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Identification by using MALDI-TOF mass spectrometry of lactic acid bacteria isolated from non-commercial yogurts in southern Anatolia, Turkey

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Summary. Yogurt is a dairy product obtained by bacterial fermentation of milk. Commercial yogurts are produced using standard starters while, in the production of non-commercial yogurt, the microbiota is quite different since yogurts are used as starter for years. To determine the final characteristics of the fermented product it is necessary to know the biochemical properties of the starter cultures, such as acidity, aroma and flavor. This can only be achieved by identifying and characterizing the bacteria in starter cultures. In our study, 208 non-commercial yogurt samples were collected from 9 different locations in Anatolia, southern Turkey. Their pH and lactic acid bacteria profiles were analyzed. Isolated bacteria were identified by MALDI-TOF MS (matrix-assisted laser sesorption-ionization time-of-flight, mass spectrometry), which is a fast and reliable method for identification of bacterial isolates compared to classical laboratory methods. In this study, 41% of the isolates were identified by using this method, which is 99.9% and 34.0% confidence. The isolates contained two genera (*Enterococcus* and *Lactobacillus*) and four species. Afterwards, the four lactic acid bacteria were characterized physiologically and biochemically and we found that they differed from lactic acid bacteria used in commercial yogurt production. [**Int Microbiol** 20(1): 25-30 (2017)]

Keywords: yogurt starters · lactic acid bacteria (LAB) · southern Anatolia (Turkey)

Introduction

Yogurt is a dairy product obtained by the bacterial fermentation of milk. Lactic acid, which is produced via the fermentation of the milk sugar lactose, is the compound that confers part of the typical textural and sensory characteristics of yogurt and acts on milk protein [2]. Yogurt, which contains high concentrations of lactic acid bacteria (LAB), has been described to have several potential health benefits: it can increase lactose tolerance, balance the intestinal microbiota, act as an antimicrobial compound, stimulate the immune system, induce anti-tumor effects, and induce anti-cholesterolemic effects [6]. Today, the vast majority of yogurt needs are met by commercially produced yogurts, and the goal of companies is

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to produce more solid, continuous sweet yogurt with a variety of additives. Commercial yogurts are produced thereby using standard starters.

During the fermentation of food stuffs, the main physical and chemical changes occur due to the growth and fermentative activity of the LAB that are used as starter cultures. LAB are also used as starter cultures for the fermentation of milk [22] and have been used for centuries as food preservatives [7,11]. Some LAB ferment lactose into lactic acid and thus help to acidify the final product. Others digest the proteins and lipids in milk producing several other substances that contribute to the formation of the unique flavor, aroma, appearance, and structure of a fermented product [18].

LAB comprise a genetically diverse group of bacteria that includes the following genera: *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* [15]. Those of industrial importance comprise *Lactococcus*, *Enterococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Leuconostoc* and *Lactobacillus* species [17]. LAB are typically classified according to categories such as morphology, Gram staining, oxidase and catalase tests [7,13,20], growth at different temperatures and salt concentrations [26,27], carbohydrate fermentation [27,31], and cell wall composition. Knowledge of the biochemical properties of the starter cultures is necessary to determine the final characteristics of the fermented food product, such as acidity, aroma and flavor. To gain such knowledge, it is crucial to identify and characterize the bacteria in a starter culture.

Analysis of protein profiles of microorganisms carried out with the MALDI-TOF (Matrix-assisted laser desorption-ionization time-of-flight) mass spectrometry are compatible with the results of identification made by using molecular methods [25].Therefore, MALDI-TOF can be considered a robust and reliable method, as well as fast and inexpensive to identify Gram-positive bacteria such as lactic acid bacteria [25,30].

The purpose of this study was to isolate and characterize LAB from collected non-commercial yogurts in southern Anatolia and find out whether they differed from the LAB used for the production of commercial yogurts.

Materials and methods

Traditional yogurt production and samples collection. A total of 208 non-commercial yogurt samples were collected from nine villages in southeastern Anatolia, Turkey, from March to May, 2015 (The various locations are indicate with codes in the Tables.). Yogurts had been produced with traditionally methods by people from those villages. They used cow milk that was heated to 85 °C for 20 min and was then cooled rapidly to 46 °C. After this sort of pasteurization, one cup the inoculum obtained from a previous fermentation was added to approximate 1 liter of cooled milk. Every yogurt sample came from a different inoculum. After inoculation, milk was incubated at room temperature (or 5 °C higher) for one night or 6–7 h. Afterwards, the yogurt obtained was kept in a refrigerator at 4 °C. The pH values of the home-made yogurts were measured with a pH meter (Martini Instruments, Romania) and recorded.

