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Dynamic profile of the microbiota during coconut water pre-fermentation for nata de coco production

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ABSTRACT

The uncontrolled coconut water pre-fermentation process represents a considerable food safety risk and can cause unstable production. In the present study, a metagenomics approach was used to explore the dynamic changes in the microbial structure and their correlation with the physicochemical indices of coconut water during pre-fermentation. At the generic level, *Leuconostoc, Lactobacillus, Acetobacter* and *Weissella* were the predominant bacteria during pre-fermentation. Using the RDP database, we detected 28 and 34 core OTUs in samples from the cities of Wenchang and Haikou (Hainan province, China) that primarily belonged to the genera *Leuconostoc* and *Lactobacillus*. The PCoA, based on Weighted Unifrac distances, demonstrated that the microbial community structure changed significantly during pre-fermentation, and these structural changes could be attributed to an increase in bacteria of the genera *Lactobacillus* and *Acetobacter*. Additionally, we observed that the pH values decreased significantly, whereas the lactic acid and acetic acid content increased during pre-fermentation. A significant positive correlation was observed between the OTUs representing *Lactobacillus* and *Acetobacter* and the lactic acid content. This study is intended as a theoretical guide for controlling and accelerating pre-fermentation, inhibiting or eliminating harmful contaminating microorganisms, especially pathogens and, finally, raising the yield of nata de coco for further research.

1. Introduction

Nata de coco is a highly popular coconut water product of the food processing industry and widely used in beverages, jellies, canned food and other edible products. The production of nata de coco, the primary component of which is bacterial cellulose, essentially involves the fermentation of coconut water by the species *Acetobacter xylinum* (currently named *Komagataeibacter xylinus*) (Makhlof, Tozuka, & Takeuchi, 2011). Due to its high fibre content, low caloric value and good texture, nata de coco has gradually become one of the most popular foods globally (Hu & Catchmark, 2010; Leroy, Grongnet, Mabeau, Corre, & Baty-Julien, 2010). Coconut water, the raw material for the production of nata de coco, is enriched in various carbohydrates (such as glucose, fructose, sucrose and sorbitol) that constitute the carbon source and amino acids for the growth of *Gluconacetobacter xylinus* (currently named *Komagataeibacter xylinus*) (Kubiak et al., 2014). Additionally, the vitamins, minerals, organic acids, and other components in the coconut water may be helpful for bacterial cellulose synthesis (Santos et al., 2013).

Hainan province, located in the southern tropical part of China, is known as a coconut island, with the area under coconut plantation being the highest in China. In Hainan, coconut processing factories can be found everywhere and nata de coco is a popular featured product. Interestingly, it is found that the nata de coco yield is significantly higher, at times up to ten times higher, when the fresh coconut water is exposed to the natural environment for several days of natural fermentation (Deng et al., 2015; Yang et al., 2015; Wang, Zhong, Wang, & Zheng, 2009)). Therefore, factories are increasingly adopting this method. However, uncontrolled pre-fermentation could significantly increase the food safety risk and cause fluctuations in productivity with very low yields occasionally because of contamination by various microorganisms (Perumpuli,

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Watanabe, & Toyama, 2014). The randomly invading microbes and their metabolites could play a key role in the pre-fermentation of coconut water. Therefore, it is important to first understand the dynamic changes in microbial diversity and structure during coconut water pre-fermentation. On the one hand, it will provide a practical approach for controlling the coconut water pre-fermentation process and thus improve the biosafety and stability of nata de coco production. On the other hand, it could offer a promising strategy for controlling bacterial cellulose synthesis in a highly efficient manner.

With the development of next generation sequencing, the metagenomic approach was widely applied to reveal the diversity and structure of micro-environmental samples. For example, numerous studies used this technology to focus on the human gut microbiota in instances of chronic disease (Forslund et al., 2015; Karlsson et al., 2013), soil microbiota for crop diseases and pests (Bhatia et al., 2015; Xu et al., 2015) and beneficial microbes in fermented foods (M. Almeida et al., 2014; Johansen, Vindelov, Arneborg, & Brockmann, 2014). In this study, coconut water samples were collected at different time points during fermentation in the Wenchang and Haikou cities of Hainan province, China. The metagenomic approach based on Illumina's high-throughput sequencing was used to explore the dynamic changes in the microbial structure and thereby their correlation with the physicochemical indices of coconut water during pre-fermentation. This study should provide a theoretical guide for controlling and reducing the time for pre-fermentation, inhibiting harmful microorganisms, eliminating pathogens, and finally raising the yield of nata de coco and bacterial cellulose, which could fuel further research on bacterial cellulose. The new insights gained from this study should help in the screening of useful microorganisms for the controlled and safe pre-fermentation of coconut water in addition to greatly improving the stability and yield of bacterial cellulose.

