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Isolation of *Cronobacter sakazakii* from different herbal teas

Izolacija *Cronobacter sakazakii* iz raznih biljnih čajeva

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Abstract

Bacground/Aim. *Cronobacter sakazakii* (*C. sakazakii*) is an emerging food-borne pathogen that has increasingly raised interest among the whole public community and food industry, especially in the production of powder infant formula. It has been isolated from water, sediment and soil. The question is whether this pathogen can be present in herbal teas. Herbal teas are widely used for great number of health problems, as an additional or sometimes only "medicine" given. The aim of this study was to investigate the presence of *C. sakazakii* in herbal teas which are traditionally used for all restricted populations, including newborns and immunocompromised infant and adults. **Methods.** In this study 150 samples of dried herbal teas were tested: *Children (Baby) tea* (11), *Althaea officinalis* (7), *Sennae folium* (4), *Mentha piperita* (8), *Hypericum perforatum* (3), *Thymus serpyllum* (5), *Matricaria recutita* (6), *Fruit tea* (18), *Black, Green and Rooibos tea* (11), *Salvia officinalis* (9), *Arctostaphylos uva ursi* (5), *Urtica dioica* (3), *Achillea millefolium* (2), *Melissa officinalis* (4), *Cynosbati fructus* (3), *Flower Herbal tea* (3) *and* 17 *different mixtures of tea* (48 samples). The presence of *C. sakazakii* was also investigated in previously positive samples of prepared teas (48 samples) after 2 h, 12 h and 24 h. *C. sakazakii* was isolated by the use of the official method ISO TS 22964 : 2006 and confirmed with the biochemical test API 20E (Biomerieux- France). **Results.** The obtained results showed that *C. sakazakii* was isolated from 48 (32%) samples dried herbal teas. *C. sakazakii* was not isolated only from 2 (4%) of the 48 tested samples of prepared tea and in 46 (96%) of the samples *C. sakazakii* remained viable after 2 h, 12 h and 24 h. **Conclusion.** Herbal teas should be carefully used, especially for infants and immunocompromited people with severe chronic diseases because of the possibility of infection by *C. sakazakii*. Better control and improve testing as well as new facts about this pathogen are necessarry.

Key words:
herbal medicine; enterobacter sakazakii; risk assessment; microbiological techniques.

Apstrakt

Uvod/Cilj. *Cronobacter sakazakii* (*C. sarazakii*) je patogeni mikroorganizam poreklom iz hrane koji je privukao pažnju celokupne javnosti i prehrambene industrije, pogotovo u proizvodnji mleka u prahu za ishranu odojčadi. Izolovan je iz vode, mulja i zemljišta. Postavlja se pitanje da li ovaj patogen može da se nađe u biljnim čajevima. Biljni čajevi se koriste kao pomoćna sredstva u lečenju različitih zdravstvenih problema, ponekad kao jedini „lek" koji se uzima. Cilj ovog rada bio je da se istraži prisustvo *C. sakazakii* u biljnim čajevima koji se po tradiciji koriste u lečenju svih rizičnih grupa, uključujući novorođenčad, imunokompromitovanu odojčad i odrasle. **Metode.** U ovom radu ispitano je 150 uzoraka biljnih čajeva**:** *Children (Baby) tea* (11), *Althaea officinalis* (7), *Sennae folium* (4), *Mentha piperita* (8), *Hypericum perforatum* (3), *Thymus serpyllum* (5), *Matricaria recutita* (6), *Fruit tea* (18), *Black, Green and Rooibos tea* (11), *Salvia officinalis* (9), *Arctostaphylos uva ursi* (5), *Urtica dioica* (3), *Achillea millefolium* (2), *Melissa officinalis* (4), *Cynosbati fructus* (3), *Flower Herbal tea* (3) i 17 različitih mešavina čajeva (48 uzoraka). Ispitano je i prisustvo *C. sakazakii* u 48 uzoraka čajeva koji su pre pripreme bili pozitivni, posle pripremanja 2, 12 i 24 sata. Za izolaciju *C. sakazakii* korišćena je zvanična preporučena metoda ISO TS 22964 : 2006 uz potvrdu biohemijskim testom API 20E (Biomerieux-Francuska). **Rezultati.** Dobijeni rezultati pokazali su da je *C. sakazakii* izolovan iz 48 (32%) uzoraka biljnih čajeva. U ispitivanju pripremljenih čajeva koji su pre pripreme bili pozitivni, samo kod 2 od 48 (4%) uzoraka posle pripreme nije moglo da se dokaže prisustvo *C. sakazakii,* dok je kod 46 uzoraka (96%) potvrđeno prisustvo ove bakterije i posle 2, 12 i 24 sata. **Zaključak.** Biljne čajeve treba oprezno koristiti posebno za decu i imunokompromitovane osobe sa teškim hroničnim bolestima, jer postoji mogućnost infekcije bakterijom *C. sakazakii*. Neophodni su bolja kontrola i testiranje, kao i nova saznanja o ovom patogenu.

