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Research Article

PRESENCE OF *Cronobacter* spp. AND SOME QUALITY CHARACTERISTICS OF THE POWDERED INFANT FORMULA

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ABSTRACT

Cronobacter species are opportunistic food-borne pathogens that can cause severe and sometimes lethal infections (meningitis, bacteraemia, and necrotizing enterocolitis) predominantly in neonates and have been frequently isolated from different environments, plant materials (cereals, herbs and spices), and various foods, including powdered infant formula (PIF). Infants and newborns may infected through contaminated PIF with *Cronobacter*. The purpose of this study was to determine *Cronobacter* spp., total mesophilic aerobic bacteria count, coliform bacteria count and evaluate physicochemical properties in infant formula as well. Sixty two PIF samples were collected from different companies. In the studied formulas, *Cronobacter* spp. was identified in three (4.8%) of all PIF samples. The average total mesophilic aerobic bacteria and coliform bacteria numbers were found as 2.66 log cfu/g and 1.32 log cfu/g, respectively. The total sugar, invert sugar and protein content of PIF examples were found 49.12%, 32.36%, and 11.24%, respectively. In conclusion, this study results showed the prevalence of *Cronobacter* spp. from PIF and demonstrating a potential risk for infants in Turkey.
Keywords: Powder infant formula, physicochemical and microbiological properties**,** *Cronobacter* spp.

1. INTRODUCTION

Cronobacter spp. (formerly known as *Enterobacter sakazakii*) is Gram-negative, peritrichous, motile, oxidase-negative, non-spore forming pathogenic, ubiquitous, non-acid-fast, straight, rod-shaped, non–spore-forming, facultative anaerobic bacteria and belongs to the Enterobacteriaceae family. The genus *Cronobacter* has been divided into seven species: *Cronobacter sakazakii*, *Cronobacter turicensis*, *Cronobacter malonaticus*, *Cronobacter dublinensis*, *Cronobacter muytjensii*, *Cronobacter universalis*, and *Cronobacter condimenti* (Healy et al., 2010; Pagotto and Abdesselam, 2013; Harouna et al., 2015).

Cronobacter spp. is a bacterium regarded as a rising opportunistic human pathogen and is the aetiological agent in grievous bacterial infections in neonates (infants<4-week-old) with predominantly low birth-weight neonates (Jung et al., 2013; Fang et al., 2015). However, immune-compromised adult infections have also been reported (Gosney, 2008). Foodborne illness outbreaks caused by *Cronobacter* spp. are implicated in a rare but life-threatening forms of

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meningitis, septicemia, bacteremia or necrotizing colitis in preterm and adults with mortality rates of 40-80 % worldwide (Chap et al., 2009; Adekunte et al., 2010; Al-Nabulsi et al., 2015; Harouna et al., 2015; Heperkan et al., 2017). Although *Cronobacter* spp. have been frequently isolated from the environment, soil, plant material (wheat, rice, herbs, and spices), vegetable origin food, dairy products, meat products, water, legumes, nuts, flours, spices and other foods, powdered milk and reconstituted powdered infant formula have been the most important vehicle or source for neonatal *Cronobacter* spp. infections (Chap et al., 2009; Reich et al., 2010; Jaradat et al., 2014; Al-Nabulsi et al., 2015).

PIF composes over 80% of the infant formula used worldwide and it was presented as a replacement for human breast milk more than 50 years ago. Powdered infant formula (PIF) is a term used for breast milk substitutes. Although their low water activity stops bacterial growth during storage, bacteria are able to grow rapidly following reconstitution (Pagotto and Abdesselam, 2013; Forsythe, 2014).

Cronobacter spp. has also been found on infant formula milk (IFM) preparation equipment in nurseries such as food blenders, spoons, and baby-bottles PIF (Pina-Pérez et al., 2009). PIF can directly or indirectly become contaminated with *Cronobacter* spp.. Directly contamination occurs during period of production infected baby food with bacteria. Indirect contamination can be welded using of dirty kitchen utensils such as blender and spoon during preparation of IF (Drudy et al., 2006). In manufacturing process of PIF, the addition of heat sensitive material, fluidized-bed-drying, spray drying, filling, and packing are the possible sources of *Cronobacter* spp. contamination (Fei et al., 2017).

