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Research Article

High-Throughput 16 S rRNA Gene Sequencing Reveals Bacterial Diversity of Infant Formula Production Line Samples in Spring and Summer

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The trend of low breastfeeding rates increases the demand for infant milk formula (IMF) worldwide, but the use of IMF may be one of the causes of bacterial infections in infants. Complete sterility in the whole production line of IMF cannot be guaranteed; therefore, it is necessary to closely monitor the microbial content in the process. In the present study, an IMF powder production line based on the wet mixing process was sampled at 27 suspicious points in spring and summer to analyze the bacterial diversity by high-throughput sequencing. We found that 70 and 69 different bacterial phyla were present in spring and summer samples, respectively, with *Proteobacteria* and *Firmicutes* being the dominant phyla (>80% relative abundance). Moreover, 13 dominant genera each were present in spring (e. g., *Pseudomonas* and *Lactococcus*) and summer (e. g., *Pseudomonas*, *Bacillus*, and *Streptococcus*). Samples associated with workers showed higher bacterial species diversity (Shannon index) and richness (Chao1 index) in summer than in spring. The bacterial community composition showed high similarity between liquid milk after pasteurization and concentrated milk after evaporation. The potential bacterial pathogens were identified as *Pseudomonas aeruginosa* in spring and *Acinetobacter baumannii* in summer. Through retrospective analysis of the two opportunistic pathogens identified, it was found that the workshop environment was the potential contamination point in spring, whereas the auxiliary ingredients were the potential source of contamination in summer. The results highlight the effect of season on bacterial diversity associated with the production process of IMF and are useful in controlling the microbial quality and safety of infant dairy products.

1. Introduction

The World Health Organization (WHO) recommends breast milk as the ideal food source for infant growth and development [1]. Trend analysis on the prevalence of exclusive breastfeeding among infants younger than 6 months of age indicated that nearly 44% of the infants were exclusively breastfed in 2020 [2]. A low rate of breastfeeding means a high demand for infant milk formula (IMF). IMF is a vital alternative when breastfeeding is not sufficient, possible, or desirable. China has become the world's largest IMF market with sales of 188.5 billion Chinese yuan (equivalent to 27.89 billion US dollars) in 2020 [3].

Newborns are particularly susceptible to bacterial infections owing to the qualitative and quantitative deficiencies of the neonatal innate immune system [4]. Therefore, IMF requires very high levels of microbiological quality and must conform to national and international microbiological criteria [5]. However, on the basis of the available manufacturing technologies, the generation of a completely sterile product remains impossible. Consequently, intrinsic contamination of IMF may be a cause of possibly serious illness in infants [4].

Indeed, there are risks of bacterial infection associated with using IMF and many contamination incidents have occurred worldwide. For example, *Clostridium botulinum* contamination of Fonterra milk powder was reported in New Zealand in 2013, followed by *Salmonella* contamination of milk powder in France in 2017. Furthermore, *Salmonella* and *Cronobacter sakazakii* contamination of milk powder was reported in the USA and the Netherlands, respectively, in 2018, whereas *Salmonella* contamination of infant rice flour was reported in France in 2019.

Generally, IMF can be produced using one of three different methods: dry mixing, wet mixing, or a combination of both. At present, the wet mixing-spray drying process remains the most widely used method of powdered IMF production [6], as illustrated in Figure 1 [7]. Because of the likelihood of introducing bacterial contamination from untreated biomass or at different points in the process, it is difficult to ensure sterility during IMF production. Although the production of a completely sterile powdered product is not feasible, every precaution is taken to reduce the possibility of IMF contamination. High standards of hygiene are maintained throughout the production process and the microbiological quality of each batch is closely monitored. Identifying the sources and points of infection in a timely manner is vital for controlling the microbiological quality and safety of IMF products.

Conventional microbiological methods for bacterial identification include laboratory culture, microscopic examination, and biochemical testing [8]. However, culture-based standard microbiological diagnosis only targets a small portion of numerous microorganisms, many of which are very difficult to culture or are considered non-culturable [9]. The difficult-to-culture or nonculturable microorganisms are in a state with viable cells that do not form colonies [10]. In some cases, a viable but nonculturable state is directly induced by food disinfection techniques [11]. Technological advances in molecular biology, especially those in next-generation sequencing (also known as high-throughput sequencing) have innovated the principles of biological research [12]. In recent years, analysis of 16 S rRNA gene sequences has been frequently used to obtain the taxonomic composition of a microbial community [13]. Because of the cost of the equipment and operation, high-throughput sequencing technology is believed to be the most economical option to characterize microbial diversity and community composition in large-size samples.

In the present study, bacterial diversity associated with IMF production in spring and summer was assessed using high-throughput 16 S rRNA gene sequencing. Twenty-seven sampling points throughout the IMF production line were selected as potential contamination points. We used 16 S rRNA gene amplicon sequence analysis to evaluate bacterial contamination risks in the wet mixing process during IMF production. Traceback studies of potential pathogenic bacteria in the samples detected using sequencing were conducted to retrospectively define the probable sources and points of infection. The study results could be useful to control the microbiological quality and safety of IMF produced using the wet mixing process.

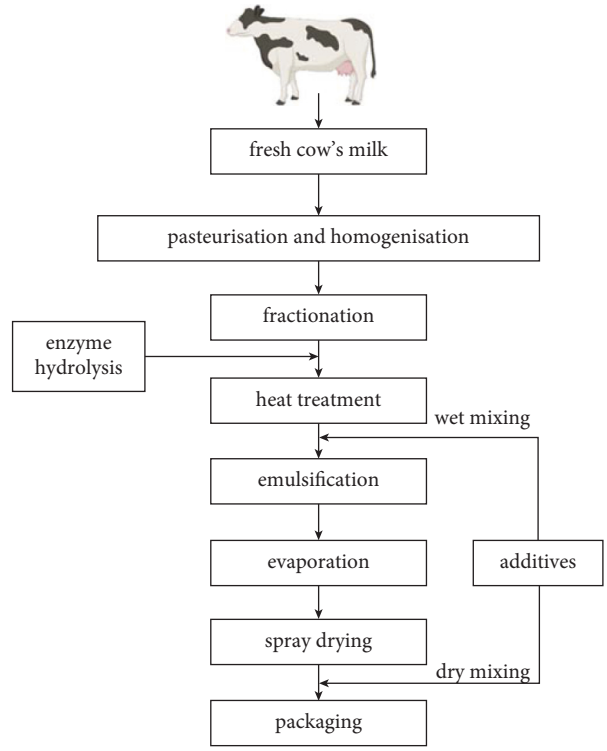


Figure 1: The process of infant milk formula production.

2. Materials and Methods

2.1. Study Design and Sampling. The dairy industry is characterized by a high level of mechanization and automation. Most of the IMF production process occurs in closed pipes. There are many disinfection and sterilization facilities with few personnel in the workshop; thus, the level of cleaning and purification is also very high. This study was therefore designed to include all the samples that can be collected from the IMF production line, mainly raw materials, auxiliary materials, production pipelines, and IMF products. All parts of the production line with valves, ingredients, accessories, and essential artificial linking parts were selected for sampling, with a total of 27 sampling points.

