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

Jiachao Zhang [a, 1], Yichong Yang [a, 1], Jian Deng [a], Yanmei Wang [a], Qisong Hu [a], Congfa Li [a, *], Sixin Liu [a, b, **]

[a] College of Food Science and Technology, Hainan University, Haikou 570228, PR China
[b] College of Materials and Chemical Engineering, Hainan University, Haikou 570228, PR China

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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,

* Corresponding author.
** Corresponding author.
E-mail addresses: congfa@vip.163.com (C. Li), sixinliu@126.com (S. Liu).
[1] These authors contributed equally to this work.

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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).

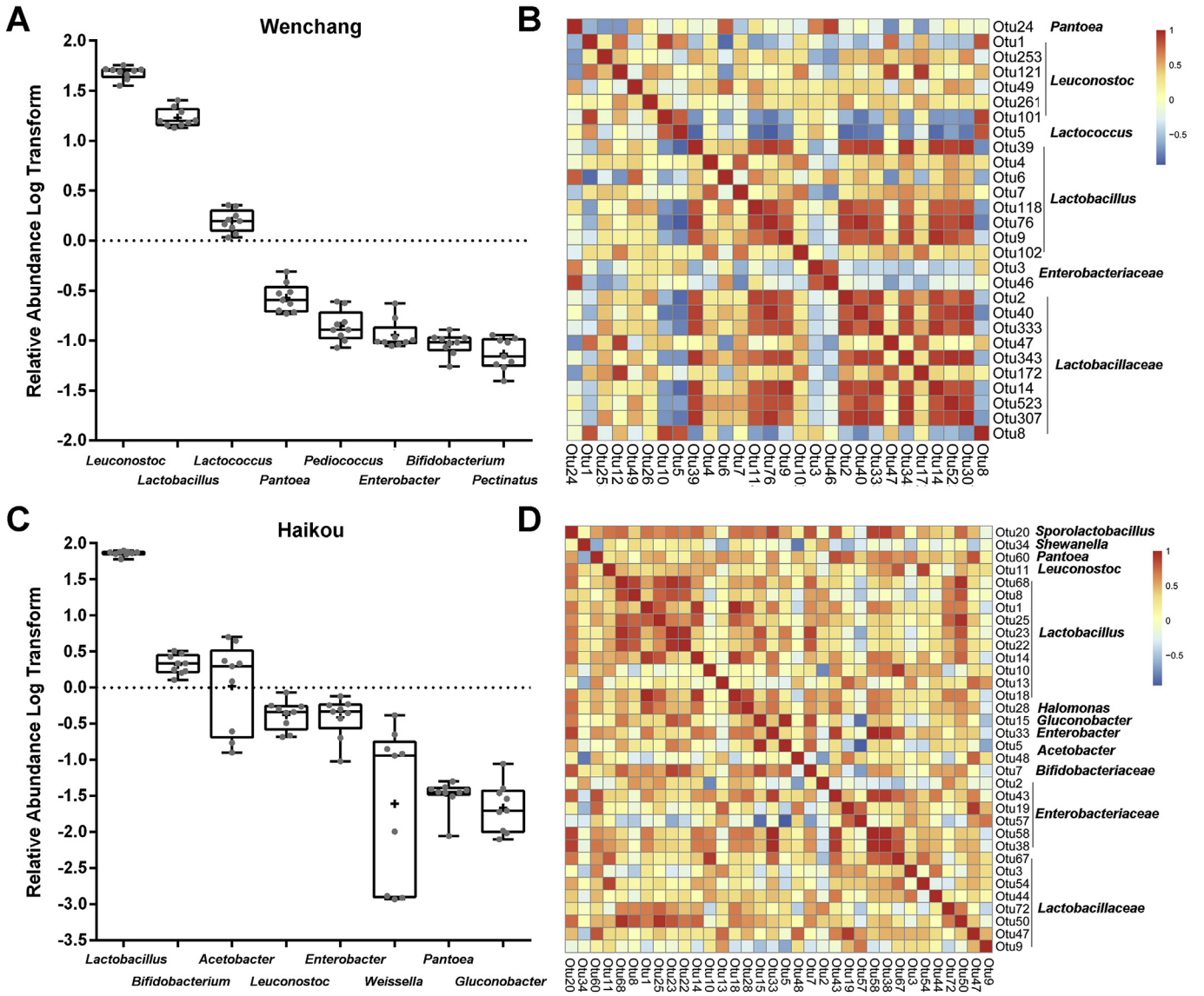

Fig. 1. The predominant genera and the core microbiota in coconut water during fermentation. (A and C) Box-plots showing bacterial composition at the generic level in the cities of Wenchang and Haikou (Hainan province of China); maximum and minimum values are indicated using whiskers. (B and D) Correlation matrix showing Spearman's rank correlation among the 28 (B, Wenchang) and 34 (D, Haikou) most abundant OTUs. Spearman's rank correlation coefficient ranges from 1.0 to −1.0 extending from a strongly positive to a strongly negative correlation.

