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RESEARCH ARTICLE

Inactivation of *Cronobacter sakazakii* in powdered infant formula with probiotics and metagenomic analysis

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Abstract
In the present study, proper manual for powdered infant formula with probiotics (PIF-P) to prevent the contamination of *Cronobacter sakazakii* was investigated. First, the population of *C. sakazakii* and LAB in three different PIF-P samples were quantitatively analyzed after reconstituted with hydrothermal treatments. When *C. sakazakii* was inoculated into reconstituted infant formula with probiotics (RIF-P), it was immediately reduced below the detection limit by 60–65 °C hydrothermal treatment whereas reduction levels of LAB was 1–2 log CFU/g. When heat resistance of *C. sakazakii* inoculated to PIF-P with 4 h drying was compared with that inoculated to RIF-P samples, the heat resistance of *C. sakazakii* increased significantly after the inoculation in PIF-P with drying. Metagenomic analysis revealed that *Lactobacillus* and *Bifidobacterium* were dominant genus in all three groups and there was no significant difference in the microbial community of untreated PIF sample and hydrothermal treated samples.

Keywords Infant formula with probiotics · Hydrothermal treatment · *Cronobacter sakazakii*, · Lactic acid bacteria · Metagenome analysis

Introduction

Cronobacter sakazakii is a gram-negative, non-spore-forming, facultative anaerobe belonging to the family *Enterobacteriaceae*. It was named *Enterobacter sakazakii* in 1980s but renamed as *C. sakazakii* in 2008 when it was reclassified as a species of *Cronobacter* (Kim et al., 2013). Infection with *C. sakazakii* can cause serious symptoms such as necrotizing enteritis, meningitis, and sepsis. Specifically, *Cronobacter* is known to cause diseases through opportunistic infection mechanism in infants and elderly people (Kim et al., 2016). Sivamaruthi et al. (2011) reported an infant mortality rate of 33–80% from *C. sakazakii* meningitis and it has been reported that up to 20% of the newborns developed serious neurological complications after infection. Although the exact infective doses of *C. sakazakii* has not yet been clarified, it is estimated that at least 3 log CFU/mL of *Cronobacter* spp. contaminated with powdered milk cause infection (Song et al., 2012).

C. sakazakii has been isolated from a variety of food sources but powdered infant formula (PIF) is commonly associated with the occurrence of *C. sakazakii* infection in infants (Alvarez-Ordóñez et al., 2014). This bacterium can survive for a long time in dry food matrix such as powdered milk because it has strong dry resistance properties (Du et al., 2018). Also, *C. sakazakii* has been frequently detected in the surrounding environment such as soil, rats, flies, milk powder factories, chocolate factories, and homes (Lee, 2016). According to a recent report from Centers for Disease Control and Prevention (CDC), *C. sakazakii* was detected from a breast pump device used at home which linked to the death of baby (McPhillips, 2023). Hwang et al. (2015) reported that *C. sakazakii* was detected in formula milk in 2006 and caused social controversy in South Korea. Also, *C. sakazakii* was detected in powdered milk of domestic brand of South Korea and more than 53,400 packages of the powered milk were discarded (Kim, 2009). In 2022, five infants were infected with *C. sakazakii* and *Salmonella* after eating milk powder produced by a United States brand milk powder

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factory, and two of them died from *C. sakazakii* infection. The US Food and Drug Administration (FDA) tests showed that *C. sakazakii* was found in a device called "scoop hopper" and it was reported that investigators also found bacteria on the floor around the product dryers (Goodman, 2022). Recently, a milk powder company headquartered in Ireland reported that some products were voluntarily recalled due to possible contamination of pathogenic *C. sakazakii* (Williams, 2023). The US FDA takes new measures in the infant formula industry to protect babies from diseases caused by *Salmonella* and *C. sakazakii* by ensuring microbiological safety in powdered milk (Hassan, 2023). European Commission also passed a number of fundamental decisions related to the veterinary sanitary and food safety area to reduce the risk represented by *C. sakazakii.*

Heat-sensitive microorganisms contaminated in PIF might be inactivated during the spray drying process by hot air. Most of *C. sakazakii* is known to be sensitive to heat, but some of them are resistant to heat which makes it difficult to completely remove the pathogen by heat (Kim and Park, 2017). World Health Organization (WHO) published guidelines for reconstituted PIF instructing parents and caregivers to clean hands, water to boiling, pour water that has been cooled to no less than 70 °C(158°F) into a clean and sterilized bottle (Chiang et al., 2023). Although the instructions from WHO, CDC, FDA, and American Academy of Pediatrics are different, hydrothermal temperature of 70–75 °C is commonly recommended to reconstitute the PIF. However, a mixture of probiotics, prebiotics, and synbiotics has been added to PIF to ensure the viability of probiotics and to exploit the synergistic effect of promoting the growth of native gut flora (Jung et al., 2015), and it has been reported that probiotics added to PIF would be affected by the hydrothermal used for reconstitution when the temperature of hydrothermal is too high (Wilkinson et al., 2019). Manuals from products recommend to reconstitute the PIF with probiotics (PIF-P) with relatively low hydrothermal temperature (40–50 **°C**) than normal PIF to prevent the inactivation of LAB. However, the lowered hydrothermal temperature (40–50 **°C**) would not inactivate the high level of *C. sakazakii* contaminated to PIF-P. Therefore, the hydrothermal treatment temperature for PIF-P should be adjusted to ensure microbiological safety and functionality of the PIF-P samples.

With the development of next-generation sequencing (NGS) technology, metagenome analysis, which identifying the microbial community existing in a specific environment, has been utilized in various field of research (Kwon et al., 2019). Among the various types of metagenomic sequencing, 16S rRNA gene amplicon sequencing is a simple and cost-effective way to identify the proportion and diversity (richness and uniformity) of microbial communities at the genus level (Peterson et al., 2021). General process of metagenomics analysis includes sample collection, DNA extraction, library preparation and data analysis (Elie et al., 2023). The microbial pipeline allows users to analyze raw DNA sequence data, generate results, apply statistics, and visualize the results. Among the various pipelines, quantitative insights into microbial ecology (QIIME2) based on Python allow users to process, manipulate and analyze large throughput datasets (Christensen, 2018). Bioformatic analysis using QIIME2 software package is performed by importing raw data, followed by sequence preprocessing (demultiplexing and denoising), taxonomic classification, visualization of taxonomic classifications, building of phylogeny, and diversity analysis (alpha and beta diversity) (Hall and Beiko, 2018). Therefore, metagenome based on QIIME2 analysis was conducted in the present study to identify the microbiota of infant formula with probiotics.

Metaganomic analysis has been widely used to identify the safety and functionality of food sample. For example, 16S rRNA amplicon based metagenomic analysis were conducted to investigate the effect of the milk powder production line on the formation and survival of anaerobic spore bacteria (Porcellato et al., 2023). Sjödin et al. (2023) also conducted 16S rRNA gene amplicon based metagenomic sequencing to determine the difference after feeding infants with prebiotics formula and synbiotic formula. The results showed that ingestion of synbiotic formula resulted in fewer *Klebsiella*, more *Bifidobacterium breve*, and increased antimicrobial metabolites. Kim et al. (2019) also used 16S rRNA sequencing to compare intestinal microbial communities in breastfed and formula fed infants. However, there is a lack of research on changes in the microbial community of infant formula with probiotics under the condition of actual baby's intake, so it is necessary to verify the infant formula with probiotics under actual situations to provide information on safety and functionality of PIF-P.