Sampling was performed in March and June 2019 using three different methods. (A) For sampling points on the production line where a valve could be directly opened, the whole valve was first wiped with a sterile gauze that had been soaked in 75% alcohol. The valve was then opened and the liquid at the front was discarded. After that, samples were collected with sterilized triangular flasks or 50 mL sterile centrifuge tubes. At least three samples were collected at each sampling point. (B) For sampling excipients, the whole package of excipients was directly and randomly sampled, and at least three packages were sampled at each sampling point. (C) At sampling points such as the feeding environment, samples were taken according to the national food safety standard for disinfection of tableware (drinking utensils) of China [14]. In brief, a cotton swab soaked with sterile normal saline was used to evenly smear the whole surface of a sampling point back and forth in two square

areas of 25 cm 2 each (5 cm * 5 cm). The sampling procedure was repeated three times. The cotton tip of the swab was cut with sterilized scissors and placed in a sterile container.

Samples from each sampling point were stored on dry ice immediately after collection and transported to the laboratory as soon as possible. Total DNA was extracted from the samples immediately after they reached the laboratory. If DNA extraction could not be performed immediately, the samples were well mixed and then divided into subsamples such that the amount of each subsample met the requirement of single DNA extraction, and the subsamples were stored at −80°C until use. The sampling methods are summarized in Table 1; the first letter of a sample's name indicates the sampling season: March (*M*) for spring and June (*J*) for summer.

2.2. DNA Extract and Next-Generation Sequencing. Total DNA was isolated from samples using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The final DNA concentration and purity were determined using the NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and the DNA quality was checked using 2% agarose gel electrophoresis.

The V4 hypervariable region of the bacterial 16 S rRNA gene was amplified by PCR using primers 515F (5′-AATGGCGCCGMCGACYGTG-3′) and 806R (5′-TAATCTWTGGGVNCATCAGG-3′) [15, 16]. PCR amplification was carried out in triplicate on a GeneAmp 9700 thermocycler (ABI, Foster City, CA, USA). PCR reactions were performed in a 25 μL reaction mixture containing 5 μL of each primer (1 μM/L), 12.5 μL of 2× KAPA HiFi HotStart Ready Mix, and 2.5 μL of template DNA (5 ng/μL). All reactions were run using the following program: 3 min of denaturation at 95°C; 27 cycles of 30 s at 95°C, 30 s for annealing at 55°C, and 45 s for elongation at 72°C; and a final extension at 72°C for 10 min. The PCR products were extracted from a 2% agarose gel. Further, the amplicons were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, Fitchburg, WI, USA) according to the manufacturer's protocol.

Purified amplicons were pooled in equimolar concentrations and paired-end sequenced (2 × 300 bp) on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) according to the standard protocols. High-throughput sequencing was completed by Hebei Food Safety Key Laboratory of Hebei Food Inspection and Research Institute (Shijiazhuang, Hebei, China). The resulting DNA sequence data were compared to data in the GenBank database using the BLAST algorithm available at the National Center for Biotechnology Information website (https://www.ncbi.nlm.nih.gov).

2.3. 16 S rDNA Sequencing Analysis and Retrospective Analysis of Aeruginosa. Raw sequences were processed for quality filtering, trimming, denoising, and amplicon sequence variants (ASVs) by using DADA2 pipelines [17] in Qiime2 [18] (https://qiime2.org/#citing). Based on Naive–Bayes classifiers and the SILVA 132 16 S SSU database [19] (https://www.arb-silva.de/), annotations were conducted. α-diversity metrics were calculated based on a rarefied feature table using rank abundance curves. The feature diversity among sample communities (β-diversity) was assessed on the basis of unweighted UniFrac distances. Statistical significance of differences in beta diversity was assessed on the basis of weighted UniFrac distance matrices using non-metric multidimensional scaling (NMDS) analysis. The results of bacterial diversity analyses were visualized using a web-based platform—Majorbio I-Sanger Cloud Platform (https://www.i-sanger.com). Traceback studies of pathogenic bacteria detected in the samples were conducted to retrospectively define the probable sources and points of infection.

2.4. Statistical Analysis. All data are expressed as mean(-s) ± standard deviation (SD) and analyzed using IBM SPSS v22.0 software (IBM Corp., Armonk, NY, USA). Permutation *t* tests were performed using QIIME scripts. *P* values were defined as statistically significant when lower than 0.05.

3. Results

3.1. Relative Abundance of Bacterial Taxa Associated with the Production Process. After quality filtering, a total of 9,505,040 high-quality sequences were obtained from 127 samples and clustered into 17,970 feature sequences. Furthermore, taxonomically annotates sequences were grouped according to their phylotypes (phyla and genera) and a heatmap of feature data relative abundance was created (Figure 2).

In total, 70 and 69 different phyla were identified in spring (Figures 2(a) and 2(c)) and summer (Figures 2(b) and 2(e)), respectively. Of these, nine phyla were observed to be dominant (>1% relative abundance) in spring (*Proteobacteria*, 45.5%; *Firmicutes*, 36.0%; *Bacteroidetes*, 5.2%; *Actinobacteria*, 4.5%; *Acidobacteria*, 1.3%; *Cyanobacteria*, 1.2%; *Gemmatimonadetes*, 1.1%; *Deinococcus-Thermus*, 1.1%; *Verrucomicrobia*, 1.0%), whereas four dominant phyla were present in summer (*Firmicutes*, 56.3%; *Proteobacteria*, 32.6%; *Bacteroidetes*, 6.0%; *Actinobacteria*, 1.5%). The two most dominant phyla, *Proteobacteria* and *Firmicutes*, together accounted for more than 81.0% and 88.5% of the bacterial community in spring and summer, respectively. The relative abundance of major bacterial phyla varied with the sampling points throughout the production line.

In total, 1726 and 1609 different genera were identified in spring (Figures 2(e) and 2(g)) and summer (Figures 2(f) and 2(h)), respectively. Thirteen dominant genera (>1% relative abundance) each were present in spring and summer. The spring samples were dominated by *Pseudomonas* (16.9%) and *Lactococcus* (13.3%), followed by *Acinetobacter* (7.6%) and *Streptococcus* (7.1%); other dominant genera included *Citrobacter* (4.3%), *Anoxybacillus* (4.0%), *Lactobacillus* (2.8%), *Geobacillus* (1.8%), *Rhodococcus* (1.6%), *Macrococcus* (1.6%), *Aeromonadaceae* (1.6%), *Enterobacteriaceae* (1.1%), and *Rhodanobacter* (1.0%). In the summer samples, the