Cronobacter spp. is nonhalophilic and grows over a wide range of temperatures (6 to 45°C) with an optimal range of 37–43°C and is inactivated at 70°C (Harouna, et al 2015) and adapt to survive in low water activity for a long time periods. *Cronobacter* species can be frozen in reconstituted PIF for over 6 months and there is no reduction in viable cell counts (Pagotto and Abdesselam, 2013). In 2007, a proposed International Code of Hygienic Infant Formula was accepted with trimming made in 2008 (CAC, 2008). The aim of this study was to investigate for *Cronobacter* spp. in powdered infant formula and to determine the microbiological and physiochemical properties.

2. MATERIAL AND METHODS

2.1. Material

A total of 62 powdered infant formula samples (33 kinds of 6 different manufacturers) were obtained from hypermarkets and a private hospital in their original packages in Turkey. The samples were kept at room temperature (25°C) and in dry condition during the study.

2.2. Methods

Physicochemical analysis of infant formula samples

Sugar content: Lane-Eynon method was used which is a titration method for determination of the concentration of invert and total sugar in PIF samples and expressed as g sugar per liter (Cemeroglu, 2007).

Protein content: Protein content was calculated from the nitrogen content of the material, using a nitrogen conversion factor. Kjeldahl method was used in this research (AOAC, 1990).

Microbiological analysis of infant formula samples

Preparing samples for analysis: Ten g of infant formula were transferred to a sterile bag or flask containing 90 mL sodium-chloride solution. The mixture was homogenized with the homogenizer

(Interscience BagMixer® 400P, France). The homogenate was then serially diluted 10^{-1} –10^{-9} and plated in duplicate on specific media to enumerate microbial groups.

Determination of total aerobic bacteria: Mesophilic bacteria counts were performed following aerobic bacterial culture on plate count agar and incubated at 30°C for 48 h. Psychrophilic, and thermophilic bacterial counts were determined by plating with overlay on Plate Count Agar (PCA, Hi-Media, India) and incubated for 7-10 days at 5-7 °C for psychrophilic bacteria, and for 2-3 days at 55°C for thermophilic bacteria. Tests were carried out in triplicate, and the results expressed as log cfu/g (Marshall, 1992).

Determination of coliform bacteria: Coliform bacteria counts were performed following aerobic bacterial culture on Violet Red Bile Agar (VRBA, Hi-Media, India) and incubated at 37 °C for 24-36 h. Tests were carried out in triplicate. Dark red colonies with a red halo larger than 0.5 mm in diameter were considered to be coliform bacteria. The results were expressed as log cfu/g (Marshall, 1992).

Isolation of Cronobacter spp.: The identification of *Cronobacter* spp. was carried out using U.S. Food and Drug Administration recommended method (FDA, 2002). Samples were weighed 1, 10, 100 g powder and rehydrated (9, 90, 900 mL) preheating at 45°C sterile distilled water and incubated at 36°C overnight. For enrichment; 10 mL from each suspension was added to 90 mL of Enterobacteriaceae Enrichment Broth (EEB, Hi-Media, India) and incubated at 36 C for overnight. The enrichment broth is then streak on to Violet Red Bile Glucose Agar (VRBGA, Hi-Media, India) in duplicate. Petri dishes were incubated at 36°C for 24 h. Five potential *Cronobacter* spp. colonies were selected and each isolate was subcultured by streaking onto a single Tryptic Soy Agar (TSA, Hi-Media, India) plate for confirmation and then incubated at 25°C for 48-72 h. Yellow pigmented colonies were picked and affirmed using the oxidase test and API 20E biochemical identification kits (Biomerieux, France). All samples were also detected according to the procedure set by Iversen et al. (2008). The green-blue colonies on Modified HiCrome *Enterobacter sakazakii* Agar chromogenic medium (Hi-Media, India) after 24 h at 37°C were considered presumptive *Cronobacter* spp.

3. RESULTS

3.1. Physicochemical properties of infant formula samples

Infant formulas are an alternative substitute for mother's milk when breast-feeding is not possible. Most conventional formulas are based on cow's milk products. Lactose is the basic carbohydrate in human and cow's milk and forms the majority or all of the carbohydrate in milk-based formulas. In most formulas 40% to 50% of calories are provided as carbohydrate (Barness et al., 1976; Guerra-Hernández et al., 2002). In this study, average chemical compositions of some infant formula samples are given in Table 1. As shown in Table 1 although IF 33 (17.99 g/100 g) had lowest invert sugar value, IF 35 (52.00 g/100 g) had highest value. Total sugar contents of the samples ranged from 33.04 g/100 g to 70.68 g/100 g. This study found the protein contents of infant formula could have a wide range of concentrations, 6.47 % (IF 30) to 14.92 % (IF 51). These results were similar to Ferrer et al. (2000) and Alpsan (2008)'s findings of 11.6 and 10.85, respectively. Hernandez-Ledesma et al. (2007) found this value between 3.35 % and 8.10 % in seven infant formulas, which were lower than those, used in other comparisons found in the literature.

