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RESEARCH ARTICLE **ARAŞTIRMA MAKALESİ**

Identification of *Propionibacterium* spp. Isolated from Mihaliç Cheeses by MALDI-TOF MS

Mihaliç Peynirlerinden İzole Edilen *Propionibacterium* Türlerinin MALDI-TOF MS ile Tanımlanması

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Abstract

Propionic acid bacteria (PAB) are responsible for characteristic properties of Mihaliç cheese, which is one of the most prevalent traditional cheese types produced in Turkey. Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been reliable and rapid method for identifying bacterial isolates. The aim of this study was to evaluate the PAB profile of Mihaliç cheese by their identification by MALDI-TOF MS system. In this study, a total of 25 cheese samples were analyzed and 95 isolates were determined as probable PAB based on their morphological characteristics, gram staining, catalase activity and pigment production. All isolates were analyzed by MALDI-TOF MS and 21 of them were belong to PAB. Isolates were identified as *Propionibacterium freudenreichii* ssp. *freudenreichii* (57%), *Propionibacterium freudenreichii* ssp. *shermanii* (33%) and *Propionibacterium thoenii* (10%). This study indicates that the diversity of PAB found in Mihaliç cheese can be determined rapidly and economically by MALDI-TOF MS.

Keywords: Mihaliç cheese, Propionic Acid Bacteria, MALDI-TOF MS

Öz

Türkiye'de üretilen önemli geleneksel peynirlerden biri olan Mihaliç peyniri, karakteristik özelliklerini Propiyonik asit bakterileri (PAB) sayesinde kazanmaktadır. Matris destekli lazer desorpsiyon / iyonizasyon uçuş süresi kütle spektrometresi (MALDI-TOF MS), son yıllarda bakteriyel izolatları tanımlamak için güvenilir ve hızlı bir yöntem olarak popüler olmuştur. Bu çalışmanın amacı Mihaliç peynirinde bulunan propiyonik asit bakterilerini MALDI-TOF MS sistemi ile tanımlamaktır. Bu çalışmada toplam 25 adet geleneksel Mihaliç peynir örnekleri incelenmiş ve morfolojik özelliklerine, gram boyamaya, katalaz aktivitesine ve pigment üretimlerine dayanarak 95 adet izolat elde edilmiştir. Bu izolatlar MALDI-TOF MS ile tanımlanmış ve 21 tanesi PAB olarak belirlenmiştir. Tanımlanan izolatlar; *Propionibacterium freudenreichii* ssp. *freudenreichii* (% 57), *Propionibacterium freudenreichii* ssp. *shermanii* (% 33) ve *Propionibacterium thoenii* (% 10) dir. Bu çalışma, Mihaliç peynirinde bulunan PAB çeşitliliğinin ilk kez MALDI-TOF MS tarafından hızlı ve ekonomik olarak belirlenebileceğini göstermiştir.

Anahtar Kelimeler: Mihaliç peyniri, Propiyonik Asit Bakterileri, MALDI-TOF MS

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Mihalic cheese is a traditional cheese produced in Turkey, especially in Bursa-Balıkesir region. It is characterized by a salty, hard and slightly acidic cheese (Demirci, Şimşek, & Taşan, 1994). It is preferred for its characteristic natural taste, texture, and flavor. Mihaliç cheese is usually produced by natural contaminating microorganisms, which grow during the ripening period and support maturation (Hayaloglu, Ozer, & Fox, 2008; Ozer, 2015). Typical properties of this cheese are mainly associated with dairy Propionibacteria, which are also key organisms in the production of Emmental and Swiss-type cheeses (Freitas et al., 2015). Mihaliç cheese has many common properties including texture and eye structure with Swiss-type cheeses. Since the products of lactate metabolism are responsible for the specific eye formation and distinctive flavor and texture, the *Propionibacteria* used in maturation process is essential in the production of those cheeses (Britz & Riedel, 1994).

