Food Microbiology 82 (2019) 11–19

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Species identification and molecular characterization of *Cronobacter* spp. isolated from food imported over nine years into Beijing, China

Qi Wang[a], Stephen James Forsythe[b], Xiao-Juan Zhao[a], Zi-Wei Wang[a], Dan Li[a], Dan Ma[a], Jia-Yue Cao[a], Jing Zeng[a,*]

[a] Beijing Customs, Tianshuiyuan Street 6, Beijing, 100026, China
[b] Foodmicrobe.com, Adams Hill, Keyworth, Nottinghamshire, NG12 5GY, UK

ARTICLE INFO

Keywords:
Cronobacter
MALDI-TOF MS
MLST
PCR-serotyping
Species identification

ABSTRACT

Cronobacter spp. are associated with serious infections in neonates with the clinical presentations of necrotizing enterocolitis, bacteraemia and meningitis. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to identify 203 *Cronobacter* isolates from imported food during 2006–2015 with an optimized in-house database. The isolates were predominantly *C. sakazakii* (88.18%), followed by *C. malonaticus* (8.37%), *C. muytjensii* (1.48%), *C. turicensis* (0.99%) and *C. dublinensis* (0.99%). The result was totally consistent with that of *fusA* allele sequencing. 12.32% (25/203) of isolates gave inconsistent spectra following separate protein extractions. Sixty *C. sakazakii* isolates and 24 isolates from the other four species were chosen for multi-locus sequence type analyses (MLST) and PCR-serotyping. Thirty-one sequence types were identified. The common sequence types were ST1 (19/60) and ST4 (13/60) for *C. sakazakii* and ST7 (12/17) for *C. malonaticus*. The primary serotypes were Csak O:1 (30/60), Csak O:2 (25/60) and Cmal O:2 (16/17) for *C. sakazakii* and *C. malonaticus* isolates, respectively. In conclusion, appropriate in-house database could make MALDI-TOF MS method identifying *Cronobacter* spp. isolates to the species level. But the spectra data were not sufficiently consistent for subtyping, unlike MLST. The *Cronobacter* spp. isolates have a high diversity including recognized pathovars.

1. Introduction

The *Cronobacter* genus consists of seven species, namely *Cronobacter sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. turicensis*, *C. universalis*, *C. condimenti* and *C. dublinensis*. It has been isolated not only from powdered infant formula (PIF), but also from environmental samples, foods and food ingredients, especially foods of plant origin (Forsythe, 2018). While infant infections by *C. sakazakii* have attracted the main attention, the majority of *Cronobacter* spp. infections occur in the adult population due to *C. malonaticus* but are less severe (Alsonosi et al., 2015; Patrick et al., 2014). *C. sakazakii* and *C. malonaticus* have been the majority of clinical isolates in all age groups. *C. dublinensis*, *C. muytjensii* and *C. condimenti* are probably of little or no clinical significance (Forsythe, 2018). This highlights the need for reliable methods to distinguish between the seven *Cronobacter* species.

Commercially available phenotyping kits have been reported as insufficient for the identification to the *Cronobacter* species level due to high false-negative and false-positive results even at the genus level (Cetinkaya et al., 2013; Iversen et al., 2006). For example, API20E (version 5.0) and ID32E (version 4.0) have 82.3% and 43.2% reliability, respectively (Jackson and Forsythe, 2016). Molecular methods, such as sequencing based on 16S rRNA gene, *rpoB* gene and *fusA* have been used to identify *Cronobacter* spp. isolates (Akineden et al., 2017; Alsonosi et al., 2015; Brandao et al., 2017; Zhu et al., 2011). Distinguishing between *C. malonaticus* and *C. sakazakii* using 16S rRNA gene is not reliable because there are seven copies of the 16S rRNA gene in *Cronobacter* and the sequence diversity between these multiple copies can introduce discrepancies (Forsythe, 2018). *RpoB* sequencing is also reported to give false-positive results, including misidentifying outbreak associated *Enterobacter* strains and PIF manufacturing site isolates of *Citrobacter koseri* as *C. sakazakii* (Forsythe, 2018; Jackson et al., 2014, 2015). The method also cannot reliably differentiate between *C. malonaticus* and *C. sakazakii* (Akineden et al., 2017). Instead, the use of *fusA* allele (438bp) sequencing has been confirmed as reliable for

* Corresponding author.
E-mail addresses: wangqi_221@163.com, wangqi@bjciq.gov.cn (Q. Wang), steve.forsythe@foodmicrobe.com (S.J. Forsythe), dtwenchao@sina.cn (X.-J. Zhao), 13911325904@139.com (Z.-W. Wang), 113674758@qq.com (D. Li), dofree1002@163.com (D. Ma), 15810113788@139.com (J.-Y. Cao), jingzeng_cn@163.com, zengj@bjciq.gov.cn (J. Zeng).

https://doi.org/10.1016/j.fm.2019.01.011
Received 6 June 2018; Received in revised form 18 January 2019; Accepted 18 January 2019
Available online 22 January 2019
0740-0020/

Cronobacter speciation by whole genome phylogenetic analysis, since none of the *fusA* profiles were shared between two or more species (Forsythe et al., 2014; Joseph et al., 2012). Nevertheless, it is still laborious and time-consuming. Hence a simple, rapid, and precise method that is able to reliably distinguish among all species of this genus is still desirable.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) can rapidly discriminate bacterial species at the level of genus, species, and in some cases, subspecies (Giacometti et al., 2018; Stephan et al., 2010; van Belkum et al., 2015). The advantages are mainly the rapid analyses, simple sample preparation, ability to detect minor differences among strains, and the possibility of direct comparison between strain-characteristic patterns. Stephan et al. (2010) developed a highly sensitive and specific MALDI-TOF MS method for *Cronobacter* using the BioMérieux instrument and the analysis software SARAMIS. However, the current Bruker 7311 MSP library is given as only reliable to the *Cronobacter* genus level. Previously, we generated an in-house database containing six species of *Cronobacter* spp. and used it to identify 43 *Cronobacter* isolates, 40% isolates gave uncertain results. We subsequently optimized the analysis method and successfully identified all 43 isolates to species level using MALDI-TOF MS(Wang et al., 2017). However, the optimized analysis method was laborious and not amenable for routine analysis.

In order to adequate monitoring sources and vehicles of the *Cronobacter* spp. isolates and reduce the risk of neonatal exposure, laboratory-based molecular methods such as pulse-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), PCR-RFLP based on *gyrB* gene and *rpoB* gene, ERIC-PCR and PCR-serotyping have been developed as useful subtyping techniques (Baldwin et al., 2009; Brengi et al., 2012; Vlach et al., 2017; Yan et al., 2015). Among them, MLST was more discriminatory than other genotyping methods. The results were easy to compare among laboratories as there is a centralized curated database and the scheme is congruent with whole genome phylogenetic analysis (Forsythe et al., 2014; Ogrodzki and Forsythe, 2017). More than 2000 *Cronobacter* isolates have been divided into > 650 sequence types using MLST, details of which are recorded in the open access MLST database http://pubmlst.org/cronobacter/(Ogrodzki and Forsythe, 2017). These studies led to the recognition of specific pathovars associated with particular neonatal and adult infections: *C. sakazakii* clonal complex 4 (CC4) with neonatal meningitis, *C. sakazakii* ST12 with necrotizing enterocolitis and *C. malonaticus* CC7 with adult infections (Forsythe et al., 2014; Joseph and Forsythe, 2011; Masood et al., 2015; Ogrodzki and Forsythe, 2017). More discriminatory methods are achievable using whole genome analysis (ribosomal-MLST, COG-MLST, capsule gene profiles and CRISPR-*cas* array analysis), but these are not feasible in routine microbiological laboratories at present (Forsythe, 2018; Ogrodzki and Forsythe, 2016, 2017).

PCR-serotyping is also widely used to type *Cronobacter* strains (Mullane et al., 2008; Sun et al., 2011, 2012). There are five serotypes in *C. sakazakii* and two serotypes in *C. malonaticus* (Yan et al., 2015). However, the total number of designated O serogroups across the *Cronobacter* genus is only 24, compared with over 650 for 7-loci MLST (Forsythe, 2018) and not all strains give PCR products and therefore cannot be assigned a serotype (Blazkova et al., 2015; Yan et al., 2015). An additional limitation of PCR-serotyping is that some *Cronobacter* serogroups occur across more than one species and even more than one genus. In order to overcome these difficulties, Ogrodzki and Forsythe (2015, 2017) proposed an alternative scheme based on the sequence analysis of the flanking genes *galF* and *gnd*. However, this requires whole genome analysis which is not accessible for routine strain analysis.

The aim of this study was to establish a rapid, accurate and easy-to-use MALDI-TOF MS method for *Cronobacter* species identification and to further investigate the diversity of the *Cronobacter* spp. isolates using both the 7-loci MLST and PCR-based O-antigen serotyping. We also evaluated whether MALDI-TOF MS spectra could be used to subtype different strains of the same *Cronobacter* species.

2. Materials and methods

2.1. Bacterial strains

Two hundred and three presumptively identified *Cronobacter* spp. isolates and two reference strains (*C. sakazakii* ATCC 12868, *C. sakazakii* ATCC 29004) were used in this study. The reference strains were kindly donated by the Chinese Academy of Inspection and Quarantine. The 203 isolates, from food imported to Beijing from 2006 to 2015, had been previously identified phenotypically as *Cronobacter* spp. according to VITEK 2 Compact (BioMérieux).

