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Investigate of the Ability of *Cronobacter sakazakii* Isolated from Clinical Samples of Children Under Two Years to Induce Swimming, Swarming and Biofilm

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Abstract

The study included 200 samples were collected from children under two years included (50 samples from each of Cerebrospinal fluid, Blood, Stool and Urine) from, Central Children Hospital and Children's Protections Educational Hospital. Isolates bacterial were obtained cultural, microscopic and biochemical examination and diagnosed to the species by using vitek2 system. The results showed there were contamination in 6.5% of clinical samples. The diagnosed colonies which gave pink color on the MacConkey agar, golden yellow color on the Trypton Soy agar and green color on the Birillent *Enterobacter sakazakii* agar and gave a probability of 99% in the vitek 2 and were identified as *Cronobacter sakazakii.* The identification revealed of thirteen isolates: 6(46.16%) isolated from Cerebrospinal fluid samples, 7(53.84%) isolated from blood samples and not isolated bacteria from stool and urine samples. The results of the investigation of some virulence factors showed that all bacteria isolates were able to swimming with a diameter ranging (1-9 mm) and swarming with a diameter ranging (1-40 mm) and their ability to biofilm formation by using three methods. The results show the ability of isolates to form biofilm by using Congo red media methods where it is 12 (92.30 %) out of 13 isolated bacteria belonging to *C. sakazakii* able to form biofilm on the Congo red media which is 3 (23.07%) were strong production biofilm , 8 (61.53%) were intermediate production biofilm and 1 (7.69%) were weak biofilm formation , while the 1 (7.69%) unable to form biofilm. Tubes method were all isolates were able to form biofilm, it were found that 3 (23.07%) isolates strong, and 8 (61.53%) intermediate and 2 (15.38%) weak biofilm formation. Microtiter plate method gave 5 (38.46 %) isolates strong, 6 (46.15%) intermediate and 1 (7.69%) weak biofilm formation.

Keywords: *Cronobacter sakazakii*, clinical samples, swarming, biofilm

1. Introduction

The genus *C.sakazakii* (formerly known as *Enterobacter sakazakii*), which belongs to the intestinal family Enterobactereacea. *C.sakazakii* is a Gram negative, cells are rod shaped, not formation of spores, moving by Peritrichous flagella, Is a formation of capsule, and its other properties are facultative anaerobic, negative for the tests of Indol, Oxidase and positive of Catalase, and its characteristic properties production of Glycosidase. Therefore, this enzyme was used differential characteristic on Chromogenic agar [1] . Cronobacter grows at a temperature ranging between 6-45˚C and optimal growth 37˚C [2]. *C.sakazakii* bacteria are present in the children's thermally tolerant milk, which varies from 58°C for 0.4 min [2] to 71 ° c for 2.6 min [3]. The bacterial resistance to dry, is characterized by high resistance compared to intestinal family (Enterobacteriacae) [4, 5] The generation time of the gene was 40 min at 23 ° c and 75 min at 25°C. Yellow colonies of *C.sakazakii* (TSA) are shown on the Trypton soy agar medium to produce yellow pigment. It produces a bright yellow after 24 hours of incubation at 25 ° c, more than 36 ° c and colonies (1-2) mm and (2-3) mm respectively for both degrees [6, 7]. One of the most significant symptoms of these bacteria is diarrhea and high fever [8]. These bacteria cause meningitis, necrotizing enterocolitis and septicemia in infants [9], especially children under the age of 28 days with more mature children and newborns weighing 2.5 kg. [10]. The study aimed to detection of some of the virulence factors including the formation of Biofilm, swimming and swarming.

2. Experimental

2.1. Materials and Methods

2.1.1. Sampling:

200 samples from different clinical sources were collected from the City of Medicine Complex (Child Protection Hospital) and the Central Child Hospital (50 Stool samples, 50 Blood samples, 50 Urine samples, 50 Cerebrospinal fluid sample(CSF).