2. Materials and methods

2.1. Experimental design and collection of coconut water samples

In this study, longitudinal design was used to investigate the changes in microbial diversity and structure during coconut water pre-fermentation. Fresh coconut water was poured directly into plastic bottles in the lab or into plastic pails in the plant after cutting the coconut shell, covered with lids, and left at ambient temperature (approximately 30 °C) for several days in the plant or in the lab. Three samples of coconut water were collected in parallel at different fermentation time points in the cities of Wenchang (Group N) and Haikou (Group L) in the Hainan province in China. The temperature during fermentation kept at 30 ± 2 °C. Detailed information about the sample with its alpha diversity is provided in Table 1.

2.2. Microbial metagenomic DNA isolation

The three parallel coconut water samples collected at each fermentation time point were pooled together. The bead-beating method (Tanaka et al., 2009) was used to disrupt bacterial cell walls, and this was followed by metagenomic DNA extraction using the QIAamp DNA Mini-Kit (QIAGEN, Hilden, Germany). The isolated microbial metagenomic DNA was used as a template for sequencing.

2.3. High-throughput sequencing

The V3-V4 region of bacterial 16S ribosomal RNA (rRNA) genes was amplified as described previously (Dethlefsen & Relman, 2011).

Table 1
Sample information and α-diversity statistics of bacteria from samples.

Group	Sample	Time point	Chao	Ace	Shannon	Simpson	OUT #
Haikou	L1	Day 1	73.0	74.4	1.80	0.253	55
	L2	Day 2	63.7	65.4	1.89	0.267	62
	L3	Day 3	60.8	63.7	1.67	0.344	60
	L4	Day 4	50.0	53.0	1.78	0.322	48
	L5	Day 5	52.5	53.1	1.81	0.324	51
	L6	Day 6	70.0	75.0	1.90	0.249	55
	L7	Day 7	55.6	68.4	1.86	0.319	50
	L8	Day 8	56.7	58.3	2.10	0.238	55
Wenchang	NH	12 Hours	531	538	2.79	0.179	183
	N1	Day 1	247	252	2.74	0.173	114
	N2	Day 2	293	308	2.75	0.157	120
	N3	Day 3	292	294	2.69	0.179	121
	N4	Day 4	286	295	2.73	0.166	118
	N5	Day 5	260	277	2.53	0.208	105
	N6	Day 6	310	328	2.93	0.118	125
	N7	Day 7	302	309	2.84	0.137	128
	N8	Day 8	250	257	2.99	0.100	119

A set of 6-nucleotide barcodes was added to the universal forward primer 518F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Next, the Agilent DNA 1000 Kit and an Agilent 2100 Bioanalyser (Agilent Technologies, America) were used to quantify the PCR products according to the manufacturer's instructions. Finally, the PCR products were pooled together in equimolar ratios with a final concentration of 100 nmol/L each. These pooled samples were sequenced using the Illumina-MiSeq platform.

2.4. Measurement of physicochemical indices

The physicochemical indices of pre-fermented coconut water, including its pH values and the lactic acid and acetic acid content, were determined at different fermentation time points. The pH values were measured using a pH metre. The lactic acid and acetic acid content were determined by high-performance liquid chromatography as described previously (Ahmed, Wang, Ali, Smillie, & Khan, 2015; Curiel et al., 2015).

2.5. Bioinformatic and statistical analyses

The QIIME (v1.6) (Caporaso, Kuczynski, et al., 2010) platform was chosen for bioinformatic analysis of the high-quality sequence data. First, the trimmed sequences were aligned by PyNAST (Caporaso, Bittinger, et al., 2010), and those with less than 100% sequence identity were clustered using UCLUST (Edgar, 2010) to obtain the unique V4 sequence set. Next, the representative sequences were extracted and classified into Operational Taxonomic Units (OTUs). The programme ChimeraSlayer (Haas et al., 2011) was used to remove any potentially chimeric sequences in the representative set of OTUs. The Ribosomal Database Project (RDP) (Cole et al., 2007) was used to assign the taxonomy to each OTU representative sequence. The phylogenetic tree of OTUs was built by FastTree (Price, Dehal, & Arkin, 2009) and used for downstream analyses, including alpha and beta diversity calculations. The alpha diversity metrics including Simpson and Shannon indices were calculated, and the UniFrac (Lozupone & Knight, 2005) metrics were computed to estimate the sample's beta diversity.

The programme R was used for all statistical analyses, including Principal Coordinate Analysis (PCoA) analysis (ade4 package), correlation analysis (Spearman rank correlation coefficient) and heatmap construction (pheatmap package). The sequence data reported in this paper have been deposited in the NCBI database (BioProject ID: PRJNA305322).