



Microbial analysis of the production line for roasted seaweed snacks

Feifei Zhou^{a,b,c,d,1}, Zhen Zhu^{a,b,c,d,1}, Chenlong Wang^{a,b,c,d}, Siyao Zhao^{a,b,c,d}, Shuting Han^{a,b,c,d}, Likun Chen^{a,b,c,d}, Yi Ding^{a,b,c,d}, Chao Liao^f, Xiran Li^f, Yan Xu^e, Yuanxia Chen^{a,b,c,d}, Jie Yang^{a,b,c,d}, Saikun Pan^{a,b,c,d}, Wenbin Wang^{a,b,c,d,**}, Lingzhao Wang^{a,b,c,d,***}, Luxin Wang^{f,*}

^a Jiangsu Key Laboratory of Marine Bioresources and Environment, Jiangsu Ocean University, Lianyungang, 222005, Jiangsu, China

^b Co-Innovation Center of Jiangsu Marine Bio-industry Technology, Jiangsu Ocean University, Lianyungang, 222005, Jiangsu, China

^c Jiangsu Key Laboratory of Marine Biotechnology, Jiangsu Ocean University, Lianyungang, 222005, Jiangsu, China

^d College of Marine Food and Bioengineering, Jiangsu Ocean University, Lianyungang, 222005, Jiangsu, China

^e Comprehensive Inspection and Testing Center of Ganyu District, Lianyungang, Jiangsu, 222005, China

^f Department of Food Science and Technology, University of California Davis, Davis, CA, 95618, USA

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ABSTRACT

Roasted seaweed snacks are getting popular worldwide. To reveal the main origins of microorganisms present in the roasted seaweed snacks, samples of raw materials, finished products, and environment (both food-contact and nonfood-contact surfaces) were collected from four plants. The total aerobic plate (APC), coliform, and mold counts of these samples were analyzed and the selected samples were also used for 16S rRNA sequencing. Results showed that dried laver contained the highest levels of APC among all raw ingredients, ranging from 2.72 to 6.95 Log CFU/g. Crushed pumpkin seeds and almond flakes had the highest coliform (up to 5.57 Log CFU/g) and mold counts (up to 2.16 Log CFU/g). The addition of seasoning to seaweed sandwich snacks increased the total microbial loads. The roasting process decreased the APC, coliforms, and mold counts of seaweed snacks by 0.22–1.73 Log CFU/g, >0.18–>2.46 Log CFU/g, and >0.18–>0.88 Log CFU/g respectively. The 16S rRNA sequencing analysis showed that the microbiota of roasted seaweed snacks was closely correlated with that of dried laver and the microbial load of roasted sandwich seaweed was primarily from the dried laver while less from microorganisms present in the processing plant environment and line workers.

1. Introduction

Roasted seaweed food products are a healthy seafood made with macroalgae (*Porphyra yezoensis* or nori). *P. yezoensis* is rich in health-benefiting polysaccharides (dietary fiber), proteins, taurine, and the vitamin B family (Chen & Zhang, 2019; Cho & Rhee, 2020). Seaweed foods are popular in Asian countries, primarily Japan, Korea, and China, which are dominant countries breeding and processing nori (Li et al., 2017; Park et al., 2021). In recent years, seaweed foods are increasing in popularity in the Western market (Banach et al., 2022; Cavallo et al., 2021). The annual output of the *P. yezoensis* cultivation and processing industry has increased from \$1.8 billion to \$6 billion (Li et al., 2014;

Løvdal et al., 2021). Popular seaweed products on the market include one-layer seasoned roasted seaweed, roasted seaweed sandwich snacks (two layers of laver filled with crushed or sliced nuts and seasonings in the middle), Sushi rolls (Japanese seaweed rice roll that consists of raw fish, seafood, rice, vegetables, and seaweed), seaweed rolls, and kimbap (Korean seaweed rice roll that consists of rice, vegetables, meat, and seaweed) (Cho & Rhee, 2020). Compared with the roasted seaweed used for sushi, kimbap, and seaweed rolls, seaweed sandwich snacks or single layer-seaweed snacks are typically prepared by adding seasoning powder, sesame seeds, and/or special ingredients like almond flakes to dried laver with additional roasting or baking before cooling, cutting, and packaging (Qian, et al., 2022).

* Corresponding author.

** Corresponding author. Jiangsu Key Laboratory of Marine Bioresources and Environment, Jiangsu Ocean University, Lianyungang, 222005, Jiangsu, China.

*** Corresponding author. Jiangsu Key Laboratory of Marine Bioresources and Environment, Jiangsu Ocean University, Lianyungang, 222005, Jiangsu, China.

E-mail addresses: wenbin66@jou.edu.cn (W. Wang), wanglz@jou.edu.cn (L. Wang), lxwang@ucdavis.edu (L. Wang).

¹ These authors contributed equally to this work.

As a ready-to-eat food, seaweed products have been reported to have microbial contamination and food safety concerns (Banach et al., 2020; Løvdal et al., 2021). Dried, shredded seaweed was found to be related to the seven outbreaks of norovirus illnesses in Japan from 2016 to 2017 (Sakon, et al., 2018). Korea's most popular ready-to-eat food kimbap has been implicated in several food poisoning outbreaks caused by *Staphylococcus aureus* and *Salmonella* (Rho & Schaffner, 2007). Roasted seaweed sandwich snacks have also been frequently reported to exceed the total aerobic plate count ($n = 5$, $c = 2$, $m = 4.48 \text{ Log CFU/g}$, $M = 5 \text{ Log CFU/g}$) and coliform count limits ($n = 5$, $c = 1$, $m = 1.30 \text{ Log CFU/g}$, $M = 1.48 \text{ Log CFU/g}$) associated with the national standards in China (National health commission of the PRC, 2016a; ICMSF, 1986). Qian et al. (2022) reported that the disqualification rate of roasted seaweed products had been averaging 10.00%. This microbial contamination problem brought huge economic losses to the local seaweed processing industry in coastal cities (Tiwari & Troy, 2015; Zheng et al., 2019).

Unfortunately, due to the limited available literature and microbial examination, the main microbial population structures of seaweed products and their sources remain unclear, which challenge the microbial control practice implemented by seaweed processing facilities. Through available studies, it has been shown that the total aerobic plate counts of dried laver, one major raw ingredient for seaweed snacks, range from 4.4 to 7.8 Log CFU/g (Choi et al., 2014). A recent study by Wang et al. (2021) indicated that personnel, machineries, raw materials, processing methods, and environment could all contribute to the microbial contamination in final products. However, both previous studies relied on culture-dependent plating methods, providing limited information about the uncultivable microbial populations present in the seaweed snack production line.

The next generation sequencing (NGS) using 16S rRNA gene analysis is a culture-independent microbial analysis method that can unbiasedly identify both culturable and uncultivable microorganisms in different food products, such as Spring Mix salad (Liao & Wang, 2021), poultry carcasses (Yu et al., 2019), and Chinese fermented vegetables (Xiao, et al., 2020). Tan et al. revealed the persistence of *L. monocytogenes* in the apple processing plants and the potential contribution of persisting *L. monocytogenes* to the final product contamination (Tan, et al., 2019), by combining the culture-dependent method and the 16S rRNA sequencing.