2.1.2. Culture media:

Brain Heart infusion broth (BHIA) Brilliant Enterobacter sakazakii agar (BESA) , MacConkey agar and Trypton soy agar (TSA) ,swimming media(10g tryptose, 5 g Nacl and 1.5 g agarose in 1 L distilled water and swarming media (8 g nutrient broth ,5 g glucose and 5 g agar in 1 L distilled water)[11]

2.1.3. Culture of Sampling:

Blood samples and CSF samples were inoculated on the brain Heart Infusion broth and incubated for 24 hours at 37 ° C, then inoculated on the MacConkey agar medium. The stool samples and urine samples were inoculated directly on MacConkey agar and incubated for 24 hours at 37 ° C, after which all colony were inoculated on the Trypton soy agar medium and incubated at 25 ° C for 72 hours. Finally, they were cultured on the Brilliant Enterobacter sakazakii agar and incubated at 37 ° C for 24 hours, according to [12].

2.1.4 Diagnosis with Vitek-2 diagnostic kit

2.1.4.1. Biofilm formation:

The samples were cultured on Congo red agar medium and incubated for 24 hours at a 37 ° C according to [13] . Tube method and Microtiter plate method according to [14] and [15], respectively.

2.1.4.2. Swimming and Swarming

Swimming test, its ability the *C.sakazakii* to induce movement, the isolates were cultured on swimming media and incubated for 8 hours at 37 ° C. It was also cultured on the swarming media to test its ability to induce the swarming and incubated for 12 hours at 37 m. Then measured the movement of the colonies in millimeters using the ruler for both tests [13].

3. Results and Discussion

The results were shown after the samples were cultured on the MacConkey agar, then diagnosed as a preliminary diagnosis on the TSA and on the BESA medium. The colonies were diagnosed by microscopic and using the biochemical tests and the confirmation of the use of the Vitek 2-Vitek. (200) of the sample and found that there were 13(6.5%) samples gave a positive result of *C.sakazakii* while there were 187(93.9%) samples gave a negative result Figure (1).

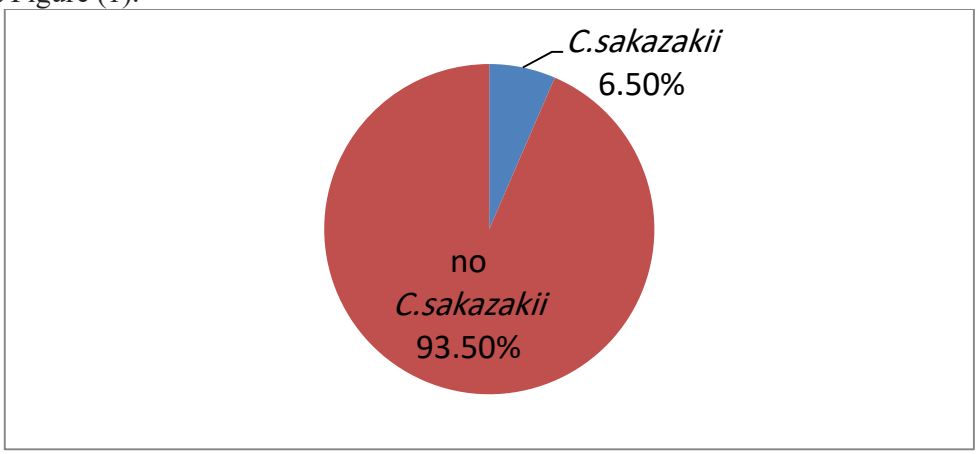

Figure (1): The presence of *C.sakazakii* bacteria in clinical samples.

6(46.16%) isolates of spinal cord fluid and 7(53.84%) isolates of blood were obtained from *C.sakazakii*. The bacteria were not obtained from the stool and urine samples shown in Table (2)

Table (1): Source of *C.sakazakii* isolates, the number of isolates of each source and the percentage of clinical isolates.

Source	No. Isolation	*C.sakazakii*	
		NO.	%
CSF	50	6	46.16
BLOOD	50	7	53.84
STOOL	50	0	0
URINE	50	0	0
total	200	13	-

Culture of *C.sakazakii*, The pink isolates appeared on the MacConkey agar. This indicates that it is fermentation of lactose and gold yellow pigment on the TSA as shown in Figure 2. When cultured on the BESA It appeared in green as in Figure (3). The colonies were distinguished in circular form as having a head with moderate edge. These features were attributed to the colonies of *C.sakazakii*.

