BOLU ABANT İZZET BAYSAL UNIVERSITY
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

PREVALENCE, MOLECULAR CHARACTERIZATION, VIRULENCE FACTORS AND ANTIMICROBIAL RESISTANCE OF *CRONOBACTER* SPP. IN READY-TO-EAT FOODS

MASTER OF SCIENCE

HAFİZE GİZEM ERTÜRK

BOLU, JUNE 2018

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APPROVAL OF THE THESIS

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To my dear family

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

HAFİZE GİZEM ERTÜRK

ABSTRACT

PREVALENCE, MOLECULAR CHARACTERIZATION, VIRULENCE FACTORS AND ANTIMICROBIAL RESISTANCE OF *CRONOBACTER* SPP. IN READY-TO-EAT FOODS
MSC THESIS
HAFİZE GİZEM ERTÜRK
BOLU ABANT İZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY
(SUPERVISOR: PROF. DR. SEZA ARSLAN)

BOLU, JUNE 2018

The *Cronobacter* spp. are a member of the family *Enterobacteriaceae*, Gram-negative, non-spore forming and facultative anaerobic bacteria. *Cronobacter* species are opportunistic pathogens that can cause severe diseases such as meningitis, sepsis, and necrotizing enterocolitis in immunocompromised individuals, neonates, infants and elderly adults. In the present study, a total of 340 ready-to-eat foods were tested for the presence of *Cronobacter* spp. The 59 (17.4%) of the 340 tested samples were positive for *Cronobacter* spp. Out of the contaminated samples with *Cronobacter*, the 64 *Cronobacter* isolates were obtained. The phenotypically identified *Cronobacter* isolates were also confirmed genotypically by the 16S rRNA, *gluA* gene and species-specific *rpoB* gene amplification. Overall, out of all samples, 64 isolates were identified as 39 (61%) *Cronobacter sakazakii*, 15 (23.4%) as *Cronobacter malonaticus*, 3 (4.7%) as *Cronobacter muytjensii*, 3 (4.7%) as *Cronobacter dublinensis*, 2 (3.1%) as *Cronobacter turicensis* and 2 (3.1%) *Cronobacter universalis*. All *Cronobacter* isolates were examined for the presence of genes encoding some virulence factors including outer membrane protein A (*ompA*) and zinc-containing metalloprotease (*zpx*). Of the 64 *Cronobacter* isolates, 63 (98.5%) were positive for both the *ompA* and *zpx* gene. In all *Cronobacter* isolates, other virulence determinants such as biofilm formation and siderophore production, which have a role in adhesion, invasion, and survival of bacteria, were detected. Out of the 64 *Cronobacter* isolates, 64 (100%) and 56 (87.5%) were found to be positive for siderophore production and biofilm formation, respectively. In this study, the results of ERIC-PCR showed the amplification bands, ranging from 3 to 12, among the 64 *Cronobacter* isolates and *C. sakazakii* ATCC 29544 as a reference strain. ERIC-PCR fingerprints revealed that all the *Cronobacter* divided into 12 genotypes. The results demonstrated that there was no a direct relationship between ERIC-PCR fingerprints and sources of the isolation. In this study, the antimicrobial susceptibility of the 64 *Cronobacter* spp. from ready-to-eat foods to commonly used 18 antimicrobial agents was examined. Most *Cronobacter* spp. with a rate of 81.3% were resistant to cephalothin, 32.8% to cefoxitin and 20.3% to ampicillin. All the *Cronobacter* isolates were susceptible to gentamicin and trimethoprim/sulfamethoxazole. The present study is remarkable that it provides a current data on the isolation and identification of *Cronobacter* spp.,

some virulence characteristics, and genetic variation by the ERIC-PCR and antimicrobial resistance profiles of *Cronobacter* spp. The monitoring the presence of *Cronobacter* spp. as an emerging pathogen in food is essential for the health of consumers.

KEYWORDS: *Cronobacter* spp., Ready-to-eat Food, Isolation, Identification, Molecular Characterization, Virulence Factors, Antimicrobial Resistance

ÖZET

TÜKETİME HAZIR GIDALARDA CRONOBACTER SPP.'NİN YAYGINLIĞI, MOLEKÜLER KARAKTERİZASYONU, VİRÜLANS FAKTÖRLERİ VE ANTİMİKROBİYAL DİRENÇLİLİĞİ
YÜKSEK LİSANS TEZİ
HAFİZE GİZEM ERTÜRK
BOLU ABANT İZZET BAYSAL ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ
BİYOLOJİ ANABİLİM DALI
(TEZ DANIŞMANI: PROF. DR. SEZA ARSLAN)

BOLU, HAZİRAN - 2018

Cronobacter spp. *Enterobacteriaceae* ailesinin, Gram-negatif, spor oluşturmayan ve fakültatif anaerobik bir üyesidir. Birçok *Cronobacter* türü, bağışıklık sistemi zayıflamış bireylerde, yeni doğanlarda, bebeklerde ve yaşlılarda menenjit, sepsis ve nekrotizan enterokolit gibi ciddi hastalıklara neden olan fırsatçı patojenlerdir. Bu çalışmada, toplam 340 tüketime hazır gıda örneği *Cronobacter* spp.'nin varlığı için test edilmiştir. Test edilen 340 örneğin 59 (17,4%)'u *Cronobacter* spp. için pozitif bulunmuştur. *Cronobacter* ile kontamine örneklerden toplam 64 izolat elde edilmiştir. Fenotipik olarak adlandırılan *Cronobacter* izolatları, 16S rRNA, *gluA* ve türe özgü *rpoB* gen amplifikasyonu ile genotipik olarak da doğrulanmıştır. Genel olarak, 64 izolatın 39'u (% 61) *Cronobacter sakazakii*, 15'i (% 23,4) *Cronobacter malonaticus*, 3'ü (% 4,7) *Cronobacter muytjensii*, 3'ü (% 4,7*) Cronobacter dublinensis*, 2'si (% 3,1) *Cronobacter turicensis* ve 2'si (% 3,1) *Cronobacter universalis* olarak adlandırılmıştır. Tüm *Cronobacter* izolatları, dış zar protein A (*ompA*) ve çinko içeren metalloproteaz (*zpx*) dahil bazı virülans faktörleri kodlayan genlerin varlığı için incelenmiştir. 64 *Cronobacter* izolatının, 63'ü (% 98,5) *ompA* ve *zpx* genleri için pozitif olarak tespit edilmiştir. Tüm *Cronobacter* izolatlarında, bakterilerin adezyon, invazyon ve hayatta kalmasında rol oynayan biyofilm ve siderofor üretimi gibi diğer virülans faktörleri incelenmiştir. 64 *Cronobacter* izolatından tümü (% 100) siderofor üretimi için ve 56'sı (% 87,5) biyofilm oluşumu için pozitif bulunmuştur. Bu çalışmada, 64 *Cronobacter* izolatı ve *C. sakazakii* ATCC 29544 referans suşunu içeren ERIC-PCR sonuçları, 3 ile 12 arasında değişen amplifikasyon bantları göstermiştir. Tüm *Cronobacter* izolatları ERIC-PCR paternlerine göre 12 genotipe ayrılmıştır. Sonuçlar, ERIC-PCR paternleri ve izolasyon kaynakları arasında doğrudan bir ilişki olmadığını göstermiştir. Bu çalışmada, tüketime hazır gıdalardan izole edilen 64 *Cronobacter* spp.'nin yaygın olarak kullanılan 18 antimikrobiyal ajana karşı antimikrobiyal duyarlılığı incelenmiştir. Çoğu *Cronobacter* spp. % 81,3 oranında sefalotin'e karşı direçli bulunurken, sefoksitine % 32,8 ve ampisiline % 20,3 direnç tespit edilmiştir. Tüm *Cronobacter* izolatları gentamisin ve trimetoprim/sülfametoksazola duyarlıdır. Bu çalışma, *Cronobacter* türlerinin izolasyonu ve tanımlanması, onların bazı virülans özellikleri, ERIC-PCR ile genetik varyasyonları ve antimikrobiyal direnç profilleri üzerine güncel veri sağlaması itibariyle dikkate değerdir. Gıdada yeni ortaya çıkan bir patojen olarak

Cronobacter türlerinin varlığının takip edilmesi tüketici sağlığı için önemlidir.

ANAHTAR KELİMELER: *Cronobacter* spp., Tüketime Hazır Gıda, İzolasyon, Tanımlama, Moleküler Özellik, Virülans Faktör, Antimikrobiyal Dirençlilik

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LIST OF ABBREVIATIONS AND SYMBOLS

CAS **:** Chrome azurol S

CHO **:** Chinese hamster ovary

ERIC **:** Enterobacterial repetitive intergenic consensus

FAO **:** Food and Agriculture Organization

FDA **:** Food and Drug Administration

ISO **:** International Standard Organization

NEC **:** Necrotizing enterocolitis

PCR **:** Polymerase chain reaction

TBE **:** Tris Borate EDTA

WHO **:** World Health Organization

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1. INTRODUCTION

Microorganisms are ubiquitous in nature. Food and food products are good nutrient sources that support their growth, especially for pathogens. They have the potential to cause food spoilage, food poisoning and foodborne diseases; thus, presence of many foodborne pathogens such as *Cronobacter* spp. may threaten both public health and food quality (FAO-WHO, 2006; Yang et al., 2017).

The genus *Cronobacter* is composed of Gram-negative, rod-shaped and non-spore forming bacteria within the *Enterobacteriaceae* family (Iversen et al., 2007a, 2008). The genus *Cronobacter* contains seven species: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter muytjensii*, *Cronobacter dublinensis*, *Cronobacter universalis* and *Cronobacter condimenti* (Iversen et al., 2008; Joseph et al., 2012a). *Cronobacter* species are recognized as emerging opportunistic pathogens causing life-threatening infections in immunocompromised individuals, neonates, infants and elderly adults (Farmer et al., 1980; Van Acker et al., 2001; Iversen and Forsythe, 2003; FAO-WHO, 2006).

Cronobacter is common in both environment and foods, mostly in water, in soil, and in vegetables. In some studies, *Cronobacter* spp. have been obtained from variety of sources including food manufacturing facilities and food products including powdered milk, vegetables, salads, herbs, cereal, chocolate, milk, meat, potato flour, pasta and spices (Kandhai et al., 2004; Pagotto et al., 2007; Friedemann, 2007; Baumgartner et al., 2009; Joseph et al., 2012a; Lee et al., 2012; Xu et al.,2015). Some species of genus *Cronobacter* are pathogenic for humans and animals. *Cronobacter* spp. have also been isolated from some animals such as rats and flies (Gakuya et al., 2001; Kuzina et al., 2001; Holy and Forsythe, 2014).

The *Cronobacter* genus has been associated mainly with necrotizing enterocolitis, bacteremia, and meningitis in neonates, infants and immunocompromised people. *Cronobacter*-induced neonatal meningitis can cause brain abscess or inflammation and the growth of hydrocephalus. The other clinical

manifestation is the development of neonatal necrotizing enterocolitis qualified by intestinal necrosis and pneumatosis intestinalis. The symptoms of *Cronobacter* infections are high fever, headache with nausea, a swelling on the head, body and neck stiffness, skin rash, seizures. It is known that problems can occur during or after operations due to the *Cronobacter*. *Cronobacter* can also infect the urinary tract. The cases of *Cronobacter* are mostly associated not only with older people but also with immunocompromised people (Nazarowec-White and Farber, 1997; Gurtler et al., 2005). Whereas *Cronobacter* spp. based human infections are not common, the mortality rate of these pathogens are considerable (40-80%) in infected human. Even if these people survive, they often suffer from severe neurological complications (Nazarowec-White and Farber, 1997; Forsythe et al., 2014; Li et al., 2014).

Cronobacter genus has been identified and characterized genotypically using amplification of 16S rRNA gene, the enterobacterial repetitive intergenic consensus (ERIC) sequences, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), ribotyping and plasmid typing (Nazarowec-White and Farber, 1999; Pagotto et al., 2007). Several target genes including the *gluA*, the *rpoB*, the *dnaG*, the *gyrB*, the 16S-23S intergenic transcribed spacer gene have been also used to detect *Cronobacter* spp. (Seo and Brackett, 2005; Iversen et al., 2007b; Liu et al., 2006; Stoop et al., 2009; Huang et al., 2013).

The capability of an agent to cause disease is pathogenicity. The pathogenic bacteria include several factors enabling them to improve their virulence. The virulence factors which have a significant role for the pathogenicity of *Cronobacter* are adhesion, invasion, toxins, proteolytic enzymes, biofilm formation and siderophore production (Iversen et al., 2004; Jaradat et al., 2014; Singh et al., 2015, Ye et al., 2016). These virulence properties of *Cronobacter* spp. help invading the host, avoiding host defenses and causing disease. The biofilm formation, caused by some pathogens, has a role as physical protective barrier and is described as unity of bacterial cells. The biofilms are surrounded by a self-producing matrix and adherent to an inert or living surface (Iversen et al., 2004; Lehner et al., 2005). *Cronobacter* has been reported to form biofilms on latex, stainless steel, glass, silicon, polyvinyl chloride and polycarbonate (Iversen et al., 2004). Moreover, siderophores, low molecular weight compounds having a high affinity for iron ions, play a role in the

virulence of *Cronobacter* spp. (Grim et al., 2012). In addition, the outer-membrane protein A encoding the *ompA* gene and zinc-containing metalloprotease encoding the *zpx* gene are the best-known virulence markers of *Cronobacter* for its pathogenicity (Nair and Venkitanarayanan, 2006, 2007; Kothary et al., 2007). For pathogenic strains, the *ompA* gene serves as virulence factors for nutrient depuration and avoidance of host defense mechanisms (Nair and Venkitanarayanan, 2006). On the other hand, the *zpx* gene, one of the virulence genes is effective factor in the pathogenesis of *Cronobacter* spp. (Kothary et al., 2007).

All around the world, resistance of antimicrobial agents has hazardously increased of high levels, which is a big threat for our ability to treat common infections such as foodborne diseases. The overuse and improper usage of antimicrobials has caused the emergence of single- and multiple- drug resistant bacterial strains (Pagotto et al., 2007). *Cronobacter* spp. are susceptible to commonly used antimicrobials such as aminogylcosides, ureidopenicillins, ampicillin, and carboxypenicillins (Adamson and Rogers, 1981; Nazarowec-White and Farber, 1997; Gurtler et al., 2005). However, it has been documented that *Cronobacter* spp. are resistant against cephalothin and cefotaxime (Farmer et al., 1980; Xu et al., 2015; Yao et al., 2016; Mardaneh and Dallal, 2017). The emergence of resistant *Cronobacter* spp. and transmission of these resistant species from food to human may be a significant problem for treatment of *Cronobacter* infections.

1.1 *Cronobacter* genus

1.1.1 Classification and Taxonomy

Prior to 1980, the bacterial genus, now named as the *Cronobacter* genus, was known as the "yellow-pigmented *Enterobacter cloacae"*. The DNA-DNA hybridization has showed that "yellow-pigmented *Enterobacter cloacae*" was only 41% and 54% related to *Citrobacter freundii* and *E. cloacae*, respectively. Therefore, this organism was more similar to *E. cloacae* than *C. freundii*. These bacteria were incorporated into the *Enterobacter* genus and were named after *Enterobacter sakazakii* (Farmer et al., 1980). Before 2006 in which a 16th biogroup was added, the *Enterobacter sakazakii* had the 15 phenotypically distinct biogroups (Farmer et al.,

1980; Iversen, 2006). Phylogenetic analyses using the 16S rRNA and the *hsp60* sequences have represented that *Cronobacter* genus had a requirement of reclassification (Iversen et al., 2004).

The *Cronobacter* genus was firstly identified in 2007 and covered four species including *C. sakazakii*, *C. muytjensii*, *C. turicensis*, and *C. dublinensis*, two subspecies of *C. sakazakii*, and one genomospecies (Iversen et al., 2007a). *Cronobacter* genus was revised in following years that the two *C. sakazakii* subspecies were accepted as *C. sakazakii* and *C. malonaticus*. Besides, the three subspecies of *C. dublinensis* were defined. The taxonomy of *Cronobacter* spp. was again regulated and the novel species *C. condimenti* was added and description of *Cronobacter* genomospecies I as *C. universalis* (Joseph et al., 2012a). In addition, although three species were added in the *Cronobacter* genus in 2013, these species were removed from this genus dependent on the consequences of molecular methods and biochemical tests in 2014 (Brady et al., 2013, Stephan et al., 2014). Therefore, the genus of *Cronobacter* with seven different species includes *C. sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. dublinensis*, *C. turicensis*, *C. condimenti* and *C. universalis* (Iversen et al., 2007a; Joseph et al., 2012a). The taxonomy of *Cronobacter* genus is given in Figure 1.1.

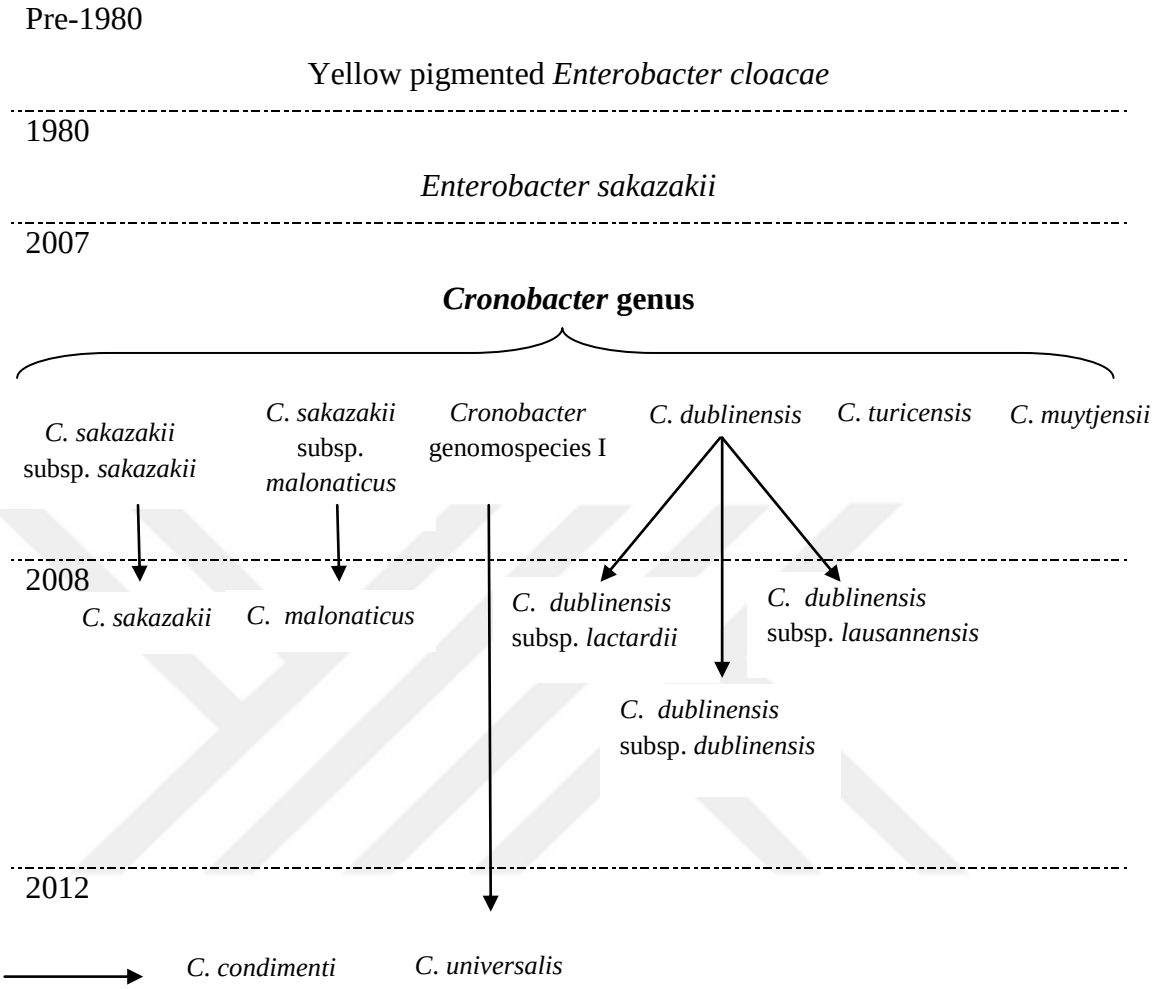

Figure 1.1. A current taxonomy of *Cronobacter* genus (Iversen et al., 2008; Joseph et al., 2012a).

