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CONTAMINATION OF MICROORGANISMS IN PEDIATRIC INFANT FORMULA MARKETED IN KARACHI

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Abstract
The worth of infant feeding is for not only growth and development in infancy but also for the long term health well into adulthood of the supreme significance. The use of pediatric infant formula (PIF) is widespread to provide nutrition for infant. Pediatric infant formula is not a sterile product and opportunistic pathogens could multiply in the reconstituted product, resulting in infections. During the manufacturing of PIF, the chances of the contamination of microbial growth has been increased many folds. In recent years, the microbes which are of mainly concern related to the contamination in PIF were Cronobacter sakazakii, Salmonella species, and Bacillus cereus. The present project has been designed to evaluate microbial contamination of twenty six selected brands of pediatric infant formula marketed in Karachi, Pakistan. It has been affirmed by the result that two samples were contaminated by Gram-negative Cronobacter sakazakii and one of sample was contaminated by Gram-positive Bacillus cereus. These potential pediatric infant formula-borne pathogens were within the range of Codex Alimentarius Commission. Moreover, two PIFs have probiotics which were Gram-positive Lactobacillus and Bifidobacterium species mainly aid in the digestion, stimulate the immune system, and inhibit the growth of pathogens, effective against bacterial induced gastroenteritis, and even recovery from acute diarrhea in children.

Keywords: pediatric infant formula**,** microorganisms, contamination, hazards, probiotics

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1. INTRODUCTION

Pediatric infant formula (PIF) has been safely consumed by infants since 1970 (Ryan, 1997). It has been forcefully recommended nearly by all the world renowned health concerned regulator bodies like WHO (FAO/WHO, 2006), FDA (FDA, 2002), CDC (2002), New Zealand Food Safety Authority (NZFSA, 2009) that there is no substitute of mother feed, except in some unusual conditions like HIV-positive mother's infants, breast surgery, risk of malnutrition. PIF is of immense importance for the cognitive and psychomotor development of infants and young children.

The first year of life is the most critical span for a new born, particularly from a nutritional view point, for the mental and physical growth and development in a child's life. Infants should be exclusively breast-fed for the first 2 years of life (WHO, 2003).

PIF has also been associated with illness even death in infants due to infections associated with PIF-borne pathogens. PIF has been manufactured by more than a dozen firms in 40 – 50 processing plants worldwide (Benkovic and Bauman, 2009). It has not possible by current technology to produce PIF that were devoid of low levels of microorganisms. Enviormental microbes have been one of the major factors for contamination in PIF (Proudy *et al.,* 2008). Personnel hygiene has been frequently ignored and poor hygienic practice has been the source of outbreaks**.** Microbial contamination can occur during the manufacturing process and/or during post-manufacture reconstitution of PIF (Mullane *et al.*, 2007). In 2007 FAO/WHO guidelines has recommended to develop the preparation of PIF and suggested international code of hygienic practice for foods for infants and children. PIF should be reconstituted at 70^{o}C for the in activation of bacterial growth and if not consume completely then it should be refrigerated. Sterile ready-to-feed and

concentrated liquid PIF are commercially available (Barron *et al.,* 2007).

The most commonly isolates found in PIF include *Cronobacter sakazakii, Salmonella enteric, Cronobacter cloacae, Citrobacter koseri, Citrobacter freundii, Escherichia coli, Escherichia vulneris, Klebsiella oxytoca, Klebsiella pneumonia, Bacteroides*, *Bifidobacterium*, *Clostridia*, *Lactobacilli*, *Streptococci* and *Salmonella* and *Pantoea* species (Arsalan *et al.,* 2013a). *Lactobacilli* and *bifidobacteria* are the most accepted microbes for probiotic application (Borriello *et al.,* 2003). Governmental and industrial standards also help to assure microbiological safety and stability. Problems of nutrient composition and adverse reactions have occurred during the evolution and development of PIFs.

