International journal of basic and applied research
www.pragatipublication.com
ISSN 2249-3352 (P) 2278-0505 (E)
Cosmos Impact Factor-5.960

Phenotypic Characterization of *Cronobacterdublinensis* isolated from different food samples

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Received: 10 July Revised: 18 July Accepted: 26 July

Abstract

*Cronobacter*species is prevalent in nature and various type of food especially milk and milk products appear to be one of the most possible reservoirs of this pathogen. This study evaluated the phenotypic characteristics of 70 samples dairy (n=40), vegetables and fruits (n=15) and herbs and spices (n=15) have been looked for the presence of *Cronobacter* species the number of *Cronobacter* species isolated from sort of food items. The typical colonies of *Cronobacter* were then identified by VITEK2. The isolates of *C. dublinensis*showed different types of morphotypes Of the 06 isolates, the most common morphotypes among the *Cronobacterdublinensis* was BDR (Brown, dry, rough), as found for 03 isolates (60%), followed by smooth and light yellow (20%) and red and smooth (20%), morphotypes. Three isolates with brown, dry and rough morphotypes were found to be positive for the Congo red dye binding assay. Isolates that exhibited other morphotypes (40%) indicated reduced binding of the Congo red dye.

Keywords:- Isolation, Biofilm, Cronobacter species, Hemolysis, Desiccation Tolerance.

Introduction

Cronobacter is an opportunistic pathogenic bacterium that can be isolated from various types of food sources and it might cause serious health issues and it could even cause death in newborn babies, children and elders too. The *Cronobacter* genus comprises gram negative rods and it includes seven species: *C. sakazakii*, *C. malonaticus,C. turicensis*, *C. muytjensii*, *C. condimenti*, *C. universalis* and *C. dublinensis* (Joseph *et al.,* 2012).

*Cronobacter*mainly infects neonates and immunocompromised individuals, and cow milk and goat milk are preferred for these groups of individuals. Besides cow milk and goat milk samples which already have medicinal properties and show resemblance with human milk, buffalo and camel milk

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samples were also tested for the occurrence of *Cronobacter*isolates. Ready to eat products such as cheese is an important source of animal protein in the vegetarian diet. Cottage cheese mainly consists of 4% fat and 11% protein; about five hundred different varieties of cheese have been recognized by the International Dairy Federation (Patrick, 2000). Cheese and curd were also selected in the present study. *Cronobacter*isolates from dairy cheeses have been also reported earlier in different nations (El-Sharoud*et al.*, 2009).United Nations Food and Agriculture Organization and WHO in 2004 estimated that the annual incidence rate due to *Cronobacter*in USA among low birth weight infants was found to be 8.7 per 100,000 and one *Cronobacter*infection has been detected per 10,470 low birth weight neonates (Mullane*et al*., 2007).This microbe has received major attention worldwide after an incident of meningitis in Tennesee in the year of 2001. *Cronobacter*causes disease like necrotizing, bacteremia and meningitis in neonates and newborns. However, the incidents of infections also been reported in elders and immune compromised persons (Healy *et al*., 2010) although the disease incidences are low but the fatality rate is high in *Cronobacter*induced infections; it may range between 40 to 80%. Neonates with low birth- weight (<2.5kg) and infants that are <28 days old are at high risk as compared with mature infants (Jaradat*et al*., 2014).Standard identification procedures for *Cronobacter*include the morphological observation of yellow pigmented colonies on TSA and mucoid colonies on VRBGA. Besides these various selective chromogen media have been developed recently such as the Druggan-Forsythe-Iversen agar, DFI and the *Enterobacter*Isolation Agar that are based on the testing for glucosidase activity. Iversensuggested various biochemical tests to study the biochemical properties of *Cronobacter*spp for its identification.Those food products which might have *Cronobacter*are imperative to find out the best feasible ways for transmission of the *Cronobacter* infection with an indication that *Cronobacter*species infects both infants and adults. Now it is necessary to check the wide range of food products for the presence of *Cronobacter*. In India there are no reports of presence of *Cronobacter* in the food items reported as far. However, single clinical case study was reported by that described two different cases of infection by *Cronobacter*species. A low birth weight neonate with meningitis and a two month infant with bacteraemia for the first time in India.The motive of present analysis were to isolate and identify *Cronobacter* species and its morphotypes from different food items and to study the effect of different stress conditions on its survival.

