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Представлено результати виявлення бактерій *Cronobacter spp.* із сирого молока корів та з об'єктів молочних ферм. З досліджених проб було виділено та відібрано 25 ізолятів для ПЛР з 16S рРНК геном. Результати доводять, що середовище молочних ферм є потенційним джерелом цих мікроорганізмів в сирому молоці. Дані є корисними для оцінки мікробіологічного ризику щодо бактерій *Cronobacter spp.*

Ключові слова: рід *Cronobacter* (*Enterobacter sakazakii*), молочні ферми, сире молоко корів, ПЛР, 16S рРНК

Представлены результаты выявления бактерий *Cronobacter spp.* из сырого молока и объектов молочных ферм. Из исследованных проб было выделено и отобрано 25 изолятов для ПЦР с 16S рРНК геном. Результаты показывают, что объекты молочных ферм являются потенциальным источником этих микроорганизмов в сыром молоке. Эти данные будут полезны для оценки микробиологического риска относительно *Cronobacter spp.*

Ключевые слова: род *Cronobacter* (*Enterobacter sakazakii*), молочные фермы, сырое молоко коров, ПЦР, 16S рРНК

IDENTIFICATION OF CRONOBACTER SPP (ENTEROBACTER SAKAZAKII) FROM RAW MILK AND ENVIRONMENTAL SAMPLES OF DAIRY FARMS

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1. Introduction

In recent years, researchers have focused on studying *Cronobacter* genus (formerly *Enterobacter sakazakii* that has been reclassified into *Cronobacter spp.* in 2007) from *Enterobacteriaceae* family [1, 2]. This is a pathogenic Gram-negative, motile, non-spore forming, facultative anaerobic bacterium that causes fatal infection in newborns and infants up to the age of one year, and is transmitted through the consumption of dry infant formula. It can cause necrotizing enterocolitis, bacteraemia and meningitis, predominantly in neonates, particularly those who are premature or immunocompromised, resulting in mortality from 40 % to 80 % [3–5]. Ways of transferring this microorganism into dry infant formula are varied. According to the World Health Organization (WHO), there are three basic routes:

- poor quality of raw milk and other ingredients or their contamination with bacteria from *Enterobacteriaceae* family;
- contamination of raw milk and other ingredients from the environment (air, equipment) during processing or after heat treatment with these bacteria, or contamination of ready product during packaging and storage;
- inadequate hygiene practice during the preparation of powdered infant formula at home [6].

Cronobacter spp. is recognized internationally as a dangerous pathogen, and its definition is required for the production of dry infant formula. Therefore, control of *Cronobacter*

should be carried out at all levels of the food chain, including the microbiological safety of raw milk for preparation of dry infant formula [6].

Cronobacter genus (formerly *Enterobacter sakazakii*) has been frequently isolated from different environmental sources, including soil, water, various foods (meat and dairy products, vegetables, grains, herbs, spices, and other), facilities of food industry factories [7–11]. After performing systematic review and meta-analysis of published articles, it was reported that *Cronobacter* genus is more prevalent in plant-related sources with an overall prevalence rate of 20.1 % than the animal-originated sources with an overall prevalence rate of 8 % [12]. Some scientists tend to believe that the gastrointestinal tract of the humans, animals, insects and rodents is the natural harborage site for *Cronobacter* genus. Fecal-carriage bacteria can survive in soil and water for up to 120 days [13, 14].

Therefore, contamination of raw materials and finished products with water, dust and other objects from the environment can occur. Thus, the potential for product contamination exists throughout the food production chain, including dairy farms and milk processing plants [6, 15].

Although *Cronobacter* genus has been isolated from a variety of food, food ingredients, food production environments, animal feed and other environmental sources in different countries, there is a lack of information about the presence of *Cronobacter* in raw cow's milk and facilities in dairy farms in Ukraine.

