ORIGINAL ARTICLE

Occurrence of total coliforms, *Escherichia coli* and *Cronobacter* species in commercially available 20 l bottled drinking water sold in Rio de Janeiro State, Brazil

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Significance and Impact of the Study: *Cronobacter malonaticus* ST440 was isolated from 20 l bottled drinking natural mineral waters sold in markets in Rio de Janeiro State, Brazil, and can be a potential threat to human health, particularly for neonates. Thirteen lots (39·4%) were unsatisfactory for human consumption due to the presence of total coliforms and/or *Escherichia coli*.

Abstract

Cronobacter infections of infants are commonly regarded as due to the ingestion of contaminated feed. The aim of this study was to determine the occurrence of *Cronobacter*, total coliforms and *Escherichia coli* in different brands of natural mineral waters as sold in 20 l returnable bottles in Rio de Janeiro, Brazil. The quantification of total coliforms and *E. coli* was performed by Most Probable Number. The detection of *Cronobacter* was as according to the ISO 22964:2017 and *Bacteriological Analytical Manual/FDA*. Molecular characterization of *Cronobacter* isolates was performed by real-time PCR and by multi-locus sequence typing. The antibiotic susceptibility profile was determined and biofilm production was evaluated in polystyrene microplates. Total coliforms and *E. coli* were detected in 13 (39·4%) and 2 (6·1%) of the 33 lots analysed respectively, and were considered unsatisfactory for human consumption according to Brazilian law. One (3·0%) lot showed contamination by *C. malonaticus* ST440 (Cronobacter MLST Databases accession no. ID 2646). The strain was susceptible to all (*n* = 13) antibiotics tested and only formed a weak biofilm. Since there is a high consumption of natural mineral waters by elderly and immunosuppressed persons, epidemiological surveillance agencies should be aware of the risk that these waters may represent for these groups.

Introduction

The *Cronobacter* genus belongs to the family Enterobacteriaceae and is composed of seven species. These species can be grouped according to their clinical relevance: Group 1 comprises *C. sakazakii* and *C. malonaticus*, which form the majority of clinical isolates in all age groups, and Group 2 comprises *C. turicensis* and *C. universalis*, which have been rarely reported clinically. The remaining three species (*C. dublinensis, C. muytjensii* and *C. condimenti*) are primarily environmental commensals and are probably of little or no clinical significance (Forsythe, 2018).

The main concern of *Cronobacter* has been life-threatening infection of neonates (FAO/WHO, 2006). Such infections may cause bacteraemia and necrotizing enterocolitis, and meningitis with life-threatening neurological sequelae (Forsythe, 2018). However *Cronobacter* infections occur in all age groups, with a greater incidence in adults (Patrick *et al.* 2014). In adults, *Cronobacter* have been associated

with bacteraemia, cholecystitis, pneumonia and urinary tract infections (Patrick *et al.* 2014). Recently, an outbreak of acute gastroenteritis due to *C. sakazakii* amongst students was reported in China (Yong *et al.* 2018).

In Brazil, *Cronobacter* infections are not subject to compulsory notification to the governmental epidemiological surveillance system. However, food-borne outbreaks are considered a "public health event" and their notification is compulsory (Brasil, 2016). No *Cronobacter* food-borne outbreaks had been reported in Brazil before 2017. However, some non-attributable clinical cases of infections were published between 1997 and 2017, with a higher occurrence in neonates (Umeda *et al.* 2017; Chaves *et al.* 2018).

The main source of *Cronobacter* infections of neonates has been identified as contaminated reconstituted powdered infant formula (PIF) (FAO/WHO, 2006). This may be intrinsically contaminated during manufacture, or extrinsically contaminated during reconstitution. *Cronobacter* isolated from water sold for the reconstitution of PIF was indistinguishable from the cerebrospinal fluid isolate from a case of neonatal *Cronobacter* meningitis (Hariri *et al.* 2013). In addition, cases of infections in neonates fed exclusively with breast milk have been reported. Together these demonstrate that sources of *Cronobacter* infection exist other than PIF which has received the most attention (Bowen *et al.* 2017).