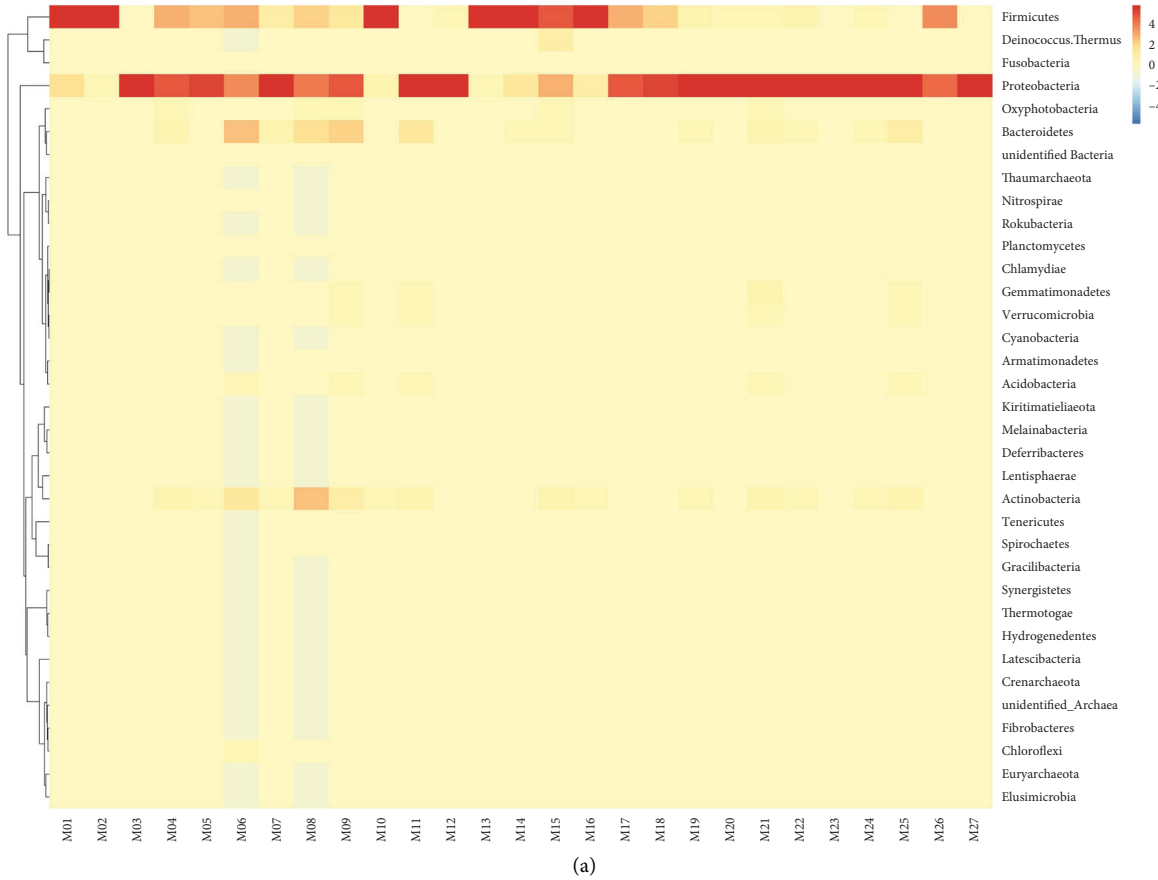
Table 1: List of sampling points in the infant milk formula production line.

Sampling point		Sample number	Sample status	Sampling purpose	Sampling method
Material	Raw milk	M/J01	Liquid	Critical Control Point (CCP) 1	
Net milk cooling	Liquid milk mixed before pasteurization	M/J02	Liquid	CCP2	A
Pasteurization	Liquid milk after pasteurization	M/J03	Liquid	To study the changes of bacterial diversity after pasteurization	
Excipients	Water	M/J04	Liquid	CCP1	B
	Soybean oil	M/J05	Liquid		
	α-Whey protein powder	M/J06	Powder		
	Polyfructans	M/J07	Powder		
	Oligofructans	M/J08	Powder		
	Mineral	M/J09	Powder		
	Arachidonic acid	M/J10	Solid		
	Multivitamins	M/J11	Powder		
	Whey protein	M/J14	Powder		
	Skim milk	M/J15	Powder		
Adding excipients	Pasteurized milk added with excipients	M/J12	Liquid	Potential risk	
Evaporation	Concentrated milk	M/J13	Liquid	CCP3	A
Cooling	Fluidized bed	M/J16	Powder	Potential risk	C
	Dust of canning	M/J17	Cotton swab	Environmental surveillance	
The dust of dry spray	Dust off feeding room table	M/J18	Cotton swab		
	Dust off feeding room ground	M/J19	Cotton swab		
Feeding environment	Feeding cover	M/J20	Cotton swab	Potential risk	
	Feeding bottom	M/J21	Cotton swab		
	Feeding table	M/J22	Cotton swab		
Cling wrap	Package film	M/J23	Cotton swab	CCP4	
	Package film exchange platform	M/J24	Cotton swab		
Workers	Hands	M/J25	Cotton swab	Potential risk	
	Soles	M/J26	Cotton swab		
	Tooling	M/J27	Cotton swab		

dominant genera were *Pseudomonas* (13.4%), *Bacillus* (13.3%), and *Streptococcus* (12.5%), followed by *Lactococcus* (8.7%), *Paenibacillus* (5.4%), *Lactobacillus* (5.3%), *Acinetobacter* (2.4%), *Anoxybacillus* (1.4%), *Bacteroides* (1.2%), and *Enterococcus* (1.0%).

Lactococcus was the dominant genus in spring raw milk samples, whereas *Yersinia* and other genera accounted for a large proportion of the bacterial community in summer raw milk samples. After pasteurization of the milk, the proportion of other bacteria increased in the spring samples, whereas *Pseudomonas* was found to be dominant in the summer samples. The spring samples showed that *Streptococcus*, *Lactococcus*, and *Lactobacillus* were introduced into the IMF production process by excipients. The summer samples showed that *Bacteroides*, *Acinetobacter*, and *Bacillus* were introduced in addition to the three genera mentioned above.

3.2. Bacterial Community Diversity Associated with the Production Process. Rarefaction curves plateaued after 15,000 reads per sample (Figure 3), approximating a saturation phase, which suggests that sufficient sampling was achieved to capture the total diversity of bacterial communities associated with the production process. The α-diversity of bacterial communities in the spring and summer samples was analyzed by calculating the Shannon species diversity index and Chao1 species richness estimator (Table 2). Among the spring samples associated with pasteurization and dry heat sterilization steps in the production pipelines, the species diversity of M01, M02, M03, and M13 milk samples was generally low, and that of M16 fluidized bed was slightly higher. The bacterial α-diversity of excipients (M04–M09 and M11) was relatively high, and environmental samples (M17–M24), especially the samples collected

