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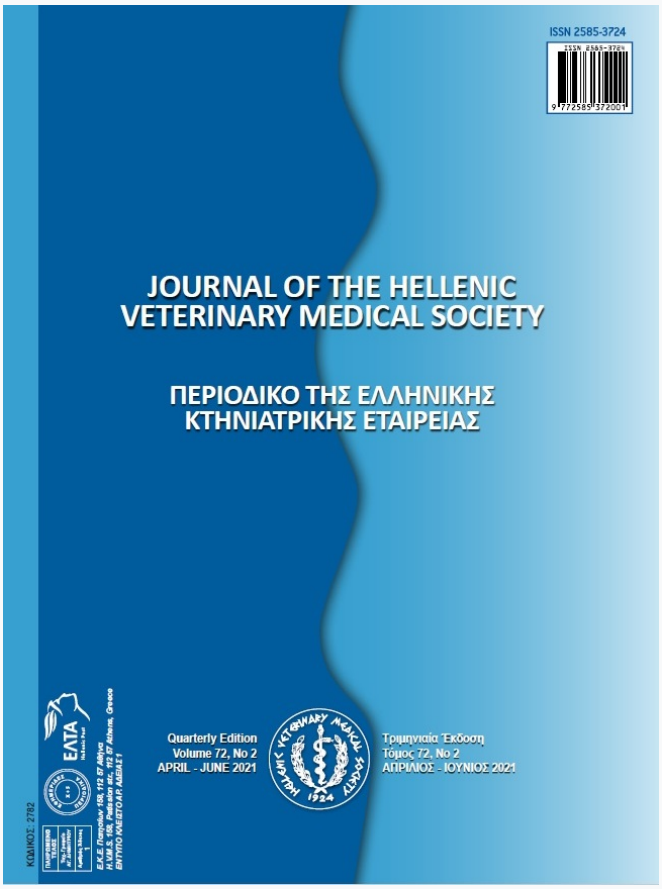

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Research article
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Microbiological evaluation of ready-to-eat foods by conventional methods and MALDI-TOF MS

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ABSTRACT: The consumption of ready-to-eat foods in daily life is increasing rapidly. Food poisoning occurs as a result of the preparation and preservation of these foods under inappropriate hygienic conditions. There is also an increased risk of getting food poisoning during the summer heat. The purpose of this study was to evaluate the microbiological properties of ready-to-eat foods by conventional methods and investigate the applicability of using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) for the identification of microorganisms in the bacteria isolates from these foods. Eighteen ready-to-eat food samples (tomato soup-G1, lentil soup-G2, tarhana soup-G3, yogurt soup-G4, ezo gelin soup-G5, chicken stew-G6, eggplant ragout-G7, green beans with meat-G8, bulgur pilaf-G9, rice pilaf-G10, macaroni-G11, rice pudding-G12, raspberry fruit dessert-G13, tomato salad G14, cucumber salad-G15, lettuce-G16, shepherd's salad-G17, and yogurt-G18) on sale in restaurants in Ankara-Turkey were evaluated. For this evaluation, the samples were analyzed in terms of staphylococcal enterotoxin (SET-RPLA), *Bacillus cereus* (EN ISO 7932), *Salmonella* spp. (EN ISO 6579-1), *Listeria monocytogenes* (EN ISO 11290-1) and *Escherichia coli* (ISO 16649-2) using conventional microbiological methods. The use of MALDI-TOF MS for the identification of bacteria isolates was also evaluated in this study. Only bulgur pilaf-G9, raspberry fruit dessert-G13, and lettuce-G16 samples exhibited microbial growth on agar plates. *Klebsiella pneumoniae* was detected following the identification of the suspected *Salmonella* spp. in the bulgur pilaf-G9 sample. *B. cereus* (50 cfu /g) and *E. coli* (80 cfu /g) were detected in raspberry fruit dessert-G13 and lettuce-G16, respectively. These bacteria, which were isolated and identified by conventional methods were also rapidly confirmed by MALDI-TOF. In conclusion, these sampled foods, which were available for public consumption, met the general hygiene criteria. Therefore, the foods complied with the Turkish national legislation. The MALDI-TOF MS method has advantages over conventional methods employed in the microbiological evaluation of these foods, in terms of shorter application time, rapidity, and greater simplicity in identifying the causes of poisoning and causative agents involved in food poisoning.

