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Research Letter

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Diversity of *Cronobacter* genus isolated between 1970 and 2019 on the American continent and genotyped using multi-locus sequence typing

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One sentence summary: *Cronobacter* spp. strains isolated on the American continent were high diverse according to MLST, and therefore could be used for microbial source tracking, including the epidemiological investigations of outbreaks.

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ABSTRACT

This study aimed to evaluate the *Cronobacter* spp. strains isolated on the American continent and characterized using multi-locus sequence typing (MLST) available in the PubMLST database and current literature. From 465 *Cronobacter* spp. strains, the majority ($n = 267$, 57.4%) was from North America, mainly from USA ($n = 234$) and 198 (42.6%) were from South America, mainly from Brazil ($n = 196$). A total of 232 (49.9%) were isolated from foods, 102 (21.9%) from environmental, 87 (18.7%) from clinical, 27 (5.8%) from PIF, one from water (0.2%) and 16 (3.5%) from unknown sources. A total of five species were represented: *Cronobacter sakazakii* (374, 80.4%), *Cronobacter malonaticus* (41, 8.8%), *Cronobacter dublinensis* (29, 6.2%), *Cronobacter turicensis* (16, 3.5%) and *Cronobacter muytjensii* (5, 1.1%). The strains with complete MLST profile ($n = 345$) were assigned to 98 STs, a ratio of 3.5 strain by ST found and the calculated Simpson`s index was 0.93. The strains showed a high diversity and after eBURST analysis, 30 STs ($n = 189$) formed 12 single and/or double-locus variant clonal complexes (CC). A total of 38 STs (38.7%) were associated with clinical cases of infection, including well established *C. sakazakii* CC 1, 4, 8 and 83; *C. malonaticus* ST60, 307, 394 and 440; and *C. sakazakii* ST 12 and 494.

Keywords: *Cronobacter*; MLST; genetic diversity; epidemiology; foodborne pathogens; bacterial infections

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INTRODUCTION

The *Cronobacter* genus belongs to the family Enterobacteriaceae, for which seven species have been described (Joseph *et al.* 2012a; Iversen *et al.* 2008). The genus has come to the attention of the food industry, especially infant formula manufacturers and regulators due to its association with life-threatening infections of neonates. According to Forsythe (2018), the *Cronobacter* species can be grouped according to their clinical relevance, with *Cronobacter sakazakii* and *Cronobacter malonaticus* forming Group 1 and is composed of the majority of clinical isolates; Group 2 comprises *Cronobacter turicensis* and *Cronobacter universalis*, which have been rarely reported clinically; Group 3 comprises *Cronobacter dublinensis*, *Cronobacter muytjensii* and *Cronobacter condimenti*, which are primarily environmental commensals and are probably of little or no clinical significance. The majority of cases are in the adult population (Patrick *et al.* 2014; Alsonosi *et al.* 2015; Kadlicekova *et al.* 2018) however only one outbreak has been attributed to contaminated food. This was due to *C. sakazakii* (Yong *et al.* 2018).

There has been considerable concern related to the presence of *Cronobacter* spp. in powdered infant formula (PIF) due to their highlighted association with neonatal infections (Flores *et al.* 2011; Pan *et al.* 2014; Fei *et al.* 2017). The Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) has undertaken three risk assessments of *Cronobacter* spp. in PIF, powdered follow-up infant formula (FUF) and other infant food (FAO/WHO 2004, 2006 and 2008). Their summary recommendations included the use of internationally validated detection and molecular typing methods for *Cronobacter* spp. to gain a better understanding of the ecology, taxonomy, virulence and other characteristics of *Cronobacter* spp. and on ways to reduce its levels in reconstituted PIF. Consequently, there has been considerable research into improved detection methods, more reliable identification procedures, genotyping schemes and analysis (Forsythe 2018).

The application of conventional (7-loci) multilocus sequence typing (MLST) has revealed considerable information on the emergent bacterial pathogen. The *Cronobacter* spp. MLST scheme develop by Baldwin *et al.* (2009) requires the partial sequence analysis of seven housekeeping genes: *atpD*, *fusA*, *glnS*, *gltB*, *gyrB*, *infB* and *ppsA*. This DNA-sequence approach, supported by an open access curated database, has established new *Cronobacter* species and recognition of the clonal lineages (pathovars) attributed to the majority of neonatal meningitis and necrotizing enterocolitis cases (Joseph *et al.* 2012b; Joseph and Forsythe 2012). As an example of the benefits of rapid DNA sequencing, even without considering Next Generation Sequencing of genomes, the study of just 7-loci is a model for modern microbial epidemiology and food microbiology (McMullan *et al.* 2018; Lepuschitz *et al.* 2019; Strysko *et al.* 2020). The majority of strains deposited in the PubMLST *Cronobacter* spp. database come from Asia, in particular China ($n = 1315$; 44.4%) and Europe ($n = 969$; 32.7%; PubMLST, 2020). However, despite the high number of entries from China ($n = 1100$; 39%), there have been few reported clinical cases in China with which to compare the distribution of *Cronobacter* spp. between different sources (Ling *et al.* 2021). In comparison, there are 492 (16.6%) strains in the database from the American continent from a wide range of sources.