2. Literature review and problem statement

Prevalence data for *Cronobacter* genus from raw milk are varied. The first report of isolation of these bacteria from raw milk was published in 1990 [16]. In later research, it was reported that 63 % of raw milk collected from different dairy farms were positive for *Cronobacter spp* [17]. Several authors [15] also isolated microorganisms of the family *Enterobacteriaceae* including bacteria *Enterobacter sakazakii* from raw cow's milk. As others have highlighted, 10.7 % of market milks were contaminated with *Enterobacter* species, including *Enterobacter gergoviae*, *Enterobacter sakazakii* and *Enterobacter cancerogenus* [18]. In contrast, some researchers were unable to isolate *Enterobacter sakazakii* (*Cronobacter* genus) from raw cow's milk [10]. Related results about the occurrence of *Cronobacter spp.* in raw milk were mentioned in research of other authors [19]. Other observations indicate cases of detection of *Cronobacter spp.* in different foods including milk powders and infant formula manufactured and marketed in the Netherlands [20]. There is still considerable uncertainty with regard to the presence of *Cronobacter spp.* in raw milk. Some investigators published their data about the presence of *Enterobacteriaceae* and especially *Cronobacter spp.* in products other than milk powders and infant formula. A total of 100 raw milk samples were tested for the presence of *Cronobacter spp.* but a variety of members of the *Enterobacteriaceae* family were observed in samples except *Cronobacter spp.* These bacteria were isolated from milk powder only [21]. As others have highlighted, *Cronobacter spp.* was isolated from the facilities of the dairy farm [22]. They investigated the objects of livestock farms where cattle and pigs were kept and it was established that these animals were sources of above-mentioned microorganisms in products of animal origin [22, 23]. According to their data, bacteria *Cronobacter spp.* were not isolated from the feces of cattle, the soil of the farm and water, but they were isolated from other objects such as animal feed, indicating a possible source of the pathogen in the animal's body. A number of studies have found that bacteria *Cronobacter spp.* were identified from the udder of cows with mastitis, and from dairy equipment for the production of dairy products [24].

As seen from the above, there is a large number of scientific reports on the detection of bacteria *Cronobacter spp.* (*E. sakazakii*) from various environmental samples and from a wide range of foods.

Methods of isolating *Cronobacter spp.* are based on isolating typical yellow colonies on Trypticase Soy Agar and to confirm its by biochemical properties. *Cronobacter* genus has broad phylogenetic relationships (Gram-negative, oxidase-negative, catalase-positive, nonspore-forming rods that, in general, are motile, able to reduce nitrate to nitrite and to produce acetoin (Voges-Proskauer test), and negative for the methyl red test) with other members of the genus *Enterobacter* (*E. cloacae*, *E. herbicola*, *E. agglomerans*) and genus *Pantoea* (*P. agglomerans*). The foregoing indicates that classical methods do not always allow to do clear differentiation of representatives of this type of bacteria. The most reliable at present are considered molecular genetic methods based on detecting specific properties of microorganisms that are encoded in their genes. These methods include polymerase chain reaction [1, 3, 4]. According to international scientific literature, several target genes (*16SrRNA*, *gluA*, *ompA*, *dnaG*, *gyrB*; *MMS atpD*, *fusA*, *glnS*, *gltB*, *gyrB infB*,

ppsA, operon (*dnaG*, *rpsU*, *rpoD*)) are used for molecular typing and identification of this pathogen [25, 26].

Due to the fact that the study of bacteria *Cronobacter spp.* in Ukraine was recently launched, in the arsenal of national laboratory methods there are no modern diagnostic tests to detect them.

3. The aim and objectives

The aim of this study was to identify the presence of the *Cronobacter spp.* in bulk tank milk and facilities of dairy farms of Sumy region (Ukraine) as a potential source or transmission routes of this pathogen from animals or environment of dairy farms to the raw material.

The following objectives were pursued in this study:

- to investigate the distribution and level of contamination of *Cronobacter spp.* in bulk tank milk and dairy farms facilities in Sumy Region of Ukraine;
- to establish morphological, cultural, biochemical properties of isolates;
- to confirm strains by identification in PCR with amplification of 16S rRNA gene.

4. Materials and methods of isolation and identification of *Cronobacter spp.*

4. 1. Samples collection for investigation

A total of 418 samples were obtained from 5 dairy farms in Ukraine (Sumy region) between 2008 and 2015. The following samples were tested: bulk tank milk (175), secret of udder of cows with a subclinical form of mastitis (53) and environmental samples (50 swabs of milking machines, 43 swabs of milk tanks, 41 swabs from the surface of cow udder, 11 samples of animal feed, 45 swabs from the floor of livestock buildings.