***Keywords*:** Conventional microbiology; MALDI-TOF MS; ready-to-eat foods

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INTRODUCTION

The consumption of ready-to-eat foods is currently growing rapidly with today's fast and busy lifestyle. Home-cooked family recipes such as soups, stews, meat dishes, vegetable dishes, olive oil dishes, pilaf, macaroni, pastries, appetizers, desserts, salads and etc. are presented as ready-to-eat foods. Food is defined as any other substance or product, including water, processed, partially processed, or unprocessed, which is intended to be consumed by humans (5996 Law on Veterinary Services Plant Health Food and Feed, 2010). The precautionary measures taken to neutralize all potential physical, chemical, biological, and other hazards in these foods are collectively known as food safety (Karabal, 2019). During the preparation of food and considering the food safety, conditions must be monitored using hazard analysis and critical control point (HACCP) procedures, and the requisite precautions must be taken to ensure that these are appropriate for consumption (Ceyhun and Artık, 2015). Contaminated foods, particularly in microbiological terms may result if these precautions and conditions are not taken and met at all stages, from food preparation to ready-to-eat food (Hachemi et al., 2019). Microbiologically hazardous foods represent health risks for babies, the elderly, and the sick individuals (Lawrence et al., 2007). Various studies have investigated the microbiological food safety (Iacumin and Comi, 2019). Pathogens play an important role in food poisoning, and *Bacillus cereus*, *Salmonella* spp., and *Listeria monocytogenes* have been the subject of thoroughly investigation (Sapkota et al., 2019). Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is a relative new generation method employed in the identification of micro-organisms in addition to conventional techniques, relies on the extraction of protein profiles from cells and consequently subjected to ionization by exposing them to an electric field (Yilmaz et al., 2014). This new generation technique has also begun being used for the identification of microorganisms present in food (Kwak et al., 2015). The purpose of this study was to evaluate the microbiological properties of ready-to-eat foods by conventional microbiological methods and investigate the applicability of MALDI-TOF MS for the identification of microorganisms in the bacteria isolates from these foods. Also, these foods were subjected in parallel to microbiological evaluation based on national legislation (Regulation on Turkish Food Codex Microbiological Criteria, 2011).

MATERIAL AND METHODS

Food Sampling

Eighteen ready-to-eat food samples (tomato soup-G1, lentil soup-G2, tarhana soup-G3, yogurt soup-G4, ezo gelin soup-G5, chicken stew-G6, eggplant ragout-G7, green beans with meat-G8, bulgur pilaf-G9, rice pilaf-G10, macaroni-G11, rice pudding-G12, raspberry fruit dessert-G13, tomato salad G14, cucumber salad-G15, lettuce-G16, shepherd's salad-G17, and yogurt-G18) on sale in restaurants in Çankaya-Ankara, Turkey, were obtained and coded. These samples were placed into sterile sample containers. They were then transported to the laboratory under cold chain and aseptic conditions for microbiological analysis.

Weighing the food samples and preparation of dilutions

For initial suspensions of *E. coli* and *B. cereus,* 10 g of each sample was placed into sterile stomacher bags and homogenized by the addition of 90 mL of sterile peptone saline (8.5 g NaCl (Merck), and 1.0 g peptone (Merck), 1000 mL distilled water) (EN ISO 6887-1, 2017). For determination of *Salmonella* spp. and *L. monocytogenes*, 25 g of each sample was placed into sterile stomacher bags for pre-enrichment and homogenized with 225 mL sterile buffered peptone water (Merck) for *Salmonella* spp., and with 225 mL Half Fraser Broth (LabM) medium for *L. monocytogenes*. After homogenization, the mixtures were incubated at 34-38 °C for 18±2 h, and at 30±1°C for 24-26 h for *Salmonella* spp. and *L. monocytogenes* detection, respectively.

