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Prevalence and identification by multiplex polymerase chain reaction patterns of *Cronobacter* spp. isolated from plant-based foods

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Abstract

Cronobacter spp. involves a group of opportunistic pathogens that cause meningitis in newborns, immunosuppressed individuals with a mortality rate of 50-80%. Seven species like *C. sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. turicensis*, *C. dublinensis*, *C. universalis*, *C. condimenti* are included in this genus which has been a subject of research especially in the bacteriologic analysis of baby foods. However, since these species were detected also in prepared foodstuffs. The objective of this study was to assert the presence of *Cronobacter* spp. in foodstuff offered for sale in Turkey. A total of 151 prepared foodstuffs including a variety of spice, flour, instant soup were purchased from different sales points. The presence of *Cronobacter* spp. were investigated in these samples. *Cronobacter* suspected isolates which were obtained by microbiological analyses were confirmed by PCR targeted to *gyrB* gene and were then identified by multiplex PCR. Prevalence of *Cronobacter* spp was estimated to be 17.88%. Out of 27 *Cronobacter* spp. isolates obtained, 13(48.1%), 6(22.2%), 5(18.5%), 3(11.1%) belonged to *C. sakazakii*, *C. muytjensii*, *C. turicensis*, *C. malonaticus* species, respectively. Consequently, the presence of the bacteria in widely consumed foodstuff revealed that *Cronobacter* spp. is subject to monitoring due to its opportunistic nature in terms of public health concern.

Keywords: *Cronobacter* spp.; flour; instant soup; PCR; spice.

Practical Application: *Cronobacter* spp in plant-based foods may compose health risks.

1 Introduction

Cronobacter spp. are gram negative, facultative anaerobic, motile and non-sporulating opportunistic bacteria which belong to the family *Enterobacteriaceae* and their optimal growth temperatures range between 37 °C and 44 °C (Iversen et al., 2004; Iversen et al., 2008; Li et al., 2014). The genus was formerly known as *Enterobacter sakazakii* and took its current name in accordance with the novel taxonomic classification system. Therefore, the genus *Cronobacter* actually consists of seven species which are *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter turicensis*, *Cronobacter dublinensis*, *Cronobacter universalis* and *Cronobacter condimenti* (Iversen et al., 2007; Iversen et al., 2008). All species except *C. condimenti* are among the causes of foodborne diseases particularly in newborns, children and immunocompromised adults (Li et al., 2014; Garbowska et al., 2015). Even though the incidence of infections caused by *Cronobacter* spp. is low, the mortality rate was reported to be 50-80% with clinical symptoms manifested by necrotizing enterocolitis, bacteremia and meningitis (Joshi et al., 2014). Microorganisms belonging to this genus along with *Listeria monocytogenes, Clostridium perfringens* type *A* and *B* and *Cryptosporidium parvum* of which infections were recognized as chronic or prolonged, life threatening and extremely dangerous for susceptible individuals by ICMSF (International Commission on Microbiological Specifications for Foods) (Iversen & Forsythe, 2003) are the members of the same class. Therefore, the fact that *Cronobacter* species share the same class with important pathogens increases their significance for public health. Outbreaks of *Cronobacter* spp. are mostly associated with contaminated powdered infant formulas and some incidents were reported in various countries (Van Acker et al., 2001; Himelright et al., 2002; Weir, 2002; Caubilla-Barron et al., 2007). In addition, *Cronobacter* species were isolated from a variety of dried foods, instant food products and their ingredients and also from domestic environments and food production facilities and environmental samples (Iversen & Forsythe, 2004; Drudy et al., 2006; Turcovsky et al., 2011; Hochel et al., 2012; Wang et al., 2012; Lee et al., 2012; Li et al., 2014; Killer et al., 2015; Garbowska et al., 2015; Vojkovska et al., 2016). It has been stated that *Cronobacter* species were more commonly isolated from plant-based foods and food compounds rather than foods of animal origin (Baumgartner et al., 2009; Turcovsky et al., 2011; Hochel et al., 2012). That the genus exists particularly in grained, dried and powdered foods and food compounds as a consequence of its resistance to the drying process, is of great importance for public health. Different types of flour may not only be found in the composition of various food products but also are the essential raw materials for bakery products. In a study, *Cronobacter* spp. was isolated