Because reporting is not mandatory in most countries, the true incidence of invasive infant *Cronobacter* spp. infections is unknown (Strysko *et al.* 2020). Strysko *et al.* (2020) reviewed all cases of blood-stream infection or meningitis among infants that had been reported to the Center for Disease Control and Prevention (CDC) and in the literature (1961–2018, $n = 183$). They reported that global annual reporting was significantly higher during the final quarter of the study period, increasing from a mean of 1.2 cases/year before 2004 to 8.7 cases/year from 2004–2018.

Many cases of *Cronobacter* spp. infection have been reported in America, including severe meningitis cases of neonates (CDC 2009; Asato *et al.* 2013; Hariri, Joseph and Forsythe 2013; Umeda *et al.* 2017; Chaves *et al.* 2018; Sundararajan *et al.* 2018; Strysko *et al.* 2020) and adult infections (Patrick *et al.* 2014; Alsonosi *et al.* 2015; Kadlicekova *et al.* 2018). Consequently, accurate microbial source tracking is needed to support epidemiological, regulatory actions surveillance and preventive measures. For example in identifying routes of contamination in foods and in particular PIF. The use of a centralized genotyping database enables further analysis of *Cronobacter* spp. strains isolates from foods, environmental and clinical samples on the American continent. This will lead to a better understanding of the epidemiology of these pathogens in this specific geographic area.

In this study, the genetic diversity and epidemiological profile of *Cronobacter* spp. strains isolated on the American continent were characterized using MLST, and compared with other *Cronobacter* spp. strains deposited in the PubMLST database.

MATERIALS AND METHODS

Bacterial strains

This study analyzed 480 *Cronobacter* spp. strains isolated on the American continent and deposited in the Cronobacter PubMLST open access curated database (Jolley, Bray and Maiden 2018; PubMLST 2020). General metadata available included ST, CC, species, country, year of isolation, source and association with human infections (last access 10/15/2020). The list and details of all the isolates included in this study, along with their MLST profile details, are in supplementary file 1.

MLST of Cronobacter spp. isolates from the American continent

The *Cronobacter* species were confirmed using the *fusA* allele sequence (Forsythe 2018). A neighbor-joining tree based on multiple alignments of the concatenated sequences of the seven loci (*atpD, fusA, glnS, gltB, gyrB, infB* and *ppsA*; 3036 nucleotides concatenated length) was constructed with the strains with complete MLST allelic profiles in the database ($n = 345$ of 465 strains) using the Interactive tree of life (iTOL) v3 (Letunic and Bork 2016). The MLST profiles were also clustered with GrapeTree (Zhou *et al.* 2018) using a categorical coefficient and visualized using the minimum spanning tree (MST) tool and also analyzed using the eBURST algorithm (Feil *et al.* 2004).

The Simpson`s index (SI) was applied to measure the genetic diversity among isolates using the *fusA* allele and also the concatenated 7-loci MLST sequences Hunter and Gaston (1988).

Investigation of *Cronobacter* spp. infections and occurrence in PIF in America

Cases of infection due to *Cronobacter* spp. and isolation of the organism from PIF in each American' country were compiled using the search terms '*Cronobacter*' or '*Enterobacter sakazakii*' in combination with 'infection', 'newborn', 'infant', 'neonate' or 'the name of each America country' to conduct a literature

search of the Medline, Scientific Electronic Library Online (SciELO), Scopus and ResearchGate databases and review associated bibliographies.

RESULTS AND DISCUSSION

A total of 465 *Cronobacter* spp. strains isolated on the American continent in the period of 1970–2019 had been deposited in the PubMLST database. The majority (57.4%) were from North America especially from USA (50.3%), followed by Canada (5.6%) and few strains (1.5%) from Mexico. Regarding South America, Brazil was the country of origin for almost all strains ($n = 196$), and Chile and Uruguay had only one strain each (Table 1). A total of 232 (49.9%) were isolated from foods, 102 (21.9%) from environmental, 87 (18.7%) from clinical samples, 27 (5.8%) from infant formula, one from water (0.2%) and 16 from unknown sources (3.4%). The distribution of *Cronobacter* spp. STs according to the source is shown in Fig. 1.

The isolation of *Cronobacter* spp. from PIF purchased in American countries and reported in the literature is shown in Table 2. A total of seven countries reported the isolation of the pathogen in the period of 1996–2018. Unfortunately, the majority of isolates were not genotyped by MLST partially due to the technique not being developed until 2009. In many studies, the isolation occurred after 2008, the year when *Codex Alimentarius* approved the microbiological criteria for PIF that include the absence of *Cronobacter* species (CAC 2008). Similarly, FAO/WHO (2006) recommended the establishment of genotyping methods for *Cronobacter* spp. to facilitate tracing the organism.

Some studies reported that contaminated PIFs had been imported from different countries within the Americas and also from Europe (Table 2). In South America, the increased commercialization of food was stimulated by the Southern Common Market (MERCOSUL 2020). Such international food trade present a challenge in order to avoid the spread of pathogens, as in the case of PIF, where the low water activity associated with the characteristics of the *Cronobacter* genus facilitates the survival of the pathogen in these products even though the microbiological criteria are very strict (Yan *et al.* 2015; Umeda *et al.* 2017).

