Original Article

Detection of *Cronobacter* spp. (formerly *Enterobacter sakazakii*) from medicinal plants and spices in Syria

Mouhammad Belal[1], Ayman Al-Mariri[2], Lila Hallab[2], Ibtesam Hamad[3]

[1]*Plant Biology Department, Faculty of Sciences, University of Damascus, Syria*
[2]*Molecular Biology and Biotechnology Department, Atomic Energy Commission, Syria*
[3]*Ecology Department, Faculty of Sciences, University of Damascus, Syria*

Abstract

Introduction: *Cronobacter* spp. (formerly *Enterobacter sakazakii*) is an emerging food-borne pathogen that causes severe meningitis, sepsis, and necrotizing enterocolitis in neonates and infants. These infections have been reported from different parts of the world. The epidemiology and reservoir of *Cronobacter* spp. are still unknown, and most strains have been isolated from clinical specimens and from a variety of foods, including cheese, meat, milk, vegetables, grains, spices, and herbs.

Methodology: Our study aimed to detect and isolate *Cronobacter* spp. from different Syrian samples of spices, medicinal herbs and liquorices, depending on the pigment production and biochemical profile of isolates and PCR technique. This PCR method, which provides a powerful tool for rapid, specific, and sensitive detection of *Cronobacter* spp., is considered a reliable alternative to traditional bacteriological methods.

Results and conclusions: This study revealed that the percentage of *Cronobacter* spp. was 94%, 52%, and 32% in liquorice, spices and medicinal herbs, respectively. In addition, it assured that the optimal enhancing growth temperature was 44°C, and optimal enhancing growth pH was 5.

Key words: biochemical reaction; Cronobacter spp.; herbs; PCR; spices; temperature

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Introduction

*Cronobacter*spp. (formerly *Enterobacter sakazakii*) is a non-spore forming, Gram-negative rod-shaped bacterium, approximately 3 x 1 μm in size. It is motile with peritrichousflagellae anaerobes facultative. It belongs to the family Enterobacteriaceae and genus *Enterobacter*, which contains a number of species, among which the differentiation is based on biochemical reactions and serological and molecular techniques [1]. *Cronobacter* spp., *E. agglomerans*, and *E. cloacae* are considered the main species of this genus that are frequently isolated from clinical samples and food products [2]. *Cronobacter* spp. is catalase positive, oxidase negative, and generally positive for α-D-glucosidase [3]. It reduces nitrates, utilizes citrates, hydrolyzes esculin and arginine, and produces acid from D-glucose, D-sucrose, D-raffinose, D-melibiose, D-cellobiose, D-mannitol, D-mannose, L-rhamnose, L-arabinose, D-xylose, D-trehalose, galacturonate and D-maltose, and it is also generally positive for acetoin production (Voges-Proskauer test) and negative for the methyl red test [4]. Traditional culture methods for identifying *Cronobacter* spp. were laborious and time-consuming and required steps of enrichment and biological tests that take 6 to 7 days to complete. PCR assay is considered a rapid, sensitive, specific and more reliable method for early detection of pathogen bacteria, including *Cronobacter* spp. [5].

The first case attributed to this organism occurred in 1958 in England and it took its name from Riichi Sakazakii, a Japanese microbiologist [6]. *Cronobacter* spp. was first described as a "yellow-pigmented *Enterobacter cloacae*" by Urmenyi and Franklin in 1961 [6]. In 1980, Farmer *et al*. designated it as a unique species [2]. The differentiation between *Cronobacter* spp. and *E. cloacae* was based on differences in biochemical reactions, the ability of *Cronobacter* spp. colonies to produce yellow pigments, and by DNA–DNA hybridization. *Cronobacter* spp. formed a microbiological hazard in the infant food chain with historic high morbidity and mortality in neonates [7]. Therefore, the name *Cronobacter* gen. nov. was proposed after the Greek mythological god Cronos, who was described as

swallowing his children at birth [8]. This genus contains the species *C. sakazakii* sp. nov.; *C. malonaticus* sp. nov.; *C. muytjensii* sp. nov.; *C. dublinensis* sp. nov.; and *C. turicensis* sp. nov. [9]. This group of bacteria are considered opportunistic pathogens that have been associated with severe forms of necrotizing enterocolitis [10] and meningitis [11], especially in neonates, and catheter-associated infections in elderly and immunocompromised people, with a mortality rate varying from 40% to 80% [12]; however, this figure has declined to about 20% in recent years [13].

