Prevalence, phenotypic and genotypic characterization, virulence potential and antimicrobial resistance of Cronobacter species from ready-to-eat foods

Seza Arslan (arslan_s3@ibu.edu.tr)
https://orcid.org/0000-0002-2478-6875
Hafize Gizem Ertürk
Abant Izzet Baysal Universitesi Fen Edebiyat Fakultesi

Research article

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Abstract

Background Cronobacter , an emerging foodborne pathogen, contaminates various foods such as ready-to-eat (RTE) food due to its ubiquitous nature. Consumption of food contaminated with Cronobacter can cause severe infections in children, elderly or immunocompromised people. Therefore, we aimed to assess the presence of Cronobacter spp. in RTE foods, popularly consumed products, and pose potential health threat for consumers, especially for risk groups.Results Out of 340 RTE food samples, 59 (17.4%) were contaminated with Cronobacter spp. in this study. The highest contamination rate was found in meat free cig koftes (51.9%, 14/27), followed by spices (46.7%, 7/15), cereals (30.8%, 4/13), and desserts (30.2%, 13/43). A total of 64 Cronobacter isolates were identified phenotypically and genotypically from the 59 ready-to-eat foods samples. Molecular characterization was accomplished by PCR targeting 16S rRNA, gluA , rpoB and cgcA genes. The 64 Cronobacter isolates were completely identified by the biochemical and rpoB , while the PCR targeting the cgcA gene failed to identify to eight isolates. In this study, we investigated major virulence characteristics contributing to the pathogenicity of Cronobacter spp. including the outer membrane protein A, zinc-metalloprotease, siderophore production and biofilm formation. Many Cronobacter isolates (>87%) had these virulence characteristics. All isolates and a type strain were characterized using ERIC-PCR and genetic profiles of cluster analysis showed that the isolates were highly heterogeneous and genetically diverse. Antimicrobial susceptibility of RTE isolates to 18 different antimicrobial agents was determined by the disc diffusion method. Most Cronobacter isolates with a rate of 81.3% were resistant to cephalothin, 32.8% to cefoxitin and 20.3% to ampicillin. All Cronobacter isolates were susceptible to gentamicin and trimethoprim/sulfamethoxazole. The multidrug resistance to at least three or more antimicrobial agents was detected in 18.8% of Cronobacter isolates.Conclusions Results indicate that RTE food harbors potential pathogenic Cronobacter species and is a possible transmission vehicle for Cronobacter infection in vulnerable person. So, there is a need to adopt hygienic practices and rigorous sanitization treatments to ensure microbiological safety of RTE food.

Background

Cronobacter spp. are recognized as Gram-negative rod, non-spore forming within the *Enterobacteriaceae* family [1]. The genus *Cronobacter* contains seven species: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter muytjensii*, *Cronobacter dublinensis, Cronobacter universalis* and *Cronobacter condimenti* [2, 3]. These organisms are extensively found in the environment and food products including powdered milk, powdered infant formula, vegetables, salads, herbs, cereal, chocolate, milk, meat, potato flour, pasta and spices [4, 5].

Cronobacter is considered to be an opportunistic pathogen implicated in several diseases including necrotizing enterocolitis, bacteremia, and meningitis [1, 6]. 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 [7]. The symptoms of *Cronobacter* infections are high fever, headache with nausea, a swelling on the head, body and neck stiffness, skin rash, seizures, urosepsis, pneumonia [8]. All *Cronobacter* species have been associated with human infections except for *C. condimenti*. *Cronobacter* infections in neonates and infant seem to be particularly concerned with species of *C. sakazakii* [8, 9, 10]. 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 [1]. Whereas *Cronobacter* spp. based human infections are not common, the mortality rate of these pathogens are considerable (40-80%) in infected human, especially in neonates. Even if these people survive, they often suffer from severe neurological complications [8, 11].

Cronobacter species have various virulence traits which are responsible for their pathogenicity [12]. The virulence factors are adhesion proteins, cell-surface proteins, toxins, proteolytic enzymes, biofilm formation and siderophore production. The adhesion and tissue invasion in the host and cell injury are considered as the main pathogenic route for *Cronobacter* infection [12, 13]. The outer membrane protein A (OmpA) encoded by the *ompA* gene is the best described virulence marker and has a significant role in *Cronobacter* invasion [14]. The OmpA of *Cronobacter* plays a role in the colonization of the gastrointestinal tract [15] and the *ompA* - positive isolates break blood-brain barrier and invade central nervous system causing clinical symptoms [16]. Furthermore, it was reported that the zinc-containing metalloprotease encoded the *zpx* gene caused rounding of Chinese Hamster Ovary cells in tissue culture in the clinical and environmental *Cronobacter* strains [13]. This gene may help as an indicator of pathogenicity [16, 17]. Biofilm formation, caused by some pathogens, has a role as physical protective barrier and is described as unity of bacterial cells [18]. The biofilms are surrounded by a self-producing matrix and adherent to an inert or living surface. *Cronobacter* has been reported to form biofilms on latex, stainless steel, glass, silicon, polyvinyl chloride and polycarbonate [18]. Moreover, siderophores, low molecular weight compounds having a high affinity for iron ions, play a role in the virulence of *Cronobacter* spp. [19].

A great many methods for identification and description of *Cronobacter* spp. have been suggested since it was defined by Farmer et al. [6]. Muytjens et al. [20] described the initial detection and isolation method developed for *Cronobacter* species. The Food and Drug Administration (FDA) and the International Organization for Standardization (ISO) improved protocols for the isolation and detection of *Cronobacter* spp. [21, 22]. *Cronobacter* species have been identified and classified genotypically using amplification of 16S rRNA gene, the *gluA* gene, the enterobacterial repetitive intergenic consensus (ERIC) sequences, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), ribotyping and plasmid typing [5, 23-25]. Moreover, the *rpoB* gene-based PCR method was improved to differentiate between the seven

Cronobacter species. 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 [26, 27]. Furthermore, gene *cgcA* encoding a diguanylate cyclase which is involved in virulence, biofilm production and survival of the organism has also been used as the genetic marker to distinguish *Cronobacter* species [28].

Cronobacter may express β-lactamases, thus conferring resistance against a range of cephalosporins and penicillins [29]. The treatment of *Cronobacter* meningitis has shifted to extended-spectrum cephalosporins, sometimes in combination with a second agent, e.g., trimethoprim [11]. *Cronobacter* spp. are susceptible to commonly used antimicrobials such as aminogylcosides, ureidopenicillins, ampicillin, and carboxypenicillins [9, 29, 30]. However, antimicrobial resistant strains of *Cronobacter* spp., some of which have multiple drug resistance, were reported in various studies [31, 32]. 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 [5, 11]

Today, a growing number of people are buying ready-to-eat foods since they are easy and practical to prepare, and delicious foods to consume [33]. Ready-to-eat foods that are usually high in protein and ready to eat without further heating or cooking are known as high-risk foods and provide excellent conditions for bacterial growth [34]. *Cronobacter*, a ubiquitous foodborne pathogen, can cause severe infections in immunocompromised people over the world. In Turkey, several studies focused on isolation and identification of *Cronobacter* spp. from samples of milk powder, powdered infant formula and cereal-based products [35, 36], but there is little data on the prevalence of *Cronobacter* spp. in ready-to-eat foods. Therefore, this study aims to determine the prevalence of *Cronobacter* species in ready-to-eat foods, to identify the *Cronobacter* species by phenotypic and genotypic methods, and to evaluate virulence factors, antimicrobial susceptibility and genetic relatedness of *Cronobacter* isolates.

