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Molecular Identification, Prevalence and Antimicrobial Susceptibility Profile of *Cronobacter spp.* Cultivated on a Chromogenic Medium in Libya

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Abstract

Background: *Cronobacter sakazakii* is associated with illness in infants from contaminated powdered infant formula (PIF) and it is frequently recovered from PIF factory environment. Limited information is available on contamination of other food such as dairy and meat products in Libya.

Methods and findings: A total of 261 samples of milk, dairy products and coarse ground meat products were collected from different localities in Libya. Samples were examined for *Cronobacter spp*. with an adapted ISO /DTS 22964 cultural protocol using HiChrome™ *Enterobacter sakazakii* modified agar coupled with 16S rDNA partial sequencing to identify the organism. The identified isolates were biochemically characterized and tested for their ability to produce yellow pigment. Out of the 261 analyzed samples, only two beef burgers, one fermented milk "Laban", one she-camel's milk, two raw cow's milk, two cereal baby food, one Maassora cheese and one ready to feed baby milk were contaminated with *Cronobacter spp*. at a total rate of 3.8%. Accuracy of HiChrome Ent. sakazakii modified agar reach 100% as all of blue-green presumptive colonies were confirmed *Cronobacter spp*. while other colorless, greenish or with blue center colonies which competed growth with *Cronobacter spp*. were predominantly *Escherichia coli* followed by *Klebsiella spp*. and to less extent *Pseudomonas luteola*, *Citrobacter freundii* and *Acinetobacter baumanii*. Moreover, the isolated strains of *Cronobacter* were resistant to Amoxicillin, Erythromycin, Vancomycin and Streptomycin, and sensitive to Doxycycline, Enrofloxacin and Gentamycin.

Conclusion: This study documents for the first time the occurrence of *Cronobacter spp*. in beef burger, raw cow's milk, fermented milk "Laban", she-camel's milk, Maassora cheese, cereal baby food and ready to feed baby milk sold in Libya, by using conventional methods, biochemical tests and molecular techniques.

Keywords: *Cronobacter spp*; 16S rDNA sequence; Dairy products; Meat products; She-camel's milk

Introduction

Cronobacter species belongs to the *Enterobacteriaceae* family. It is closely related to the genera *Enterobacter cloacae* and *Citrobacter freundii* [1]. It is an ubiquitous gram-negative bacteria, non-spore-forming, rod-shaped, oxidase, lactose and sorbitol negative, facultative anaerobes, motile by peritrichous flagella [2]. Recently, this pathogen has become more diverse that comprises ten species: *Cronobacter sakazakii, C. malonaticus, C. turicensis, C. universalis, C. muytjensii, C. dublinensis, C. condiment, C. pulveris, C. helveticus and C. zurichensis* [3].

As a hallmark distinguishing *Cronobacter spp*. from the rest of *Enterobacteriaceae* members, *C. sakazakii* is positive for -glucosidase enzyme, which considered the base for development of numerous chromogenic media proposed for sensitive, specific and accurate detection of this bacterium [4]. Chromogenic medium (Druggan-Forsythe-Iversen agar, DFI) is described for the selective detection of *C. sakazakii*. This medium is based on the -glucosidase reaction which is detected using 5-bromo-4-chloro-3-indolyl-alpha, D-glucopyranoside (X alpha Glc). *C. sakazakii* hydrolyses this substrate to an indigo pigment, producing blue-green colonies

on this medium [5]. Commercially available biochemical test panels, such as the API 20E and ID 32E, are not sufficient to identify *Cronobacter* isolates at the species level and reliance on these methods will result in false positive and false negative identifications [6]. Moreover, the 16S rRNA gene sequencing showed that there is sequence differences between *C. sakazakii* and other *Enterobacteriaceae* within the hypervariable regions V1, V2, and V3 [7].

C. sakazakii is an emerging opportunistic, foodborne pathogen associated with severe illness and high mortality in neonates and infants [8]. It has also been reported in adults especially among the elderly and patients who are immunocompromised [9]. *C. sakazakii* has been recovered from a wide range of foods and environments; also it was isolated from human clinical samples [10].

