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ORIGINAL ARTICLE

Contamination and antibiotic resistance profile of *Cronobacter sakazakii* isolated from raw milk and infant formula

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ABSTRACT

Background and Objectives: *Cronobacter sakazakii* is an opportunistic pathogen associated with severe fatal infections. This study aimed to investigate the contamination of raw milk delivered to a powdered milk factory and the produced powdered milk, with *C. sakazakii*, and also the antibiotic resistance patterns of the isolates.

Materials and Methods: During 2024, 120 raw milk and 60 powdered milk samples were collected from one of the milk powder processing plants in Shahrekord city. A peptone water medium supplemented with nutrients, followed by Cronobacter Selective Broth, was used as the enrichment medium. Chromogenic Cronobacter Isolation (CCI) agar was used to isolate the suspicious colonies. Biochemical tests were performed on the isolates. The PCR test was performed to confirm the molecular identity of the isolates. The antibiogram test was performed using the disk diffusion method on Mueller-Hinton agar.

Results: A total of 14 suspected *C. sakazakii* colonies were isolated from the raw milk. However, the PCR test confirmed that only 2 isolates (1.67%) were *C. sakazakii*. Also, the results revealed that none of the powdered milk samples were contaminated with *C. sakazakii*. The antibiogram test showed that the isolated *C. sakazakii* were resistant to erythromycin, ampicillin, amoxicillin, cephalexin, and tetracycline antibiotics.

Conclusion: According to the results, some raw milk is contaminated with *C. sakazakii*, which is resistant to certain common antibiotics. However, contamination with this bacterium was not observed in the powdered milk samples. Given the importance of *C. sakazakii* in infant health, further studies should be conducted on other powdered milk and infant food supplements produced in Iran to ensure their safety.

Keywords: Raw milk; Antibiotic resistance; *Cronobacter sakazakii*; Infant formula

INTRODUCTION

Foodborne infections are among the most important diseases at different ages (1). Infants are more susceptible to such infections due to their immature immune systems and intestinal microflora (2).

Cronobacter sakazakii, formerly known as *Enterobacter sakazakii*, is a Gram-negative bacterium within the *Enterobacteriaceae* family found in the environment, especially in the digestive tract of humans and animals. Five species of the *Cronobacter* genus, including *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, and *C. dublinensis*, were identified. *C. sakazakii* grows in a temperature range of 5 to 47°C. Moreover, it can survive refrigeration (below 4°C). *C. sakazakii* colonies on CCI agar are

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small (1–3 mm) and blue-green (3, 4). *C. sakazakii* tolerates pH (range of 4.5 to 10) and heat up to 47°C, and low water activity (aw) (5). In numerous studies, *C. sakazakii* has been isolated from various sources, including blood, sputum, urine, wounds, water, soil, sewage, and food, such as milk (6-8).

C. sakazakii is an opportunistic pathogen that is associated with severe, fatal diseases such as meningitis, necrotizing enterocolitis, and septicemia, especially in neonates and immunocompromised individuals (5). Other complications of infection with this bacterium include multiple cases of pneumonia, abscesses, urinary tract infections, diarrhea, ulcers, and conjunctivitis in adults, the elderly, and infants (9-11). The first report of infection with *C. sakazakii* was published in 1961, involving two fatal cases of meningitis in a term infant and a premature infant. Since then, several outbreaks of *C. sakazakii* infection have been reported (8). Recently, the CDC reported 2-4 severe *Cronobacter* infections in infants annually in the USA (12).

Formula milk is a major source of *C. sakazakii* for neonatal infections, and contamination of the raw milk with this bacterium is one of the main sources of contamination of milk powder. Most often, the infection occurs in neonatal intensive care units in hospitals. Storing prepared formula at room temperature for long periods increases the risk of infection with *C. sakazakii* (13, 10).

Studies have shown that the gastric pH of infants is higher than that of adults, which allows *C. sakazakii* to survive. The incidence of infection with this bacterium is generally low, but the mortality rate of sick infants has been reported to be as high as 80% (14). For this reason, the International Commission on Microbiological Specifications for Foods has classified *C. sakazakii* as a serious risk to limited populations, including infants or patients with significant or life-threatening chronic conditions (15). *C. sakazakii* is widespread in the environment and often affects sensitive population groups, such as infants, children, and immunocompromised individuals (16).

Numerous studies in Iran and other countries have indicated that infection with this bacterium is one of the most significant issues in pediatric care units (10, 13, 17, 18).

