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Novel Method for Reliable Identification of *Siccibacter* and *Franconibacter* Strains: from "Pseudo-*Cronobacter*" to New *Enterobacteriaceae* Genera

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ABSTRACT In the last decade, strains of the genera *Franconibacter* and *Siccibacter* have been misclassified as first *Enterobacter* and later *Cronobacter*. Because *Cronobacter* is a serious foodborne pathogen that affects premature neonates and elderly individuals, such misidentification may not only falsify epidemiological statistics but also lead to tests of powdered infant formula or other foods giving false results. Currently, the main ways of identifying *Franconibacter* and *Siccibacter* strains are by biochemical testing or by sequencing of the *fusA* gene as part of *Cronobacter* multilocus sequence typing (MLST), but in relation to these strains the former is generally highly difficult and unreliable while the latter remains expensive. To address this, we developed a fast, simple, and most importantly, reliable method for *Franconibacter* and *Siccibacter* identification based on intact-cell matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Our method integrates the following steps: data preprocessing using mMass software; principal-component analysis (PCA) for the selection of mass spectrum fingerprints of *Franconibacter* and *Siccibacter* strains; optimization of the Biotyper database settings for the creation of main spectrum projections (MSPs). This methodology enabled us to create an in-house MALDI MS database that extends the current MALDI Biotyper database by including *Franconibacter* and *Siccibacter* strains. Finally, we verified our approach using seven previously unclassified strains, all of which were correctly identified, thereby validating our method.

IMPORTANCE We show that the majority of methods currently used for the identification of *Franconibacter* and *Siccibacter* bacteria are not able to properly distinguish these strains from those of *Cronobacter*. While sequencing of the *fusA* gene as part of *Cronobacter* MLST remains the most reliable such method, it is highly expensive and time-consuming. Here, we demonstrate a cost-effective and reliable alternative that correctly distinguishes between *Franconibacter*, *Siccibacter*, and *Cronobacter* bacteria and identifies *Franconibacter* and *Siccibacter* at the species level. Using intact-cell MALDI-TOF MS, we extend the current MALDI Biotyper database with 11 *Franconibacter* and *Siccibacter* MSPs. In addition, the use of our approach is likely to lead to a more reliable identification scheme for *Franconibacter* and *Siccibacter* strains and, consequently, a more trustworthy epidemiological picture of their involvement in disease.

KEYWORDS *Franconibacter*, *Siccibacter*, "pseudo-*Cronobacter*", intact-cell MALDI-TOF mass spectrometry

In the last decade, the taxonomy of the Gram-negative bacteria of the family *Enterobacteriaceae* has progressed rapidly, but somewhat confusingly. In 2007, Stephan et al. isolated several *Franconibacter* and *Siccibacter* strains from fruit powder, infant formula, and the infant formula preparation environment. However, based on 16S rRNA

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and *rpoB* gene analysis, they classified these strains into the genus *Enterobacter* because their *rpoB* gene sequences were most similar to those of *Enterobacter radicincitans*, *E. cowanii*, and *E. sakazakii*. Confirming these data by DNA-DNA hybridization and biochemical testing, they consequently designated three new species: *E. pulveris* (type strain LMG 24057^T), *E. helveticus* (type strain LMG 23732^T), and *E. turicensis* (type strain LMG 23730^T) (1, 2).

Although 16S rRNA is considered to be the gold standard for phylogenetic analysis, it is insufficient for the identification of strains in the family *Enterobacteriaceae* due to the polyphyletic nature of the group (3, 4). In the search for alternative methods more suitable for *Enterobacteriaceae* strain phylogeny, Brady et al. used multilocus sequence analysis of concatenated nucleotide sequences of four protein-encoding genes (*gyrB*, *rpoB*, *infB*, and *atpD*). They defined five groups within the family and, using fatty acid and phenotypic analyses, reclassified several species into new genera. *E. pulveris*, *E. helveticus*, and *E. turicensis* became *Cronobacter pulveris*, *C. helveticus*, and *C. zurichensis*, respectively (5).

Nevertheless, when, 1 year later, Stephan et al. performed genome-scaled analysis of all *Cronobacter* species, as well as of *Enterobacter cloacae*, it emerged that the organisms that Brady et al. identified as *Cronobacter* species were actually "pseudo-*Cronobacter*," belonging neither to the genus *Cronobacter* nor to the genus *Enterobacter*. At that point, Stephan grouped these species into two new genera within *Enterobacteriaceae*: (i) *Franconibacter*, which incorporated *F. pulveris* and *F. helveticus* (previously *C. pulveris* and *C. helveticus*, respectively), and (ii) *Siccibacter*, which included *S. turicensis* (previously *C. zurichensis*) (6). More recently, Jackson et al. used average nucleotide identity (ANI) analysis and phenotypic testing to describe two strains phylogenetically close to *S. turicensis*. On the basis of their analysis, they proposed a new species within the genus *Siccibacter*, *Siccibacter colletis* (type strain LMG 28204^T) (7).

The close relationship between "pseudo-*Cronobacter*" strains and strains of the genus *Cronobacter* has implications. *Cronobacter* is associated with rare but serious infections (e.g., meningitis, necrotizing enterocolitis, and sepsis) among low-birth-weight neonates and the immunocompromised elderly (8, 9). In 2008, FAO/WHO classified all *Cronobacter* species as potentially pathogenic (10), and therefore, the authorities in food safety require identification of *Cronobacter* at the genus level only. Since then, several differences in virulence traits have been reported between *Cronobacter* species, with *C. sakazakii* and *C. malonaticus* most commonly found in clinical samples (9). Before they were mislabeled as "pseudo-*Cronobacter*," strains of the genera *Franconibacter* and *Siccibacter* had been used as negative controls for *Cronobacter* identification methods. After this misclassification, they were actually included in the *Cronobacter* genus. And then, following Stephan's reclassification, they were finally removed from the genus (6). This complicated history shows that it is relatively easy to misidentify nonpathogenic *Franconibacter* and *Siccibacter* strains as *Cronobacter* strains.

One of the main reasons for this is that the three genera share similar biochemical properties. For instance, the α-glucosidase activity of *Cronobacter* strains, encoded by two separate genes (11), has been used as a marker in some *Cronobacter* diagnostic media, such as *Enterobacter sakazakii* isolation chromogenic agar (ESIA; AES) or the Oxoid chromogenic *Enterobacter sakazakii* agar (Druggan-Forsythe-Iversen [DFI] medium) (12). However, because the reclassified *Franconibacter* and *Siccibacter* species also possess α-glucosidase activity, there is the danger of false-positive phenotypic results (12). Making it even more complicated is the fact that it has been reported that some *Cronobacter* species lack α-glucosidase activity, which also raises the possibility of false-negative results (1, 2, 13). To avoid these circumstances, another chromogenic medium, the chromID Sakazakii agar (bioMérieux) has been developed. This agar combines two *Cronobacter* enzymatic activities, the above-mentioned α-glucosidase activity and the ability to utilize cellobiose (α-D-glucopyranosidase and β-D-cellobiosidase) (14). Furthermore, a similar issue was noticed in the use of molecular biology identification methods, such as *rpoB* or *zpx* gene analysis, for *Cronobacter* genus

and species determination (15). On the other hand, several PCR-based assays targeting, for example, *dnaG* (16) or *cgcA* (17), genes that are specific for the *Cronobacter* genus and exclude the *Franconibacter* and *Siccibacter* strains, have been developed and successfully used (15, 18). Thus, it is likely that the misclassification of "pseudo-*Cronobacter*" strains has affected epidemiological statistics, with one consequence being the lack of a reliable epidemiological background of *Franconibacter* and *Siccibacter*.