Among all cases, about half of the patients die within one week of the onset of the infections and about 94% of the meningitis survivors exhibit severe neurological complications [14]. The International Commission for Microbiological Specification for Foods has ranked *Cronobacter* spp. as "Severe hazard for restricted populations, life-threatening or substantial chronic sequelae for long duration" [15]. Also, the US Food and Drug Administration has issued an alert to health-care professionals about the risk associated with *Cronobacter* spp. infections among neonates fed with milk-based infant formula. The alert stated that the most effective way to avoid *Cronobacter* spp. infections in premature babies and neonates is to prevent contamination of infant milk formula during production and bottle preparation [16]. The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) promote research that investigates ways to reduce the levels of *Cronobacter* spp. in reconstituted powdered infant formula, *e.g.*, ensuring strict time-temperature control on rehydration; decreasing the time of feeding; adding inhibitors; using biopreservatives and acidification; combining treatments; and promoting research to gain a better understanding of the ecology, taxonomy, characteristics and virulence of *Cronobacter* spp. The information gathered from this research will be important for the interpretation of epidemiology data and undertaking further risk assessments. More complex risk assessments were initiated in two FAO/WHO expert meetings and will be completed and expanded by the Joint FAO/ WHO Expert Meetings on Microbiological Risk Assessment (JEMRA) group [17]. However, knowledge of the etiological and ecological characteristics of *Cronobacter* spp. is sparse and its occurrence in factories that produce infant formulas and in hospital kitchens has not been studied in depth. *Cronobacter* spp. repeatedly have been reported as remarkably resistant to osmotic stress and dryness and moderately thermotolerant as some encapsulated *Cronobacter* spp. were still recoverable from desiccated infant formula after storage for up to 2.5 years [18]. The composition of dry foods and infant formula combined with their low water activity significantly affected the survival of *Cronobacter* spp. in these foods [19]. Previously in Syria, traditional medicine used some herbs, such as *cuminum cyminum* and *pimpinella anisum*, as additives to infant formula to treat some enterogastric confusions. The presence of *Cronobacter* spp. in these herbs was not yet been completely studied. The aim of this study is to analyze the contribution of vegetative foods, including medicinal plants, (*e.g.*, spices and medicinal plants purchased from a Syrian traditional market) in the morbidity of adults with *Cronobacter* spp.

Methodology

Food samples

A total of 144 different samples were collected from different locations across Damascus, Syria, and its countryside. The samples composed of 68 medicinal plants, 16 liquorice, and 60 (Tables 1 and 2).

Detection, isolation and identification of Cronobacter spp.

The procedure of ISO/TS 22964:2006 for detection of *Cronobacter* spp. was followed [20]. Typical colonies that appeared yellow on tryptic soy agar (TSA) were picked and subjected to further characterization by using microscope, biochemicals, and PCR analysis. and studying its ability to grow at different temperatures and pH degrees.

Morphology

Yellow colonies from TSA were examined by microscope after staining with Gram stain to study their morphology, and by dark field microscope to study their motility.

Biochemical tests

The following biochemical tests were performed: oxidase, catalase, α-glucosidase, lactose fermentation, methyl red, and Voges-Proskauer. The tests were purchased from Oxoid (Basingstoke, United Kingdom) and carried out as follows:

- The oxidase test was performed by transferring one colony to an oxidase strip. A reaction was considered positive when the strip turned dark blue or violet within three minutes.

