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Prevalence and Characterization of *Cronobacter* spp. from Various Foods, Medicinal Plants, and Environmental Samples

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Abstract Dairy or non-dairy based products were explored to determine the prevalence, molecular characterization, and antibiotic susceptibility of *Cronobacter* spp. The isolation was done as per ISO 22964:2006 on chromogenic media followed by further confirmation by biochemical- and 16S rRNA-based identification. From 219 samples, the chromogenic agar assay and biochemical tests yielded presumptive 45 isolates. Among them, only 36 isolates showed 282 bp band amplified from ITS-G gene confirming as *Cronobacter sakazakii*. The *Cronobacter* spp. prevalence was highest in herbs and spices (34 %) while environmental samples had contamination rates of 23 % indicating plants as a possible reservoir of this pathogen. All the isolates were resistant to β-lactam derivatives (68 %), macrolides (88.6 %), and aminoglycosides (79.9 %) but susceptible to phenicoles (31.6 %) and tetracyclines (15 %) derivatives. The results emphasize the screening of plant materials before their incorporation in food matrices.

Introduction

Cronobacter spp. is gram-negative facultative anaerobe, motile, rod-shaped, non-spore-forming pathogenic bacterium earlier known as *Enterobacter sakazakii* [16]. *Cronobacter* spp. is recognized world-wide as emerging opportunistic food-borne pathogens and includes eleven species: *Cronobacter sakazakii, Cronobacter malonaticus, Cronobacter dublinensis, Cronobacter turicensis, Cronobacter muytjensii, Cronobacter condimenti, Cronobacter universalis, Cronobacter helveticus, Cronobacter zurichenesis, Cronobacter pulveris,* and *Cronobacter colletis* [4, 11, 16, 18, 19, 25, 31]. The species *C. sakazakii* is reported to cause meningitis, bacteremia, and necrotizing enterocolitis in infants and neonates [30, 36, 39] and also in elderly or immunocompromised hosts [12, 35]. Neonates are at the utmost risk and suffer a high mortality rates ranging between 40 and 80 % [9, 22, 26].

The organism is ubiquitous being detected from a wide spectrum of food and food ingredients of animal and plant origin. Furthermore, it should be noted that fresh, ready-to-eat, frozen, fermented and cooked food products, and water used for the preparation of food have been found to be contaminated by *Cronobacter* spp. [10, 14]. The pathogen has also been reported to be inhabitant of various environmental samples such as water [10], insects [5, 13, 23], and spoon and blenders used to mix infant formula [3, 27]. Contamination of powdered infant formula (PIF) has been associated with severe systemic neonatal infections by *Cronobacter* spp. Kandhai et al. reported *Cronobacter* spp. in factories producing milk powder, chocolate, cereals, legumes, pasta and potato flour, as well as in domestic environments [20]. The human clinical samples such as cerebrospinal fluid, urine, respiratory secretions, digestive tract, and skin wounds samples are also infected by this pathogen [2, 6, 24].

Identifying foods that may contain *Cronobacter* is imperative to discover the possible ways for transmission of infection. With an indication that *Cronobacter* spp. infects both infants and vulnerable adults, it is significant that an extensive range of foods should now be evaluated. In Indian context, there has not been a far-reaching report on association of this pathogen with the different food commodities. However, single clinical case study was reported by Ray et al. which described two cases of *C. sakazakii*

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infections; one preterm very low birth weight neonate with meningitis and a 2-month infant with bacteraemia for the first time in India [33].

Therefore, the purpose of this study was to investigate the occurrence of *Cronobacter* spp. in an extensive range of foods including milk powder, infant foods, herbs, and spices, and environmental samples in an attempt to find the reservoir for this pathogen. The isolates were further confirmed by an array of biochemical, cultural, and molecular methods.

Methodology

Collection of Samples

A total of 219 samples of food, PIF, infant food formula, medicinal plants, herbs, and spices were purchased from local markets with additional environmental samples (soil, water) were also tested for the presence of *Cronobacter* spp. The standard strains used were *C. sakazakii* ATCC 12868 and E604 (kindly gifted by Dr. Davis Tall Ben, FDA, USA).