Raw milk is exposed to various contaminants, including pathogens, either initially or after milking, and must be treated to eliminate all pathogenic microorganisms (19, 20). Today, infant formula is widely used as a supplement or substitute for breast milk. The heat applied during its production is not high enough to eliminate all microorganisms, but rather reduces the microbial load of the formula (7). Accordingly, for the production of infant formula, choosing raw milk with a low microbial load alone is not sufficient, and the type of microorganisms present in the milk's microbial flora and their heat resistance must also be considered (21).

Since a few comprehensive studies have been conducted on the contamination of raw milk and infant formula with *C. sakazakii*, this study aimed to investigate the level of contamination and antibiotic resistance of this bacterium in raw milk and milk powders produced in Chaharmahal va Bakhtiari province.

MATERIALS AND METHODS

Sample collection. 120 samples of raw milk delivered to the Pegah factory (Shahrekord, Iran) from bulk tanks, and 60 cans of powdered milk during February 2024 to January 2025 were collected randomly to isolate *C. sakazakii*.

Isolation and identification of *C. sakazakii*. Under sterile conditions, 10 mL of raw milk or 10 g of powdered milk were added to an Erlenmeyer flask containing 100 mL of sterilized buffered peptone water and incubated for 24 hours at 37°C in aerobic conditions. Subsequently, they were subcultured into the Cronobacter Selective Broth (CSB) and incubated at 41.5°C for 24 h. Streak plating was performed on the Chromogenic Cronobacter Isolation (CCI) agar and incubated at 42°C for 24 h. Five suspicious colonies of C. sakazakii (green-blue, 1-3 mm in size) were selected (4), and biochemical tests, including oxidase, Methyl red, Voges-Proskauer, catalase, nitrate reduction, as well as Gram staining, were performed on suspicious colonies to identify the *C. sakazakii* (3, 22).

Molecular confirmation. For molecular confirmation of the suspected *C. sakazakii* colonies, PCR method was performed using specific primers for the 16S rRNA gene 832 bp (F: CCC GCATCT CTG CAG GAT TCT C) and (R: CTA ATA CCG CAT AAC GTC TAC G), GenBank (http://www.ncbi.nlm.nih.gov/), Accession number

AB004746 (13).

For DNA extraction, the boiling method was used. For this purpose, 2 mL of a 24-hour bacterial culture in TSB medium was poured into a microtube and placed in a 100°C water bath for 10 minutes. The microtubes were then centrifuged at 1200 rpm for 1 minute. The supernatant containing DNA was collected and used in the PCR test.

The PCR test was performed in a total volume of 25 µL, containing 13 µL of master mix (Cinna-Gen-Iran) containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1 U of Taq DNA polymerase, 2 µL of each primer, 1 µL of DNA template, and 9 µL of sterile distilled water. Thermal cycles of the PCR test were performed within a thermocycler (Bio-Rad, USA) (Table 1).

Gel electrophoresis was performed using 1% agarose gel containing safe red at 94 V for 40 min in TAE buffer, and then the bands were visualized using a gel dock (9).

Antibiogram test. Bacteria suspension was prepared at 0.5 McFarland concentration and swabbed onto the surface of Mueller-Hinton agar (Merck, Germany) using the antibiotic disks (erythromycin 15 mcg, gentamicin 10 mcg, chloramphenicol 30 mcg, ampicillin 10 mcg, cephalexin 30 mcg, neomycin 30 mcg, amoxicillin 25 mcg, tetracycline 30 mcg, ciprofloxacin 5 mcg, and sulfamethoxazole- trimethoprim 25 µg), (PadtanTeb-Iran) and incubated for 18-24 hours at 37°C. The diameter of the growth inhibition zone was measured using a digital caliper and compared with the CLSI reference (23).

Data analysis. The results obtained from microbial and molecular tests were described in percentages prepared using SigmaStat 4 statistical software.

RESULTS

Of the 120 raw milk samples, 14 colonies suspected of being *C. sakazakii* were isolated based on biochemical tests (Table 2 and Fig. 1). However, no colonies suspected of this bacterium were isolated from any of the powdered milk samples.

PCR results. The PCR test revealed that only two isolates among the suspected isolates were detected as *C. sakazakii*. Therefore, the contamination rate of this bacterium among all samples was 1.68% (Table 3 and Fig. 2).

Antibiogram results. Antibiogram test results showed that both *C. sakazakii* isolates were sensitive to gentamicin, ciprofloxacin, and sulfamethoxazole-trimethoprim, and resistant to erythromycin, ampicillin, amoxicillin, cephalexin, and tetracycline. The *C. sakazakii* isolates were semi-sensitive to chloramphenicol and neomycin (Table 4 and Fig. 3).