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from 8 (16%) of 50 cereals (Lee et al., 2012). Li et al. (2014) reported that 12 (14.1%) out of 85 cereal samples were positive for *Cronobacter* spp. In a study by Yao et al. (2016), 16 (12.0%) from 133 samples contained *Cronobacter* spp. Moreover, Lou et al. (2014) investigated the presence of *Cronobacter* spp. in flours and various bakery products and found out that all of 18 wheat flours and 5 dried noodle samples and 3 (42.9%) of 7 frozen ravioli samples and one (12.5%) of 8 ready-to-eat cereal based food products contained *Cronobacter* spp. Unlike these findings, Turcovsky et al. (2011) did not detect these bacteria in any of 20 cereals and cereal based food samples analyzed. The findings of Jaradat et al. (2009) were similar to those of Turcovsky et al. (2011) and the authors reported that none of 32 cereals and cereal based products were contaminated with the bacteria. Spices and culinary herbs/herbal flavourings of different types are included in the ingredients of several food formulas to enhance the taste and flavor of the products. In a study by Turcovsky et al. (2011), *Cronobacter* spp. were isolated from 13 of 21 spices. Garbowska et al. (2015) reported that out of all tested culinary herbs/herbal flavourings and spices, 10 (16.7%) samples contained *Cronobacter* spp. In another study by Li et al. (2014) the agent was isolated only from one sample of black pepper (4.5%) out of a total of 22 spices and herbs of different types. Baumgartner et al. (2009) detected *Cronobacter* spp. in 7 (26.9%) of 26 spices and herbs, whereas Jaradat et al. (2009) isolated the bacteria from 26 (38.8%) of 67 spice samples. In a study by Iversen & Forsythe (2004) 40 samples out of a total of 122 different culinary herbs/herbal flavourings and spices were positive for *Cronobacter* spp. Instant soups are widely consumed products due to their practicalness. Their ingredients involve various food compounds of plant or animal origin due to the type of soup. Instant soups as well as spices may contain contaminants including *Cronobacter* spp., hence Killer et al. (2015) reported that *Cronobacter* spp. were found in soups containing garlic powder. Turcovsky et al. (2011) showed the presence of *Cronobacter* spp. in 2 (15.3%) of 13 dry instant soup products. According to the study by Hochel et al. (2012) 52 (13.0%) of analyzed 399 different food products, mostly of plant origin, were found to be positive for *Cronobacter* spp. In addition, the prevalences of *Cronobacter* species were reported to be 28.3%, 26.4% and 15.1% in spices-herbs, different grains and cereal based product samples, respectively. The aim of this study was to determine the prevalence of *Cronobacter* spp. in food products such as flours, spices and culinary herbs/herbal flavourings and dry instant soups that were manufactured and packaged in different regions of Turkey and thus to achieve the molecular typing of the isolated agents by means of phenotypic and genotypic techniques.

2 Materials and methods

2.1 Sampling

A total of 151 food products of different types in their original packaging like instant dry soups, cereal flours and spices-herbs which were produced and sold in Turkey by various manufacturers constituted the study material. The products which comprised 50 spices and culinary herbs/herbal flavourings, 51 flour and 50 instant dry soup samples were randomly purchased from different markets. All collected samples were transferred to the lab under optimal conditions on the same day of purchase and the analyses were subsequently initiated.