2.2. Species identification of Cronobacter isolates

2.2.1. Species identification based on MALDI-TOF MS

2.2.1.1. Optimizing the in-house database. *C. sakazakii* reference strain ATCC12868 and one *C. sakazakii* isolate Cro4 were grown on TSA (Trypticase Soy Agar, Landbridge Technology Co. Ltd., Beijing, China) at 37 °C for 22–24 h before processing according to the ethanol/formic acid method (Bruker Daltonik GmbH). The procedure for generating main spectra projects (MSPs) and adding them to the in-house database was as according to the manufacturer's instructions and as described by Wang et al. (2017).

2.2.1.2. Analysis of presumptively identified Cronobacter spp. isolates. *Cronobacter* spp. isolates were grown on TSA at 37 °C for 22–24 h before preparation for MALDI-TOF analysis using ethanol/formic acid method (Bruker Daltonik GmbH). One microliter of the supernatant was spotted onto the MSP 96 target polished steel plate. Dried sample were overlaid with 1 μL freshly prepared HCCA matrix solution. For each isolate, at least two distinct bacterial colonies were spotted in duplicate onto independent steel target plates, ultimately acquiring at least four spectra. The MALDI-TOF MS data was analyzed using the software Biotyper RTC and the combined database (Bruker's database and the improved in-house *Cronobacter* database). All the spectra were imported into the Biotyper OC software for principle component analysis (PCA). The consistency of spectra for the same isolate was checked using the Flexanalysis software.

2.2.2. Species identification based on analysis of the fusA gene

Genomic DNA was extracted by the TIANamp Bacteria DNA kit (Tiangen Biotech Co. Ltd., Beijing, China) from an overnight culture on TSA at 37 °C. Analysis of the *fusA* allele was according to the protocol available from the *Cronobacter* PubMLST website (http://pubmlst.org/cronobacter). Amplicons were sequenced by the Sangon Biotech (Shanghai) company. The *fusA* sequences were queried in the online MLST *Cronobacter* database (http://pubmlst.org/cronobacter) to identify the allele number and species.

2.3. Genotyping of Cronobacter spp. isolates

2.3.1. Multilocus sequence typing

MLST analysis was performed according to the method described by Baldwin et al. (2009). Amplification and sequencing primers of the 7-loci MLST and PCR conditions were as the original and alternative protocols available from the MLST website (http://pubmlst.org/cronobacter). Amplicons were sequenced by Sangon Biotech (Shanghai) company, China. Allele numbers and ST types were assigned using tools available at the PubMLST *Cronobacter* website. New alleles and STs were assigned by the database curator (SJF). The relationship between the newly assigned STs and other STs was analyzed by downloading the respective 7-loci concatenated sequences (3036 bp) from the database and constructing the phylogenetic tree using MEGA 7.0 with the neighbor-joining method.

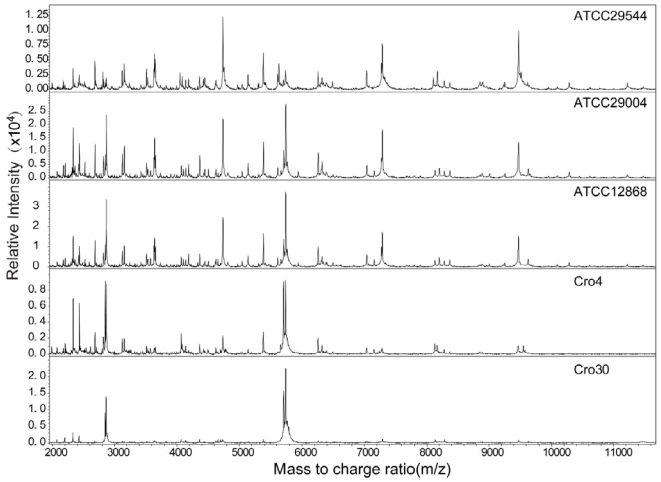

Fig. 1. MALDI-TOF MS spectra of three *C. sakazakii* reference strains (ATCC 29544, ATCC 29004 and ATCC 12868) and two *C. sakazakii* isolates (Cro4 and Cro30). The mass intensity is given on the y-axis, and the x-axis indicates the mass-to-charge ratio (m/z).

2.3.2. Molecular serotyping of O-Antigens

The serotypes of the *Cronobacter* spp. isolates were identified using the PCR-based scheme as described by Yan et al. (2015).

3. Results

3.1. Species identification

3.1.1. Species identification by MALDI-TOF MS

Seventeen of the 43 isolates gave ambiguous identification results when analyzed using the combined Bruker and in-house *Cronobacter* databases. These isolates had all been identified as *C. sakazakii* by 16S rRNA sequencing. They had similar spectra pattern to each other, and differed from the reference strain *C. sakazakii* ATCC 29544 (Wang et al., 2017).

In this study, we further collected the MALDI-TOF spectra of another two *C. sakazakii* reference strains, *C. sakazakii* ATCC 12868 and *C. sakazakii* ATCC 29004. The spectra of *C. sakazakii* ATCC 29544 and two typical *C. sakazakii* isolates Cro4 and Cro30 which gave ambiguous identification results (Wang et al., 2017) were also showed in Fig. 1. Two new reference strains ATCC 12868 and ATCC 29004 gave the same MALDI-TOF spectrum pattern. *C. sakazakii* isolates Cro4 and Cro30 had similar MALDI-TOF spectrum pattern and differed only in peak intensity. Thus *C. sakazakii* had at least three different MALDI-TOF MS patterns (ATCC 29544, ATCC 12868 and Cro4). We used *C. sakazakii* isolate Cro4 and the reference strain *C. sakazakii* ATCC 12868 to create another two *C. sakazakii* MSPs and added these two MSPs into the in-house *Cronobacter* database. Thus, we obtained an optimized in-house *Cronobacter* database comprising nine reference strains and one isolate. The 43 *Cronobacter* isolates as used by Wang et al. (2017) were re-analyzed using Biotyper OC. All the results were in accordance with the results of 16S rRNA gene. We then identified 203 *Cronobacter* isolates from foods with the combined Bruker and optimized in-house *Cronobacter* database, and Biotyper RTC automatically. The isolates were predominated by *C. sakazakii* (88.18%,179/203), followed by *C. malonaticus* (8.37%, 17/203), *C. muytjensii* (1.48%, 3/203), *C. turicensis* (0.99%, 2/203) and *C. dublinensis* (0.99%, 2/203). The identification results and LogScores were listed in Table 1.

To determine if the spectra of MALDI-TOF MS method could be used for further subtyping *Cronobacter* spp. isolates, all the spectra were imported into the Biotyper OC software. The PCA clustering suggested no meaningful clusters. Replicate profiles for the same isolates were sometimes divided into different branches, just the same as we had reported (Wang et al., 2017). Spectra of each isolate were imported into flexAnalysis software (Bruker Daltonik). It was found that the spectra of most (178/203, 87.68%) isolates were reproducible, only differing slightly in peak intensity, and therefore only differed a little in Log-score. However, for some isolates (25/203, 12.32%) the spectra after different times of extraction differed not only in peak intensity, but also in the presence or absence of peaks, sometimes even with peaks of strong intensity (Fig. 2). For these isolates, it was difficult to definite specific peaks for subtyping.

3.1.2. Species identification by fusA sequencing

The nucleotide sequences of *fusA* gene of the 203 isolates were compared with the *Cronobacter* PubMLST database to obtain their allele numbers (Table 1). Based on the *fusA* allele, the 203 isolates of *Cronobacter* spp. were identified as five species. The majority of the isolates were identified as *C. sakazakii* (n = 179, 88.18%), followed by *C. malonaticus* (n = 17, 8.37%), *C. muytjensii* (n = 3, 1.48%), *C. turicensis* (n = 2, 0.99%) and *C. dublinensis* (n = 2, 0.99%). The speciation results were in good accordance (100%) with the results of MALDI-TOF MS method (Table 1).

3.2. Subtyping

3.2.1. Subtyping of Cronobacter spp. by MLST

Totally 84 isolates consisting 60 *C. sakazakii* isolates selected randomly and all the 24 isolates of other species were selected for further MLST analysis. Amplification primers designed to amplify *atpD*, *fusA* and *infB* alleles generated amplicons for all the 84 isolates. The majority of remaining alleles (*glnS*, *gltB*, *gyrB* and *ppsA*) were sequenced using PCR products generated using the amplification primers. However, the amplicons of Cro70, Cro121 and Cro124 with both the amplification primers and sequencing primes for *glnS* gene were too weak to sequence. For these three strains, the alternative primers obtained from the *Cronobacter* PubMLST database were used which were not available at the beginning of this study. Complete sets of data obtained for all the 84 isolates (Table 2) were submitted to the *Cronobacter* MLST database with PubMLST ID 2119–2202 (Table 1).

We observed a high diversity for the 84 isolates with a total of 31 STs being identified. The 60 *C. sakazakii* isolates were divided into 20 sequence types, with ST1 (19/60, 31.67%) and ST4 (13/60, 21.67%) being the two main ST types, and to a lesser degree ST8 (3/60, 5.00%) and ST64 (1/60, 1.67%). The *C. malonaticus* isolates were from five STs, of which ST7 was the dominant ST (12/17, 70.59%). There were two STs each in *C. muytjensii*, *C. turicensis*, and *C. dublinensis* (Table 2).

Nine STs (ST614-ST619, ST624, ST625 and ST478) were newly assigned STs in the *Cronobacter* MLST database. Twelve new allele numbers assigned: *atpD* 173, *glnS* 232, *gltB* 264–267, *gyrB* 238–239, *infB* 224–225 and *ppsA* 320–321.

