



Prevalence of *Cronobacter* spp. in various foodstuffs and identification by multiplex PCR

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Abstract

Cronobacter spp. are opportunistic pathogens isolated from many different type of food and environmental samples and may cause serious health problems and even death in newborns, children and elders. The purpose of the study was to determine occurrence and prevalence of *Cronobacter* spp. in animal originated food, ready-to-eat food, fruits and vegetables, sold in Istanbul and its vicinity and to perform molecular typing of the isolated agents by multiplex PCR. Analyses were conducted on a total of 219 food samples including 11 confectionary, 50 fruits and vegetables, 52 dairy products, 50 meat and meat products and 56 ready-to-eat food. Suspected isolates were validated by PCR targeted to *gyrB* gene and multiplex PCR was performed for identification. Overall analyses revealed a prevalence of 5.48% for *Cronobacter* spp. in all food products assessed. Prevalence of the bacteria was 9%, 1.9% and 17.8% in confectionary, dairy products and ready-to-eat food, respectively whereas no bacteria were detected in fruits and vegetables, meat and meat products. Out of a total of 12 isolates determined, 8 (66%), 3 (25%) and 1 (8%) were identified as *C.sakazakii*, *C.muytjensii* and *C.malonaticus*, respectively and nine isolates belonged to cereal-based balls (meatless *cig kofte*).

Keywords: cereal-based balls (*cig kofte*); *Cronobacter* spp.; dairy product; meat product; PCR.

Practical Application: The presence of *Cronobacter* spp. in ready-to-eat-food can cause a risk of food safety.

1 Introduction

Cronobacter spp. are gram-negative, asporogenous, motile bacilli included in the family *Enterobacteriaceae*. Due to the opportunistic nature of these bacteria they may induce several clinical symptoms in different age groups. High mortality rates were documented especially in the newborns (Garbaj et al., 2017; Iversen et al., 2004; Iversen et al., 2008; Li et al., 2014; Joshi et al., 2014; Hu et al., 2018).

Six species of the genus *Cronobacter* were indicated to be foodborne pathogens causing diseases. These pathogenic species such as *C.sakazakii* (formerly known as *Enterobacter sakazakii*), *C.turicensis*, *C.universalis*, *C.dublinensis*, *C.malonaticus* and *C.muytjensii* were reported in different studies. *C. dublinensis* has recently been divided into 3 subspecies: *C.dublinensis subsp. dublinensis*, *C.dublinensis subsp.lausennensis* and *C.dublinensis subsp.lactaridi*. These six species differ from each other in terms of stress tolerance, virulence capacity and antibiotic resistance. *C.condimenti* was determined as the seventh species but was found to be of no medical significance (Li et al., 2014; Hu et al., 2018; Iversen et al., 2007; Garbowska et al., 2015).

Cronobacter species were frequently obtained from food products such as powdered infant formula (PIF) (Akineden et al., 2017; Caubilla-Barron et al., 2007; Himelright et al., 2002; Weir, 2002; van Acker et al., 2001). However, they may also be isolated frequently from environmental sources like water,

soil and vegetables and the food products can be contaminated subsequently by means of vectors such as rodents and flies (Iversen & Forsythe, 2003; Koluman, 2011; Vojkowska et al., 2016). As in the case of infant foods, the bacteria were isolated from low-water activity foods like flour, spice, cereal, instant soups and dried pasta in various studies (Aksu et al., 2016; Akineden et al., 2015). Still on the other hand, they may frequently be isolated from plant-based foods of high water activity like salad, fruits and vegetables and also foods of animal origin like milk, meat, fish and their products (Li et al., 2014; Garbowska et al., 2015; Mohammed et al., 2015; Lou et al., 2014; Gökmen et al., 2010). Reported studies revealed the occurrence of the bacteria in animal source foods particularly like egg, cheese, fish, chicken, meat products, milk, pork products, milk powder, sausage, shellfish; in plant-based food like barley, biscuit, cereal, coconut powder, squash, kidney-bean, almond, peanut, lentil, instant dried soup, processed vegetarian food, flour of various origins, spice, dried nuts and fruits, rice, dessert, tea, fermented soy bean, tomato, vegetables and salads (Aksu et al., 2016; Gökmen et al., 2010; Beuchat et al., 2009).

High tolerance of *Cronobacter* spp. to food preservation methods such as drying, acidic pH, heating and osmotic pressure; the property of biofilm formation and the long-term survival of the bacteria on the surfaces during food processing may be listed among the reasons for the occurrence of bacteria

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in various foods and environmental products. Poor hygienic conditions and contaminated tools and equipment may result in the contamination of the food and food ingredients with this type of bacteria (Jaradat et al., 2014).

