



MALDI-TOF MS IDENTIFICATION OF *CITROBACTER YOUNGAE* ISOLATED FROM FOOD

Dubravka S. Milanov^{*1}, Milan D. Đilas², Jelena M. Babić¹, Bojana Z. Prunić¹, Maja J. Velhner¹

¹ Scientific Veterinary Institute 'Novi Sad', Rumenački put 20, 21000 Novi Sad, Serbia

² Institute of Public Health of Vojvodina, Futoška 121, Novi Sad, Serbia

*Corresponding author
Tel.: +381 21 4895 346
E-mail: dubravka@niv.ns.ac.rs

ABSTRACT: *Salmonella*-like bacteria, such as certain species of the *Citrobacter* genus are sometimes isolated from food and feed. By testing their biochemical and, partially, serological characteristics (according to the regulations given in the ISO 6579-1:2017 Standard) it is impossible to completely reject their affiliation to the *Salmonella* genus. In addition to this, the food microbiology laboratories usually lack diagnostic tools and procedures for precise identification of these isolates to the species level. Thus, it is considered useful to describe the identification of *Citrobacter youngae* species using MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry). Besides differential diagnostic criteria for distinguishing between *Salmonella* spp. and *Citrobacter youngae*, in this communication the relevant data on *Citrobacter* spp., the emerging opportunistic pathogenic member of *Enterobacteriaceae* family were presented.

Key words: *Salmonella*-like bacteria, cheese

A REPORT ON A LABORATORY CASE

A sample of soft, medium-fat cheese sprinkled with spices, produced in a small scale plant, was examined for the presence of *Salmonella* spp. in the Laboratory of Food Microbiology of the Scientific Veterinary Institute in Novi Sad. The sample was tested in accordance with the requirements of the ISO 6579-1:2017 Standard. Characteristic pink colonies with a black centre of *Salmonella* species on XLD agar (Biokar Diagnostics, France) were isolated from the sample and further examined.

With tests of catalase (positive) and oxidase (negative) it was confirmed that the isolate belonged to the *Enterobacteriaceae* family. The biochemical characteristics of the isolate were assessed in

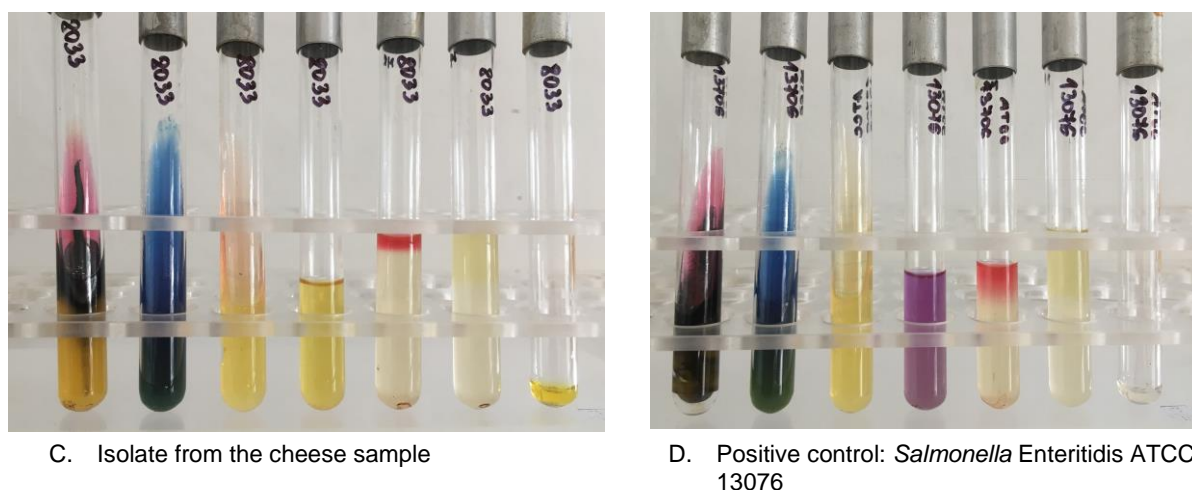
accordance with the ISO 6579-1:2017 Standard (Table 1), plus using the methyl-red and citrate utilization tests, which were both positive (Figure 2).