Cronobacter sakazakii

Cronobacter sakazakii is a Gram-negative, non-spore forming *Enterobacteriaceae* (Farmer *et al.,* 1980), named after the Japanese microbacteriologist Riichi Sakazakii. A number of studies have been found the contamination of *C. sakazakii* in PIF (Bar-Oz *et al.,* 2001; Arsalan *et al.,* 2013b) has been associated with necrotizing enterocolitis (van Acker *et al.,* 2001), bacteraemia (Muytjens *et al.,* 1988) and meningitis (Bar-Oz *et al.,* 2001). Mortality rates vary from 10 to 80% because of its virulent pathogenicity (Lai, 2001; van Acker *et al.,* 2001).

Cronobacter sakazakii has ubiquitous and chiefly found in food processing area, milk powder production area, hospital environment (FAO/WHO, 2004) as well as in households utensils (Kandhai *et al.,* 2004). *C. sakazakii* can affix to intestine and even plastic and silicon surfaces of feeding bottles and nipples and multiply in a biofilm (Zogaj *et al.,* 2003). Noriega and co-workers (1990) have been observed PIF preparation equipment might be contaminated by *C. sakazakii*. Although *C. sakazakii* occurring in dry PIF are very low, reconstituted PIF provides good medium for growth. *C. sakazakii* infections have not only been occurred in infants but may also occur in adults (Lai, 2001). Immuno-compromised infants and neonates have been considered to be at greatest risk, especially neonates of low birth weight and pre-mauture (Arsalan *et al.,* 2013b). The less acidic environment of stomach of premature babies is an important factor for the survival of *Cronobacter sakazakii* (van Acker *et al.,* 2001; Lai, 2001).

The two main routes by which *C. sakazakii* can enter reconstituted PIF intrinsic contagion either through contaminated ingredients added after drying or from the processing environment following drying and before packing; through external contagion of the formula during reconstitution and handling (e.g. through poorly cleaned utensils) Gurtler *et al.,* 2005).

Farmer *et al.* (1980) have reported *C. sakazakii* has been shown growth at 25°C, 36°C and 45°C but not shown multiplication at 4°C or 50°C**.** It has been found that minimum growth temperatures for *C. sakazakii* varied from 5.5°C to 8°C and at 4°C microbes has been started to kill while show major growth at temperature 41°C to 45°C in Brain Heart Infusion broth. Iversen and Forsythe (2004) observed that improper storage of contaminated reconstituted powdered PIF might help rapid growth of *C. sakazakii*.

Various studies have been showed that standard pasteurization practices are effective for the inactivation of *C. sakazakii* (Nazarowec-White *et al.,* 1999; Iversen and Forsythe, 2004). Jaspar *et al.* (1990) suggested rehydrated PIF should be stored at refrigerator and better to heat PIF in the microwave oven just earlier to feed. Holding feed in bottle warmers for lengthy periods was reported as one of the feasible grounds of an eruption of *C. sakazakii* infection**.** It has been evaluated the effect of microwave heating on the destruction of microorganisms in milk. The mechanism by which microwaves cause the death of microbial cells is thought to involve thermal as well as non-thermal effects associated with electromagnetic radiation. The bactericidal efficacy of microwave treatment of milk is recommended over traditional methods to rewarm rehydrated PIF (FDA, 2005).

PIF manufactured by Wyeth in 2002 was contaminated with *C. sakazakii*, which led to casualty rates of 33 to 80% in infected children (Lai, 2001). The Centres for Disease Control and Prevention has been reported 16 invasive cases of *C. sakazakii* in infants less than 1 year between January 1999 and April 2002 (CDC, 2002). In the United States of America, an incidence rate of 1 per 100 000 infants for *C. sakazakii* infection has been reported. This incidence rate increases to 9.4 per 100 000 in infants of very low birth weight, i.e. <1.5 kg. A survey in USA in 2002 has estimated the rate of infection among infants as 1/100,000 and the rate among low birth weight neonates as 8.7/100,000 (FAO/WHO, 2004). Since 1958, more than 70 cases are reported with a mortality of 24 in the outbreak of *C. sakazakii*, out of these cases 23 cases are reported in USA with a causality of only 3 babies. In last decade, total twenty major outbreaks of *E.sakazakii* are reported in France (2004), NewZealand (2004), Belgium (2002) (FDA, 2005), and USA (2001) (Himelright *et al.,* 2002) with a death tool of 2, 1, 1 and 1 respectively. In the era of 1990s, major outburst of in Belgium in 1998 with 12 cases was reported with two causalities (van Acker *et al.,* 2001). But in the period of 1980s, two main outbreaks were reported in Denmark in 1983 (Muytjens *et al*., 1988) and Greece in 1984 (Arseni *et al*., 1987), 8 and 11 cases were reported with mortality of 6 and 4 respectively.