1.1.2 Characteristics of *Cronobacter* spp.

Cronobacter species belong to the *Enterobacteriaceae* family which is found in the *Gamma-proteobacteria* subdivision. The *Cronobacter* spp. are Gram negative, generally motile by means of peritrichous flagella, non-sporing rods, nonhalophilic and facultative anaerobe bacteria (Farmer et al., 1980; Iversen, 2006).

The *Cronobacter* organisms have dimensions of 0.3 to 1.0 by 1.0 to 6.0 μm. This organism is capable of growing between 6 °C and 45 °C. The range of pH ranges from 5.0 to 10.0, and there is no growth below pH 4.5. Whereas they can grow up to 7% (w/v) NaCl, this cannot occur in 10% (Iversen et al., 2008). After overnight incubation, colonies of *Cronobacter* on standard laboratory growth media including Tryptic soy agar (TSA) measure 2 to 3 mm and 1 to 1.5 mm in diameter at 36 and 25 °C, respectively (Pagotto et al., 2007). This organism has two distinct colony types as A and B. Colonies of type A are defined as dry or mucoid, and rubbery. Type B appears to be a typical smooth colony (Yan et al., 2013).

Hydrogen sulphide (H_2S) production, urea hydrolysis, fermentation of sorbitol, lysine decarboxylation and β-D-glucuronidase activity are negative for *Cronobacter* spp. Generally, they have a positive acetoin test and a negative methyl red test. *Cronobacter* spp. can also utilize citrate, reduce nitrate, hydrolyze aesculin, and arginine. This genus can use various carbonhydrates as a sole carbon source: D-galactose, D-glucose, sucrose, raffinose, melibiose, cellobiose, D-mannitol, glycerol, D-mannose, L-rhamnose, L-arabinose, D-xylose, trehalose, galacturonate, and maltose. *Cronobacter* spp. can use variety of amino acids as an energy sources: L-ornithine, D-alanine, L-alanine, L-glutamate, L- proline, L-serin.

For the differentiation between seven species of *Cronobacter*, indole, dulcitol, *cis*-aconitate, *trans*-aconitate, inositol, lactulose, malonate, maltitol, melezitose, palatinose, putrescine, turanose, 4-aminobutyrate, and 1-0-methyl α-$_D$-glucopyranoside can be used (Iversen et al., 2008; Joseph et al., 2012a).

1.1.3 Reservoir of *Cronobacter* spp.

Cronobacter is extensively found in the environment. *Cronobacter* spp. have been obtained from mainly plant and organic material (Osaili et al., 2009; Holy and Forsythe, 2014). *Cronobacter* species have been isolated from dry foods including powdered infant formula, spices and herbs (Yan et al., 2013). Kuklinsky-Sobral et al. (2004) reported presence of *Cronobacter* from ready-to-eat salads. The twenty of *Cronobacter* strains from 141 samples of infant formula and other foods, including cheeses, fermented breads, meats, and minced beef were isolated by Muytjens et al. (1988). It has also been documented that this genus was found in rice seeds, lettuce, alfalfa sprouts, and tomatoes. It has been concluded from the over 500 food products that most herbs and spices harbored *Cronobacter* (Iversen and Forsythe, 2004). The contaminated foods with *Cronobacter* could be raw and processed food. Moreover, foods including the beverages, water, fresh, ready-to-eat and fermented food products were contaminated with *Cronobacter* (Friedemann, 2007).

Furthermore, the genus has been found in not only humid but also desiccated conditions. During the production process, the addition of ingredients contamination and environmental pollution can make powdered infant formula (PIF) contaminate with *Cronobacter* (Nazarowec-White and Farber, 1997). Also, due to the ability of the strong resistance of the bacteria, the risk of contamination after pasteurization increases in the environment of PIF factory (Riedel and Lehner, 2007). In addition, resistance to the lack of water, enterotoxin and being capable of producing capsule are among the factors that make *Cronobacter* spp. in powdered infant formula live during the shelf life as much as 24 months (Iversen and Forsythe, 2003; Lehner et al., 2004).

It has been concluded from the two studies that the isolation of *Cronobacter* can also be obtained both from rats and Mexican fruit flies (Gakuya et al., 2001; Kuzina et al., 2001). Hamilton et al. (2003) isolated from the intestine of the flies, named *Stomoxys calcitrans.* Furthermore, the nasopharynx and gastrointestinal tract of humans, the home and clinical environments are among the areas that this genus can be found (Holy and Forsythe, 2014).

1.2 Isolation and Identification of *Cronobacter* spp.

Cronobacter has been found naturally in the environment and obtained from many different types of foods. A series of steps is necessary for isolation of bacteria in food, such as *Cronobacter* spp., to resuscitate stressed cells, to culture and record them. Developed phenotyping databases and DNA-sequence-based methods can make defined identification possible.

1.2.1 Conventional Protocols

A great many methods for identification and description of *Cronobacter* spp. have been suggested since it was defined by Farmer et al. (1980). Muytjens et al. (1998) described the initial detection and isolation method developed for *Cronobacter* species in 1988. The Food and Drug Administration (FDA, 2002) and the International Organization for Standardization (Anonymous, ISO, 2006 ISO/TS 22964) improved protocols for the isolation and detection of *Cronobacter* spp.

Cronobacter can cultivate easily on several culture media, including non-selective media, selective and chromogenic media including Tryptic Soy Agar, Violet Red Bile Glucose Agar, Laury Sulfate broth, Druggan-Forsythe-Iversen agar, *Enterobacter sakazakii* chromogenic plating medium (Iversen and Druggan, 2012). The defined seven species along with the three sub-species can identify using some biochemical tests including the fermentation of dulcitol, lactulose, maltitol, palatinose, putresine, melezitose, turanose, *myo*-inositol, *trans*-aconitate, *cis*-aconitate, 1-O-methyl α-D-glucopyranoside and 4- aminobutyrate (Farmer et al., 1980; Iversen et al., 2006, 2008).

1.2.2 Molecular Based Protocols

It is the molecular-based study that can support and widen the vision of epidemiology of any given bacterium. It was in 1980's that a new standard for identification of bacteria started to be developed. It has been revealed that the phylogenetic relations between the bacteria could be defined with comparison of stabile parts of the genetic code (Woese et al., 1985; Woese, 1987).

Molecular based methods for example, polymerase chain reaction (PCR) could provide to fast, particular, and delicate identification of foodborne pathogens (Mashoufi et al., 2017). Several conventional PCR assay formats have been developed to detect *Cronobacter* genus. These take advantage of unique sequences. Identified targets and genotypic methods include 16S rRNA gene, the enterobacterial repetitive intergenic consensus (ERIC) sequences, the pulsed-field gel electrophoresis (PFGE), the multilocus sequence typing (MLST), ribotyping and plasmid typing. Target genes including the *gluA*, the *dnaG*, the 16S-23S internal-transcribed spacer (ITS) and the *gyrB* have also been utilized to detect *Cronobacter* spp. (Seo and Brackett, 2005; Iversen et al., 2007b; Liu et al., 2006; Huang et al., 2013). Moreover, the *rpoB* gene-based PCR method was improved to differentiate between the seven *Cronobacter* species (Stoop et al., 2009; Lehner et al., 2012). It has been known that the bacterial RNA polymerase β-subunit is encoded by this universal gene and its suitability in species definition has been evaluated (Mollet et al., 1997).

It has been approved that for definition and classification of bacterial species, the 16S rRNA gene sequencing is the "gold standard". The genetic method has been established depending on comparison of the bacterial 16S rRNA sequences (Böttger, 1989; Tortoli, 2003). The method can also use to the definition of emerging pathogens such as *Cronobacter*. The α-glucosidase activity has also a significant role in the definition of *Cronobacter*. A great number of studies have shown the presence of α-glucosidase genes (Jaradat et al., 2009; Mofokeng et al., 2010; Iversen and Druggan, 2012; Jackson et al., 2014; Mohammed et al., 2015).

1.3 Virulence Factors of *Cronobacter* spp.

The members of *Cronobacter* belong the family of *Enterobacteriaceae* and are oppurtunistic pathogens. The infections caused by *Cronobacter* are rare and sporadic. These pathogens also cause severe infections in neonates, infants and adults. *Cronobacter* species have various virulence traits which are responsible for their pathogenicity (Ye et al., 2016). The virulence factors of *Cronobacter* may aid adhesion, invasion and host cell injury. Enzymes, toxins, adhesion proteins, cell-surface proteins, biofilm formation, antimicrobial resistance contribute to the bacteria

for evasion of the immune defense and tissue invasion in the host (Jaradat et al., 2014). The presence of heat stable enterotoxin that could be resistant to pasteurization practices. The proteolytic enzymes produced by *Cronobacter* lyse cell and cause tissue damage and the outer membrane proteins have a critical role in bacterial invasion. The specific numerous virulence genes and plasmids of *Cronobacter* spp. have been identified. These are included zinc-containing metalloprotease (*zpx*) gene, haemolysin (*hly*), siderophore-interacting protein (*sip*) and plasminogen activator (*cpa*). The complete cation efflux system (*cusA, cusB, cusC* and *cusF*) and its regulatory gene *cusR* were present in *Cronobacter* strains associated with neonatal infections (Kucerova et al., 2010). RepFIB plasmids in *Cronobacter* code two iron acquisition systems. One of them is siderophore-mediated iron acquisition system (*iucABCD*/*iutA* operon) and known as Cronobactin. The other one is ATP-binding cassette transport-mediated iron uptake and siderophore system (*eitCBAD* operon) (Franco et al., 2011).

1.3.1 Outer Membrane Protein A

The presence of double membrane in Gram-negative bacteria helps for protection and for supplying the nutrient requirements for viability. The outer membrane of double membrane includes membrane proteins provided curial functions of the cell such as nutrition uptake, adhesion, signalling and waste removal. These outer membrane proteins as virulence factors also help for nutrient depuration and avoidance of host defense mechanisms (Rollauer et al., 2015). Many studies indicated that outer membrane protein A contributes to virulence of *Cronobacter*. The outer membrane protein A encoded by the *ompA* gene is the best described virulence marker and has a significant role in *Cronobacter* invasion. Two primers containing specific sequences of the *ompA* gene of *Cronobacter* were synthesized to improve and optimize a *Cronobacter*-specific PCR (Nair and Venkitanarayanan, 2006, 2007).

The outer membrane protein A of *Cronobacter* spp. plays a role in the colonization of the gastrointestinal tract (Kim et al., 2010; Franco et al., 2011) Furthermore, it has been concluded from the studies on invasion that with human intestinal cells, not only microfilaments but also microtubules contribute to the outer

membrane protein A. It has also been reported in the study of Mittal et al. (2009) that outer membrane protein A - positive isolates break blood-brain barrier and invade central nervous system causing clinical symptoms.

1.3.2 Zinc-containing Metalloprotease

In pathogenic organisms, numerous extracellular proteases have been demonstrated to play important roles in pathogenesis of bacteria. It hypothesized that bacterial proteases, as proteolytic enzymes, lead to extensive cellular damage in neonates involved necrotizing enterocolitis and may allow the organism to cross the blood-brain barrier (Nair and Venkitanarayanan, 2006). One of microbial proteases is the metalloprotease and most metalloprotease are zinc-containing proteins. Zinc has a importance in cellular metabolism and is a component of many proteins which are involved in virtually all aspects of metabolism of the species (Hase et al., 1993). Kothary et al. (2007) demonstrated that *Cronobacter* secretes a zinc-containing metalloprotease.

The *zpx* gene, a virulence gene, was used as a genus-specific gene for determination of *Cronobacter*. This gene may help as an indicator of pathogenicity. In the study of Kothary et al. (2007) the gene locus of zinc-containing metalloprotease (*zpx*) was determined that this protease encoded the *zpx* gene caused rounding of Chinese Hamster Ovary (CHO) cells in tissue culture in the clinical and environmental *Cronobacter* strains. Several reports have documented the presence of the *zpx* gene, a virulence gene, for *Cronobacter* (Fakruddin et al., 2014; Jackson et al., 2014; Eshwar et al., 2016).

1.3.3 Siderophore Production

Iron is necessary for most bacteria in metabolic and cellular pathways. The iron is a cofactor for various enzymes concerned in cellular respiration, electron transfer, and superoxide metabolism, which means that it has a significant role for pathogenesis of bacteria (Grim et al., 2012). As the low bioavailability of iron in the environment can be bacteriostatic or even lethal for microorganisms, bacteria develop specific uptake strategies such as production of siderophores. Siderophores

are low molecular mass compounds produced by bacteria. This iron acquisition system, siderophores, is also important in bacterial pathogenesis of *Cronobacter* spp.

Siderophores in Gram negative bacteria are transported by an outer membrane receptor, a periplasmic binding protein, and a complex of ATP-binding cassette. When it is transported to the cytoplasm, involves the reduction of the Fe^{3}-siderophore complex to Fe^{2}, which means it is more easily accessible to microorganisms (Faraldo-Gómez and Sansom, 2003; Miethke and Marahiel, 2007). Iron can be released not only by siderophore resolution but also by other biological mechanisms (Lin et al., 2005). The siderophore production is accepted as virulence determinant for bacteria. Furthermore, it has been concluded from the study of Kim et al. (2014) that all *C. sakazakii* strains which produce siderophore, as a virulence factor, can cause necrotizing enterocolitis.

1.3.4 Biofilm Formation

A biofilm is a community of microorganisms that can grow on living or many different surfaces and surrounded in a matrix of primarily polysaccharides (Mullane et al., 2006). When bacteria grow in a biofilm, they may show varied physiological replies to nutrient conditions. Although transportation of many nutrients to the biofilm matrix happens with diffusion, it is indicated from the researches that less oxygen and fewer nutrients are enough for biofilm-forming bacteria to grow in suspension. This supplies advantage in growth, changed physiology, and increased resistance to an array of stress compared with their planktonic forms. Through diffusional mass transfer, biophysical and cell-to-cell interactions, mutual and commensal communities of bacteria manage to survive with the fewer nutrient and in low temperature that are found in food handling and storage environments (Hodgson et al., 1995; Kuchma et al., 2000; Sauer et al., 2002).

Biofilm formation has a particular significance in the food industry. Therefore, biofilms are reservoir of bacterial contamination that might cause food spoilage, shelf-life reduction and contamination of food products undergoing processing. Biofilms are considered as a potential way of pathogen transmission (Lehner et al., 2005; Jaradat et al., 2009). Biofilms of *Cronobacter*, produced on the

surfaces of the equipments of both food and food production area, were thought to have caused to several outbreaks (Kim et al., 2006). The production of biofilms by *Cronobacter* spp. on inanimate environments consisted of silicon, latex, stainless steel and glass has been documented (Iversen et al., 2004; Lehner et al., 2005). Moreover, Cruz-Cordova et al. (2012) suggested that the flagella of *C. sakazakii* were involved in biofilm formation. Colanic acid of *Cronobacter* spp. as an extracellular polysaccharides component contributes to attachment to various surfaces and enhanced resistance to environmental harsh conditions (Kothary et al., 2007).

1.3.5 Other Virulence Factors

The *Cronobacter* pathogenesis has been broadly worked at a genotypic grade. *Cronobacter* has many virulence factors including adhesion, invasion, and enzymes as well as toxins such as endotoxin and enterotoxins. Endotoxin is found in structure of the outer membrane in Gram negative bacteria. Endotoxin may increase the permeability of the neonatal intestinal epithelium and induce potent pathophysiological effects in the host, following digestion (Almajed and Forsythe, 2016). Townsend et al. (2008) reported that the presence of endotoxin along with *C. sakazakii* in infant formula enhanced the translocation of *C. sakazakii* from rat guts through the blood-brain barrier. As the endotoxin can be resistant at 100 °C, its presence in infant formulas may be important in enhancing the *Cronobacter* pathogenesis in infants (Townsend et al., 2008). Additionally, it is known that the O-antigen, a component of the lipopolysaccharide (endotoxin) structure, has responsibility for serological diversity. It has been established that *Cronobacter* has different lipopolysaccharide structures. Therefore, *Cronobacter* also shows differences in pathogenicity depending on differences in structure and composition of lipopolysaccharide (MacLean et al., 2009). Pagotto et al. (2003) firstly documented that the four strains had enterotoxin production in their research. They investigated the virulence factors of *Cronobacter* from both clinical and food sources using the suckling mouse assay.

As being virulence factor for *Cronobacter*, sialic acid, in the form of sialyloligosaccharides, is found in milk of human and in infant formula (Wang,

2009). Sialic acid cannot be digested in neonates and infants, therefore the sialic acid can increase in the intestinal microvilli of neonates and the remnants of N-acetylglucosamine cause proliferation of gut microbiota (Sprenger and Duncan, 2012). It has been reported by Joseph et al. (2013) that there is an acceptable connection between sialic acid metabolism and the pathogenicity of *C. sakazakii* possessing the *nanAKT* gene cluster encoding for utilization of sialic acid. On the other hand, active efflux system is a defined as virulence mechanism promoting to survival of microorganism in the gastrointestinal tract of host (Touze et al., 2004). Interestingly, the coding region of the *ibeB* gene in *C. sakazakii* has been documented that it ultimately allows invading the brain microvascular endothelial cells.

1.4 Clinical Significance

Cronobacter spp. is considered to be an opportunistic pathogen implicated in several diseases including necrotizing enterocolitis, bacteremia, and meningitis in neonates, infants and immunocompromised people (Nazarowec-White and Farber, 1997; Van Acker et al., 2001). *Cronobacter* infections cause neonatal meningitis and necrotizing enterocolitis which results in brain abscess or inflammation and the growth of hydrocephalus, intestinal necrosis and pneumatosis intestinalis (Nazarowec-White and Farber 1997; Gurtler et al. 2005).

In 2002, the International Commission for Microbiological Specifications for Foods (ICMSF) classified *Cronobacter* as a serious threat for restricted populations, causing life-threatening or considerable illness of extended period, with the high-risk populations being newborns and immunocompromised infants. As a result of the occurrence of *Cronobacter* infections especially in newborn infants; it has been shown that the stomach acidity of newborn babies is less acidic than adults. This is a significant determinant in the survival of the agent in infants with *Cronobacter* infections (FAO-WHO, 2006). *Cronobacter* infections in neonates and infant seem to be particularly concerned with species of *C. sakazakii* (Joseph and Forsythe, 2012; Holy and Forsythe, 2014). In addition, *C. malonaticus* has been commonly linked to adult infections. Moreover, a single case of neonatal meningitis has been relevant with *C. turicensis* infection (Iversen et al., 2007a, 2008).