Thus, to reveal the main origin of microbial populations present in roasted seaweed snacks, raw materials, finished products (seaweed snacks), and environmental samples of four active seaweed snack production plants were collected. The microbial load (APC, coliform, and mold counts) of these samples were firstly evaluated by culture-dependent plating method. After that, a subgroup of samples was analyzed using culture-independent 16S rRNA sequencing.

2. Materials and methods

2.1. Study design

Four roasted seaweed processing plants located in Lianyungang, Jiangsu, China, were chosen in this study. All plants were equipped with epoxy flooring, air-conditioning system, air shower, changing room, hand washing and disinfection stations/devices. The workers wear working clothes, working cap, and disposable gloves at the plant. All plants were cleaned and disinfected after every daily operation. Two of the processing plants (Plants A and B) produce seaweed sandwich snacks, a snack made with two layers of lavers and filled with crushed or sliced nuts and flavoring ingredients in the middle. The production lines in these two roasted seaweed sandwich plants were semi-automated as shown in Fig. 1A. Plant A produced seaweed sandwich with crushed pumpkin seeds; Plant B produced seaweed sandwich with almond flakes. The other two processing plants (Plants C and D) made one-layer roasted seaweed snacks. The production lines in the two roasted seaweed plants were similar with the roasted seaweed sandwich plants, except no nut

filling was added besides seasonings or food-grade oil. Plant C produced one-layer roasted seaweed with no seasoning; Plant D produced one-layer roasted seaweed with seasoning. Samples of raw ingredients (dried laver, crushed or sliced nuts, and seasoning ingredients), final products, environmental samples collected from food contact surfaces (surface of the dried laver distributor, the conveyor belt of the dried laver distributor, inner surface of seasoning hopper, scale of roasted seaweed sandwich, grinding table for seaweed sandwich, inner surface of transit bag, surface of the slicing gear, placement tray of seaweed sandwiches, inner surface of packaging can) and non-food contact (handle of pumpkin seed adding scoop, handle of seasoning adding scoop, workbench for seaweed sandwich slicing, floor of processing areas), and swab samples collected from the gloves of processing plant personals. Samples were independently collected on three working days (three visits per plant) from March to June 2021. Right after collection, all samples were placed on ice and brought to the microbiology laboratory within 4 h of collection. Samples were stored at -80°C before DNA extraction and 16S rRNA sequencing. Fig. 1B provides a graphic abstract of this paper, showing the analytical steps applied to samples and the major results.

2.2. The production of roasted seaweed snacks and seaweed sandwich snacks

Fig. 1A shows the general workflow of the preparation of roasted seaweed snacks. It started with the first layer of dried laver being manually loaded and continuously distributed through a conveyor belt. As the first layer of laver going through the conveyor belt, seasoning ingredients, such as sugar, salt, powdered soy sauce, maltose powder, and sesame seeds, were added on top of the first layer of laver through a hopper. After that, a layer of almond flakes or crushed pumpkin seeds were added/sprinkled on top manually by line workers. In the end, the second layer of laver was added to complete the sandwich preparation. After assembly, all seaweed snacks (both single layer and the sandwich snacks) receive far-infrared heating at $180\text{--}240^{\circ}\text{C}$ for 30–50 s. Roasted products were cooled by using a fan and the uneven edges were manually removed by grinding/cutting on the stainless-steel tables. After a temporary ambient storage (25°C for 2–4 h), seaweed snacks were cut using a slicer (Dayang Machinery Co., Ltd, Lianyungang, Jiangsu, China) into smaller sizes (e.g., 9 cm \times 2.5 cm). The sliced seaweed sandwiches were weighed and packaged into cans or plastic bags before sealing. The typical composition of roasted seaweed sandwich snacks includes two layers (ca. 6.00 g) of dried lavers (23.08% of the total weight), maltose (ca. 6.25 g, 24.04%) and compound seasoning powder (ca. 6.25 g, 24.04%), sesame seeds (ca. 6.00 g, 23.08%), and crushed pumpkin seeds or almond flakes (ca. 1.50 g, 5.77%). The seasoning ingredients, including the maltose powder and compound seasoning powder (salt, sugar, powdered soy sauce), account for half of the weight of the final product. The proportion of maltose powder and compound seasoning powder varied between plants to produce different flavor. The composition of the flavored roasted seaweed snacks (single layer) includes one piece of laver (ca. 2.60 g, 61.91%), food-grade soybean oil (ca. 1.30 g, 30.95%), and seasonings (ca. 0.3 g, 7.14%).

2.3. Sample collection

All food and environmental samples (swab samples of food contact surfaces and non-food contact surfaces) were collected from the seaweed snack processing lines after 4 h of operation. This was to make sure that the sampling was a true representation of a working production line. Food samples, including raw materials (lavers, maltose powder, compound seasoning, sesames, crushed pumpkin seeds, almond flakes, and seasoning salt), unroasted and roasted seaweed snacks, sliced seaweed snacks, and the packaged seaweed snacks were randomly grabbed with sterile gloves and transferred to sterile stomach bags. Food contact surfaces and non-food contact surface environmental areas (5 cm \times 5