Materials and methods

Collection of Samples

A total of 70 samples including dairy products (40), vegetables and fruits (15), herbs and spices (15) were collected from local markets across Dehradun Uttarakhand, India.

Isolation of *Cronobacter* spp.

The solution of samples, were prepared by adding 1g of each in 10ml of buffered peptone water. 1ml from above was then transferred to Enterobacteriaceaeenrichment broth (ESE) and incubated for 24 h at 37°C. A loopful of each culture broth was streaked onto violet red bile glucose agar plates and incubated for 24 h at 37°C. This is called the selection step. The purple or pink colonies were picked and streaked on tryptic soy agar and incubated for 48 h at 37°C to look for the characteristic

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pigmented colonies of *Cronobacter*spp. The pigmented colonies on TSA were picked and subjected to further characterization.

Isolation and Identification

Preliminary identification of bacterial isolates was done on the basis of following morphological and biochemical properties. Gram staining, motility test, endospore staining, oxidase test, catalase test, hemolysis. The above tests were performed as per standard protocol. The gram -negative, rod, catalase positive, oxidase negative, motile isolates were then further characterized biochemically using VITEK 2.

Analysis of morphotypes by Congo Red Method

The colony morphology of *Cronobacter*species on Congo red agar was examined for the binding of the Congo red dye. Congo red agar plates were prepared using LB agar without salt. LB agar without salt, was mixed in 500 ml distilled water and then autoclaved. Congo red solution dye was prepared by dissolving 32 mg of Congo red powder in 8 ml sterile distilled water and the solution was filtered. After this, LB agar without salt was cooled to 55°C. 5 ml of filtered Congo red solution was added, gently mixed and dispensed into petridishes.

Microtiter plate assay for Biofilm analysis

Biofilm forming ability of the isolates were performed in a microtiter plate as follows briefly, the isolates were inoculated in 5 ml of tryptic soya broth (TSB) and then incubated at 37^0C for 18 hours. In a 96 well microtiter plates, 600 µL of each inoculated tryptic soya broth was dispensed into 3 wells (200 µL into each well) of each 96 well plate. Loading plates were incubated at 37°C for 24h. After 24 hours of incubation the plates were emptied from the TSB and washed twice with sterile distilled water the plates were then left for 10 minutes at room temperature to dry. Two hundred microliter of 1% crystal violet (CV) was added to each well and left for 30 minutes. After half hour, all the wells were washed three times with sterile distilled water. Finally, 200 µL of absolute ethanol was added to each well and after 15 minutes the content of the plates was transferred into new plates. The plates were read using microplate reader at absorbance 600 nm.

Analysis of Isolates for Desiccation Tolerance

Desiccation tolerance analysis of the strains was performed as in previous reports with some modifications. After culture of the strains on plates, a single colony was cultured in 10 mL LB broth until the bacterial cells reached logarithmic phase. "The OD_{600} value of different bacterial culture was measured, and a 100 µL portion of the culture was transferred into each well of a 96-well microtiter plate. Subsequently, the plate was transferred into a sterile dryer with dehydrated silica gel. The dryer was placed in a sterile incubator which was controlled at a temperature of 37^0C. After 6 days of drying, the 96-well microtiter plate was removed, 100 µL/well of fresh medium was added, with continuous shaking at regular intervals upto three hours. The liquid in each well was transferred to a new 96-well microtiter plate, and the OD_{600} was measured. The above experiment was performed in triplicates". (Du X *et al*., 2018).

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Resistance of isolates to acid stress

To investigate the resistance to acid and alkaline stresses, Tryptic soy broth (TSB) test media were adjusted to the target pH of 3.5 and 1.5 using sterile 1N of HCl or 1N of NaOH prior to autoclaving, assisted by a pH metre test strains were cultured for 12 hours in TSB to the late exponential phase. The isolates were inoculated into TSB with different pH (3.5 & 1.5) levels and incubated at 37°C. After incubation, the tubes were examined for the turbidity and subsequently OD was also taken at 600 nm.