Enrichment and Isolation

A 25 g of each sample was added to 225 ml buffered peptone water (BPW) and mixed well for pre-enrichment (ISO 22964:2006). Following an overnight incubation at 37 °C, 10 ml of the pre-enrichment culture was inoculated into 90 ml of *Enterobacteriaceae* enrichment (EE) broth and incubated overnight at 37 °C. A 10 µl volume of the selective enrichment was then streaked onto a tryptone soya agar (TSA). All colonies that appeared on TSA were picked and subjected to further characterization using chromogenic media *E. sakazakii* agar (Himedia laboratories, India) containing 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside, a chromogenic substrate which upon hydrolysis of the substrates gives blue/green colonies typical for *Cronobacter* spp. The presumptive isolates were confirmed by biochemical tests.

Biochemical Differentiation of *Cronobacter* Species

The isolates were identified by biochemical tests for the differentiation of *Cronobacter* spp., including Indole production, methyl red, Voges–Proskauer, and citrate utilisation test as per the standard microbiological protocol. All the results were compared with the standard strains.

16S rRNA Target PCR

The isolates were confirmed by genus-specific and species-specific PCR. The DNA was isolated as per the method described by Sambrook et al. [34] from overnight grown culture. Isolated DNA was assessed for PCR amplification of the 16S rRNA gene and ITS region according to Jaradat et al. [17]. The PCR reaction for genus-specific was achieved by mixing 1 µl of extracted DNA with a 49 µl of PCR mixture containing the following: 1×PCR buffer, 5 U *Taq* DNA polymerase (Intron), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 1 pmol from primers P_f (5′-ACAGGGAGCCAGCTTGC TGC-3′) and P_r (5′-TCCCGCATCTCTGCAGGA-3′). PCR amplification was performed as follows: 10 min at 95 °C; 30 cycles of 30 s at 95 °C, 30 s at 56 °C, 2 min at 72 °C; 5 min at 72 °C. For the species-specific PCR, the partial nucleotide internal transcribed spacer (ITS) region was amplified using the ITS-G forward primer (5′-GGGTTGTCTGCGAAAG CGAA-3′) and ITS-G reverse primers (5′-GTCTTCGT GCTGCGAGTTTG-3′) with following reaction conditions: 10 min at 94 °C for denaturation, 30 cycles each of 30 s at 94 °C for denaturation, 1 min at 57 °C for annealing, 1 min at 72 °C for extension followed by the final extension at 72 °C for 5 min. The PCR products were then analyzed by electrophoresis in 1.5 % (w/v) agarose gel in 1X Tris–acetate-EDTA buffer with 0.5 mg/ml of ethidium bromide at a constant voltage of 80 V for 45 min, and then visualized under UV light to confirm the presence of the amplified DNA. A non-template control was included in each run. ITS sequences of our isolates and standard sequences were analyzed by drawing the phylogenetic tree (Neighbor-joining method with 1000 bootstrap replication) using MEGA 5.1 (http://www.megasoftware.net).

Antibiotic Susceptibility Test

The disk diffusion method for antimicrobial susceptibility testing as described by the National Committee for Clinical Laboratory Standards [28] was performed on TSA using commercial antibiotic disks (Himedia Laboratories, India). *Cronobacter* spp. isolates were tested against eight antibiotic derivatives which includes β-lactams (ampicilln, cephalothin, amoxyclav, and penicillin-G), macrolides (rifampicin, clindamycin, and erythromycin-G), aminoglycosides (gentamicin, amikacin, tobramycin, neomycin, kanamycin, and streptomycin), coumarin-glycoside (novobiocin), tetracyclines (tetracycline), glycopeptides (vancomycin), peptide (bacitracin and carbencillin), and phenicoles (chloramphenicol). The zone of inhibition was measured and interpreted according to NCCLS guidelines for *Enterobacteriaceae* [28].

Results and Discussion

The present study reported the isolation of *Cronobacter* spp. from a wide variety of food and environmental samples in an attempt to pinpoint their reservoir. The prevalence of

Table 1 Number of samples analyzed for presence of *Cronobacter* spp.