Neonatal meningitis as the first case caused by *Cronobacter* was indicated in 1958 in England (Urmenyi and Franklin, 1961). The pathogen received worldwide attention after an epidemic of meningitis in 2001 (MMWR, 2002). Until 2008, the meeting has identified around many of documented cases of *Cronobacter* infection and at least 27 deaths from all around the world, in the published literature (FAO-WHO, 2008). Several outbreaks and cases caused by *Cronobacter* spp. are shown in Table 1.1.

Table 1.1. Several cases and outbreaks associated with *Cronobacter*

Years	**Cases**	**Results**	**Location and Reference**
1958	2	Meningitis and death	England, Urmenyi and Franklin, (1961)
1965	1	Meningitis and extreme mental impairment	Denmark, Joker et al. (1965)
1977-1981	8	Meningitis and death	The Netherland, Muytjens et al. (1983)
1995-1996	5	Immunosuppressive illnesses	Baston, Lai (2001)
1998	12	Meningitis and death	Belgium, Van Acker et al. (2001)
2001	10	Meningitis and death	Knoxville, Tennessee, US CDC-MMWR, 2002
2004	10	Meningitis and death	France, Acoignard et al. (2006); New Zealand, Ministry of Health (2005)
2008-2011	5	Meningitis, bacteremia and death	New Mexico, US CDC (2009); Florida, Illinois CDC (2012)

1.5 Genetic Variation of *Cronobacter* spp.

Molecular typing of foodborne pathogens including *Cronobacter* spp. is essential for tracking the infection's source and determination of virulent strains as well as for detecting the geographic and host distribution of possible variants (Mullane et al., 2007; Chen et al., 2013). At present, various methods including 16S rRNA and housekeeping gene sequencing, multilocus sequence typing (MLST), polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), pulse-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD)-PCR, and enterobacterial repetitive intergenic consensus sequence (ERIC)-PCR have been successfully utilized for differentiating and typing of *Cronobacter* species (Lehner et al., 2004; Iversen et al., 2008; Ye et al., 2008; Jaradat et al., 2009; Joseph et al., 2012a; Joseph et al., 2012b; Li et al., 2014; Cui et al., 2014; Chen et al., 2016). Molecular techniques may provide valuable information on taxonomic relationship of strains (Strydom et al., 2011). Among the genetic tools, ERIC as a simplified marker has been utilized in epidemiologic studies to follow up the contamination and infection of pathogens such as *Cronobacter* spp. ERIC is known as highly conserved DNA sequences which are about 124–127 bp long located mainly within the intergenic regions of chromosomes of *Enterobacteriaceae*. It has the inverted repeat sequences that can form stable stem-loop structure (Sharples and Lloyd, 1990). Recently, in several studies, ERIC-PCR has been performed as a useful method for the epidemiological investigation of *Cronobacter* from different food sources including vegetables (Chen et al., 2016), powdered milks and infant formulas (Ye et al., 2008), milk powder, biscuit, spice, malted milk drink (Fakruddin et al., 2014).

1.6 Antimicrobial Susceptibility of *Cronobacter* spp.

Exposing microorganisms to concentration of antimicrobials potentially leads to an increase in the resistance to antimicrobials available for treatment. Several researches have described the susceptibility or resistance of *Cronobacter* spp. The natural antibiotic susceptibilities of 35 clinical isolates of *Cronobacter* to 69 different antimicrobial agents and comparison of them to the relevant clinical breakpoints were investigated by Stock and Wiedemann (2002). The results indicated that the *Cronobacter* isolates were a relatively high natural resistance to benzylpenicillin and

oxacillin, although the bacterium showed susceptibility to most -lactam and cephalosporin antibiotics. Furthermore, the results of study of Stock and Wiedemann (2002) demonstrated that some strains were resistant to amoxicillin, cefalozin, cefoxitin, and cefixime. To sum up, these data classify *Cronobacter* among the Gram negative pathogens that may express -lactamases, thus conferring resistance against a range of cephalosporins and penicillins. It was observed in 1983 that the treatment of *Cronobacter* meningitis by ampicillin and gentamicin was not very effective (Muytjens et al., 1983), but this was attributed to the low level of penetration of these antibiotics into the central nervous system (Willis and Robinson, 1988) and was not due to antibiotic resistance. In either case, to circumvent antibiotic resistance and to achieve better penetration, the treatment of *Cronobacter* meningitis has shifted to extended-spectrum cephalosporins, sometimes in combination with a second agent, e.g., trimethoprim (Lai, 2001; Block et al., 2002).

2. AIM AND SCOPE OF THE STUDY

The present study aimed to determine the prevalence of *Cronobacter* species in ready-to-eat foods, to identify the *Cronobacter* species by phenotypic and genotypic methods, and to detect virulence factors of the *Cronobacter* species using the PCR and phenotypic techniques such as outer membrane protein A, zinc-containing metalloprotease, biofilm formation and siderophore production. Moreover, the objective of present study was to determine the genetic diversity of the *Cronobacter* isolates obtained from ready-to-eat foods using ERIC-PCR and to investigate the antimicrobial resistance of the *Cronobacter* isolates from ready-to-eat foods.

3. MATERIALS AND METHODS

3.1 Sample Collection

A total of 340 ready-to-eat food samples, including doner (n=49), dessert (n=43), pastrami (n=41), cheese (n=40), salad (n=38), kavurma (n=35), meat free cig kofte (n=27), ice cream (n=27), spices (n=15), cereal (n=13), herb (n=7), and vegetables (n=5) were purchased from markets, local bazaar, patisserie and herbalist. All of the samples put into the ice container in sterile bag and transported to the laboratory under aseptically conditions. The samples were analyzed within 1 h of sampling.

3.2 Isolation of *Cronobacter* spp.

Cronobacter spp. from ready-to-eat foods was isolated and detected according to the methods of the Food and Drug Administration (FDA, 2002) and the International Standard Organization (ISO/TS 22964, Anonymous, 2006) with some modification. In this study, the isolation methods including FDA and ISO are given in Figure 3.1 and Figure 3.2, respectively.

FDA: 2002

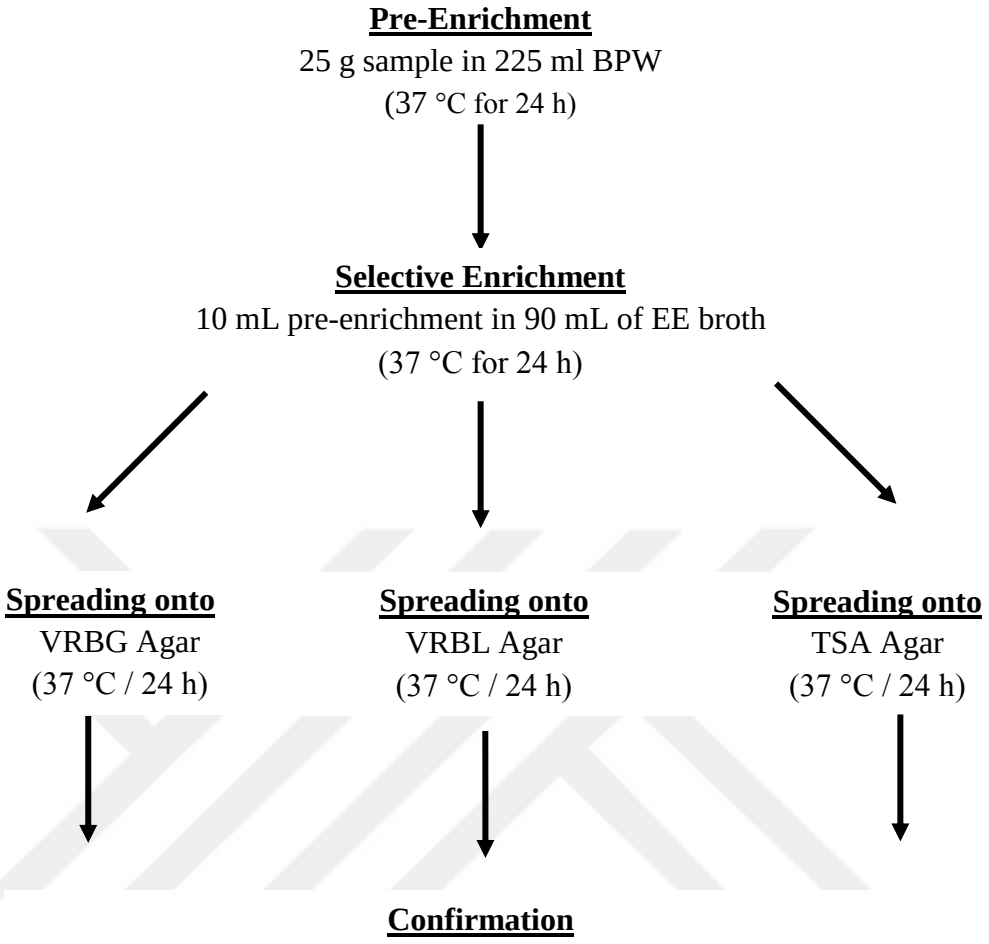

Figure 3.1. The isolation methods used in this study for determination of *Cronobacter* spp. according to FDA: 2002 method. BPW, Buffered Peptone Water; EE, *Enterobacteriaceae* Enrichment; VRBG, Violet Red Bile Glucose Agar; VRBL, Violet Red Bile Lactose Agar; TSA, Tryptic Soya Agar.

ISO/TS 22964: 2006 METHOD WITH SOME MODIFICATION

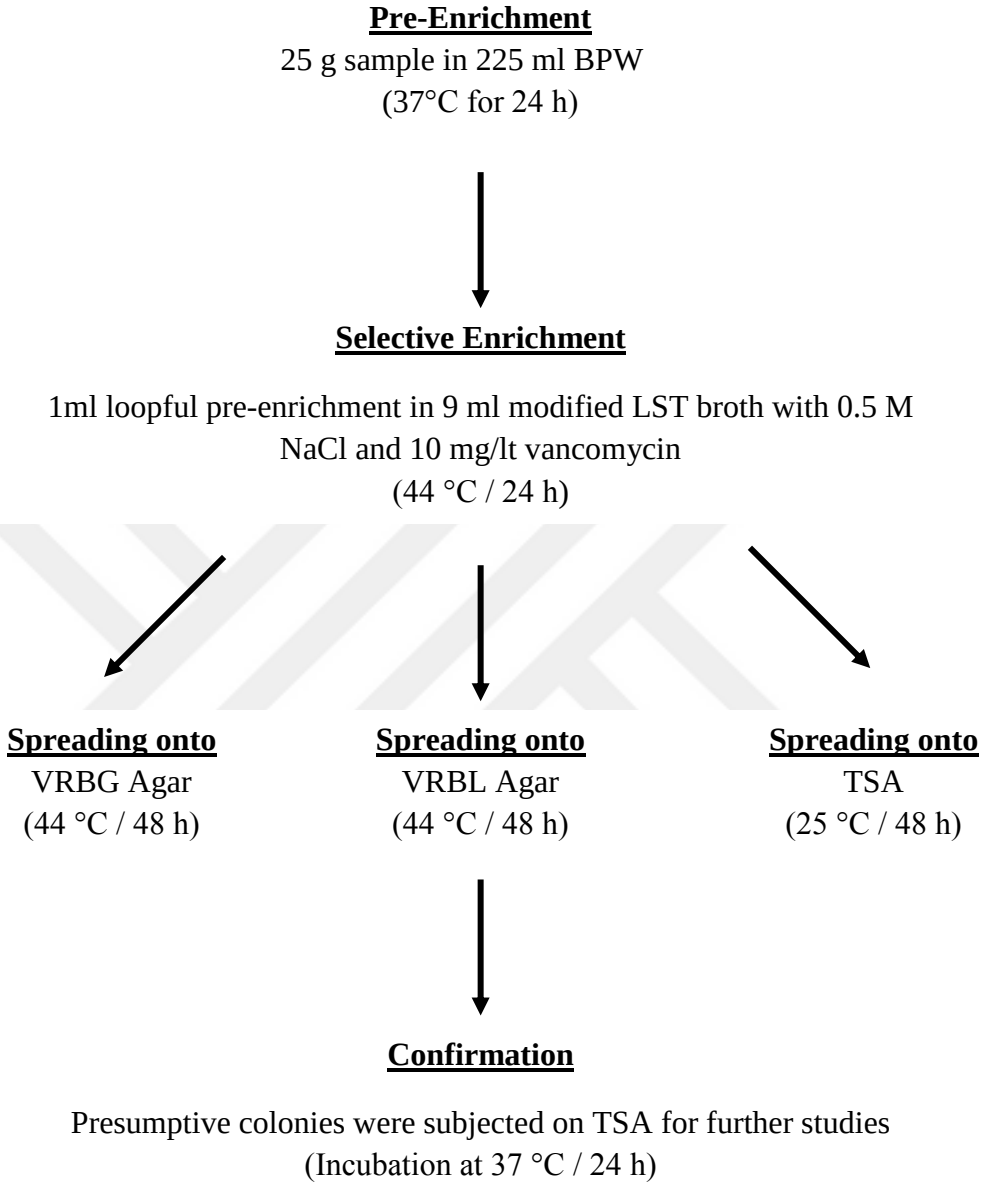

Figure 3.2. The isolation method used in this study for determination of *Cronobacter* spp. according to ISO/TS 22964: 2006 method with some modifications. BPW, Buffered Peptone Water; LST, Lauryl Sulfate Tryptose; VRBG, Violet Red Bile Glucose Agar; VRBL, Violet Red Bile Lactose Agar; TSA, Tryptic Soya Agar.

In the present study, ready-to-eat foods including doner, desserts, pastrami, cheese, salad, kavurma, meat free cig kofte, ice creams, spices, cereals, herbs, and vegetables were analyzed to detect the presence of *Cronobacter* spp. According to the FDA method, a 25 g sample was aseptically homogenizated with 225 mL Buffered Peptone Water (BPW) (Merck, Darmstadt, Germany) by stomacher (Bagmixer 400, Interscience, Paris, France) and incubated at 37 °C for 24 h. After that, 10 mL of BPW pre-enrichment was added into 90 mL *Enterobacteriaceae* enrichment (EE) broth (Merck) and incubated for 24 h at 37 °C. The EE broth was streaked onto Violet Red Bile Glucose (VRBG) agar (Fluka, Steinheim, Germany), Violet Red Bile Lactose (VRBL) agar (Fluka, Steinheim, Germany), and Tryptic Soy Agar (TSA, Merck). The samples were incubated at 37 °C for 24 h.

The other procedure which was accepted for the isolation of *Cronobacter* spp. from ready-to-eat foods samples had some modifications of the ISO method; a 25 g of each sample was homogenizated with 225 mL BPW (Merck, Germany) and incubated for 18-20 h at 37 °C. One milliliter of BPW pre-enrichment was added to 10 ml modified Lauryl Sulfate Tryptose (mLST) broth (Fluka) supplemented with 0.5 M NaCl (Merck) and 0.1 mg/L vancomycin (Hospira, Germany) and incubated for 24 h at 44 °C. After incubation, a loopful of mLST broth was taken and streaked onto VRBG agar (Fluka, India) and VRBL agar (Fluka). Then, the plates were incubated (44 °C / 24 h). At the same time, a loopful of mLST was streaked on TSA and incubated (25 °C/48 h).

Typical suspected colonies obtained according to both FDA and modified ISO methods which appeared pink or red on VRBG agar and VRBL agar were chosen and subcultured on TSA medium. After incubation, a yellow pigment colony on TSA medium was counted as *Cronobacter* spp.

3.3 Identification of *Cronobacter* spp.

3.3.1 Phenotypic Protocols

A progressive steps are necessary for isolation of the *Cronobacter* spp. Presumptive *Cronobacter* spp. colonies were taken and examined for biochemical

tests; Gram staining, motility at 37 °C, citrate utilization, acid reaction with methyl red, acetoin production, urease test, indole and H_2S production, lysine decarboxylase, fermentation of various carbohydrates including sorbitol, sucrose, dulcitol, myo-inositol, trans-aconitate and malonate (Farmer et al., 1980; FDA, 2002; Iversen et al., 2006, 2008; Joseph et al., 2012a). The *Cronobacter sakazakii* ATCC 29544 as a reference strain was used in all biochemical. Some biochemical tests performed in our study for differentiation of *Cronobacter* spp. were shown in Table 3.1.

Table 3.1. Biochemical tests for identification of the *Cronobacter* spp.

Biochemical Tests	***Cronobacter* spp.**						
	C. sakazakii	*C. malonaticus*	*C. muytjensii*	*C. turicensis*	*C. dublinensis*	*C. universalis*	*C. condimenti*
Gram stain	-	-	-	-	-	-	-
Motility	+	+	+	+	+	V[a]	-
Oxidase	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+
Indol production	-	-	+	-	V	-	+
Acetoin production	+	+	+	+	+	+	+
H_2S from TSI	-	-	-	-	-	-	-
Methyl Red	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-
Lysine decarboxylase	-	-	-	-	-	-	-
Malonate	-	+	+	+	V	-	+

Table 3.1. Biochemical tests for identification of the *Cronobacter* spp. (continued)

Biochemical Tests	***Cronobacter* spp.**						
	C. sakazakii	*C. malonaticus*	*C. muytjensii*	*C. turicensis*	*C. dublinensis*	*C. universalis*	*C. condimenti*
Fermentation of							
Sucrose	+	+	+	+	+	+	+
Dulcitol	-	-	+	+	+	-	-
Myo-inositol	V[a]	V	+	+	V	+	-
Trans-aconitate	-	+	V	-	+	-	-
Sorbitol	-	-	-	-	-	-	-
1-0-Methyl α-D-glucopyranoside	+	+	-	+	+	+	+

[a], variable

3.3.2 Genotypic Protocols

In this study, the genomic DNA extraction was performed using a previously described method (Ausubel et al. 1991). All the *Cronobacter* isolates were grown on TSA (Merck) for 24 h at 37 °C. The one colony of the each culture was added into 5 mL Brain Heart Infusion (BHI) broth (Merck) and was incubated at 37 °C for 18 h. The extracted DNA was kept at -20 °C for further use.

In the current study, the *Cronobacter* isolates were identified with phenotypic methods and were also confirmed with genotypic methods. The 16S rRNA gene and the *gluA* target gene were used for this confirmation. Moreover, the *rpoB* gene was carried out to differentiate between the seven *Cronobacter* species. Besides, as

virulence genes, the *zpx* and the *ompA* were examined using the PCR. *Cronobacter sakazakii* ATCC 29544 as a reference strain was used in all molecular studies.

3.3.2.1 The 16S rRNA gene

In this study, PCR amplification of the 16S rRNA gene of the *Cronobacter* isolates, obtained from ready-to-eat foods, was performed as described previously (Hassan et al., 2007). Subsequently, a pair of PCR primer Saka-1 (ACA GGG AGC AGC TTG CTG C) and Saka-2b (TCC CGC ATC TCT GCA GGA) were used for *Cronobacter* spp. PCR amplification was performed in a total volume of 30 µL, with the following reagent concentration: 3 µL of 10 X PCR buffer (100 mM Tris-HCl pH 8.8, 500 mM KCl, 0.8% (v/v) nonidet P40; Vivantis Techologies Sdn. Bhn., Kuala Lumpur, Malaysia), 1.8 µL of 25 mM $MgCl_2$ (Vivantis), 0.6 µL of 10 mM dNTP mix (Vivantis), 1 µL of 10 µM each of the primers (Biomers, Ulm, Germany), 2.5 µL of template DNA (50 ng/ µL) and 1 U Taq DNA polymerase (Vivantis). The thermal cycling conditions were as follows: initial denaturation at 94 ºC for 4 min, followed by 30 cycles of denaturation at 95 ºC for 1 min; annealing at 57 ºC for 1 min, and elongation at 72 ºC for 1 min 30 s, and a final extension at 72 ºC for 4 min. PCR reactions were carried out on a XP Thermal Cycler (Bioer Technology Co., Ltd., Japan). A total of 3 µL of gel loading dye (Fermentas, Life Science, Canada) was added to 5 µL of each PCR product and 8 µL of the obtained mixture were analyzed in agarose gel (1.5%) (Merck) using Tris-borate-EDTA (TBE) (1X) supplemented with ethidium bromide (Applichem), and then electrophoresis (Bio-Rad) was carried out. The amplification product size of 952 bp was visualized with ultraviolet transilluminator (DNR Minilumi).

