J. Dairy Sci. 97:632–641
http://dx.doi.org/10.3168/jds.2013-7147

Comparison of methods for the microbiological identification and typing of *Cronobacter* species in infant formula

Y. Lu,*† Y. Chen,‡ X. A. Lu,‡ J. Lv,‡ C. X. Man,† Y. L. Chai,* and Y. J. Jiang*†§[1]
*Key Laboratory of Dairy Science, Ministry of Education, Department of Food Science, Northeast Agricultural University, Harbin 150030, China
†National Dairy Engineering and Technical Research Center, Harbin 150086, China
‡Chinese Academy of Inspection and Quarantine, Beijing 100123, China
§Synergetic Innovation Center of Food Safety and Nutrition, Harbin 150030, China

ABSTRACT

Cronobacter species represent an emerging opportunistic foodborne pathogen associated with meningitis and necrotizing enterocolitis in infants. Current evidence indicates that powdered infant formula (PIF) is the main source of *Cronobacter* contamination. A total of 75 strains of *Cronobacter* spp. from different geographic regions, as well as from PIF processing environments, were identified and typed with different methods, including biochemical profiling by the API 20E system (bioMérieux, Marcy l'Etoile, France), protein profiling by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and genotypic profiling by ribotype. Analysis by MALDI-TOF MS and biochemical identification was more accurate compared with ribotype analysis. However, MALDI-TOF MS typing and ribotype analysis showed more discriminatory ability compared with biochemical phenotyping. In conclusion, MALDI-TOF MS is a rapid and reliable tool to identify *Cronobacter* spp. in PIF and has the potential to trace dissemination of *Chronobacter* along the production chain.
Key words: *Cronobacter* species, powdered infant formula, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), ribotype

INTRODUCTION

Cronobacter is a newly emerging, gram-negative, motile, non-spore-forming, and peritrichous rod-shaped opportunistic foodborne pathogen (Lai, 2001). The organism can be detected in various types of foods [powdered infant formula (**PIF**), dairy milk, cheese products, meat, and vegetables), food production environments, and households (Iversen and Forsythe, 2004; Forsythe, 2005; Gurtler et al., 2005). *Cronobacter* infections in PIF for neonates and infants have received attention, with sporadic and outbreak cases reported globally (Block et al., 2002; Himelright et al., 2002). Infection with *Cronobacter* is associated with meningitis and necrotizing enterocolitis in infants (Caubilla-Barron et al., 2007; Hartmann et al., 2010).Current evidence indicates that PIF is the main medium of *Cronobacter* spp. dissemination (van Acker et al., 2001; Javůrková et al., 2012). To date, there are no reports of *Cronobacter* spp. infecting infants in China.

The definition of the *Cronobacter* genus was revised in 2012. The species *Enterobacter sakazakii* was formally replaced with 7 species of the *Cronobacter* genus: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter turicensis*, *Cronobacter dublinensis*, *Cronobacter universalis*, and *Cronobacter condimenti* (Iversen et al., 2008; Joseph et al., 2012). The isolates examined in this study were all *C. sakazakii* strains.

For this study, 75 *C. sakazakii* strains were isolated from samples deposited in the National Dairy Testing Center (Harbin, China) from 2009 to 2012 and from a wet-mixing PIF-manufacturing facility over a 10-mo period, including samples from raw materials to final products in the factory environment (Mullane et al., 2007; Table 1). Several microbiologic identification methods have been used to identify *Cronobacter* spp. isolates (Caubilla-Barron et al., 2007). The isolates in the current study were identified using the API20E system (bioMérieux, Marcy l'Etoile, France), and classification was based on different biochemical characters. The present study evaluated the application of matrix-assisted laser desorption ionization-time of flight mass spectrometry (**MALDI-TOF MS**) for identification and protein profiling of *C. sakazakii* strains (Karamonová et al., 2013). With the rapid development of bacterial analysis by MALDI-TOF MS, through single-component and multi-component determination in the group, the identification and classification of bacteria was performed on the basis of the acquired mass spectra matched to the MALDI-TOF database (Lay, 2000). Barbuddhe et al. (2008) were able to separate