Research studies have been carried out in Turkey with respect to the presence of *Cronobacter* spp. in milk, dairy products and infant food; however no studies are available in terms of the occurrence of these microorganisms in ready-to-eat food (delicatessen and cereal-based balls), confectionary, fruits and vegetables, meat and meat products. Therefore, the current study aimed to determine the prevalence of *Cronobacter* spp. in a variety of ready-to-eat food, meat and meat products, dairy products, fruits and vegetables and confectionary and to achieve species-based molecular identification of these bacteria.

2 Materials and methods

2.1 Sampling

A total of 219 different types of food products in their original packaging or as in non-packed form were purchased from miscellaneous sales points in Istanbul and its vicinity, using random selection method. The study material consisted of 11 confectionary, 50 fruits and vegetables, 52 dairy products, 50 meat and meat products and 56 ready-to-eat food. The collected materials were submitted to the laboratory under cold storage conditions and assessed for the presence of *Cronobacter* spp. by conventional methods.

2.2 Isolation of *Cronobacter* spp. in the analyzed samples

Isolation of *Cronobacter* spp. was performed according to the method (International Organization for Standardization, 2006) modified by Li et al. (2014). Initially, 25 g of the sample was homogenized in 225 mL of buffered peptone water (Oxoid) and incubated at 37 °C for 18h. Then, 1 mL of the content was transferred into a test tube containing 10 mL of modified Lauryl Sulfate Tryptose Broth (Oxoid) and incubated at 42 °C for 24h. Followed by the incubation period, the homogenate was streaked on the surface of Chromogenic *Cronobacter* Isolation Agar (Oxoid) plate by a loop and then the plates were incubated at 42 °C for 24h. Suspected colonies appeared as blue-greenish

coloring on the agar were purified in Tryptic Soy Agar medium. Various biochemical tests (indole, methyl red, Voges-Proskauer, citrate, oxidase, catalyze and carbohydrate fermentation tests) were run and gram staining was performed on the isolates for the identification of *Cronobacter* spp. Verified isolates were preserved in EE Broth containing 20% glycerol and kept at -80 °C for further analyses.

2.3 Genomic DNA Extraction

The isolates obtained from *Cronobacter* spp. analysis were incubated in 5 mL of Tryptic Soy Broth for 24h at 37 °C prior to the extraction process. DNA extraction was carried out according to manufacturer's instructions by using spin column technology based Genomic DNA Purification Kit (Thermo Fisher).

2.4 Verification and molecular typing of the isolates by Polymerase Chain Reaction (PCR)

For the identification of *Cronobacter* spp. by PCR, *gyrB* gene fragment with a length of 438bp (*gyrB-F: ATGGATAAAGAGGGCTACAG ve gyrB-R: CGCCTGATTCTTACGGTTAC*) was targeted. Followed by pre-denaturation for 5 min at 95 °C, the amplification process was carried out in 35 cycles: 30 sec at 94 °C, 30 sec at 62 °C and 30 sec at 72 °C, respectively. Elongation was performed at 72 °C for 10 min (Chen et al., 2013).

PCR products obtained after amplification were run on a 1.5% agarose gel in 1x TAE for 1h at 100 volt. Non-toxic, non-mutagen SYBRsafe DNA stain was used for the visualization of the products. The lengths of the amplicons obtained on the gel were assessed with the DNA ladder by using a gel visualization system (BIO RAD Gel Doc[™] EZ Imager).

Molecular identification of *Cronobacter* spp. was conducted by using *multiplex* PCR Method suggested by Carter et al. (2013) (Table 1). After pre-denaturation for 3 min at 94 °C, amplification process of the DNA fragments included 25 cycles: 30 sec at 94 °C, 30 sec at 62 °C and 60 sec at 72 °C, respectively. Elongation step was adjusted to 5 min at 72 °C Carter et al. (2013). *C.sakazakii* (ATCC 29544) and *C.muytjensii* (ATCC 51329) were used as the positive controls.

Table 1. Primers used for the molecular typing of *Cronobacter* spp. (Carter et al., 2013).