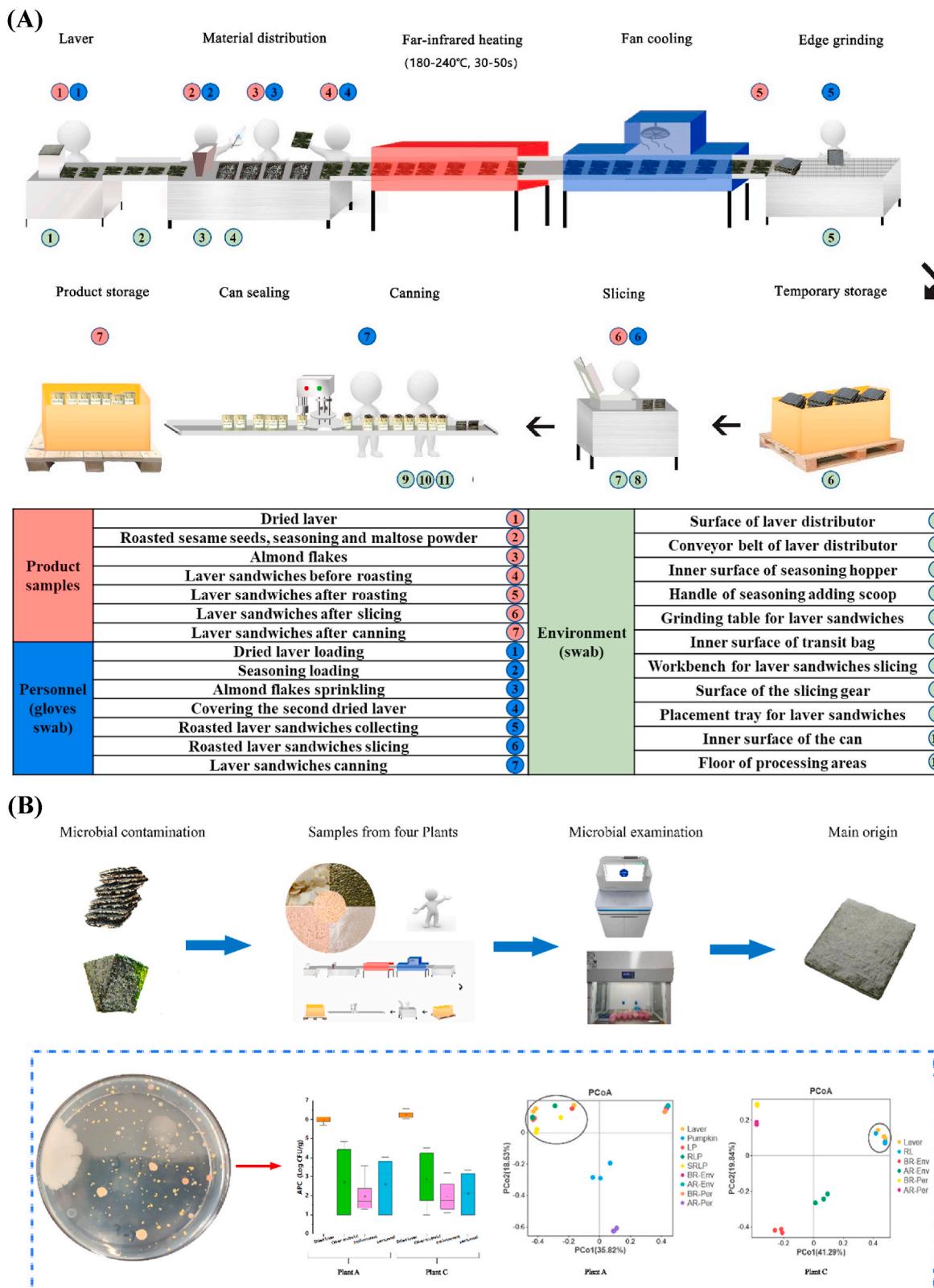


Fig. 1. (A) The processing workflow of roasted seaweed sandwich snack and sampling locations; (B) graphic abstract indicating the analyses applied to collected samples. While all samples went through culture-dependent analyses, selected samples collected from Plants A and C were sent for 16S sequencing. Results indicated that dried laver (the raw ingredient) contributed the most significantly to the final microbial populations present in roasted seaweed snacks (both seaweed snacks and single layer seaweed snacks).

cm) were sampled by swabbing using pre-moistened (each with 10 ml of sterile saline) sponges (HK-PS, Huankai Microbial, Guangzhou, Guangdong) with 10 horizontal and 10 vertical strokes. Disposable latex gloves of operating personals working at the loading stations of laver and seasoning powder, and in areas of roasted seaweed collecting, grinding, slicing, and packaging were sampled by swabbing with aforementioned pre-moistened sponges. All samples were collected in duplicate with one set used for APC, coliform, and mold counts analysis and the other set of samples used for sequencing analysis.

2.4. Culture-dependent microbial analysis

At the laboratory, the APC, coliform, and mold counts were evaluated following the standards issued by the Chinese National Health Commission (2016b, 2016c, 2016d). Briefly, 25 g of each sample were weighed and transferred into a sterile stomacher bag containing 225 mL of sterile saline. The mixture was homogenized in a smasher (HX-4GM, Huxi Industry Co., Ltd, Shanghai) for 1 min. A series of 10-fold dilutions was made and two 1 mL of each dilution were taken out from each dilution and used for mixing with duplicate liquid Plate Count Agar (PCA, 15–20 mL), crystal violet neutral red bile salt agar (VRBA, 15–20 mL), or Rose Bengal Chloramphenicol agar (RBC, 20–25 mL) for pour plating. The APC and coliform plates were incubated at 35 °C for 48 h and 24 h, respectively. After 24 h of incubation, 10 colonies on VRBA were picked and inoculated into the brilliant green bile lactose broth (BGLB) and cultured at 35 °C for 24 h–48 h. Positive BGLB tubes further confirmed suspect coliform colonies. For total mold counts, RBC plates were cultured at 28 °C for 5 days before enumeration.

2.5. 16S rRNA sequencing of the V3–V4 region

Total DNA was extracted from food and environmental samples by using the HiPure Soil DNA Kits (Magen, Guangzhou, China) following the manufacturer's protocols. The 16S rRNA V3–V4 region (~466 bp) of the ribosomal RNA gene was amplified with primers 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACHVGGGTATCTAAT) (Guo, et al., 2017). Purified amplicons were pooled and paired-end sequenced (PE250) on an Illumina Novaseq 6000 according to the standard protocols by Gene Denovo Biotechnology Co., Ltd (Guangzhou, China). All sequencing data has been uploaded in the NCBI database (PRJNA960839).

2.6. Bioinformatics analysis

Raw data was filtered using FASTP version 0.18.0 (Chen, et al., 2018). Specifically, reads containing more than 10% of unknown nucleotides or less than 50% of bases with quality score > 20 were removed. After the initial filtering, the paired-end clean reads were merged using FLASH version 1.2.11 (Magoč & Salzberg, 2011) with a minimum overlap of 10 bp and mismatch error rates of 2%. Noisy sequences of raw tags were filtered under specific filtering conditions (Bokulich, et al., 2013) to obtain high-quality clean tags. The filtering conditions were set as follows: first, break raw tags from the first low-quality base site where the number of bases in the continuous low-quality value (the default quality threshold ≤ 3) reached the set length (the default length 3 bp); second, filter tags whose continuous high-quality base length was less than 75% of the tag length. The clean tags were clustered into operational taxonomic units (OTUs) of $\geq 97\%$ similarity using UPARSE version 9.2.64 (Edgar, 2013). All chimeric tags were removed using the UCHIME algorithm (Edgar, et al., 2011), and effective tags (100,000) were acquired for further analysis. The most abundant tag sequence was chosen as a representative sequence within each cluster. SILVA (version 132) was used for taxonomy annotation. The host contamination caused by chloroplast and mitochondria genes from the laver were excluded (Beckers, et al., 2016). Further analysis, including microbiota composition, α -diversity, and β -diversity, was

performed using Omicsmart (<http://www.omicsmart.com>), a real-time interactive online platform for data analysis (Zhong, et al., 2022).

2.7. Statistical analysis

Experiments were performed in triplicate (three visits for each plant) and duplicated for samples taken for microbiological analysis. Bacterial counts were converted to Log_{10} CFU/g or Log_{10} CFU/cm² or per glove and expressed as mean \pm standard deviation. The analysis of variance (ANOVA) of microbial levels between samples was performed with the software SPSS® 26 (IBM Co.) with a significant level of $p < 0.05$. For α -diversity, the Kruskal-Wallis method was used to test the variance among all groups and the Tukey-honestly significant difference HSD test was used to test the difference between two groups. For β -diversity, Adonis (PERMANOVA) was used to test the variance between all the groups and the Welch's T test was used to test the difference between two groups.

3. Results

3.1. Microbial levels of product and environmental samples

Tables 1–4 summarize the culture-dependent analyses of all samples. As explained earlier, Plants A and B produce roasted seaweed sandwich snacks which require more raw ingredients than single layer roasted seaweed snacks produced by Plants C and D. Dried laver (the common ingredient used by all four plants) exhibited the highest APC counts compared to other raw ingredients with high variations, ranging from 2.72 to 6.95 Log CFU/g. The other raw ingredients that contained high APC counts included crushed pumpkin seeds (5.94 Log CFU/g, Plant A) and almond flakes (4.52 Log CFU/g, Plant B). Crushed pumpkin seeds also had the highest coliform counts compared to all other raw ingredients, 5.57 ± 0.14 Log CFU/g. The addition of crushed pumpkin seeds to the seaweed sandwich snacks (before roasting) brought the coliform levels of seaweed sandwich snacks to 3.46 ± 0.27 Log CFU/g. Both almond flakes and crushed pumpkin seeds had higher mold counts compared to other raw ingredients. The addition of almond flakes (2.16 ± 0.11 Log CFU/g) and crushed pumpkin seeds (1.95 ± 0.31 Log CFU/g) made the seaweed sandwich containing mold levels of approximately 1.64 Log CFU/g.