In Argentina, Asato *et al.* (2013) reported two cases of neonatal infections associated with *C. malonaticus* and one case associated with *C. sakazakii*. A total of two of these were fatal, however the route of transmission was not identified. Similarly in Brazil, where cases of *Cronobacter* spp. infections in neonates were reported but neither PIF nor other types of foods were identified as the vehicle of contamination (Brandao *et al.* 2015; Chaves *et al.* 2018). Further studies in Brazil have reported an occurrence of *Cronobacter* spp. in 44.4% of corn-based farinaceous foods samples (Costa *et al.* 2020a) and 56.7% of oat and linseed samples (Silva *et al.* 2019). In Cuba, Leyva *et al.* (2008) isolated *Cronobacter* spp. from one imported skimmed powdered milk sample. In Colombia, Vanegas, Rugeles and Martinez (2009) analyzed 222 samples from milk feeders including sterile and non-sterile surfaces, utensils used for the formula preparation and food handlers and *Cronobacter* spp. was identified in eight samples. Morato-Rodríguez *et al.* (2018) analyzed 102 samples of breast milk substitutes made from corn and plantain starch and identified 27 samples contaminated by *Cronobacter* spp. The presence of this organism in these foods and their potential risk to neonates could be a significant public health problem in America, especially in developing countries, where a large proportion of neonates are fed breast milk substitutes (Morato-Rodríguez *et al.* 2018; Silva *et al.* 2019; Costa *et al.* 2020a).

A total of five *Cronobacter* species were reported: *C. sakazakii* (374, 80.4%), *C. malonaticus* (41, 8.8%), *C. dublinensis* (29, 6.2%), *C. turicensis* (16, 3.5%) and *C. muytjensii* (5, 1.1%). The diversity of *Cronobacter* species observed was similar to the data from the PubMLST database for other countries; highlighting the domination of Group 1 species (*C. sakazakii* and *C. malonaticus*) (Forsythe 2018).

A total of 452 strains (97.2%) had been identified based on *fusA* allele sequencing and the calculated SI was 0.84. *Cronobacter* spp. genetic diversity using neighbor-joining tree analysis indicating the species, source and country of origin is presented in Fig. 2. The 345 strains were assigned to 98 STs, a ratio of 3.5 strain per ST and the calculated SI was 0.93. *C. sakazakii* strains were found in almost all countries and represented 64 out of a total of 98 STs (65.3%). The SI is used to measure the genetic diversity among isolates, since it calculates the probability of unrelated strains being classified into different groups (Hunter and Gaston 1988). The acceptable level of discrimination will depend on a number of factors, but an index of greater than 0.90 would seem to be desirable for a typing scheme (Hunter and Gaston 1988). Consequently, the MLST technique was considered efficient for typing strains isolated within America. This diversity was also observed using the eBURST algorithm. A total of 30 STs formed 12 single-locus variant (SLV) and/or double-locus variant (DLV) groups which shared five or more allelic profiles, and nine CCs had already been defined in the database (Table 3; Fig. 1). A total of 68 STs were identified as singletons and belonged to *C. sakazakii* ($n = 100$), *C. malonaticus* ($n = 24$), *C. dublinensis* ($n = 18$), *C. turicensis* ($n = 9$) and *C. muytjensii* ($n = 5$).

Group 1 ($n = 69$) comprised *C. sakazakii* of six STs from CC4; 4, 15, 107, 108, 121 and 218. ST4 is a dominant ST in the PubMLST database, consisting of 64 strains isolated from three countries over a period of more than 40 years and the majority of strains was isolated from clinical specimens and infant formula (Joseph *et al.* 2012b; Siqueira *et al.* 2013). STs 15, 107, 108, 121 are SLVs of ST4, differ in the *fusA* allele, whereas ST 218 is another SLV but differs in the *gltB* allele. A high number of strains in the PubMLST database have been reported as CC4, and it has been recognized as a stable clonal lineage which is particularly associated with neonatal meningitis (Joseph and Forsythe 2011; Joseph *et al.* 2012b; Joseph and Forsythe 2012; Hariri, Joseph and Forsythe 2013). ST15 was isolated in 1990 from a hospitalized child in Canada (Baldwin *et al.* 2009; Joseph and Forsythe 2012). ST107 was isolated from cerebrospinal fluid in a brain abscess of a term infant in USA (Joseph *et al.* 2012b; Joseph and Forsythe 2012). ST108 was isolated from an infant formula in USA and had previously been isolated from PIF in Germany. ST121 and ST218 had been isolated in USA from soy protein and from an infant formula, respectively (Ivy *et al.* 2013).

Group 2 ($n = 61$) comprised *C. sakazakii* STs 1, 391 and 693. These belong to CC1. ST1 was a dominant ST (59/61) isolated from four countries over a period of more than 30 years, including strains from clinical cases and infant formula (Siqueira *et al.* 2013; Pan *et al.* 2014). ST1 is the central ST and has been associated with infections in infants and adults in many countries (Joseph *et al.* 2012b; Shi *et al.* 2018; Li *et al.* 2020). ST391 and ST693 are DLV and SLV of ST1, respectively. They had been isolated from an unknown source in Canada and from a flake corn flour in Brazil, respectively (Paula *et al.* 2020).