Primer	Oligonucleotide Chain	Length(bp)	Species
Cdm-469R ^a	CCACATGGCCGATATGCACGCC		
Cdub-40F	GATACCTCTCTGGGCCGCAGC	430	<i>C. dublinensis</i>
Cmuy-209F	TTCTTCAGGCGGAGCTGACCT	260	<i>C. muytjensii</i>
Cmstu-825F ^b	GGTGGCSGGGTATGACAAAGAC		
Ctur-1036R	TCGCCATCGAGTGCAGCGTAT	211	<i>C. turicensis</i>
Cuni-1133R	GAAACAGGCTGTCCGGTACAG	308	<i>C. universalis</i>
Csak-1317R	GGCGGACGAAGCCTCAGAGAGT	492	<i>C. sakazakii</i>
Cmal-1410R	GGTGACCACACCTTCAGGCAGA	585	<i>C. malonaticus</i>

^a The PCR primer Cdm-469R was used in multiplex reactions with Cdub-40F and Cmuy-209F primers for identifying *C. dublinensis* and *C. muytjensii* strains, respectively. ^b The PCR primer Cmstu-825F was used in multiplex reactions with Ctur-1036R, Cuni-1133R, Ccak-1317R and Cmal-1410R primers for identifying *C. turicensis*, *C. universalis*, *C. sakazakii* ve *C. malonaticus* strains, respectively.

3 Results

In the study, a total of 219 food products were microbiologically analyzed for *Cronobacter* spp. Twelve isolates were obtained. Prevalence of *Cronobacter* spp. was estimated to be 5.48%. Prevalences of the bacteria in terms of the type of the food products were 9%, 1.9% and 17.8% for confectionary, dairy products and ready-to-eat food, respectively. *Cronobacter* spp. were not detected in fruits-vegetables, meat and meat products. Isolates were molecularly identified to the species level and 8 (66%), 3 (25%) and 1 (8%) isolates were found to be *C.sakazakii*, *C.muytjensii* and *C.malonaticus*, respectively. The number of the analyzed food products and the identified strains were given in Table 2.

Cronobacter spp. positive samples were determined in dehydrated fruit-based leather (traditionally named as *pestil*) (*C. muytjensii*) from the confectionary group; in plaited cheese (*C. muytjensii*) from dairy products; in cereal-based balls (*C. sakazakii*, *C. muytjensii*, *C. malonaticus*) and in cooked liver salad (*C. sakazakii*) from ready-to-eat food.

Gel image of DNA bands obtained from *Cronobacter* spp. positive isolates by PCR assay was given in Figure 1.

Gel image of Multiplex PCR products for certain *Cronobacter* spp. isolates identified to the species level was shown in Figure 2.

Table 2. Occurrence and prevalence of *Cronobacter* spp. in a variety of foods analyzed.

Type of food product	Number of the samples (n)	<i>C. sakazakii</i>	<i>C. muytjensii</i>	<i>C. malonaticus</i>	Positive samples	Percentage of positive samples (%)
Confectionary	11	- ^a	1	- ^a	1	9.0
Fruit and vegetables	50	- ^a	- ^a	- ^a	-	0.0
Dairy products	52	- ^a	1	- ^a	1	1.9
Meat and Meat products	50	- ^a	- ^a	- ^a	-	0.0
Ready-to eat food	56	8	1	1	10	17.8
Total count	219	8	3	1	12	5.48

-^a: not detected

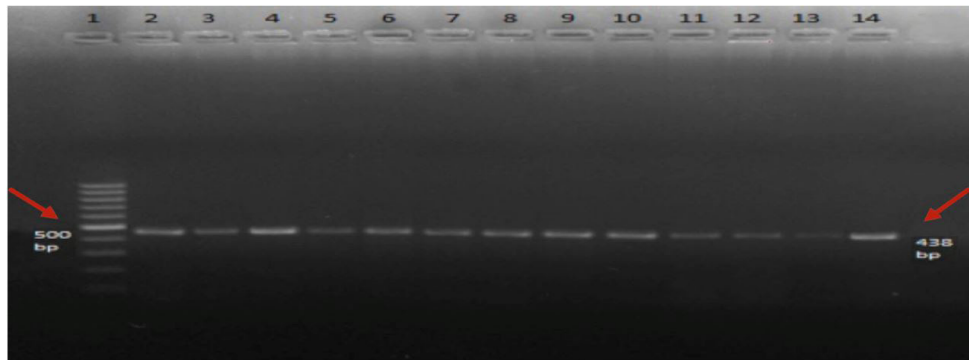


Figure 1. Gel image of 438-bp-long DNA fragments of targeted *gyrB* gene in *Cronobacter* spp. positive isolates. 1st loading well: 100 bp DNA ladder; 2nd-13th loading wells: *Cronobacter* spp. positive isolates obtained from food samples of different types; 14th loading well: positive control (438 bp *C. sakazakii*, ATCC 29544).