3.3.2.2 The *rpoB* gene

In the studies of both Stoop et al. (2009) and Lehner et al. (2012), an *rpoB*-gene-targeted PCR assay was improved, which allows the identification of members of the genus *Cronobacter* to the species level. The different set of primers was performed in the species-specific *rpoB* gene assay to identify each species of *Cronobacter* including *C. sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. turicensis*, *C. universalis*, *C. dublinensis*, and *C. condimenti*. The PCR reaction mixture included 5 µL of 10 X PCR buffer (Vivantis), 4 µL of 25 mM $MgCl_2$ (Vivantis), 1 µL of 10 mM dNTP mix (Vivantis), 2 µL 10 µM each of the primers (Biomers), 3 µL of template DNA (50 ng/ µL) and 1 U Taq DNA polymerase (Vivantis). Primer pairs, PCR conditions and product size of the *Cronobacter* species-specific amplification are given in Table 3.2. The PCR products were analyzed on %1 agarose gel (Merck) that was examined under a UV transilluminator (DNR Minilumi).

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Table 3.2. Primer sequences, PCR conditions and product size of the species-specific *rpoB* gene for the *Cronobacter* species

Species	Primer sequence	PCR cycling condition	Size (bp)	Reference
C. sakazakii	Csakf 5′-ACG CCA AGC CTA TCT CCG CG-3′ Csakr 5′-ACG GTT GGC GTC ATC GTG-3′	94 ºC, 3 min; 30 × (94 ºC, 1 min; 67 ºC, 30 s; 72 ºC, 1 min); 72 ºC, 5 min	514 bp	Stoop et al. (2009)
C. malonaticus	Cmalf 5′-CGT CGT ATC TCT GCT CTC-3′ Cmalr 5′-AGG TTG GTG TTC GCC TGA-3′	94 ºC, 3 min; 30 × (94 ºC,1 min; 60 ºC, 30 s; 72 ºC, 30 s); 72 ºC, 5 min	251 bp	Stoop et al. (2009)
C. turicensis	Cturf 5′-CGG TAA AAG AGT TCT TCG GC-3′ Cturr 5′-GTA CCG CCA CGT TTC GCC-3′	94 ºC, 3 min; 30 × (94 ºC, 1 min; 61 ºC, 30 s; 72 ºC, 1 min); 72 ºC, 5 min	628 bp	Stoop et al. (2009)
C. dublinensis	Cdublf 5′-GCA CAA GCG TCG TAT CTC C-3′ Cdublr 5′-TTG GCG TCA TCG TGT TCC-3′	94 ºC, 3 min; 30 × (94 ºC, 1 min; 62 ºC, 30 s; 72 ºC, 30 s); 72 ºC,5 min	418 bp	Stoop et al. (2009)
C. muytjensii	Cmuyf 5′-TGT CCG TGT ATG CGC AGA CC-3′ Cmuyr 5′-TGT TCG CAC CCA TCA ATG CG-3′	94 ºC, 3 min; 30 × (94 ºC, 1 min; 61 ºC, 30 s; 72 ºC, 30 s); 72 ºC, 5 min	289 bp	Stoop et al. (2009)
C. *universalis*	Cgenomof 5′-ACA AAC GTC GTA TCT CTG CG-3′ Cgenomor 5′-AGC ACG TTC CAT ACC GGT C-3′	94 ºC, 3 min; 30 × (94 ºC, 1 min; 61 ºC, 30 s; 72 ºC, 30 s); 72 ºC, 5 min	506 bp	Stoop et al. (2009)
C. condimenti	Ccon-f 5′- AAC GCC AAG CCA ATC TCG-3′ Ccon-r 5′-GTA CCG CCA CGT TTT GCT-3′	95 ºC, 2 min; 30 × (95 ºC, 1 min; 58 ºC, 30 s; 72 ºC, 1 min); 72 ºC, 5 min	689 bp	Lehner et al. (2012)

3.3.2.3 The *gluA* gene

The α-glucosidase activity has been encoded by the *gluA* gene. The *gluA* gene amplification, using primers EsAgf (TGA AAG CAA TCG ACA AGA AG) and EsAgr (ACT CAT TAC CCC TCC TGA TG) were performed, obtaining a product of 1680 bp according to Iversen et al. (2007b).

All PCR mixtures contained 5 µL 10 X PCR buffer (Vivantis), 4 µL of 25 mM $MgCl_2$ (Vivantis), 1 µL of 10 mM dNTP mix (Vivantis), 0.75 µL of 10 µM each primers (Biomers), 1 µL of target DNA (50 ng/ µL) and 1 U Taq DNA polymerase (Vivantis). PCR mixtures were subjected to an initial denaturation at 94 ºC for 2 min, followed by 29 cycles of 94 ºC for 30 s, 58 ºC for 1 min, 72 ºC for 90 s and final extension at 72 ºC for 5 min. Amplicons of PCR reactions were separated by electrophoresis (Bio-Rad) and visualized under UV transilluminator (DNR Minilumi).

3.1 Detection of Genotypic Virulence Characteristics of *Cronobacter* spp.

In this research, for the virulence detection of *Cronobacter* isolates obtained from ready-to-eat foods, PCR was targeted to *ompA* gene (ESSF: GGA TTT AAC CGT GAA CTT TTC C; ESSR: CGC CAG CGA TGT TAG AAG A), which was previously suggested by Nair and Venkitanarayanan (2006). Reactions were performed in 50 µL volume, 5 µL 10 X PCR buffer (100 mM Tris-HCl pH 8.8, 500 mM KCl, 0.8% (v/v) nonidet P40; Vivantis), 2 µL $MgCl_2$ (25 mM), 1 µL dNTP mix (10 mM) (Vivantis), 5 µL primers (10 µM) (Biomers, Ulm, Germany), 3 µL template DNA (50 ng/µL) and 1 U Taq DNA polymerase (Vivantis). PCR cycling conditions for amplification of DNA fragments included an initial denaturation (94 ºC, 2 min), 30 cycles of denaturation (94 ºC, 15 s), annealing (60 ºC, 15 s), extension (72 ºC, 30 s) followed by final extension (72 ºC, 5 min) (Nair and Venkitanarayanan, 2006). Then, the amplified products were analyzed via gel electrophoresis (Bio-Rad) (1.5% agarose (Merck) concentration). They were illustrated with UV transilluminator (DNR Minilumi).

In this study, the PCR amplification of the *zpx* gene, a virulence gene, was achieved by 3 µL template DNA (50 ng/ µL) with a 47 µL of PCR mixture

containing the following: 5 µL 10 X PCR buffer (100 mM Tris-HCl pH 8.8, 500 mM KCl, 0.8% (v/v) nonidet P40; Vivantis), 2 µL $MgCl_2$ (25 mM), 1 µL dNTP mix (10 mM) (Vivantis), 1 U Taq DNA polymerase (Vivantis), 5 µL from primers (10 µM) (Biomers) Es-ProF (GAA AGC GTA TAA GCG CGA TTC) and EsPro-R (GTT CCA GAA GGC GTT CTG GT) (Kothary et al., 2007). The amplification conditions of the *zpx* gene were as follows: an initial denaturation of 95 ºC for 15 s, followed by 35 cycles of 94 ºC for 1 min, 62 ºC for 1 min, 72 ºC for 1 min, with a final elongation at 72 ºC for 10 min (Kothary et al., 2007). The PCR products were then examined by electrophoresis (Bio-Rad) in agarose gel (1.5%) (Merck) and viewed under UV light (DNR Minilumi).

3.2 Detection of Phenotypic Virulence Characteristics of *Cronobacter* spp.

Siderophore production of *Cronobacter* spp. was studied based on the Chrome azurol S (CAS) assay of Schwyn and Neilands (1986) with some modifications made by Fiss and Brooks (1991). Firstly, 60.5 mg of CAS was added in 50 mL distilled water, and then 10 mL iron solution (1 mM $FeCl_3.6H_2O$, 10 mM HCl) added in this preparation. This solution was mixed slowly with 72.9 mg of hexadecyltrimethylammonium bromide dissolved in 40 mL of distilled water. After the dark blue solution was autoclaved, the basal medium of the CAS indicator solution was prepared. The CAS indicator solution includes KH_2PO_4, 0.3 g/L; NaCl, 0.5 g/L, NH_4Cl, 1.0 g/L; PIPES (piperazine-*N*, *N*'-bis [2-ethanesulfonic acid]), 0.1 M, pH 6.8; L-asparagine, 5.0 g/L; and agar, 15 g/L. The basal medium autoclaved, and then the sterile CAS indicator solution was added slowly, which was followed with 20 mL of sterile glycerol. The plates were incubated for 5 days at 37 °C. After incubation, siderophore positive isolates were seen as an orange halo around a colony (Schwyn and Neilands, 1986).

Biofilm formation of the *Cronobacter* isolates was analyzed using the microtiter plate technique described by Stepanovic´ et al. (2000) and Lee et al. (2017) with some modifications. The *Cronobacter* isolates were incubated in 5 mL of Tryptic Soy Broth (TSB, Merck) at 37°C for 24 h. Cultures were diluted until reaching 0.5 on the McFarland scale (approximately 10^8 cells/mL). Then, 200 µL of

each bacterial suspension were transferred into wells of flat bottomed, 96-well polystyrene microplate. The microtiter plates were incubated statically at 37°C for 48 h. The sterile TSB, as a negative control, was used for determination of the ability of the *Cronobacter* isolates to biofilm formation. Following incubation, wells were washed once sterile phosphate-buffered saline (PBS, pH 7.2), fixed with 200 µL methanol (Merck). After microplate were stained with crystal violet 200 µL of 0.1% (Merck) for 15 min, dried and resolubilized with 200 µL glacial acetic acid (33% (v/v)) (Merck). The optical density (OD) was measured at 570 nm (Multiskan Spectrum, Thermo Electron Corporation). The result for each isolate was calculated by subtracting the control OD_{570} from the average of both two parallels and two replicate isolates OD_{570}. The isolates were classified as strong, moderate and weak for biofilm formation in respect to Stepanovic´ et al. (2000). In this study, *C. sakazakii* ATCC 29544 was also used as a positive control for biofilm formation and siderophore production.

3.3 Enterobacterial repetitive intergenic consensus (ERIC)-PCR

In this study, ERIC-PCR was performed using primers ERIC1R (ATG TAA GCT CCT GGG GAT TCA C) and ERIC2 (AAG TAA GTG ACT GGG GTG AGC G) as previously described (Versalovic et al., 1991). All of *Cronobacter* isolates from ready-to-eat foods were tested for genotyping by the ERIC-PCR method. PCR reaction was optimized in a 50 µL reaction mixture consisting of 3 µL of the bacterial genomic DNA solution (50 ng), 5 µL PCR buffer, 4 µL $MgCl_2$ of 25 mM, 1 µL 10 mM dNTPs, 1 µL (200 nM each) primers, 1U Taq polymerase and 34.75 µL nuclease free water. PCR cycling conditions were as follows: initial denaturation at 94 °C for 8 min, followed by 35 cycles of 30 s at 94 °C, annealing for 1 min at 55 °C, and extension for 8 min at 72 °C. PCR was completed with a final extension for 10 min at 72 °C. After amplification, the PCR amplicons were separated on agarose gel (1%), stained with ethidium bromide and visualized under UV light (DNR Minilumi). The band patterns of ERIC-PCR were analyzed using the NTSYS-pc (version 2.10) software package. Each band of ERIC-PCR products was marked as 1 for presence and 0 for absence. When the *Cronobacter* isolates was calculated as a similarity with Dice coefficient, the dendogram was obtained by means of unweighted pair group method using average (UPGMA) clustering.

3.4 Antimicrobial Susceptibility Test

Antimicrobial susceptibility testing of the 64 *Cronobacter* isolates from ready-to-eat foods was performed by the disk diffusion method on Mueller Hinton Agar (Merck), according to the Clinical and Laboratory Standards Institute (CLSI, 2014). In this study, the 18 antimicrobial agents were selected for analysing. They belonged to the following groups: penicillins (ampicillin – 10 µg; piperacillin – 100 µg), carbapenems (imipenem – 10 µg; meropenem – 10 µg), beta-lactamase inhibitors (amoxicillin-clavulanic acid – 30 µg; piperacillin-tazobactam – 110 µg), cephalosporins (cefotaxime – 30 µg; cefoxitin – 30 µg; cephalothin – 30 µg; cefepime – 30 µg), aminoglycosides (gentamicin – 10 µg; amikacin – 30 µg), tetracyclines (tetracycline – 30 µg), folate pathway inhibitors (trimethoprim/sulfamethoxazole – 25 µg), monobactam (aztreonam – 30 µg), quinolones (nalidixic acid – 30 µg; ciprofloxacin – 5 µg) and phenicols (chloramphenicol – 30 µg).

The turbidity of each bacterial concentration was adjusted to 0.5 McFarland in Mueller-Hinton broth (Merck), and the bacterial inoculums were plated onto Mueller-Hinton agar. The disks of antimicrobial agents were placed on the agar sufficiently separated. The agar plates were incubated for 24 h at 37 ºC. Then, the diameter of inhibition zones was measured. The results of all the inhibition zones of antimicrobial agents were interpreted according to *Enterobacteriaceae* table in CLSI (CLSI, 2014).

4. RESULTS

4.1 Isolation and Identification of *Cronobacter* spp. using Phenotypic and Genotypic Methods

In this study, *Cronobacter* spp. was isolated from ready-to-eat foods according to FDA and ISO with some modifications. The 59 ready-to-eat food samples out of the 340 samples were contaminated by *Cronobacter*. The 64 *Cronobacter* isolates were obtained from these contaminated foods. These *Cronobacter* isolates were identified using both phenotypic and genotypic methods. Firstly, the identification of *Cronobacter* spp. was carried out phenotypically using biochemical tests which were previously described by Bergey's Manual of Systematic Bacteriology (2005), Farmer et al. (1980), Iversen et al. (2006, 2008) and Joseph et al. (2012a). Then, genotypically, some targets including the 16S rRNA gene, the *gluA* gene and the *rpoB* species-specific gene were used for the identification of *Cronobacter* spp.

In the present study, by using the method proposed by Hassan et al. (2007) for determination of 16S rRNA gene, all isolated *Cronobacter* were specifically identified (Figure 4.1.). Additionally, PCR on the *gluA* gene was performed on *Cronobacter* isolates. In our research, PCR amplification of the *gluA* gene was positive for the 59 (92.2%) out of the 64 *Cronobacter* isolates (Figure 4.1.). However, the *gluA* gene was not detected in the 5 (7.8%) isolates.

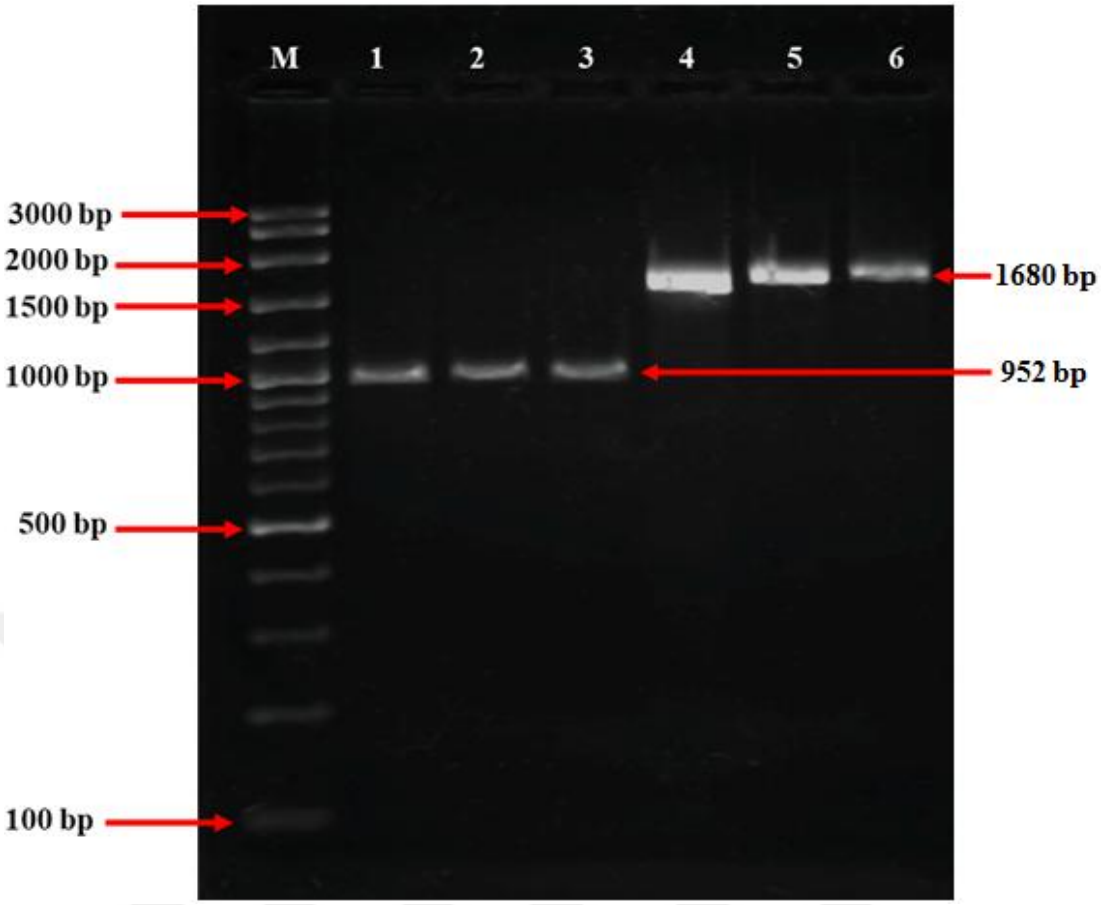

Figure 4.1. Amplification of the 16S rRNA and the *gluA* gene of the *Cronobacter* isolates. Lane M: 100 bp molecular size marker (Thermo Scientific, California, USA). Lane 1: Positive control for the 16S rRNA gene (*C. sakazakii* ATCC 29544) (952 bp). Lane 2 and 3: Representative *Cronobacter* isolates carried the 16S rRNA gene in this study. Lane 4: Positive control for the *gluA* gene (*C. sakazakii* ATCC 29544) (1680 bp). Lane 5 and 6: Representative *Cronobacter* isolates carried the *gluA* gene in this study.

The species-specific PCR primers of the *rpoB* gene which were useful for direct identification of bacterial species were used in this study. Out of 64 isolates, 39 (61%) *C. sakazakii*, 15 (23.4%) *C. malonaticus*, 3 (4.7%) *C. muytjensii*, 2 (3.1%) *C. turicensis*, 2 (3.1%) *C. universalis* and 3 (4.7%) *C. dublinensis* were identified using the *rpoB* species-specific genes. With the primers *Csak*, *Cmal*, *Ctur*, *Cmuy*, *Cdubl* and *Cgenomo* all isolates showed a correctly size amplification product. The species-specific bands were observed for *Cronobacter* species except for the *C. condimenti* encoded by *Ccon* primer. The results of PCR were documented by photographs of gels with the presence of appropriate size bands which is shown in Figure 4.2.