Table 1. Medicinal plants that were used in the study

Medicinal plants	No. samples	Medicinal plants	No. samples	Medicinal plants	No. samples
Pimpinella anisum	1	*Equisetum* sp.	1	*Urtica dioica*	1
Cassia acutifolia	3	*Rosa damascena*	2	*Erica vulgaris*	1
Matricaria chamomilla	3	*Thymus serpyllum*	2	*Mentha sylvestris*	1
Salvia officinalis	4	*Althea officinalis*	2	*Mentha viridis*	3
Hibiscus sabdariffa	3	*Melissa officinalis*	2	*Ferula harmoni*	1
Trigonellafoenum-graecum	2	*Eleagnus angustifolia*	1	*Cyperus rotundus*	1
Cinnamomum sp.	2	*Zea mays*	1	*Crataegu soxycantha*	2
Artemisia herba-alba	1	*Ecballium elaterium*	1	*Ocimum basilicum*	1
Capsella bursa-pastori	1	*Marrhubium vulgare*	2	*Prosopis farcta*	1
Sambucusniger	1	*Artemisia argentea*	1	*Ephorbia helioscopia*	1
Stipa tenacissima	1	*Rubia* sp.	1	*Artemisia rupestris*	1
Centauria sp.	1	*Cynodon dactylon*	1	*Chelidonium majus*	1
Oenanthe aquatic	1	*Bee-Pollen*	1	*Galega officinalis*	1
Spartium sp.	1	*Lavande stoechas*	1	*Thymus capitatus*	2
Paronychia argentea	1	*Anethum graveolens*	1	*Nigella sativa*	1
Arum sp.	1	*Linum* sp.	1	*Avena sativa*	1

Table 2. Spices that were used in the study

Spices	No. samples	Local spices	No. samples
Curry	3	Kabseh spices	4
Coriander sativum	3	Kegen	1
Cuminum cyminum	3	Magi	3
Piper nigrum	2	Tahi spices	1
Carum carvi	1	Hot dog spices	1
Myristica fragrams	1	Chicken spices	1
Carthamus sp.	1	Shawarma spices	2
Indian chili	1	Cheese spices	1
Alpinia officinarum	2	Sheesh spices	1
Oregano spices	1	White pepper	1
Orchis mascula	1	Salad spices	1
Cerasus mahaleb	1	Fish spices	1
Zingiber officinalis	2	Ozi spices	1
Elettaria cardamomum	1	Falafel spices	1
Eugenia caryophyllata	1	Muskrose	1
Ammi sp.	1	Turmeric	3
Chili	2	Mardakosh	2
Rhuscoriaria	3	Hamburger spices	1
		Faheeta spices	1
		Breani spices	1
		Babreeka spices	1

Table 3. Percentage of the presence of *Cronobacter* spp. in the specimens

Specimens	Number of specimens	Cronobacter spp. isolates	Percentage
Spices	60	31	51.7%
Medicinal plants	68	22	32.4%
Liquorice	16	15	93.8%
Total	144	68	47.2%

Our results were accepted according to biochemical reactions and PCR.

- The catalase test was performed by using hydrogen peroxide 3%. A reaction was determined to be positive if the catalase reaction showed gas bubbles, whereas no gas bubbles appeared in a negative reaction.
- The α-glucosidase test was carried out using ESIA plates which included the substrate 5-bromo 4-chloro 3-indolyl-α, D-glucopyranoside (XαGlc). The production of a blue-green colony indicated a positive reaction while a negative reaction was indicated by the production of a violet or transparent colony.
- Lactose fermentation was conducted using MacConkey plates containing lactose. The bacteria that ferment lactose produce acidic compounds which reduce the pH to less than 6.8, resulting in a change of the medium colour due to the presence of neutral red.
- Methyl red and Voges-Proskauer tests were used to differentiate among the Gram-negative bacilli in the family Enterobacteriaceae. The tests were performed by incubation of the bacteria in MR-VP medium at 37ºC for 24 hours; half of this medium was then used for the methyl red test by adding some drops of methyl red, and the other half for a Voges-Proskauer test by adding alpha-naphtol (5%) and potassium hydroxide (40%). Positive results for the methyl red and Voges-Proskauer tests were indicated by the appearance of red in the medium, whereas the medium in negative tests remained yellow.