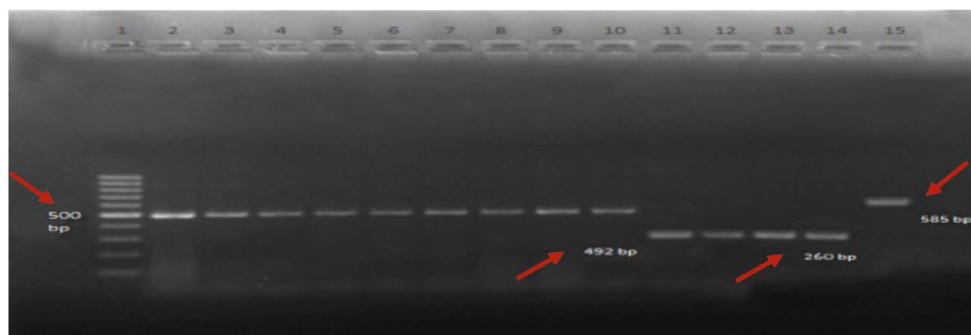


Figure 2. Gel image of some isolates identified to the species level according to their DNA fragments of different lengths by Multiplex PCR assay. 1st loading wells: 100 bp DNA ladder; 2nd loading well: Positive control (492 bp *C. sakazakii*, ATCC 29544); 3rd to 10th loading wells 492 bp *C. sakazakii*; 11th loading well: Positive control (260 bp *C. muytjensii*, ATCC 51329); 12th to 14th loading wells: 260 bp *C. muytjensii*; 15th loading wells: 585 bp *C. malonaticus*.

4 Discussion

Since *Cronobacter* spp. give rise to serious health issues and may even cause high mortality in susceptible individuals like newborns and children, they have been extensively investigated in food products appealing to this age group, like milk powder and infant foods (Gökmen et al., 2010; Yao et al., 2016; Hoque et al., 2010). However, no comprehensive studies are available with respect to the occurrence and prevalence of these bacteria in meat and meat products, fruits and vegetables and ready-to-eat foods in Turkey. For this purpose, 219 miscellaneous food products collected from Istanbul and its vicinity were investigated in terms of occurrence of *Cronobacter* spp. Suspected isolates were verified by PCR targeted to the specific gene fragment of the bacteria and identification was achieved by multiplex PCR.

In the study, the most noteworthy type of foods in terms of the occurrence and prevalence of *Cronobacter* spp. were detected to be ready-to-eat food. In this group, 56 food samples, 18 of which were cereal-based balls, were investigated and 10 isolates were determined. Nine (50%) out of 18 cereal-based ball samples were positive for *Cronobacter* spp. *C.sakazakii*, which was the most prevalent species was detected in 7 samples. *C.muytjensii* was identified in 1 sample and one other sample was positive for *C.malonaticus*. Cereal-based ball (named as *cig kofte*) is a local dish, which consists mostly of plant-based foodstuffs like bulgur, (it contains fat free minced meat in some regions), a variety of spice, tomato and red pepper paste, onion, garlic and salt. All ingredients are thoroughly kneaded together and it is consumed uncooked. Despite the lack of sufficient literature information available in terms of the prevalence of *Cronobacter* spp. in cereal-based balls, microbiological quality of the product has previously been investigated in Turkey. However, the products were found to be of poor quality (especially in terms of total aerobic mesophilic bacteria and *Enterobacteriaceae*) and it was indicated by several researches that the products were contaminated with numerous microorganisms due to non-hygienic conditions as well as the poor quality of the raw materials (Sancak & İşleyici, 2006; Elmalı & Yaman, 2005).

Other product positive for *Cronobacter* spp. from ready-to-eat food was liver salad which is a cooked side dish served with a variety of spices before consumption. It was considered that the source of contamination in this food product was the spices used. Hence, in the previous studies carried out with respect to prevalence of *Cronobacter* spp. in spices, Aksu et al. (2016) found 14% positivity. According to Li et al. (2014) the percentage value of positive products was 4.5% and Garbowska et al. (2015) reported prevalence rate of 16.7% for *Cronobacter* spp. Turcovský et al. (2011) pointed out that particularly plant-based foods contained higher levels of *Cronobacter* spp. when compared with other types of food products. Likewise, meat products flavored with miscellaneous spices and condiments revealed increased levels of *Cronobacter* spp. In a study carried out in Korea, prevalence of *Cronobacter* spp. in *Sunsik* and *Sangsik*, which are cereal-based spicy local foods, was found to be 36% and *C.sakazakii* was identified in 26.7% of the isolates (Lee et al., 2012). In this study, prevalence of *Cronobacter* spp. in raw meat balls which also contained cereal and spice was 50% and the most prevalent strain was *C.sakazakii*. Our findings were compatible with those

of Lee et al. (2012). As can be seen in many studies with respect to high prevalence of *Cronobacter* spp. in spice and cereal, spice are demonstrated to be one of the most important sources of contamination in the food which contain these ingredients. Cereal-based balls are food that are widely and fondly consumed in Turkey by different age groups. Schoolchildren also consume this product as snack. It is considered that such contaminants in ready-to-eat foods may give rise to various health problems in immunocompromised individuals.