Determination of the presence of staphylococcal enterotoxin in samples

For staphylococcal enterotoxin determination, 10 g of each sample was homogenized with 10 mL 0.85% sterile peptone salt solution (Merck) and centrifuged at 4 °C for 30 min. After centrifugation, the supernatant was filtered through a 0.20 μm-diameter sterile filter (Sartorius). After filtration, the staphylococcal enterotoxin kit procedure (SET-RPLA, Oxoid) was applied according to the manufacturer's instruction.

Detection and enumeration of *B. cereus* in the samples

The EN ISO 7932 (2004) method was used for the detection and enumeration of *B. cereus* in the food samples. Briefly, 1.0 mL of the initial suspension prepared was distributed on three dishes with MYP Agar

medium (Oxoid), prepared in duplicates by using six plates and then incubated at 30±1 °C for 21±3 h. After incubation, pink colonies surrounded by an opaque zone due to high lecithinase production in the medium were analyzed further by the conventional method as suspicious colonies. The hemolysis test was performed on Sheep Blood Agar (Merck) for confirmation of the colonies, and incubation was performed at 30±2 °C for 24±2 h. After incubation, the presence of a hemolysis zone on the Sheep Blood Agar (Merck) medium was interpreted as a positive reaction.

Determination of *Salmonella* spp. in the samples

The EN ISO 6579-1 method (2017) was used to determine *Salmonella* spp. in the samples. After the pre-enrichment, the samples were placed into incubation at 41.5±1 °C for 24±3 h in Rappaport Vassilliadis Broth (Biokar) and at 37±1 °C for 24±3 h in Muller-Kauffmann Tetrathionate-Novobiocin Broth (MKTTn broth) (Biokar) media. At the end of incubation, they were incubated in Xylose Lysine Deoxycholate (XLD) Agar (Merck) and Brilliant Green Agar Modified (Condalab) at 37±1 °C for 24±3 h. After the incubation, in order to confirm the colonies identified, black-centered and colorless suspicious colonies growing in the medium were placed into Nutrient Broth (Merck) medium and allowed to incubate at 34-38 °C for 24±3 h. After incubation, culturing was performed in Triple Sugar Iron agar (TSI) (Merck) followed by incubation at 37±1 °C for 24±3 h. The medium was examined for the usage of glucose, non-usage of lactose, non-usage of sucrose, gas formation from glucose, and formation of H_2S. Suspicious colonies also were examined for urea hydrolysis, L-lysine decarboxylase activity and the indole test. For these identification tests, EnteroPluri-Test (Liofilchem) was used, and the manufacturer's instructions were followed. Agglutination control, O antigens, Vi antigens, H antigens were applied for serological confirmation analysis. These antigens were obtained from the Turkish Ministry of Health.

Determination of *L. monocytogenes* in the samples

The EN ISO 11290-1 method (2017) was used for determination of *L. monocytogenes*. After the pre-enrichment process, the sample was placed into Fraser Broth (Biokar) medium and enrichment was performed at 37±1 °C for 24±2 h. After the enrichment process, the samples were inoculated on Agar Listeria according to Ottaviani and Agosti (Merck) and Oxford Listeria Selective Agar medium (Merck), and then incubated at 37±1 °C for 48±2 h. Presumptive *L. monocytogenes* colonies were considered as blue-green colonies surrounded by an opaque halo on Agar Listeria, and grayish-green colonies with collapsed centers, surrounded by a black halo on Oxford Listeria Selective Agar medium. For confirmation of presumptive *L. monocytogenes* colonies, biochemical analyses such as "beta-hemolysis, L-rhamnose, D-xylose, the catalase test, Gram staining" and Microbact *L. monocytogenes* were employed using an identification kit (Oxoid).