DISCUSSION

Raw milk could be contaminated with various microorganisms, including *C. sakazakii*, during the production process, and this contamination may be transferred to other dairy products, such as milk powder. In the present study, the contamination of raw milk delivered to the Pegah powdered milk factory with *C. sakazakii* was investigated using culture and PCR methods. The results showed that 2 (1.68%) samples of raw milk were contaminated with *C. sakazakii*. But none of the milk powder samples were contaminated with this bacterium.

Few studies have been conducted in Iran on the contamination of milk and its powder with *C. sakazakii*. For example, Abbasi Bafitrot et al. (2016) in Isfahan reported that 3 out of 100 baby food samples (3%) and 5 out of 100 infant powdered milk samples (5%) were contaminated with *C. sakazakii* (7).

In other countries, studies have been conducted to determine the contamination of raw milk and milk powder with *C. sakazakii*. However, the results indicate that the contamination rate is not high. Yu et al. (2017) in China conducted a study on raw milk, peanut milk with walnuts, and wheat milk, as well as 1165 milk product samples. The results of the study

Table 1. Thermal cycles of the PCR test for identification 16srRNA gene of *C. sakazakii*

Initial Denaturation	Denaturation Cycle	Annealing T	Extension	Final Extension
94°C	94°C	58°C	72°C	72°C
4 min	60 sec	60 sec	60 sec	6 min

Table 2. Results of biochemical tests on suspected *C. sakazakii* colonies

Suspected colonies	Biochemical tests				
Blue-green colonies on CCI medium	Catalase	Oxidase	Nitrate reduction	MR	VP
	+	-	+	-	+
	14	14	14	14	14

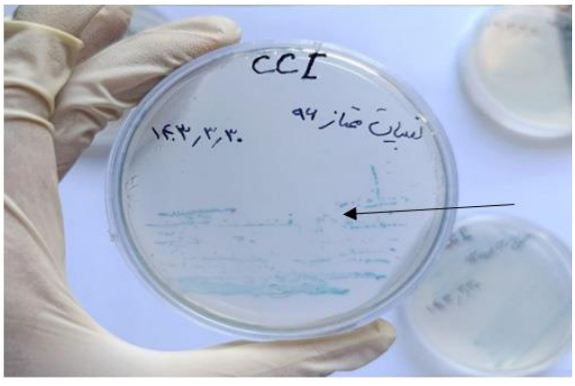

Fig. 1. Blue-green colonies suspected of being *C. sakazakii* bacteria on CCI medium

Table 3. Microbial and molecular results for identification and confirmation of *C. sakazakii* isolated from raw milk and milk powder

Sample Type	Number of samples	Suspicious colonies	Positive (PCR)
Raw milk	120	14	2 (1.68%)
Milk powder	60	0	0

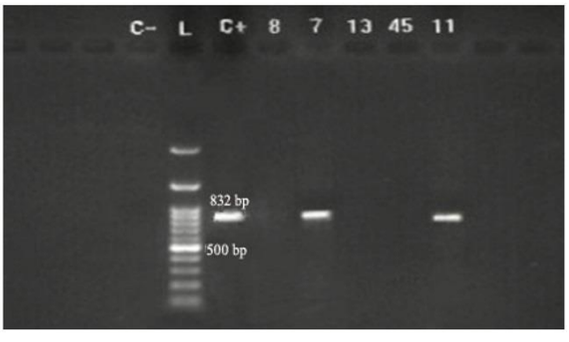

Fig. 2. Identification of *C. sakazakii* by PCR test
L: Leader (100bp). C+: Positive control of *C. sakazakii* (832bp). C-: Negative control (distilled water). Samples 7 and 11: Positive

Table 4. The results of the antibiogram test on *C. sakazakii* isolates

Antibiotics	Sensitive	Semi-sensitive	Resistant
Erythromycin (15 mcg)			✓
Gentamycin (10 mcg)	✓		
Chloramphenicol (30 mcg)		✓	
Ampicillin (10 mcg)			✓
Cephalexin (30 mcg)			✓
Neomycin (30 mcg)		✓	
Amoxicillin (25 mcg)			✓
Tetracycline (30 mcg)			✓
Ciprofloxacin (5 mcg)	✓		
Trimethoprim -Sulfamethoxazole (25 µg)	✓		