Results and discussion

Isolation of *Cronobacter* species from food samples

In the current study total of 70 samples dairy (n=40), vegetables & fruits (n=15) and herbs and spices (n=15) have been looked for the presence of *Cronobacter* species the number of *Cronobacter* species isolated from sort of food items examined is summarized in **Table 1**.below

Table 1: The number of Samples that have been analyzed for isolation of *Cronobacter* species

Samples	Number of samples	Number of positive isolates
Milk and milk products	40	03 (02 from infant milk powder and 01 from raw milk)
Vegetables and fruits	15	01(fresh tomato)
Herbs and Spices	15	01 (Asafoetida)

Among the milk and milk products, infant milk formula and raw milk sample have high frequency of contaminated with *Cronobacter*species. Meanwhile no *Cronobacter* species was present in open curd, pasteurized milk, packed curd, open cheese, packed cheese, packed milk powder and chocolate. Rest of the isolate were recovered from tomato andasafoetida.

Appearance of the isolates on different culture media used for the isolation

After growing the strains on violet red bile glucose agar for 24 hrs at 37°C all produced dark pink to purple colonies and surrounded by a pink to purple halo, indicated fermentation of glucose. In addition to above, the isolates also produced mucoid colonies on VRBGA (**Figure 1**). On tryptic soya agar (TSA), isolates form creamish to slightly yellowish colonies. The typical *Cronobacter*colonies are yellow on TSA but none of the tested isolates showed yellow colour even after incubating for more than the desired period (**Figure 1**). All the isolates showed beta hemolytic activity when cultured on sheep blood agar (**Figure2**)

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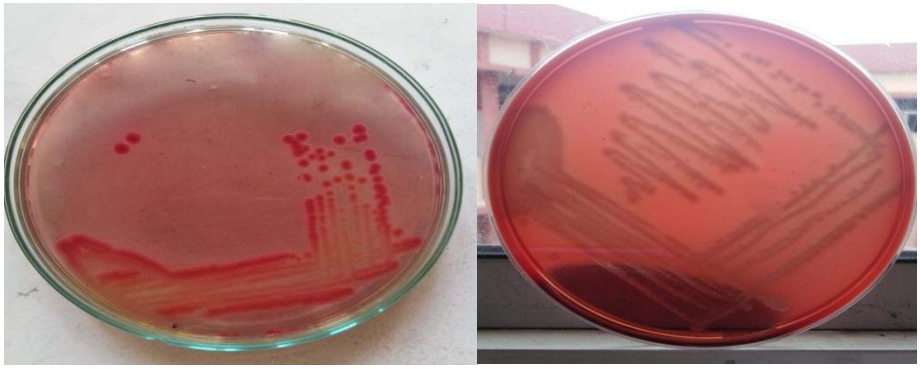

Isolation and identification

Based on the appearance of colonies on different detection media, isolates with dark pink, slightly yellowish and beta-hemolytic were selected for the identification. Gram staining of above isolates showed Gram-negative rods. These isolates showed motility when tested by hanging drop method. On performing catalase and oxidase tests, only 06 isolates showed the characteristic combination of catalase positive and oxidase negative. These isolates were tentatively considered as *Cronobacter*spp.and were carried for further identification using VITEK 2 system. The VITEK 2 system revealed all the tentative isolates were *Cronobacterdublinensis*(Report Attached).

Identification Information	Analysis Time:	3.75 hours	Status:	Final
Selected Organism	99% Probability Bionumber:	Cronobacter dublinensis ssp lactaridi 0627734151722011		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	+	9	BGAL	+
10	H2S	-	11	BNAG	+	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	+	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	+	37	MNT	-	39	5KG	-
40	ILATk	+	41	AGLU	+	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	-	53	IHISa	-	56	CMT	-	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	+	64	ILATa	-			

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Identification Information	Card:	GN	Lot Number: 2410844103	Expires:	Mar 19, 2020 12:00 GMT-06:00
	Completed:	Jun 10, 2019 01:15 GMT-06:00	Status: Final	Analysis Time:	3.75 hours
Selected Organism	99% Probability		Cronobacter dublinensis ssp lactaridi		
	Bionumber:	062773415172201		Confidence:	Excellent identification
SRF Organism					

Analysis Organisms and Tests to Separate:

Cronobacter sakazakii group	
Cronobacter dublinensis ssp dublinensis	dMLZ(90),IND(90),DUL(10),MNTk(90),
Cronobacter malonaticus	dMLZ(10),IND(10),DUL(10),MNTk(90),
Cronobacter muytjensii	dMLZ(10),IND(90),DUL(90),MNTk(90),
Cronobacter turicensis	dMLZ(90),IND(10),DUL(90),MNTk(90),
Cronobacter dublinensis ssp lausannensis	dMLZ(10),IND(50),DUL(10),MNTk(10),
Cronobacter dublinensis ssp lactaridi	dMLZ(10),IND(90),DUL(10),MNTk(10),
Cronobacter sakazakii	dMLZ(10),IND(10),DUL(10),MNTk(10),
Cronobacter genomospecies 1	dMLZ(10),IND(10),DUL(90),MNTk(50),