Concerns rose due to an increasing number of cases of neonatal meningitis related to the consumption of infant formula contaminated with *C. sakazakii* [11]. Moreover, the International Commission for Microbiological Specifications for Foods ranked the organism as 'severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration [12,13].

Cronobacter species are widely distributed in nature occurring in fresh water, soil and sewage, plants, vegetables, animal and human feces. Among various food samples, *C. sakazakii* has been found in milk and dairy products [10]. Furthermore, *C. sakazakii* is an important competitor to *E. coli* O157:H7 and Salmonella on their selective media and has recently been isolated from coarse ground beef products at contamination rates of 15% [14].

Different protein products have been prepared from milk for use in meat product formulations, these dairy protein ingredients are used to improve moisture retention, fat-binding, and textural characteristics of cooked meats. Both caseins and whey proteins have been used in comminuted and emulsified meat products, such as frankfurters and bologna, and in coarse ground meat products, such as fresh sausage, meat patties, and meatballs [15].

In Libya, consumption of raw milk and dairy products is common especially by elderly people. Cow's milk, she-camel's milk and locally made dairy products, such as Maassora, Ricotta and fermented milk are manufactured at small scale dairy parlors, where hygienic measures are not applied, moreover, traditionally, she-camel's milk is consumed raw, neither pasteurized nor boiled. Studies regarding the isolation of *C. sakazakii* from powdered infant formula (PIF) and food products are scarce in Libya [16], which makes such a study of value not only locally but also worldwide, as infant food can be risk factor for infant illness. Therefore, the aim of this study was to investigate the prevalence of *C. sakazakii* in PIF, dairy and meat products in Libya with a special reference to its antibiotic resistant profile.

Materials and Methods

Collection of samples

A total of 261 samples of milk, PIF, dairy and ground meat products were randomly collected from different geographic localities in Libya **(Table 1).**

Table 1 Type and number of the examined samples contaminated with confirmed *Cronobacter* spp. (blue green colonies on HiChrome Ent. sakazakii modified agar).

Type of Sample	Total Number of Samples	Positive Samples	
		Chromogenic Agar	16S rRNA
Raw cow's milk	46	2 (4.3%)	*C. pulveris*
Raw she camel's milk	5	1(20%)	*C. sakazakii*
Raw fermented milk (Laban)	28	1(3.5%)	*C. sakazakii*
UHT milk	8	0	-
Yoghurt	5	0	-
Maassora cheese	21	1(4.7%)	*C. pulveris*
Ricotta cheese	13	0	-
Imported soft cheese	6	0	-
Butter	4	0	-
Ice cream	6	0	-
Full cream milk powder	10	0	-
Skimmed milk powder	6	0	-
Ground beef	11	0	-

Beef burger	12	2 (16.6%)	*C. sakazakii*
Powdered infant formula	36	0	-
Growing up formula	18	0	-
Ready to feed baby milk	10	1(10%)	*C. pulveris*
Cereal baby food	16	2 (12.5%)	*C. sakazakii*
Total	**261**	**10 (3.8%)**	-