The source(s) of *Cronobacter* infections in age groups other than infants is unknown, but may include food and water as the organism is both food- and water-borne (Forsythe, 2018). *Cronobacter* can be isolated from a wide range of food sources, especially vegetables and seasonings. However, few studies have considered its occurrence in drinking water (Lu *et al.* 2013; Hariri *et al.* 2013).

Cronobacter has been isolated from different categories of retail foods in Brazil (Brandao *et al.* 2017; Vasconcellos *et al.* 2018), but no research has been undertaken on drinking water. Ayaz *et al.* (2017) evaluated the cleaning practices and contamination status of contents and teats of feeding bottles used by children admitted in a hospitals in Pakistan and observed that 5·6% of the mothers/caretakers use natural mineral waters to prepare feeding bottle for children. It is notable therefore that previously studies in Brazil reported that 50–72% of samples of natural mineral waters sold in 20 l bottles were unsatisfactory for human consumption due to microbiological contamination, including the presence of indicators for faecal contamination (Brandao *et al.* 2012; Pedrosa *et al.* 2014).

According to Brazilian Ministry of Health data from 2000 to 2017, drinking water was implicated as contamination vehicle in 770 (6·16%) outbreaks from a total of 12 503; and *E. coli* was one of the most frequent aetiologic agent identified (Brasil, 2018).

Given that *Cronobacter* can cause infections in adults, the microbiological evaluation of drinking water is important as it is a potential route for colonization and transmission (Liu *et al.* 2013). Due to the high consumption of natural mineral waters sold in 20 l returnable bottles by the adult population and previous studies have reported their contamination by bacterial indicators of faecal contamination (Brandao *et al.* 2012; Pedrosa *et al.* 2014), monitoring the quality of these products is very important.

Considering the absence of data for the occurrence of *Cronobacter* in natural mineral waters, the current study aimed to determine the occurrence of *Cronobacter* species, as well as total coliforms and *Escherichia coli* from these products, and to characterize any *Cronobacter* isolates using both phenotypic and molecular assays.

Results and discussion

Total coliforms and *E. coli* were detected in 16 (16·2%) and 2 (2·0%) of the 99 individual samples analysed respectively (Table 1). These 16 (16·2%) individual samples are considered unsatisfactory for human consumption according to the Brazilian RDC no. 275/2005 which requires the absence of total coliforms and *E. coli* per 100 ml drinking natural mineral water (Brasil, 2005). Furthermore, considering each representative sample, from the 33 lots analysed, 13 (39·4%) lots were considered unsatisfactory according to Brazilian law (Table 2). The number of unsatisfactory lots (39·4%) was much higher when the samples were evaluated individually (16·2%). These results emphasize the importance of collecting multiple samples from the same lot for analysis, since the contamination is not homogeneous. This can be attributed to the use of different returnable 20 l bottles in the same lot of natural mineral water, or intermittent contamination, such as during packaging, sealing or other points in the production line (Brandao *et al.* 2012; Pedrosa *et al.* 2014). Coliforms can originate from unhygienic practices or from the environment, and *E. coli* is considered one of the most suitable indicators of faecal contamination (Codex Alimentarius Commission 2011). Coliforms which can occur naturally in soil, water and vegetation, indicate possible contamination from airborne sources or from product contact surfaces that have not been effectively disinfected (Codex Alimentarius Commission 2011). As coliforms are normally not present in natural mineral water sources, their presence are considered as an indicator of contamination of the water at source or during the packaging process (Codex Alimentarius Commission 2011).

Of the 15 brands analysed, nine (60·0%) gave an unsatisfactory result for at least one of the lots analysed.