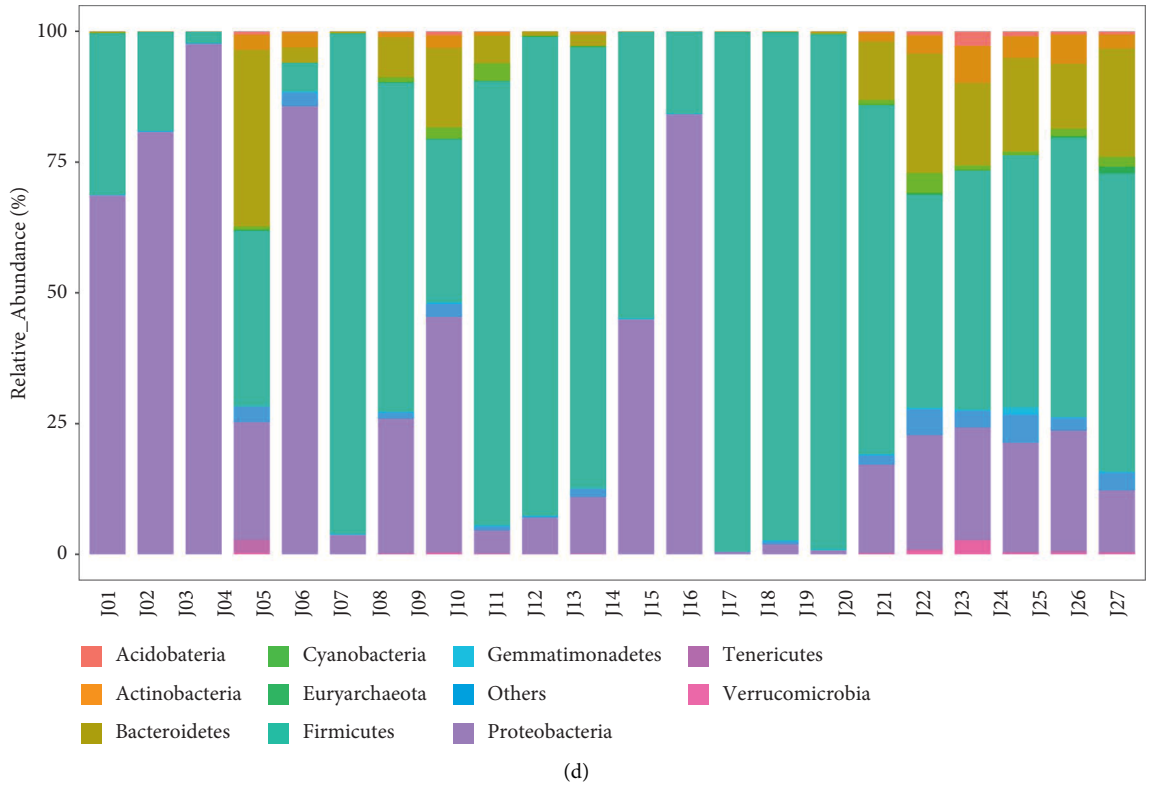


(a)

(b)

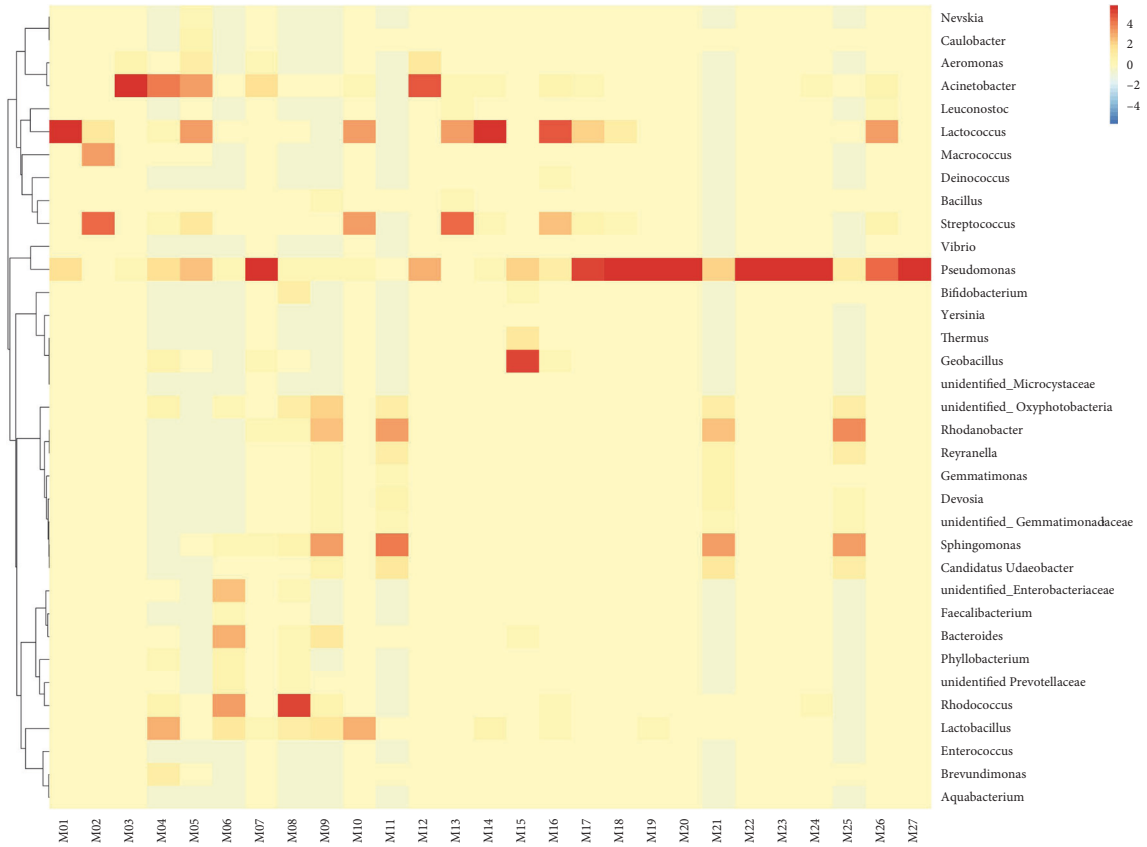
Figure 2: Continued.

(c)



(d)

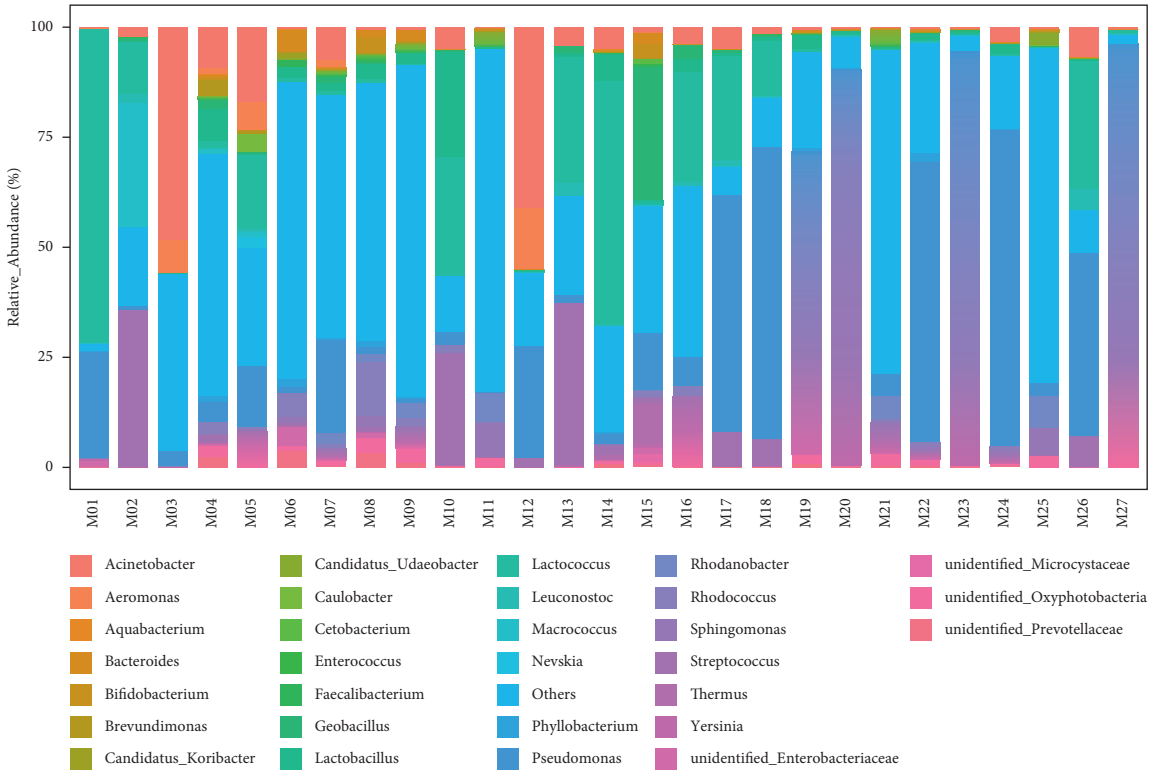
Figure 2: Continued.



