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DETERMINATION OF THE PHYLOGENETIC RELATEDNESS OF *CRONOBACTER* SPP. ISOLATED FROM POWDERED INFANT FORMULA RETAILED IN NIGERIA USING PAN–GENOMIC DNA MICROARRAY

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Abstract

Cronobacter spp. are emerging, opportunistic, food-borne pathogens associated with infections like meningitis, necrotizing enterocolitis and septicaemia in premature and immunocompromised neonates and infants. The phylogenetic relatedness of three Cronobacter species isolated from powdered infant formula retailed in Nigeria was carried out using a Pan-Genomic DNA Microarray constituting 19,287 independent genes representing 15 Cronobacter genomes and 18 plasmids and 2,371 virulence genes of phylogenetically related Gram-negative bacteria. The hybridization results showed that Cronobacter malonaticus (CS14) and Cronobacter sakazakii (CS17 and CS124) clustered with powdered infant formula environmental and clinical strains of C. malonaticus and C. sakazakii isolated from countries like Jordan, Czech Republic, Ireland and USA with a significant relatedness greater than 80%. The sequence types of C. malonaticus CS14 was ST303 and C. sakakakii CS17 and CS124 were ST304 and ST296, respectively. Some virulence genes (integrase of Shigella flexnerri bacteriophage X, hypothetical protein z1655, dihydrofolate reductase, and formate acetyltransferase 1) were detected in CS124 and CS17. Adequate regulatory measures should be applied to monitor imported and locally produced powdered infant formulae to prevent contamination with Cronobacter spp. and other food borne pathogens to ensure the safety of vulnerable neonates and infants.

Keywords: *Cronobacter sakazakii*; *C. malonaticus*; Powdered infant formula; Virulence; Microarray Hybridization.

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1. Introduction

Cronobacter spp. are an emerging, opportunistic pathogen that cause infections such as septicaemia, meningitis and necrotizing enterocolitis in neonates and infants, and can sometimes lead to death. Although they were initially publicized for their connections to neonatal infections, they are now recognized as predominantly causing infections in adults (Forsythe, 2018). *Cronobacter* spp. are recognized to be more globally and economically widespread than was once thought, and have been found associated with foods such as infant foods [powdered infant formula (PIF), follow-up formula], dried milk protein products, cheese, licorice, candies, dried spices, teas, nuts, herbs, ready to eat foods such as pastas, vegetables as well as filth and stable flies. Also implicated are milk powder production facilities and house hold environments (Tall *et al.*, 2017). There is zero tolerance for the presence of *Cronobacter* spp. in all powdered infant formulae because of the high mortality rate (80%) associated with infections caused by their consumption by infants (Jackson *et al.*, 2014). The genus contains seven species: *Cronobacter sakazakii, Cronobacter malonaticus, Cronobacter turicensis, Cronobacter muytjensii, Cronobacter dublinensis, Cronobacter universalis,* and *Cronobacter condimenti* with *C. sakazakii* and *C. malonaticus* forming the majority of clinical isolates in all age groups with a greater incidence in the very young and elderly, particularly the immunocompromised (Patrick *et al.*, 2014). The FDA *Cronobacter* microarray is a next generation sequence-based, custom-designed pan genomic microarray platform that has been used as a highly discriminatory characterization, identification and dataset mining tool for public health laboratorian use and source attribution (Tall *et al.*, 2017). The phylogenic divergence of the genus *Cronobacter* and the genomic diversity among each member of the genus has been reported (Tall *et al*., 2015), with the pan genomic microarray evaluating the global genomic diversity among the seven species of *Cronobacter*, and also discriminating among individual and closely related strains within each species. This is important in microbial source tracking investigations during food borne outbreaks. The pan genomic DNA microarray with its concise annotation can differentiate each *Cronobacter* species and correctly identify and characterize the phylogenetic relatedness among strains isolated during surveillance and outbreak investigations.

The goal of this study was to determine the phylogenetic relatedness of *Cronobacter* spp. isolated from PIF retailed in Nigeria using Pan–Genomic DNA Microarray.

2. Materials and Methods

Bacterial Genomic DNA preparations.
The bacterial strains were grown overnight at 37°C in 5 ml of Trypticase soy broth (BBL, Becton Dickinson, Franklin Lakes, New Jersey) supplemented with 1% NaCl (final conc.), shaking at 160 rpm. Genomic DNA was isolated from 2 ml of the culture using a robotic QIAcube workstation with its automated QIAGEN DNeasy chemistry (Qiagen, Germantown, MD) for purification of DNA following the manufacturer's recommendations. Purified genomic DNA (5-15 µg) was recovered in a final elution volume of 200 µl. The purified DNA was further concentrated using an Amicron Ultracel-30 membrane filter (30,000 molecular weight cut-off, 0.5 ml, MILLIPORE Corp. Billerica, MA) to a final volume of approximately 10-25 µl.