Ključne reči:
medicina, biljna; enterobacter sakazakii; rizik, procena; mikrobiološke tehnike.

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Introduction

Cronobacter sakazakii (*C. sakazakii*) is Gram-negative, nonspore-forming bacterium belonging to the *Enterobacteriaceae* family. This is an emerging food-borne pathogen that has increasingly raised interest among the whole public community and food industry, especially in the production of powdered infant formula. The natural habitat of *C. sakazakii* is presently unknown. It has been isolated from water, sediment and soil. In a large survey of over 500 foodstuffs and ingredients, a large proportion (~25%) of herbs and spices were shown to contain *C. sakazakii* [1].

C. sakazakii has subsequently been implicated in neonatal and infant cases of meningitis [2, 3], septicemia [4] and necrotizing enterocolitis [5]. Among infants, those most at risk of infection with *C. sakazakii* are: neonates (first 28 days), particularly infants born prematurely; low birth-weight infants; immunocompromised infants; infants of HIV-positive mothers [6]. Among adults, 50% of reported cases had underlying malignancy, or some other severe chronic disease [7]. Severe consequences of infection in some cases may be linked to the production of enterotoxins by *C. sakazakii*. More than 20% of the 18 strains tested in a research project produced enterotoxins [8]. There would appear to be variations in virulence among *C. sakazakii* strains, and some strains may be non-pathogenic [9]. Although fecal carriage may last for 8–18 weeks, secondary transfer is not known to occur [10]. A survey of powdered infant formula from 35 countries showed that more than 14% of samples were positive for *C. sakazakii*. The levels of contamination were low – less than 1 cfu/100 g in 17 of the 20 positive samples [11]. Infective dose is unknown, but it depends on infected organism – its age, health condition, secondary and chronic disease. The survival of *C. sakazakii* in powdered infant formula may be partially due to the ability of the microorganism to survive very low water activity (0.14–0.27 after the process of drying) during manufacture and afterwards [1, 11]. The stomach of neonates, especially of premature babies, is less acidic than that of adults, and this may be an important factor contributing to the development of infection in infants [12]. Better understanding of environmental reservoir(s) of a microorganism will help facilitate control of this pathogen. Some recent studies have suggested flies (fruit flies, stable flies) and rats as carriers [13, 14]. Pest control in manufacturing or production facilities may therefore be particularly important. One study in 2004 found *C. sakazakii* in 8 out of 9 manufacturing facilities producing a variety of dried foods, which suggests that this opportunistic pathogen is more ubiquitous than had been previously assumed, thereby making control more difficult. The highest percentage of positive samples were those of corn, soy, wheat and rice flours (17.9%), dried cereals (~30%) and dried vegetables and spices (20%) [15].