Group 3 ($n = 20$) comprises *C. sakazakii* STs 8 and 226 that belong to CC8, and were isolated from clinical ($n = 13$), environmental ($n = 4$) infant formula ($n = 2$) and food ($n = 1$) samples. ST8 is associated with neonatal infections in neonates over a long time period (1977–2019) across the world, including

Table 1. *Cronobacter* strains ($n = 465$) isolated from the American continent and deposited in the PubMLST database (http://www.pubmlst.org/cronobacter/, last access 10/15/2020).

Continent (Number of strains)	Country (Number of strains)	Species (Number of strains)	Source (Number of strains)
North America (267)	United States of America (234)	*C. sakazakii* (193)	Clinical (55), environment[1] (70), food (48), infant formula (11), unknown (8), water (1)
		C. dublinensis (15)	Environment (14), clinical (1)
		C. malonaticus (13)	Clinical (9), environment[1] (3), food (1)
		C. turicensis (10)	Environment[1] (6), clinical (2), food (2)
		C. muytjensii (3)	Environment[1] (1), unknown (2)
	Canada (26)	*C. sakazakii* (24)	Clinical (11), unknown (5), environment (3), infant formula (3), food (2)
		C. malonaticus (2)	Clinical (1), unknown (1),
	Mexico (7)	*C. sakazakii* (7)	Infant formula (4), clinical (2), environment (1)
South America (198)	Brazil (196)	*C. sakazakii* (148)	Food (136), infant formula (6), clinical (3), environment[2] (3)
		C. malonaticus (26)	Food (21), clinical (3), infant formula (1), environment (1)
		C. dublinensis (14)	Food (14)
		C. turicensis (6)	Food (6)
		C. muytjensii (2)	Food (2)
	Chile (1)	*C. sakazakii* (1)	Infant formula (1)
	Uruguay (1)	*C. sakazakii* (1)	Infant formula (1)

[1]Including strains isolated from insects;
[2]Including strains isolated from utensils.

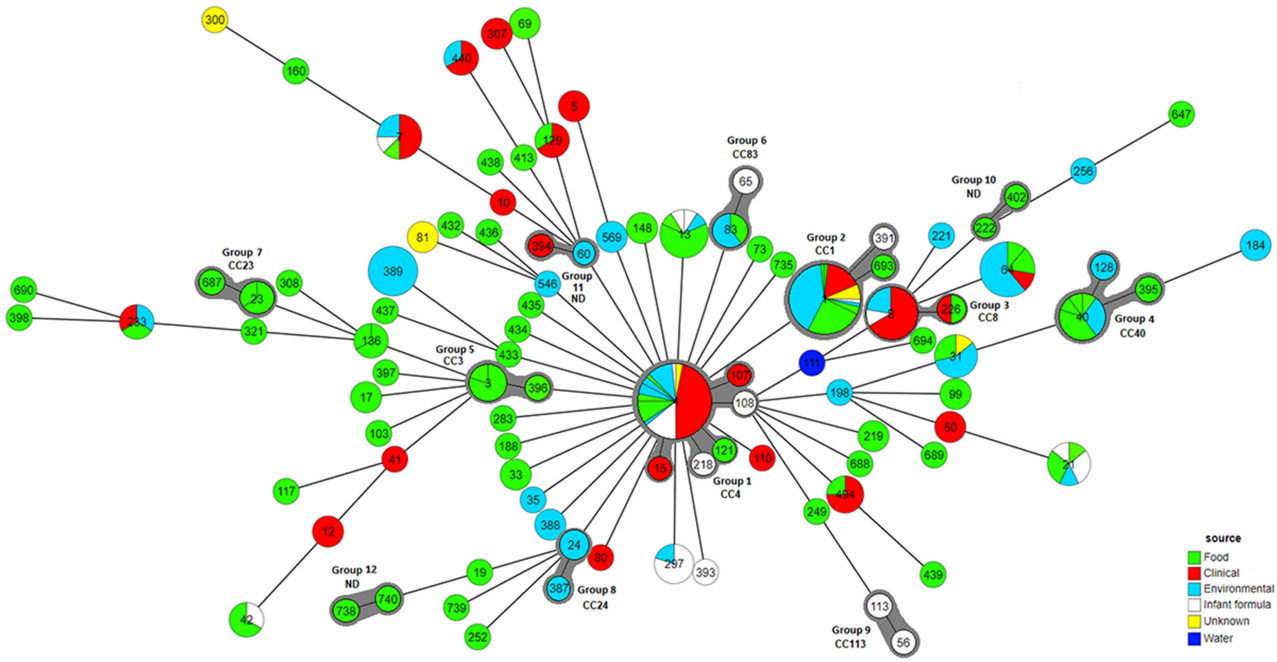

Figure 1. Minimum spanning tree showing the genetic relationship of 345 *Cronobacter* strains isolated from the American continent and deposited in the PubMLST database (http://www.pubmlst.org/cronobacter/, last access 10/15/2020). Strains are distributed according to ST classification. The size of the circle is proportional to the number of the strains; strains sharing five or more alleles are surrounding by a gray halo and represents the 12 groups found in the eBURST algorithm analyses and clonal complex (CC) described in the database. The groups that not belongs to any CC described in the database are represented as 'not determined' (ND). Lines connecting ST groups, indicate that they differ in one allele (thick solid line), or three to five locus (thin and dotted lines).