DNA isolation and PCR

DNA was isolated by using the cetyltrimethylammonium bromide/NaCl (CTAB /NaCl) method [21], and PCR was performed with an automated thermal cycler (Techne TC-512, Staffordshire, UK). Three pairs of specific primers (SG-F: 5'-GGG-TTG-TCT-GCG-AAA-GCG-AA-3', SG-R: 5'-GTC-TTC-GTG-CTG-CGA-GTT-TG-3'; SI-F: 5'-CAG-GAG-TTG-AAG-AGG-TTT-AAC-T-3', SI-R: 5'-GTG-CTG-CGA-GTT-TGA-GAG-ACT-C-3') designated for the sequences between 16S rDNA and 23S rDNA (internal transcribed spacer ITS) as described by Liu *et al*. [22] were used in the study. Variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions. The EsAg-F: 5'-TGA-AAG-CAA-TCG-ACA-AGA-AG-3', EsAg-R: 5'-ACT-CAT-TAC-CCC-TCC-TGA-TG-3' primer was previously described by Lehner*et al*. and designated for the gluA gene [23]. The reaction was performed at the volume of 25 µl consisting of 2 µl of bacterial genomic DNA 100 ng (with SI and SG primers) or 10 µl of bacterial genomic DNA 100 ng (with EsAg primer), 1X buffer 10X, 3 mM MgCl2, 0.2 mMdNTPs, 1 U Taq DNA polymerase (5 U), 2 µl of each pairs of primers (0.4 µM each), and nuclease free water (Fermentas, Vilnius, Lithuania). PCR conditions were, hot start for 5 minutes at 95˚C followed by 35 cycles of 1 minute at 95°C; 57°C for 1 minute; 72°C for 1.5 minutes; and a final extension of 10 minutes at 72˚C. PCR products were then analyzed by electrophoresis in 1.5% (w/v) agarose gel in 1X TAE buffer at a constant voltage of 65 V for 1 hour, then visualized under UV light to confirm the presence of the amplified DNA.

Growth at different temperatures and pH degrees

Six typical yellow colonies confirmed as *Cronobacter* spp. were chosen for further analysis as follows: two from medicinal plants (*C. acutifolia* and *M. chamomilla*) two from spices (curry and black pepper) and two from liquorice. Each of these colonies was then subcultured in 3 ml BPW at 44° for 24 hours. Then, to determine the optimal temperature and the optimal pH level for enhancing the growth of bacteria, 0.6 x 109 cfu/ml of each sample was re-cultured at a fixed pH (7.0) and at different temperatures as follows: 25-30-37-44-48°C for 24 hours. Optical densities (O.D) were read at 600 nm. Next, using the same conditions mentioned above, the optimal enhancing growth temperature was fixed, and different