Across all plants, roasting, in general, reduced the microbial load, although not significantly. For seaweed sandwich snacks, the roasting step reduced APC counts by 0.34 (Plant A) and 1.73 (Plant B) Log CFU/g respectively. The roasting reduced >2.46 Log CFU/g of coliforms and >0.88 Log CFU/g of molds in Plant A, and 1.01 Log CFU/g of coliforms and >0.4 Log CFU/g of molds in Plant B. For single layer seaweed snacks, the roasting reduced 0.22 (Plant C) to 1.72 Log CFU/g (Plant D) of APC, 0.18 Log CFU/g of coliform, and up to 0.5 Log CFU/g of molds (Plants C and D). All consecutive operations, including slicing and canning, did not appear to alter the APC counts by a significantly amount ($p < 0.05$). Coliforms were only detected in the raw ingredients but not in the finished laver products (Tables 1–4).

When looking at the environmental samples, no discernible pattern emerges when comparing the APC (Aerobic Plate Count) values of samples collected from food contact surfaces (FCS) with those collected from non-food contact surfaces (NFCS). In Plant A, the APC for FCS ranged from <1 to 3.58 log CFU/cm², while for NFCS, it ranged from 1.58 to 2.00 log CFU/cm². Plant B exhibited an overall higher APC compared to the other three plants, with a range of <1 to 3.37 log CFU/cm² for FCS and 2.79 to 4.82 log CFU/cm² for NFCS. The APC values for FCS in plant C ranged from <1 to 2.22 log CFU/cm² while NFCS had values ranging from <1 to 1.64 log CFU/cm². In Plant D, the APC for FCS ranged from <1 to 0.7 log CFU/cm². Only one NFCS sample was examined in Plant D, specifically the floor of the processing areas, and it had an APC of 1.48 log CFU/cm². Coliforms and molds were not detected in the majority of the environmental samples. However, it is

Table 1

Microbial levels of raw ingredients and product samples, as well as food contact and nonfood contact surfaces collected from Plant A.

Sample types/samples	APC	Coliforms	Molds
Ingredients and products			
Dried laver	Log CFU/g		
Dried laver	6.95 ± 0.24	1.00 ± 0.07	1.00 ± 0.24
Roasted sesame seeds	2.00 ± 0.18	<1	0.70 ± 0.07
Seasoning	1.40 ± 0.09	<1	<1
Maltose powder	0.70 ± 0.13	<1	0.70 ± 0.23
Crushed pumpkin seeds	5.94 ± 0.19	5.57 ± 0.14	1.95 ± 0.31
Laver sandwiches ^a before roasting	6.17 ± 0.33	3.46 ± 0.27	1.88 ± 0.30
Laver sandwiches after roasting	5.83 ± 0.21	<1	<1
Laver sandwiches after slicing	5.46 ± 0.16	<1	<1
Laver sandwiches after canning	5.12 ± 0.22	<1	1.16 ± 0.24
Environment			
Surface of laver distributor	Log CFU/cm ²		
Surface of laver distributor	2.68 ± 0.23	1.04 ± 0.26	<1
Conveyor belt of laver distributor	3.58 ± 0.18	1.26 ± 0.23	<1
Inner surface of seasoning hopper	<1	<1	1.05 ± 0.34
Handle of pumpkin seed adding scoop	2.00 ± 0.11	0.41 ± 0.11	<1
Handle of seasoning adding scoop	1.04 ± 0.05	<1	0.86 ± 0.06
Scale for roasted laver sandwiches	2.42 ± 0.46	<1	<1
Grinding table for laver sandwiches	1.60 ± 0.25	<1	0.45 ± 0.22
Inner surface of transit bag	0.70 ± 0.15	<1	<1
Workbench for laver sandwiches slicing	2.23 ± 0.31	0.79 ± 0.17	0.51 ± 0.24
Surface of the slicing gear	0.66 ± 0.12	<1	<1
Placement tray for laver sandwiches	0.15 ± 0.27	<1	0.26 ± 0.27
Inner surface of the can	<1	<1	<1
Floor of processing areas	1.58 ± 0.31	<1	0.20 ± 0.23
Personnel (Gloves of line workers at following stations)			
Dried laver loading	Log CFU/glove		
Dried laver loading	3.61 ± 0.15	2.16 ± 0.25	2.10 ± 0.05
Seasoning sub packaging	2.02 ± 0.15	<1	0.70 ± 0.11
Seasoning loading	2.56 ± 0.06	<1	1.70 ± 0.15
Crushed pumpkin seeds sprinkling	3.77 ± 0.11	<1	1.60 ± 0.11
Covering the second dried laver	2.33 ± 0.25	<1	1.94 ± 0.25
Roasted laver sandwiches collecting	5.62 ± 0.30	1.78 ± 0.13	2.76 ± 0.11
Roasted laver sandwiches slicing	2.52 ± 0.10	1.48 ± 0.30	1.65 ± 0.18
Desiccant loading	2.56 ± 0.16	<1	1.98 ± 0.24
Laver sandwiches canning	2.85 ± 0.29	<1	2.41 ± 0.12

^a Laver sandwiches represent dried laver snacks filled with seasoning, maltose powder, roasted sesame seeds, and crushed pumpkin seeds.

worth noting that a substantial 2.95 log CFU/cm² of coliforms in Plant B were detected at the handle of the seasoning adding scoop, where the highest APC across all environmental samples in all plants was observed (Tables 1–4).

Table 2

Microbial levels of raw ingredients and product samples, as well as food contact and nonfood contact surfaces collected from Plant B.