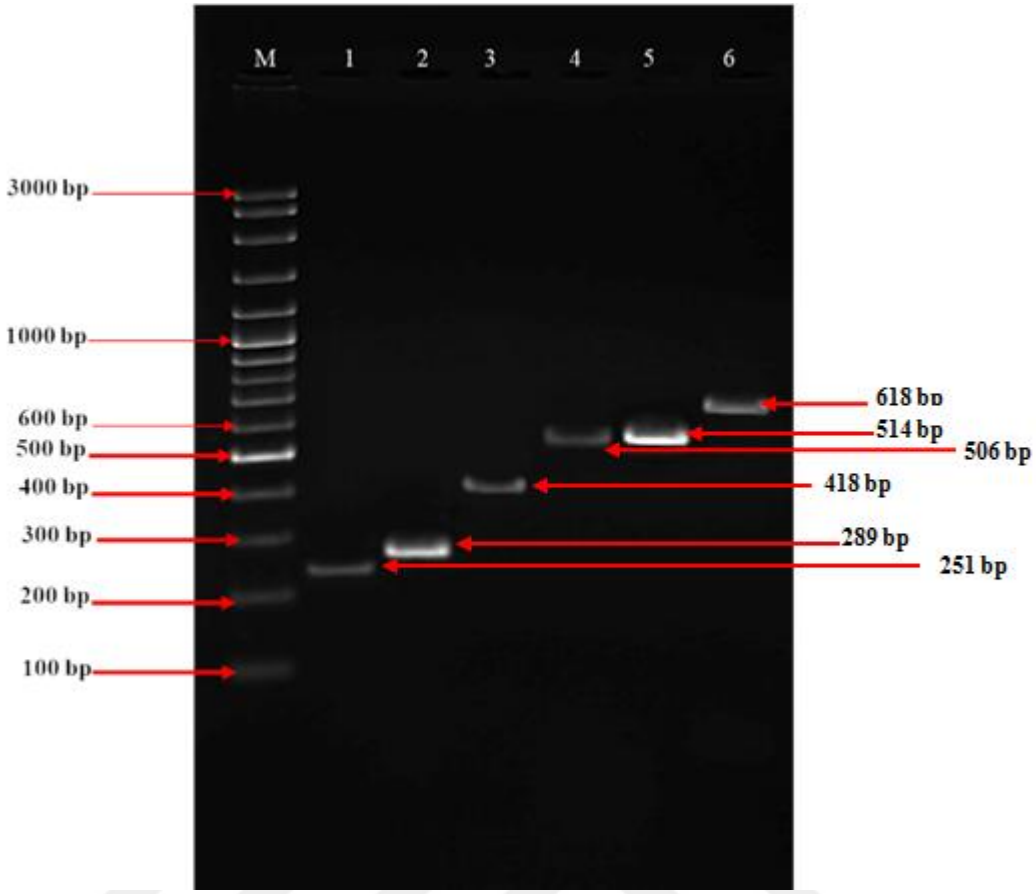

Figure 4.2. Gel image of the representative *Cronobacter* isolates identified in this study based on the *rpoB* gene. Lane M: 100 bp DNA ladder (Thermo Scientific, California, USA). Lane 1: *C. malonaticus*, Lane 2: *C. muytjensii*, Lane 3: *C. dublinensis*, Lane 4: *C. universalis*, Lane 5: *C. sakazakii*, Lane 6: *C. turicensis*.

4.2 Prevalence of *Cronobacter* spp. in Ready-to-eat Foods

In this research, a total of 340 ready-to-eat foods; doners (n=49), desserts (n=43), pastramis (n=41), cheeses (n=40), salad (n=38), kavurmas (n=35), meat free cig koftes (n=27), ice creams (n=27), spices (n=15), cereals (n=13), herbs (n=7), and vegetables (n=5) were examined for the presence of *Cronobacter* spp. Out of the 340 food samples, 59 (17.4%) were contaminated with *Cronobacter* spp. Of the contaminated samples, the 64 isolates were identified. The prevalence of *Cronobacter* spp. was 6 (12.2%) in the 49 doners, 13 (30.2%) in the 43 desserts, 4 (9.8%) in the 41 pastramis, 3 (7.5%) 40 cheeses, 1 (2.6%) in the 38 salad, 3 (8.6%) in the 35 kavurmas, 14 (51.9%) in the 27 meat free cig koftes, 4 (14.8%) in the 27 ice creams, 7 (46.7%) in the 15 spices and 4 (30.8%) in the 13 cereals. Overall, *Cronobacter* were detected in ten of twelve food categories. Herbs and vegetables revealed no positive results. The prevalence of the *Cronobacter* isolates among ready-to-eat food samples is given in Figure 4.3.

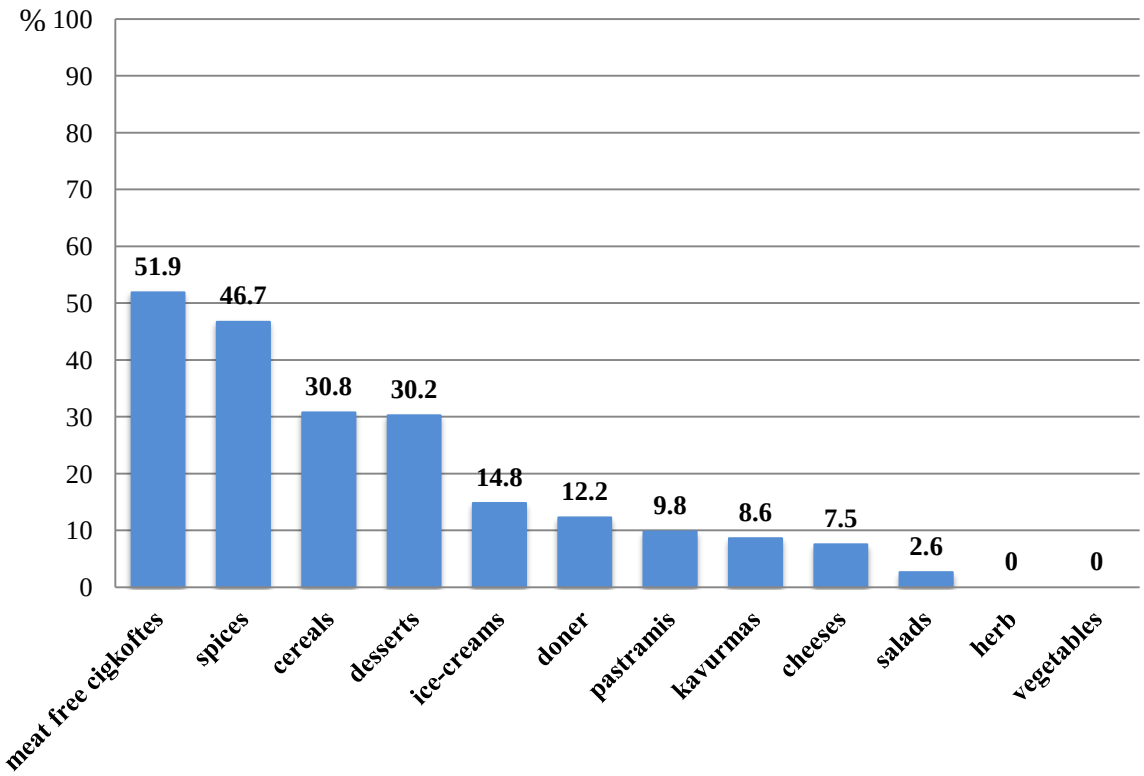

Figure 4.3. Prevalence of the *Cronobacter* isolates among ready-to-eat foods.

In this study, the *Cronobacter* spp. were found mostly in meat free cig koftes, spices and cereals. The *C. sakazakii*, as a significant pathogen, was common in meat free cig koftes, desserts and doners. The *C. malonaticus* associated with some adults infections, was predominated in meat free cig koftes and desserts. The distribution of *Cronobacter* species according to ready-to-eat foods is shown in Table 4.1.

Table 4.1. The distribution of *Cronobacter* species according to the types of ready-to-eat foods

Foods	**No. of samples/ No. of isolates**	***C. sakazakii***	***C. malonaticus***	***C. turicensis***	***C. muytjensii***	***C. universalis***	***C. dublinensis***
Meat free cig koftes	27/15	10	4	0	0	1	0
Spices	15/8	4	2	0	1	0	1
Cereals	13/4	1	1	1	0	1	0
Desserts	43/14	8	3	0	1	0	2
Ice-creams	27/5	3	1	0	1	0	0
Doners	49/7	6	1	0	0	0	0
Pastramis	41/4	1	2	1	0	0	0
Kavurmas	35/3	3	0	0	0	0	0
Cheeses	40/3	2	1	0	0	0	0
Salads	38/1	1	0	0	0	0	0
Herbs	7/0	0	0	0	0	0	0
Vegetables	5/0	0	0	0	0	0	0
Total	**340/64**	**39**	**15**	**2**	**3**	**2**	**3**

Overall, out of the 59 contaminated samples, the 64 isolates were identified 39 (61%) as *C. sakazakii*, 15 (23.4%) as *C. malonaticus*, 3 (4.7%) as *C. muytjensii*, 2 (3.1%) as *C. turicensis*, 3 (4.7%) as *C. dublinensis* and 2 (3.1%) as *C. universalis*, which means there were more than one species in some samples. The distribution among *Cronobacter* species isolated from ready-to-eat foods is given in Figure 4.4.

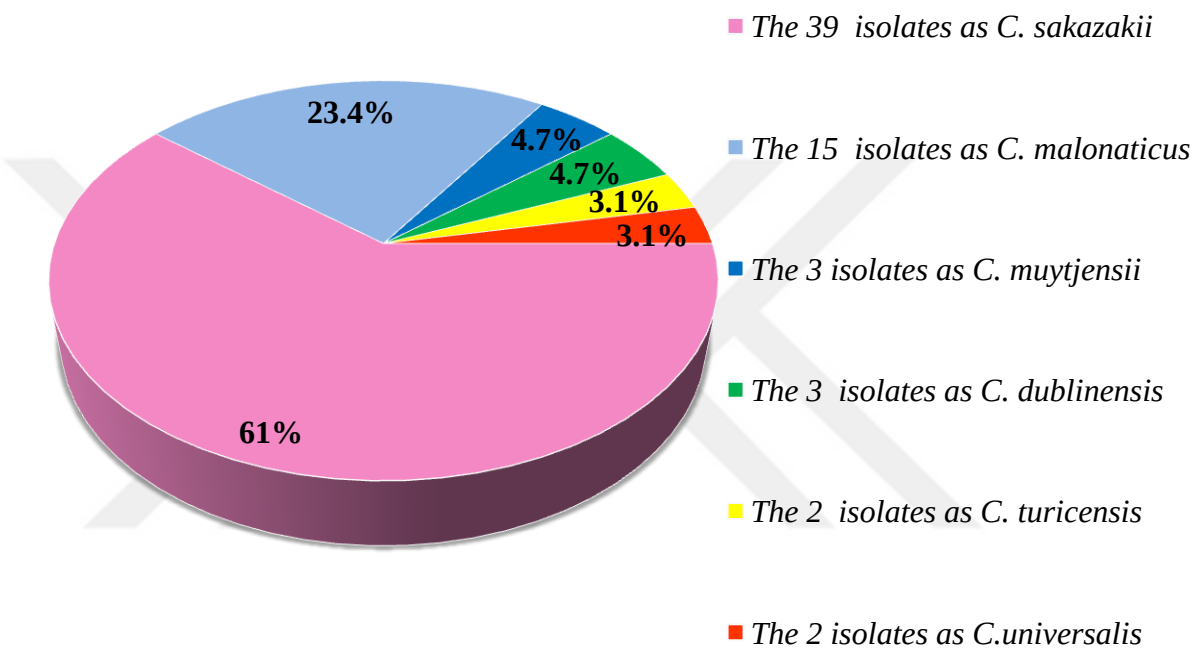

Figure 4.4. The distribution of identified *Cronobacter* species from ready-to-eat foods.

4.3 Genotypic Virulence Characteristics of *Cronobacter* spp.

Cronobacter carry a basic set of virulence characteristics and have the potential for invading the host cells. These virulence properties have significant roles in the pathogenicity of *Cronobacter* spp. In the present study, genotypic virulence characteristics of *Cronobacter* were examined by PCR for the existence of virulence genes such as the *ompA* and the *zpx.* In this research, out of the 64 *Cronobacter* isolates, 63 (98.5%) were found to be positive for the *ompA* gene, but 1 (1.5%) isolate did not harbor the *ompA* gene that it was *C. malonaticus* isolated from meat free cigkofte. We found that 63 (98.5%) of all *Cronobacter* isolates were positive for the *zpx* gene. However, 1 (1.5%) isolate, *C. malonaticus* isolated from dessert was found to be negative for the *zpx* gene. Photograph of agarose gel (1%) for PCR products of the *ompA* and *zpx* genes of the *Cronobacter* isolates is exhibited in the Figure 4.5.

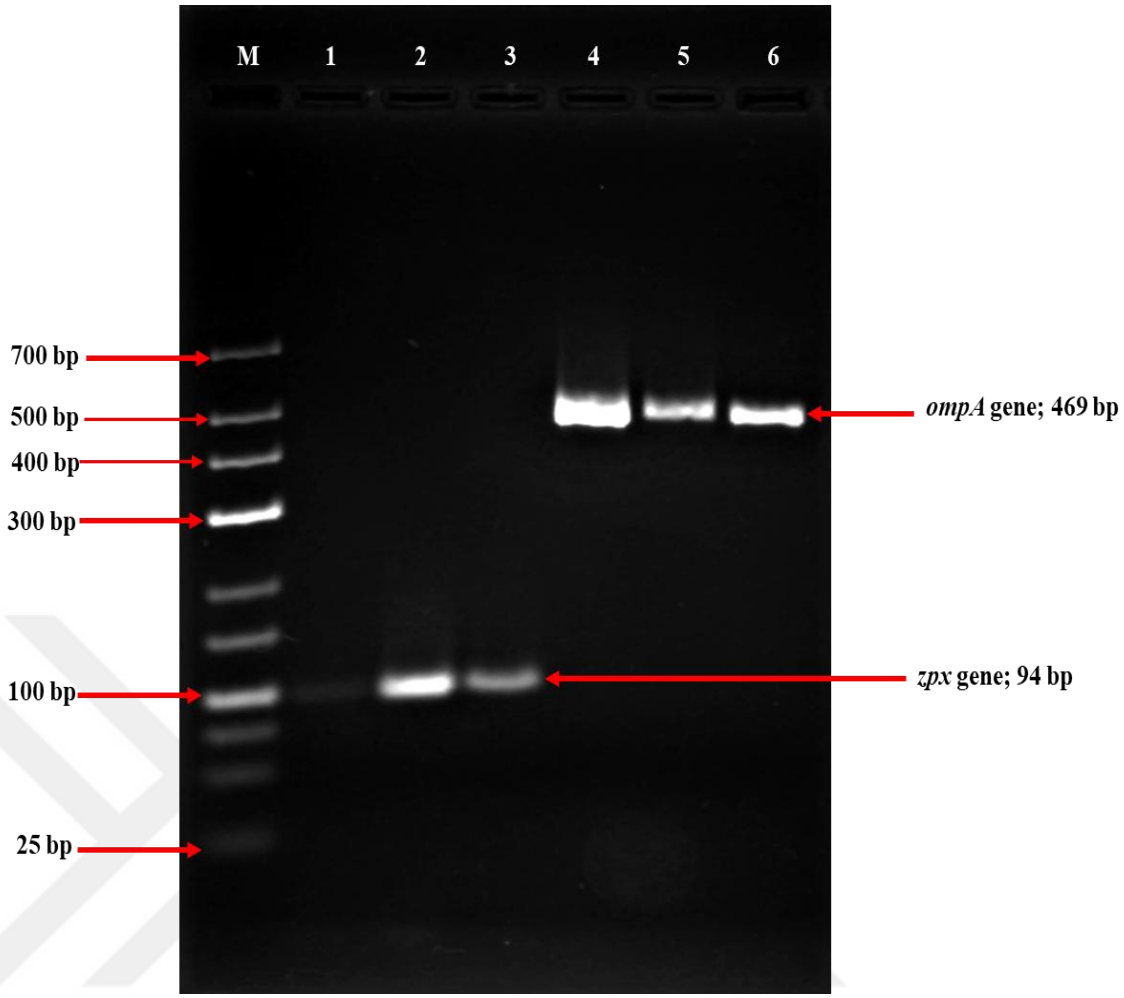

Figure 4.5. Agarose gel electrophoresis of the *ompA* and *zpx* genes. Lane M: GeneRuler Low-Range DNA ladder (Thermo Scientific, California, USA). Lane 1: Positive control for the *zpx* gene (*C. sakazakii* ATCC 29544). Lane 2 and 3: Representative *Cronobacter* isolates carried the *zpx* gene in this study. Lane 4: Positive control for the *ompA* gene (*C. sakazakii* ATCC 29544). Lane 5 and 6: Representative *Cronobacter* isolates carried the *ompA* gene in this study.

4.4 Phenotypic Virulence Characteristics of *Cronobacter* spp.

In the present study, the 64 *Cronobacter* isolates from ready-to-eat foods were examined to siderophore production by using the chrome azurol S (CAS) assay. The production of siderophore as a significant virulence property was detected in all of the *Cronobacter* isolates (100%). In the study, some positive *Cronobacter* isolates for siderophore production on CAS agar are shown in Figure 4.6.

In our study, biofilm formation by the all *Cronobacter* isolates from ready-to-eat food samples was analyzed using a microtiter plate assay. Out of the 64 *Cronobacter* isolates, the 56 (87.5%) were found to be positive for biofilm formation. However, the biofilm formation was not detected in 8 (12.5%) of isolates. The results of biofilm formation of the *Cronobacter* spp. are seen in Table 4.2.

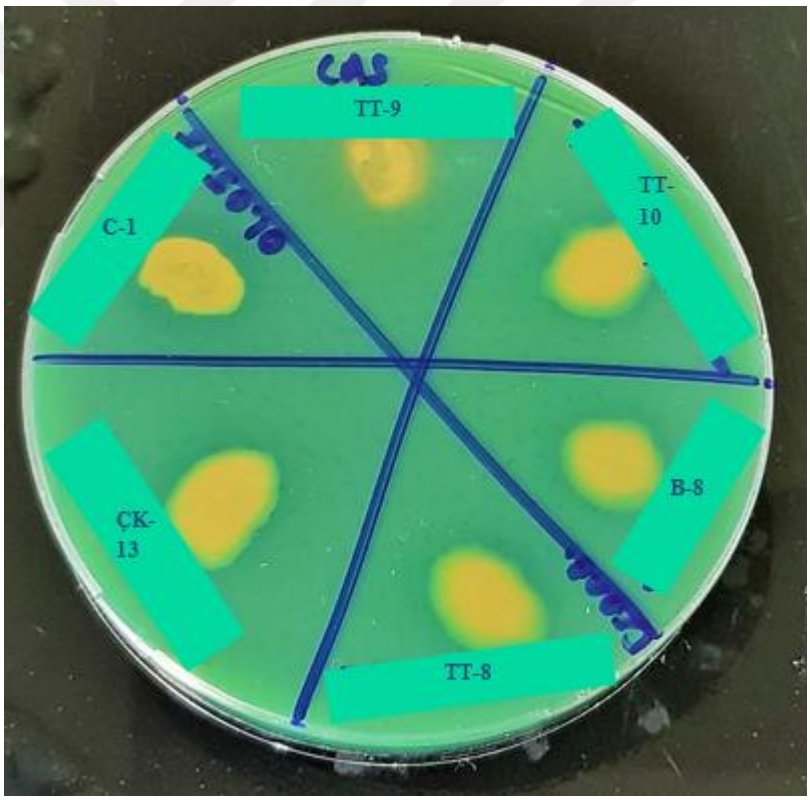

Figure 4.6. Siderophore production of *Cronobacter* spp. using the CAS agar. In this figure, all of the isolates were positive for the produce of siderophore.