Currently, the genera *Franconibacter* and *Siccibacter* are not included in the ID32E test (bioMérieux), the widely used commercial identification kit for *Enterobacteriaceae* (15). Moreover, no PCR detection system has yet been published for these genera. Therefore, the only ways to correctly identify strains from the genera *Franconibacter* and *Siccibacter* are by biochemical testing (6, 7) and by sequencing of the *fusA* gene as part of the *Cronobacter* multilocus sequence type (MLST) scheme (19, 20). However, in terms of the proper identification of *Cronobacter*, *Franconibacter*, and *Siccibacter* genera, the former method is highly difficult and the latter remains expensive.

In this study, we use a comprehensive approach to retrospectively characterize *Franconibacter* and *Siccibacter* strains using biochemical tests, the *Cronobacter* PCR detection systems, and MLST. Having demonstrated the issues related to these approaches, we then present a new cost-effective and rapid identification method based on intact-cell matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). We create 11 *Franconibacter* and *Siccibacter* main spectrum projections (MSPs) using a novel procedure that includes principal-component analysis (PCA). In this way, we establish an "in-house" database that extends the current MALDI Biotyper database and use it to identify seven previously unclassified strains, thereby validating our method.

RESULTS

Biochemical tests. The 11 strains of the *Franconibacter* and *Siccibacter* genera, as well as the *Cronobacter* strains used as controls in this study, were tested using the commercial ID32E biochemical system. In addition, to scale up the strain profile, several biochemical tube tests suitable for the *Cronobacter* genus were also used for all strains. The combined results are shown in Table 1.

The ID32E results showed that the *Franconibacter* and *Siccibacter* strains had a "doubtful" or "unacceptable" profile, with most of them being misidentified as *Cronobacter* species except SI 45, which was misidentified as *Escherichia vulneris*. Similarly, strains FR 29 and FR 33 were misidentified as *C. malonaticus* or *Buttiauxella agrestis*. Together with the *Franconibacter* and *Siccibacter* strains, the type strain for each *Cronobacter* species was also analyzed for control purposes. Compared to the *Franconibacter* and *Siccibacter* strains, the *Cronobacter* strains were clearly identified at the genus level. Conversely, *Cronobacter* species identification was not correct in many cases. Although strain CB 51 was correctly identified as *C. malonaticus*, an additional dulcitol test (see below) was needed to properly determine strains CB 55 and CB 62 as *Cronobacter muytjensii* and *C. dublinensis*, respectively. Furthermore, in the case of CB 03, it was not possible to distinguish between *C. malonaticus* and *C. sakazakii*. As well, several strains were misidentified: CB 59 as *C. turicensis*, CB 78 as *C. muytjensii*, and CB 61 as *C. malonaticus*.

Remarkably, the ID32E results showed that all *Franconibacter* and *Siccibacter* strains were positive for RP (phenol red test), while the *Cronobacter* strains were not (data not shown). Additionally, only *Franconibacter pulveris* and the *Cronobacter* species utilized saccharose (SAC), while *Franconibacter helveticus* and *S. turicensis* did not possess that ability (data not shown).

Following the ID32E tests, the strains were further characterized using 10 additional biochemical tube tests selected according to the literature (21–24). These tests are listed in Table 1 with the exception of the putrescine and turanose utilization tests, which were added to the analysis in order to differentiate between *C. turicensis* and *C. universalis* (data not shown). The selected biochemical tube tests were applied to the

TABLE 1 Strain characterization and identification using ID32E and selected biochemical tube tests

		ID32E			Test result using additional biochemical tube test[a]									
Strain ID	Species	Identification results	Profile no.	Identification level	GLU	INO	DUL	MNT	VP	MR	NIT	ORN	MOT	IND
FR 29	*F. helveticus*	*C. malonaticus/Buttiauxella agrestis*	34774563051	Unacceptable profile	−	−	+	+	+	+	+	−	+	−
FR 31	*F. helveticus*	*C. malonaticus*	34674563051	Doubtful profile	−	−	+	+	+	+	+	−	+	−
FR 32	*F. helveticus*	*C. malonaticus*	34674563051	Doubtful profile	−	−	+	+	+	+	+	−	+	−
FR 33	*F. helveticus*	*C. malonaticus/Buttiauxella agrestis*	34774563051	Unacceptable profile	−	−	+	−	+	+	+	−	+	−
FR 35	*F. helveticus*	*C. malonaticus*	34674563051	Doubtful profile	−	−	+	+	+	+	+	−	+	−
FR 38	*F. pulveris*	*C. dublinensis/C. malonaticus/ C. sakazakii*	34676773050	Doubtful profile	−	−	+	−	−	+	+	−	+	−
FR 39	*F. pulveris*	*C. malonaticus/C. sakazakii*	34634773050	Unacceptable profile	−	−	+	−	−	+	+	−	+	−
FR 41	*F. pulveris*	*C. malonaticus*	34674563051	Doubtful profile	−	−	+	−	−	+	+	−	+	−
FR 42	*F. pulveris*	*C. malonaticus*	34674563051	Doubtful profile	−	−	+	−	−	+	+	−	+	−
SI 44	*S. turicensis*	*C. malonaticus*	34676773051	Doubtful profile	−	−	+	+	−	+	+	−	+	−
SI 45	*S. turicensis*	*E. cloacae/Buttiauxella agrestis/ E. vulneris*	30474573051	Unacceptable profile	+	−	−	+	−	+	+	−	+	−
CB 03	*C. sakazakii*	*C. sakazakii/C. malonaticus*	34276767010	Very good identification of the genus	+	+	−	−	+	−	+	+	+	−
CB 51	*C. malonaticus*	*C. malonaticus*	34276763251	Acceptable identification	+	−	−	+	+	−	+	+	+	−
CB 55	*C. muytjensii*	*C. muytjensii/C. dublinensis*	34277767051	Very good identification of the genus	−	+	+	+	+	−	+	+	+	+
CB 59	*C. universalis*	*C. turicensis/C. malonaticus/ C. sakazakii*	24276767251	Excellent identification of the genus	+	+	+	+	+	−	+	+	+	−
CB 61	*C. turicensis*	*C. malonaticus*	34274767611	Doubtful profile	+	+	+	+	+	−	+	+	+	−
CB 62	*C. dublinensis*	*C. muytjensii/C. dublinensis*	34277767051	Very good identification of the genus	+	+	−	+	+	−	+	+	+	+
CB 78	*C. condimenti*	*C. muytjensii*	34277767011	Very good identification	−	+	−	+	+	−	+	+	+	+

[a]GLU, utilization of methyl-α-D-glucopyranoside; INO, utilization of inositol; DUL, utilization of dulcitol; MNT, malonate utilization test; VP, Voges-Proskauer test; MR, methyl red test; NIT, nitrate reduction test; ORN, ornithine decarboxylase test; MOT, motility; IND, indole test; +, positive test result; −, negative test result.

