ZNANSTVENA BILJEŠKA / SCIENTIFIC NOTE

Identification of bacteria species among *Enterobacteriaceae* found in confectionery cakes

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Abstract

The goal of this research was to identify which Enterobacteriaceae species are present in cakes from confectionery establishments and whether these are species that can be considered pathogenic for humans and what their harmful impact on human health could be.
The sampling of confectionery cakes was carried out in the area of the City of Zagreb, and the samples were analysed for the presence of Enterobacteriaceae in accordance with the HRN ISO 21528-2:2017 standard. In cases where the samples contained the number of Enterobacteriaceae greater than 102 CFU/g, the MALDI-TOF method was used to identify individual species of enterobacteria.
The results of the analyses determined the presence of 10 Enterobacteriaceae species: Enterobacter kobei, Enterobacter cloacae, Pantoea agglomerans, Serratia liquefaciens, Enterobacter asburiae, Klebsiella oxytoca, Buttiauxella gaviniae, Buttiauxella warmboldiae, Raoultella and Cedecea neteri. All species of Enterobacteriaceae determined in this research, according to the literature, were previously isolated from food or water, and all species except Buttiauxella warmboldiae were isolated from humans and the environment. However, for none of the species of Enterobacteriaceae determined in this research a literature reference to support food as a vehicle in case of human illness was found.

Key words: confectionery cakes, Enterobacteriaceae, MALDI-TOF, human disease

Sažetak

Cilj ovog istraživanja bio je utvrditi prisutnost vrsta Enterobacteriaceae u slastičarskim kolačima te jesu li to vrste koje se mogu smatrati patogenima za ljude i kakav bi mogao biti njihov štetan utjecaj na ljudsko zdravlje.
Uzorkovanje slastičarskih kolača provedeno je na području Grada Zagreba, a uzorci su analizirani na prisutnost Enterobacteriaceae sukladno normi HRN ISO 21528-2:2017. U slučajevima kada su uzorci sadržavali broj Enterobacteriaceae veći od 102 CFU/g, korištena je MALDI-TOF metoda za identifikaciju pojedinih vrsta enterobakterija.
Rezultatima analiza utvrđena je prisutnost 10 vrsta Enterobacteriaceae: Enterobacter kobei, Enterobacter cloacae, Pantoea agglomerans, Serratia liquefaciens, Enterobacter asburiae, Klebsiella oxytoca, Buttiauxella gaviniae, Buttiauxella warmboldiae, Raoultella i Cedecea neteri. Sve vrste Enterobacteriaceae utvrđene u ovom istraživanju, prema literaturi, prethodno su izolirane iz hrane ili vode, a sve vrste osim Buttiauxella warmboldiae izolirane su iz ljudi i okoliša. Međutim, niti za jednu od vrsta Enterobacteriaceae utvrđenih u ovom istraživanju nije pronađen literaturni navod prema kojem je bolest uzrokovana ovim patogenima bila porijeklom iz hrane.

Ključne riječi: slastičarski kolači, Enterobacteriaceae, MALDI-TOF, bolest

Introduction

Confectionery cakes, due to their high content of carbohydrates, fats and proteins, are a suitable medium for the growth and reproduction of various microorganisms. Contamination of this type of food is possible at all stages of the production process: through contaminated raw materials, especially if the cakes are not thermally processed before consumption, after thermal processing, during improper storage and/or transportation, and due to unhygienic handling (Kumar et al., 2001; Shahbaz et al., 2013; Chaudhari et al., 2017; El-Kadi et al., 2018).
Determining the number of enterobacteria in food is a common way of monitoring production hygiene. Microbiological criteria values are set in the Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs only for some food, and they are listed in detail in the Guide for microbiological criteria for food issued by the Ministry of Agriculture in Croatia in 2011 as recommendations. In cases where the results for enterobacteria are higher than the values specified in the Regulation and/or the Guide, it is possible to identify rare pathogenic or facultative pathogenic species of enterobacteria for which it is uncertain whether they have harmful consequences for human health.
Application of the Guide is voluntary, and the microbiological criterion for Enterobacteriaceae related to confectionery cakes is m=102 CFU/g, and it is listed in the Guide under category 4.2. Confectionery products, subcategory 4.2.6. Desserts (confectionery cakes) with filling and ready-made creams. According to the research results presented by Hengl et al. (2022) a third of cakes from confectionery establishments contained Enterobacteriaceae in an amount higher than 102 CFU/g.
The family Enterobacteriaceae (enterobacteria) belong to the group of gram-negative bacteria that do not form spores. They are facultative anaerobes