Because of its presence in the environment, there is a great risk of contamination of fresh herbal teas still growing on meadows. Its ability to survive high temperatures, and very dry conditions, can raise concern of its presence in dry herbal teas. It has been shown that this pathogen has high tolerance to desiccation. It has ability to grow at as low temperatures as 5.5°C and to grow at different storage temperatures according to Kim and Beuchat [16]. Their number can increase during the time on storage, but it depends of other factors like: pH, temperature, water activity [17–21]. Biofilm formation may enhance the resistance to sanitizers and allow a long-term presence in the manufacturing environment [1, 22]. Prepared herbal teas have neutral pH, high water activity and they are usually stored at room temperature for a few hours, so it can be a good media for growth and survival of *C. sakazakii*. It has the ability to adhere to a wide variety of surfaces used in food manufacturing. A recent United Kingdom study found that the number of *C. sakazakii* in reconstituted infant formula doubled every 10 hours in a fridge, while at room temperature it took just half an hour [16, 20, 23]. This means that any drink left out overnight could result in microbial growth to a dangerous level. This fact was used for this study to determine a possible presence of this bacterium in herbal teas.

Methods

The presence of *C. sakazakii* in herbal teas and its survival in the prepared tea were investigated in two parts: determination of the presence of *C. sakazakii* in 10 g of different types of dried herbal tea; determination of the presence of these bacteria after 2 h, 12 h and 24 h of storage of the prepared teas at room temperature.

Dried herbal tea

In this study, 150 samples of 17 different kinds of dried herbal teas were tested: *Children (Baby) tea* (11), *Althaea officinalis* (7), *Sennae folium* (4), *Mentha piperita* (8), *Hypericum perforatum* (3), *Thymus serpyllum* (5), *Matricaria recutita* (6), *Fruit tea* (18), *Black*, *Green and Rooibos tea* (11), *Salvia officinalis* (9), *Arctostaphylos Uva ursi* (5), *Urtica dioica* (3), *Achillea millefolium* (2), *Melissa officinalis* (4), *Cynosbati fructus* (4), *Flower Herbal tea* (3) *and* 17 *different mixtures of tea (*48 samples), but only those prescribed to be used for health problems, such as: for help in pain relief, for respiratory symptoms of those with some kind of flu, those with gastrointestinal, gynecology or endocrine symptoms.

Preparation of tea

Infusion. Fresh or dried herbs are mixed with boiled water (~100°C), and left for minimum 10 minutes. In some cases, liquid prepared like this can be poured into a jug, covered and stored in fridge for up to 2 days.

Decoction. Roots, berries and bark are covered with cold water, then boiled and left 25–40 minutes. It can be stored in fridge for days.

Macerations. Roots or dried herbs are mixed with cold and left to soak in it for few hours, usually overnight.

Isolation of C. sakazakii

The official method ISO TS 22964 : 2006 was used for isolation *Cronobacter sakazakii*.

Pre-enrichment

10 g of each sample of dried herbs or 10 mL of prepared tea were mixed with 90 mL of buffered peptone water

(BPW, Merck, Germany), and incubated at 37C° for 18 ± 2 hours.

Enrichment (selective step)

From the cultured buftered peptone meter 0.1 mL was transferred to a tube containing modified lauryl sulphate tryptose (mLST, Biokar- Italy) broth containing antibiotic vancomycin. This mLST selective broth was incubated for a period of 24 ± 2 hours at 44°C ± 0.5°C.

Selective + differential step

Streaking from incubated mLSTV broth onto *C. sakazakii* isolation media- COMPASS *E. sakazakii* agar (Biokar-Italy) and Chromocult *E. sakazakii* agar (Merck- Germany). The plates were incubated at 44°C ± 0,5°C for a period of 24 ± 2 h.

Identification of presumptive colonies

Typical colonies grown on cromogenic media were plating on a tryptic soya agar (Merck-Germany) plates following incubation at 25°C ± 1°C for 48 ± 4 h to enhance the production of yellow pigment, and then confirmed by biochemical tests API 20 E (BioMerieux- France). This test requires further 18–24 h to complete. This was a biochemical test kit, with sensitivity 98.3% and specificity 88.9%. Of course, whatever biochemical test kit is used, there is always a possibility to misidentify colonies which do not produce enough yellow pigment or remain totally white on trypticase soy agoir. The main reason for choosing API 20 E test was better detection of gelatinase positive colonies, which are totally unidentified with other biochemical tests (Biolog Micrologg 3 4.20).