Determination of *E. coli* in the samples

The ISO 16649-2 method (2012) was used to determine *E. coli* in the samples. One milliliter of the initial suspension prepared was inoculated on duplicate plates, poured into each Petri dish of the TBX Agar medium (Himedia) and incubated at 44±1 °C for 18-24 h. After incubation, typical blue colony β- glucuronidase-positive *E. coli* colonies on the medium were counted.

Identification of suspected colonies in the samples using MALDI-TOF MS

Suspected *Salmonella* spp. colonies detected on the XLD Agar medium from the bulgur pilaf-G9 sample, and speculated *B. cereus* colonies on MYP Agar medium from the raspberry fruit dessert-G13 sample were also analyzed using MALDI-TOF MS and compared to the conventional methods. *E. coli* colonies in the lettuce-G16 sample were also identified using MALDI-TOF MS.

A MALDI-TOF MS spectrometer (Microflex LT, Bruker Daltonics, Germany) operating by the Flexcontrol software (v.3.0, Bruker Daltonics) for acquiring mass spectra was employed. HCCA (α-cyano-4-hydroxy cinnamic acid) (Bruker) was used as a MALDI-TOF MS matrix. ACN (acetonitrile, HPLC grade) (Sigma-Aldrich), TFA (trifluoroacetic acid) (Sigma-Aldrich), ultra-pure water with a 0.1 μm filter without DNAse and RNAse (Sigma-Aldrich) and a Bruker BTS (bacterial test solution) containing (Sigma-Aldrich) *E. coli*, RNAase and myoglobin protein profiles were used. For microbial biomass analysis with MALDI TOF MS, culture from a single colony with sterile toothpick tip was applied to a special steel 96 MTP-MALDI (Bruker Daltonics) plate (direct transfer method), to which was added 1.0 μL of HCCA matrix solution (12.5 mg/mL HCCA in a mixture of 50% ACN and 2.5% TFA) and the mixture was allowed to dry completely at room temperature. The

MALDI steel plate was loaded onto the MALDI TOF MS. Three studies were performed for each colony and the highest score was taken into consideration by repeated reading. Calibration with BTS was performed simultaneously with the suspected colony.

MALDI-TOF MS was operated with a linear positive ion mode (60 Hz nitrogen laser at 337 nm as ion source) and the method for identifying microorganisms in the mass range of 2,000-20,000 Da. Identification was performed by comparing the mass spectra of suspicious colonies consisting of proteins that were positively ionized by the laser beam (λ = 337 nm) with the most compatible mass profiles in the device library (MALDI Biotyper 3.1; 8500 entires; Bruker Daltonics). In order to obtain the spectra, laser pulses consisting of 40 packets of 240 were performed in the measurement of each colony. The MALDI-TOF MS microbial identification threshold value was set at a 1.70 score value. For identification of micro-organisms, score values between 1.70 to 1.99 were considered as possible genus identification, score values 2.00 between 2.30 indicated secure genus, probable species identification, and score values between 2.30 to 3.00 indicated a higher possible secure species identification (Szabados et al., 2012).

RESULTS

Eighteen ready-to-eat food samples were examined in this study. No growth of atypical or typical *L. monocytogenes* colonies was observed on medium. No staphylococcal enterotoxin was also detected. However, four yellow blackening suspicious *Salmonella* spp. colonies growing on the XLD Agar medium from the bulgur pilaf-G9 sample were subjected to biochemical and serological tests according to EN ISO 6579-1 (2017). The colonies were also identified using MALDI-TOF MS and thus rapidly confirmed. No *Salmonella* spp. were detected in 25 g of bulgur pilaf-G9 sample by either method. Nevertheless, *K. pneumoniae* had already been detected in this sample by MALDI-TOF MS (Figure 1) while using the conventional method. *K. pneumoniae* was reconfirmed as a result of this conventional method.