(with the exception of Saccharobacter, some strains of Yersinia and Erwinia) and reduce nitrates to nitrites. Enterobacteriaceae family includes a large number of genera, including some species that appear in the environment where food is produced and do not pose a threat to human health. Of the bacteria that belong to this family as causes of digestive infections and intoxication, the following are significant: Escherichia coli and members of the genera Salmonella, Shigella, Yersinia, Klebsiella, Enterobacter, Proteus, Citrobacter, Aerobacter, Providencia and Serratia. In addition to the abovementioned, there are also other genera from the Enterobacteriaceae family whose representatives can be associated with pathogenic effects on humans.

Initial contamination with Enterobacteriaceae in raw food is a consequence of contamination in primary production. Further contamination with enterobacteria during the food production chain can be prevented by implementing one of the quality assurance and/or food safety systems, such as Good Manufacturing Practice (GMP) and HACCP (Hazard Analysis and Critical Control Point) (ILSI, 2011). Enterobacteria in some food have the ability to reproduce even during cold chain storage, which makes it difficult to determine the place of initial contamination, because it does not necessarily mean that the food was contaminated during the production process, storage or subsequent handling.

The monitoring of enterobacteria in the production process is a good way to control the GMP during the production itself, however, the analysis of food in the market, during or at the end of shelf life, cannot be unambiguously used to determine the location of contamination during the production process.

The growth and metabolic activity of enterobacteria in food can lead to organoleptic changes like the formation of inappropriate tastes, smells, colour changes and other. These changes could be the result of enzymatic degradation of proteins or lipids, production of volatile components or production of gases (ILSI, 2011).

Analysis of food samples during the production process according to the Enterobacteriaceae criterion is used to assess food hygiene status. All Enterobacteriaceae are sensitive to the thermal processing used in food production and can be prevented by the application of appropriate cleaning procedures that effectively remove enterobacteria from facilities, equipment and work surfaces (ILSI, 2011). Their presence in food that has undergone thermal process indicates inadequate cleaning and sanitation of the production area, poor control of temperature and production time, inadequate thermal treatment and/or subsequent contamination (FSAI, 2016), and may also indicate poor personal hygiene of workers.

According to the results of the research on microbiological contamination of confectionary cakes in Croatia presented by Hengl et al. (2022), a third of cakes from confectionery establishments contained enterobacteria in an amount higher than 102 CFU/g, therefore the aim of this study was to identify which Enterobacteriaceae species are present in cakes from confectionery establishments and whether these species can be considered pathogenic for humans as well what their harmful impact on human health could be.

Materials and methods

The research was conducted on 201 samples of cakes obtained from confectionery establishments in the area of the City of Zagreb, Croatia. Sampling was carried out according to the ISO/TS 17728 standard. Samples were taken in the amount of minimum of 250 g and packed into sterile plastic bags. Transportation to the laboratory was done using transport refrigerator and keeping the temperature up to 8 °C. The samples were processed and analysed according to the HRN ISO 21528-2:2017 standard, immediately upon receipt in the laboratory. Cake samples were analysed in two accredited laboratories in Zagreb.

After the first analysis for the presence of Enterobacteriaceae, in cases where the samples contained the number of enterobacteria greater than 102 CFU/g, five randomly selected grown bacterial colonies were taken and prepared for analysis by the MALDI-TOF method (VITEK® MS, Biomeriux) by preparing cell lysates and isolating proteins. The MALDI-TOF method was used to determine Enterobacteriaceae species that were present in that sample.

Enterobacteriaceae strains were identified with the VITEK MS system (bioMérieux, Lyon, France), according to the manufacturer's instructions. A fresh colony was smeared with a toothpick onto a 48-well plate and covered with 1 μL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution. After drying, the target plate was loaded into the MALDI-TOF VITEK MS system. Escherichia coli ATCC 8739 was used as quality control strain.