Table 1 Detection of coliforms, *Escherichia coli* and *Cronobacter* in samples of commercially available drinking waters

Sample identifier	No. samples (%)	Microorganism		
		Total coliforms (MPN per 100 ml)	*Escherichia coli* (MPN per 100 ml)	*Cronobacter* (per 100 ml)
A-1-1, A-1-2, A-1-3, A-2-1, A-2-2, A-2-3, A-3-1, A-3-2, A-3-3, B-1-1, B-1-2, B-1-3, B-2-1, B-2-2, B-2-3, B-3-1, B-3-2, B-3-3, C-1-1, C-1-2, C-2-1, C-2-2, C-2-3, D-1-2, D-2-2, D-3-3, E-1-1, E-1-2, E-1-3, E-2-1, E-2-2, E-2-3, E-3-1, E-3-2, E-3-3, F-1-1, F-1-2, F-1-3, G-1-1, G-1-2, G-1-3, H-1-2, H-1-3, I-1-1, I-1-2, I-1-3, I-2-1, I-2-2, J-1-1, J-1-2, J-1-3, J-2-1, J-2-2, J-3-1, J-3-2, K-1-1, K-1-2, L-1-1, L-1-2, L-1-3, M-1-1, M-1-3, M-2-1, M-2-2, M-3-1, M-3-2, M-3-3, N-1-1, N-1-2, N-2-1, N-2-2, N-2-3, N-3-1, N-3-2, N-3-3, O-1-1, O-1-2, O-2-1, O-2-2, O-2-3, O-3-1, O-3-2, O-3-3	83 (83·8)	<1·1	<1·1	Absent
C-1-3	1 (1·0)	2·2	<1·1	Absent
D-1-1	1 (1·0)	>23	>23	Absent
D-1-3	1 (1·0)	16	12	Absent
D-2-1, D-2-3, H-1-1, I-2-3, J-3-3, K-1-3, M-1-2	7 (7·1)	1·1	<1·1	Absent
D-3-1, D-3-2, J-2-3, N-1-3, O-1-3	5 (5·1)	>23	<1·1	Absent
M-2-3	1 (1·0)	>23	<1·1	Present
Total (n = 99)		16 (16·2%)	2 (2·0%)	1 (1·0%)

These results were similar of those reported by Pedrosa *et al.* (2014) who analysed 33 brands and observed that 57·6% gave unsatisfactory results. These results indicate that these products continue to represent risk for human consumption and agrees with earlier Brazilian Ministry of Health data (Brasil 2018).According to Pedrosa *et al.* (2014), the water marketed in returnable bottles should be more closely controlled, especially in terms of its microbiological characteristics. Studies performed in water marketed in not returnable bottles in others countries revealed low occurrence or absence of bacterial contamination (Caskey *et al.* 2018; Totaro *et al.* 2018).

Only the sample M-2-3 showed contamination by *C. malonaticus*. This sample had a high presence of total coliforms but *E. coli* was not detected (Table 1). As *Cronobacter* belongs to total coliforms group, positive detection by the total coliforms procedure was expected. However, this group is environmentally very heterogeneous and not necessarily faecal, and therefore gives no indication of the source of contamination (Forsythe, 2018). Liu *et al.* (2013) reported that total coliforms can be used as indicator of *Cronobacter* contamination in drinking water, with a correct positive rate of 96%. However due to the single occurrence of *Cronobacter* in the 99 samples analysed, no statistical correlation between the presence of fecal indicators and *Cronobacter* can be made. In addition, neither *Cronobacter*, total coliforms nor *E. coli* were recovered from samples M-2-1 and M-2-2 which were from the same lot as sample M-2-3, indicating that the contamination was not

homogeneous. Other authors have reported a higher occurrence of *Cronobacter* in water samples for human consumption (Liu *et al.* 2013; Fei *et al.* 2018) but no research specifically on mineral waters could be found in the literature. Liu *et al.* (2013) analysed 248 samples of drinking water from municipal water supply on premises and small community water supply for premises in China and detected *Cronobacter* in 32 (12·9%) of them. Fei *et al.* (2018) isolated *Cronobacter* from nine (9·0%) of 100 samples of drinking water. In the present study, *Cronobacter* was isolated in one (3·0%) of the 33 lots analysed. The lower occurrence obtained in the present study can be attributed to the type of water analysed, and the differences in the methodologies used, since there is no reference method for screening *Cronobacter* species in drink water samples. Liu *et al.* (2013) used a membrane filter method combining a pre-incubation of the filter membranes on TSA before incubation on Druggan-Forsythe-Iversen (DFI) agar. Fei *et al.* (2018) performed a pre-enrichment in BPW followed by a selective enrichment into modified lauryl sulphate tryptose broth with vancomycin, a medium which is no longer recommend by the ISO standard methodology (Anonymous, 2017). In the present study, the BPW pre-enrichment was concentrated by centrifugation as described by Chen *et al.* (2012) before the selective enrichment into CSB/v and isolation on CCI agar.