Bacterial isolates. Agar plates of media M17 (Oxoid, UK) (50 ml of 10% lactose solution was added) and MRS (Oxoid, UK) were inoculated by dilution. The plates were incubated aerobically at 37 °C and 30 °C for 72 h. The numbers of the resulting colonies were determined as CFU (colony forming units)/g. Phenotypic characterization (colony morphology, Gram staining and catalase assay) of LABs isolated from colonies was performed. Afterwards, they were identified by using MALDI-TOF mass spectroscopy.

Isolation and total bacterial counts. The samples were diluted before inoculating them on M17 agar (Oxoid, England) and MRS (de Man, Rogosa and Sharpe) agar (Oxoid, England). The M17 and MRS plates were incubated for 3 days in aerobic environments at 37 °C and 30 °C, respectively [10,12,14,21]. All of the suspect colonies in petri dishes were recorded as CFU/g [21].

Stock culture preparation. A sterile loop was used to sample colonies and to inoculate them into Eppendorf tubes containing 1 ml of M17 Broth (Oxoid, England) or MRS Broth (Oxoid, England). Bacterial samples inoculated into M17 were incubated for 48 h. Stocks were prepared from 500 µl of LAB cultured in MRS broth or M17 broth and 500 µl of sterile liquid glycerol that were mixed in a 1 ml Eppendorf tube and stored at –20 °C [5].

Identification of LAB. The phenotypic identification of colonies was conducted according to their culture properties (Gram staining), their cell morphology (cocci or rods) under the light microscope and their biochemical properties (catalase test) [4,5,24]. For these tests, stock cultures were grown on M17 agar and MRS agar as described in the previous paragraph.

The identification of LAB colonies isolated from non-commercial yogurts was carried out according to their protein and peptide registered information analysis using MALDI-TOF MS, which is a new technology for identifying LAB cultures. The advantages of this embodiment are that it has rapid and routine properties. For these tests, stock cultures were grown on M17 and MRS agar, and applied according to Susakol et al. [5]. The isolates were tested against various surface proteins. All suspicious isolates were selected for LAB identification with the MALDI-TOF MS test kit. Protein patterns of these isolates were analyzed by MALDI. Bruker MS (Becton-Dickinson USA) was used to identify the microorganisms. In the MALDI-TOF method, the Bruker device transforms the so-called matrix into a molecule of interest (DNA or proteins) by absorbing the matrix light after the ion beam is applied to a chemical surface that has been exposed to light for 24 h.

This method, which is used for extracting protein products from microorganisms, is easily recognizable by the result of comparing these profiles with a reference spectrum. In definition: 1 drop of physiological saline was sprayed onto the matrix, and fresh (24 h) samples were taken from the cultures. After

mixing with the serum physiological solution in the matrix and drying in the air, identification was made in the MS device.

Characterization of LAB. The growth properties of LAB species that were isolated from the yogurt samples were determined at 4 °C, 25 °C and 45 °C [8] at pH 2, pH 5 and pH 7 [29] and at 3%, 6% and 10% NaCl (table salt) [20].

Results

pH measuring. The pH values of the 208 yogurt samples analized ranged from 3.9 to 6.0. The average pH from same sources ranged between 4.35 and 4.97 (Table 1).

Isolation and total bacterial counts. The total bacterial counts on MRS agar medium were 0–2.3 × 10^{10} CFU/g, while the total bacterial counts on M17 agar medium were 3.7 × 10^{8}–3.1 × 10^{10} CFU/g following the isolation of LAB from the yogurt samples. Out of the 731 variety analyzed isolates, 503 were cocci (360 cocci were identified on M17 medium and 143 cocci were identified on MRS medium) while 228 of the analyzed LAB were rods (100 on M17 medium and 128 on MRS medium). Overall, 69% of the isolates were cocci and 31% were rods. All of the LAB were non-motile.