Figure (2): *C.sakazakii* on TSA

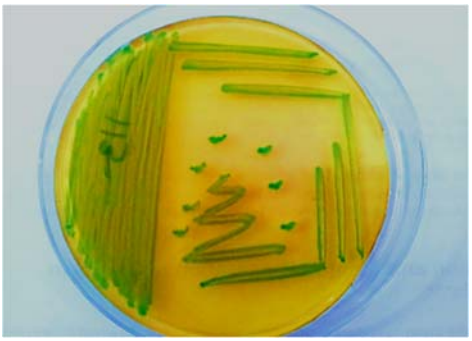

Figure (3): *C.sakazakii* on BESA

The bacterial cell was then dyed by gram stain, as they were characterized as small shaped gram negative, whose cells were either single or double shaped, not formation spores [5]. Bacterial isolates were then identification by biochemical tests [16]. Biochemical tests all isolates *C.sakazakii* gave negative indol test and oxidase test, but gave a positive test for amino acid such as Ornithine and Ariginine which show the production of an enzyme ornithine decarboxylase and ariginine decarboxylase ,and gave a test negative for the amino acid lysin, suggesting the lack of production of the enzyme lysin decarboxylase ,showed all isolates their ability to ferment sugars (Rhamnose, Melibios- D and Raffinose) and be yellow color indication of the ability to produce acid from suger [18], and use vitek2 for the purpose of diagnosis of bacterial isolates, vitek-2 showed that all isolates belonged to *C.sakazakii* , with a probability of 99%.The results of the current study conducted on Iraqi children under two years in the hospitals mentioned in the study that the presence of *C.sakazakii* in the blood was 14% while 12% of the scf out of 200 samples of children under two years .There were 13 cases of *C.sakazakii* infection and 26%. in a local study of *C.sakazakii by*(17)confirmed the presence of these bacteria (11.9%) in environmental and clinical samples ,and another study indicated the presence of *C.sakazakii* in the stool of people with diarrhea 3%(18) .While the World Health Organization (WHO,19) confirmed in 2006 that the annual rate of infection in the United States of America among infants under normal weight was 8.7 per 100,000 children. At the global level, there is no effective control system to overcome this pathogen. However, from 1961 to 2008, WHO recorded 120 cases of Cronobacter infection among infants and children under 3 years of age. [20]. Although only 120 cases have been reported worldwide, the number of cases of these bacteria is increasing. [21] A study confirmed that the infection of these bacteria threatens the lives of newborn infants (infants 4 weeks old) [22, 23] .Death rate between 40-80% [24, 25]. *C.sakazakii* causes meningitis in infants and is fatal within hours or days of birth [26] and surviving children often suffer from neurological disorders [27]. The incidence of children with *C.sakazakii* is 13% in newborns. Infection of children under the age of 2 years is due to their low weight, weak immune system and feeding on dried baby milk, which leads to bowel necrosis, Ranging between 5-10% and meningitis at 80-40% [28]. This is indicated by [29] that Cronobacter bacteria have the potential to cause meningitis, necrosis of the intestines and bacteremia, or transmission from mother to child during cesarean delivery [30]. While [31, 32] reported that the infection may occur by vagina during normal delivery and that Cronobacter may be part of vaginal infections.

3.1. Swimming and swarming motility of C.sakazakii

The results showed that the ability of *C.sakazakii* isolates to motility, 13 isolates able to swimming and swarming in different diameters on different media.

The results showed that 3 isolates (B5, CSF1, and CSF3) had a diameter of 5 mm and two isolates (B6 and B7) had a diameter 6 mm and 5 isolates (B1, B2, CSF4, CSF5 and CSF6) with a diameter of 7 mm and two isolates (B4 and CSF2) with a diameter of 8 mm and one isolation of B3 with a diameter of 9 mm.

Swarming showed that the isolation of one (B7) was a diameter of 40 mm and (CSF6) was 36 mm, this is a strong movement. While the 4 isolates (B3 and B4 and CSF1 and CSF5) were 24, 20 and 26 mm respectively, this movement is moderate, while the movement of 7 isolates (B1, B2, B5, B6, CSF2, CSF3 and CSF4) was 18, 12, 10, 13, 18, 10 and 13 mm respectively, Figure (3).