All samples were collected in sterile conditions and carried at 4 °C to the laboratory.

Swabs of milking machines were taken from the inside part of the rubber which can have microorganisms in micro-cracks. Swabs from the surface of cow udder were taken after washing the udder before milk processing.

4. 2. Methods of isolation and identification of *Cronobacter spp.* from samples

All these samples were analyzed by a standard isolation method as outlined by the FDA [28]. In brief, the samples were first enriched in *Enterobacteriaceae* enrichment broth at 36 °C overnight; a loopful of enriched cultures was streaked on VRBG agar (violet red bile glucose agar; Himedia M581) plates and incubated overnight at 36 °C. Five presumptive *Cronobacter spp.* colonies producing yellow pigmentation were picked and re-streaked on TSA (Trypticase Soy Agar; Himedia M290) and incubated at 25 °C for 48–72 h. Typical yellow colonies were picked from TSA, resuspended in physiological saline and subjected to subsequent biochemical characterization using API 20E test strips (BioMerieux, France) according to the manufacturer's instructions.

4. 3. PCR reaction conditions for confirmation of *Cronobacter spp.* isolates

Twenty isolates of *Cronobacter* representing five isolates of bulk tank milk, nine environment isolates, one animal

feed, and five udder isolates of cows with a subclinical form of mastitis were verified by PCR amplification of 16S rRNA gene. All oligonucleotide primers derived from the genome of *Cronobacter spp* strain BAA-894 (Genbank accession number CP000783-5) that flank the gene fragments 16S rRNA were calculated using the VectorNTI, v.11.0.1 software (Invitrogen) and synthesized by ThermoHybaid BioSciences (Germany). To analyze the DNA nucleotide sequence homology, *Cronobacter* genes from other bacteria were compared using e-service BLAST 2.0 (NCBI) and the US module AlignX Software VectorNTI (Fig. 1).

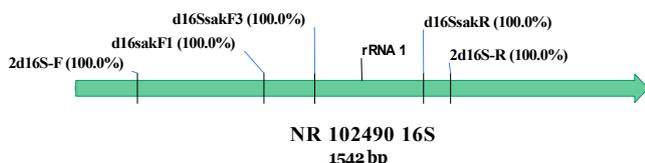


Fig. 1. Characteristics of homology of oligonucleotide primers at gene 16S rRNA *Cronobacter spp* (genome of *Cronobacter sakazakii* strain BAA-894):

- 16sakF1 – TAACTCCGTGCCAGCAGCC (451 bp),
- d16SsakR – TAAACACATGCTCCACCGCT (451 bp),
- d16SsakF3 – GGCAGGCTTGAGTCTCGTAG (314 bp),
- 2d16S-F – AGCTAATACCGCATAACGTCTACG (863 bp),
- 2d16S-R – AGGCACTCCCGCATCTCTG (863 bp)

For amplification, reaction mixtures containing primers were prepared by using *Taq* reaction mixture as before (reference or given details including the amount of template DNA, primers, dNTPs, and other components). Thermal cycling was carried out by using an initial denaturation step of 94 °C for 3 min (1 cycle), followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at (given temperature) for 1 min, and elongation at 72 °C for 1 min 30 sec. Cycling was completed by a final elongation step at 72 °C for 4 min. The reaction products were resolved on a 1.5 % agarose gel followed by ethidium bromide staining and examination under UV light.

5. Research findings on isolation and identification of *Cronobacter spp*

All presumptive *Cronobacter spp* colonies produce yellow pigment on VRBG and exhibited positive glucosidase activity when tested in API 20E test strips. Ukraine has established national standards for raw milk that are intended for dry infant formula production. The raw milk should be of grade (class) Extra or Higher and the total microbial counts in Extra and Higher grades should not exceed 1×10⁶CFU ml⁻¹, 3×10⁶CFU ml⁻¹, respectively. Here we analyzed 175 samples of raw cow’s milk that are graded Extra class and Higher class. The total microbial counts in these milk samples varied from 7.8×10⁴ CFU ml⁻¹ to 2.7×10⁵ CFU ml⁻¹. Analysis of 75 grade Extra milk revealed that 12 samples (16 %) were positive for *Cronobacter spp*, while 22 (22 %) samples from 100 Higher grade milk samples were positive. Altogether, 19.4 % of raw milk samples were positive for *Cronobacter spp*, as shown in Table 1.