In this study, (1) *C. sakazakii* and LAB were quantitatively analyzed according to the hydrothermal temperature and treatment time in reconstituted infant formula with probiotics (RIF-P), (2) *C. sakazakii* was inoculated into PIF-P with 4 h dyring for adaption and the heat resistance change was investigated (3) the metagenome of PIF-P before and after hydrothermal treatment was evaluated by sequencing in the V3-V4 region of 16S rRNA gene.

Materials and methods

Sample preparation

Three different PIF-P samples purchased from the online market (South Korea) were used for analysis. The specifications of samples used in the present study were described in Table S1. Briefly, the LAB content of infant formula labelled

on packaging was more than 5×10^6 CFU/g in sample A, more than 3×10^6, CFU/g in sample B, and 1.3×10^6 CFU/g in sample C. Each sample was stored at room temperature (20–25 °C) before experiments.

Bacterial cell preparation

C. sakazakii ES15 and *C. sakazakii* KCTC 2949 obtained from the bacterial culture collection of the School of Food Engineering, Dankook University (Cheonan, South Korea) were used in the present study. Frozen stocks of *C. sakazakii* ES15 and *C. sakazakii* KCTC 2949 were transferred to 5 mL of tryptic soy broth (TSB, Difco, Franklin Lakes, New Jersey, USA) for cell revival and inoculated at 37 °C for 24 h. After incubation, the cultures were mixed using a vortex mixer (Vortex VM-10, DAIHAN Scientific Company, Seoul, South Korea) and centrifuged (Union 55R, Hanil science Company, Gimpo, South Korea) at $4470 \times g$ for 20 min at 4 °C. The final pellets were resuspended in 9 ml of 0.2% peptone water (PW; Difco; Becton Dickinson Co., Sparks, MD, USA) corresponding to approximately 10^9 CFU/mL of *C. sakazaki*i ES15 and 10^7 CFU/mL of *C. sakazakii* KCTC 2949.

Quantitative analysis of *C. sakazakii* and lactic acid bacteria (LAB) after hydrothermal treatment

C. sakazakii and LAB populations in PIF-P samples were analyzed quantitatively using Chromogenic Enterobacter Sakazakii Agar (CES agar, MB cell; KisanBio Co., Ltd., Seoul, South Korea) and Plate Count Agar with Bromocresol Purple (PCA with BCP, MB cell; KisanBio Co., Ltd., Seoul, South Korea), respectively. For analysis, RIF-P samples were prepared by adding 200 mL of hot water (37 °C for control, and 40–70 °C for hydrothermal treatment) with PIF-P according to each sample manual. When the experimental temperature (40–70 °C) is reached, *C. sakazakii* (1 mL) was inoculated to RIF-P sample (200 mL) in the 250 mL beaker (PYREX®, USA). For the quantification of LAB, an infant formula that was not inoculated with *C. sakazakii* was used. The pathogen-inoculated RIF-P and non-inoculated RIF-P were stirred and hydrothermal treated for 0, 2, 4, and 8 min. The temperature of the sample was measured for each treatment time with the surface of the beaker containing the sample after hydrothermal treatment for 0, 2, 4, and 8 min using an infrared thermometer (BLUETECBO-350) (Table S2). After treatment, 25 mL of RIF-P sample was mixed with 225 mL of 0.2% peptone water in stomacher bag and homogenized using a stomacher (Stomacher LS 700A; BNF Korea Co., South Korea) for 30 s. Subsequently, ten-fold serial dilutions were conducted, and 100 µL of diluted solution was spread plated using CES medium followed by the incubation at 37 °C for 24 h. Typical blue-green colonies were enumerated as the number of *C. sakazakii*. For LAB quantification, 15 mL of PCA with BCP was poured into 1 mL of diluted solution by the pour plate method. BCP medium was incubated at 35 °C for 72 ± 3 h in a rectangular jar (MGC AnaeroPack® System, 7.0 L; Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) with the addition of GENbox Anaer Gaspack (bioMériux, Marcy-l'Étoile, France) for anaerobic counts. After incubation, typical white colonies around yellow zone were enumerated as the number of LAB.

Quantitative analysis of *C. sakazakii* treated with hydrothermal after inoculation with PIF-P

Thermal resistance of *C. sakazakii* was analyzed by inoculating on the PIF-P samples and dried for 4 h in clean bench. More specifically, 1 ml of prepared *C. sakazakii* was inoculated into a high-density polyethylene bag containing PIF-P followed by hand massage for homogenization. Inoculated PIF-P was dried for 4 h inside a biosafety hood (23 ± 2 °C) with the fan running. The inoculated PIF-P was subjected to 200 mL of hydrothermal treatment. Treatment temperatures were selected as 60 °C and 65 °C for sample A and B, 55 °C and 60 °C for sample C, considering the results of RIF-P experiments with the treatment time of 0—8 min. Sample treatment of 37 °C for 10 s was used as control. After treatment, 25 mL of RIF-P sample was mixed with 225 mL of 0.2% peptone water in stomacher bag and homogenized using a stomacher (BNF Korea Co., South Korea) for 30 s. Enumeration of *C. sakazakii* is not different from the experiment of RIF-P samples.

Quantitative analysis of LAB with powdered infant formula with probiotics storage period

The population levels of LAB according to storage period of PIF-P were quantitatively analyzed using PCA with BCP agar. Specifications of sample A, B, and C indicated the maximum storage periods as 21, 30, and 20 days, respectively. The day open the sample was set as day 1, and the levels of LAB were identified for following days: sample A (1, 5, 10, 15, 21 days), sample B (1, 7, 14, 21, 30 days), sample C(1, 5, 10, 15, 20 days). To mimic the consumer's behavior, the PIF-P lid was opened once a day during the storage period, and after removing 3 scoops, the lid was closed and stored at room temperature. At designated day, each PIF-P was reconstituted by adding 200 mL of hydrothermal (37 °C), and the RIF-P was stirred for 10 s. After treatment, 25 mL of RIF-P sample was mixed with 225 mL of 0.2% peptone water in stomacher bag and homogenized using a stomacher (BNF Korea Co., South Korea) for 30 s. Subsequently, ten-fold serial dilutions were conducted, and 15 mL of PCA with BCP was poured into 1 mL of diluted solution by the pour plate method.

DNA extraction and 16S rRNA gene amplification

DNA of total 6 samples (two time repeat of 3 samples) was extracted using the Qiagen DNeasy PowerSoil Pro extraction kit (Qiagen, Hilden, Germany). Untreated powder sample (250 mg) was designated as P and used to identify the microbiota of untreated sample C. Sample after treatment of 200 mL of 60 °C hydrothermal for 8 min followed by the treatment of the pellet (130 mg) with propidium monoazide (PMA, Sigma-Aldrich) was designated as HP to identify the microbiota of hydrothermal treated sample C after removing dead cell. Finally, sample subjected to 200 mL of 60 °C hydrothermal for 8 min followed by removal of supernatant was designated as HC to identify the microbiota of hydrothermal treated sample C containing live and dead cell.