The results of the real time PCR assay (Ct = 25·3) agreed with the results of the Vitek 2.0 (Identification as "C.sak group Bionumber 0625734153622210) and MLST identified the isolate as *C. malonaticus* ST 440

Table 2 Occurrence of total coliforms, *Escherichia coli* and *Cronobacter* species in different brands of drinking waters and their evaluation according to Brazilian law (RDC no. 275/2005)

Sample identifier	Samples that did not meet the RDC no. 275/2005 (Micro-organisms found)
A-1-1, A-1-2, A-1-3	0
A-2-1, A-2-2, A-2-3	0
A-3-1, A-3-2, A-3-3	0
B-1-1, B-1-2, B-1-3	0
B-2-1, B-2-2, B-2-3	0
B-3-1, B-3-2, B-3-3	0
C-1-1, C-1-2, C-1-3	C-1-3 (total coliforms)
C-2-1, C-2-2, C-2-3	0
D-1-1, D-1-2, D-1-3	D-1-1 and D-1-3 (total coliforms and *E. coli*)
D-2-1, D-2-2, D-2-3	D-2-1 and D-2-3 (total coliforms)
D-3-1, D-3-2, D-3-3	D-3-1 and D-3-2 (total coliforms)
E-1-1, E-1-2, E-1-3	0
E-2-1, E-2-2, E-2-3	0
E-3-1, E-3-2, E-3-3	0
F-1-1, F-1-2, F-1-3	0
G-1-1, G-1-2, G-1-3	0
H-1-1, H-1-2, H-1-3	H-1-1 (total coliforms)
I-1-1, I-1-2, I-1-3	0
I-2-1, I-2-2, I-2-3	I-2-3 (total coliforms)
J-1-1, J-1-2, J-1-3	0
J-2-1, J-2-2, J-2-3	J-2-3 (total coliforms)
J-3-1, J-3-2, J-3-3	J-3-3 (total coliforms)
K-1-1, K-1-2, K-1-3	K-1-3 (total coliforms)
L-1-1, L-1-2, L-1-3	0
M-1-1, M-1-2, M-1-3	M-1-2 (total coliforms)
M-2-1, M-2-2, M-2-3	M-2-3 (total coliforms)*
M-3-1, M-3-2, M-3-3	0
N-1-1, N-1-2, N-1-3	N-1-3 (total coliforms)
N-2-1, N-2-2, N-2-3	0
N-3-1, N-3-2, N-3-3	0
O-1-1, O-1-2, O-1-3	O-1-3 (total coliforms)
O-2-1, O-2-2, O-2-3	0
O-3-1, O-3-2, O-3-3	0
Total of brands (*n* = 15)/Total of lots (*n* = 33)	Total of unsatisfactory lots (%) *n* = 13 (39·4%)

*Sample was contaminated by *Cronobacter malonaticus* ST440.

(Cronobacter MLST Databases accession no. ID 2646). The species *C. malonaticus* is more prevalent in adult infections (Forsythe, 2018) although it has also been associated with serious neonatal infections (Hariri *et al.* 2013; Umeda *et al.* 2017). In Brazil, *C. malonaticus* ST440 strains have already been isolated from two cases of infections in neonates (Umeda *et al.* 2017). The isolation of *C. malonaticus* strains in sequence types ST7, 159, 160, 169, 172 and 408 from drinking water have already been reported in China (Liu *et al.* 2013; Fei *et al.* 2018). Fei *et al.* (2018) also reported the isolation of *C. sakazakii* ST 4 strains, a clonal lineage associated with neonatal meningitis (Joseph and Forsythe 2011). Additionally, Hariri *et al.* (2013) reported the isolation of *C. sakazakii* ST111 from water sold for PIF reconstitution which had been used to feed a neonate who had *C. sakazakii* meningitis. These findings highlight the potential health hazard from *Cronobacter* strains in water.