By downloading the concatenated sequences (3036 bp) from the database and constructing the phylogenetic tree using MEGA 7.0 with the neighbor-joining method, it was found that ST616 belonged to CC13, and ST615 clustered with ST380 and ST381.

3.2.2. Subtyping of Cronobacter spp. by O-antigen serotype analysis

Sixty *C. sakazakii* isolates were divided into four serotypes, Csak O:1, Csak O:2, Csak O:3 and Csak O:4 (Table 2). Csak O:1 (30/60, 50.00%) and Csak O:2 (25/60, 41.67%) were the primary serotypes. Csak O:1 was composed of nine sequence types: ST1, ST8, ST21, ST99, ST125, ST219, ST378, ST615 and ST618. Csak O:2 contained nine sequence types: ST3, ST4, ST17, ST22, ST23, ST31, ST64, ST96 and ST616. Csak O:3 was composed of two sequence types: ST4 and ST619. Csak O:4 was composed of two sequence types: ST1 and ST13. Conversely, *C. sakazakii* ST1 (n = 19) contained two serotypes: Csak O:1 (17/19, 89.47%) and Csak O:4 (2/19, 10.53%). *C. sakazakii* ST4 (n = 13) also contained two serotypes: Csak O:2 (12/13, 92.31%) and Csak O:3 (1/13, 7.69%).

Table 1
Speciation of 203 presumptive *Cronobacter* isolates according to MALDI-TOF MS and *fusA* allele sequencing.

No.	PubMLST ID	Isolates	Origin	Isolation Date (DD/MM/YY)	MALDI-TOF MS			*fusA* gene sequencing	
					LogScore[a]	n[b]	Species	Allele number	Species
1	2119	Cro2	unknown	15/05/2006	2.490 ± 0.028	4	*C. sakazakii*	1	*C. sakazakii*
2	2120	Cro3	milk powder	25/04/2006	2.492 ± 0.078	4	*C. sakazakii*	1	*C. sakazakii*
3	2121	Cro4	unknown	17/07/2006	2.507 ± 0.047	4	*C. sakazakii*	1	*C. sakazakii*
4	2122	Cro5	unknown	17/05/2006	2.493 ± 0.002	4	*C. sakazakii*	1	*C. sakazakii*
5	2123	Cro6	unknown	17/07/2006	2.481 ± 0.074	4	*C. sakazakii*	1	*C. sakazakii*
6	2124	Cro7	unknown	25/05/2006	2.525 ± 0.040	4	*C. sakazakii*	1	*C. sakazakii*
7	2125	Cro8	unknown	11/07/2006	2.544 ± 0.021	4	*C. sakazakii*	1	*C. sakazakii*
8	2126	Cro9	unknown	11/07/2006	2.471 ± 0.073	4	*C. sakazakii*	1	*C. sakazakii*
9	2127	Cro10	unknown	11/07/2006	2.490 ± 0.014	4	*C. sakazakii*	1	*C. sakazakii*
10		Cro11	milk powder	27/07/2007	2.389 ± 0.053	4	*C. sakazakii*	36	*C. sakazakii*
11	2128	Cro12	ice cream	16/08/2007	2.497 ± 0.056	4	*C. sakazakii*	1	*C. sakazakii*
12		Cro13	wheat powder	14/09/2007	2.343 ± 0.071	4	*C. sakazakii*	18	*C. sakazakii*
13		Cro14	wheat powder	14/09/2007	2.365 ± 0.038	4	*C. sakazakii*	11	*C. sakazakii*
14		Cro15	wheat powder	14/09/2007	2.324 ± 0.103	4	*C. sakazakii*	18	*C. sakazakii*
15	2129	Cro16	powdered infant formula	14/09/2007	2.363 ± 0.051	4	*C. sakazakii*	18	*C. sakazakii*
16		Cro17	PIF	14/09/2007	2.359 ± 0.115	4	*C. sakazakii*	18	*C. sakazakii*
17		Cro18	milk powder	14/09/2007	2.319 ± 0.088	4	*C. sakazakii*	18	*C. sakazakii*
18		Cro19	food	14/09/2007	2.304 ± 0.070	4	*C. sakazakii*	18	*C. sakazakii*
19	2130	Cro20	milk powder	14/09/2007	2.402 ± 0.086	4	*C. sakazakii*	1	*C. sakazakii*
20		Cro22	milk powder	14/09/2007	2.328 ± 0.051	4	*C. sakazakii*	1	*C. sakazakii*
21		Cro23	protein powder	21/10/2007	2.299 ± 0.093	4	*C. sakazakii*	1	*C. sakazakii*
22		Cro24	food	17/11/2007	2.384 ± 0.109	4	*C. sakazakii*	18	*C. sakazakii*
23	2131	Cro25	vanilla seed powder	17/11/2007	2.529 ± 0.035	4	*C. sakazakii*	8	*C. sakazakii*
24		Cro26	food	29/12/2007	2.333 ± 0.068	4	*C. sakazakii*	1	*C. sakazakii*
25		Cro27	food	29/12/2007	2.377 ± 0.057	4	*C. sakazakii*	1	*C. sakazakii*
26		Cro28	food	29/12/2007	2.371 ± 0.149	4	*C. sakazakii*	1	*C. sakazakii*
27		Cro29	food	29/12/2007	2.397 ± 0.111	4	*C. sakazakii*	1	*C. sakazakii*
28		Cro30	food	29/12/2007	2.350 ± 0.059	4	*C. sakazakii*	1	*C. sakazakii*
29	2132	Cro31	food	29/12/2007	2.358 ± 0.084	4	*C. sakazakii*	1	*C. sakazakii*
30	2133	Cro32	food	29/12/2007	2.296 ± 0.044	4	*C. sakazakii*	1	*C. sakazakii*
31		Cro33	food	29/12/2007	2.389 ± 0.235	4	*C. sakazakii*	1	*C. sakazakii*
32		Cro34	food	29/12/2007	2.322 ± 0.173	4	*C. sakazakii*	1	*C. sakazakii*
33		Cro35	food	29/12/2007	2.338 ± 0.119	13	*C. sakazakii*	1	*C. sakazakii*
34		Cro36	food	29/12/2007	2.302 ± 0.181	8	*C. sakazakii*	1	*C. sakazakii*
35		Cro37	cheese	29/12/2007	2.368 ± 0.054	4	*C. sakazakii*	14	*C. sakazakii*
36	2134	Cro38	milk powder	29/12/2007	2.134 ± 0.091	4	*C. sakazakii*	14	*C. sakazakii*
37		Cro39	milk powder	29/12/2007	2.330 ± 0.028	4	*C. sakazakii*	14	*C. sakazakii*
38		Cro40	milk powder	29/12/2007	2.302 ± 0.185	4	*C. sakazakii*	14	*C. sakazakii*
39		Cro41	milk powder	29/12/2007	2.127 ± 0.155	4	*C. sakazakii*	14	*C. sakazakii*
40	2179	Cro42	food	29/12/2007	2.330 ± 0.201	4	*C. malonaticus*	13	*C. malonaticus*
41	2135	Cro43	food	25/01/2008	2.356 ± 0.021	8	*C. sakazakii*	1	*C. sakazakii*
42		Cro44	ice cream	06/06/2008	2.525 ± 0.088	11	*C. sakazakii*	1	*C. sakazakii*
43		Cro45	ice cream	01/07/2008	2.431 ± 0.127	8	*C. sakazakii*	1	*C. sakazakii*
44		Cro46	protein powder	11/07/2008	2.431 ± 0.127	4	*C. sakazakii*	8	*C. sakazakii*
45	2136	Cro48	unknown	17/07/2008	2.397 ± 0.053	8	*C. sakazakii*	1	*C. sakazakii*
46		Cro49	unknown	17/07/2008	2.477 ± 0.133	4	*C. sakazakii*	1	*C. sakazakii*
47		Cro50	unknown	17/07/2008	2.603 ± 0.032	4	*C. sakazakii*	1	*C. sakazakii*
48		Cro51	milkshake powder	07/08/2008	2.436 ± 0.040	4	*C. sakazakii*	1	*C. sakazakii*
49		Cro52	unknown	22/10/2008	2.560 ± 0.002	6	*C. sakazakii*	1	*C. sakazakii*
50	2137	Cro53	unknown	04/11/2008	2.438 ± 0.078	6	*C. sakazakii*	1	*C. sakazakii*
51		Cro54	unknown	04/11/2008	2.487 ± 0.051	4	*C. sakazakii*	11	*C. sakazakii*
52		Cro55	unknown	04/11/2008	2.381 ± 0.063	5	*C. sakazakii*	17	*C. sakazakii*
53	2196	Cro56	unknown	04/11/2008	2.372 ± 0.061	13	*C. turicensis*	22	*C. turicensis*
54	2138	Cro58	unknown	04/11/2008	2.377 ± 0.066	4	*C. sakazakii*	1	*C. sakazakii*
55		Cro59	unknown	04/11/2008	2.388 ± 0.155	6	*C. sakazakii*	15	*C. sakazakii*
56		Cro61	unknown	21/11/2008	2.280 ± 0.069	4	*C. sakazakii*	1	*C. sakazakii*
57		Cro63	unknown	21/11/2008	2.310 ± 0.198	6	*C. sakazakii*	36	*C. sakazakii*
58		Cro65	unknown	21/11/2008	2.346 ± 0.042	4	*C. sakazakii*	38	*C. sakazakii*
59	2139	Cro66	unknown	21/11/2008	2.312 ± 0.056	6	*C. sakazakii*	8	*C. sakazakii*
60	2140	Cro67	unknown	21/11/2008	2.414 ± 0.065	6	*C. sakazakii*	1	*C. sakazakii*
61	2197	Cro68	unknown	18/12/2008	2.556 ± 0.031	8	*C. turicensis*	22	*C. turicensis*
62	2198	Cro70	unknown	07/01/2009	2.233 ± 0.146	5	*C. muytjensii*	64	*C. muytjensii*
63		Cro71	unknown	07/01/2009	2.364 ± 0.111	4	*C. sakazakii*	1	*C. sakazakii*
64		Cro72	whey	21/01/2009	2.408 ± 0.182	4	*C. sakazakii*	8	*C. sakazakii*
65		Cro73	food ingredient	21/01/2009	2.222 ± 0.161	4	*C. sakazakii*	8	*C. sakazakii*
66	2141	Cro74	food ingredient	21/01/2009	2.351 ± 0.071	4	*C. sakazakii*	8	*C. sakazakii*
67		Cro75	food ingredient	21/01/2009	2.332 ± 0.076	4	*C. sakazakii*	8	*C. sakazakii*
68		Cro76	food ingredient	21/01/2009	2.455 ± 0.08	4	*C. sakazakii*	8	*C. sakazakii*
69		Cro77	food ingredient	21/01/2009	2.292 ± 0.068	4	*C. sakazakii*	8	*C. sakazakii*
70		Cro78	food ingredient	21/01/2009	2.356 ± 0.105	4	*C. sakazakii*	8	*C. sakazakii*
71		Cro79	food ingredient	21/01/2009	2.368 ± 0.049	5	*C. sakazakii*	8	*C. sakazakii*
72		Cro80	food ingredient	21/01/2009	2.260 ± 0.052	4	*C. sakazakii*	8	*C. sakazakii*