Methods

Sample collection

A total of 340 ready-to-eat food samples were purchased from markets, local bazaar, patisserie and herbalist from January 2016 to December 2016 in Bolu (Western Turkey). The samples included 49 doners (thinly sliced doner kebab from chicken and beef), 43 desserts, 41 pastrami (seasoned, air-dried, cured, smoked, and non-fermented beef cut), 40 cheeses, 38 salads, 35 kavurma (sliced and fried meat from beef or mutton), 27 cigkoftes (meatless and uncooked bulgur product), 27 ice cream, 15 spices, 13 cereals, 7 herbs, and 5 vegetables. These samples were transported to the laboratory in a cold box (below 4°C) and immediately analyzed.

Isolation and phenotypically identification of *Cronobacter* spp.

Cronobacter spp. were isolated according to the methods of the Food and Drug Administration [21] and the International Standard Organization [22] with some modification. According to FDA [21], for each sample, 225 mL of buffered peptone water (BPW; Merck, Darmstadt, Germany) were added to 25 g of sample. After homogenizing in a stomacher (Interscience, Saint-Nom-la-Breteche, France) for 60 s, samples were incubated at 37ºC for 24 h. Then, 10 mL of pre-enrichment broth was added to 90 mL of *Enterobacteriaceae* Enrichment Broth (Merck). Following an overnight incubation at 37ºC, these cultures were streaked onto Violet Red Bile Glucose agar (VRBG; Merck, Germany), Violet Red Bile Lactose agar (VRBL; Merck, Germany), and Tryptic Soy Agar (TSA; Merck, Germany) and incubated for 24 h at 37°C. The ISO/ TS 22964 [22] method with some modification; a 25 g of each sample was homogenizated with 225 mL BPW and incubated for 24 h at 37°C. 1 mL of BPW pre-enrichment was added to 10 mL modified Lauryl Sulfate Tryptose (Merck) broth supplemented with 0.5 M NaCl and 0.1 mg/L vancomycin and incubated for 24 h at 44°C. After incubation, a loopful of mLST broth was streaked onto VRBG agar and VRBL agar at 44°C/ 24 h, while TSA incubated at 25°C/ 48 h. Then, all colonies counted as *Cronobacter* spp. were subcultured on TSA medium. Five suspect yellow pigment colonies of *Cronobacter* spp. were subjected to the following biochemical tests for identification: Gram staining; oxidase and catalase test; motility assessment; indole production; utilization of citrate and malonate; Voges-Proskauer (acetoin production) reaction; lysine decarboxylase; utilization of carbohydrates such as dulcitol, sucrose, trans-aconitate, cis-aconitate, myo-inositol, lactulose, maltitol, melezitose, 4-aminobutyrate, and 10-methyl α-D-glucopyranoside [2, 3, 6]. *Cronobacter sakazakii* ATCC 29544 was used as a reference strain in all biochemical and molecular experiments.

Genus-specific identification of *Cronobacter* spp. using PCR

The genomic DNA extraction to use in all PCR experiments was performed using a previously described method [59]. All *Cronobacter* isolates were grown on TSA for 24 h at 37°C. One colony of each culture was added into 5 mL Brain Heart Infusion (Merck) broth and was incubated at 37°C for 18 h. The extracted DNA was kept at -20°C for further use.

For the genus-specific identification, the 16S rRNA gene [25] and the *gluA* gene [24] were used in all isolates. The primer sequences, expected size of amplification products and references are presented in Table 3. PCR amplification of 16S rRNA was performed in a total volume of 30 µL, with the following reagent concentration: 3 µL of 10× PCR buffer (500mM KCl, 100mM Tris-HCl, pH 9.1, 0.1% Triton™X-100, Vivantis Techologies Sdn. Bhn., Kuala Lumpur, Malaysia), 1.8 µL of 50 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), 1.25 U *Taq* DNA polymerase (Vivantis) and 19.85 µL molecular grade water

(AppliChem, Darmstadt, Germany). The thermal cycling conditions were as follows: initial denaturation at 94ºC for 4 min, followed by 30 cycles of denaturation at 95ºC for 1 min; annealing at 57ºC for 1 min, and elongation at 72ºC for 1 min 30 s, and a final extension at 72ºC for 4 min.

For the *gluA* gene amplification, cycling conditions for amplification of DNA fragments was performed as described by Lehner et al. [24]. All PCR mixtures contained 5 µL 10× PCR buffer (Vivantis), 4 µL of 50 mM $MgCl_2$ (Vivantis), 1 µL of 10 mM dNTP mix (Vivantis), 2 µL of 10 µM each primers (Biomers), 3 µL of target DNA (50 ng/ µL), 3 U *Taq* DNA polymerase (Vivantis) and 32.4 µL molecular grade water (AppliChem).

Species-specific identification of *Cronobacter* spp. using PCR

Two PCR assays targeting the *rpoB* gene and the *cgcA* gene were used to identify *Cronobacter* species. Identification of the seven species of *Cronobacter* including *C. condimenti*, *C. dublinensis*, *C. malonaticus*, *C. muytjensii*, *C. sakazakii*, *C. turicensis* and *C. universalis* was carried out with the appropriate primer sets for the *rpoB* gene [26, 27]. The PCR reaction mixture included 5 µL of 10× PCR buffer (Vivantis), 4 mM $MgCl_2$ (Vivantis), 0.2 mM dNTP mix (Vivantis), 0.4 µM of each primer (Biomers), 3 µL of template DNA (50 ng/ µL) and 2 U *Taq* DNA polymerase (Vivantis, Malaysia). A total of 32.60 µl of molecular grade water (AppliChem) was added to a final volume of 50 µl. The cycling condition of the *rpoB* PCR assay was performed as described by Stoop et al. [26] and Lehner et al. [27].

Species-specific detection and differentiation of *Cronobacter* species by Multiplex-PCR (M-PCR) assay employing amplification of the *cgcA* gene were performed as described earlier [28]. PCR was performed in a reaction volume of 50 µL containing 5 µL of 10× PCR buffer (Vivantis), 4 mM $MgCl_2$ (Vivantis), 0.2 mM dNTP mix (Vivantis), 0.4 µM of each primers (Biomers), 5 µL of DNA template (50 ng/µL) and 2 U of *Taq* DNA polymerase (Vivantis). The final volume to 50 µL was adjusted by adding molecular grade water (AppliChem). The PCR condition of the *cgcA* M-PCR assays was carried out as described by Carter et al. [28]. The primer sequences, expected size of amplification products and references for both methods are given in Table 3.