four continents (North America, South America, Europe and Asia). The most recent cases were reported in 2019 in China (Kadlicekova *et al.* 2018). ST8 has been isolated from PIF in Uruguay and USA (Forsythe 2015), and from insects (*Musca domestica*) in USA, indicating that these flies can act as reservoir of this pathogen (Pava-Ripoll *et al.* 2012). ST226 is a SLV from ST8 and was associated with clinical infection in Mexico and also isolated from food samples in China, Turkey, South Korea, Czechoslovakia and Brazil but not in PIF (Xu *et al.* 2015; Vojkovska *et al.* 2016; Brandao *et al.* 2017; Ling *et al.* 2018; Li *et al.* 2019).

Table 2. Presence of *Cronobacter* in powdered infant formula commercialized in American countries 1996–2018.

Country	Year of isolation	Microorganism (Number of strains)	Source	Comments	Reference
Canada	1996	*Cronobacter* spp. (8)	PIF[1]	A total of 24 samples from five different companies were analysed ($n = 120$). *Cronobacter* was isolated from four companies.	Nazarowec-White and Farber (1997)
USA[2]	2001	*Cronobacter* spp. (not determined)	PIF and reconstituted PIF	*Cronobacter* spp. was isolated from both opened and unopened cans during the investigation of an outbreak.	CDC (2002)
Argentina	2005–2008	*C. sakazakii* (22) *C. malonaticus* (1)	PIF	The PIF samples were from three separate companies. Samples had been imported from two different Latin American countries and one from Europe.	Terragno *et al.* (2009)
Brazil	2006	*C. sakazakii* (6) *C. malonaticus* (1)	PIF and reconstituted PIF	A total of 99 samples were analyzed, of which 42 were unopened cans of powdered infant formula (PIF), 25 reconstituted infant formulas in feeding bottles, 27 utensils used in the preparation of infant formula and five samples of fortified cows milk	Siqueira *et al.* (2013)
Chile	2008	*Cronobacter* spp. (4)	PIF	A total of 80 samples were analyzed	Sáez, Llanos and Tamayo (2012)
	2013–2014	*C. sakazakii* (2)	PIF	A total of seven sample for premature neonates and 65 samples for neonates 0–6 months. 21 produced in Chile, 44 in México and seven in Holland. The two positive samples for *C. sakazakii* were produced in Chile.	Parra *et al.* (2015)
	2016–2017	*C. sakazakii* (8)	PIF	A total of 90 samples from four countries (United States, Singapore, Chile and Holland), from three manufacturers (1, 2 and 3), seven commercial dairy brands, of which three were powdered and four were liquid products. *C. sakazakii* was isolated from seven samples produced in Chile and one in Singapore, and all PIF.	Parra-Flores *et al.* (2018)
Mexico	2011	*C. sakazakii* (2)	PIF and reconstitute PIF	A total of 21 samples of PIF, 10 for premature infants and 11 for children aged 0–6 months; and 29 samples of reconstitute PIF, 11 for premature infants and 18 for infants aged from 0–6 months. *C. sakazakii* was isolated from one sample of PIF and one of reconstitute PIF.	Flores *et al.* (2011)
Honduras	2018	*C. sakazakii* (2)	PIF and reconstitute PIF	A total of 100 samples of imported PIF from five commercial brands, from six different countries. *C. sakazakii* was isolated from two samples of reconstitute PIF.	Márquez *et al.* (2019)

[1]Powdered infant formula;
[2]United States of America.

Group 4 ($n = 12$) comprises *C. sakazakii* STs 40, 128 and 395 that belong to CC40 and were isolated from food and environmental samples. ST40 have been isolate from samples of human clinical samples (feces, urine and cervix) in Europe and Asia (Lepuschitz *et al.* 2019) and foods (Gičová *et al.* 2014; Fei *et al.* 2017; Li *et al.* 2019). STs 128 and 395, both SLV of ST40, were isolated from environmental in USA (Ivy *et al.* 2013) and from flaxseed flour in Brazil (Brandao *et al.* 2017), respectively.

Group 5 ($n = 6$) comprised of *C. sakazakii* STs 3 and 396. These belong to CC3 and were food isolates. ST396 is a DLV of ST3 and had been isolated from a milk powder in Brazil (Brandao *et al.* 2017). ST3 has been isolated from enteral feeding tube of neonates in a neonatal intensive care unit survey in United Kingdom (Hurrell *et al.* 2009) and also from foods and environmental samples in China (Xu *et al.* 2015; Ling *et al.* 2018; Hu *et al.* 2019; Li *et al.* 2019).