A total of 53 samples of the secret of the udder of cows with a subclinical form of mastitis were analyzed, and 17 (32.1 %) of them were positive, as shown in Table 2. Rapid mastitis test (California mastitis test, CMT) was used for

detection of mastitis in cows. The results were confirmed by the counting of somatic cells13366-1(IDF 148-1:2008).

Table 1

Detection of *Cronobacter spp* from raw cow milk samples

Farms	Ranges of total bacterial counts (CFU ml ⁻¹)	Class of milk	No. of investigated samples	No. of positive samples	Percentages, %
Farm 1	7.8×10 ⁴	Extra	13	1	7.7
Farm 2	8.3×10 ⁴	Extra	15	2	13.3
	2.8×10 ⁵	Higher	57	13	22.8
Farm 3	9.5×10 ⁴	Extra	17	3	17.6
	2.7×10 ⁵	Higher	43	9	20.9
Farm 4	9.3×10 ⁴	Extra	15	2	13.3
Farm 5	9.4×10 ⁴	Extra	15	2	13.3
–	–	Extra	75	12	16
–	–	Higher	100	22	22
Total			175	34	19.4 %

Table 2

Results of detection of *Cronobacter spp* from secret of udder of cows with a subclinical form of mastitis

Farms	No. of investigated samples	No. of positive samples	Percentages, %
Farm 1	7	1	14.7
Farm 2	10	3	30.0
Farm 3	14	5	35.7
Farm 4	10	4	40.0
Farm 5	12	4	33.3
Total	53	17	32.1

A total 190 environmental samples (50 swabs of milking machines, 43 swabs of milk tanks, 41 swabs from the surface of cow udder, 11 samples of animal feed, 45 swabs from the floor of livestock buildings) were examined. As shown in Table 3, 73 samples (38.4 %) were positive.

Table 3

Results of detection of *Cronobacter spp* from environmental samples

Farms	No. of investigated samples	No. of positive samples	Percentages, %
Farm 1	30	5	16.7
milking machines	8	0	0
milk tanks	6	0	0
skin of the cow udder	5	0	0
animal feed	2	0	0
floor of livestock buildings	9	5	55.6
Farm 2	37	12	32.4
milking machines	12	2	16.7
milk tanks	7	0	0
skin of the cow udder	6	2	33.3

Initially, 20 isolates were identified as suspected isolates with typical cultural and biochemical properties (5 from milk, 1 from the udder and 14 from the milk production facility) (Fig. 2, a, b, Fig. 3, a, b).

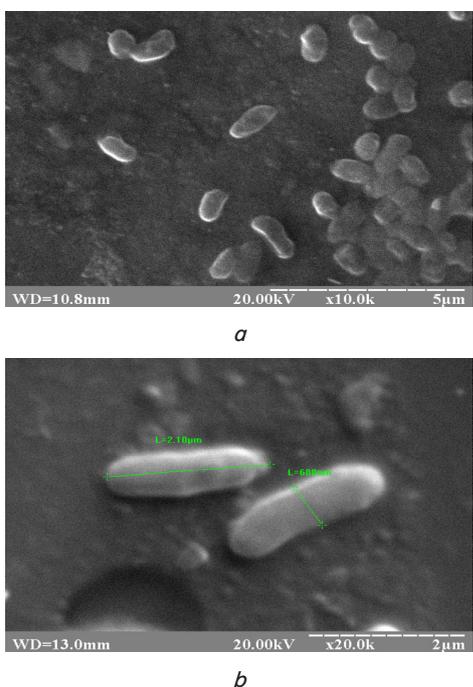


Fig. 2. Bacteria cells of *Cronobacter spp* (*Enterobacter sakazakii*): a – cell cluster; b – measurements of cells (canning electron microscope)