2.2 Isolation of Cronobacter spp. from food samples

The isolation of *Cronobacter* spp. was carried out according to the method (International Organization for Standardization, 2006) modified by Li et al. (2014). For this purpose, 25 g of each sample was homogenized in 225 mL of buffered peptone water (Oxoid) and incubated at 37 °C for 18h. Following the incubation period, 1 mL of the homogenate was transferred into the test tube containing 10 mL of Modified Lauryl Sulfate Tryptose Broth (Oxoid) and then incubated at 42 °C for 24h. After the incubation, the homogenate was spread onto Chromogenic Cronobacter Isolation Agar plates (Oxoid) by a loop and the plates were incubated at 42 °C for 24h. The blue-green coloring on Chromogenic Cronobacter Isolation Agar medium revealed growth of suspicious bacterial colonies, which were then purified on Tryptic Soy Agar medium. Gram staining and several biochemical tests (indole, methyl red, voges-proskauer, citrate, oxidase, catalase and carbohydrate fermentation tests) were performed on these purified cultures for the identification of *Cronobacter* spp. The verified isolates were preserved in EE Broth containing 20% glycerol and then stored at –80 °C for further analyses.

2.3 Genomic DNA extraction

Prior to DNA extraction, the isolates obtained through the above-mentioned procedure were incubated in 5 mL of Trpytic Soy Broth at 37 °C for 24h. DNA extraction was performed by a commercially available Spin Column Technology Based Genomic DNA Purification Kit (ThermoFisher) according to the manufacturer's instructions.

2.4 Identification of Cronobacter isolates by Polymerase Chain Reaction (PCR) assay

For the identification of *Cronobacter spp*. PCR was targeted to *gyrB* (438 bp in length) gene (*gyrB*-F: ATGGATAAAGAGGGCTACAG; *gyrB*-R: GCCTGATTCTTACGGTTAC), which was previously suggested by Chen et al. (2013). PCR cycling conditions for amplification of DNA fragments included an initial denaturation for 5 min at 95 °C followed by 35 cycles of 30 sec at 94 °C, 30 sec at 62 °C and 30 sec at 72 °C and finally 10 min at 72 °C for elongation (Chen et al., 2013).

2.5 Typing of Cronobacter spp.

Species specific primer sets were applied by multiplex PCR method for the typing of *Cronobacter spp.* as previously described by Carter et al. (2013) (Table 1). Amplification procedure involved an initial denaturation for 3 min at 94 °C followed by 25 cycles of 30 sec at 94 °C, 30 sec at 62 °C and 60 sec at 72 °C and finally 5 min at 72 °C for elongation (Carter et al., 2013).

PCR products were visualized on a 1.5% agarose gel in running buffer 1×TAE (Tris Acetate EDTA) with a non-toxic, non-mutagen DNA loading dye (SYBR Safe DNA Stain) under

UV illuminator (Running voltage: 100V/cm, running time: 1h). The DNA fragments of different lengths which appeared as migrating colored bands on the gel were evaluated comparatively with the DNA ladder by a gel visualization system (BIO RAD Gel Doc™ EZ Imager).

3 Results

In this study, *Cronobacter* spp. were isolated from 27 (17.88) of 151 food products on the basis of microbiologic analyses. Prevalences of *Cronobacter* spp. were 21.56%, 18% and 14% in flour, dry instant soup samples and spices and culinary herbs/herbal flavourings, respectively. Thirteen (48.1%), six (22.2%), five (18.5%) and three (11.1%) of all obtained *Cronobacter* isolates were identified as *C. sakazakii*, *C. muytjensii*, *C. turicensis* and *C. malonaticus*, respectively. Existence of *Cronobacter* species in different types of food products and the species identified were given in Table 2.