Group 6 ($n = 6$) comprises *C. sakazakii* STs 65 and 83 that belong to CC83. ST83 strains were isolated from environmental and food samples in Brazil and USA (Brandao *et al.* 2017). However, this ST have been associated bacteremia cases in Israel and China (Block *et al.* 2002). ST83 is recognized as a persistent contaminant in PIF manufacturing facilities (Chase *et al.* 2017) and have been isolated from infant formula samples in many countries (Gičová *et al.* 2014; Fei *et al.* 2017). ST 65 is a SLV from ST83 (differ in *infB* allele) and was isolated from an infant formula in 1988 in USA.

The Groups 7–12 have only few strains each one ($n \leq 4$) but Group 11 comprise relevant STs associated with severe clinical

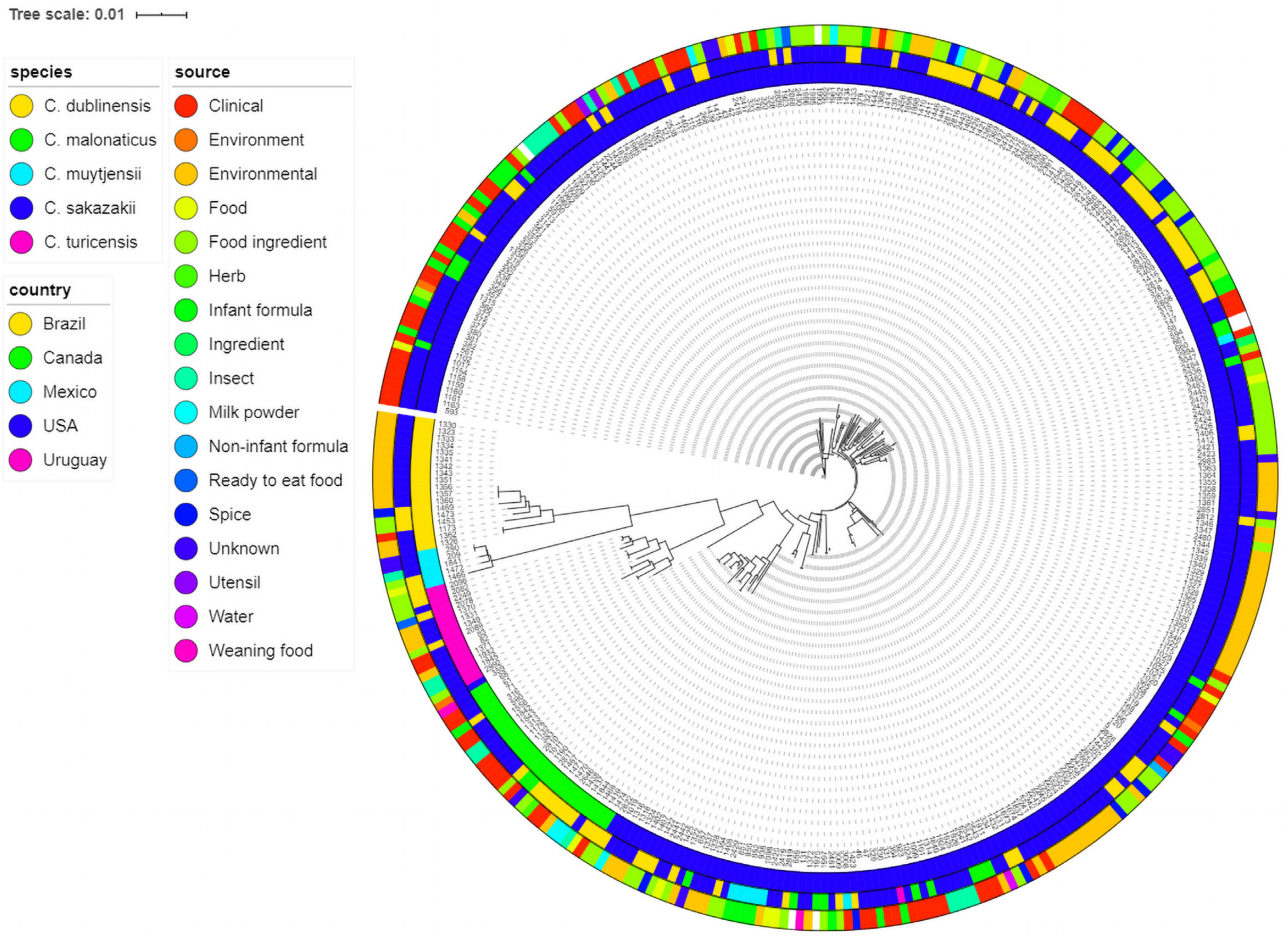

Figure 2. Neighbor-joining phylogenetic tree based on the seven MLST loci (3036 base pair concatenated length) of the 345 *Cronobacter* strains isolated from the American continent and deposited in the PubMLST database (http://www.pubmlst.org/cronobacter/, last access 10/15/2020). This tree was generated using iTOL v3. The first inner circle shows *Cronobacter* species, the second circle shows the country from which strain was isolated; the outer circle refers to the source of the isolation from each strain.

infections in neonates. Groups 7–10 and 12 comprises STs that were isolated from foods and environmental samples but not associated with human infections (Joseph and Forsythe 2012; Siqueira *et al.* 2013; Gičová *et al.* 2014; Pan *et al.* 2014; Ling *et al.* 2018; Silva *et al.* 2019; Paula *et al.* 2020).