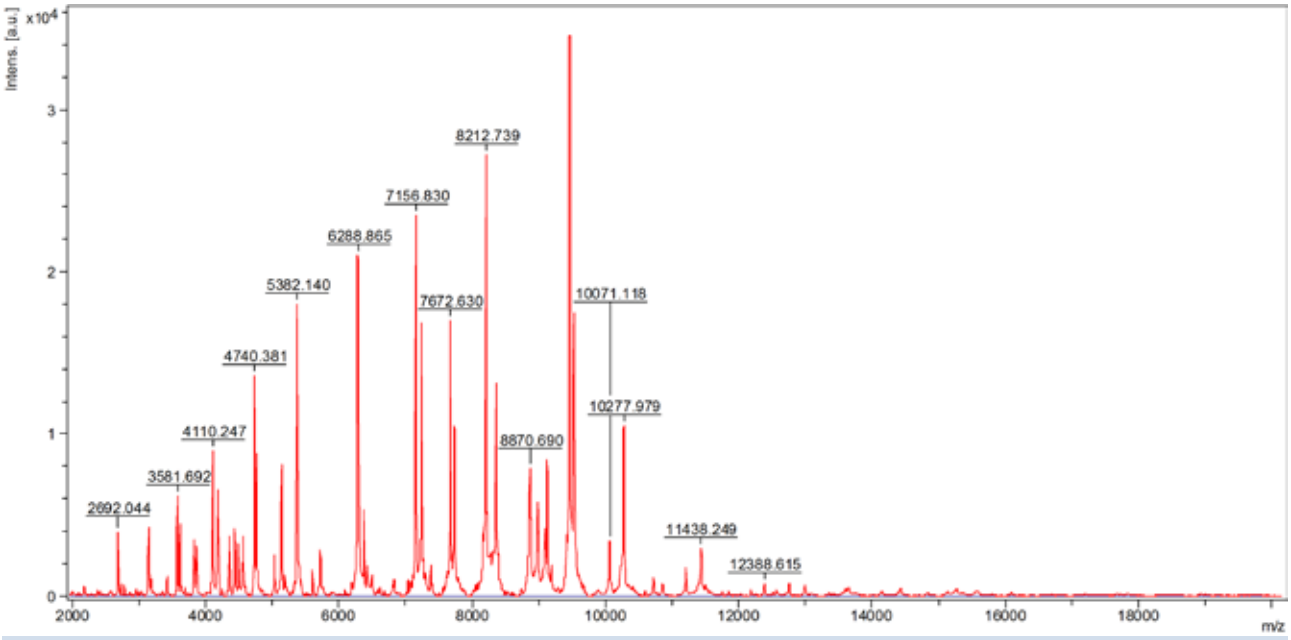

Figure 1. Mass spectrum of *K. pneumoniae* in the bulgur pilaf-G9 (score value=2.322)

Five pink suspicious *B. cereus* colonies surrounded by an opaque zone due to high lecithinase production in MYP Agar medium in the raspberry fruit dessert-G13 sample were confirmed using a conventional method (EN ISO 7932, 2004) and were also identified with MALDI-TOF MS (Figure 2). After these analyses, the number of *B. cereus* colonies per gram of raspberry fruit dessert-G13 sample was 50 cfu. Additionally, typical blue positive β-glucuronidase *E. coli* colonies were observed on TBX Agar from the lettuce-G16 sample, and the number of *E. coli* colonies detected was 80 cfu/g. *E. coli* colonies were also identified by MALDI-TOF MS (Figure 3). In our study, no microbiological growth was observed in the other ready-to-eat food samples, apart from bulgur pilaf-G9, raspberry fruit dessert-G13 and lettuce-G16 samples.