In the group of spices-herbs, samples tested positive for *Cronobacter* spp. belonged to 2 coriander (*C. sakazakii* and *C. turicensis*) and one for nutmeg (*C. muytjensii*), dried dill (*C. muytjensii*), mustard powder (*C. sakazakii*), dried parsley (*C. muytjensii*) and aniseed (*C. sakazakii*) samples. Positive isolates in the group of flours were obtained from 5 of wheat flour (*C. malonaticus*, *C. sakazakii*, *C. turicensis*), 4 of corn flour (*C. malonaticus, C. sakazakii, C. turicensis*) and 2 of rice flour (*C. malonaticus, C. turicensis*) samples. *Cronobacter* spp. positive instant soup samples included 4 mixed vegetable soup traditionally-called *ezogelin* (*C. sakazakii, C. muytjensii*), 2 mixed soup traditionally-called *yayla* (*C. sakazakii*) and one for mushroom soup with cream (*C. muytjensii*), lentil soup (*C. turicensis*) and mixed soup called *tarhana* (*C. muytjensii*) soup samples.

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.

4 Discussion

Cronobacter spp. comprise a group of opportunistic pathogen species, which may induce various health issues particularly in newborns. Findings regarding the infections caused by this genus in children, adults and elder people strengthened the significance of the presence of these bacteria in food products in terms of microbiological safety of foods. Numerous research studies have been carried out regarding the presence of *Cronobacter* spp. in infant foods and baby formulas however, it has yet recently gained recognition in various food products and environmental conditions.

In this study, a total of 151 samples comprising 3 different types of food products were collected from various consumption points/markets at different intervals and then microbiologically analyzed. Twenty-seven isolates of *Cronobacter* spp. were obtained from all tested samples. Overall prevalence of *Cronobacter* spp. was found to be 17.88%. Eleven (21.56%) out of 51 flour; 9 (18.0%) out of 50 instant dry soup and finally 7 (14.0%) out of 50 spices-herbs samples were detected to be positive for *Cronobacter* spp.

Most commonly isolated species was determined to be *Cronobacter sakazakii* in numerous studies (Lee et al., 2012; Turcovsky et al., 2011; Vojkovska et al., 2016; Hochel et al., 2012). Our findings also revealed the predominance of this species. Of all 27 isolates, 13 (48.1%), 6 (22.2%), 5 (18.5%) and 3 (11.1%) were classified as *C. sakazakii*, *C. muytjensii*, *C. turicensis*

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

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

[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* species, respectively. [b] The PCR primer Cmstu-825F was used in multiplex reactions with Ctur-1036R, Cuni-1133R, Csak-1317R and Cmal-1410R primers for identifying *C. turicensis*, *C. universalis, C. sakazakii* and *C. malonaticus* species, respectively.

Table 2. Presence of *Cronobacter* species in different types of food products and the species identified.

Type of food product	Number of the samples (n)	*C. sakazakii*	*C. muytjensii*	*C. turicensis*	*C. malonaticus*	Positive samples	Percentage of positive samples (%)
Flour	51	5	-[a]	3	3	11	21.56
Instant dry soup	50	5	3	1	-	9	18.0
Spices / herbs	50	3	3	1	-	7	14.0
Total count	**151**	**13**	**6**	**5**	**3**	**27**	**17.88**

[a]: not detected.