TABLE 2 Detection and identification of tested strains using *Cronobacter* genus- and species-specific PCR assays and *fusA* gene sequencing[c]

Strain	Genus-specific PCR targeting 16S rRNA gene[a]			Species-specific PCR targeting *rpoB* gene[b]							*Cronobacter* MLST *fusA* allele	Genus and species identification results based on *fusA* allele
	Lehner	Hassan	Keyser	Ccon	Cdub	Cmal	Cmuy	Csak	Ctur	Cuni		
FR 29	−	−	−	−	+	−	+	−	−	−	71	*F. helveticus*
FR 31	−	−	−	−	+	−	+	−	−	−	71	*F. helveticus*
FR 32	−	−	−	−	+	−	+	−	−	−	71	*F. helveticus*
FR 33	−	−	−	−	+	−	+	−	−	−	71	*F. helveticus*
FR 35	−	−	−	−	+	−	+	−	−	−	71	*F. helveticus*
FR 38	−	−	−	−	+	−	+	+	−	−	73	*F. pulveris*
FR 39	−	−	−	−	+	−	+	+	−	−	74	*F. pulveris*
FR 41	−	−	−	−	+	−	+	+	−	−	74	*F. pulveris*
FR 42	−	−	−	−	+	−	+	+	−	−	73	*F. pulveris*
SI 44	−	−	−	−	+	−	+	−	−	−	76	*S. turicensis*
SI 45	−	−	−	−	+	−	+	−	−	−	76	*S. turicensis*
CB 03	+	+	+	−	−	+	−	+	−	−	8	*C. sakazakii*
CB 51	+	+	+	−	−	+	−	−	−	−	7	*C. malonaticus*
CB 55	+	+	+	−	−	−	+	−	−	−	24	*C. muytjensii*
CB 59	+	+	+	−	−	−	−	−	−	+	19	*C. universalis*
CB 61	+	+	+	−	−	−	−	−	+	−	22	*C. turicensis*
CB 62	+	+	+	−	+	−	−	−	−	−	30	*C. dublinensis*
CB 78	+	+	−	+	−	−	−	−	−	−	86	*C. condimenti*

[a]PCR protocols according to Lehner et al. (46), Hassan et al. (44), and Keyser et al. (42).
[b]Ccon, specific for *C. condimenti*; Cdub, specific for *C. dublinensis*; Cmal, specific for *C. malonaticus*; Cmuy, specific for *C. muytjensii*; Csak, specific for *C. sakazakii*; Ctur, specific for *C. turicensis*; Cuni, specific for *C. universalis*.
[c]+, positive result; −, negative result.

seven *Cronobacter* strains in order to confirm their identity at the species level. Several differences were observed between the actual *Cronobacter* strains and the "pseudo-*Cronobacter*" strains (i.e., the ones believed to be *Franconibacter* and *Siccibacter* strains). None of the "pseudo-*Cronobacter*" strains were able to produce indol or to utilize inositol and ornithine. Furthermore, unlike the *Cronobacter* strains, they tested positive to methyl red. Apart from the *Cronobacter* strains, only *F. helveticus* tested positive to the Voges-Proskauer (VP) test. These results suggest that such biochemical tube tests can help us to reliably discriminate between *Cronobacter*, *Franconibacter*, and *Siccibacter* genera.

***Cronobacter* genus- and species-specific PCRs.** Further characterization of all strains except the unclassified ones was performed using PCR systems designed for *Cronobacter* detection; namely, three *Cronobacter* genus-specific PCR assays targeting the 16S rRNA gene and seven *Cronobacter* species-specific PCR assays targeting the *rpoB* gene were used. The representatives of the seven *Cronobacter* species were included as positive controls. The results are summarized in Table 2. All of the tested *Franconibacter* and *Siccibacter* strains gave negative results for all three genus-specific PCRs. Surprisingly, the strains belonging to *F. pulveris* produced an amplicon of the appropriate size detected by electrophoresis for the PCRs specific to *C. dublinensis*, *C. muytjensii*, and *C. sakazakii*. Equally surprisingly, the *F. helveticus* and *S. turicensis* strains produced a specific amplicon for both the *C. dublinensis*- and the *C. muytjensii*-specific PCRs.

Sequencing of the *fusA* gene. The accurate identification of all 25 strains used in this study was performed by partial sequencing of the *fusA* gene. For all of the tested strains, the *fusA* allele was sequenced and the results were compared to entries in the *Cronobacter* pubMLST database (https://pubmlst.org/), which also provides species determination in the case of the *fusA* gene (7, 25). The alleles are enumerated in Table 2: *fusA* alleles 73 (FR 38, FR 42) and 74 (FR 39, FR 41) indicate *F. pulveris*; *fusA* allele 71 (FR 29, FR 31, FR 32, FR 33, FR 35) indicates *F. helveticus*; and *fusA* allele 76 indicates *S. turicensis* (SI 44, SI 45). Using partial sequencing of the *fusA* gene (part of the *Cronobacter* MLST seven loci), the unclassified strains suspected of belonging to the genus *Franconibacter* or *Siccibacter* were identified. The results are summarized in Table 3.

TABLE 3 Identification results of the unclassified strains used for evaluation of the newly created in-house MALDI Biotyper database

Unclassified strain ID	Identification according to *fusA* allele	MS identification[a]
V 01	*F. pulveris* (*fusA* 74)	*F. pulveris*
V 02	*F. helveticus* (*fusA* 71)	*F. helveticus*
V 03	*F. pulveris* (*fusA* 73)	*F. pulveris*
V 04	*F. pulveris* (*fusA* 73)	*F. pulveris*
V 05	*F. pulveris* (*fusA* 74)	*F. pulveris*
V 06	*F. helveticus* (*fusA* 71)	*F. helveticus*
V 07	*F. helveticus* (*fusA* 71)	*F. helveticus*

[a]All identifications received a score of ≥2.3 (+++), defined as "highly probable species identification."

Among these isolates, three *fusA* alleles were detected: *fusA* allele 71 (strains V 02, V 06 and V 07), *fusA* allele 73 (strains V 03 and V 04), and *fusA* allele 74 (strains V 01 and V 05).