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Microarray Hybridization

The microarray hybridization of DNA from the *Cronobacter* spp. was carried out using the Affymetrix MyGeneChip Custom Array (Affymetrix design number: FDACRONOa520845F). Hybridizations were performed according to the Affymetrix GeneChip Expression Analysis Technical Manual for the 49-format array (Bolstad *et al*., 2003). Five µg of the genomic DNA was fragmented by incubating at 37°C for 1 min in a 20 µl of reaction containing 1× One-Phor-All Plus Buffer (GE Healthcare) and 0.01 U DNase I (GE Healthcare). The fragmentation was heat-inactivated at 99°C for 15 min. The fragmented DNA was 3'-end labelled by adding 4 µl of 5× terminal transferase buffer (Promega), 1 µl of 1 mM biotin-11-ddATP (PerkinElmer NEL508), and 2 µl (60 u) of terminal transferase enzyme (Promega). Labelling was carried out for 4 h at 37°C followed by heat inactivation at 98°C for 1 min. Hybridizations were performed according to the Affymetrix GeneChip Expression Analysis Technical Manual for the 49-format array (Affymetrix, 2014). Briefly, 146 µl of a hybridization buffer comprised of 100 µl of 2X hybridization buffer, 3.3 µl of a 3nM B2 oligonucleotide solution, 2 µl each of a 10 mg /ml Salmon DNA and 50 mg/ml Bovine Serum Albumin (BSA) solutions, and 15.5 µl of Dimethyl sulfoxide (DMSO) (SIGMA-ALDRICH, Inc. St. Louis, MO) per reaction followed by denaturation at 98°C for 1 minute. The denatured samples were added onto the Affymetrix arrays, which were then incubated at 45°C, with rotation (60 rpm) for 16 h in a hybridization oven. Following hybridization, wash and stain procedures were carried out on an Affymetrix FS-450 fluidics station using the mini_prok2v1_450 fluidics. Reagents for washing and staining were prepared according to the GeneChip® Expression Analysis Technical Manual (Affymetrix, 2014). The following exceptions were made to the wash and stain procedure: Streptavidin solution mix (vial 1) was replaced with SAPE solution mix (LIFE TECHNOLOGIES, Grand Island, NY). Arrays were scanned using Affymetrix GeneChip® Scanner 3000 running AGCC software (Tall *et al*., 2015).

Microarray data analysis

Probe set intensities for each gene represented on the microarray were summarized using the Robust MultiArray Averaging (RMA) function in the Affymetrix package of R-Bioconductor as described by Bolstad *et al.* (2003). The RMA summarization of probe level data was done by carrying out three individual treatments on all of the experimental data (CEL file). The probe specific correction of the perfect match (PM) probes was done using a model based on the observed intensities being the sum of signal and noise. Second, quantile normalization was performed on the corrected PM probe intensities. Finally, a median polishing algorithm was used to summarize the background-corrected, normalized probe intensities to generate a final probe set value.

Calculating gene differences and generating dendrograms

Robust MultiArray Averaging-summarized probe set intensities were compared across all strains for each gene. If the same gene in different strains had an RMA intensity difference greater than eightfold ($\log_2 = 3$), then that gene was considered to be "different." With this criterion, a strain versus strain gene-difference matrix was generated; where the difference matrix represents the number of genes/alleles that differs between any two isolates. Gene-difference matrices were converted to dendrograms using the *hclust* function in the base package as well as the *phylo* function in the *ape* package of R-Bioconductor. Hierarchical clustering was performed using the RMA-summarized probe set intensities using the MADE4 package of R-Bioconductor. Phylogenetic trees were made using the nearest neighbour-joining method via the MEGA 5

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software package as described by Jackson *et al*. (2011). Scatter plots were used to verify with the RMA-summarized probe set intensities as described by Jackson *et al*. (2011).

3. Results and Discussion

Results

The strains analysed in this study were from different sources and different countries as shown on Table 1.