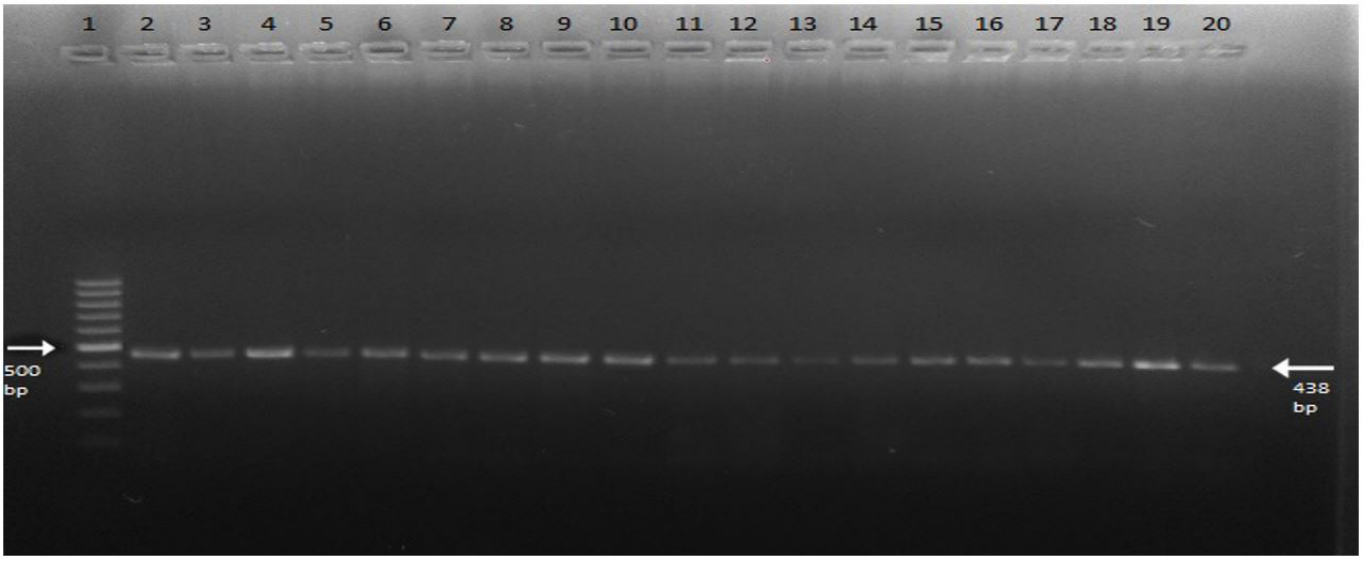

Figure 1. Gel image of 438-bp-long DNA fragments of targeted *gyrB gene* in *Cronobacter* spp. positive isolates. 1st loading well: 100 bp DNA ladder, 2nd loading well: positive control (438 bp *C. sakazakii,* ATCC 29544), 3rd-20th loading wells: *Cronobacter* spp. positive isolates obtained from food samples of different types.