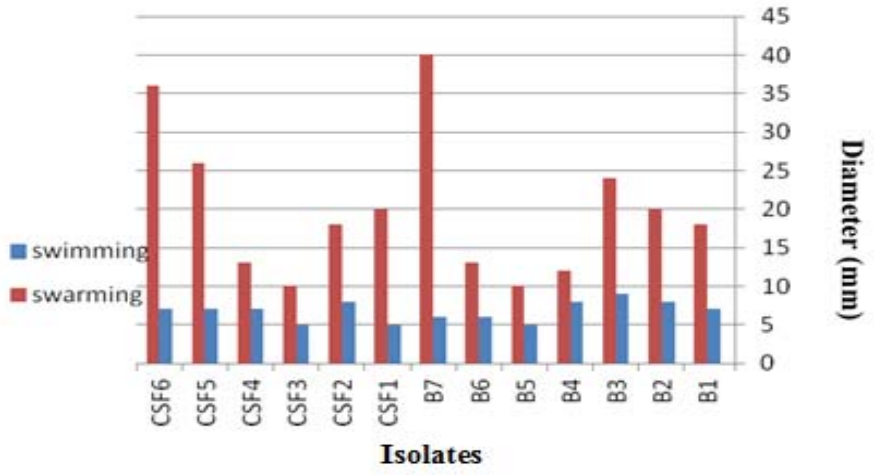

Figure (4): The ability of *C.sakazakii* isolates to swimming and swarming B: Blood, CSF: Cerebrospinal fluid.

The results showed that *C.sakazakii* bacteria have the ability to induce swarming greater than their ability to induce swimming (9-5 mm) for all isolates. There are significant differences between swimming and swarming [11]. [33] showed that there was no significant difference between bacterial isolates when measuring the swimming on a medium containing 0.3% of agar, while there were significant differences between the same isolates when measuring swarming on the media contains 0.5% of the agar. [34] noted that the *C.sakazakii* bacteria had the ability to swarm and diameter 73 mm after 6 hours of incubation in the media containing 0.3% of the agar. While [35] reported that the ability of *C.sakazakii* bacteria on the swimming was 15.7 mm in wild strain.

The results showed that the isolates (B3, B4, and CSF2) showed a decrease in swarming ability while high in swimming. [11] showed The *C.sakazakii* l3101 strain was the most swimming of the *C.sakazakii* G362 which gave the swarming more than the *C.sakazakii* strain L3101, which also indicated [36] that ST1 *C.sakazakii* isolated from the milk samples had a greater ability to swarm more than the strain isolated from clinical samples, while the strain *C.Sakazakii* ST4 isolated from clinical samples has the ability to swim more than the isolated strain of milk samples has been this is due to adaptation.Bacteria have the ability to swarming because it is able to form Biofilm formation [37]. This is what is indicated by [11] and [32] that flagella have a role in swimming which has a significant role in the formation of the biofilm and the swarming. Several studies have confirmed that

there are genes that are encoded into the methyl-accepting chemotaxis protein (mcp), which regulates the movement of *C.sakazakii* bacteria as it acts to induce changes in the whiplash circulation of bacteria [38, 39]. And [40] reported that the movement is a virulent agent for many pathogenic bacteria. The mutation in the mcp gene in *C.sakazakii* ATCC29544, which plays a major role in the colonization and invasion of the organism**.**

3.2. Formation of the biofilm:

Three methods were used to test the ability of bacteria to form the biofilm and to identify the difference in the three methods.

The isolation of *C.sakazakii* isolates from clinical samples was tested on the Congo red agar. The result was based on giving dark brown colonies with dry crystalline density a positive result, whereas colonies that gave color pink is a negative result of this test.

The results of the study showed that 12(92.30%) isolates out of 13 isolates belonging to *C.sakazakii* were able to form the biofilm. Three (23.07%) isolates were active, which gave colonies of high blackness, 8(61.53%) isolates, which gave colonies of medium blackness, and one(7.69%) isolation , which gave colonies with very little blackness, while a one (7.69%) isolation was unable to form the biofilm, which gave pink colonies as shown in Table (2) and (4**).**

Table (2): The ability of *C.sakazakii* bacteria to form of biofilm on the Congo red media

Congo red agar	Isolation	Congo red agar	Isolation
(+++)	CSF1	(+++)	B1
(++)	CSF2	(++)	B2
(+)	CSF3	(++)	B3
(++)	CSF4	(++)	B4
(++)	CSF5	(+++)	B5
(++)	CSF6	(-)	B6
		(++)	B7

(+++): Black colonies,(++): colonies of medium black,(+): colonies of a few black, (-): pink colonies
B: Blood, CSF: Cerebrospinal fluid

[36] indicated that 31% of the bacterial isolates of *C.sakazakii* had the ability to bind red Congo, while 12.5% of the bacterial isolates showed a reduction in the association with the red Congo dye and 56.5% did not have the ability to bind to the dye The association with the red dye of the Congo is also a factor of virulence, indicating that isolated bacterial isolates from clinical samples had a high correlation with red pigment compared with isolated bacteria from PIF samples due to environmental adaptation.