Identification of bacteria by the MALDI Biotyper database. We used the current Biotyper database (standard software, default settings) to identify the seven *Cronobacter* control strains. Although the Biotyper database does include only one *Siccibacter colletis* strain and no *Franconibacter* or *Siccibacter turicensis* strains, we also tested the possibility of it identifying the 9 *Franconibacter* and 2 *Siccibacter* strains as "pseudo-*Cronobacter*" strains. The mass spectra measured on 93 spots for each *Franconibacter* and *Siccibacter* strain were matched to the Biotyper database, again according to the default settings. All obtained spectra of the 11 strains were identified, but either as "probable genus identification" (low scores ranging between 1.7 and 2.0; indicated by a plus sign [+]) or as "not reliable identification" (score under 1.7; indicated by a minus sign [−]). The bacterial genus or species determined for each measurement was highly variable (Table 4). Despite the fact that some measurements misidentified *Franconibacter* and *Siccibacter* strains as *Cronobacter* strains, *F. helveticus* was primarily misidentified as *Raoultella ornithinolytica*, *F. pulveris* was misidentified as *Enterobacter radicincitans*, and the majority of the tested *Siccibacter* strains were not even identified. In addition, 12 spots of each *Cronobacter* species type strain were measured (Table 4). All of the strains were identified as the genus *Cronobacter*, with six of them having a high score, over 2.3 (+++), and only *C. condimenti* strain CB78 having a lower score,

TABLE 4 *Franconibacter*, *Siccibacter*, and *Cronobacter* strain identification by MALDI-TOF MS before application of extended MALDI Biotyper database

Strain ID[a]	Species	Most frequent identification (%)[b]	Reliability score[c]
FR 29	*F. helveticus*	Not reliable identification (37)	−
FR 31	*F. helveticus*	*Raoultella ornithinolytica* (53)	+
FR 32	*F. helveticus*	*Raoultella ornithinolytica* (22)	+
FR 33	*F. helveticus*	*Raoultella ornithinolytica* (44)	+
FR 35	*F. helveticus*	Not reliable identification (32)	−
FR 38	*F. pulveris*	*Enterobacter radicincitans* (44)	+
FR 39	*F. pulveris*	*Enterobacter radicincitans* (28)	+
FR 41	*F. pulveris*	*Enterobacter radicincitans* (26)	+
FR 42	*F. pulveris*	*Enterobacter radicincitans* (54)	+
SI 44	*S. turicensis*	Not reliable identification (29)	−
SI 45	*S. turicensis*	Not reliable identification (21)	−
CB 03	*C. sakazakii*	*C. sakazakii* (100)	+++
CB 51	*C. malonaticus*	*C. sakazakii* (100)	+++
CB 55	*C. muytjensii*	*C. sakazakii* (100)	+++
CB 59	*C. universalis*	*C. sakazakii* (100)	+++
CB 61	*C. turicensis*	*C. sakazakii* (100)	+++
CB 62	*C. dublinensis*	*C. sakazakii* (100)	+++
CB 78	*C. condimenti*	*C. sakazakii* (100)	++

[a]CB, *Cronobacter*; FR, *Franconibacter*; SI, *Siccibacter*.
[b]The percentage was determined from a total of 93.
[c]+++, score of ≥2.3, defined as "highly probable species identification"; ++, score of ≥2.0, defined as "secure genus, probable species identification"; +, score of ≥1.7, defined as "probable genus identification"; −, score of <1.7, defined as "not reliable identification."

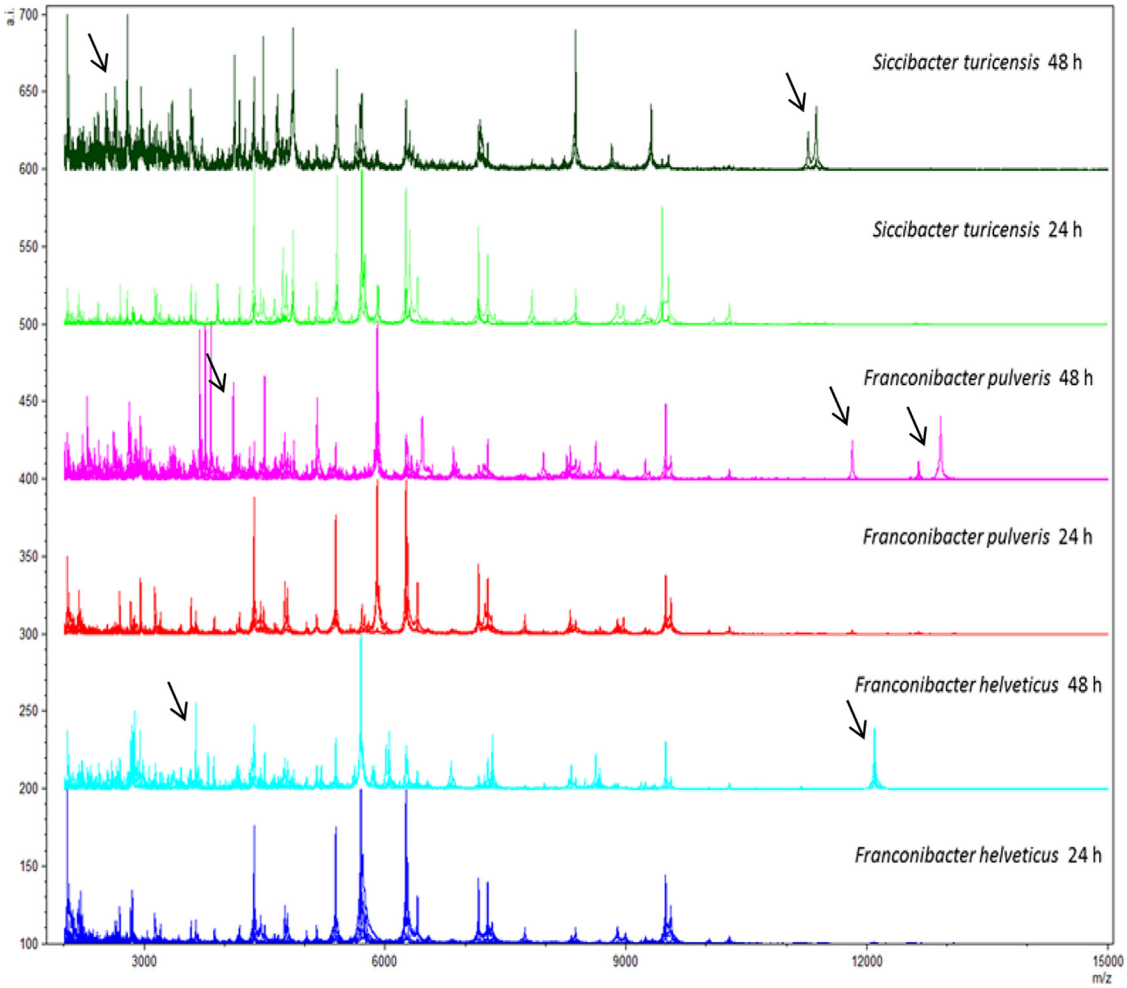

FIG 1 Comparison of 24-h and 48-h mass spectra summarized for particular *Franconibacter* and *Siccibacter* species. Mass spectral fingerprints of "pseudo-*Cronobacter*" strains are shown as summarized data using mMass software. *F. helveticus*, strains FR 29, FR 31, FR 32, FR 33, FR 35; *F. pulveris*, strains FR 38, FR 39, FR 41, FR 42; *S. turicensis*, strains SI 44, SI 45. The 48-h mass spectra show significantly more peaks, both in high- and small-peak areas (labeled with black arrows) than the 24-h ones; nevertheless, the 24-h mass spectra had a lower S/N ratio.

between 2.0 and 2.3 (++). The classification of all strains as belonging to *C. sakazakii* reflects the fact that *Cronobacter* strains included in the Biotyper database are currently unsatisfactorily all defined as *C. sakazakii*. Because of this, it is not possible to use the Biotyper database for *Cronobacter* species identification at the moment.