Propionibacteria are from the *Actinobacteria* class with high G+C % (64–68 %). They are gram positive, catalase positive, non-motile, mesophilic, pleomorphic rods, non- spore-forming, anaerobic/aerotolerant bacteria. The optimal temperature for growth is 30 °C, but they grow at 15–40 °C at pH 5.1–8.5 (Cummins & Johnson, 2003; Patrick & McDowell, 2015). Besides fermentation properties, dairy *Propionibacteria* are used as probiotic, silage inoculum and in the production of vitamin B12 and propionic acid (Argañaraz-Martínez, Babot, Apella, & Perez Chaia, 2013; Ibrahim, Effat, Tawfik, Mehanna, & Soliman, 2017; Martens, Barg, Warren, & Jahn, 2002; Pillai, Prakash, & Lali, 2017).

Probiotic properties of *Propionibacteria* make them health-promoting microorganisms (Ibrahim et al., 2017). *Propionibacteria* can produce various food components that contribute to the health-benefits of fermented foods (Luiz et al., 2017; Plé et al., 2015). Propionic acid bacteria used as a starter culture in cheese production can exhibit beneficial health effects; this may lead to improvement of new fermented dairy products with the help of identified strains having beneficial properties. It is crucial to have a reliable, quick and economical technique for the identification of productive and valuable strains(Vorob'eva, Khasaeva, Vasilyuk, & Trenquil, 2011).

It is fundamental to identify PAB in order to determine strains, which can produce functional compounds, so provide advantages to industry. There are many genotypic methods like polymerase chain reaction (PCR), random amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) used in characterization of microorganisms (Giraffa & Neviani, 2001). However, they do not meet the need of an economic, fast and precise method for identification. Classification of these microorganisms using classical microbiological methods is time- consuming and interpretation of the results is often difficult.

Rapid and reliable identification of food-related bacteria is essential for food safety and quality. Recently, MALDI-TOF MS has been used as a tool for quick and correct characterization of microorganisms (Wieser, Schneider, Jung, & Schubert, 2012). MALDI-TOF MS has been employed as a tool for not only identification of bacteria, but also yeast, mycobacteria, and molds (Chalupová, Raus, Sedlářová, & Šebela, 2014; Pavlovic, Huber, Konrad, & Busch, 2013). The method is based on the determination of distinctive proteins, like ribosomal proteins and identification through automatic matching of the generated mass spectrum with spectra in the database (Lay, 2001).The principle of MALDI-TOF MS basically relies on thermal desorption caused by laser energy leading to ionization of proteins and then ions can move in the flight tube according to their degree of ionization and the masses. Therefore, they can be separated easily based on their time of flight. Flight times of the ions provides a characteristic spectrum which is unique for each species (Fenselau & Demirev, 2001). Since MALDI-TOF MS fingerprints a large spectrum of proteins, it has the ability to classify closely related species and to identify organisms at the species level (Fox, 2006; Murray, 2010).

There are many advantages that MALDI-TOF MS provides for microbial identification include easy pretreatment of samples, quick results, and lower costs, compared to conventional phenotypic techniques (Pavlovic et al., 2013). The suitability of MALDI-TOF MS for identification of propionic acid bacteria has been studied before and it has been proved that MALDI profiles of the bacteria indicate high correlation with the genotypic identity (Vorob'eva et al., 2011). Therefore, the aim of the present study was to determine the most abundant propionic acid bacteria in Mihaliç cheese by MALDI-TOF MS, which is a rapid and reliable technique. Considering that propionic acid bacteria can be used in numerous food-related applications, it is important to identify them to make use of beneficial properties.

Materials and Methods

Isolation of Propionic Acid Bacteria

A total of 25 Mihaliç cheese samples were provided from 10 different local producers in Balıkesir–Bursa region. Cheese sample were at least 3 months maturated. Samples were brought to the laboratory without breaking cold chain. They were stored at 4 °C until analyses start.