Based on the TOF data, a characteristic distribution spectrum was created, which is interpreted using a database of more than 25,000 described spectra, which enables the identification of microorganisms according to genus, species or strain.

Results and discussion

Depending on the composition of the cakes, the results of the confectionary cakes analyses obtained in the research were divided into three groups: chocolate, fruit and cream cakes. The chocolate cake group includes those samples that contained chocolate in their composition, such as chocolate cake, brownie cake, Zagreb cake, Sacher cake, Hungarian cake, etc. The fruit cake group includes cake samples that necessarily contained one or more types of fruit in their composition or topping, such as Black Forest cake, fruit cake, strawberry cake, cheesecake with forest fruits, fruit meringue cake, Spanische Windtorte, etc. All other cake samples are classified into the group of cream cakes, such as Cremeschnitte ("krempita", puff pastry cake with custard), tiramisu, cheesecake, Jaffa cake, walnut cake, hazelnut cake, etc. Out of the total number of cakes, there were 31 chocolate cakes (15.42%), 34 fruit cakes (16.91%) and 136 cream cakes (67.66%).

The research results determined the presence of enterobacteria above 102 CFU/g in 49 (24.38%) of the analysed samples. In the fruit cake group of samples, the number of enterobacteria above 102 CFU/g was determined in 11 samples (32.35%), in the group of chocolate cake samples in 7 (22.58%) and 31 (22.79%) in the cream cake group of samples.

Using the MALDI TOF method, the presence of 10 different bacteria species within the genus Enterobacteriaceae was determined (Table 1), of which the presence of the bacterium Enterobacter (E.) kobei was most frequently determined (in 4.0% of the samples). Followed by Enterobacter (E.) cloacae, Pantoea (P.) agglomerans and Serratia (S.) liquefaciens, which were present in 3.5% of samples, and Enterobacter (E.) asburiae and Klebsiella (K.) oxytoca (2.5% each), Buttiauxella (B.) gaviniae (2.0%) and Buttiauxella (B.) warmboldiae (1.5%). Raoultella and Cedecea (C.) neteri species were detected in the smallest number of samples, 1.0% and 0.5% respectively. In the literature, all established species of enterobacteria, with the exception of the bacterium B. warmboldiae, are listed as possible human pathogens, found in water, food or environment (Table 1).

In the group of chocolate cakes, five (5) different species of enterobacteria were found (E. asburiae, P. agglomerans, S. liquefaciens, K. oxytoca and C. neteri), seven (7) in the group of fruit cakes (E. kobei, E. cloacae, E. asburiae, B. gaviniae, B. warmboldiae, P. agglomerans and S. liquefaciens) and nine (9) in the group of cream cake samples (E. kobei, E. cloacae, E. asburiae, B. gaviniae, B. warmboldiae, P. agglomerans, S. liquefaciens, K. oxytoca and Raoultella) (Figure 1).

Three bacteria species, E. asburiae, P. agglomerans and S. liquefaciens were determined in all cake groups (Figure 1). The bacteria E. asburiae was previously found in infant milk powder (Mardaneh et al., 2014), sweet potatoes and cotton roots (McInroy and Kloepper, 1995) and rice (Elbeltagy et al., 2001), while P. agglomerans was found in trout (Loch and Faisal, 2007), raw turkey (Martínez-Laorden et al., 2023) and rice, corn, bamboo, cotton, sweet onion and walnuts (Dutkiewicz et al., 2016).