The density and purity of the extracted DNA were evaluated using Qubit 4.0 (Invitrogen Corporation, Carlsbad, CA, USA) and Nanodrop™ Fluorospectrometer (Thermo Fisher Scientific Inc., WalthamMA, USA) in accordance with the manufacturer's instructions. After confirming the DNA quality, the bacterial 16S rRNA V3-V4 hypervariable region was amplified with polymerase chain reaction (PCR) using KAPA HIFI Hot Start Ready Mix (2x) (Roche Cat. No. 07958935001; Basel, Switzerland). Following 16S rRNA gene amplification primer sequence including Illumina overhang adapter sequences were used for amplification.

(341F:5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′)

805R:5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC -3′).

The reaction mixture used in PCR was as follows: extracted genomic DNA 2.5 µL; amplicon PCR forward primer (1 µM) 5 µL; amplicon PCR reverse primer (1 µM) 5 µL; 2 × KAPA HiFi Hot Start Ready Mix 12.5 µL (total 25 µL). For PCR, T100 Thermal Cycler (Cat. No.1861096; Hercules, CA, USA) was used, and using the following program: one cycle of denaturation at 95 °C for 3 min followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s, and a final extension at 72 °C for 5 min.

16S gene library construction

The free primers and primer dimer species in the amplification products were purified using AMPure XP beads (Cat. No. A63881; Beckman Coulter, Pasadena, CA, USA). After that, the Nextera XT Index kit (Illumina, San Diego, CA, USA) was used to attach dual indices and Illumina sequencing adapters, and the amplicon was purified again using AMPure XP beads. Prior to sequencing, the DNA concentration of each PCR product was determined using a Qubit 3.0 fluorometer, and the quality control was performed using a bioanalyzer (Agilent 2100, Santa Clara, CA, USA). A total of six genomic libraries was pooled together for a single Illumina sequencing. Sequencing was performed using the Illumina Nextseq system (Illumina, CA) according to the manufacturer's instructions.

Sequencing data processing and bioinformatics analysis

The sequencing data of Illumina Nextseq (2 × 300 bp) were analyzed using the QIIME2 (version 2023.05) software package. For bioinformatic analysis, deblur in the QIIME2 pipeline was used to prepare high-quality sequences by quality control and denoising (Amir et al., 2017). The denoising task removes the artificial sequence, cuts the low-quality part of the read, and merges the reliable high-quality forward and reverse read. Based on the quality scores, initial quality filtering was performed (Bokulich et al., 2013), and then the sequence was trimmed with the length of 200 bp (–p-trim-length 200). As a result of deblur, amplicon sequence variants were generated and used for diversity analysis. In order to compare the abundance of operational taxonomic units (OTUs) between different samples, the sequencing depth of each sample was standardized to 97,266 reads. Taxonomic classification was performed by aligning the representative sequence to the SILVA 138 database. MicrobiomeAnalyst (version 2.0) pipeline was used to visualize the results of alpha, beta diversity analysis, and taxonomy analysis.

Statistical analysis

Experiments for the quantitative analysis of *C. sakazakii* and LAB were repeated three times and are represented as the average ± standard deviation. In the case of the experiment for quantitative analysis of LAB by storage period and metagenome analysis were repeated two times and are also represented as the average ± standard deviation. One-way ANOVA of IBM SPSS Statistics 26 (SPSS lnc, IBM, NY, USA) was used to compare the significant differences. The mean values were separated using Duncan's multiple comparison test, and the significant difference was determined at the $p < 0.05$ level. T-test was conducted to identify the significant between the two groups.

Results

Quantitative analysis of *C. sakazakii* and LAB by hydrothermal treatment in RIF-P

The effect of hydrothermal treatment (40–70 °C) on the reduction of *C. sakazakii* inoculated in RIF-P was represented in Tables 1–3. The reduction level of *C. sakazakii* increased as treatment temperature and time increased.

Table 1 Population (log CFU/g) of *C. sakazakii* ES15 and KCTC 2949, and lactic acid bacteria (LAB) to in RIF-P (sample A) subjected to 40–70 °C hydrothermal treatment[1,2]

Experimental temperature (℃)	min	*C. sakazakii* ES15	*C. sakazakii* KCTC 2949	Lactic acid bacteria (LAB)
Control	0	6.18 ± 0.05^{Aa}	5.90 ± 0.07^{ABCDb}	6.14 ± 0.05^{AB}
40	0	5.97 ± 0.05^{Ba}	6.04 ± 0.05^{Aa}	6.13 ± 0.03^{AB}
	2	5.95 ± 0.03^{Bca}	6.04 ± 0.05^{Aa}	6.12 ± 0.02^{AB}
	4	5.86 ± 0.05^{BCDa}	5.98 ± 0.04^{ABCb}	6.15 ± 0.02^{AB}
	8	5.87 ± 0.01^{BCDa}	5.86 ± 0.14^{BCDa}	6.14 ± 0.05^{AB}
50	0	5.83 ± 0.05 Da	6.09 ± 0.10^{Ab}	6.10 ± 0.04^{AB}
	2	5.84 ± 0.02^{CDa}	5.82 ± 0.10^{CDa}	6.17 ± 0.03^{AB}
	4	5.84 ± 0.05^{CDa}	5.78 ± 0.04^{Da}	6.19 ± 0.03^{A}
	8	5.98 ± 0.04^{Ba}	5.84 ± 0.01^{CDb}	6.18 ± 0.02^{AB}
55	0	5.71 ± 0.01^{Ea}	4.47 ± 0.11^{Eb}	6.07 ± 0.06^{B}
	2	5.26 ± 0.08^{Ga}	4.24 ± 0.11^{Fb}	6.18 ± 0.03^{A}
	4	5.40 ± 0.02^{Fa}	4.26 ± 0.06^{Fb}	6.19 ± 0.08^{A}
	8	5.21 ± 0.18^{Ga}	3.64 ± 0.32^{Gb}	6.16 ± 0.02^{AB}
60	0	4.22 ± 0.05^{Ha}	$< 1.00^{Hb}$	5.99 ± 0.06^{CD}
	2	2.10 ± 0.06^{Ja}	$< 1.00^{Hb}$	5.98 ± 0.02^{CD}
	4	$< 1.00^{K}$	$< 1.00^{H}$	5.97 ± 0.02^{CD}
	8	$< 1.00^{K}$	$< 1.00^{H}$	5.90 ± 0.02^{D}
65	0	3.45 ± 0.10^{Ia}	$< 1.00^{Hb}$	5.22 ± 0.03^{E}
	2	$< 1.00^{K}$	$< 1.00^{H}$	5.21 ± 0.11^{E}
	4	$< 1.00^{K}$	$< 1.00^{H}$	4.59 ± 0.07^{F}
	8	$< 1.00^{K}$	$< 1.00^{H}$	4.32 ± 0.07^{G}
70	0	$< 1.00^{K}$	$< 1.00^{H}$	2.63 ± 0.05^{H}
	2	$< 1.00^{K}$	$< 1.00^{H}$	2.27 ± 0.08^{I}
	4	$< 1.00^{K}$	$< 1.00^{H}$	$< 1.00^{J}$
	8	$< 1.00^{K}$	$< 1.00^{H}$	$< 1.00^{J}$