Origin	Sample category	No. of samples	Number of *Cronobacter* spp. isolates	% of total samples in the category	% of total isolates
Dairy products	Raw milk	7	2 (Raw milk)	8.7	4.5
	Pasteurized milk	9			
	Curd	5			
	Yoghurt	2			
Cereals and cereal products	Gram flour	4	1 (Gram flour)	11.2	2.3
	Wheat flour	3			
	Corn flour	1			
	Legume	1			
Reconstituted Infant formula and milk powder	Powdered infant formula (0–6 months)	5	2 (Opened packet)	18.2	4.4
	Follow-on formulae (6–12 months)	3			
	Growing-up milk (12–36 months)	3			
Medicinal plants	*Saraca indica, Rauwolfia serpentine, Tribulus terrestris, Plantago ovate, Amomum subulatum, Coriandrium sativum, Carum carvi, Cuminum cymirium, Cassia augustifolia, Nux vomica, Urtica dioica, Roylea cinerea, Citrus, Tylophora indica, Withania somnifera, Stevia, Trachyspermum ammi, Syzygium aromaticum, Nicotiana tabacum, Picrorhiza kurroa, Andrographis paniculata, Aconitum, Jatropha, Valeriana jatamansi, Notha podytes, Ocimum tenuiflorum, Malus domestica, Lilium, Hypericum perforatum, Orchid, Camellia sinensis*	1 each	3 (*Coriandrium sativum, Cuminum cymirium, Syzygium aromaticum*)	9.7	6.7
Herbs and spices	Cumin	4	12 (Cumin, fenugreek, coriander, black pepper, clove, chilli powder, large cardamom, ginger, turmeric and mango powder)	34.3	26.7
	Fenugreek	3			
	Coriander	3			
	Black pepper	4			
	Clove	2			
	Cinnamon	2			
	Chilli powder	3			
	Poppy seeds	1			
	Large cardamom	2			
	Nutmeg	2			
	Ginger powder	4			
	Turmeric powder	2			
	Bishop's weed	2			
	Mango powder	1			
Environmental samples	Soil	9	3	23.0	13.3
	Water	10	2		
	Vaccum dust	7	1		
Vegetables and fruits	Vegetables	12	3	26	15.4
	Fruits	15	4		
Clinical sample	Stool samples	15	2	13.3	4.5
Miscellaneous food products		42	10	23.8	22.2
Total		219	45		100

Table 2 Confirmed isolates of *Cronobacter* spp. by biochemical testing and PCR analysis

Isolate		IMViC test				PCR primers	
ID	Source	Indole	Methyl red	Vogus Proskauer	Citrate utilisation	16S rRNA	ITS-G
E604	Standard strain	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
ATCC 12868	Standard strain	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N1	Raw milk	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N2	Vegetable	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N3	Vegetable	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N4	Raw milk	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N5	Milk powder	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N6	Stool sample	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N7	*Coriandrium sativum*	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N8	*Syzygium aromaticum*	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N9	Milk powder	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N10	*Cuminum cymirium*	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N11	Stool sample	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N12	Fruit	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N13	Fruit	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N14	Turmeric powder	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N15	Soil	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N19	Legumes	–	–	+	+	–	–
N24	Market snacks	–	–	+	+	*Cronobacter* spp.	–
N27	Biscuits	–	–	+	+	–	–
N31	Citrus fruit	–	–	+	+	*Cronobacter* spp.	–
N35	Baked bread	–	–	+	+	–	–
N41	Pasteurized milk	–	–	+	+	–	–
N46	Coriander	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N48	Gram flour	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N50	Black pepper	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N51	Large cardamom	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N52	Nutmeg	–	–	+	+	–	–
N53	Cumin	–	–	+	+	–	–
N54	Clove	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N56	Mango powder	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N57	Ginger	–	–	+	+	–	–
N61	Soil	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N63	Fenugreek	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N64	Cardamom	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N65	Fenugreek	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N69	Market soup powder	–	–	+	+	–	–
N74	Vacuum dust	–	–	–	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N75	Soil	–	–	–	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N77	Chilli powder	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N79	Cumin	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N81	Pond water	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N83	Sugar	–	–	+	+	*Cronobacter* spp.	–
N85	Rain water	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N87	Vegetable	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N88	Fruit	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*