Table 1: Strains analyzed by Microarray

Strains	*Species*	Source	Country of Origin	Serotype	ST
LMG26250	*condimenti*	Food, Spiced Sausage	Slovakia	ND	98
51329B	*muytjensii*	Unknown	USA	Cmuy O:2	81
CFS237	*dublinensis*	Environmental, Milk powder production facility	Ireland	Cdub O:1	106
464	*dublinensis*	Environmental, Milk powder production facility	Zimbabwe	Cdub O:1	79
5960−70	*dublinensis*	Clinical, Blood	USA	ND	5
3032	*turicensis*	Clinical, Blood	Switzerland	Ctur O:1	19
254N	*sakazakii*	Clinical	Ireland	Csak O:1	1
BAA 894	*sakazakii*	PIF	USA	Csak O:1	1
Csak18−01	*sakazakii*	Clinical, Stool	USA	Csak O:1	4
Csak18−07	*sakazakii*	Clinical, Stool	USA	Csak O:1	8
Csak4.01C	*sakazakii*	Food, PIF	USA	Csak O:2	218
Csak2010−13−32	*sakazakii*	Clinical, CSF	USA	Csak O:2	4
Csak2010−13−33	*sakazakii*	Clinical, CSF	USA	Csak O:2	4
Csak200−205	*sakazakii*	Clinical, CSF	USA	Csak O:2	4
Csak2010−16−01	*sakazakii*	Clinical, Brain exudate	USA	Csak O:2	4
Csak2010−16−11−8	*sakazakii*	Environmental, Baby Pacifier	USA	Csak O:2	4
Csak2151	*sakazakii*	Clinical, CSF	USA	Csak O:2	4
Csak2156−3	*sakazakii*	Clinical, Blood	USA	Csak O:3	4
207NC.sak	*sakazakii*	Clinical	Ireland	Csak O:2	4
208NC.sak	*sakazakii*	Clinical	Ireland	Csak O:2	4
CQ6	*sakazakii*	PIF Manufacturing Environment	Ireland	Csak O:2	4
CQ5	*sakazakii*	PIF Manufacturing Environment	Ireland	Csak O:2	4

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CQ4	*sakazakii*	PIF Manufacturing Environment	Ireland	Csak O:2	4
CQ2	*sakazakii*	PIF Manufacturing Environment	Ireland	Csak O:2	4
CQ3	*sakazakii*	PIF Manufacturing Environment	Ireland	Csak O:2	4
CS124	*sakazakii*	PIF	Nigeria	Csak O:4	296
CDC1121−73A	*sakazakii*	Clinical, Bronchial wash	USA	Csak O:2	64
CS17	*sakazakii*	PIF	Nigeria	Csak O:4	304
CmalCI825	*malonaticus*	Clinical, Breast abscess	USA	Cmal O:2	7
Cmal2149	*malonaticus*	Clinical, CSF	USA	Cmal O:2	7
Cmal2153	*malonaticus*	Clinical, Blood	USA	Cmal O:2	7
CmalE831	*malonaticus*	Clinical	Czech Republic	Cmal O:1	60
CmalJ160	*malonaticus*	Vacuum dust	Jordan	Cmal O:2	443 or 451
CS14	*malonaticus*	PIF	Nigeria	Cmal O:2	303
Cuni797−2	*universalis*	Environmental, Water	UK	Cuni O:1	54
SalSTM	*Salmonella enterica Typhimurium*	Unknown	Unknown	ND	ND
Kpneumoniae214	*Klebsiella pneumoniae*	Unknown	Unknown	ND	ND
Cfreundii576	*Citrobacter freundii*	Unknown	Unknown	ND	ND
Stur508	*Siccibacter turicensis*	Fruit Powder	Switzerland	ND	ND
Fhelz1159	*Franconibacter helveticus*	Fruit Powder	Switzerland	ND	ND
Fhelz513	*Franconibacter helveticus*	Fruit Powder	Switzerland	ND	ND
Fpul1160	*Franconibacter pulveris*	PIF Manufacturing Environment	Switzerland	ND	ND

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Cronobacter species were identified according to the proposed classification scheme as suggested by Iversen *et al*. (2008) and Joseph *et al.* (2012). All of the *Cronobacter* strains possessed the zinc metalloprotease (zpx) gene, a genus-specific target previously reported by Kothary *et al*. (2007). The *Cronobacter* species identity of the isolates was also confirmed using the species-specific rpoB PCR assays as described by Stoop *et al*. (2009) and Lehner *et al.* (2012) and the cgcA species-specific PCR assay as described by Carter *et al*. (2013).

Legend: ND - Not Determined, PIF – Powdered Infant Formula, ST – Sequence Type

Microarray analysis of the three *Cronobacter* strains in relation to 42 other *Cronobacter* and phylogenetically-related strains using the neighbour-net function of Splits Tree is shown in Figure 1.