(*continued on next page*)

Table 1 (*continued*)

No.	PubMLST ID	Isolates	Origin	Isolation Date (DD/MM/YY)	MALDI-TOF MS			*fusA* gene sequencing	
					LogScore[a]	n[b]	Species	Allele number	Species
73		Cro81	whey protein	21/01/2009	2.283 ± 0.052	4	*C. sakazakii*	8	*C. sakazakii*
74	2142	Cro82	powdered infant formula	12/03/2009	2.433 ± 0.112	4	*C. sakazakii*	8	*C. sakazakii*
75		Cro83	powdered infant formula	12/03/2009	2.401 ± 0.058	4	*C. sakazakii*	8	*C. sakazakii*
76		Cro84	powdered infant formula	12/03/2009	2.343 ± 0.143	4	*C. sakazakii*	8	*C. sakazakii*
77		Cro85	wheat flour	12/03/2009	2.338 ± 0.256	4	*C. sakazakii*	1	*C. sakazakii*
78	2143	Cro86	infant rice powder	12/03/2009	2.360 ± 0.063	4	*C. sakazakii*	8	*C. sakazakii*
79		Cro87	wheat flour	12/03/2009	2.296 ± 0.136	4	*C. sakazakii*	8	*C. sakazakii*
80		Cro88	infant wheat flour	12/03/2009	2.282 ± 0.056	4	*C. sakazakii*	8	*C. sakazakii*
81		Cro89	infant millet powder	12/03/2009	2.365 ± 0.032	4	*C. sakazakii*	8	*C. sakazakii*
82		Cro90	wheat powder	12/03/2009	2.333 ± 0.052	4	*C. sakazakii*	8	*C. sakazakii*
83		Cro91	infant mashed carrot	12/03/2009	2.243 ± 0.062	4	*C. sakazakii*	8	*C. sakazakii*
84	2144	Cro92	protein powder	23/03/2009	2.442 ± 0.099	4	*C. sakazakii*	1	*C. sakazakii*
85	2145	Cro93	food ingredient	23/03/2009	2.489 ± 0.017	4	*C. sakazakii*	1	*C. sakazakii*
86	2180	Cro95	milk powder	24/03/2009	2.360 ± 0.074	4	*C. malonaticus*	7	*C. malonaticus*
87	2181	Cro96	milk	23/04/2009	2.340 ± 0.032	4	*C. malonaticus*	13	*C. malonaticus*
88	2146	Cro97	ice cream	26/05/2009	2.430 ± 0.082	4	*C. sakazakii*	12	*C. sakazakii*
89		Cro98	food	01/06/2009	2.544 ± 0.049	4	*C. sakazakii*	1	*C. sakazakii*
90	2147	Cro99	ice cream powder	02/07/2009	2.555 ± 0.052	4	*C. sakazakii*	1	*C. sakazakii*
91		Cro100	food ingredient	03/07/2009	2.460 ± 0.191	4	*C. sakazakii*	1	*C. sakazakii*
92		Cro101	milk	06/07/2009	2.553 ± 0.017	4	*C. sakazakii*	17	*C. sakazakii*
93		Cro102	protein powder	06/07/2009	2.430 ± 0.055	4	*C. sakazakii*	8	*C. sakazakii*
94	2148	Cro103	protein powder	07/07/2009	2.560 ± 0.025	4	*C. sakazakii*	8	*C. sakazakii*
95		Cro104	food	31/07/2009	2.320 ± 0.026	4	*C. sakazakii*	15	*C. sakazakii*
96		Cro105	protein powder	31/07/2009	2.504 ± 0.016	4	*C. sakazakii*	1	*C. sakazakii*
97	2149	Cro106	ice cream	05/08/2009	2.423 ± 0.097	4	*C. sakazakii*	1	*C. sakazakii*
98	2150	Cro107	ice cream	05/08/2009	2.500 ± 0.063	4	*C. sakazakii*	1	*C. sakazakii*
99		Cro108	milk powder	06/08/2009	2.492 ± 0.053	4	*C. sakazakii*	17	*C. sakazakii*
100	2151	Cro110	whey albumen powder	18/09/2009	2.564 ± 0.020	4	*C. sakazakii*	1	*C. sakazakii*
101	2152	Cro111	food	29/10/2009	2.419 ± 0.056	4	*C. sakazakii*	1	*C. sakazakii*
102		Cro112	food	29/10/2009	2.495 ± 0.100	4	*C. sakazakii*	1	*C. sakazakii*
103		Cro113	milk powder	29/10/2009	2.348 ± 0.113	4	*C. sakazakii*	1	*C. sakazakii*
104		Cro114	food	29/10/2009	2.429 ± 0.035	4	*C. sakazakii*	1	*C. sakazakii*
105	2182	Cro115	ice cream	03/11/2009	2.505 ± 0.042	4	*C. malonaticus*	7	*C. malonaticus*
106	2183	Cro116	ice cream	03/11/2009	2.543 ± 0.041	4	*C. malonaticus*	7	*C. malonaticus*
107		Cro117	food	12/11/2009	2.497 ± 0.098	4	*C. sakazakii*	8	*C. sakazakii*
108	2153	Cro119	food	12/11/2009	2.541 ± 0.043	4	*C. sakazakii*	8	*C. sakazakii*
109	2199	Cro121	food	12/11/2009	2.466 ± 0.071	4	*C. muytjensii*	25	*C. muytjensii*
110		Cro122	food	17/11/2009	2.565 ± 0.082	4	*C. sakazakii*	1	*C. sakazakii*
111		Cro123	ice cream	17/11/2009	2.489 ± 0.043	4	*C. sakazakii*	8	*C. sakazakii*
112	2200	Cro124	food	17/11/2009	2.472 ± 0.015	4	*C. muytjensii*	25	*C. muytjensii*
113	2154	Cro125	food	17/11/2009	2.499 ± 0.049	4	*C. sakazakii*	8	*C. sakazakii*
114		Cro126	food	17/11/2009	2.499 ± 0.098	4	*C. sakazakii*	8	*C. sakazakii*
115		Cro127	food	19/11/2009	2.451 ± 0.118	4	*C. sakazakii*	8	*C. sakazakii*
116		Cro128	food	19/11/2009	2.286 ± 0.048	4	*C. sakazakii*	8	*C. sakazakii*
117		Cro129	food	19/11/2009	2.509 ± 0.092	4	*C. sakazakii*	8	*C. sakazakii*
118	2184	Cro130	ice cream	20/11/2009	2.512 ± 0.047	4	*C. malonaticus*	7	*C. malonaticus*
119	2155	Cro131	ice cream	20/11/2009	2.473 ± 0.036	4	*C. sakazakii*	8	*C. sakazakii*
120	2185	Cro132	ice cream	20/11/2009	2.488 ± 0.035	4	*C. malonaticus*	7	*C. malonaticus*
121	2186	Cro133	ice cream	24/11/2009	2.524 ± 0.033	4	*C. malonaticus*	7	*C. malonaticus*
122	2187	Cro134	ice cream	24/11/2009	2.533 ± 0.034	4	*C. malonaticus*	7	*C. malonaticus*
123	2156	Cro135	food	24/11/2009	2.520 ± 0.064	4	*C. sakazakii*	1	*C. sakazakii*
124		Cro136	food	24/11/2009	2.515 ± 0.070	4	*C. sakazakii*	8	*C. sakazakii*
125		Cro137	food ingredient	25/11/2009	2.488 ± 0.106	4	*C. sakazakii*	8	*C. sakazakii*
126		Cro138	food	26/11/2009	2.516 ± 0.025	4	*C. sakazakii*	8	*C. sakazakii*
127	2188	Cro139	food	26/11/2009	2.443 ± 0.030	4	*C. malonaticus*	7	*C. malonaticus*
128	2189	Cro140	food	26/11/2009	2.500 ± 0.017	4	*C. malonaticus*	7	*C. malonaticus*
129	2190	Cro141	food	26/11/2009	2.402 ± 0.042	4	*C. malonaticus*	7	*C. malonaticus*
130	2191	Cro142	food	26/11/2009	2.455 ± 0.128	4	*C. malonaticus*	7	*C. malonaticus*
131	2192	Cro143	food	27/11/2009	2.520 ± 0.035	4	*C. malonaticus*	7	*C. malonaticus*
132		Cro144	food ingredient	08/12/2009	2.298 ± 0.090	4	*C. sakazakii*	11	*C. sakazakii*
133	2157	Cro145	food	08/12/2009	2.475 ± 0.089	4	*C. sakazakii*	8	*C. sakazakii*
134		Cro147	food	08/12/2009	2.504 ± 0.071	4	*C. sakazakii*	1	*C. sakazakii*
135	2158	Cro148	food	08/12/2009	2.504 ± 0.046	4	*C. sakazakii*	8	*C. sakazakii*
136		Cro149	food	08/12/2009	2.500 ± 0.041	4	*C. sakazakii*	11	*C. sakazakii*
137	2159	Cro150	food	08/12/2009	2.337 ± 0.085	4	*C. sakazakii*	11	*C. sakazakii*
138	2160	Cro151	food	08/12/2009	2.467 ± 0.056	4	*C. sakazakii*	8	*C. sakazakii*
139	2193	Cro152	milk powder	08/12/2009	2.508 ± 0.046	4	*C. malonaticus*	7	*C. malonaticus*
140	2161	Cro153	protein powder	11/12/2009	2.504 ± 0.057	4	*C. sakazakii*	116	*C. sakazakii*
141		Cro154	milk	19/01/2010	2.601 ± 0.042	4	*C. sakazakii*	1	*C. sakazakii*
142		Cro155	ice cream	19/01/2010	2.511 ± 0.031	4	*C. sakazakii*	1	*C. sakazakii*
143	2162	Cro156	food ingredient	21/01/2010	2.491 ± 0.026	4	*C. sakazakii*	1	*C. sakazakii*
144		Cro157	food ingredient	21/01/2010	2.439 ± 0.044	4	*C. sakazakii*	18	*C. sakazakii*
145	2201	Cro158	food	03/02/2010	2.220 ± 0.061	4	*C. dublinensis*	125	*C. dublinensis*