Mean ± Standard deviations (SD)

< 1.00: inactivated below the detection limit

[1]Different uppercase letters in the column represent the significant different ($p < 0.05$)

[2]Different lowercase letters in the row represent significant difference ($p < 0.05$)

Sample A

The number of *C. sakazakii* ES15 inoculated to sample A was reduced to 2.10 ± 0.06 log CFU/g from 6.18 ± 0.05 log CFU/g (control) by 60 °C hydrothermal treatment for 2 min, and the pathogen was inactivated below the detection limit after 4 min of 60 °C hydrothermal treatment (Table 1). When the treatment temperature increased to 65 °C, the population of pathogen decreased to 3.45 ± 0.10 log CFU/g immediately, and the number of pathogen decreased below the detection limit (1 log CFU/g) after 2 min treatment. When 70 °C hydrothermal used, the pathogen was inactivated below the detection limit immediately. Similar trend was observed for *C. sakazakii* KCTC 2949, but thermal resistance was not exactly same. The number of *C. sakazakii* KCTC 2949 inoculated to sample A was reduced to 3.64 ± 0.32 log CFU/g from 5.90 ± 0.07 log CFU/g (control) by 55 °C hydrothermal treatment for 8 min. When the treatment temperature increased to 60 °C, the pathogen was inactivated below the detection limit immediately. When the population of LAB was measured, thermal resistance of LAB was significantly higher than that of *C. sakazakii*. The number of LAB in sample A was reduced to 2.27 ± 0.08 log CFU/g from 6.14 ± 0.05 log CFU/g (control) by 70 °C hydrothermal treatment for 2 min. The population of LAB decreased below the detection limit after 4 min of 70 °C hydrothermal treatment.

Sample B

Similar trend with sample A was observed in the sample B, but the specific level of cell population was different. Overall, thermal resistance of *C. sakazakii* was lower in sample B than in sample A. The number of *C. sakazakii* ES15 inoculated to sample B was immediately reduced to 2.16 ± 0.27 log CFU/g from 5.91 ± 0.26 log CFU/g

(control) by 60 °C hydrothermal treatment, and the number of pathogens decreased below the detection limit after 2 min (Table 2). In sample B, the thermal resistance of *C. sakazakii* KCTC 2949 was not significantly different compared to that of *C. sakazakii* ES15. Similar with *C. sakazakii* ES15, the number of *C. sakazakii* KCTC 2949 inoculated to sample B was immediately reduced to 2.95 ± 0.21 log CFU/g from 5.98 ± 0.18 log CFU/g (control) by 60 °C hydrothermal treatment, and the number of pathogens decreased below the detection limit after 2 min. When the treatment temperature increased to above 65 °C, both *C. sakazakii* ES15 and *C. sakazakii* KCTC 2949 were reduced to below the detection limit. Thermal resistance of LAB was higher in sample B than in sample A. For example, the number of LAB in sample B was reduced to 2.53 ± 0.09 log CFU/g from 6.12 ± 0.03 log CFU/g (control) by 8 min of 70 °C hydrothermal treatment.

Sample C

Unlike the samples A and B, both *C. sakazakii* and LAB showed low thermal resistance in sample C. The number of *C. sakazakii* ES15 was immediately reduced to 3.96 ± 0.15 log CFU/g from 5.67 ± 0.08 log CFU/g (control) by 55 °C hydrothermal treatment, and the number of pathogens decreased below the detection limit after 2 min (Table 3). In the case of *C. sakazakii* KCTC 2949 in sample C, it was reduced to 2.27 ± 0.19 log CFU/g from 5.76 ± 0.05 log CFU/g (control) by 55 °C hydrothermal treatment for 4 min, and the number of pathogens decreased below the detection limit after 8 min. The number of LAB in sample C was reduced to 2.86 ± 0.03 log CFU/g from 5.65 ± 0.07 log CFU/g (control) by 65 °C hydrothermal treatment for 2 min, and the population decreased below the detection limit after 4 min. When the higher temperature (70 °C) of hydrothermal was used, the population of LAB immediately decreased to

Table 2 Population (log CFU/g) of *C. sakazakii* ES15 and KCTC 2949, and lactic acid bacteria (LAB) to in RIF-P (sample B) subjected to 40–70 °C hydrothermal treatment[1,2]

Experimental temperature (℃)	min	*C. sakazakii* ES15	*C. sakazakii* KCTC 2949	Lactic acid bacteria (LAB)
Control	0	5.91 ± 0.26 ^{Aa}	5.98 ± 0.18 ^{ABCDa}	6.12 ± 0.03 ^{A}
40	0	6.05 ± 0.17 ^{Aa}	6.03 ± 0.14 ^{ABCDa}	6.09 ± 0.06 ^{A}
	2	6.05 ± 0.14 ^{Aa}	6.06 ± 0.11 ^{ABCa}	6.10 ± 0.03 ^{A}
	4	6.06 ± 0.14 ^{Aa}	6.13 ± 0.12 ^{ABa}	6.01 ± 0.16 ^{A}
	8	6.06 ± 0.09 ^{Aa}	6.19 ± 0.08 ^{Aa}	6.09 ± 0.01 ^{A}
50	0	5.98 ± 0.10 ^{Aa}	5.94 ± 0.14 ^{BCDa}	6.13 ± 0.04 ^{A}
	2	5.99 ± 0.12 ^{Aa}	5.86 ± 0.08 ^{CDa}	6.13 ± 0.03 ^{A}
	4	6.01 ± 0.12 ^{Aa}	5.93 ± 0.18 ^{BCDa}	6.14 ± 0.04 ^{A}
	8	6.00 ± 0.18 ^{Aa}	6.05 ± 0.14 ^{ABCa}	6.14 ± 0.02 ^{A}
55	0	5.91 ± 0.04 ^{Aa}	5.81 ± 0.08 ^{Da}	5.97 ± 0.05 ^{A}
	2	5.89 ± 0.01 ^{Aa}	5.41 ± 0.05 ^{Eb}	6.01 ± 0.05 ^{A}
	4	5.81 ± 0.03 ^{Aa}	5.47 ± 0.14 ^{Eb}	5.97 ± 0.03 ^{A}
	8	5.59 ± 0.06 ^{Ba}	5.48 ± 0.12 ^{Ea}	6.07 ± 0.05 ^{A}
60	0	2.16 ± 0.27 ^{Ca}	2.95 ± 0.21 ^{Fb}	5.96 ± 0.06 ^{A}
	2	< 1.00 ^{D}	< 1.00 ^{G}	5.97 ± 0.09 ^{A}
	4	< 1.00 ^{D}	< 1.00 ^{G}	5.97 ± 0.13 ^{A}
	8	< 1.00 ^{D}	< 1.00 ^{G}	5.96 ± 0.13 ^{A}
65	0	< 1.00 ^{D}	< 1.00 ^{G}	4.87 ± 0.20 ^{B}
	2	< 1.00 ^{D}	< 1.00 ^{G}	3.60 ± 0.45 ^{C}
	4	< 1.00 ^{D}	< 1.00 ^{G}	3.42 ± 0.50 ^{CD}
	8	< 1.00 ^{D}	< 1.00 ^{G}	3.07 ± 0.39 ^{DE}
70	0	< 1.00 ^{D}	< 1.00 ^{G}	2.80 ± 0.11 ^{EF}
	2	< 1.00 ^{D}	< 1.00 ^{G}	2.51 ± 0.19 ^{F}
	4	< 1.00 ^{D}	< 1.00 ^{G}	2.62 ± 0.08 ^{F}
	8	< 1.00 ^{D}	< 1.00 ^{G}	2.53 ± 0.09 ^{F}