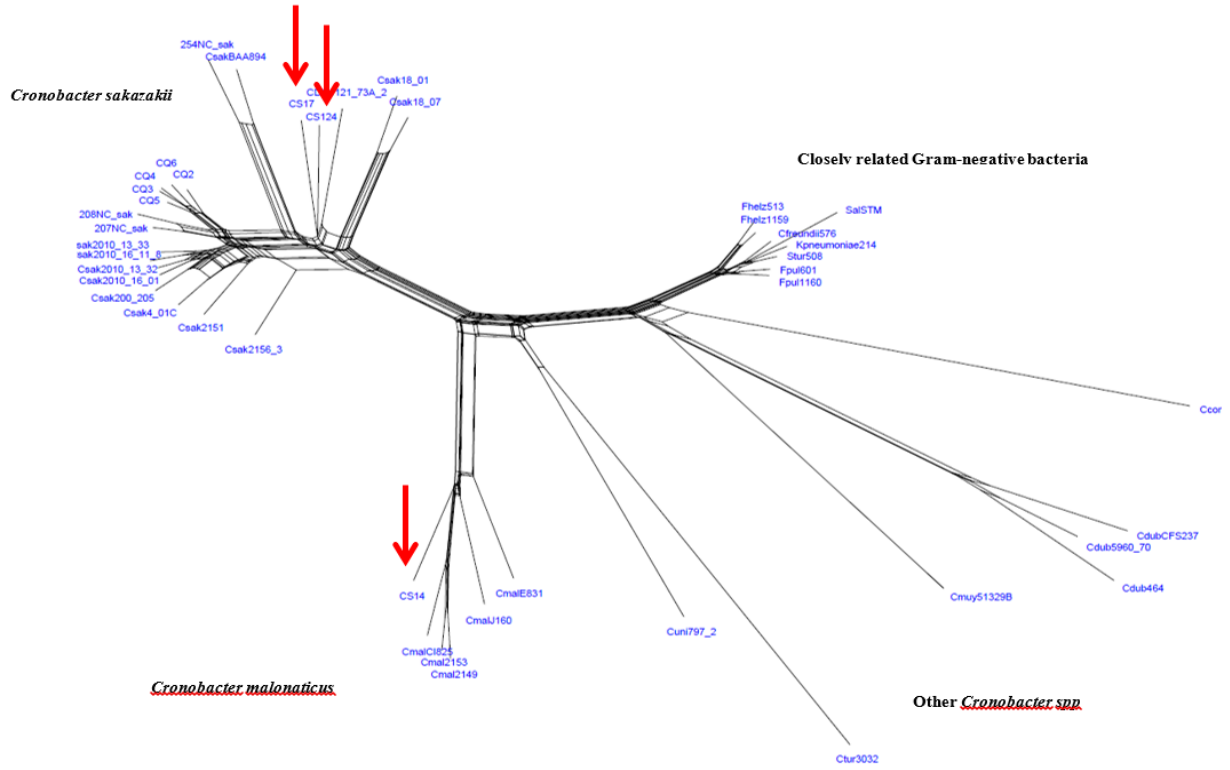

Figure 1: Neighbour net (Splits Tree4) analysis of 42 *Cronobacter* and phylogenetically related strains, generated from the gene-difference matrix

The microarray analysis was able to correctly identify the *Cronobacter* strains to each species epiphet. For example, *C. malonaticus* (CS 14) was identified as *C. malonaticus* and clustered with environmental and clinical strains of *C. malonaticus* which were isolated from different countries like Jordan, Czech Republic, and USA (Figure 1), with a significant relatedness of more than 80% as shown by Pearson's correlation coefficient analysis (Table 2). Accordingly, microarray analysis of the *C. sakazakii* strains CS 17 (ST304) and CS 124 (ST296) correctly identified these strains as *C. sakazakii* and phylogenetically placed them within the *C. sakazakii* species cluster alongside a