Bacillus cereus

Bacillus cereus is one of the most frequently isolate found in PIF Wong *et al*., 1988; Becker *et al*., 1994). It has been found ubiquitously and widely distributed in the soil, dust, air, and water and are resistant to environmental destructive factors (Stenfors *et al*., 2008). Usually, PIF have been contaminated with *B. cereus* via raw milk. The initial heat treatment step applied in the production of dried milk is very important for the activation and germination of *B. cereus* spores. Where raw milk has not generally supported the germination of spores, a high temperature short time pasteurisation treatment renders the milk as a good germination medium (Wong *et al*., 1988). *B. cereus* has an optimum growth temperature between 30°C and 37°C. However, the organism has been shown to be able to grow at much lower (4-5°C) or higher temperatures (55°C). To kill all *B. cereus* spores in milk heating for 10-20 seconds at 125°C is claimed to be necessary (Van Netten *et al*., 1990). *B. cereus* strains have been isolated from milk and milk products showed cytotoxicity after incubation in laboratory media and, therefore, have to be considered potentially enterotoxigenic (Wong *et al*., 1988). It could be prevented, for instance, by temperature below 4°C and pH-values not exceeding 5.0 (Stadhouders *et al*., 1982).

There are two main routes linked in the contamination of *B. cereus* spores in PIF is due to the detachment of microorganisms from biofilms on stainless steel surfaces and spores that formed in milk pre-pasteurisation (Van Netten *et al*., 1990). Becker with his colleagues (1994) has been reported that about 70% PIF are contaminated by *Bacillus cereus* in 1992, but in 1994, only 18%. It has been proved that the processing and packaging practices in the PIF manufacturing plant have been improved to reduce microbial contamination. It has been found that heat treatment initiates the production and germination of *B. cereus* spores. Bactofugation removes bacteria, especially spores, from milk in a high speed centrifuge and is applied by some producers of infant food. Up to 95% of the spores are removed.

***Lactobacilli* Species**

Lactobacilli are Gram-positive rods commonly found in gut but mainly present in the large intestine and mainly used as probiotics (Wall *et al.,* 2008). It has been proven that the nearly all probiotics reduced diarrhea and gastroenteritis in infants (Isolauri *et al.,* 1995; Gonzalez *et al*., 1995; Engelbrektson *et al*., 2009). *Lactobacilli*, more acid tolerant as compared to *Bifidobacteria*. *Lactobacilli* have stimulated the immune system, help in digestion, and inhibit the growth of pathogenic bacteria such as *Helicobacter pylori* by decrease in pH of

stomach due to accrual of lactic acid (Haarman and Knol, 2006). Parracho *et al.* (2007) observed that *Lactobacilli* also hamper the growth of other bacteria by contending with them for nutrients and hold the place on the epithelial lining of the intestine. Gonzalez with his colleagues (1995) found that mixture of *Lactobacillus* species have been used as bacteriotherapy against the three diarrhea-causing microbes. It has been found *Lactobacillus acidophilus* is added in PIF to improve weight gain of infant (Isolauri *et al.*, 1995). Infants are mainly suffered from watery diarrhea and/or excessive flatulence. *Lactobacillus* species have been increased β - galactosidase [lactase] which may develop lactose digestibility Rastall *et al.*, 2000).

Bifidobacterium Species

Gram positive anaerobe *Bifidobacterium* is mainly colonized in the infant's intestine rather than stomach. *Bafidobacterium* species are found the infant is on either breast-fed or formula-fed. The most common *Bifidobacterium* species found in infants' intestine are *Bifidobacterium infantis*, *Bifidobacterium breve*, and *Bifidobacterium longum*. *Bifidobacterium infantis* has specifically unique to the infant's digestive tract (Matsuki *et al.*, 2003). *Bifidobacterium* has been helped in the digestion of glucose and oligosaccharides, which not only provide energy and nutrients for growth but also help in eradication of *Clostridium* species (Ward *et al.*, 2006). It has been observed that by addition of prebiotics in PIF reduces the pH of of infant's stool like the pH of breast-fed infants, indicates the growth of beneficial microbes like *Bifidobacteria* (Costalos *et al.*, 2007).