Several colonies were randomly selected from all the cheese samples and then purified on the same medium. Isolates of propionic acid bacteria were grown at the optimum growth temperature (30°C), in yeast extract lactate broth (YEL Broth) for 48 hours under anaerobic conditions. After incubation, samples were examined under a microscope to determine the morphological characteristics. Gram staining was performed to separate gram positive colonies, then catalase activity test was done to determine the catalase positive isolates. Moreover pigment production after ten days on yeast extract lactate agar (YEL Agar) plates was observed and white/cream, yellow/orange and red/brown colonies were evaluated as "possible PAB" (Britz & Riedel, 1994).

Identification of Isolates by MALDI-TOF MS

MALDI-TOF MS analysis was performed by following the instructions given by Bruker Daltonics, (Bremen, Germany). It was performed for 95 isolates. Each sample was placed in duplicate onto the Micro scout 96 target plate.

A single colony was picked from a petri dish and transferred into a tube, then suspended with distilled water. It was centrifuged for 5 min at 6000 g. The pellet was mixed with acetonitrile/formic acid/water (50:35:15, v/v) solution for the extraction process. α- cyano-4-hydroxycinnamic acid (CHCA) was chosen as the matrix solution. For analysis, the cell suspension in the matrix (1: 1) was applied dropwise with an automatic dispenser onto the target plate and then dried at room temperature. After matrix and sample dried on the target plate, it was inserted in the MALDI-TOF MS instrument and the proteomic spectrums were generated.

The mass spectrometry was calibrated with the manufacturer bacterial test standard *E. coli* DH5α extracts (Bruker Daltonics, Bremen, Germany). Ion mass spectra in the linear mode were executed on a Microflex LT mass spectrometer (Bruker Daltonics, Bremen, Germany). The range of recorded masses was 3,6–17 kDa in the positive ion mode. The spectrum of the sample was then matched with reference spectrum in the MALDI Biotyper Reference Library version 4.0.0.1 (Bruker Daltonics). The integrated software generates an outcome list.

Results obtained from the Microflex LT system were given as score values. Score values > 2.0 (green color) and from 1.7 to 2 (yellow color) were recognized as reliable at the species and genus level, respectively. When the given score was < 1.7, the fingerprint was not reported as 'reliable identification', meaning that it did not match sufficiently with any reference species in the database.

Result and Discussion

Characterization studies of dairy propionic acid bacteria are important to realize their diversity in various samples (Vorob'eva et al., 2011). Since food industry are looking for biotechnologically important strains for being used as culture in ripening process of cheese, research about this topic become significantly important.

Dairy *propionibacteria* do not exist in human microbiota normally however they can be found naturally in raw milk, dairy products, soil and fermented foods and plant materials like fermented olives (Patrick and McDowell, 2012). Dairy *propionibacteria* traditionally isolated from milk and dairy products were described as: *P. freudenreichii*, *P. acidipropionici*, *P. jensenii* and *P. thoenii* (Cumins & Johnson, 1986).

For this study 25 Mihaliç cheese from 10 different local producers were provided YEL agar was chosen for growth of ''target'' microorganisms which are propionic acid bacteria, (Britz & Riedel, 1994). Among the different isolates from these cheese, 95 isolates were selected according to their morphologic properties including being Gram-positive, non-motile, non-spore former, anaerobic to aerotolerant cocci to short rods and catalase positive. However, only 21 of them were identified as *Propionibacterium* spp. according to MALDI-TOF MS analysis. The rest was identified as mostly *Lactobacillus* sp. and *Enterococcus* sp.. Quantitative information on the identified isolates from the samples is given in Table 1.

Table 1: Identification of Isolates from Mihaliç Cheeses Cultured on YEL Agar Plates Incubated Anaerobically at 30 °C.