In the present study, 11 samples were investigated in the confectionary group and *Cronobacter* spp. (*C.muytjensii*) was isolated in one product (dehydrated fruit-based leather traditionally named as *pestil*) with 9% prevalence. In a similar study, Baumgartner et al. (2009) reported 7.1% prevalence, which was compatible with our findings. Dehydrated fruit-based leather, which was positive for *Cronobacter* spp. in this study, is local confectionary which is dried naturally under environmental conditions. The studies carried out with respect to occurrence of *Cronobacter* spp. in domestic and environmental dust particles supported the idea that the natural drying process might have had increased the risk for contamination (Killer et al., 2015).

In the study, 50 food samples were investigated in fruits and vegetables group and none of them was positive for *Cronobacter* spp. This finding was compatible with those of Li et al. (2014) and Turcovský et al. (2011) in the studies conducted on fruits/vegetables and fruits, respectively. However, Lee et al. (2012) reported that *Cronobacter* isolates were obtained from 3 (7.3%) out of 41 fruit samples and 19 (14.8%) out of 128 vegetable samples. In a similar study by Vojkowska et al. (2016), 38 out of 396 vegetables and 1 out of 49 fruits were positive for *Cronobacter* spp. in the samples collected from both supermarkets and farms. In the present study, it was considered that the absence of *Cronobacter* spp. positive products in this type of foods might have been associated with the rather small number of the samples, the selection of samples from different sources and the improvement in fruit and vegetable growing conditions.

In the dairy products group, 52 samples were analyzed and one sample (plaited cheese) was found to be positive for *Cronobacter* spp. (*C.muytjensii*) with the prevalence rate of 1.9%. Survival of the bacteria in this product might have been attributed to the insufficient salt concentration in the brine when compared with the equivalent products. Hence, *Cronobacter* spp. was observed in none of 98 dairy products and 32 cheese samples in a study by Turcovský et al. (2011). Likewise, Heperkan et al. (2017) demonstrated no positivity in 19 cheese samples tested. Our findings were compatible with those of these researches. Pasteurization and fermentation processes applied in these products were shown to constitute biological inhibition technologies. However, it must be considered that there are always risk factors regarding these technologies due to the biofilm formation ability of the bacteria on silicon, latex, polycarbonate, stainless steel, glass and polyvinyl chloride surfaces (Beuchat et al., 2009).

Fifty samples were analyzed in the meat and meat products group and *Cronobacter* spp. were isolated in none of them. In this category, 25 raw meat and 25 processed meat products (fermented sausage, salami, frankfurter type sausage and pastrami) were

tested. Researches indicated that these bacteria were found in lower levels in animal-based food than plant-based food. Hence, prevalence of *Cronobacter* spp. was found to be quite low in meat and meat products in the studies (Wang et al., 2012). However, despite the low prevalence in raw meat, risk factors for contamination were shown to have increased in spiced meat products and thus unfavorable microbiological profile of the spices constituted the source of contamination in a variety of both raw and ready-to-eat food (Turcovský et al., 2011).

5 Conclusion

In conclusion, the present study put forward a high prevalence for *Cronobacter* spp. in plant-based balls that are frequently consumed by different age groups and are available on the market in Istanbul and its vicinity. That the seven out of nine isolates were *C.sakazakii*; one *C.muytjensii* and one *C.malonaticus*, which are the pathogenic strains, indicated that these food products should be taken into account in terms of public health. Occurrence of *C.sakazakii* and *C. muytjensii* in spiced cooked liver salad and dehydrated fruit leather, respectively were found noteworthy since these products are ready-to-eat foodstuffs. Particularly, identification of *C.muytjensii* in one plaited cheese sample was a significant finding. The unfavorable outcomes of the contamination with these species are already associated with the immunocompromised individuals like infants and children however they may give rise to miscellaneous health issues in different age groups, as well. Therefore, it was suggested that the presence of pathogenic agents should be monitored in foods and food manufacturers should take the necessary precautions to provide the food safety from the raw material to the end product under the system of HACCP.

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