Preparation of samples for cultivation of *C. sakazakii*

The culturing technique of *Cronobacter spp*. was performed according to the reference method ISO/TS 22964:2006 [17] for the detection of *C. sakazakii* in milk powder and PIF. Briefly, 25 g/mL from each sample was aseptically transferred into a sterile polyethylene stomacher bag and blended with 225 mL of Buffer peptone water (Park Scientific, M 0063, Northampton Limited, UK), homogenized in a stomacher (Stomacher 400, Seaward Medicals, UK) at 230 rpm for 1 min, then incubated at 37°C for 18 ± 2 h. Ten mL of the pre-enrichment was cultured by inoculation into 90 mL of *Enterobacteriaceae* enrichment broth (EEB, Lot: 082610202 – Liofilchem- Italy) then incubated at 37°C for 24 h. Only 0.1 mL of the selective enriched broth was streaked onto a chromogenic media (HiChrome Ent. sakazakii modified agar, HiMedia, M1641, India); the inoculated plates were incubated at 44°C for 24 h. The presumptive colonies (blue green) were picked up for further investigation. HiChrome™ *Enterobacter sakazakii* modified agar contains a chromogenic substrate (5-Bromo-4-chloro-3-indolyl α-D-glucopyranoside) which is cleaved specifically by *C. sakazakii* resulting in the formation of blue green colonies [5]. Other organisms, which do not cleave this substrate, produce colorless, green or colorless with blue center colonies. Further, a presumptive *C. sakazakii* isolates were streaked onto Tryptone soya agar (TSA, Park, # M 266, U.K) incubated at 25°C for at least 72h to detect yellow pigmented colonies produced by *C. sakazakii* [18].

Biochemical characterization

Hi25™ *Enterobacteriaceae* identification kit (KB003 Hi 25TM *Enterobacteriaceae* identification kit, HiMedia) was used according to the manufacturer's instruction.

Identification of *C. sakazakii* by PCR and partial sequencing of 16S rDNA

Suspected colonies cultivated on HiChrome Ent. sakazakii modified agar were picked up and purified several times. The procedure of DNA extraction of *Cronobacter* isolates was carried out as described before [19]. Partial 16S rDNA was amplified using the universal oligonucleotides primers; Forward: S-D-Bact-0341-b-S-17 and Reverse: S-D-Bact-0785-a-A-21 [20]. The amplified 16S rDNA PCR fragment (464 bp) was excised from the gel, and the DNA was extracted from the gel using GF-1 AmbiClean kit (Cat. # GF-GC-100, Vivantis, Malaysia). The purified 16S rDNA amplicons were then sequenced in IZSLER - Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy.

BLAST search was carried out for the obtained consensus sequences by both NCBI (http://www.ncbi.nlm.nih.gov/pubmed) and 16S bacterial cultures Blast Server for the identification of prokaryotes (http://orygenesdb.cirad.fr/blast.html).

Antimicrobial susceptibility profile

PCR confirmed *Cronobacter* isolates were tested against nine antibiotics by applying the disc-diffusion method as described in the Clinical and Laboratory Standards Institute (CLSI) [21]. These antibiotics included Gentamycin (Mast discs 10 µg), Streptomycin (Oxoid 10 µg), Amoxicillin (Arcomex 25 µg), Colistin (Mast discs 10 µg), Oxytetracycline (Oxiod 30 µg), Doxycycline (Mast discs 30 µg), Vancomycin (Oxoid 30 µg), Enrofloxacin (Arcomex 5 µg) and Erythromycin (Mast discs 10 µg).

Results

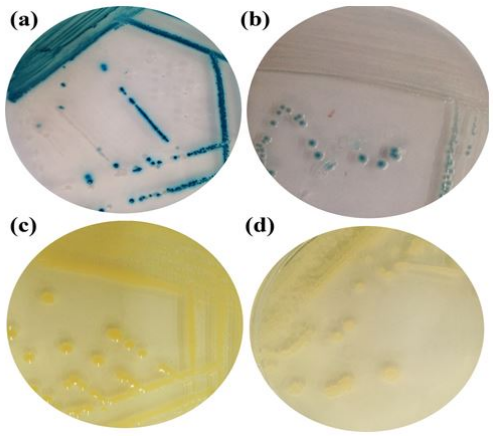

Figure 1 Cultural characteristics of *C. sakazakii* colonies on HiChrome Ent. sakazakii modified agar plate showing blue green characteristic colonies of *C. sakazakii* (a), the luxuriant growth of *E. coli* colorless colonies with blue center (b), Tryptone soya agar plate showing yellow pigment colonies of *C. sakazakii* (c), while *E.coli* appearing white colonies (d).