Creation and evaluation of an in-house MALDI database. The 11 *Franconibacter* and *Siccibacter* strains were used for the creation of an in-house MALDI database. For each strain, 93 spots were measured at the end of both the 24-h and 48-h cultivation periods. The mass fingerprints of both (Fig. 1) demonstrated important differences between the two cultivation times. We found that the 48-h mass spectra had significantly more peaks, both in high- and small-peak areas. Although this means that the 48-h mass fingerprints were enriched with desirable higher peaks, the 24-h mass spectra had a higher signal-to-noise (S/N) ratio (lower noise), thereby enabling better strain determination in half the time. Thus, we used the 24-h mass spectra for the creation of an in-house MALDI database, and only these mass fingerprints were used in the analysis described below.

The mass fingerprints of each strain were preprocessed by mMass software before undergoing principal-component analysis using the R project for statistical computing. For each strain, we selected 20 mass spectra on the basis of their variability. We searched for the most similar fingerprints representative enough to subsequently

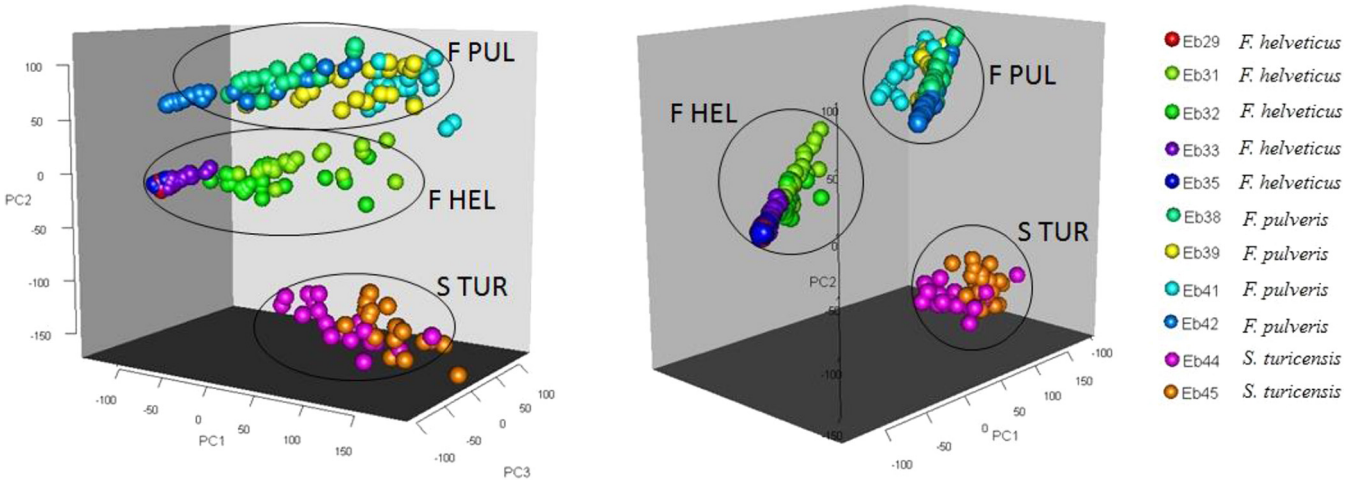

FIG 2 Three-dimensional principal-component analysis plots demonstrating variation between *F. helveticus* (F HEL), *F. pulveris* (F PUL), and *S. turicensis* (S TUR) strains. The 3D plots of the first three principal components (PC1 versus PC2 versus PC3) were generated in the R project for statistical computing using the rgl package (the 3D Real-Time Visualization Device System for R). Twenty mass spectrum fingerprints of each *Franconibacter* and *Siccibacter* strain chosen for creation of an in-house MALDI Biotyper database were separated into 3 individual clusters representing each species.

obtain the best possible MSPs in the MALDI Biotyper software. The final combined analysis of the 220 selected spectra (11 strains) is shown in Fig. 2 as a three-dimensional (3D) plot. This visualization clearly reveals three main groups within all 11 strains corresponding to species level identification.

MALDI Biotyper software was then used to create MSPs for each strain from the 20 mass spectra. To obtain better-quality MSPs, we modified the default software settings (lower bound, 2,000; desired peak frequency minimum, 50%). The final MSP for each tested strain was used to create a dendrogram that demonstrates the relationships between the particular strains (Fig. 3). Again, the strains were clustered in three main groups, with genus determination clearly shown at a distance level of 700 to 800 and species determination at 400 to 500.

Our in-house MALDI database was then combined with the current MALDI Biotyper database for the identification of six food isolates and one environmental isolate. As described above, we had previously identified all of these strains by sequencing the *fusA* gene as part of *Cronobacter* MLST. The summarized data are listed in Table 3. All of the tested strains were correctly identified with a high score of ≥2.3 (+++) using our combined data, thus extending the MALDI Biotyper database. This score enables secure strain detection at the species level. The strains were identified as follows: V 01, V 03, V 04, and V 05 as *F. pulveris* and V 02, V 06, and V 07 as *F. helveticus* (Table 3). We analyzed additional strains of genus *Cronobacter* and other related genera to exclude false-positive results. We tested 109 strains in total (38 *C. sakazakii*, 7 *C. malonaticus*, 6 *C. turicensis*, 2 *C. muytjensii*, 12 *C. dublinesis*, 3 *C. universalis*, 1 *C. condimenti*, 1 *S. colletis*, 5 *E. cloacae*, 5 *Enterobacter aerogenes*, 2 *Enterobacter ludwigii*, 4 *Enterobacter kobei*, 5 *Enterobacter asburiae*, 2 *Escherichia coli*, 5 *Klebsiella pneumoniae*, 1 *Klebsiella oxytoca*, 1 *Hafnia alvei*, 1 *Pantoea septica*, 4 *Pantoea agglomerans*, 3 *Citrobacter freundii*, and 1 *M. morganii* strains), and no false-positive identification results occurred (data not shown).