Mixture of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* beneficial for reduced *C. difficile*-associated disease, similarly, *Bifidobacterium animalis* produce effect on the gastrointestinal system, moreover, supplementation with *Lactobacillus rhamnosus GG* and with *Bifidobacterium bifidum* and *Streptococcus thermophilus* aid in preventing rotavirus diarrhoea in infants (Saavedra *et al.*, 1994).

It is important to ensure that PIF has been prepared using good hygienic procedures, along minimization of the time between preparation and consumption to reduce the risk of contamination.

Table 1: Biochemical Test of *Bacillus cereus* and *Cronobacter sakazakii*

MICRORGANISMS	DIFFERENTIAL/ SELECTIVE MEDIUM	CHROMOGENIC MEDIUM	MICROBE COLOR & COLONY TYPE	BIOLOGICAL TEST	
				+ve Test	-ve Test
Bacillus cereus	Mannitol-egg yolk-polymyxin [MYP] (Tallent *et al.*, 2012)	Bacara agar (Tallent *et al.*, 2012)	Pink-orange uniform colonies (Tallent *et al.*, 2012)	Hemolysis [sheep rbc], Egg yolk reaction, Anaerobic utilization of glucose, Tyrosine decompo-sition, Motility, Reduction of nitrate (Tallent *et al.*, 2012)	Acid produced from mannitol (Tallent *et al.*, 2012)
Cronobacter sakazakii	Tryptone soy agar [TSA] *C. sakazakii* show Yellow colour with Mucoid/matte (Mullane *et al.*, 2007)	Druggan-Forsythe Iversen [DFI] agar (Mullane *et al.*, 2007)	Blue-green color with smooth texture (Mullane *et al.*, 2007)	α-glucosidase and Tween 80 esterase show positive (Mullane *et al.*, 2007)	D-sorbitol fermentation show negative result (Mullane *et al.*, 2007)

2. MATERIAL AND METHODS

Twenty six selected brands of pediatric infant formulas (PIF), Mannitol-egg yolk-polymyxin (MYP) agar**,** Bacara (differential agar)**,** Sterile distilled water, Enterobacteriaceae Enrichment broth (EEb)**,** Violet red bile glucose agar (VRBG), Tryptic soy agar (TSA). All glass wares were sterile like sampling spatula, pipette, test tubes, stirrer, pertiplate and beakers.

The samples were prepared and evaluated according to FDA, 2002. Samples were prepared and inoculated by direct spreading, streaking and direct pour method. For detection of *Cronobacter sakazakii* in PIF, required 5 days to complete. Dilute 100 g of each brand of PIF sample in sterile water. Mix 10 ml samples into 90 ml Enterobacteriaceae enrichment broth [EEb]. Mix suspensions and surface plate 0.1 ml on VRBG agar, streak on VRBG agar with inoculating loop onto 60^{o} angle of each petriplate dish for isolation and incubate overnight at 36^{o}C. Now isolate presumptive-positive *C. sakazakii* colonies from both sets of VRBG plates and confirmed by streaking onto TSA and incubate for 48-72 h at 25^{o}C. *Bacillus cereus* has been confirmed by mannitol-egg yolk-polymyxin (MYP) and Bacara has been used as a chromogenic selective and differential medium, Bacara has aided and promote the growth of *B. cereus* and has inhibited the growth of background flora (Rowan and Anderson, 1998; Tallent *et al.*, 2012). Differential / selective medium were used to detect the contaminants, moreover, biological test are performed and mentioned in Table 1.

3. RESULTS AND DISCUSSION

During the present procedure, twenty six selected brands of PIF marketed in Karachi, Pakistan were purchased for microbial evaluation both locally and imported manufactured. FDA suggested that microbial specification of PIF should be not less than six. Each sample was given a code number in order to maintain the secrecy and results of evaluation are given in Table 2 and Fig. 1.