(*continued on next page*)

Table 1 (*continued*)

No.	PubMLST ID	Isolates	Origin	Isolation Date (DD/MM/YY)	MALDI-TOF MS			*fusA* gene sequencing	
					LogScore[a]	n[b]	Species	Allele number	Species
146		Cro159	milk powder	09/02/2010	2.600 ± 0.067	4	*C. sakazakii*	1	*C. sakazakii*
147		Cro160	food	02/03/2010	2.474 ± 0.107	4	*C. sakazakii*	1	*C. sakazakii*
148	2163	Cro161	food	02/03/2010	2.483 ± 0.038	4	*C. sakazakii*	1	*C. sakazakii*
149		Cro162	food	02/03/2010	2.629 ± 0.073	4	*C. sakazakii*	1	*C. sakazakii*
150		Cro163	ice cream	02/03/2010	2.569 ± 0.058	4	*C. sakazakii*	1	*C. sakazakii*
151	2164	Cro164	ice cream	02/03/2010	2.336 ± 0.072	4	*C. sakazakii*	8	*C. sakazakii*
152		Cro165	ice cream	02/03/2010	2.505 ± 0.063	4	*C. sakazakii*	1	*C. sakazakii*
153	2165	Cro166	food	03/03/2010	2.330 ± 0.025	4	*C. sakazakii*	18	*C. sakazakii*
154	2166	Cro167	food	03/03/2010	2.474 ± 0.034	4	*C. sakazakii*	17	*C. sakazakii*
155		Cro168	food	05/03/2010	2.633 ± 0.023	4	*C. sakazakii*	1	*C. sakazakii*
156	2167	Cro169	food	10/03/2010	2.412 ± 0.091	4	*C. sakazakii*	1	*C. sakazakii*
157	2168	Cro170	food	11/03/2010	2.491 ± 0.037	4	*C. sakazakii*	1	*C. sakazakii*
158		Cro171	food	17/03/2010	2.558 ± 0.061	4	*C. sakazakii*	1	*C. sakazakii*
159		Cro172	food	24/03/2010	2.342 ± 0.069	4	*C. sakazakii*	11	*C. sakazakii*
160		Cro173	food	26/03/2010	2.453 ± 0.101	4	*C. sakazakii*	18	*C. sakazakii*
161		Cro174	food	30/03/2010	2.595 ± 0.049	4	*C. sakazakii*	1	*C. sakazakii*
162		Cro175	food	30/03/2010	2.452 ± 0.042	4	*C. sakazakii*	18	*C. sakazakii*
163		Cro176	food	30/03/2010	2.450 ± 0.041	4	*C. sakazakii*	18	*C. sakazakii*
164		Cro177	food	01/04/2010	2.578 ± 0.051	4	*C. sakazakii*	1	*C. sakazakii*
165		Cro178	food	01/04/2010	2.602 ± 0.061	4	*C. sakazakii*	1	*C. sakazakii*
166		Cro179	food	01/04/2010	2.518 ± 0.038	4	*C. sakazakii*	1	*C. sakazakii*
167		Cro180	food	09/04/2010	2.428 ± 0.044	4	*C. sakazakii*	1	*C. sakazakii*
168		Cro183	food	09/04/2010	2.656 ± 0.033	4	*C. sakazakii*	1	*C. sakazakii*
169	2202	Cro184	food	09/04/2010	2.481 ± 0.089	4	*C. dublinensis*	63	*C. dublinensis*
170		Cro185	milk powder	19/04/2010	2.309 ± 0.093	4	*C. sakazakii*	11	*C. sakazakii*
171	2169	Cro187	ice cream	20/04/2010	2.439 ± 0.057	4	*C. sakazakii*	1	*C. sakazakii*
172		Cro189	ice cream	29/04/2010	2.551 ± 0.037	4	*C. sakazakii*	1	*C. sakazakii*
173		Cro190	food	13/05/2010	2.474 ± 0.067	4	*C. sakazakii*	1	*C. sakazakii*
174		Cro191	food	13/05/2010	2.534 ± 0.039	4	*C. sakazakii*	1	*C. sakazakii*
175		Cro192	milk powder	13/05/2010	2.614 ± 0.023	4	*C. sakazakii*	1	*C. sakazakii*
176		Cro193	food	13/05/2010	2.487 ± 0.05	4	*C. sakazakii*	1	*C. sakazakii*
177		Cro194	milk powder	21/05/2010	2.495 ± 0.134	4	*C. sakazakii*	1	*C. sakazakii*
178		Cro195	food	08/06/2010	2.623 ± 0.030	4	*C. sakazakii*	1	*C. sakazakii*
179		Cro197	food	30/06/2010	2.638 ± 0.084	4	*C. sakazakii*	1	*C. sakazakii*
180		Cro198	ice cream powder	15/07/2010	2.574 ± 0.019	4	*C. sakazakii*	1	*C. sakazakii*
181		Cro199	ice cream powder	15/07/2010	2.448 ± 0.101	4	*C. sakazakii*	17	*C. sakazakii*
182	2194	Cro200	ice cream powder	15/07/2010	2.350 ± 0.066	4	*C. malonaticus*	7	*C. malonaticus*
183	2195	Cro201	ice cream powder	20/07/2010	2.392 ± 0.029	4	*C. malonaticus*	7	*C. malonaticus*
184	2170	Cro202	milk powder	06/08/2010	2.536 ± 0.039	4	*C. sakazakii*	1	*C. sakazakii*
185	2171	Cro203	milk powder	06/08/2010	2.432 ± 0.014	4	*C. sakazakii*	8	*C. sakazakii*
186	2172	Cro204	proficiency test sample	30/08/2010	2.608 ± 0.038	4	*C. sakazakii*	8	*C. sakazakii*
187	2173	Cro205	food	25/07/2011	2.382 ± 0.071	4	*C. sakazakii*	8	*C. sakazakii*
188		Cro207	food	25/07/2011	2.437 ± 0.124	4	*C. sakazakii*	8	*C. sakazakii*
189		Cro208	food	25/07/2011	2.321 ± 0.077	4	*C. sakazakii*	14	*C. sakazakii*
190		Cro210	food	11/07/2012	2.406 ± 0.056	4	*C. sakazakii*	3	*C. sakazakii*
191		Cro212	powdered infant formula	10/08/2012	2.443 ± 0.148	4	*C. sakazakii*	1	*C. sakazakii*
192	2174	Cro213	powdered infant formula	10/08/2012	2.373 ± 0.045	4	*C. sakazakii*	1	*C. sakazakii*
193	2175	Cro214	powdered infant formula	27/12/2012	2.432 ± 0.071	4	*C. sakazakii*	3	*C. sakazakii*
194	2176	Cro215	powdered infant formula	27/12/2012	2.383 ± 0.065	4	*C. sakazakii*	3	*C. sakazakii*
195	2177	Cro216	powdered infant formula	27/12/2012	2.400 ± 0.069	4	*C. sakazakii*	3	*C. sakazakii*
196		Cro217	powdered infant formula	17/06/2013	2.261 ± 0.056	4	*C. sakazakii*	3	*C. sakazakii*
197	2178	Cro218	powdered infant formula	26/06/2013	2.323 ± 0.070	4	*C. sakazakii*	37	*C. sakazakii*
198		Cro219	powdered infant formula	26/06/2013	2.471 ± 0.076	4	*C. sakazakii*	37	*C. sakazakii*
199		Cro220	powdered infant formula	26/06/2013	2.427 ± 0.080	4	*C. sakazakii*	3	*C. sakazakii*
200		Cro221	powdered infant formula	26/06/2013	2.586 ± 0.057	4	*C. sakazakii*	3	*C. sakazakii*
201		Cro222	powdered infant formula	26/06/2013	2.472 ± 0.035	4	*C. sakazakii*	3	*C. sakazakii*
202		Cro224	food ingredient	30/04/2015	2.524 ± 0.034	4	*C. sakazakii*	15	*C. sakazakii*
203		Cro225	food ingredient	30/04/2015	2.461 ± 0.055	4	*C. sakazakii*	10	*C. sakazakii*

[a] Logscore, average ± standard deviation.
[b] n, number of the spectra analyzed.