Rapid slide agglutination test with the polyvalent serum for *Salmonella* groups A, B, C, D and E (Institute for Public Health of Serbia, Belgrade, Serbia) was positive but not with the group-specific sera B (O:4), D1 (O:9), C1 (O:7), C2-C3 (O:8), B (O:4) and E1 (O:3,10).

Based on the negative reaction of lysine decarboxylase and the production of β -galactosidase, with 95% and 99% probability, respectively, the hypothesis that the isolate belonged to *Salmonella* genus was ruled out.



Figure 1. Colonies on XLD agar medium after 24h incubation at 37 °C



From left to right: Kligler, citrate, urea, lysine decarboxylase, methyl red, indole, β -galactosidase (ONPG disk)

Figure 2. Results of biochemical testing

Table 1.
Results of biochemical tests

Test	Isolate	Other strains*	
		Reaction	%**
TSI*** acid from glucose	+	+	100
TSI gas from glucose	-	+	92
TSI acid from lactose	-	-	1
TSI hydrogen sulfide produced	+	+	92
Urea hydrolysis	-	-	1
Lysine decarboxylation	-	+	95
Production of indole	-	-	2
β -galactosidase	+	-	1

*Interpretation of biochemical tests for other *Salmonella* strains (does not refer to the following species: *S. typhi*, *S. paratyphi* A, B and C; *S. Gallinarum* biovar *gallinarum* and biovar *pullorum*);

**Percentages indicate that not all isolates of *Salmonella* serovars show the reactions marked + or -. Reaction may also vary between and within serovars;

***TSI-Triple sugar iron

(Source: ISO 6579-1:2017,p.10)