Table 1. Protein, total sugar and invert sugar content of infant formulas samples**.**

No of samples	Protein (g 100 g^{-1})	Invert sugar (g 100 g^{-1})	Total sugar (g 100 g^{-1})	No of samples	Protein (g 100 g^{-1})	Invert sugar (g 100 g^{-1})	Total sugar (g 100 g^{-1})
IF1	13.74±1.12	23.89±2.87	33.85±1.49	**IF32**	13.93±1.76	43.69±2.13	52.37±4.41
IF2	12.50±1.86	22.68±3.06	33.04±4.72	**IF33**	11.20±1.12	17.99±0.55	35.87±0.57
IF3	9.85±1.04	31.17±2.19	50.38±5.56	**IF34**	10.78±1.12	21.65±3.97	34.21±3.69
IF4	9.78±0.70	34.70±4.40	62.27±4.83	**IF35**	13.09±0.58	52.00±5.64	65.12±4.30
IF5	10.18±0.72	28.83±2.81	39.92±3.60	**IF36**	11.82±1.90	51.35±3.81	62.86±3.68
IF6	10.64±1.28	34.64±2.36	53.88±4.94	**IF37**	12.82±1.02	23.49±1.33	37.17±4.13
IF7	10.61±0.24	26.99±2.93	66.52±3.76	**IF38**	12.32±1.34	24.74±2.08	36.58±2.64
IF8	9.05±1.66	23.97±1.15	65.77±5.81	**IF39**	13.52±0.60	23.83±0.69	42.90±4.62
IF9	10.09±1.30	30.21±2.37	55.69±3.13	**IF40**	13.56±1.12	25.79±2.35	41.32±3.84
IF10	9.93±2.02	31.70±4.94	57.84±4.28	**IF41**	14.09±1.56	28.88±3.24	48.14±4.00
IF11	14.40±0.48	22.51±3.25	49.05±2.33	**IF42**	14.52±2.72	26.71±1.67	48.19±3.25
IF12	14.55±1.20	26.45±1.29	45.68±5.82	**IF43**	13.35±1.44	31.01±4.23	55.45±5.91
IF13	7.31±0.14	28.69±2.33	36.43±0.71	**IF44**	12.55±1.18	37.16±4.10	53.28±2.34
IF14	7.10±0.94	27.08±1.62	33.11±3.95	**IF45**	12.46±1.04	29.25±1.37	45.96±4.42
IF15	10.70±2.26	34.66±2.80	50.64±2.36	**IF46**	11.58±0.78	27.36±2.04	41.13±3.81
IF16	10.73±1.38	38.40±3.68	58.26±4.02	**IF47**	13.53±1.42	28.89±2.63	43.54±4.12
IF17	9.35±1.10	30.87±4.27	40.63±5.25	**IF48**	14.29±1.26	29.64±1.28	40.98±1.60
IF18	9.01±1.44	37.74±3.26	51.99±5.79	**IF49**	10.71±0.50	25.56±3.44	35.72±4.34
IF19	11.21±1.82	36.70±3.74	52.86±4.30	**IF50**	9.63±1.94	26.39±0.83	41.08±4.28
IF20	11.17±0.68	33.46±0.98	56.77±2.13	**IF51**	14.92±2.18	39.39±2.65	48.14±2.72
IF21	9.00±1.08	36.99±2.23	53.98±4.48	**IF52**	14.02±1.30	37.63±3.17	47.95±4.55
IF22	9.06±0.36	43.29±3.59	59.74±4.62	**IF53**	12.52±0.10	33.38±4.50	48.01±3.89
IF23	7.64±0.54	29.10±1.22	54.33±5.69	**IF54**	10.13±0.86	33.11±3.43	46.85±2.73
IF24	7.14±0.90	28.09±2.81	51.03±2.87	**IF55**	10.77±1.24	43.90±5.26	48.84±4.10
IF25	10.15±1.74	30.29±2.35	41.63±2.23	**IF56**	9.46±0.50	29.45±1.69	34.27±1.93
IF26	11.84±1.02	30.74±1.68	41.26±3.50	**IF57**	10.03±1.54	26.67±1.83	46.24±3.52
IF27	10.92±0.96	49.47±2.87	61.32±5.84	**IF58**	9.48±0.84	24.95±2.87	47.31±4.11
IF28	11.92±1.18	45.78±5.05	64.84±2.76	**IF59**	11.56±1.16	42.90±2.04	58.00±3.82
IF29	7.08±0.28	49.90±4.36	68.95±5.11	**IF60**	11.73±1.38	30.88±4.58	41.38±4.20
IF30	6.47±0.44	42.86±4.40	70.68±4.58	**IF61**	12.58±1.12	27.15±2.33	53.95±4.47
IF31	13.53±1.12	42.95±3.03	51.24±5.92	**IF62**	13.73±2.04	27.08±1.62	49.28±2.86