A total of 261 isolates were successfully cultivated on HiChrome Ent. sakazakii modified agar. Ten of them showed the characteristic blue-green colonies, while the rest of isolates ranged from colorless to greenish, and colorless with blue center colonies **(Figure 1).**

Out of 261 samples of milk, baby food, dairy and ground meat products only 10 (3.8%) yielded characteristic blue-green colonies on HiChrome Ent. sakazakii modified agar suggested to be *C. sakazakii* **(Figure 1).** Three samples out of 80 baby food samples were contaminated with *C. sakazakii* at a rate of (3.7%). Among the examined dairy products samples, *C. sakazakii* was recovered from one raw fermented milk (Laban) (3.5%), one she-camel's milk (20%), two cow's milk (4.3%) and one Maassora cheese (4.7%) respectively. Only two out of twelve examined beef burger samples (16.6%) were found to contain *C. sakazakii* **(Table 1).**

Moreover, only three characteristic isolates were identically *C. sakazakii* and produced yellow pigment colonies **(Figure 1).** All positive samples subjected to biochemical tests (Hi25TM identification kit, HiMedia) were positive for xylose and cellobiose but gave negative results to nitrate, indole and phenylalanine deamination tests **(Table 2).**

All purified isolates were subjected to PCR and partial sequencing of 16S rDNA to identify the presumptive bacteria and to ensure the selectivity of the used medium. Interestingly, this assay confirmed all above isolates as *Cronobacter spp*. with 99% nucleotide identity. In particular six isolates were identified as *C. sakazakii*, while the remaining four were *C. pulveris*. She-camel's milk and beef burger could represent an important vehicles for transmission of *C. sakazakii* at rate of contamination of 20% and 16.6%, respectively **(Table 1 and Figure 2).**

Table 2 Biochemical characterization of *Cronobacter* species.

Biochemical Test	Isolate Code								
	10305.2	10322.1	10324.1	10429	10456	10460.6	10492	2204	6208
OPNG	+	+	+	+	+	+	+	+	+
Lysine utilization	-	-	-	-	-	-	-	-	-
Ornithine utilization	+	-	-	-	-	+	-	+	-
Urease	-	-	-	-	-	-	-	-	-
Phenylalanine deamination	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-
H2S production	-	-	-	-	-	-	-	-	-
Citrate utilization	+	-	-	-	-	+	-	+	+
Voges proskauers	+	-	-	-	-	+	-	+	-
Methyl red	-	+	+	+	+	-	+	-	+
Indole	-	-	-	-	-	-	-	-	-
Malonate utilization	-	+	+	+	+	+	+	+	+
Esculin hydrolysis	-	+	+	+	+	+	+	+	+
Arabinose	-	+	+	+	+	-	+	-	+
Xylose	+	+	+	+	+	+	+	+	+
Adonitol	-	-	-	-	-	-	-	-	-
Rhamnose	+	-	-	-	-	+	-	+	-
Cellobiose	+	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	-	-
Saccharose	-	-	-	-	-	+	-	-	+
Raffinose	-	-	-	-	-	+	-	+	-
Trehalose	-	+	+	+	+	-	+	-	+
Glucose	-	+	+	+	+	+	+	-	+
Lactose	+	+	-	-	-	+	+	-	+