Sample types/samples	APC	Coliforms	Molds
Ingredients and products			
Dried laver	Log CFU/g		
Dried laver	5.80 ± 0.20	<1	<1
Roasted sesame seeds	1.74 ± 0.16	<1	1.05 ± 0.18
Seasoning	2.75 ± 0.08	<1	1.30 ± 0.11
Maltose powder	0.35 ± 0.15	<1	0.70 ± 0.23
Almond flakes	4.52 ± 0.13	0.70 ± 0.14	2.16 ± 0.11
Laver sandwiches ^a before roasting	4.35 ± 0.24	1.30 ± 0.07	1.40 ± 0.09
Laver sandwiches after roasting	2.62 ± 0.58	0.29 ± 0.11	<1
Laver sandwiches after slicing	2.67 ± 0.16	1.00 ± 0.16	<1
Laver sandwiches after canning	2.61 ± 0.17	<1	1.60 ± 0.13
Environment			
Surface of laver distributor	Log CFU/cm ²		
Surface of laver distributor	2.32 ± 0.07	<1	<1
Conveyor belt of laver distributor	2.95 ± 0.16	<1	0.34 ± 0.21
Inner surface of seasoning hopper	2.82 ± 0.15	<1	<1
Handle of almond flakes adding scoop	2.80 ± 0.08	<1	<1
Handle of seasoning adding scoop	4.82 ± 0.12	2.95 ± 0.15	<1
Scale for roasted laver sandwiches	1.01 ± 0.21	<1	<1
Grinding table for laver sandwiches	1.53 ± 0.22	<1	<1
Inner surface of transit bag	0.38 ± 0.07	<1	<1
Workbench for laver sandwiches slicing	3.03 ± 0.18	<1	<1
Surface of the slicing gear	2.37 ± 0.16	<1	<1
Placement tray for laver sandwiches	3.37 ± 0.09	<1	<1
Inner surface of the can	<1	<1	<1
Floor of processing areas	2.79 ± 0.76	<1	0.75 ± 0.30
Personnel (Gloves of line workers at following stations)			
Dried laver loading	Log CFU/glove		
Dried laver loading	4.35 ± 0.18	<1	0.70 ± 0.11
Seasoning sub packaging	4.84 ± 0.12	1.48 ± 0.35	3.10 ± 0.08
Seasoning loading	3.54 ± 0.22	<1	1.88 ± 0.03
Almond flakes sprinkling	5.38 ± 0.19	2.66 ± 0.14	2.11 ± 0.14
Covering the second dried laver	4.20 ± 0.61	<1	1.00 ± 0.30
Roasted laver sandwiches collecting	3.40 ± 0.23	0.43 ± 0.21	0.76 ± 0.56
Roasted laver sandwiches slicing	3.86 ± 0.13	1.88 ± 0.22	1.00 ± 0.04
Desiccant loading	2.63 ± 0.24	<1	0.70 ± 0.08
Laver sandwiches canning	4.00 ± 0.31	1.35 ± 0.65	0.50 ± 0.70

^a Laver sandwiches represent dried laver snacks filled with seasoning, maltose powder, sesame seeds, and almonds.

Due to the variations in production procedures across different plants, samples collected from the gloves of the workers also varied. In Plants A and B, where laver sandwiches were produced, Plant B consistently had higher APC counts compared to Plant A. The APC

Table 3

Microbial levels of raw ingredients and product samples, as well as food contact and nonfood contact surfaces collected from Plant C.

Sample types/samples	APC	Coliforms	Molds
Ingredients and products			
Dried laver	Log CFU/g		
	6.58 ±	<1	1.88 ±
	0.31		0.25
Laver after roasting	6.36 ±	<1	1.30 ±
	0.06		0.22
Laver after packaging	6.12 ±	<1	1.54 ±
	0.29		0.12
Environment			
Surface of laver distributor	Log CFU/cm ²		
	1.78 ±	<1	<1
	0.26		
Conveyor belt of laver distributor	2.22 ±	<1	<1
	0.23		
Conveyor belt of roasted laver	<1	<1	<1
Workbench for roasted laver packaging	<1	<1	<1
Placement tray for roasted laver	<1	<1	0.45 ±
			0.14
Inner surface of plastic bags	<1	<1	<1
Floor of the processing areas	1.64 ±	<1	<1
	0.34		
Personnel (Gloves of line workers at following stations)			
Dried laver loading	Log CFU/glove		
	3.56 ±	<1	1.30 ±
	0.30		0.07
Roasted laver collecting	1.00 ±	<1	1.44 ±
	0.11		0.18
Roasted laver bagging	1.35 ±	<1	1.77 ±
	0.35		0.24
Plastic packaging	3.01 ±	<1	0.70 ±
	0.16		0.21

Table 4

Microbial levels of raw ingredients and product samples, as well as food contact and nonfood contact surfaces collected from Plant D.

Sample types/samples	APC	Coliforms	Molds
Ingredients and products			
Dried laver	Log CFU/g		
	2.72 ±	1.18 ± 0.05	1.18 ±
	0.28		0.20
Seasoning salt	2.50 ±	<1	<1
	0.05		
Laver after roasting	1.00 ±	<1	<1
	0.30		
Laver after slicing	1.33 ±	<1	1.00 ±
	0.15		0.40
Laver after packaging	0.98 ±	<1	<1
	0.28		
Environment			
Surface of laver distributor	Log CFU/cm ²		
	<1	<1	<1
Conveyor belt of laver distributor	0.20 ±	<1	<1
	0.05		
Inner surface of salt hopper	<1	<1	<1
Conveyor belt of roasted laver	<1	<1	<1
Scale for roasted laver	0.62 ±	<1	<1
	0.11		
Surface of the slicing gear	<1	<1	<1
Placement tray for roasted laver	0.70 ±	<1	<1
	0.07		
Inner surface of plastic bags	<1	<1	<1
Floor of the processing areas	1.48 ±	1.29 ± 0.08	0.51 ±
	0.19		0.07
Personals (Gloves of line workers at following stations)			
Dried laver loading	Log CFU/glove		
	2.91 ±	<1	<1
	0.11		
Roasted laver collecting	1.82 ±	0.70 ± 0.13	<1
	0.13		
Roasted laver bagging	2.16 ±	<1	<1
	0.11		
Plastic packaging	2.26 ±	<1	1.43 ±
	0.27		0.13

ranged from 2.02 to 5.62 Log CFU/glove for Plant A and 2.63 to 5.38 Log CFU/glove for Plant B. On the other hand, relatively lower microbial loads were detected in Plants C and D compared to Plants A and B, where roasted single layer laver was the primary product. The APC values for glove samples from Plants C and D ranged from 1.0 to 3.56 Log CFU/glove and 1.82 to 2.91 Log CFU/glove, respectively (Tables 1–4).

3.2. Microbiota of material, environmental, and operating personnel samples

Samples collected from Plants A and C were further analyzed via 16S rRNA sequencing given their higher microbial loads on raw ingredients, assembled and finished products. The average number of reads per sample was 12,316 reads (range: 8294–15,847) for plant A and 19,813 reads (range: 18,361–21,311) for plant C, respectively.

Chao1 richness and Shannon indices were used to quantify microbial species richness and diversity in collected samples. In Plant A, alpha diversity indices in BR-Env, AR-Env, and BR-Per samples were significantly higher ($p < 0.050$) than those in the raw ingredient and finished product samples (i.e., laver, pumpkin, LP, RLP, and SRLP), as indicated by the Tukey-HSD test. Interestingly, the alpha diversity of AR-Per samples was significantly lower ($p < 0.050$) compared to that of BR-Per samples (Fig. 2A). In contrast, in Plant C, the alpha diversities of BR-Env were comparable to those of the raw ingredients and finished product (i.e., laver and RL). AR-Env displayed considerably lower alpha diversities compared to the aforementioned three types of samples ($p < 0.050$). BR-Per exhibited the highest diversities, which were comparable to the AR-Per samples (Fig. 2B).