Table 4.2. Distribution of biofilm producers among the *Cronobacter* species from ready-to-eat foods

***Cronobacter* spp.**	**No. of isolates**	**Weak producer**	**Moderate producer**	**Strong Producer**	**No producer**
C. sakazakii	39	18 (46.2%)	15 (38.5%)	1 (2.6%)	5 (12.8%)
C. malonaticus	15	12 (80%)	1 (6.7%)	1(6.7%)	1(6.7%)
C. turicensis	2	2 (100%)	-	-	-
C. muytjensii	3	2 (66.6%)	1 (33.4%)	-	-
C. universalis	2	-	1 (50%)	-	1 (50%)
C. dublinensis	3	2 (66.6%)	-	-	1(33.4%)
Total	**64**	**34 (53.1%)**	**20 (31.2%)**	**2 (3.1%)**	**8 (12.5%)**

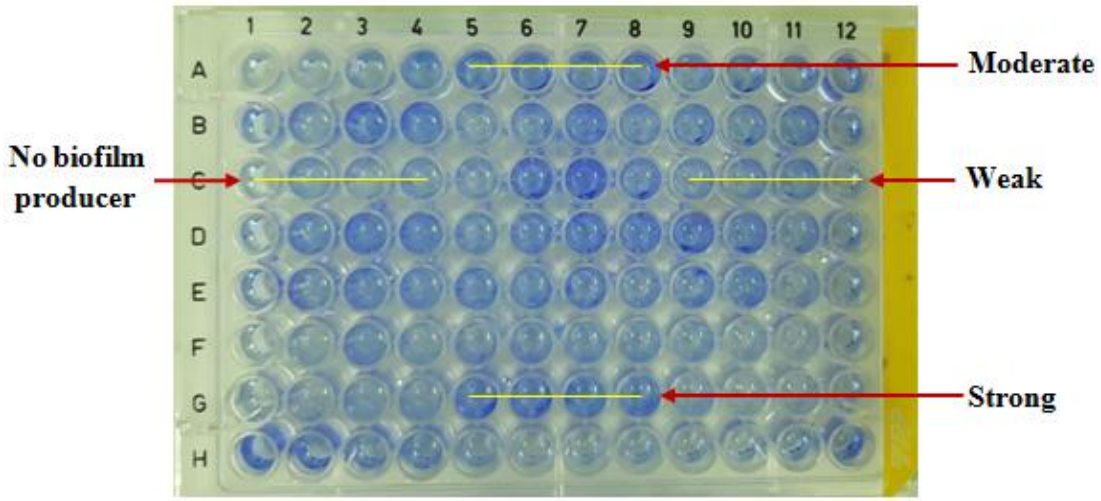

Figure 4.7. Screening of biofilm producers of representative *Cronobacter* isolates by the microtiter plate assay in this study. Moderate, *C. sakazakii* TT-1; No biofilm producer, *C. dublinensis* P-2; Weak, *C. sakazakii* KV-1; Strong, *C. malonaticus* PS-3.

4.5 Genetic Variation of *Cronobacter* spp. using ERIC-PCR

In this study, the results of the ERIC-PCR showed the 3 to 12 amplification bands among the 64 *Cronobacter* isolates and *C. sakazakii* ATCC 29544 as a reference strain. The dendogram of the ERIC-PCR fingerprints revealed that the all *Cronobacter* could be divided into the 12 genotype. The ERIC-PCR revealed 7 clusters consisting of the 60 isolates at the 100% similarity level (Figure 4.9). Visual comparison of the banding patterns showed 10 distinct ERIC profiles for the *Cronobacter* isolates. The number of amplified bands and its size were presented on gel electrophoresis as observed in the Figure 4.8. The number of amplified bands varied from 3 to 12 which included the profile 1 to profile 10 as seen in the Figure 4.8, respectively. Of the 64 *Cronobacter* isolates, 33 belonged to profile 4. According to ERIC-PCR analysis, *Cronobacter* isolates and *C. sakazakii* ATCC 29544 strain produced different DNA fragments of sizes ranging from 100 to 3000 bp.

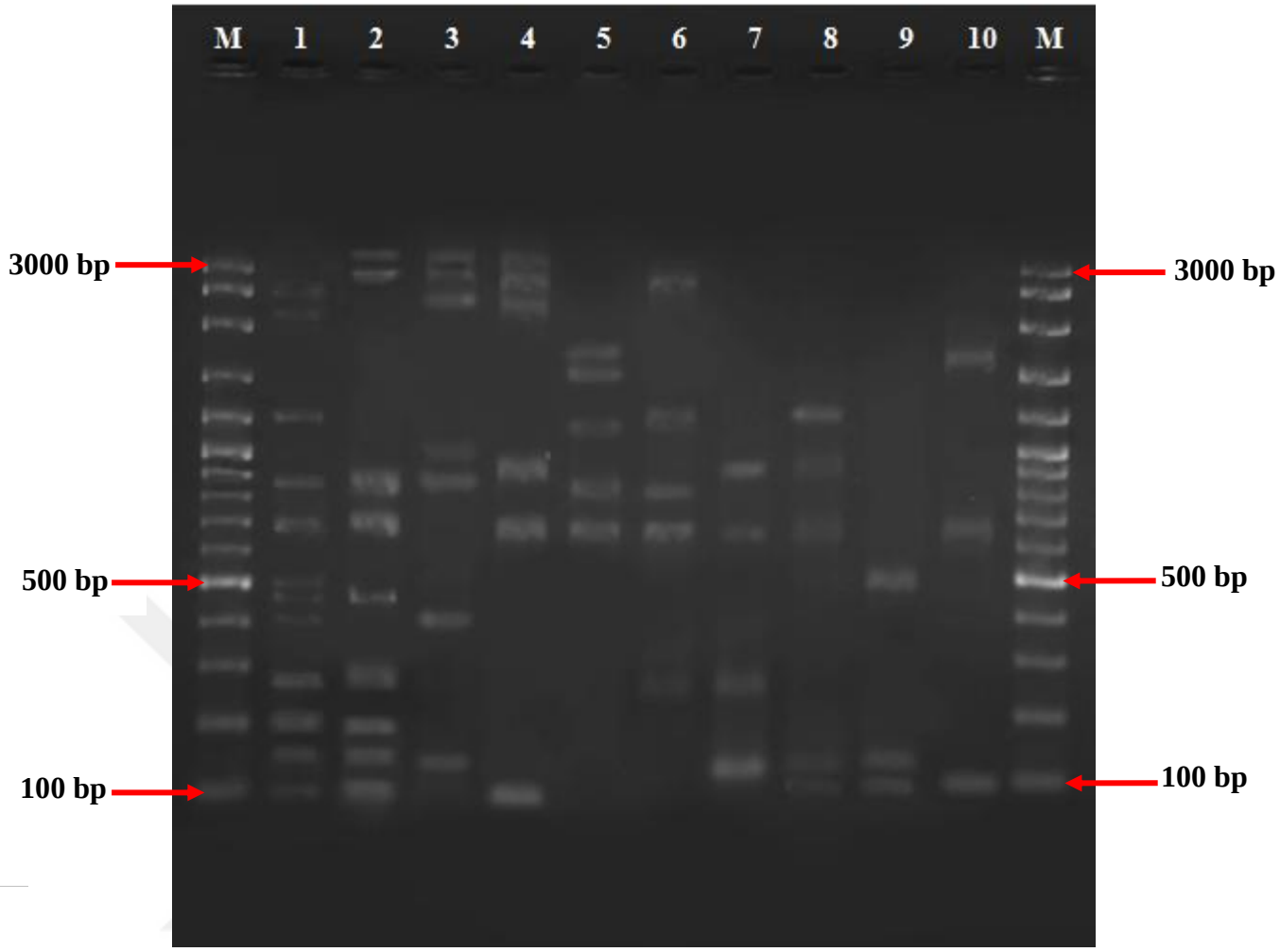

Figure 4.8. ERIC-PCR profiles of *C. sakazakii* ATCC 29544 and *Cronobacter* isolates from ready-to-eat foods on 1% agarose gel. M: Molecular weight marker 100 bp DNA Ladder (Thermo Scientific). Lanes 1 to 10 includes 3 to 12 bands of ERIC-PCR, respectively.

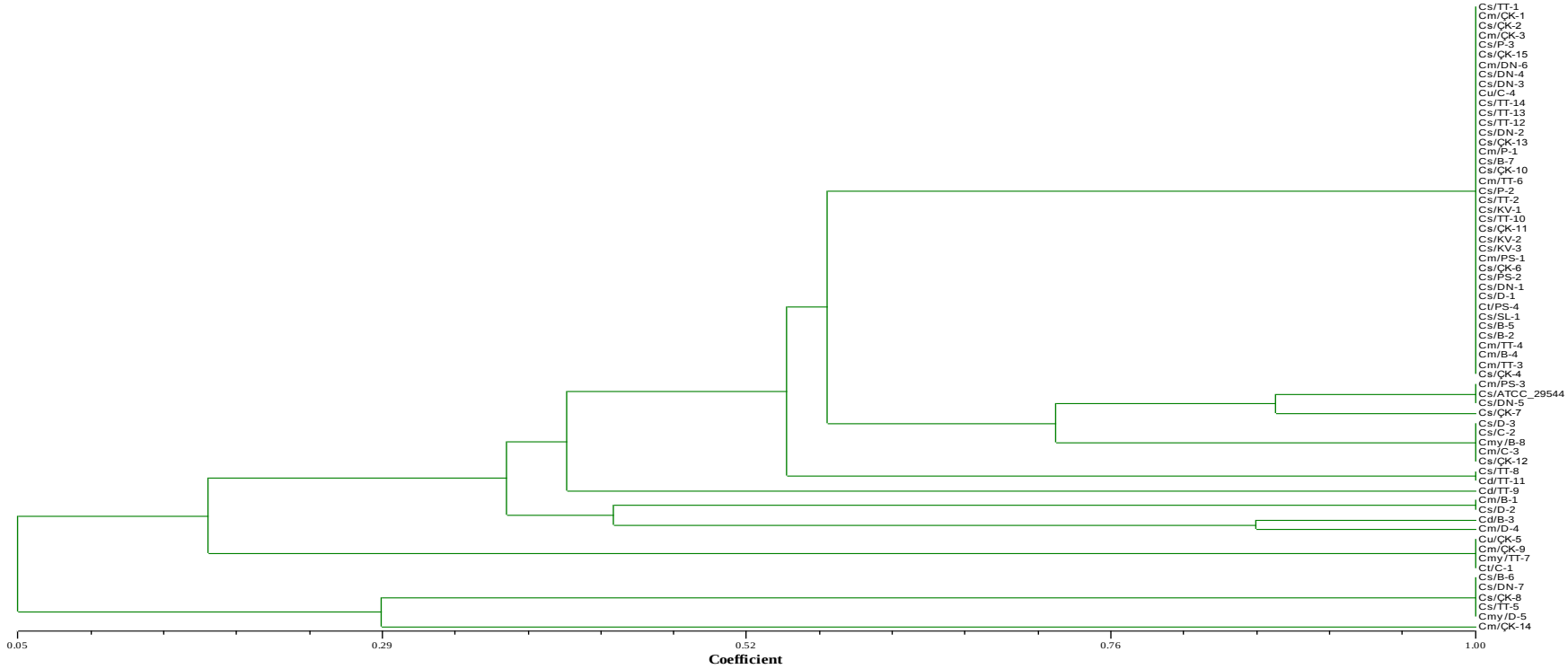

Figure 4.9. Dendogram showing genetic relationships among the 64 *Cronobacter* isolates originated from ready-to-eat foods and the reference strain based on ERIC-PCR fingerprints. Cluster analysis was performed by UPGMA method on a Dice similarity coefficient. Cs, *C. sakazakii*; Cm, *C. malonaticus*; Cu, *C. universalis*; Ct, *C. turicensis*; Cmy, *C. muytjensii*; Cd, *C. dublinensis*. ÇK, meat free cig kofte; TT, dessert; DN, doner; PS, pastrami; KV, kavurma; SL, salad; C, cereal; B, spice; P, cheese; D, ice cream.

4.6 Antimicrobial resistance

In this research, the antimicrobial susceptibility of the 64 *Cronobacter* spp. isolated from ready-to-eat foods to 18 various antimicrobial agents was examined using the disk diffusion method. The *Cronobacter* isolates indicated distinct levels of antimicrobial resistance. Most isolates had resistance of 81.3% against the cephalothin. Besides, the isolates showed resistance of 32.8% and 20.3% for cefoxitin and ampicillin, respectively. All the *Cronobacter* isolates were sensitive to trimethoprim/sulfamethoxazole (100%) and gentamicin (100%). Among the all *Cronobacter* isolates, the highest susceptibility was found to meropenem (96.9%), chloramphenicol (96.9%) and tetracycline (96.9%) followed by amikacin (95.3%), ciprofloxacin (95.3%) imipenem (93.8%), aztreonam (93.8%), piperacillin/tazobactam (93.8%). Besides, all the *Cronobacter* isolates susceptible to amoxicillin clavulanic acid (87.5%), piperacillin (81.3%), nalidixic acid (81.3%), cefepime (73.4%), cefotaxime (67.2%) (Table 4.3). In addition, the *C. sakazakii* isolates were also resistant to cephalothin with the rate of 92.3% and to cefoxitin with the rate of 30.8% (Table 4.4). The profiles of multidrug resistance were detected in 12 (18.8%) of the *Cronobacter* isolates to at least three or more antimicrobial agents. Among the 60 (93.75%) *Cronobacter* isolates that were resistant to at least one or more antimicrobial agents, the 15 different resistance patterns were found (Table 4.5). Out of the 64 *Cronobacter* isolates, the four isolates were not resistant against these 18 antimicrobial agents in our study. The percentage of antimicrobial susceptibilities of the 64 *Cronobacter* spp. is shown in Figure 4.10. The 39 *C. sakazakii* isolates which are resistant to 18 antimicrobial agents tested is presented in Figure 4.11.

Table 4.3. Antimicrobial susceptibilities of the 64 *Cronobacter* isolates from ready-to-eat foods

Antimicrobial class	Antimicrobial agents (Concentration)	No. (%)		
		Resistant	Intermediate	Susceptible
Penicillins	Ampicillin (10 μg)	13 (20.3)	11 (17.2)	40 (62.5)
	Piperacillin (100 μg)	1 (1.5)	11 (17.2)	52 (81.3)
β-lactamase inhibitors	Amoxicillin clavulanic acid (20/10 μg)	3 (4.7)	5 (7.8)	56 (87.5)
	Piperacillin-tazobactam (110 μg)	0 (0)	4 (6.2)	60 (93.8)
Cephalosporins	Cephalothin (30 μg)	52 (81.3)	7 (10.9)	5 (7.8)
	Cefotaxime (30 μg)	8 (12.5)	13 (20.3)	43 (67.2)
	Cefoxitin (30 μg)	21 (32.8)	21 (32.8)	22 (34.4)
	Cefepime (30 μg)	3 (4.7)	14 (21.9)	47 (73.4)
Carbapenems	Imipenem (10 μg)	0 (0)	4 (6.2)	60 (93.8)
	Meropenem (10 μg)	0 (0)	2 (3.1)	62 (96.9)
Monobactam	Aztreonam (30 μg)	4 (6.2)	0 (0)	60 (93.8)
Aminoglycosides	Gentamicin (10 μg)	0 (0)	0 (0)	64 (100)
	Amikacin (30 μg)	0 (0)	3 (4.7)	61 (95.3)
Tetracyclines	Tetracycline (30 μg)	0 (0)	2 (3.1)	62 (96.9)
Quinolones	Nalidixic acid (30 μg)	3 (4.7)	9 (14)	52 (81.3)
	Ciprofloxacin (5 μg)	0 (0)	3 (4.7)	61 (95.3)
Folate pathway inhibitors	Trimethoprim-sulphamethoxazole (1.25/23.75 μg)	0 (0)	0 (0)	64 (100)
Phenicols	Chloramphenicol (30 μg)	0 (0)	2 (3.1)	62 (96.9)

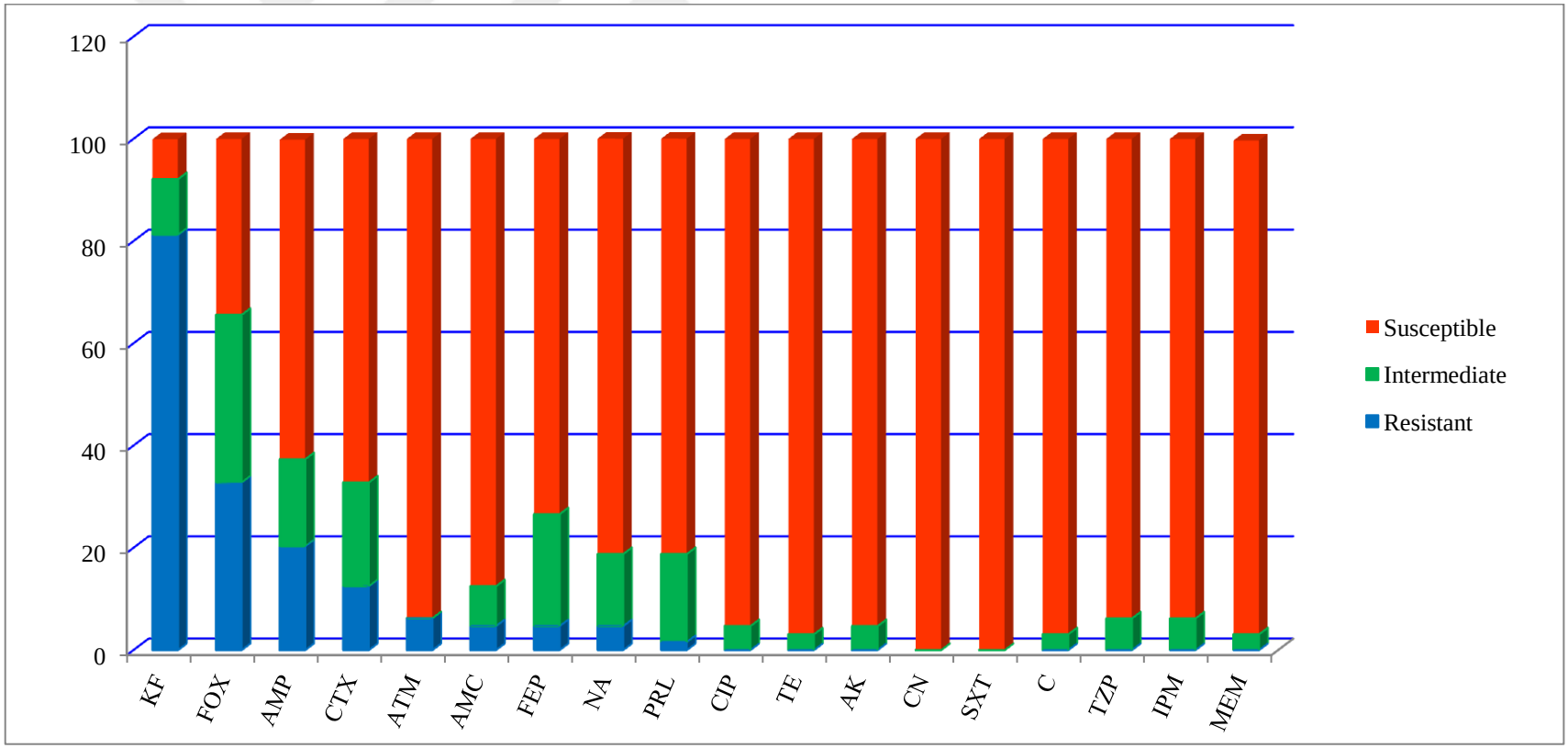

Figure 4.10. Antimicrobial susceptibility of the *Cronobacter* isolates from ready-to-eat foods. AMP, ampicillin; AMC, amoxicillin-clavulanic acid; KF, cephalothin; FOX, cefoxitin; CTX, cefotaxime; FEP, cefepime; NA, nalidixic acid; CN, gentamicin; AK, amikacin; TE, tetracycline; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; C, chloramphenicol; IPM, imipenem; MEM, meropenem; PRL, piperacillin; TZP, Piperacillin/tazobactam; ATM, aztreonam.