Defined Microorganisms	Number of Isolates	Percent of Isolates (%)
Propionibacterium freudenreichii spp.	19	20
Propionibacterium thoenii sp.	2	2,1
Lactobacillus paracasei spp.	22	23,2
Lactobacillus plantarum sp.	13	13,7

Lactobacillus fermentum sp.	10	10,5
Enterococcus faecalis sp.	4	4,2
Enterococcus faecium sp.	4	4,2
Enterococcus durans sp.	3	3,2
not reliable identification	18	18,9

Recently, a study investigating microbiota of local cheese from Turkey published similar results obtained by MALDI-TOF MS (Kanak & Yilmaz, 2018). Kanak (2018) specially focused on lactic acid bacteria and identified as *Enterococcus durans* (6), *E. faecalis* (18), *E. faecium* (24), *E. italicus* (2), *Lb. brevis* (1), *Lb. paracasei* (2), *Lb. plantarum* (1), *Lactococcus lactis* (3), *Leuconostoc lactis* (1), *Leu. mesenteroides* (11), and *Streptococcus parauberis* (2) at species levels using MALDO-TDF MS analysis. Similarly, a study conducted to determine lactic acid bacteria isolated from a French cheese by MALDI-TOF (Nacef, Chevalier, Chollet, & Flahaut, 2017). They also indicated that *Lactobacillus* was the most abundant genus with seven species: *Lb. plantarum*, *Lb. paracasei*, *Lb. curvatus*, *Lb. rhamnosus*, *Lb. fructivorans*, *Lb. parabuchneri*, *Lb. brevis* found in cheese samples.

In this study, 81,1% of isolates were identified at the species and genus level reliably. It was identified that 18 isolates were unreliably identified at the genus level. *Lactobacillus* paracasei spp. were most abundant bacteria isolated from Mihaliç cheese. *Propionibacterium*, the main focus of this study, were 27,8% of the all identified isolates.

The MALDI-TOF MS analysis of the isolates allowed differentiation of 21 propionic acid bacteria at the species level. All 21 isolates identified were from the 'classical' group of the genus *Propionibacterium*. The three different species of dairy *propionibacteria* determined in that study were represented as a pie chart in Figure 1. The most predominant species was *Propionibacterium freudenreichii* ssp. *freudenreichii* (57 %) which was reported as the main species in Swiss-type cheese (Baer & Ryba, 1992). Moreover, a study performed to determine the *Propionibacterium* species in Mihaliç cheese showed that *Propionibacterium freudenreichii* subsp. *freudenreichii* was ubiquitous species (Önal Darılmaz, 2010). *Propionibacterium freudenreichii* ssp. *shermanii* was second highest (33%) propionic acid bacteria found in cheese samples. It has been difficult to distinguish between the subspecies in identification by conventional methods since P. *freudenreichii* subspecies *freudenreichii* and *shermanii* can be differentiated by lactose fermentation and nitrate reduction, while *shermanii* can ferment lactose and not have reductase activity for nitrate (De Freitas et al., 2015). Although molecular methods have failed up to separate P. *freudenreichii* subspecies, MALDI-TOF analysis has been successful method for characterization of intraspecific differences (subspecies, strains) (Vorob'eva et al., 2011). Because MALDI-TOF MS provides more detailed protein profile than what is signified by the 16S rRNA gene, it can detect strain-level differences among isolate samples (Seuylemezian et al., 2018). The MALDI spectra of the samples revealed that there were characteristic protein groups belong to *Propionibacterium* as marker biomolecules (Vorob'eva et al., 2011); therefore, they can be differentiated easily.