For Plant A, the overall dominant bacterial families in dried laver and finished products were similar. The major bacterial family include Flavobacteriaceae (45.44%–58.35%), Moraxellaceae (3.04%–8.47%), Enterobacteriaceae (1.98%–7.73%), Staphylococcaceae (3.62%–7.41%), Bacillaceae (1.74%–2.62%), Burkholderiaceae (2.04%–3.32%), Chitinophagaceae (1.92%–2.92%), Rhodobacteraceae (1.91%–2.47%), Pseudomonadaceae (1.01%–1.49%), and Vibrionaceae (0.69%–1.47%). The microbial composition of pumpkin seeds differed from that of laver, with lower percentage of Flavobacteriaceae (2.52%), higher percentages of Enterobacteriaceae (24.37%) and Staphylococcaceae (15.84%). However, the addition of this ingredient did not significantly impact the taxonomical profile of the finished products ($p > 0.4$). Additionally, roasting did not appear to have a significant ($p > 0.4$) influence on the microbial composition of the finished products (Fig. 4A). Notably, the reduced alpha diversity observed in AR-Per (Fig. 3A) could be a result of the dominance of Moraxellaceae (58.96%), Enterobacteriaceae (13.72%), and Bacillaceae (15.68%) (Fig. 4A). A few human-pathogenic species, such as *Acinetobacter baumannii*, *Salmonella*, *Shigella*, and *Bacillus cereus*, are known to be members of these families. Ordination analyses supported these findings, with laver, LP, RLP, and SRLP samples forming one group and distinctly separating from pumpkin seed and AR-Per samples (Fig. 5A).

In terms of Plant C, similar observations were made; the raw ingredient laver and the finished product RL exhibited similar microbial compositions, with the dominant families being Flavobacteriaceae (35.55%–38.66%), Rhodobacteraceae (25.87%–32.60%), Moraxellaceae (2.17%–5.45%), Staphylococcaceae (0.24%–4.19%), and Saprospiraceae (2.72%–8.72%). Enterobacteriaceae was found in high abundance in BR-Env (21.14%) but significantly ($p < 0.05$) reduced in AR-Env (3.09%). A similar trend was also observed when comparing BR-Per and AR-Per (Fig. 4B). The principal-coordinate analysis (PCoA) plot (Fig. 5B) demonstrated that laver and RL samples clustered together, while other types of samples formed distinct groups. This alignment with the results obtained from the microbial composition analysis (Fig. 4B) further validates the findings.

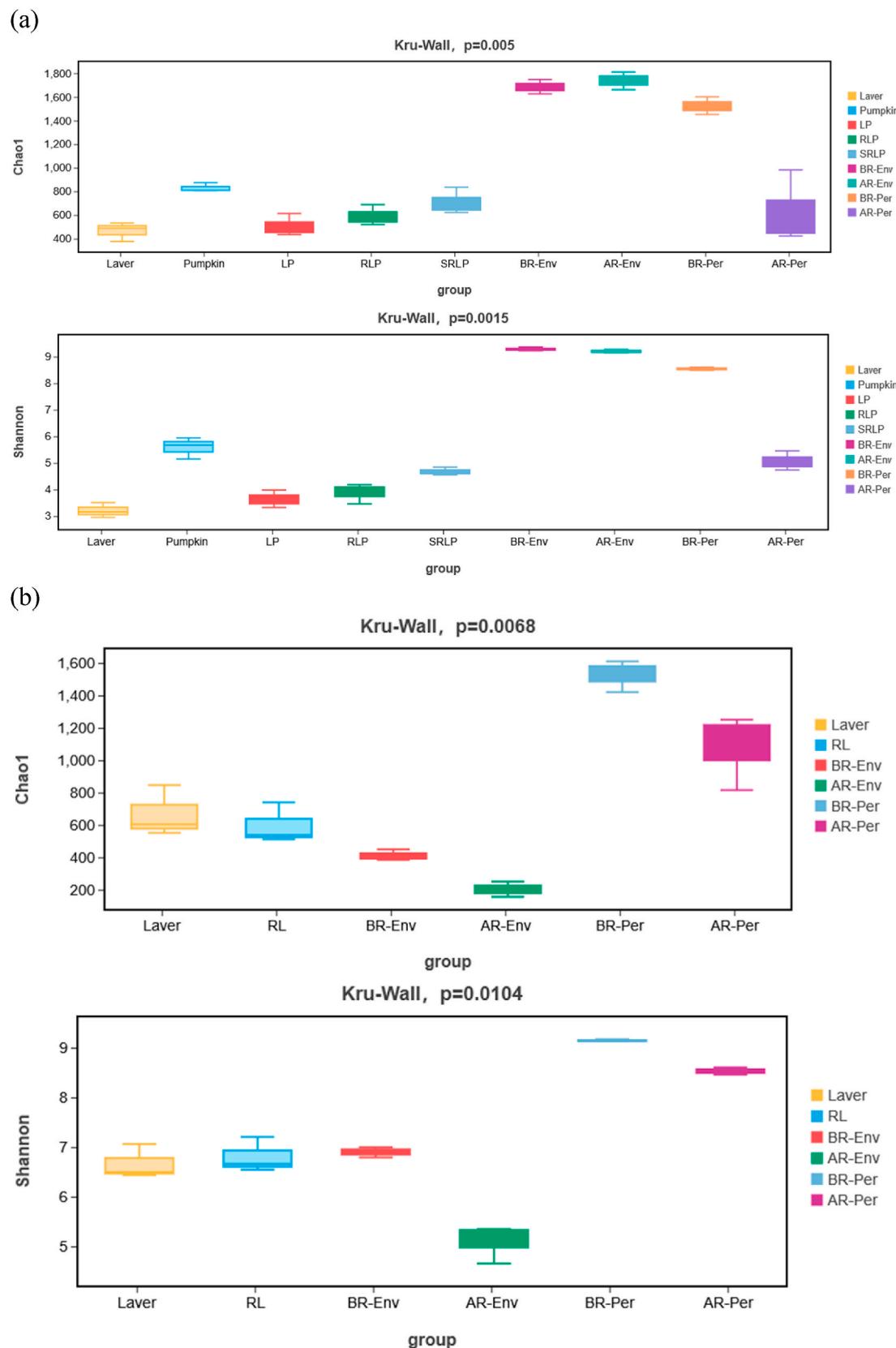


Fig. 2. The α -diversity (Chao1 and Shannon indices) of microbiota present in raw materials or product, environmental, and operating personnel samples collected from Plant A (a) and Plant C (b). Pumpkin represents the crushed pumpkin seeds; LP represents pumpkin seeds laver sandwiches before roasting; RLP represents roasted pumpkin seeds laver sandwiches; SRLP represents sliced roasted pumpkin seeds laver sandwiches. RL represents roasted laver (single layer seaweed snacks after heat treatment); BR-Env represents swab samples collected from food contact surfaces before the roasting process. AR-Env represents swab samples from food contact surfaces after the roasting process. BR-Per represents swab samples of line workers' hands before the roasting process. AR-Per represents swab samples of line workers' hands after the roasting process.