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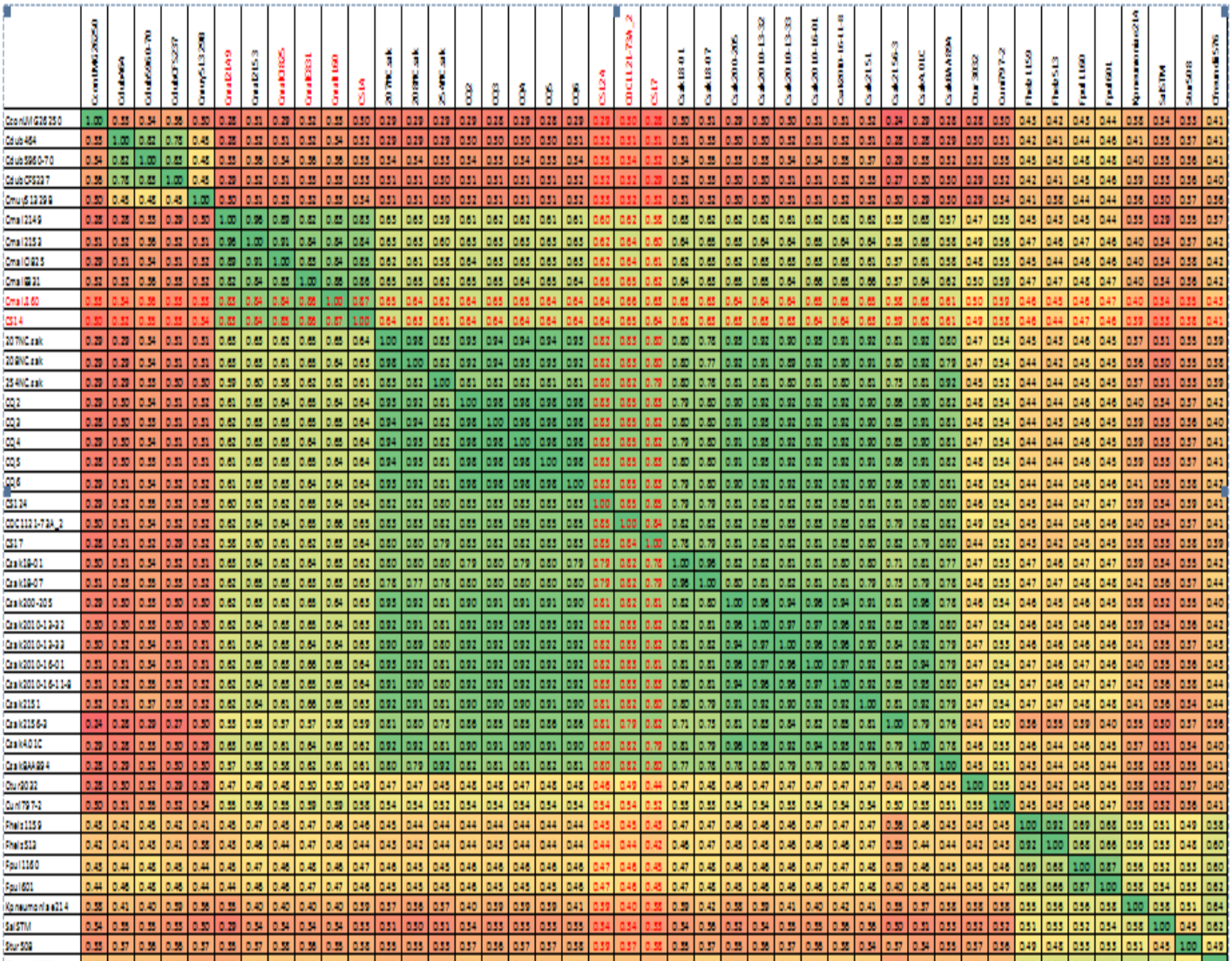
C. sakazakii clinical strain CDC 1121-73 (Figure 1). These results also suggest that strains possessing ST304 and ST296 share phylogeny with ST64 strains and may represent a new clonal complex. The larger *C. sakazakii* cluster contained isolates from different countries like Ireland and USA with a significant relatedness of more than 80% as shown in Table 2. The relatedness of CS 14, CS 17, CS 124 and other closely related Gram-negative bacteria ranged from 33 - 47% as shown in Table 2.

Table 2: Pearson's Correlation coefficient of gene relatedness between CS 14, CS 17, CS 124, other *Cronobacter* spp. and closely related Gram-negative bacteria based on their RMA probe set summarized values



Legend: RMA = Robust MultiArray Averaging; 1.00 = identical; 0.80-0.99 = closely related; <0.80 = not so closely related

C. malonaticus strain CS 14 was more related to *C. malonaticus* strain J160 which was isolated from vacuum dust from Jordan and differed by 260 genes (Table 3). The *Cronobacter sakazakii*