3.2. Microbiological properties of infant formula samples

The method recommended by FDA was applied for the determination of *Cronobacter* spp. in PIF; three isolates (4.83%) were found to be *Cronobacter* spp. positive. The microbiological results were given in Table 2. Some researchers have detected this pathogen in similar proportions. Palcich et al. (2009) found 0.03 MPN / 100 g *Enterobacter (Cronobacter) sakazaki* in 186 powdered infant formulas purchased at retail in Brazil. In an American survey, *Cronobacter* was isolated from 2 of 82 (2.4 %) samples of PIF (Iversen and Forsythe, 2004). In a research the overall frequency was found 2.5 % in intermediate and final product *Cronobacter* that characterized by pulsed-field gel electrophoresis (Mullane et al., 2007). Two thousand and twenty PIF samples were collected from different companies and *Cronobacter* spp. strains were isolated from 56 out of 2,020 (2.8 %) PIF samples in Chinese retail markets (Fei et al., 2017). *Cronobacter* spp. was isolated from 3 of 91 (3%) follow up formulas (Chap et al., 2009). Xu et al. (2014) found the *Cronobacter* contamination rate 4.3%, 23 out of 530 using conventional biochemical methods and duplex PCR. Nazarowec-White and Farber (1997) examined 120 samples of PIF from five different manufacturers, enumerated *Cronobacter* from 8 samples (6.7 %). Zhang et al. (2017) found 42/1032 samples positive, including; 1.6 % (8/509) in PIFs, 6.5 % 34/523 in follow-up formulas using chromogenic media and biochemical test methods. Mardaneh

and Dallal (2017) examined 125 PIF samples, nine (7.2 %) of the samples were found positive for *Cronobacter*.

In contrast, other researchers have isolated the pathogen at higher frequencies. A study, which was conducted in Switzerland; *Cronobacter* spp. were detected in 9 % of ready to eat food samples. Fauziah et al., (2008) conducted a study to assess the presence of *Cronobacter* spp. and Enterobacteriaceae in powdered infant formula and children's milk, which were taken from three nurseries. *Cronobacter* spp. was isolated from 4 samples from 38 (10.5 %). Pan et al. (2014) found the contamination rate 12.3 %, 49 out of 399 in their research. In an Indonesian research, *Cronobacter* were isolated in 10/74 (13.5 %) samples of PIF (Estuningsih et al., 2006). Leuschner and Bew (2004) isolated *Cronobacter* from infant formula cans collected from 11 countries at 13.8 % (8 of 58). Santos et al. (2005), found that 12 of 86 (13.95 %) dried infant formula samples examined in Brazil were positive for *Cronobacter* spp. In a survey of 141 different powdered infant formulas from 35 countries, 20 (14.2 %) samples were positive for *Cronobacter* spp. (Muytjens et al., 1988). Minami et al. (2012) found that 5 of the 17 (29.4 %) PIFs were positive by DqPCR when they were not treated with EMA on the contrary both the FDA method and their rapid assay, which consists of DqPCR combined with gEMA, produced negative results for all 17 PIF for *Cronobacter* spp. A research in Shaanxi province *Cronobacter* spp. were positive in 67/632 samples of goat milk powder factories. The prevalence rates in finished products, intermediate powder, and manufacturing environment were 1.5 %, 6.0 %, and 92.5 %, respectively. 280 ready to eat foods samples tested, 52 (18.6 %) were positive for *Cronobacter* spp in China (Xu et al., 2015). Torres-Chavolla et al. (2007) determined *Cronobacter* in two types of powdered infant milk formula; 92 % and 32%, respectively. Many researchers reported that they could not find *Cronobacter* spp. in the powder infant formula samples examined (Baumgartner et al., 2009; Heperkan et al., 2017).

Table 2. Presence of *Cronobacter* spp., total mesophilic aerobic bacteria (TMAB) and coliform bacteria counts in powder infant formula samples.

