted for *Weissella*, *Enterococcus*, and *Lactococcus*. These results suggest that while the major genera responsible for fermentation are all LABs and are similar between FP1 and FP2, the dominant genera that act to liberate the VCO during the latter stages of fermentation may be different for the two fermentation processes examined. This is clarified by a comparison of similar sampling points between FP1 and FP2, where statistically ($P < 0.05$) higher abundances of *Lactobacillus* and *Streptococcus* were seen in L1, L2, and L3, for FP1, and for *Enterococcus* in L3 for FP2. These results suggest that the *Lactobacillus* and *Streptococcus* genera may have significant fermentative roles in FP1, while *Enterococcus* may have a significant role in the fermentation process of FP2 during late-stage fermentation. An examination of a greater number of samples and processing lines would further enhance our understanding of which microbial communities are involved in the fermentation production processes specific for VCO.

The dominant LAB assemblage during the fermentation processes may have impacts on the quality of the VCO produced due to the release of organic acids such as lactate and acetate (Nuryana et al., 2019) as well as ethanol and hydrogen peroxide, among several other substances (Florou-Paneri et al., 2013). Organic acid production has been found to be increased during greater moisture levels where microbial action is prominent and may be a contributory factor in VCO degradation (Dimzon et al., 2011). Several of the major genera identified in this work such as *Enterococcus*, *Streptococcus*, and *Lactococcus* are generally homolactic and produce primarily lactic acid (Gänzle, 2015). Heterofermentative genera produce ethanol and acetic acid apart from lactate, which has been documented in *Leuconostoc* (Endo et al., 2020) and *Weissella* (Fusco et al., 2015). *Lactobacilli* have been shown to have both homo- and hetero-fermentative groups which produce a variety of different acids (De Angelis and Gobbetti, 2011). These organic acids, if not removed properly, may be contributory to perceived VCO sensory properties such as the sour aroma (Villarino et al., 2007) and organoleptic qualities like acidity and rancidity noted in fermented VCO (Villarino et al., 2020). The identities of the major microbial groups described in this study thus provide a practical basis for recommending the use of industrial practices such as settling, filtering, centrifugation, and vacuum-drying to separate and reduce both organic acids and excess moisture present in freshly harvested raw VCO.

Despite these considerations in terms of the impact of organic acids on VCO quality, it is generally accepted that these acids, when secreted together with bacteriocins, hydrogen peroxide, diacetyl, and carbon dioxide, can act as inhibitory compounds against harmful microorganisms, making *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus* species suitable as LAB probiotics (Vieco-Saiz et al., 2019). Some LABs belonging to the genera described in this work have also been used as starter cultures and have been documented to exert positive features

contributory to good quality VCO produced via fermentation. Virgin coconut oil produced using *Lactobacillus plantarum* starter cultures showed a lowered moisture content that passed the international Codex Alimentarius standards which is a major consideration in prolonging VCO shelf-life (Satheesh and Prasad, 2012). Supplementation of coconut milk with *Lactobacillus pentosus*, *Enterococcus faecium*, and *Leuconostoc mesenteroides* has also been shown to produce VCO having higher crude protein content, lower free fatty acids and steroids, and similar acceptable sensory evaluation results in terms of taste, aroma, color, and flavor compared to spontaneously fermented VCO (Olateru et al., 2020). Even VCO produced via natural non-supplemented fermentation has been shown to have four times the total phenolic content compared to VCO produced using several non-fermentative means, giving it a high antioxidant capacity (Ghani et al., 2018). Most importantly, LAB culture inocula have been shown to decrease oil separation time as well as increase oil yield, both critical for the VCO industry (Mesias and Tan, 2014).

Other non-LAB genera which may exert impacts on VCO found in the core microbiome for FP1 and FP2 include the lipolytic genera *Serratia*, *Pseudomonas*, *Bacillus*, and *Acinetobacter* (Sangeetha et al., 2011), as well as members of the genera *Enterococcus* and *Enterobacter* (Javed et al., 2018). The action of lipases produced by these microbes could change the composition of free fatty acids in VCO, as was demonstrated by thermostable lipases isolated from *Candida rugosa* (Nguyen et al., 2018) and *Mucor miehei* (Chua et al., 2012). Since VCO is generally not heat-sterilized and bacterial lipases have better stability profiles at lower temperatures (Chandra et al., 2020), if lipases are secreted, these may remain active for extended periods long after the growth of microorganisms has ceased (Braun et al., 1999). If these are not efficiently removed in the final VCO product, these may potentially cause changes in the quality of VCO over time. Verification of the presence of these lipases in the various stages of VCO production presents an interesting research direction which can contribute to prolonging the shelf-life of commercially sold VCO.