Table 4. Positive specimens for *Cronobacter* spp. and their biochemical tests

Specimen	No. of positive	Oxidase	Catalase	α-glucosidase	Methyl red	Voges-Proskauer	Lactose fermenting	Yellow pigment	PCR
Curry	2	-	+	+	-	+	+	+	+
Coriander sativum	1	-	+	+	-	+	+	+	+
Cuminum cyminum	2	-	+	+	-	+	+	+	+
Piper nigrum	1	-	+	+	-	+	+	+	+
Myristica fragrams	1	-	+	+	-	+	+	+	+
Indian chili	1	-	+	+	-	+	+	+	+
Alpiniacofficinarum	1	-	+	+	-	+	+	+	+
Cerasus mahaleb	1	-	+	+	-	+	+	+	+
Ammi sp.	1	-	+	+	-	+	+	+	+
Rhus coriaria	2	-	+	+	-	+	+	+	+
Kabseh spices	2	-	+	+	-	+	+	+	+
Magi	1	-	+	+	-	+	+	+	+
Tahi spices	1	-	+	+	-	+	+	+	+
Chicken spices	1	-	+	+	-	+	+	+	+
Shawarma spices	1	-	+	+	-	+	+	+	+
Cheese spices	1	-	+	+	-	+	+	+	+
Sheesh spices	1	-	+	+	-	+	+	+	+
White pepper	1	-	+	+	-	+	+	+	+
Salad spices	1	-	+	+	-	+	+	+	+
Ozi spices	1	-	+	+	-	+	+	+	+
Falafel spices	1	-	+	+	-	+	+	+	+
Musk rose	1	-	+	+	-	+	+	+	+
Tumeric	2	-	+	+	-	+	+	+	+
Mardakosh	2	-	+	+	-	+	+	+	+
Babreeka spices	1	-	+	+	-	+	+	+	+
Liquorice	15	-	+	+	-	+	+	+	+
Cassia acutifolia	3	-	+	+	-	+	+	+	+
Matricariachamomilla	3	-	+	+	-	+	+	+	+
Hibiscus sabdariffa	1	-	+	+	-	+	+	+	+
Trigonellafoenum-graecum	2	-	+	+	-	+	+	+	+
Cinnamomum sp.	1	-	+	+	-	+	+	+	+
Avena sativa	1	-	+	+	-	+	+	+	+
Equisetum sp.	1	-	+	+	-	+	+	+	+
Thymus serpyllum	1	-	+	+	-	+	+	+	+
Althea officinalis	1	-	+	+	-	+	+	+	+
Eleagnusangustifolia	1	-	+	+	-	+	+	+	+
Artemisia argentea	1	-	+	+	-	+	+	+	+
Linum sp.	1	-	+	+	-	+	+	+	+
Mentha viridis	2	-	+	+	-	+	+	+	+
Cyperus rotundus	1	-	+	+	-	+	+	+	+
Thymus capitatus	1	-	+	+	-	+	+	+	+
Nigella sativa	1	-	+	+	-	+	+	+	+

Table 5. Specimens used in the experiments of temperature and pH

Medicinal plants	Spices	Liquorice
Cassia acutifolia (67d)*	Curry (18d)	29b
Matricaria chamomilla (71d)*	Black pepper (41d)	38b

*Brackets refer to the specimen lab ID number

pH levels (pH = 3-5-7-9) were examined to assess the optimal pH level at the optimal temperature, in. Each sample was processed five times in this manner.

Results and discussion

This research isolated *Cronobacter* spp. from different spices and medicinal plants. There are many differences in phenotype among the isolates of *Cronobacter* spp; thus it is difficult to confirm the identity of isolates by using only one method or one set of specific PCR primers [24]. Therefore, this study depended on the use of chromogenic, biochemical, and molecular techniques for detection, isolation, and identification of *Cronobacter* spp. from food samples. Our results concur with those described by Farmer and colleagues [2], who showed that freshly isolated *Cronobacter* spp. produce two distinct colony morphologies, the first type dry and the second type mucoid.

All isolated strains of *Cronobacter* spp. appeared by microscopical examination as Gram-negative, rod shaped, and motile bacteria. They were also lactose

fermenting, catalase positive, oxidase negative, α-glucosidase positive, methyl red negative, and Voges-Proskauer positive. However, several previous studies have produced conflicting results [25].

PCR was performed by using three pairs of specific primers as described above and all strains of *Cronobacter* spp. showed a correct-sized amplification product of 1680, 282, and 251 bp according to the primers EsAg, SG, and SI respectively (Figure 1). However, no amplification product was obtained for all *E. cloacae* strains by using the same primers (100% specificity).

It was observed that the highest percentage of *Cronobacter* spp. (≈ 94%) was found in liquorice (herbal drink), while the percentage of *Cronobacter* spp. found in spices and medicinal plants was about 52% and 32%, respectively [Table 3]. The large amount of these bacteria in liquorice (roots) corresponds s with the similar large amount of *Cronobacter* spp. present in the roots of some agriculture plants such as corn, cucumber, rough lemon, and tomato [26,27,28,29].

The medicinal plants and spices that were positive for the presence of *Cronobacter* spp. are listed in table [4]. In addition, biochemical test results for these isolates are given in the table.