Analysis of morphotypes by Congo Red Method

The isolates of *C. dublinensis*showed different types of morphotypes by Congo red method (**Figure 4.**)

Isolate 1- Brown, rough and dry — Isolate5-Brown, rough and dry

Isolate 9 - light yellow and Smooth — Isolate 14 – Brown, rough and dry

Isolate 16- Red and smooth — Control

Figure 4: Different cell morphologies on Congo red medium

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Distribution of the 06 isolates, the most common morphotypes among the *Cronobacterdublinensis* was BDR (Brown, dry, rough), as found for 03 isolates (60%), followed by smooth and light yellow (20%) and red and smooth (20%), morphotypes (**Figure 5**). Three isolates with brown, dry and rough morphotypes were found to be positive for the Congo red dye binding assay. Isolates that exhibited other morphotypes (40%) indicated reduced binding of the Congo red dye.

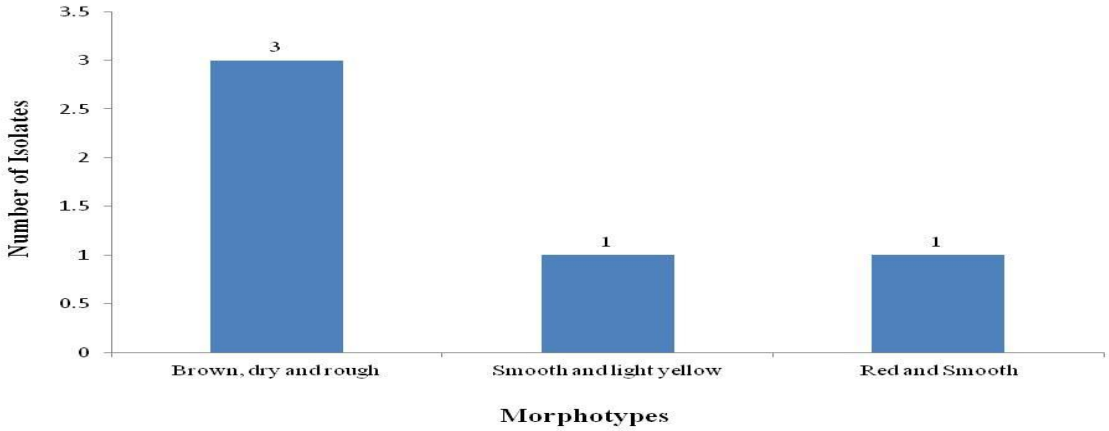

Figure 5: Distribution of isolates according to morphology

Microtiter plate assay for Biofilm analysis

The ability of the isolates to form biofilm was studied by crystal violet assay using microtiter plate (**Figure 6**) and was differentiated as strong, moderate, weak and no biofilm producers