Virulence factors of *Cronobacter* spp.

a) Detection of the *ompA* gene

The PCR was targeted to *ompA* gene, which was previously suggested by Mohan-Nair and Venkitanarayanan [14] (Table 3). Reactions were performed in 50 µL volume, 5 µL 10× PCR buffer (Vivantis), 2 mM of $MgCl_2$ (50 mM, Vivantis), 0.2 mM dNTP mix (10 mM, Vivantis), 1 µM (each) primer (10 µM, Biomers), 1.25 U of *Taq* DNA polymerase (Vivantis), 3 µL of 50 ng (template DNA) and 28.75 µL molecular grade water (AppliChem,). PCR cycling conditions for amplification of DNA fragments was performed as described by Mohan-Nair and Venkitanarayanan [14].

b) Detection of the *zpx* gene

The PCR protocol which amplifies a 94 bp region of the *zpx* gene was applied to all isolates [13] (Table 3). The PCR amplification of the *zpx* gene was achieved by 3 µL template DNA (50 ng/ µL) with a 47 µL of PCR mixture containing the following: 5 µL 10× PCR buffer (Vivantis), 2 µL of 50 mM $MgCl_2$ (Vivantis), 1 µL of 10 mM dNTP mix (Vivantis), 1.25 U *Taq* DNA polymerase (Vivantis), 5 µL from primers (10 µM, Biomers) and 28.75 of molecular grade water (AppliChem). The amplification conditions of the *zpx* gene were as follows: an initial denaturation of 95ºC for 15 s, followed by 35 cycles of 94ºC for 1 min, 62ºC for 1 min, 72ºC for 1 min, with a final elongation at 72ºC for 10 min [13].

All PCR reactions were carried out on a XP Thermal Cycler (Bioer Technology Co., Ltd., Japan). The PCR products were analyzed in agarose gel (1.5%) (Merck) and visualized with ultraviolet transilluminator (DNR Minilumi Bio-imaging Systems Ltd., Mahale HaHamisha, Jerusalem, Israel).

c) Siderophore production of *Cronobacter* spp.

Siderophore production was studied based on the Chrome azurol S (CAS) assay of Schwyn and Neilands [60] with some modifications made by Fiss and Brooks [61]. Briefly, overnight culture of *Cronobacter* isolates were spot inoculated onto a chrome azurol S (CAS) agar plate and incubated for 5 days at 37°C. After incubation, siderophore positive isolates were seen as an orange halo around a colony [60].

d) Biofilm formation of *Cronobacter* spp.

Biofilm formation of the *Cronobacter* isolates was analyzed using the microtiter plate technique described by Stepanovic et al. [62] and Ye et al. [53] with some modifications. The *Cronobacter* isolates were incubated in 5 mL of Tryptic Soy Broth (Merck) at 37°C for 24 h. Cultures were diluted until reaching 0.5 on the McFarland scale (approximately 10^8 cells/mL) and 200 µL of each suspension were transferred into wells. Following incubation at 37°C for 48 h, wells were washed once sterile phosphate-buffered saline (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 Ascent spectrophotometer, Thermo Electron Corporation, Vantaa,

Finland). All the experiments were performed triple times. The isolates were classified as strong, moderate and weak for biofilm formation with respect to Stepanovic et al. [62].

Enterobacterial repetitive intergenic consensus (ERIC)-PCR

ERIC-PCR was performed using the primer pair, ERIC1R and ERIC2 as described previously by Versalovic et al. [23] (Table 3). PCR reaction was optimized in a 50 µL reaction mixture consisting of 3 µL of the bacterial genomic DNA solution (50 ng), 5 µL of 10× PCR buffer (Vivantis), 4 µL of 50 mM $MgCl_2$ (Vivantis), 1 µL of 10 mM dNTPs (Vivantis), 1 µL of 10 µM primers (Biomers), 1.25 U *Taq* polymerase and 34.75 µL nuclease free water. ERIC-PCR was performed as follows: 8 min at 94°C, followed by 35 cycles of 30 s at 94°C, 1 min at 55°C, and 8 min at 72°C, and a final extension at 72°C for 10 min. The PCR amplicons were separated on agarose gel (1%), stained with ethidium bromide and visualized under a UV transilluminator (DNR Minilumi Bio-imaging Systems Ltd., Israel).

Data analysis

The band patterns generated by ERIC-PCR were analyzed by using the BioNumerics version 7.6 software (Applied Maths, Sint-Martens-Latem, Belgium) based on the Dice's similarity coefficient with a 1% position tolerance and the unweighted pair group method using arithmetic averages (UPGMA).

Antimicrobial susceptibility test

All *Cronobacter* spp. were tested for antimicrobial susceptibility to 18 antimicrobials using the disk diffusion method according to the Clinical and Laboratory Standards Institute [63]. Antibiotic disks (Oxoid, Basingstoke, U.K.) with the following concentrations were used: penicillins (ampicillin- 10 µg; piperacillin- 100 µg), carbapenems (imipenem- 10 µg; meropenem- 10 µg), β-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 results of all the inhibition zones of antimicrobial agents were interpreted according to *Enterobacteriaceae* table in CLSI [63].

Results

Isolation and identification of *Cronobacter* spp.

Out of the 340 ready-to-eat foods tested, phenotypically, a total of 17.4% (59/340) of samples, categorized as 51.9% (14/27) of meat free cig koftes, 46.7% (7/15) of spices, 30.8% (4/13) of cereals, 30.2% (13/43) of desserts, 14.8% (4/27) of ice creams, 12.2% (6/49) of doners, 9.8% (4/41) of pastramis, 8.6% (3/35) of kavurmas, 7.5% (3/40) of cheeses and 2.6% (1/38) of salads were considered contaminated with *Cronobacter* species. *Cronobacter* spp. were not detected in herbs and vegetables. Of the 59 contaminated samples, the 54 samples contained only one species, whereas 5 samples contained two different species. Thus, the 64 isolates were identified. The occurrence of *Cronobacter* isolates in the 59 ready-to-eat food samples analyzed is summarized in Table 1.

In this study, all phenotypically-identified isolates were completely confirmed by the genus-specific 16S rRNA gene while the presence of *gluA* gene was 93.8% among the *Cronobacter* isolates. All *Cronobacter* species differentiated using species-specific the *rpoB* gene and the *cgcA* gene along with the results of the biochemical identification, the 16S rRNA and *gluA* are shown in Table 2.

Virulence characteristics of *Cronobacter* spp.

The pathogenic potential of the obtained *Cronobacter* isolates was confirmed by the presence of virulence related genes: *ompA* is associated with the invasion ability; *zpx* encodes the zinc-containing metalloprotease that contributes pathogenicity of *Cronobacter*. All 64 *Cronobacter* isolates (100%) were found to be positive for the *ompA* gene while 63 (98.4%) isolates harbored the *zpx* gene. The biofilm formation was detected in the 56 (87.5%) of the 64 isolates by the microtiter plate assay. Overall, 3.1%, 31.3% and 53.1% of the *Cronobacter* isolates were strong, moderate and weak biofilm producer, respectively. On the other hand, the production of siderophore as a significant virulence property was detected in all *Cronobacter* isolates (100%) (Table 2).