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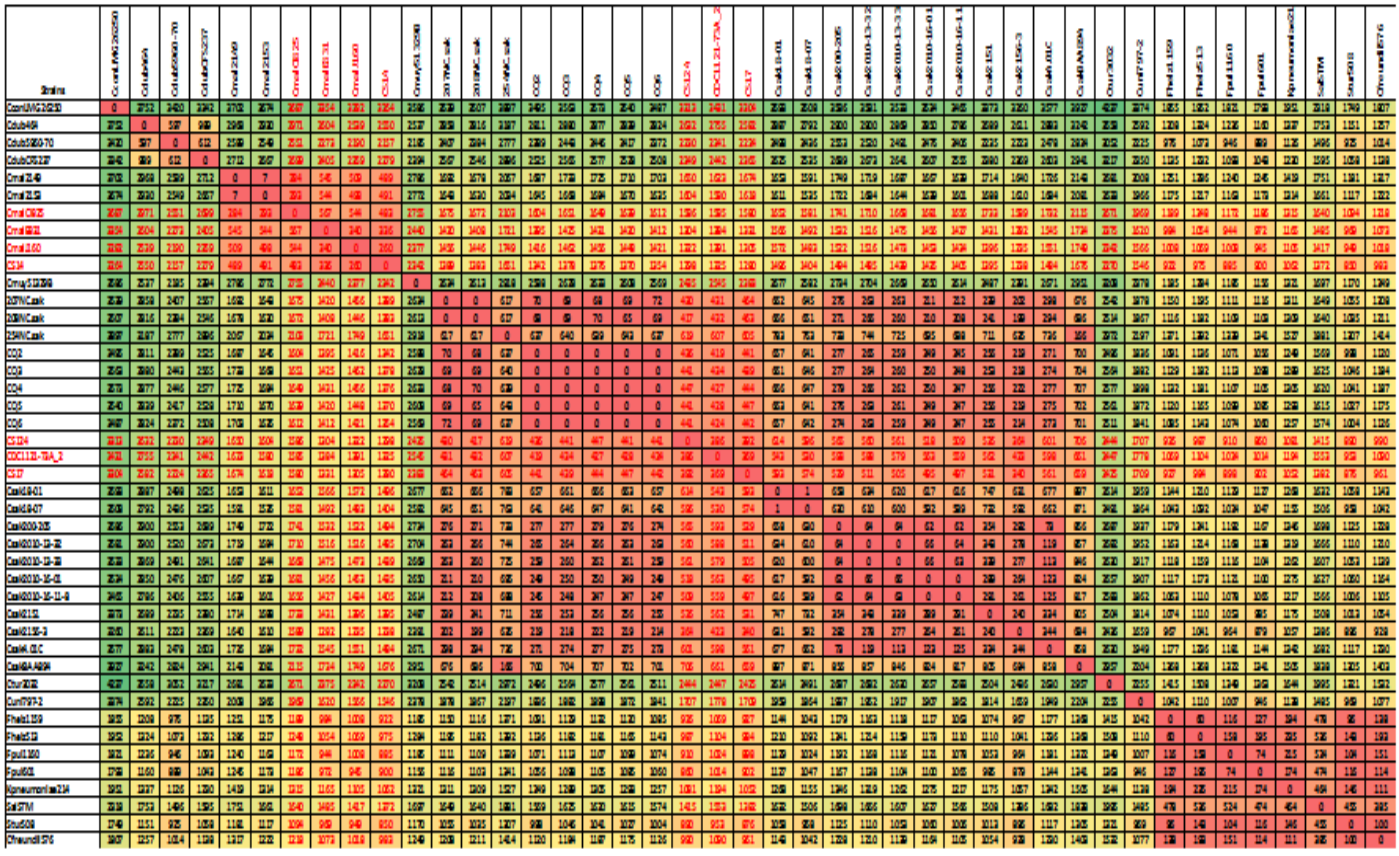
strains CS 17 and CS 124 had 369 and 386 genes respectively that were different from clinical strain CDC1121-73A_2 that was isolated from bronchial wash (Table 3).

Table 3: Number of Genes Different between CS 14, CS 17, CS 124, other *Cronobacter* spp. and closely related Gram-negative bacteria



Some of the genes encoding for virulence factors in phylogenetically related enteric pathogens were detected in the two *C. sakazakii* isolates as shown on Table 4. CS 124 possessed dihydrofolate reductase, integrase of *Shigella flexnerri* bacteriophage and a hypothetical protein z1655 while CS 17 possessed dihydrofolate reductase and formate acetyltransferase 1.

Table 4: Virulence Factor type of genes acquired from closely related Gram-negative bacteria.

NCBI Annotation	Virulence Factor Type	Source Bacteria	Cronobacter spp.
Formate acetyl transferase 1	Pathogenicity Island	*Salmonella enterica* subspp. *enterica* serovar Typhi str	CS 17
Dihydrofolate reductase	Antibiotic resistance	*E. coli*	CS 17, CS 124
Integrase of *Shigella flexneri* bacteriophage X	Virulence Protein	*Shigella flexneri*	CS 124
Hypothetical protein z1655	Pathogenicity Island	*E. coli* 0157:H7	CS 124

Legend: NCBI = National Center for Biotechnology Information

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Table 5 shows the phage related genes acquired by CS 14 and CS 17 from *Cronobacter dublinensis.*