DISCUSSION

Bacteria of the genera *Franconibacter* and *Siccibacter* are close relatives of those of the pathogenic genus *Cronobacter*, so close that they can easily be misidentified. Despite the fact that they were reclassified into new genera in 2014, we refer to them here as "pseudo-*Cronobacter*" because of their checkered classification history. *Cronobacter* bacteria can cause serious infections, particularly among premature neonates and the immunocompromised elderly (26, 27). When such infections have been associated with *Cronobacter*, contaminated powdered infant formula (PIF) or the contamination of utensils used in PIF preparation have generally been implicated (28). Con-

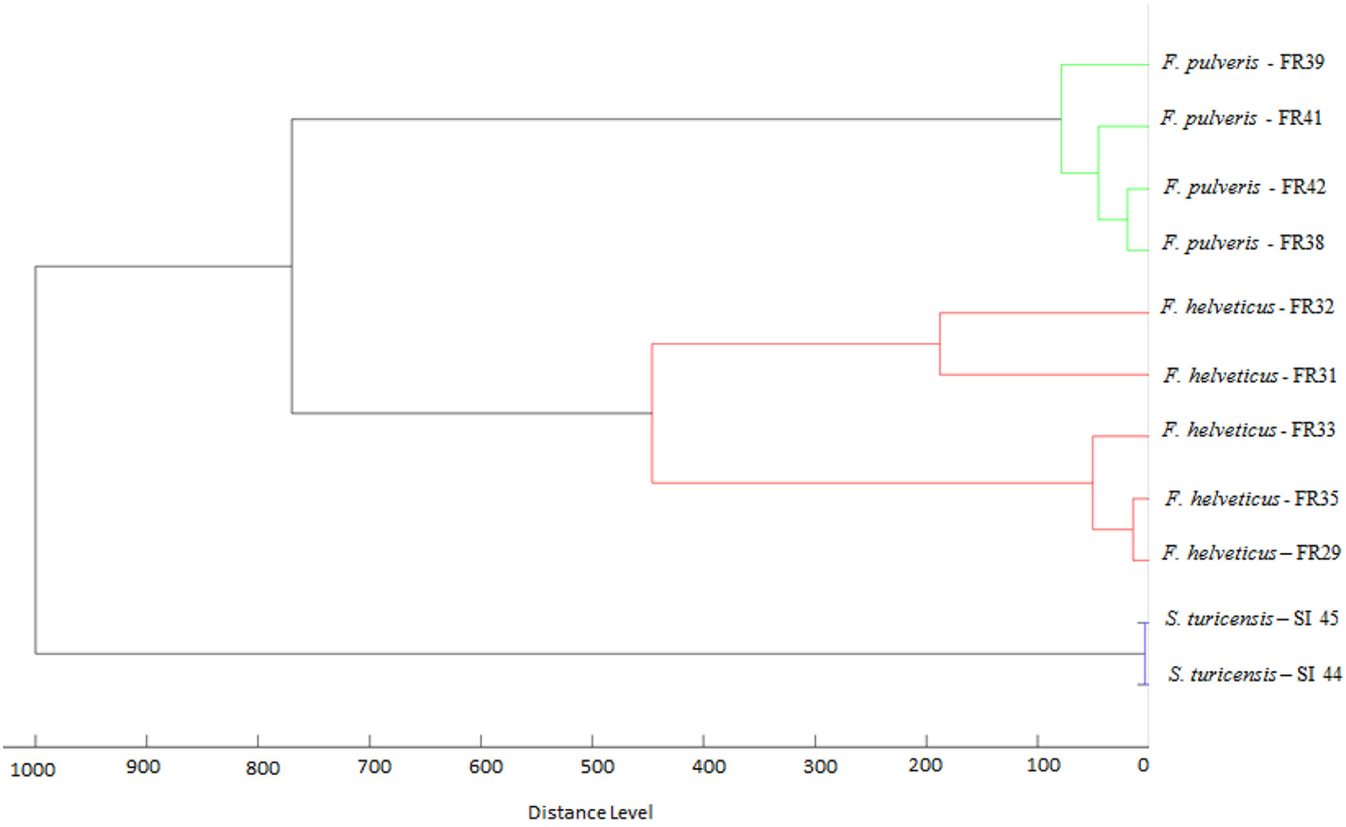

FIG 3 Dendrogram created in the Biotyper software of 11 MSPs of *Franconibacter* and *Siccibacter* strains used in this study. The MSPs are clearly separated into 2 main clusters representing 2 individual genera. Subsequently, the *Franconibacter* MSPs are further divided into 2 branches, which represent strain identification at the species level.

sequently, the incidence of *Cronobacter* in both PIF and follow-up formula (FUF) is carefully monitored. Perhaps surprisingly, thus far there has been no evidence of such infections being caused by *Franconibacter* or *Siccibacter* strains, but these have been isolated mainly from fruit powders, spices, and herbs rather than from powdered infant formula. However, in the absence of separate *Franconibacter* and *Siccibacter* databases, the *Cronobacter* pubMLST database now contains 29 *Franconibacter* and 11 *Siccibacter* strains, 2 of which (both *F. helveticus* strains) were isolated from FUF and 1 of which (*F. pulveris*) was isolated from PIF. As yet, there have been no reports of *Franconibacter* or *Siccibacter* isolates from clinical samples in the *Cronobacter* pubMLST database, but this may be because no study has focused on the incidence of *Franconibacter* and *Siccibacter* in clinical samples or on their virulence properties.

Methods for the identification of *Cronobacter* isolated from PIF are still primarily based either on biochemical tests (ISO 22964:2006) or on a combination of biochemical tests and real-time PCR (U.S. Food and Drug Administration). For biochemical identification, the Rapid ID32E or Vitek 2 GN systems are generally recommended. Although the *Cronobacter* genus is currently included in these identification systems, *Franconibacter* and *Siccibacter* genera are not part of their strain collections.

Together with the fact that *Franconibacter* and *Siccibacter* bacteria have biochemical properties very similar to those of *Cronobacter* bacteria, this all means that it is quite possible that they could be misidentified in the analysis of food or powdered formula samples. In this study, similarly to Jackson et al. (29), we too have shown that ID32E misidentifies *Franconibacter* and *Siccibacter* strains, predominantly as *Cronobacter* strains. However, we have also shown that the use of certain biochemical tube tests enables a "pseudo-*Cronobacter*" strain misidentified by ID32E to be either differentiated from an actual *Cronobacter* strain or identified as an actual *Franconibacter* or *Siccibacter* strain. All of the above data support Jackson's view that these tests should be replaced

by a more reliable identification technique, such as PCR or another molecular biology method (29).

In our study, we tested the use of several *Cronobacter* PCR techniques for the identification of both *Cronobacter* and "pseudo-*Cronobacter*" strains. As expected, all three *Cronobacter*-genus-specific PCRs targeting 16S rRNA were negative when testing *Franconibacter* and *Siccibacter* strains for the PCR amplicon. On the other hand, when the same strains were tested using seven *Cronobacter* species-specific PCRs targeting the *rpoB* gene, a nonspecific amplicon was obtained in the case of the *C. dublinensis*-, *C. muytjensii*-, and *C. sakazakii*-specific PCRs. Thus, both genus- and species-specific PCRs should be performed before the determination of *Cronobacter* species.