Genetic variation among the *Cronobacter* isolates from ready-to-eat foods

The 64 *Cronobacter* isolates and type strain *C. sakazakii* ATCC 29544 were subjected to ERIC-PCR. All isolates amplified with the ERIC primers. The ERIC-PCR generated the 3 to 12 amplified bands with sizes ranging from 100 to 3000 bp. We identified 64 different ERIC patterns with a similarity index ranging from 10% to 100% using the BioNumerics software (version 7.6) (Fig. 1). The ERIC patterns were grouped into two major clusters (A and B). The predominant cluster was cluster B, which contained 56 (86.2%) isolates, 2 of which had the same pattern. Cluster A was

composed of 9 (13.8%) of the isolates that presented 9 distinct patterns. The *Cronobacter* isolates from the same food categories were represented in the distinct patterns; therefore, there was no correlation between the ERIC-PCR profiles and origin of the isolates (Fig. 1).

Antimicrobial resistance

The antimicrobial susceptibility results of all 64 *Cronobacter* isolates from RTE food to 18 antimicrobial agents interpreted as susceptible, intermediate or resistant are shown in Fig. 2. All *Cronobacter* isolates were susceptible to trimethoprim/sulfamethoxazole and gentamicin. The most frequently detected resistances were to cephalothin (81.3%), followed by cefoxitin (32.8%), and ampicillin (20.3%). The profiles of multidrug resistance were detected in 12 (18.8%) of the *Cronobacter* isolates to at least three or more antimicrobial agents. The antimicrobial resistances and intermediate results for each of the 64 isolates are exhibited in Table 2.

Discussion

Cronobacter species as pathogens cause severe infections in vulnerable adults and infants. These bacteria which are ubiquitous have been isolated from a wide range of sources such as contaminated ready-to-eat foods [5, 6, 34]. In this study, the prevalence of *Cronobacter* spp. in RTE foods was detected to be 17.4%. In previous studies, the level of contamination with *Cronobacter* spp. in RTE foods ranged from 9.0% to 45% [4, 37-39]. The different rates of the presence of the *Cronobacter* in various foods have been documented in many different countries such as in Ireland, China, India, Brazil and Egypt [9, 40-42]. The variation in the occurrence of bacteria can be explained by different factors such as source of the isolation, nature of samples and geographic location [5].

In recent studies, among food products, cereal, flour, pasta, herbs and spices have been highlighted as being most frequently contaminated by *Cronobacter* spp. [9, 38, 41]. In the current study, *Cronobacter* spp. were commonly isolated from 51.9%, 46.7%, 30.8% and 30.2% samples of meat-free cigkoftes, spices, cereals and desserts, respectively. In Turkey, meat-free cig kofte is a popular food consisting of bulgur, various spices and vegetables and it is consumed uncooked. In particular, a high occurrence of *Cronobacter* spp. (51.9%) in meat free cig koftes may be due to contaminated ingredients including cereal (46.7%) and spices (30.8%). In the study of Aksu et al. [10] in Turkey, the 50% of the *Cronobacter* isolates obtained from cig kofte (cereal-based ball) was similar to our research. Four samples of cereals (bulgur, red lentil, peanut, and goji berry) and seven samples of spices (cumin, chili pepper, thyme, mint, flower flour, coconut) were contaminated with *Cronobacter* spp. (Table 1). These findings were similar to some studies focused on isolation from cereals and spices [38, 40, 41]. The reported isolation rates of *Cronobacter* spp. in cereal samples varied from 4.9% to 45% [40, 41, 43], while in spices samples varied from 3.6% to 34% [37, 41, 43].

In several studies, *Cronobacter* were isolated from flours and dairy products such as milk-based desserts, cheeses, raw milk, curd, ice-cream [38, 41-43]. In this study, *Cronobacter* spp. were detected from desserts including milky desserts (2/16) and dough sweets (11/27) (Table 1). Desserts can be made by using ingredients such as milk, flour, cream, flavors, sweeteners, cereals, nuts and fruits. Our study indicates that contamination of desserts may be resulted from contaminated ingredients. In the study of Saad and Ewida [42], the prevalence of *Cronobacter* spp. was detected in 5.5% of milk-based desserts in accordance with the result of our study. The occurrence of *Cronobacter* in cheeses (7.5%) was found to be higher than results of studies of Iversen and Forsythe [44] and Singh et al. [41]. In another study, *Cronobacter* was not isolated (0/20) from cheeses [40]. Saad and Ewida [42] isolated *Cronobacter* from 1/30 samples of ice-cream, but Kandhai et al. [43] did not found *Cronobacter* in ice-cream (0/89). These findings were lower than our results (14.8%) in ice-cream samples.

The occurrence of *Cronobacter* spp. was 12.2% in doner, 9.8% in pastramis and 8.6% in kavurmas as retail meat products in this research. Several studies demonstrated varying prevalence of *Cronobacter* spp.in meat and meat products: 0.0% in Turkey [10], 3.2% in the Netherlands [43], and 14.5% in China [4]. In this study, *Cronobacter* spp. were isolated from 2.6% samples of salads, while were not detected in herbs and vegetables. The result in salads (1/38) was lower than the result (13/30) obtained by Vasconcellos et al. [39], while was similar to the result (1/15) obtained by Lee et al. [45]. *Cronobacter* were not detected from dried herbs and vegetables in some studies, similar to our results [10, 40]. In another research, the *Cronobacter* spp. was detected in 25% of vegetables [41]. In the light of our results and results of many previous studies, it has been considered that the contamination of RTE foods may be the result from the contaminated ingredients, improper food handling practices, storage conditions and environmental factors.

In the present study, all *Cronobacter* spp. to the genus level were identified using the 16S rRNA and the *gluA* gene. Many researchers have been used the *gluA* gene as an additional tool for identification of *Cronobacter* spp. [24, 46]. Iversen et al. [46] reported that *gluA* gene were 100% sensitive and specific for determination and confirmation of *Cronobacter* spp. However, in this research, out of the 64 isolates, the 60 (93.8%) isolates were positive for the *gluA* gene (Table 2). Some other researchers did not also detect the *gluA* gene in some of *Cronobacter* strains, similar to our findings [47, 48].