Bacterial isolates. Of the 503 cocci isolates, 86 (17%) belonged to the genus *Enterococcus*: 45 were *Enterococcus hirae* (9%), 36 were *Enterococcus faecium* (7%), 5 were *Enterococcus durans* (1%). Of the 228 rod isolates, 54 (24%) belonged to the genus *Lactobacillus*, the sepcies *Lactobacillus casei* or *Lactobacillus paracasei*. We attempted to classify the rest of cocci and rod isolates, but the data obtained were not reliable. The distribution of different LAB among the yogurts collected is shown in Table 2.

All of the LAB were Gram-positive. Gram (+) bacteria were selected by Gram staining. Additionally, LAB do not encode a catalase enzyme, and thus they cannot degrade hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) molecules. The results of catalase tests were negative (–) for the isolated LAB. The LAB identification results revealed that the LAB microbiota in the Turkish yogurts analyzed contained similarity 99,9%; 7% *Enterococcus faecium*, 9% *E. hirae*, 1% *E. durans*; contained similarity 34,0%; 24% *Lactobacillus casei* or *L. paracasei* (rod isolates are presented with either of them by 34,0% similarity). Spectra of four species obtained by MALDI-TOF MS are shown in Fig. 1.

Table 1. The pH values of yogurts collected from villages in Turkey

Villages	n	pH values		
		Smallest	Biggest	Average
S1	30	4.00	5.90	4.40
S2	30	4.00	6.00	4.53
O	30	4.10	4.90	4.35
K	15	4.30	5.00	4.62
I	15	4.90	5.00	4.97
N1	15	4.40	4.80	4.63
N2	28	4.30	5.20	4.55
A	15	3.90	4.90	4.43
Y	30	4.30	4.70	4.43

(n = sample counter; S1, S2, O, K, I, N1, N2, A, Y = codes of nine locations, villages)

The most broadly growing isolates were *Enterococcus durans* and *E. faecium* (the latter grew under all conditions except pH 2; 3%, 6% NaCl), *Enterococcus hirae* (it grew under all conditions except pH 2, 5; 3% NaCl). In contrast, the most sensitive isolates were *Lactobacillus casei* and *L. paracasei* (they former failed to grow at 4, 45 °C, at 10% NaCl and at pH 2; and the latter failed to grow at 4, 45 °C, at 6%,

Table 2. The distribution of different LAB among yogurts collected from villages in Turkey

Villages	n	*E. faecium*	*E. hirae*	*E. durans*	*L. casei ?* *L. paracasei ?*
S1	30	7	2	–	1
S2	30	18	3	–	1
O	30	11	8	–	5
K	15	–	20	–	11
I	15	–	7	5	3
N1	15	–	5	–	–
N2	28	–	–	–	18
A	15	–	–	–	9
Y	30	–	–	–	6
Total		36 isolates	45 isolates	5 isolates	54 isolates
		86 isolates cocci			54 isolates rod

(n = sample counter; S1, S2, O, K, I, N1, N2, A, Y = codes of nine locations, villages)