Oxidase	-	-	-	-	-	-	-	-	-

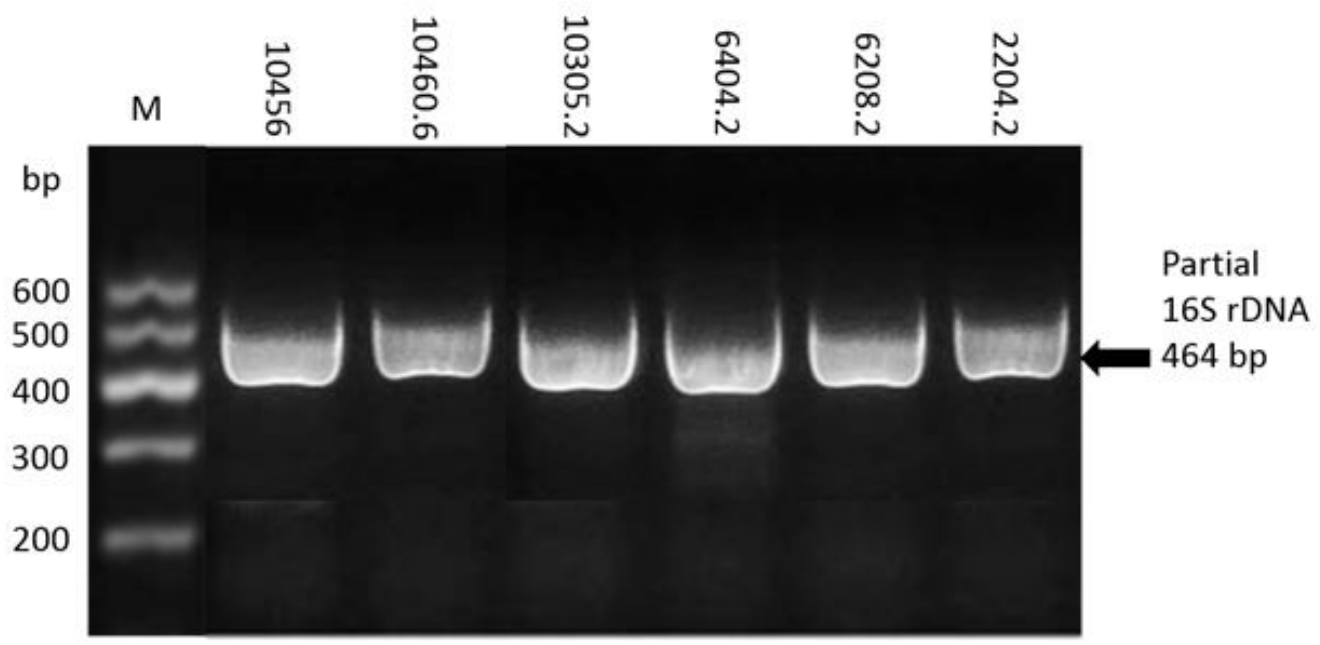

Figure 2 Partial amplification of 16S rDNA (464 bp) gene of blue green colonies cultivated on HiChrome Ent. sakazakii modified and the PCR products were sequenced and identified as *C. sakazakii*. (M): DNA marker; (Lane 1-2): isolates (10456 and 10460.6) from PIF; (Lane 3): isolate (10305.2) from she-camel's milk; (Lane 4): isolate (6404.2) from Laban; (Lane 5-6): isolates (6208.2 and 2204.2) from beef burgers.

Many bacterial species appeared colorless to colorless with blue center and greenish colonies were competing growth with *C. sakazakii* on HiChrome Ent. sakazakii modified agar. However, bacterial identification on basis of 16S rRNA Blast server for the identification of prokaryotes showed that *Escherichia coli* was predominant followed by *Klebsiella spp*. and to less extent *Pseudomonas luteola, Citrobacter freundii* and *Acinetobacter baumanii* **(Table 3).**

Table 3 Bacterial species that compete growth with *C. sakazakii* on HiChrome Ent. sakazakii modified agar as identified on basis of 16S rRNA Blast server for the identification of prokaryotes.

Bacterial Species	Number of Isolates	Colony Color
Escherichia coli	76	Colorless with blue center
Klebsiella spp.	12	Slightly violet
Cronobater pulveris	4	Slightly violet
Pseudomona luteola	3	Brownish
Citrobacter freundii	3	Slightly violet
Acinetobacter baumanii	1	Slightly violet

Antimicrobial susceptibility profile of *C. sakazakii* isolates was evaluated by using 9 antibiotics against *Cronobacter spp*. Results showed in **Table 4** revealed that the isolates were sensitive to Doxycycline, Enrofloxacin, Gentamycin, Colistin and Oxytetracycline with the exception of two isolates that were resistant to Colistin and Oxytetracycline, respectively. In contrast, all isolates were resistant to Amoxicillin, Erythromycin, Vancomycin and Streptomycin **(Table 4).**