Received June 15, 2013.
Accepted October 6, 2013.
[1]Corresponding author: yujun_jiang@163.com

Table 1. Analyzed bacterial strains and isolates from the commercially available powdered infant formulas and raw materials and their production facilities in China

Bacterial strain	No. of strains	Strain(s) or source
Cronobacter spp.	5	ATCC[1] 29544 ATCC 51329 ATCC 12868 ATCC 29004 ATCC BAA-894
ES60, ES65, ES66	3	Environment
ES36, ES62, ES63, ES68, ES74, ES79	6	Powder lumps[2]
ES5, ES33, ES58, ES59	4	Raw material
ES30, ES31, ES32, ES61, ES63, ES64, ES68, ES70, ES71, ES72, ES73, ES75, ES76, ES77, ES78	15	Final products
ES11, ES12, ES13, ES14, ES15, ES16, ES17, ES18, ES19, ES20, ES22, ES23	12	Milk powders
ES1, ES2, ES3, ES4, ES8, ES9, ES10, ES21, ES24, ES25, ES26, ES27, ES28, ES29, ES34, ES35, ES41, ES43, ES44, ES47, ES48, ES49, ES50, ES51, ES52, ES53, ES54, ES55, ES56, ES69	30	Powdered infant formula

[1]American Type Culture Collection (Manassas, VA).

[2]The residual powder at end of the processing, gathered together for *Cronobacter* detection.

Listeria monocytogenes isolates to the subtype level. In their study, the pulsed-field gel electrophoresis lineages were in complete agreement with the MALDI-based groupings. The RiboPrinter Microbial Characterization System (DuPont/Qualicon Inc., Wilmington, DE) has also been used to analyze specific genetic fingerprints and to type *C. sakazakii* strains (Gaston, 1988; Poilane et al., 1993).

The objective of this study was to identify and type *Cronobacter* isolates from PIF and related production environment. Several different methods, including biochemical identification, mass spectrometry, and ribosome analysis, were used to identify and characterize these strains and the results compared. The advantages and disadvantages of these methods and the relationship between them were analyzed to find a more powerful method to reduce *Cronobacter* contamination during dairy production and to trace the contamination source along the processing chain.

MATERIALS AND METHODS

Detection and Isolation of Bacterial Strains

Cronobacter spp. isolates in this study were detected based on GB4789.40–2010 (Ministry of Health of the People's Republic of China, 2010), the national food safety standard method for food microbiological examination used in China. *Cronobacter sakazakii* strains were isolated from commercially available PIF and their production factories in China, and none was associated with confirmed clinical cases. Specifically, *C. sakazakii* was isolated in PIF production facilities in powder samples obtained from the drying tower, raw materials for PIF production, final PIF products, waste powder lumps in the dryer, swabs taken outside the facility drying tower, an operator's hands, carriers' wheels, vacuum, ground leakage, powder miller, bag-filling platform, and packing materials in the factory environment. The strains used are summarized in Table 1.

Reference Strains

The biochemical characteristics of *C. sakazakii* strain ATCC 29544 were used as the standard biotype to standardize the sample preparation protocol for MALDI-TOF MS analysis. *Cronobacter muytjensii* strain ATCC 51329 was used as a reference strain to ensure reproducibility between ribotype experiments.

Bacterial Growth

To standardize the analysis protocol, several experimental parameters, including culture medium and culture time (Smole et al., 2002), were evaluated. Nutrient agar, tryptic soy agar (**TSA**), violet red bile agar, and *C. sakazakii* chromogenic medium were tested to determine the influence of different medium on strain identification (Lynn et al., 1999; Madonna et al., 2000). Extended culture periods of *C. sakazakii* on agar at 36°C for 18, 24, and 48 h were evaluated to select the optimal culture time.

Biochemical Identification

The API20E system (bioMérieux), consisting of a strip of 20 individual, miniaturized plastic test tubes (cupules) and each containing different reagents, was used according to the manufacturer's instructions. This system determines the metabolic capabilities and allows for the identification of the genus and species of enteric bacteria in the family *Enterobacteriaceae*. Appropriate positive and negative controls were included.