The presence of the bacterium S. liquefaciens was determined in fresh turkey meat (Martínez-Laorden et al., 2023), chicken meat (Kačániová et al., 2021), cold smoked salmon, vacuum-packed chilled meat (Gram et al., 1999) and raw cow's milk (Salgado et al., 2021).
The bacteria E. kobei, E. cloacae, B. gaviniae and B. warmboldiae were found in fruit and cream cakes, and K. oxytoca in chocolate and cream cakes (Figure 1). Thanh et al. (2020) identified E. kobei bacteria in potatoes, and Nyenje et al. (2012) E. cloacae in ready-to-eat food (RTE food) from South Africa. B. gaviniae has been detected in snails and drinking water (Muller et al., 1996) and raw salmon (Mace et al, 2012). Martinez-Laorden et al. (2023) identified the bacterium K. oxytoca in fresh turkey meat. In this study, Raoultella was found only in cream cakes, and previously Manhique et al. (2020) found it in ready-to-eat salad (RTE) and drinking water, and Khater et al. (2021) in minced meat. The bacterium C. neteri was found only in chocolate cakes (Figure 1), while Tan and Chan (2015) found it in mackerel sashimi.
To our knowledge, so far some species were only, or mostly, found in raw food like P. agglomerans, S. liquefaciens, E. kobei , B. gaviniae, K. oxytoca, Raoultella and C. neteri, but in this research, all of them were found in cakes which had undergone high thermal treatment. Even if we take into consideration that some bacteria species could be introduced by fresh fruit, all of the mentioned bacteria species are probably easily found in the environment, which in this case serves as a continuous source of contamination.

Table 1. The occurrence of established enterobacteria, in other studies

Bacteria spp.	ISOLATED FROM	LITERATURE SOURCE
Buttiauxella gaviniae	Food	Müller et al., 1996; Brenner et al., 2007; Macé et al., 2012; Kačániová et al., 2021; Martínez-Laorden et al., 2023
	Humans	De Baere et al., 2002; Brenner et al., 2007; Patra et al., 2018; Zgheib et al., 2022
	Environment	Brenner et al., 2007; Lauková et al., 2018
Buttiauxella warmboldiae	Food	Müller et al., 1996; Patra et al., 2018; Martínez-Laorden et al., 2023
Cedecea neteri	Food	Tan and Chan, 2015
	Humans	Abate et al., 2011; Ginn et al., 2018; Ahmad & Sur, 2021
	Environment	Chan et al., 2017; Hegde et al., 2019
Enterobacter asburiae	Food	McInroy and Kloepper, 1995; Elbeltagy et al., 2001; Mardaneh et al., 2014
	Humans	Harbarth et al., 1999; Mardaneh et al., 2014; Salimiyan Rizi et al., 2019
	Environment	Mardaneh et al., 2014
Enterobacter cloacae	Food	Shaker et al., 2006; Nyenje i sur, 2012; Rady i sur, 2022; Martínez-Laorden et al., 2023
	Humans	Salimiyan Rizi i sur, 2019; Nyenje et al., 2012; Bejuk et al., 2013
	Environment	Nyenje et al., 2012
Enterobacter kobei	Food	Kosako et al., 1996; Thanh et al., 2020
	Humans	Kosako et al., 1996
	Environment	Glushakova et al., 2022
Klebsiella oxytoca	Food	Martínez-Laorden et al., 2023
	Humans	Podschun and Ullmann, 1998; Hogenauer et al., 2006
	Environment	Kao et al., 2003
Pantoea agglomerans	Food	Loch and Faisal, 2007; Dutkiewicz et al., 2016; Büyükcam et al., 2017; Patel et al., 2020; Martínez-Laorden et al., 2023
	Humans	Loch and Faisal, 2007; Cruz et al., 2007; Dutkiewicz et al., 2016; Büyükcam et al., 2017; Lorenzi et al., 2022
	Environment	Loch and Faisal, 2007; Cruz et al., 2007; Büyükcam et al., 2017; Patel et al., 2020; Lorenzi et al., 2022
Raoultella	Food	Manhique et al., 2020; Khater et al., 2021
	Humans	Gajdács, 2019; Appel et al., 2021
	Environment	Gajdács, 2019; Appel et al., 2021
Serratia liquefaciens	Food	Gram et al., 1999; Kačániová et al., 2021; Salgado et al., 2021; Martínez-Laorden et al., 2023
	Humans	Grohskopf et al., 2001; Mahlen et al., 2011; Ikumapayi et al., 2016
	Environment	Mahlen et al., 2011