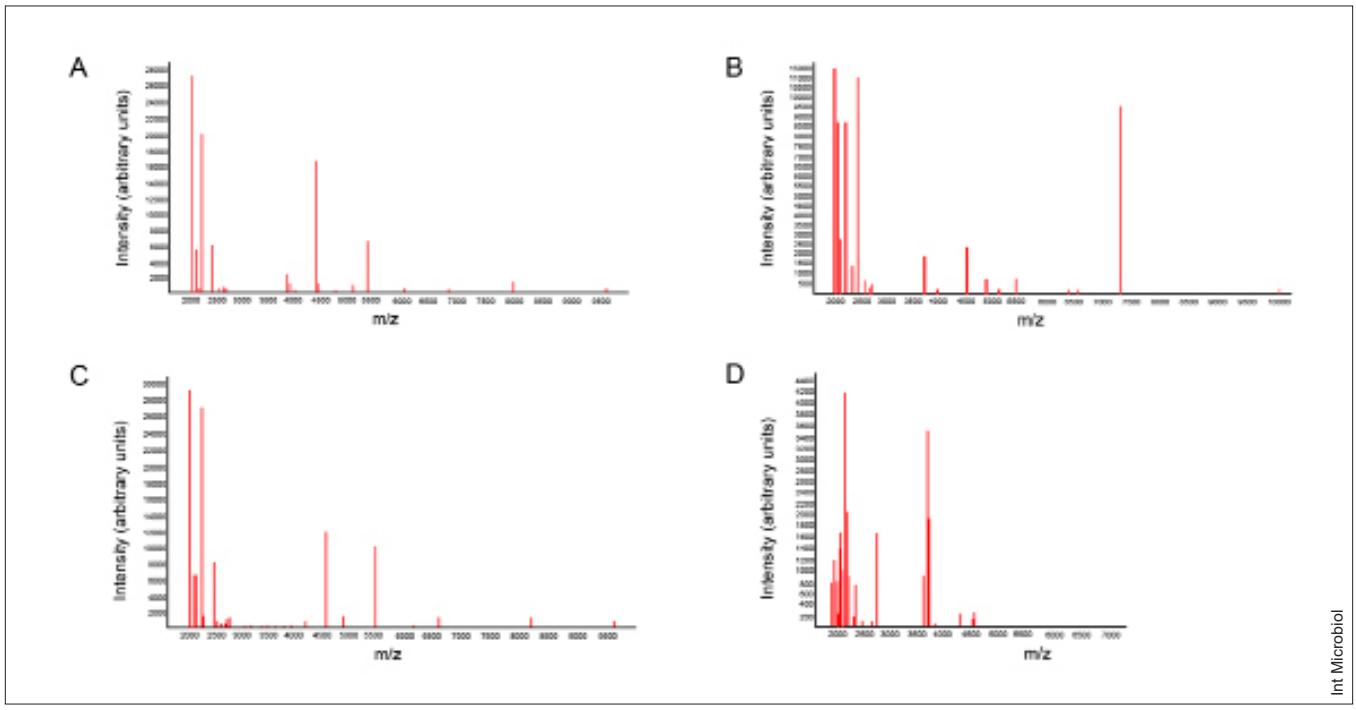

Fig. 1. Spectra of four species obtained by MALDI-TOF-MS. **(A)** Spectrum of *Enterococcus hirae* (99,9% similarity). (**B)** Spectrum of *Enterococcus faecium* (99,9% similarity). (**C**) Spectrum of E*nterococcus durans* (99,9% similarity). (**D)** Spectrum of *Lactobacillus casei* (34,0% similarity), or *Lactobacillus paracasei* (34,0% similarity).

10% NaCl and at pH 2). Almost all LAB could grow, either weakly or strongly, at 25 °C and at pH 7. The characteristics of the LAB species isolated are shown in Table 3.

Discussion

Lactic acid bacteria (LAB) play an important role in food technology. They are the microorganisms that typically used for the production and long-term preservation of various fermented dairy products such as yogurt, buttermilk and cheese. LAB are considered generally recognized as safe (GRAS) microorganisms [16,23]. They are cocci or rod-shaped bacteria that primarily produce lactic acid as the end product of carbohydrate fermentation [1,3,23]. The results of this study showed that the pH levels of then non-commercial yogurts from villages in Turkey that we analyzed were acidic (below 7) as a result of lactic acid fermentation; (their pH ranged from 3.9–6.0). As no standard inocula were applied in this study, the relatively high pH of some yogurts could be related to both yeasts and bacteria present in the yogurt total microbiota. In fact, 80 colonies of yeasts were also found under the microscopy in 20 yogurt samples. With striking resemblance, yeasts were observed in the samples from S2 (30 colonies) and S1 (7 colonies) locations, which also had the highest pH values. These yeasts, however, have not been identified yet. The animal sources of the milk from which the yogurts were obtained varied; they included cow milk, sheep milk and goat milk. Erkaya and Sengul [9] have described pH levels of yogurts produced from different milk types ranged between 4.02 and 4.26 and they have reported that these pH values were related to the metabolic activities of the starter cultures and the fermentation time of the yogurt [9]. In addition, Omafuvbe and Enyioha [20] have found pH values ranging from 3.80 to 4.48 in yogurts obtained from villages in Nigeria [20]. They have also reported that these levels were lower than pH levels of commercial yogurts. In several countries, LAB are present in yogurt in numbers of at least 10^6 to 5×10^8 CFU/ml, depending on the type of yogurt [6]. According to the Turkish Food Codex, the total microorganism count in yogurt must exceed 10^6 CFU/g [19]. In this study on non-commercial yogurts, we found LAB counts of at least 3.7×10^8 CFU/g when the counts from M17 agar and MRS agar were considered together

Table 3. The characteristics of the LAB species isolated from non-commercial yogurts from nine locations in Turkey.