Mean ± Standard deviations (SD)

< 1.00: inactivated below the detection limit

[1]Different uppercase letters in the column represent the significant different ($p < 0.05$)

[2]Different lowercase letters in the row represent significant difference ($p < 0.05$)

Table 3 Population (log CFU/g) of *C. sakazakii* ES15 and KCTC 2949, and lactic acid bacteria (LAB) to in RIF-P (sample C) subjected to 40–70 °C hydrothermal treatment[1,2]

Experimental temperature (℃)	min	*C. sakazakii* ES15	*C. sakazakii* KCTC 2949	Lactic acid bacteria (LAB)
Control	0	5.67 ± 0.08 ^{BCa}	5.76 ± 0.05 ^{Aa}	5.65 ± 0.07 ^{CDE}
40	0	5.72 ± 0.03 ^{ABa}	5.75 ± 0.15 ^{Aa}	5.67 ± 0.06 ^{BCD}
	2	5.76 ± 0.02 ^{Aa}	5.74 ± 0.10 ^{Aa}	5.85 ± 0.10 ^{A}
	4	5.65 ± 0.02 ^{BCa}	5.63 ± 0.14 ^{Aa}	5.64 ± 0.12 ^{CDE}
	8	5.36 ± 0.13 ^{Ea}	5.69 ± 0.04 ^{Ab}	5.61 ± 0.01 ^{DE}
50	0	5.59 ± 0.20 ^{CDa}	5.76 ± 0.17 ^{Aa}	5.52 ± 0.05 ^{E}
	2	5.55 ± 0.07 Da	5.20 ± 0.07 ^{Bb}	5.58 ± 0.05 ^{DE}
	4	5.40 ± 0.03 ^{Ea}	2.12 ± 0.08 ^{Eb}	5.68 ± 0.08 ^{BCD}
	8	5.72 ± 0.07 ^{Aba}	2.19 ± 0.03 ^{Eb}	5.74 ± 0.11 ^{ABC}
55	0	3.96 ± 0.15 ^{Fa}	4.73 ± 0.26 ^{Cb}	5.76 ± 0.04 ^{ABC}
	2	< 1.00 Ga	2.52 ± 0.22 ^{Db}	5.79 ± 0.04 ^{AB}
	4	< 1.00 Ga	2.27 ± 0.19 ^{Eb}	5.84 ± 0.02 ^{A}
	8	< 1.00 ^{G}	< 1.00 ^{F}	5.82 ± 0.05 ^{A}
60	0	< 1.00 ^{G}	< 1.00 ^{F}	4.19 ± 0.02 ^{F}
	2	< 1.00 ^{G}	< 1.00 ^{F}	3.61 ± 0.07 ^{G}
	4	< 1.00 ^{G}	< 1.00 ^{F}	3.20 ± 0.01 ^{H}
	8	< 1.00 ^{G}	< 1.00 ^{F}	3.08 ± 0.04 ^{I}
65	0	< 1.00 ^{G}	< 1.00 ^{F}	3.31 ± 0.01 ^{H}
	2	< 1.00 ^{G}	< 1.00 ^{F}	2.86 ± 0.03 ^{J}
	4	< 1.00 ^{G}	< 1.00 ^{F}	< 1.00 ^{L}
	8	< 1.00 ^{G}	< 1.00 ^{F}	< 1.00 ^{L}
70	0	< 1.00 ^{G}	< 1.00 ^{F}	2.30 ± 0.09 ^{Ka}
	2	< 1.00 ^{G}	< 1.00 ^{F}	< 1.00 ^{Lb}
	4	< 1.00 ^{G}	< 1.00 ^{F}	< 1.00 ^{Lb}
	8	< 1.00 ^{G}	< 1.00 ^{F}	< 1.00 ^{Lb}

Mean ± Standard deviations (SD)

< 1.00: inactivated below the detection limit

[1]Different uppercase letters in the column represent the significant different ($p < 0.05$)

[2]Different lowercase letters in the row represent significant difference ($p < 0.05$)

2.30 ± 0.09 log CFU/g, and the number of LAB decreased below the detection limit after 2 min.

Quantitative analysis of *C. sakazakii* by hydrothermal treatment inoculated in PIF-P

The heat resistance of *C. sakazakii* inoculated to PIF-P with 4 h drying was identified and compared with that inoculated to RIF-P (Table 4). The temperatures of hydrothermal in PIF-P experiments were set to 55, 60, and 65 °C considering the results of RIF-P experiments. The results of PIF-P indicated that the thermal resistance of the pathogen increased significantly when inoculated to PIF-P with 4 h drying compared to that of RIF-P samples.

More specifically, in sample A, *C. sakazkaii* ES15 was inactivated below the detection limit after more than 4 min with 60 °C hydrothermal treatment in RIF-P inoculation whereas the pathogen inoculated in PIF-P exhibited 2.37 ± 0.08 log CFU/g at same treatment condition. It is noteworthy that even after 8 min of 60 °C hydrothermal treatment, 1.84 ± 0.07 log CFU/g of the *C. sakazakii* ES15 were survived in PIF-P sample. The thermal resistance of PIF-P inoculated pathogen was more significant for *C. sakazakii* KCTC 2949. When the pathogen inoculated in RIF-P, pathogen was immediately inactivated below the detection limit with 60 °C hydrothermal, whereas 2.24 ± 0.12 log CFU/g was survived even after 4 min treatment of 65 °C. hydrothermal treatment. The pathogen decreased below the detection limit after 8 min treatment of 65 °C hydrothermal treatment.