In this study, in addition to phenotypic identification, the genotypic identification of *Cronobacter* isolates were performed using the species-specific *rpoB* and *cgcA* genes [26-28]. Although the *Cronobacter* isolates were completely identified by the biochemical test and the *rpoB* PCR method, the eight isolates were not identified by the *cgcA* M-PCR method. Compared to the results of identification using the *rpoB* gene, failure results in the *cgcA* method were associated with the species *C. malonaticus*, *C. turicensis*, *C. muytjensii* and *C. dublinensis* (Table 2). Of 64

Cronobacter isolates, 42 (65.6%) were completely consistent according to the identification results of the biochemical tests, *rpoB* PCR method and *cgcA* M-PCR method. Among the remaining *Cronobacter* isolates, there was inconsistency to phenotypic and genotypic identification methods. In this study, the identification of 41 *C. sakazakii* isolates was same in *rpoB* PCR and *cgcA* M-PCR method. Brandao et al. [38] identified successfully *Cronobacter* isolates to the species level using *cgcA* primers. However, some reports documented the inefficiency of *cgcA* gene to identify some *Cronobacter* isolates for the species-specific identification [30, 39].

C. sakazakii is most common among the species of the *Cronobacter* genus and plays a significant role in human diseases [5, 20]. In this study, *C. sakazakii* was also the most common *Cronobacter* species isolated, followed by *C. malonaticus* and other species of *Cronobacter*. Many researchers found *C. sakazakii* as the most common species among the *Cronobacter* genus, similar to our result [10, 30, 38]. Moreover, in many previous studies, the *C. malonaticus* andother species of *Cronobacter* were isolated from various foods [4, 30, 39].

In the present study, various virulence characteristics such as outer membrane protein A (OmpA), zinc-containing metalloprotease, siderophore production, and biofilm formation were found among *Cronobacter* isolates from RTE foods. The *ompA* gene encoding OmpA, has a significant role in *Cronobacter* invasion, was detected in 100% of the *Cronobacter* isolates. Similarly, the occurrence of *ompA* gene was found 100% among the *Cronobacter* isolates by many researchers[43, 49]. On the contrary, in some studies, the incidence of *Cronobacter* spp. containing the *ompA* gene has been reported as 64.7% in the USA [50] and 33.3% in the Bangladesh[17]. In addition, in this study, many isolates of *Cronobacter* (98.4%) harbored the *zpx* gene encoding zinc-containing metalloprotease as an indicator of pathogenicity. The prevalence of *zpx* gene in *Cronobacter* spp. ranging from 1.3% to 65.5% was reported in previous studies [17, 47, 51]. Siderophore required for the iron uptake is a virulence factor for *Cronobacter* [19]. In several researches, all *Cronobacter* isolates exhibited siderophore production, similar to the result in this research [17, 52]. Biofilms are considered to be a potential way of pathogen transmission [17, 18]. In this study, the biofilm formation of *Cronobacter* (87.5%) was higher than the result obtained by Fakruddin et al. [17] and Lee et al. [45], while was similar to the result obtained by Ye et al. [53].

The genetic diversity of the 64 *Cronobacter* isolates in RTE food products and type strain *C. sakazakii* ATCC 29544 was assessed by ERIC-PCR in the present research (Fig. 1). The 64 distinct ERIC-PCR profiles were categorized in two major clusters (A and B) at a similarity threshold of 10%. Most isolates (>86%) harbored virulence factors and antibiotic resistance were distributed on the cluster B. In this study, the results of ERIC-PCR indicated that there was no apparent clustering tendency among the genotypes related to the origin of isolates, virulence profile and antimicrobial resistance. In many studies, genetic variation of *Cronobacter* isolates from various food samples have been also detected using ERIC-PCR method [17, 54, 55].

In this study, the resistance rates of the *Cronobacter* isolates ranging from 1.5% to 20.3% to piperacillin, ampicillin, amoxicillin-clavulanic acid, nalidixic acid, aztreonam were obtained. Previous reports revealed the occurrence of resistance to the antimicrobial agents in *Cronobacter* spp. isolated from different sources [31, 32, 56]. Increasing resistance of *Cronobacter* against a range of cephalosporins and penicillins due to the production of β-lactamases has been reported [11, 29, 30]. In our study, the cephalosporins that are used in the treatment of *Cronobacter* infections had resistance ranging from 4.7% to 81.3% through the first- second- third- fourth generation. In addition, in the present study, it is noteworthy that *Cronobacter* isolates displayed a high intermediate resistance to cefoxitin (32.8%), cefepime (21.9%), cefotaxime (20.3%), ampicillin (17.2%), piperacillin (17.2%) and cephalothin (10.9%) (Fig. 2). In several studies, the resistance to ampicillin, cephalothin, cefoxitin, and cefotaxime in *Cronobacter* was reported to range from 5.6% to 50.0% [31, 32]. *Cronobacter* infections have been treated with carbapenems or the newer cephalosporins in concert with an aminoglycoside or trimethoprim-sulfamethoxazole [11, 12]. As prescribed for the treatment, in our study, more than 90% of the *Cronobacter* isolates were also susceptibility to piperacillin-tazobactam, gentamicin, amikacin, imipenem, meropenem, tetracycline, trimethoprim/sulfamethoxazole, ciprofloxacin, and chloramphenicol. Susceptibility to these antimicrobials has been reported in many countries: Brazil [30], China [9], and Iran [56]. 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. The findings of Kim et al. [57] and Li et al. [58] were contrary to our results that any isolates were resistant against three or more antimicrobial agents.

Conclusions

Cronobacter spp. were isolated from samples of ready-to-eat (RTE) foods, with a higher presence of *C. sakazakii*, an emerging foodborne pathogen. The present study provides current knowledge on the incidence, identification, genotyping, potential virulence and antimicrobial resistance of *Cronobacter* spp. in RTE foods. The consumption of RTE foods contaminated with antimicrobial resistant or potential pathogenic *Cronobacter* may cause a serious threat to human health especially for children, elderly and immunocompromised people. As a result, RTE foods may be a possible transmission vehicle for *Cronobacter* infection in vulnerable person, and need adoption of hygienic practices and rigorous sanitization treatments to ensure microbiological safety.

Abbreviations

BPW: Buffered peptone water; CAS: Chrome azurol S; CLSI: Clinical and Laboratory Standards Institute; ERIC-PCR: Enterobacterial repetitive intergenic consensus-PCR; FDA: Food and Drug Administration; ICMSF: International Commission for Microbiological Specifications for Foods;

ISO: International Organization for Standardization; MLST: Multilocus sequence typing; M-PCR: Multiplex-PCR; OD: Optical density; OmpA: Outer membrane protein A; PFGE: Pulsed-field gel electrophoresis; RTE: Ready-to-eat; TSA: Tryptic Soy Agar; UPGMA: Unweighted pair group method with arithmetic mean; VRBG: Violet Red Bile Glucose; VRBL: Violet Red Bile Lactose

Declarations

Authors' contributions

SA designed the study and analyzed the data. SA revised the manuscript. HGE performed the experiments and interpretation of the results and wrote the draft. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Prevalence of the *Cronobacter* spp. among ready-to-eat foods