Table 4 Antimicrobial susceptibility profile of the isolated *C. sakazakii.*

Isolate Code	Type of Antibiotics								
	AMX	CO	DOX	ENR	E	G	OXT	VA	St
10305.2	R	S	S	S	R	S	S	R	R
10322.1	R	S	S	S	R	S	R	R	R
10324.1	R	S	S	S	R	S	S	R	R
10429	R	S	S	S	R	S	S	R	R
10429	R	S	S	S	R	S	S	R	R
10460.6	R	S	S	S	R	S	S	R	R
10492	R	R	S	S	R	S	S	R	R
2204.2	R	S	S	S	R	S	S	R	R
6208.2	R	S	S	S	R	S	S	R	R
6404.2	R	S	S	S	R	S	S	R	R

AMX: Amoxicillin, **CO:** Colistin, **DOX:** Doxycylin, **ENR:** Enerofloxacin, **E:** Erythromycin, **G;** Gentamycin, **OXT:** Oxytetracycline, **VA:** Vancomycin, **St:** Streptomycin; **S:** Sensitive, **R:** Resistance.

Discussion

Cronobacter strains were recovered from a widespread range of foods including dried foods, meat, milk, cheese, vegetables, water, tea, rice, spices and herbs, various food production environments and household [22-27]. This wide spectrum of *Cronobacter*-contaminated foods covers both raw and processed foods and the type of food processing is not limited to dry products [23].

Our study revealed that beef burger samples represent a considerable source of *C. sakazakii*, this may be attributed to the spices added to enhance flavor or due to milk powder which is used as functional dairy protein ingredients in this product. This is consistent with recent survey study showing that 16% (8/50) and 15% (6/40) of ground beef and beef burger samples were contaminated with *C. sakazakii* [14].

The contamination of dairy products may occur during handling, processing, inadequate refrigeration or poor personal hygiene and probable use of polluted water in cleaning of dairy utensils [28]. *C. sakazakii* was occasionally detected in (6.6%) of examined raw cow' milk based only on the conventional methods and biochemical tests [28]. Other investigators found only (0.5%) of bulk tank cow's milk samples were contaminated with *C. sakazakii* [29].

As shown in **Table 1 and Figure 1,** *C. sakazakii* was found in two samples (4.3%) of raw cow's milk and one sample (20%) of raw she-camel's milk. While the observed results in this study were slightly lower than those reported before [28] where *C. sakazakii* (6.6%) was isolated in raw cow' milk based only on the conventional methods and biochemical tests. These findings were higher than that recorded by another study [29] were only (0.5%) of *C. sakazakii* strain was detected in bulk tank cow's milk. Moreover, seven out of 14 samples of she-camel's milk were contaminated by *C. sakazakii* which were identified on the basis of their cultural characteristics and the biochemical reactions [30]. In contrast, in other studies, *C. sakazakii* were not detected in cow's milk samples [31,32].

Interestingly, this is the first report that documents the contamination of she-camel's milk with *C. sakazakii* in Libya. These results supported those studies that reported *C. sakazakii* is a possible cause of diarrhea, wound infections, and urinary tract infections among immunocompromised people, particularly the elderly [33]. This is very important especially in developing countries such as Libya where raw she-camel's milk is traditionally consumed without pasteurization or boiling.

Results of the present study showed that *C. sakazakii* was not detected in examined samples of UHT milk. The reason for this may be due to its heat treatment. However, another study reported the presence of *C. sakazakii* in UHT milk [28]. On the other hand, one sample out of 28 samples of fermented milk (Laban) was contaminated with *C. sakazakii* and none of the tested 5 samples of yoghurt contained any *C. sakazakii*, these results are similar with that reported in another study [34]. A possible reason for *C. sakazakii* not being detected in yoghurt samples could be due to its sensitivity to low pH [10]. In addition, *C. sakazakii* could not be detected from ice cream as previously described [9,35]. As indicated from some researchers that *C. sakazakii* is unable to grow in ice cream during frozen storage [10]. Although *C. sakazakii* had been reported in ice cream samples at range of (4% - 26.6%) [10,28].