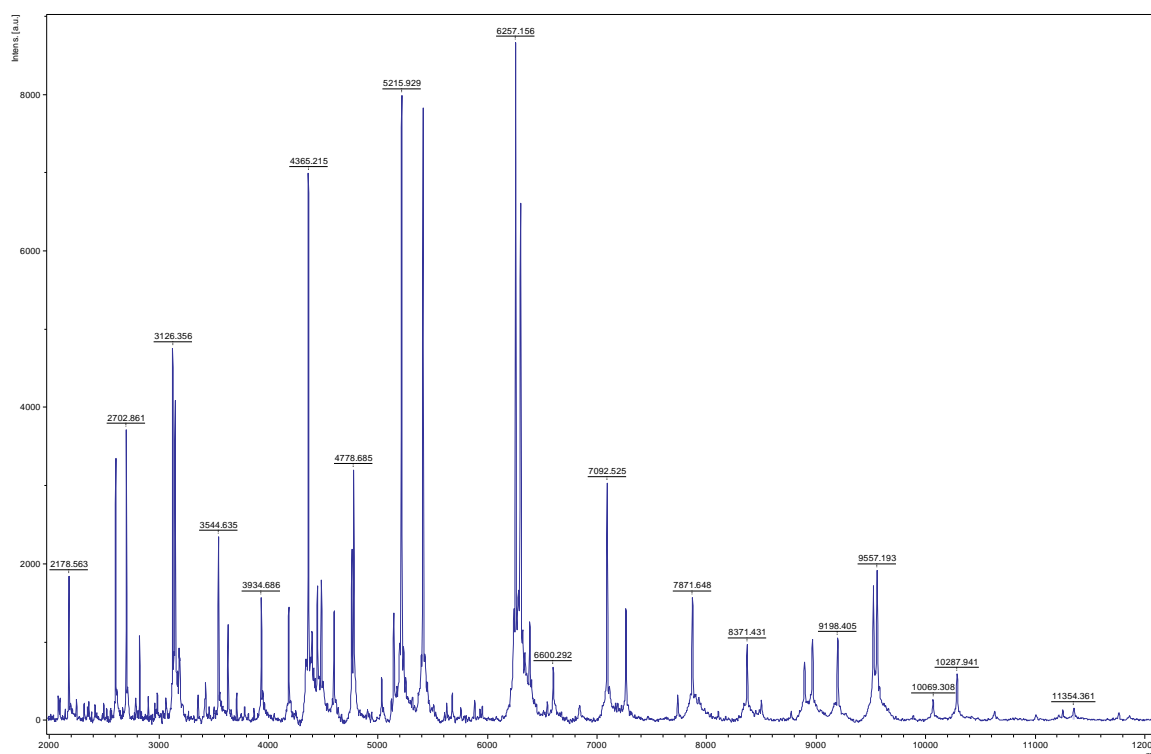


Figure 3. Spectra of *Citrobacter youngae* isolate generated by the MALDI-TOF Bruker Biotyper. The x-axis shows the masses (m/z) of the ions, and the y-axis shows the absolute intensities of the ions. The m/z values represent the mass-to-charge ratio

Based on the positive test for glucose fermentation, citrate utilization test, methyl red and β -galactosidase activity, as well as the characteristic appearance of the colonies on the XLD agar medium, the diagnostic assumption was that the isolate belonged to *Citrobacter* genus. Moreover, the bacterial culture produced a putrefaction smell, which is not characteristic of *Salmonella* species. In order to identify the species, the isolate was further processed at the Institute for Public Health of Vojvodina in Novi Sad, Serbia.

MALDI-TOF MS analysis

The isolate was prepared using the standard Bruker's direct transfer sample preparation procedure for MALDI-TOF MS. A single bacterial colony was spotted directly onto a MALDI target plate (Bruker Daltonics, Germany), allowed to dry and immediately overlaid with 1.0 μ L of the matrix solution (Bruker Matrix HCCA; α -Cyano-4-hydroxycinnamic acid).

MALDI-TOF mass spectra were obtained using Microflex LT/SH Biotyper spectrometer (Bruker Daltonics, Germany) equipped with a nitrogen laser (337 nm) under

the control of flexControl software ver. 3.4 (Bruker Daltonics). Spectra in the mass range of 2 to 20 kDa were collected by accumulating 240 laser shots (laser frequency, 60 Hz; ion source 1 voltage, 20 kV; ion source 2 voltage, 18.15 kV; lens voltage, 6 kV) at 30–40% of maximum laser power.

COMMENT

The most important food borne pathogens belong to the *Enterobacteriaceae* family. These are *Salmonella* spp., *Yersinia enterocolitica*, pathogenic *Escherichia coli* and *Shigella* spp. Some members of the family, for instance, *Providencia alcalifaciens*, *Klebsiella* spp., *Serratia* spp. and *Citrobacter* spp., are emerging opportunistic pathogens (Baylis et al., 2011). Based on the results of DNA hybridization assay, *Citrobacter* are classified into 11 species: *C. amalonaticus*, *C. braakii*, *C. farmeri*, *C. freundii*, *C. gillenii*, *C. koseri*, *C. murlinae*, *C. rodentium*, *C. sedlakii*, *C. werkmanii* and *C. youngae* (Brener et al., 1993). *Citrobacter* spp. are typically motile bacteria, owing the peritrichous flagellae, facultative anaerobic, usually oxidase ne-

gative, and positive for catalase and methyl red (Doran, 1998; Duman et al., 2017). They generally utilize citrate as the only carbon source, which explains the etymology of the name of the genus (Arens et al., 1996). Two additional properties of citrobacteria which help to distinguish them from other members of the family *Enterobacteriaceae* are their failure to produce acetylmethylcarbinol (negative Voges-Proskauer test) and the lack of lysine decarboxylase (Janda et al., 1994).

Citrobacter species are present in water, soil, food and the intestinal tracts of animals and humans (Doran, 1998; Baylis et al., 2011; Clermont et al., 2015), and are commonly recovered from human faecal/intestinal flora (Duman et al., 2017). They can cause urinary infections, hospital-acquired bacteremias, brain abscesses, neonatal meningitis, wound infections, pneumonias, abscess, septicaemias, endocarditis and diarrhoea (Arens et al., 1996; Doran, 1998; Clermont et al., 2015). *Citrobacter* spp. are also considered to be capable of causing gastrointestinal infections especially in infants and young children (Janda et al., 1994).