Laboratory detection and recognition methods for infectious agents have developed at a remarkable pace in recent years, and several conventional *polymerase chain reaction* (PCR) assay formats have been developed to detected 16 S RNA gene of *C. sakazakii*. After standard enrichment, a sample is lysed to release nucleic acid which is then amplified and detected. Processing of a sample takes less than 4 hours. Molecular techniques are fast to perform with results generated in 1 day instead of 1 week with the classic method [15, 24].

Results

All the examined teas were ordinary samples which came for testing in the Center for Food Analysis in Belgrade, Serbia. There were all kinds of different teas, from teas especially made for children, to commercially made teas with different aroma and teas made for people with chronic diseases for additional help treatment. All the samples (150) were purchased from the local retailed stores in Belgrade.

In this study 150 samples of herbal teas were examined for the presence of *C. sakazakii* and 32% (48 samples) were positive (Table 1).

Table 1

The results of examination of dried herbal teas for the presence of *Cronobacter sakazakii*

Name of a dried herbal tea	Samples				
	Examined	Positive		Negative	
	n	n	%	n	%
Children (Baby) tea	11	5	46	6	54
Althaea officinalis	7	2	29	5	71
Sennae folium	4	3	75	1	25
Mentha piperita	8	4	50	4	50
Hypericum perforatum	3	1	33	2	67
Thymus seryllum	5	2	40	3	60
Matricaria recutita	6	3	50	3	50
Fruit tea	18	2	11	16	89
Black, Green and Rooibos tea	11	3	27	8	73
Salvia officinalis	9	2	22	7	78
Arctostaphylos uva ursi	5	0	0	5	100
Urtica dioica	3	0	0	3	100
Achillea millefolium	2	0	0	2	100
Melissa officinalis	4	2	50	2	50
Cynosbati fructus	3	2	67	1	33
Flower herbal tea	3	0	0	3	100
Mixtures of tea	48	17	35	31	65
Total	150	48	32	102	68

Of all the examined samples, only 3 dried herbal teas: *Arctostaphylos uva ursi, Urtica dioica, Achillea millefolium* and *Flower Herbal Tea* did not contain *C. sakazakii.*

Those positive samples were again tested, but after reconstitution as prescribed on labels, and only 2 (~4%) of the prepared teas were negative for the presence of *C. sakazakii* (Table 2). Both samples were herbal teas of *Melissa officinalis* and all other teas showed the growth of *C. sakazakii* after 2 h, 12 h and 24 h storage at room temperature. It is possible that it was very low number of *C. sakazakii* in the sample of *Melissa officinalis* tea and after reconstitution with warm water, these bacteria had not survived.

Special care and attention was focused on teas for children and babies. Of 11 teas tested, 5 (45%) were positive. Those 5 (Baby tea for digestive problems – abdominal cramps

Table 2

The results of the presence of viable *Cronobacter sakazakii* in the prepared teas after 2 h, 12 h and 24 h of storage at room temperature

Name of a prepared tea	Samples						
	Examined	Positive				Negative	
	n	after 2 hours (n)	after 12 hours (n)	after 24 hours (n)	%	n	%
Children (Baby) tea	5	5	5	5	100	0	0
Althaea officinalis	2	2	2	2	100	0	0
Sennae folium	3	3	3	3	100	0	0
Mentha piperita	4	4	4	4	100	0	0
Hypericum perforatum	1	1	1	1	100	0	0
Thymus seryllum	2	2	2	2	100	0	0
Matricaria recutita	3	3	3	3	100	0	0
Fruit tea	2	2	2	2	100	0	0
Black, Green and Rooibos tea	3	3	3	3	100	0	0
Salvia officinalis	2	2	2	2	100	0	0
Arctostaphylos uva ursi	0	0	0	0	100	0	0
Urtica dioica	0	0	0	0	100	0	0
Achillea millefolium	0	0	0	0	100	0	0
Melissa officinalis	2	0	0	0	0	2	100
Cynosbati fructus	2	2	2	2	100	0	0
Flower herbal tea	0	0	0	0	100	0	0
Mixtures of tea	17	17	17	17	100	0	0
Total	48	46	46	46	96	2	4

in the first 3 months of life) were examined after a storage time and all remained positive for the presence of *C. sakazakii*.