Similar trend was observed in sample B. In RIF-P inoculation, the number of *C. sakazakii* ES15 decreased below the detection limit after 2 min of 60 °C hydrothermal treatment. After PIF-P inoculation and 4 h drying, 1.95 ± 0.10 log CFU/g of the pathogen survived after 4 min of 60 °C hydrothermal treatment. It is noteworthy that 2.13 ± 0.06 log CFU/g of pathogen survived at 65 °C hydrothermal treatment. Similar with sample A, the thermal resistance of the

Table 4 Populations (log CFU/g) of *C. sakazakii* ES15 and KCTC 2949 (log CFU/g) inoculated to RIF-P samples and PIF-P samples subjected to 60 °C and 65 °C hydrothermal treatment[1,2]

Type	Temperature (℃)	Time (min)	*C. sakazakii* ES15		*C. sakazakii* KCTC 2949	
			RiF-P inoculation	PiF-P inoculation	RiF-P inoculation	PiF-P inoculation
Sample A	Control	0	6.18 ± 0.05^{Aa}	4.57 ± 0.07^{Ab}	5.90 ± 0.07^{Aa}	4.73 ± 0.07^{Ab}
	60	0	4.22 ± 0.05^{Ba}	2.23 ± 0.12^{Bb}	$< 1.00^{Ba}$	2.54 ± 0.50^{BCb}
		2	2.10 ± 0.06 Da	2.43 ± 0.19^{Ba}	$< 1.00^{Ba}$	2.55 ± 0.04^{BCb}
		4	$< 1.00^{Ea}$	2.37 ± 0.08^{Bb}	$< 1.00^{Ba}$	2.85 ± 0.03^{Bb}
		8	$< 1.00^{Ea}$	1.84 ± 0.07^{Cb}	$< 1.00^{Ba}$	2.72 ± 0.21^{Bb}
	65	0	3.45 ± 0.10^{Ca}	2.21 ± 0.21^{Bb}	$< 1.00^{Ba}$	2.06 ± 0.17^{Db}
		2	$< 1.00^{E}$	$< 1.00^{D}$	$< 1.00^{Ba}$	1.98 ± 0.11^{Db}
		4	$< 1.00^{E}$	$< 1.00^{D}$	$< 1.00^{Ba}$	2.24 ± 0.12^{Db}
		8	$< 1.00^{E}$	$< 1.00^{D}$	$< 1.00^{B}$	$< 1.00^{E}$
Sample B	Control	0	5.91 ± 0.26^{Aa}	4.59 ± 0.06^{Ab}	5.98 ± 0.18^{Aa}	4.71 ± 0.09^{Ab}
	60	0	2.16 ± 0.27^{Ba}	2.08 ± 0.09^{Ba}	2.95 ± 0.21^{Ba}	2.09 ± 0.13^{Cb}
		2	$< 1.00^{Ca}$	1.86 ± 0.10^{Cb}	$< 1.00^{C}$	$< 1.00^{D}$
		4	$< 1.00^{Ca}$	1.95 ± 0.10^{Cb}	$< 1.00^{C}$	$< 1.00^{D}$
		8	$< 1.00^{C}$	$< 1.00^{D}$	$< 1.00^{C}$	$< 1.00^{D}$
	65	0	$< 1.00^{Ca}$	2.13 ± 0.06^{Bb}	$< 1.00^{Ca}$	2.68 ± 0.05^{Bb}
		2	$< 1.00^{C}$	$< 1.00^{D}$	$< 1.00^{C}$	$< 1.00^{D}$
		4	$< 1.00^{C}$	$< 1.00^{D}$	$< 1.00^{C}$	$< 1.00^{D}$
		8	$< 1.00^{C}$	$< 1.00^{D}$	$< 1.00^{C}$	$< 1.00^{D}$
Sample C	Control	0	5.67 ± 0.08^{Aa}	5.07 ± 0.03^{Ab}	5.76 ± 0.05^{Aa}	4.93 ± 0.12^{Ab}
	55	0	3.96 ± 0.15^{Ba}	4.37 ± 0.10^{Bb}	4.73 ± 0.26^{Ba}	4.07 ± 0.06^{Ba}
		2	$< 1.00^{Ca}$	3.98 ± 0.05^{Cb}	2.52 ± 0.22^{Ca}	4.23 ± 0.03^{Bb}
		4	$< 1.00^{Ca}$	3.97 ± 0.15^{Cb}	2.27 ± 0.19^{Ca}	4.06 ± 0.10^{Bb}
		8	$< 1.00^{Ca}$	3.85 ± 0.06^{Cb}	< 1.00 Da	2.88 ± 0.08^{Cb}
	60	0	$< 1.00^{Ca}$	2.61 ± 0.14^{Db}	$< 1.00^{Da}$	2.46 ± 0.15^{Db}
		2	$< 1.00^{C}$	$< 1.00^{E}$	$< 1.00^{D}$	$< 1.00^{E}$
		4	$< 1.00^{C}$	$< 1.00^{E}$	$< 1.00^{D}$	$< 1.00^{E}$
		8	$< 1.00^{C}$	$< 1.00^{E}$	$< 1.00^{D}$	$< 1.00^{E}$

Mean ± Standard deviations (SD)

< 1.00: inactivated below the detection limit

[1]Different uppercase letters in the column represent the significant different within the same sample ($p < 0.05$)

[2]Different lowercase letters in the row represent significant difference within the same strain ($p < 0.05$)

PIF-P inoculated pathogen was significantly higher for *C. sakazakii* KCTC 2949 than that of ES15 strain. The pathogen inoculated to RIF-P sample was inactivated below the detection limit with 65 °C hydrothermal treatment whereas 2.68 ± 0.05 log CFU/g still survived for the pathogen inoculated in PIF-P samples.

Similar trend was observed in the sample C and the heat resistance of pathogen inoculated to PIF-P samples increased more significantly. *C. sakazakii* ES15 inoculated in RIF-P decreased below the detection limit after 2 min of 55 °C hydrothermal treatment. On the other hand, 3.85 ± 0.06 log CFU/g of the pathogen survived after 8 min of 55 °C hydrothermal treatment. It is noteworthy that 2.61 ± 0.14 log CFU/g of pathogens were survived even after 60 °C hydrothermal treatment. Similar trend was observed for *C. sakazakii* KCTC 2949. In the case of RIF-P inoculation, the pathogen decreased below the detection limit after 55 °C hydrothermal treatment for 8 min. When the pathogen inoculated in PIF-P, 2.88 ± 0.08 log CFU/g of pathogen survived after same treatment condition. When the temperature of hydrothermal treatment increased to 60 °C, 2.46 ± 0.15 log CFU/g of the pathogen were survived.

Metagenome analysis before and after hydrothermal treatment

Prior to the metagenomic analysis, the levels of LAB by storing the three PIF-P samples at room temperature were identified (Table S3). In the case of sample A, the number of LAB reduced to 4.36 ± 0.36 log CFU/g from 6.13 ± 0.02 log

CFU/g after 15 days of storage, but increased to 5.59 ± 0.43 log CFU/g after 21 days. The population of LAB in sample B was significantly reduced to 4.79 ± 0.18 log CFU/g from 6.15 ± 0.05 log CFU/g after 30 days of storage. Unlike samples A and B, the number of LAB in sample C maintained almost the same level from 6.20 ± 0.04 log CFU/g after opening to 6.20 ± 0.03 log CFU/g 20 days after storage. Therefore, 16S rRNA amplicon sequencing in sample C was performed to compare and analyze the changes in the probiotics in PIF-P before and after hydrothermal treatment at 60 °C for 8 min.