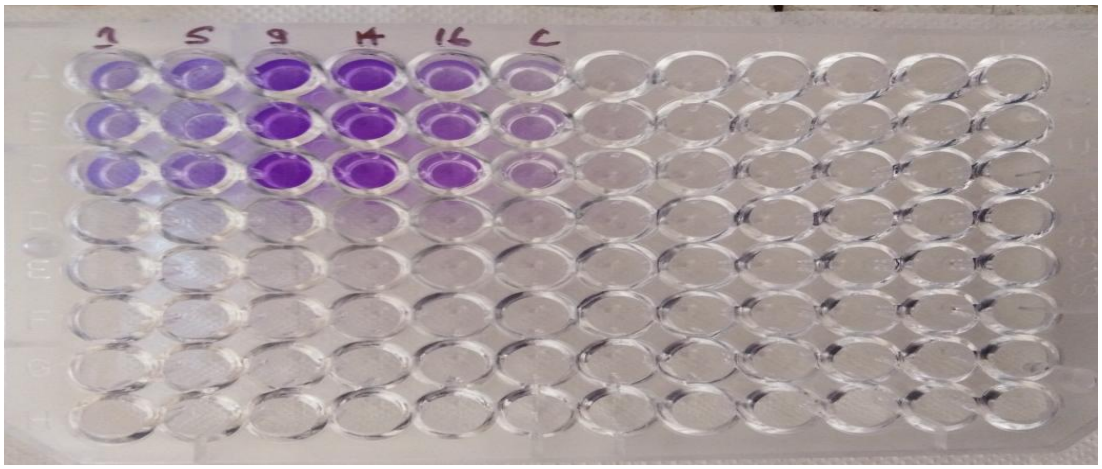

Figure 6: Microtiter plate assay for biofilm

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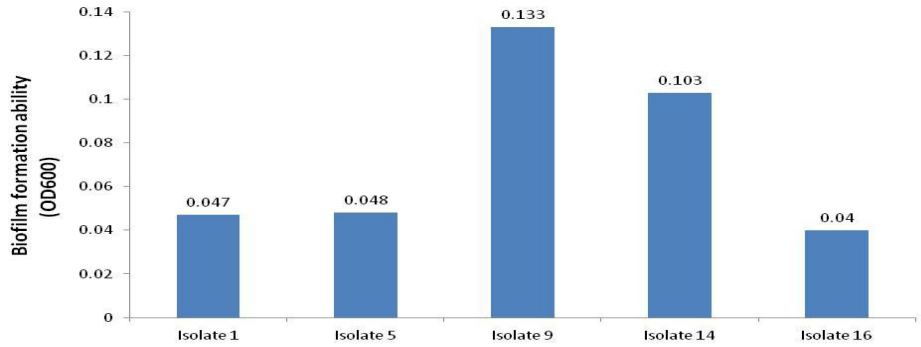
There was variation observed among the *C. dublinensis* strains in the quantity of biofilm produced. The results showed that 02 (40%) of the 05 tested isolates, were capable to produce biofilm on polystyrene microtiter plates. Further, results showed that, two isolates (9 & 14) are the most biofilm producing strains, isolate (16) has ability to produce moderate quantity of biofilm and rest of the 02 (1 & 5) isolates were weak biofilm producers.



Isolate 5, Isolate 5, Isolate 9, Isolate 14 and Isolate 16 – all represent *Cronobacterdublinensis* recovered from different food items

Figure 7: Biofilm forming ability of isolates

Desiccation Tolerant ability of Isolates

After exposing the strains in dry environment, the desiccation tolerant ability for the two isolates was found to be less than 40% in terms of inactivation rate. **(Figure 8)** Of the tested strains, the isolate 16 confirmed the best functionality for capability of desiccation tolerance, and the inactivation rate was 35.3%. On the other hand, one isolate showed inactivation rate of 45.3% (weak desiccation). All in all, the examined strains confirmed < 50% inactivation rate suggesting good desiccation tolerance with minimum inactivation rate of 39.8 and 35.5%.

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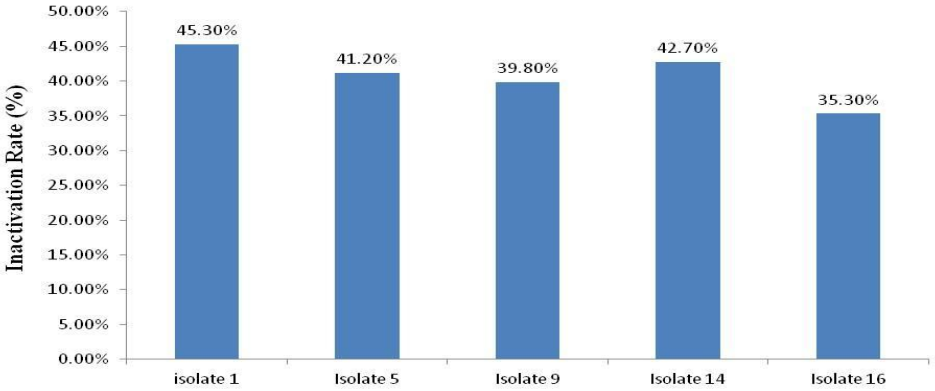
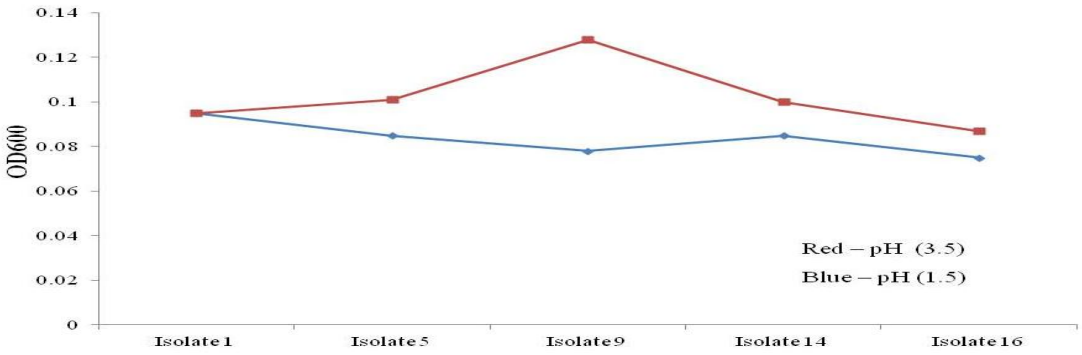