The use of hot water at a temperature of 70°C in the dilution of PIF is recommend in order to prevent the *Cronobacter* contamination and growth (FAO/WHO, 2006). However, some parents believe that mineral waters are safe products and did not follow this recommendation (Ayaz *et al.* 2017). Hence, despite the low occurrence of *Cronobacter*, as found in the present study, the use of these natural mineral waters without the correct thermal treatment to reconstitute PIF can represent a risk which should be minimized.

The *C. malonaticus* ST440 strain was susceptible to the 13 antibiotics tested. This result was similar to those obtained by other authors who reported that *Cronobacter* strains isolated from food and water samples were susceptible to almost all the antibiotics tested (Brandao *et al.* 2017; Fei *et al.* 2018; Vasconcellos *et al.* 2018).

Biofilms enable the survival and persistence of bacteria in different environments (Iversen and Forsythe 2004). As biofilm formation is associated with the contamination of natural mineral waters as sold in returnable 20 l bottles (Pedrosa *et al.* 2014), the capacity of biofilm formation by the *Cronobacter* isolate was evaluated. In this study, according to the parameters used, the *C. malonaticus* ST440 strain produced a weak biofilm on polystyrene plates. These results were similar to the reported by Umeda *et al.* (2017) that evaluated 10 *C. malonaticus* strains from six different ST, including two ST440 clinical isolates, and also observed that these strains only formed weak biofilms on polystyrene plates.

In conclusion, 13 lots (39·4%) of 20 l volumes of natural mineral water sold in markets in Rio de Janeiro State, Brazil, were unsatisfactory for human consumption due to the presence of total coliforms and/or *E. coli*. Since these natural mineral waters are commonly consumed by elderly and immunosuppressed persons, epidemiological surveillance agencies should be aware of the risk that these waters may represent for these groups. Of particular interest was the recovery of *C. malonaticus* ST 440. This pathovar is already associated with neonatal infections. Hence, the use of these waters to reconstitute PIF without an efficient thermic treatment can represent a risk to neonates and may also be a direct source of adult infections.

Materials and methods

Water samples

A total of 33 representative samples (three from each lot, totalizing 99 individual samples and 15 brands) of natural mineral waters, non-carbonated, sold in returnable 20 l bottles, were collected at gas stations and commercial establishments located in Rio de Janeiro State, Brazil, from October 2017 to August 2018. The samples were codified adding the letter of the brand, number of the lot and then the replicate of the lot (i.e.: A-1-1). The samples were transported individually to the laboratory and stored at ambient temperature until analysis.

The overall aim was to analyse three samples from three different lots of each brand of water, giving a total of nine samples per brand over an 11 months time period. However, over the collection period such sampling was not always possible due to limited sample availability. Consequently, the number of samples and lots for each brand varied. Nine samples were obtained for brands A, B, D, E, J, M, N and O, six samples from brands C and I, and three samples for brands F, G, H, K and L.

Quantification of total coliforms and E. coli

The Most Probable Number (MPN) of total coliforms and *E. coli* analysis was performed on 100 ml water samples using COLITAG™ enzyme substrate (CPI International, California City, CA) according to manufacturer's instructions.

Isolation of Cronobacter species

The isolation of *Cronobacter* was based in the procedures described by the ISO 22964:2017 (Anonymous 2017) and the *Bacteriological Analytical Manual* (Chen *et al.* 2012). *C. sakazakii* ATCC 29544 (INCQS 00578) and *Escherichia coli* ATCC 25922 (INCQS0033) were used as positive and negative controls respectively.