3. Results

3.1. Sequencing coverage and estimation of bacterial alpha diversity

In our research, the microbial diversity and composition of the coconut water at different time points during fermentation were evaluated by the metagenomic sequencing approach. A dataset consisting of 353,279 filtered high-quality sequences (with an average of 20,782 sequences) was obtained for each sample. All sequences were clustered with representatives using a cutoff value of 97% sequence identity. The number of Operational Taxonomic Units (OTUs) varied between 50 and 183 (Table 1), and the bacterial alpha diversity (Chao 1, Simpson, and Shannon and Ace indices) in each sample was calculated (Table 1). From the table, it can be observed that the microbial alpha diversity increased along with the fermentation time, which indicated changes in the abundance and the species in the microbiota; however, these changes were not significant.

3.2. Composition of the microbiota during coconut water pre-fermentation

To probe the bacterial diversity during coconut water fermentation, we listed the predominant bacteria (relative abundance>0.1%) at the generic level. For samples from the Wenchang area, the relative abundance of *Leuconostoc, Lactobacillus, Lactococcus, Pantoea, Pediococcus, Enterobacter, Bifidobacterium* and *Pectinatus* was greater than 0.1% (Fig. 1A). For samples from Haikou, *Lactobacillus, Bifidobacterium, Acetobacter, Leuconostoc, Enterobacter* and *Weissella* were the predominant genera (Fig. 1C).

3.3. Core microbes during coconut water pre-fermentation

The core microbiota could be defined as the microbes that were likely to play a key role in a biological process. To explore the common core microbiota in all coconut water samples during pre-fermentation, we identified high frequency OTUs in each sample. A