Isolate 5, Isolate 5, Isolate 9, Isolate 14 and Isolate 16 – all represent *Cronobacterdublinensis* recovered from different food items

Figure 8: Desiccation tolerance ability of the isolates

Resistance of isolates to acidic environment

It was observed that the isolates have shown resistance to very low pH conditions. Average OD_{600} of isolate 9 was the highest at pH 3.5. Of the tested isolates, majority of isolates showed resistance ability at pH 3.5. As the pH further drops to 1.5 (at more acidic condition), the resistance ability of isolates decreased except for one isolate which showed tolerance at both pH 1.5 and 3.5 (**Figure 9**)



Isolate 5, Isolate 5, Isolate 9, Isolate 14 and Isolate 16 – all represent *Cronobacterdublinensis* recovered from different food items

Figure 9: Response of isolates towards acidic pH

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Discussion

The prevalenceof *Cronobacter* species from spices and herbs is only 6.6%. Similar low prevalence in spices samples (3.6%, 1/28) was also reported by Kandhai*et al.,* 2010.HoweverSingh *et al.,* in 2015 showed a little high prevalence of 26.8% of herbs and spices in our country. Although the percentage of bacteria in spices and herbs is low but its presence recommend to take extra precaution while giving home remedies made up of herbs in order to prevent gastrointestinal discomfort in infants.Of the six tentatively identified *Cronobacter* isolates, full confirmation of identification was done using VITEK 2. Majority of studies reported other *Cronobacter*spp. as like *C. sakazakii*, *C. malonaticus* as the commonly identified with *C. dublinensis* as least prevalent (Molloy *et al*. 2009). It is likely to be ecologically significant that the bacteria isolated from different food samples belonging to only a few biotypes. Iversen*et al* in 2004 found that among the biotypes & subtypes of *C.sakazakii*, only biotypes 1, 2, 2a, 3 and 4 have been found, with biotypes 5 and 5a of *C. malonaticus*. Less frequent *C. dublinensis* strains were categorized into *C. dublinensis* subspecies *dublinensis* and C. *dublinensis* subspecies *lactaridi.* The Congo red binding assay was used in this study analysis to assess the expression of curli fimbriae. The *Cronobacter* isolates showed different cell morphologies depending on their ability to bind to congo red when cultured in a medium red. Three types of cell morphologies have been seen namely brown dry and rough, red and smooth & smooth and light yellow. Our studies are similar to the study done by Yan *et al*., 2015 who reported 4 types of cell morphology with Congo red dye binding namely red, dry, and rough (RDAR), brown, dry, and rough (BDAR), red and smooth (RAS), brown and smooth (BAS). In the current investigation, all *Cronobacter*isolates had ability to form a biofilm on microtiter plates. Our results shows that all of the five tested isolates had ability produce biofilm on microtiter plates with two isolates (40%) isolated from as most efficient biofilm producers.*Cronobacter* showed overall a higher desiccation rate in terms of inactivation rate ranged from 35 to 45% with two isolates recovered from herbs and tomato has 35 and 39%. Rest of the three isolate have more than 40%. Another important feature of the *Cronobacter* isolates is their ability to survive at high acidic conditions and causing infections in neonates and other immuno-compromised persons. In our study, *Cronobacter* isolates were tested at acidic (3.5) and very acidic (1.5) pH. All the isolates showed turbidity at pH of 3.5 but as the condition become more acidic, their turbidity decreases. It shows that these strains are acid tolerant.In 2009, Dancer *et al*reported*Cronobacter*to be more acid-tolerant (able to grow at pH 3.9) than most closely related enteric pathogens.

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