Table 2. Bacteriological growth in PIF

S. NO.	CODE NO.	GRAM STRAINING	NO OF CFU/100g	MICROORGANISMS
1	F01	NIL	0	NIL
2	F02	NIL	0	NIL
3	F03	NIL	0	NIL
4	F04	NIL	0	NIL
5	F05	NIL	0	NIL
6	F06	NIL	0	NIL
7	F07	NIL	0	NIL
8	F08	Gram-Positive	7	*Bacillus cereus* (Contaminant)
9	F09	NIL	0	NIL
10	F10	NIL	0	NIL
11	F11	Gram-Negative	6	*Cronobacter sakazakii* (Contaminant)
12	F12	NIL	0	NIL
13	F13	NIL	0	NIL
14	F14	NIL	0	NIL
15	F15	NIL	0	NIL
16	F16	NIL	0	NIL
17	F17	Gram-Positive	13	*Lactobacilli* and *Bifidobacterium* species (Probiotic)
18	F18	NIL	0	NIL
19	F19	NIL	0	NIL
20	F20	NIL	0	NIL
21	F21	Gram-Negative	9	*Cronobacter sakazakii* (Contaminant)
22	F22	NIL	0	NIL
23	F23	Gram-Positive	11	*Lactobacilli* species (Probiotic)
24	F24	NIL	0	NIL
25	F25	NIL	0	NIL
26	F26	NIL	0	NIL