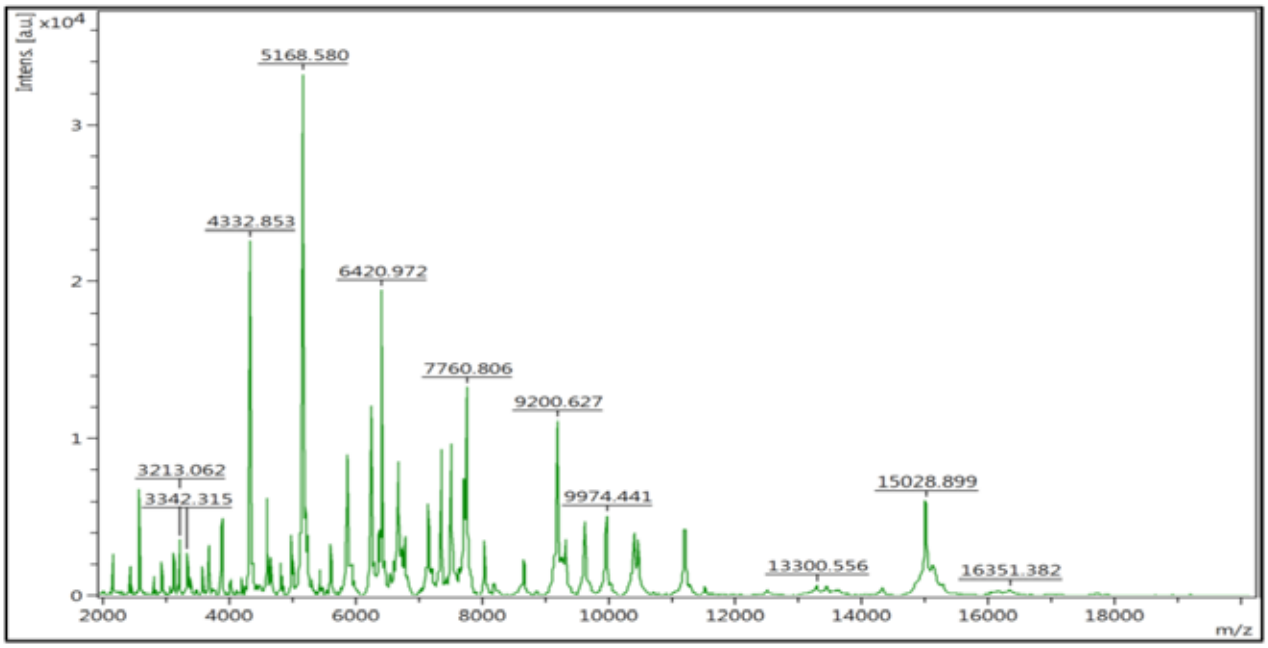

Figure 2. Mass spectrum of *B. cereus* in raspberry fruit dessert-G13 (score value= 2.242)