Table 2 continued

Isolate		IMViC test				PCR primers	
ID	Source	Indole	Methyl red	Vogus Proskauer	Citrate utilisation	16S rRNA	ITS-G
N92	Chilli powder	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N96	Vegetable	–	–	+	+	–	–
N101	Flattened rice	–	–	+	+	–	–
N106	Fruit	–	–	+	+	*Cronobacter* spp.	–
N108	Coriander	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N112	Fruit	–	–	+	+	*Cronobacter* spp.	*Cronobacter sakazakii*
N118	Fruit	–	–	+	+	–	–
N119	Water	–	–	+	+	–	–
N121	Biscuit	–	–	+	+	*Cronobacter* spp.	–
N134	Chocolate	–	–	+	+	*Cronobacter* spp.	–
N148	Chips	–	–	+	+	–	–
N156	Fruit	–	–	+	+	*Cronobacter* spp.	–
N162	Salt	–	–	+	+	*Cronobacter* spp.	–
N174	Fruit	–	–	+	+	*Cronobacter* spp.	–

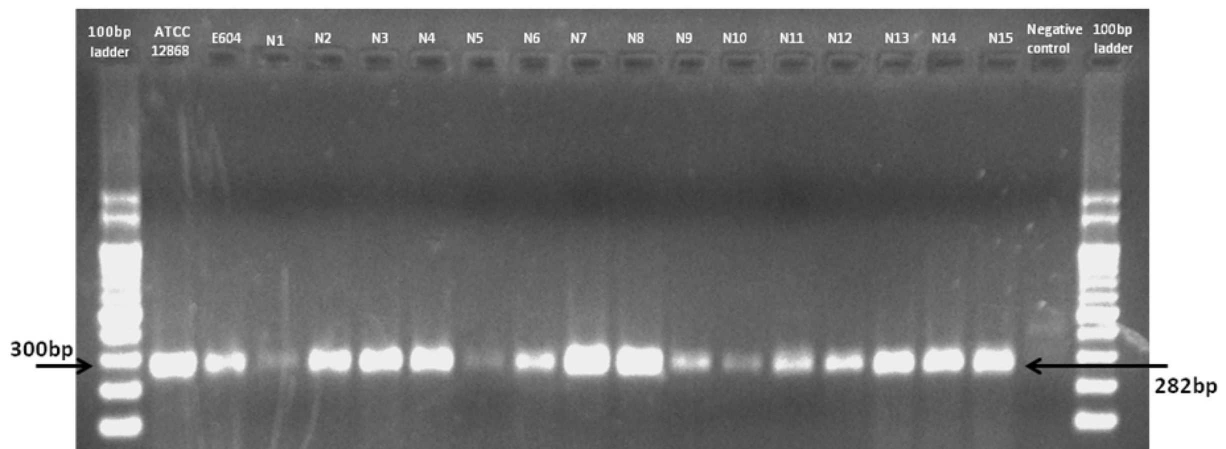

Fig. 1 A 1.5 % agarose gel showing the PCR products derived from ITS-G (282 bp). *Lane 1*, 20 size marker; *Lane 2–3*, *Cronobacter sakazakii* ATCC 12868 and E604 used as reference strain; *Lane 4–18*, *Cronobacter* isolates; *Lane 19*, negative control (master mix without DNA template)

Cronobacter spp. isolated from different commodities examined is summarized in Table 1. From 219 samples, among 85 presumptive isolates on TSA, only 58 isolates resulted in blue-green coloration on chromogenic ESA. Although the chromogenic substrate is a diagnostic tool for the detection of *Cronobacter* spp., it is not consistent because these studies have given different results [7, 8, 15]. The biochemical tests carried out in the study revealed that all the 58 isolates as well as the reference strain ATCC 12868 and E604 were positive for citrate utilisation. All *Cronobacter* spp. isolates were negative for the production of Indole and Methyl red. However, two isolates out of 58 isolates were negative for VP test (Table 2).