Group 11 ($n = 2$) comprises the SLV *C. malonaticus* STs 60 and 394. ST60 was first reported in clinical cases in Czechoslovakia in 1983. In 2014, a ST60 strain (Chcon-9, ID 1494) was isolated from cerebral spinal fluid of an infant born in China, who had been fed fortified breast milk before developing clinical symptoms of meningitis (Ogrodzki and Forsythe 2017). Ogrodzki and Forsythe (2015) reported that ST60 had the same capsular profile (K2:CA2:Cell+) as *C. sakazakii* CC4 strains which are associated with meningitis cases. ST60 have been isolated from foods in Europe and Asia (Fei *et al.* 2018; Ling *et al.* 2018; Li *et al.* 2019), including follow up formula and PIF (Joseph and Forsythe 2012). In America, ST60 was isolated from an insect (*Musca domestica*) in USA highlighting the potential risk of these flies in the contamination of food chain with this pathovar (Pava-Ripoll *et al.* 2012). The ST394 is a SLV of ST60 and was isolated from hemoculture of a neonate with bacteremia in Brazil (Brandao *et al.* 2015; Umeda *et al.* 2017). A further phenotypical characterization of the strain showed the ability to producing of biofilm in glass tubes, degradation of casein in milk agar and β-hemolysis against erythrocytes from guinea, pig, horse and rabbit (Umeda *et al.* 2017). However, no study evaluating if ST394 strains also encode for the K2:CA2:Cell + capsular profile has been undertaken, which will be important to predict if ST394 could also presented the same virulence potential as ST60.

Regarding the singletons, from the 68 STs, 26 (38.2%) were associated with clinical cases, and 14 (20.6%) of them occurred in America and are discussed below. The majority was found in *C. sakazakii* ($n = 7$) and *C. malonaticus* ($n = 5$) strains.

C. sakazakii ST12 was isolated from a clinical sample in 1975 in USA, from clinical cases in former Czechoslovakia (Baldwin *et al.* 2009), from a fatal outbreak in a neonatal intensive care unit in France and the most recent was from a hemoculture of 65-years-old patient in Ireland (Masood *et al.* 2015). *C. sakazakii* ST41 (CC281) was isolated from a foot wound sample in 1975 in USA (Joseph *et al.* 2012b) and no other clinical reported was found. *C. sakazakii* ST50 were isolated from a sputum or spinal fluid sample in 1977 (Joseph *et al.* 2012b) and septum in 2009, both in USA. No other clinical case was reported, and the ST were found in foods from China and Czechia, including infant formula (Killer *et al.* 2015; Fei *et al.* 2017; Li *et al.* 2017, 2019; Ling *et al.* 2018). *C. sakazakii* ST64 (CC64) was isolated from a bronchial sample in 1973 in USA (Joseph *et al.* 2012b). Later it was isolated from clinical samples in Czechia and Slovakia, in 2013 and 2016, respectively (PubMLST, 2020). Regarding food samples, it has been isolated from in many countries (including Brazil) in different type

Table 3. Groups resulting among the 75 STs of the *Cronobacter* strains ($n = 345$) isolated from the American continent and deposited in the PubMLST database (http://www.pubmlst.org/cronobacter/, last access 10/15/2020) following eBURST analysis.

Group (CC[1])	Species	ST[2]	Number of isolates	Source (Number of isolates)	Geographic distribution	Period of isolation
1 (CC4)	*C. sakazakii*	4	64	Clinical (30), environmental[3] (13), infant formula (9), food (9), unknow (3)	Brazil, Canada, USA	1973–2014
		15	1	Clinical (1)	Canada	1990
		107	1	Clinical (1)	USA	2011
		108	1	Infant formula (1)	USA	2011
		121	1	Food (1)	USA	2003
		218	1	Infant formula (1)	USA	Unknown
2 (CC1)	*C. sakazakii*	1	59	Environmental (25), food (19), clinical (10), unknown (4), infant formula (1)	Brazil, USA, Canada, Mexico	1988–2019
		391	1	Unknown (1)	Canada	Unknown
		693	1	Food (1)	Brazil	2018
3 (CC8)	*C. sakazakii*	8	18	Clinical (12), infant formula (2), environmental[3] (4)	USA, Canada, Uruguay	1977–2012
		226	2	Clinical (1), food (1)	Brazil, Mexico	2014–2016
4 (CC40)	*C. sakazakii*	40	10	Food (7), environmental (3)	Brazil, USA	2013–2018
		128	1	Environmental (1)	USA	Unknown
		395	1	Food (1)	Brazil	2014
5 (CC3)	*C. sakazakii*	3	5	Food (5)	Brazil, USA	2014–2018
		396	1	Food (1)	Brazil	2009
6 (CC83)	*C. sakazakii*	65	1	Infant formula (1)	USA	1988
		83	5	Environmental (3), food (2)	Brazil, USA	2006–2014
7 (CC23)	*C. sakazakii*	23	3	Food (3)	Brazil, USA	2005–2013
		687	1	Food (1)	Brazil	2018
8 (CC24)	*C. turicensis*	24	2	Environmental (2)	USA	2014
		387	1	Environmental (1)	USA	2014
9 (CC113)	*C. sakazakii*	56	1	Infant formula (1)	Brazil	2007
		113	1	Infant formula (1)	Brazil	2007
10 (ND[4])	*C. sakazakii*	222	1	Food (1)	Brazil	2018
		402	1	Food (1)	Brazil	2014
11 (ND)	*C. malonaticus*	60	1	Environmental[3] (1)	USA	2012
		394	1	Clinical (1)	Brazil	2013
12 (ND)	*C. turicensis*	738	1	Food (1)	Brazil	2016
		740	1	Food (1)	Brazil	2016

