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# Characterization of *Cronobacter* spp. isolated from food of plant origin and environmental samples collected from farms and from supermarkets in the Czech Republic

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ABSTRACT

The *Cronobacter* genus (previously known as *Enterobacter sakazakii*) comprises seven species (*Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter turicensis*, *Cronobacter dublinensis*, *Cronobacter universalis* and *Cronobacter condimenti*) which cause serious infections in neonates and immunocompromised people. Most of the documented outbreaks of these bacteria have been associated with consumption of contaminated powdered infant formula. The plant environment is considered to be the natural habitat of these bacteria. Therefore, a total number of 563 samples of vegetables, fruit, water and environmental swabs were collected from local farms and supermarkets in the Czech Republic and investigated for the presence of *Cronobacter* spp. The obtained 45 isolates (8.0%) were further characterized by phenotyping (antimicrobial resistance, capsule and pigment production) and genotyping (*fusA* sequencing, MLST, PCR-serotyping) methods. Most of the *Cronobacter* isolates (42.2%) were identified as *C. sakazakii*, followed by *C. turicensis* (31.1%), *C. dublinensis* (22.2%), *C. malonaticus* (2.2%) and *C. universalis* (2.2%). The 25 identified sequence types, out of which 17 were unique for only one strain, indicated a high diversity of strains. *C. sakazakii* sequence type 4 (ST 4), which has been associated with many cases of meningitis, was isolated only in one case. A strong association of *C. turicensis* and *C. dublinensis* with the plant environment can be deduced from our results.



## 1. Introduction

*Cronobacter* species comprises a group of opportunistic pathogens associated with serious infections in neonates with the manifestation of necrotizing enterocolitis, bacteraemia and meningitis with case fatality rates ranging between 40 and 80%. The majority of *Cronobacter* infections are in the adult population, especially in the immunocompromised, the elderly, patients with medical implants, patients with extended hospital visits and those with acute, chronic, or serious illnesses (Alsonosi et al., 2015; Patrick et al., 2014; Yan et al., 2012). Non-infant population can be affected by *Cronobacter* in plant material being ingested as part of normal diet; *Cronobacter* spp. pose furthermore part of the normal flora carriage (Alsonosi et al., 2015; Patrick et al., 2014).

The *Cronobacter* genus was established in 2007 as a substitute for *Enterobacter sakazakii* (Iversen et al., 2007a). The genus passed through several reclassifications (Brady et al., 2013; Joseph et al., 2012a; Iversen et al., 2008a; Jackson et al., 2014; Stephan et al., 2014) and currently it contains seven species (*Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter turicensis*, *Cronobacter dublinensis*, *Cronobacter universalis*, and *Cronobacter condimenti*). Strains belonging to three species (*C. sakazakii*, *C. malonaticus*, and *C. turicensis*) are predominantly isolated from human infections and some clones with enhanced virulence potential have also been described within these species (Joseph and Forsythe, 2011).

Although the outbreaks of *Cronobacter* spp. have been associated with the consumption of contaminated powdered infant formula (PIF) and with the equipment and utensils used for the preparation of rehydrated product (FAO and WHO, 2008; Gurtler et al., 2005), infections have also occurred among persons who did not consume or handle formula (Alsonosi et al., 2015; Patrick et al., 2014). Bacteria have been isolated from a wide range of foods and environments (Edelson-Mammel et al., 2005; Friedemann, 2007; Iversen and Forsythe, 2004; Turcovsky et al., 2011). The plant environment is considered to be the natural habitat for *Cronobacter* spp. *Cronobacter* spp. strains typically produce yellow pigment, form a gum-like extracellular polysaccharide and resist desiccation during long dry periods (Schmid et al., 2009). High resistance to environmental stresses, e.g. resistance to dry and osmotic conditions, heat resistance and tolerance to low pH are linked to the spread of *Cronobacter* spp. in food (Caubilla-Barron et al., 2007; Norberg et al.,

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2012; Orieskova et al., 2013, Osaili and Forsythe, 2009; Walsh et al., 2011; Yan et al., 2012).

Although *Cronobacter* spp. occur in several matrices, the technical standard protocol for the detection of *Cronobacter* species is available only for milk-based powdered infant formula (ISO/TS 22964, 2006; Yan et al., 2012). Consequently, combinations of standard methods described in ISO/TS 22964 and a new approach using different selective media were employed for the isolation of *Cronobacter* strains from food in several studies (Iversen et al., 2008b; Turcovsky et al., 2011; Yan et al., 2012).

Presumptive *Cronobacter* isolates are necessary to be validly identified at genus and species levels. Commercially available phenotyping kits (e.g. API20E, ID32E) have been reported as insufficient tools for the identification to the species level and several false-negative as well as false-positive results were observed even at the genus level (Cetinkaya et al., 2013, Iversen et al., 2006). By reason of this fact, PCR and real time PCR methods for the identification of *Cronobacter* genus have been designed (Drudy et al., 2006; Kothary et al., 2007; Lehner et al., 2006; Liu et al., 2006; Malorny and Wagner, 2005; Nair and Venkitanarayanan, 2006; Seo and Brackett, 2005; Zhou et al., 2008). The design of PCR system for the differentiation between various species within the *Cronobacter* genus represents a challenge for molecular biology because of closely related sequences of some *Cronobacter* species, for example *C. sakazakii* and *C. malonaticus*. The species-specific assays covering all seven species which are based on *rpoB* PCR were described recently (Lehner et al. 2012; Stoop et al., 2009; Yan et al., 2012). Currently, *fusA* sequencing enables most accurate speciation because it follows the whole genome phylogeny and adjusts to taxonomic changes (Alsonosi et al., 2015; Jackson et al., 2014; Forsythe et al., 2014).