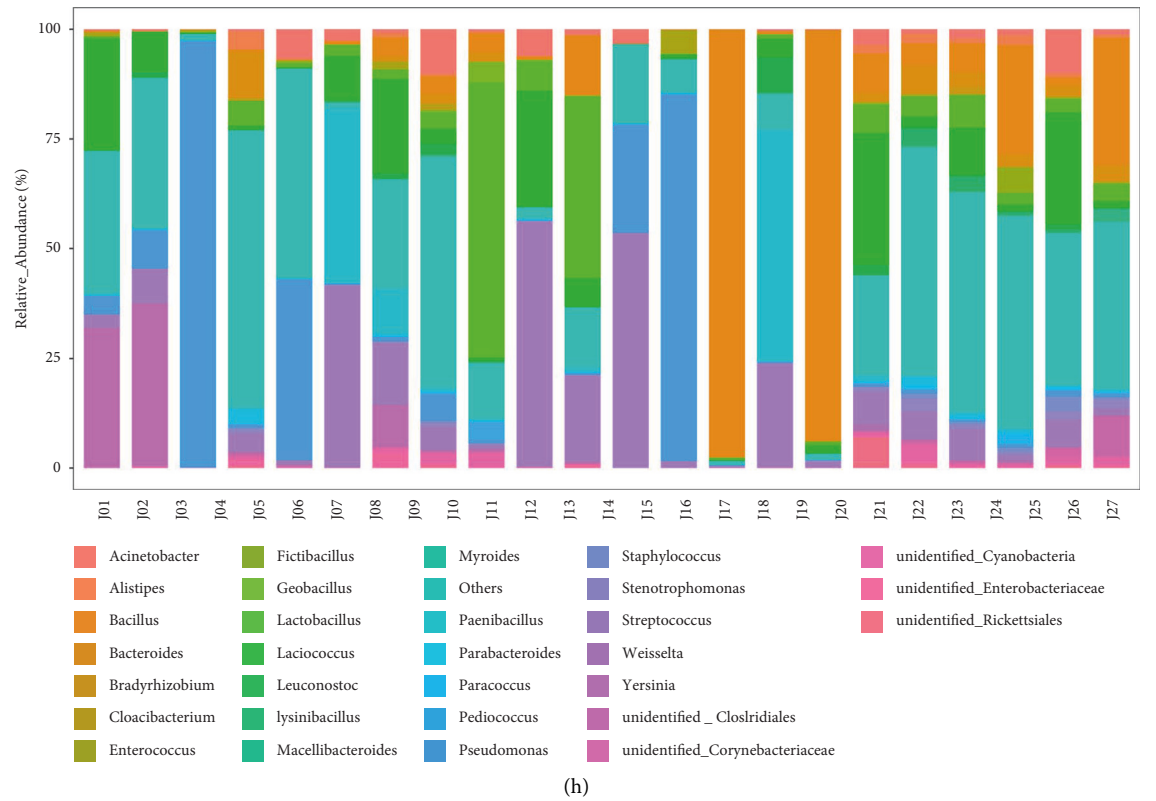
(e)

(f)

Figure 2: Continued.



(g)



(h)

Figure 2: Heatmaps of bacterial operational taxonomic unit (OTU) abundance at the phylum level and genus level in spring (a) and (e), respectively, and summer (b) and (f), respectively; red indicates higher relative abundance. Relative abundance of bacterial species at the phylum and genus levels in spring (c) and (g), respectively, and summer (d) and (h), respectively. The diagrams show the mean relative abundance of top 10 phyla and top 30 genera in each sample.

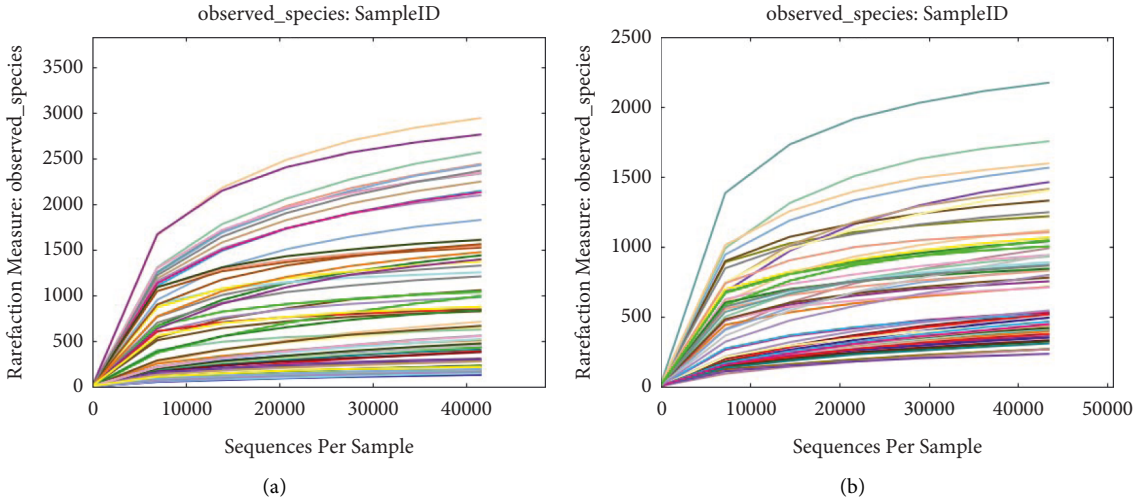


Figure 3: Dilution curve of bacteria of infant milk formula production line samples in spring ((a), $n = 70$) and summer ((b), $n = 69$).

from the bottom of feeding cover (M21 : 2891.06), contained abundant bacterial species. Among the samples from workers (M25–M27), the species richness of samples associated with hands (M25) was more prominent than that of the remaining samples in the production line. On the whole, the bacterial α-diversity of samples collected in summer was lower than that of samples collected in spring. The species diversity of J01 raw milk and J23 package film (summer samples) was higher than that of the corresponding spring samples, whereas the species richness of J02, J03, J12, and J13 exhibited an inverse trend. The bacterial α-diversity of cling wrap (J23 and J24) was higher than that of the corresponding spring samples. Moreover, the bacterial α-diversity of J26–J27 samples from workers was higher than that in the corresponding spring samples, and an even higher species richness was associated with samples taken from workers' hands (J25).