Samples	Number of samples	Number of contaminated samples	Percentage of samples contaminated with *Cronobacter* spp.	Number of *Cronobacter* isolates
Doners (n=49)				
Chicken	28	1	12.2 (6/49)	1 (DN-3)
Beef	21	5		6 (DN-1, DN-2, DN-4, DN-5, DN-6, DN-7)
Pastramis	41	4	9.8 (4/41)	4 (PS-1, PS-2, PS-3, PS-4)
Cheeses	40	3	7.5 (3/40)	3 (P-1, P-2, P-3)
Kavurmas	35	3	8.6 (3/35)	3 (KV-1, KV-2, KV-3)
Meat-free cigkoftes	27	14	51.9 (14/27)	15 (CK-1 to CK-15)
Vegetables (n=5)				
lettuce	1	0	0.0 (0/5)	0
pepper	1	0		0
dill	1	0		0
fresh mint	1	0		0
parsley	1	0		0
Salads (n=38)				
Salads (vegetables and sauces)	28	1	2.6 (1/38)	1 (SL-1)
Salads (meat, cereals, or dough)	10	0		0
Desserts (n=43)				
Milky desserts	16	2	30.2 (13/43)	2 (TT-6, TT-14)
Dough sweets	27	11		12 (TT-1, TT-2, TT-3, TT-4, TT-5, TT-7, TT-8, TT-9,TT-10, TT-11, TT-12, TT-13)
Spices (n=15)				
cumin	2	1	46.7 (7/15)	2 (B-1, B-2)
chili pepper	2	1		1 (B-5)
thyme	1	1		1 (B-3)
turmeric	1	0		0
ginger	1	0		0
mint	2	2		2 (B-4, B-8)
black pepper	1	0		0
cinnamon	1	0		0
flower flour	1	1		1 (B-6)
flaxseed	1	0		0
sesame	1	0		0
coconut	1	1		1 (B-7)
Cereals (n=13)				
rice	1	0	30.8 (4/13)	0
wheat	1	0		0
red lentil	1	1		1 (C-1)
bulgur	1	1		1 (C-2)
cashew nut	1	0		0
peanut	3	1		1 (C-3)
hazelnut	1	0		0
raisins	1	0		0
dried apricot	1	0		0
oleaster	1	0		0
goji berry	1	1		1 (C-4)
Ice creams (n=27)				
nuts	4	2	14.8 (4/27)	2 (D-1, D-2)
vanilla	5	0		0
fruity	12	1		1 (D-3)
cacao	6	1		2 (D-4, D-5)
Herbs (n=7)				

sage	2	0	0.0 (0/7)	0
licorice	1	0		0
rosehip	1	0		0
linden	1	0		0
black tea	2	0		0
Total	340	59	17.4 (59/340)	64

Table 2 Molecular characterization, virulence characteristics and antimicrobial resistance of *Cronobacter* species from ready-to-eat foods

				Molecular characterization				Virulence characterization				Antimicrobial Resistance	
			Biochemical identification	Genus-specific		Species-specific		Genotypic		Phenotypic			
No	Isolate No	Origin	[a]	16S rRNA	*gluA*	PCR *rpoB*	M-PCR *cgcA*	*ompA*	*zpx*	Biofilm formation	SP[b]	Resistance	Intermediate
1	TT-1	Dessert	*C. malonaticus*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Moderate	+	KF	-
2	TT-2	Dessert	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Moderate	+	-	FOX, KF, PRL
3	TT-3	Dessert	*C. dublinensis*	+	+	*C. malonaticus*	NI[c]	+	-	Weak	+	KF	AMP
4	TT-4	Dessert	*C. malonaticus*	+	+	*C. malonaticus*	*C. malonaticus*	+	+	Absent	+	FOX, KF	PRL, TZP
5	TT-5	Dessert	*C. malonaticus*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Moderate	+	-	FOX, KF
6	TT-6	Dessert	*C. malonaticus*	+	+	*C. malonaticus*	*C. malonaticus*	+	+	Weak	+	FOX, KF	-
7	TT-7	Dessert	*C. muytjensii*	+	+	*C. muytjensii*	NI	+	+	Moderate	+	AMP, KF	PRL
8	TT-8	Dessert	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	KF	FOX
9	TT-9	Dessert	*C. dublinensis*	+	-	*C. dublinensis*	NI	+	+	Weak	+	AMC, FOX, KF	IPM
10	TT-10	Dessert	*C. malonaticus*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Moderate	+	CTX, KF	-
11	TT-11	Dessert	*C. dublinensis*	+	+	*C. dublinensis*	NI	+	+	Weak	+	KF	CTX, PRL
12	TT-12	Dessert	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	CTX, FOX, KF	AMC, NA
13	TT-13	Dessert	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	CTX, FOX, KF	FEP, PRL
14	TT-14	Dessert	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Moderate	+	KF	AMP, FEP, FOX
15	CK-1	Cig kofte	*C. malonaticus*	+	+	*C. malonaticus*	*C. malonaticus*	+	+	Weak	+	ATM, KF, NA	CIP, FOX, PRL
16	CK-2	Cig kofte	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Moderate	+	KF	AMC, AMP, FEP
17	CK-3	Cig kofte	*C. malonaticus*	+	+	*C. malonaticus*	*C. malonaticus*	+	+	Weak	+	KF	AMC, CTX, FOX
18	CK-4	Cig kofte	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	AMP, FEP, FOX, KF, NA	TE
19	CK-5	Cig kofte	*C. turicensis*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Moderate	+	AMP	KF
20	CK-6	Cig kofte	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	AMP, KF	FOX
21	CK-7	Cig kofte	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Absent	+	ATM, KF, NA	CTX, FEP
22	CK-8	Cig kofte	*C. malonaticus*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Moderate	+	KF	-
23	CK-9	Cig kofte	*C. malonaticus*	+	-	*C. malonaticus*	NI	+	+	Moderate	+	AMC, AMP, FOX, KF	-
24	CK-10	Cig kofte	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Moderate	+	FEP, KF	IPM
25	CK-11	Cig kofte	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	KF	-
26	CK-12	Cig kofte	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	FOX, KF	FEP
27	CK-13	Cig kofte	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	FOX, KF	-
28	CK-14	Cig kofte	*C. malonaticus*	+	+	*C. malonaticus*	*C. malonaticus*	+	+	Moderate	+	-	KF
29	CK-15	Cig kofte	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	FEP, KF	FOX, NA
30	P-1	Cheese	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	FOX, KF	CTX
31	P-2	Cheese	*C. sakazakii*	+	+	*C. malonaticus*	*C. malonaticus*	+	+	Absent	+	FOX, KF	AMP, CTX, FEP
32	P-3	Cheese	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	KF	CTX, FEP, FOX
33	KV-1	Kavurma	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	FOX, KF	AMP, NA, TZP
34	KV-2	Kavurma	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	FOX, KF	CIP