A total of 3,415,188 reads was generated in the 16S rRNA gene V3-V4 region. The total number of amplicon sequence variants identified through the deblur process was 1,311,778, and the read count and sequencing quality of all samples were judged to be appropriate. Taxonomic analysis at the genus level of each sample group was shown in Fig. 1. The microbial flora ratios between the P group and the HP and HC groups treated with 60 °C hydrothermal were observed to be very similar. All three groups showed the highest proportion of *Lactobacillus* followed by *Bifidobacterium*. The average ratios of *Lactobacillus* in P, HP, and HC samples were 69%, 68%, and 70%, respectively. Likewise, *Bifidobacterium* showed similar trends among samples showing 29%, 27%, and 27% values in P, HP, and HC groups, respectively.

Additionally, diversity analysis was performed at the genus level to verify the significant difference between the groups (Fig. 2). Diversity analysis was performed with alpha diversity indicating in species richness and evenness within the sample and beta diversity measuring the similarity of microbial composition between different samples. More specifically, Shannon (representing both species richness and evenness) indices were used for alpha diversity. As a result, the HP group showed the highest values (0.83 ± 0.01), followed by P group (0.76 ± 0.05), and HC group (0.72 ± 0.04). However, there was no significant difference in the abundance and evenness of the microbial flora between the groups ($p > 0.05$). Beta diversity was represented by a principal coordinate analysis (PCoA) plot at the genus level based on Bray–Curtis. As a result, there was

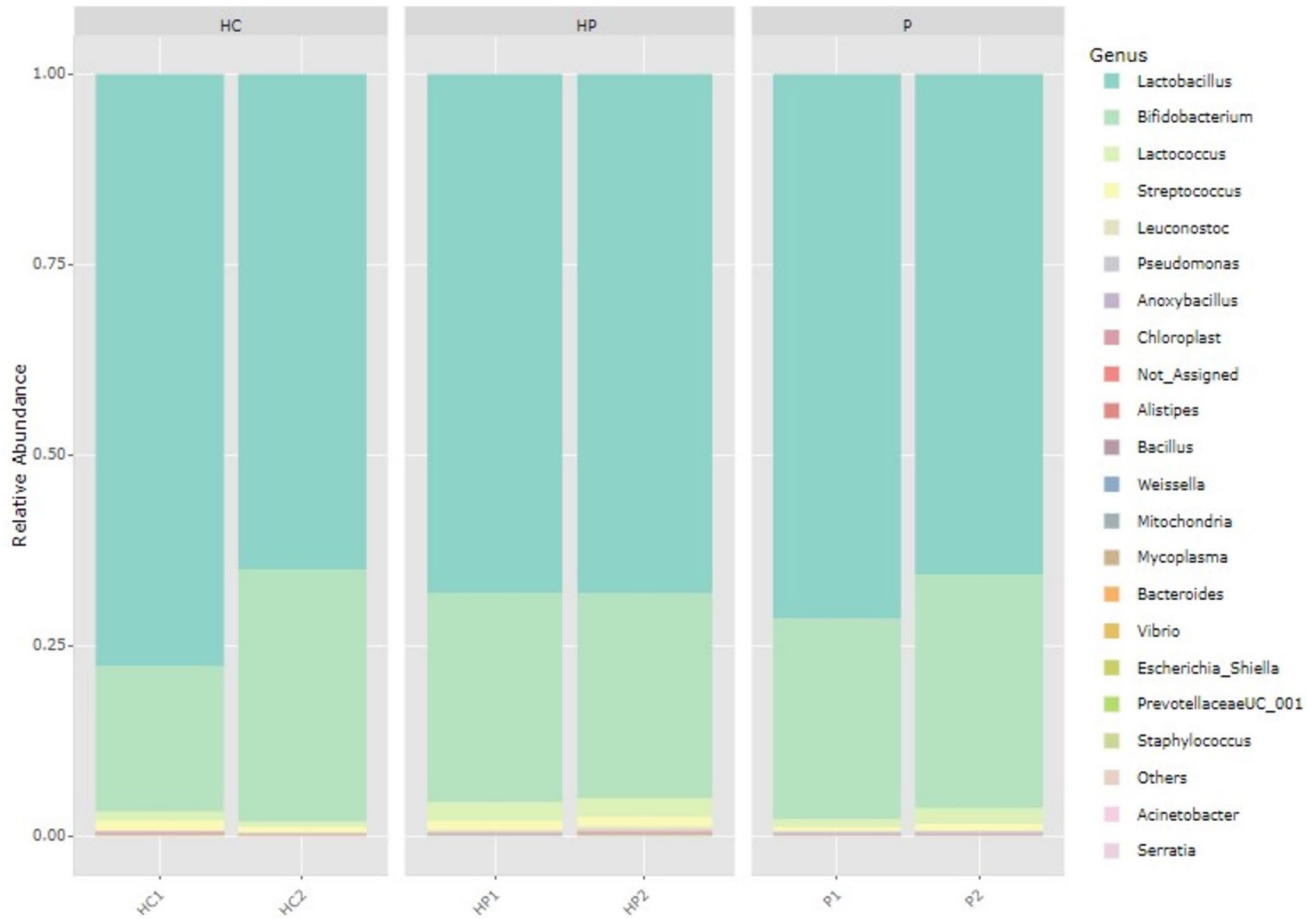

Fig. 1 Taxonomic analysis of hydrothermal treatment before and after of infant formula with probiotics using 16S rRNA amplicon sequencing. The analyzed infant formula with probiotics samples were repeated twice each and grouped as Powder "P", Hydrothermal with PMA "HP", and Hydrothermal with Centrifuge "HC"

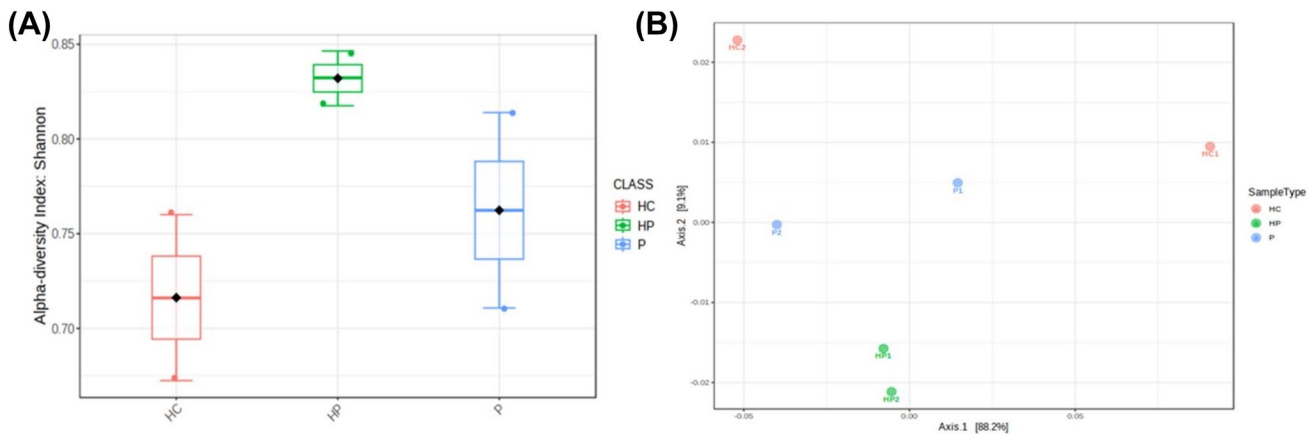

Fig. 2 Diversity analysis of hydrothermal treatment before and after of infant formula with probiotics samples grouped by Powder (P), Hydrothermal with PMA (HP), Hydrothermal with Centrifuge (HC). Alpha diversity analysis with Shannon **A** indices were represented, Beta diversity analysis with Bray–Curtis **B** indices was represented

no significant difference in microbial composition between the groups ($p > 0.05$).