Seventeen *C. malonaticus* isolates were divided into two serotypes, Cmal O:1 and Cmal O:2. Cmal O:2 (16/17, 94.12%) was the predominant serotype. Of the two *C. turicensis* isolates, one isolate was assigned to Ctur O:1, however the remaining isolate could not be assigned to any serotype due to lack of PCR product. Three *C. muytjensii* isolates belonged to Cmuy O:1. As for the two *C. dublinensis* isolates, one isolate was assigned to Cdub O:2, the other one could not be assigned to any serotype, also due to no PCR product.

4. Discussion

Reference database is the most important factor for accurate and precise species identification using MALDI-TOF MS because it is used to compare with the mass spectra of the unknown bacteria. It was not possible to identify *Cronobacter* isolates to the species level using the database provided by Bruker because all the nine reference MSPs of *Cronobacter* spp. were named as *C. sakazakii* in the recent database (containing 7311 MSPs). Subsequently Cetinkaya et al. (2013)

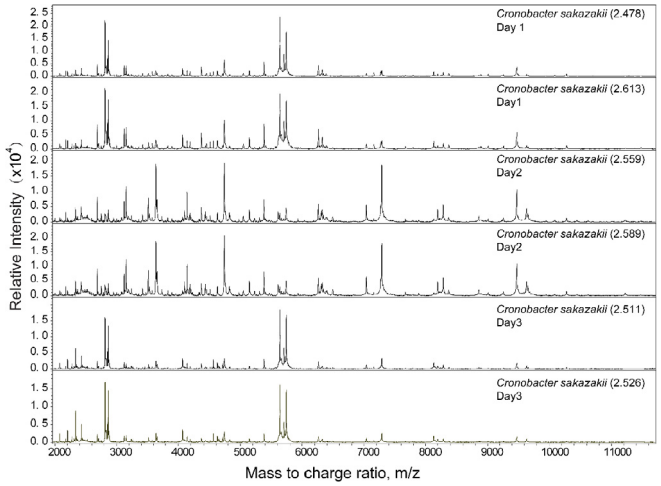

Fig. 2. MALDI-TOF MS spectra of the *C. sakazakii* isolate Cro44 after three separate extractions. The mass intensity is given on the y-axis, and the x-axis indicates the mass-to-charge ratio (m/z). The identification results according to the highest score by Biotyper RTC are shown on the upper right corner of each spectrum.

identified six *Cronobacter* strains as *C. sakazakii* by MALDI-TOF MS, whereas a more accurate detection method revealed they were four strains of *C. sakazakii*, one strain of *C. malonaticus* and one strain of *C. universalis*. Consequently, an in-house database for *Cronobacter* species is needed for species identification. Krasny et al. (2014) developed a *Cronobacter* database including 52 reference spectra and tested on a set of 45 samples, but the overall accuracy was only about 80%. The main reason for the low accuracy maybe because they did not use the formic-acid protein extraction method and thus the quality of the spectra was low and reduced the discrimination.

Our initial in-house database containing eight reference strains across six *Cronobacter* species correctly identified all isolates of *Cronobacter* species other than *C. sakazakii*. However 17 isolates of *C. sakazakii* gave two or more identification results (Wang et al., 2017). This is because *C. sakazakii* has at least three different spectra (Fig. 1). By adding the other two additional spectra of *C. sakazakii* into the in-house database, all the 43 isolates were identified to species level with good agreement with that of *fusA* sequencing (Table 1). Extend the database with more reference strains is a routine way to get more specific species identifications with higher matching scores and enhance the discriminatory power of MALDI-TOF MS-based classification (Erler et al., 2015; Xiao et al., 2014). It is because more MSPs were provided to compare and in-house established MSPs can reduce the influence of instrument and reagent. As Biotyper only shows the top 10 matched results, it is better adding different MSPs (e.g. Logscore < 2.300) into the database to afford more information.

A number of studies using MALDI-TOF MS for epidemiological typing have been published (Fagerquist et al., 2014; Wolters et al., 2011). However, other studies have described a failure to achieved satisfying resolution (Lasch et al., 2014). For some successful reports in MALDI-TOF MS typing, it becomes evident that for some bacterial taxa, the available data are not consistent (Spinali et al., 2015; Szabados et al., 2011). It seems that the discriminatory potential for MALDI

Table 2
Comparison of 7-loci MLST and PCR-serotyping results for 84 strains from 5 *Cronobacter* species.

Species	No. of isolates		Serotype	*atpD*	*fusA*	*glnS*	*gltB*	*gyrB*	*infB*	*ppsA*	ST	CC
C. sakazakii (60 strains)	17	Cro2, Cro3, Cro4, Cro5, Cro6, Cro7, Cro8, Cro9, Cro10, Cro12, Cro67, Cro92, Cro99, Cro156, Cro169, Cro170, Cro202	Csak O:1	1	1	1	1	1	1	1	1	1
	3	Cro25, Cro204, Cro205	Csak O:1	11	8	7	5	8	15	10	8	8
	3	Cro145, Cro148, Cro151	Csak O:1	15	8	3	84	161	162	210	**615**	
	2	Cro119, Cro125	Csak O:1	69	8	13	95	86	105	47	219	155
	1	Cro16	Csak O:1	3	18	9	**267**	1	**225**	210	**618**	
	1	Cro103	Csak O:1	3	8	52	54	21	65	73	99	99
	1	Cro131	Csak O:1	3	8	52	54	21	65	209	378	99
	1	Cro150	Csak O:1	3	11	13	18	11	17	13	21	21
	1	Cro167	Csak O:1	10	17	30	59	57	66	83	125	100
	12	Cro20, Cro31, Cro32, Cro43, Cro48, Cro53, Cro58, Cro106, Cro107, Cro110, Cro135, Cro213	Csak O:2	5	1	3	3	5	5	4	4	4
	4	Cro74, Cro82, Cro86, Cro164	Csak O:2	3	8	37	22	29	36	32	31	31
	3	Cro214, Cro215, Cro216	Csak O:2	3	3	3	5	3	3	3	3	3
	1	Cro66	Csak O:2	16	8	13	40	15	15	10	64	64
	1	Cro93	Csak O:2	16	1	19	19	26	5	26	22	
	1	Cro97	Csak O:2	3	12	16	5	16	20	14	17	17
	1	Cro153	Csak O:2	15	116	15	13	22	5	16	**616**	13
	1	Cro166	Csak O:2	20	18	16	10	3	20	27	23	23
	1	Cro218	Csak O:2	16	37	48	61	5	56	77	96	
	1	Cro111	Csak O:3	5	1	3	3	5	5	4	4	4
	1	Cro203	Csak O:3	16	8	52	143	22	15	**321**	**619**	
	2	Cro161, Cro187	Csak O:4	1	1	1	1	1	1	1	1	1
	1	Cro38	Csak O:4	15	14	15	13	22	5	16	13	13
C. malonaticus (17 strains)	1	Cro96	Cmal O:1	124	13	172	204	77	183	244	440	
	12	Cro115, Cro116, Cro130, Cro132, Cro133, Cro134, Cro139, Cro140, Cro141, Cro142, Cro143, Cro152	Cmal O:2	10	7	6	7	9	14	9	7	7
	2	Cro200, Cro201	Cmal O:2	57	7	25	8	72	40	89	138	
	1	Cro42	Cmal O:2	10	13	64	75	**239**	14	247	**617**	
	1	Cro95	Cmal O:2	10	7	25	23	10	40	29	25	
C. turicensis (2 strains)	1	Cro56	Ctur O:1	**173**	22	57	**264**	46	18	180	**614**	
	1	Cro68	NP[a]	14	22	14	20	13	18	28	24	24
C. muytjensii (3 strains)	2	Cro121, Cro124	Cmuy O:1	26	25	**232**	**266**	45	39	217	**625**	
	1	Cro70	Cmuy O:1	35	64	36	**265**	50	**224**	**320**	**624**	
C. dublinensis (2 strains)	1	Cro158	NP	113	125	141	164	**238**	159	201	**478**	
	1	Cro184	Cdub O:2	58	63	75	76	73	76	108	167	

[a] NP = No PCR product generated.
New assigned allele number and STs are in bold.

typing is likely not the same for all bacterial species. Given the diversity and taxonomic re-evaluations of the *Cronobacter* genera, it was of interest to consider the method for epidemiological typing.