An outbreak of haemolytic uraemic syndrome (HUS) in Germany was linked to the consumption of butter containing parsley contaminated with *C. freundii* carrying the *vtx2* gene (Tschape et al., 2011). The infections most often occur in neonates and immunocompromised people (Thurm and Gericke, 1994; Doran, 1998). *C. freundii* and *C. koseri* are the two most common clinical isolates. The other nine *Citrobacter* species, including *C. youngae*, rarely cause infections (Samonis et al., 2009).

The species identification of isolates belonging to the genus *Citrobacter* can be tentatively made using biochemical profiles or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Clermont et al., 2015; Kwak et al., 2015). MALDI-TOF MS has been described in the identification of *Citrobacter* isolates from clinical samples (Kolinska et al., 2015). Online search retrieved a single report on the use of

MALDI-TOF MS in the identification of *Citrobacter* species in food samples originating from pork (Kwak et al., 2015).

Within less than a decade MALDI-TOF MS has become a gold standard in clinical microbiology laboratories, as it represents a powerful, rapid, precise, and cost-effective method for identification of bacteria, mycobacteria, and fungi, compared to conventional phenotypic techniques or molecular biology.

MALDI-TOF MS is able to detect a large spectrum of proteins, allowing the discrimination between closely related species and identification at the species and subspecies levels (Biswas and Rolan, 2012; Murray, 2012).

This technique allows desorption of peptides and proteins from both whole different cultured bacteria and crude bacterial extracts (Biswas and Rolan, 2012).

Usually, a single bacterial colony is enough for MALDI-TOF MS analysis (Dekker and Branda, 2011). Bacterial colonies are removed from agar culture plates, mixed with a matrix compound, and dried on steel target plates. The dried preparations are exposed to laser pulses, resulting in energy transfer from the matrix to the nonvolatile analyte molecules, with desorption of analyte into the gas phase.

The ionized molecules are accelerated by electric potentials through a flight tube to the mass spectrometer, with separation of the biomarkers determined by their mass/charge ratio (m/z). The profile of biomarkers is then compared with profiles of a collection of well characterized organisms included in the database (Murray, 2012).

This short communication can serve as a practical guide to the laboratories for food and feed control as to why and when to suspect the presence of this bacterial species. In order to achieve food safety monitoring and obtain precise data on the opportunistic pathogen species which can be transferred to consumers by food a continuous cooperation between laboratories for microbiological control of food and those dealing with public health is inevitable.

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ИДЕНТИФИКАЦИЈА *CITROBACTER YOUNGAE* ИЗОЛОВАНОГ ИЗ ХРАНЕ ПРИМЕНОМ MALDI TOF МЕТОДЕ

Дубравка С. Миланов*¹, Милан Д. Ђилас², Јелена М. Бабић, Бојана З. Прунић¹, Маја Ј. Велхнер¹

¹ Научни институт за ветеринарство „Нови Сад“, 21000 Нови Сад, Руменачки пут 20, Србија

² Институт за јавно здравље Војводине, 21000 Нови Сад, Футошки пут 121, Србија

Сажетак: Из хране и хране за животиње понекада се изолују бактерије које су веома сличне салмонелама, као што су поједине врсте из рода *Citrobacter*. Испитивањем њихових биохемијских, а делимично и серолошких карактеристика (према одредбама Стандарда ISO 6579-1:2017), није могуће у потпуности искључити припадност роду *Salmonella*. Осим тога, лабораторије за микробиолошки преглед хране уобичајено немају дијагностичке могућности за прецизну идентификацију ових изолата до врсте. Из тих разлога сматрамо да је корисно приказати идентификацију врсте *Citrobacter youngae* применом MALDI TOF методе (*Matrix-Assisted-Laser-Desorption Ionization Time-of-Flight Mass Spectrometry*). Поред диференцијално-дијагностичких критеријума у односу на *Salmonella* spp., у овом саопштењу укратко приказујемо релевантне податке о *Citrobacter* spp., опортунистичким патогенима из фамилије *Enterobacteriaceae*.

Кључне речи: *Salmonella* имитирајуће врсте бактерија, суп

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