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

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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
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 (Narayankutty 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 17 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 12 h. 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 12 h. 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 husked nuts which were inspected, 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 12 h, 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 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 polystyrene sulfone (PES) membrane (Membrane Solutions, Chiba-ken, Japan) to collect bacteria present (Nnadzie 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 SPECTROrstar 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′-TGTCGCGGACGTCAGATGTGTATAAGAGA-3′; and (R), 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-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, Santa 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).
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 Bioinformatics pipeline at standard settings unless mentioned below (Bolten 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), Chao 1 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 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 PASwv4.03 (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 physico-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](image_url)

**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.
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, Kurbhia, Acinetobacter, Kocuria, Pseudomonas, Streptococcus, and Klebsiella.

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.

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
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.
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, while *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.

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 $P < 0.05$. Z.A. Maini, C.M. Lopez Heliyon 8 (2022) e10154
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, Pantoaea,* 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, Pantoaea,* 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 microbiion 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
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 Lactobacillaceae. 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 for 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 ≤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 demonstrated that the total biomass of Leuconostoc species in early stages of fermentation leads to the liberation of high amounts of CO2 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 (Bjorkroth 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 to control less desirable 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 accept-able sensory evaluation results in terms of taste, aroma, color, and flavor compared to spontaneously fermented VCO (Olateju 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 anti-oxidant capacity (Ghani et al., 2018). Most importantly, LAB culture inocula have been shown to decrease oil separation time as well as in-crease 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., 2014). 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 li-pases 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 modula-tion 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 fer-mentations 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* (Celendroni et al., 2016). Since coconut meat has long been established to be sterile (Kais 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 commer-cially 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 *Serratia* 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 Arts 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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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 phyllum to the family level (Figure A1-A) compared to all raw materials and fermented products (P < mean). At the Phylum level (Figure A1-A), Firmicutes was the most dominant in all sampling points from FP1 (P < 72.9%) and FP2 (P < 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 (P < 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 (P < 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 class and with A-PW composed chiefly of Corynebacteria (67.7%) and B-PW composed of Sphingomonadales (30.7%) and Flavobacterales (29.1%). All the remaining sampling points for both FP1 and FP2 show a predominant abundance of the Lactobacillales (P < 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 (29.2%) and Sphingomonadaceae (29.1%) (Figure A1-D). This contrasts with other steps of both FFs that were dominated by Enterobacteriales (P < 15.2%), Enterococccaceae (P < 13.7%), Lactobacillaceae (P < 12.4%), Leuconostocaceae (P < 39.7%), and Streptococccaceae (P < 10.2%). Pseudomonaceae was also present in all sampling points except A-PW (P < 1.38%).

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