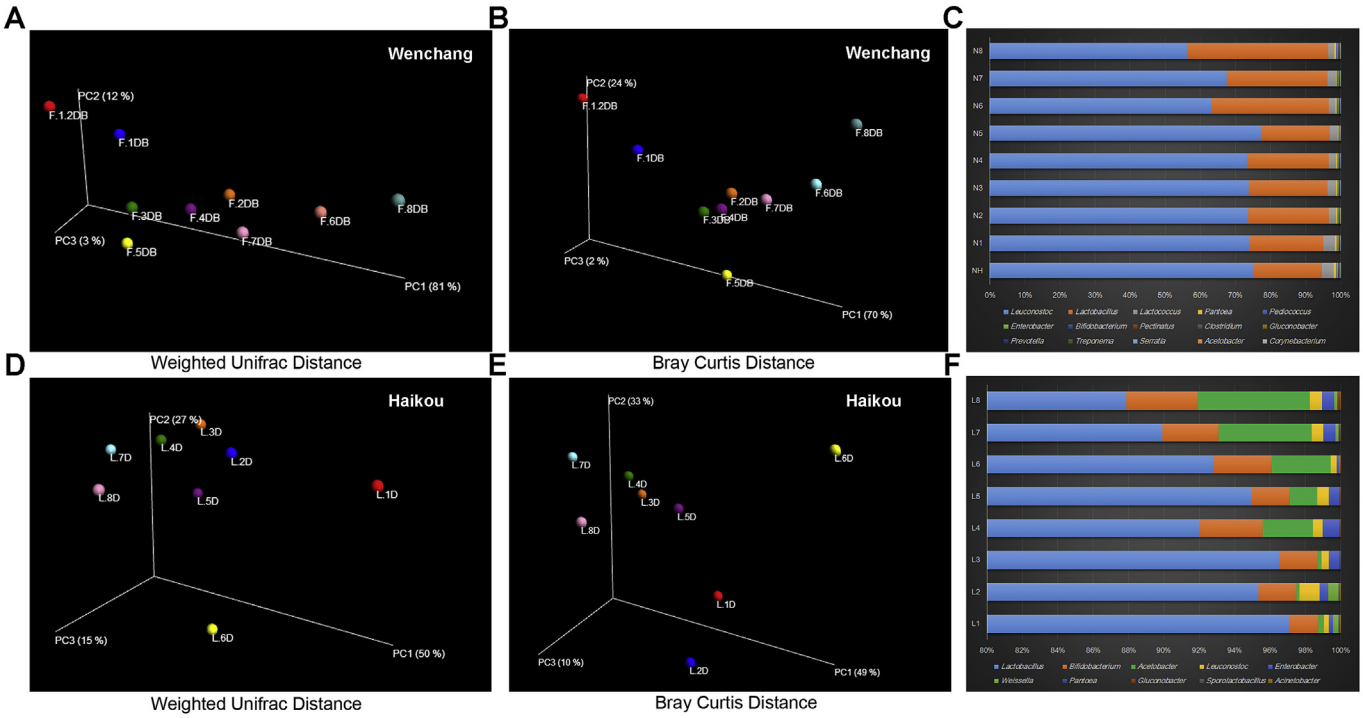

Fig. 2. The composition of and changes in the microbiota in coconut water during fermentation. (A and D) PCoA score plot based on Weighted UniFrac metrics (A, Wenchang; D, Haikou). Each point represents the composition of the microbiota of one sample at a single time point. (B and E) PCoA score plot based on Bray Curtis metrics (B, Wenchang; E, Haikou). Each point represents the composition of the microbiota of one sample at a single time point. (E and F) The predominant changes at the generic level (C, Wenchang; F, Haikou) during coconut water fermentation.

total of 28 and 34 core OTU candidates (the frequency of occurrence of these OTUs was greater than 95%) were identified in naturally fermented coconut water samples from Wenchang and Haikou, respectively. Each OTU had an average abundance greater than 0.1% in all samples. The correlations among these OTUs were reflected in Spearman's rank correlation coefficients (heatmap form, Fig. 1B and D). Identified using the RDP database, the 28 core OTUs in the samples from Wenchang primarily belonged to the genera *Leuconostoc, Lactococcus, Lactobacillus* and *Pantoea*. The 34 core OTUs in the samples from Haikou primarily belonged to the genera *Leuconostoc, Sporolactobacillus, Acetobacter, Gluconobacter, Enterobacter, Lactobacillus* and *Pantoea*.

3.4. Dynamic changes in the structure and composition of microbiota during pre-fermentation

To explore the changes in the structure and the composition of microbiota during coconut water fermentation, PCoA based on Weighted Unifrac distances (Fig. 2A and D) and Bray Curtis distances (Fig. 2B and E) was performed using the high-throughput sequencing data obtained from the coconut water samples at different fermentation time points from Wenchang and Haikou. The coloured points labelled with sample names represent the microbial community structure in coconut water samples at different time points from day 1 (or 12 h) to day 8. It can be observed that the structure of the microbial community in coconut water changed significantly during the 8-day fermentation. The results show that both the Weighted UniFrac distances and the Bray Curtis distances between samples on day 1 (or 12 Hours) and samples at other time points increased gradually, which indicated that changes in the microbial composition became increasingly significant with fermentation time. Since there were intrinsic differences in the microbial composition during coconut fermentation, we proceeded to identify these differences at the generic level (Fig. 2C and F). For samples from Wenchang, compared with the microbial composition on day 1, the genus *Lactobacillus* increased sharply. For samples from Haikou, *Acetobacter* and *Bifidobacterium* increased significantly while *Lactobacillus* decreased during fermentation.

3.5. Physicochemical indices and their correlations with the core microbes in coconut water during fermentation

We determined the pH and the lactic acid and acetic acid content at different time points during fermentation (Fig. 3A). It was observed that the pH values decreased significantly whereas the lactic acid and acetic acid increased significantly during the fermentation of coconut water. Notably, the most remarkable changes in the physicochemical indices (pH, lactic acid and acetic acid) occurred in the initial stages of fermentation (from day 0 to day 2).

To examine the mechanism of the coconut water fermentation, the correlations between the physicochemical indices and the core microbes were examined using Spearman's rank correlation coefficient (heatmap form, Fig. 3B and C). A significant positive correlation was observed between the OTUs representing *Lactobacillus* and *Acetobacter* and lactic acid. Furthermore, these OTUs were significantly negatively correlated with the pH value. Additionally, we found a significant negative correlation between the family *Enterobacteriaceae* and lactic acid.