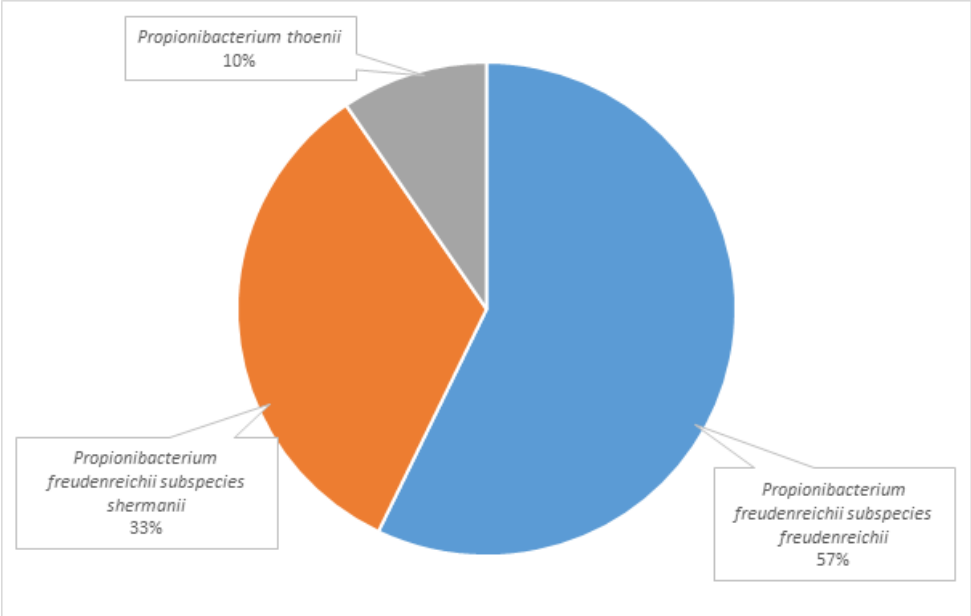

Figure 1: Diversity of *Propionibacterium* Strains Determined in Mihaliç Cheese

Propionibacterium freudenreichii has been considered as a must culture in Swiss-type cheese production during the ripening period and is taking attention recently because of its beneficial probiotic effects. In cheese manufacture, it has an important role since it produces a variety of flavor compounds as well as it causes lipolysis milk fat and degradation of amino acids (Thierry, Maillard, Richoux, Kerjean, & Lortal, 2005). Furthermore, the probiotic effects of microorganisms are highly strain-dependent. A study about probiotic properties of dairy *propionibacteria* studying acid tolerance, resistance to bile salts, antimicrobial activity, fermentation of carbohydrates, phenol resistance, acid production, exopolysaccharide production and antibiotic susceptibility and then indicated that 8 tested strains of *propionibacteria* had various technological and probiotic properties (Ibrahim et al., 2017). So, it is important to identify microorganisms at the strain level.

The other *Propionibacterium* detected by MALDI-TOF was *Propionibacterium thoenii* sp. which represented 10% of the all propionic acid bacteria found in Mihaliç cheese. In contrast with this study, Onal Darilmaz (2012) did not identify any P. thoenii in their study investigated traditional Turkish cheese. It demonstrates that especially traditional cheese from different producer may have different microbiota which directly affects the texture, flavor and quality of cheeses.

Since functional properties are peculiar to specific strains, the characterization of microorganisms at species or better strain level is essential for starter culture and cheese producers. Even though molecular identification methods are successful enough, MALDI-TOF systems have been preferred more because it is faster and less expensive. A study about the cost analysis of MALDI-TOF process revealed that it was 20-30 % cheaper than conventional identification methods. Moreover, MALDI-TOF required 5-6 min for identification of one isolate (Seng et al., 2009). It is obvious that MALDI-TOF can reduce the time, cost and workload for identification of bacterial cultures. When it is used as the first step identification method especially for slow growing and anaerobic microorganisms, it may lead to an increase in laboratory efficacy because of advantages of MALDI system such as being very quick and economical.

Conclusion

Recently, there is an increasing demand for artisanal cheese for the reason that wild strains can grow in that kind of products and produce unique taste and flavor. The large diversity of microorganisms in traditionally produced cheese makes it possible to determine them having potential functional properties. In this study, it was seen that MALDI-TOF MS can be imperative method to identify microorganisms in short time and in an economic way. *Propionibacterium* species obtained in this study were examined in details to discover the specific properties, which are topic of another study. Strains with specific properties may be utilized to encounter the need for traditional fermentations or be used in the production of new functional food dairy products. Therefore, reliable identification and high discrimination of *propionibacteria* strains are significantly fundamental in food research. MALDI-TOF based identification, which is cost-effective, robust, reliable method, can allow the selection of appropriate strains for specific starter cultures for standardization and improvement of the cheese quality and safety.

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