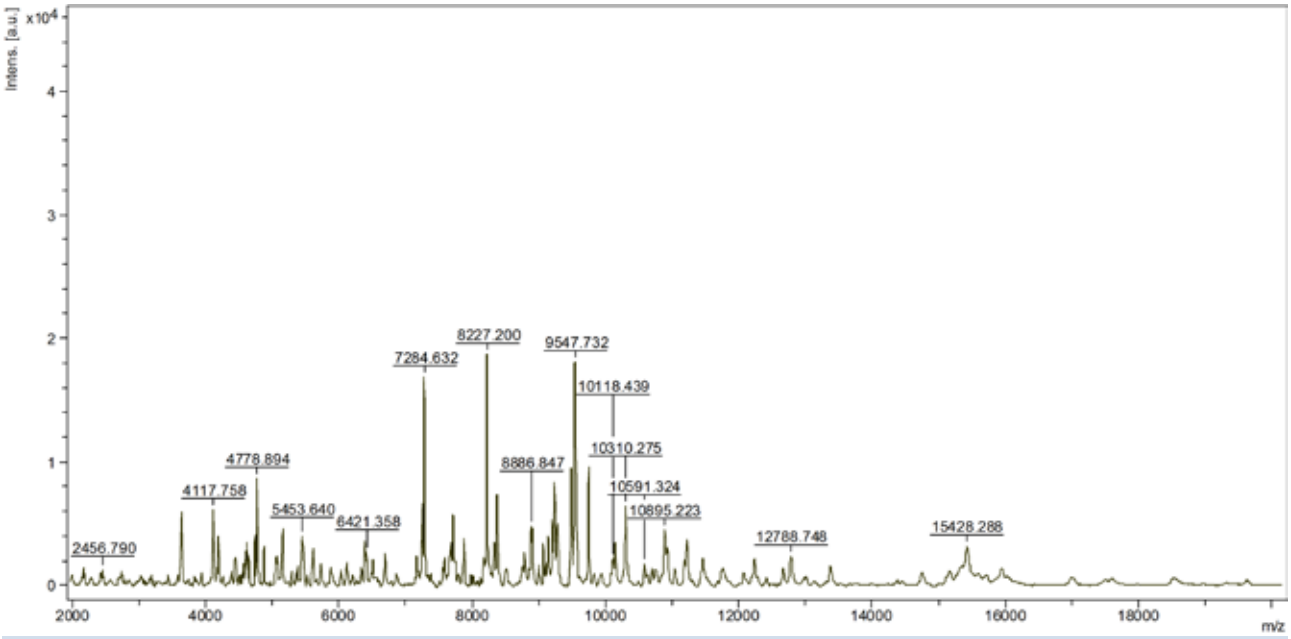

Figure 3. Mass spectrum of *E. coli* in lettuce-G16 (score value= 2.261)

DISCUSSION

Food safety is incredibly important for public health. Cases and/or outbreaks of gastroenteritis may occur due to the consumption of unsafe foods (Aijuka et al., 2018). Microbial agents of food poisoning have therefore been the subject of considerable research (Polański and Sadkowska-Todys, 2018). In this study, the microbiological properties of ready-to-eat food samples available for public consumption in restaurants in Çankaya-Ankara were therefore evaluated. The results of this evaluation were then examined in terms of their compatibility with the Regulation on Turkish Food Codex Microbiological Criteria (2011). In our study, conventional, standard microbiological methods were applied for detecting *Salmonella* spp. (EN ISO 6579-1, 2017), *B. cereus* (EN ISO 7932,

2004), *L. monocytogenes* (EN ISO 11290-1, 2017) and *E. coli* (ISO 16649-2, 2012). Bacteria isolates were also analyzed by MALDI-TOF MS and compared with the results from the conventional microbiological methods. MALDI-TOF MS has previously been used to identify microbiological hazards in foods (Pavlovic et al., 2013; Singhal et al., 2015; Koster and Stanley, 2016; Jadhav et al., 2018). This method has been described as a reliable and rapid technique for the identification and classification of microorganisms (Stahl and Schröder, 2017). The results of this study showed that no *L. monocytogenes* was isolated from any of the samples examined. Moreover, no staphylococcal enterotoxin was also detected. Various studies have investigated the presence of staphylococcal enterotoxin and *L. monocytogenes* in different foods (Leong et al., 2017; Mahfoozi et al., 2019).

Outbreaks caused by *Salmonella* spp. agents have occurred following the consumption of ready-to-eat foods (Donachie et al., 2018). In the present study, suspected *Salmonella* spp. colonies in the bulgur pilaf-G9 sample were isolated and subjected to confirmation tests. *Salmonella* spp. was not detected in any of the ready-to-eat food samples, examined in this study.

However, *K. pneumoniae* was detected in the bulgur pilaf-G9 sample by MALDI-TOF MS (Figure 1). *K. pneumoniae* detection in these foods is not compulsory under the Regulation on Turkish Food Codex Microbiological Criteria (2011). The bulgur pilaf G9 sample was thus compliant with the food safety criteria defined in this regulation, which adopted the Codex microbiological criteria into the national system for use. Nevertheless, it may be hypothesized that hygiene rules were neglected at some stages during the production, preparation, storage, and distribution of the bulgur pilaf-G9 sample. Wang et al. (2019) found that *Salmonella* was commonly present in pickled ready-to-eat meats, a meat product frequently consumed in Shaanxi, China. They recommended that greater attention should be paid to the processing and storage of this ready-to-eat food to prevent bacterial contamination and foodborne outbreaks. Another study indicated that *Salmonella* spp. were found in collected ready-to-eat food samples in middle Thailand (Ananchaipattana, et al., 2016). It was reported that the risk of foodborne illnesses caused by the consumption of these foods after applying suitable hygienic practices in the small food businesses could be reduced (Paul et al., 2017).