PCA was unable to precisely subtype *Cronobacter* isolates. The PCA clustering results were different when using replicates of the same isolate. This was due to the replicates of the same isolate generating different peak lists according to the flexAnalysis software (Wang et al., 2017). In this paper, we further found that the MALDI-TOF MS data itself was also not sufficiently consistent for strain subtyping. 12.32% (25/203) isolates gave inconsistent spectra following separate protein extractions. Additionally, the spectra of *C. sakazakii* ATCC 29544, *C. sakazakii* ATCC 29004 and *C. sakazakii* ATCC 12868 differed from the spectra in previous literature (Lu et al., 2014). Spectra differences may not affect the species identification results, but could create difficulties when using spectra for subtyping.

Compared to MALDI-TOF MS method, 7-loci MLST is a more reliable method for subtyping *Cronobacter* spp. isolates. Our study indicates that the 84 *Cronobacter* spp. isolates from imported foods have been subtyped into 31 STs. Sixty *C. sakazakii* isolates belong to 20 STs and four serotypes. Seventeen *C. malonaticus* isolates belonged to five STs and two serotypes. The discrimination power of PCR serotyping method is also relatively low compared to the 7-loci MLST method and not all *Cronobacter* isolates generated amplification products and therefore could not be serotyped (Table 2). This is in accordance with previous studies (Jarvis et al., 2011; Yan et al., 2015).

The main sequence types in our study were ST1 (31.67%, 19/60) and ST4 (21.67%, 13/60) for *C. sakazakii* and ST7 (70.59%, 12/17) for *C. malonaticus*, which were major *Cronobacter* pathovars. The result agrees with previous studies (Fei et al., 2015, 2017; Ogrodzki and Forsythe, 2017; Sonbol et al., 2013; Vojkovska et al., 2016). ST1 belongs to *C. sakazakii* clonal complex 1 (CC1), which is the second major ST in the PubMLST *Cronobacter* database (accessed on January 14, 2019). In our study, Csak O:1 and Csak O:4 serotypes were found in ST1 and Csak O:1 (17/19, 89.47%) was the predominant serotype. This has previously been reported in an earlier study of over 1000 strains (Forsythe et al., 2014). *C. sakazakii* ST4 are strongly associated with neonatal meningitis, and have previously been isolated from milk powder, food, ice cream and powdered infant formula. In our study the major (12/13, 92.31%) of *C. sakazakii* ST4 strains were serotype Csak O:2. *C. malonaticus* ST7 is associated with adult infections (Forsythe, 2018; Forsythe et al., 2014). The main serotype of our *C. malonaticus* ST7 strains were serotype Cmal O:2.

The remaining 18 *C. sakazakii* ST types included ST64 (n = 1), ST13 (n = 1) and four new STs (ST615, ST616, ST618, ST619). It is interesting to find that ST615 is clustered with ST380 and ST381, which were submitted by a Chinese Entry-Exit Inspection and Quarantine Bureau and also isolated from imported food in the same year (2009). It was also found that ST616 belongs to CC13, which is associated with clinical cases.

In conclusion, spectral data from MALDI-TOF MS were not consistent enough for subtyping *Cronobacter* spp. isolates, but the method is still a rapid and precise method for *Cronobacter* species identification when an appropriate in-house database has been established. PCR-based O-antigen serotype could only subtype isolates to limited serotypes. Currently, 7-loci MLST is a more reliable method for subtyping *Cronobacter* spp. and has a centralized, curated database accessible by laboratories in different countries. The *Cronobacter* spp. isolates from imported foods during 2006–2015 were highly divergent, and included recognized *Cronobacter* pathovars.

Acknowledgements

We thank Prof. Wan-Jin Liao for his valuable help with experimental design and paper writing. This work was supported by the National Key Research and Development Program, China (2016YFD0401102); and the Science and Technology Program of General Administration of Quality Supervision, Inspection and Quarantine, China (2017IK145).

References

Akineden, O., Heinrich, V., Gross, M., Usleber, E., 2017. Reassessment of *Cronobacter* spp. originally isolated as *Enterobacter sakazakii* from infant food. Food Microbiol. 65, 44–50. https://doi.org/10.1016/j.fm.2017.01.021.

Alsonosi, A., Hariri, S., Kajsik, M., Orieskova, M., Hanulik, V., Roederova, M., Petrzelova, J., Kollarova, H., Drahovska, H., Forsythe, S., Holy, O., 2015. The speciation and genotyping of *Cronobacter* isolates from hospitalised patients. Eur. J. Clin. Microbiol. Infect. Dis. 34, 1979–1988. https://doi.org/10.1007/s10096-015-2440-8.

Baldwin, A., Loughlin, M., Caubilla-Barron, J., Kucerova, E., Manning, G., Dowson, C., Forsythe, S., 2009. Multilocus sequence typing of *Cronobacter sakazakii* and *Cronobacter malonaticus* reveals stable clonal structures with clinical significance which do not correlate with biotypes. BMC Microbiol. 9, 223. https://doi.org/10.1186/1471-2180-9-223.

Blazkova, M., Javurkova, B., Vlach, J., Goeselova, S., Karamonova, L., Ogrodzki, P., Forsythe, S., Fukal, L., 2015. Diversity of O-antigens within the genus *Cronobacter*: from disorder to order. Appl. Environ. Microbiol. 81, 5574–5582. https://doi.org/10.1128/aem.00277-15.

Brandao, M.L.L., Umeda, N.S., Jackson, E., Forsythe, S.J., de Filippis, I., 2017. Isolation, molecular and phenotypic characterization, and antibiotic susceptibility of *Cronobacter* spp. from Brazilian retail foods. Food Microbiol. 63, 129–138. https://doi.org/10.1016/j.fm.2016.11.011.

Brengi, S.P., O'Brien, S.B., Pichel, M., Iversen, C., Arduino, M., Binsztein, N., Jensen, B., Pagotto, F., Ribot, E.M., Stephan, R., Cernela, N., Cooper, K., Fanning, S., 2012. Development and validation of a PulseNet standardized protocol for subtyping isolates of *Cronobacter* species. Foodborne Pathogens and Disease 9, 861–867. https://doi.org/10.1089/fpd.2012.1161.

Cetinkaya, E., Joseph, S., Ayhan, K., Forsythe, S.J., 2013. Comparison of methods for the microbiological identification and profiling of *Cronobacter* species from ingredients used in the preparation of infant formula. Mol. Cell. Probes 27, 60–64. https://doi.org/10.1016/j.mcp.2012.10.003.

Erler, R., Wichels, A., Heinemeyer, E.-A., Hauk, G., Hippelein, M., Reyes, N.T., Gerdts, G., 2015. VibrioBase: a MALDI-TOF MS database for fast identification of *Vibrio* spp. that are potentially pathogenic in humans. Syst. Appl. Microbiol. 38, 16–25. https://doi.org/10.1016/j.syapm.2014.10.009.

Fagerquist, C.K., Zaragoza, W.J., Sultan, O., Woo, N., Quinones, B., Cooley, M.B., Mandrell, R.E., 2014. Top-down proteomic identification of Shiga toxin 2 subtypes from Shiga toxin-producing *Escherichia coli* by matrix-assisted laser desorption ionization-tandem time of flight mass spectrometry. Appl. Environ. Microbiol. 80, 2928–2940. https://doi.org/10.1128/aem.04058-13.

Fei, P., Jiang, Y., Jiang, Y., Yuan, X., Yang, T., Chen, J., Wang, Z., Kang, H., Forsythe, S.J., 2017. Prevalence, molecular characterization, and antibiotic susceptibility of *Cronobacter sakazakii* isolates from powdered infant formula collected from Chinese retail markets. Front. Microbiol. 8, 2026. https://doi.org/10.3389/fmicb.2017.02026.

Fei, P., Man, C., Lou, B., Forsythe, S.J., Chai, Y., Li, R., Niu, J., Jiang, Y., 2015. Genotyping and source tracking of *Cronobacter sakazakii* and *C. malonaticus* isolates from powdered infant formula and an infant formula production factory in China. Appl. Environ. Microbiol. 81, 5430–5439. https://doi.org/10.1128/aem.01390-15.

Forsythe, S.J., 2018. Updates on the *Cronobacter* genus. Annual Review of Food Science and Technology 9, 23–44. https://doi.org/10.1146/annurev-food-030117-012246.

Forsythe, S.J., Dickins, B., Jolley, K.A., 2014. *Cronobacter*, the emergent bacterial pathogen *Enterobacter sakazakii* comes of age; MLST and whole genome sequence analysis. BMC Genomics 15, 1121. https://doi.org/10.1186/1471-2164-15-1121.

Giacometti, F., Piva, S., Vranckx, K., De Bruyne, K., Drigo, I., Lucchi, A., Manfreda, G., Serraino, A., 2018. Application of MALDI-TOF MS for the subtyping of *Arcobacter butzleri* strains and comparison with their MLST and PFGE types. Int. J. Food Microbiol. 277, 50–57. https://doi.org/10.1016/j.ijfoodmicro.2018.04.026.

Iversen, C., Waddington, M., Farmer III, J.J., Forsythe, S.J., 2006. The biochemical differentiation of *Enterobacter sakazakii* genotypes. BMC Microbiol. 6, 94. https://doi.org/10.1186/1471-2180-6-94.

Jackson, E.E., Forsythe, S.J., 2016. Comparative study of *Cronobacter* identification according to phenotyping methods. BMC Microbiol. 16, 146. https://doi.org/10.1186/s12866-016-0768-6.

Jackson, E.E., Parra Flores, J., Fernandez-Escartin, E., Forsythe, S.J., 2015. Reevaluation of a suspected *Cronobacter sakazakii* outbreak in Mexico. J. Food Protect. 78, 1191–1196. https://doi.org/10.4315/0362-028x.jfp-14-563.