When the typical colonies were confirmed by biochemical tests and PCR, characterization of optimal growth temperatures and pH levels were determined as described in the methodology section. Figures 2 and 3 show the effects of different temperatures and different pH levels, respectively, on enhancing the growth of bacteria. It was observed that the optimal temperature was 44˚C and the optimal pH level was 5.

These results conform with the fact that the pH level of an infant's stomach is 5 [30], which would allow the growth of *Cronobacter* spp., whereas this bacteria likely would not survive at the pH level found in an adult's stomach (pH3). Most studies recommend not preparing food for infants at room temperature for longer than 20 minutes as doing so allows bacteria to grow very quickly [31]. These findings reflect the possibility of coexisting *Cronobacter* spp. in foods other than infant formula, infant food, and milk powder. Despite the death of the bacteria at high temperatures, secreted endotoxins which tolerate high temperatures used in food preparation still remain and can cause illness.

Our results are in agreement with those reported in a review of endophytic bacteria in agriculture crops by Hallmann *et al*., 1997, who noted that the genus *Enterobacter* is associated with the phytic flora [32]. Also *Enterobacter* species have been isolated from corn roots and stems [26], cucumber roots [27], rough lemon roots [28], and grapevine stems [33]. Mossel and Struijk hypothesized that just as the primary reservoir for the coliform *E. coli* is feces, the reservoir for *E. sakazakii* (*Cronobacter* spp.), in addition to other coliforms, such as *Klebsiella oxytoca*, *K. pneumoniae*, *E. cloacae* and *Citrobacter* species, may primarily be environmental and from plant materials [34]. Iversen and Forsythe hypothesize that the principal environmental sources of *E. sakazakii* (*Cronobacter* spp.) are water, soil and vegetables, and

a secondary means of contamination may be vectors such as flies and rodents [13]. Cottyn *et al*. analyzed rice harvested from various sites in the Philippines for bacterial flora; of the 428 bacterial isolates examined, 184 were Gram-positive and 244 were Gram-negative. The most prevalent (25%) of the Gram-negative isolates were from the family Enterobacteriaceae, with the genus *Pantoea* and *Enterobacter* predominating. Four seed lots yielded 20 *E. sakazakii* (*Cronobacter* spp.) isolates and five lots yielded 9 isolates of *E. cloacae* [35]. In another study, the highest percentage of *Cronobacter* spp. isolates (39%) was found in herbs and spices; for instance, the four samples tested of a traditional herbal drink (liquorice) contained *Cronobacter* spp. (100%) while 11 out of 15 samples (73.3%) of mixed spices contained *Cronobacter* spp. [36]. However, our results differ from those of several reports that have implicated rehydrated powdered infant formula as a source of *Cronobacter* spp. in neonatal infections [10,37]. It might be that infant formula and infant foods become contaminated at certain stages during the processing, particularly after sterilization during vitamin or supplement fortification steps. Furthermore it is worthwhile to mention that *Cronobacter* spp. may be associated with the foods other than infant formula, infant food, and milk powder. These results are in agreement with those reported by Forsythe and Friedemann, who emphasize that the majority of *E. sakazakii* (*Cronobacter* spp.) isolates are from plant sources, irrespective of the claims of most studies which confirmed that powdered infant formula is a source of this pathogen [38,39]. Our results also indicate that plants possibly incarnate the major reservoir of *Cronobacter* spp., and its ability to survive in dry foods, herbs, spices and the general manufacturing environment may be due to its thermotolerant and osmotolerant nature [40].

The high association of this pathogen with herbs and spices suggests that extra precautions should be taken when home medications containing herbs or herbal beverages are given to infants to allay gastrointestinal disturbance. Extra care should also be taken in hospital kitchens when preparing meals for immuno-compromized persons who may be at risk for *Cronobacter* spp. infection from contaminated.

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Corresponding author
Ayman Al-Mariri
Researcher
Molecular Biology and Biotechnology Department
Atomic Energy Commission of Syria
Damascus, Syria
Telephone: +963 11 2132580
Fax: +963 11 6112289
Email: ascientific1@aec.org.sy

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