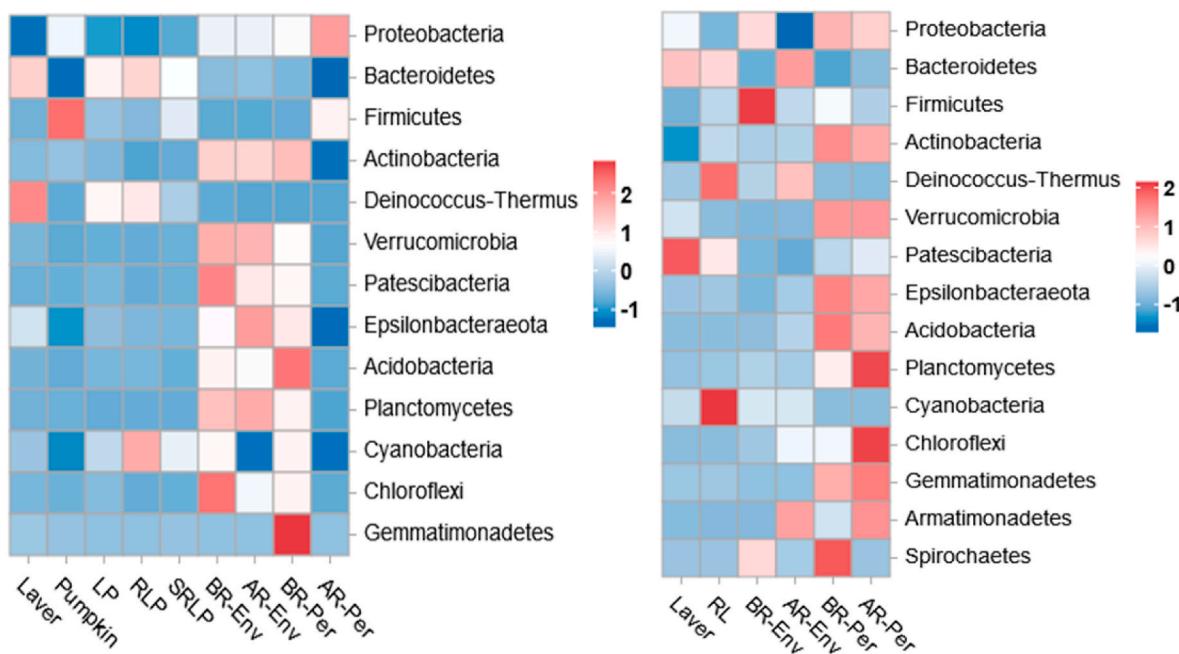


Fig. 3. Microbial composition at the phylum level (abundance heatmap) of raw materials or product, environmental, and operating personnel samples collected from processing Plant A (left) and Plant C (right). Refer to [Fig. 2](#) caption for explanation of the sample labels and abbreviations.

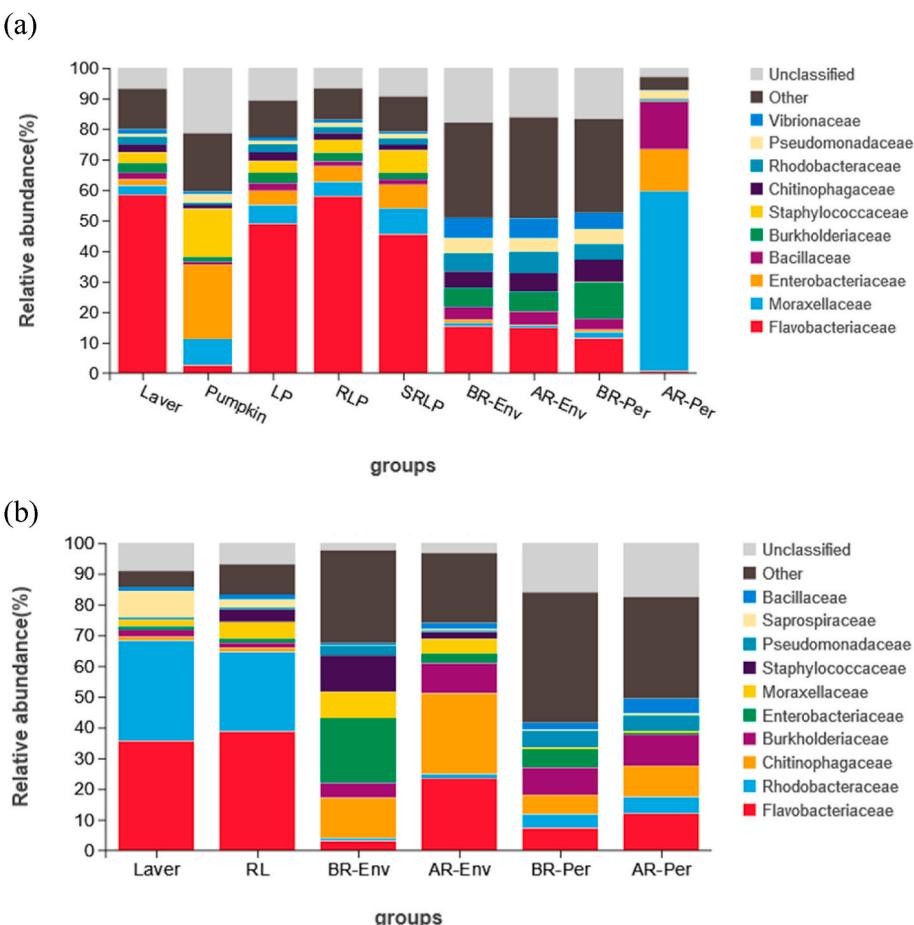


Fig. 4. The microbial composition at the family level of raw materials, and product, environmental, and operating personnel samples collected from processing Plant A (top) and Plant C (bottom). Refer to [Fig. 2](#) caption for explanation of the sample labels and abbreviations.

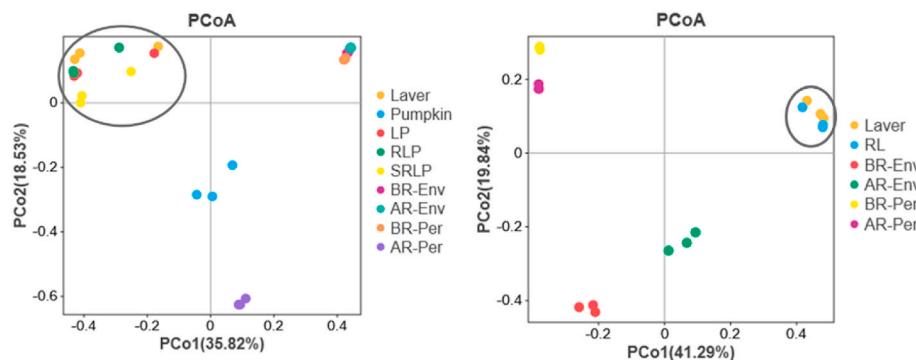


Fig. 5. Principal co-ordinate analyses (β -diversity) of the raw materials, and product, environmental, and operating personnel samples collected from Plant A (left) and Plant C (right). Refer to Fig. 2 caption for explanation of the sample labels and abbreviations.

4. Discussion

In this study, the microbial levels and composition of seaweed snacks (sandwich snacks and single layer seaweed snacks) were analyzed by using culture-dependent and culture-independent methods. The high APC levels identified with dried laver were consistent with previous microbial studies (Choi et al., 2014; Kim et al., 2015; Kwang-Tae et al., 2014), in which the APC of dried laver ranged 6.06–7.96 Log CFU/g. Our recent study showed that the first drying step (around 45 °C, 2.5 h) could increase the APC in dried laver to 1.87–3.33 Log CFU/g when the second gradient air drying (35–90 °C, 3–4 h) provided very limited decontamination (Wang, et al., 2023). The development of heat resistance or heat tolerance by bacteria can be explained from the following perspectives. First, the first hot-air drying step could promote the proliferation of heat-resistant bacteria. Second, the low water activity (a_w) in dried laver enhanced the thermal tolerance of surviving microorganisms such as *Salmonella* (Syamaladevi et al., 2016). The observed microbial data of seaweed sandwiches before and after roasting also indicated the limited microbial control efficacy of hot air drying, especially on low moisture foods. Chen et al. (2021) showed that hot air heating reduced the APC load of off-ground harvested almonds by 0.8 Log CFU/g. In the study conducted by Liu et al. (2021), hot air heating reduced the APC counts of dry Sichuan pepper by 1.5 ± 0.4 Log CFU/g.