In the current study, only one sample of tested Maassora cheese was contaminated by *C. sakazakii*. This finding was lower than that reported in other studies [2,9,10,28] where *C. sakazakii* was observed in (30%), (4%), (40%) and (23.3%) of cheese samples respectively. In contrast, other studies failed to isolate this bacterium from cheese samples [34,36].

The present investigation showed that baby food (cereal baby food and ready to feed baby milk) was contaminated with *C. sakazakii* at rate of (3.7%). This finding is in agreement with another study that found *C. sakazakii* in two cereal-based

infant drinks [37]. However, no *C. sakazakii* was found in wheat-based infant food [30]. Moreover, *C. sakazakii* was not detected in milk powder samples because of its heat treatment used in the final stage which could eliminate most of pathogenic bacteria [38].

The contamination of cereal baby food and ready to feed baby milk with *C. sakazakii* may result from addition of some ingredients sensitive to the heat after pasteurization step such as dried fruit, also the infant rice cereal when reconstituted with water, milk or infant formula supports the growth of *C. sakazakii* [39].

Molecular confirmation of the recovered *Cronobacter* isolates was done by application of the PCR assay based on the partial amplification of 16S rDNA and using of universal oligonucleotides primers where the specific 464 bp band has been documented according with that reported before for the same genus *Cronobacter* using the same specific primers and 16S rDNA PCR protocol [20]. Results showed in **Table 1** confirming the first report for detection of *Cronobacter spp*. by cultural and molecular techniques from baby food, milk and meat product samples collected from different localities in Libya. The partial sequencing of 16S rDNA confirmed that all 10 isolates were *Cronobacter spp*. with 99% nucleotide identity. Only 6 out of 261 (2.2%) isolates were *C. sakazakii* that obtained from raw she-camel's milk, cereal baby food, beef burger and fermented milk (Laban) while the remaining 4 (1.5%) isolates were C. pulveris that were isolated from raw cow's milk, Maassora cheese and ready to feed baby milk.

First isolation of *Cronobacter pulveris* was from baby food [40]. It was suggested that these isolates have a similarity with *C. sakazakii* depending on the chromogenic media, biochemical tests and comparative assay between 16S rRNA and rpoB gene sequence analysis. Later they re-classified these strains as a novel species *Cronobacter pulveris*.

The antibiotics susceptibility test of the *Cronobacter spp*. revealed that most isolates were resistant to most of the tested antibiotics. This is an alarm indicating the increasing resistance of *Cronobacter spp*. to common used antibiotics and raise concerns for the possible consequence of that on public health.

Conclusion

In conclusion, this work on genus *Cronobacter*, reveals the important consideration for the food industry since this bacterium can cause severe illness to exposed people in particular the highly vulnerable neonates, infants and the elderly. The presence of *Cronobacter spp*. in dairy and meat products considered as potential risk to the public health and transmission vehicle of this organism, especially in countries such as Libya. In this study, ten *Cronobacter* isolates were recovered from 261 samples of milk and milk products based on the conventional method, yellow pigmentation and biochemical tests. These results indicate that conventional method, yellow pigmentation and commercially biochemical test panels are not sufficiently reliable for speciation of *Cronobacter* isolates at the species level and reliance on these methods will result in false positive and false negative identifications. Furthermore, the application of molecular technique plays a major role in identification of bacteria; also it improves and facilitates the control of most pathogenic bacterial disease including the foodborne disease. Interestingly, this assay could differentiate between species in the same genus. Moreover, this is first document reporting the presences of *C. sakazakii* in she-camel's milk. This is very important especially in developing countries such as Libya where raw she-camel's milk is traditionally consumed as raw milk without heat treatment.

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