Discussion

In the present study, thermal resistances of *C. sakazakii* inoculated in RIF-P were investigated and compared with those inoculated in PIF-P and dried for 4 h. Even though the thermal resistances were various depending on the microbial strain and type of samples, most pathogens inoculated in RIF-P were inactivated below detection limits with hydrothermal temperatures of 60 °C or 65 °C. These results were similar to previous study reported that > 60 oC hydrothermal treatment was effective to inactivate *E. sakazakii* (the former name of *C. sakazakii*) (Kim et al., 2008). Osaili et al. (2009) also reported that over 70 °C of hydrothermal treatment was effective for the inactivation of *Cronobacter* in powdered products such as powdered milk and feeding formula. Moreover, WHO (2007) suggested that preparation of PIF with water at a temperature of higher than 70 °C dramatically reduces the microbiological risk. On the other hand, Losio et al. (2018) insisted that the temperature higher than 85 °C is lethal to pathogens because hydrothermal treatment of 70 °C reaching the formula is likely to cool rapidly, which is not lethal to pathogens such as *C. sakazakii* and *Salmonella* spp. in powdered products. In this regard, the temperature of hydrothermal needed to inactivate *C. sakazakii* in RIF-P was investigated in the present study revealing that over than 60 °C hydrothermal treatment was enough to control about 6 log CFU/mL *C. sakazakii* inoculated in RIF-P.

When the heat resistance of *C. sakazakii* inoculated to PIF-P with 4 h drying was compared with that directly inoculated to RIF-P, the thermal resistance of the pathogen inoculated to PIF-P was higher than that directly inoculated to RIF-P. These results suggest that *C. sakazakii* increased thermal resistance in dry stress environment. Previously, Fakruddin et al. (2014) showed significantly ($p < 0.05$) higher survival rate when *C. sakazakii* was grown and dried in a PIF than that was grown in TSB. Huertas et al. (2015) also reported that when the pathogen artificially inoculated PIF and reconstituted with hydrothermal (50, 55, 60, 65, 70 °C), *C. sakazakii* survived for a long time in reconstituted infant formula and could proliferate during cooling after reconstitution. Additionally, Garbaj et al. (2023) reported that *C. sakazakii* can grow and proliferate at cooling temperature over time by showing the heat resistance as D-value and the z-value. These results indicated that the resistance of *C. sakazakii* increased in the dry conditions and the pathogen can proliferate during cooling period implicating the careful intake of RIF is needed.

Thermal resistance in the dry stress environment of *C. sakazakii* would be associated with biofilm formation. Previously, Lee et al. (2009) reported that most *Cronobacter* isolated from domestic food is resistant to drying, and most have the biofilm forming characteristics under drying stress. Biofilm showed significant high resistance to hostile environmental conditions in bacteria, and these biofilms have a spongy structure that can absorb water and prevent bacteria from desiccation (Abebe, 2020). Even though further study is needed to confirm the biofilm formation of *C. sakazakii* inoculated PIF with 4 h drying, the present and previous studies indicated that the temperature and treatment time of hydrothermal used for reconstitution should be adjusted carefully considering the heat resistance change of *C. sakazakii* contaminated to PIF-P.

The number of food products containing probiotics has been increased and various PIF products containing probiotics have been produced in market. The recommended intake of probiotics has known to 10^6 CFU/g with daily

consumption of at least 100 g food because at least 10^8–10^9 viable cells can demonstrate the effectiveness of probiotics (Sidira et al., 2014). Eom et al. (2005) reported that intake of 10^8 CFU/day *Lactobacillus reuteri* was effective in treating diarrhea in infants between 6 and 36 months of age who suffer from acute diarrhea. Kajander et al. (2007) also confirmed that intake of $8–9 \times 10^9$ CFU/day multispecies probiotic capsules (*Lactobacillus rhamnosus* GG, *Lactobacillus rhamnosus* Lc705, *Propionibacterium freudenreichii* ssp., *Bifidobacterium breve*) were effective in alleviating chronic colorectal syndrome symptoms in adult patients with irritable bowel syndrome. In this regard, infant formula with probiotics must maintain a certain level of viable cell during the actual PIF-P consumption period and even after the reconstitution with hot water to ensure functionality.

In the present study, it was confirmed that the LAB tends to decrease over time in sample A and B. In the previous studies, Song et al. (2021) reported that the viable cell of six samples containing LAB was significantly decreased in both MRS and BCP agar during 7 months storage period. Lim et al. (2015) also reported that the survival rate of LAB in freeze-dried milk decreased after 16 weeks of storage. It was identified that lower storage temperature ensured the the higher survival rate of LAB while the storage at room temperature seems to accelerate the death rate of LAB in the product, and the levels of LAB decreased when exposed to air (Park et al., 2002). Considering these points, it is expected that the change of LAB in samples A and B during the storage period is due to the air exposure and storage environment of the samples.

Different from sample A and B, the levels of LAB populations (6.20 log CFU/g) in sample C were not significantly changed after 20 days of storage. Therefore, microbiota of sample C before and after hydrothermal treatment was analyzed to identify the change of LAB levels. The aim of 16S rRNA amplicon sequencing in sample C was to identify the ratio of LAB that changes when PIF-P is manufactured at the appropriate hydrothermal temperature (60 °C for 8 min) set to prevent *C. sakazakii* infection. Taxonomic analysis showed that *Lactobacillus* and *Bifidobacterium* were dominant species (Fig. 1) which is in line with the LAB indicated in the label of sample C; *Lactobacillus reuteri* DSM 17938, *Lactobacillus fermentum* Lc 40, *Lactobacillus rhamnosus* GG, *Bifidobacterium animalis* spp. *lactis* BB-12®. It is noteworthy that the diversity analysis indicated that there is no change in the abundance (%) of major microbial flora before and after 60 °C hydrothermal treatment of PIF-P (Fig. 2). From the media-based experiments we confirmed that 60 °C for 8 min hydrothermal treatment decreased the levels of both *C. sakazakii* ES15 and *C. sakazakii* KCTC 2949 to below the detection limit but more than 3 log CFU LAB (3.08 ± 0.04 log CFU/g) survived after the treatment. These results indicated that the proposed hydrothermal treatment conditions (60 °C for 8 min) to ensure the microbiological safety of infant formula with probiotics can reduce the number of LAB but have no significant effect on the ratio of microbiota.

The results of this study suggest that even if the temperature is higher than the recommendation of manufacturer's manual, microbiota is not significantly changed after hot water reconstitution. The result of the present study is considered to provide a scientific basis for suggesting safety and functionality to consumers purchasing infant formula with probiotics.

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Declarations

Conflict of interest No conflict of interest to declare.

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