No of samples	TMAB log cfu/g	Coliform log cfu/g	Growth in EE Broth	Growth in VRBGA	TSA	*Cronobacter* spp. (API 20E)
IF 1	2.32		-	-	*	*
IF 2	2.41	2.08	+	+	+	+
IF 3	2.17	-	-	-	*	*
IF 4	3.05	-	-	-	*	*
IF 5	3.16	-	-	-	*	*
IF 6	2.96	-	-	-	*	*
IF 7	2.38	-	-	-	*	*
IF 8	2.24	-	-	-	*	*
IF 9	3.05	-	-	-	*	*
IF 10	3.07	-	-	-	*	*
IF 11	<1	-	-	-	*	*
IF 12	2.51	-	-	-	*	*
IF 13	<1	-	-	-	*	*
IF 14	3.25	-	-	-	*	*
IF 15	2.39	1.07	+	+	-	*
IF 16	2.25	-	-	-	*	*
IF 17	2.11	-	-	-	*	*
IF 18	1.07	-	-	-	*	*
IF 19	2.89	-	-	-	*	*
IF 20	<1	-	-	-	*	*
IF 21	2.13	-	-	-	*	*
IF 22	2.68	-	-	-	*	*
IF 23	2.37	1.27	+	+	+	-
IF 24	2.14	1.27	+	+	+	+
IF 25	2.58	-	-	-	*	*

IF 26	2.56	-	-	-	*	*
IF 27	2.23	1.20	-	-	*	*
IF 28	2.06	-	-	-	*	*
IF 29	3.40	1.07	+	+	+	+
IF 30	3.06	1.17	+	+	+	-
IF 31	2.19	-	-	-	*	*
IF 32	<1	-	-	-	*	*
IF 33	<1	-	-	-	*	*
IF 34	1.11	-	-	-	*	*
IF 35	-	-	-	-	*	*
IF 36	2.43	-	-	-	*	*
IF 37	2.45	-	-	-	*	*
IF 38	2.42	-	-	-	*	*
IF 39	2.19	-	-	-	*	*
IF 40	2.13	-	-	-	*	*
IF 41	2.06	-	-	-	*	*
IF 42	2.25	-	-	-	*	*
IF 43	1.57	-	-	-	*	*
IF 44	1.23	-	-	-	*	*
IF 45	1.66	-	-	-	*	*
IF 46	1.85	-	-	-	*	*
IF 47	<1	-	-	-	*	*
IF 48	1.04	-	-	-	*	*
IF 49	1.27	-	-	-	*	*
IF 50	2.37	-	-	-	*	*
IF 51	1.17	-	-	-	*	*
IF 52	1.38	-	-	-	*	*
IF 53	2.57	-	-	-	*	*
IF 54	3.42	1.39	+	+	+	-
IF 55	2.51	-	-	-	*	*
IF 56	2.03	-	-	-	*	*
IF 57	<1	-	-	-	*	*
IF 58	2.29	-	-	-	*	*
IF 59	2.18	-	-	-	*	*
IF 60	2.72	-	-	-	*	*
IF 61	<1	-	-	-	*	*
IF 62	2.72	-	-	-	*	*

- not found

* Analysis were not made since there was no bacterial growth VRBG Agar and TSA.

The microbiological profile of infant formula sample was also determined in this study. The number of mesophilic aerobic bacteria count were between <1 log cfu/g and 3.42 log cfu/g. No mesophilic aerobic bacteria were detected in eight samples. Eight out of sixty two PIF were also positive for coliforms. This bacteria's average count was 1.32 log cfu/g. eleven samples were positive for thermophilic bacteria and their counts were between 1.07 log cfu/g and 2.21 log cfu/g.

4. CONCLUSION

In this study, three out of 62 samples were found to be *Cronobacter* spp. positive performed according to FDA (2002) methods. Infection caused by *Cronobacter* spp. can cause significant public health problems because of the excessive use of baby products. Especially microbiological safety of PIF is very important in this regard. The presence of *Cronobacter* spp. in PIF can lead to high mortality rates. Therefore, in the production of PIF, the necessary controls from raw material procurement to packing stage must be carried out appropriately and carefully. Due to the

excessive use of baby products, *Cronobacter* infections can cause serious health problems. Future studies should further focus on *Cronobacter* infection susceptibilities. There is no regulation on *Cronobacter* in the Notification on Infant Formulas (Notification Nr: 2014/31) of the Turkish Food Codex, and this is seen as an important deficiency.

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