[1]Clonal complex;
[2]Sequence type;
[3]Including strains isolated from insects and/or utensils;
[4]Not determined.

of foods that include PIF and FUF (Pan *et al.* 2014; Xu *et al.* 2015; Brandao *et al.* 2017; Li *et al.* 2019; Costa *et al.* 2020a). *C. sakazakii* ST110 (CC4), unique in the database, was isolated from a CSF of a neonate in 2011 in USA and is a triple locus variant (TLV) of ST4 (Joseph *et al.* 2012b). *C. sakazakii* ST233 was isolated from a faecal human sample in USA. After, this ST was found in environmental and food samples in Slovenia, Czechoslovakia, China (Ling *et al.* 2018; PubMLST, 2020), USA and Brazil (Brandao *et al.* 2017). *C. sakazakii* ST494 has previously been isolated from clinical samples related to a fatal case of meningitis in a newborn in Brazil in 2017 (Chaves *et al.* 2018). Costa *et al.* (2020b) reported that this ST can produce cytotoxic compounds that induced several cell death characteristics, including loss of cell-cell contact, microvilli reduction and cellular lysis. This ST was also isolated from spices and edible mushroom in China (Li *et al.* 2019) and in 2018 from a corn meal sample in Brazil (Costa *et al.* 202a).

C. malonaticus ST7 (CC7) strains were isolated in clinical cases in 1973 and 1977 in USA (Baldwin *et al.* 2009), from PIF samples in Brazil (Siqueira *et al.*2013), as well as insects and weaning foods in USA (Baldwin *et al.* 2009; Pava-Ripoll *et al.* 2012). In other countries, ST7 strains were isolated from clinical, environmental and foods (Pan *et al.* 2014; Xu *et al.* 2015; Ling *et al.* 2018; Hu *et al.* 2019; Li *et al.* 2019), and from clinical samples from adults in Europe (Alsonosi *et al.* 2015; Kadlicekova *et al.* 2018). *C. malonaticus* ST10 (CC63) has only one strain isolated from a clinical sample in Canada and the others four strains were isolated from foods in China and Czechoslovakia (Baldwin *et al.* 2009; Li *et al.* 2019). *C. malonaticus* ST129 (CC129) strains were isolated from a blood culture in USA in 1977 (Joseph *et al.* 2012b; Ivy *et al.* 2013). Afterwards, this ST was found in environmental samples from PIF production facilities in Ireland (Yan *et al.* 2015) and from foods in China and Brazil (Li *et al.* 2017; Costa *et al.* 2020a). *C. malonaticus* ST307 (CC112) was isolated from blood culture samples of a <1 month infant preterm that died due to fatal meningitis in USA in 2011 (Hariri, Joseph and Forsythe 2013). *C. malonaticus* ST440 (CC611) strains have already been isolated from two cases of infections in neonates in Brazil in 2013 (Brandao *et al.* 2015; Umeda *et al.* 2017). ST440 was found in milk and edible mushrooms in China (Li *et al.* 2019) and natural mineral drinking water in Brazil (Vasconcellos *et al.* 2019). In 2020, one strain isolated

from CSF of a neonate not premature was reported in Japan, but no more information is available in the PubMLST database (PubMLST 2020).

Regarding the remaining species, *C. dublinensis* ST80 (CC80) was isolated from an abscess in 1979 in USA (Joseph *et al.* 2012b). It was also found in water, foods and infant formula samples in Europe (Grim *et al.* 2013; Gičová *et al.* 2014; Vojkovska *et al.* 2016). *C. turicensis* ST5 is the main ST of the species isolated from clinical infections. The strains were isolated in the USA from blood in 1970 and from bone marrow in 1975 in the USA. Furthermore, it was isolated from UK milk powder in 2004 (Joseph *et al.* 2012b; Czerwicka *et al.* 2013).

Cronobacter spp. strains isolated on the American continent showed a high diversity when characterized by MLST and therefore a powerful tool for epidemiological investigations. In the majority of reported *Cronobacter* spp. clinical cases, the source of contamination could not be determined. This could be due to many reasons such as inadequate investigation, lack of appropriate analytical methodologies, or insufficient sample collection. This reinforces the need for a more effective risk communication strategy in relation to the risk to neonates from *Cronobacter* spp. The notification of *Cronobacter* spp. infections in American countries should be encouraged in order to understand the real incidence in the continent. The sending of strains to reference laboratories to perform molecular characterization is also of great importance so that investigations and routes of contamination of pathogenic strains can be defined and control measures can be better planned and implemented.

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SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

Conflicts of Interest. None declared.

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