The β-diversity of bacterial communities was analyzed using NMDS based on weighted (quantitative) UniFrac distance metrics. We found that the distribution of spring samples at the genus level was relatively scattered (Figure 4(a)), indicating distinct differences in the bacterial community composition of spring samples (Figure 4(a)). Summer samples J03, J06, J10, J12, J13, J14, J15, J16, J25, and J27 were far away from most of the remaining samples, whereas J03 and J13 were adjacent to each other (Figure 4(b)); these results indicate that there was little difference in the bacterial species retained in the summer samples after pasteurization and concentration by evaporation.

3.3. Retrospective Analysis of Pseudomonas aeruginosa and Acinetobacter baumannii. According to the analysis of sequencing results, two opportunistic pathogens, *Pseudomonas aeruginosa* (Figure 5(a)) and *Acinetobacter baumannii* (Figure 5(b)) were detected at the species level in the spring and summer samples, respectively. *Pseudomonas aeruginosa* was found in excipient samples in spring, at 41.9% in M07DJGT3, 13.7% in M15TZRF3, and 13.0% in M15TZRF2. The proportion of *P. aeruginosa* in the environmental samples (M17–M20, M22–M24 and M26–M27) was high (33.8%–77.8%), which indicates that the bacterial species was highly abundant in the workshop environment in spring. *Pseudomonas aeruginosa* was also found in M16, M05, M10, M14, and other excipient samples, which would introduce contamination into the production line in spring. In summer, *A. baumannii* was found in J10, with a relative abundance of 5.4%–6.6%. The proportion of *A. baumannii* in the environmental samples (J23 and J25–J27) was relatively low (0.1%–0.9%), indicating that the environment was not the major source of contamination. *Acinetobacter baumannii* was mainly introduced into the production line in summer by excipients, including J06 and J10 (1.5%–6.6%).

4. Discussion

In this study, the IMF powder production line was selected to investigate the diversity of bacterial communities associated with the process control and final products. The whole production line was preliminarily studied using 16 S rRNA gene sequencing analysis. A set of sampling points throughout the production line were selected, including milk, excipients, valves, environment, and personnel. Samples were collected in spring and summer to explore the seasonal distribution of bacteria within the production line. *Proteobacteria* and *Firmicutes* were found to be the main dominant bacterial phyla associated with IMF production in both sampling seasons; however, their relative abundance differed between seasons. At the genus level, the spring samples mainly contained *Pseudomonas*, *Lactobacillus*, *Acinetobacter*, *Streptococcus*, *Citrobacter*, and anoxic bacteria, whereas the summer samples mainly contained *Pseudomonas*, *Bacillus*, *Streptococcus*, *Lactococcus*, and *Paenibacillus*. There were distinct differences in species diversity and richness between the two seasons. The taxonomic distribution of bacterial communities was explained on the basis of a heatmap of feature sequences relative abundance in combination with the production method.

Table 2: Estimates of bacterial α-diversity in spring (*M*) and summer (*J*) samples.

Sample_ID	Shannon	Mean	Chao1	Mean	Sample_ID	Shannon	Mean	Chao1	Mean
M01YN1	1.78		334.19		J01YN1	3.60		587.65	
M01YN2	1.87	1.81	243.00	270.27	J01YN2	4.49	3.98	522.87	566.16
M01YN3	1.77		233.63		J01YN3	3.84		587.94	
M02BSQ1	3.23		396.98		J02BSQ1	3.24		320.37	
M02BSQ2	3.61	3.35	454.12	414.41	J02BSQ2	3.23	3.33	311.70	297.56
M02BSQ3	3.20		392.12		J02BSQ3	3.53		260.60	
M03BSH1	3.32		377.59		J03BSH1	1.03		241.60	
M03BSH2	3.57	3.48	358.46	380.57	J03BSH2	0.99	1.09	257.90	244.88
M03BSH3	3.53		405.67		J03BSH3	1.25		235.13	
M04PLS1	8.70		1797.76		J04PLS1	7.79		957.76	
M04PLS2	7.47	7.77	1787.46	1502.28	J04PLS2	7.79	7.79	957.76	957.76
M04PLS3	7.15		921.62		J04PLS3	7.79		957.76	
M05DDY1	3.85		631.00		J05DDY1	5.83		935.01	
M05DDY2	5.36	5.29	649.00	1013.06	J05DDY2	6.65	4.88	1476.82	1029.44
M05DDY3	6.67		1759.17		J05DDY3	2.17		676.48	
M06aRQF1	9.74		3126.49		J06aRQF1	3.52		565.47	
M06aRQF2	8.14	8.57	1535.68	2019.06	J06aRQF2	2.90	3.15	708.71	644.09
M06aRQF3	7.84		1395.01		J06aRQF3	3.03		658.09	
M07DJGT1	8.92		2662.33		J07DJGT1	6.39		834.15	
M07DJGT2	7.50	7.05	2659.77	2182.33	J07DJGT2	6.38	6.39	991.68	915.79
M07DJGT3	4.74		1224.90		J07DJGT3	6.39		921.54	
M08FOS1	8.69		2925.31		J08FOS1	6.94		1041.58	
M08FOS2	6.96	8.17	1188.08	1941.39	J08FOS2	7.55	7.27	1156.48	1122.04
M08FOS3	8.88		1710.78		J08FOS3	7.34		1168.05	
M09KWZ1	8.87		2982.72		J09KWZ1	4.32		1051.73	
M09KWZ2	9.66	9.19	3377.52	3182.48	J09KWZ2	5.17	4.96	1280.52	1150.81
M09KWZ3	9.03		3187.19		J09KWZ3	5.39		1120.17	
M10ARA1	5.79		1106.43		J10ARA1	2.81		809.38	
M10ARA2	3.30	4.22	537.94	795.72	J10ARA2	2.84	2.89	636.40	646.01
M10ARA3	3.55		742.78		J10ARA3	3.03		492.26	
M11WSS1	8.66		2537.29		J11WSS1	4.04		695.07	
M11WSS2	8.56	8.68	2609.40	2603.90	J11WSS2	3.86	4.58	607.88	964.78
M11WSS3	8.81		2665.01		J11WSS3	5.86		1591.39	
M12BSJF1	4.71		429.68		J12BSJF1	2.91		236.03	
M12BSJF2	4.87	4.82	408.00	411.56	J12BSJF2	3.25	2.91	265.00	263.38
M12BSJF3	4.89		397.00		J12BSJF3	2.58		289.11	
M13NS1	3.50		639.33		J13NS1	1.94		247.31	
M13NS2	3.41	3.45	628.56	673.52	J13NS2	1.25	1.74	246.69	265.04
M13NS3	3.45		752.67		J13NS3	2.02		301.13	
M14NSRQ1	8.27		1654.03		J14NSRQ1	2.28		374.73	
M14NSRQ2	2.39	4.41	811.32	1110.00	J14NSRQ2	3.18	2.46	304.22	395.14
M14NSRQ3	2.56		864.65		J14NSRQ3	1.92		506.47	
M15TZRF1	7.34		1703.33		J15TZRF1	3.07		516.02	
M15TZRF2	3.77	5.27	1075.80	1493.12	J15TZRF2	3.17	3.16	493.02	527.20
M15TZRF3	4.71		1700.22		J15TZRF3	3.22		572.57	
M16LHC1	5.84		1040.01		J16LHC1	3.63		408.27	
M16LHC2	4.28	4.72	889.69	936.15	J16LHC2	2.52	2.86	820.12	683.35
M16LHC3	4.04		878.75		J16LHC3	2.43		821.64	
M17GZLDF	3.67		916.01		J17LDF1	5.75		936.04	
M18LDFTM	3.73		928.30		J17LDF2	6.83	5.82	1197.02	992.21
M19LDFDM	4.80		1399.04		J17LDF3	4.87		843.57	
M20GTOP	2.63		1507.74		J23BZMO3	8.05		1358.12	
M21GBot	8.83		2891.06		J24HMPT1	6.21	7.15	1105.70	1220.51
M22PLPT	5.36		2105.40		J24HMPT2	7.20	7.56	1197.72	1599.20
M23BZMO	2.41		986.86		J24HMPT3	9.26		2494.17	
M24HMPT	4.28		800.21		J25GRSH1	7.24		1539.69	
M25GRSH	8.71		2433.06		J25GRSH2	7.96	6.87	1449.69	1438.18
M26GRXIE	4.30		996.70		J26GRXIE1	5.42		1325.16	
M27GRYF	2.06		739.57		J26GRXIE2	7.52	6.69	975.91	1386.58
					J26GRXIE3	7.14		1858.68	