4. Discussion

In our study, the metagenomic method was used to explore changes in the structure and diversity of microbiota during coconut water pre-fermentation. A dataset consisting of 353,279 filtered

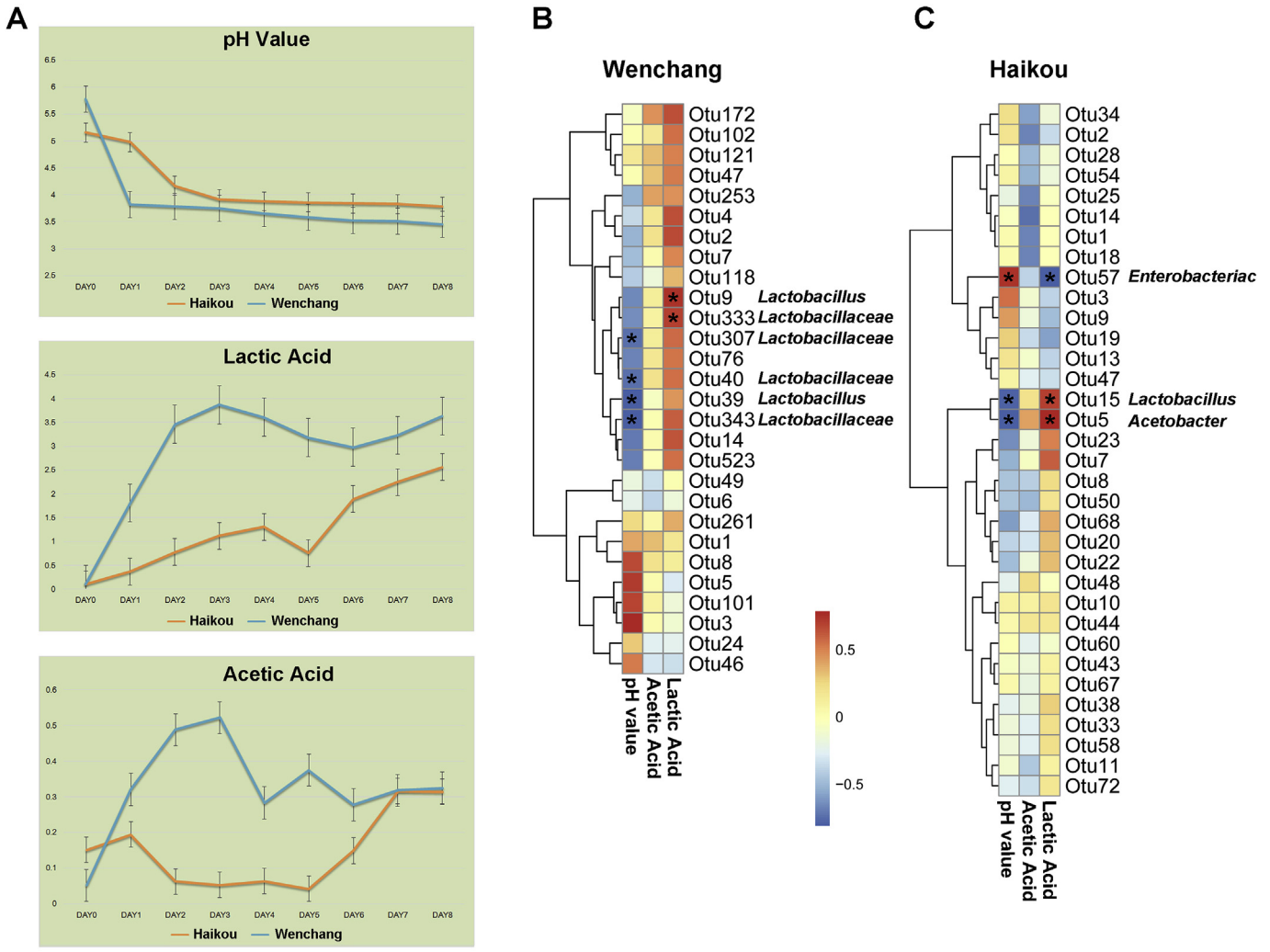

Fig. 3. The determination of physicochemical indices and their correlations with the core microbes during coconut water fermentation. (A) The changes in pH and lactic acid and acetic acid content at different time points during the course fermentation. (B and C) The correlations between the physicochemical indices and the core microbes revealed by Spearman's rank correlation coefficient (B, Wenchang; C, Haikou). Spearman's rank correlation coefficient ranges from 0.5 to −0.5 extending from a strongly positive to a strongly negative correlation.