Jackson, E.E., Sonbol, H., Masood, N., Forsythe, S.J., 2014. Genotypic and phenotypic characteristics of *Cronobacter* species, with particular attention to the newly reclassified species *Cronobacter helveticus, Cronobacter pulveris*, and *Cronobacter zurichensis*. Food Microbiol. 44, 226–235. https://doi.org/10.1016/j.fm.2014.06.013.

Jarvis, K.G., Grim, C.J., Franco, A.A., Gopinath, G., Sathyamoorthy, V., Hu, L., Sadowski, J.A., Lee, C.S., Tall, B.D., 2011. Molecular characterization of *Cronobacter* lipopolysaccharide O-antigen gene clusters and development of serotype-specific PCR assays. Appl. Environ. Microbiol. 77, 4017–4026. https://doi.org/10.1128/aem.00162-11.

Joseph, S., Forsythe, S.J., 2011. Predominance of *Cronobacter sakazakii* sequence type 4 in neonatal infections. Emerg. Infect. Dis. 17, 1713–1715. https://doi.org/10.3201/eid1709.110260.

Joseph, S., Sonbol, H., Hariri, S., Desai, P., McClelland, M., Forsythe, S.J., 2012. Diversity of the *Cronobacter* genus as revealed by multilocus sequence typing. J. Clin. Microbiol. 50, 3031–3039. https://doi.org/10.1128/jcm.00905-12.

Krasny, L., Rohlova, E., Ruzickova, H., Jiri, s.A., Hynek, R., Hochel, I., 2014. Differentiation of *Cronobacter* spp. by tryptic digestion of the cell suspension followed by MALDI-TOF MS analysis. J. Microbiol. Methods 98, 105–113. https://doi.org/10.1016/j.mimet.2014.01.008.

Lasch, P., Fleige, C., Staemmler, M., Layer, F., Nuebel, U., Witte, W., Werner, G., 2014. Insufficient discriminatory power of MALDI-TOF mass spectrometry for typing of *Enterococcus faecium* and *Staphylococcus aureus* isolates. J. Microbiol. Methods 100, 58–69. https://doi.org/10.1016/j.mimet.2014.02.015.

Lu, Y., Chen, Y., Lu, X.A., Lv, J., Man, C.X., Chai, Y.L., Jiang, Y.J., 2014. Comparison of methods for the microbiological identification and typing of *Cronobacter* species in infant formula. J. Dairy Sci. 97, 632–641. https://doi.org/10.3168/jds.2013-7147.

Masood, N., Moore, K., Farbos, A., Paszkiewicz, K., Dickins, B., McNally, A., Forsythe, S., 2015. Genomic dissection of the 1994 *Cronobacter sakazakii* outbreak in a French neonatal intensive care unit. BMC Genomics 16, 750. https://doi.org/10.1186/s12864-015-1961-y.

Mullane, N., O'Gaora, P., Nally, J.E., Iversen, C., Whyte, P., Wall, P.G., Fanning, S., 2008. Molecular analysis of the *Enterobacter sakazakii* O-antigen gene locus. Appl. Environ. Microbiol. 74, 3783–3794. https://doi.org/10.1128/aem.02302-07.

Ogrodzki, P., Forsythe, S., 2015. Capsular profiling of the *Cronobacter* genus and the association of specific *Cronobacter sakazakii* and *C. malonaticus* capsule types with neonatal meningitis and necrotizing enterocolitis. BMC Genomics 16, 758. https://doi.org/10.1186/s12864-015-1960-z.

Ogrodzki, P., Forsythe, S.J., 2016. CRISPR-cas loci profiling of *Cronobacter sakazakii* pathovars. Future Microbiol. 11, 1507–1519. https://doi.org/10.2217/fmb-2016-0070.

Ogrodzki, P., Forsythe, S.J., 2017. DNA-sequence based typing of the *Cronobacter* genus using MLST, CRISPR-cas array and capsular profiling. Front. Microbiol. 8, 1875. https://doi.org/10.3389/fmicb.2017.01875.

Patrick, M.E., Mahon, B.E., Greene, S.A., Rounds, J., Cronquist, A., Wymore, K., Boothe, E., Lathrop, S., Palmer, A., Bowen, A., 2014. Incidence of *Cronobacter* spp. infections, USA, 2003-2009. Emerg. Infect. Dis. 20, 1520–1523. https://doi.org/10.3201/eid2009.140545.

Sonbol, H., Joseph, S., McAuley, C.M., Craven, H.M., Forsythe, S.J., 2013. Multilocus sequence typing of *Cronobacter* spp. from powdered infant formula and milk powder production factories. Int. Dairy J. 30, 1–7. https://doi.org/10.1016/j.idairyj.2012.11.004.

Spinali, S., van Belkum, A., Goering, R.V., Girard, V., Welker, M., Van Nuenen, M., Pincus, D.H., Arsac, M., Durand, G., 2015. Microbial typing by matrix-assisted laser desorption ionization-time of flight mass spectrometry: do we need guidance for data interpretation? J. Clin. Microbiol. 53, 760–765. https://doi.org/10.1128/jcm.01635-14.

Stephan, R., Ziegler, D., Pflueger, V., Vogel, G., Lehner, A., 2010. Rapid genus- and species-specific identification of *Cronobacter* spp. by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J. Clin. Microbiol. 48, 2846–2851. https://doi.org/10.1128/jcm.00156-10.

Sun, Y., Wang, M., Liu, H., Wang, J., He, X., Zeng, J., Guo, X., Li, K., Cao, B., Wang, L., 2011. Development of an O-antigen serotyping scheme for *Cronobacter sakazakii*. Appl. Environ. Microbiol. 77, 2209–2214. https://doi.org/10.1128/aem.02229-10.

Sun, Y., Wang, M., Wang, Q., Cao, B., He, X., Li, K., Feng, L., Wang, L., 2012. Genetic analysis of the *Cronobacter sakazakii* O4 to O7 O-antigen gene clusters and development of a PCR assay for identification of all *C. sakazakii* O serotypes. Appl. Environ. Microbiol. 78, 3966–3974. https://doi.org/10.1128/aem.07825-11.

Szabados, F., Becker, K., von Eiff, C., Kaase, M., Gatermann, S., 2011. The matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS)-based protein peaks of 4448 and 5302 Da are not associated with the presence of Panton-Valentine leukocidin. International Journal of Medical Microbiology 301, 58–63. https://doi.org/10.1016/j.ijmm.2010.05.005.

van Belkum, A., Chatellier, S., Girard, V., Pincus, D., Deol, P., Dunne Jr., W.M., 2015. Progress in proteomics for clinical microbiology: MALDI-TOF MS for microbial species identification and more. Expert Rev. Proteom 12, 595–605. https://doi.org/10.1586/14789450.2015.1091731.

Vlach, J., Javurkova, B., Karamonova, L., Blazkova, M., Fukal, L., 2017. Novel PCR-RFLP system based on rpoB gene for differentiation of *Cronobacter* species. Food Microbiol. 62, 1–8. https://doi.org/10.1016/j.fm.2016.08.004.

Vojkovska, H., Karpiskova, R., Orieskova, M., Drahovska, H., 2016. Characterization of *Cronobacter* spp. isolated from food of plant origin and environmental samples collected from farms and from supermarkets in the Czech Republic. Int. J. Food Microbiol. 217, 130–136. https://doi.org/10.1016/j.ijfoodmicro.2015.10.017.

Wang, Q., Zhao, X.-J., Wang, Z.-W., Liu, L., Wei, Y.-X., Han, X., Zeng, J., Liao, W.-J., 2017. Identification of *Cronobacter* species by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with an optimized analysis method. J. Microbiol. Methods 139, 172–180. https://doi.org/10.1016/j.mimet.2017.05.016.

Wolters, M., Rohde, H., Maier, T., Belmar-Campos, C., Franke, G., Scherpe, S., Aepfelbacher, M., Christner, M., 2011. MALDI-TOF MS fingerprinting allows for discrimination of major methicillin-resistant *Staphylococcus aureus* lineages. International Journal of Medical Microbiology 301, 64–68. https://doi.org/10.1016/j.ijmm.2010.06.002.

Xiao, D., Ye, C., Zhang, H., Kan, B., Lu, J., Xu, J., Jiang, X., Zhao, F., You, Y., Yan, X., Wang, D., Hu, Y., Zhang, M., Zhang, J., 2014. The construction and evaluation of reference spectra for the identification of human pathogenic microorganisms by MALDI-TOF MS. PLoS One 9, e106312. https://doi.org/10.1371/journal.pone.0106312.

Yan, Q., Jarvis, K.G., Chase, H.R., Hebert, K., Trach, L.H., Lee, C., Sadowski, J., Lee, B., Hwang, S., Sathyamoorthy, V., Mullane, N., Pava-Ripoll, M., Iversen, C., Pagotto, F., Fanning, S., Tall, B.D., 2015. A proposed harmonized LPS molecular-subtyping scheme for *Cronobacter* species. Food Microbiol. 50, 38–43. https://doi.org/10.1016/j.fm.2015.03.003.

Zhu, S., Ratering, S., Schnell, S., Wacker, R., 2011. Matrix-assisted laser desorption and ionization-time-of-flight mass spectrometry, 16S rRNA gene sequencing, and API 32E for identification of *Cronobacter* spp.: a comparative study. J. Food Protect. 74, 2182–2187. https://doi.org/10.4315/0362-028x.jfp-11-205.