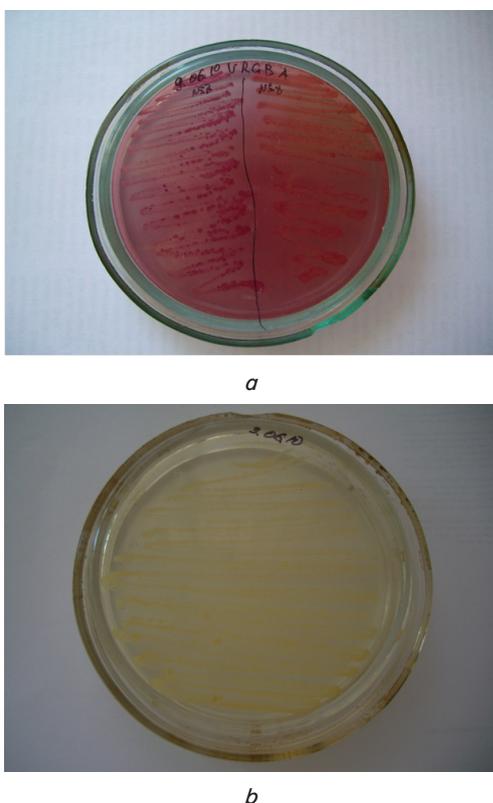


Fig. 3. Typical colonies of *Cronobacter spp* (*Enterobacter sakazakii*): a – on Violet Red Bile Glucose Agar (the left half of Petri dish has typical dark red colonies, the right half of Petri dish has no typical colonies); b – on Trypticase Soy Agar

Several pairs of oligonucleotide primers specific for different domains of gene 16SrRNA were developed and used in PCR for identifying bacteria *Cronobacter spp.* (*Enterobacter sakazakii*) by polymerase chain reaction. Some positive results with 20 isolates of bacteria were obtained (Fig. 4).

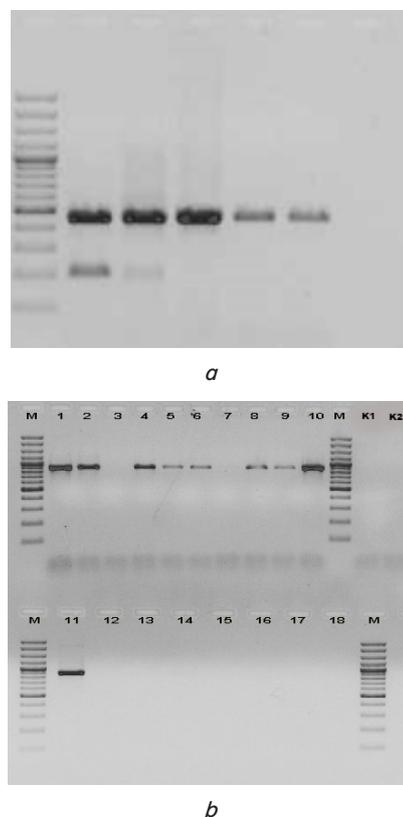


Fig. 4. The results of electrophoretic analysis of PCR products with primers d16sakF1/d16SsakR: a – M-marker “100 bpPlusDNALadder” (Fermentas); 1, 2 – deposited strain of *Cronobacter spp*; 3 – isolate (milk); 4 – isolate (udder); 5 – isolate (animal feed); C(-) – negative control; b – M-marker “100 bpPlusDNALadder” (Fermentas); 1–18 – selected isolates of *Cronobacter spp*; K1, K2 – heterologous negative controls (*B. subtilis*, *E. coli*)

As shown in Fig. 4, a, b, *Cronobacter spp* (formerly *Enterobacter sakazakii*) can be detected from samples with using 16SrRNA gene in PCR.

6. Discussion of the research findings on isolation and identification of *Cronobacter spp*

Quality and safety of raw milk depend on the quantity and qualitative composition of the microflora that get into it during production. It is known that microflora of raw milk affects the health of dairy cows, sanitary conditions of farms and milking equipment [7, 10, 13, 15, 18, 25]. In recent years, special attention of researchers has been focused on studying new pathogens. New pathogens include bacteria of *Cronobacter* genus (formerly *Enterobacter sakazakii*), which is especially dangerous for babies up to a year. These microorganisms are opportunistic pathogens that can cause serious infections (necrotizing colitis and meningitis) in neonates after consumption of PIF and mortality ranges from 40 % to 80 % [2, 5, 11, 23, 24].