The microbial qualities of the dominant microbial assemblages discussed previously must be taken into consideration in future studies that would aim to explore the use of the genera identified in this work for modulation and improvement of VCO production through assisted fermentation. Apart from these characteristics, it must be noted that both *Weissella* and *Enterococcus* have not yet received the generally regarded as safe (GRAS) and Qualified Presumption of Safety (QPS) statuses due to debates over their safety (Hanchi et al., 2018; Fessard and Remize, 2017; Abriouel et al., 2015) despite their widespread use in various food fermentations over a long historical timespan (Plavec and Berlec, 2020). Moreover, several members of the core microbiome identified in this work can sporadically pose as risks to food safety, such as in the case of *Stenotrophomonas* (Ryan et al., 2009), *Pseudomonas* (Silby et al., 2011), *Serratia* (Khanna et al., 2013), *Pantoea* (Walterson and Stavrinides, 2015), *Kosakonia* (Mertschnigg et al., 2020), *Klebsiella* (Paterson et al., 2014), *Enterobacter* (Davin-Regli et al., 2019), and *Bacillus* (Celandroni et al., 2016). Since coconut meat has long been established to be sterile (Kajs et al., 1976), and these microbes are either absent or present at $<1\%$ in the process water samples examined, this suggests that these are environmentally derived during the handling of the raw materials. The same observations were seen in coconut water where *Pantoea*, *Serratia*, and *Enterobacter* were detected in the metagenome of freshly collected coconut water, where these were thought to have used abundant nutrient sources in the substrate to easily proliferate (Zhang et al., 2017). Considering all these, despite not being detected in the final commercially sold products from which no DNA was isolated in this work, additional focus must still be placed on ensuring workplace cleanliness to avoid unnecessary microbial contaminants in the production of VCO. This is important especially during the preparation of the raw materials prior to the actual fermentation process as well as the handling of the raw VCO prior to subsequent clarification and moisture-removal steps during which human handling is inevitable.

5. Conclusions

The results of this work showed that LABs are the most abundant microbes in both raw materials and fermented products of the two VCO processing lines examined. The core microbiome for both processes included 17 genera, with majority of the relative abundances for all samples coming from members of the Lactobacillales order. Transitions in the microbial community populations were driven by the changes in relative abundances of *Leuconostoc*, *Weissella*, *Enterococcus*, *Lactobacillus*, *Lactococcus,* and *Streptococcus* as the raw materials were fermented, similar to the canonical transitions in microbial communities of other fermented vegetables. Differences in the abundance of the major LAB groups present in the samples as well as other members of the core microbiome may have consequences on how fermentation is carried out, as well as on the quality of VCO produced in terms of the possible influence of acid and lipase production, and the presence of microbial contaminants in the processing intermediates. These results could provide the basis for examining the specific roles of members of these genera in the modulation and improvement of VCO production processes.

Declarations

Author contribution statement

Zomesh Artus Maini and Crisanto M. Lopez conceived and designed the experiments and analyzed and interpreted the data.

Crisanto M. Lopez contributed reagents, materials, analysis tools or data.

Zomesh A. Maini performed the experiments and wrote the paper.

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Data availability statement

Data associated with this study has been deposited at NCBI Sequence Read Archive (SRA) under BioProject PRJNA774430.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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Appendix

Noticeable differences exist between the microbial composition of the various samples at the phylum, class, order, and family taxonomic levels, shown in Figure A1.