Table 2: Continued.

Sample_ID	Shannon	Mean	Chao1	Mean	Sample_ID	Shannon	Mean	Chao1	Mean
					J27GRYF1	8.65		1729.19	
					J27GRYF2	7.12	6.43	1879.29	1605.53
					J27GRYF3	3.53		1208.11	

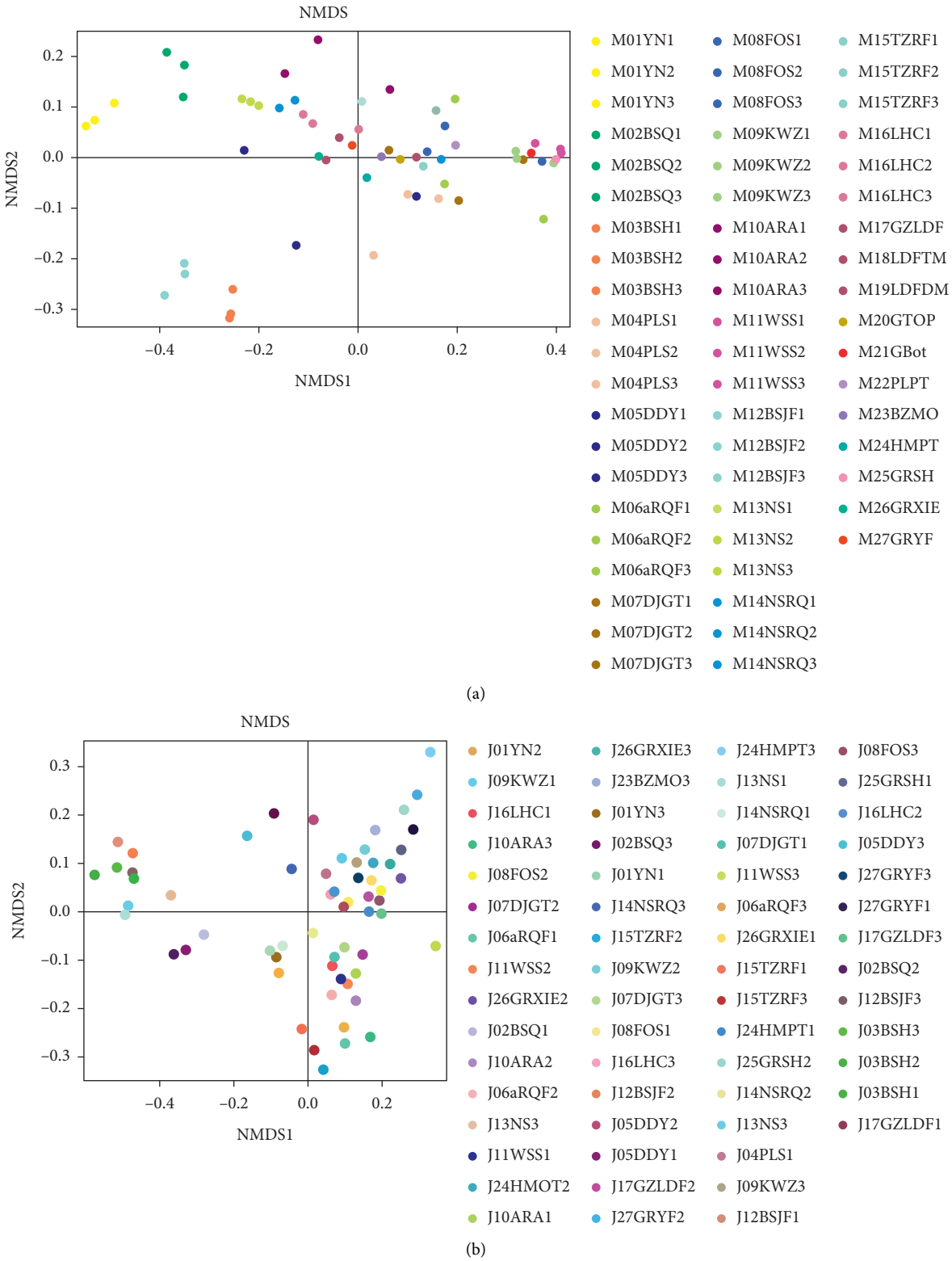


Figure 4: Nonmetric multidimensional scaling (NMDS) analysis of bacterial community composition in infant milk formula production line samples in spring (a) and summer (b) at the genus level.