The natural reservoir of *Cronobacter* genus and way of transmission are still not clear but infant formula has been epidemiologically implicated in several clinical cases. There are mixed reports of *Cronobacter* genus presence in raw milk. There were unsuccessful attempts to isolate this bacterium from raw milk [16]. Later, the same investigations reported that 63 % of raw milk samples from dairy farms were positive [17]. The aim of our study was to establish the presence of *Cronobacter* genus in bulk tank milk. Also, our aim was to examine possible sources of these bacteria in dairy farms. With this, 190 environmental samples (swabs of milking machines, milk tanks, from the surface of cow udder, from the floor of livestock buildings and samples of animal feed) were collected from 5 dairy farms in Sumy Region, Ukraine. They were investigated for establishing their role in the transmission of *Cronobacter* spp. Samples of bulk tank milk were obtained from cows that are kept on 2 types of dairy farms. In one type of farms (2 farms), milking of cows was conducted in stalls in the milk line with portable milking machines. In the second type of farms, milking of cows was conducted in milking parlors by using two-stroke milking machines (DeLaval). The average milk yield for a cow is 8–9.5 thousand liters. A wide range of samples ($n=418$) was collected and examined for the presence of *Cronobacter* spp. using the FDA method and then confirmed by PCR targeting of the 16S rRNA gene. The cultural method helped to isolate presumptive positive colonies and API 20E test helped to identify the organism.

PCR assay targeting of the 16S rRNA gene sequence has been used for *Cronobacter* identification before [21, 24, 23]. In this study, we tested 20 isolates with PCR and all were accurately identified. It was determined that 19.4 % of raw milk samples were positive for *Cronobacter* spp and the pathogen is widespread in the dairy environment. We also isolated the pathogen from the udder secretion of cows (32 %) that were suffering from subclinical mastitis. Not all dairy farms in Ukraine have indicators of subclinical mastitis in milk equipment. Therefore, subclinical mastitis in cows in Ukraine is determined once a week with using rapid mastitis test (CMT) and confirmed by the microscopic method for the counting of somatic cells in cows' milk. Unfortunately, milk from cows with a subclinical form of mastitis may fall into the total bulk milk.

Similar results were also reported by researchers who were able to isolate this bacterium from the secretions of the affected mammary gland in heifers [28]. *Cronobacter* spp enters the milk through the udder, when the cattle are reared in the unhygienic condition. This bacterium is also isolated

from mastitis infected raw milk around the Chikmagalur District in India [25]. Several other researchers failed to isolate the pathogen from raw cow's milk [16, 19, 21].

Same research data indicate that the environment of livestock could be a source of this pathogen in animal origin products [13]. They determined that the feces of cattle, farm soil and water were negative while the animal feed was positive, which may be the possible source of the microorganism in animal body. Other observations indicate the presence of the bacteria in animal feed [28]. Several researchers found *Cronobacter* genus (formerly *Enterobacter sakazakii*) from various objects in the dairy farm [13, 29]. Although several reports have been published describing the prevalence of *Cronobacter* spp in food and environmental samples, a similar study on the prevalence of these bacteria in raw cow's milk and dairy farm environment has not been undertaken to date in Ukraine.

7. Conclusions

1. The results indicate that the dairy farm environments are a potential source of *Cronobacter* in raw milk. It was found that detection of these bacteria from raw cow's milk on average is 19.4 %.

2. Several pairs of oligonucleotide primers specific for different domains of gene 16SrRNA were developed and used in PCR for identifying bacteria *Cronobacter* spp. (*Enterobacter sakazakii*) by polymerase chain reaction. Positive results with 20 isolates of bacteria were obtained.

3. We suggest that control of bacteria *Cronobacter* spp should be carried out at all levels of the food chain, including the process of obtaining raw milk, which as a raw material for dry infant formula may be a potential contaminant of the finished products. These data will be valuable for microbiological risk assessment and help authorities to develop strategies to mitigate health risk.

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