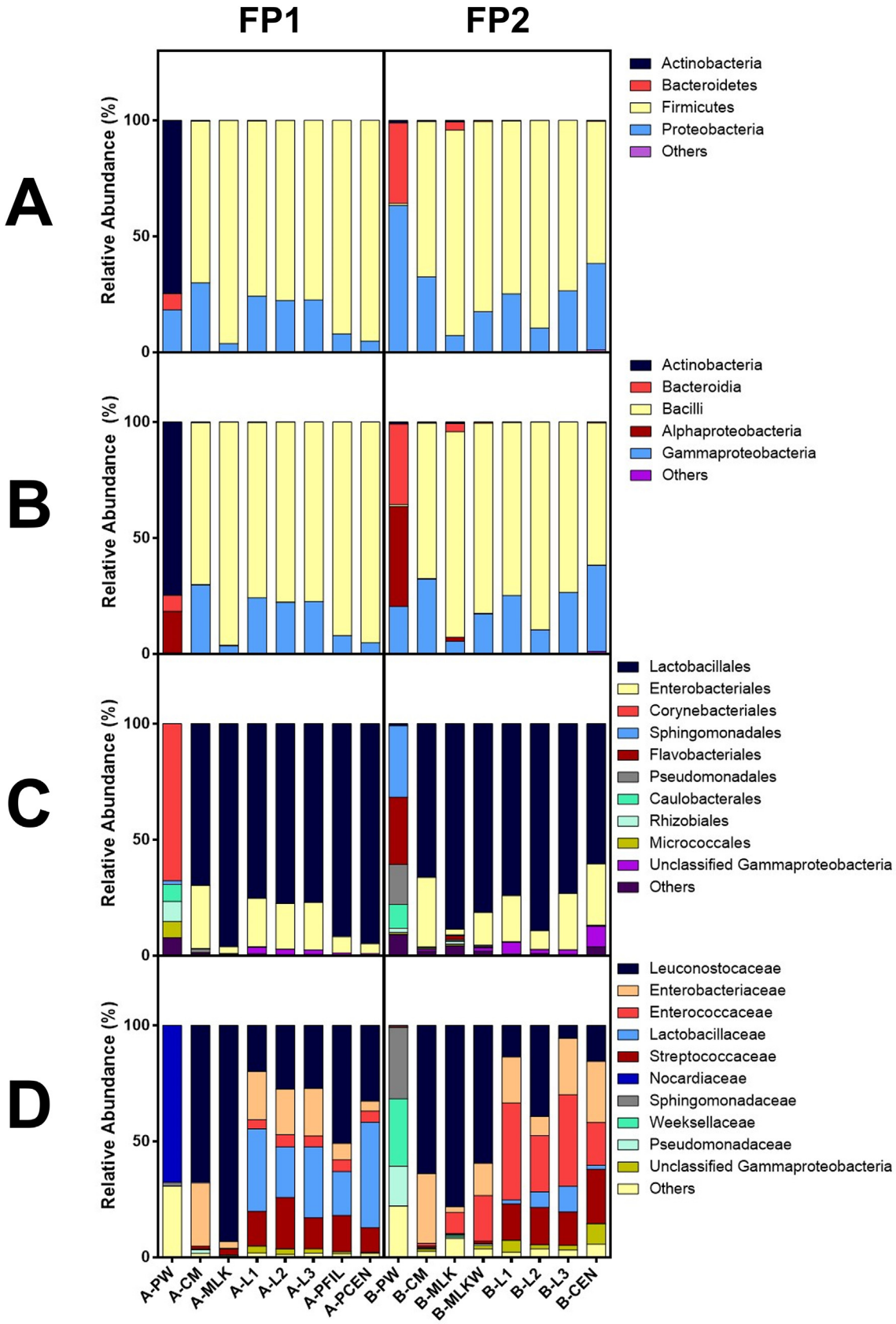

Figure A1. Microbiome structure at various taxonomic levels. Stacked bar graphs representing the microbiome structure in terms of % relative abundance of all samples across the two fermentation processes at the (A) Phylum, (B) Class, (C) Order, and (D) Family level.

For both FP1 and FP2, process water samples contained different microbes from the phylum to the family level (Figure A1, A-D) compared to all raw materials and fermented products ($\bar{x}$ = mean). At the Phylum level (Figure A1-A), Firmicutes was the most dominant in all sampling points from FP1 ($\bar{x}$ = 72.9%) and FP2 ($\bar{x}$ = 67.1%) except for A-PW which was composed mostly of Actinobacteria (74.7%) and FP2 B-PW which was composed mostly of Proteobacteria (63.3%) and Bacteroidetes (34.7%). Proteobacteria were present in all samples from FP1 and FP2 ($\bar{x}$ = 21.9%).

A similar trend in microbial composition was seen at the class level (Figure A1-B), where A-PW showed an abundance of Actinobacteria (74.7%) and B-PW showed a composition mostly of alpha-Proteobacteria (43.1%) and Bacteroidia (34.8%), compared to all the other remaining sampling points from FP1 and FP2 which were dominated by Bacilli ($\bar{x}$ = 80.0%). Gamma-proteobacteria were present in all samples in varying abundances while alpha-proteobacteria were present in all samples at <1% except for A-PW (17.9%) and B-PW (43.1%) and was completely absent in samples A-L1, A-L3, B-L1, and B-L3.

Examination of the order level (Figure A1-C) microbiome structure reinforce the previous trends for phylum and class, with A-PW composed chiefly of Corynebacteriales (67.7%) and B-PW composed of Sphingomonadales (30.7%) and Flavobacteriales (29.1%). All the remaining sampling points for both FP1 and FP2 show a predominant abundance of the Lactobacillales ($\bar{x}$ = 83.7%). The Lactobacillales order was not detected (0%) for A-PW and was <1% for B-PW.

The predominant family in A-PW was Nocardiaceae (67.6%), while for B-PW, it was Weeksellaceae (29.1%) and Sphingomonadaceae (30.7%) (Figure A1-D). This contrasts with other steps of both FPs that were dominated by Enterobacteriaceae ($\bar{x}$ = 15.2%), Enterococcaceae ($\bar{x}$ = 13.7%), Lactobacillaceae ($\bar{x}$ = 12.4%), Leuconostocaceae ($\bar{x}$ = 39.7%), and Streptococcaceae ($\bar{x}$ = 10.2%). Pseudomonaceae was also present in all sampling points except A-PW ($\bar{x}$ = 1.38%).

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