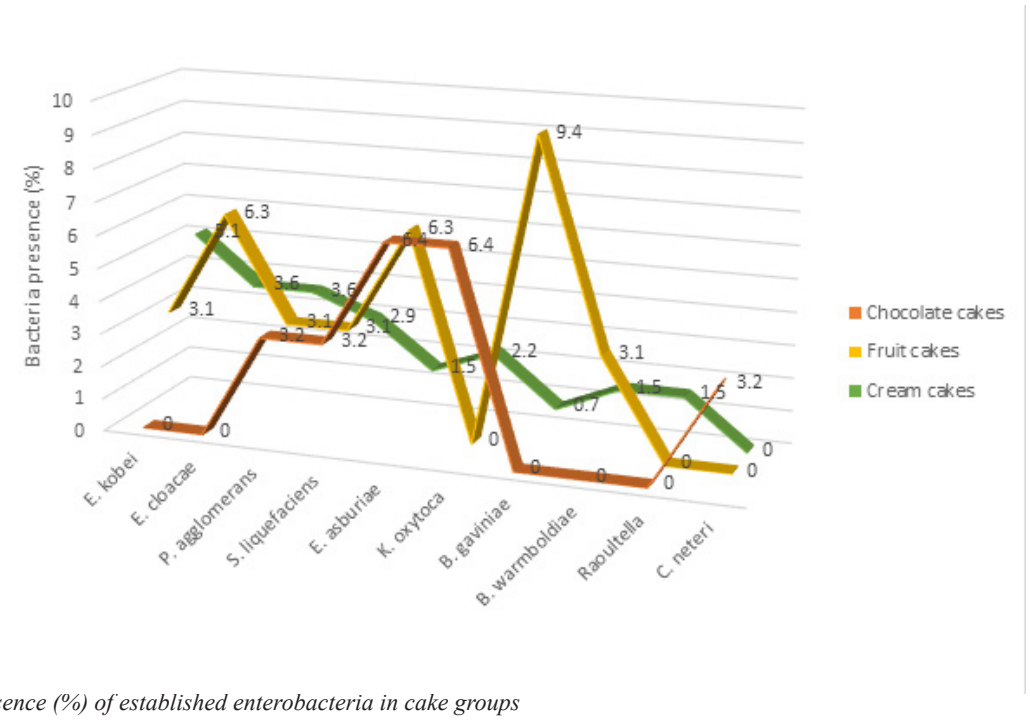

Figure 1. The presence (%) of established enterobacteria in cake groups

Conclusions

The analysis of confectionery cakes determined the presence of enterobacteria above 102 CFU/g in 22.79% of cream, 22.58% of chocolate, and 32.35% of fruit cakes, and the MALDI-TOF method identified the presence of 10 species from the family of enterobacteria, 9 of which, except for B. warmboldiae, are described in the scientific literature as facultative human pathogens.

The results of the research suggest that if no pathogenic species from the genus Enterobacteriaceae (e.g. Salmonella, Shigella, Yersinia or similar) are found in the sample, the remaining species that contribute to the total number of enterobacteria, although in some cases they have a pathogenic character, cannot cause a harmful effect on health if consumed orally. However, it is not possible to rule out another route of transmission of infection that occurs when handling food contaminated with enterobacteria, which is not necessarily oral intake.

If Enterobacteriaceae are present in a larger number, one bacterial species dominates the sample. However, according to the total number of Enterobacteriaceae in sample, it cannot be assumed which species could predominate.

Although the results of our research indicate that not all Enterobacteriaceae species were identified in all three groups of cakes, and considering that the species identified in this research were previously detected in different foods, it cannot be concluded that the identified species are characteristic of a certain type of food. It can be assumed that they occur depending on the microflora of the production facility, i.e. from the surfaces of equipment, the environment and employees. Therefore, it can be expected, if a similar study is conducted, that the species of enterobacteria present will be different from those determined in this study. This also indicates that the reduction of contamination could be influenced even more by conducting thorough, consistent and detailed cleaning and sanitation of the equipment.

Finally, and in accordance with currently available scientific evidence, the species of the Enterobacteriaceae determined in this research, with the exception of B. warmboldiae, have so far been isolated not only from water and food, but also from people and the environment. However, no scientific evidence was found to indicate that any Enterobacteriaceae species identified in this study, regardless of being described as the pathogen that caused the disease, was of food origin. We can assume that such a transfer is possible, but it is extremely rare in real life.

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