Table 4.4. Antimicrobial susceptibilities of the 39 *C. sakazakii* from ready to-eat foods

Antimicrobial class	Antimicrobial agents (Concentration)	No. (%)		
		Resistant	Intermediate	Susceptible
Penicillins	Ampicillin (10 μg)	4 (10.3)	7 (18)	28 (71.7)
	Piperacillin (100 μg)	0 (0)	2 (5.1)	37 (94.9)
β-lactamase inhibitors	Amoxicillin clavulanic acid (20/10 μg)	0 (0)	3 (7.7)	36 (92.3)
	Piperacillin-tazobactam (110 μg)	0 (0)	1 (2.6)	38 (97.4)
Cephalosporins	Cephalothin (30 μg)	36 (92.3)	3 (7.7)	0 (0)
	Cefotaxime (30 μg)	6 (15.4)	9 (23.1)	24 (61.5)
	Cefoxitin (30 μg)	12 (30.8)	18 (46.1)	9 (23.1)
	Cefepime (30 μg)	3 (7.7)	11 (28.2)	25 (64.1)
Carbapenems	Imipenem (10 μg)	0 (0)	2 (5.1)	37 (94.9)
	Meropenem (10 μg)	0 (0)	1 (2.6)	38 (97.4)
Monobactam	Aztreonam (30 μg)	1 (2.6)	0 (0)	38 (97.4)
Aminoglycosides	Gentamicin (10 μg)	0 (0)	0 (0)	39 (100)
	Amikacin (30 μg)	0 (0)	3 (7.7)	36 (92.3)
Tetracyclines	Tetracycline (30 μg)	0 (0)	1 (2.6)	38 (97.4)
Quinolones	Nalidixic acid (30 μg)	2 (5.1)	7 (18)	30 (76.9)
	Ciprofloxacin (5 μg)	0 (0)	3 (7.7)	36 (92.3)
Folate pathway inhibitors	Trimethoprim-sulphamethoxazole (1.25/23.75 μg)	0 (0)	0 (0)	39 (100)
Phenicols	Chloramphenicol (30 μg)	0 (0)	0 (0)	39 (100)

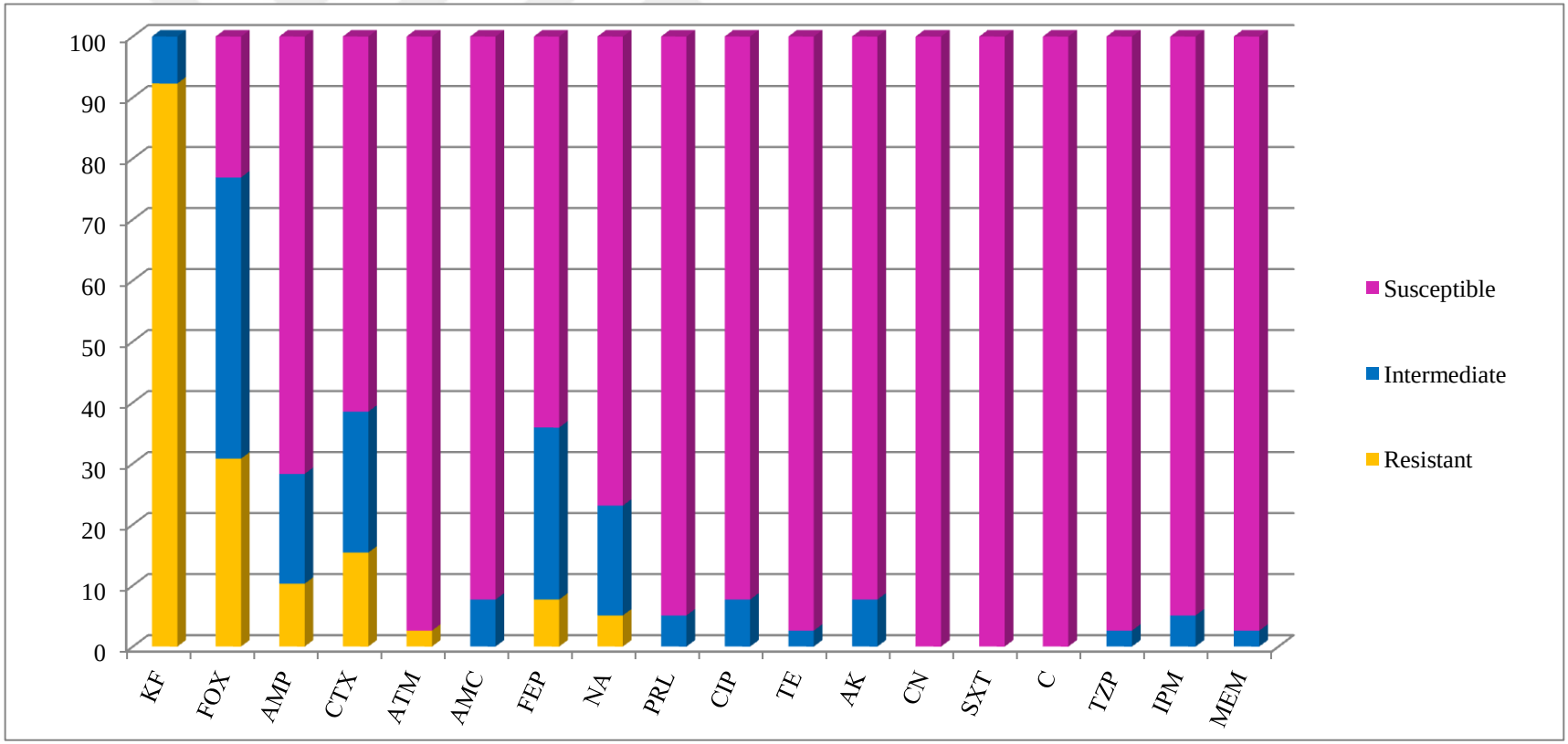

Figure 4.11. Antimicrobial susceptibility of the *C. sakazakii* isolates from ready-to-eat foods. AMP, ampicillin; AMC, amoxicillin-clavulanic acid; KF, cephalothin; FOX, cefoxitin; CTX, cefotaxime; FEP, cefepime; NA, nalidixic acid; CN, gentamicin; AK, amikacin; TE, tetracycline; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; C, chloramphenicol; IPM, imipenem; MEM, meropenem; PRL, piperacillin; TZP, Piperacillin/tazobactam; ATM, aztreonam.

Table 4.5. Resistance pattern among *Cronobacter* spp. isolated from ready-to-eat foods

Resistance phenotype	Resistance pattern	Number of isolates (%)
P1	KF	21 (32.9%)
P2	FOX	1(1.6%)
P3	CTX	1(1.6%)
P4	AMP	5 (7.8%)
P5	AMP, ATM	1(1.6%)
P6	KF, CTX	2 (3.1%)
P7	KF, FOX	11 (17.2%)
P8	KF, FEP	2 (3.1%)
P9	KF, AMP	4 (6.25%)
P10	KF, ATM, NA	2 (3.1%)
P11	KF, FOX, CTX	5 (7.8%)
P12	KF, ATM, PRL	1 (1.6%)
P13	KF, AMC, FOX	1 (1.6%)
P14	KF, AMP, AMC, FOX	2 (3.1%)
P15	KF, AMP, FOX, FEP, NA	1 (1.6%)

AMP, ampicillin; AMC, amoxicillin-clavulanic acid; KF, cephalothin; FOX, cefoxitin; CTX, cefotaxime; FEP, cefepime; NA, nalidixic acid; PRL, piperacillin; ATM, aztreonam

Table 4.6. Distribution of antimicrobial resistance among *Cronobacter* species from ready-to-eat foods

***Cronobacter* isolates (n)**	AMP	AMC	KF	FOX	CTX	FEP	NA	CIP	AK	CN	TE	C	IPM	MEM	PRL	TZP	ATM	SXT
C. sakazakii (n=39)	4	0	36	12	6	3	2	0	0	0	0	0	0	0	0	0	1	0
C. malonaticus (n=15)	3	2	12	8	2	0	1	0	0	0	0	0	0	0	1	0	2	0
C. turicensis (n=2)	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
C. muytjensii (n=3)	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. universalis (n=2)	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. dublinensis (n=3)	1	1	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	13	3	52	21	8	3	3	0	0	0	0	0	0	0	1	0	4	0

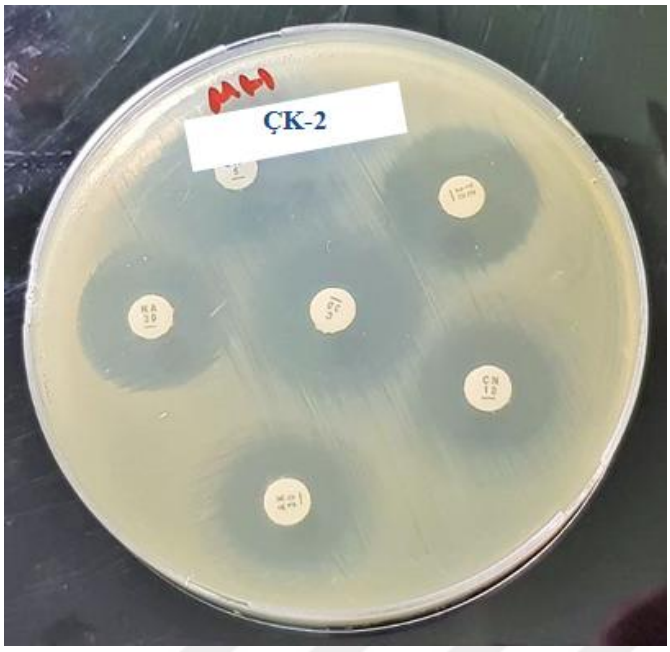

Figure 4.12. Antimicrobial susceptibility test to amikacin, gentamicin, nalidixic acid, tetracycline, chloramphenicol and ciprofloxacin. Representative *Cronobacter* isolate, *C. sakazakii* ÇK-2.

Figure 4.13. Antimicrobial susceptibility test to meropenem, imipenem, aztreonam, piperacillin, trimethoprim-sulfamethoxazole and piperacillin-tazobactam. Representative *Cronobacter* isolate, *C. sakazakii* ÇK-2.

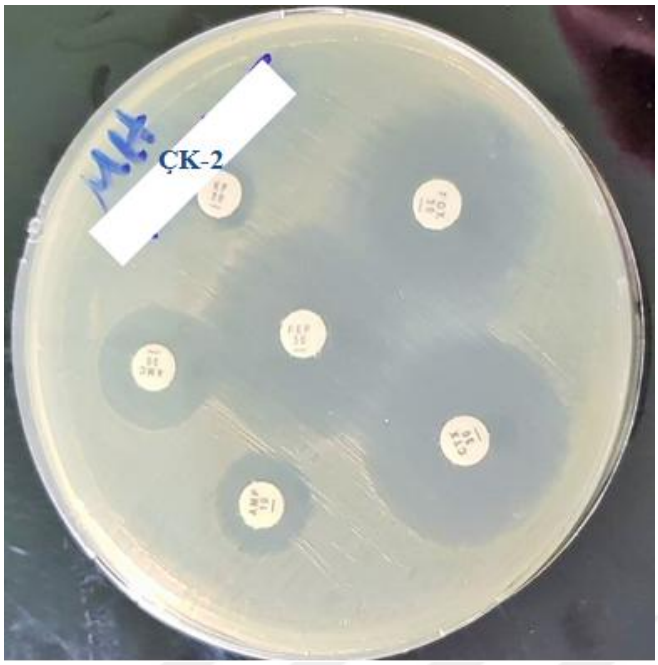

Figure 4.14. Antimicrobial susceptibility test to ampicillin, amoxicillin clavulanic acid, cefotaxime, cefepime and cephalothin. Representative *Cronobacter* isolate, *C. sakazakii* ÇK-2.

5. DISCUSSION

Foodborne diseases can occur due to the consumption of food contaminated with bacteria. *Cronobacter* spp., which causes the important diseases among which there are meningitis, bacteremia and necrotizing enterocolitis, threats the public health. The monitoring the presence of *Cronobacter* in foods, a foodborne pathogen, may contribute to decrease the threat of human health.

There have been many ways to define and identify the *Cronobacter* spp. Using both molecular and conventional methods together strengthen the confirmation of *Cronobacter*. In this study, therefore, it was preferred to identify isolated bacteria not only with biochemical tests but also with three unique target genes for specific determination of *Cronobacter* spp.; the 16S rRNA, *gluA* and *rpoB* species-specific genes. In this research, the 64 *Cronobacter* isolates, obtained from the 340 samples of ready-to-eat food, were detected using the FDA and modified ISO methods. These isolates were identified as 39 (61%) *C. sakazakii*, 15 (23.4%) as *C. malonaticus*, 3 (4.7%) as *C. muytjensii*, 2 (3.1%) as *C. turicensis*, 3 (4.7%) as *C. dublinensis* and 2 (3.1%) *C. universalis* by the confirmation of target genes and biochemical tests.

It is very useful to apply the 16S rRNA gene for identification and taxonomic differentiation of bacterial species. In this research, all tested *Cronobacter* was specifically identified by using the 16S rRNA. These findings were similar to results of Hassan et al. (2007), Cawthorn et al. (2008) and Mohammed et al. (2015) who reported that the primer pair Saka1/Saka2b were able to detect all the isolates they tested. Though the 16S rRNA gene is the most frequently used gene, it is not the only gene method in molecular microbial ecology. It is considered that the *gluA* gene is an additional tool for identification of *Cronobacter* spp. It was reported in the study of Iversen et al. (2007b) that *gluA* gene PCR assays were 100% sensitive and specific for determination and confirmation of *Cronobacter* spp. However, in this research, out of the 64 isolates, the 59 (92.2%) isolates were positive for the *gluA* gene and this gene was not detected in the five isolates. These results were also similar to the findings of Jaradat et al. (2009) and Mofokeng et al. (2010) who reported that the

gluA gene was not detected in some of *Cronobacter* strains tested. On the other hand, species-specific primers of the *rpoB* gene which encodes the bacterial RNA polymerase β-subunit were used for describing the species of *Cronobacter*. In this research, the species of *Cronobacter* were successfully defined according to *rpoB* gene. Several studies were reported to describe the identification of *Cronobacter* using the species specific PCR targeting *rpoB* gene (Jackson and Forsythe, 2016; Ezeh et al., 2016; Svobodová et al., 2017).

In the present study, the 340 ready-to-eat foods in twelve different groups were analyzed and in the 59 samples *Cronobacter* spp. contamination was detected. The prevalence of *Cronobacter* spp. in ready-to-eat food samples was detected to be 17.4%. Of the contaminated samples, the 64 isolates were identified. Out of twelve ready-to-eat food categories, ten were contaminated with the *Cronobacter*. The prevalence of *Cronobacter* spp. was 6 (12.2%) in the 49 doners, 13 (30.2%) in the 43 desserts, 4 (9.8%) in the 41 pastramis, 3 (7.5%) 40 cheeses, 1 (2.6%) in the 38 salad, 3 (8.6%) in the 35 kavurmas, 14 (51.9%) in the 27 meat free cig koftes, 4 (14.8%) in the 27 ice creams, 7 (46.7%) in the 15 spices and 4 (30.8%) in the 13 cereals, but herbs and vegetables did not harbor *Cronobacter* spp. It was similar to the findings of this study that in the study of Xu et al. (2015) the prevalence of *Cronobacter* spp. contamination in 280 ready-to-eat foods was 52 (18.6%). In Turkey, Aksu et al. (2016) reported that prevalence of *Cronobacter* spp. were found to be 17.8%. In some of the previous studies, the prevalence of *Cronobacter* spp. in ready-to-eat foods was detected lower than the results of this study. For instance, in Switzerland, *Cronobacter* spp. were detected in 9.0% from ready-to-eat foods (Baumgartner et al., 2009). In Prague, it was reported by Hochel et al. (2012) that the prevalence of *Cronobacter* was 13% in retail foods. Moreover, in the researches of Yao et al. (2016) and Li et al. (2014), *Cronobacter* was isolated in other food types in rate of 12% and 6.7%, respectively. On the other hand, compared with the studies of Restaino et al. (2006) and Singh et al. (2015), the prevalence of *Cronobacter* was higher than the present research. The different incidence rates of bacteria in food samples have been reported in previous studies (Kandhai et al., 2004; Restaino et al., 2006; Mullane et al., 2007; Baumgartner et al., 2009; Hochel et al., 2012; Xu et al., 2015; Singh et al. 2015). The variation in the occurrence of bacteria can be explained by different factors such as source of the isolation, nature of samples, geographic

location and contamination from human handlers, environment and water (Fanning and Forsythe, 2008; Kanyerere et al., 2012; Osman et al., 2012).

C. sakazakii is most common among the species of the *Cronobacter* genus and plays a significant role in human diseases (Muytjens et al., 1988). Furthermore, *C. sakazakii* has been implicated in the most common pathogens (Iversen and Forsythe, 2003). In the present study, out of 64 *Cronobacter* isolates obtained, 39 (61%) belong to the *C. sakazakii* which was clearly much higher than the other species among the identified all of the *Cronobacter* isolates. Generally, *C. sakazakii* has been reported as the dominant species in foods in the previous studies (Strydom et al., 2011; Aksu et al., 2016), similar to our results. *C. malonaticus* is also one of the species of *Cronobacter* commonly connected to adult infections. In the present study, 15 (23.4%) of the 64 *Cronobacter* isolates were identified as the *C. malonaticus*. On contrary to this research, the presence of the *C. malonaticus* in various foods was found in lower levels in the studies of Terragno et al. (2009) and Strydom et al. (2011). In this study, out of all isolates, 3 (4.7%) as *C. muytjensii*, 2 (3.1%) as *C. turicensis*, 3 (4.7%) as *C. dublinensis* and 2 (3.1%) *C. universalis* were also found in ready-to-eat food. When it is compared with the study of Aksu et al. (2016), 13 (48.1%), 6 (22.2%), 5 (18.5%) and 3 (11.1%) of the 27 *Cronobacter* isolates were defined as *C. sakazakii*, *C. muytjensii*, *C. turicensis* and *C. malonaticus*, respectively. In the study of Strydom et al. (2011), it was found at the level of 82.5% *C. sakazakii*, of 8% *C. malonaticus*, of 5% *C. muytjensii*, of 3% *C. dublinensis* and of 1.5% *C. turicensis*. In the study of Hochel et al. (2012), 399 samples of retail foods were analyzed and 52 samples of *Cronobacter* contamination, of which 54.7% were by *C. sakazakii*, 28.4% were by *C. malonaticus*, and 7.5% were by *C. muytjensii* and *C. dublinensis*, were found.