The tubes method was used to test bacterial isolates from clinical samples on the formation of the biofilm. The positive result was based on the appearance of adherent cells on the walls and bottom of the test tubes. The absence of adherent cells on the walls and bottom of the tubes indicates the negative result as shown in Figure 4.

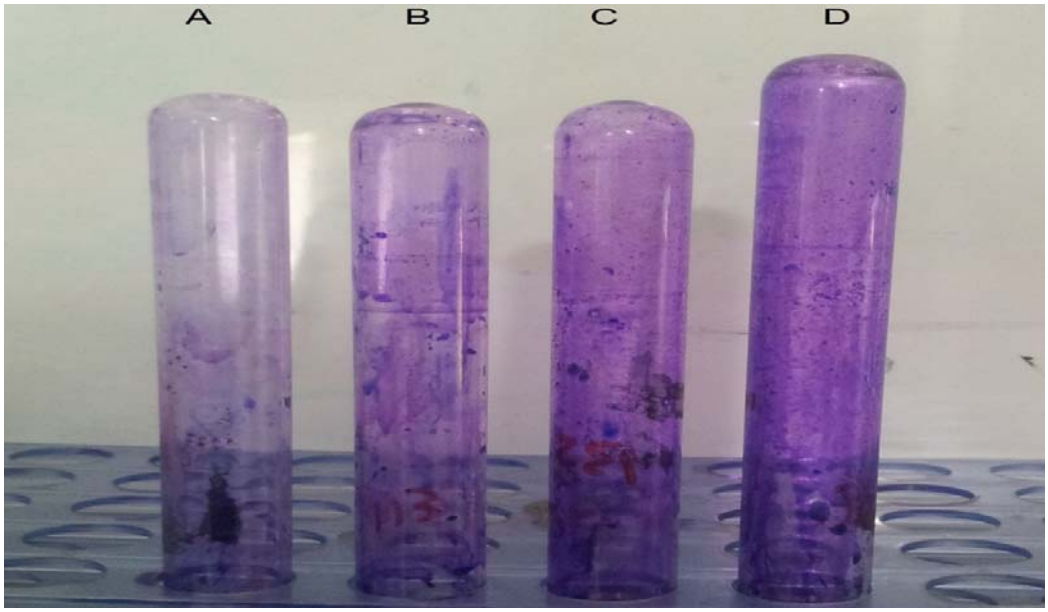

Figure (5): the ability of *C.sakazakii* bacteria for the formation of biofilm by tubes method A: Negative control, B: (+) weak biofilm formation: C: (++)medium biofilm formation D: Strong biofilm formation.(+++)

The results showed that 13 isolates of *C.sakazakii* showed 100% ability to form the biofilm. Three (23.07%) isolates were highly bioactive, 8(61.53%) isolates were medium and 2 (15.83%) were weak, shown in Table (3) and (4).

Table (3): The ability of *C.sakazakii* bacteria to form the Biofilm by tubes method.

Tube method	Isolation code	Tube method	Isolation code
(+++)	CSF1	(+++)	B1
(++)	CSF2	(++)	B2
(++)	CSF3	(+)	B3
(++)	CSF4	(++)	B4
(++)	CSF5	(+++)	B5
(++)	CSF6	(+)	B6
		(++)	B7

(+): weak biofilm formation, (++): medium biofilm formation, (+++): strong biofilm formation
B: Blood, CSF: Cerebrospinal fluid.

The bacterial isolates were examed to the formation of biofilm by using microtiter plate method and measured using ELISA with a wavelength of 630 nm.
The results showed that 13 isolates of *C.sakazakii* were 100% showed their ability to form the biofilm. Five (38.46%) isolates were highly biofilm formation, 7(53.84%) isolates were medium and one (7.69%) isolation was a weak as shown in Figure 5.