It is interesting to examine other teas which are used as additional therapy to cure all kinds of health problems. *Althaea officinalis* is used to cure respiratory problems, especially for small babies, sometimes as a totall change in medical therapy. For this purposes, the root of this herb is usually prepared as previously described decoction, but left minimum overnight and then used for drinking or throat and nose flushing. *Althaea officinalis* tea samples were positive in 28%. Two more herbal teas are prepared usually with cold water: *Salvia officinalis* – used for healing a sore throat (22% positive for the presence of *C. sakazakii*) and 75% positive samples of *Sennae folium,* the drug which is used for better digestion and usually prepared with cold water (overnight).

Discussion

Herbal teas can be very contaminated products, with a various number of present microorganisms: *Clostridium spp*., *Enterobacteriaceae* – from *Salmonella* spp. to *Escherichia coli, Bacillus cereus*, yeasts and moulds [25, 26].

C. sakazakii produces a viscous extrapolysaccharide capsular material that enhances attachment and adherence to surfaces and provides a physical barrier protecting cells from environmental stresses: UV light, osmotic stress, detergent and antibiotics [27, 28]. This fact explains the ability of survival of *C. sakazakii* in a very dry environment, as shown by Breeuwer et.al [19] and Caubilla-Barron and Forsythe [29].

Tea production is a high by developed chain of industry and tea is sold worldwide. There are hundreds of different species and aromas. It is used traditionally for ages in some countries – as drink (hot and cold) and as medicine.

Because of tradition and old customs, tea is widely used in Serbia for all kinds of health problems in all categories of population. Many consumers are not aware that herbal teas can be contaminated and potentially very dangerous to human health, especially those prepared with cold water and left at room temperature. These conditions will increase a number of already present bacteria, including *C. sakazakii.* Herbal teas are known only as a cure for some health problems, so they are widely used as common drink for everyone, including infants. Among those consumers, most of them are immunocompromised people, people with different kinds of chronic diseases, tumors, HIV infected.

This study showed a high percentage of the presence of *C. sakazakii* in all kinds of herbal teas, especially those prescribed on label for infants and children. Some of those teas can be found on the open market, unpackaged, without traceability. Labels on tea packages for babies and children do not contain precise prescription for use, especially the age of children it is recommended to.

It is important for the manufactures of powdered infant formule to have a clearly defined criteria for production, in order to design the necessary preventive measures in a correct and appropriate way. Such references also are necessary to establish appropriate monitoring, as well as to validate the complex internal procedures to ensure the release of safe products that comply with regulatory requirements. There are microbiological criteria in the EC Regulation 2073/2005 only for the powdered infant formula, for a 10 g sample, n = 30, c = 0, m=0. All samples must be negative. If *C. sakazakii* appears in some of the tested samples, there are recommendations for testing environment samples to increase knowledge about the presence of this pathogen in the factory area [30].

Conclusion

The results in this study showed a high percentage of samples of herbal teas with the confirmed presence of *C.*

sakazakii. This fact can raise new questions and dilemma – whether and how to use herbal teas for infants or immunocompromised people, who already suffer from other severe chronic diseases. Theoretically, it is possible that some kinds of the prepared teas cause infection, but this have never been considered as a possibility. In Serbia there are no microbiological criteria for this pathogen, and it is not tested in any kind of foodstuff or environment samples. Improved surveillance, based on robust detection methods will facilitate much better control and improve testing the presence of *C. sakazakii* in a greater number of different food samples. New facts and knowledge about this pathogen will contribute to better control of its distribution and infectivity.

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