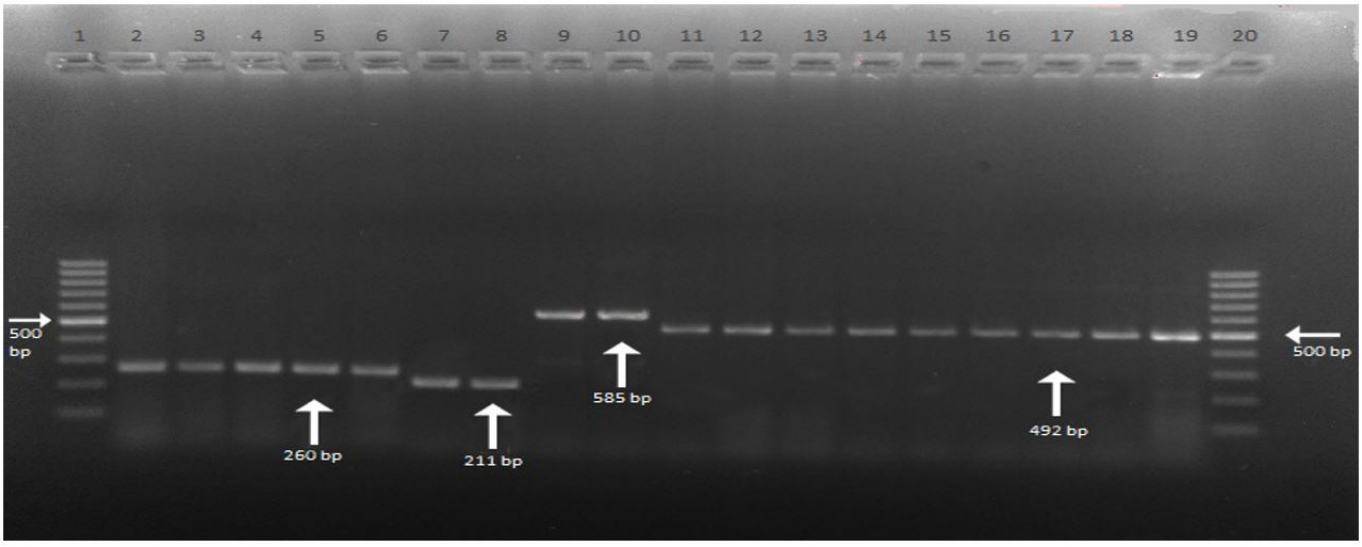

Figure 2. Gel image of some isolates identified to the species level according to their DNA fragments of different lengths by Multiplex PCR assay. 1st and 20th loading wells: 100 bp DNA ladder; 2nd loading well: Positive control (260 bp *C. muytjensii,* ATCC 51329); 3rd to 6th loading wells: 260 bp *C. muytjensii*; 7th and 8th loading wells: 211 bp *C. turicensis*; 9th and 10th loading wells: 585 bp *C. malonaticus*; 11th and 18th loading wells: 492 bp *C. sakazakii*; 19th loading well: Positive control (492 bp *C. sakazakii,* ATCC 29544).

and *C. malonaticus*, respectively. The proportional differences of the isolates at the species level when compared with those of the previous studies were associated with the number and type of selected product samples. The majority of dried food products included in the selected samples might have asserted the predominance of *Cronobacter sakazakii* among the isolates due to the potential growth of this particular species in dried food products. *Cronobacter* species are often isolated from foods of plant origin (Lee et al., 2010, 2012). Cereals, cereal based flours and bakery products made from these types of foods constitute a group of food products in which *Cronobacter* species are frequently encountered. In this study, the largest quantity of *Cronobacter* spp. was detected in flour and bakery products with a ratio of 21.56%. Predominance of the genus in food products of this type was previously indicated by Lee et al. (2012), Li et al. (2014) and Yao et al. (2016) at ratios of 16.0%, 14.1% and 12.0%, respectively. The prevalence ratios of *Cronobacter* spp. in our study were consistent with those reported in these studies (Lee et al., 2012; Li et al., 2014; Yao et al., 2016) except for the study by Lou et al. (2014) from which our results differed by a lower ratio. In particular, microorganisms that contaminate cereals and so forth may come from soil during harvesting and

environmental conditions contribute to the contamination of this type of foods which may partially carry their microbiological load into processed final food products. It was considered that the variety of raw materials and the diversity of processing techniques might have affected the prevalence ratios of the bacteria.

Spices and dried herbs may frequently be contaminated with microbiologic agents during harvesting and further procedures (Salari et al., 2012). In our study, prevalence of *Cronobacter* spp. in the spices and culinary herbs/herbal flavourings was detected to be 14%. In a similar study, Turcovsky et al. (2011) reported that the occurrence of *Cronobacter* spp. was more prevalent in the products of this type (13 of 21 samples were positive for *Cronobacter* spp). The differences between the findings might be associated with the diversity of the type of tested spices and also with the fact that herbal flavors were included in the spices group in our study. Prevalence ratio of *Cronobacter* species in our study was compatible with that (16.7%) in a study by Garbowska et al. (2015). On the other hand, it was detected to be lower than that of Li et al. (2014) while higher than the prevalence rations reported by Iversen & Forsythe, (2004); Baumgartner et al. (2009); Jaradat et al. (2009). Overall, microbial agents were found to be more prevalent in the foods of plant origin in the studies conducted. Hence, Turcovsky et al. (2011) reported that prevalence ratio of *Cronobacter* spp. in plant-originated foods (31.29%) was higher than that of foods of animal origin (6.15%). That the spices are dried products that are prone to dust-soil contamination and the fact that *Cronobacter* spp. are known to survive for prolonged periods in dried food products must be taken into account for the risk of contamination. The diversity of prevalence rate among different studies including ours might be associated with the diversity of isolation methods and packaging procedures. Our samples consisted of food products in their original packaging which might have affected the results.