Further, the molecular characterization targeting 16S rRNA region resulted in 952 bp amplified product in the 45 isolates in addition to standard strains. The other 13 isolates did not give the expected PCR product although they were identified as *Cronobacter* spp. by chromogenic assay which imposes the relevance of more than one technique in precise identification of the *Cronobacter* spp. The studies by Iversen et al. and Barron et al. have also suggested a new system for categorizing *Cronobacter* spp. isolates based on combination of DNA–DNA hybridization, f-AFLP and 16S rRNA gene sequences, and phenotypic characteristics [2, 15, 16]. The species-specific PCR based on the ITS-G sequences confirmed 36 numbers of isolates

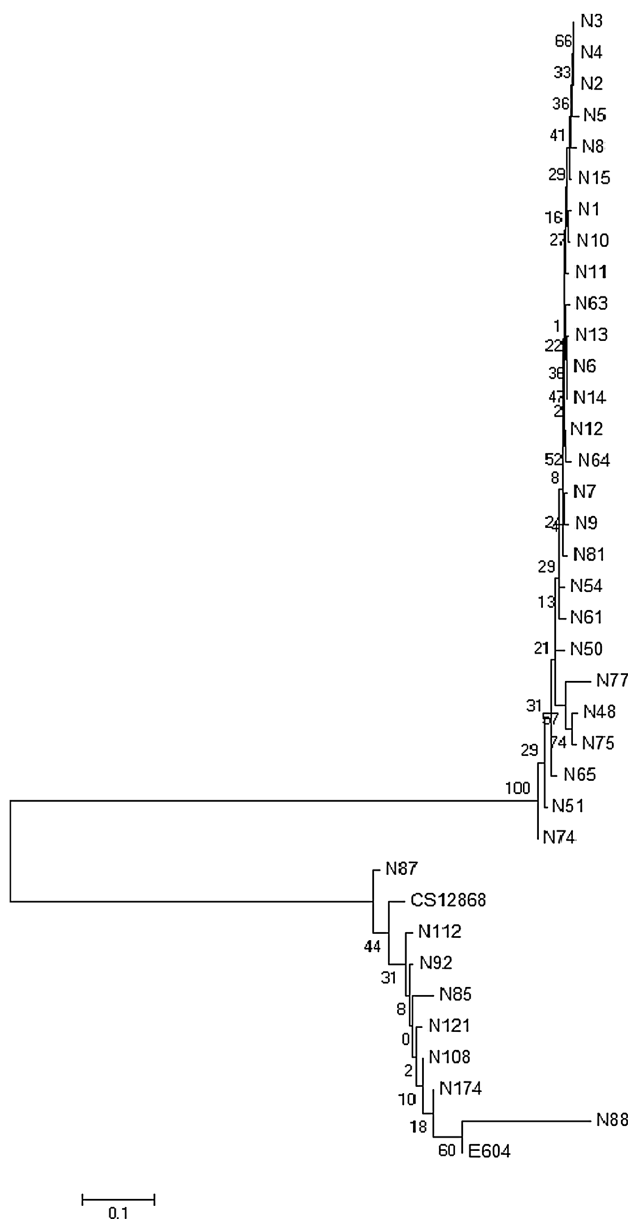

Fig. 2 Phylogenetic tree inferred using neighbor-joining method for query and control ITS sequence

as *C. sakazakii* (Fig. 1). The other isolates may belong to other species of *Cronobacter* which might play a role mostly as environmental commensals and are probably of modest clinical significance.

The plant-based products were the most frequently contaminated with *Cronobacter* spp. (n = 102, 22 %) with herbs and spices (34 %) as a potential reservoir of this pathogen. The high prevalence of this pathogen with herbs and spices suggests that more precautions should be taken when home remedies containing herbs or herbal beverages are given to infants to alleviate gastrointestinal discomfort. The pervasiveness on plant-based materials could be due to the resistance of the pathogen to various environmental stresses which might be due to the organism possibly colonizes plant material, and the yellow carotenoid-based pigmentation may shield it from sunlight-generated oxygen radicals [9]. Studies by Forsythe and Friedemann also reported that plant origin samples are most repeatedly contaminated with *Cronobacter* spp. irrespective of the world region of analysis [9, 10]. *Cronobacter* spp. were also detected in environmental samples (23 %) which supports the hypothesis of the role played by environmental contamination. Among dairy-based products, only 9 % raw milk samples were found to be contaminated with *Cronobacter* spp. with no occurrence in sealed PIF, milk powder, and pasteurized milk samples indicating that proper hygiene and healthy practices were implemented in the industrial units manufacturing these products. These findings are in agreement with reports by Iverson and Forsythe and Nazarowec-White and Farber who put forwarded that application of pasteurization at final treatment stage eliminates all pathogens from such products [14, 29]. We classified 38 *Cronobacter* spp. isolates by comparing the ITS sequence. The neighbor-joining tree reveals that numbers of eight isolates are closely identical to standard strains (Fig. 2).