MLST enables deeper strain characterization because of the partial sequencing of multiple loci rather than a single locus. The *Cronobacter* MLST uses seven genes (20), of which the *fusA* gene, because it reflects the whole-genome phylogeny, independently enables proper species identification (7, 30, 31). Thus, with the "pseudo-*Cronobacter*" strains still included in the *Cronobacter* pubMLST database, partial sequencing of the *fusA* gene can be applied for *Franconibacter* and *Siccibacter* species identification. The current numbers of the *fusA* alleles deposited in the pubMLST database for particular species are as follows: alleles 71 and 72 for *F. helveticus*; alleles 73, 74, and 94 for *F. pulveris*; alleles 76 and 91 for *S. turicensis*; alleles 70 and 97 for *S. colletis*. While *fusA* gene sequencing currently seems to be the only technique that reliably identifies *Franconibacter* and *Siccibacter* species, it must be noted that the use of MLST is still expensive and time-consuming, even for the partial sequencing of just one gene, in comparison to MS-based methods.

The protein profiling of whole bacteria by MALDI-TOF MS is another possible identification method. This method enables rapid and cost-effective bacterial identification based on a comparison of bacterial mass spectra with reference fingerprints containing the signals of the most abundant bacterial components, such as ribosomal or DNA-binding proteins (32, 33). The main advantages of intact-cell MALDI-TOF MS are the simple and rapid analysis of very high numbers of samples and the ability to group strains without knowledge of their taxonomic positions (34).

To date, only two studies of the MALDI-TOF MS of intact *Cronobacter* cells have been published. Each study was performed using a different evaluation system. Using the SARAMIS SuperSpectrum tool, Stephan et al. (35) identified six of the seven current *Cronobacter* species. In contrast, Karamonova et al. used MALDI-TOF MS and the Biotyper identification system to characterize *C. sakazakii* biogroups (36). While MALDI Biotyper is undoubtedly the most commonly used system for bacterial identification by MALDI-TOF MS, the bacterial entries in the current version include only 9 *Cronobacter strains*, all of which are named *C. sakazakii* (*C. sakazakii* is used as an unsatisfactory synonym for the genus *Cronobacter*). The confusion in the designations *C. sakazakii*, *E. sakazakii*, and *Cronobacter* species has been pointed out in the literature (37). No *Franconibacter* or *Siccibacter turicensis* strains are currently included. Only one strain of *Siccibacter colletis* is presented in the database. However, as we have shown, this system does enable the creation of a so-called in-house database through the generation of MSPs from the mass spectra of the desired strains. To establish a new intact-cell MALDI-TOF MS database, it is crucial to correctly determine the cultivation conditions (38, 39) and to thoroughly check the obtained MSPs (40, 41). MSP reliability is influenced by two main factors: (i) the quality of the input data and (ii) the preprocessing and MSP creation settings in Biotyper itself.

In Materials and Methods, we provide simple instructions for MSP creation and the development of an in-house database for the identification of *Franconibacter* and *Siccibacter*. In terms of input data, the key is to select consistent fingerprints with a satisfactory number of peaks. Because this process becomes complicated when handling large numbers of fingerprints from multiple measurements, we implemented two steps: (i) the data were preprocessed using mMass software, and (ii) principal-component analysis was carried out using the R project for statistical computing in order to select the 20 mass fingerprints most suitable for MSP creation. Then, with only

TABLE 5 Bacterial strains used in this study

Strain ID[a]	Organism name	Reference strain	Source, country
CB 03	*C. sakazakii*	ATCC 29544	Human, USA
CB 51	*C. malonaticus*	LMG 23826	Human, USA
CB 55	*C. muytjensii*	ATCC 51329	Unknown, USA
CB 59	*C. universalis*	NCTC 9529	Environment, UK
CB 61	*C. turicensis*	LMG 23827	Human, Switzerland
CB 62	*C. dublinensis*	LMG 23823	Environment, Ireland
CB 78	*C. condimenti*	LMG 26250	Food, Slovakia
FR 29	*F. helveticus* (earlier, *C. helveticus*)		Environment, unknown
FR 31	*F. helveticus* (earlier, *C. helveticus*)		Food, Switzerland
FR 32	*F. helveticus* (earlier, *C. helveticus*)		Food, Germany
FR 33	*F. helveticus* (earlier, *C. helveticus*)		Food, unknown
FR 35	*F. helveticus* (earlier, *C. helveticus*)		Food, Switzerland
FR 38	*F. pulveris* (earlier, *C. pulveris*)		Food, Switzerland
FR 39	*F. pulveris* (earlier, *C. pulveris*)		Food, unknown
FR 41	*F. pulveris* (earlier, *C. pulveris*)		Food, USA
FR 42	*F. pulveris* (earlier, *C. pulveris*)		Food, unknown
SI 44	*S. turicensis* (earlier, *C. zurichensis*)		Food, unknown
SI 45	*S. turicensis* (earlier, *C. zurichensis*)		Food, unknown
V 01	*F. pulveris*		Environment, Portugal
V 02	*F. helveticus*		Food, India
V 03	*F. pulveris*		Food, unknown
V 04	*F. pulveris*		Food, unknown
V 05	*F. pulveris*		Food, unknown
V 06	*F. helveticus*		Food, Czech Republic
V 07	*F. helveticus*		Food, Czech Republic

[a]CB, *Cronobacter* strains; FR, *Franconibacter* strains; SI, *Siccibacter* strains; V, unclassified strains used for validation of the MALDI in-house database: species of these strains were determined in this study using the sequencing of the *fusA* gene.

a minor modification to Biotyper's own preprocessing method, it was possible to create MSPs of significantly higher quality. During this work, we also analyzed the effect of cultivation time on mass spectrum quality. Despite the occurrence of seemingly helpful higher mass peaks after 48 h of cultivation, we used the 24-h cultivation fingerprints because of the reduced noise and the speed of analysis.

Ultimately, we have created an in-house MALDI database containing the MSPs of 11 *Franconibacter* and *Siccibacter* strains and combined it with the current MALDI Biotyper database to identify seven strains, six of which are food isolates and one of which is an environmental isolate. All of the strains were successfully identified as *F. pulveris* or *F. helveticus* species, and this was confirmed by *fusA* gene sequencing.

MATERIALS AND METHODS

Bacterial strains. A total of 25 strains from the genera *Cronobacter*, *Franconibacter*, and *Siccibacter* were used in this study: seven *Cronobacter* strains, one for each *Cronobacter* species, were used as positive controls for *Cronobacter* identification methods; nine *Franconibacter* strains and two *Siccibacter* strains ("pseudo-*Cronobacter*" strains) were tested using common *Cronobacter* identification methods and subsequently used for the development of an in-house MALDI database of *Franconibacter* and *Siccibacter* genera; and seven unclassified strains were used for the sole purpose of evaluating the newly created MALDI database. None of these strains had been fully identified at the beginning of this study, so the accurate species identification of these strains was confirmed by sequencing the *fusA* gene.