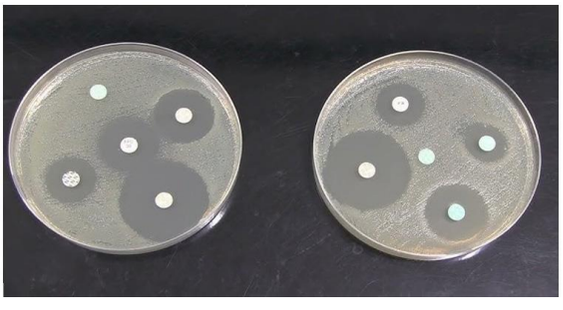

Fig. 3. The result of the antibiogram test of isolated *C. sakazakii* from raw milk

showed that 2 samples of infant formula were contaminated with *C. sakazakii*, and 9 cfu/mL of viable *C. sakazakii* were detected after 7 h of enrichment in spiked pure milk, walnut peanut milk, and whole-wheat milk (24). Hu et al. (2022) in China showed that 13% of raw milk samples were contaminated with *C. sakazakii* (15). Tutar et al. (2018) also analyzed 25 raw milk and 20 cheese samples in Turkey using the Multiplex RT-PCR method. *C. sakazakii* was not isolated from the raw milk samples, but 4 out of 20 cheese samples were contaminated with this bacterium (25).

Kim HR et al. (2020) conducted a study on infant formula in South Korea and isolated *Cronobacter* species, including *C. sakazakii*, from 4 infant formula samples (16). Also, Holy et al. (2020) conducted a study on the 1450 formula samples in Mexico, of which 6 were contaminated with *C. sakazakii* (9). Stevens et al. (2023) in Switzerland isolated 26 strains of *C. sakazakii* by examining formula milk over 15 years (26). Shakeir et al. (2015) conducted a study on formula milk and stool samples from infants with severe diarrhea, and swab samples from hospital equipment and environments (floors, walls, etc.). The results showed that 15% of environmental samples, 16% of infant formula samples, and 10% of infant stool samples were contaminated with *C. sakazakii* (18). In another study by ElGamal et al. (2013) in Egypt, 140 samples of milk products were examined for contamination with *C. sakazakii*. The results showed that 30% of cheese, 4% of ice cream, 4% of yogurt, and 24% of infant formula samples were contaminated with *C. sakazakii* (27).

The results of the present study showed that both isolates of *C. saccasakii* were resistant to erythromycin, ampicillin, amoxicillin, cephalexin, and tetracycline. Other studies also indicate the presence of antibiotic resistance in *C. saccasakii* isolates. Abbasi Bafitrot et al. (2016) reported resistance to amoxicillin, ampicillin, tetracycline, aztreonam, and ceftazidime in isolates of *C. saccasakii* from infant formula, which is consistent with the results of the present study (7). Also, Pakbin et al. (2022) in the Netherlands showed that 6.8% of infant formula was contaminated with *C. saccasakii*, and all of these isolates were resistant to amoxicillin, clavulanic acid, ampicillin, cefoxitin, azithromycin, ceftriaxone, and ciprofloxacin (10).

Also, Shakeir et al. (2015) in Iraq showed that 100% of *C. sakazakii* isolates from formula milk, infant feces, and hospital environments were resistant to ampicillin, ceftazidime, cephalothin, and cefotaxime, and 75% were resistant to amoxicillin (18). Wang et al. (2016) in China showed that the *C. sakazakii* isolates from formula milk were resistant to cefoxitin, chloramphenicol, and ceftriaxone, which is consistent with the results of the present study (6).

Given the circumstances, consumers are increasingly inclined to consume raw milk outside of its industrial processing cycle (pasteurized or sterilized), and in some cases, due to the shortage of powdered milk or its high cost, they may use raw milk that has not been properly sterilized for children. Therefore, regulatory or public health organizations such as the Food and Drug Administration and the Centers for Disease Control and Prevention have expressed significant concerns about the risk of milk-borne diseases if raw milk is contaminated with pathogens (28). *C. sakazakii* is a bacterium that does not cause systemic infection or mastitis in livestock, so contamination of raw milk with this organism is often due to fecal contamination or contact with contaminated water, the environment, or people's hands. Additionally, failure to observe hygiene principles, such as water hygiene, personal hygiene, and environmental control during the production process of milk products including powdered milk can lead to contamination with various pathogens (29). In this regard, advice and guidance are provided to reduce the risk of infection in infants from the consumption of powdered infant formula (PIF) (30).

CONCLUSION

Based on the results of the present study, some raw milk is contaminated with the *C. sakazakii*, which is resistant to certain common antibiotics. Although contamination of powdered milk with this bacterium was not observed in the present study, given its importance in infant health, further studies should be conducted on other powdered milk and baby food supplements produced in Iran to ensure their safety.

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