Fiedler et al. (2019) investigated food-poisoning by toxin-producing from *B. cereus* strains in ready-to-eat mixed salads in German retail markets. They emphasized the need for monitoring of the presence of these bacteria to ensure that the salads are safe to eat. In the present study, suspected *B. cereus* colonies were identified only in the raspberry fruit dessert-G13 sample and analyzed using conventional methods (EN ISO 7932, 2004) and reconfirmed by MALDI-TOF MS (Figure 2). *B. cereus* was detected at 50 cfu/g in the raspberry fruit dessert-G13 sample. However, *B. cereus* in ready-to-eat foods is not specified in the Regulation on Turkish Food Codex Microbiological Criteria (2011), which adopted the Codex microbiological criteria into the national system for use. *B. cereus* is found in raw and cooked foods, and especially in puddings, cakes, creams, and milk desserts, and has caused significant health problems in public consumption areas serving ready-to-eat foods (Forero et al., 2018). Yu et al. (2019) evaluated *B. cereus* in ready-to-eat food samples including cooked meats, cold vegetable dishes in sauce and rice/noodles in different regions of China, demonstrating the potential hazards of *B. cereus* isolated from these foods. Based on our study results, we have considered that milk desserts should be subjected to a rapid cooling process at +4 °C after cooking to prevent food poisoning caused by *B. cereus. B. cereus* is found in milk desserts as a resulting of poor hygiene during production, storage, and sale (Kaynar, 2020). Amin (2018) recommended that these desserts be prepared in small batches, cooled rapidly, and stored at 4°C.

In this study, *E. coli* (80 cfu /g) was found in the lettuce-G16 sample. However, the amount detected (80 cfu /g) was lower than the threshold limit value for *E. coli* (10^1 cfu /g) which is permitted in these foods by the Regulation on Turkish Food Codex Microbiological Criteria (2011), which adopted the Codex microbiological criteria into the national system for use. The lettuce-G16 sample thus complied with that regulation. Castro-Rosas et al. (2012) investigated the presence of *E. coli* in ready-to-eat salads purchased from restaurants in Pachuca-City, Hidalgo, Mexico. They reported that the salads analyzed were of poor microbiological quality since diarrheagenic *E. coli* pathotypes (DEPs) were identified in up to 6% of salad samples and that raw vegetables should continue to be screened. In addition, they suggested that the irrigation of raw vegetables with untreated sewage water should also be forbidden by national legislation. Mira Miralles et al. (2019) did not detect the presence of *E.*

coli in salads, one component of ready-to-eat foods. Ready-to-eat raw salad can be easily contaminated by *E. coli* originating from water sources (Temelli et al., 2005). In the present study, we hypothesized that cross-contamination may have occurred due to inadequate washing of the ready-to-eat raw lettuce-G16 sample and due to poor hygiene of personnel and equipment during salad preparation.

CONCLUSION

In conclusion, the tested ready-to-eat foods in this study intended for public consumption complied with general hygiene criteria. These foods complied with the national legislation, which adopted the Codex microbiological criteria into the national system for use. However, it is important that good hygiene rules and practices must be applied in all stages of food supply chain, from preparation to consumption to safeguard the public health. It is also essential that the raw materials used in the preparation of ready-to-eat foods should not pose a health risk. MALDI-TOF MS can be employed in addition to conventional methods used in the microbiological evaluation of these foods, as well as for identifying the source of food poisoning. This method also has the advantages of greater simplicity, rapidity, and shorter application times.

CONFLICT OF INTEREST

None declared by the authors.

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