Table 5: Phage related genes acquired from *Cronobacter dublinensis*

Probe id	Probe set id	NCBI annotations	CS124	CS14	CS17
7513	C_413500.3.2241_at	Phage terminase2C small subnunit2C putative2C P27 family	A	P	P
7514	C_413500.3.2242_at	Phage terminase large subunit	A	P	P
7515	C_413500.3.2243_at	Phage portal protein	A	P	P
7516	C_413500.3.2244_at	Phage head maturation protease	A	P	P
7517	C_413500.3.2245_at	Phage major capsid protein	A	P	P
7518	C_413500.3.2246_at	"FIG111678: IS	A	A	P
7519	C_413500.3.2247_at	Bacteriophage tail sheath protein	A	P	P
7520	C_413500.3.2248_at	Phage tail tube protein	A	P	P
7523	C_413500.3.2250_at	Fels-2_prohage_protein	A	A	P
7524	C_413500.3.2251_at	Phage tail/DNA circulation protein	A	A	P
7525	C_413500.3.2252_at	FIG003269:Prophage tail protein	A	P	P
7526	C_413500.3.2253_at	Prophage baseplate assembly protein V	A	A	P
7527	C_413500.3.2254_at	Bacteriophage protein GP46	A	P	P
7528	C_413500.3.2255_at	Hypothetical protein	A	P	P
7529	C_413500.3.2256_at	FIG121501: Prophage tail protein	A	A	P
7530	C_413500.3.2257_at	Prophage tail fiber protein	A	A	P
7531	C_413500.3.2258_at	Fels-2_prophage_protein	A	A	P

Legend: NCBI = National Center for Biotechnology Information; id = identification

Table 6 shows the phage related genes acquired by CS 17 from *C. sakazakii* 2151.

Table 6: Phage related genes acquired from *C. sakazakii* 2151

Probe id	Probe set id	NCBI annotations	CS124	CS14	CS17
4406	C_28141.3.1398_at	Fels-2_prohage_protein	A	A	P
4407	C_28141.3.1399_at	Prophage_baseplate_assembly_protein	A	A	P
4408	C_28141.3.1400_at	Probable_phage_baseplate_assembly_protein	A	A	P
4409	C_28141.3.1401_at	Putative_phage_tail_protein	A	A	P

Legend: NCBI = National Center for Biotechnology Information; id = identification

4. Discussion

Infants are the most vulnerable group of the human population and so attempt to protect them from health hazards should be done with utmost priority. PIF is not a sterile product but it should be free from all potential pathogens because neonates and infants possess under-developed immune

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systems and lack a competing intestinal flora (Townsend *et al*., 2008). Because infant formula products are primarily imported into the Nigerian market, the relatedness of the isolated Nigerian strains was examined in relation to other strains from other countries. Out of the 154 samples of PIF analysed in this study, *Cronobacter* species was isolated from 2 per cent (3). *Cronobacter sakazakii* was isolated from 1.30% while *C. malonaticus* was isolated from 0.65% of samples.

Both of these *Cronobacter* spp. have been isolated from PIF samples from different countries around the world (Farmer, 2015). Gicova *et al*. (2013) isolated *Cronobacter* strains from 0.9 % of powdered infant samples (N = 916). Ashfaqul *et al.* (2010) isolated *Cronobacter* spp. from 3% of powdered infant samples (N = 32) in Bangladesh. Fu *et al*. (2011) isolated *Cronobacter* spp. from 4.35% of powdered infant samples (N = 23) and Li *et al.* (2016) isolated *Cronobacter* spp. from 16.9% of powdered infant samples (N = 119) in China. Mardaneh and Dallal (2016) isolated *Cronobacter* spp. from 7.2% of powdered infant samples (N = 125) in Iran. Muytjens *et al*. (1988) examined 141 different powdered formulae from 35 countries and reported that 14% contained *Cronobacter* spp. The level of contamination ranged from 0.36 to 66.0 cfu/100 g. Simmons *et al*. (1989) isolated *Cronobacter* spp. from PIF associated with an outbreak in Memphis, Tennessee. Biering *et al*. (1989) also isolated *C. sakazakii* from five different lot numbers of unopened packages of PIF after an outbreak of neonatal meningitis in Iceland. A survey in Canada (Nazarowec and Farber 1997) isolated *C. sakazakii* from 8 out of 120 cans from 5 different manufacturers at levels of 0.36 cfu/100 g. Heuvelink *et al*. (2001), detected *Cronobacter* spp. in 1 of 40 infant formula powders and 7 of 170 milk powders. Santos, (2006) reported levels of isolation of *Cronobacter* at 0.22 – 1.61 cfu/100 g product. The joint FAO/WHO (2008) call for data on follow-up formula, reported the isolation of *Cronobacter* spp. from 1 of 84 samples of follow-up formula and 30 of 203 weaning foods. There is zero tolerance for all *Cronobacter* spp. in all infant formula because of the high rate of mortality (80%) and long-lasting sequela associated with infections caused by them (Jackson *et al.*, 2014).