An additional 69 strains of the genus *Cronobacter* and 40 clinical isolates of other *Enterobacteriaceae* genera were used for the purpose of evaluation of the newly created MALDI database (data not shown). Some strains (CB 03 to CB 78) were purchased from the American Type Culture Collection (ATCC, Virginia, USA), the Czech Collection of Microorganisms (CCM, Brno, Czech Republic), the Laboratorium voor Microbiologie (LMG, Ghent, Belgium), and the National Collection of Type Cultures (NCTC, Salisbury, UK). Some strains (FR 29 to FR 42, SI 44, and SI 45) were kindly donated by Carol Iversen (University of Dundee), Stephen Forsythe (Nottingham Trent University), and Igor Hochel (University of Chemistry and Technology, Prague, Czech Republic). Further, two strains (V 06 and V 07) were isolated from food according to standard method ISO 22964:2006 (data not shown). See Table 5 for more details.

Biochemical tests. All tested strains were able to grow on ESIA at 42°C, exhibiting typical blue-green colonies after 24 h of incubation. For further analysis, the bacteria were cultivated on tryptic soy agar (TSA) at 37°C for 24 h. The strains were characterized by ID32E (version 4.0; bioMérieux). This test was performed according to the manufacturer's recommendations, with substrate utilization being determined after 24 h at 37°C. For the identification, Api web (version 1.3.0; bioMérieux) was used.

In addition to the phenotypes derived from the commercial test kit and for the purpose of identifying *Cronobacter* at the species level, 10 additional biochemical tube tests (motility; malonate, methyl-α-D-glucopyranoside, inositol, and dulcitol utilization; production of indol; methyl red test; Voges-Proskauer test; nitrate reduction to nitrite; ornithine decarboxylation) were performed according to the method described by Karamonova et al. (36). Moreover, to distinguish between *C. turicensis* and *C. universalis*, putrescine and turanose utilization tests were incorporated into the individual tube tests (21).

***Cronobacter* genus- and species-specific PCRs.** Each extraction of bacterial DNA for use in the molecular biology tests was performed using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) with the Gram-negative bacterial protocol. Three PCRs targeting different parts of the16S rRNA gene were used to identify the *Cronobacter* genus (42–44). Furthermore, the species within *Cronobacter* were identified by PCR in accordance with the protocols published by Stoop et al. (45) and Lehner et al. (46), both of which target the *rpoB* gene.

Sequencing of the *fusA* gene. Accurate species identification of the strains was confirmed by the partial sequencing of elongation factor G (*fusA*) as part of the *Cronobacter* MLST scheme. This was carried out according to the method of Joseph et al. (19).

Intact-cell MALDI-TOF MS. All bacterial strains were streaked onto tryptic soy agar and incubated at 37°C for 24 h or 48 h. Each strain was smeared onto an MTP 384 polished-steel target plate (Bruker Daltonics) in three independent spots, and the spots were allowed to dry at room temperature. The samples were overlaid with 1 μl of freshly prepared matrix solution (saturated α-cyano-4-hydroxycinnamic acid [HCCA] in 50% acetonitrile and 2.5% trifluoroacetic acid). Each spot was air dried within minutes at room temperature. MS analysis was performed using an Autoflex Speed MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). External calibration was carried out using the Bruker bacterial test standard (Bruker Daltonics). The mass spectra were automatically collected by Flex Control software (version 3.4; Bruker Daltonics) in linear positive mode at an accelerated voltage of 19 kV for the range of 2,000 to 20,000 Da.

Identification of bacteria by the Biotyper database. The mass spectra obtained for the bacteria incubated for 24 h were initially identified using Biotyper database (version 3.1; Bruker Daltonics), which contains 6,903 entries, including 566 entries for *Enterobacteriaceae*. The MALDI Biotyper MSP identification standard method was used. The identification was classified into four groups: (i) "highly probable species identification," score of ≥2.3 (+++); (ii) "secure genus, probable species identification," score of ≥2.0 (++); (iii) "probable genus identification," score of ≥1.7 (+); and (iv) "not reliable identification," score of <1.7 (−).

Creation and evaluation of in-house MALDI database. Nine *Franconibacter* strains and two *Siccibacter* strains were chosen for the extension of the Biotyper database. For each strain, 93 spots were prepared by the smear method (described above) at both cultivation times (24 h and 48 h) and measured. The obtained spectra were processed in mMass software (version 5.5.0) (47, 48) as follows: the mass spectra were cropped (from 2,000 to 15,000 Da), baseline correction was performed with a relative offset of 100 and a precision of 20, the mass spectra were smoothed according to the Savitzky-Golay algorithm (window size, 0.3 *m/z* in two cycles), the peaks were automatically picked using a signal-to-noise (S/N) ratio of 3.0, and the mass spectra were normalized. Then, the 10 highest peaks for each strain were used for internal calibration of the relevant mass spectra.

From each set of 93 processed mass spectra, only the 20 most suitable were chosen to extend the Biotyper database. For reliable spectrum selection, principal-component analysis (PCA) was carried out using the R project for statistical computing version 3.3.1 (https://www.R-project.org/). This statistical method is widely used to illustrate variability among data sets. PCA also makes data easy to explore and visualize (49). For graphic visualization and plotting, the rgl (https://r-forge.r-project.org/projects/rgl/) and ggplot2 (http://ggplot2.org) packages were used. Individual spectrum processing and peak extraction were performed using ms-alone, a stand-alone command line utility, according to its user guide (50). The data subsequently underwent principal-component analysis using the multiMS-toolbox script in the R software, also according to the user guide (50).

Next, the 20 most similar and abundant spectra of each strain were used to create MSPs. To obtain high-quality MSPs, two changes were implemented from the normal procedure: (i) the MALDI Biotyper Preprocessing Standard Method was modified by setting the lower bound to 2,000 instead of the normal 3,000, and (ii) the MALDI Biotyper MSP Creation Standard Method was used with a minor modification; namely, the desired peak frequency minimum was increased from 25% to 50%. For each strain, one MSP for the 24-h cultivation time was created and added to the database.

To evaluate the functioning of our thus-established database, we used the seven unclassified strains believed to be *Franconibacter* or *Siccibacter* strains and 109 nontarget strains. These isolates were analyzed by the smear method after cultivation on TSA for only 24 h. Then, the MALDI Biotyper preprocessing and the MALDI Biotyper MSP identification standard methods were used to identify them from the Biotyper database, which, as described above, had already been extended by the MSPs for the initial 9 *Franconibacter* and 2 *Siccibacter* strains.

The "pseudo-*Cronobacter*" MSPs are available for free downloads at the website of the Laboratory of Bioaffinity Techniques at the Department of Biochemistry and Microbiology, UCT Prague (http://biomikro.vscht.cz/pseudocronobacter/).

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This work made use of the PubMLST website (http://pubmlst.org/cronobacter/) developed by Keith Jolley (25) and situated at the University of Oxford. The MLST scheme was developed by Adam Baldwin (20) (Warwick University, United Kingdom) in close collaboration with Stephen Forsythe (Nottingham Trent University, United Kingdom). The development of this site has been funded by the Wellcome Trust.

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