The antibiotic susceptibility of *Cronobacter* spp. isolates to eight antibiotic derivatives is shown in Fig. 3. All the 45 *Cronobacter* isolates obtained in this study were resistant to bacitracin, clindamycin, kanamycin, penicillin-G, tobramycin, and vancomycin. Al-Nabulsi et al. also reported the resistance of *Cronobacter* spp. to glycopeptides (vancomycin) and coumarin-glycosides (novobiocin) derivatives [1]. Majority of the *Cronobacter* isolates were resistant to β-lactam derivatives (68 %), macrolides (88.6 %), and aminoglycosides (79.9 %). Stock and Wiedemann also reported the similar trend of resistance for *Cronobacter* spp. toward these antibiotics [37]. Terragno et al. outlined the susceptibility of *Cronobacter* spp. to amoxicillin, ampicillin, chloramphenicol, gentamicin and tetracycline [38]. Kim et al. summarized the susceptibility of *Cronobacter* spp. to tetracycline, and resistance toward ampicillin or cephalothin [21]. Our results also indicate that *Cronobacter* spp. exhibited moderate susceptible response toward phenicoles including chloramphenicol (31.6 %). *Cronobacter* spp. showing tetracycline and chloramphenicol susceptibility were reported to be found at a higher ratio by other investigators [21, 29, 37]. *Cronobacter* spp. infections are conventionally treated with ampicillin-chloramphenicol or ampicillin-gentamicin [7, 24]. However, ampicillin-chloramphenicol or gentamicin-resistant *Cronobacter* spp. have appeared because of the production of β-lactamases, the acquisition of transposable elements, and the presence of multiple antibiotic resistance operons [11, 32].

Cronobacter spp. is ubiquitous in nature, and foods of plant origin appear to be one of the most possible natural

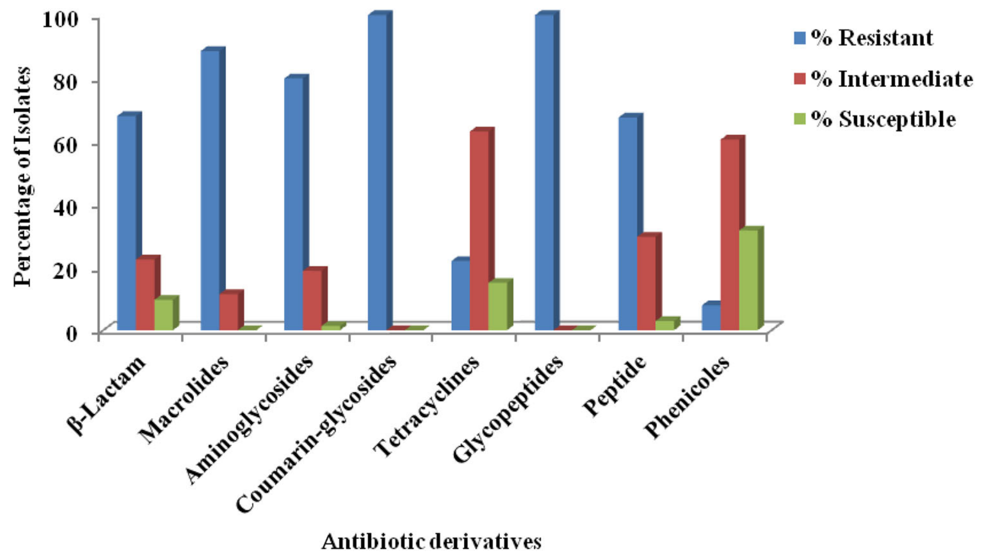

Fig. 3 Antibiotic susceptibility of *Cronobacter* spp. by agar disk diffusion method [antibiotic susceptibility limits: 0–15 mm: resistant (R); 15–20 mm: intermediate (I); 20–30 mm: susceptible (S)]

reservoirs of this pathogen. From our findings, a single method of identification of this pathogen is not sufficient to confirm its presence. Therefore, a combination of confirmation methods is necessary to completely eliminate false positives and false negatives. The antibiogram studies indicate emergence of resistance toward commonly used drugs emphasizing the need on preventing the resistance and to track the resistance pattern.

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