Microarray studies have been used to understand the genomic diversity within *Cronobacter*. Tall *et al*. (2015) surveyed specific genes from different *Cronobacter* species and within each species group. The FDA *Cronobacter* microarray is a pan genomic array which assesses the total gene content of each strain without the need to compare hybridization intensities to a reference genome which was necessary prior to this new microarray designed for accurate comparative genomic hybridization (CGH) studies (Kucerova et al., 2010). Yan *et al*. (2015) used the microarray to investigate the genomic diversity of several clinical and environmental strains of *C. sakazakii* which were isolated from the environment of a group of European PIF manufacturing facilities. The results of that study showed that the microarray could separate 25 *C. sakazakii* ST4 strains into two distinct subclades which suggested that there may be two evolutionary lineages associated with ST4 strains. The microarray analysis also showed that these two lineages differed in a total of 95 unique genes, of which many were phage-related genes (seven related genes) and 17 of these unique genes were associated with the pESA3-encoded type six secretion system (T6SS) gene cluster as described by Franco *et al*. (2011a).

The three (3) isolates in this study were compared directly with nearest neighbors and other *Cronobacter* species. The results showed that CS 17 and CS 124 had significant relatedness (> 80%) to *C. sakazakii* strains isolated from blood, CSF and breast abscess from different countries such as USA and Ireland. The CS 14 also had greater than 80% relatedness to the clinical isolates

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of *C. malonaticus* from countries like Czech Republic, USA and Jordan. The number of gene differences is based on strain-to-strain comparisons and gene difference is defined as an eightfold difference in the RMA-summarized probe set intensities for each gene (Tall *et al*., 2015). Speculatively, the difference in the *Cronobacter* strains isolated in this study may be due to bacterial adaptation to Nigerian tropical environment or the acquisition of genes from indigenous bacteria. This study also illustrates the global nature and spread of *Cronobacter* spp., in infant formula products which may be produced in one part of the world and consumed in another part.

Microarray analysis of *C. malonaticus* strain CS 14 and *C. sakazakii* strain CS 17 showed that these strains had acquired some phage related genes which were found in *Cronobacter dublinensis* while *C. sakazakii* strain CS 17 had acquired some phage related genes which were found in *C. sakazakii* 2155. Whole-genome analyses have revealed that many bacterial genomes contain foreign genes, especially phage genes (Ochman *et al.,* 2000). The phage genes in bacterial genomes include genes for virulence or fitness factors such as extracellular toxins, super antigens, lipopolysaccharide-modifying enzymes, and proteins conferring serum resistance, etc. (Brussow *et al.,* 2004). The horizontal transfer of phage genes has contributed significantly to the acquisition of new genetic traits and to the genetic diversity of bacteria (Brussow *et al.,* 2004, Ochman *et al.,* 2000).

Pathogenicity islands which contain one or more virulence genes, are present in the genomes of pathogenic bacteria but are absent from the non-pathogenic variant of the same species and often exist in the size range of 10-200 kb (Schmidt and Hensel, 2004). They contain clusters of functionally related genes necessary for virulence in bacteria. *Salmonella* spp. contains a wide variety of mobile genetic elements from pathogenicity islands to conjugative transposons, (Kelly *et al*., 2009). One of these pathogenicity island gene, formate acetyl transferase 1, was found in CS 17 (*C. sakazakii*). A pathogenicity island gene from *E. coli* 0157:H7, hypothetical protein z1655, was also found in CS 124 (*C. sakazakii*). The presence of these pathogenicity islands genes in CS 17 and CS 124 could enhance their virulence. The presence of reputable virulence genes in the *C. sakazakii* isolates indicates the potential risk of consumption of these *Cronobacter* contaminated powdered infant formula (PIF) by neonates and infants; hence the need for intensive and continuous monitoring of potential pathogens in powdered infant milk formula to ensure the safety of vulnerable infants.

Antibiotic resistance gene (dihydrofolate reductase) from *E. coli* was also detected in *C. sakazakii* strains CS 17 and CS 124. Resistance to clinically relevant, front-line antimicrobials such as fluoroquinolones, extended-spectrum β-lactams (including extended-spectrum cephalosporins) has been reported among *E. coli* strains and they are believed to be an important reservoir of transferable antimicrobial resistance genes (Singh *et al*., 2005). The transfer of this antibiotic resistance to indigenous non-resistant bacteria could contribute to an increase in the rate of resistance of bacteria to drugs especially in the Nigerian environment where there is no regulation on the use of antibiotics.

5. Conclusion

Infant formula producers must enforce the use of guidelines aimed at decreasing the risks of product contamination with foodborne pathogens. The control of primary populations of

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Cronobacter spp. during the PIF production process and prevention of post processing contamination can be ensured by using suitable microbiological guidelines for quality control and assurance. Sanitary practices for the preparation of infant formula in both the home and hospitals should be carefully controlled through the regular creation of the awareness that PIF are not sterile but that they may contain potential pathogens. The use of hygienic measures during preparation and reconstitution of PIF are essential. The risk of foodborne illness in neonates and infants fed infant formula can be reduced if guidelines for the preparation, storage and handling of PIF are strictly adhered to (Silano *et al.*, 2016; Juan-Pablo, 2015; Norberg *et al*., 2012; WHO 2007).

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