LAB species	Characteristics		
	Growth of temperature (0C)	Growth in medium with NaCl (%)	Growth at pH (acidity)
	4, 25, 45	3, 6, 10	2, 5, 7
L. casei (R) or *L. paracasei*	–, +, –	+, –,–	–, +, +
E. durans (C)	w, +, w	–, –, +	–, +, +
E. hirae (C)	+, +, w	–, +, w	–, –, w
E. faecium (C)	w, +, w	–, –, +	–, w, w

Note: C: cocci, R: rod, +: positive growth, –: no growth, w: weak growth

(that is, adding the lowest number of CFU on MRS agar [zero growth] and the lowest number of CFU on M17 agar [3.7×10^8 CFU/g]). Therefore, the their LAB numbers were within the range of LAB abundancec in other yogurts in Turkey and in other countries. *Enterococcus faecium*, *E. hirae*, *E. durans* and *Lactobacillus casei* or *L. paracasei* were the most frequent members of the yogurt LAB microbiota. These results suggested that non-commercial yogurts might develop in different ways and have a more wide profile of LAB species than commercial yogurts. *Enterococcus faecium*, *E. hirae*, *E. durans* (sensitive to low acid and low salinity) and *Lactobacillus casei* or *L. paracasei* (sensitive low acid and high salinity to low or high temperature) were the most interesting species among the isolated LAB species in terms of their characteristic properties. These LAB strains could be used for the fermentation process of yogurt production and are good candidate starter cultures.

MALDI has been successfully applied in recent times. It is a convenient and routine technique used to identify with 99% similarity of MALDI-TOF isolates. With only one colony, it is possible to identify it in just 10–15 min. MALDI is seen as a method that can be used in the food industry, in which rapid diagnostic procedures are needed. In addition, this method can be an inexpensive alternative to conventional methods. In fact, the identification fee for a single isolate is 1.2 US $. In our study, this method was strengthened by phenotypic identification and 2 different genus and 4 different species were identified. Rodríguez-Sánchez et al. [25] identified 3 *L. casei* [25] by same method such as 54 *L. casei* or *Lactobacillus paracasei* (similarity 34%), which we identified by MALDI-TOF MS in our study or 54 isolates Susakul Palakawong et al. [28] defined two new bacterial strains of the *Actinomyces* genus, such as Gram-positive (+) anaerobic bacterial strains [28] that we identified with MALDI-TOF MS in our study. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, which are used as starter cultures in commercial yogurt production, were not isolated in this study. The reason why they not were found in our study is that both of them need microaerophilic or anaerobic conditions for isolation. Incubation on MRS at 30 ºC under aerobic conditions, as used in our experiment, might have hindered the growth of *L. delbrueckii*, especially when it had to compete with the mesophilic bacteria found. Although lactose has to be present in the M17 medium, growth of *S. thermophilus* may be stunted under aerobic conditions. However, except for these species, other five species that we isolated had not been previously described to have the potential or ability to produce yogurt. These additional species might represent factors underliying the flavors and properties of non-commercial yogurts, different from commercial yogurts. Important results have been obtained for the provision of yogurts to Turkish consumers, who enjoy their local yogurt flavors. This study suggests different functions for different bacteria during the yogurt production, as we isolated bacterial species that have not often been isolated in other several studies. For instance, we isolated *E. hirae*, *E. faecium*, *E. durans*, *Lactobacillus casei* and *L. paracasei* strains from yogurts in which we could expect to find *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* strains. Other previous yogurt-related research in Turkey have identified only *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* species.

Our study focused on the basic development of starter cultures for non-commercial yogurt production. The yogurt bacteria isolated in this study are used in several combinations that allow the development of a starter culture that is the basis for the unique taste of yogurt. To our knowledge, our study is the first to specifically analyze non-commercial yogurt microbiota in southeastern Anatolia, Turkey.

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Competing interests. None declared.

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