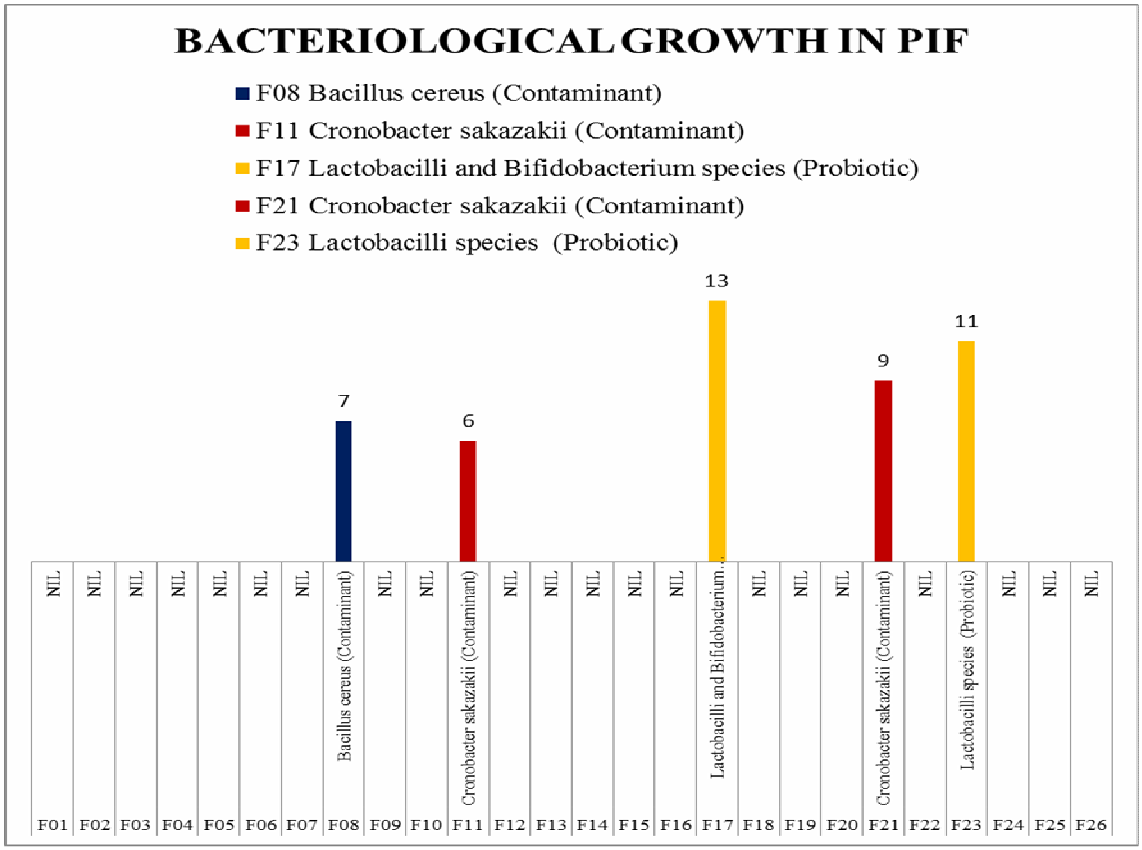

Fig. 1. Bacteriological growth in PIF

It has been found that one of sample is contaminated by Gram-positive *Bacillus cereus* (3.85%). The risk assessment concluded that levels of *B. cereus* greater than 100 cfu/g may not provide adequate public health and safety for infants. A regulatory limit is of 100 cfu/g is therefore recommended. It has been found 3.8% samples of PIF were contaminated by *B. cereus* (Rowan and Anderson, 1998). *B. cereus* found in 17% of fermented milks 2% of pasteurized milks (Wong *et al.*, 1988). Becker *et al.* observed in 261 samples of infant food distributed in 17 countries 54% were contaminated with *B. cereus* reaching levels from 0.3 to 600 CFU/g (Becker *et al.*, 1994). Formula reconstituted with cooled boiled water (25^{o}C) and stored for 24 hours at 4^{o}C. Formula reconstituted from powder with levels of 1000 cfu/g and then stored at 10^{o}C for 24 hours may pose a risk to infants (Rowan and Anderson, 1998).

Out of twenty six samples, two samples were contaminated by Gram-negative *Cronobacter sakazakii* (7.69%) were within the limit assigned by Codex Alimentarius Commission. The Food and Agricultural Organization of the United Nations advocates bacterial counts for coliforms in PIF of less than 3 cfu/g (FAO/WHO, 2006). Muytjens and co-workers (1988) found 14% *C. sakazakii* of 141 PIF samples with concentration ranged from 0.36 to 66 cfu/100g. It has been observed by Aigbekaen and Oshoma (2010) that 33.9% samples were contaminated by *C. sakazakii* out of 70 samples of PIF. Nazarowec-White and Farber (1997) have surveyed of the incidence of *C. sakazakii* in commercial PIF powder found that eight of 120 cans (6.7%) tested positive for *C. sakazakii* (Sani and Yi, 2011). These eight positive *C. sakazakii* cans with levels of 0.36 cfu/ 100 g. However, no data was provided on the number of samples actually taken from Australia in that survey and it was likely that this result has not reflected recent changes in the production of PIF that were likely to influence the microbiological quality

of PIF (NSW, 2011). Thompson (2010) also has not found any pathogens in the twenty samples tested. Similarly, Sani and Yi (2011) supported Thompson (2010) that even in thirty PIF samples of eight manufacturers no *C. sakazakii* has been detected; only one of the samples contained probiotic. It has also been found in a survey conducted on the New Zealand market, *C. sakazakii* was not present in any of the thirty-four samples of PIF (NZFSA, 2009).

Furthermore, two PIFs have probiotics which are Gram-positive *Lactobacilli* and *Bifidobacterium* species as probiotics support in the digestion, stimulate the immune system, and inhibit the growth of pathogens, effective against gastroenteritis induced by pathogenic bacteria, and even recovery from acute diarrhea by pathogenic bacteria in infants (Isolauri *et al.,* 1995; Engelbrektson *et al.*, 2009). Out of thirty PIF samples tested by Sani and Yi (2011) found only one sample contain probiotic. Thus, probiotics has been added in PIF to provide beneficial effect in digestion and improvement in immune system.

4. CONCLUSIONS

It has been concluded from the present study that PIF has enormous importance for the mental and physical growth of infants. Although, the microbial contamination have been found in some of PIF marketed in Karachi, Pakistan. These microbes are within the range of regulatory authorities but still some pathogenic microbial contamination has also been found in PIF. On the basis of the present study, we concluded mother-feed is still the best for neonates and infants. Moreover, FDA, Health Canada, FAO/WHO, and CDC forcefully advocate mother-feed over bottle-feed to avoid the possible life threatening illness to neonates and infants caused by the microbial contamination.

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