The 16S rRNA sequencing results suggested a higher abundance of heat-resistant *Deinococcus-Thermus* were found in dried laver, roasted seaweed semi-products, and the final products, but not in the other samples. *Deinococcus-Thermus* has been known for its stress resistance including tolerance to high temperatures, desiccation, radiation, and oxidation (Ho et al., 2016; Tian & Hua, 2010). Furthermore, *Moraxellaceae* (*Acinetobacter*, 4.52%–8.27%) and *Staphylococcaceae* (*Staphylococcus*, 2.82%–6.96%) also survived in the roasted seaweed sandwiches or dried laver. An opportunistic pathogen, *Acinetobacter* spp. including *A. radioresistens* and *A. baumannii*, had been shown to be able to survive desiccation and radiation (Jawad et al., 1998; Zeidler & Müller, 2019). *Staphylococcus aureus* has a strong tolerance to desiccation stress (Wang, et al., 2022) and strong heat resistance in walnut shells at low a_w (Zhang et al., 2018). *Vibrio* spp., known as marine pathogen to marine animals and human (Bi et al., 2016; Li et al., 2021), were identified in the dried laver and roasted seaweed products (<1%) by 16S rRNA sequencing. Microbial examination of dried laver, roasted seaweed products by traditional plating method did not find any suspicious colony on the Thiosulfate citrate bile salts sucrose.

Coliforms were not detected in roasted sandwiches sampled at Plants A and B; however, coliforms had been detectable prior to roasting and after packaging. A previous study indicated that coliforms were sensitive to heat treatment (Denis et al., 2006). A more significant reduction of coliforms instead of APC has been obtained after the infrared drying or air drying in other studies (Bourdoux et al., 2016; Sánchez-Maldonado et al., 2018). The 16S rRNA sequencing showed that significantly higher

($p < 0.05$) abundance of Enterobacteriaceae were detected on gloves of personnel working in post-roasting area than pre-roasting area and environment swabs in Plant A.

The personnel collecting roasted seaweed sandwiches showed high APC level, which may be related with the use of heat-resistant gloves lacking regular disinfection. The conveyor belt of laver distributor, and the workbench for roasted seaweed sandwich slicing showed higher APC level than the other FCS. These areas were frequently and closely contacted with raw and finished products. The swab samples of the line worker's gloves collected in the post-roasting area in Plant A showed higher abundance of Enterobacteriaceae and *Acinetobacter*, which indicated inadequate personnel hygiene and potential cross-contamination. Attention should be paid to glove disinfection and the regular change of the disposable gloves during a work shift. Our results were consistent with the previous report that nonpathogenic Gram-negative bacteria, especially *Pseudomonas* spp., Enterobacteriaceae, and *Acinetobacter* spp. dominated on food processing surfaces (Moretrø & Langsrød, 2017).

The β -diversity of the 16S rRNA sequencing provides a measure of the degree to which samples differ from one another and has been shown to be robust to addressing issues such as low sequence counts and noise (Goodrich et al., 2014). Nakatsu et al. (2019) demonstrated that it is possible to identify sources of aquatic contamination using a consortium of microbes as an index rather than using traditional indicators (e.g., *E. coli*, *enterococci*). Their results showed that the Grand Calumet River had minimal influence on shoreline water quality at the study sites. In another study of the microbial diversity analysis of yellow-feathered broiler carcasses at select stages of slaughter, PCoA analysis showed bacterial communities of scalder tank water distanced from carcasses, indicating the limited effect of scalding water on the bacterial communities of broiler carcasses (Wang et al., 2019).

In our study, the β -diversity analysis from Plants A and C revealed that the microbiota of dried laver and that of the pre-roasting and post-roasting seaweed snacks clustered together, and differed from the microbiota of other raw ingredients, such as the facility swabs and swabs of personnel's gloves. These results indicated that the microbiota of roasted seaweed products was shaped mainly by dried laver. Checking laver and determining materials' microbial loads before acceptance should be an important control point for roasted seaweed products. Unfortunately, the majority of the dried laver produced in Asian counties are detected with high APC levels (>6 Log CFU/g) (Kim, et al., 2015; Løvdal et al., 2021). Although there are no microorganism limitations for the dried laver, the highly variable APC levels of dried laver highlight the importance of microbial control in dried laver production. Most importantly, several studies have shown that roasting, which directly determined the ripening, sensory, and decontamination of the final products, had limited microbial control efficacy.

5. Conclusions

By using both culture-dependent and culture-independent analysis methods, this study showed that dried laver with high APC counts contributed significantly to the roasted seaweed snacks' microbial populations, though ingredients such as crushed pumpkin seeds and almond flakes had relatively high microbial loads. Our results were consistent with the previous finding that the dried laver has high microbial load and, for the first time, illustrated the main origin of high microbial load in roasted seaweed snacks. The different contribution from raw materials to final products' microbiota were affected by their proportion in the ingredient list and the abundance of heat-resistant bacteria. The roasting process applied after the seaweed snacks are assembled can reduce the APC but the reduction was not adequate. Decontamination of laver in the processing plants where wet laver was shredded, molded and dried, and raw material check for seaweed snack production plants hold the promise to control the microbial contamination of roasted seaweed products. Although FCS, non-FCS, and line workers did not contribute as much as the laver to the final products' microbial population, the detection of higher microbial load on the reusable heat-resistant gloves indicated the need for more structured cleaning and sanitizing programs.

CRediT authorship contribution statement

Feifei Zhou: mainly conducted the experiment. **Zhen zhu:** mainly conducted the experiment. **Chenlong Wang:** participated in the experiment. **Siyao Zhao:** participated in the experiment. **Shuting Han:** participated in the experiment. **Likun Chen:** participated in the experiment. **Yi Ding:** participated in the data analysis. **Chao Liao:** participated in the data analysis. **Xiran Li:** participated in the data analysis. **Yan Xu:** give valuable suggestion to the experiments. **Yuanxia Chen:** give valuable suggestion to the experiments. **Jie Yang:** give valuable suggestion to the experiments. **Saikun Pan:** give valuable suggestion to the experiments. **Wenbin Wang:** Funding acquisition, acquired funding, designed and participated in the experiment, and wrote the manuscript. **Lingzhao Wang:** contacted the roasted seaweed processing plants and participated the experiment design. **Luxin Wang:** Formal analysis, participated in the experiment design, data analysis, and revised the manuscript. All the authors read and approved the manuscript.

Declaration of competing interest

All Authors, Feifei Zhou, Zhen Zhu, Chenlong Wang, Siyao Zhao, Shuting Han, Likun Chen, Yi Ding, Chao Liao, Xiran Li, Yan Xu, Yuanxia Chen, Jie Yang, Saikun Pan, Wenbin Wang, Lingzhao Wang, and Luxin Wang, certify that there is no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. There is no conflict of interest from any of the authors.

Data availability

Data will be made available on request.

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