35	KV-3	Kavurma	*C. malonaticus*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Absent	+	FOX, KF	CTX, NA
36	PS-1	Pastrami	*C. malonaticus*	+	-	*C. malonaticus*	*C. malonaticus*	+	+	Weak	+	AMC, AMP, FOX, KF	CTX
37	PS-2	Pastrami	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Moderate	+	CTX	FEP, FOX, KF, NA
38	PS-3	Pastrami	*C. sakazakii*	+	+	*C. malonaticus*	*C. malonaticus*	+	+	Strong	+	ATM, KF, PRL	AMP, MEM
39	PS-4	Pastrami	*C. turicensis*	+	+	*C. turicensis*	*C. turicensis*	+	+	Weak	+	-	FOX, KF, TZP
40	DN-1	Doner	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Moderate	+	AMP, KF	FOX, PRL
41	DN-2	Doner	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	KF	FEP, FOX, NA,PRL
42	DN-3	Doner	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Absent	+	KF	-
43	DN-4	Doner	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	CTX, KF	FOX
44	DN-5	Doner	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Absent	+	KF	CTX, FOX
45	DN-6	Doner	*C. malonaticus*	+	+	*C. malonaticus*	*C. malonaticus*	+	+	Weak	+	FOX	KF, PRL
46	DN-7	Doner	*C. malonaticus*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	CTX, FOX, KF	-
47	B-1	Spice	*C. sakazakii*	+	+	*C. malonaticus*	*C. malonaticus*	+	+	Weak	+	KF	AMP
48	B-2	Spice	*C. malonaticus*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Moderate	+	KF	AK, AMC, AMP, CIP, CTX,MEM, PRL
49	B-3	Spice	*C. dublinensis*	+	+	*C. dublinensis*	*C. dublinensis*	+	+	Absent	+	AMP	-
50	B-4	Spice	*C. malonaticus*	+	+	*C. malonaticus*	*C. malonaticus*	+	+	Weak	+	CTX, FOX, KF	AMC, AMP, FEP, TZP
51	B-5	Spice	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	KF	AK, AMP, CTX, FOX, PRL
52	B-6	Spice	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Strong	+	AMP, KF	FOX
53	B-7	Spice	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Moderate	+	KF	FOX
54	B-8	Spice	*C. muytjensii*	+	+	*C. dublinensis*	*C. dublinensis*	+	+	Weak	+	KF	-
55	C-1	Cereal	*C. turicensis*	+	+	*C. turicensis*	NI	+	+	Weak	+	AMP, ATM	NA, TE
56	C-2	Cereal	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Moderate	+	KF	FOX
57	C-3	Cereal	*C. malonaticus*	+	+	*C. malonaticus*	*C. malonaticus*	+	+	Weak	+	CTX, FOX, KF	FEP, IPM
58	C-4	Cereal	*C. universalis*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Absent	+	AMP	-
59	D-1	Ice cream	*C. malonaticus*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	FOX, KF	AMP, FEP
60	D-2	Ice cream	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Moderate	+	KF	AK, CTX, FEP, FOX, IPM
61	D-3	Ice cream	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Moderate	+	FOX, KF	NA
62	D-4	Ice cream	*C. sakazakii*	+	+	*C. malonaticus*	NI	+	+	Moderate	+	AMP	C, FEP
63	D-5	Ice cream	*C. muytjensii*	+	-	*C. muytjensii*	NI	+	+	Weak	+	AMP	C
64	SL-1	Salad	*C. sakazakii*	+	+	*C. sakazakii*	*C. sakazakii*	+	+	Weak	+	KF	CTX, FOX

[a]Biochemical identification according to Iversen et al. [2] and Joseph et al. [3]. [b]Siderophore Production. [c]Not identified due to non-PCR amplification or non-specific PCR amplification.

Abbreviations of antimicrobials: AK, amikacin; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; ATM, aztreonam; C, chloramphenicol; CIP, ciprofloxacin; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; IPM, imipenem; KF, cephalothin; MEM, meropenem; NA, nalidixic acid; PRL, piperacillin; TE, tetracycline; TZP, piperacillin/tazobactam.

Table 3 Primer sequences used in PCR assays

Target Gene	Primer	Primer Sequence (5′→ 3′)	Product Size (bp)	Reference
16S rRNA	Saka-1	ACA GGG AGC AGC TTG CTG C	952	[25]
	Saka-2b	TCC CGC ATC TCT GCA GGA		
gluA	EsAgf	TGA AAG CAA TCG ACA AGA AG	1680	[24]
	EsAgr	ACT CAT TAC CCC TCC TGA TG		
rpoB	Csakf	ACG CCA AGC CTA TCT CCG CG	514	[26]
	Csakr	ACG GTT GGC GTC ATC GTG		
	Cmalf	CGT CGT ATC TCT GCT CTC	251	
	Cmalr	AGG TTG GTG TTC GCC TGA		
	Cmuyf	TGT CCG TGT ATG CGC AGA CC	289	
	Cmuyr	TGT TCG CAC CCA TCA ATG CG		
	Cdublf	GCA CAA GCG TCG TAT CTC C	418	
	Cdublr	TTG GCG TCA TCG TGT TCC		
	Cturf	CGG TAA AAG AGT TCT TCG GC	628	
	Cturr	GTA CCG CCA CGT TTC GCC		
	Cgenomf	ACA AAC GTC GTA TCT CTG CG	506	
	Cgenomr	AGC ACG TTC CAT ACC GGT C		
	Ccon-f	AAC GCC AAG CCA ATC TCG	689	[27]
	Ccon-r	GTA CCG CCA CGT TTT GCT		
cgcA	Cdm-469R[a]	CCA CAT GGC CGA TAT GCA CGC C		[28]
	Cdub-40F	GAT ACC TCT CTG GGC CGC AGC	430	
	Cmuy-209F	TTC TTC AGG CGG AGC TGA CCT	260	
	Cmstu-825F[b]	GGT GGC SGG GTA TGA CAA AGA C		
	Ctur-1036R	TCG CCA TCG AGT GCA GCG TAT	211	
	Cuni-1133R	GAA ACA GGC TGT CCG GTC ACG	308	
	Csak-1317R	GGC GGA CGA AGC CTC AGA GAG T	492	
	Cmal-1410R	GGT GAC CAC ACC TTC AGG CAG A	585	
ompA	ESSF	GGA TTT AAC CGT GAA CTT TTC C	469	[14]
	ESSR	CGC CAG CGA TGT TAG AAG A		
zpx	Es-ProF	GAA AGC GTA TAA GCG CGA TTC	94	[13]
	Es-ProR	GTT CCA GAA GGC GTT CTG GT		
ERIC	ERIC1R	ATG TAA GCT CCT GGG GAT TCA C		[23]
	ERIC2	AAG TAA GTG ACT GGG GTG AGC G		

[a]The PCR primer Cdm-469R was used in multiplex reactions, with primers Cdub-40F and Cmuy-209F identifying *C. dublinensis* and C. *muytjensii* strains, respectively. [b]The PCR primer Cmstu-825F was used in multiplex reactions, with primers Ctur-1036R, Cuni-1133R, Csak1317R and Cmal-1410R identifying *C. turicensis*, *C. universalis*, *C. sakazakii* and *C. malonaticus* strains, respectively.