*Cronobacter* isolates can be further characterized by several genotyping techniques, e.g. PFGE (Alsonosi et al., 2015; Cetinkaya et al., 2013; Cui et al., 2014; Miled-Bennour et al., 2010; Mullane et al., 2007), AFLP (Iversen et al., 2007b, Turcovsky et al. 2011), O-antigen serotyping (Blazkova et al., 2015) and by DNA sequencing (Forsythe et al., 2014). The multilocus sequence typing (MLST) scheme using seven housekeeping genes developed by Baldwin et al. (2009) enables both species identification and strain sub-species discrimination due to a database containing defined sequence types covering all *Cronobacter* spp. (Joseph et al., 2012b; Yan et al., 2012).

Recently, several studies investigated food and environmental strains of *Cronobacter* isolated mainly from powdered infant formula (Gicova et al., 2014; Sonbol et al., 2013), flour (Cetinkaya et al., 2013), dry powdered foods (Lee et al., 2012), cereals, cereal products, spices, herbs (Chon et al., 2012; Li et al., 2014; Turcovsky et al., 2011) and vegetables (Chon et al., 2012; Lee et al., 2012; Osterblad et al., 1999), but also from various foods of animal origin (Chon et al., 2012; Friedemann, 2007; Turcovsky et al., 2011). Some studies dealing with the occurrence of *Cronobacter* spp. in retail foods accessible in the Czech Republic have been conducted (Hochel et al., 2012; Killer et al., 2015).

The aim of this study was to detect *Cronobacter* spp. in vegetables, fruit and environmental samples collected on local farms and at supermarkets in the Czech Republic. The obtained 45 isolates were further characterized by phenotyping (antimicrobial resistance, capsule and pigment production) and genotyping (*fusA* sequencing, MLST, PCR-serotyping) methods, which enables their comparison with clinical and other food strains.

## 2. Material and methods

### 2.1. Strain collection

Samples of vegetables, fruit, water (10 l of irrigation or rinsing water in a sterile container) and environmental swabs from a standard sample area (10 × 10 cm) of equipment, tools and hands of workers were collected from 11 local farms within the surveillance programme for safety of food of plant origin at the Veterinary Research Institute, Brno, Czech Republic in 2012–2014. Samples of vegetables and fruit were further collected from eight different supermarkets. Analysed sample categories are listed in Table 1.

### 2.2. Species determination

#### 2.2.1. Isolation and identification of Cronobacter spp.

According to the standard method specified in ISO/TS 22964:2006 and by Iversen et al. (2008b) the two step enrichment consisting of pre-enrichment (1/10) in buffered peptone water (BPW; Oxoid, Hampshire, UK) and selective enrichment (1/100) at 42 °C in *Cronobacter* screening broth (CSB; Oxoid) was used for *Cronobacter* spp. isolation. Namely, 25 g of food samples, swabs and microbiological filters after filtration of 100 ml of water were used for enrichment. Presumptive *Cronobacter* strains were isolated as typical green-blue colonies on chromogenic *E. sakazakii* agar, DFI formulation (Oxoid) and *E. sakazakii* Agar, modified (ESIA; Fluka, St. Gallen, Switzerland) at 37 °C and 44 °C.

#### 2.2.2. PCR confirmation of Cronobacter isolates

Presumptive *Cronobacter* isolates were identified by PCR targeted to the α-glucosidase gene according to Lehner et al. (2006).

#### 2.2.3. Multilocus sequence typing

In our study, the multilocus sequence typing method (MLST) published by Baldwin et al. (2009) and Gicova et al. (2014) was adopted. The bacterial DNA was isolated from an overnight culture using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). PCR with primers and conditions according to a protocol available at the MLST *Cronobacter* web database (http://pubmlst.org/cronobacter/) was performed. Allele numbers were assigned using tools available at the MLST *Cronobacter* website (http://pubmlst.org/cronobacter/) in Bionumerics software (Applied Maths, Belgium). Species identification was performed by means of *fusA* sequencing.

**Table 1**
The occurrence and characterization of *Cronobacter* spp. in analysed sample categories.

| Sample category | Origin | Type | Number of analysed samples | Number of positive samples | Species and serotypes |
|---|---|---|---|---|---|
| Vegetables | Farm | Carrot | 118 | 16 (13.6%) | *C. sakazakii*, *C. turicensis*, *C. dublinensis*, *C. universalis* |
| | | Cucumber | 110 | 14 (12.7%) | *C. sakazakii*, *C. dublinensis* |
| | | Leafy greens | 90 | 1 (1.1%) | *C. turicensis* |
| | Supermarket | Mixed vegetables (RTE) | 35 | 0 | – |
| | | Whole vegetables | 27 | 1 (3.7%) | *C. turicensis* |
| | | Frozen vegetables | 6 | 2 (33.3%) | *C. sakazakii*, *C. malonaticus* |
| | | Sprouts | 10 | 4 (40.0%) | *C. dublinensis*, *C. turicensis* |
| Fruit | Farm | Strawberry | 40 | 1 (2.5%) | *C. sakazakii* |
| | Supermarket | Frozen fruit | 9 | 0 | – |
| Water | Farm | – | 16 | 1 (6.3%) | *C. turicensis* |
| Env. swabs | Farm | – | 102 | 5 (4.9%) | *C. sakazakii*, *C. turicensis* |