One hundred millilitres of water were added to 900 ml of buffered peptone water (BPW; Merck, Darmstadt, Germany) and incubated at 37°C for 18 h. The enrichment was mixed and one aliquot of 40 ml was centrifuged at 3000*g*/10 min (Eppendorf 5810R, A-4-81, Germany).The supernatant was dispensed and the pellet was suspended in 0·2 ml of phosphate buffered saline (PBS) pH 7·4. A 0·1 ml aliquot was inoculated into 10 ml of *Cronobacter* Screening Broth supplemented with vancomycin 10 μg ml^{-1} (CSB/v; Oxoid, CM1121, Basingstoke, Hampshire, England). The sample was incubated at 41·5°C for 24 h. The CSB/v enrichments were streaked (10 μl) onto the surface of the Chromogenic *Cronobacter* Isolation Agar (CCI; Oxoid, CM1122, Basingstoke, Hampshire, UK) and the plates were incubated at 41·5 1°C for 24 2 h. Typical colonies isolated from CCI were then transferred to Trypticase Soy Agar (TSA; Merck, Darmstadt, Germany) and incubated at 36°C for 24 2 h. After incubation, the colonies were submitted for presumptive phenotypical identification by semi-automatized Vitek 2.0 system using GN TEST KIT VTK2 (GRAM NEG) (bioMérieux, Durham, NC) and real time PCR targeting the *dnaG* gene (Chen *et al.* 2012).The real time PCR reactions were carried out in a total volume of 25 μl containing 12·5 μl of TaqMan® Universal Master Mix (Applied Biosystems®, California City, CA), 2·0 μl of DNA template (20-60 ng μl^{-1}), 10·0 pmol of each primer (Invitrogen, Carlsbad, CA) and 0·3 μmol l^{-1} of the probe (Applied Biosystems®). Reactions were carried out on ABI 7500 Real-Time PCR System (Applied Biosystems®).

Multi-locus sequence typing (MLST) and sequence analysis

DNA samples of presumptive *Cronobacter* isolates were extracted from overnight cultures in BHI broth using Dneasy Blood & Tissue kit (Qiagen®, Valencia, CA) in accordance with the manufacturer's instructions. The DNA concentration was measured using NanoDrop 2000c Spectrophotometer (Thermo Scientific, California City, CA).

The MLST gene set was amplified using primers and PCR conditions according to protocol available at MLST *Cronobacter* database (http://pubmlst.org/cronobacter/info/protocol.shtml; Baldwin *et al.* 2009, last accessed date: 10/14/2019). The PCR reactions, amplification product purification and sequencing reactions were as described by Vaconcellos *et al.* (2018). The nucleotide sequences of each gene were trimmed to the appropriate length and then searched for in the MLST database. Finally, allele numbers were assigned and the sequence type (ST) were determined by tools available at MLST *Cronobacter* database (Cronobacter MLST Databases accession no. ID 2646).

Phenotypic characterization of Cronobacter isolates

Antimicrobial susceptibility

The antimicrobial susceptibility profile of isolates was determined using the standardized Bauer-Kirby agar disc diffusion method using Mueller-Hinton agar (Oxoid, CM0337, Basingstoke, Hampshire, England) following the instructions of the Clinical Laboratory Standards Institute (CLSI, 2018). The following antimicrobials (BIO-RAD Laboratories Inc, Boulevad Raymond, France) were tested ($n = 13$): ampicillin (10 μg), ampicillin-sulbactam

(10 : 10 μg), amoxicillin-clavulanic acid (20:10 μg), ceftriaxone (30 μg), tetracycline (30 μg), ciprofloxacin (5 μg), trimethoprim-sulfamethoxazol (1·25:23·75 μg), piperacillin-tazobactam(100/10 μg), meropenem (10 μg), gentamicin (10 μg), nalidixic acid (30 μg), aztreonam (30 μg) and nitrofurantoin (300 μg). *E. coli* ATCC 25922 (INCQS0033) was used as the quality control organism.

Biofilm formation assay

The biofilm formation assay was performed in 96-well polystyrene plates on brain heart infusion broth (BHI; Merck, Darmstadt, Germany) and sterile natural mineral water (Nestlé, Brazil, autoclaved at 121°C/15 min) for 48 h in the temperatures of 25 and 36°C, according to the procedure described by Umeda *et al.* (2017).

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest.

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