Figures

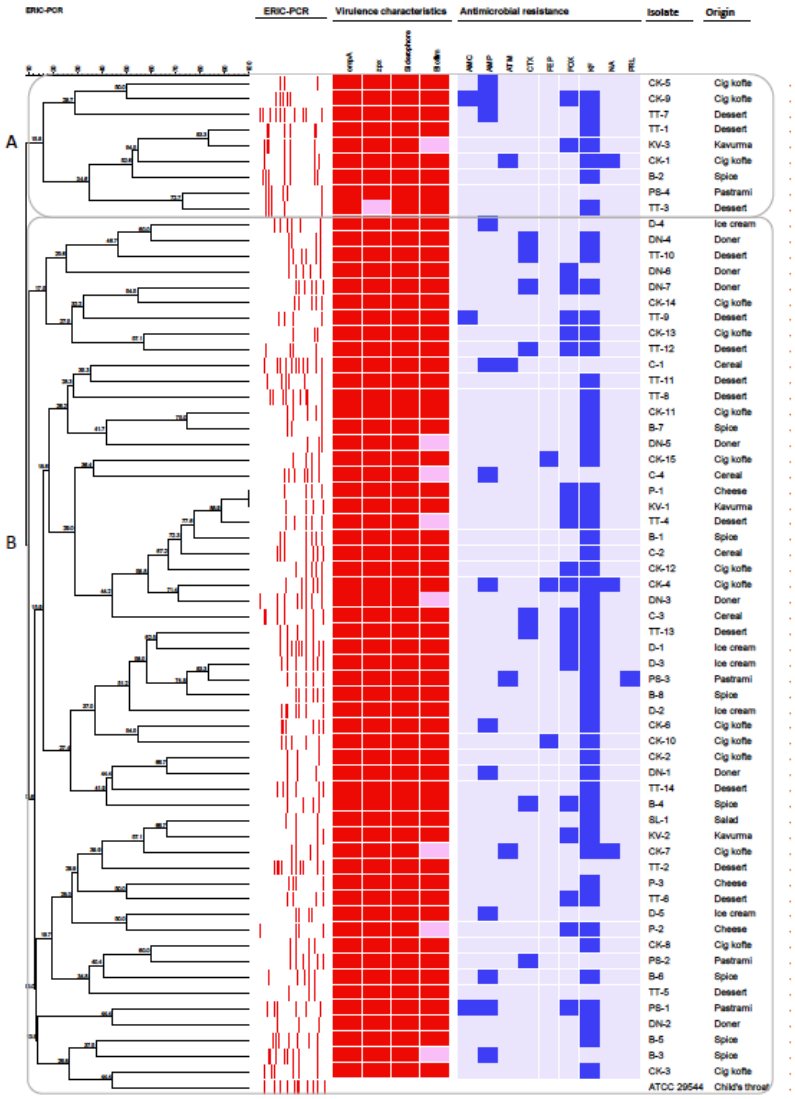

Figure 1

ERIC-PCR patterns, virulence characteristics and antimicrobial resistance profiles of Cronobacter isolates in this study. Dendrogram was constructed using BioNumerics version 7.6. The red box indicates the presence of the gene while the pink box indicates the absence of the gene. The dark blue box indicates resistance to a particular antimicrobial whereas the light blue box indicates negative result of resistance to a particular antimicrobial. Abbreviations of antimicrobials: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; ATM, aztreonam; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; KF, cephalothin; NA, nalidixic acid; PRL, piperacillin.

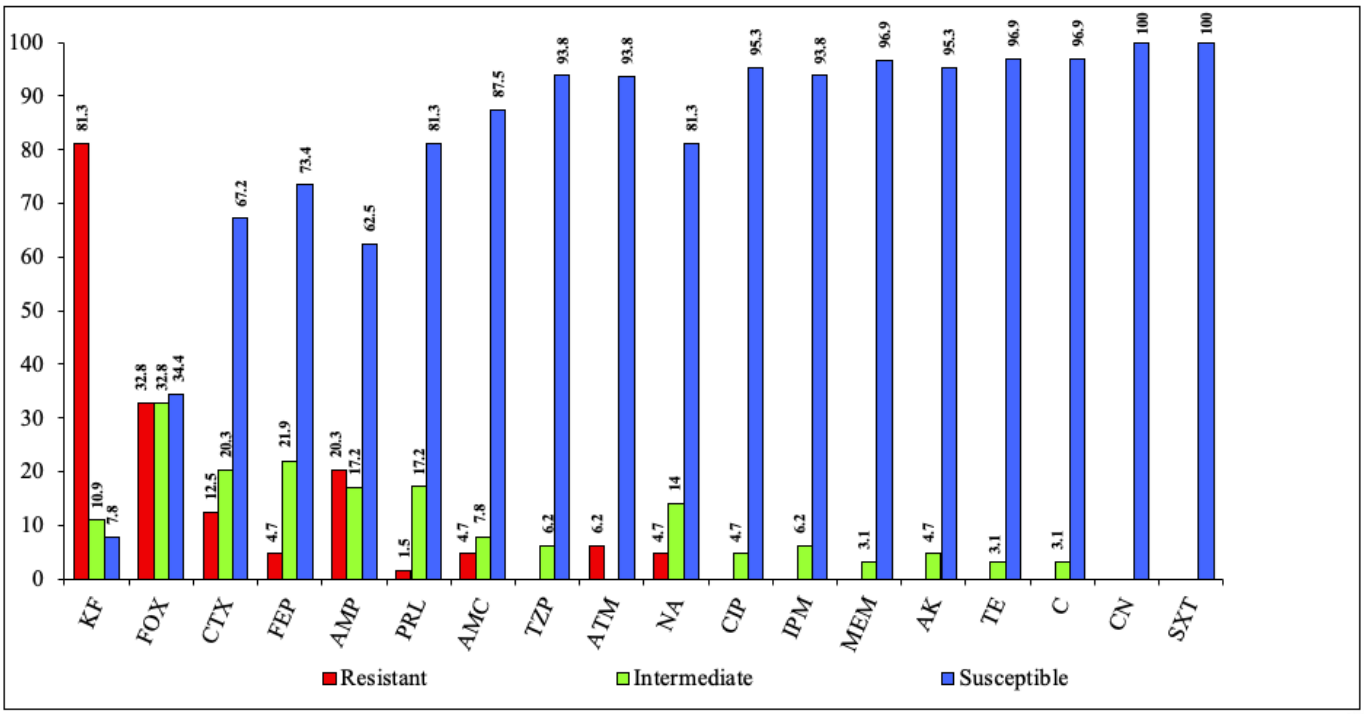

Figure 2

Antimicrobial susceptibility of the 64 Cronobacter isolates from ready-to-eat foods. Abbreviations of antimicrobials: AK, amikacin; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; ATM, aztreonam; C, chloramphenicol; CIP, ciprofloxacin; CN, gentamicin; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; IPM, imipenem; KF, cephalothin; MEM, meropenem; NA, nalidixic acid; PRL, piperacillin; SXT, trimethoprim/sulfamethoxazole; TE, tetracycline; TZP, piperacillin/tazobactam.