In the previous studies, different rates of the presence of the *Cronobacter* in food has been documented in many countries such as in Korea, Slovakia, South Africa, Holland, Spain, Egypt, Germany by several researchers (Iversen et al., 2004; Hassan et al., 2007; Oh et al., 2007; Turcovsky et al., 2011; Lee et al., 2012; Mohammed et al., 2015). Furthermore, *Cronobacter* spp. have been frequently isolated from the environment (Kandhai et al., 2004; Mullane et al., 2007), clinics (Lai, 2001; Kandhai et al., 2010), powdered infant formula and milk powders

(Muytjens et al., 1988; Iversen and Forsythe, 2004, 2007; Cawthorn et al., 2008), animals (Kuzina et al., 2001; Hamilton et al., 2003) as well as from ready-to-eat foods and other foods (Gurtler et al., 2005; Mullane et al., 2006; Friedemann et al., 2007; Baumgartner et al., 2009; Lee et al., 2012; Li et al., 2014).

Recently, several researchers have also found that *Cronobacter* could be isolated from cereals, spices and meat products (Lee et al., 2012; Li et al., 2014; Yao et al., 2016). *Cronobacter* was found in 5.5% of cereal products such as starch, rice, semolina, oats and flour (Gurtler et al., 2005). In other study, *Cronobacter* was isolated from 6 of 18 (33.3%) dry infant cereals (Restaino et al., 2006). Molloy et al. (2009) isolated *C. sakazakii* from nine of twenty organic cereals. *Cronobacter* isolates were obtained from dry cereals (6/123), raw minced meats (7/222) and spices (1/28) according to Kandhai et al. (2010). In a study of Jaradat et al. (2009), *Cronobacter* had a high prevalence (38.8%) in herbs and spices. According to Mohammed et al. (2015), the 16% of ground beef and 15% of beef burger were positive for *Cronobacter*. According to results of this study, *Cronobacter* spp. were particularly high, 30.8% (13/4) and 46.7% (15/8), in cereals and spices, respectively. Indeed, the *Cronobacter* had also a higher prevalence (51.9%) in meat free cig koftes in this study. In the light of our results and results of several studies mentioned above, it was believed that the high level of contamination of meat free cig koftes may be the result from the contamination ingredients cereal and spices together. Furthermore, the high incidence rate of *Cronobacter* spp. in meat free cig koftes may be due to improper food handling practices, storage conditions and environmental factors.

Cronobacter, as a pathogen microorganism, can have virulence properties and these are very important for its pathogenicity. *Cronobacter* has able to produce many virulence determinants including lipases, DNase, endotoxins, exotoxins, hemagglutinin and attachment proteins (Livrelli et al., 1996; Keller et al., 1998, Pagotto et al., 2003). The outer membrane proteins as a virulence factor help for nutrient depuration and avoidance of host defense mechanisms (Rollauer et al., 2015). Many studies indicated that outer membrane protein A contribute to virulence of *Cronobacter* and reported that *ompA* gene was used to determine the pathogenicity of *Cronobacter* spp. (Terragno et al., 2009; Kandhai et al., 2010;

Fakruddin et al., 2014). In the present study, the *Cronobacter* harbored the *ompA* gene of 63 (98.5%) but the *ompA* gene was not detected in only one isolate. Terragno et al. (2009) detected the *ompA* gene in all *Cronobacter* isolates, similar to our results. Similarly, the *ompA* gene was harbored in all *C. sakazakii* isolates from milk and milk product by study of Sharma and Prakash (2013a) in Agra region. All the *Cronobacter* strains expressed the *ompA* gene by real-time PCR (Kandhai et al., 2010). On contrary to our study, Fakruddin et al. (2014) revealed that only one *C. sakazakii* strain had the *ompA* gene. In the study of Yan et al. (2011), the *ompA* gene was harbored level of 64.7% in *Cronobacter* strains, obtained from the clinical, food and environmental, were lower than the levels in our research. Moreover, Kothary et al. (2007) announced that *Cronobacter* has a zinc-containing metalloprotease and this protein is factor for virulence of *Cronobacter*. Several reports have documented the presence of the *zpx* gene, a virulence gene, for *Cronobacter* (Fakruddin et al., 2014; Jackson et al., 2014; Eshwar et al., 2016). In this study, the *zpx* gene was found in 63 (98.5%) isolates. However, 33.3% of the *Cronobacter* strains from various food samples carried the *zpx* gene (Fakruddin et al., 2014). In another study, the detection rate of *zpx* gene was so low that only 18.2% of the *Cronobacter* strains were positive (Jackson et al., 2014). In the research of Awadallah et al. (2018), the *zpx* gene was harbored in *Cronobacter* isolates, obtained from the animal feces, was extremely lower than the levels in our research.

Cronobacter has some virulence properties which may help its abilities to adhesion, invasion, and survival in the host (Jaradat et al., 2014). Siderophore which is required for the iron uptake is virulence factor for *Cronobacter* (Grim et al., 2012). In this research, all the isolates were positive for siderophore production. There were several studies which reported siderophore production of *Cronobacter* species (Schmid et al., 2009; Fakruddin et al., 2014). In the study of Fakruddin et al. (2014), all *C. sakazakii* isolates from food samples were found to be positive for the siderophore production, similar to the results of this research. In other study, Schmid et al. (2009) reported that siderophore production was observed for all of the *Cronobacter* strains except one strain from plant roots. Furthermore, biofilms can lead to bacterial contamination that may cause food spoilage, shelf-life reduction or contamination of food products undergoing processing. Biofilms are considered to be a potential way of pathogen transmission (Lehner et al., 2005; Jaradat et al., 2009). It

has been documented in the studies of Iversen et al. (2004) and Lehner et al. (2005) that the production of biofilms by *Cronobacter* spp. could occur on the surface of inanimate environments consisted of silicon, latex, stainless steel and glass. In the present study, out of the 64 *Cronobacter* isolates, the 56 (87.5%) were found to be positive for biofilm formation that the data of the *Cronobacter* isolates for the biofilm formation were interpreted according to Stepanovic´ et al. (2000). Therefore, the biofilm formation of the isolates was as followed: 2 (3.1%) as strong, 20 (31.2%) as moderate, 34 (53.1%) as weak and 8 (12.5%) non-biofilm producer in this research. Biofilm formation was found at the rate of 50% according to the study of Fakruddin et al. (2014) whose results were lower than our study. In Korea, in the study of Lee et al. (2012) was found that out of 33 *C. sakazakii* isolates, 11 showed intermediate biofilm formation and 8 indicated strong biofilm formation from various foods in contrary to our research. In the study of Oh et al. (2007), it was reported that biofilm formation of *Cronobacter* was detected as a lower ratio (16%) in diluted Tryptic Soy Broth, in contrast to our study.

The genetic diversity of *Cronobacter* isolates in ready-to-eat food products was assessed by ERIC-PCR in the present research. Among the molecular techniques, ERIC-PCR is simple, rapid and inexpensive with an acceptable outcome. ERIC-PCR is a useful method for genotyping of the bacteria including *Cronobacter* spp. In several studies, ERIC-PCR has been used for typing and discriminating the strains of *Cronobacter* from food such as dry food samples (Ye et al., 2008), milk, powdered milk and infant food (Chen et al., 2013), milk powder, malted milk drink, honeys, chutney, chocolates and biscuits (Fakruddin et al., 2014). Using ERIC-PCR, the determination the level of genetic diversity of food isolates can be important to detect a virulent strain with a known fingerprint and to trace its dissemination (Burr et al., 1998). The results of a study reported by Ye et al. (2009) showed that ERIC-PCR analysis of *Cronobacter* strains can be useful for monitoring the microbial contamination and tracking of pathogens. Fakruddin et al. (2014) demonstrated that all *C. sakazakii* strains from food belonged to different genotypes based on the ERIC-PCR, similar to our results. In other study, genetic patterns of cluster analysis from ERIC-PCR showed that there were differences among *Cronobacter* spp. from different vegetables (Chen et al., 2016). In China, genetic variability of the *Cronobacter* isolates has been demonstrated from many sources including food

(Chen et al., 2013). In this study, data indicated the absence of a direct relationship between the ERIC-PCR fingerprint patterns in terms of their sampling origin. In this research, the *C. sakazakii*, *C. malonaticus* and *C. turicensis* isolates harbored virulence factors associated with infections were grouped into the profiles 1 to 9 except the profile 10 using ERIC-PCR, indicating that there was no correlation between the presence of virulence determinants and a specific ERIC-PCR profile. Consequently, the ERIC-PCR may be beneficial in the molecular characterization of *Cronobacter* spp. from various foods (Casarez et al., 2007; Fakruddin et al., 2014).

The resistance of the bacteria against the antimicrobial agents, used for human treatment, is increased by exposition of bacteria to extremely concentration of antimicrobial. The occurrence of antimicrobial resistant bacteria in food causes an increasing risk for human health. *Cronobacter* spp. have been susceptible to commonly used antimicrobials such as aminogylcosides, ureidopenicillins, ampicillin, and carboxypenicillins (Adamson and Rogers, 1981; Nazarowec-White and Farber, 1997; Gurtler et al., 2005). However, it has been documented that *Cronobacter* spp. were resistant to cephalothin, cefotaxime, and streptomycin (Farmer et al., 1980; Xu et al., 2015; Yao et al., 2016). In the present research, the antimicrobial susceptibility of the 64 *Cronobacter* isolates from ready-to-eat foods to 18 various antimicrobial agents was examined. In our study, the *Cronobacter* isolates obtained from ready-to-eat food were resistant against cephalothin at the level of 81.3%. In accordance with the finding reported in literature (Chon et al., 2012), the *Cronobacter* isolates from desiccated food were resistant to cephalothin (77.8%). However, Li et al. (2014) indicated susceptible to cephalothin in the *Cronobacter* spp. from various foods, contrast to our results. In addition, Xu et al. (2015) reported that in *Cronobacter* from ready-to-eat foods, level of resistant to cephalothin was lower than our study. The susceptibility level of cefotaxime (100%) and cefoxitin (89.5%) in the study of Huang et al. (2015) were higher than the levels in our research. High levels of meropenem and aztreonam resistance in *Cronobacter* isolates have been reported by Mardaneh and Dallal (2017). However, susceptibility to cefepime and imipenem was found to be slightly lower in the present study than the obtained by Mardaneh and Dallal (2017). The resistance of chloramphenicol, tetracycline and ciprofloxacin, which were not determined in our study, was revealed by Yao et al. (2016). Whereas the levels of piperacillin and amikacin in the study of

Fakruddin et al. (2014) were lower than our research, piperacillin/tazobactam and nalidixic acid levels of it were higher. *C. sakazakii*, as an opportunistic pathogen, carries an important risk for human health. In this study, the resistance to cefoxitin and nalidixic acid was detected in 30.8% and 5.1% of the *C. sakazakii* isolates, respectively. These isolates were found to be susceptible to gentamicin (100%), tetracycline (97.4%) and ciprofloxacin (92.3%). Several studies have reported the resistance at different rates to some antimicrobial agents for *C. sakazakii* (Kilonzo-Nthenge et al., 2012; Fei et al., 2017). In contrast to our results, in the study of Kilonzo-Nthenge (2012), the *C. sakazakii* isolates obtained from domestic kitchens were highly resistant to tetracycline, ciprofloxacin and nalidixic acid, but the resistance of cefoxitin was significantly low. However, in the research of Sharma et al. (2013b), susceptibility to gentamicin and ciprofloxacin was found in all *C. sakazakii*, similar to this study.

Prompt and efficient treatment is required for meningitis. It is very important to know antimicrobial susceptibility for *Cronobacter* infections of neonates and for their treatment. Lai (2001) and, Willis and Robinson (1988) suggested the β-lactams and the combinations of either ampicillin with gentamicin or chloramphenicol for the treatment of the infections of *Cronobacter*, but the efficiency of this treatment decreased in the following years (Muytjens et al., 1983). However, this circumstance, attributed to the low level of penetration of these antibiotics into the central nervous system (Willis and Robinson, 1988), was not the result of the antibiotic resistance. In either case, to circumvent antibiotic resistance and achieve better penetration, the treatment of *Cronobacter* infections has shifted to extended-spectrum cephalosporins, sometimes in combination with a second agent such as trimethoprim (Block et al., 2002). As mentioned before, in the present study, the cephalosporins used for *Cronobacter* treatment had resistance until the fourth generation. According to the findings of Nazarowec-White and Farber (1999) and Kuzina et al. (2001), all the *Cronobacter* strains were susceptible to ampicillin, contrary to our findings. Similarly, in the research of Lee et al. (2012) that the isolates, identified as *Cronobacter*, were susceptible to ampicillin (98.7%). In the study of Yao et al. (2016), it was indicated that the isolates were resistant to trimethoprim/sulfamethoxazole while no resistance to this antimicrobial was identified in our research. However, Li et al. (2014) reported that all the *Cronobacter*

isolates from various foods were susceptible to trimethoprim/sulfamethoxazole, similar to our results. The gentamicin were found susceptible for all the *Cronobacter* isolates in the study of Huang et al. (2015), similar to this researh, but Xu et al. (2015) documented that the *Cronobacter* isolates from Chinese ready-to-eat foods were resistant to gentamicin at the level of 1.4%.

In the current study, the multidrug resistance to at least three or more antimicrobial agents was detected in the 12 (18.8%) of *Cronobacter* isolates. Moreover, the 15 different resistance patterns were obtained among the 60 (93.75%) *Cronobacter* isolates which were resistant to at least one or more antimicrobial agents (Table 4.5). These patterns representing all the *Cronobacter* isolates showed resistance between one to five antimicrobial agents. The findings of Kim et al. (2008) were contrary to our results that any isolates were resistant against more than one antibiotic.

6. CONCLUSIONS

Microorganisms have the ability of growing and surviving on food as a good source. Pathogenic bacteria are the main cause of food spoilage and foodborne diseases. *Cronobacter* species as a pathogen cause critical diseaeses in immunocompromised individuals, infants and elderly adults. *Cronobacter* spp. can be found in the environment, water, soil, and food. The presence of *Cronobacter* spp. has also been reported in diverse foods including meat, vegetables, milk powder, cheese, kefir, dried foods and spices.

The genus *Cronobacter* is composed of seven species such as C. *sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, *C. universalis* and *C. condimenti*. *Cronobacter* species, especially C. *sakazakii* as an emerging opportunistic pathogen cause life-threatening infections such as necrotizing enterocolitis, bacteremia, and meningitis in immunocompromised individuals, neonates, infants and elderly adults. The common symptoms of *Cronobacter* infections are high fever, headache with nausea, a swelling on the head, stiffness of the body and neck, skin rash, and seizures. The prevalence of infections in human beings caused by *Cronobacter* spp. is not high. However, *Cronobacter* as pathogen has a high mortality rate in involved people.

At present, various methods are availabe for identification and typing of *Cronobacte*r spp. These methods, among which there are 16S rRNA and housekeeping gene sequencing, multilocus sequence typing (MLST), polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), pulse-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD)-PCR, and enterobacterial repetitive intergenic consensus sequence (ERIC)-PCR, have been successfully used for the differentiation and typing of *Cronobacter* species.

In this study, identification of the *Cronobacter* isolates was performed by the phenotypic and genotypic methods including the 16S rRNA, *rpoB* and *gluA* gene amplification. Of the 340 ready-to-eat food samples including doners, desserts,

pastramis, kavurmas, salad, meat free cig koftes, cheeses, ice creams, spices, cereals, herbs, vegetables, 59 were positive for contamination of *Cronobacter* spp. A total of 64 isolates of *Cronobacter* from these contaminated samples were examined for further studies. The number of *Cronobacter* species identified were 39 *C. sakazakii*, 15 *C. malonaticus*, 3 *C. muytjensii*, 2 *C. turicensis*, 2 *C. universalis* and 3 *C. dublinensis*. In this study, the results for identification of *Cronobacter* species by phenotypic methods were consistent with the genotypic methods.

Several virulence factors produced by *Cronobacter* species play an important role in a variety of infections. These factors include enzymes, toxins, adhesion proteins, cell-surface proteins, siderephore production and biofilm formation. Virulence factors help the bacteria to evade the innate immune defense, and antimicrobial resistance mediate survival of the bacteria and tissue invasion at the site of infection. Biofilms play an important role in spoilage of foods, reduction of shelf-life and contamination of foods undergoing processing. Siderophores plays a vital role to overcome the Fe limitations so iron acquisition is a recognized factor related to microbial pathogenicity.

In the present study, the 63 (98.5%) *Cronobacter* isolates were found to be positive for both the *ompA* and the *zpx* virulence genes at same rate. Siderophore production as a virulence determinant was detected in all *Cronobacter* isolates (100%). Of the 64 isolates, 56 (87.5%) were biofilm producers.

Genetic variation of the food-originated isolates was detected by ERIC-PCR as an important molecular marker that has been utilized in epidemiologic studies for tracing the pathogen contamination and infections. In this study, ERIC-PCR fingerprinting analysis demonstrated the variation among the isolates of *Cronobacter* from ready-to-eat food samples. The dendogram of the ERIC-PCR fingerprints revealed that all the *Cronobacter* could be divided into the 12 genotype. ERIC-PCR revealed 7 clusters consisting of the 60 isolates at the 100% similarity level. The results of this study indicated the absence of a direct relationship between the ERIC-PCR fingerprints in terms of their sampling origin. It can be concluded from the current researches that ERIC-PCR type of molecular analysis ought to be developed in details and sustained to improve current monitoring of contamination and the programs of food safety.

In this study, the 64 *Cronobacter* isolates were observed to be against common used 18 antimicrobials at different levels. Multidrug resistance profile for at least three or more antimicrobial agents was observed in 12 (18.8%) of the *Cronobacter* isolates from ready-to-eat food. Among the 60 (93.75%) *Cronobacter* isolates which were resistant to at least one or more antimicrobial agents, the 15 different resistance phenotypes were obtained. The presence of antimicrobial resistant pathogens in foods is significant problem for human health. The emergence of these resistant pathogens and their transmission from food to human is major concern for treatment of infections. It has a great importance to do research for evaluating and discussing the effects of antimicrobial resistance on the health of human beings and animals. More information must be gathered about the risks that can occur with the usage of antimicrobial from the view of constructing the more appropriate risk management strategies.

The present study provides a current data on the presence, identification, genotyping, some virulence properties and antimicrobial resistances of *Cronobacter* spp. including *C. sakazakii*, an emerging foodborne pathogen, in ready-to-eat foods. The consumption of food contaminated with antimicrobial resistant or potential pathogenic *Cronobacter* may cause a serious threat to public health. Therefore, monitoring the prevalence of foodborne pathogens is necessary for improving the food quality, for reducing the incidence of infections and ensuring appropriate hygiene. It is not only effective control measures of food safety hazards for food producers and handlers, but also effective education for consumers to ensure food safety. The desired shelf life and storage conditions are very important to be taught to the consumers for healthy life.

7. REFERENCES

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