Similarly, instant soups may also contain various contaminants including *Cronobacter* spp. Hence, Killer et al. (2015) reported the occurrence of *Cronobacter* spp. in instant soups with garlic powder out of all other retail foods. In a study by Turcovsky et al. (2011) prevalence of *Cronobacter* spp. (*C. malonaticus* and *C. dublinensis*) was detected to be 15.3% in tested instant soup samples. Nine (18.0%) of 50 instant soup samples were positive for *Cronobacter* spp. in our study and the findings regarding the prevalence ratios were found to be compatible. Turcovsky et al. (2011) did not isolate *C. sakazakii* species in their instant food samples unlike our samples from which *C. sakazakii* (n:5), *C. muytjensii* (n:3) and *C. turicensis* (n:1) species were isolated. The difference might be associated with the type of the soup tested as well as it might have resulted from the raw material ingredients of the samples. In our study, all *Cronobacter* spp. isolates were obtained from the soup samples of plant origin. Since spices and dried vegetables that are used as the raw materials of these instant food products carry high risks of contamination it was an anticipated outcome for this study. Turcovsky et al. (2011) indicated that positive samples were obtained from the foods composed of spices or marinated food products out of all tested food samples of different type. Kim et al. (2011) reported that contamination risk was higher (70%) in root vegetables like carrot and sweet potato and thus indicated that the root vegetable ingredient of several food products might be an important source of contamination.

As a result of resistance of *Cronobacter* species to drying process, they have high survival capacity in foods with low moisture (0.3-0.8) content (Lin & Beuchat, 2007; Beuchat et al., 2009; Hochel et al., 2012). *C. sakazakii* was reported to have survived more than 12 months in low moisture cereal based products at different temperatures under different storage conditions (Beuchat et al., 2009). Microbial quality of equipment surfaces in contact with foods is of great importance for quality food production. Occurrence of cross-contamination is possible with unclean and non-disenfected equipments and surfaces (Shaker et al., 2007). Kuo et al. (2013) reported that *Cronobacter* species survived for certain time intervals on stainless steel, teflon and glass surfaces and hence, proper sanitation and disinfection are highly crucial for food hygiene.

Although *Cronobacter* spp. do not survive after pasteurization process, the products are highly likely to be contaminated with the bacteria due to inaccurate heating and the subsequent processes (Baumgartner et al., 2009; Lee et al., 2010; Hochel et al., 2012). Cold storage (4 °C) is a requisite and sufficient for food formulas produced from powdered products so as to prevent the growth of *Cronobacter* species (Osaili et al., 2009).

Since *Cronobacter* spp. are included in the family *Enterobacteriaceae*, the existence potential of these species in the environment is as high as that of other types of gut-associated bacteria. Accordingly *Cronobacter* spp. were detected in dust, soil and other environmental samples at various quantities (Turcovsky et al., 2011; Vojkovska et al., 2016; Jaradat et al., 2009; Lee et al., 2012; Killer et al., 2015; Wang et al., 2012). These findings revealed the crucial role of environmental contaminants upon the microbiologic safety of end products.

5 Conclusion

This study revealed the considerable amount of *Cronobacter* spp. in a variety of spices and culinary herbs/herbal flavourings, flours and dry instant soups which were produced and offered for sale by different manufacturers in Turkey. That almost fifty percent of isolated strains comprised *C. sakazakii*, known as an opportunistic pathogen, was considered to be a public health issue worthy of concern. Infections caused by these species affect not only the health status of infants and children but also those of susceptible individuals including immunocompromised adults. Therefore, further studies should be designed regarding the isolation and identification of *Cronobacter* species notably in food products of high potential risk as well as in a variety of food products, raw materials, and environmental samples. In addition, possible routes of contamination are to be determined and prevented and appropriate food processing techniques should be applied. In food industry, it is feasible to restrain prevalence of *Cronobacter* spp, which are likely to cause serious health concerns particularly in individuals of a specific age range, by following the principles of HACCP (Hazard Analysis Critical Control Point) system with respect to providing the high sanitary standards for raw materials, production processes and storage of food products as wells as for hygiene of staff and equipment.

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