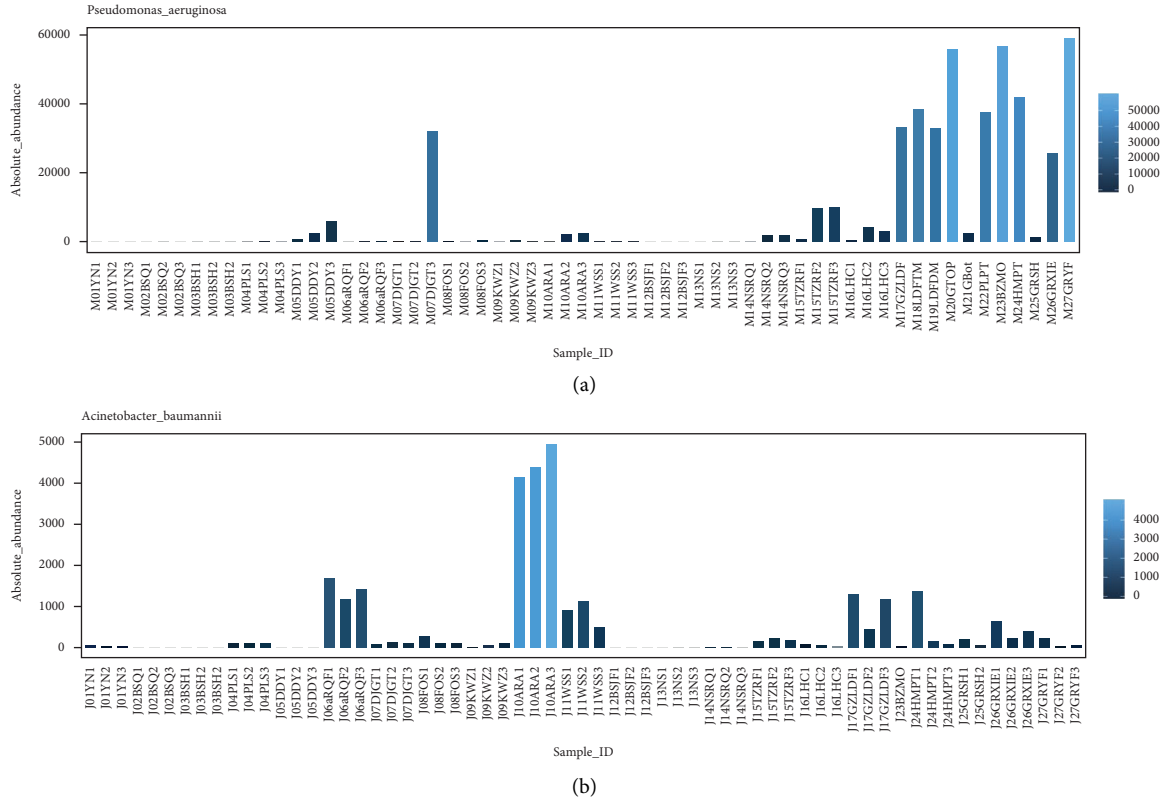


Figure 5: Number of feature sequences classified as *Pseudomonas aeruginosa* in spring samples (a) and *Acinetobacter baumannii* in summer samples (b) of the infant milk formula production line.

Pasteurization and concentration by evaporation can reduce the abundance of bacteria in IMF products but not completely eliminate them. Previously, a joint FAO/WHO consultation group (2004–2006) identified the primary bacteria associated with IMF contamination as *Cronobacter* sp., *Salmonella enteritidis*, *Enterobacter agglomerans*, *Enterobacter cloacae*, *Hafnia alvei*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Citrobacter koseri*, *Citrobacter freundii*, *Escherichia coli*, *Serratia* sp., *Acinetobacter* sp., *Bacillus cereus*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium botulinum*, *Listeria monocytogenes*, and *Staphylococcus* sp. [20]. In the present study, we found that *Pseudomonas*, a group of cold-resistant bacteria, was the most dominant genus in both spring and summer samples associated with IMF production. *Pseudomonas* grow and produce thermostable enzymes in cooled raw milk before heat processing. These thermostable enzymes withstand ultrahigh temperature treatment and cause spoilage of dairy products [21]. Furthermore, we found that the genus *Bacillus*, which belongs to a group of aerobic, psychrotrophic, endospore-forming bacteria, was dominant in the summer samples obtained from the IMF production line. Because the endospores resist the pasteurization process, the main spoilage mechanism is their subsequent germination and outgrowth accompanied by the production of spoilage enzymes in the pasteurized milk [22].

Bacterial α-diversity analysis indicated that the species richness of excipients in spring was relatively high, which suggests that we should pay attention to the microbial indicators of excipients and water. The bacterial α-diversity of raw milk samples in summer (J01) was higher than that in spring (M01), which indicates that the bacterial community in the main raw materials varies with season. Additionally, the α-diversity indices of the membrane exchange platform were higher in summer (J24) than in spring (M24), indicating that the seasonal temperature had a profound impact on the bacterial community in the workshop environment. We found that the species richness of bacteria associated with the hands of batching operators was high over the two seasons; therefore, further attention needs to be paid to strengthening the operation of personal disinfection. Furthermore, the species diversity of bacteria at different sampling points was compared, but no specific patterns were found. The contaminant microbiota may persist in water, teat cups, and milking equipment over time, providing a continuous source of microbial contamination [23, 24]. This persistence of bacteria may be explained by biofilm formation and the consequent high resistance to disinfection. It is well established that storage equipment is commonly colonized by bacterial biofilms [25–28]. This may be the reason for the change in bacterial diversity and richness after storage in the IMF production pipelines.

β-Diversity analysis using NMDS showed distinct differences in the bacterial community composition across sampling points in spring. In summer, the bacterial community composition associated with workers was markedly

different from that associated with the production pipelines; therefore, more attention should be paid to the disinfection of workers during this season. Moreover, the bacterial community composition showed high similarity between J03 and J13 samples. Taking into account the main production processes, we found that dry heat sterilization effectively reduced the diversity of associated bacterial communities. Subsequent changes in the bacterial community composition associated with the production pipelines may be related to the equipment, environment, and workers.

Finally, we selected the potential pathogenic bacteria detected through sequencing for traceability analysis, namely, *P. aeruginosa* in spring and *A. baumannii* in summer. These two species are widespread opportunistic pathogens in nature. *Pseudomonas aeruginosa* is a metabolically versatile, ubiquitous, Gram-negative bacterium that can cause infections in animals and plants. Owing to its intrinsic resistance to multiple antibiotics, *P. aeruginosa* infections are difficult to treat [29, 30]. *Acinetobacter baumannii* is an important pathogen that causes nosocomial infections associated with several types of diseases, including pneumonia, meningitis, septicemia, and urinary tract infections [31]. In the present study, we found that all the possible sources of pathogenic bacteria were distinctly different between spring and summer. This distinction could be attributed to the seasonal changes in temperature, as the optimum growth temperature for *P. aeruginosa* and *A. baumannii* is approximately 30°C and 35°C, respectively. Although the sources of pathogenic bacteria introduced in spring and summer were different, most of them were concentrated in excipients and workshop environmental factors. It is therefore imperative to further standardize the operating procedures of IMF production workshops. Furthermore, more research is needed to analyze the possible pathogenic bacteria and their sources associated with IMF production in autumn and winter.

Data Availability

The data used to support the findings of this study are included within the article.

Disclosure

Xian Li and Yongbo Li are co-first authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Conceptualization was done by G.S. and W.Z.; methodology was performed by X.L. and Y.L.; software was done by Y.L.; validation was done by Y.Z., R.Z., and H. W.; formal analysis was done by Y.Z. and T.Z.; investigation was done by R.Z. and T.Z.; resources were gathered by H.W. and Z.Z.; data curation was done by Y.L.; X.L. wrote the original draft; X.L. and Y.L. reviewed and edited the article; visualization was done by Y.L. and T.Y.; supervision was done by G.S.; project administration was done by G.S. and W.Z.; and funding acquisition was done by W.Z. All authors have read and agreed to the published version of the manuscript. X.L. and Y.L. contributed equally to this work.

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