high-quality sequences (with an average of 20,782 sequences) was obtained for each sample. Although the individual rarefaction curve plateaued with the sequencing data used, it failed to reach the saturation phase; the Shannon diversity estimates of the samples reached stable values. These results indicate that although new phylotypes would be expected with additional sequencing, most of the microbial diversity had already been captured. We found that the genera *Lactobacillus, Leuconostoc* and *Lactococcus* were the predominant microbes during pre-fermentation in the samples from Wenchang and Haikou. Furthermore, the relative abundance of the sequences of the above genera reached 45–60% of all reads. Interestingly, *Lactobacillus, Leuconostoc* and *Lactococcus* belong to the lactic acid group of bacteria. Generally, the lactic acid bacteria are a group of Gram positive bacteria that can ferment glucose, fructose and other sugars to lactic acid; these bacteria are beneficial to human heath (Wagar, Champagne, Buckley, Raymond, & Green-Johnson, 2009). Upon measuring the physicochemical indices, we observed that the pH declined even as the amount of lactic acid and acetic acid increased. Moreover, a significant positive correlation was found between the core OTUs representing *Lactobacillus* and lactic acid. Therefore, we can attribute the production of lactic acid and acetic acid in coconut water during fermentation to the existing lactic acid bacteria. The production of lactic acid and acetic acid would create a favourable acidic environment for further fermentation by *Acetobacter* (D. M. Almeida, Prestes, da Fonseca, Woiciechowski, & Wosiacki, 2013; Verschuren, Cardona, Nout, De Gooijer, & Van den Heuvel, 2000). Additionally, the lactic acid and acetic acid are the main sources of flavour in the fermented product (Sawada et al., 2015).

Interestingly, it has been found that the nata de coco yield is much higher, occasionally greater than ten times, when the fresh coconut water is exposed to the natural environment for several days for natural fermentation. Drawing on the results of the present study, we attribute this phenomenon to the interactions between *Lactobacillus* and *Acetobacter*. In previous studies, researchers found that co-cultivation of *Acetobacter xylinum* (currently called *Komagataeibacter xylinus*) and *Lactobacillus mali* in corn steep liquor resulted in a three-fold higher cellulose yield when compared to a monoculture of *Acetobacter xylinum* (Seto et al., 2006). An explanation for the enhancement of cellulose production is that certain enzymes involved in sucrose metabolism and/or the exocellular polysaccharide formation in *Lactobacillus mali* facilitate metabolic flow leading to cellulose formation in *Acetobacter xylinum* (Branda, Vik, Friedman, & Kolter, 2005). Additionally, we identified

pathogenic bacteria and conditional pathogenic bacteria in pre-fermented coconut water, such as *Pantoea, Serratia and Enterobacter,* which are associated with infectious disease in humans and constitute a risk to food safety (Dutkiewicz, Mackiewicz, Lemieszek, Golec, & Milanowski, 2015; Martins, Raposo, Baptista, & Almeida, 2015; Montagnani et al., 2015). These harmful microbes were likely derived from the natural environment during pre-fermentation, with the abundant sources of carbon and nitrogen in coconut water providing suitable conditions for multiplication. Therefore, the present method of uncontrolled pre-fermentation used by the industry should be replaced by a fermentation method that incorporates a full understanding of the changes in the microbial community structure and diversity during coconut water pre-fermentation.

Natural pre-fermentation has enormous advantages, which include a stable micro-ecosystem, abundant microbial diversity, various beneficial metabolic products and the production of flavour-imparting substances. However, the natural fermentation process is hard to replicate and carries the risk of various unintended contaminants and pathogenic microorganisms. These factors are difficult to control and may cause instability and even failure in bacterial cellulose and nata de coco production. In this study, a high-throughput sequencing based metagenomic approach was used to examine the microbial diversity and composition of coconut water at different pre-fermentation time points. These results could be the basis for simulating and controlling the pre-fermentation process of coconut water. During the pre-fermentation process and subsequently the production of bacterial cellulose, we need to focus on the personal hygiene of operators who could bring exogenous contaminating bacteria. Therefore, a standard operating procedure needs to be developed (e.g., first, inoculate using a pure culture of the predominant bacterium). The data presented here could provide the theoretical basis for controlling and accelerating pre-fermentation, inhibiting harmful microbial contaminants, eliminating pathogens and finally, raising the yield of nata de coco and thereby establish the foundation for further research on the mechanisms bacterial cellulose synthesis. In this research, the analysis of microbiota presented here could provide a promising method for controlling the upstream pre-fermentation process of coconut water and help shed new light on the process of bacterial cellulose synthesis. This would significantly improve our understanding of the predominant microbes and their metabolites in pre-fermented coconut water and thus enhance the stability and yield in the industrialized production of bacterial cellulose.

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