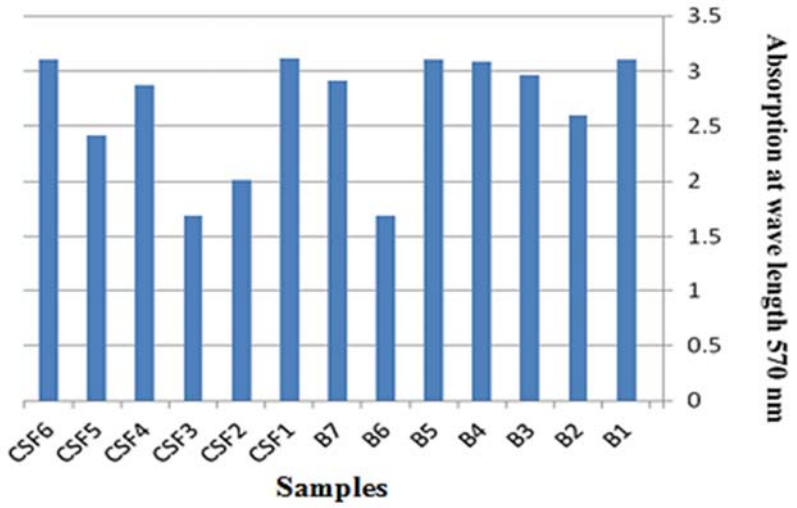

Figure (6): The ability of isolates of *C.sakazakii* bacteria to form the bioflim by the Microtiter plate method, B: Blood, CSF: Cerebrospinal fluid.

Table 8 shows that the method of microtiter method is more efficient in measuring the ability of *C.sakazakii* to form the biofilm than the other methods, as indicated by [11] when comparing the methods of measuring the formation of the biofilm .The microtiter was 14.4% while 11.8% and 1.97%for TM and Congo method respectively, this was due to the difficulty of measuring the biofilm by TM because it isnt able to distinguish the growth in tube when the isolates are weak and isolates that are unable to form the biofilm and there is no relationship between Congo red method and the tube method and the Microtiter plate.

Table (4): Percentage of the ability of *C.sakazakii* isolates to form Biofilm

The ability of bacteria to form a biofilm	Congo red method		Tubes method		Microtiter plate method	
	number	The ratio%	Number	The ratio%	number	The ratio%
Strong	3	23.07	3	23.07	5	38.46
Medium	8	61.53	13	68.42	7	53.84
Weak	1	7.69	2	10.53	1	7.69
Do'nt ability to form	1	7.69	-	-	-	-
Total	13	100%	13	100%	13	100%

It is clear from the above that the isolates have shown a difference in their ability on the formation of the biofilm, as most of the isolates have a medium active to form the biofilm. This is indicated by [36] the ability of *C.sakazakii* to form the biofilm differently, 119(70.8%) isolates showed high ability to biofilm formation, while 32(19.1%) isolates were medium and 17(10.1%) have weak ability to the formation of the biofilm when using the microtiter plate method, indicating that the temperature has an effect on the formation of the biofilm, where 15 isolates out of 35 isolates have a high ability to form the biofilm at 28 ° C and medium at 37 ° C and showed that 20 isolates were highly ability of forming the biofilm at 37 ° C and medium at 28 ° C . The formation of the biofilm depended largely on conditions such as temperature, pH and incubation time [41].
The formation of the biofilm in *C.sakazakii* and other bacteria varies between of one the species and this depends on the media and the nature of the surface bacteria [42] . [41,43,44,45 and 46] formation of biofilm is affected by the availability of nutrients for

bacteria, and *C.sakazakii* have the ability to form biofilm in the equipment used in the preparation of powdered baby milk and adhesion to different surfaces including glass, silicon and others .

The presence of a number of genes on the bacterial genome and plasmid can effect on biology processes and virulence factors such as movement, biofilm formation [42] . [35] The presence of methyl-accepting chemotaxis protein (mcp) in *C.sakazakii* (ATCC29544) plays a role in some of the virulence factor , movement and biofilm,

[11] showed that the difference in virulence of *C.sakazakii* strains was due to the presence of some proteins called membrane proteins. When comparing *C.sakazakii* G362 with *C.sakazakii* L310, there was an increase in the amount of proteins in the G362 strain compared with the L310

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